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CHAPTER II.6.7 BONE TISSUE ENGINEERING

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INTRODUCTION

The use of biocompatible polymeric materials for orthopedic applications, such as bone graft substitutes, has been under investigation since the 1940s (Blaine, 1946; Leveen and Barberio, 1949). By the 1970s the importance of attaining appropriate mechanical properties, an interconnected porosity, and a microstructure that promotes tissue ingrowth was realized (Hench et al., 1971; Weber et al., 1971). Recent research has highlighted the necessity for a subcellular dimension, or nanostructure, in synthetic bone grafts to promote the appropriate organization of bone cells in an effort to generate or regenerate bone tissue (Christenson et al., 2007; Horii et al., 2007; Hu et al., 2008).

This chapter begins with an overview of the biology of bone to provide a framework for what the application of biomaterials strives to recreate. The chapter then proceeds through a discussion of bone tissue engineering, beginning with the natural bone grafts, moving through bone graft substitutes, and finishing with a discussion of bioreactors used in bone tissue engineering.

BONE BIOLOGY

Bones are vascularized and innervated organs that are composed of bone tissue, bone marrow, and a surrounding connective tissue called periosteum. Bones serve a number of functions such as: support for muscles; protection of internal organs; production of blood; calcium homeostasis; acid/base buffering; and transmission of sound (Bilezikian et al., 2002; Rauschecker and Shannon, 2002). Bone tissue is the rigid calcified portion of the bone organ, and is critical for many of the functions.

Types of Bone Tissue

Bone tissue is classified as either cortical bone or trabecular bone. Cortical bone is dense and highly mineralized bone tissue that is found on the peripheral regions of bone. Cortical bone is 80–90% mineralized, and constitutes 80% by mass of the bone tissue in the body (Bilezikian et al., 2002). The high density of cortical bone makes it well-suited for the mechanical and structural properties of bone. The thickness and density of cortical bone is loosely correlated to mechanical loading; however, many other variables are also involved (Pearson and Lieberman, 2004). Trabecular bone is found on the interior of bones adjacent to the marrow cavity. It is approximately 80% porous, and exhibits less than 10% of the compressive strength and less than 5% of the compressive modulus of cortical bone (Bilezikian et al., 2002; Miyakoshi, 2004; Rezwan et al., 2006). However, trabecular bone exhibits higher surface area than cortical bone, and is considered more important for bone functions such as calcium homeostasis and acid/base regulation. Figure II.6.7.1 illustrates the hierarchical organization of bone, moving from the tissue level down to the subcellular level.

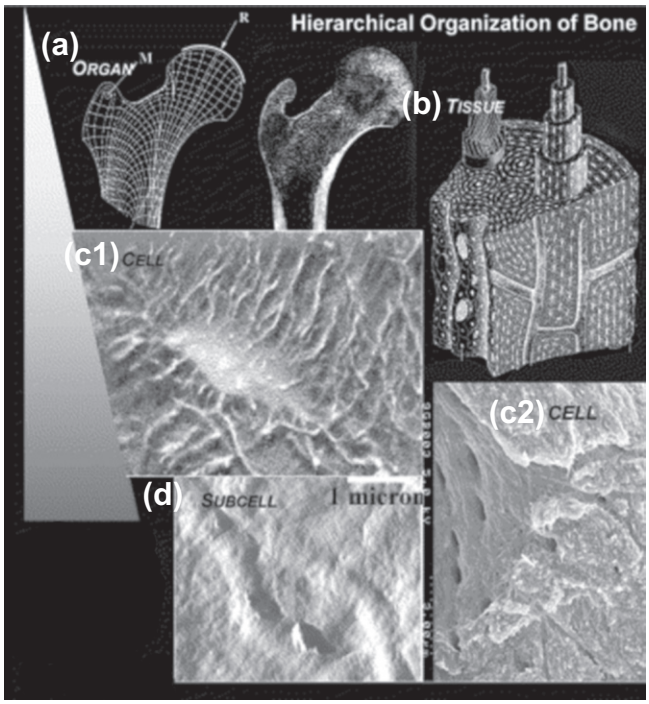

FIGURE II.6.7.1 Hierarchical organization of bone. This figure provides a view of the organization of bone beginning with organ level: (a) and moving to a depiction of the tissue level (b), which illustrates the network of osteocytes organized radially around Haversian canals. An individual osteocyte with the mineralized matrix removed is depicted in (c1) and clearly illustrates multiple processes extending away from the cell body; on the contrary, (c2) depicts the mineralized matrix without the cell, and illustrates on the left-hand side the interior of the lacuna where the osteocyte cell body would reside, and shows canals called canaliculi where the processes extending away from the osteocyte cell body ultimately connect with other osteocyte process. Finally, (d) depicts the interior of canaliculi and shows the presence of striations created by collagen fibers that formed the initial framework for the mineralized matrix (Knothe Tate, 2003).

CELLS INVOLVED

Osteoblasts

There are several distinct cell types involved in the formation and remodeling of bone tissue. These cells are osteoblasts, bone lining cells, osteocytes, and osteoclasts. Osteoblasts are the workhorses of bone formation. Osteoblasts are fully-differentiated cells derived from preosteoblasts or osteoprogenitor cells, which are progenitor cells derived from mesenchymal stem cells found in the bone marrow or the periosteum (Franz-Odendaal et al., 2006; Marie, 2008). The transition from a preosteoblast to an osteoblast occurs when the preosteoblast is stimulated to differentiate via soluble factors, such as bone morphogenetic proteins and wingless-int proteins (Franz-Odendaal et al., 2006; Zaidi, 2007). Once stimulated to differentiate, the preosteoblasts cease proliferation and begin to secrete proteins indicative of an osteoblast phenotype. The new osteoblasts are found at the surface of developing bone tissue, and exhibit a cuboidal morphology. They actively secrete a nonmineralized osteoid matrix at the location of newly forming bone. This osteoid matrix is the organic portion of bone extracellular matrix, and is composed primarily of collagen type I, which makes up approximately 90% of the matrix (Toole and Linsenmayer, 1977; Bilezikian et al., 2002). The remaining portion of the osteoid matrix is composed of proteoglycans and noncollagenous proteins, such as osteopontin, osteocalcin, and osteonectin.

The active production of the osteoid matrix, as well as the presence of the membrane protein alkaline phosphatase, distinguishes the osteoblast phenotype. The osteoid matrix around the osteoblast begins to calcify, and approximately 20% of the buried osteoblasts transition to osteocytes (Franz-Odendaal et al., 2006). The osteoblasts that do not transition to osteocytes undergo apoptosis.

Bone Lining Cells

Bone lining cells, much as their name suggests, are found lining the surface of bone. Unlike osteoblasts on the bone surface, bone lining cells have a long, slender, and flat morphology. Bone lining cells were initially considered to be preosteoblasts (Bilezikian et al., 2002); however, this is no longer thought to be the case. Instead, the current opinion is that osteoblasts that do not undergo apoptosis or differentiate to osteocytes become bone lining cells (Karsdal et al., 2002; Khosla et al., 2008; Matsuo and Irie, 2008). Two of the key phenotypic differences between the bone lining cells and osteoblasts are that bone lining cells express intercellular adhesion molecule 1, and they do not express osteocalcin (Everts et al., 2002). Recent research has shown that bone lining cells

anchor hematopoietic stem cells, and provide these stem cells with appropriate signals to keep them in an undifferentiated state (Kollet et al., 2006). The bone lining cells then play a crucial role in the transitions involved with bone remodeling by communicating through gap junctions with osteocytes deep in the bone matrix, promoting differentiation of hematopoietic stem cells into osteoclasts (Kollet et al., 2006; Matsuo and Irie, 2008).

Additionally, the bone lining cells are responsible for preparing the surface of the bone by removing nonmineralized collagen fibrils through the use of matrix metalloproteinases. After remodeling, the bone lining cells deposit a smooth layer of collagen over the bone surface (Everts et al., 2002; Karsdal et al., 2002; Khosla et al., 2008; Matsuo and Irie, 2008).

Osteocytes

Osteocytes are terminally differentiated cells derived from mature osteoblasts that have become encased within a calcified matrix. In the transformation from an osteoblast to an osteocyte, the expression of many of the proteins that constitute an osteoblast phenotype, such as type I collagen, alkaline phosphatase, osteocalcin, and bone sialoprotein, are no longer produced (Franz-Odendaal et al., 2006; Zaidi, 2007). Additionally, osteocytes create a network among themselves by extending many long processes to adjacent osteocytes. This network, the lacunar–canalicular network, is used for nutrient and waste transfer, as well as for communication between the osteocytes via gap junctions (Franz-Odendaal et al., 2006).

The osteocyte cell body resides in the lacuna, and the osteocyte's processes extend out through the canaliculi to adjacent osteocytes and Haversian canals. The Haversian canals provide vasculature to suuply and remove nutrients. The concentric arrangement of the lacunar–canalicular network of osteocytes around a Haversian canal is referred to as an osteon (Bilezikian et al., 2002). The organization of the osteocytes within bone is illustrated in Figure II.6.7.1. Additionally, osteocytes have been implicated as the primary mechanosensors in bone. The mechanotransduction that occurs in osteocytes is believed to be initiated by fluid flux within the canaliculi, created by pressure gradients between lacunae when the bone is loaded. The fluid motion triggers depolarization of the osteocyte process, and is propagated to other osteocytes via gap junctions. The mechanotransduction in osteocytes contributes to the recruitment of osteoblasts or osteoclasts, depending on the loading condition (Wang et al., 2000; Goulet et al., 2008).

Osteoclasts

Osteoclasts, unlike the cells discussed thus far, are multinucleated cells derived from hematopoietic stem cells, as opposed to mesenchymal stem cells. It has been established that hematopoietic stem cells anchored to bone lining cells are induced to differentiate into osteoclasts in response to osteocyte–bone lining cell signaling (Kollet et al., 2006; Matsuo and Irie, 2008). The role of osteoclasts in bone metabolism is the resorption of bone. Osteoclasts exhibit a polarized plasma membrane. Osteoclasts involved in resorbing bone exhibit two distinct plasma membrane regions on the basal surface of the osteoclast; a ruffled portion of the plasma membrane which is where the resorption of the bone occurs, and a sealing region that binds the ruffled border to the bone extracellular matrix (Bilezikian et al., 2002; Vaananen and Laitala-Leinonen, 2008). The combination of the ruffled and sealing regions of the plasma membrane forms the resorption lacuna.

