Chapter 5

Functional Tissue Engineering of Bone: Signals and Scaffolds

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Summary

Tissue engineering is the science of design and manufacture of tissues including bones and other musculoskeletal tissues. The three key ingredients for both tissue engineering and morphogenesis are signals for morphogenesis, responding stem cells and the scaffolding. Regeneration of musculoskeletal tissues recapitulates embryonic development and morphogenesis. Morphogenesis is the developmental cascade of pattern formation, body plan establishment leading to adult form and function. Therefore, signals involved in morphogenesis will be useful for tissue engineering of bones. BMPs have pleiotropic roles in initial pattern formation, cell differentiation and maintenance of bone and articular cartilage. The regenerative potency of bone is due to bone morphogenetic proteins (BMPs) in the bone matrix. BMPs act via BMP receptors and Smads 1, 5 and 8 to initiate lineage of cartilage and bone. The homeostasis of tissue engineered bone and cartilage is dependent on the maintenance of extracellular matrix and biomechanics. The use of BMPs by gene therapy and isolation of stem cells in a biomimetic scaffold of extracellular matrix will lead to functional bone tissue. In conclusion, these are exciting times in functional tissue engineering of bone using signals, scaffolds and stem cells.

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Introduction

One of the challenges confronted by an orthopaedic surgeon is the repair and restoration of large segmental skeletal bone defects resulting from resection of a malignant bone neoplasms and trauma. Although large-segment bone allografts have gained increasing acceptance, it has the drawbacks of potential fractures (1, 2). The problem of bone fractures in patients with postmenopausal osteoporosis, metastases due to breast and prostate cancer and metabolic diseases such as diabetes requires the application of principles of tissue engineering to bone (3-7).

Tissue engineering is the science of design and fabrication of new tissues for functional restoration of impaired organs and replacement of lost parts due to cancer, disease and trauma (3, 8). Among the many tissues in the body, bone has the highest potential for regeneration and therefore is a prototype paradigm for the enunciation of principles of tissue engineering in general. The accumulating knowledge in tissue engineering will lead to the design of bone with predetermined shapes for orthopaedic surgery applications.

The three key ingredients for tissue engineering and tissue regeneration are signals, stem cells and scaffolding. The specificity of signals is dependent on tissue morphogenesis and inductive cues in the embryo and they are generally recapitulated during regeneration (9). Bone grafts have been utilized by orthopaedic surgeons for over a century. Urist made the key discovery that intermolecular implantation of demineralized, lyophilized segments of allogeneic rabbit bone induced new bone formation (10). Bone induction is a sequential multistep cascade and the three key steps are chemotaxis, mitosis and differentiation (9, 11, 12). Chemotaxis is the directed migration of cells in response to a chemical gradient of signals released from the demineralized bone matrix (13). The migration and attachment of osteo-progenitor cells to the collagenous matrix is mediated by fibronectin. On day 3 there is a peak in proliferation of cells in response to growth factors released from the insoluble demineralized matrix (14). Chondrogenesis is maximal on days 7-8 and is followed by vascular invasion and osteogenesis on day 9. Bone formation is maximal on days 10-12 as indicated by alkaline phosphatase activity and is followed by increases in osteocalcin, the bone γ-carboxyglutamic acid containing protein (BGP). The newly formed ossicle is filled with

hematopoietic marrow on day 21 (12). The demineralized bone matrix-induced bone morphogenesis system led to the isolation of bone morphogenetic proteins (BMPs) the primordial signals for morphogenesis of bone and a variety of organ systems beyond bone such as brain, heart, kidney, lungs, liver, skin and teeth. Hence, one can refer to BMPs as body morphogenetic proteins.

Bone Morphogenetic Proteins

Demineralized bone matrix is an insoluble scaffolding. The demineralized bone matrix was extracted by dissociative agents such as 4 M guanidine HCL, 8 M urea or 1% sodium dodecyl sulfate at pH 7.4 (15, 16). Approximately three percent of the proteins were solubilized and the residue was predominantly type I insoluble bone collagen scaffolding. Although the soluble extract or insoluble collagen scaffolding were not osteoinductive singly, when recombined and reconstituted together it restored bone induction. Thus, there is a collaboration between a soluble signal and an insoluble substratum of collagen to initiate new bone formation. The soluble signal was purified by heparin affinity chromatography, hydroxyapatite columns, and molecular since chromatography. The final purification was accomplished by preparative gel electrophoresis and novel BMPs were isolated, cloned and expressed (3, 17-19).

