1 Tissue Engineering: The Therapeutic Strategy of the Twenty-First Century

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CONTENTS

1.1 INTRODUCTION

The loss or failure of an organ or tissue is one of the most frequent, devastating, and costly problems in health care. Tissue engineering was born largely of the need for investigators to turn to multidisciplinary approaches to solve this long-standing problem in medicine. Advances in medicine have been paralleled by increased interactions among multiple disciplines such as biology, material sciences, and engineering, which led to progress in diagnostics, monitoring, and emergence of implanted devices and tissue grafts. Moreover, as medicine continued to advance, and the survivability of major disorders and injuries increased, so did the number of patients receiving and awaiting these critical treatments, and the need for alternative therapies became clearly apparent.

Clinicians have been a powerful driving force for innovation in medicine. The origin of tissue engineering stems from the demands by surgeons in regenerating functionally active tissue to replace those lost due to trauma, congenital malformations, or various disease processes. Current methods for organ and tissue replacement mainly utilize autografts, allografts, or metallic devices. Effective as these approaches are, they are associated with clear limitations including donor site morbidity, shortages in supply, poor integration, and potential immunologic reactions. These limitations further emphasize the importance of a timely development and successful translation of therapies based on tissue engineering principles. Internists also have historically turned to more complex therapies, from pharmacological administration of small molecules, to use of proteins, DNA, and other macromolecules, to extracorporeal devices for the replacement of lost cell or tissue function. Cell therapies became attractive for their ability to carry out numerous complex biochemical functions. Thus, these difficult problems in clinical medicine have continually inspired scientists and physicians in their quest to uncover biological mechanisms for exploitation at the bedside.

Our research in collaboration with Vacanti's group started seeking an alternative for patients awaiting liver transplants. Together, we sought ways to expand the cell seeding concept to three dimensions as an effort toward whole organ replacement. Our collaboration led to a publication describing the use of synthetic, resorbable, polymeric meshes for cell transplantation (Vacanti et al. 1988). This approach was adopted by a number of chemical engineers and others working with synthetic polymers, influencing many to employ similar techniques with degradable polymers.

Many turned their skills in biology or engineering toward tissue engineering, and the excitement felt in the academic sector was closely paralleled by that in the private sector. Due in part to federal agencies' early predilection toward funding hypothesis-driven research and in part to a contemporaneous flurry of corporate investment in biotechnology, tissue engineering research enjoyed a large influx in private funding. From the mid-1980s though the end of the millennium, over $3.5 billion was invested worldwide in research and development, and over 90% of those funds were supplied by the private sector. At the end of 2000, over 70 companies were participating in tissue engineering research and development and/or manufacturing. They were spending an estimated $600 million annually and employed about 3300 full-time equivalent scientists and support staff, all while only two products had received FDA approval (Lysaght and Reyes 2001).

In the first decade of the twenty-first century, scientific advances continue at a steady pace. Federal agencies have been increasingly stalwart in their nurturing of the field, both in the United States and abroad, not only by increasing funding but also by sponsoring workshops and studies and helping to define its future (McIntire 2003; Viola et al. 2003). Most importantly, as the complexities and challenges of engineering living tissue have become more fully understood, research has

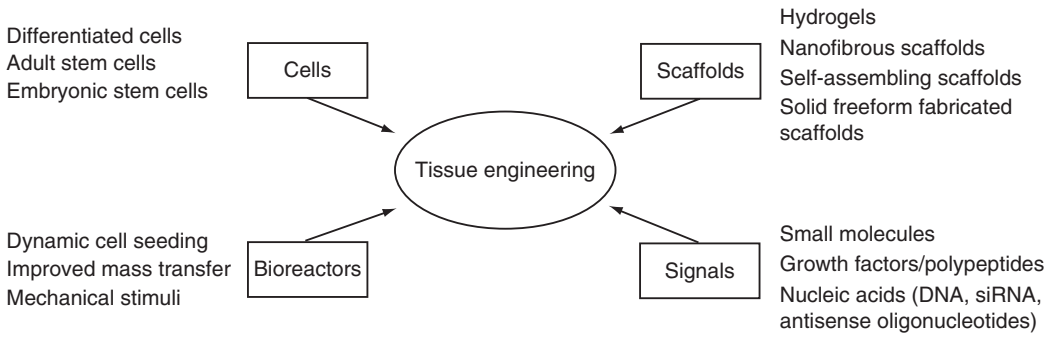

FIGURE 1.1 Key components of tissue engineering.

plumbed ever greater depths of innovation and technology. Tissue engineers have become increasingly drawn toward tangential fields, particularly stem cell and developmental biology and nanotechnology (Ingber et al. 2006; Vunjak-Novakovic and Kaplan 2006). So, even as first-generation products continue to come to market, the scientific foundation is being laid for engineering ever more complex and functional tissues. In this chapter, we briefly review a number of important advances in the field from its inception to the present day, including cells, scaffolds, tissue-inducing factors, and bioreactors (Figure 1.1).

1.2 CELLS

1.2.1 Cell Sources for Tissue Engineering

Cells are the building blocks of tissue, and cells present in the grafted tissue are believed to play a critical role in promoting tissue healing and regeneration. Therefore, most tissue engineering approaches involve isolating and expanding cells in vitro. Cell source is an important parameter to consider when applying tissue engineering strategies to restore lost tissue and functions. One of the major obstacles in engineering tissue constructs for clinical use is the shortage of available human cells. Conventional approaches usually utilize the fully differentiated adult cell types that make up the target organ or tissue. This often requires harvesting tissue such as autogenous or allogeneic tissue, enzymatically digesting the tissue to release cells, and culturing the dissociated cells in tissue culture flasks to initiate cell expansion. For example, autologous chondrocyte transplantation (ACT) is a cell-based procedure for cartilage repair that involves obtaining chondrocytes from the patient, expanding the cells in vitro, and transplanting the cells back into the same patient (Brittberg et al. 1994; Peterson et al. 2000). However, tissue engineering approaches need large numbers of cells, whereas the proliferation capability of fully differentiated cells is very limited. Furthermore, fully differentiated adult cells tend to lose their phenotype or dedifferentiate during in vitro expansion (Schnabel et al. 2002). Given the limitations of the fully differentiated cells, scientists and clinicians have collaborated to harness the potential of stem cells, which many believe hold the key to unlocking the secrets of tissue regeneration. In this chapter, we will mainly focus on advances in the stem cell field that have generated significant excitement in the past decade.

1.2.2 Potential of Stem Cells for Tissue Engineering Applications

Stem cells provide alternative cell sources for tissue engineering, such as craniofacial repair (Bruder et al. 1994; Aubin 1998; Shamblott et al. 1998; Thomson et al. 1998; Pittenger et al. 1999; Sottile et al. 2003; Cowan et al. 2004; Kim et al. 2005a). Unlike other types of cells in the body, stem cells

are unspecialized cells that are capable of self-renewal for long periods yet maintain their capacity to differentiate into multiple specialized cell types upon exposure to specific induction cues. Development of techniques for culturing and regulating human stem cells could lead to unprecedented regenerative treatments and cures for diseases that cannot presently be treated via other means. It has been estimated that approximately 3000 people die every day in the United States from diseases that could have been treated with stem cell–derived tissues (Lanza et al. 2001). In addition to the generation of tissues and organs to treat cancer, trauma, inflammation, or age-related tissue deterioration, stem cells are also potentially useful for treatment of numerous diseases including Parkinson's disease, Alzheimer's disease, osteoporosis, and heart disease. Stem cells are currently being tested therapeutically for the treatment of liver diseases, coronary diseases, autoimmune and metabolic disorders, chronic inflammatory diseases, and other advanced cancers. Stem cells may be xenogenic, allogeneic, or autologous, where autologous cells are preferred as they will not evoke an immunologic response, and thus the harmful side effects of immunosuppressive agents can be avoided. Autologous stem and progenitor cells may be derived postnatally in adulthood or early in life from umbilical cord blood (Cetrulo 2006) or tissue (Baksh et al. 2007). Autologous-like cells may also be generated using therapeutic cloning or somatic cell nuclear transfer (SCNT), the process through which Dolly the sheep was cloned in 1997 (Hwang et al. 2004). Studies to date have demonstrated that cells derived from SCNT can be expanded in culture and can organize into tissue structures after transplantation in vivo in combination with biodegradable scaffolds (Lanza et al. 1999).