Initially, the osteoclast dissolves the mineralized portion of the bone matrix by secreting hydrochloric acid. After the mineral content is removed, the protein portion of the matrix is degraded by proteolytic enzymes (Kollet et al., 2006; Coxon and Taylor, 2008; Vaananen and Laitala-Leinonen, 2008). The resulting matrix fragments, and potentially the ions created from matrix dissolution, are transported through the osteoclast in vesicles that are emptied into the extracellular space on the basolateral side of the osteoclast. Much like the basal plasma membrane, the basolateral plasma membrane of the osteoclast is polarized and exhibits a functional secretory domain in the middle of the basolateral membrane. The functional secretory domain of the basolateral membrane is rich in microtubules, and is the site where the vesicles are emptied after the transcytotic transportation of the bone matrix degradation products (Kollet et al., 2006; Coxon and Taylor, 2008; Vaananen and Laitala-Leinonen, 2008).

BONE TISSUE DEVELOPMENT

Calcified bone tissue is formed by two distinct modes of ossification or calcification. These methods of ossification are classified as either intramembranous ossification or endochondral ossification (Bilezikian et al., 2002; Franz-Odendaal et al., 2006). The method of ossification depends on the type of bone being formed. Intramembranous ossification is involved in the formation of flat and irregularly shaped bones, such as the cranial bones. Endochondral ossification is involved in the formation of long bones (bones that are longer than they are wide), such as the femur, humerus, and metacarpal (Bilezikian et al., 2002).

Intramembranous Ossification

Intramembranous ossification begins without a preexisting cartilage model. Instead, mesenchymal stem cells form clusters. The mesenchymal stem cells then differentiate into osteoblasts, and the newly formed osteoblasts start to secrete an osteoid matrix. The osteoid matrix

is calcified to form bone spicules. Osteoblasts trapped within the bone spicules either differentiate to osteocytes or undergo apoptosis. The bone spicules radiate outward from where the mesenchymal cluster originally formed. Eventually, spicules initiated by separate mesenchymal stem cell clusters join together to create a layer of calcified bone. Mesenchymal stem cells apical to the calcifying tissue differentiate to form the periosteum, whereas those basal to the calcifying tissue differentiate to osteoblasts which form subsequent layers of calcified tissue. The resulting bone tissue is classified as woven bone. Woven bone is formed quickly, and is characterized by randomly oriented collagen fibrils; however, it is not as mechanically viable as lamellar bone. Woven bone will be remodeled over time through resorption and deposition by osteoclasts and osteoblasts to form lamellar bone (Bilezikian et al., 2002; Franz-Odendaal et al., 2006; Shapiro, 2008).

Endochondral Ossification

Endochondral ossification occurs in several steps. Initially, endochondral ossification begins with a pre-existing cartilage template. The cartilage template begins to be calcified. As the cartilage template calcifies, the chondrocytes in the cartilage become hypertrophic and undergo apoptosis (Bilezikian et al., 2002; Tuan, 2004; Franz-Odendaal et al., 2006). Then, mesenchymal stem cells in the membrane surrounding the calcifying cartilage, periosteum, differentiate to osteoblasts. These osteoblasts lay down an osteoid matrix around the exterior of the cartilage template. At the same time, a bud of cells originating from the periosteum invades the interior of the partially calcified cartilage template. This periosteal bud leads to vascularization and innervation of the developing bone. The periosteal bud also supplies mesenchymal and hematopoietic stem cells to the center of the cartilage template. The mesenchymal stem cells differentiate to osteoblasts, and the hematopoietic stem cells differentiate to osteoclasts. These osteoblasts and osteoclasts remodel the partially calcified cartilage into woven bone, which is ultimately remodeled to become lamellar bone. Lamellar bone contains collagen fibrils that are arranged in parallel areas, and exhibits greater strength compared to woven bone (Shapiro, 2008). As the bone tissue created from cells originating from the periosteal bud increases, it radiates outward and eventually joins the bone tissue created by the osteoblasts on the surface of the cartilage template (Bilezikian et al., 2002; Franz-Odendaal et al., 2006; Shapiro, 2008).

Bone Tissue Engineering

Tissue engineering is the application of biological, chemical, and engineering principles toward the repair, restoration or regeneration of living tissue using biomaterials, cells, and factors alone or in combination (Laurencin et al., 1999). Strategies for tissue engineering often focus on one of the three elements; using biomaterials, cells or factors. For example, a common tissue engineering strategy involves fabricating biomaterials into porous scaffolds to facilitate cell growth and the eventual repair, restoration or regeneration of the tissue (Langer and Vacanti, 1993). These biomaterial scaffolds can be used without any further modification *in vivo*. The next iteration in the application of biomaterial-focused tissue engineering strategies involves culturing the biomaterial scaffold seeded with cells *in vitro*. The ultimate strategy for the *in vitro* use of a biomaterial scaffold involves seeding the scaffold and culturing *in vitro* to develop a replacement tissue that, on implantation, functions exactly as did the original host tissue (Langer, 2000).

Applying this tissue engineering strategy, a paradigm for a successful bone graft emerges. This paradigm is that the graft or construct should be osteoconductive, osteoinductive, osteogenic, resorbable or degradable, and possess mechanical properties near to that of the implant site. Osteoconduction refers to the ability of a scaffold or implant to promote attachment of osteoblastic cells on the surface and throughout the interior of the scaffold or implant. In an *in vitro* setting, osteoconduction is seen as an ability to promote the attachment, migration, and proliferation of osteoblasts (Kneser et al., 2002). Osteoinduction refers to the ability of a scaffold or implant to promote the differentiation of mesenchymal stem cells down an osteoblastic lineage, ultimately leading to the formation of mineralized tissue. Osteoinduction can also be viewed as an ability to promote phenotype progression of an osteoblast from an early osteoblast to a mature osteoblast, followed by differentiation to an osteocyte (Kneser et al., 2002).

Osteogenicity refers to the ability of a scaffold or implant to promote *de novo* bone formation, which would occur in the absence of host cell invasion. For a scaffold to be osteogenic, cells would need to be seeded on the scaffold prior to implantation (Kneser et al., 2002). The necessity of a bone tissue engineering construct to be degradable arises from the fact that bone is constantly remodeling. A non-resorbable or non-degradable implant would impede the natural remodeling process of bone, and extend the time it takes for the organ to return to natural function (Hutmacher, 2000). Finally, the graft, scaffold or implant should have mechanical properties that match that of the native bone tissue. The range for the mechanical properties depends on whether the bone tissue is cortical or trabecular. For trabecular bone the compressive strength varies from 4–12 Mpa, and the compressive modulus varies from 100–500 Mpa (Rezwan et al., 2006). Cortical bone exhibits a compressive strength from 130–180 Mpa, and a compressive modulus from 12–18 Gpa (Rezwan et al., 2006).

A problem with mechanical properties that exceed these ranges is stress shielding. Stress shielding results

when the load on the bone is redistributed, with the scaffold or implant being the loadbearing region, and the surrounding bone being unloaded. The result of stress shielding is osteopenia of the bone surrounding the implant (Pitto et al., 2007). To solve the bone tissue engineering paradigm, there are presently a range of bone grafts and bone graft substitutes that fulfill all or some of the bone tissue engineering paradigm.

BONE GRAFTS

Autograft

Bone grafts are pieces of bone that are harvested from the patient, a donor or a cadaver, and placed at the desired site of bone repair, regeneration or restoration as needed. The long standing, and considered "gold standard," bone graft solution to the bone tissue engineering paradigm is an autograft (Greenwald et al., 2001; Kneser et al., 2002; Laurencin et al., 2006). Autografts are sections or fragments of bone removed from one site on the patient, typically the iliac crest, and implanted to another site based on need. Figure II.6.7.2 depicts an example of where two autografts were harvested from a patient, and shows the harvest site beginning to heal and reform bone after five months, with the help of a metal plate to serve as a template for the new bone growth.

Autografts harvested from the iliac crest are mostly trabecular bone with a thin shell of cortical bone. Since autografts originate within the patient, they are readily incorporated at the implant site and rarely elicit any immune responses, which allow autografts to have excellent wound healing properties. Autografts fulfill all four elements of the bone tissue engineering paradigm, primarily because they consist of native bone tissue moved from one region of a patient's body to another region. However, autografts have a few drawbacks; there is often donor site morbidity indicated by necrosis and infection at the location of autograft harvest that may cause the patient more pain from the harvest site than the implant site (Greenwald et al., 2001; Laurencin et al., 2006). Additionally, autografts are limited in availability to the amount of tissue that can be harvested from the donor site (Greenwald et al., 2001; Laurencin et al., 2006). The problem of autograft bone tissue availability increases in cases where the need is the highest; those that involve osteoporotic, pediatric or patients afflicted with bone cancer (Meister et al., 1990). It is these shortcomings plaguing the autograft that has increased the effort to find other bone graft substitutes.

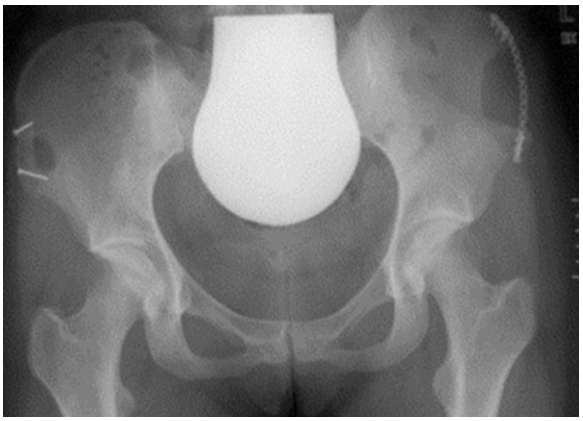

FIGURE II.6.7.2 X-ray of an individual that required an autograft harvest from both iliac crests. The right hip (left side of image) was harvested five months prior to the left hip. Both were reconstructed with a metal plate fixed with screws (most visible on the left hip). Notice how even five months post-harvest there persists a significant amount of bone that has not regrown (Huemer et al., 2004).