BMP Subfamily	***BMP* Designation***
BMP 2/4	BMP 2 BMP 4
BMP 3	BMP 3 BMP 3B
OP-1 / BMP 7	BMP 5 BMP 6 BMP 7 BMP -8 BMP 8B
Others	BMP 9 BMP 10 BMP 11 BMP 15
Cartilage-Derived Morphogenetic	BMP 14/CDMP1/GDF5
Proteins (CDMPs), Growth/Differentiation	BMP13/CDMP2/GDF6
Factors (GDF)	BMP12/CDMP3/GDF7

* BMP 1 is procollagen C-proteinase related to Drosophila Tolloid and does not contain the canonical seven cysteines of classical BMPs listed in this Table. BMP 1, copurified with the osteogenic BMPs such as BMP2.

Table 1. The Superfamily of BMPs

Table 1 summarizes the fifteen known BMPs in mammals that are related to members of the TGF-ß superfamily. BMPs are dimers and are held together by a critical intermolecular disulfide linkage. The dimeric conformation is critical for bone induction and morphogenesis. Each of the two monomers is biosynthesized as a precursor molecule of over 400 amino acids. However, mature BMP monomer derived by proteolytic processing is an approximately 120 amino acid polypeptide. BMPs are pleiotropic signals. Pleiotropy is the property of a gene or protein to act in a multiplicity of steps. BMPs act on the three key steps in the sequential cascade of bone morphogenesis such as chemotaxis, mitosis and differentiation of transient stage of cartilage and the permanent induction of bone.

Although BMPs were first isolated, cloned and expressed from bone, they have actions beyond bone. Genetic evidence based on gene knockouts has implicated BMPs in development and morphogenesis of brain, eye, heart, kidney, liver, lung, ovary, skin, teeth, testis and in a variety of tissues during various steps of epithelial-mesenchymal interactions during embryogenesis. It is indeed gratifying to note that BMPs are at the core of key developments in morphogenesis of many tissues (3).

BMPs elicit their biological actions by their interaction with types I and II BMP receptors. There are two kinds of type I BMP Receptors, types IA and IB (3, 20). BMPs receptors are protein kinases that phosphorylate cytoplasmic substrates called Smads 1, 5 and 8. The phosphorylated Smads 1, 5 and 8 partner with a co-Smad called Smad 4 and enter the nucleus to turn on BMP-response genes. The phosphorylation of Smads 1, 5 and 8 by BMP receptors is inhibited by inhibitory Smad 6. Thus, the BMP signaling system is an intricately regulated homeostatic machine such as a thermostat in an air conditioner (3). BMP-BMP receptor signaling system in the mesenchymal stem cells results in bone induction and morphogenesis.

Natural Scaffolds: Extracellular Matrix

The isolation, cloning and expression of BMPs and the advances in stem cell research will permit the rational design of the bones of predetermined shapes using scaffolds for tissue engineering of bone. A scaffold in the context of bone tissue engineering is the extracellular matrix (ECM) of bone, the unique microenvironmental niche for bone morphogenesis. What are biomimetic biomaterials in the context of the extracellular matrix scaffolding? Biomaterials that mimic native extracellular matrix scaffolding are biomimetic as they imitate NATURE. The biomimetic biomaterials in the musculoskeletal tissues include collagens, proteoglycans, component glycosamigolycans and hyaluronan. The adhesive proteins fibronectin and laminin are critical in the attachment of cells to ECM. Hydroxyapatite in the mineral phase of bone is a natural biomimetic biomaterial. BMPs bind to collagens I and IV, heparin sulfate, heparin and hydroxyapatite (3, 21). The geometry of the hydroxyapatite is critical for delivery of BMPs for bone induction. Consistently, optimal bone morphogenesis was observed by hydroxyapatic discs compared to beads. This profound difference is independent of pore size in the range from 200 to 500 µm. The chemical composition of the hydroxyapatites were identical illustrating the key role of three-dimensional architecture of the substratum the geometry for tissue engineering (3, 22-24). The role of bioceramics in medical applications is well known (25). In subhuman primates hydroxyapatite appears to be "osteoinductive" (26). It is likely that BMPs in circulation in the vascular system may bind to hydroxyapatite and secondarily induce bone formation. Thus, an osteoconductive biomaterial such as hydroxyapatite progressively becomes an osteoinductive substratum.