1.2.3 Stem Cell Source

Depending on the development stage of the tissues from which the stem cells are isolated, stem cells can be broadly divided into two categories: adult stem cells and embryonic stem cells (Shamblott et al. 1998; Thomson et al. 1998; Pittenger et al. 1999). Adult stem cells can be found in many adult tissue types including bone marrow, peripheral blood, adipose tissue, nervous tissue, muscle, dermis, etc. (Table 1.1). Adult stem cells are considered to be multipotent, which can give rise to several other cell types. Among the adults stem cells, bone marrow–derived stem cells (MSC) have been shown to have the capability of differentiating into multiple tissue types, including bone, cartilage, muscle, tendon, etc., and hold great potential for autologous cell-based therapy (Pittenger et al. 1999). Another important characteristic of MSCs for regenerative medicine is their potential allogenic use without immunosuppressive therapy (Le Blanc et al. 2003; Maitra et al. 2004; Aggarwal and Pittenger 2005). In addition to the adult tissues mentioned above, stem cells have

TABLE 1.1
Types and Sources of Human Stem Cells

Origin	Types of Stem Cells	Sources of Isolation
Adult	Mesenchymal stem cells	Bone marrow
	Hemopoietic stem cells	Bone marrow and peripheral blood
	Neural stem cells	Neural tissue
	Adipose-derived stem cells	Adipose tissue
	Muscle-derived stem cells	Muscle
	Epidermal-derived stem cells	Skin, hair
	Umbilical cord blood stem cells	Umbilical cord blood
	Umbilical cord matrix stem cells	Wharton's jelly
Embryonic	Embryonic stem cells	Inner cell mass of 5–7 day blastocyst
	Embryonic germ cells	Gonadal ridge of 6–11 week fetus

also been identified in fetal tissues such as umbilical cord blood and Wharton's jelly. Although it was originally believed that stem cells derived from a particular tissue could only regenerate that specific tissue, numerous studies have disproved this idea (Macpherson et al. 2005). For example, both bone marrow and adipose tissue–derived mesenchymal stem cells may differentiate into cells and tissues of mesodermal origin including adipocytes, chondrocytes, osteoblasts, and skeletal myocytes and can be used to generate respective tissues including fat, cartilage, bone, and muscle (Caplan and Bruder 2001; Zuk et al. 2001; Baksh et al. 2003; Izadpanah et al. 2006). Unlike isolates of bone marrow, which typically require multiple punctures with a large bore needle, subcutaneous adipose tissue can be obtained through surgical removal with scalpels or through liposuction, which some patients may view as advantageous. However, despite their ability to differentiate into multiple cell types, adult stem cells are generally considered to give rise to only a limited range of differentiated cell types in comparison to embryonic stem cells.

Compared with MSCs, which can only be expanded in an undifferentiated state for limited passages, embryonic stem (ES) cells or embryonic germ (EG) cells can self-renew without differentiation for much longer. This property makes them attractive candidates as cell sources for tissue engineering, where large cell numbers are often needed. ES cells are derived from the inner cell mass of blastocysts, and EG cells are isolated from developing gonadal ridge. Since these cells are isolated from embryonic stage, they are considered to be pluripotent and can develop into any of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system). It was only in 1998 that the political and ethical controversy surrounding stem cells erupted with the creation of human ES cells derived from discarded human embryos (Thomson et al. 1998). In addition to direct therapeutic use, ES cells represent an attractive cell source for the study of developmental biology, for drug/toxin screening studies, and for the development of therapeutic agents to aid in tissue or organ replacement therapies. Although ES cells may hold the secret to multiple cures and groundbreaking advancements in the field of regenerative medicine, they raise significant ethical concerns because they are harvested from embryos.

1.2.4 Pure Stem Cell–Based Therapies

Although stem cell research is still in its infancy, there are some remarkable success stories including blood transfusions and bone marrow transplantation that have been used in thousands of patients to successfully treat low blood volume and diseases of the blood and bone marrow such as lymphoma. Bone marrow transplantation represents the most common clinically approved method of stem cell–based therapy. Here growth factors such as granulocyte colony-stimulating factor (G-CSF) are initially administered to amplify and mobilize hematopoietic stem cells into the peripheral circulation where they can easily be collected using leukapheresis techniques. The transplantation of bone marrow which has been used since the 1960s involves infusion of the stem cells into the recipients' peripheral circulation through an intravenous catheter. The stem cells home to the bone marrow where they proliferate and start to produce blood cells. Remarkably, even a single hematopoietic stem cell can be used to fully reconstitute the lymphohematopoietic system (Osawa et al. 1996). Numerous pure stem cell–based therapies are currently in clinical trials. For example, Osiris Therapeutics Inc. is working on a product called Prochymal, which is a treatment for a life-threatening disease called acute graft versus host disease (AGHD) which attacks the gastrointestinal tract, skin, and liver. AGHD affects half of all patients who receive a bone marrow transplant for anemia and other diseases. A trial is also currently under way using Prochymal to assess its ability to reduce the symptoms of moderate-to-severe Crohn's disease. Australia's adult stem cell company, Mesoblast Ltd., recently commenced a phase 2 clinical trial of its allogeneic adult stem cell therapy for patients with heart attacks. This therapy involves injection of stem cells via catheter into damaged heart muscle and aims to improve heart function and reduce congestive heart failure.

1.2.5 Scaffold-Based Stem Cell Therapies

Unlike pure cell-based therapies where stem cells are injected directly into the peripheral circulation or a specific tissue, many applications require a cell carrier to transport and/or arrange the stem cells within a 3D configuration, or to isolate them within a particular location in the body. Moreover, certain applications require differentiation of the cells down particular lineages prior to transplantation. These approaches are common in the field of tissue engineering where stem cells are combined with engineered matrices either to build transplantable tissues ex vivo or to inject or implant viable constructs that are programmed to promote or initiate regeneration. Whereas fundamental research centers on developing an understanding of the mechanisms that regulate stem cells, applied fields such as tissue engineering aim to harness this knowledge to initiate tissue-specific regeneration. The field of tissue engineering has promoted the transition of standard two-dimensional (2D) stem cell culture systems to 3D platforms in an attempt to mimic the in vivo 3D culture environment which may be more conducive for regulating stem cell function.

Previous research on ES cells mainly focused on the understanding of stem cell biology, and tissue engineering applications using ES cells or EG cells in combination with scaffolds is still at its infancy (Elisseeff et al. 2006; Hwang et al. 2006). Scaffolds can promote cell and tissue development by providing a 3D environment in which cells can proliferate, attach, and deposit extracellular matrix (ECM) (Peppas and Langer 1994; Hubbell 1995; Langer and Tirrell 2004; Lutolf and Hubbell 2005). Various biological signals such as growth factors or peptides can also be incorporated into scaffolds to promote the desired differentiation (Hubbell 1999; Healy et al. 1999). For example, it has been shown that bone MSCs can undergo osteogenesis in a 3D hydrogel scaffold, and incorporation of cell adhesion peptide YRGDS into the scaffold promotes the osteogenesis of MSCs in a dosage-dependent manner, with 2.5 mM being the optimal concentration (Yang et al. 2005b).

One of the major challenges with scaffold-based transplantation of cells is lack of engraftment that typically results within damaged avascular tissue due to a deficiency in mass transport of oxygen and nutrients, a requirement for cell survival and for proper cell function. Maximal reported rates of angiogenesis are $\sim$1 mm/day (Folkman 1971; Li et al. 2000) and cells need to be within $\sim$100–200 μm of the nearest blood vessel (Muschler et al. 2004). Thus, transplanted cells within the core of large defects ($>$1–2 cm) do not survive long enough to contribute to the healing process. Specifically, it may take many weeks or months for complete vascularization of the defect (Mooney et al. 1994; Sanders et al. 2002) leading to tissue ischemia and necrosis (Helmlinger et al. 1997) (cell and tissue death) in graft sites as small as 1–2 mm (Muschler et al. 2004). This significantly reduces the capacity for an exogenous cell source to contribute to the regenerative process. Furthermore, most scaffold-based tissue engineering strategies passively permit filling of the scaffold pores with blood clot (Karp et al. 2004), which represents a static and potentially harsh environment for the transplanted cells. Although hematomas contain factors such as vascular endothelial growth factor (VEGF) that induce neovascularization (Street et al. 2000), hematomas are acidic and hypoxic and exhibit elevated levels of phosphorous, potassium, and lactic acid, which are cytotoxic to multiple cell types (Wray 1970). Therefore, transplanted cells are susceptible to death given their distance from host vasculature, and their position within the static and relatively harsh environment of the blood clot. This has significantly limited advancement in the field of tissue and organ replacement. After three decades of substantial research in this area, the potential to provide tissues and organs to millions of patients suffering from trauma, congenital defects, and chronic diseases has yet to be fully realized (Mikos et al. 2006). Although this is partly due to uncertainty and difficulties with clinical markets, typical results in preclinical animal models remain highly variable with poor rates of success in larger defects and in higher animal species likely due to poor survival of the transplanted cells (Petite et al. 2000; Muschler et al. 2004). Although it is not surprising that the effectiveness of cell-based therapies relies on the retention of cell viability after implantation (Wilson et al. 2002; Kruyt et al. 2003), little attention has been focused on this issue.

