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Modulating the Release of Therapeutic Agents from PLGA Coated Cancellous Allografts

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Modulating the Release of Therapeutic Agents from PLGA Coated Cancellous Allografts

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Abstract

Bone grafts are used in 2 million annual surgeries with that statistic expected to rise due to the increasing elderly population as well as sports related injuries. At a certain size bone fracture or defect, the body is unable to heal spontaneously, and a bone graft must be implanted into the defect site. Allografts are used as bone grafts due to their availability and elimination of donor site morbidity, however they show poor bioactivity and bone regeneration. This study utilizes a degradable poly(lactic-co-glycolide) (PLGA) polymer coating applied to cancellous allografts, loaded with a variety of therapeutic agents aimed to improve the bioactivity of the allografts. Bone morphogenetic protein-2 (BMP-2) was physically encapsulated in the polymer coating, the antibiotic gentamicin was physically encapsulated and surface adsorbed, and previous studies surface adsorbed the growth factor vascular endothelial growth factor (VEGF). Results show that encapsulated proteins followed the trend of a moderate burst release within the first day of release, followed by sustained release for the 6 week time period that followed. Surface adsorbed tests additionally show a larger burst release in the first day, followed by a less gradual, faster release of 100% of protein. Qualitative results of an *in vivo* study using cortical allografts with dual coating of encapsulated BMP-2 and surface adsorbed VEGF showed enhanced bone callus formation in the combined group compared to BMP-2 alone, with X-Ray and MicroCT imaging showing a near bony union by 8 weeks in the combined group.

1.0. Introduction

1.1. Physiology of Bone

The human skeletal system provides an array of functions, including mechanical tasks such as proving structural support, aiding in locomotion, and protection of internal organs [1]. In addition, bone function includes metabolic tasks such as serving as a host of growth factors or other signaling molecules, a reservoir for mineral homeostasis, and an environment for bone marrow [2]. Bone tissue is composed of approximately 60% inorganic components, 30% organic components, and 10% water [3]. The inorganic component is mainly low crystallinity hydroxyapatite, a calcium phosphate mineral substance [3]. Over 90% of the organic matrix of bone is type I collagen, a triple-helical molecule that provides tensile bone strength. Though collagen is the most abundant, an array of noncollagenous proteins are also found in the organic matrix such as proteoglycans, osteocalcin, osteonectin, osteopontin, and a multitude of cytokines and growth factors [3].

Bones can be categorized by shape, size, and location, and are generally classified by shape into one of the following groups; long bones such as the femur, short bones including the carpal and tarsal bones, flat bones such as the skull, and irregular bones which form the vertebrae and sacrum among other bones. Bone can also be classified by tissue type, the two types being cortical and cancellous bone. Cortical bone is dense, compact bone that makes up 80% of the bones mass in the body [1], providing compressive strength. Cortical bone is composed of osteons. Osteons are columns containing multiple layers of osteoblast and osteocyte bone cells, surrounding a central Haversian canal. Cancellous bone, also referred to as trabecular or spongy bone, is similar in matrix composition to cortical bones, but has different structures and functions. It is able to absorb forces and deforms more easily than cortical bone. Cancellous bone

has 50-90% porosity, compared to the 5-10% porosity of cortical bones, resulting in cancellous bones having a much greater surface area to volume ratio [1]. Due to its porosity, cancellous bone is much more vascularized than cortical bone, has a higher rate of metabolic activity, and is remodeled more actively. Rather than osteon columns, cancellous bone is formed from rod and plate like elements, as well as circular packets of lamellae - organized collagen sheets.

Long bones in particular are comprised of three distinct regions; the diaphysis, metaphysis, and epiphysis [1]. The diaphysis is a hollow, central shaft comprised of cortical bone tissue, the epiphysis is the trabecular ends of the bone, while the metaphysis is the trabecular meshwork in between the two. Both the epiphysis and metaphysis are primary cancellous bone, surrounded by a thin cortical shell layer [1]. Figure 1.1.1 below details the structure and different regions of long bones [4].