Synthetic Scaffolds: Degradable Polymers

Scaffolds for bone tissue engineering are designed to act as artificial matrices that temporarily recapitulate the major roles of the extracellular matrix in bone. Specifically, these scaffolds are meant to function as support structures to the surrounding bone tissue, adhesion sites for invading bone

cells, platforms for the delivery of matrix-bound signaling molecules, delivery vehicles for transplanted cell populations, and devices for the controlled release of biologically active molecules. Additionally, the temporal aspect of tissue engineering scaffolds is critical. Tissue engineering scaffolds must be designed to degrade into biocompatible products throughout the bone healing process eventually leaving repaired or regenerated bone tissue.

Synthetic Polymers

Surprisingly few polymers have been investigated for bone tissue engineering scaffold applications (See Table 2 for a description of some degradable polymers). Most of those degradable polymer gels that have been investigated are based on an ester polymer backbone, such as poly(L-lactic acid) (PLA) (27-30), poly(glycolic acid) (PGA), poly(D,L-lactic acid-co-glycolic acid) (PLGA) (30-34), poly(caprolactone) (27 ,35), and poly (propylene fumarate) (36-39). The Food and Drug Administration's approval of PLGA for specific clinical uses have probably led to the numerous research studies involving these polymers. Furthermore, polyesters have been widely investigated because esters react with water, and thus water can slowly add to polyester so as to break, or degrade, the polymer. Ester hydrolysis is the basic mechanism by which most polymers under study for tissue engineering applications degrade. The hydrophobicity of most polyesters, demonstrated by their limitedly wettable surfaces, allows for protein adsorption and cell adhesion, thus these materials are well suited as scaffolds for cell transplantation when cells are seeded on the surface of the scaffold. However, the hydrophobicity of many of these polymers prevents the encapsulation of cells within the polyester.

Name	***Repeating Unit***	***Curing Method***	***Degradation Mechanism***	***Degradation Products***	***Degradation Type***
PLLA poly(L-lactic acid)	O O n	entanglement	ester hydrolysis	lactic acid	bulk
PGA poly(glycolic acid)	O O n	entanglement	ester hydrolysis	glycolic acid	bulk
PLGA poly(D,L-lactic acid-co-glycolic acid)	O O n O O m	entanglement	ester hydrolysis	lactic acid and glycolic acid	bulk
P(CL) poly(caprolactone)	O $(CH_2)_5$ O n	entanglement	ester hydrolysis	caproic acid	bulk / surface
PPF poly(propylene fumarate)	HO O O O O n OH	crosslinking	ester hydrolysis	fumaric acid and propylene glycol	bulk / surface
P(MSA) poly(methacrylated sebacic anhydride)	O O $(CH_2)_8$ O n	crosslinking	anhydride hydrolysis	sebacic acid	surface
P(MCPH) poly(methacrylated 1,6-bis(carboxyphenoxy) hexane)	O O O $(CH_2)_6$ O O n	crosslinking	anhydride hydrolysis	1,6-bis (carboxyphenoxy) hexanoic acid	surface
P(DTR carbonate) tyrosine-derived polycarbonate	N O COOR O O O n	entanglement	ester and carbonate hydrolysis	alkyl alcohol and desaminoyrosyl -tyrosine	bulk

Table 2. Degradable synthetic polymers currently under investigation as scaffold materials for bone tissue engineering applications

While polyesters have been vigorously studied and considerable achievements made in their fabrication into tissue engineering scaffolds, a fundamental problem that is associated with their use is their degradation. The degradation products of polyesters are acids and alcohols. This acidity has been implicated in both the catalysis of further scaffold degradation and the eliciting of a pronounced inflammatory response leading to inhibition of tissue formation. While strategies have been developed to overcome this limitation, such as the inclusion of buffering agents into the scaffold, the future clinical use of polyesters for bone tissue engineering is unclear. It is ironic that the property of polyesters which has allowed their widespread investigation, also contributes to their major disadvantage.

Other degradable polymers have been studied for use in bone tissue engineering applications. Polyanhydrides such as poly(methacrylated sebacic anhydride) and poly(methacrylated 1,6-bis(carboxyphenoxy) hexane) have been shown to possess a surface degradation mechanism, which may be well suited for bone tissue engineering applications (40-42). However, polyanhydrides also form acidic degradation products, and thus their application may be associated with some limitations. Polycarbonates, and especially tyrosine derived polycarbonates, have been extensively studied for bone tissue engineering applications (43-45). These polymers, sometimes described as pseudo-poly(amino acids) for their repeating unit is based upon the amino acid tyrosine, have been shown to be biocompatible and nonimmunogenic. Furthermore, by alterations in the structure of the repeating unit's side chain, the degradation kinetics may be tailored for a specific application.