Recently, an advanced cell-instructive tissue engineering approach was successfully employed that utilized (1) high density arginine, glycine, aspartic acid (RGD)-containing cell adhesion ligands, (2) an exogenous differentiated myoblast cell source, and (3) growth factors to enhance the regenerative capacity of the transplanted cells through promoting their survival, preventing their terminal differentiation, and promoting their outward migration (Figure 1.4) (Hill et al. 2006). AQ1
Specifically, cells were delivered on porous alginate/calcium sulfate scaffolds that contained both hepatocyte growth factor (HGF) and fibroblast growth factor-2 (FGF-2), which were employed to maintain the cells in an activated, proliferating, but nondifferentiated state. Whereas control groups had only a modest effect on muscle regeneration, a combination strategy employing controlled release of HGF and FGF-2 in combination with scaffolds and cells dramatically enhanced the participation of transplanted cells leading to significant tissue regeneration. Despite the relatively small size of the scaffolds employed here (50 mm^3) and the uncertainties in translating this strategy into larger clinically relevant defects, the work demonstrates a proof of concept for cell-based therapies that can be designed to direct tissue regeneration.

1.3 SCAFFOLDS AND FABRICATION

1.3.1 Importance of Scaffolds to Promote Tissue Formation

Three common strategies employed in tissue regeneration are infusion of isolated cells, treatment with tissue-inducing substances, and implantation of a cell–scaffold composite (Langer and Vacanti 1993). Of the three strategies, the use of cell–scaffold composites generally leads to a more successful outcome. These scaffolds are often critical, both in vitro as well as in vivo, to recapitulating the normal tissue development process and allowing cells to formulate their own microenvironments. In contrast to using cells alone, a scaffold provides a 3D matrix on which the cells can proliferate and migrate, produce matrix, and form a functional tissue with a desired shape. The scaffold also provides structural stability for developing tissue and allows incorporation of biological or mechanical signals to enhance tissue formation. The biological and mechanical properties of scaffolds may vary depending on the application, and can be designed to provide an environment with appropriate signals that stimulate cells to proliferate and/or differentiate.

The importance of the ECM scaffold in cell development should not be underestimated. Nearly 30 years ago, Bissell proposed dynamic reciprocity, which states that a tissue achieves a specific function in part through interactions of the cells with the ECM (Bissell et al. 1982). Subsequent work demonstrated that gene expression can be mediated by the ECM binding to ECM receptors on the cell surface, which provide a link to the cytoskeleton and eventually the nuclear matrix (Nickerson 2001). The inclusion of neighboring cell interactions and soluble signals originating systemically or from cells in the immediate or distant vicinity provides a more complete model of tissue environment (Nelson and Bissell 2006).

Much attention has been given to the simulation of the extracellular environment. Of particular interest is the creation of scaffolds as substitutes for the ECM. Scaffolds for tissue regeneration occupy a fundamental role in tissue development since they must support the proliferation and differentiation of cells as they mature into a functional tissue. Regeneration to the native state necessitates removal of the artificial scaffold, most commonly by bioabsorption. To this end, numerous natural and synthetic materials have been proposed for use in tissue scaffolds (Nair and Laurencin 2006; Velema and Kaplan 2006). Drawbacks do exist for the use of existing materials in particular tissue engineering applications; however, 3D scaffold fabrication and incorporation of biofactors into the scaffold comprise the central challenges in the field today. Furthermore, although biomaterials received much attention during the 1990s, the current emphasis on hybrid living–artificial systems requires continued development of fabrication methods.

As a substitute for the ECM, a scaffold in general should impart a 3D geometry, have appropriate mechanical properties, enable cell attachment, and facilitate the development of a

functional tissue. At the microscopic level, a highly porous structure is needed for diffusion of nutrients and waste products through the scaffold. The optimal pore size should be tailored to the specific cell type and be large enough to allow for cell migration and ECM formation yet not be so small that pore occlusion occurs. The scaffold surface architecture and chemistry should facilitate cell migration through the scaffold, provide developmental signals to the cells, and promote cell recruitment from the surrounding tissue. Additionally, in most cases the scaffold should be constructed from a degradable nontoxic material (Leong et al. 2003). Recent advancements in scaffold fabrication technologies are discussed below.

1.3.2 Scaffold Fabrication

1.3.2.1 Conventional Methods and Limitations

The formation of a porous structure constitutes a central goal of scaffold fabrication and a number of techniques were developed to achieve this aim including phase separation (Lo et al. 1995) (nonsolvent-induced phase separation and thermally induced phase separation), gas foaming (Mooney et al. 1996), solvent casting/particulate leaching (Wald et al. 1993), and freeze drying (Dagalakis et al. 1980). Because of the relative ease in using these techniques to fabricate scaffolds, they are still commonly used. A core limitation of these technologies is the lack of precise control over scaffold specifications such as pore size, shape, distribution, and interconnectivity as well as the overall scaffold shape. Numerous studies note the importance of pore size in the ability of cells to adhere and proliferate on a scaffold (Hulbert et al. 1970), but recent work with scaffolds produced using solid freeform fabrication (SFF) techniques where the pore size is precisely controlled suggests that eliminating the variability in the pore size and structure decouples the dependence of cell adhesion and proliferation on pore characteristics (Itala et al. 2001; Hollister 2005) (Figure 1.2). However, the porosity of the material, which is defined as the proportion of void space in a solid, is still a critical factor (Karageorgiou and Kaplan 2005). The fabrication of hierarchical porous structures, which consist of both a nano- or microscopic pore structure and a macroscopic pore structure, is more readily realized using SFF methods. These techniques allow the reproducible fabrication of scaffolds directly from a computer-aided design (CAD) file. The ability to translate an electronic data set into a scaffold opens up the possibility for patient-specific scaffolds based on computed tomography (CT) or MRI data (Mankovich et al. 1990; Hollister et al. 2000; Wettergreen et al. 2005).

1.3.2.2 Solid Freeform Fabrication Methods

1.3.2.2.1 Fused Deposition Modeling

Fused deposition modeling (FDM) (Crump 1992) is a process whereby a molten material is extruded through a nozzle and deposited as a layer on a surface. At the completion of the layer deposition, the sample stage is lowered and a new layer is deposited. In this fashion, the technique fabricates a 3D structure. A benefit of this method is the absence of organic solvents in the fabrication process. The process is computer controlled, which allows the use of CAD data in the design of the scaffold. The technique has been used to prepare porous scaffolds from polymers such as PCL (Hutmacher et al. 2001), PEG-PCL-PLA (Hoque et al. 2005), and HA/PCL composite (Sun et al. 2007). The requirement of a melt feed limits the range of materials that can be used and excludes sensitive molecules such as proteins from being directly incorporated into the scaffold.

1.3.2.2.2 3D Printing

The technique of 3D printing (Sachs et al. 1993) consists of applying a layer of powder onto a surface and using an inkjet printer head to spray the surface precisely with a binder to join the powder particles. The process is repeated after spreading a new layer of powder on top of the previous layer, which results in the creation of a 3D structure. In the past, organic solvents