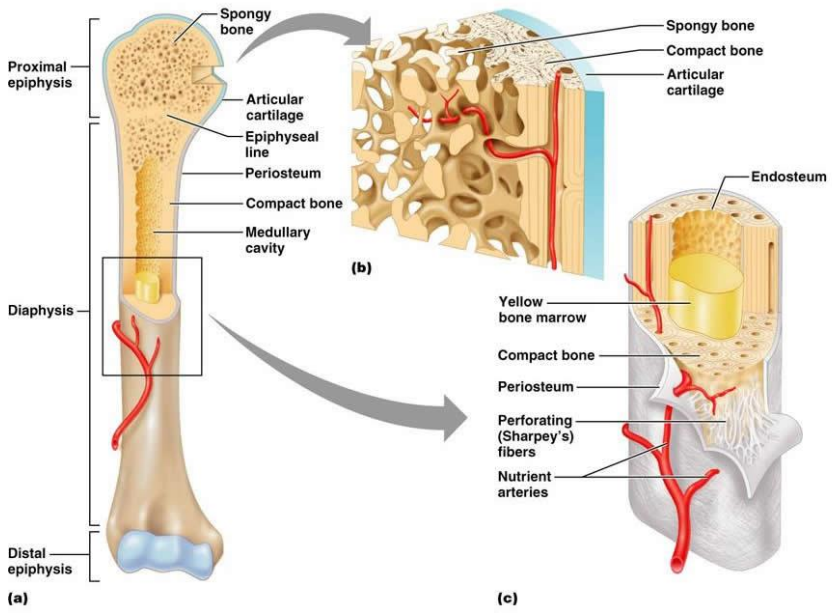

Figure 1.1.1: The structure and different regions of long bone. The metaphysis is not labeled in this image.

Bone tissue contains three primary bone cells; osteoclasts, osteoblasts, and osteocytes. Osteoclasts are responsible for the resorption of bone. Osteoclasts bind to integrin receptors, and upon the secretion of hydrogen ions and cathespin K enzymes, dissolves the mineral and collagen matrix respectively [1]. Osteoblast cells are responsible for the synthesis of new bone matrices and bone formation [1]. Osteocytes are a subset of bone cells that serve a regulatory function in the body. They express protein whose function relates to the regulation and exchange of minerals in bone, as well as the transduction of mechanical bone stresses in biologic activity [1].

1.2. Fracture Healing

In non-critical size defects, the human body is able to facilitate new bone formation and the repair of the defect site. Surgery and immobilization are common techniques to expedite recovery, however bone healing is physiological process. The bone healing process occurs in three phases; the inflammatory stage, the repair stage, and the remodel stage [5]. The first process is the inflammatory stage and bone healing is the formation of a hematoma, or a swelling and collection of clotted blood at the fracture site. Following the formation of the hematoma, inflammatory cells such as monocytes and macrophages are recruited to the defect site, as well as mesenchymal stems cells (MSCs) [5]. Fibroblast cells migrate and proliferate at the defect site, forming granulation tissue. The granulation tissue begins the process of the formation of a vascular network for the transport of oxygen and nutrients to the wound site.

During the repair stage of fracture healing, fibroblasts continue to lay down a vascular network, as well as begin to develop into chondroblasts that form cartilage. Osteoclasts begin to break down the bone matrix in preparation for new bone formation. The differentiation of MSCs into osteoblasts occurs due to the presence of the growth factor BMP-2, leading to mineralization

and woven bone formation, resulting in the formation of a callus surrounding the defect site. At early stages of callus formation, the bone tissue is very weak, thus immobilization is used as a mechanical technique to aid in bone formation [5]. The last step in fracture healing, the remodeling step, is a long term process that restores healed bone to its original shape and structure. Osteocytes constantly facilitate the resorption of unnecessary bone and formation of new bone in required locations in response to mechanical cues [5].