The polymer which has been most widely investigated for use in the fabrication of hydrogels is poly(ethylene glycol) (PEG), a nondegradable polymer (39, 46-50). PEG is a highly hydrophilic molecule, and this hydrophilicity is often cited as the property responsible for its biocompatibility. The repeating unit of PEG ($-CH_2CH_2O-$) is generally not reactive, so any functionality of the polymer must be added to the polymer backbone. For example, PEG hydrogels are often formed from acrylated PEG, such as poly(ethylene glycol) diacrylate where the terminal acrylate groups ($H_2C=CHCOO-R$) react with one another to form a large polymer network. Furthermore, while PEG itself is not degradable, it can be made either hydrolytically or enzymatically degradable with the insertion of functional groups within the polymer backbone. Proteolytically degradable PEG hydrogels may be formed from acrylated PEG with protease labile groups, such as collagenase or

elastinase sensitive peptide sequences, dispersed throughout the PEG polymer chain length (49, 51). Hydrolytically degradable PEG may be formed by the addition of lactic acid units into the polymer chain.

Scaffold Fabrication Techniques

After a degradable polymer has been identified as a candidate for bone tissue engineering applications, it must be fabricated into a porous scaffold (36, 52-58). Two major steps are required. First a method must be developed that forms the polymer into a bulk material. Second, a method is needed to make this material porous.

Material Fabrication

The proper material fabrication method, or curing, depends in part upon the chemical nature of the polymer. Long, linear, saturated polymers, such as PLGA, are typically formed into bulk materials by entangling the individual polymer chains to form a loosely bound polymer network. Polymer chain entanglement is often achieved by casting the polymer within a mold. Here, the polymer is dissolved into a solvent, the solution is then poured into a mold or film, and the solvent is subsequently removed by evaporation, leaving the polymer as a bulk material in the form of the mold. Alternatively, polymer casting may be accomplished with the use of heat, pressure, or both. Here, the polymer is placed into a mold, heated above its glass transition temperature, and with the application of pressure, formed into the shape of the mold. The advantage to these methods is that they are relatively simple. However, since the material is elastic solid only because of entangled polymer chains, the material is generally lacking significant mechanical strength. This disadvantage is difficult to overcome without altering the chemical structure of the polymer.

Another curing method to form a bulk material from a linear polymer involves forming chemical bonds between polymer chains, known as polymer cross linking (39, 42, 50-59). Cross linking is most often performed between unsaturated carbon-carbon double bonds, and thus this moiety, or a similarly reactive one, is required to exist on somewhere along the polymer chain. An initiation system, typically either radical or ionic, is also needed to promote cross-linking. The initiator system is combined with the polymer and, in response to a signal such as heat, light, a chemical accelerant,

or simply time, the initiator forms species that propagate cross-linking. As these polymers are formed into bulk materials by covalent cross-linking, they typically posses significant mechanical strength. Furthermore, their ability to cure in response to an applied signal allows these materials to be injected into the defect site and cure *in situ*. The major disadvantage of crosslinked materials is that the growing complexity of the material, in terms of the number of components and presence of a chemical reaction, often leads to problems with cytotoxicity and biocompatibility.

It should also be noted that the starting point of the material does not need to be a polymer, but may be a smaller molecule such as an oligomer or monomer. With these smaller molecules, materials can be formed by initiating their polymerization. The polymerized monomers can then form bulk materials by means such as entanglements of the long polymer chains, in the case of bifunctional monomer, or branching networks, in the case of multifunctional monomers. The advantages and disadvantages associated with monomer polymerization are similar to those of polymer cross-linking.

The curing methods described above may be applied both to hydrophobic and hydrophilic polymers. The general advantage of hydrophobic polymers, such as PLA, over hydrophilic polymers, such as PEG, is the comparative strength of the resulting gel. However, hydrophobic polymers generally cannot be used for cell encapsulation for the gel prevents the transport of water, nutrients, and waste to and from the cell. Gels formed from hydrophobic polymers are typically utilized as a skeleton, where cells and tissues adhere to the surface of the material rather than existing within the material. For cell encapsulation applications, hydrophilic polymers are extremely useful (39, 46-51, 59-61). These polymers form gels that often contain water contents in excess of 90-wt%, allowing for considerable passive diffusion of molecules to and from the cell. The large water content, unfortunately, does often result in inferior mechanical properties of the gel. For bone tissue engineering applications, hydrogels may be utilized in non-load bearing environments or as a component within a scaffold which does possess suitable mechanical properties. The choice of hydrophobic or hydrophilic polymer depends primarily upon the tissue engineering strategy under consideration as well as the tissue itself.