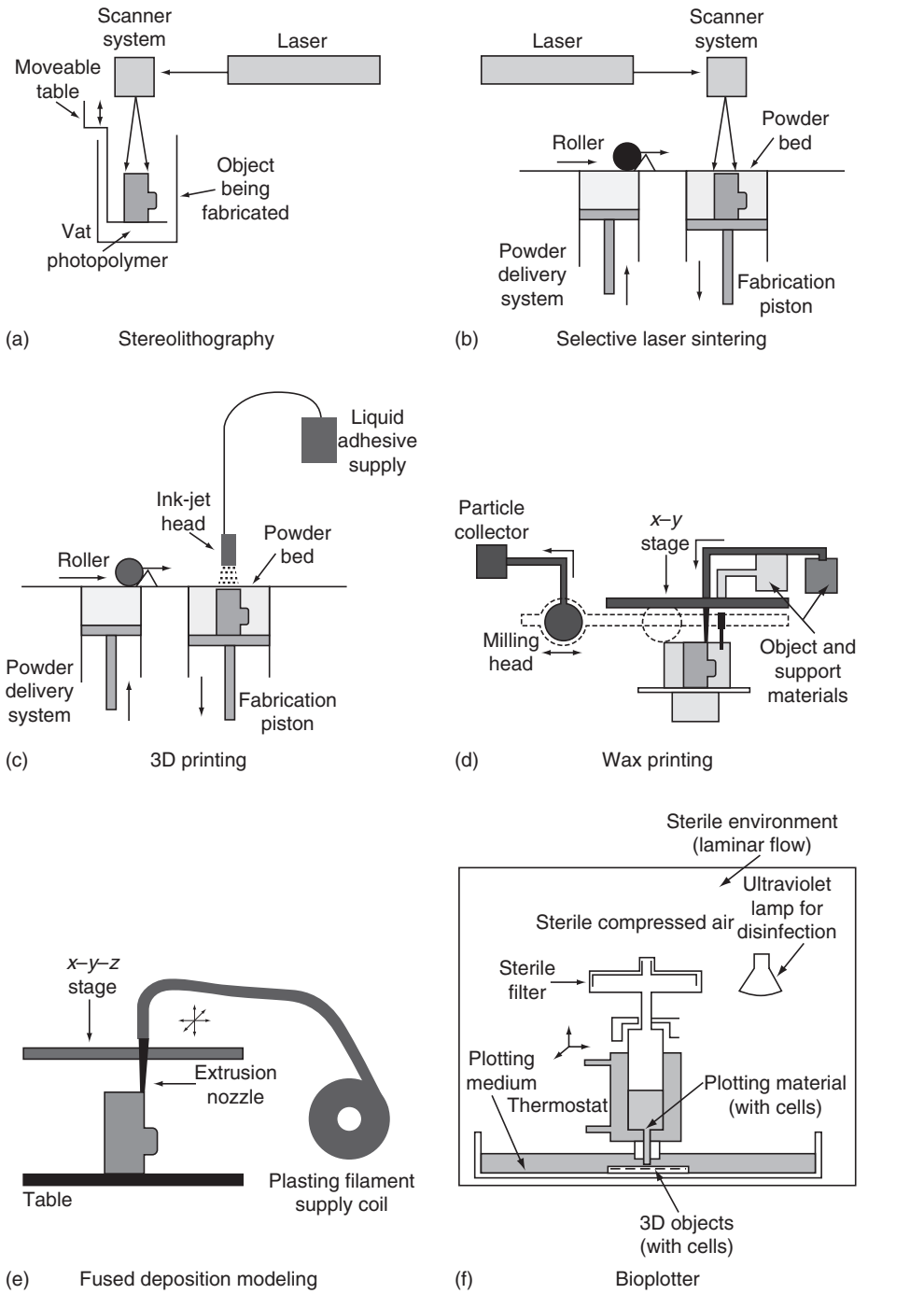

FIGURE 1.2 Schematics of solid freeform fabrication (SFF) systems categorized by the processing technique. (a,b) Laser-based processing systems include (a) the stereolithography system, which photopolymerizes a liquid and (b) the selective laser sintering (SLS) systems, which sinter powdered material. In each system, material is swept over a built platform that is lowered for each layer. (c,d) Printing-based systems, including (c) 3D printing and (d) a wax printing machine. 3D printing of a chemical binder onto a powder bed. The wax-based system prints two types of wax material in sequence. (e,f) Nozzle-based systems. (e) The fused deposition modeler prints a thin filament of material that is heated through a nozzle. (f) The Bioplotter prints material that is processed either thermally or chemically. (From Hollister, S.J., *Nat. Mater.*, 4, 7, 518, 2005. With permission.)

have been used as binders (Giordano et al. 1996); however, recent examples stress the use of biocompatible materials. In one example, hydroxyapatite powder was used to prepare bone repair scaffolds using a binder composed of 25% v/v polyacrylic acid in a water–glycerol mixture (Dutta Roy et al. 2003). Aqueous citric acid solution has also been used as a binder in the preparation of calcium phosphate–based ceramics (Khalyfa et al. 2007).

1.3.2.2.3 Selective Laser Sintering

Resembling 3D printing, the selective laser sintering (SLS) process also begins by applying a thin layer of powder to a surface. A laser beam sinters the powder particles together in the desired pattern. Upon completion of the layer patterning, a new layer of powder is deposited and the process repeated. This technique has been used to prepare scaffolds from the biodegradable polymers polyetheretherketone, poly(vinyl alcohol), polycaprolactone (Williams et al. 2005), and poly(L-lactic acid) (Tan et al. 2005). Composites of some of these polymers and hydroxyapatite have also been prepared using SLS (Chua et al. 2004; Tan et al. 2005; Wiria et al. 2007).

1.3.2.2.4 Wax Printing

In the fabrication of 3D scaffolds using a wax printer, a negative mold is created by printing droplets of a build wax and a support wax on a surface, which harden after cooling. Once a layer is printed, the surface is milled flat and another layer is printed. This process continues until the structure is complete at which point the support wax is dissolved to yield a porous negative mold. The desired scaffold material is added to the mold as a casting solution and allowed to solidify, and the negative mold is dissolved or melted to release the scaffold. This technique has been used to prepare, for example, scaffolds for bone and cartilage replacement (Manjubala et al. 2005). Like most SFF processes, this technique was not originally designed for use in biological systems. The waxes and solvents used are often proprietary formulations and contain dyes, both of which can contaminate the scaffold with nonbiocompatible agents (Sachlos et al. 2003). Recent reports have made use of apparently biocompatible proprietary waxes (BioBuild and BioSupport) that can be orthogonally dissolved using ethanol and water (Sachlos et al. 2006); however, the identities of these materials have not been disclosed in the literature.

1.3.2.2.5 Stereolithography

Stereolithography relies on light-mediated chemical reactions to create a 3D object from a liquid polymer. In this process, a surface is lowered into a vat of photocurable polymer and the resultant layer of liquid polymer on the top of the surface is exposed to a laser to harden the polymer. The surface is then submerged slightly, which covers it with a new layer of liquid polymer that can be exposed to the laser. The surface can be raised or lowered as needed to create the 3D object. Biomaterials that have been in this application include poly(propylene fumarate) (Cooke et al. 2003; Lee et al. 2007), which contains photocrosslinkable double bonds, and acrylated poly(ethylene glycol) (Dhariwala et al. 2004; Arcaute et al. 2006).

1.3.2.3 Nanofibrous Scaffolds

1.3.2.3.1 Electrospinning

This technique produces nanofibers in a continuous fashion that are interconnected. The fiber diameter can range from 5 nm to more than 1 μm (Murugan and Ramakrishna 2006). Electrospinning differs from the current SFF technologies in that it produces a nanofibrous scaffold. Such a construct mimics the ECM by possessing high surface area, high aspect ratio, high porosity, small pore size, and low density (Murugan and Ramakrishna 2007). Due to the nature of the electrospinning process, randomly oriented fibers are produced (Matthews et al. 2002). Recent efforts have focused on electrospinning aligned fibers (Yang et al. 2005a). Both natural and synthetic materials have been electrospun into random and aligned meshes including collagen, gelatin, and chitosan (Murugan and Ramakrishna 2006).

1.3.2.3.2 Self-Assembling Scaffolds

Self-assembly relies on noncovalent interactions to achieve the goal of a spontaneously assembling 3D structure. Possessing this property, biopolymers such as peptides and nucleic acids are ideally suited for this role. Rationally designed peptides that spontaneously form 3D scaffolds in response to specific environmental triggers may have great potential in tissue engineering. Several elegant methods have been reported making use of peptides (Hartgerink et al. 2001, 2002; Beniash et al. 2005). Furthermore, these designer self-assembling peptide scaffolds have recently been demonstrated to repair nervous tissue, to stop bleeding in seconds, to repair infarctuated myocardia, as well as being useful medical devices for slow drug release (Zhang et al. 2005; Gelain et al. 2007). This concept has also been applied to DNA, where branched molecules were designed so that the arms of the DNA can hybridize with each other. In the presence of a DNA ligase, which serves to crosslink the DNA, the DNA molecules self-assembled into a hydrogel (Um et al. 2006).

1.3.2.4 Hybrid (Cell/Scaffold) Constructs

1.3.2.4.1 Conventional Cell-Laden Hydrogels

Hydrogels are swollen, typically crosslinked networks that are particularly useful for suspending cells in 3D. A variety of synthetic and natural polymers have been utilized for this application including polyethylene glycol (PEG) and copolymers containing PEG (Tessmar and Gopferich 2007), hyaluronic acid (Baier Leach et al. 2003), chitosan (Leach et al. 2004), and alginate (Mosahebi et al. 2001). Photocrosslinkable systems have been used extensively to form the gels, and other methods have been developed including enzymatic (Um et al. 2006) and thermosensitive (Park et al. 2007) systems to avoid the use of potentially cytotoxic UV light and radicals. Hydrogels have been used extensively to prevent adhesions due to their relative lack of cell adhesiveness (Sawada et al. 2001; Yeo et al. 2006). Consequently, cell adhesion proteins have been incorporated into hydrogels to promote cell adhesion (Hern and Hubbell 1998; Rowley et al. 1999; Shu et al. 2004). Degradation of hydrogels generally occurs by hydrolysis; however, enzymatically degrading hydrogels have also been reported (He and Jabbari 2007). The mechanical properties of hydrogels are generally weak so there have been efforts to create strong hydrogels (Kaneko et al. 2005). In order to form a 3D structure, conventional cell-laden hydrogels utilize a mold into which the cell-laden hydrogel is cast. AQ2

