RICE UNIVERSITY

Targeted Delivery of Osteogenic Drugs for Bone Tissue Engineering

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Abstract

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To create a more efficient and effective method of osteogenic drug delivery in vivo, drugs were modified with high calcium affinity moieties including pamidronate, poly(aspartic acid), and poly(glutamic acid). To test the initial hypothesis that modified drugs can demonstrate the same bone binding capabilities of pamidronate, poly(aspartic acid), and poly(glutamic acid), these motifs were conjugated to model peptides and exhibited high affinity to hydroxyapatite (HA).

An in vitro controlled release experiment was conducted for native and modified TP508. Native and modified TP508 drugs were loaded in PLGA-PEG microparticles. Porous PPF scaffolds were injected with these drug-loaded particles, and in some instances with HA microparticles (20-50 or 50-100 μm). Less mineral surface resulted in less binding of drugs after release from the PLGA-PEG carriers and therefore a greater release than with the large HA particles. A final study was performed in the presence of 383 ng/mL collagenase,
which cleaved the TP508 from the bone-binding domains at the point of the degradable peptide linker sequence.

The dose effect of TP508 was established by delivering 0, 25, 50, and 100 μg TP508 loaded into PPF scaffolds and implanted in a sized rat cranial defect. After 4 weeks, microCT analysis of the skulls revealed a statistically significant increase in bone formation for the 50 μg dose compared to controls and the 25 μg dose. Based on these findings, an equivalent of 50 μg TP508 or modified drugs were delivered from PLGA-PEG microspheres in the presence of 20-50 μm HA microparticles in the PPF scaffolds' pore network, which revealed no significant differences between drug groups. These results were promising in that this strategy of drug modification had no apparent negative effect on the bioactivity of TP508. Another finding of this work was that the incorporation of HA into PPF composites resulted in significantly greater bone formation, even after subtraction of the initial amount of HA. The addition of this osteoconductive material stimulated an increase in new bone over 4 weeks for both the control and 50 μg TP508 groups.
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List of Abbreviations

Agg: Aggrecan peptide sequence, IPENFFGV

Asp₈: Eight consecutive aspartic acid residues

BP: Bisphosphonate

ECM: Extracelluar matrix

Glu₈: Eight consecutive glutamic acid residues

HA: Hydroxyapatite, the mineral component of bone, Ca₁₀(PO₄)₆(OH)₂

MicroCT: Microcomputed tomography, a technique used to evaluate the three-dimensional structure of radio-opaque samples.

Mₐ: Number average molecular weight

Mₘ: Weight average molecular weight

Pam: Pamidronate, a nitrogen-containing bisphosphonate

PEG: Poly(ethylene glycol), also known as poly(ethylene oxide)

PLGA: Poly(lactic-co-glycolic acid)

Poly(Asp): Consecutive aspartic acid residues, typically numbering six or eight

Poly(Glu): Consecutive glutamic acid residues, typically numbering six or eight

PPF: Poly(propylene fumarate), a hydrophobic polymer used in orthopedic tissue engineering applications
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Chapter 1. Introduction

I. Biology of Bone

The two types of bone, cortical and trabecular, are composed of the same matrix materials and a woven or lamellar structure. Healthy, mature bone is comprised of hematopoietic marrow surrounded by lamellar tissue and periosteum, the fibrous membrane surrounding bone. Woven bone tissue consists of collagen fibers randomly weaved together, while the lamellar tissue comes in tight, parallel sheets of collagen. Woven bone is typically observed in embryonic development and wound repair [1]. The lamellar bone is made up of cells and extracellular matrix (ECM). The ECM accounts for over 90% of bone volume and is responsible for the acquisition of bioactive molecules as well as mechanical strength [2]. Bone ECM is a composite of mineral (hydroxyapatite) and organic molecules (mostly type I collagen).

The cells in bones arise from either mesenchymal stem cells (MSCs) or hematopoietic stem cells (HSCs). Totally undifferentiated MSCs have the ability to turn into osteoblasts (bone forming cells). Mature osteoblasts encompassed with ECM become osteocytes. More than 90% of bone cells are osteocytes. HSCs can differentiate into osteoclast (bone resorbing cells). Osteoclasts are responsible for the degradation of ECM and demineralization of bone.
II. Bone Formation and Repair

Bone formation arises via intramembranous ossification, endochondral ossification, or apposition. In intramembranous ossification, the MSCs change into osteoprogenitor cells that exude bone matrix. Upon further differentiation, the cells become osteoblasts and deposit new ECM [3]. Endochondral ossification involves MSCs differentiating into chondrocytes that deposit a cartilaginous matrix. After mineralization within the matrix, there is a penetration of blood vessels. Blood transports osteoprogenitor cells to the matrix, which differentiate into osteoblasts to continue the formation of bone ECM. Apposition is simply the formation of new bone matrix on the surface from existing osteoblasts within the bone [4].

The three typical stages of bone healing are inflammation, repair, and remodeling. In the inflammation phase, a hematoma is created and there is an acute inflammatory response. This acute response can happen after minutes to days. Edema and the migration of macrophages and neutrophils to the wound site are characteristic of acute inflammation [5]. The macrophages and neutrophils begin the process of chemotaxis, secreting growth factors that recruit other cells and promote the proliferation of MSCs. During angiogenesis, new blood vessels sprout from existing nearby vessels and penetrate the granulated tissue of the wound site. During repair, more MSCs and osteoblasts arrive to create a repair blastema. If the fracture is mechanically stable, MSCs can differentiate to
osteoblasts directly \[^6\]. The new bone deposited by these cells forms a hard callus. Typically the MSCs become chondrocytes, secreting collagen that helps form a soft callus \[^5\]. After endochondral ossification, this cartilaginous tissue gives rise to new bone. The hard bony callus forms permanent lamellar bone via standard remodeling.

In some instances, fractured bone fails to fully heal and is known as a non-union. Non-unions great enough to never heal and rejoin are termed “critically sized defects” and are an interesting subject to the field of tissue engineering. Often, non-unions are due to insufficient vascularity from uninitiated or poor angiogenesis \[^7\]. Age, gender, prior injuries at or near the site of fracture, location/structure of the bone in question, disease, and other medication/treatments (e.g. cancer treatments such as radiation and chemotherapy) can affect the formation of new blood vessels and the repair of bone \[^8\].
III. Bone Tissue Engineering

Due to several aspects of modern society, including advanced medical technology and longer life expectancy, there is an ever-present need for organ and tissue replacements, either donated from another person (allografts) or synthetically produced via tissue engineering. In the field of tissue engineering, the central paradigm consists of combining cells, desirable growth factors, and a scaffold to direct growth into mature replacement tissue. The cells involved can migrate into the scaffold from surrounding tissues or be provided within the scaffold after culturing and encapsulation. The choice of growth factor is specific to the tissue and cell type. Not only must one choose the correct growth factor(s), but also the delivery mechanism and rate of each growth factor plays a critical role in the differentiation of cells and development of new tissue.

In the specific realm of bone tissue engineering, growth factors are typically selected which can induce osteogenesis (bone formation) and/or angiogenesis (blood vessel formation). Osteogenic factors include several from the transforming growth factor-beta (TGF-β) family, especially bone morphogenic proteins (e.g. BMP-2) [9]. These proteins can aid in the differentiation of mesenchymal stem cells into osteoblasts that build new bone tissue. They are also capable of enhancing the production of other bone-forming proteins in differentiating and mature bone cells. There exists a spectrum of angiogenic growth factors in tissue engineering, including the fibroblast growth
factors (FGF) and vascular endothelial growth factor (VEGF) [10, 11]. Angiogenic factors can enhance the number, size, and growth rate of new blood vessels within the healing tissue defect. A promising new drug in this arena is thrombin peptide 508, which will be discussed later.

The selection of a scaffold material is also critical in developing quality, healthy tissue. Ideally, the material (and its byproducts) would be biocompatible, biodegradable, possess mechanical properties comparable to that of the native tissue, and have degradation rates similar to the rate of new tissue formation. For materials containing growth factors, controlled release of the drug from the polymer is also essential. Scaffolds are typically made of polymeric materials, either in solid or gel-like (hydrogel) forms.

IV. DRUG DELIVERY

A key component of the “tissue engineering paradigm” remains growth factors influential in the recruitment, proliferation, and/or differentiation of cells within the tissue engineering construct. A controlled release system is desirable to dictate the supply of growth factors at different stages of tissue regeneration. The earliest drug delivery systems focused on encapsulating drugs within biodegradable polymer microparticles (e.g. PLGA) [12]. PLGA has an
established degradation pattern dependent on molecular weight and composition and has been approved by the U.S. Food and Drug Administration for use in humans. More recent work has focused on the delivery of growth factors and DNA from gelatin microparticles [13, 14]. Gelatin can be advantageous because it is a more natural material and its degradation is controlled by enzymatic degradation by proteins from nearby cells. Other methods include drug entrapment within a hydrogel network or the hydrophobic polymer scaffold itself [15, 16].

V. References


Chapter 2. Biomaterials for Tissue Engineering

I. Background of Biomaterials

The field of biomaterials, or materials used for medical or biological applications, is an emerging area of biotechnology key in the development of new treatments for a wide array of ailments. Applications include bone substitutes and cement, dental implants and fixatives, synthetic ligaments and tendons, blood vessels, heart valves, skin replacements, and contact lenses. Biomaterials used for tissue engineering typically require three key characteristics: biocompatibility, biodegradability, and/or bioactivity. Biocompatibility refers to the compliance of materials with organs, tissues, or cells. Although there are numerous materials with mechanical or degradative properties consistent with various bio-applications, many synthetic materials are toxic to living organisms and not feasible for use in tissue regeneration or drug delivery. Biodegradability describes the controlled or predictable degradation of materials in vivo. While some materials may be designed as a permanent implant, optimal tissue regeneration occurs as the material resorbs at rates equivalent to new tissue formation that effectively replaces the synthetic material. Bioactivity refers to the induction of a biological response of implanted or recruited cells to chemical or molecular signals on the surface of the material. Some materials possess intrinsic bioactivity while others are supplemented by the conjugation to
or release of bioactive molecules that stimulate cells to proliferate, differentiate, or attach to the implant.

II. Poly(Propylene Fumarate)

For bone tissue engineering, materials are sought that conduct bone growth, withstand mechanical forces comparable to those of native bone, and degrade on the order of weeks to months. One such promising polymer is poly(propylene fumarate) (PPF), an unsaturated linear polyester that hydrolytically degrades into fumaric acid and propylene glycol, both biocompatible byproducts [1]. A viscous liquid at room temperature, it can be injected and crosslinked in situ to form a solid and sturdy implant within a regular or irregular bone defect, thus minimizing the need for an invasive surgical procedure.

A transesterification reaction of diethyl fumarate and propylene glycol yield the linear PPF material, the molecular weight of which is determined by the reaction conditions (temperature, time, catalysts, etc.) [2]. The chemical structure of PPF is shown in Figure 2.1. A unique advantage of PPF over other polymers is the double bond present in the fumaric acid repeat unit. These double bonds can be used to crosslink the polymer with other chains of PPF or various
molecules possessing double bonds mixed into the *in situ* crosslinking solution [3]. This provides scientists and doctors the ability to crosslink PPF with more hydrophilic polymers such as poly(ethylene glycol), the resultant having potential for soft tissue applications.

![Structure of PPF](image)

**FIGURE 2.1. STRUCTURE OF PPF**

Composites of PPF and other materials have been examined thoroughly *in vitro* and *in vivo* for drug delivery and tissue engineering applications. Compounds such as single-walled carbon nanotubes and functional alumoxane have been chemically incorporated into PPF composites to provide additional mechanical strength to the material [4, 5]. A copolymer of PPF and PEG was developed for vascular tissue engineering [6]. Other compilations have been produced for ocular implants and drug delivery systems [7, 8].
III. Poly(lactic-co-glycolic acid) Polymers

Over 30 years ago, scientists began working with polymers composed of glycolic and lactic acids. Both glycolic and lactic acids appear naturally in the body as metabolic intermediates [9]. They are readily polymerized into linear, aliphatic polyesters. In fact, poly(glycolic acid) (PGA) is the simplest of all polyesters. PGA was utilized to produce the first completely biodegradable sutures. To adjust the properties of the polymer, PGA was copolymerized with poly(lactic acid) (PLA) to form poly(lactic-co-glycolic acid) (PLGA). PLA is more hydrophobic than PGA, limiting the hydrolytic degradation along the chain backbone. However, incorporation of PLA into the three-dimensional polymer networks decreases the crystallinity and creates unnatural geometries. These structural changes can lead to higher rates of hydrolysis. Strangely, there is no linear correlation between the biological, degradative, and mechanical properties of PLGA copolymers [10].

\[
\begin{align*}
\text{Poly(glycolic acid)} & \quad \text{Poly(lactic acid)} \\
\begin{array}{c}
\text{CH}_2 \quad \text{C} \quad \text{O} \\
\end{array} & \quad \\
\begin{array}{c}
\text{CH} \quad \text{C} \quad \text{O} \\
\text{CH}_3
\end{array}
\end{align*}
\]
Figure 2.2. Chemical Structures of PGA, PLA, and PLGA Polymers

After undergoing hydrolysis, PLGA breaks down into lactic and glycolic acid, which are easily excreted by the body. A less than desirable result of the degradation is a decrease in the local pH due to the acid formation. PLGA dissolution can often lead to an inflammatory response, but not radically different than the response of the immune system to other synthetic polymers.

As previously discussed, PLGA is a linear polyester that undergoes hydrolytic degradation at the ester bond site. The most relevant chemical property of the polymer is the hydrophilicity of PGA and hydrophobicity of PLA components. Use of a 50:50 ratio tends to yield the greatest degradation rate due to poor crystallinity, which exposes more of the glycolic acid residues to water [9]. Three-dimensional scaffolds do not require crosslinking, as there is a high adhesion among chains. These structures can be formed by a number of methods, which will be discussed later. Other chemicals are often incorporated into PLGA, such as poly(ethylene glycol) (PEG). These compounds can improve hydrophilicity and biocompatibility to the polymer network. PEG is hydrophilic
and relatively non-toxic (toxicity decreases as molecular weight increases). Further, PEG can be functionalized for additional covalent modification of the scaffold, a property not available to pure PLGA.

The biodegradation of PLGA scaffolds depends largely on the composition, molecular weight, and local physiological conditions. Increased lactic acid moiety results in slowed degradation due to the mentioned hydrophobic interactions. Scaffolds composed of high molecular weight PLGA actually degrade faster, as the average chain molecular weight decreases quickly [11]. Polymer scaffolds typically degrade by either surface or bulk erosion. In acidic and near-neutral environments, PLGA degrades via bulk erosion by acid autocatalysis. As water permeates the polymer, hydrolysis occurs producing acidic byproducts. These products propagate the reaction while increasing osmotic pressure. The pressure flux causes swelling in the polymer and more higher water permeation. Conversely in basic conditions (pH > 13), hydrolysis on the surface occurs much faster than water can penetrate the polymer, leading to surface erosion. Physiological conditions (pH = 7.4) dictate bulk erosion to be the most likely form of degradation in vivo [12]. Figure 2.3 illustrates the bulk degradation of PLGA films at pH 5.0, 7.4, and 9.24.
Figure 2.3. Weight-average Molecular Weight of PLGA (50:50) vs. Time at different pH [11]

Crystalline forms of PLGA have the mechanical strength required for bone screws and other orthopedic fixative devices [13]. However, processing technique ultimately determines the bulk of the mechanical properties of the polymer. Continuous scaffolds of porosity up to 94% can support stresses of $10^4$ Pa, while scaffolds made by fiber-weaving techniques may only resist $10^2$ Pa [14]. Inclusion of minerals, glass, or metal also provides further mechanical strength to PLGA scaffolds. PLGA loaded with calcium phosphate (1:2 weight ratio) exhibits compressive strengths ranging from 4-16 $10^4$ Pa [15].
The positive biological effects of PLGA products are well documented. Culture of hepatocytes on PLGA mesh scaffolds showed high cell attachment and interaction [16]. Osteoblasts, chondrocytes, fibroblasts, and intestinal epithelial and smooth muscle cells have all shown to be capable of culture and transplantation on PLGA constructs [17]. In vivo studies have shown that PLGA is conducive for tissue regeneration in bone [18] and articular cartilage [19]. This is convenient given the desirable mechanical properties of PLGA for these high-stress tissues. As noted, PLGA-PEG polymers can also be modified with proteins conducive of cell attachment, growth, and/or differentiation.

PLGA blends are also used to form microparticles, which can encapsulate proteins and/or DNA for drug or gene delivery. Because the degradation of PLGA can be controlled with lactic to glycolic acid ratio and the inclusion of other chemical motifs, the microparticles are tailored to the desired release profile of the drug/DNA. The most common method of PLGA microparticle production is by water-in-oil-in-water double emulsion. This involves emulsifying a drug/DNA aqueous solution with an organic phase (typically methylene chloride) and PLGA/PLGA-PEG, sonication of the first emulsion, then addition to a dilute alcohol in water solution. By adjusting parameters such as temperature, concentration of polymer and drug, and the ratio of phase volumes, one can control the size and weight of microparticles [20].
The future of PLGA biomaterials lies in drug delivery and tissue engineering. PLGA composite microparticles, loaded with proteins or DNA, are effective for controlled delivery as the polymer erodes. These microparticles can be used independently at a defect site or incorporated within another structure (e.g. polymer or metal scaffold) [20]. Porous PLGA scaffolds can be fabricated into the proper architecture for guided tissue replacement. The molding of specific formulations of PLGA (with appropriate mechanical and degradative properties) can produce complex structures, such as ear and nose cartilage, heart valves, and blood vessels [17].

IV. References


Chapter 3. Polymer Scaffold Fabrication for Tissue Engineering Applications

Matthew B. Murphy and Antonios G. Mikos

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I. INTRODUCTION

In the modern age of medicine, tissue engineering has become a viable option for the replacement of tissue and organ function. The creation of such substitutes requires a three-dimensional, porous, biocompatible, and preferably biodegradable scaffold. Tissue engineering scaffolds should have geometries that direct new tissue formation and mass transport properties sufficient for the exchange of biological nutrients and waste. The scaffolds also provide temporary mechanical support to the regenerating tissue. They must degrade into
biocompatible byproducts, ideally on a time scale comparable to that of new tissue development. Such scaffolds are typically fabricated with biocompatible polymers, proteins, peptides, and inorganic materials. Aside from the properties of the raw material, the major factor determining the final scaffold characteristics is the fabrication technique utilized to produce the scaffold. Mechanical strength, porosity, degradation rates, surface chemistry, and the ability to incorporate biologically active molecules are all aspects affected by the manner of fabrication. This chapter discusses many established processing and fabrication methods using various polymeric components, including fiber bonding, electrostatic fiber spinning, solvent casting and particulate leaching, melt molding, membrane lamination, extrusion, freeze-drying, phase separation, high-internal-phase emulsion, gas foaming, polymer/ceramic composite fabrication, rapid-prototyping, peptide self-assembly, and in situ polymerization.

In an era of decreasing availability of organs for transplantation and a growing need for suitable replacements, the emerging field of tissue engineering gives hope to patients who desperately require tissue and organ substitutes. Scaffolding is essential in this endeavor to act as a three-dimensional template for tissue ingrowth by mimicking the extracellular matrix (ECM) for cell adhesion and proliferation (Freed et al., 1994). Since the mid-1980s, researchers have developed many novel techniques to shape polymers into complex architectures that exhibit the desired properties for specific tissue-engineering applications. These fabrication techniques result in reproducible scaffolds for the regeneration
of specific tissues. Polymer scaffolds can provide mechanical strength, interconnected porosity and surface area, varying surface chemistry, and unique geometries to direct tissue regeneration (Hutmacher, 2001). These key scaffold characteristics can be tailored to the application by careful selection of the polymers, additional scaffold components, and the fabrication technique.

Patient safety is the paramount concern for any tissue engineering application. The bulk material and degradation products of the scaffold must be biocompatible and clearable by the body. It is equally critical that the elected processing strategy not affect the biocompatibility and biodegradability of the scaffolding materials. Restoring the function of a tissue or replacing an organ entirely requires a porous scaffold that degrades on an appropriate time scale so that the new tissue replaces the resorbing scaffold. The primary function of the scaffold is to direct the growth and migration of cells from surrounding tissues into the defect or to facilitate the growth of cells seeded into the scaffold prior to implantation. Surface chemistry favorable to cell attachment and proliferation is desirable. Large pore diameters and high pore interconnectivity are essential for confluent tissue formation, transport of nutrients and metabolic wastes, and sufficient vascularization of the new tissue. Increased porosity and pore diameter can result in increased surface-area-to-volume ratios within the scaffold or more surfaces for cell adhesion. Control over the scaffold's size and shape provides increased utility for differing tissue engineering applications.
The mechanical properties of a scaffold arise from a combination of the properties of the bulk polymer, the geometry of the scaffold, incorporation of strength enhancing materials, and the scaffold fabrication technique. For example, polymers with higher crystallinity exhibit increased tensile strength at the expense of slower degradation rates. Processing methods that reduce crystallinity or the molecular weight of polymer chains diminish the strength of the scaffold and reduce the scaffold’s lifetime. Elevated mechanical strength is preferable in the regeneration of load-bearing tissues such as bone and cartilage. Mechanical stimulation via force transduction can be beneficial in the differentiation of many cell types (Tan et al., 1996). While hydrophobic polymers typically offer greater mechanical properties, adsorbing proteins may become denatured through interaction with the surface (Gray, 2004). Typical materials utilized in tissue engineering scaffolds include synthetic polymers [e.g., poly(glycolic acid) (PGA), poly(l-lactic acid) (PLLA), poly(d,l-lactic-co-glycolic acid) (PLGA) copolymers, poly(ε-caprolactone) (PCL), and ethylene glycol-based copolymers], natural polymers (e.g., collagens, gelatins, fibrin, carbohydrates, peptides, and nucleic acids), and inorganic materials (e.g., hydroxyapatite, tricalcium phosphate, and titanium).