Allograft

The next bone graft solution to the bone tissue engineering paradigm is the allograft. Allografts are bone fragments obtained from donors or cadavers that are typically first frozen, irradiated and/or lyophilized. These steps are implemented to reduce the likelihood of disease transmission. Allografts are readily available in an array of shapes and sizes. Since allografts originate from a donor or cadaver there is no additional surgical site on the patient, which removes complications such as donor site morbidity (Greenwald et al., 2001; Kneser et al., 2002; Laurencin et al., 2006). However, the sterilization procedures performed on allograft bone tissue are not without consequence. In comparison to autografts, allografts are less osteoconductive, less osteoinductive, and are not osteogenic (Greenwald et al., 2001; Laurencin et al., 2006). Additionally, allografts that have been lyophilized exhibit much less mechanical integrity compared to autografts (Laurencin et al., 2006). Due to allografts being non-native tissue, they have been shown to occasionally produce an immune response, which requires allograft recipients to be placed on immunosuppressant drugs to prevent rejection of the allograft tissue (Paskert et al., 1987). The complications that arise from autografts and allografts have fueled the search for bone graft substitutes.

BONE GRAFT SUBSTITUTES

Bone graft substitutes can offer solutions to the bone tissue engineering paradigm, and are based on the tissue engineering concepts that arise from the definition of tissue engineering. These tissue engineering principles lead to bone graft substitutes that can be classified as those based on biomaterials, cells, factors or any combination of the three.

Allograft-Based Substitutes

Biomaterial-based bone graft substitutes can be further subdivided into allografts, natural polymers, synthetic polymers, and ceramics. Allograft-based bone graft

substitutes use allograft bone tissue that has been thoroughly sterilized, decellularized, and demineralized. The methods by which manufacturers sterilize, decelluarize, and demineralize the bone graft substitutes are carefully controlled to create a product that retains the collagen, non-collagenous proteins, and some of the growth factors present in the original bone tissue (Gazdag et al., 1995). The result is demineralized bone matrix (DBM), which has been used in a variety of commercially available bone graft substitutes either as is or mixed with glycerol, hyaluronic acid or calcium phosphates to improve the handling and performance characteristics of the product (Martin et al., 1999; Schwartz et al., 2007). Osteotech, Inc.® has a line of DBM products under the trade name Grafton®. The Grafton® line of products contain materials that are simple DBM fragments, such as Grafton® DBM Crunch, as well as intact pieces of DBM that has been precut to a desired shape, such as Grafton® DBM Matrix PLF, DBM Matrix Plugs, and DBM Matrix Strips. Osteotech, Inc.® also has a number of commercially available DBM products that have been mixed with glycerol and other proprietary agents to create an injectable gel, Grafton® DBM Gel, moldable putty, Grafton® DBM Putty, and flexible strips, Grafton® DBM Flex and A-Flex™. Figure II.6.7.3 depicts a representative image of DBM formed into a simple rectangular solid, and also shows the presence of cells growing on the DBM. DBM-based bone graft substitutes exhibit a variable degree of osteoinduction based on the processing parameters (Kneser et al., 2006; Laurencin et al., 2006).

There is evidence that supports improved osteoinduction with demineralization due to exposure of soluble factors that would be occluded in mineralized bone (Gazdag et al., 1995; Peterson et al., 2004); however, DBM-based bone graft substitutes have limited osteoconductivity, no osteogenicity, and mechanical properties that are less than the desirable range (Gazdag et al., 1995; Peterson et al., 2004).

Natural Polymer-Based Substitutes

Natural polymers are gaining interest among the research community for bone tissue engineering applications, and there are also commercial bone graft substitutes derived from natural polymers available. One example of a commercially viable natural polymer product is Healos® from DePuy Orthopaedics, Inc. Healos® is a collagen microfiber matrix that has been coated with hydroxyapatite (Neen et al., 2006). The recommended use of Healos® involves coating it with bone marrow aspirate prior to implantation. By supplying the matrix with bone marrow aspirate there are progenitor cells present on the Healos® matrix, which makes it osteogenic in addition to being osteoconductive (Neen et al., 2006). There is no evidence that Healos® is osteoinductive, and it has poor mechanical properties (Neen et al., 2006). In addition to collagen, fibrin and chitosan are two other natural polymers that are being investigated for bone tissue engineering applications (Khan et al., 2008; Osathanon et al., 2008). The structures created by these polymers are typically fibers or foams (Wahl and Czernuszka, 2006; Osathanon et al., 2008; Song et al., 2008). These structures provide excellent osteoconduction; however, osteoinduction, osteogenicity, and mechanical properties are less than those provided by autograft tissue (Wahl and Czernuszka, 2006; Khan et al., 2008).

Synthetic Polymer-Based Substitutes

Synthetic polymer solutions for bone tissue engineering applications are varied and abundant in current research. The use of synthetic polymers provides control over the surface chemistry, degradation kinetics, and geometry in much finer detail than can be accomplished with natural polymers. Clinically, only a handful of synthetic polymers are US Food and Drug Administration (FDA) approved for use in non-life-threatening applications, such as bone graft substitutes. These are the following poly(α-hydroxy esters): poly(lactide); poly(glycolide); poly(lactide-*co*-glycolide); and poly(caprolactone). Figure II.6.7.4 illustrates a lattice structure made of poly(caprolactone) rods that has been implanted into a pig, and imaged three months post-implantation with μCT (micro-computed tomography) to demonstrate where new bone formation is occurring, which is not adjacent to existing bone tissue (Jones et al., 2004).

The mechanical properties of the construct are not documented, and the cracking and plastic deformation

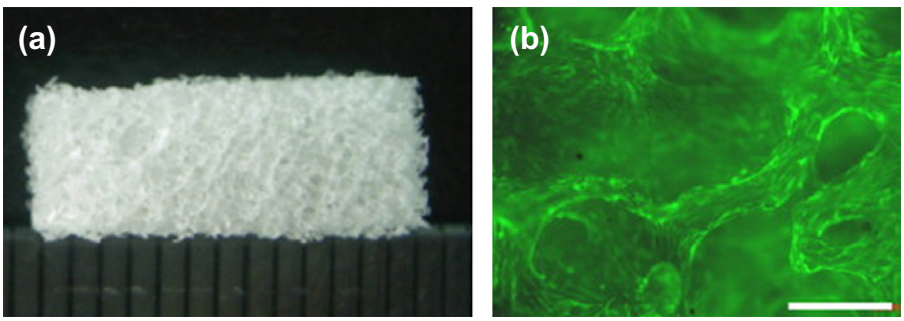

FIGURE II.6.7.3 Demineralized bone cut into a rectangular solid (a); and a high magnification fluorescent image showing the presence of viable cells on the demineralized bone matrix (b) (Ma et al., 2007).

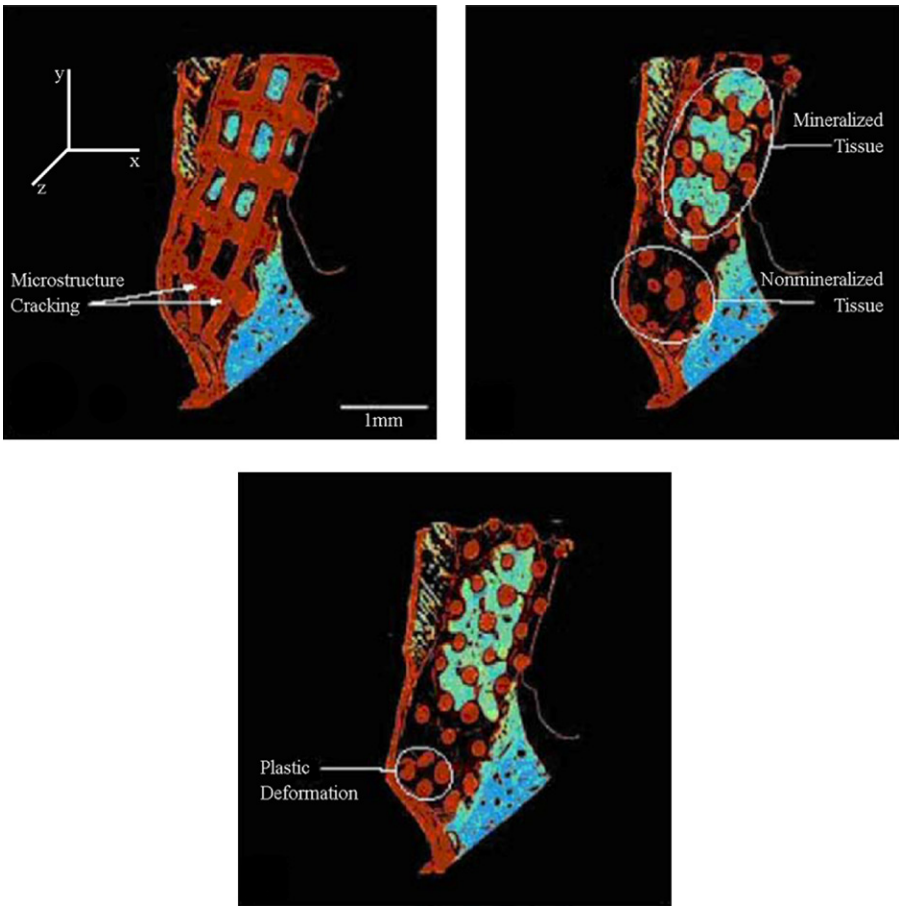

FIGURE II.6.7.4 Representation obtained of a synthetic bone graft after three months *in vivo* using μCT. The synthetic bone graft is a simple poly(caprolactone) (PCL) scaffold (red), with nonmineralized tissue (black), and mineralized tissue (blue green). The native bone is visible in the lower right corner of each image. The three images are different sections separated by 280 μm. The poly(caprolactone) scaffold was made by a rapid prototyping process, and consists of a simple three-dimensional lattice of connected rods. Also visible in the series of images are two defects caused by either degradation of the PCL, which the authors note that after three months has caused the diameters of the rods to shrink from 500 μm to 300 μm, or due to excessive loading on the scaffold compromising the material prior to degradation (Jones et al., 2004).

visible after three months indicates the material may not be mechanically viable. The first porous bone tissue engineering constructs fabricated from poly(α-hydroxy esters) were foams created by various porogen leaching techniques (Thomson et al., 1995). The porogen in these foams is typically a substance that readily dissolves in water, such as gelatin, salt or sugar (Thomson et al., 1995; Mooney et al., 1996). These scaffolds are created by dissolution of the poly(α-hydroxy esters) in an organic solvent. The dissolved polymer is then mixed with the porogen and cast. After the solvent has evaporated, the construct is immersed in water to remove the porogen (Thomson et al., 1995; Mooney et al., 1996; Rezwan et al., 2006). This technique can produce scaffolds that are highly porous.