1.3.2.4.2 3D Patterning of Cell-Laden Hydrogels

In order to achieve more control over the 3D placement of cells within hydrogels and realize patient-specific geometries, a number of SFF technologies have been adapted for use with cell-laden hydrogels. Laser-guided direct writing uses a weakly focused laser beam to trap cells and then deposit them on a surface (Odde and Renn 2000; Nahmias et al. 2005). This technique allows single-cell resolution patterning and has been used to directly write endothelial cells that self-assemble into vascular structures (Nahmias et al. 2005). Using a modified inkjet printer and thermosensitive gels, multiple layers of different cell types can be printed to create a 3D organ (organ printing) (Boland et al. 2003). This technology attempts to mimic the architecture of organs, which consist of complex structures containing many cell types positioned in precise locations. Bioplotter, a commercially available instrument, utilizes a needle to dispense a material in a layer-by-layer fashion into a plotting medium, which causes the material to solidify. A polymer–cell mixture can be dispensed using this technique leading to the formation of a cell-laden hydrogel (Landers and Mülhaupt 2000). Microfluidics have also been used to create 3D structures using a layer-by-layer approach (Tan and Desai 2004). In this method, pressure-driven microfluidics are used to transport cell–polymer solutions, which are deposited as layers within the microchannels. Photolithography has been utilized to create patterned hydrogel structures that encapsulate cells (Liu and Bhatia 2002). Cell-laden hydrogels have also been created from photopolymerizable polymer solutions where the cells are first localized using dielectrophoretic forces and then locked into place by light-mediated hydrogel

formation (Albrecht et al. 2006). Recent efforts have also focused on adapting traditional SFF technologies for use with hydrogels, for example by using stereolithography to create complex PEG hydrogels (Arcaute et al. 2006).

1.4 DELIVERY OF TISSUE-INDUCING FACTORS

1.4.1 Potential of Controlled Release System to Enhance Tissue Formation

Since the inception of molecular biology, biologists have steadily worked on identifying and isolating molecular agents responsible for tissue formation and repair. Mechanisms of development and wound healing are continually being elucidated and their molecular bases constantly being explored for therapeutic exploitation. Cellular therapies important for tissue engineering are also highly dependent on cell-signaling factors, as the culture of cells appropriately differentiated or undifferentiated often requires the addition of isolated molecular agents that promote maintenance of specific cell phenotypes. For example, progenitor cells isolated from the retina can be maintained in an undifferentiated state for long periods of time in the presence of recombinant epidermal growth factor (EGF). These cells can then be cultured with the EGF replaced by nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and basic fibroblast growth factor (bFGF) to induce differentiation into cells that express neuronal and glial markers (Tomita et al. 2006). In tissue engineering, the goal is to supply cells with the factors necessary to induce proliferation and/or differentiation so the cells then may secrete the appropriate extracellular components for tissue formation. The supplication of tissue-inducing factors in growth medium may be sufficient for growth of tissues in vitro, but administration of such biomolecules to induce tissue formation in vivo is generally not sufficient, as the molecules diffuse rapidly away from the desired location and are quickly degraded. For this reason, controlled drug-delivery systems are usually necessary.

As tissue engineering approaches were taking shape in the mid-1970s, advancements were being made in a seemingly disparate field that would eventually be considered one of the most important enabling technologies in tissue engineering. In 1976, Langer and Folkman, seeking experimental methods to assay angiogenesis factorsin vivo, demonstrated sustained release of an enzyme from synthetic polymers (Langer and Folkman 1976). This advance helped pave the way for the development of versatile delivery systems capable of delivering biomolecules in a controlled fashion. Polymeric delivery systems have been reported in the form of tablets, wafers, fibers, extruded implants, films, microparticles, and many others (Wise 2000). Indeed, polymeric controlled release technology has progressed such that for nearly every macromolecule undergoing therapeutic development, polymeric release has probably at least been considered (Schwendeman 2002). Researchers have frequently experimented with drug delivery systems fabricated from biodegradable polymers which have been approved by the FDA for other uses. Coincidentally, early tissue engineering research groups began to use the same materials for scaffold fabrication, and it was not long before the scaffold began to be utilized as a delivery device for both cells and tissue-inducing biomolecules (Sokolsky-Papkov et al. 2007).

1.4.2 Types of Tissue-Inducing Factors

A large range of biomolecules have been investigated for the controlled induction of tissue formation, but the majority of important factors can be divided into three classes: small molecules, proteins and polypeptides, and oligonucleotides.

1. Small molecules (arbitrarily lumped into one category here for convenience) are important components in numerous cell signaling cascades, both in intercellular communication, as in the case of corticosteroids and other hormones, and in intracellular signaling. They typically trigger intracellular signaling cascades by binding to specific protein receptors, which leads to gene transcription.

2. Polypeptides, most often as whole proteins, may act on cells as mitogens, morphogens, growth factors, survival factors, and cytokines (these terms are technically neither synonymous nor mutually exclusive; however, for the purposes of this chapter they will not distinguished). These tissue-inducing proteins may be soluble or bound to the ECM, and typically act upon cells through receptor–ligand binding.
3. Oligonucleotides, either as DNA or RNA, can either bind to DNA to affect gene transcription, RNA to affect gene translation, or when delivered as whole genes, become incorporated directly into the cell's genome.

The challenges and strategies to deliver these classes of compounds vary due to the differences in their physical and chemical properties. Here, we briefly survey the important considerations of delivering each and provide some prominent examples. However, because the vast majority of tissue-inducing factors investigated for tissue engineering have been macromolecules, the greater part of this section will be devoted to discussing proteins and gene delivery.

1.4.3 Small Molecule Delivery for Tissue Engineering

Due to their size, small molecules tend to diffuse rapidly, so controlled delivery depends largely on strategies to slow or prevent diffusion. These strategies vary widely depending on the structure of the molecule to be delivered and the target environment. Some methods to retard diffusion include making use of ionic interactions to form insoluble complexes or matching hydrophilic and lipophilic properties of the drug and delivery device to retard release. More advanced techniques involve chemically modifying the molecule or attaching it to the delivery device. For example, Nuttelman and colleagues recently synthesized PEG hydrogels containing dexamethasone covalently linked to the hydrogel backbone with degradable lactide units. Dexamethasone is a corticosteroid which reliably promotes osteogenic differentiation of human mesenchymal stem cells (hMSCs). The authors showed that dexamethasone was released slowly from the hydrogel and induced hMSCs to express osteocytic phenotypes (Nuttelman et al. 2006).

Small molecules serve as important components of intercellular communications, as in the case of hormones, and intracellular signaling, particularly as second messengers. It is often in this second messenger capacity that small molecules have been utilized for tissue induction, as evidenced by their codelivery with other factors. For example, cyclic adenosine monophosphate (cAMP) has been found to act synergistically when coadministered with Schwann cell implants in the injured spinal cord (Pearse et al. 2004). Another research group delivered cAMP along with a neuronal growth factor via microparticles injected into the eye and found the combination to be effective for promoting optic nerve regeneration (Yin et al. 2006). As delivery of multiple factors with distinct release profiles becomes more common, it is likely that the role of small molecules in tissue engineering will be augmented.

1.4.4 Protein Delivery for Tissue Engineering

1.4.4.1 Challenges for Controlled Protein Delivery

The controlled delivery of proteins and polypeptides has been investigated extensively for a wide range of applications and has been met with some success; however, significant challenges remain. Because proteins are highly complex, ordered molecules whose functions depend on chemical and structural integrity, the greatest difficulties in devising controlled protein release systems arise from instability of the protein, formulation, storage, and release (Fu et al. 2000). Much work has been undertaken to elucidate mechanisms of protein degradation and inactivation and therefore to devise methods to mitigate these processes. The major mechanisms of protein inactivation in polymeric delivery systems include aggregation due to dehydration and rehydration, protein unfolding or aggregation along hydrophobic surfaces or at aqueous–organic interfaces, and acidification of the

microclimate within the delivery system (Schwendeman 2002). Reported methods of overcoming these challenges include zinc complexation (Johnson et al. 1997) or addition of lyoprotectants (Prestrelski et al. 1993) to inhibit moisture-related aggregation, choosing protein-friendly processing techniques to prevent aggregation at polymer surfaces and interfaces (Herbert et al. 1998; Burke 2000), and addition of antacids (Zhu et al. 2000) or pore-forming excipients such as PEG (Jiang and Schwendeman 2001) to prevent microclimate acidification. While these advances have been instructive, different proteins are susceptible to different forms of instability; thus, it is necessary to optimize each delivery system for its specific application.

1.4.4.2 Strategies for Protein Delivery

Controlled protein delivery for tissue engineering can be achieved using multiple delivery vehicles, including transplanted cells that are genetically modified (Chang et al. 1999; Tresco et al. 2000), polymer microparticles (Edelman et al. 1991; Krewson et al. 1996; Oldham et al. 2000; Lu, Yaszemski, and Mikos 2001), and scaffolds. The main advantage of the scaffold-free approach is that requirements of the delivery system may be met independently from those of the scaffold. However, researchers have increasingly turned toward utilizing the scaffolds themselves as delivery vehicles. In order to accomplish this, proteins have been adsorbed to the surface of the scaffold, encapsulated in the bulk of the scaffold, or covalently attached to the scaffold (Tessmar and Gopferich 2007). Maintaining stability of the protein in these cases is far from trivial and obviously essential. Protein release kinetics will naturally vary among these techniques, and utilizing combinations of release techniques has proved to be advantageous. For example, one research group demonstrated release of two growth factors with distinct kinetics by incorporating gelatin microparticles within a hydrogel scaffold (Holland et al. 2005). Another group demonstrated dual release kinetics by using a sequential emulsion technique to form protein-containing coatings on a preformed scaffold (Sohier et al. 2006).