The inclusion of bioactive molecules is another major consideration in the design of porous scaffolds. Bioactive molecules include proteins, ECM-like peptides, and DNA. Because the bioactive molecules are incorporated for cell adhesion, cell signaling, or drug/gene delivery, fabrication techniques that do not
inactivate the molecules must be utilized. Local drug and gene delivery to promote cell migration, proliferation, and differentiation is an enormous tool to improve the required time and quality of tissue regeneration (Jang et al., 2004).

The fabrication technique for tissue-engineering scaffolds depends almost entirely on the bulk and surface properties of the material and the proposed function of the scaffold. However, the cost and time of manufacturing scaffolds must be considered for the viability of patient treatment. Most techniques involve the application of heat and/or pressure to the polymer or dissolving it in an organic solvent to mold the material into its desired shape. Evolving techniques have been studied that reduce potentially harsh conditions of older scaffold fabrication schemes to protect incorporated cells and bioactive molecules. While each method presents distinct advantages and disadvantages, the appropriate technique must be selected to meet the requirements for the specific type of tissue.

II. FIBER BONDING

Polymer fibers exhibit an excellent surface-area-to-volume ratio for enhanced cell attachment, making them a viable option as a scaffold material. The earliest tissue engineering scaffolds were fiber mesh, nonbonded PGA tassels or felts that lacked the mechanical integrity to be used for in vivo organ
regeneration (Cima et al., 1991). To overcome this problem, fiber-bonding techniques were developed to bind the fibers together at points of intersection.

The original examples of fiber-bonded scaffolds used PGA and PLLA polymers (Mikos et al., 1993a). Briefly, PGA fibers are arranged in a nonwoven mesh. At temperatures above the melting point of the polymer, the fibers will bond at their contact points. To prevent a structural collapse of the melting polymer, PGA fibers are encapsulated prior to heat treatment. PLLA, dissolved in methylene chloride (not a solvent for PGA), is cast over the meshed fibers and dried, resulting in a PGA–PLLA composite matrix. After heat treatment and fiber bonding, the PLLA is dissolved in methylene chloride and the solvent is removed from the scaffold by vacuum drying. Another method involves rotating a nonwoven PGA fiber mesh while spraying it with an atomized PLLA or PLGA solution (Mooney et al., 1996a). The polymer solution builds up on the PGA fibers and bonds them at contact points. This method provides the mechanical properties of PGA while exposing cells to the surface properties of PLLA or PLGA. This method is excellent for producing tubular structures, but it lacks the ability to create complex three-dimensional structures and increases the original fiber diameter. Similar methods exist for other biocompatible polymer fibers. The fiber-bonding scaffold fabrication technique is desirable for its simplicity, the retention of the PGA fibers' original properties, the use of only biocompatible materials, and the structural advantages over tassel or felt arrangements. The drawbacks of fiber bonding are the lack of control over porosity and pore size, the
availability of suitable solvents, immiscibility of the two polymers in the melt state, and the required relative melting temperatures of the polymers

III. ELECTROSPINNING

A modern method for creating porous scaffolds composed of nano- and microscale biodegradable fibers employs electrostatic fiber spinning, or electrospinning, a technology derived from the electrostatic spraying of polymer coatings. Electrospinning fabricates highly porous scaffolds of nonwoven and ultrafine fibers. Many biocompatible polymers, including PGA, PLGA, and PCL, can be electrospun into scaffolds of nanofibers with porosities greater than 90% (Yoshimoto et al., 2003). Scaffolds are prepared by dissolving the selected polymer in an appropriate solvent (e.g., PCL in chloroform). The polymer solution is loaded into a syringe and then expelled through a metal capillary at a constant rate via syringe pump. A high voltage (10–15 kV) is applied to the capillary, charging the polymer and ejecting it toward a grounded collecting surface. As the thin fibers assemble on the plate, the solvent evaporates, leaving a nonwoven porous scaffold. Fiber thickness, scaffold diameter, and average pore diameter are adjusted by factors including polymer concentration, choice of solvent, ejection rate, applied voltage, capillary diameter, collecting plate material, and the distance between the capillary and the collecting plate. Examples of electrospun P(LLA-CL) fiber meshes are shown in Figure 3.1.
Electrospun scaffolds exhibit promise in mesenchymal stem cell culture for bone and cartilage tissue engineering (Pham et al., 2006).

**Figure 3.1.** Scanning electron micrographs of P(LLA-CL) fibers electrospun at an applied voltage of 12 kV from different polymer concentration solutions: (A) 3 wt.%; (B) 5 wt.%; (C) 7 wt.%; (D) 9 wt.%. Reprinted from X. M. Mo, C. Y. Xu, M. Kotaki, and S. Ramakrishna (2004), Electrospun P(LLA-CL) nanofiber: a biomimetic extracellular matrix for smooth muscle cell and endothelial cell proliferation, *Biomaterials* 25, pp. 1883–1890. Copyright 2003, with permission of Elsevier Science.
IV. SOLVENT CASTING AND PARTICULATE LEACHING

For enhanced control over porosity and pore diameter as compared to most fabrication methods, a solvent-casting and particulate-leaching technique was developed. With careful system selection, porous scaffolds can be manufactured with specific pore size, porosity, surface-area to volume ratio, and crystallinity. This technique involves casting a dissolved polymer around a suitable porogen, drying and solidifying the polymer, and leaching out the porogen to yield a polymer scaffold with an interconnected porous network. Early systems utilized PLLA and PLGA polymers with sieved salt particles as a porogen (Mikos et al., 1994). To adjust the crystallinity, the composite material is heated above the polymer melting temperature and annealed at the appropriate rate prior to porogen leaching. Afterwards, the composite is immersed in water to remove the salt particles, leaving a porous PLLA membrane. Similar techniques have utilized alternative biocompatible porogens, such as sugars (Holy et al., 1999) and lipids (Hacker et al., 2003). A solvent exchange system, where the second organic phase dissolves the porogen but is a nonsolvent for the polymer, eliminates the traditional leaching step and presents an advantage in the total leaching time required.
For polymers preloaded with bioactive molecules, salt leaching can remove molecules or decrease their bioactivity during the leaching process. This technique can produce scaffolds with controlled porosity (up to 93%), pore size (up to 500 μm), and crystallinity. By adjusting the fabrication parameters and the type, amount, and size of porogen, porous scaffolds can be tailored to the tissue-engineering application of interest. The primary advantage to this technique is the relatively small amount of polymer required to create a scaffold. PLGA and poly(ethylene glycol) (PEG) blends have been utilized to produce porous foams with the solvent casting and particulate leaching technique that are less brittle and more suitable for soft-tissue regeneration (Wake et al., 1996). To overcome problems with cell seeding due to the polymer’s hydrophobicity, scaffolds can be prewetted using ethanol (Mikos et al., 1994). The scaffolds are submerged first in ethanol, followed by water. Prewet scaffolds show higher cell attachment for chondrocytes and hepatocytes. As an alternative, PLGA scaffolds have been soaked and coated with more hydrophilic polymers, such as poly(vinyl alcohol) (PVA) (Mooney et al., 1994). The attachment of hepatocytes was greatly increased for PVA-coated scaffolds as compared to untreated PLGA scaffolds.

V. MELT MOLDING

An alternative method for the production of three dimensional scaffolds is melt molding. This technique calls for polymer and porogen particles to be
combined in a mold and heated above the polymer's glass transition temperature (for amorphous polymers) or melting temperature (for semicrystalline polymers). After the reorganization of the polymer, the composite material is removed from the mold, cooled, and soaked in an appropriate liquid to leach out the porogen. The resulting porous scaffold has the exact external shape as the mold. PLGA/gelatin microparticle composites have been formed in this fashion with gelatin leaching in distilled-deionized water (Thomson et al., 1995a). Melt molding allows for the formation of scaffolds of any desired geometry by altering the size and shape of the mold. Adjusting the amount and size of porogen used, respectively, can control the porosity and pore size of the scaffold. The melt molding protocol can be adapted to incorporate materials such as hydroxyapatite fibers (Thomson et al., 1995b). Such fibers provide additional mechanical support and a bioactive surface for cells when uniformly distributed throughout the polymer prior to melting. Melt molding is advantageous for the inclusion and delivery of bioactive molecules because the materials are not exposed to harsh organic solvents, although excessively high molding temperatures can degrade and inactivate the molecules.

VI. MEMBRANE LAMINATION

Tissue engineering often requires precise three dimensional anatomical geometries for hard tissues with shape-dependent function like bone and cartilage.
Thin layers of porous polymer produced in the previously mentioned manners can be cut, stacked, and bonded by means of membrane lamination (Mikos et al., 1993b). The layers are chemically joined, but there is no distinguishable boundary at the interface of two adjacent membranes. The key to this method is the creation of a three-dimensional contour plot of the desired scaffold shape. Each layer of the scaffold is cut from a highly porous membrane into its corresponding shape for that level. A small amount of solvent, such as chloroform, is coated on the interfacial surface, and a bond is formed between membranes. This process is repeated for all subsequent layers until the completion of the final three-dimensional structure.

Porous polymers used in membrane lamination include PLLA and PLGA membranes formed by solvent casting and particulate leaching. As previously mentioned, there is no detectable boundary between layers in the finished scaffold. Membrane lamination provides a method for fabricating three-dimensional anatomical shapes with identical bulk properties to the individual membranes. Membrane lamination has also been utilized in the preparation of degradable tubular stents (Mooney et al., 1994). Porous membranes of PLGA are produced by solvent casting and particulate leaching and wrapped around a Teflon cylinder. The overlapped edges are bonded with a small volume of solvent, and the Teflon is removed, yielding a hollow cylinder of porous PLGA for applications such as intestinal and vascular regeneration.
VII. EXTRUSION

While extrusion is a well-documented processing method for industrial polymers such as polyethylene, this method is relatively new for biocompatible porous scaffold production. The first extrusion of polymers for tissue engineering utilized PLGA and PLLA to form tubular scaffolds for peripheral nerve regeneration (Widmer et al., 1998). Extruded tubular PLGA scaffolds are illustrated in Figure 3.2. The polymers were fabricated into membranes using solvent casting, with sodium chloride as a porogen. The membranes were cut to appropriate sizes and loaded into a customized extrusion tool. The extruder applies heat and pressure to the composite material and forces it through a die and out the nozzle to form cylindrical conduits. After the conduits are cooled, they are soaked in water, to leach the salt, and vacuum dried. Higher temperatures require less pressure, and vice versa. While high pressures may require a powerful hydraulic press, high temperatures can adversely affect the crystallinity and porosity of the scaffold and the activity of incorporated biomolecules. As with other methods, porogen content and size are the most important parameters of porosity and average pore diameter. Extruded polymer scaffolds can be fabricated to support the loading of cells or growth factors for tissue engineering. PLGA, PCL, and most biocompatible polymers can be extruded at appropriate temperatures and pressures.
**Figure 3.2.** Optical micrograph of a conduit fabricated by extrusion from PLGA and salt crystals, a salt weight fraction of 90%, and an extrusion temperature of 250°C. Reprinted from Widmer et al. (1998). Copyright 1998, with permission of Elsevier Science.

**VIII. FREEZE-DRYING**

Another method for rapid fabrication of scaffolds with controllable porosity and average pore diameter employs emulsion and freeze-drying. An organic solution containing dissolved polymer is combined with a suitable amount
of water and emulsified until homogeneity is achieved (Whang et al., 1995). The resulting emulsion is poured into a metal mold of specified dimensions and frozen with liquid nitrogen. Freeze-drying removes the water and solvent to yield scaffolds of highly interconnected pores, porosities up to 90%, and median pore diameters from 15 to 35 μm. This technique has been utilized with many biocompatible polymers, including PGA, PLLA, PLGA, and poly(propylene fumarate) (PPF) blends. Inclusion of polymers like PPF in composite scaffolds is beneficial for adjustment of compressive strength and properties related to hydrophobicity (e.g., water penetration, scaffold degradation rates, and drug diffusion) (Hsu et al., 1997). PLGA/PPF foam scaffolds exhibit a closed-pore morphology, however, an unattractive quality for most tissue-engineering applications. PLGA and PLGA-blend polymer scaffolds of greater than 1 cm thickness can be manufactured by emulsion and freeze-drying. Non-emulsion-based freeze-drying is also capable of producing porous polymer scaffolds.

Synthetic polymers dissolved in glacial organic solvents are frozen, and then the solvent is removed by freeze-drying (Hsu et al., 1997). Similar techniques were utilized to create collagen scaffolds by dispersing the protein in water and freeze-drying the suspension (Yannas et al., 1980). Sublimed ice crystals generate pores, with pore size being controlled by solution parameters such as freezing rate, temperature, ionic concentrations, and pH.
IX. PHASE SEPARATION

The potential to deliver drugs and other bioactive molecules from a degradable tissue-engineering scaffold is advantageous for modulating cell differentiation and guiding tissue regeneration. Such scaffolds can be produced by a phase separation technique that does not expose the bioactive molecules to harsh organic chemicals or temperatures (Lo et al., 1995). Briefly, a biocompatible polymer such as PLGA or a poly(phosphoester) is dissolved in an appropriate solvent (e.g., phenol at 552°C, dioxane at 63°C, or naphthalene at 85°C). While stirring, the bioactive molecules are added and dispersed into a homogeneous mixture and cooled below the solvent melting point until the liquid phases separate (Hua et al., 2002). The polymer and solvent are quenched with liquid nitrogen, resulting in a two-phase solid. The solvent is removed by sublimation, which yields a porous scaffold with bioactive molecules embedded inside the polymer. Porosity and architecture are affected by the cooling rate and the melting temperature of the solvent relative to the polymer. Tailoring specific drug-release rates and incorporating large proteins are the major obstacles with phase separation methods of polymer scaffold fabrication.
X. HIGH-INTERNAL-PHASE EMULSION

Porous scaffolds are typically prepared by bulk polymerization or condensation with the use of porogenic materials. An alternative method of fabrication is the polymerization of the continuous phase around aqueous droplets in an emulsion (Busby et al., 2001). The setup involves a water-in-oil emulsion system with an organic phase containing the specified monomers. When the internal (droplet) phase volume fraction exceeds 74%, the emulsion is defined as a high-internal-phase emulsion (HIPE) (Lissant, 1974). Under desired HIPE conditions, polymers are synthesized and/or cross-linked to yield a solid network with interconnected pores. Polymers derived from HIPEs are dubbed PolyHIPEs. PolyHIPE foams resemble the structure of emulsion-formed scaffolds at the gel point. The morphology of the structure depends primarily on the volume fraction and the droplet radius, which can be controlled by the physical conditions of the emulsion. Total porosity is based on phase volume fraction, and scaffolds of more than 90% porosity have been produced from PolyHIPE systems. Porogens can also be incorporated into PolyHIPEs for additional porosity. Early research with PolyHIPE scaffolds used nondegradable polymers like poly(styrene), but recent work has utilized biodegradable polymers such as PLLA and PCL (Busby et al., 2002). Images of PLA-MMA PolyHIPEs are shown in Figure 3.3.
Figure 3.3. Scanning electron micrographs of PLA-MMA PolyHIPEs: (A) 0.2-M at low magnification; (B) 0.2-M at high magnification; (C) 0.4-M at low magnification; (D) 0.4-M at high magnification. Reprinted from Busby et al. (2002). Copyright 2002, with permission of John Wiley & Sons, Ltd.
XI. GAS FOAMING

A major concern with typical solvent-casting and particulate-leaching strategies is the use of organic solvents, remnants of which might lead to an inflammatory response after implantation. A method that avoids any organic solvents is gas-foaming scaffold fabrication (Mooney et al., 1996b). Compressed polymer disks (e.g., PLGA) are treated with high-pressure CO2. As the pressure is decreased, nucleation and pore formation occur in the polymer matrix based on the amount and reduction rate of pressure. The average pore size ranges from 100 to 500 μm; however, a drawback of this method remains its closed-pore morphology. Incorporation of a particle-leaching technique has been shown to create an open-pore network in scaffolds produced by gas foaming (Harris et al., 1998). Smooth muscle cells have exhibited enhanced adhesion and proliferation to scaffolds fabricated in this manner.

XII. POLYMER/CERAMIC COMPOSITE FABRICATION

Tissue engineering strategies for bone replacement are unique, in that they must account for the irregular shape of most bone defects and the required mechanical strength of the scaffold. While scaffolds of polymers such as the poly(α-hydroxyester) family provide sufficient support in orthopedic applications,
increasing the scaffold porosity drastically reduces the compressive strength (Thomson et al., 1995). PLGA scaffolds containing hydroxyapatite (HA, the mineral component of bone) fibers have been assembled using melt molding and solvent-casting techniques (Thomson et al., 1998). The greatest effects of HA on the scaffold’s mechanical properties are observed when the fibers are fully dispersed throughout the polymer to maximize polymer–HA contact.

More recent methods incorporate microparticles of HA, rather than fibers, into the scaffold network. One technique uses an emulsion of PLGA and HA dissolved in chloroform with an aqueous PVA solution (Devin et al., 1996). After the mixture is emulsified, it is cast into molds and vacuum dried to yield a porous PLGA/HA composite foam. The compressive strength of the scaffold was found to be proportional to its HA content. Such scaffolds exhibited compressive strengths on the same order of magnitude as cancellous bone (10–1000 MPa) (Hollister, 2005). Another method that integrates HA powder into PLGA scaffolds employs phase separation (R. Zhang and Ma, 1999). HA is dispersed in a PLGA/dioxane solution; then the blend is injected into molds and frozen. Following phase separation, the material is freeze-dried to remove the solvent. The resulting composite scaffolds contain an interconnected-pore network, with pore sizes from 30 to 100 μm and porosity up to 95%. PLGA/HA composite scaffolds produced by solvent casting or gas foaming, followed by particulate leaching, are pictured in Figure 3.4.
Figure 3.4. Scanning electron micrographs of (A,C) surfaces and (B,D) cross sections of the PLGA/HA composite scaffolds fabricated by (A,B) the solvent-casting/particulate-leaching method and (C,D) the gas-foaming/particulate-leaching method. Reprinted from Kim et al. (2005). Copyright 2005, with permission of Elsevier Science.

XIII. RAPID PROTOTYPING OF SOLID FREE FORMS

Another technique for the creation of scaffolds with specific three dimensional structures is the rapid prototyping of solid free-form structures, which includes three dimensional printing, laser sintering, and stereolithography.
These methods require a computer model of the desired scaffold architecture from computer-assisted design (CAD) or computed tomography (CT). Although there are several approaches to this family of scaffold production, the result is a three dimensionally accurate structure with a fully interconnected network of pores (Lam et al., 2002). These methods have an advantage over conventional fabrication techniques due to their ability to create geometries with complex architectures on the micron scale. Three-dimensional printing utilizes a simple inkjet printing system directed by the CAD program. Briefly, a thin layer of polymer powder, such as PLGA, is spread over a piston surface. The inkjet dispenses a binding liquid, which is a solvent for the polymer, in the desired pattern of the scaffold layer. After a short bonding time, the piston is lowered by the thickness of a single layer and the subsequent layers of powder and binding liquid are applied. Unbound polymer remains in the network during the fabrication process to support disconnected sections in the layer. PLLA and PLGA scaffolds produced in this manner have properties similar to those made via compression molding (Giordano et al., 1996) and show great promise in cell transplantation and vascular penetration into the implanted structure (Kim et al., 1998). Fused-deposition modeling (FDM) combines the elements of extrusion and melt molding with free-form scaffold fabrication (Leong et al., 2003). Polymer stock is heated and extruded through a computer-controlled nozzle. With each layer deposited and cooled, the nozzle changes the direction of deposition to yield a porous, honeycomb-type structure. Scaffolds produced via FDM have controlled pore size, porosity, and total pore interconnectivity. FDM
is used with many synthetic polymers, including PCL, PLGA, and high-density polyethylene. An FDM-fabricated scaffold with three-dimensional pore interconnectivity is shown in Figure 3.5.

![Figure 3.5. Scanning electron micrograph of scaffold with three dimensional pore interconnectivity fabricated by means of FDM. Reprinted from Leong et al. (2003). Copyright 2003, with permission of Elsevier Science.](image)

Laser sintering is similar to three-dimensional printing, but it uses a high-powered laser to sinter the polymer instead of dispensing a binding liquid. The laser selectively scans the powder polymer surface, directed by the CAD or CT
computer program (K. H. Tan et al., 2003). The laser beam heats the polymer above its melt temperature and fuses particles into a solid structure. Additional layers of polymer are added to the top surface and sintered accordingly. This technique has been used with biocompatible materials such as PLLA, PCL, PVA, and hydroxyapatite (K. H. Tan et al., 2005). Such scaffolds were shown to be biocompatible, highly porous, and accurate to design specifications.