The primary issue with porogen leaching is that the increase in porosity is directly correlated with a decrease in mechanical integrity. Additionally, at low porosities, which are more robust mechanically, the polymer surrounds each individual porogen, resulting in poor interconnectivity among the pores (Mooney et al., 1996; Rezwan et al., 2006). The sintered microsphere scaffold fabricated from poly(α-hydroxy esters) succeeded the scaffolds created with porogen leaching, and also resolved several of the problems with scaffolds created by porogen leaching. The maximum porosity achievable by the sintered microsphere scaffold is only around 45%, based on random packing of spheres; however, the interconnectivity of the porosity is 100%, which was a dramatic improvement over the scaffolds fabricated with porogen leaching (Devin et al., 1996; Mooney et al., 1996; Borden et al., 2002a,b; Rezwan et al., 2006). More recently, microscale scaffolds have been fabricated with a very specific architecture from poly(α-hydroxy esters) by using solid free-form fabrication techniques (Sachlos and Czernuszka, 2003; Ge et al., 2008). Solid free-form fabrication uses computer aided design to build structures layer by layer through techniques such as stereolithography, selective laser sintering, and three-dimensional printing (Hutmacher et al., 2004).

All of the scaffolds created with the above microscale technologies provide similar performance. The mechanical integrity of the scaffolds can be fabricated into a range that suits bone tissue applications, the scaffolds degrade

in a controllable manner based on the selection of the poly(α-hydroxy esters), and they are all osteoconductive. However, for the preparation of microscale structures, each of the techniques is limited to a resolution an order of magnitude larger than a cell, and the resulting materials are not osteoinductive nor are they osteogenic without supplementing the structure with growth factors or osteoblastic cells (Rezwan et al., 2006). The research community has moved to examine scaffolds composed of poly(α-hydroxy esters) that exhibit a subcellular dimension. These scaffolds are almost invariably fiber based, and are made either with electrospinning, phase separation for crystalline poly(L-lactide), and precipitation in a non-solvent of a continuous fiber stream from a polymer solution (Yoshimoto et al., 2003; Smith and Ma, 2004; Tuzlakoglu et al., 2005). The diameter of these fiber scaffolds can vary from 50 nanometers to several micrometers (Yoshimoto et al., 2003; Smith and Ma, 2004). The subcellular dimension provided by nano- and microfibers has illustrated improved osteoconductivity as compared to the supercellular dimension of the microstructures covered previously. Additionally, there is some evidence that nanofibers may promote osteoinduction; however, the gains that nano- and microfibers make with osteoconduction and osteoinduction are compromised by the mechanical properties under compression of the nano- and microfiber structures (Patel et al., 2007; Hu et al., 2008).

Nano- and microfiber scaffolds exhibit near negligible compressive strength when compared to the microscale structures created by sintered microspheres, solid free-form fabrication, and porogen leaching (Kjelstrup-Hansen et al., 2006; Rezwan et al., 2006). The next degree of control being explored for bone tissue engineering constructs involves moving away from the FDA approved poly(α-hydroxy esters) and on to other biodegradable polymers that provide better degradation by-products or improve the osteoconductivity of polymers through the incorporation of integrin binding peptides, such as RGD (Arginine-Glycine-Aspartic Acid peptide) or, within the synthetic polymer structure. A noted drawback of poly(α-hydroxy esters) is that they degrade into carboxylic acids, which can be immunogenic (Bostman and Pihlajamaki, 1998; Mosier-Laclair et al., 2001).

An additional drawback to the usage of poly(α-hydroxy esters) is that they undergo bulk degradation, which results in sudden failure of the scaffold. Similar to the poly(α-hydroxy esters), poly(propylene fumarate) is a polyester that can be used to fabricate similar scaffolds to the poly(α-hydroxy esters), and also shares the same problems with bulk degradation and acidic degradation products (Lee et al., 2006; Rezwan et al., 2006). Poly[(amino acid ester)phosphazenes] address the negative degradation products found with poly(α-hydroxy esters), and have recently been investigated for bone tissue engineering applications. Poly[(amino acid ester)phosphazenes] degrade into amino acids, which are much easier for the body to metabolize, and a buffer solution consisting of ammonia and phosphate, which prevents any change in the pH potentially brought about by the increase in the concentration of amino acids. Poly[(amino acid ester) phosphazenes] have also exhibited a more favorable surface erosion degradation mechanism (Allcock et al., 1994; Ibim et al., 1997). Additionally, poly[(amino acid ester)phosphazenes] are suitable for many of the fabrication procedures discussed previously, such as sintered microsphere scaffolds, porogen leaching scaffolds, and electrospun nanofibers (Laurencin et al., 1996; Kumbar et al., 2006a; Brown, et al., 2008b).

The scaffolds fabricated from poly[(amino acid ester) phosphazenes] have illustrated osteoconduction, but studies investigating the osteoinductivity and mechanical properties have yet to be reported (Conconi et al., 2006; Kumbar et al., 2006a). Figure II.6.7.5 provides a representative image of sintered microsphere scaffolds, porogen leaching scaffolds, and electrospun nanofiber scaffolds created with degradable polyphosphazenes. Polyanhydrides are surface eroding polymers similar to polyphosphazenes; however, little research has been performed investigating porous polyanhydride scaffolds for bone tissue engineering (Muggli et al., 1999; Rezwan et al., 2006).

Ceramic-Based Substitutes

Ceramic-based biomaterials are prevalent and widespread as bone graft substitutes. These ceramic biomaterial bone graft substitutes are made primarily from calcium phosphates, calcium sulfate, and Bioglass®, which is a glass formulation containing lower amounts of silicon dioxide, and higher amounts of sodium oxide and calcium oxide compared to conventional glass. This specific glass formulation is bioactive, and undergoes dissolution in the body. Calcium phosphate bone graft substitutes are usually either tricalcium phosphate or hydroxyapatite, which is the primary mineral in bone. One example of a commercially available hydroxyapatite-based bone graft substitute is Pro-Osteon™ from Biomet® Osteobiologics. The Pro-Osteon™ bone graft substitute begins as natural coral, which is primarily calcium carbonate. The coral is then treated with a hydrothermal process in the presence of ammonium phosphate, to convert the calcium carbonate structure to hydroxyapatite (Ben-Nissan, 2003). The resulting structure exhibits a pore structure similar to trabecular bone. Figure II.6.7.6 is a depiction of the coralline hydroxyapatite resulting from the above hydrothermal process. Osteohealth® produces a product through the removal of the organic portion of bovine bone called Bio-Oss®. Bio-Oss® retains the structure of the hydroxyapatite formed in the bovine trabecular bone from which it is obtained.

A similar product to Bio-Oss® is Orthograf® which is manufactured by Dentsply, and which is also derived from bovine bone that has been treated to remove all

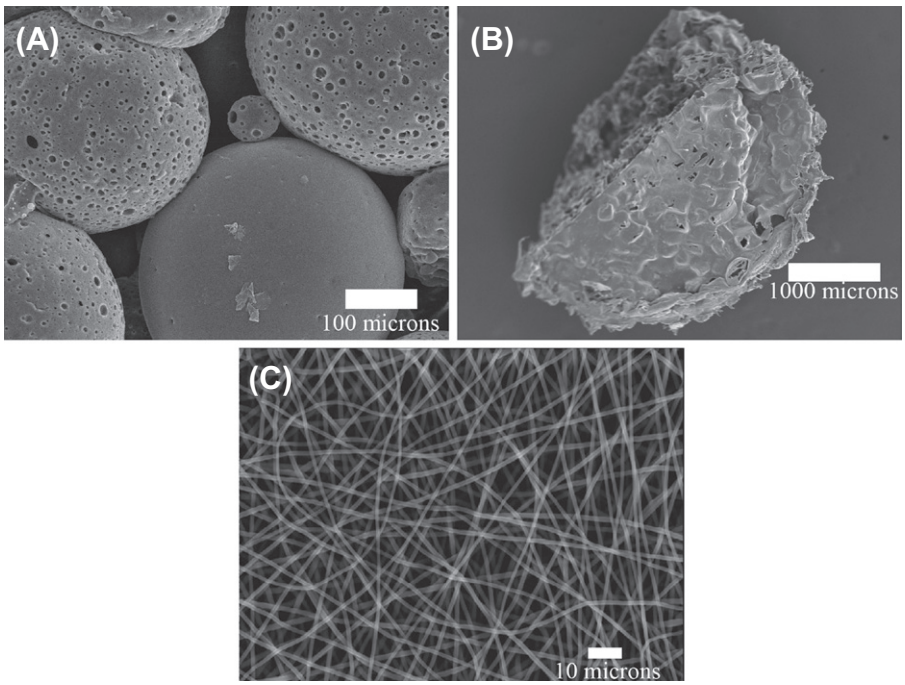

FIGURE II.6.7.5 Scanning electron microscope (SEM) images of three scaffold architectures fabricated from poly[(amino acid ester)phosphazenes]. (A) Sintered microsphere scaffold composed of poly[bis(ethyl alaninato)phosphazene]; (B) Scaffold made from leaching salt from poly[(ethyl alaninato-co-methylphenoxy)phosphazene]; (C) Nanofibers created by electrospinning poly[bis(methylphenoxy)phosphazene] (Nair et al., 2004). Images (A) and (B) are previously unpublished.

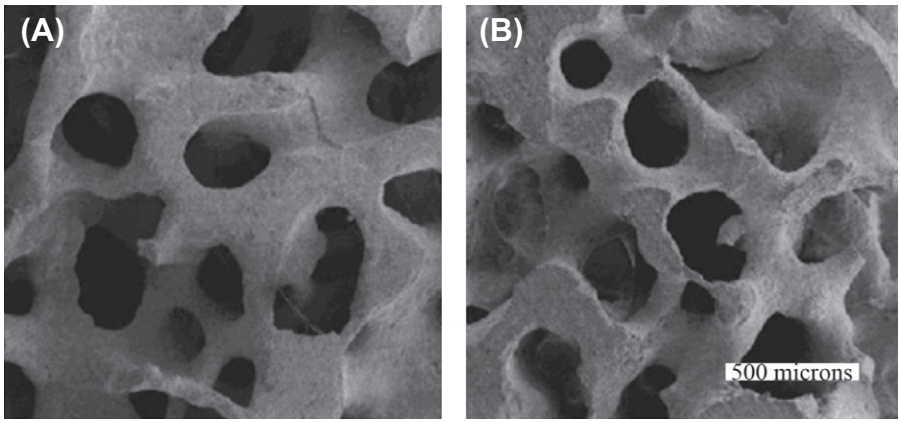

FIGURE II.6.7.6 SEM image of coral prior to conversion to hydroxyapatite (A); and after hydrothermal conversion to coralline hydroxyapatite (B) (Ben-Nissan, 2003).

organic components. All of the hydroxyapatite-based ceramic bone graft substitutes are slowly resorbed by the body as the bone surrounding the implant remodels. Hydroxyapatite-based ceramic bone graft substitutes are also osteoconductive; however, the porous hydroxyapatite bone graft substitutes typically have compressive strength and moduli that fall below the range of trabecular bone (Rezwan et al., 2006). Whether hydroxyapatite-based biomaterials are osteoinductive is a controversial topic. Osteoinduction appears to occur with hydroxyapatite and other calcium phosphate-based biomaterials.