Biomimetic Materials

Recent studies have focused on the development of biomimetic materials (39, 50, 51, 60). Biomimetic materials, developed to more closely recapitulate the structure of the extracellular matrix, are typically hydrogels designed to specifically interact with a predetermined cell type so as to create an artificial tissue that performs a desired function. In general, these materials are first developed by creating a material which prevents nearly all cell adhesion. Next, signaling molecules, most often short peptide sequences derived from adhesion proteins and known to participate in specific cell adhesion, are covalently bonded to the material. The result is a material which allows only a specified cell type to adhere to its surface or enter its porosity. The critical factor, which is often overlooked, is that the initial material must prevent random cell adhesion so that the final material has cell adhesion specificity. This is often accomplished by using a hydrogel as the base material, for the hydrophilicity of hydrogels is generally thought to prevent the adsorption of hydrophobic proteins required for cell adhesion. Additional factors that determine the success of this strategy include the incorporation of the peptide sequence in the bulk, rather than on the surface, of the material, the tethering distance given to the peptide sequence so that it is available for binding to cell surface receptors, and the density of peptide sequences within the material. Finally, further discovery of peptide sequences which are truly specific for the adhesion of distinct cell populations is required for the future success of this strategy.

Pore Formation

After a strategy has been developed for curing the polymer into a solid material, a method for forming a porous architecture within the material must be developed. The most straightforward strategy is to include a porogen into the material before curing, and then remove the porogen after curing (62, 63). The volume that was once filled by the porogen is then left void, forming pores within the material. With knowledge of the density of both the material and porogen, the porosity can be predetermined by controlling the material to porogen weight ratio. This method, known as porogen leaching, is most easily accomplished by utilizing a water soluble porogen, such as salt, sugar, or gelatin particles, which can be removed by soaking the cured construct in water. The key to this method is that enough porogen must be incorporated so that the individual pores are in contact with one another, forming an interconnected pore structure within the material. An interconnected porosity is not only a requirement for the subsequent removal of the porogen, but also generally

necessary for a viable tissue engineering scaffold. The amount of porogen required for interconnectivity varies with the curing material and porogen, but generally exists when the construct is approximately 70 wt% porogen. Finally, the porogen method does have the advantage that pore interconnectivity can be determined by simply measuring the weight of the scaffold before and after the removal of the porogen; if the weight of porogen included within the scaffold is similar to the weight lost to porogen leaching, interconnectivity is generally assured.

A second general strategy for forming a porous structure involves the use of a gas to form pores within the curing material (33, 54, 57, 58). Conventionally, gases such as nitrogen or carbon dioxide are incorporated into the bulk material during its curing, either by purging the material with the gas or by forming gas as a product of a chemical reaction. Another method is the formation of frozen solvent bubbles, which are subsequently removed by sublimation, to form a porous structure within a curing material (30). Again, the key aspect to this strategy is the incorporation of sufficient gas volume so as to form an interconnected pore structure.

Recently, more elegant techniques have been developed so as to fabricate scaffolds with defined architectures. Up to this point in time, the methods most often used to create porous scaffolds, such as the ones described above, form a scaffold with a random architecture. This uncontrolled porous architecture has two downsides. First, it dramatically diminishes the mechanical properties of the scaffold from those of the material. This results in the need to fabricate materials of extremely high mechanical properties so that the resulting scaffold is suitable for bone tissue engineering applications, and thus limits the possible materials for this application. Second, and equally important, the uncontrolled porous architecture prevents serious investigation of the effects of scaffold architecture upon tissue formation, a issue of critical importance for bone tissue engineering. The leading methods of creating scaffolds of defined architecture involve rapid prototyping techniques such as three dimensional printing and stereolithography (37, 58, 64).

Characterization Techniques

Biocompatibility

The biocompatibility of a material is dependent on purity of the materials. Primarily the material must be found to be biocompatible, a concept that is simple in principle, but considerably

complicated in practice (31, 38, 59, 65). If the material is novel, initial work should involve cytotoxicity studies, initially with cell lines and then moving to primary cells of the tissue of interest. These studies investigate the changes in cellular phenotype when cultured (a) in media containing soluble products from the uncured material, (b) on the surface of the cured material, and (c) within the bulk of the material. Such work is typically followed with *in vivo* studies which examine the tissue response to the implanted material. A significant concern lies in the curing of the material, and specifically with the toxicity of the chemical species, as well as their reactive forms, that are involved with materials which are formed by polymerization or cross-linking, especially if this is intended to be accomplished *in vivo*. For this aspect of development, advantages lie in both limiting the number of species involved in the fabrication of a scaffold as well as fabricating scaffolds without the use of chemical reactions (i.e., by polymer chain entanglement). This is not to mean that chemically cross-linked scaffolds, for example, are always toxic, but simply that by increasing the complexity of the system, either by increasing the number of components or by including chemical reactions, will increase the likelihood of toxicity problems. Toxicity concerns should be addressed early, for later work would be fruitless if the material induces a significant inflammatory or immunological response.