1.4.4.3 Controlled Release of Growth Factors to Enhance Tissue Formation

Delivery of an enormous variety of proteins has been investigated for tissue engineering applications, due in part to the extreme importance of proteins in cellular signaling. The groups of tissue-inducing proteins often collectively called growth factors have been widely studied (Tessmar and Gopferich 2007). Growth-factor strategies for tissue formation include promotion of cell proliferation, differentiation into the desired tissue-forming cell types, migration into the desired locations, cell growth along with secretion of matrix for tissue formation, and generation of a blood supply (Boontheekul and Mooney 2003; Tabata 2003; Tessmar and Gopferich 2007). Growth factors often work in concert with one another, and each may act upon numerous tissue types and produce varying effects on different cell types. Moreover, they act in diverse manners, for example by binding cell surface receptors before internalization or binding the ECM before cell interaction, and cells often respond to soluble growth factors according to a concentration gradient (Boontheekul and Mooney 2003). For these reasons, the engineering of a delivery system used to present these biomolecules to the tissue-forming cells is of utmost importance.

Scaffolds and delivery systems have proven effective for generating relatively simple neotissue constructs in vitro and in vivo. However, developing and healing tissues often respond to transient or gradient concentrations of signaling molecules, so the formation of spatially complex tissues will likely require presentation of biomolecules in a spatiotemporally controlled manner (Saltzman and Olbricht 2002). While difficult, this challenge has spurred impressive innovations. New methods of scaffold seeding such as layer-by-layer film deposition can help lead to complex temporal files within tissues, by incorporating growth factors, proteins, and other important cellular components within specific layers (Wood et al. 2005). This can help control the speed and time spans of proliferation and differentiation during the culture process. Scaffolds endowed with a spatial

gradient of NGF have shown promise in directing axonal outgrowth (Moore et al. 2006), and those with a gradient of bFGF promoted the directed migration of vascular smooth muscle cells (DeLong et al. 2005). One research group developed a method of sintering protein-containing microspheres that allowed multiple proteins to be released with distinct rates from distinct zones within a scaffold (Suciati et al. 2006). Spatiotemporal control has also shown effectiveness in vivo. For example, Chen and colleagues demonstrated release of two angiogenic growth factors with distinct release kinetics from within specific regions of a scaffold. When implanted in an ischemic hindlimb, the region of the scaffold that released the two factors sequentially promoted development of a mature vascular network that was superior to that found in the region delivering only one growth factor (Chen et al. 2007). Furthermore, for tissues that are subject to mechanical stimuli, such as bone, muscle, and blood vessels, Lee et al. demonstrated a controlled growth factor release system that can respond to repeated mechanical stimuli (Lee et al. 2000). VEGF encapsulated in alginate hydrogels was released in response to compression both in vitro and in vivo, and mechanical stimulation was shown to upregulate the blood vessel formation in vivo.

1.4.5 Nucleic Acid Delivery for Tissue Engineering

1.4.5.1 Techniques for Gene Delivery

Advances in gene delivery have provided an alternative venue to recapitulate the natural tissue development process for tissue engineering purposes. For various tissue engineering applications, a target gene can be transferred to specific cell types, such as stem cells, to promote the desired cell differentiation and tissue formation. Cells can either be transfected ex vivo and then seeded onto 3D scaffolds for in vivoimplantation, or they can be transfected in vivodirectly.

The techniques for delivering genetic materials into mammalian cells can be broadly divided into two categories: viral-based and synthetic nonviral methods. Both approaches have their own advantages and disadvantages, and no single vector is suitable for all gene delivery applications. The viral approach employs a key property of viruses, which deliver their genome into target cells. Many different types of viruses, such as retrovirus, adenovirus, and lentivirus, can be transformed into gene delivery vehicles by replacing part of the viral genome with a target gene (Vile et al. 1996; During 1997). As the viral approach essentially utilizes the naturally evolved mechanism for viral self-replication, it is typically very efficient and thus has been the major approach undertaken for most applications. In fact, 69% of the ongoing clinical trials employ a viral-based approach (Gene Therapy Clinical Trials online, http://www.wiley.co.uk/genetherapy/clinical/, Accessed 2005). Despite the high transfection efficiency, the viral-based approach is also associated with several major limitations. As viruses are inherently immunogenic and potentially pathogenic, safety concerns have always been a major issue for the clinical applications of viral-based gene delivery. Furthermore, viral vectors do not facilitate the design of target cell specificity and are associated with relatively high manufacturing costs.

Nonviral synthetic vectors, mostly cationic polymers and lipids, provide another attractive vehicle for gene delivery. In general, synthetic vectors are cationic materials that can electrostatically bind to DNA or RNA to form condensed nanoparticles (polyplexes or lipoplexes). The biomaterials that have been explored include cationic polymers, cationic lipids, liposomes, chitosans, dendrimers, and inorganic nanoparticles (Merdan et al. 2002; Partridge and Oreffo 2004; Wagner et al. 2004). These synthetic vectors overcome the problems associated with a viral-based approach and are nonimmunogenic. They also enable greater flexibility in structure design and integrating a targeting moiety, as well as relatively easy synthesis and lower manufacturing costs. However, nonviral-based vectors have suffered from low transfection efficiency and occasional toxicity, and most synthetic vectors are unstable in the presence of serum, thus severely hindering their applications in vivo.

1.4.5.2 Major Barriers in Gene Delivery and Conventional Solutions

To enhance the delivery of target genes into the cell nucleus, it is very important to understand the major barriers that gene vectors need to overcome. Before reaching the target cell nucleus, polyplexes must first attach to cell surface, be internalized through endocytosis, escape from the resulting endosome/lysosome, navigate through the cytoplasm toward cell nucleus, and finally cross the nuclear membrane (Pack et al. 2005). Furthermore, polyplexes must be unpackaged at a certain time point so that the DNA can be released.

Overcoming the extracellular barriers requires efficient condensing of plasmid DNA, stability of nanoparticles in the blood stream and surrounding tissue, and specific targeting to the cells of interest. Polyplexes form spontaneously upon mixing of cationic polymers with DNA and condense into nanoparticles with a size ranging from thirty to several hundred nanometers. Polyplexes protect the naked DNA from being degraded by DNase. The stability of polyplexes in serum depends on the polymer chemistry and the DNA/polymer charge ratio. In general, positively charged polyplexes show better stability under physiological salt conditions in comparison to neutral polyplexes. However, in the presence of serum, negatively charged proteins such as albumin can adsorb onto the nanoparticles and cause aggregation, which leads to clearance of nanoparticles by phagocytic cells (Dash et al. 1999).

Once attached to the cell surface, polyplexes are internalized, either by cell-surface receptor-mediated endocytosis or by adsorptive pinocytosis (Mislick and Baldeschwieler 1996). Polyplexes will then become localized in endosomes, which are vesicles that rapidly acidify to pH 5–6 due to the action of an ATPase proton-pump enzyme in the vesicle membrane. Polyplexes can subsequently be transported to lysosomes, which are organelles with an internal pH of ~4.5 and an abundance of degradative enzymes. Significant amounts of DNA are believed to be degraded during the endosome/lysosome phase, and only those that escape into the cytoplasm can reach the cell nucleus. One way to overcome this barrier is by using ''proton-sponge'' polymers, such as polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimers (Haensler and Szoka 1993; Boussif et al. 1995). These polymers contain many secondary and tertiary amines, and undergo large changes in protonation during endocytic trafficking. This process is accompanied by an increased influx of counterions, increased osmotic pressure, and vesicle rupture, which releases the polyplexes into the cytoplasm (Behr 1997). In an effort to mimic the endosomal escape mechanisms utilized by viruses, membrane-active peptides, such as the HIV TAT sequence and influenza virus hemagglutinin subunit HA-2, have also been incorporated into polycationic polymers (Plank et al. 1998; Beerens et al. 2003). In addition, nuclear localization sequence can also be conjugated to the polymer vector to enhance DNA targeting to the cell nucleus (Cartier and Reszka 2002; Chan and Jans 2002).