Another popular method of fabrication by rapid prototyping is stereolithography. Stereolithography uses light to polymerize, crosslink, or harden a photosensitive material (Dhariwala et al., 2004). Typically for tissue engineering applications, a fine layer of a solution of biocompatible polymer, photo-crosslinking initiating agent, porogen, and an appropriate solvent is placed beneath the laser. Like previous methods, the CAD software guides the laser in the desired pattern for the designed scaffold. The laser’s ultraviolet light reacts with the photo-initiator to form chemical bonds between polymer chains in the specified locations. Subsequent layers of polymer solution are added and photo-crosslinked. The final product is washed to remove unreacted polymer and yield a three-dimensional structure with specific microarchitectures.
XIV. PEPTIDE SELF-ASSEMBLY

Since the mid-1990s, new research has studied the use of peptide nanofibers as a synthetic ECM in a tissue engineering scaffold (S. Zhang et al., 2006). While other biologically derived materials, such as collagen, gelatin, and fibrin, can interact favorably with cells as compared to synthetic polymers, designer peptide fibers can self-assemble to form stable, highly ordered scaffolds on the nanoscale (Yokoi et al., 2005). Self-assembling peptides typically consist of ionic, self-complementary sequences with alternating hydrophobic and hydrophilic domains (S. Zhang et al., 1995). They can also include motifs favorable to cell attachment, such as the popular arginine-glycine-aspartate (RGD) peptide. Peptide-based scaffolds have shown promise in the *in vitro* culture of osteoblasts, chondrocytes, and hepatocytes. Self-assembling peptide structures form on the nanoscale, allowing attached cells to remain in their native three-dimensional shape and not flattened like cells attached to some microscale surfaces. While the individual fibers can be as small as 5 nm, the aggregate scaffolds can reach sizes in the centimeters (Hartgerink et al., 2002). A scanning electron micrograph of self-assembling peptide nanofibers is seen in Figure 3.6.
Figure 3.6. Transmission electron microscopy images of peptide nanofibers. (a) Self-assembled by drying without adjusted pH; (b) self-assembled by mixing with CaCl₂. Reprinted from Hartgerink et al. (2002). Copyright 2002, with permission of the National Academy of Sciences.

By controlling the spacing of charged and hydrophobic residues in the amino acid sequence, the geometries of the forming scaffold can be manipulated. Noncovalent bonds and ionic interactions within and between peptide molecules create functional and dynamic structures in these synthetic biological systems. Adjacent fibers can be permanently cross-linked with disulfide bonds by the strategic placement of cysteine residues. Self-assembling peptides typically form stable β-sheets in water or physiological solutions. Peptide amphiphiles have also been shown to form more complex architectures, such as sheets, rods, spheres, and disks. Scaffold assembly and size can be controlled by pH, peptide concentration, and divalent ion induction.
XV. **IN SITU POLYMERIZATION**

The previous scaffold fabrication techniques discuss the production of prefabricated scaffolds for surgical implantation within a defect. Although these scaffolds are useful in most tissue engineering applications, many orthopedic procedures require immediate treatment in defects of irregular or unpredictable shape. In such situations, an injectable, *in situ* polymerizing or hardening polymer is advantageous. Early bone cements composed of PMMA were injected into the bone fracture space (Yaszemski *et al.*, 1996). A degradable alternative for cementing bone defects is PPF, which can be thermally cross-linked with the addition of N-vinyl pyrrolidone. Unlike PMMA, injected PPF may not result in necrosis of local tissues from the elevated temperatures of polymerization or any residual toxic monomer. Incorporation of mineral into the polymer mixture can provide added mechanical properties to the scaffold. PPF with β-tricalcium phosphate has shown strength similar to that of human trabecular bone (Peter *et al.*, 1997). More self-cross-linkable macromers, such as poly(ε-caprolactone-fumarate) (PCLF), have been developed to harden *in situ* without the aid of low molecular weight crosslinking agents, but with the addition of an initiator and accelerator, to form porous, biodegradable scaffolds (Jabbari *et al.*, 2005). A cross section of a thermally crosslinked PCLF scaffold is presented in Figure 3.7.
Figure 3.7. Scanning electron micrograph of the cross section of a PCLF scaffold thermally cross-linked with 75 vol% salt content. Reprinted from Jabbari et al. (2005). Copyright 2005, with permission of the American Chemical Society.

For cartilage and most soft tissues, less compressive strength is required during tissue repair. Water-based polymer gels, or hydrogels, are often favorable for promoting cell migration, angiogenesis, high water content, and rapid nutrient diffusion (Bryant and Anseth, 2001). Most hydrogels are formed by the aqueous cross-linking of poly(ethylene glycol) (PEG)–based synthetic polymers or biologically derived molecules such as gelatin and fibrin. Prior to injection, cells cultured in vitro can be loaded into the polymer solution and encapsulated within the crosslinked hydrogel to accelerate tissue regeneration. Like PPF, modified
PEG and oligo(poly(ethylene glycol) fumarate) (OPF) can be \textit{in situ} crosslinked with inclusion of a thermal initiator (Temenoff \textit{et al.}, 2004).

There are a variety of strategies to create porosity within \textit{in situ} crosslinked scaffolds. Salts or other small biocompatible molecules included in the polymer solution are able to leach out \textit{in vivo} to create a pore network over time (Peter \textit{et al.}, 1997). Gelatin microparticles incorporated into hydrogels are enzymatically degraded to leave pores for tissue penetration (Kasper \textit{et al.}, 2005). Hydrogels can utilize gas bubbling to form pores during cross-linking (Behravesh \textit{et al.}, 2002). Carbon dioxide produced from the reaction of l-ascorbic acid with sodium bicarbonate, both mixed into the polymer solution prior to injection, has been used in the synthesis of poly(propylene fumarate-\textit{co}-ethylene glycol) hydrogels with greater than 80\% porosity.

\textbf{XVI. CONCLUSIONS}

To meet the diverse needs of tissue reconstruction and replacement, tissue-engineering strategies attempt to provide artificial, yet permanent, biological solutions. As a key component of any tissue-engineering application, scaffolds require a high porosity, adequate pore size for cell migration and nutrient diffusion, biocompatibility, biodegradability, and mechanical integrity. The
selected scaffold processing technique can have a profound effect on the final properties and geometry of the scaffold. The fabrication schemes in this chapter offer a practical and promising solution for scaffolds to repair and regenerate different tissues. Each method presents distinctive advantages (e.g., the ease of processing, the ability to incorporate bioactive molecules, or increased structural properties) and limitations (e.g., applicable polymers, cost of materials or equipment). Thus there is no universal scaffold fabrication technique for all tissue-engineering applications (see Table 1). Depending on the tissue type and extent of regeneration, scaffold properties must be prioritized in order to select the most appropriate manufacturing method. At present, tissue engineers are working to incorporate bioactive molecules into the scaffolds, develop new scaffold materials, produce constructs with mechanical properties that match those of the specific tissue, and improve the time and costs of scaffold production.
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<td>Limited mechanical properties; temperatures unsuitable for biomolecules</td>
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<td>Good porosity and pore interconnectivity</td>
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<td>High porosity; ability to incorporate biomolecules</td>
<td>Limited pore sizes; residual solvents; no control over micro-geometry</td>
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<td>High Internal Phase Emulsion</td>
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<tr>
<td>Rapid Prototyping</td>
<td>CAD-controlled fabrication using solvent dispensing, fused deposition, laser sintering, or stereolithography</td>
<td>Excellent control over geometry (macro and micro) and porosity</td>
<td>Limited polymer types; high equipment cost</td>
</tr>
<tr>
<td>Peptide Self-Assembly</td>
<td>Designer peptide sequences are self-assembled into spheres, fibers, or complex scaffolds</td>
<td>Control over porosity, pore sizes, and fiber diameter; bioactive degradation products</td>
<td>Expensive materials; complex design parameters; limited macro-sizes and mechanical properties</td>
</tr>
<tr>
<td>In Situ Polymerization</td>
<td>Polymers are polymerized or crosslinked; scaffolds are formed post-implantation</td>
<td>Injectable; control over mechanical properties; ability to incorporate biomolecules</td>
<td>Limited porosity; residual monomers and crosslinking agents</td>
</tr>
</tbody>
</table>
XVII. ACKNOWLEDGMENTS

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XVIII. REFERENCES


poly(propylene fumarate)/B-tricalcium phosphate injectable composite scaffold. 
*Tissue Eng.*** 3, **207–215.**


Chapter 4. Thrombin Peptide 508, Bisphosphonates, and Other Calcium-binding Moieties

I. Thrombin Peptide 508

Thrombin Peptide 508 (TP508) represents the active segment of the human thrombin protein. TP508 is a 23 amino acid sequence and shows a high affinity for thrombin binding fibroblasts. Interaction with TP508 causes a signaling cascade that stimulates chemotaxis and mitogenesis to accelerate the wound healing process [1]. The amino acid sequence of TP508 is AGYPDENGKRGDACEGDSGGPFV (amino to carboxyl terminus) with a molecular weight of 2,311 Da [2]. It is hypothesized that monocytes and T-cells are actively involved in the cell recruitment and gene up-regulation of cytokines [3]. Thrombin effectively has two binding sites to cells; a proteolytic (PAR) and non-proteolytic (NPAR) activated receptor. PAR-bound thrombin tends to deal with tissue breakdown (inflammation, platelet activation, and matrix degradation), while NPAR-bound thrombin aids in the repair process (angiogenesis, chemotaxis, and matrix synthesis). TP508 binds to the NPAR receptor sites only and initiates the listed repair mechanisms [4].

Because TP508 initiates a systemic healing cascade, in vitro studies have yielded little results. TP508 decreased ALPase activity of human MSC’s significantly over the first 5 days and showed little effect after 10 days.
Treatment of human MSC's with TP508 did not significantly affect cell proliferation rates [5]. However, human osteoblasts were shown to experience increased proliferation at doses of 1-100 μg/mL. TP508 in this dosage range also increased the chemotaxis to osteoblasts [6]. There is evidence that TP508 may boost BMP-7 expression and alkaline phosphatase secretion in vitro [4].

TP508 treatment resulted in accelerated, but not increased, branching and sprouting of rat adipose blood vessels in vitro. TP508 also increased the average length of new vessels and number of sprouts compared to VEGF, an accepted angiogenic growth factor [7]. Delivery of 0.1 μg TP508 to normal tissue wounds, bipedal flaps, and cranial flaps resulted in significant wound healing over controls within 7 days. Dermal wounds treated with TP508 also developed a significantly increased number of large, functional blood vessels [8]. Full thickness wound closures and closure rates were also improved (approximately 40%) with 0.1 μg TP508 treatment compared to controls through 7 days in rat models [9]. TP508 delivered via PLGA microparticles exhibited enhanced repair over controls in articular cartilage defects. The optimum dosage range was 10-50 μg encapsulated within the PLGA [10].

In specific cases of skeletal defects, TP508 has shown the ability to increase healing in critically and non-critically sized defect models. TP508 administered to rat femoral fractures doubled the breaking strength of the bone after two and four weeks compared to saline controls [11]. In another study,
PLGA microparticles loaded with 50, 100, and 200 μg TP508 were placed in radial (non-critically sized) and ulnar (critically sized) defects in rabbits for 6 and 9 weeks, respectively. All dosage levels yielded improved bone regeneration over unloaded microparticles, while the 100 μg TP508 group resulted in the best observed healing and mechanical properties in both models [12]. The release kinetics of TP508 from PLGA-PEG blend microparticles has also been studied, showing the loading and release efficiency decreased with PEG content [13]. It appears that a large burst release of TP508 is better for tissue regeneration in skeletal defects than a slow sustained release [14]. This is likely due to the recruitment of inflammatory cells, which leads to faster matrix fabrication and blood vessel formation. This effect will only be enhanced by specific bone targeting of the drug.

II. Bisphosphonates and Bone

Bisphosphonates, or diphosphonic acids, are synthetic analogues of the organic molecule pyrophosphate (PCP). Their structure is that of C(R₁)(R₂)(PO₃H₂)₂, with R₁ typically as −H or −OH and R₂ as a unique motif that provides the bisphosphonate with its own properties and characteristics. Typically bisphosphonates are divided into two classes based on their R₂ group: nitrogen-containing and non-nitrogen-containing bisphosphonates. Popular bisphosphonates for pharmaceutical research are listed in Table 4.1 below.
Table 4.1. Prominent Bisphosphonates and their Structures

<table>
<thead>
<tr>
<th>Name</th>
<th>R₁ Group</th>
<th>R₂ Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etidronate</td>
<td>−OH</td>
<td>−CH₃</td>
</tr>
<tr>
<td>Clodronate</td>
<td>−Cl</td>
<td>−Cl</td>
</tr>
<tr>
<td>Pamidronate</td>
<td>−OH</td>
<td>−(CH₂)₂NH₂</td>
</tr>
<tr>
<td>Alendronate</td>
<td>−OH</td>
<td>−(CH₂)₃NH₂</td>
</tr>
<tr>
<td>Ibandronate</td>
<td>−OH</td>
<td>−(CH₂)₂N(CH₃)((CH₂)₄CH₃)</td>
</tr>
<tr>
<td>Risedronate</td>
<td>−OH</td>
<td>−H₂C—N</td>
</tr>
</tbody>
</table>

III. Bisphosphonates’ Affinity for Bone

Bisphosphonates, like pyrophosphate, exhibit a remarkable affinity to bone. This affinity arises from the attraction of the diphosphonate moiety to the calcium ions present in hydroxyapatite, the mineral component of bone [15]. Specifically for terminal amino-bisphosphonates like pamidronate and alendronate at physiological pH, the bisphosphonate is a zwitterion, possessing a
negative charge on each –PO$_3$ group while the terminal amine is protonated [16]. In fact, bisphosphonates are totally ionized at physiological pH. Initial accumulation in the kidney is expected, as excretion is the only method of eliminating bisphosphonates from the body [17]. Alendronate displayed a volume of distribution of approximately 28 L while 50% of the drug introduced via IV bolus was excreted in urine within 72 hours (none in feces). Plasma concentrations drop 95% over the 6 hours following injection, indicating rapid excretion or bone-binding. Also of great importance, alendronate (like most bisphosphonates) has no natural metabolism and therefore will remain active for its duration of time in the body [18]. At pH 7, the affinity of alendronate for crushed bone particles is described by an apparent dissociation constant of 1 mmol/L and a binding constant of 100 nmol/mg [17]. According to the pharmacokinetic data of Fosamax (alendronate drug for osteoporosis), alendronate is transiently spread through nearby soft tissues, then quickly redistributed to bone or excreted. The ratio of alendronate in non-calcified to tissue to alendronate in plasma ranged from 0.05 to 0.7 one hour after IV administration.

The systemic or extraskeletal delivery of many drugs is undesirable due to possible adverse effects at the unintended reception sites as well as the obvious inefficiency in drug delivery. Specifically targeting drugs to bone is difficult because of the high mineral content and lack of natural biological targets. However, bisphosphonates can utilize this perceived disadvantage in order to
attach itself (as well as any conjugated agents) to the bone’s mineral surface. Early studies linking bisphosphonates to protein-like molecules for systemic skeletal delivery have shown great promise [15].

IV. Osteoclast Inactivation and Death by Bisphosphonates

As part of the normal physiological process, skeletal tissue is continuously remodeled to maintain homeostasis and proper mechanical strength. Osteoclasts tear away at the collagen and hydroxyapatite structure, creating free proteins, calcium, and crystal fragments. Opposing this function, osteoblasts are responsible for rebuilding the complex mesh of bone components. The rate of new bone formation to old bone resorption is called bone turnover. A widely investigated group of compounds called bisphosphonates have been shown improve bone turnover and maintain bone mineralization and structural integrity [18]. Bisphosphonates are an organic analogy to pyrophosphate, which is present and degraded normally in the body. In bisphosphonates, the central oxygen between the phosphate groups is replaced with a carbon. Unlike pyrophosphate, there is no known metabolism for the P-C-P bond. The two side groups on the central carbon determine the unique properties of the bisphosphonate. The FDA-approved, nitrogen-containing pamidronate is the chief bisphosphonate of interest for this study and its chemical structure is shown below in Figure 4.1.
Osteoporosis represents a common bone disease in older people, especially women. It is classified as the loss of total bone mass and deterioration of the complex micro-architecture in skeletal tissue. Treatment of patients with bisphosphonates gives a systemic delivery highly specific to bone. Bisphosphonates have shown to reduce the rate of bone fracture in osteoporosis patients by 60-84%, the most effective being the nitrogen-containing bisphosphonate alendronate [18]. One mechanism of enhanced bone turnover is the interference bisphosphonates have with glucocorticoids. Glucocorticoids prolong the normal lifespan of osteoclasts while preventing osteoblastogenesis and/or promoting osteoblast apoptosis. The presence of bisphosphonates can cancel out over-abundant or over-active glucocorticoids [19]. Another critical mechanism utilized by nitrogen-containing bisphosphonates is the interruption of the mevalonate pathway. In this metabolic process, mevalonate is eventually converted to cholesterol or geranylgeranyl diphosphate, a substrate for the
prenylation of most GTP proteins. Nitrogen containing bisphosphonates were found to interrupt this pathway after mevalonate, between isopentyl diphosphate and farnesyl diphosphate [18]. This belief was later confirmed when rat and rabbit osteoclasts were treated with alendronate. Alendronate halted both proliferation and function in these cultures. Heavy treatment with mevalonate eventually overwhelmed the bisphosphonates' effect and cells returned to normal osteoclastic behavior. However, addition of low doses of geranylgeraniol (intermediate after bisphosphonate blocking) was sufficient for normal osteoclast function. Nitrogen-containing bisphosphonates also caused apoptosis of rapidly dividing, macrophagic cells (osteoclasts, tumor cells) at higher doses [20, 21]. Bisphosphonates lacking nitrogen were not as efficient in osteoclast deactivation in vivo [22]. Bisphosphonates have also been found to alter lactic acid production and disrupt the osteoclast cytoskeleton [23]. These findings are promising as alendronate may inactivate osteoclasts in a regenerating bone defect site.

V. Osteoblast Proliferation and Differentiation by Nitrogen-Containing Bisphosphonates

While all bisphosphonates are effective in osteoclast inactivation at some concentration, nitrogen-containing bisphosphonates also influence the proliferation, differentiation, and mineralization of osteoblastic cells. Early studies with alendronate and pamidronate revealed a biphasic effect; enhanced
osteoblastic properties up to a peak concentration followed by a negative response. Alendronate stimulated osteoblast colony formation (with calcium nodules) from bone marrow cells in the concentration range of $10^{-7}$ to $10^{-10}$ mol/L [24] (illustrated in Figure 2). A second study confirmed an increase in colony formation and osteoblastic properties (alkaline phosphatase activity, collagen accumulation, and calcification) with treatment of alendronate and risedronate in the range of $10^{-7}$ to $10^{-9}$ mol/L. It was also observed that bisphosphonate treatment in conjunction with geranylgeraniol could stop the negative portion of the biphasic effect observed earlier (cytotoxicity). As previously discussed, bisphosphonates interfere with the mevalonate pathway, although the cytotoxicity is much greater to osteoclasts than osteoblasts, osteocytes, or marrow stromal cells [25].
Figure 4.2. Osteoblastic Colony-Forming Units of Marrow Stromal Cells Treated with Alendronate and no dexamethasone [24].

Nitrogen-containing bisphosphonates have also been studied in conjunction with total joint replacements. This is logical due to their pro-osteoblast, anti-osteoclast functionality while bone resorption is common in bone near replacements because of the stress shielding of the implant. Human trabecular bone cells were cultured with alendronate to study the anabolic effects over three days of treatment. In agreement with previous studies, these cell cultures also experienced the biphasic effect with an optimal range between $10^{-7}$ and $10^{-9}$ mol/L, depending on the measured property (alkaline phosphatase activity, gene expression for osteoblast markers such as BMP-2, collagen-1, and
ostocalcin) [26]. Because the first few days are critical in bone healing, the results seen in Figure 4.3 are critical to the design of a new novel biomaterial.

![Graph showing Alkaline Phosphatase Activity in MG-63 OB's](image)

**Figure 4.3. Alendronate and Risedronate Effects on Alkaline Phosphatase Activity [26].**

An exciting discovery in the field of bisphosphonates is the effects of bound nitrogen-containing bisphosphonates on osteoblasts. Rat osteoblasts were exposed to clodronate (no nitrogen) and pamidronate in solution as well as bound to hydroxyapatite crystal. Pamidronate bound to hydroxyapatite caused greater protein production than pamidronate in solution. Additionally, total DNA and cell number also increased drastically when pamidronate was bound to the crystal.
This infers that the pro-osteoblastic effects of nitrogen-containing bisphosphonates are the result of external, cell surface interactions, while the anti-osteoclastic and osteoblast-biphase effects are due to disruption of the mevalonate pathway after cell internalization [23].

VI. Negatively-charged Oligopeptides and Bone

Based on the same principles as bisphosphonate bone affinity, poly(aspartic acid) binds to bone due to the attraction to calcium in hydroxyapatite. For each aspartic acid, there is an ethanoic acid side chain exposed. At physiological pH, the acid is deprotonated and the two oxygen atoms display a resonance form where both share the negative charge. This motif, when demonstrated by several consecutive amino acids, shows an affinity for hydroxyapatite.

Early work in the use of poly(aspartic acid) for bone targeting has shown promise. Over two percent of fluorescein isothiocyanate (FITC) conjugated to six aspartic acids (Asp₆) and injected intravenously accumulated in the bones of rats after 24 hours. After subcutaneous injection in mice, FITC-Asp₆ conjugates bound to bone had a half-life of 14 days. Unconjugated FITC was immediately excreted and is not accumulated by soft or bony tissues [27]. FITC-poly(ethylene
glycol) (PEG) conjugated to eight aspartic acids (Asp₈) exhibited 90% binding by weight to hydroxyapatite in vitro, opposed to 73% for FITC-PEG-alendronate conjugates. To test molecules of significantly larger molecular weight, N-(2-Hydroxypropyl) methacrylamide (HPMA) was conjugated to Asp₈ and alendronate and successfully bound to hydroxyapatite in vitro at 80% and 66% by weight, respectively [28].