Degradation

The next step in scaffold characterization should involve study of its degradation. Degradation studies may be conducted either *in vitro* or *in vivo*. *In vitro* studies, for hydrolytically degrading polymers, are typically performed with the scaffold immersed in phosphate buffered saline (pH 7.4) at 37°C. Enzymatically degrading polymers would require the functional enzyme to be included in the saline. The buffer solution is changed hourly in the beginning of the study and less frequently (daily to weekly) as the study progresses, with care taken that the acidity of the system does not significantly deviate from neutrality. Neutrality is typically demanded since most degrading polymers are polyesters and, as the reaction of water with ester bonds is catalyzed by acidic conditions, their acidic degradation products may thus catalyze the degradation of the scaffold. Since it is assumed that *in vivo* neutrality would be maintained, degradation studies are then typically performed in buffer saline. Results from *in vitro* studies clearly do not give direct information on the scaffolds implanted *in vivo*, but rather a baseline from which *in vivo* properties may be inferred. In general, the degradation of a scaffold is not going to occur at a slower rate *in vivo*

than *in vitro*. For more relevant information, an *in vivo* study is required. Here, scaffolds are implanted into a suitable animal model, typically subcutaneously, and then retrieved at predetermined time points. The difficulty with *in vivo* studies, beyond obvious obstacles, is that generally fewer properties may be monitored or significantly greater numbers of samples, and therefore animal models are required. The properties of interest throughout a degradation study are briefly discussed next.

Polymer Molecular Weight

The change in polymer molecular weight is a critical factor in describing the rate at which hydrolytic (or enzymatic) degradation is occurring. To analyze this property, the degrading scaffold is collected, dissolved into a solvent, and the polymer chains are analyzed by any of a variety of techniques, such as gel permeation chromatography. As the scaffold degrades, the mean molecular weight of the polymer chains that constitute the scaffold will decrease. The rate at which this occurs depends upon factors such as the molecular structure of the polymer, scaffold fabrication technique, and scaffold porous properties. This work is only relevant to gels that are formed into solids by chain entanglement, as the polymer chains remain individual molecules. When a material is cured by cross-linking of the polymer, the chains are no longer isolated molecules and thus their molecular weight can not be determined. Techniques such as Fourier transform infrared spectroscopy and solid-state nuclear magnetic resonance spectroscopy may be investigated as methods for monitoring the molecular changes involved in the degradation of a cross-linked network, but would depend heavily upon the molecular structure of the polymer of interest.

Scaffold Mass, Volume, and Water Absorption

Changes in the physical properties of the scaffold during degradation, while probably the most simple to carry out, are often the most informative. Changes in mass are measured simply by monitoring the weight change of the scaffolds throughout the study. Care must be taken to account for the porogen, if used in the fabrication of the scaffold, as well as for moisture from the degradation solution. Volume change is difficult to assess with a high level of precision.

Nevertheless, measurement of the scaffold's bulk dimensions, and their change during degradation, do clearly indicate if scaffold swells or disintegrates significantly during degradation. Finally, water absorption can be assessed by weighing the wet scaffold immediately after retrieval from the degradation study and then three days later after drying.

A question of critical importance is how quickly or slowly should a bone tissue engineering scaffold degrade (52, 54, 58). In general, the answer is not known and proposals, such as the ones described next, should be considered with a degree of caution. Fast degrading scaffolds, those that degrade within days to weeks after implantation, seem to be preferred clinically as they would allow for tissue growth into the porous volume and quick subsequent transfer of mechanical forces from the scaffold to the new tissue. On the other hand, slowly degrading scaffolds, those that degrade in many weeks to months, may be preferred because of tissue response issues. Specifically, the degradation products of the scaffold must be taken up by the host environment and, while some extent of inflammatory response will be associated with any degrading scaffold, a slow rate of their production should help to lower the adverse response. Certainly the metabolic activity of the surrounding tissue would influence the outcome, with bone tissue which undergoes remodeling at a high rate probably accepting higher scaffold degradation rates.