1.4.5.3 High-Throughput Approach to Identify Novel Biodegradable Materials for Gene Delivery

To improve the biocompatibility and DNA release, recent research efforts in polymer-based gene delivery have incorporated biodegradable components, such as hydrolyzable ester bonds, into the structural design. Several biodegradable polymeric gene vectors have been synthesized including poly(amino-ester) (Lim et al. 2002), poly-amino acid (Guping et al. 2005), and poly(β-amino esters) (Lynn and Langer 2000; Lynn et al. 2001; Akinc et al. 2003a, b; Anderson et al. 2004; Kim et al. 2005b). Among these, poly(β-amino esters) are particularly attractive due to their facile synthesis, high transfection efficiency, and low toxicity. In contrast to the conventional approach for synthesizing polymers, which often involves multistep purifications and protection/deprotection steps, these polymers can easily be synthesized by the conjugate addition of primary amines or bis-secondary amines to diacrylate compounds (Lynn and Langer 2000). Furthermore, polymers used to be synthesized and screened on an individual basis, a process that is slow, labor intensive, and inefficient. High throughput synthesis and screening of a large polymer library using combinatorial

methods enables faster discovery of potential polymer vectors, better understanding of the structure/property relationship, and rational design of novel polymers for gene delivery. Recently, Anderson et al. reported a semiautomated, solution-phase parallel synthesis and screening of a large library of 2350 structurally diverse, degradable poly(β-amino esters) using commercially available monomers (Anderson et al. 2003). High throughput screening discovered 47 polymers that demonstrate better transfection efficiency than PEI, the best commercially available polymer transfection reagent (Anderson et al. 2003). Further structure/property analyses demonstrated structural similarity in the top-performing polymers, which are all formed from amino alcohols. Such structural convergence offers valuable insight on rational design of polymer vectors for gene delivery (Anderson et al. 2005). The polymer with the highest transfection efficiency, C32, is an aminopentanol-terminated polymer with a molecular weight around 18 kDa relative to polystyrene standards. When injected AQ3
intratumorally in vivo in mice, the C32 polymer demonstrates high biocompatibility and significantly reduces tumor size, a property that is attributable to cell apoptosis (Anderson et al. 2004; Peng et al. 2007). The top-performing poly(β-amino esters) also showed great efficacy and low cytotoxicity in transfecting primary human vascular endothelial cells (HUVEC) in the presence of serum, which have been a great challenge (Green et al. 2006). These results demonstrated great potential of using these polymers for vascular tissue engineering applications.

Amine-terminated poly(β-amino esters) have been shown to be generally more efficient in transfection efficiency. To examine further the effect of the type of amine group at the end chain on gene delivery, a generalized method has been presented to modify poly(β-amino esters) without the need for purification (Zugates et al. 2007). This system enables the rapid synthesis and screening of many structural variations at the polymer chain terminus. End modification of C32 significantly enhances its in vitro transfection efficiency. Most notably, the end-modification strategy has led to the discovery of many effective polymers that work very well in the presence of serum, which overcomes a great obstacle in using nonviral vectors for gene delivery. In vivo, intraperitoneal (IP) gene delivery using end-modified C32 polymers leads to expression levels over one order of magnitude higher than the levels attained by using unmodified C32.

1.4.5.4 Sustained DNA Release from Polymeric Scaffolds for Tissue Engineering

The delivery of DNA plasmids using polymeric scaffolds allows for localized delivery over an extended time period. For this approach, DNA plasmids, either naked or condensed, can be encapsulated into polymeric scaffolds and used with or without cells (Figure 1.3). Polymeric scaffolds have been extensively used for controlled drug release purposes and knowledge learnt from those applications can also be applied to DNA delivery. For example, local delivery of growth factors can promote desired cell differentiation and tissue formation, but is usually associated with problems such as burst release profile and loss of protein activity after encapsulation. In contrast, controlled release of DNA plasmids encoding those growth factors using polymeric scaffolds can overcome the above limitations. Both biocompatible synthetic and natural polymers have been employed for DNA delivery purposes such as poly(lactide-co-glycolide) (PLGA), collagen and hyaluronan (Cohen et al. 2000; Walter et al. 2001; Eliaz and Szoka 2002; Huang et al. 2003, 2005a; Segura et al. 2005). Localized gene delivery using scaffold has been shown to improve bone regeneration (Huang et al. 2005b), angiogenesis (Shea et al. 1999), as well as skin and nerve regeneration (Tyrone et al. 2000; Berry et al. 2001).

1.4.5.5 Targeted Gene Delivery for In Vivo Applications

For in vivo gene delivery, specificity is critical and one approach to achieve specificity is to attach targeting ligands to the surface of the nanoparticles so that only the targeted cell type will be transfected. One major challenge concerns the change in biophysical properties of nanoparticles after coating (Suh et al. 2002; Kursa et al. 2003). Recently, a general method of coating polymer/DNA nanoparticles was developed, and peptide coated nanoparticles were found to have

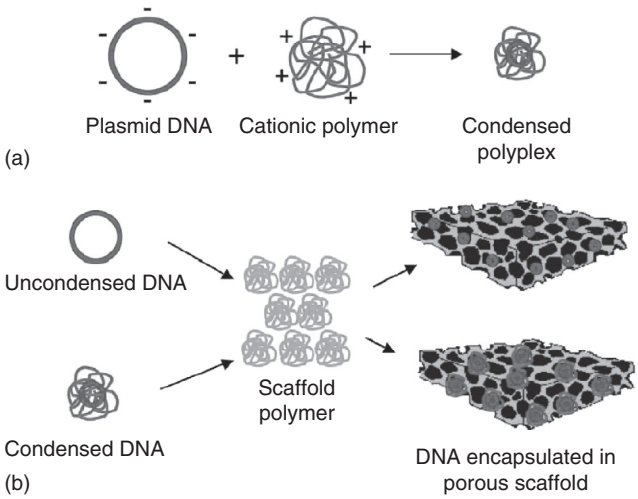

FIGURE 1.3 Schematic of DNA condensation and encapsulation into polymeric depot systems. (a) DNA complexation with cationic polymers leads to the formation of nanometer sized polyplexes. (b) Condensed or uncondensed DNA can be encapsulated into polymeric scaffolds for sustained delivery. (From Sorrie, H. et al., *Adv. Drug Deliv. Rev*., 58, 500, 2006. With permission.)

favorable biophysical characteristics including small particle size, near-neutral zeta potential, and stability in serum (Green et al. 2007). At appropriate formulation conditions including near-neutral charge ratio, the coated nanoparticles enable effective ligand-specific gene delivery to human primary endothelial cells in serum-containing media. As this nanoparticulate drug delivery system has high efficacy, ligand-based specificity, biodegradability, and low cytotoxicity, it potentially may be useful in several clinical applications.

1.4.5.6 Antisense Oligonucleotides and siRNA Delivery

In contrast to DNA delivery, where certain genes are turned on, oligonucleotides containing a sequence complementary to certain gene or mRNA can also be delivered to initiate their degradation and knockdown. Antisense oligonucleotides have been shown to silence the expression of specific genes to achieve a desired cellular function (Tsuboi et al. 2007; Wilton et al. 2007). Recent discovery of the RNA interference (RNAi) pathway has broadened the area of gene delivery and opened up new venues to regulate cell phenotype. The RNAi pathway was first discovered and described in *Caenorhabditis elegans* in 1998 (Fire et al. 1998), and the phenomenon was then found to be present in mammalian cells as well (Elbashir et al. 2001). RNAi is the process of sequence-specific, posttranscriptional gene silencing mediated by small interfering RNA (siRNA), a class of double-stranded RNA molecules that are typically 20–25 nucleotides long. Several groups have demonstrated that RNAi can be elicited in mammalian cells using exogenously derived siRNA (Caplen et al. 2001; Elbashir et al. 2001). Due to its high efficiency in silencing gene expressions and ease of use, siRNA has rapidly drawn significant attention in functional genomics, pathway analysis, and drug target validation experiments.

The safe and efficient delivery of nucleic acids remains the major challenge for nucleic acid-based therapeutics. Several methods can be employed to deliver siRNA to mammalian cells including electroporation, reverse transfection, and chemical transfection. Nonviral delivery using lipids or cationic polymers are safe and promising, but the current collection of available delivery materials is still limited and the transfection efficiency is not yet ideal. In the future, high throughput approaches to the synthesis and screening of large libraries of potential nonviral delivery molecules

hopefully will lead to identification of novel materials that can have broad applications for delivery of siRNA or antisense oligonucleotide therapeutics.

1.5 BIOREACTORS IN TISSUE ENGINEERING

1.5.1 Requirements for Bioreactors in Tissue Engineering

With the rapid advancement in the field of tissue engineering, bioreactors have gained increasing attention as a powerful tool to provide additional exogenous stimuli for the engineered tissue construct to achieve long-term success. As a vessel in which various parameters can be precisely controlled, bioreactors can be designed to provide the desired conditions for the cells to regenerate functional tissue. Examples of such variables include mechanical signal, temperature, media flow rate, oxygen and carbon dioxide concentrations, and other tissue-specific stimuli. To promote tissue regeneration in vitro, several requirements need to be satisfied when designing bioreactors. These are best described as (1) a need to simulate the in vivo environment required for cell proliferation and differentiation, (2) the importance of a uniform cellular distribution in 3D scaffolds, (3) necessary maintenance of adequate nutrient concentrations, (4) appropriate mass transfer of nutrients to developing tissues, and (5) exposure to physical stimuli simulating in vivo conditions in tissues (Freed and Vunjak-Novakovic 2000; Ellis, Jarman-Smith, and Chaudhuri 2005).