Initially, the osteoinductivity of hydroxyapatite and other calcium phosphates was believed to be a property of the ceramic itself (Damien and Parsons, 1991). However, recent evidence suggests that it is a property of the microstructure of the material, which would be similar to results observed with polymer scaffolds (Yuan et al., 2002; Giannoudis et al., 2005; Li et al., 2008). Two calcium phosphate structures, one composed of hydroxyapatite and the other of a blend with 85% hydroxyapatite and 15% tricalcium phosphate, were fabricated into similar macrostructures; however, the hydroxyapatite/tricalcium phosphate structure also had a subcellular microstructure, and was found to be more osteoinductive than the hydroxyapatite with no subcellular microstructure (Yuan et al., 2002). Additionally, an examination of several distinct calcium phosphates with different

chemical and crystal structures fabricated into similar macrostructures illustrated no significant difference in the expression of phenotype markers (Wang et al., 2004). Furthermore, examination of chemically identical types of hydroxyapatite formed into different geometries produced pronounced differences in the expression of phenotype markers, with the hydroxyapatite structure exhibiting a subcellular microstructure being more osteoinductive than the hydroxyapatite with no subcellular microstructure (Li et al., 2008).

An additional type of calcium salt is calcium sulfate. Wright Medical Technology produces a dense bone graft substitute from calcium sulfate called Osteoset®. Osteoset® has been shown to be resorbed nine months post-implantation, and has also been illustrated to be osteoconductive; however, conflicting evidence is found over whether Osteoset® and other calcium sulfate bone graft substitutes improve healing in bone (Clokie et al., 2002; Petruskevicius et al., 2002). Additionally, calcium sulfate bone graft substitutes have been found to be potentially immunogenic (Robinson et al., 1999; Lee et al., 2002).

Of the ceramic-based bone graft substitutes, those fabricated from Bioglass® are the most intriguing. Bioglass® was developed in the late 1960s and has been shown to fit the bone tissue engineering paradigm better than the other ceramics covered (Hench et al., 1971). Porous scaffolds have been fabricated from Bioglass® in a number of ways. One way, that produces a 90–95% porous structure, involves a foam replacement technique where a polyurethane foam is coated with a Bioglass® slurry. The coated foam is then heated, which burns the polyurethane out and processed through a heat-treatment schedule to fuse the Bioglass® particles and obtain the desired density and crystallinity (Vargas et al., 2009). Another technique sinters Bioglass® fibers to form porous mats of Bioglass® fibers. These Bioglass® fiber mats have been shown to be osteoconductive, and promote phenotype progression of preosteoblast cells, which suggests they may be osteoinductive. The compressive strength and modulus of these Bioglass® fiber rafts are in the range of trabecular bone, and they exhibit adequate porosity of around 44% (Brown et al., 2008a). Despite the advantages of Bioglass® over the other ceramics covered, there are only a few commercial applications. Perioglas® is a Bioglass® particulate produced by Novabone™ to serve as bone filler or graft extender. The other commercial Bioglass® product is Biogran®, which is also a particulate and is currently produced by Biomet® 3i.

Cell-Based Substitutes

Cell-based strategies for bone tissue engineering, similar to biomaterial-based strategies for bone tissue engineering, fall into several categories. These are transplantation of autogenous progenitor cells, transplantation of autogenous progenitor cells that have been expanded and/or differentiated in culture prior to implantation, transplantation of genetically modified cells, and transplantation of *ex vivo* generated tissue (Muschler et al., 2004). The simplest of these strategies is the transplantation of autogenous progenitor cells. Typically this is performed by aspirating bone marrow and placing the aspirate at the defect or surgical site, unmodified or centrifuged to remove red blood cells and hematopoietic cells, including monocytes, from the bone marrow. The transplantation of autogenous stem cells provides osteogenic potential to the defect, but does not provide osteoconductivity, osteoinductivity or mechanical strength (Connolly et al., 1989).

The second method involves culturing the progenitor cells extracted in the bone marrow in culture. This can increase the number of progenitor cells available to implant, but it also comes with risks. The added time in culture *in vitro* increases the likelihood of bacteria contaminating the progenitor cell population. Successful expansion of the progenitor cells *in vitro* can ultimately improve the healing at the defect or surgical site. Similar to the non-expanded cells, progenitor cells expanded *in vitro* prior to implantation are osteogenic, but not osteoconductive, osteoinductive or mechanically viable (Patterson et al., 2008).

The third strategy for cell-based bone tissue engineering is the use of genetically modified cells. This is similar to the use of *in vitro* expanded autogenous progenitor cells, except the progenitor cells have been treated with an adenovirus to express a protein of interest. For bone tissue, often the progenitor cells will be transfected to express bone morphogenetic protein 2, (BMP-2). BMP-2-expressing progenitor cells provide the cell-based construct to be osteoinductive as well as osteogenic; however, the construct would still not improve in regard to osteoinductivity or mechanical viability (Lieberman et al., 1999; Cui et al., 2006).

The final strategy for cell-based bone tissue engineering is the most ambitious and the least clinically relevant at present. This is the *in vitro* culture of autogenous or embryonic stem cells with the aid of a bioreactor to ultimately produce a viable piece of bone tissue *ex vivo*. The major limitation in this strategy is material transport in developing tissue, since the issue of developing a vascularized construct has not been solved (Muschler et al., 2004). Despite this limitation there is evidence that mesenchymal stem cells can be induced to form small spheroids and differentiate into osteoblasts (Kale et al., 2000). If successful, the *ex vivo* formation of bone tissue would meet all five criteria outlined in the bone tissue engineering paradigm.

Growth Factor-Based Substitutes

The next strategy for bone tissue engineering is based on the use of factors. Two factors that have been shown to be effective in a clinical setting and are approved by

TABLE II.6.7.1 Summary of the Properties Illustrated by the Different Types of Non-Composite Bone Graft Substitutes and How They Relate to the Desirable Characteristics of an Ideal Bone Graft Substitute

	Osteoconductive	Osteoinductive	Osteogenic	Mechanical Match
Allograft-based	Yes	Yes	No	No
Microscale biomaterials	Yes	No	No	Yes, within the range of trabecular bone
Nanoscale biomaterials	Yes	Potentially, supported by evidence with nanofibers made by self-assembly or phase separation	No	No
Ceramics	Yes	Potentially, supported by evidence for Bioglass®	No	Yes, range from less than trabecular bone to more than cortical bone depending on porosity
Cells	No	No	Yes	No
Growth factors	No	Yes	No	No

the FDA are recombinant human bone morphogenetic proteins 2 and 7, rhBMP-2 and rhBMP-7, respectively. Bone morphogenetic proteins are part of the transforming growth factor-β super family, and have been shown to exhibit osteoinductive potential (Fouletier-Dilling et al., 2007). Between the two rhBMPs, rhBMP-2 has been shown to be more osteoinductive than rhBMP-7 (Govender et al., 2002b; Ripamonti et al., 2007). Due to the soluble nature of both rhBMPs, they are typically packaged with some sort of carrier, often as simple as a collagen sponge (Gautschi et al., 2007). RhBMP-2 combined with a collagen sponge has been shown to perform better in spinal fusions than the "gold standard" autograft (Geiger et al., 2003).

In addition to spinal fusions, rhBMP-2 combined with a collagen sponge has illustrated effectiveness in fracture healing, where the rhBMP-2/collagen construct illustrated a significant improvement in fracture healing after 10 weeks (Govender et al., 2002a). RhBMP-2/collagen constructs have illustrated improved healing of critical size defects in animal models (Geiger et al., 2003). The use of rhBMP-7 in spinal fusions has been proven more effective than the autograft in high risk patients (Govender et al., 2002b; Gautschi et al., 2007). Medtronic®, Inc. developed a collagen sponge that is combined with rhBMP-2 to improve spinal fusion. This collagen sponge combined with rhBMP-2 and a spinal cage is marketed under the trade name of INFUSE®. The primary limitation to the usage of rhBMPs in bone tissue engineering applications is that they often cost a significant amount more than comparable procedures, and consequently the use of rhBMPs is primarily only in high risk cases where all other options have been exhausted (Gautschi et al., 2007).

Composite Substitutes

The final strategy for bone tissue engineering is based on composites which combine two or more of the elements detailed above. The goal of creating composites is to combine the benefits of each component. Table II.6.7.1 summarizes how the different bone tissue engineering strategies correlate to the bone tissue engineering paradigm. A common composite encountered consists of a biomaterial structure seeded with osteoblasts or osteoblastic cells. This is typically accomplished by either seeding of the biomaterial structure with osteoblast progenitor cells *ex vivo* just prior to implantation *in vivo*, or seeding of the biomaterial structure with osteoblasts or osteoblast progenitor cells followed by culture *in vitro* for several days to weeks prior to implantation.

An example of this technique that is presently used clinically is seeding Grafton® DBM with bone marrow aspirate from the patient *ex vivo*, followed by implantation of the Grafton® DBM/bone marrow aspirate composite at the desired surgical site. This composite combines the osteogenic potential of the progenitor cells in the bone marrow aspirate with the osteoinductive and osteoconductive properties of the Grafton® DBM, and has been shown to be comparable to an autograft in formation of bone at the surgical site (Russell, 2000; Lindsey et al., 2006).