1.3. Bone Grafting

The bone healing process is only effective in certain capacities. At certain defect sizes, the body is unable to stimulate natural bone regeneration and healing. This defect size is characterized as a critical sized defect, and represents the point at which healing will not spontaneously occur in the human body. Many factors determine if a defect is critical, such as age, defect location, health of the bone tissue, and mechanical loads placed on the bone, thus an exact size of a critical defect is relatively arbitrary to define [7], and is considered any defect large enough that won't heal on their own, resulting in a non-union. For the purpose of animal models, critical size defects have been considered as any deficiency at least 2 times the diameter of the bone [6]. Any untreated defect that is critically sized will remain non-union, meaning limited integration of bone around the defect site. One of the most common treatments for a critical sized defect is the implantation of a bone graft. Bone grafts must meet a certain criteria for effective healing, such as able to fill the defect site, being biocompatible to ensure it does not elicit an immune response or rejection, and offer mechanical support while simultaneously serving as a bioactive substrate to facilitate the formation of new bone between the graft and host bone. Further, ideal bone grafts should possess the following properties aimed to mimic the natural bone healing process [8]:

Osteogenicity – The presence of osteoblasts or osteoblast progenitor cells, aiding the formation of new bone and mineralization.

Osteoconductivity – The ability of the scaffold to allow cells to infiltrate into the graft, as well as attach and proliferate throughout the graft.

Osteoinductivity – An osteoinductive graft stimulates the differentiation of progenitor cells into osteoblast or osteoclast cells to aid in bone resorption or formation.

1.3.1. Autografts

An autograft is a bone graft that has been harvested from the patient's own bone tissue. The most common harvesting locations are from the patient's iliac crest due to its density and non-essential functions, as well as mandibular bone for facial grafts. Due to native tissue being used, autografts will not be rejected or cause a foreign body response. Autografts are seen as the current gold standard for bone grafting, due to their biocompatibility, as well as their retention of naturally occurring bone cells and proteins [9]. The porous nature of the harvested bone retains osteoconductive properties, while these cells and proteins that remain in the graft result in a bone substitute that is osteoinductive and osteogenic [8], and will contribute to bridging and new bone formation.

Though autografts remain the gold standard for bone grafting, they introduce a variety of setbacks and shortcomings. The primary drawback is additional donor site morbidity at the site of harvested bone. This has the potential to lead to longer recovery time, increased risk of infection, or an increased risk of other complications. Additionally, because tissue is harvested from the patient themselves, there is a limited supply available, and furthermore an autograft

becomes impossible to use for very large sized defects, as it is not reasonable to take a large portion of the patients own bone to fill the defect site.

1.3.2. Allografts

The use of allografts addresses the current shortcomings of autografts. Allografts are the main focus of this research, and are bone grafts that are harvested from a separate human donor, mainly a cadaver. The allografts harvested are typically taken from the same location as an autograft, however due to the use of cadaveric donors, additional donor site morbidity and limited supply is not an issue. Moreover, the number of harvest locations increases in a cadaveric donor, as there is less of a need to harvest non-essential bones. While allografts address the shortcomings of autografts, they introduce a variety of issues that prevent them from being the gold standard used in bone grafting. For instance, biocompatibility is a concern with allograft implantation, ensuring that the graft does not elicit an immune response or become rejected [10]. A related concern is the rare transmission of disease or contaminants as well through allograft implantation. In order to guarantee there is no immune rejection or disease transmission, allografts are often sterilized prior to implantation, typically using ethylene oxide, radiation, or an autoclave [11]. However, the side effect of the sterilization process is the loss of natural bone cells and proteins that generate new bone formation. This causes allografts to lose their osteogenic as well as osteoinductive properties [8]. Due to the loss of osteogenic and osteoinductive components, the natural healing process and union of the host bone and the graft is less likely, resulting in a large percent of long term failures and complications [12]. Up to 60% of cortical allografts have reported failures or complications after 10 years post-implantation, as well as a 50% loss in allograft tissue strength in the same time period [12]. This is thought to be

due to the bone graft's inability to fully bridge between the graft and host bone, and not fully achieving the natural process of bone healing. The current research focuses on methods to revitalize allografts, to improve their bioactivity and bone healing, to ultimately lead to a greater long term success rate.