VII. Matrix Metalloproteinase-Sensitive Sequence

Drug modification will require the use of a linker between the active pharmaceutical agent and the bone-binding entity. While there are several viable options based on size and degradation, it was hypothesized that an enzymatically cleavable peptide sequence would be optimal. Such a peptide linker would provide space between the domains, chain flexibility, and secondary control of release based on the interaction with secreted enzymes. Because the aim of this research is to deliver an osteogenic/angiogenic drug to the cells entering the wound site, the most universally and easily cleaved peptide sequence is desired. The first cells at the bone/polymer network interface, like all cells, secrete matrix metalloproteinases (MMP’s) to degrade biological material. MMP-2 (gelatinase A), MMP-9 (gelatinase B), and MMP-13 (collagenase 3) are readily produced by cells native to the bone and are critical in the remodeling of bone tissue. MMP-1
(collagenase 1) and MMP-8 (collagenase 2/neutrophil collagenase) are also present in bone, but at lesser concentrations [29].

Cleavage sequences for each of these MMP’s have been well studied for many substrates such as collagens, gelatins, and link proteins. While there are no common amino acid cleavage sequences for MMP-2, 9, and 13, MMP-2 and 9 share four common sequences (aggrecan, galectin 3, link protein, and α2-macroglobulin). Comparison of MMP-1, 2, and 9 yields three common cleavage sequences (aggrecan, link protein, and α2-macroglobulin). Inclusion of MMP-8 into search for a single common cleavage sequence leaves only the aggrecan sequence (IPENFFGV) [30]. Because this sequence is easily cleavable by four MMP’s present in the bone healing site, it is the most appropriate to use as a linkage between the bisphosphonate/poly(aspartic acid) and the drug.

VIII. References


Chapter 5. Objectives

I. Overview

The goal of this research was to investigate the effects of drug modification for targeting on the binding of drugs to bone mineral and the in vivo repair of skeletal defects. To improve the delivery of osteogenic growth factors to the site of healing, hydroxyapatite (the mineral component of bone)-binding agents were chemically linked to the drug in order to anchor it to the surface of bone upon release. For this purpose, bisphosphonates, poly(aspartic acid), and poly(glutamic acid) were selected due to their established attraction to calcium within hydroxyapatite crystal. Thrombin Peptide 508 (TP508), a 23 amino acid pro-inflammatory peptide that aids in osteogenesis, was chosen as a model drug for this investigation. TP508 was linked to pamidronate, a nitrogen-containing bisphosphonate, or a negatively charged oligopeptide of aspartic acid or glutamic acid residues by an enzymatically degradable eight amino acid sequence.

II. Hypothesis

It was believed that a controlled release of TP508 conjugated to a bone-binding agent from a biodegradable, biocompatible polymer scaffold would result
in enhanced bone regeneration compared to native TP508. The attraction between bone and the bisphosphonate, poly(aspartic acid), or poly(glutamic acid) should effectively anchor the drug to nearby bone tissue as it is released from the scaffold. If true, a smaller dose of modified drug would be required than unaltered TP508 to yield similar results, due to its affinity to bone and localization to the defect area, rendering it more efficient in its delivery. This increased delivery efficiency meant less TP508 escaping from the wound environment, which is not a major concern with TP508, but could be significant for other growth factors that may have undesirable side-effects in other parts of the body. The specific targeting would obviously require less total drug, making patient treatment more cost efficient as well. It is further believed that the integration of HA within the composite scaffold will provide a local target for modified drug binding and an osteoconductive surface for recruited cells.
Chapter 6. Synthesis and *in vitro* Hydroxyapatite Binding of Peptides Conjugated to Calcium-Binding Moieties

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I. Abstract:

To confer bone-binding properties to proteins and other biological agents that lack specific targeting capacity, model peptide-based molecules were synthesized containing poly(aspartic acid), poly(glutamic acid), or a bisphosphonate (pamidronate). These motifs have well-documented affinities to
hydroxyapatite, a property desirable for the targeting of molecules to bone for drug delivery and tissue engineering applications. Model peptides of increasing molecular mass (5-33 amino acids) were directly conjugated to eight aspartic acids (Asp₈), eight glutamic acids (Glu₈), or pamidronate, purified by high-performance liquid chromatography, and characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy. The modified peptides were incubated with hydroxyapatite in phosphate-buffered saline at physiological conditions over 24 h. This study revealed a significant amount (>90%) of conjugated peptides adsorbed to the hydroxyapatite as compared to unmodified peptides (<5%). It was found that while there were significant differences between the different hydroxyapatite-binding and control groups for all time points, the size of the peptide had no statistical effect on peptide-hydroxyapatite binding. These results demonstrate that bisphosphonate and oligopeptide conjugates hold great promise for the development of new bioactive molecules for bone-specific applications.

II. Introduction

With an ever-increasing need for organ and tissue replacements, bone loss due to disease or trauma remains a particularly serious health concern. In the field of orthopedic tissue engineering, the central paradigm consists of combining
cells, osteogenic growth factors, and a biocompatible scaffold to direct the repair and replacement of tissue [1]. While choosing the appropriate growth factor(s) is of paramount importance, the delivery mechanism and rate also play a critical role in the recruitment and differentiation of cells and the development of new tissue. Most present tissue engineering strategies rely on diffusion of growth factors from a degrading polymeric system, which can lead to the spread of molecules systemically as well as a loss of function during prolonged delivery [2]. The systemic or extraskeletal delivery of many drugs is undesirable due to possible adverse effects at unintended reception sites as well as the obvious inefficiency in drug delivery. Specifically targeting drugs to bone is difficult because of its high mineral content and lack of natural biological target molecules.

Extensive research has been conducted on various bisphosphonates, especially with regard to their affinity for and effects on bone. Bisphosphonates and their drug conjugates are hydrophilic and resistant to chemical and enzymatic degradation [3]. Bisphosphonates present a popular therapy for bone pathologies including osteoporosis and osteogenesis imperfecta [4]. It is believed that internalized bisphosphonates disrupt the mevalonate pathway in the cell metabolism, leading to apoptosis [5]. These effects were seen in higher rates for osteoclasts (bone-resorbing cells) and metastatic tumor cells than osteoblasts (bone-forming cells). Bisphosphonates also reduce the resorption of mineralized bone during the revascularization of a bone defect [6]. In addition to an uncanny
attraction to bone tissue, nitrogen-containing bisphosphonates have shown pro-
osteoblastic potential in vitro [7-9]. Because osteoblasts are responsible for
incorporating the mineral component into mature bone tissue, differentiation of
migrating mesenchymal stem cells from the bone marrow down the osteoblast
lineage should lead to increased bone formation. This is a favorable property
given the ultimate goal of this research: bone regeneration.

Bisphosphonates have demonstrated a remarkable attraction to bone. This
affinity arises from the attraction of the diphosphonate moiety to calcium ions
present in HA crystal, the mineral component of bone [10]. Specifically for
amino-bisphosphonates like pamidronate and alendronate at physiological pH, the
bisphosphonate is a zwitterion, possessing negative charges on each phosphonate
group while the terminal amine is protonated [11]. The protonated form of
pamidronate is shown in Figure 6.1. Plasma concentrations drop 95% over the 6
h following injection, indicating rapid excretion or bone-binding. Also of great
importance, alendronate (like most bisphosphonates) has no natural metabolism
and therefore will remain active for its duration of time in the body [12]. At pH 7,
the affinity of alendronate for crushed bone particles is described by an apparent
dissociation constant of 1 mmol/L and a binding constant of 100 nmol/mg [13].
These high bone-binding, pro-osteoblastic characteristics make nitrogen-
containing bisphosphonates, such as pamidronate, a promising candidate for use
in bone-targeting applications. Nevertheless, there are some concerns reported
with the heavy systemic use of bisphosphonates that must be addressed before the
development of any bisphosphonate-based treatment [14].

\[
\begin{align*}
H_2N^+ & \quad PO_3H_2 \\
& \quad \quad OH \\
& \quad \quad PO_3H_2 \\
\end{align*}
\]

\[pK_A \approx 11\]

**Figure 6.1.** Pamidronate fully protonated under acidic conditions.

Poly(aspartic acid) (Asp₈) and poly(glutamic acid) (Glu₈) oligopeptides
(eight consecutive residues of aspartic acid and glutamic acid, respectively) have
also exhibited a remarkable affinity to hydroxyapatite (HA) mineral. Not only do
they show preferential attraction to bone but also they are enzymatically
degraded, do not form colloids with metallic ions, and have no long-term health
effects [3]. It has been shown that, with nonspecific conjugation to 6-8
consecutive negatively charged amino acid residues, molecules readily adsorb to
certain calcium-containing surfaces [3, 15]. Molecules modified with Asp₆
exhibited high accumulation in bone in vivo after systemic delivery, while others
conjugated to Asp₈ showed greater than 90% binding to HA in vitro [16, 17]. An
inherent advantage to using these oligopeptides is that no unnatural, synthetic
chemicals are necessary to achieve the desired calcium affinity for the proposed
drug conjugates. The conjugation chemistry of these molecules to peptides is also established and relatively straightforward.

Previous work has been conducted linking molecules to bisphosphonates and acidic oligopeptides in order to confer their bone-binding properties to the conjugate. Uludag and co-workers have utilized numerous methods to link bisphosphonic acid moieties to proteins, including the synthesis of small bisphosphonate functional groups and tethering of such groups to larger molecules using thiol and N-hydroxysuccinimide (NHS) chemistry [10, 18]. However, that work required bisphosphonates that are not registered as pharmaceutically actively agents (such as alendronate and pamidronate), as well as use of biologically unnatural chemical linkers. The only work to use an FDA-approved, medically prescribed bisphosphonate (alendronate) required 11.2-18.4 mol of nitrogen-containing bisphosphonate/mol of conjugate product [17]. The present work made use of a recognized pharmaceutically active bisphosphonate (pamidronate) and more efficient conjugation chemistry for the development of bone-specific bioactive molecules.

The primary objective of this study was to assess the effects of peptide size on the HA-binding capacity of Asp₈, Glu₈, and pamidronate bound to peptides. It is hypothesized that these groups, attached at the N-terminus of all
peptides, will provide modified drugs with their previously observed high affinity for mineralized surfaces. Another goal of this work was to determine variation in HA binding between the different moieties (Asp$_8$, Glu$_8$, and pamidronate) and control groups. A depiction of the chemical structures of these peptide-bound agents is provided in Figure 6.2.

Figure 6.2. Structures of peptides ($n$ amino acids in length) modified with (A) Asp$_8$, (B) Glu$_8$, or (C) Glu-pamidronate. Each modification was performed on the terminal amine group of the resin-bound peptide.
III. Materials and Methods

Materials

Amino acids (L isomers only, when applicable), water-soluble carbodiimide (WSC), N-hydroxsuccinimide (NHS), o-benzotriazole-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBT), Wang resins, and other peptide synthesis supplies were purchased from NovaBiochem (San Diego, CA). Anisole, calcium hydroxide, dichloromethane (DCM), dicyclohexylcarbodiimide (DCC), diisopropylethylamine (DiEA), dimethylformamide (DMF), phosphoric acid, tetrafluorophenol (TFP), triethylamine (TEA), trifluoroacetic acid (TFA), and triisopropylsilane (TIS) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. HA granular microparticles were prepared from calcium hydroxide and phosphoric acid in a pH-controlled reaction [19, 20]. Pamidronate disodium salt was graciously provided by the University Hospital, University of Regensburg, Germany.
Peptide Synthesis

Peptides were produced by solid-state, fluorenlymethoxycarbonyl- (Fmoc) based peptide chemistry on an Advanced Chemtech Apex 396 peptide synthesizer (Louisville, KY). Acylation was obtained by reacting the N-termini of the peptides with 2 equiv of HOBT, 2 equiv of HBTU, and 4 equiv of DiEA in DMF at room temperature. For peptides conjugated to pamidronate, Fmoc-Glu(O-2-PhiPr)-OH was added as the final amino acid. Its side-chain protecting group was removed with 1% TFA in DCM with mild shaking for 1 h.

Bisphosphonate-Peptide Conjugation

To provide a carboxylic acid for reaction with pamidronate, the peptide (still bound to its synthesis resin) was extended by addition of a special glutamic acid [Fmoc-Glu(O-2-PhiPr)-OH]. This glutamic acid (abbreviated E') possessed a very weak protecting group on its carboxylic acid side chain (R-group) that can be selectively removed with a 1% TFA solution while the Fmoc remains to protect
the terminal amine. It is also important to note that the protecting groups remain intact for all other amino acids of the peptide to prevent side reactions and the formation of undesired products.

Resin-bound peptides with deprotected Glu residues (as described above) were dissolved in 5 mL of a DMF/chloroform solution, to which 2 molar equiv of TFP was added. One molar equivalent of DCC was dissolved in 1 mL of the DMF/chloroform solution and added dropwise to the reaction mixture. The solution was shaken at room temperature for 2 h, and then the liquid phase was removed from the resin-bound peptide by vacuum filtration. Resin-bound peptides with a TFP-activated carboxylic acid (TFP-peptides) were dried by vacuum filtration and stored at 0 °C until further reaction.

In a separate vessel, 2 molar equiv of pamidronate disodium were dissolved in 4 mL of Millipore water and 0.5 mL of TEA and sonicated for 10 min. The amount of TEA was based on bisphosphonate concentration such that the final solution pH was greater than 11. The amounts of each reactant used in the conjugation trials can be seen in Table 6.1. After the bisphosphonate was totally dissolved, the solution was added to TFP-peptides, and the mixture was vortexed for 2 min and shaken at room temperature. To maximize the yield, 2 molar equiv of WSC and NHS were added after 2 h of shaking to reactivate any
remaining hydrolyzed or unconjugated carboxylic acids. Exposure of successfully conjugated peptides to WSC does not reverse the reaction, as the amide bond between pamidronate and the peptide was very stable. After a total of 24 h, the liquid was removed by vacuum filtration and the peptide was washed twice with 10 mL of DCM. The resin-bound conjugates were again stored at 0 °C until the time of cleavage. This method was performed twice for each model peptide group to verify reproducibility.

<table>
<thead>
<tr>
<th>reactant</th>
<th>amount (mmol)</th>
<th>mass (mg)</th>
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<tbody>
<tr>
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<td>$a$</td>
</tr>
<tr>
<td>TFP</td>
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<td>50</td>
</tr>
<tr>
<td>DCC</td>
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</tr>
<tr>
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<td>40</td>
</tr>
<tr>
<td>NHS</td>
<td>0.15</td>
<td>20</td>
</tr>
</tbody>
</table>

$a$ Varies with sequence.
**Asp₈** and **Glu₈-Peptide Conjugation**

Unlike bisphosphonate conjugation, the addition of Asp₈ or Glu₈ can be achieved in organic solutions. For resin-bound peptides, the addition of Asp₈ or Glu₈ was achieved by extending the peptide with eight additional aspartic acid or glutamic acid molecules, respectively, by use of standard solid-state peptide synthesis techniques. Like bisphosphonate conjugates, Asp₈- and Glu₈-conjugated peptides were washed, dried, and stored at 0°C until cleavage from the resin.

**Product Cleavage, Purification, and Characterization**

Peptide products were cleaved from the resin by exposure to a TFA-TIS-anisole-water (91:3:3:3 by volume) solution for 1 h. The peptide-containing solution was collected via vacuum filtration and dried in a rotary evaporator (37°C, <25 mm Hg). The product was precipitated by addition of cold diethyl ether, centrifuged at 2500 rpm for 5 min, filtered, dried under vacuum, and stored at 0°C. The crude product was dissolved in DI water to a concentration of 0.5 wt % and purified by high-performance liquid chromatography (HPLC) with a C18
column and using a linear gradient of acetonitrile and DI water containing 0.05% TFA. Peptides and conjugates were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry to determine molecular mass. MALDI-TOF was also utilized to verify the addition of pamidronate, Asp₈, and Glu₈ to the peptide conjugation products.

**In Vitro Binding of Peptides and Peptide Conjugates to HA**

Peptides were synthesized with an aromatic residue as a signaling agent (UV absorbance calibrated to determine peptide concentration) and a semirandom repeat pattern. Four different peptide sizes were tested, from 5 to 33 amino acids in length. The sequences were Y*GNAE, Y*GNAEGRN, Y*[GNAEGRN]₂, and Y*[GNAEGRN]₄ (Y* represents tyrosine for peptides in the control, Asp₈, and Glu₈ groups, and tryptophan for the pamidronate group), yielding 16 total groups. Hydrophobic/hydrophilic interactions were balanced by use of glycine and alanine (hydrophobic residues) with asparagine, glutamic acid, and arginine (hydrophilic residues). To balance the net charge within the model peptide sequence, glutamic acid (negative at physiological pH) and arginine (positive) were alternated. All tests were performed in triplicate (n = 3).
Peptides were dissolved (0.25 mM) in a phosphate-buffered saline solution (PBS, pH 7.4) at 37°C. In Eppendorf centrifuge tubes, 10 mg of HA (100-300 μm microparticles) and 200 L of peptide solution were mixed via vortex for 1 min and placed in a shaker bath at 37°C. Each formulation was cultured for 0.5, 1, 2, 4, 8, and 24 h. After the prescribed time, tubes were removed from the water bath and centrifuged for 2 min at 2500 rpm. Aliquots (100 μL) of the supernatant solution were removed and placed in 96-well flat-bottom plates. Standard peptide solutions were also prepared from 0.01 to 1 mM to generate a concentration calibration curve. Concentrations for each solution were determined by UV absorbance on a Tecan GENios plate reader (Durham, NC) at 280 nm.

Statistics

Data were interpreted by two-way analysis of variance (ANOVA) for the effects of each binding group (control, Asp₈, Glu₈, and pamidronate) and peptide length on HA binding at all time points. Tukey's HSD test was performed for factors determined to have a significant effect on HA binding. ANOVA and
Tukey's tests were conducted with JMP IN version 5.1 statistical discovery software.

IV. Results

Bisphosphonate-Peptide Conjugation

Pamidronate was covalently linked to resin-bound peptides via amide bond between the primary amine of the bisphosphonate and the deprotected carboxylic acid on the peptide's final glutamic acid residue. Peptides before and after bisphosphonate conjugation were analyzed via MALDI-TOF to determine their molecular mass. MALDI-TOF spectra for the WGNAAEE-pamidronate and W[GNAEGNAR]₄E-pamidronate conjugated products are shown in Figure 6.3. The shift in primary peaks was roughly 216 Da (corresponding to the molecular mass addition of the bisphosphonate and loss of one water molecule) for each peptide, with a secondary peak shifted approximately 22 Da more due to sodium ionization. The lack of peak at the molecular mass of the respective unmodified peptide should also be noted.
Figure 6.3. MALDI-TOF mass spectrographs indicating molecular masses of the smallest and largest peptide groups, as well as the corresponding peptide-bisphosphonate and peptide-Asp$_8$ conjugates examined: (A) WGNAE-E, (B) WGNAE-E-pamidronate, (C) YGNAE-D$_8$, (D) W[GNAEGNAR]$_4$-E, (E) W[GNAEGNAR]$_4$-E-pamidronate, and (F) Y[GNAEGNAR]$_4$-D$_8$. 
Effects of Peptide Size on HA Binding in Vitro

Modified peptides exhibited immediate binding to HA, with over 90% of initial peptides bound after only 2-4 h (binding data for the smallest and largest peptide sequences are shown in Figures 6.4 and 6.5). The concentration of unmodified and conjugated peptides (5-33 amino acids in length) was measured over 24 h in culture with HA. It was observed that, for each HA-seeking domain, molecular mass of the peptide had no significant effect on the conjugate's binding rate ($p > 0.05$). The only contributor to significant differences in binding was the HA binding group.
Figure 6.4. Change of percent peptide bound to hydroxyapatite for short (5 amino acids, Y*GNAE) peptides conjugated to (A) Asp₈, (B) Glu₈, and (C) pamidronate and (D) unmodified peptides. Error bars designate standard deviation for n = 3.
Figure 6.5. Change of percent peptide bound to hydroxyapatite for long (33 amino acids, Y*[GNAEGNAR]₄) peptides conjugated to (A) Asp₈, (B) Glu₈, and (C) pamidronate and (D) unmodified peptides. Error bars designate standard deviation for n = 3.
Comparing the HA Affinities of Asp$_8$, Glu$_8$, and Pamidronate-Conjugated Peptides

The choice of HA binding group had a significant effect on the percentage of bound peptides ($p < 0.01$). For each peptide length, the bisphosphonate conjugate initially exhibits less HA binding than the oligopeptide analogues (the difference between pamidronate and Asp$_8$/Glu$_8$ conjugates was statistically significant at 0.5 and 1 h time points). From 2 to 24 h, all conjugates for each model sequence (all lengths) are not statistically different from one another. All modified peptides reached equilibrium between 90% and 95% bound by 24 h. Unmodified control peptides showed no greater than 4% mean binding. The binding of various-sized peptides for each targeting moiety at 0.5 and 24 h is shown in Figures 6.6 and 6.7.
Figure 6.6. Percentage of peptides bound to hydroxyapatite at 0.5 h for control, Asp₈⁻, Glu₈⁻, and pamidronate-modified groups (all peptide sizes). Error bars indicate standard deviation for $n = 3$. *Controls were significantly different than all binding groups for each peptide size; #pamidronate conjugates were significantly different than Asp₈ and Glu₈ groups for each peptide size.
Figure 6.7. Percentage of peptides bound to hydroxyapatite at 24 h for control, Asp₈-, Glu₈-, and pamidronate-modified groups (all peptide sizes). Error bars indicate standard deviation for n = 3. *Controls were significantly different than all binding groups for each peptide size.