Another example of a cell/biomaterial composite involves seeding a porous polymer scaffold created by selective laser sintering of poly(caprolactone) with fibroblasts transfected to express bone morphogenetic protein-7, BMP-7 (Williams et al., 2005). The selective laser sintered poly(caprolactone) scaffold provides a structure with millimeter scale features that is mechanically adequate and osteoconductive, and the incorporation of the BMP-7-expressing fibroblasts makes the composite construct osteoinductive. However, the fibroblasts are not expected to differentiate to osteoblasts, and consequently this composite is not osteogenic. A second example of composite scaffolds for bone tissue engineering involves the incorporation of a ceramic calcium phosphate with a polymer scaffold. This can be accomplished through several different methods, such as suspension of calcium phosphate particles with the polymer phase

prior to processing to form a scaffold, spontaneous formation of calcium phosphates within the polymer phase during processing, and coating of calcium phosphates on the surface of a biomaterial scaffold through soaking in simulated body fluid. A composite created by incorporation of calcium phosphates with the polymer phase during processing is illustrated by electrospinning a slurry of β-tricalcium phosphate particles in dissolved poly(caprolactone) to create a scaffold consisting of nanofibers exhibiting β-tricalcium phosphate particles on the surface of the fibers (Erisken et al., 2008). The incorporation of the β-tricalcium phosphate particles improves the mechanical properties of the nanofiber scaffold, and may also improve the osteoinductivity of the scaffold; however, an improvement in the osteoinductivity of the nanofiber/β-tricalcium phosphate composite has not been illustrated (Erisken et al., 2008).

A composite created by spontaneous formation of calcium phosphates during processing is illustrated by a technique where microspheres are created by an emulsion technique where the organic phase contains the polymer, and the aqueous phase contains calcium and phosphate salts, which through careful control of temperature and pH precipitate onto the microsphere surface as amorphous hydroxyapatite (Khan et al., 2004). The microspheres are heat sintered to form a porous scaffold, and could yield promising results as a bone graft substitute; however, no published evaluation of these composite scaffolds' performance *in vitro* or *in vivo* is currently available. An example of a ceramic/polymer composite formed by precipitation of calcium phosphates on the surface of a scaffold is demonstrated by a technique where PLAGA (poly(lactic-co-glycolic acid)) microspheres are formed and placed in simulated body fluid, which cause nucleation of calcium phosphates on the surface of the microspheres. The microspheres are then compression molded into porous scaffolds (Davis et al., 2008). These scaffolds illustrate improved osteoconductivity *in vitro*, but do not show any improvement in osteoinductivity over the uncoated microsphere control (Davis et al., 2008).

A third example of a composite scaffold for bone tissue engineering involves the incorporation of growth factors with a polymer or ceramic scaffold. An advanced example of this concept involved the development of cross-linked poly(vinyl pyridine) microspheres containing either rhBMP-2 or rhBMP-7, which were then suspended in a PLAGA foam (Buket Basmanav et al., 2008). The concentration of the poly(vinyl pyridine) and the degree of cross-linking allowed for a staged release of rhBMP-2 and rhBMP-7. The rhBMP-2 was entrapped in the lower concentration and less cross-linked microspheres, and was released more rapidly than the rhBMP-7. The result of this rhBMP-microsphere-loaded PLAGA foam was an increase in the differentiation of bone marrow-derived mesenchymal stem cells *in vitro* as compared to the PLAGA foam loaded with control microspheres not containing any rhBMPs (Buket Basmanav et al., 2008). The results of this investigation are typical of what is expected by including BMPs into scaffolds, and provides a strategy for making an osteoconductive scaffold osteoinductive as well (Jeon et al., 2007; Buket Basmanav et al., 2008; Kempen et al., 2008). A critical issue with constructs containing growth factors, which is shared by growth factor strategies in general, is that they are prohibitively expensive for developing medical strategies to treat typically non-life-threatening injuries. The above examples of strategies for composite structures for bone tissue engineering represent only a small portion of the composite structures that have been investigated; however, the above examples do provide an accurate representation of the desired outcomes in preparing composite structures for bone tissue engineering.

POROSITY IN BONE GRAFT SUBSTITUTES

An important characteristic of a successful biomaterial-based bone graft substitute that promotes all the features of the bone tissue engineering paradigm involves the pore structure of the scaffold or construct. Without an adequate pore structure, migration into the scaffold is restricted, which subsequently limits the potential of the scaffold to be osteoconductive, osteoinductive, and osteogenic. The pore structure of the scaffold, both pore diameter and porosity, is a critical component in allowing cellular migration.

The concept of pore structure and cellular migration is similar across cell lines, and as such, this discussion focuses primarily on the characteristic dimension of the scaffold and the subsequent requirements to promote cell migration into the scaffold. For microscale scaffolds fabricated through techniques such as microsphere sintering, gas foaming, and particulate leaching the critical design aspects involve maintaining interconnected pores and pore diameters above 40 μm (Akay et al., 2004; Karageorgiou and Kaplan, 2005). An interconnected porosity is necessary to facilitate migration of cells throughout the scaffold, as well as to maintain a supply of nutrients and removal of waste from the cells on the scaffold interior. It was found that scaffolds with large, 100–300 μm, pore diameters facilitated faster migration throughout the scaffold (Borden et al., 2002b; Karageorgiou and Kaplan, 2005); however, despite the decreased rate of migration, the lower, 40 μm, pore diameter scaffolds achieved the same level of cellular penetration as those with larger pore diameters (Karageorgiou and Kaplan, 2005).

When the characteristic dimension of the scaffold shrinks to the nanoscale, these same concepts do not apply (Stevens and George, 2005). No longer does pore diameter appear to be as influential, since nanofiber scaffolds fabricated from self assembly and thermally-induced phase separation have illustrated cell invasion into and throughout the interior of the scaffold, despite pore diameters ranging from 300 nm–10 μm (Zhang, 2003; Semino et al., 2004; Silva et al., 2004; Chen et al., 2006; Horii et al., 2007). Two characteristics shared by

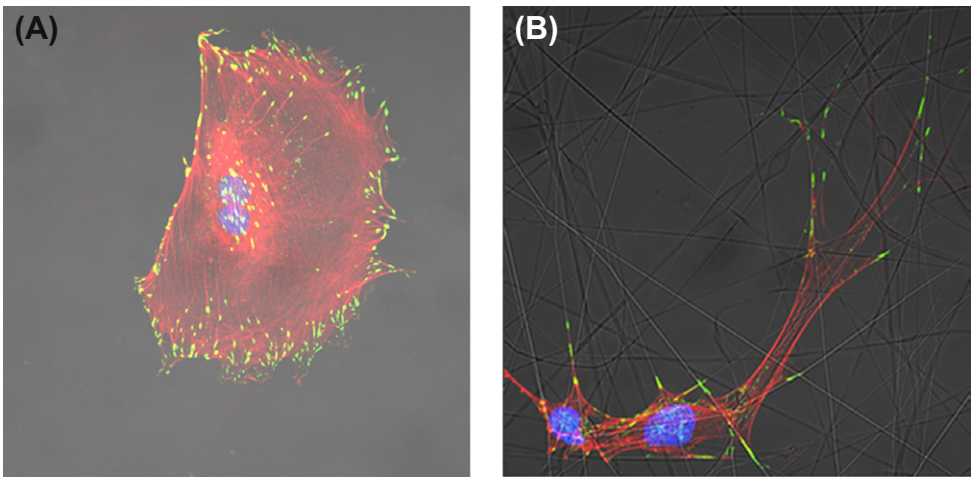

FIGURE II.6.7.7 Flourescent microscopy depicting preosteoblasts on either a flat poly(methyl methacrylate) surface (A); or poly(methyl methacrylate) nanofibers (B). The actin cytoskeleton of each cell is red, the protein vinculin, that is localized to points where the cell is adhered to the substrate, is green and the cell nucleus is stained blue. The image was taken 24 hours after the cells were seeded on the substrates. Of interest is how the preosteoblast on the flat surface spreads out radially, whereas the preosteoblasts on the nanofibers extend out along the fibers. What is not clear by the two-dimensional images provided is that the preosteoblast in (A) is much flatter in the z-direction than the preosteoblast in (B). Previously unpublished images.

both of these scaffold architectures are that they illustrate porosities greater than 95%, and that the fibers are randomly oriented in three dimensions (Ma and Zhang, 1999; Zhang, 2003; Horii et al., 2007). Likewise, electrospun nanofibers, which typically exhibit total porosities ranging from 40–75%, with isolated reports up to 90%, and pore diameters ranging from a few microns to 30 μm, have also exhibited cellular infiltration throughout the interior of the scaffold (Kim et al., 2003; Boland et al., 2004; Zhang et al., 2005; Venugopal et al., 2007).

These results illustrating cellular migration into the small pores contradict those previously found for microscale scaffolds, and consequently have raised questions regarding how cells are able to infiltrate nanoscale scaffolds. The ability of cell infiltration with a nanoscale scaffold starts with a fundamental difference in how cells respond to nanodimensional surfaces (Stevens and George, 2005). On nanostructures, cell morphology changes to exhibit pronounced pseudopodia-like processes which extend along individual fibers (Tan and Saltzman, 2004; Patel et al., 2007). Figure II.6.7.7 illustrates preosteoblasts seeded on a flat surface, which mimics a microstructure, and preosteoblasts seeded on nanofibers illustrating the aforementioned pseudopia-like processes. These extensions on randomly orientated fibers produce an image appearing to be a well-spread cell; however, it is important to consider that most imaging techniques depict a two-dimensional field when there is a third dimension not necessarily seen. This implies that cells are extending down into the field of view as much as they are extending out within the field of view, and this theory has been corroborated through the use of confocal fluorescence imaging illustrating cells extending in three dimensions (Silva et al., 2004; Horii et al., 2007). These well-spread cells exhibiting pseudopodia-like processes have led researchers to conclude that cells utilize ameboidal migration to reach the interior of the scaffold (Friedl and Brocker, 2000a,b; Zhang et al., 2005). Presumably, this ameboidal migration is driven by soluble factors and proteins adsorbed to the surface of fibers below the cells, providing a gradient and driving infiltration to establish a uniform density of cells throughout the construct (Stevens and George, 2005; Patel et al., 2007). In conclusion, microscale scaffolds rely on pore diameter to allow cell migration; whereas nanoscale scaffolds rely on chemical gradients created by adsorbed factors and ameboidal motion to allow cell migration, which suggests that porosity is the critical property of nanoscale scaffolds.

DIMENSION IN BONE GRAFT SUBSTITUTES

In addition to porosity, the characteristic dimension of a polymer scaffold can have tremendous implications to the success of the scaffold as a bone graft substitute. Following are detailed descriptions of two scaffold types with dramatically different characteristic dimensions. The first, sintered microspheres, has a characteristic dimension an order of magnitude larger than that of a cell; while the second, nanofibers, demonstrate a characteristic dimension two orders of magnitude smaller than that of a cell.