To evaluate the performance of a designed bioreactor, the engineered tissues cultivated within the bioreactor should be evaluated both structurally and functionally, and specific assessment depends on the target tissue type. In general, all tissues must have an adequate distribution of cells, ECM, and certain characteristic components of the tissue. For example, a specific structural requirement for engineered cartilage is the amount of glucosaminoglycan (GAG) present (Freed and Vunjak-Novakovic 2000). To determine the functional properties of specific tissues, such as cartilage and cardiac tissue, mechanical and electrophysiological properties, respectively, should also be examined (Bursac et al. 1999; Vunjak-Novakovic et al. 1999). Aside from the general requirements for all bioreactors, specific structural and functional requirements of target tissues must be considered when designing and deciding which bioreactor to use.

1.5.2 Bioreactors for Dynamic Cell Seeding

Bioreactors can be used for the engineered construct in various aspects including enhancing cell seeding, increasing construct size and cellularity, and promoting ECM deposition. Generally, the initial step in engineering a tissue includes seeding the appropriate cells onto scaffolds. These scaffolds are usually biodegradable 3D polymeric constructs and are typically comprised of the synthetic polymers: polyglycolide, polylactide, and polylactide coglycolide (Griffith and Naughton 2002). The conventional approach of seeding cells onto scaffolds is done in a static manner, often resulting in low seeding efficiency and a heterogeneous distribution of the seeded cells. However, uniform tissue growth requires a high yield process in cell seeding and a great degree of uniformity in cell attachment. It has been shown that uniform cell seeding at high densities leads to optimized 3D tissue formation upon cultivation (Martin et al. 2004). In contrast to static seeding, dynamic seeding would induce flow in the vessel, either by convective mixing using spinner flask bioreactors or convective flow using perfused bioreactors. This method causes mass transfer to occur through convection to the scaffold surface and then primarily by diffusion through the scaffold. Due to the incorporation of convective mass transfer, dynamic seeding has been shown to yield higher seeding efficiencies (Martin et al. 2004). Despite these higher efficiencies, convective mass transfer may not lead to highly uniform distributions of cells. To ensure uniformity, flow conditions or rotational speeds, amongst several other parameters, must be optimized based on the desired tissue characteristics and scaffolds type, etc. It has been shown, for example, that direct perfusion in seeding systems increases the level of uniformity of seeding and subsequent tissues, compared to static and

stirred-flask vessels (Wendt et al. 2003). In this study, the direct perfusion took place within the bioreactor used for subsequent cultivation of the tissue as well. This approach eliminates all difficulties associated with transferring seeded scaffolds to the bioreactor for cultivation. Additionally, for cells that are shear sensitive, the time spent in suspension during seeding must be minimized (Freed and Vunjak-Novakovic 2000). The exact mode of cell seeding chosen is heavily dependent on the cell and tissue type. Studies have shown that mixed flasks work well for cartilage, where the kinetic rate and cell deformation rates are minimized (Vunjak-Novakovic et al. 1998). In the case of cardiac tissues, rotating bioreactor vessels have been accompanied with high metabolic activity and thus are the seeding and culture method of choice (Carrier et al. 1999).

1.5.3 Bioreactors to Improve Mass Transfer

One of the greatest challenges in engineering tissue constructs is enabling adequate mass transfer of nutrients to the seeded scaffold. It has been shown that nutrient supplying vasculature is usually within 100–200 μm of a living tissue in vivo (Yang et al. 2001) whereas engineered tissues must be at least on the scale of several millimeters in size to be useful (Martin et al. 2004). Ensuring oxygen and nutrient transport over this length scale is very important in creating healthy multilayer tissues. To address this problem, many different bioreactors have been developed including static/mixed spinner flasks, slow turning lateral vessels (STLV), high aspect ratio rotating vessels (HARV), rotating wall perfused vessels (RWPV), perfused columns, and perfused chambers (for a review, see Freed and Vunjak-Novakovic (2000)). In perfusion vessels, cells are retained within a chamber rather than continually removed and have a continuous supply of nutrients leading to high cell densities within the tissue. All stirred vessels also lead to high cell densities but care must be taken to ensure that mixing is not too vigorous in the case of shear-sensitive cells. Rotating vessels, such as the STLV and RWPV, were initially developed by the National Aeronautics and Space Administration (NASA) to use in microgravity experiments, yielding free-floating scaffolds and highly laminar flow conditions. With highly laminar flows, mass transfer limitations to the scaffold surface are minimized and efficient nutrient transfer can occur (Ellis et al. 2005). The effects of using STLVs and RWPVs have been examined in cartilage and skin cultures, both of which yielded tissues of better overall properties than when other vessels were used (Ellis et al. 2005).

When designing and deciding which bioreactors to implement, it is important to consider the necessary length scales and balances between convective and diffusive mass transfer (Peclet number, Pe), especially in the case of cell seeding. During the cultivation process, it is critical to consider the balance between reaction rates and diffusional mass transfer (Damköhler number, Da), the dominant mode of mass transfer within the scaffold. The relevant reactions rates are those of nutrient consumption. Considering the tissue requirements, experimenting with different Pe and Da can help determine the optimal values of bioreactor parameters, such as residence times and nutrient feed compositions.

1.5.4 Bioreactors to Provide Mechanical Stimuli for Enhanced Tissue Formation

Bioreactors also allow studies of mechanical stimuli on the cells and 3D tissue structures. Mechanical stimuli, such as shear stress due to flow characteristics, have been shown to have a great effect on the development of tissues (Ellis et al. 2005). For example, cardiac muscle in vivoencounters strong pulsatile flows, whereas bone constantly encounters mechanical stress and compression. Exposure to various mechanical stimuli during seeding and cultivation in bioreactors, in attempts to simulate in vivo conditions, has led to significant enhancement in the functions of engineered tissues. For example, exposure of cardiac cells to cyclical mechanical stretching results in a marked improvement in cell proliferation and distribution as well as ECM organization, which ultimately leads to a greater increase in the strength of the tissue (Akhyari et al. 2002). In the case of chondrocytes, both dynamic compression and shearing improve ECM production and the tissue

mechanical properties (Waldman et al. 2003). Interestingly, it was also noted that imparting shear stresses on the developing cartilage tissue leads to a greater improvement on the mechanical properties as compared to the compression of subjected tissues (Waldman et al. 2003). This result supports the notion that the exact effects of different mechanical stimuli on these tissues vary greatly with the type of stimuli applied and the tissue type, and it also emphasizes the need to study in seclusion the effects of individual parameters on the development of tissues.

1.5.5 Future Directions for Using Bioreactors in Tissue Engineering

Bioreactors are powerful tools to provide a more favorable environment for engineering tissue constructs in vitro. Their applications for cultivating engineered tissues for use in biomedical applications represent current and future directions that have gained large appeal. The first product which demonstrated large-scale use of bioreactors was Dermagraft, a skin graft developed by Advanced Tissue Sciences (Martin et al. 2004). In addition to its great potential to create tissue grafts for clinical applications, bioreactors have and will continue to be very useful tools for studying tissue growth in general. Unlike conditions in vivo, bioreactors enable the control and examination of the effects of certain factors on tissue development individually or in various combinations. The knowledge gained from such mechanistic studies will in turn provide guidance for research in tissue manufacture for clinical uses. Another interesting area where bioreactors can be used as a valuable tool is for studying the effect of different parameters on tissue development during pathological processes and various diseases (Griffith and Naughton 2002). Novel ideas such as these along with development of new on-sight control systems and computational fluid dynamic studies will lead to development of more advanced bioreactors and engineered tissues in the future. AQ4

1.6 CONCLUSIONS

The field of tissue engineering has been growing rapidly for the past two decades, driven by the enormous demand and realistic potential of this new discipline. Much progress has been made including the isolation and utilization of adult and ES cells, development of biodegradable scaffolds, delivery of various tissue-inducing factors, and applications of bioreactors to promote tissue formation. Although significant advances have been accomplished, most regenerative therapies are still in the developmental phase. Understanding the fundamental biology associated with normal tissue development is critical for the development of more powerful approaches to achieve controlled cell differentiation and tissue formation. More quantitative approach such as system biology and computational modeling may also shed light on deciphering the complex signaling network. Advances in microfabrication technology might also help design artificial scaffolds and enable mechanistic studies of spatial cues and gradients, etc. Generating vascularized tissues is an essential prerequisite for most tissue types to be clinically useful. In summary, further progress in the field will rely on the advancement and close interactions among multiple disciplines, such as developmental biology, nanotechnology, material sciences, immunology, and computational biology.

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