V. Discussion

The objective of this research was to produce model peptides, chemically linked to calcium-binding moieties that demonstrate high attraction to bone. While bones contain few biologically active targets, the compounds examined in
this study can utilize this perceived targeting disadvantage in order to adhere (and anchor any conjugated agents) to bone's mineral surface. Early studies employing similar strategies with protein-like molecules for systemic skeletal delivery have shown great promise in bone binding \textit{in vivo} [21].

The exact mechanisms for the preferential binding bisphosphonates and poly(aspartic acid) to bone have never been completely explained in the literature. It is hypothesized that the structures provide favorable ionic interactions between the negative charges of these groups (Asp\textsubscript{8}, Glu\textsubscript{8}, and pamidronate) and calcium ions within the mineral component of bone (hydroxyapatite) at physiological pH [22]. This study was not intended to discover the mechanisms of this attraction but rather to impart the known bone-binding properties of these chemicals to model peptides.

Pamidronate was successfully coupled directly to peptides via an amide bond between the primary amine of the bisphosphonate and a carboxylic acid on the terminal amino acid of the resin-bound peptides. The method described here used a 2:1 bisphosphonate to peptide ratio with only an amide bond between the molecules, which is significantly more efficient than previously reported methods [17]. Significant obstacles presented themselves in conjugating pamidronate to a resin-bound peptide. First, this design strategy calls for linkage of the primary
amine of pamidronate to a terminal amino acid of the model peptide via amide bond (highly stable under physiological conditions). Unfortunately, solid-state peptide synthesis is based on building peptides from the carboxyl to the amine terminus, meaning the amine is the exposed functionality at the end of the model peptide. Amine-amine conjugation is generally nonspecific, meaning some peptides would conjugate to each other, as would bisphosphonate molecules.

A more selective method explored in this work transformed the terminal functional group of the peptide to a carboxylic acid to form a stable amide bond with the amine of the bisphosphonate. An available carboxylic acid on the terminal amino acid was activated with an electronegative leaving group, making the carbon atom susceptible to nucleophilic attack by the pamidronate amine's electron pair. A depiction of this scheme is shown in Figure 6.8. To achieve this, Fmoc-Glu(O-2-PhiPr)-OH was added to the N-terminal end of resin-bound peptides as described in the Materials and Methods section.

![Diagram of conjugation reaction](image)

**Figure 6.8.** Conjugation reaction of pamidronate to the carboxylic acid of glutamic acid (X = leaving group).
An alternative method for generation of a new carboxylic acid group was conversion of the terminal amine of the peptide. The reaction of succinic anhydride with primary amines is an established method to achieve this conversion. Model peptides were reacted in this manner to form corollary conjugates (data not shown), but this approach was deemed less desirable due to the use of biologically unnatural materials (succinic anhydride) and the requisite extra mass added to the final product.

Another challenge of this chemistry was the lack of swelling of the polystyrene peptide resins in water. Bisphosphonate salts were insoluble in mixtures of >95% organic solvents, rendering traditional peptide chemistry techniques ineffective. When the bisphosphonate conjugation was performed in an aqueous solution, the polystyrene resin can fold on itself to minimize thermodynamically unfavorable contact with water. This compacted resin left many or most of the peptide chains unexposed to the reaction solution. To overcome this dilemma, the standard Wang resin was replaced with a NovaSyn TGA resin (NovaBiochem, San Diego, CA). The NovaSyn resin contained a poly(ethylene glycol) (PEG) spacer between the polystyrene and the cleavage site prior to the first amino acid. This PEG spacer was soluble in both aqueous and organic solutions, ideal for peptide synthesis and conjugation in dual phases.
The most critical impediment to this conjugation chemistry is the highly acidic nature of pamidronate (and alendronate) sodium salts. There are five ionizable protons on these bisphosphonates (two on each phosphonate group and one on the primary amine) [23]. While the primary amine is protonated, it will not act as a nucleophile to attack the activated carboxylic acid and form a peptide bond. The pKₐ of the amine's proton is above 11, meaning an equal or higher reaction pH is necessary for the conjugation. Typical amino acid coupling by amide bond formation is conducted by reaction of the carboxyl group with an activating molecule such as DCC or WSC. The activation agent is electron-withdrawing, making the carboxyl vulnerable to nucleophilic attack by the amine's electron pair under mildly basic conditions. The primary amine of pamidronate is protonated (as -NH₃⁺) below pH 11 and unable to attack the carboxyl without its free electron pair. However, at such a high pH, standard carboxyl activating agents are hydrolyzed and rendered useless. To overcome this dilemma, the deprotected carboxylic acid of E' is activated to a significantly more stable intermediate with TFP by a modified version of a previously described reaction [24]. The TFP intermediates were considered very stable and could be preserved for substantial amounts of time and in different solvents. Although the Asp₈ and Glu₈ peptide conjugates synthesized in this study employed previously established peptide chemistry methods (addition during synthesis), use of the TFP-activated intermediate technique can be applied to the conjugation of poly(Asp) and poly(Glu) oligopeptides to primary amines without the requirement
of elevated pH or an aqueous reaction solution used in the reaction with pamidronate.

Peptides of four different lengths/molecular masses were conjugated to \textit{Asp$_8$}, Glu$_8$, or pamidronate as described previously. Each group was incubated with HA in PBS over 24 h to determine the effects of (a) the different calcium-binding moieties and (b) peptide molecular mass on the conjugate's HA affinity. These studies were conducted at physiological conditions (pH 7.4, 37 °C) to draw conclusions about the likely binding properties of modified peptides in vivo. All modified peptides demonstrated rapid adsorption during the first 2 h. Unmodified peptides exhibited practically no affinity for HA, especially when compared to the conjugated versions. The molecular mass of the peptides had no statistically significant effect on the molecule's attraction to HA. Asp$_8$- and Glu$_8$-conjugated peptides appeared to show greater or more rapid binding than bisphosphonate-incorporated molecules for all peptide masses, with differences between pamidronate and Asp$_8$/Glu$_8$ conjugates statistically significant at 0.5 and 1 h time points. By 8 h, all modified groups exhibited greater than 90% binding and approached their respective binding plateaus. The added HA affinity of peptides modified with Asp$_8$, Glu$_8$, or pamidronate, compared to unmodified counterparts, was significant at all time points and for all peptide lengths and is promising for the modification of other biologically active molecules.
The precise mechanisms for the early binding advantage of the Asp₈ and Glu₈ oligopeptides over pamidronate are not yet fully understood, but it could be a result of the larger region of negative charges to associate with calcium in the mineral crystal. The greater total binding at later time points for bisphosphonate conjugates could be a result of more specific binding to HA than for the Asp₈- or Glu₈-conjugated molecules. Regardless of the mechanism, the combining of all these molecules to larger-sized peptides resulted in the conference of HA attraction to the product.

The addition of Asp₈, Glu₈, and bisphosphonates such as pamidronate to larger molecules to confer HA affinity is promising for the future of bone drug delivery, both systemically and locally. Some drugs influence cells via interactions with receptors on the cell surface and need not be released from the chemical anchoring them to bone. For drugs that must be internalized by the cell or require conformational flexibility, it is envisioned that hydrolytically or enzymatically degradable linkers will be used to connect the drug with the bone-binding moiety.
VI. Conclusion

In order to more efficiently deliver molecules to bone tissue, moieties with high calcium affinity were covalently linked to model peptides. The ability of Asp₈, Glu₈, and pamidronate to confer their attraction to HA in vitro to larger conjugated molecules was observed for peptides ranging from 5 to 33 amino acids in length. Asp₈ and Glu₈ conjugates exhibited a faster initial binding than those of pamidronate, but after 2 h the bisphosphonate-linked peptides achieved equivalent binding to their counterparts (>90% bound) from hours 4 through 24. The differences between the bisphosphonate and oligopeptide conjugates were significant only at the 0.5 and 1 h time points. The molecular mass of the peptide was found to have no statistically significant effect on HA binding. It can be reasoned that such HA affinity can be provided to other molecules of similar (and potentially larger) size and molecular mass of these model peptides. The findings of this work are critical in the development of future drugs with the capacity to localize in bone tissue as well as improved bone tissue engineering scaffolding materials.
VII. Acknowledgment

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Chapter 7. The Controlled Release of Native and Modified Osteogenic Peptide from Biodegradable Polymer-Hydroxyapatite Composite Scaffolds

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Abstract

Endeavoring to achieve greater control over the release of osteogenic drugs in orthopedic tissue engineering applications, a model drug (thrombin peptide 508 (TP508)) was modified with compounds exhibiting high affinity to the bone mineral hydroxyapatite (HA). TP508 was covalently linked to poly(glutamic acid) (Glu₈) or the nitrogen-containing bisphosphonate...
pamidronate via an eight amino acid, enzyme-degradable peptide sequence. Native TP508 and the modified versions of the drug were encapsulated in poly(lactic-co-glycolic acid)-poly(ethylene glycol) (PLGA-PEG) blended microparticles for the first level of controlled release. Greater than 96% of all drugs were released over four weeks. Next, the drug-loaded PLGA-PEG microparticles were injected into porous poly(propylene fumarate) (PPF) scaffolds, some of which also containing HA microparticles. The release profiles of all drug types were altered when released from these scaffolds. Inclusion of HA microparticles, as well as variation of the size or surface area of HA within the scaffold, had a statistically significant effect on total release of modified drugs over the course of the study. Larger HA particles most hindered the release of native TP508 peptide molecules due to diffusional limitations, while smaller HA particles (which possess greater surface area per mass HA for drug binding) caused the most decrease in modified drug release. Modified drugs released at nearly half of the original rate by incorporating HA into the PPF construct. A final level of control was attained by releasing the drugs in the presence of collagenase, which cleaved the enzyme-degradable linker sequence and freed the modified drug previously bound to HA. The collagenase-rich media elevated modified drug release to rates more comparable with native TP508 molecules in the same tissue engineering system. By selecting the appropriate drug delivery materials, the amount and size of HA microparticles within a scaffold, and a degradable peptide linker sequence with desirable sensitivity to enzymatic
activity, a higher order of controlled osteogenic drug release can be realized than previously possible.

1. Introduction

Modern strategies for the repair or regeneration of severe skeletal defects include the implantation of donor tissue or synthetic materials in order to maintain the voided space and/or promote the growth of new bone tissue. Donor tissue can be supplied by the patients themselves (autografts), from human cadavers (allografts), or from another mammalian source (xenografts) [1]. Autografts are considered the gold standard in bone replacement because there is no risk of tissue rejection and the transplanted bone should contain viable cells, a mature network of blood vessels, and extracellular matrix (ECM) proteins capable of stimulating its incorporation into the surrounding bone. The primary drawback of an autograft is the requisite creation of a new defect at the donor site, often the iliac crest of a hip or a rib. Also due to shape mismatch, the repaired defect may heal into an unnatural form. Allografts are dependent on donor tissue availability and are processed (decellularized) to limit rejection by the recipient. After processing, the tissue is deficient of a viable cell population and existing vascular network, and may potentially lack functional bioactive molecules or growth factors present in the ECM which stimulate cell migration, differentiation, and/or tissue incorporation. Disease transmission also remains a concern with the use of
any allogenic materials. Xenografts possess no advantages over allografts other than availability and cost [2]. Donor tissue from other species is likely to experience problems with structure and/or compliance, while xenophobic concerns and ethical issues render xenotransplantation a less than ideal solution. The field of tissue engineering and regenerative medicine offers an alternative to these methods for orthopedic tissue repair [3]. Development of new biocompatible and biodegradable materials, cellular incorporation techniques, and delivery methods for bioactive factors offer great promise for the future of synthetic tissue replacement.

The delivery of bioactive factors for bone tissue engineering includes osteogenic (bone-forming) and angiogenic (blood vessel-forming) growth factors, cell recruiting agents, and gene vectors for desired proteins [4-5]. Controlled release is desirable to sustain and optimize the effects these molecules have on tissue regeneration. This control can be achieved by loading the molecules within biodegradable materials that erode at a predictable and desired rate. An established example of this is the use of synthetic biodegradable polymers to form microparticles embedded with drugs. Popular polymers include members of the poly(α-hydroxy ester) family such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and poly(DL-lactic-co-glycolic acid) (PLGA) [6]. The polymers experience hydrolytic degradation in a controlled manner into natural byproducts that are metabolized by the body. The degradation rates depend on material properties such as molecular weight ($M_w$) and crystallinity. Incorporation of more
hydrophilic polymers like poly(ethylene glycol) (PEG) into the microparticles allows for varied or sustained kinetics of the total drug release [7-8]. These approaches have been used in orthopedic tissue engineering for the delivery of drugs like bone morphogenic protein 2 (BMP-2), transforming growth factor β1 (TGF-β1), and vascular endothelial growth factor (VEGF).

Traditional drug delivery strategies focus on the degradation or alteration of the delivery vehicle (e.g. PLGA microparticles) to control the rate at which the molecules are able to diffuse away from the material [9]. After a typical release, the drug’s fate is controlled by diffusional parameters such as the local anatomy, blood supply, and the porosity or permeability of the implant. The aim of this study is to provide further control to tissue engineers, scientists and physicians by modifying drugs with targeting agents so that they might selectively bind (or unbind) to molecular targets within or near the defect. This strategy will localize drugs at the targeted tissue and may significantly increase drug delivery efficiency [10]. The principles of this design can be applied to any molecular marker specific to a tissue of interest. In the field of bone tissue engineering, hydroxyapatite (HA), the mineral component of bone, is an obvious binding target.

Selected targeting moieties for HA must demonstrate consistent affinity to the mineral in order to anchor the conjugated drug to bone’s surface. Ample research has been conducted in this area and provided several intriguing options.
One such class of chemicals is the bisphosphonate family. Bisphosphonates contain two phosphonic acids in a characteristic “P-C-P” (phosphonate-carbon-phosphonate) backbone and are classified according to the other two side chains of the central carbon. The “P-C-P” bond is believed to possess a favorable molecular structure to interact with calcium ions in HA crystal [11-12]. Their remarkable affinity to calcium and bone has been well documented, as well as their anti-bone resorption effects. Bisphosphonates are typically grouped as “nitrogen-containing” or “non-nitrogen-containing.” When consumed by an osteoclast (bone-resorbing cell), those without nitrogen compete with ATP and result in apoptosis of the cell [13]. However, nitrogen-containing bisphosphonates bind to enzymes involved in the mevalonate pathway, such that protein prenylation is hindered. This is believed to alter osteoclastic proteins and cytoskeletal properties essential for survival and bone resorption [14]. Because of the decreased bone resorption observed with their use, bisphosphonates are commonly prescribed for the treatment of osteoporosis, Paget’s disease, and bone metastasis.

Another prominent bone-binding agent is oligopeptides of consecutive negatively charged amino acids. Poly(aspartic acid) and poly(glutamic acid) exhibit high affinity to calcium-containing materials with as few as six aspartic or glutamic acid residues in series [15-16]. It is hypothesized, like with bisphosphonates, that favorable charge distributions and bond angles exist in these oligopeptides to strongly associate with the calcium in the HA crystal
structure. Poly(glutamic acid) was determined to serve as a nucleation agent for HA crystal formation in mineralizing bone tissue as well as an HA binding domain for proteins such as bone sialoprotein [17]. It has been theorized that Asp$_8$ binding to HA is more sensitive than bisphosphonate binding and could be highly dependent on the crystallinity of the mineral [18]. It is important to note that unlike bisphosphonates, poly(aspartic acid) and poly(glutamic acid) are natural, biodegradable compounds with no other known pharmacological effects.

Prior work in this area has shown that compounds conjugated to bisphosphonates or the negatively charged oligopeptides demonstrate the similar preferential binding to HA as the targeting compounds alone [19]. Kasugai et al. demonstrated that Asp$_6$-modified fluorescein isothiocyanate (FITC) subcutaneously injected in mice bound to bone had a half-life of 14 days, while unconjugated FITC was immediately excreted and is not accumulated by soft or bony tissues [20]. Wang et al. also showed that N-(2-Hydroxypropyl) methacrylamide (HPMA) conjugated to Asp$_8$ and alendronate successfully fixed to hydroxyapatite in vitro at 80% and 66% by weight, respectively [19]. Poly(aspartic acid)-linked drugs have demonstrated preferential binding to sites of both bone formation and resorption in vivo when administered systemically [18]. Our laboratory is currently exploring the conjugation of peptide-based drugs to the negatively charged oligopeptides and nitrogen-containing bisphosphonates in order to impart their bone-binding capacity to the drug. In a previous study, we demonstrated that eight consecutive aspartic acids (Asp$_8$), glutamic acids (Glu$_8$),
or pamidronate were all highly capable of providing this ability to conjugated peptides irrespective of the peptides' molecular weight [21]. This was obviously quite promising for small osteogenic drugs to be targeted toward bone tissue.

As a model drug for this new strategy in osteogenic drug delivery, thrombin peptide 508 (TP508, AGYPDEGKRDACEGDSGGPFV) was chosen due to its known effects in bone regeneration, relative small size, and simple secondary structure. TP508 is a 23 amino acid peptide corresponding to the non-proteolytic active sequence of the protein thrombin categorized as the fibroblast receptor binding domain [22-23]. TP508 mimics many of the functions of natural thrombin without affecting normal clot formation in a wound site. Such functions include stimulating cell migration, proliferation, and differentiation for endothelial cells and fibroblasts, as well as chemotaxis for neutrophils, monocytes, and lymphocytes [24]. TP508 is also chemotactic for endothelial cells and induces further protein syntheses than thrombin [25]. Its ability to recruit and differentiate cells in the wound repair site leads to accelerated vascularization, which in turn hastens the bone regenerative processes. Unlike natural thrombin, TP508 molecules are not degraded after interaction with one of the aforementioned cells, making its potency several folds greater than thrombin. Earlier studies have shown that TP508 accelerates fracture repair in long bone fractures and bone bridging in critical sized rabbit tibia defects [26-27]. More recent studies have determined that the peptide upregulates factors dealing with cell growth, proliferation, and death [22]. The overall function is a stimulation of
multiple bone repair pathways. Thus, TP508 was found to be a suitable model drug for this study and future in vivo work.

In this study, fluorescently labeled native and modified versions of TP508 were released from PLGA-PEG microparticles under physiological conditions. Drug-loaded microparticles were also injected into the pore network of poly(propylene fumarate) (PPF) scaffolds, some of which contained HA microparticles of various sizes. A final release was conducted from PPF-HA composites in the presence of the peptide degradative enzyme collagenase. The effects of drug modification, HA incorporation, and enzyme interaction on the release of all drug types were studied over 28 days. The goals of this study were to assess: (1) the benefits of modifying TP508 with Glu₈ or pamidronate moieties on controlled release; (2) the effects of HA microparticles' incorporation and surface area on the release of all peptides; and (3) the ability to achieve a secondary release by cleavage of the degradable linker peptide that connected TP508 with its bone-binding agent.
2. Materials and Methods

2.1 Materials

Amino acids (L isomers only, where applicable), water-soluble carbodiimide (WSC), N-hydroxysuccinimide (NHS), o-benzotriazole-N,N,N,N'-tetramethyluroniumhexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBT), Wang resins, ethylenediaminetetraacetic acid (EDTA), and other peptide synthesis supplies were purchased from NovaBiochem (San Diego, CA). TP508 and TP508-aggrecan-Glu₈ peptides were synthesized by the GenScript Corporation (Piscataway, NJ). Alexa Fluor® 488 carboxylic acid, succinimidyl ester was purchased from Invitrogen (Carlsbad, CA). Zeba™ micro desalt spin columns were purchased from Pierce (Rockford, IL). Acryloyl chloride, anisole, benzoyl peroxide (BP), calcium hydroxide, chloroform, collagenase (clostridiopeptidase A), dichloromethane (DCM), dicyclohexylcarbodiimide (DCC), diethyl fumarate, diisopropylethylamine (DiEA), dimethylformamide (DMF), hydroquinone, fumaric acid, poly(ethylene glycol) (PEG, Mₙ 4,600), phosphoric acid, poly(vinyl alcohol) (PVA, Mₙ 13,000-23,000), tetrafluorophenol (TFP), triethylamine (TEA), trifluoroacetic acid (TFA), triisopropylsilane (TIS), and N-vinyl-2-pyrrolidone (NVP) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Propylene glycol, zinc chloride, propylene oxide, pyridine, hydrochloric acid, sodium hydroxide, and sodium sulfate were purchased from Fisher-Acros (Fair Lawn, NJ). F127 Pluronic gel was purchased
from the BASF Corporation (Mount Olive, NJ). Medisorb® poly(DL-lactic-co-glycolic acid) (PLGA, $M_w$ 47,600) with a 50:50 lactic to glycolic acid composition was purchased from Alkermes (Cincinnati, OH). HA granular microparticles were prepared from calcium hydroxide and phosphoric acid in a pH-controlled reaction [28-29]. Pamidronate disodium salt was supplied by the University Hospital at the University of Regensburg, Germany.