Sintered Microspheres

Sintered biodegradable microsphere scaffolds were first developed by Cato Laurencin's laboratory in 1996, and were composed of PLAGA. These initial scaffolds were fabricated both with and without hydroxyapatite, and exhibited mechanical properties and porosity suitable for bone tissue engineering (Devin et al., 1996). Since

that time the microsphere scaffold has undergone several compositional iterations. Microsphere scaffolds have been fabricated from other types of biodegradable polyesters, such as polylactide and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (Nof and Shea, 2002; Zhu et al., 2007). Sintered microsphere scaffolds have been fabricated from a polymer/ceramic composite microsphere composed of poly(lactide-*co*-glycolide) microspheres fabricated in such a way as to spontaneously induce calcium phosphate precipitation on the surface of the microspheres (Khan et al., 2004). Additionally, microsphere scaffolds have been combined with growth factors to provide differentiation cues to the seeded cells (Jaklenec et al., 2007).

A final recent iteration of the microsphere scaffold has dealt with the blend of poly(lactide-*co*-glycolide) with the biopolymer chitosan, as well as blends with other polyesters, such as poly(propylene fumarate) (Jiang et al., 2006; Kempen et al., 2006). The tremendous interest in the sintered microsphere scaffold is due to it being advantageous over other microscale scaffolds produced by such techniques as gas foaming and porogen leaching (Sachlos and Czernuzska, 2003; Rezwan et al., 2006). These advantages are reproducibility, scalability, and controllability over pore size, porosity, and mechanical properties. Microsphere scaffolds rely on the chemical properties of a polymer to sinter uniform spheres into scaffolds by elevating the polymer above the glass transition point, which causes the adjacent polymer chains to migrate and intertwine, forming a cohesive bond when the polymer is cooled (Borden et al., 2002a). Maintaining the temperature, size of microsphere, and time to sinter has proven to produce uniform scaffolds (Borden et al., 2002a). Gas foaming and porogen leaching do not have the same level of reproducibility, due to variation introduced by having multiple phases, either a heterogeneous mixture of a solid and liquid or a liquid and gas. These heterogeneous mixtures can undergo demixing, which introduces inconsistencies in the mixing of the phases and decreases reproducibility (Sachlos and Czernuzska, 2003; Rezwan et al., 2006).

Microsphere scaffolds maintain a very high level of interconnectivity among the pores, and allow control over the total interconnected porosity, as well as the average pore diameter (Borden et al., 2002a). Gas foaming and particle leaching provide control over the total porosity and pore size as well; however, they often have very poor interconnectivity (only 10–30% interconnectivity with gas foaming) of the pores, rendering a percentage of the pores inaccessible (Mooney et al., 1996). The inaccessible pores are formed when the initial heterogeneous mixture lacks contact between the solid or gaseous phases, which causes the resulting scaffold to contain pores surrounded by solid polymer. Another advantage of microsphere scaffolds, when compared to gas foaming and porogen leaching, is that they can be fabricated to exhibit mechanical properties that mimic that of trabecular bone (Borden et al., 2002a, 2004).

High mechanical properties are achievable with gas foaming and porogen leaching; however, to achieve this, the scaffold density must be increased, which causes the internal pore structure to be further compromised (Sachlos and Czernuzska, 2003; Rezwan et al., 2006). Microsphere scaffolds composed of polylactide and poly(lactide-*co*-glycolide) have been produced previously, and have yielded reasonable results in *in vitro* studies, as well as in *in vivo* studies examining critical size defect healing (Borden et al., 2002b, 2004; Botchwey et al., 2003; Yu et al., 2004; Jiang et al., 2006). *In vitro* studies have illustrated that rat calvarial cells demonstrated a phenotype with earlier expression of matrix proteins in a greater magnitude when cultured on microsphere scaffolds, compared with the same cells on tissue culture plastic (Borden et al., 2002b).

Nanofibers

Recent developments in tissue engineering have indicated that nanoscale structures are more advantageous for cellular phenotype expression and morphology when compared to microscale structures (Tuzlakoglu et al., 2005; Wan et al., 2005). A frequently employed type of nanoscale scaffold is based on polymeric nanofibers. Nanofibers can be fabricated through several different techniques. The initial and most common technique is through electrospinning, which has been used for over 70 years (Formhals and Schreiber-Gastell, 1934). Electrospun nanofibers are created by applying a voltage gradient between a target and a drop of polymer, either dissolved or melted (Formhals and Schreiber-Gastell, 1934; Nair et al., 2004; Smith and Ma, 2004). Once the voltage gradient overcomes the surface tension of the droplet, a polymer stream extends toward the target. As this stream travels, it is thinned out and eventually strikes the target as a nanofiber, with the accumulation of these fibers leading to the production of a nonwoven nanofiber mat (Nair et al., 2004; Smith and Ma, 2004).

Initially, electrospun nanofibers were made from non-degradable polymers for applications in filtration; however, recent developments have led to the use of electrospun nanofibers made of biodegradable polymers for tissue engineering applications (Nair et al., 2004). Nanofibers created by electrospinning typically have diameters ranging from 300–1200 nm, depending on the spinning conditions used (Kim et al., 2003; Yoshimoto et al., 2003; Nair et al., 2004; Smith and Ma, 2004; Tuzlakoglu et al., 2005; Kumbar et al., 2006b). Electrospun nanofibers are oriented lengthwise in only two dimensions, with the third dimension created from the stacking of the fibers on top of each other. This is why electrospun nanofiber scaffolds are typically referred to as mats. Nanofibers have also recently been developed using synthetic peptides designed to self-assemble into a three-dimensional

nanofiber network. Self-assembly nanofibers often have a characteristic diameter that is within the range of 5–10 nm (Zhang, 2003; Semino et al., 2004; Silva et al., 2004; Smith and Ma, 2004; Horii et al., 2007). The very thin diameter of the fibers in the network, and the high porosity created with self-assembly, produces a scaffold poor in mechanical properties making them suitable primarily for hydrogel applications.

A final method of nanofiber fabrication is thermally-induced phase separation, which relies on the spinodal liquid–liquid phase separation of a polymer solution into a polymer poor phase and a polymer rich phase when the solution is rapidly cooled. Spinodal phase differs from binodal phase separation in that the two phases separate and exist continuously throughout the original mixture; whereas bimodal phase separation occurs via nucleation sites that build spherical particles. The selection of an appropriate polymer, one with a high degree of crystallinity, will allow the polymer rich phase to crystallize into nanofibers; whereas polymers with low degrees of crystallinity form microstructured foams (van de Witte et al., 1996; Chen et al., 2006). Nanofibers created with the thermally-induced phase separation technique exhibit a fiber diameter of 50–500 nm, which is similar to collagen, and a three-dimensional fiber structure making them very different to that of electrospun nanofibers (Smith and Ma, 2004). Evidence suggests that nanofibers exhibiting a three-dimensional spatial arrangement promote phenotype progression of osteoblasts, and may be osteoinductive (Hu et al., 2008). Producing a three-dimensional nanofibrous mechanically viable implant could be a tremendous leap forward in the field of bone tissue engineering.

Osteoinduction

Osteoinduction is the ability of a substance to cause stem cell differentiation down an osteoblastic lineage. Osteoinduction is known to occur when certain growth factors, such as bone morphogenetic proteins 2 and 7, and to a lesser extent vascular endothelial growth factor, are present (Urist, 1983; Geiger et al., 2003; Habibovic and de Groot, 2007; Dawson et al., 2009). What is less clear is whether a biomaterial can be osteoinductive in the absence of growth factor supplements. Current research has provided contradictory evidence as to whether or not a biomaterial is osteoinductive (Ye et al., 2007; Catros et al., 2009). No particular biomaterial has been conclusively shown to be osteoinductive, independent of structure and growth factors (Habibovic and de Groot, 2007; Habibovic et al., 2008).

This suggests that what may be more important than a particular biomaterial is the structure that the biomaterial is fabricated into. Research suggests that macroscale concavities (Graziano et al., 2008) in a surface, a microporosity (Habibovic et al., 2005), as well as subcellular structures (e.g., nanofibers) (Hu et al., 2008) may all promote osteoinduction. However, each of these elements has yet to be thoroughly challenged to definitively ascertain if they are indeed osteoinductive. For instance, the study investigating macroscale concavities included flat and convex surfaces as controls; however, all three surfaces were made from different materials, and the stem cells used were differentiated to osteoblast progenitors prior to seeding on the substrates. Another intriguing osteoinductive quality of substrates is that mesenchymal stem cell differentiation has demonstrated a dependence on the rigidity of a substrate (Engler et al., 2006; Khatiwala et al., 2007). These elements suggest that developing an osteoinductive bone graft substitute may depend on design and material considerations. Moving forward, research should first investigate how osteinduction occurs, and then deconstruct the independent elements of biomaterial chemistry, surface topography, and porosity to determine which, if any, element provides non-growth factor initiated osteoinduction.

IN VITRO CULTURE TECHNIQUES FOR BONE GRAFT SUBSTITUTES

A critical limitation of static culture conditions is that waste efflux and nutrient influx are governed by diffusion, which becomes exponentially more problematic with large three-dimensional constructs. As time passes, the accumulation of waste within a construct can lead to an acidic microenvironment that impedes calcification of the developing tissue, and compromises the viability of osteoblasts within a construct (Bushinsky et al., 1983; Han et al., 2009). To alleviate these issues the use of bioreactors becomes necessary when trying to achieve significant tissue growth *in vitro*. The bioreactor provides fluid flux that replenishes nutrients and removes waste; additionally, for tissues such as bone, bioreactors can provide mechanical stimulation to encourage the development of a mechanically viable tissue.

The earliest bioreactor that has been applied to bone tissue engineering involves dialysis membranes that are either gas or small molecule permeable (Figure II.6.7.8A). The bioreactor is divided into two compartments that are separated by small molecule permeable membrane, and each compartment also contains a gas permeable membrane (Vogler, 1989). The gas permeable membrane provides a liquid–air boundary to provide oxygen and CO_2, and the membrane between compartments serves as liquid–liquid boundary between the culture media surrounding the cells and a reservoir of fresh media. This boundary provides fresh nutrients while removing waste, but also allows the soluble factor gradients to remain intact during media changes. Recently, this bioreactor design has supported the development of simple osteoid tissue after culturing osteoprogenitor cells for 10 months (Mastro and Vogler, 2009). Despite the development of osteoid tissue, this bioreactor design still relies on passive diffusion to supply nutrients, and therefore limits the ultimate thickness of the construct to only a few cell layers (Mastro and Vogler, 2009).