1.4. Growth Factors

The processes of bone healing are initiated and stimulated by the release of growth factors. Growth factors are proteins that are secreted by a signaling cell to elicit a specific response. Growth factors bind to their respective cellular receptors causing a cascade of signaling to produce a response. In bone healing, vascularization and new bone formation are the two main aims for wound site repair. These events are driven by the secretion of angiogenic and osteogenic growth factors respectively [13].

Osteogenesis is the process of new bone formation. The growth factors that are the most crucial in this step are the bone morphogenetic proteins (BMPs). BMP-2 and BMP-7 are the commonly used and studied BMPs, and both are currently available in certain clinical applications [14]. BMPs are classified under the TGFβs family, and influence many different events during the bone healing process, mainly stimulating the formation of new bone. BMPs elicit the proliferation and differentiation of osteoprogenitor cells from mesenchymal stem cells (MSCs). The MSCs are then differentiated into osteoblasts and osteocytes, which form mineralized woven bone, as well as maintain bone tissue homeostasis respectively. Additionally, BMPs stimulate chemotaxis, which promotes the formation of cartilage and new bone. Osteogenic and angiogenic growth factors have shown to have synergistic effects when delivered together, one example showing that BMPs also aid in the synthesis of extracellular matrices and are seen during the stages of angiogenesis.

Angiogenesis is the process of forming new blood vessels, and is critical in bone repair, and one of the initial events that occurs. Newly formed blood vessels allow the access of nutrients, oxygen, and cells to the defect site, as well as a method for the removal of metabolic waste. Without this step, bone formation becomes more difficult and delayed union or non-union is much more likely, as seen in research that showed limited bone formation in groups without angiogenic growth factors present [38]. Angiogenesis is stimulated by a variety of signaling growth factors. One angiogenic growth factor is fibroblast growth factor (FGF). FGF stimulates proliferation and differentiation into endothelial and smooth muscle cells that are essential for new blood vessel formation. Another angiogenic growth factor, VEGF, is thought to be of the most important growth factor to stimulate new vessel formation. VEGF is present in 4 different isoforms: A, B, C, D, however VEGF-A has been studied as the most significant for angiogenesis. VEGF is secreted and is present throughout many steps of the natural bone healing process. VEGF is seen in early stages of wound healing, as a role of vascularizing bone tissue from a cartilaginous matrix. VEGF in combination with angiopoietin causes the formation and differentiation of mature blood vessels at the wound site. However as previously stated, VEGF is an angiogenic growth factor that has synergistic results when used in combination with osteogenic growth factors [16]. VEGF receptors are found on many cells related to the process of bone formation, such as osteoblasts and osteoclasts. VEGF can bind to these receptors to increase blood vessel density at their location. Additionally, VEGF in combination with RANKL, stimulates the differentiation of progenitor cells into osteoclasts *in vitro* [41, 42], which are later present in the resorption of bone and osteoclastogenesis, a key process in bone remodeling. The table below based on [17] details the growth factors and cytokines that are present in the natural bone healing response.