2.2 Drug conjugate design

The drugs examined in this study were native TP508 and TP508 modified with eight consecutive glutamic acid residues (Glu₈) or the nitrogen-containing bisphosphonate pamidronate. To provide flexibility and access to the drug when bound to a calcium-rich surface, a spacer is required between TP508 and the targeting moiety. While any number of spacers could serve this purpose, a degradable linker would provide a secondary control over the drug’s delivery. Current systems for modifying molecules with bisphosphonates or other bone-binding agents do not provide a means by which the drug can be liberated at a desirable time or location [30]. A matrix metalloproteinase (MMP)-sensitive peptide sequence was selected such that when inflammatory cells or osteoclasts approach the drug carrier, secreted enzymes could cleave the linker sequence and liberate the drug to interact with the nearby cells. There are several MMPs known to be readily secreted by cells present in a skeletal repair site, including
collagenase 1 (MMP-1), gelatinase A (MMP-2), collagenase 2 (MMP-8), and gelatinase B (MMP-9) [31]. The amino acid sequence most susceptible to all four of these enzymes is the aggrecan sequence IPENFFGV [32]. This eight amino acid linker should provide TP508 with adequate space and flexibility while tethered to bone-bound Glu₈ or pamidronate. It should also be easily cleaved by enzymes that are readily secreted by the cells known to invade such a wound environment. The final modified drug configuration was “Targeting Moiety”-IPENFFGV-TP508.

2.3 Peptide synthesis

Bisphosphonate-linked peptides were synthesized and modified with pamidronate as previously described [21]. Briefly, peptides were produced by solid-state, fluorenylmethoxycarbonyl (Fmoc) based peptide chemistry. For peptides conjugated to pamidronate, Fmoc-Glu(O-2-PhiPr)-OH was added at the N-terminal end. Its carboxylic acid side-chain protecting group was detached by 1% TFA in DCM with mild shaking for 1 h. Peptide-bisphosphonate conjugation was achieved by activation of the unprotected carboxylic acid of the resin-bound peptides with TFP followed by reaction with the primary amine of pamidronate disodium (2:1 bisphosphonate to peptide molar ratio) in a basic environment (pH > 11). The reaction was driven to completion by addition of WSC and NHS after
two hours. The mixture was shaken for a total of 24 hours at room temperature. All peptides were cleaved by the described methods and stored at 0°C. The completed drug was of the form Pamidronate-EIPENFFGV-TP508.

2.4 Synthesis of PPF

PPF was synthesized in a two-stage reaction by established methods as described by Shung et al. [33] Chemical structures of the products were verified by nuclear magnetic resonance (NMR, Bruker, Billerica, MA). The molecular weights of polymers were measured against polystyrene standards (Fluka, Buchs, Switzerland) by gel permeation chromatography (GPC) using a differential refractive index detector (Waters Corporation, Milford, MA) and a Styragel HR 4E 7.8 mm × 300 mm column (Waters Corporation). The PPF synthesized for the in vitro experiment had a number average molecular weight of 1920 and a polydispersity index of 2.6.
2.5 Fluorescent labeling of peptides

All peptides were labeled with Alexa Fluor® 488 dye (AF488, Mₐ 643). The carboxylic acid, succinimidyl ester of the dye molecule forms a stable amide bond with any primary amine on the peptide. AF488 was found to be superior to traditional fluorescein isothiocyanate dyes because of its photostability, reactivity, bond stability, pH insensitivity (between pH 4 and 10), and water solubility. All peptides contained three primary amines (two lysine residues and the terminal amine). Peptides were dissolved in DMSO at a concentration of 2 mg/180 μL (increased to the molar equivalent for peptides conjugated to Glu₈ and pamidronate). EDTA was added to the solution (0.05 mg/180 μL) and nitrogen was bubbled through to minimize dimerization of the peptides at their cysteine residues. EDTA was selected based on its lack of primary amines and relative ease of removal during purification.

A stock solution of 1 mg AF488 in 200 μL DMSO was prepared in a dark room and vortexed for two minutes. 20 μL of the AF488 stock solution was added to each peptide mixture in a foil wrapped vial and shaken for one hour at room temperature. The labeled peptides were run through a Zeba™ micro desalt spin columns that retain >95% of molecules below 1000 Da (i.e. unreacted AF488 and EDTA). The columns were centrifuged at 4500 rpm, then washed with 50 μL fresh DMSO and respun. The products were stored in foil wrapped tubes at 0°C.
2.6 Fluorescence measurement

The concentration of AF488-labeled peptides was determined by fluorescence measurement on a Molecular Devices SpectraMax M2 plate reader. Liquid samples and standards were injected into 96 well plates and read at an excitation wavelength of 495 nm and emission wavelength of 519 nm. Linear calibration curves were established for concentrations in the range of 0.01 to 100 μg/mL.

2.7 PLGA-PEG microparticle fabrication

Blended microparticles of PLGA (50:50) and PEG were produced using an emulsion-solvent extraction technique [(water-in-oil)-in-water] similar to previously described methods [7-8, 34]. Briefly, 47.5 mg PLGA and 2.5 mg PEG (Mw 1000) were dissolved in 100 μL DCM. For microparticles receiving fluorescently labeled peptides, nitrogen gas was bubbled through the solution for 15 minutes to purge oxygen.
A volume of 112.5 µL of pure DMSO or labeled peptide solution (as prepared above) was added and the resulting solution was vortexed for 2 minutes. Then, 200 µL of cold aqueous PVA solution (3% by mass) was added and vortexed for 30 seconds to create an emulsion. This emulsion was cast on 2 mL aqueous isopropanol (2% by volume) on ice. The emulsion container was rinsed with an addition 200 µL PVA solution and cast onto the isopropanol. The mixture was agitated for one hour on ice and then centrifuged for 5 minutes at 4000 rpm. The supernatant was drained, washed on 20 µm filter paper (Whatman® 41 ashless filters) under vacuum, and the solid microparticles were lyophilized for 24 hours. Dry microparticles were separated based upon physical size by high velocity sieve shaking using 53 µm and 106 µm pore diameter sieves (Fisher Scientific).

2.8 Drug encapsulation efficiency

The efficiency of drug loading into the PLGA-PEG microparticles was determined by first dissolving the 5 mg microparticles in chloroform (200 µL) then adding 5 mL water to the solution in order to extract the drug. The amount of drug in this solution was again determined via fluorescence spectroscopy.
2.9 PLGA-PEG and HA microparticle characterization

PLGA-PEG and HA microparticles were characterized by scanning electron microscopy (SEM), light microscopy, and coulter counter measurement after physical separation with sieves. Briefly, microparticles were sieved under high vibration and separated into three groups based on particle size (20-50, 50-100, and 100+μm). To establish the approximate particle shape within each range, microparticles were added to water and dropped on an SEM stage or glass slide. After drying, microparticles on SEM stages were sputter coated in gold and examined via SEM. The glass slides were observed under light microscope to confirm the shape of microparticles under wet conditions. To assess the size distribution of microparticles within each size range, microparticles were diluted to 10 μg per mL of isotonic buffer and processed by a Beckman Coulter Multisizer™ 3 coulter counter with 100 μm aperture.

2.10 Composite scaffold fabrication

Earlier research in our laboratory has demonstrated the effectiveness of PPF-based scaffolds for in vivo tissue engineering applications [27, 35]. Porous scaffolds were fabricated by combining 1.5 g PPF and 1.5 g NVP in a 200 mL
beaker. The polymer mixture was stirred for 24 hours to achieve homogeneity. To serve as a thermal crosslinking initiator, 150 μL BP solution (0.1 g BP per mL acetone) was injected into the mixture and vortexed for 1 minute. Next, 22 g NaCl (300-500 μm) were added and the mixture was thoroughly stirred until homogenous. The resulting paste (88% salt by mass) was inserted into cylindrical Teflon molds (diameter = 8 mm, height = 10 mm) and incubated at 60°C for 60 hours to ensure complete crosslinking. Crosslinked scaffolds were removed from the molds and cut to a height of 1 mm with a diamond saw. The scaffolds were leached for 5 days in 500 mL Millipore water to remove the salt porogen. The solution was exchanged with fresh Millipore water each day. The scaffolds were lyophilized for 2 days then stored at room temperature.

To provide the modified drugs an internal target within an implanted tissue engineering construct, as well as to potentially enhance the osteoconductive properties of the material, HA microparticles were loaded into the pore network for some experimental groups. Scaffolds containing HA received 5 mg total, regardless of HA particle size (sieved into 20-50 and 50-100 μm size ranges). Two HA size ranges were studied to determine the effect, if any, surface area played to the release or binding of modified drugs. All scaffolds received a total of 5 mg PLGA-PEG microparticles (loaded and blank microparticles mixed to yield the appropriate dose of peptide). Microparticles were delivered into the pore network by injecting them within a Pluronic gel solution. For the described scaffold formulation, there is approximately 43 μL of pore volume per scaffold,
determined via microcomputed tomography (microCT). Thusly, a suspension of
5 mg microparticles per 40 μL Pluronic F127 solution (PF127, 20 wt % in
ddH₂O) was prepared and refrigerated to prevent gelation. This solution has the
desirable property of phase reversibility based on temperature (liquid below 10°C,
gel above 25°C). Prior to application of this Pluronic microparticle suspension to
the scaffold, the solution was stored on ice. On both faces of the scaffold, 20 μL
of the Pluronic-PLGA-(HA) solution was dispensed and allowed ten minutes for
it to penetrate the pores and gel.

2.11 Scaffold characterization

Scaffolds were weighed prior to and after leaching to insure complete salt
removal. Porous scaffolds and scaffolds containing PLGA-PEG and HA
microparticles were analyzed via microCT. Blank PPF scaffolds, scaffolds loaded
with PLGA-PEG microparticles, and scaffolds loaded with PLGA-PEG and HA
microparticles (20-50 μm) were scanned by microCT at 8 μm per pixel. Scans
were reconstructed in three dimensions and projections were made based on
signal intensity.
2.12 Experimental design

A study to assess the release kinetics of native TP508 and TP508 modified with Glu₈ or pamidronate under various conditions was designed based on previous studies involving the release of TP508 from similar tissue engineering constructs [34]. A total of 50 µg of TP508 (or the molar equivalent of TP508-agg-Glu₈ or TP508-agg-Pam) was released from a total of 5 mg drug-loaded and blank PLGA-PEG blended microparticles per scaffold. Based on the determined loading efficiency of each drug, 2.55, 2.73, and 2.64 mg of drug-loaded microparticles were used for the native TP508, Glu₈-modified, and pamidronate-modified groups, respectively, with the balance of the 5 mg comprised of blank microparticles for each group. The primary effects tested were the targeting moiety, time, incorporation of HA microparticles, surface area of HA microparticles, and the presence of collagenase. The design of 12 total groups is shown in Table 7.1. A release of each type of peptide from PLGA-PEG microparticles (no scaffold) was also conducted as a control.
<table>
<thead>
<tr>
<th>Group</th>
<th>Targeting Moiety</th>
<th>HA microparticles</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>Glu₈</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>Pamidronate</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>20-50 μm</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>Glu₈</td>
<td>20-50 μm</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>Pamidronate</td>
<td>20-50 μm</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>50-100 μm</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>Glu₈</td>
<td>50-100 μm</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>Pamidronate</td>
<td>50-100 μm</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>None</td>
<td>20-50 μm</td>
<td>Collagenase</td>
</tr>
<tr>
<td>11</td>
<td>Glu₈</td>
<td>20-50 μm</td>
<td>Collagenase</td>
</tr>
<tr>
<td>12</td>
<td>Pamidronate</td>
<td>20-50 μm</td>
<td>Collagenase</td>
</tr>
</tbody>
</table>
2.13 In vitro peptide release

A total of 60 scaffolds (n=5 per group) were prepared as described. Each scaffold was loaded with the prescribed amount of peptide-loaded PLGA-PEG microparticles (and HA microparticles for Groups 4-12) and placed in a well of a 48 well plastic plate. Each well received 1 mL phosphate buffered saline solution (PBS, pH 7.4). The plate was covered with a lid and wrapped in foil to minimize photobleaching of the labeled peptides. The plates were placed on a shaker table at 37°C. To determine the cumulative release of peptides, all release media was removed at time points of 0.5, 1, 2, 4, 6, 8, 12, 16, 20, 24, and 28 days and was replaced with fresh PBS. Samples incubated with collagenase (373 ng/mL in PBS) received fresh enzyme-containing media at each time point. This concentration was selected based on previous degradation studies conducted in our laboratory corresponding to collagenase concentrations in vivo at repair sites [36-37]. The release samples and concentration standards were placed into a 96 well plates for fluorescence measurement. PLGA-PEG microparticle release (no scaffold) was conducted by incubating the drug-loaded microparticles in Eppendorf centrifuge tubes. At the specified time points, the tubes were centrifuged and the release media replaced with 1 mL fresh PBS.
2.14 Statistical analysis

Data were interpreted by a two factor analysis of variance (ANOVA) to assess the main effects of each binding motif (control, Glu₈, and pamidronate), HA incorporation (none, small, or large particles), and the presence of collagenase on total peptide release at all time points. Tukey's HSD test was performed for factors determined to have a significant effect on total peptide release. ANOVA and Tukey's tests were conducted with JMP IN® version 5.1 statistical discovery software.

3. Results

3.1 Drug entrapment efficiency

Initially, 1.125 mg TP508, 1.980 mg TP508-Glu8, or 1.733 mg TP508-Pam (the molar equivalent for modified peptides) were loaded per 50 mg PLGA-PEG polymer. The drug was not totally loaded within the polymer, which was evidenced by the discoloration of the emulsion media due to the orange AF488 dye. A sample of 5 mg TP508-loaded microparticles was dissolved in 200 µL chloroform. After the polymer had dissolved, the solution was diluted with 5 mL water to extract the peptide drug. The concentration of each peptide was
measured and compared to the amount originally loaded into the microparticles during fabrication to determine its loading efficiency. The loading efficiency of TP508, Glu8-modified TP508, and pamidronate-modified TP508 was 78.5 ± 3.8%, 73.2 ± 2.6%, and 75.7 ± 3.0%, respectively.

3.2 PLGA-PEG and HA microparticle characterization

Microparticles of PLGA-PEG and HA were fabricated as previously described. After synthesis and lyophilization, all microparticles were sieved and separated by physical size. Particles were collected in the size ranges of 20-50 μm and 50-100 μm. A coulter counter assessed the microparticle size distributions (n = 8). The results of this characterization are shown in Table 7.2. The reported error for each average particle size corresponds to the calculated error from the standard deviations of each tested sample. To confirm the particles spherical shape for surface area estimation, microparticles were dispersed in water, dropped onto SEM stages and glass microscope slides, and observed via SEM or light microscopy, respectively. Images of SEM-analyzed PLGA-PEG and HA microparticles are displayed in Figure 7.1.
Table 7.2. Size and shape distribution of PLGA-PEG and HA microparticles

<table>
<thead>
<tr>
<th>Material</th>
<th>Size Range (μm)</th>
<th>Average Particle Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA-PEG</td>
<td>20-50</td>
<td>27.9 ± 1.8</td>
</tr>
<tr>
<td>HA</td>
<td>20-50</td>
<td>33.1 ± 3.5</td>
</tr>
<tr>
<td>HA</td>
<td>50-100</td>
<td>79.3 ± 5.0</td>
</tr>
</tbody>
</table>

Figure 7.1. Scanning electron microscopy images of PLGA-PEG microparticles (20-50 μm) (A), HA microparticles (20-50 μm) (B), and HA microparticles (50-100 μm) (C).

Microparticles of all types were approximated as spherical or semi-spherical. For purposes of surface area comparison between HA particle sizes, the surface area per volume ratio of a sphere was assumed (S.A. per V = 3/R
where $R$ is the average microparticle radius). By more than doubling the average particle radius (16.6 to 39.6 $\mu$m), the available surface area per mg HA was reduced by more than 58.2%.

### 3.3 Scaffold characterization

Blank and composite scaffolds were scanned using microCT to evaluate the distribution of microparticles throughout the pore network ($n = 8$). Figure 7.2 contains coronal and sagittal images of scaffolds loaded with PLGA-PEG microparticles only (A) and the mineral component of scaffolds loaded with both PLGA-PEG and HA microparticles (B). The colors in Figure 7.2 were representative of the signal intensity, which is directly related to the opacity of the material. Due to having a similar density to PPF, PLGA-PEG microparticles were not very distinguishable from the scaffold. However, HA microparticles were quite visible coating the pore walls in Figure 7.2B. The broad distribution of HA observed in both the coronal and sagittal views of the scaffold is desirable for its intended purpose of serving as a molecular target for modified drugs and as an osteoconductive surface for bone-forming cells. Micro-computed tomography measurements ($n=3$) of microparticle-free scaffolds revealed an average porosity of $84.6 \pm 2.2\%$ by volume and a volumetric interconnectivity of $94.8 \pm 1.7\%$. 
Figure 7.2. MicroCT images of PPF scaffolds loaded with PLGA-PEG only (A), and PLGA-PEG and HA microparticles (B). The polymer component of the construct is seen as green, while the more radio-opaque mineralized material appears blue.

3.4 *In vitro* release of drugs from PLGA-PEG microparticles

The cumulative release of TP508, TP508-aggrecaGlu₈, and TP508-aggrecaG-pamidronate from PLGA-PEG microparticles was measured over 28 days (Figure 7.3). An initial burst release was seen for all drugs over the first two days (>85% of total dose) with a sustained release over the remainder of the study (approximately 100% release by day 28). There were no statistically significant differences in cumulative release between the drugs at any time point, suggesting
that the additional size of the modified drugs did not hinder their release from nor alter the degradation of the PLGA-PEG microparticles.

Figure 7.3. Percent cumulative release of native (• TP508), Glu8-modified TP508 (■ Glu8), and pamidronate-modified TP508 (△ Pamidronate) from PLGA-PEG microparticles over 28 days. Error bars represent means ± standard deviation at each time point for n=5.

3.5 *In vitro* release of drugs from PPF-based composite scaffolds

Native and modified TP508 peptides were released from PLGA-PEG microparticles loaded within PPF scaffolds for 28 days under various conditions described in Table 7.1. For all groups under all conditions, an initial burst release
was observed followed by a slow and sustained release of the drug (Figure 7.4). The profiles of TP508 released without HA, with the small (20-50 μm) HA particles, and with the large (50-100 μm) HA particles are illustrated in Figure 7.4A. The analogous release profiles of TP508-aggrecaGlu₈ and TP508-aggreca-Pamidronate are shown in Figures 4B and 4C, respectively.
Figure 7.4. Percent cumulative release of native TP508 (A), Glu₈-modified TP508 (B), and pamidronate-modified TP508 (C) from PLGA-PEG microparticles in polymer scaffolds without HA (• No HA), with 5 mg 20-50
μm HA ( ■ Small HA), or 5 mg 50-100 μm HA ( △ Large HA) over 28 days. Error bars represent means ± standard deviation at each time point for n=5.

HA microparticles retarded the release of modified drugs from the composite scaffolds. Interestingly, the presence of HA also hindered the release of native TP508 peptides. It is critical to recognize that the incorporation of larger HA microparticles resulted in the greatest decrease in TP508 release, but the small HA microparticles caused the most hindrance to the drugs modified with Glu8 and pamidronate. The effect of the presence of HA mineral on cumulative release for modified drugs after the initial burst (t > 2 days) was statistically significant (p < 0.05). The size (and therefore surface area) of HA also had a statistically significant effect on the release of all drugs after the initial burst release.

3.6 In vitro release of drugs from scaffolds with collagenase

To test the feasibility of the enzyme-degradable linker sequence, Groups 4-6 were repeated in Groups 10-12 with the presence of the MMP collagenase. The release of all drugs in the presence of collagenase is illustrated in Figure 7.5. Collagenase had no effect on the release of native TP508 from PPF scaffolds containing 20-50 μm HA microparticles, comparing the release of TP508 in
Figure 7.5 to its release with the small HA particles in Figure 4A. For the drugs modified with Glu₈ or pamidronate, the characteristic initial burst release was again observed followed by a sustained release. At each time point after the burst release (t > 2 days), there was a statistically significant increase in drug release caused by the collagenase enzyme cleaving the MMP degradable sequence and liberating the TP508 segment from the targeting moieties bound to HA (p < 0.05). The release of native TP508 in the presence of small HA microparticles is significantly greater than this of modified drugs in the same construct with collagenase at all time points (Figures 7.4A and 7.5).

Figure 7.5. Percent cumulative release of native TP508 (●TP508), Glu₈-modified TP508 (■Glu₈), and pamidronate-modified TP508 (▲Pamidronate) drugs released in media with collagenase from microparticles within polymer scaffolds over 28 days. Collagenase concentration was 383 ng/mL in PBS. Error bars represent means ± standard deviation at each time point for n=5.
4. Discussion

Drug delivery remains a critical variable in the concept of tissue engineering. Most drug delivery systems rely on the degradation of the carrier material to control the release of drugs into the local environment. To maximize the efficiency of drug delivery in tissue engineering applications, it became necessary to modify drugs with molecular targeting agents for the intended tissue. This modification provided greater control in the release of osteogenic drugs from biocompatible, osteoconductive polymer scaffolds. Not only that, during the course of this study it was revealed that multiple levels of control been obtained.