Another question of interest concerns the type of degradation: surface or bulk (40, 66). Surface degradation typically involves a material which absorbs little water and therefore degrades only at the interface between the material's surface and the surrounding water. Surface degradation can be observed experimentally by a degrading scaffold whose dimensions slowly decrease while its mechanical properties are generally retained, until a critical point where both fall dramatically. Bulk degradation, on the other hand, involves a material which can absorb water and thus degrades throughout its entire volume. Bulk degradation is observed experimentally by a degrading scaffold whose dimensions are retained, but whose mechanical properties decrease. Whether bulk or surface degradation is preferred is unclear. Surface degradation may be preferred because the scaffold's mechanical properties need to be retained, while bulk degradation may be preferred because the maintenance of the scaffold's surface facilitates enhanced cell adhesion and tissue response. Finally, it should be noted that scaffold degradation in practice is most likely not due either to surface degradation or bulk degradation, but a mechanism that lies somewhere between these two extremes.

Scaffold Porosity

Prior to a discussion of scaffold porosity, it should be made clear that two levels of porosity exist within porous polymer scaffolds. Solid polymeric materials contain a porosity (often known as microporosity) that describes the volumes that are not occupied by individual polymer chains. The size of this porosity is generally on the scale of nanometers to micrometers. For example, in hydrogels, this volume is defined by polymer chains and filled with water. For tissue engineering applications, microporosity is generally only of interest in hydrogels, as opposed to conventional polymer gels, for the aqueous environments of hydrogels allow for cell migration and protein diffusion. In addition to microporosity, polymer gels can possess a porosity on the scale of micrometers to millimeters, similar to the porosity of a common sponge. To differentiate it from microporosity, this size of porosity can be referred to as macroporosity, but it is commonly known as simply porosity and is the subject of the following discussion.

Information about the porous structure of a tissue engineering scaffold can actually provide a number of different parameters that are of interest, with the most notable including porous volume (volume of void space defined by the scaffold), skeletal volume (volume of material contained in the scaffold), porosity (the percent of porous volume when compared to total volume), pore size (the average size of the pores), and surface area (area of the surface of the scaffold). Scaffold porosity is traditionally measured by intrusion or adsorption techniques, and most commonly by mercury porosimetry. In this measurement, the porous volume of a scaffold under vacuum is filled with mercury by application of pressurized nitrogen. (Note that the need to place the scaffold into a vacuum often creates practical problems for some scaffolds, such as macroporous hydrogels.) The total volume of mercury intruded into the scaffold determines the porous volume of the scaffold; with knowledge of the scaffold mass and the material's density, porosity can then be calculated. Furthermore, the intruded volume as a function of pressure can provide information on pore size and surface area, though the applicability of the assumptions underlying these theories to the porous architectures described here should be considered carefully.

Advances in imaging techniques have provided alternate methods for determining scaffold porosity (67, 68). Micro-computed tomography (μCT) allows for creation of a three dimensional image of the

entire scaffold, from which not only porosity, but also skeletal volume, porous volume, and surface area may be determined. While the images of scaffolds are often stunning, both the monetary and time costs required for µCT currently prevents its wider use. Alternatively, image analysis may be utilized to determine porosity. Here, a thin section of the scaffold is first obtained, typically by histological techniques. Using an image analysis software, the two dimensional porous void area is compared to the total area to determine porosity. This method is especially useful for studying scaffolds that have been implanted in animal models and subsequently prepared for histological analysis. An advantage of porosimetry over imaging methods, however, is that since it physically fills the pores of the scaffold, it only measures pores that are connected by an open path to the surface and thus can assess pore interconnectivity, while this is more difficult to resolve with imaging methods.

The proper porosity and pore size for a bone tissue engineering scaffold remains unclear. There has been a number of investigations which sought to identify the proper pore size for a tissue engineering scaffold, with results showing that pores ranging from 80 to 500 µm to be viable (69). For the example of a rabbit cranial defect model, one study found scaffold porosity (ranging from 57% to 75%) and pore size (either 300 - 500 µm or 600 - 800 µm) to have little effect on tissue response and bone formation (38), while another study found that pore size less than or equal to 350 µm produced the most bone ingrowth (70). Finally, it has been suggested by some that blood clots may promote bone formation in a defect and, furthermore, that the ability of a material to retain a blood clot may be critical for proper bone regeneration within a tissue engineering strategy (71, 72).

Mechanical Strength

Mechanical strength studies are required to provide information on the ability of a scaffold to resist mechanical forces in the implanted environment as well as to support surrounding tissue, especially important for bone tissue engineering applications. Typically, compressive mechanical tests are performed. These tests, which require cylindrical samples whose height is twice the diameter, monitor the force required to compress the sample so as to determine parameters such as modulus and strength to fracture. In general, the material should be tested first as a solid so that the material properties may be determined, and then subsequent tests with porous materials performed. Studies on porous scaffolds should be conducted on dry scaffolds as well as on scaffolds which have been

soaked in an appropriate solution, as wet scaffolds may have significantly different mechanical properties.