Cytokines/Growth Factors	Bone healing stages	Overall action
• Interleukin-1, 6 (IL) • Tumor necrosis factor α (TNF) • Platelet-derived growth factors (PDGF) • Growth differentiation factor-8 (GDF) • RANKL, macrophage colony-stimulating factor (M-CSF) • Osteoprotegerin	1.Inflammation	• Hematoma • Inflammation • MSCs recruitment
• Vascular endothelial growth factors (VEGF) • Bone morphogenetic growth factors (BMP) • Transforming growth factor βs (TGF) • Angiopoietin • Fibroblast growth factors (FGF)	2.Cartilage Formation	• Chondrogenesis • Endochondral ossification • Osteoblast/Osteoclast precursors recruitment • Vascular ingrowth • New angiogenesis
• VEGFs • BMP-2, 7 • RANKL and M-CSF • Angiopoietin	3.Cartilage resorption and primary bone formation	• Chondrocyte apoptosis • Cartilage resorption • Osteoblast/Osteoclast precursors differentiation • Woven bone formation
• IL-1, IL-6 • RANKL and M-CSF	4.Secondary bone formation and remodeling	• Bone remodeling • Osteoblast activity • Marrow establishment

Table 1.4.1: Growth factors and cytokines and their roles in natural bone healing.

1.5. Growth Factor Loading for Delivery

The goal of successful tissue engineering applications is to mimic the natural biological response for the specific tissue healing type. Due to growth factor secretion being a key aspect of natural bone healing, incorporating growth factors into a bone graft system has the potential to increase the bioactivity and wound healing on the graft. The growth factors must be attached to the graft in a manner that can protect the growth factor from degrading in the body prior to being effective. VEGF has an estimated half-life of 30 minutes [18], while the half-life of BMP-2 has been determined to be up to 16 minutes in animal models [19] when administered intravenously. As a result, the dosage used often exceeds physiological levels of the natural growth factor, and can lead to ectopic vessel and bone formation in the non-target area [18, 31, 32]. Thus, the desired design is to load the growth factors to the graft in a manner that protects them from degradation, and provides sustained release of the growth factor, eliminating the need for exceeding dosages to elicit a response.

One method to incorporate growth factors or proteins in a system, and the method used in this research, is to integrate them into a degradable polymer coating. Polymer coatings can be synthesized with varying properties, to alter and control degradation rates. Poly(lactic-co-glycolic acid) (PLGA) was the polymer of choice for the allograft coating for a variety of reasons; including its FDA approval, affinity to surface bind proteins, as well as its controllable degradation, allowing for long term sustained delivery [37]. In addition to long term delivery, proteins released from PLGA show biphasic behavior, with an initial burst release from interactions with its surrounding media, as well as the extended, sustained release from degradation via hydrolysis [37]. The release kinetics of therapeutic agents from PLGA can also be altered by varying the drug concentration, polymer concentration, molecular weight, ratio of

lactic acid to glycolic acid, as well as the methodology of protein loading, hypothesizing that the physical amount of growth factor released can be controlled with different PLGA parameters, a significant aspect for potential future clinical applications.

Two strategies to load growth factors or other therapeutic agents within a polymer coating is through physical encapsulation or surface adsorption. Growth factors or agents can be physically encapsulated to a scaffold, by mixing the drug solution with the polymer solution prior to the synthesis of the scaffold. This method will release the entrapped drugs as the polymer degrades, thus release kinetics can be altered by varying the degradation rate of the polymer. For instance, PLGA, a copolymer of polylactic acid (PLA) and polyglycolide (PGA) can have varying degradation rates based on the ratio of PLA to PGA [20], and additional factors such as molecular weight or polymer concentration can also vary degradation times. Another method of loading growth factors or other proteins onto a scaffold is through surface adsorption. This technique is completed by the incorporation of the drug solution to the preexisting scaffold, attaching the growth factor directly to the surface of the polymer scaffold. The main mechanism behind drug release in this case is diffusion. This results in a burst release of polymer, as the proteins on the surface of the scaffold desorb at a rate based on solubility and molecular weight, allowing for a more immediate delivery and availability of the growth factor or therapeutic agent.