Biodegradable and biocompatible porous PPF-based scaffolds were infused with drug-loaded PLGA-PEG microparticles in a manner consistent with previous in vivo studies [27]. To supply a local drug target and an osteoconductive material for new bone formation within the construct, some scaffolds received HA microparticles of 20-50 μm or 50-100 μm. The larger HA particles were utilized to test drug binding with less available surface area. A final release was conducted from PPF scaffolds with 20-50 μm HA incubated with the MMP enzyme collagenase. This release was to determine the feasibility of linking osteogenic drugs to bond-binding agents via a MMP-sensitive peptide sequence for the purpose of obtaining a secondary controlled release of the drugs into the neighboring environment in the presence of enzyme-secreting cells.
Release of peptides from PPF scaffolds without HA did not achieve the maximum theoretical release over the course of the study. The release of native and modified TP508 from PLGA-PEG microparticles without scaffolds was observed and found to be significantly higher than with scaffolds (approximately 100% cumulative release at 28 days). This demonstrated that peptides were released from the microparticles, but the release could have been hindered by incomplete PLGA-PEG degradation in the polymer composite, adsorption of the peptides to the PPF scaffold, or diffusional limitations related to the Pluronic gel carrier material within the pores.

The release of native and modified types of TP508 was also studied with HA microparticles injected with the PLGA-PEG microparticles into PPF scaffolds. The initial study utilized HA microparticles of the same size range as the PLGA-PEG microparticles (20-50 μm) and found that the release of all drugs was dampened. While a decreased release was expected for modified drugs capable of binding to HA, the reduced discharge of native TP508 raised the possibility that physical diffusional limitations could be obstructing the drugs from escaping into the release media.

Another release was performed using an equal mass of larger HA particles (50-100 μm) to reduce the available surface area for modified drugs to target. While there was not a significant change in the release of native TP508
molecules, modified drugs experienced a statistically significant increase in cumulative release at all time points. As the HA particles were approximated to be spherical, the surface area per volume was $3/R$, where $R$ is the average particle radius. By increasing the size of HA within the scaffold, but not the volume or mass, the surface area was significantly decreased to approximately 45.5% of the original surface area. In this system with larger HA particles, the release of natural TP508 was diminished while both modified drugs experienced a significant increase in release at all time points during the study. This indicated that while there were some diffusional limitations at play resulting from the existence of HA in the pore network of scaffolds, modified drugs underwent specific binding to the mineral and their release rates were related to the available surface of HA.

The final release experiment featured a collagenase enzyme in the release media. Conditions were repeated as in Groups 4-6 (20-50 μm HA) with a collagenase concentration of 373 ng/mL in PBS at 37°C. This enzyme was representative of the degradative enzymes secreted by the inflammatory cells and osteoclasts present in the wound environment [10]. As previously discussed, the peptide linker sequence (IPENFFVG) was sensitive to multiple forms of collagenase and was believed to be ideal for the secondary release of osteogenic drugs in the presence inflammatory cells or osteoclasts near the implant or defect. As predicted, the enzyme had no effect on the release of unmodified TP508 from the construct at any time point. For Glu₈ and pamidronate-modified peptides,
collagenase caused a significant increase in drug release after one day and for all time points thereafter. Because there were no changes in the release of TP508, it can be inferred that the enzyme did not considerably alter the degradation of the PLGA-PEG microparticles. Therefore, variation in the release profiles of modified drugs was due to the cleavage of peptide linker sequences and liberation of the TP508 segment previously anchored to HA. This additional form of control may make this system superior to those in which the binding agents and drugs are permanently attached. Other systems for drug modification focus on systemic, rather than local, drug delivery, and do not provide additional control in the form of microparticle/scaffold degradation, provision of local HA, and a mechanism for the eventual separation of the drug from its bone-binding moiety. The long-term objective of this research is to achieve maximum control and efficiency in osteogenic drug delivery for bone tissue engineering applications.
5. Conclusion

Constructs for bone tissue applications were created by fabrication of porous PPF scaffolds containing drug-loaded PLGA-PEG and HA microparticles. This study evaluated the effects of drug modification, HA incorporation, and enzyme interaction on osteogenic drug release from these tissue engineering constructs over four weeks. By using different sized HA microparticles, it was determined that the HA surface area was a significant factor in the rate that modified drugs were released. Also, the presence of a collagenase enzyme resulted in amplified drug release after one day in incubation, proving that the MMP-sensitive degradable peptide linker sequence functioned correctly. The results of this study are significant to the field of drug delivery as multiple forms of control have been demonstrated. By modifying drugs with molecular targeting agents, released drugs can bind to and localize in desired local tissues. Secondly, inclusion of HA into tissue engineering constructs provides an immediate binding target for modified drugs, as well as a more osteoconductive surface for cells and a resource for future mineralization. Variation in the amount and size of HA microparticles yields another form of control in this drug delivery system. Thirdly, selection of an appropriate chemical linker, in this case the enzyme-sensitive IPENFFVG peptide, dictates the rate at which the active drug is freed from the HA-bound Glu8 or pamidronate segment.
6. Acknowledgements

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7. References


Chapter 8. The Effects of Native, Glu₈-modified, and Bisphosphonate-modified Osteogenic Peptide on Bone Regeneration In Vivo

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Abstract

In this study, we examined the dose effects of thrombin peptide 508 (TP508) released from biodegradable polymer constructs on bone regeneration in a critically sized rat cranial defect model after 4 weeks. It was observed that the
medium dose (50 μg) resulted in significantly greater amounts of bone regeneration than controls or the low dose (25 μg). A larger dose of TP508 (100 μg) did not produce significantly different bone formation. After the dose response was assessed, equal molar doses of native TP508 and modified, bone-targeting versions of the drug were released from polymer-hydroxyapatite (HA) composite scaffolds in the rat cranial model. We found that modification of TP508 with bone-binding moieties had no negative effects on the drug’s ability to regenerate bone. Native and modified versions of TP508 caused significantly greater bone formation than blank drug-free polymer-HA constructs. The effect of HA incorporation on bone regeneration was also studied for blank controls and 50 μg native TP508 doses. In both cases, HA inclusion yielded significantly more bone after 4 weeks in vivo. This study demonstrates that our drug modification process does not result in a loss of activity or a decrease in bone regeneration. It also confirms that incorporating HA into polymer constructs contributes to a higher amount of bone formation.

1. Introduction

In the field of regenerative medicine, strategies for tissue engineering typically incorporate a combination of a biocompatible scaffold, cells, and bioactive factors to induce the formation or repair of a desired tissue [1]. The scaffolding material serves as a pattern for guided tissue growth and provides
mechanical support for the regenerating tissue. Cells are essential for recreating functional tissue and organs and can be provided by implantation with the biomaterial or by recruitment from surrounding tissue. Bioactive factors, including growth factors, hormones, and gene vectors, are generally released in influence cell differentiation and/or proliferation. Most drug delivery strategies in tissue engineering rely on simple diffusion of drug molecules from the carrier material to the surrounding tissue. While this is often effective for constructs with a local or implanted cell population, it is inefficient for sustained delivery because drug molecules that reach the blood stream are quickly removed from the repair site and metabolized by the body. The goal of this study was to test the biological function of drugs modified with bone-binding agents that can target mineralized surfaces in or near the implant and reduce or eliminate systemic drug loss.

Previous work has been conducted on peptide drugs modified with negatively charged oligopeptides and bisphosphonates [2]. It was observed that peptides in excess of 30 amino acids, when modified with these calcium-binding moieties, demonstrated a high affinity for hydroxyapatite (HA), the mineral component of bone. As a model drug for this work, thrombin peptide 508 (TP508) was selected for its established use in orthopedic repair and manageable molecular size. TP508 functions as a chemo-attractant and initiates various wound healing cascades that accelerate tissue repair. A representation of native TP508 can be seen in Figure 8.1. It has previously been used in the repair of bone and cartilage in various animal models [3, 4]. Native TP508, as well as versions
of the peptide conjugated to eight consecutive glutamic acid residues (Glu₈) or pamidronate via an eight amino acid, matrix metalloproteinase (MMP) cleavable peptide linker sequence, was released from composite polymer-HA scaffolds. It was observed that HA incorporation in the pore network of scaffolds significantly reduced the apparent release of modified drugs, and that the reduction in release was related to the surface area of HA microparticles.

Figure 8.1. Thrombin Peptide 508 Structure

In this study, we implanted the composite scaffolds that released native, Glu₈-modified, or pamidronate-modified TP508 in a critically-sized rat cranial defect model. The amount of bone formed in each animal after four weeks was measured using microcomputed topography (microCT). First, a dose-effect of unmodified TP508 was established to select an appropriate base dose at which to study modified versions of the drug. Composites for certain groups included HA
to provide modified drugs with a local binding target within the defect and to study the effects of HA incorporation on in vivo bone regeneration.

2. Materials and Methods

2.1 Materials

Amino acids (L isomers only, where applicable), water-soluble carbodiimide (WSC), $N$-hydroxysuccinimide (NHS), $o$-benzotriazole-$N,N,N',N'$-tetramethyluroniumhexafluorophosphate (HBTU), $N$-hydroxybenzotriazole (HOBT), Wang resins, ethylenediaminetetraacetic acid (EDTA), and other peptide synthesis supplies were purchased from NovaBiochem (San Diego, CA). TP508 and TP508-aggreca-Glu₈ peptides were synthesized by the GenScript Corporation (Piscataway, NJ). Acryloyl chloride, anisole, benzoyl peroxide (BP), calcium hydroxide, chloroform, collagenase (clostridiopeptidase A), dichloromethane (DCM), dicyclohexycarbodiimide (DCC), diethyl fumarate, diisopropylethylamine (DiEA), dimethylformamide (DMF), hydroquinone, fumaric acid, poly(ethylene glycol) (PEG, $M_w$ 4,600), phosphoric acid, poly(vinyl alcohol) (PVA, $M_w$ 13,000-23,000), tetrafluorophenol (TFP), triethylamine (TEA), trifluoroacetic acid (TFA), triisopropylsilane (TIS), and $N$-vinyl-2-pyrrolidone (NVP) were purchased from Sigma-Aldrich (St. Louis, MO) and used
as received. Propylene glycol, zinc chloride, propylene oxide, pyridine, hydrochloric acid, sodium hydroxide, and sodium sulfate were purchased from Fisher-Acros (Fair Lawn, NJ). F127 Pluronic gel was purchased from the BASF Corporation (Mount Olive, NJ). Medisorb® poly(DL-lactic-co-glycolic acid) (PLGA, M<sub>n</sub> 47,600) with a 50:50 lactic to glycolic acid composition was purchased from Alkermes (Cincinnati, OH). HA granular microparticles were prepared from calcium hydroxide and phosphoric acid in a pH-controlled reaction [5, 6]. The product was washed over 20 μm filter paper, dried, and physically sieved to collect microparticles in the size range of 20-50 μm (53 μm sieve, Fisher Scientific). Pamidronate disodium salt was supplied by the University Hospital, University of Regensburg, Germany. Surgical supplies were purchased from Henry Schein Dental (Milwaukee, WI).

2.2 Peptide Synthesis and Drug Design

Peptides modified with pamidronate were synthesized as previously described [2]. Briefly, peptides were produced by solid-state, fluorenylmethoxycarbonyl (Fmoc) based peptide chemistry. For peptides conjugated to pamidronate, Fmoc-Glu(O-2-PhiPr)-OH was added at the N-terminal end. Its carboxylic acid side-chain protecting group was detached by 1% TFA in DCM with mild shaking for 1 h. Peptide-bisphosphonate conjugation was achieved by activation of the unprotected carboxylic acid of the resin-bound
peptides with TFP followed by reaction with the primary amine of pamidronate disodium (2:1 bisphosphonate to peptide molar ratio) in a basic environment (pH > 11). The reaction was driven to completion by addition of WSC and NHS after two hours. The mixture was shaken for a total of 24 hours at room temperature. All peptides were cleaved by the described methods and stored at 0°C.

Modified drugs were designed as previous described [7]. The Glu₈ and pamidronate groups were covalently linked to TP508 via an eight amino acid, enzymatically degradable peptide sequence. For maximum release in the presence of cells, the most easily degraded sequence was selected for MMPs found in sites of bone repair. The bovine aggrecan sequence (IPENFFGV) is susceptible to cleavage by MMP-1, 2, 8, and 9 [8, 9].

2.3 PLGA-PEG Microparticle Fabrication

Microparticles of PLGA (50:50) and PEG were produced using an emulsion-solvent extraction technique [water-in-oil-in-water] similar to previously described methods [7, 10]. Briefly, 47.5 mg PLGA and 2.5 mg PEG (M₆ 1000) were dissolved in 200 µL DCM. A desired ratio of 25, 44, and 38.5 µg (native TP508, Glu₈-modified, and pamidronate-modified, respectively) drug per mg polymer was added to solutions intended for drug-loaded microparticles
and vortexed for two minutes. Then 200 μL of cold aqueous PVA solution (5% by mass) was added and vortexed for 30 seconds to create an emulsion. This emulsion was cast on 2 mL aqueous isopropanol (2% by volume) on ice. The emulsion contain was rinsed with an addition 200 μL PVA solution and cast onto the isopropanol. The mixture was agitated for one hour on ice and then centrifuged for 5 minutes at 4000 rpm. The supernatant was drained and the solid microparticles were lyophilized for 24 hours.

PLGA-PEG microparticles were characterized for shape and drug loading as previously described [7]. Briefly, the isopropanol solution containing microparticles was filtered at 20 μm under vacuum. Dried microparticles were then sieved to separate by physical size and collected in the range of 20-50 μm. Microparticles were dispersed in water, and then analyzed by coulter counter to assess the distribution of particle size. A sample of the product dropped onto a scanning electron microscopy (SEM) stage and a glass slide for light microscopy observation to confirm size and spheroid shape. In order to determine the loading efficiency of the drugs within the PLGA-PEG microparticles, samples were prepared using fluorescently labeled analogues of each drug. A sample of 5 mg of each type of drug-loaded microparticles was dissolved in 200 μL chloroform. After dissolution, 5 mL water was added to extract the drug from the organic phase, then the concentration of peptide in solution was measured to calculate the mass ratio of peptide drug per polymer. It was determined that the loading
efficiency of native TP508 was $78.5 \pm 3.79\%$, Glu8-modified TP508 was $73.2 \pm 2.61\%$, and pamidronate-modified TP508 was $75.7 \pm 3.01\%$.

2.4 Composite Scaffold Fabrication

Porous PPF-based scaffolds were fabricated by combining 2 g PPF and 2 g NVP in a 200 mL beaker. The polymer mixture reached confluency after 24 hours. To serve as a thermal crosslinking initiator, 200 μL BP solution (0.1 g BP per mL acetone) was injected into the mixture and vortexed for 1 minute. To generate a porous network, 28 g NaCl (300-500 μm) were added and the mixture thoroughly stirred until homogenous. The resulting paste (88% salt by mass) was inserted into cylindrical Teflon molds (diameter = 8 mm, height = 10 mm) and incubated at 70°C for 36 hours to ensure complete crosslinking. Crosslinked scaffolds were removed from the molds and cut to a height of 1 mm with a diamond saw. The scaffolds were leached for 5 days in 500 mL Millipore water to remove the salt porogen, exchanging the leaching solution with fresh water each day.

All scaffolds received a total of 5 mg PLGA-PEG microparticles (20-50 μm in diameter) using a combination of drug-loaded and blank microparticles based on the drug loading efficiency and desired dose. The amounts of drug-
loaded and blank microparticles used for composites of each group are stated in Table 8.1. Scaffolds containing HA received 5 mg of particles, also in the size range 20-50 μm. One hour prior to surgery, microparticles were dispersed in a F127 Pluronic gel solution (24% by wt. in phosphate buffered saline, pH 7.4). Microparticle concentration was based on the pore volume of scaffolds (40 μL). Microparticles were delivered by injecting 20 μL of the Pluronic solution on both faces of the scaffold. The solution filled the pore network and formed a gel at room temperature within minutes. Scaffolds, microparticles, and pluronic gel materials were sterilized with ethylene oxide for 12 hours. Pluronic solutions and composite scaffolds were prepared under sterile conditions and kept sterile until the time of implantation. A microCT analysis of the scaffolds is displayed in Figure 8.2.

Figure 8.2. Microcomputed tomography images of the polymer component of PPF scaffold loaded with PLGA-PEG microparticles only (A), the mineral
component only (B), and the maximum intensity projection of the entire composite scaffold.

2.5 Surgical Implantation

Sixty-four healthy, 12-week-old male Fischer-344 rats received one cranial implant each, for an $n = 8$ for each experimental implant group. Doses were based on scaled down results previously published [11]. Eight total implant groups were examined: (1) polymer scaffold composites with 5 mg blank PLGA-PEG microparticles (primary control), (2) composites with 25 μg TP508 loaded into 5 mg PLGA-PEG microparticles, (3) composites with 50 μg of TP508 loaded into 5 mg microparticles, (4) composites with 100 μg of TP508 loaded into 5 mg microparticles, (5) composites with 5 mg blank microparticles and 5 mg HA (secondary control), (6) composites with 50 μg of TP508 loaded into 5 mg microparticles and 5 mg HA, (7) composites with 88 μg of Glu₆-modified TP508 loaded into 5 mg microparticles with 5 mg HA, and (8) composites with 77 μg of pamidronate-modified TP508 loaded into 5 mg microparticles with 5 mg HA. Descriptions of the groups can be seen in Table 8.1.
Table 8.1. Drug groups examined *in vivo*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>Dose (µg)</th>
<th>HA (mg)</th>
<th>Drug-loaded PLGA (mg)</th>
<th>Blank PLGA (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (control)</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>TP508</td>
<td>25</td>
<td>-</td>
<td>1.28</td>
<td>3.72</td>
</tr>
<tr>
<td>3</td>
<td>TP508</td>
<td>50</td>
<td>-</td>
<td>2.55</td>
<td>2.45</td>
</tr>
<tr>
<td>4</td>
<td>TP508</td>
<td>100</td>
<td>-</td>
<td>5.10</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>None (control)</td>
<td>-</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>TP508</td>
<td>50</td>
<td>5</td>
<td>2.55</td>
<td>2.45</td>
</tr>
<tr>
<td>7</td>
<td>Glu₅-modified</td>
<td>88</td>
<td>5</td>
<td>2.73</td>
<td>2.27</td>
</tr>
<tr>
<td>8</td>
<td>Pamidronate-modified</td>
<td>77</td>
<td>5</td>
<td>2.64</td>
<td>2.36</td>
</tr>
</tbody>
</table>

Critically sized cranial defect surgeries were conducted similarly to Kasper et al. [12, 13]. Briefly, each animal underwent anesthesia in an isolated chamber receiving 4% isoflurane in oxygen. The surgical site was shaved and sterilized with a povidine iodine solution. Each animal was given an intraperitoneal injection of buprenorphine (0.05 mg/kg) for post-operative analgesia and 3 mL sterile saline to compensate for fluid loss during surgery. The animal was then transferred onto a heated surface (maintained at 37°C) on the operation table and remained under anesthesia with a 2% isoflurane in oxygen gas through a breathing mask. Just prior to surgery, the animal was given 0.1 mL of
1% lidocaine solution for local anesthesia. An incision was made through the skin and periosteum over the cranium from the nasal bone to the midsagittal crest. The scalp and periosteum were split, elevated, and removed from the underlying bone. A critical-sized 8-mm cranial defect was created with a 3i dental drilling unit equipped with a trephine burr. The defect site was washed with saline and an epinephrine solution to minimize bleeding as necessary. Once free, the bony disc was carefully lifted in order to circumvent tearing of the superior sagittal sinus and dura. A microCT rendering of a cranial defect prior to scaffold implantation is shown in Figure 8.3. The appropriate composite implant was placed into the vacated defect, and then the periosteum and scalp were stitched closed using resorbable 5-0 Vicryl sutures (Ethicon, Somerville, NJ).

Figure 8.3. A microCT projection of an empty rat cranial defect prior to construct implantation; coronal (A), transaxial (B), and sagittal reconstructions (C). Scans were performed using a 1 mm aluminum filter.
After the operation, each animal was given pure oxygen in a heated recovery chamber until it regained consciousness. Blood and iodine outside of the wound was cleaned with hydrogen peroxide. Three subcutaneous injections of buprenorphine were given at 12, 24, and 36 hours post-surgery to ensure the comfort of each animal. All subjects were immediately provided food and water and were monitored by trained veterinary staff. Animals were euthanized after 28 days by isoflurane anesthesia and CO$_2$ inhalation. All experimental protocol was reviewed and approved by the Rice University Institutional Animal Care and Use Committee (IUCAC) and were performed according to the Principles of Laboratory Animal Care (NIH publication 85-23, 1985).

2.6 Microcomputed Tomography (microCT)

The animal scalps were carefully removed to expose the cranium. Skull caps were removed and fixed in a formalin solution for 5 days. After fixation, the samples were stored in a 70% ethanol solution until the time of analysis. For microCT analysis, skull caps were removed from ethanol and wrapped in Parafilm®. Samples were mounted on a stage vertically with the top of the defect facing the x-ray source. A SkyScan 1172 high-resolution imaging system was used at a resolution of 10 $\mu$m with a 0.5 mm aluminum filter at 100 kV and 100 $\mu$A. Thresholds of 45 to 255 were used to identify mineralized tissue within the defect region. A region of interest (ROI) was created in the site of the original
defect (8 mm diameter by 1.5 mm in height) and the percent bone (by volume) within was calculated. Because groups 5-8 contained HA at the time of implantation, scaffolds of the group 5 composition (n=8) were soaked in 200 μL of fresh rat blood that was allowed to clot. Dried scaffolds were analyzed by microCT to determine the amount of mineralization in the defect at the time of implantation.

To achieve greater insight in the healing process, the original ROI’s from each specimen were resliced vertically into smaller discs and radially into rings. The percent of each shape’s volume filled with bone was determined by the preceding method to assess the effects of each drug on bone formation in the various regions of the defect. Vertical sections resulted in three cylinders (8 mm diameter by 0.5 mm in height), while radial slices created two inner cylinders (2.67 and 5.33 mm in diameter). The volume of the two circumferential ringed cylinders was determined by subtraction of the inner volume(s).