While the mechanical properties of tissue engineering scaffolds can vary significantly, even for bone applications, general guidelines are the properties of bulk, trabecular bone: 50 – 100 MPa modulus and 5 – 10 MPa fracture strength (73). The focus, however, should be the scaffold strength required to promote wound healing and the formation of bone tissue. Whether this requires scaffolds with properties above, equal, or below those of trabecular bone is still unclear. Finally, the primary reason that bone tissue engineering scaffolds often possess poor mechanical properties is the random nature of their porous architecture. In general, the bulk materials possess significant mechanical properties, but these properties are lost when the material is formed into a porous scaffold using techniques such as porogen inclusion. This clearly indicates that more elegant techniques described earlier, such as rapid prototyping and stereolithography, could significantly advance the development of novel bone tissue engineering scaffolds.

The controlled release of morphogenes and growth factors from biodegradable polymers of poly (DL-lactic-co glycolyic acid, PLGA) and polyethylene glycol (PEG) is a critical area for tissue engineering (74). Biodegradable block copolymers of PLGA and PEG are optimal delivery systems for BMP2 (75). Recombinant BMP4 and purified BMP3 bind to types I and IV collagen and heparin (21). A comparison of several delivery systems indicated collagen is the most optimal delivery system for bone induction (76). It is likely in the native demineralized bone matrix BMPs are bound to collagenous extracellular matrix scaffolding. The role of the biomimetic material in the delivery of recombinant BMPs for bone tissue engineering is critically dependent on the pharmacokinetics of BMP release (77). The local retention of BMPs by biomimetic materials such as collagen sponges, hydroxyapatite, or composites of collagen and hydroxyapatite may have profound influence on the osteoinduction by a tissue engineering device. Cells may be transplanted in various matrices (78).

Stem Cells

Mesenchymal stem cells derived from mesoderm are the common progenitors for the various lineages of the musculoskeletal system such as bone, cartilage, ligaments, muscle and tendon. The exciting advances in stem cell biology is opportune for the introduction of BMP genes by gene therapy into responding stem cells. The fundamental work of Friedenstein and Owen (79, 80) laid the foundations for recent excitement in bone marrow-derived mesenchymal stem cells for bone tissue engineering (81, 82).

The characterization of stem cells including unique markers will permit isolation by fluorescent-activated cell sorters (FACs). These isolated stem cells can be transduced by gene therapy (83, 84). Thus stem cell and BMP gene therapy in combination is a platform which can be applied to other tissues beyond bone in tissue engineering. Refinement of viral and non-viral vectors and novel physical techniques including electroporation, sonoporation of plasmid DNA into cells may enhance the efficiency and efficacy of gene therapy for bone tissue engineering.

Clinical Applications of BMPs

The proof of concept that an osteoinductive composite of BMPs and scaffolding can be used to fabricate a tissue engineered bone was demonstrated (85). In this experiment a vascularized muscle flap was placed in a mold mimicking the head of the femur of rat and was injected with BMPs and collagenous matrix. It is noteworthy that a true transformation of muscle into bone mirroring the shape of the femur was accomplished demonstrating the proof of principle for tissue engineering of bone (85). The outstanding regenerative potential of bone is common knowledge. However, in the repair of massive segmental bone loss due to tumors, trauma or fractures due to metabolic diseases such as diabetes and osteoporosis, it is common orthopaedic practice to aid and abet the healing site with autogenous bone graft. The limited supply of autograft bone, the associated donor site

morbidity (86) including infections and pain is a major challenge. The availability of recombinant BMPs and biomimetic biomaterials and stem cells has set the optimal stage for tissue engineering to enter the operating suites in orthopaedic surgery.

An auspicious beginning was made by the use of BMP 7 in treatment of tibial nonunions (87, 88). In addition to orthopaedics BMPs have been used in clinical dentistry in the realms of maxillofacial surgery, bone augmentation, and integration of dental implants (89, 90). Despite these positive advances, many clinical challenges remain. In addition to optimization of the dose of BMPs, pharmacokinetics of release, the optimal delivery from biomimetic biomaterials and the optimal sterilization including irradiation need to be investigated (91, 92). The recent approval by the Food and Drug Administration of recombinant BMP2 for spine fusion appears to be the first use of a recombinant human morphogen in orthopaedic surgery for tissue engineering of bone.

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