The next iteration of the bioreactor involved rotation, either of the reactor itself or of the construct within the bioreactor (Figure II.6.7.8C). NASA developed rotating wall bioreactors to simulate microgravity. These bioreactors consist of a cylinder and a gas exchange membrane, either on one face of the cylinder

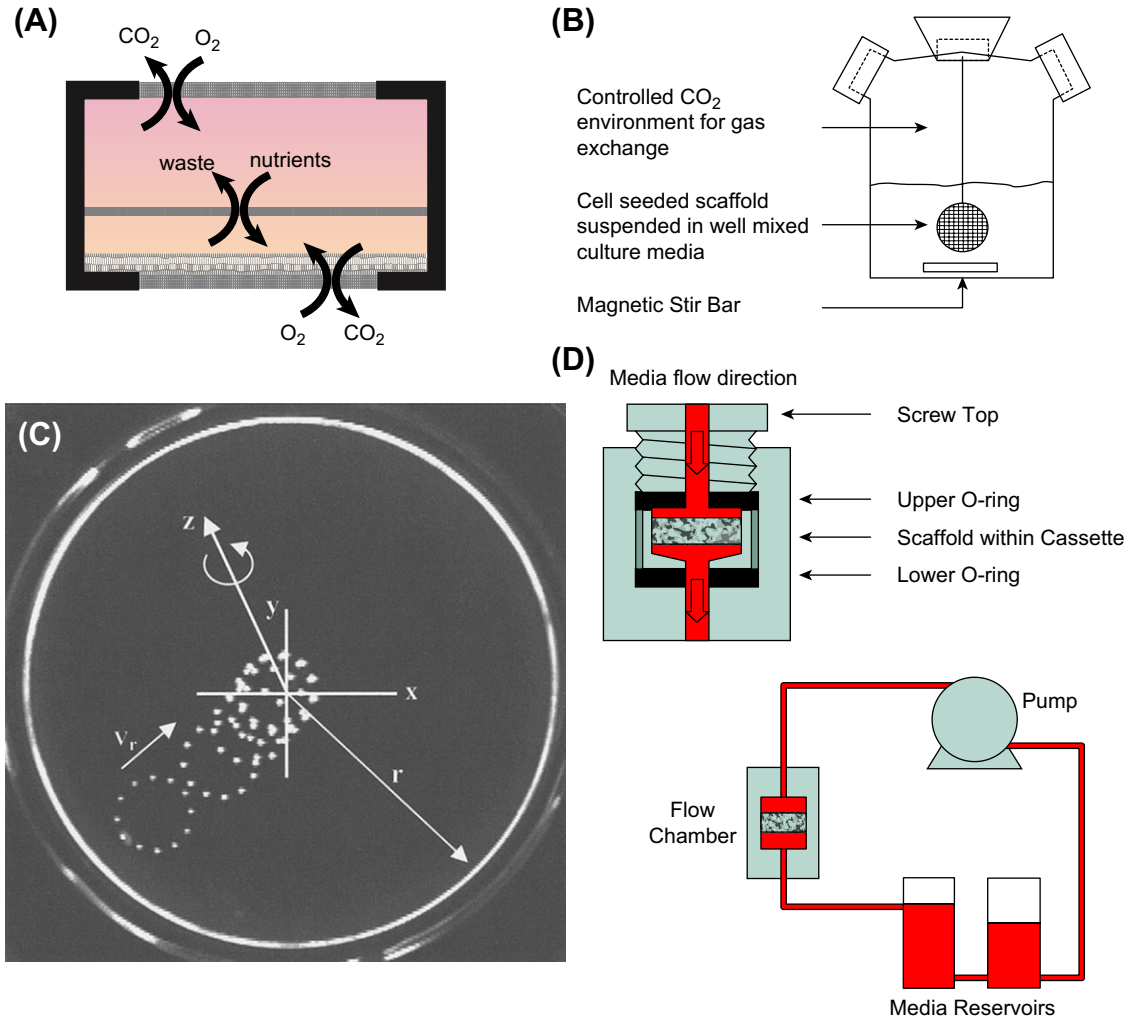

FIGURE II.6.7.8 Graphical depiction of four bioreactors. The simplest bioreactor that supports multi-layered growth of cells on the bottom surface and consists of two gas permeable membranes on the top and bottom and a dialysis membrane in the middle (A). A spinner flask bioreactor that utilizes a stir bar to force nutrient flux through the constructs, which remain in a fixed position (B) (Martin and Vermette, 2005). A high aspect ratio rotating vessel bioreactor demonstrating the trajectory of a lighter than water construct within the rotating wall bioreactor, the fluid shear on the scaffold is low; however, the back panel of the bioreactor is gas permeable membrane and the rotation maintains a large volume of well-mixed media to support nutrient supply (C) (Botchwey et al., 2004). Finally, a schematic illustrating a perfusion bioreactor that utilizes a pump to force media through a construct, which research suggests also promotes bone tissue formation through mechanical stimulation provided by fluid shear forces on the construct (D) (Bancroft et al., 2002). Copyright (2002) National Academies of Sciences, U.S.A.

or as a separate inner cylinder. In this design the shear force exerted by fluid rotation negates the effect of gravity on the constructs, and also provides a well-mixed volume of culture media. This strategy was employed by tissue engineers to culture constructs seeded with osteoblasts, in the hope that the rotating well-mixed volume of culture media would overcome diffusion limitations and allow tissue development throughout the thickness of the construct (Botchwey et al., 2004). Research suggests this is certainly the case, with osteoblasts found throughout the thickness of the construct (Yu et al., 2004); however, the simulation of microgravity seems to (not surprisingly due to a lack of mechanical stimulation) discourage bone tissue development; this is demonstrated both by lower levels of phenotype markers, and by lower mineralization as compared to static cultured constructs (Sikavitsas et al., 2002; Yu et al., 2004).

The next type of bioreactor that involves rotating fluid is the spinner flask (Figure II.6.7.8B). Spinner flask bioreactors suspend the constructs on thin needles that are extended into a stirred flask of media. This configuration not only provides a well mixed environment to avoid accumulation of waste in local environment of the construct, but also provides mechanical stimulation through shear forces on the constructs. Since the spinner flask also provides a well-mixed environment, it is not surprising to see that cells migrate throughout the construct suspended in the spinner flask, similar to those cultured in rotating wall bioreactors (Sikavitsas et al., 2002; Stiehler et al., 2008). Additionally, because the spinner flask provides much higher shear forces than the rotating wall bioreactor, there is a marked increase in the calcification of the construct and the differentiation of osteoblasts in the

spinner flask (Sikavitsas et al., 2002; Stiehler et al., 2008; Wang et al., 2008).

The final bioreactor for bone tissue engineering is a perfusion bioreactor (Figure II.6.7.8D). The perfusion bioreactor works by forcing culture media through a construct with a pump. This system provides intricate control over the shear experienced by the cells in culture. Similar to the spinner flask, osteoblasts generated more calcium and exhibited increased expression of phenotype markers in the perfusion bioreactor (Gomes et al., 2003; Pham et al., 2008).

CONCLUSION

The concepts of tissue engineering have made profound advances in developing clinically relevant solutions for tissue such as skin, bladder, and to some extent bone; however, where the clinical strategies for skin and bladder exist as a straightforward solution based on the requirements of those tissues, bone presents a more complicated situation. Skin and bladder tissue are essentially a uniform two-dimensional sheet; whereas, bone often takes many unusual three-dimensional and non-uniform geometries (Bannasch et al., 2003; Vats et al., 2003; Atala et al., 2006; Bolland and Southgate, 2008).

The evolution of bone tissue engineering began with osteoblasts cultured on rudimentary polymer foams, and has progressed to include an array of bone graft substitutes cultured in bioreactors to drive the development of *de novo* bone tissue. Significant clinical achievements have occurred in using biodegradable scaffolds, with or without growth factors or cells, as synthetic bone grafts to heal large defects in bone tissue (Damron, 2007; Rosa et al., 2008; Wlodarski et al., 2008; Dawson et al., 2009; Gosain et al., 2009). However, despite the ground that has been covered thus far, there persist unmet goals and challenges still ahead. No research has yet demonstrated the capability to grow *de novo* bone in an *in vitro* setting; so far only rudimentary calcified cell masses approaching bone tissue have been developed. Moving forward, future researchers should consider the flexibility in design of the macroscale structure to accommodate the unusual architectures and mechanics necessary for bone graft substitutes, biodegradability of the structure such that natural healthy bone is ultimately all that persists, the osteoconductivity of the bone graft substitute to promote proliferation of progenitor cells and osteoblasts throughout the bone graft substitute, and the osteoinductivity of the bone graft substitute to promote the maturation of the progenitor cells and osteoblasts into organized bone tissue.

To date, the one aspect the earliest bioreactor got right has been lacking in all future designs; the ability to maintain gradients in soluble growth factors secreted by cells. The development of the bioreactor for bone tissue engineering has moved from a series of static chambers separated by dialysis membranes to reactors that provide mechanical stimulation; the future success of the bioreactor will depend on the ability to incorporate aspects of the existing bioreactors: nutrient influx; waste efflux; mechanical stimulation; and the establishment of soluble factor gradients. Providing all four of these bioreactor elements with a bone graft substitute may lead to the development of hierarchical bone tissue, and ultimately the *de novo* formation of bone *in vitro*.

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CHAPTER II.6.8 CARTILAGE AND LIGAMENT TISSUE ENGINEERING: BIOMATERIALS, CELLULAR INTERACTIONS, AND REGENERATIVE STRATEGIES

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INTRODUCTION TO CARTILAGE AND LIGAMENT TISSUE ENGINEERING

The musculoskeletal system is responsible for complex movements that are performed many thousands of times over a lifetime. Two connective tissues of the musculoskeletal system, cartilage and ligament, protect the body from injuries during these movements, primarily by absorbing loads and maintaining joint stability, respectively. Relative to other musculoskeletal tissues, cartilage and ligament have low oxygen and nutrient requirements, low cell density, and poor regenerative capacity, yet they experience some of the highest mechanical loads in the body. When these loads exceed a critical threshold that causes permanent tissue damage, or if diseases cause severe tissue degeneration, these problems often result in a significant locomotive impairment. For the repair of both tissues, given their very low self-regenerative capacity, typically the only recourse is surgical intervention. Current surgical reparative techniques rely upon total joint replacement or grafting, and are often accompanied with further musculoskeletal problems; a more ideal solution would be to use a biological approach to repair the defects and fully restore the cartilage or ligament tissue to its pre-injured state. This is the promise of tissue engineering, a new field