2.7 Statistical Analysis

MicroCT and histological scoring data were interpreted by two factor analysis of variance (ANOVA) to assess the effects of each drug (control, TP508 Glu₈, and pamidronate), dose, and HA incorporation with α = 0.05. Tukey’s HSD
test was performed for factors determined to have a significant effect on bone regeneration. ANOVA and Tukey's tests were conducted with JMP IN version 5.1 statistical discovery software.

3. Results

3.1 TP508 Dose Effects on Bone Regeneration

The percent volume filled by bone within each defect was analyzed via microCT. The results for each of the 8 groups can be seen in Figure 8.4 and Table 8.2. To assess the dose effect of TP508 released from PLGA-PEG microparticles, the amount of bone in groups 1-4 were evaluated and compared. It was determined that the medium (50 µg) dose caused statistically significantly greater bone formation than controls and the low (25 µg) dose (Figure 8.5). There were no significant differences observed between the medium and high dosages. The differences in new bone formed by the high dose compared to controls or the low dosage was not statistically significant. For comparison, pristine skulls (Fisher 344 rats, age 8 weeks) without surgical defects were scanned (n=3) and found to be only 41.8 ± 4.6% filled with bone by volume. Representative microCT images of groups 1-4 are seen in Figure 8.6.
Figure 8.4. Percent volume bone fill at 4 weeks for each drug group. Bone volume determined via microCT. Error bars represent standard deviation for $n = 8$. 
Table 8.2. Average percent defect volume filled with bone 4, weeks post implantation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>% Volume filled with Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (control)</td>
<td>1.70 ± 0.67</td>
</tr>
<tr>
<td>2</td>
<td>TP508</td>
<td>2.71 ± 1.42</td>
</tr>
<tr>
<td>3</td>
<td>TP508</td>
<td>4.86 ± 2.03</td>
</tr>
<tr>
<td>4</td>
<td>TP508</td>
<td>3.83 ± 0.76</td>
</tr>
<tr>
<td>5</td>
<td>None (control)</td>
<td>4.41 ± 2.19</td>
</tr>
<tr>
<td>6</td>
<td>TP508</td>
<td>9.29 ± 1.85</td>
</tr>
<tr>
<td>7</td>
<td>Glu8-agg-TP508</td>
<td>9.62 ± 2.50</td>
</tr>
<tr>
<td>8</td>
<td>Pamidronate-agg-TP508</td>
<td>10.12 ± 2.90</td>
</tr>
</tbody>
</table>

Figure 8.5. Percent volume bone fill vs. TP508 dose. Bone volume determined via micro CT. Error bars represent standard deviation for n = 8, where * represents a statistically significant difference (\( \alpha = 0.05 \)).
Figure 8.6. Microcomputed tomography images of rat skulls (euthanized at 4 weeks) treated with a blank control scaffold (A), scaffold containing a low (25 μg) dose of TP508 (B), scaffold containing a medium (50 μg) dose of TP508 (C), and scaffold containing a high (100 μg) dose of TP508 (D). The fourth best of eight specimens from each group in terms of bone growth is shown.
3.2 Effects of Drug Modification on Bone Regeneration

The second phase of in vivo study was based around the most effective dosage observed (50 µg). Molar equivalents of the two modified drugs were also released. All drugs and controls were loaded in scaffolds containing HA to determine the effects of HA incorporation on new bone formation. The microCT results for groups 5-8 are presented in Figure 8.7. Representative microCT images from this study are illustrated in Figure 8.8. The average percent volume of detectable mineral in the blood-clotted scaffolds was 0.77%. TP508, as well as both modified versions of the drug, resulted in statistically significantly more bone than the polymer-HA, drug-free controls. ROI’s for all samples were resliced vertically (into top, middle, and bottom layers) and radially (into outer ring, inner ring, and center) (see Figure 8.9). The average percent volume filled with bone for each sector of the defect for all groups are given in Table 8.3. For all groups, a trend was observed that bone grew from anterior to the posterior, and from the outer ring towards the center. A standard effects test revealed that both the type of drug and the analyzed zones had significant effects on bone formation. The statistical significance of these effects are shown in Figure 8.10.
Figure 8.7. Percent volume bone fill for natural and modified TP508 released from polymer-HA composite scaffolds. Bone volume determined via micro CT. Error bars represent standard deviation for n = 8, where * represents a statistically significant difference from all other groups (α = 0.05).
Figure 8.8. Microcomputed tomography images of rat skulls (euthanized at 4 weeks) treated with a polymer scaffolds containing 5 mg hydroxyapatite and blank PLGA-PEG microparticles (A), PLGA-PEG microparticles containing 50 μg TP508 (B), PLGA-PEG microparticles containing 88 μg Glu8-modified TP508 (C), and PLGA-PEG microparticles containing 77 mg pamidronate-modified TP508 (D). The fourth best of eight specimens from each group in terms of bone growth is shown.
Figure 8.9. Regions of interest (ROI, 8 mm diameter by 1.5 mm height) were resliced vertically (A) and radially (B).
Table 8.3. Average ROI volume filled with bone in each defect zone.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Bone (mm$^3$)</th>
<th>Vertically Sliced</th>
<th>Radially Sliced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Upper (mm$^3$)</td>
<td>Middle (mm$^3$)</td>
</tr>
<tr>
<td>Control</td>
<td>4.21 ± 2.09</td>
<td>0.61 ± 0.40</td>
<td>1.43 ± 0.79</td>
</tr>
<tr>
<td>TP508</td>
<td>8.87 ± 2.38</td>
<td>1.73 ± 0.46</td>
<td>3.42 ± 1.14</td>
</tr>
<tr>
<td>Glu8</td>
<td>9.18 ± 1.77</td>
<td>1.79 ± 0.89</td>
<td>2.81 ± 1.20</td>
</tr>
<tr>
<td>Pam</td>
<td>9.66 ± 2.77</td>
<td>1.82 ± 1.25</td>
<td>3.52 ± 1.15</td>
</tr>
</tbody>
</table>

Figure 8.10. Average volume of bone formation in each defect zone for control, TP508, Glu8-modified TP508 (Glu8), and pamidronate-modified TP508 (Pamidronate). Error bars indicate standard deviation for n=8. Unique letters indicate statistically significant differences (α=0.05).
3.3. Effects of HA Incorporation on Bone Regeneration

The effects of HA incorporation on bone formation were analyzed by comparing groups 5 and 6 with groups 1 and 3, respectively. The volume of bone formed in composites initially provided with HA was significantly different than the polymer-only constructs with and without TP508 (Figure 8.11). The amount of new bone was also shown less the amount of mineralized material present at the time of implantation.

![Bar chart showing percent volume bone fill](chart.jpg)

Figure 8.11. Percent volume bone fill for controls and 50 μg TP508 doses with and without HA. Control-HA* and TP508-HA* indicate normalized
bone formation with background HA from implants subtracted. Bone volume determined via micro CT. Error bars represent standard deviation for n = 8, where unique letters represent statistically significant differences between groups (α = 0.05).

4. Discussion

The objective of this project was to determine the effects of modifying a drug with bone-targeting agents on in vivo bone regeneration. Before this could be done, we first had to assess the relationship between native TP508 dose and bone formation in a critically sized rat cranial defect. PPF-based scaffolds with TP508-loaded PLGA-PEG microparticles were implanted for four weeks in four experiment groups (blank control and low, medium, and high doses). It was observed that the medium dose (50 μg) incited the most new bone in the defect volume, significantly more so than controls or the low dose (25 μg).

Dosages for the second phase of this study, which examined the effects of drug modification on bone formation, were based upon the dose-effect results of native TP508. With 50 μg TP508 shown to produce a significant amount of bone in a critically sized cranial defect, it was selected as the base dose for the next phase of in vivo work. Molar equivalents of TP508 modified with Glu₈ or
pamidronate were also delivered by PLGA-PEG microparticles loaded into porous PPF-based scaffolds. To provide modified drugs with a local target and recruited cells with an osteoconductive surface and raw materials for mineralization, HA microparticles were injected into the scaffolds with the drug-loaded polymer microparticles. Again, the composites were implanted in rat cranial defects for four weeks and then harvested for tissue analysis. Drug modification resulted in no apparent loss of function; perhaps a decrease in diffusional loss was countered by a decrease in drug interaction with cells for drugs anchored to mineralized surfaces. Regardless of the mechanism, drugs linked to Glu$_8$ and pamidronate via an eight amino acid peptide linker displayed similar results in new bone formation after four weeks. Further analysis was performed to find potential differences in bone formation caused by the various forms of the drug. The digital reconstructions of bone in the defect were subdivided radially and vertically for all 32 rats. It was found that for all groups (control, native TP508, and both modified versions of TP508), each method of reslicing the data resulted in three significantly different zones of bone formation. The new bone grew from the perimeter edges inward and from the dural side above the brain upwards toward the scalp.

The last findings of this study focused on the benefits of HA incorporation in the polymer scaffolds. The inclusion of HA into scaffolds prior to implantation results in greater mineralization not only from the provided HA, but there are still significant differences in total bone formation in controls and TP508 groups with
and without HA after subtraction of the pre-implantation mineral material (Figure 8.10). The introduction of an osteoconductive material such as HA into the pore network of the scaffold may have influenced or aided the production of mineralized tissue and the activity of bone-forming cells.

It is believed that TP508 releases chemical attractants that hasten the onset and extent of angiogenesis [3, 13-14]. TP508 mimics the function of thrombin after clot dissolution, whereby the interaction of certain thrombin fragments activates cell receptors that accelerate the healing process. There is evidence that the release of factors from platelets may stimulate the production of proteins such as VEGF and bFGF [15-17]. The enhanced angiogenic effect should result in greater osteogenesis and bone formation by providing a stronger vascular network for the transport of cells as well as nutrients and waste products. The amount of new bone formed by the 50 μg dose of TP508 was superior to that produced by the controlled release of the established angiogenic factor VEGF from similar materials in the same animal model [18]. Patel et al. examined the effects of dual release of angiogenic and osteogenic drugs from PPF scaffolds. It was found that BMP-2 was significantly more powerful for new bone formation than VEGF, however the combination of the two drugs was effective in the generation of new bone at early time points. It is possible that the release of TP508 in conjunction with an osteogenic factor such as BMP-2 might lead to accelerated repair of skeletal defects. Because the modified versions of the TP508 exhibited equivalent bone formation to the native drug, it is reasonable to assume that drug
modification would not negatively affect other bone-forming growth factors such as BMP-2 or FGF-2. For drugs released in larger doses than those examined in this study, the targeting effects of modified drugs (drug distribution and bone regeneration) may be more apparent. Although most of these growth factors interact with surface receptors of cells and need not be internalized, a longer and/or more flexible linker sequence between the drug and bone-binding moiety may be favorable for maximal interaction with cells in the wound repair site.

5. Conclusion

Drug modification can be useful in the targeting and delivery of growth factors for tissue engineering applications. In this study, an inflammatory peptide and analogs modified with calcium-targeting agents were released in vivo from composite scaffolds in a skeletal defect model to determine the effects of modification on the bioactivity of the drug. There was a significant dose-effect of the native drug on bone formation. Further, drug modification had no ill effects on the regenerative properties when compared to the native drug. By closer examination of bone formation by vertical and radial sections, a trend was revealed that bone formation originates from the outer perimeter towards the center, and from the dural side upward. Lastly, incorporation of HA into the implanted construct produced significantly more bone in both the control and 50 μg TP508 doses. Future studies should examine the effects of modifying more osteogenic drugs, such as BMP-2, with moieties like Glu₈ and pamidronate on in
vivo bone regeneration, while taking advantage of the benefits of HA incorporation in tissue engineering scaffolds.

6. Acknowledgements

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7. References


[13] Y Wang, C Wan, G Szoke, JT Ryaby, and G Li. Local injection of thrombin-related peptide (TP508) in PPF/PLGA microparticles-enhanced bone


Chapter 9. Future Directions of Targeted Drug Delivery

The modification of drugs for targeted delivery is both novel and exciting for numerous medical applications, including tissue engineering. This work has shown potential for the modification of drugs used in bone regeneration. Enhanced targeting results in greater delivery efficiency, which in turn would reduce many concerns presented by current controlled release technologies. First, normal drug delivery strategies rely on diffusion of molecules away from the carriers to the nearby tissue. However, these drugs often travel away from the implant to distant tissues. Many physicians and scientists are apprehensive of the providing pro-angiogenic drugs in this manner that might lead to tumor formation. There are also concerns of ectopic bone formation and mineralization from the spread of osteogenic factors such as BMP-2. Secondly, increased drug delivery efficiency would require less total drugs to be used in tissue engineering applications. In general less foreign biochemical factors is more desirable for the aforementioned reason, but also in terms of the costs of treatment. Many bioactive factors are very expensive, so a significant reduction in required dosage may dramatically decrease long-term healthcare costs associated with tissue regeneration.

Future work should focus on the modification of more powerful drugs such as BMP-2 and vary the amount of HA incorporated within polymer constructs for bone regeneration. Although TP508 was an appropriate model drug
for preliminary investigation, modified versions of established growth factors like BMP-2 and TGF-β could yield even more significant findings. In terms of the animal model, as long as implants are cell-free, a longer time point such as 8 or 12 weeks may be more suitable to determine the effects and mechanism of modified targeting drugs. TP508 causes the release and cascade of many bioactive factors that recruit cells and influence their function. The eventual effects of the drugs and dosages used in this work may not be fully understood at 4 weeks. Next, the presence of HA in polymer scaffolds appeared to produce significant gains in bone formation. Variation of the amount of HA in bone tissue engineering constructs is worthy of further exploration. Lastly, targeted delivery of growth factors is not reserved solely for bone applications. The principles of this work should be considered for the targeted delivery of growth factors in many tissue engineering endeavors.
Chapter 10. Summary

This research was conducted to produce new drugs with specific bone-targeting capabilities to increase the efficiency and potency of bone tissue engineering strategies. A known osteogenic factor, TP508, was modified with completely biocompatible molecules with high attraction to calcium to form a new class of drug that is feasible for eventual human applications. The enzymatically degradable peptide linker sequence serves as an additional level of control in the release of drugs anchored to bony or mineralized surfaces, but is easily substitutable for other biologically inert or favorable and potentially degradable materials (e.g. PEG, PLGA, citric acid, etc.).

After identifying potential agents with remarkable calcium affinity (bisphosphonates and poly-negatively charged amino acids) for use in this scheme, model peptides were conjugated to pamidronate, Asp₈, and Glu₈. It was found that modified drugs displayed the same attraction for HA as the agents themselves. Based on these results, TP508 was linked to pamidronate or Glu₈ by the bovine aggrecan sequence, which is susceptible to degradation by four enzymes commonly present in bone repairs sites. The drugs displayed repeatable release patterns from PLGA-PEG microparticles loaded in the pore network of PPF constructs prepared for rat cranial defects. Scaffolds loaded with HA microparticles retained more of the modified drugs due to their ionic attraction to the mineral. In the presence of the enzyme collagenase, modified drugs released
at rates comparable to native TP508, illustrating the feasibility of the degradable peptide linker sequence.

Pamidronate and Glu₈ modified versions of TP508 were impregnated within PLGA-PEG microparticles loaded in PPF composites with HA. Although the new drugs did not significantly out-perform native TP508 in this study, it is promising that the modification process did not have a negative effect on the activity of the osteogenic factor. It is possible that four weeks was too short to observe differences in response to the various drugs and dosages, based on the relatively low amount of new bone formed by all groups. The additional bone formation resulting from HA incorporation in controls and TP508 groups was promising for the improvement of current tissue engineering constructs and the development of new biomaterials for bone regeneration. This research provides new ideas to the development of better drugs, materials, and tissue engineering strategies for the future of orthopedic regenerative medicine.
APPENDIX

1. The Effect of Amino-bisphosphonates on Rat MSC Differentiation in vitro

Research Design

To confirm literature results and test the in vitro effects of nitrogen-containing bisphosphonates on the differentiation of rat mesenchymal stem cells (MSC's, the likely target for future in vivo work), cells were cultured in media containing different concentrations of amino-bisphosphonate, pamidronate, and alendronate. Collagen production per well was determined via hydroxyproline assay. Mineralization was observed via Von Kossa staining for calcium. Differentiation of rat MSC's in the presence of the three amino-bisphosphonates was examined in two sets of culture. Each culture set included a control group (cells cultured in Dulbecco's Modified Eagle Medium (DMEM) only) and a standard group (cells cultured in standard induction cocktail without bisphosphonates). This experiment was conducted in the lab of Achim Goepferich (University of Regensburg, Germany).
Experimental Methods

Rat MSC's were extracted from the femurs of adult male rats and passaged once. Cells were seeded in 24 well plates (3.8 cm² per well) at a density of 50,000 cells/cm². Each well contained 2 mL culture solution. Bisphosphonate culture solutions consisted of amino-bisphosphonate (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸ M), pamidronate (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ M), and alendronate (10⁻⁷, 10⁻⁸, 10⁻⁹ M) dissolved in standard induction cocktail (90% DMEM, 10% fetal bovine serum (FBS), 1% antibiotics (penicillin and streptomycin), and 0.1% ascorbic acid). Standard groups were cultured in the standard induction cocktail and control groups were cultured in DMEM only. Half the media was exchanged with fresh, bisphosphonate-free culture solution every three days to mimic the decreasing in vivo release concentration. Cells were cultured for a total of 15 days. Each group had a total of 6 wells, n = 3 for collagen assay and Von Kossa staining.

After 15 days of culture, the wells were drained of media and washed twice with phosphate buffered saline (PBS). Cells were fixed by addition of 1 mL 10% formaldehyde. Total collagen production was assessed by determining the hydroxyproline content, a proportional component of collagen. Fixed samples are scraped and suspended in PBS. Red 4-dimethyl aminobenzaldehyde is added to the solution and reacts with hydroxyproline. Absorbance in a UV-Vis spectrophotometer is measured at 560 nm. The amount of hydroxyproline is determined by comparing the sample's absorbance to known standards of
hydroxyproline. For Von Kossa staining, fixed cells are washed twice with H₂O and treated with 2 mL silver nitrate solution (5 g AgNO₃ per 100 mL H₂O). The cells are exposed to sunlight/UV for one hour to exchange calcium with silver ions. The silver nitrate solution is removed and samples are counterstained with 2 mL sodium thiosulfate (5 g Na₂S₂O₃ per 100 mL H₂O). Calcified areas appear black under light microscopy.

Conclusions

The first experimental set included a control, a standard, amino-bisphosphonate (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸ M), and pamidronate (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸ M) groups. The second experimental set included a control, a standard, pamidronate (10⁻⁷, 10⁻⁸, 10⁻⁹ M), and alendronate (10⁻⁷, 10⁻⁸, 10⁻⁹ M) groups. The hydroxyproline content, corollary to collagen content, for each group is shown in Figures 11 and 12. All cells cultured in 10⁻⁵ M amino-bisphosphonate died before the first media exchange.
Figure A1. Total Collagen Assay after Culture with Amino-bisphosphonate and Pamidronate.
In agreement with published research, MSC’s treated with nitrogen-containing bisphosphonates generally produced more collagen and deposited more mineral than cells cultured in media-only or standard induction cocktail. In particular, pamidronate and alendronate in the range of $10^{-8}$ to $10^{-9}$ M showed the largest contrast in hydroxyproline content and calcium staining. Although bound nitrogen-containing bisphosphonates may provide more osteogenic differentiation than those in solution, concentrations in this range for pamidronate and alendronate are also stimulatory to undifferentiated MSC’s. This information will be considered in planning the in vivo release of bisphosphonates in a bone defect
model. Note: Von Kossa staining images are not available due to unforeseeable complications.

2. Differential Scanning Calorimetry

In order to assess the glass transition temperature of PLGA polymers (75:25 and 50:50 lactic to glycolic acid ratio) available in the lab, differential scanning calorimetry was performed in a two-pass fashion under nitrogen gas. The T_g values for 75:25 and 50:50 were approximately 47.8°C and 44.6°C, respectively. The DSC output graphs are seen below. These T_g values are acceptable for the temperatures (40°C, 50°C) of the hexane bathes during scaffold fabrication.
Figure A3. DSC for PLGA (50:50)

Figure A4. DSC for PLGA (50:50)
3. MALDI-TOF Spectroscopy of Peptide-Bisphosphonate Conjugates

To confirm the conjugation chemistry, small peptides were linked to pamidronate by the previously described technique. Below are the MALDI-TOF spectrographs for three formulations; one using the sequence Phe-Ala-Gly-Glu and two with Phe-Ala-Gly-Asp. The first two peptides used glutamic and aspartic acids with tBOC protected amines and unprotected carboxylic acid side chains and were provided at more than twice the molar requirement during synthesis to avoid crosslinking peptides. The third sequence uses the specialty aspartic acid, Asp(O$_2$-PhiPr)-FMOC, to avoid crosslinking peptides at the expense of higher synthesis costs. Phe-Ala-Gly-Asp-Pamidronate and Phe-Ala-Gly-Glu-Pamidronate conjugates can be seen at the 684 and 699 Da peaks, respectively. Peaks at 137 and 154 are background associated with the dihydroxybutyric acid matrix and the peak observed at 237 is likely unreacted pamidronate.
Figure A5. Mass Spectrograph for FAGE(BOC)-Pam Conjugates
Figure A6. Mass Spectrograph for FAGD(BOC)-Pam Conjugates

Figure A7. Mass Spectrograph for FAGD(O2PhiPr)-Pam Conjugates