1.6. Bone Infection

Bone infection, or osteomyelitis is a common concern post-surgery. It causes the inflammation of bone tissue, which can hinder the recovery and healing process. Patients are at an elevated risk of developing osteomyelitis post-surgery. Thus, there is a benefit to being able to incorporate and controllably release antibiotics to the surgical site, to prevent infection or fight infection that begins to develop. Furthermore, common osteomyelitis treatment protocols suggest

4 to 6 weeks of antibiotic usage [21, 22], and additionally it has been determined that local administration of antibiotics had lesser instances of side effect reactions that systemic administration, while still demonstrating comparable treatments [23]. Thus, the long term local delivery of antibiotics is an aspect of bone grafting that is addressed in this current research. A common method of antibiotic release is the use of poly(methylmethacrylate) (PMMA) beads containing antibiotics [23], however the non-degradable nature of the beads has gathered concerns about toxicity or future complications, and release patterns were shown to be erratic and non-controllable. Release kinetics of antibiotics should be aimed to mimic the current treatment of antibiotics following surgery. Antibiotic treatment is started immediately in the operating room, and often preventative antibiotics are given prior to surgery [24]. While long term controlled delivery will follow in accordance with the 4 to 6 week treatment protocol, burst release of antibiotics will mimic the treatment given in the operating room, and provide antibiotic release immediately upon graft implantation. The antibiotic used in these studies is gentamicin. Gentamicin is effective against many bacterial infections, particularly gram negative infection [25]. It is an aminoglycoside antibiotic, which inhibits bacteria from synthesizing protein [25]. Side effects of gentamicin include nerve, ear, and kidney problem [25], thus the controlled local release of gentamicin is beneficial for reducing side effects while maintaining the therapeutic efficacy.

1.7. Specific Aims

The project was broken down into 4 specific aims. The first specific aim was to characterize and qualitatively assess the polymer degradation *in vitro* of the PLGA coated allografts. This was used in conjunction with release studies to verify and observe the relationship between polymer degradation and drug release. Specific aim 2 was to analyze the release kinetics of encapsulated BMP-2 using various polymer concentrations. The goal was to

show how varying the coating parameters would alter the amount of drug released, as well as show encapsulated BMP-2 was able to continue to provide sustained, long term release after a slight burst effect. The objective of specific aim 3 was to compare the release kinetics of both encapsulated and surface adsorbed gentamicin from varying polymer concentrations. The multiple concentrations would again be compared to assess the controllability of the allograft, as well as attempt to achieve the desired release kinetics over 4 to 6 weeks of controlled gentamicin delivery, in addition to the burst effect to immediately begin to fight and prevent infection. The final specific aim was to observe wound healing upon allograft implantation *in vivo.* Within the scope of this project, qualitative results only were analyzed from an *in vivo* animal study headed up by Farzana Sharmin, a PhD candidate. Femoral segmental defects were made in rat models, where control allografts, allografts encapsulated with BMP-2, and allografts with a dual coating of encapsulated BMP-2 and surface adsorbed VEGF were implanted, and wound healing was compared between the 3 groups using X-ray imaging and MicroCT.

1.8. Preliminary Studies

Preliminary studies for this research were done by last year's Master's student Casey McDermott, and his research and results are summarized in this section. Two PLGA coating techniques were performed, and SEM imaging and MicroCT were used to determine which coating method resulted in a more continuous and uniform coating that was able to coat not only the surface of the allograft, but within the porous network as well. A static technique was employed, by submerging the cancellous allografts in a 50:50 ratio (PLA:PGA) PLGA solution for 24 hours, followed by 24 hours of solvent evaporation, concluded with 24 hours of lyophilization. A dynamic method followed similar techniques, however for the first 24 hours of coating, the allografts were placed in a rotating rocker machine, for continuous 3D motion,

following by the same evaporation and lyophilization process. The dynamic coating method is explaining in further detail in section 2.1. X-ray and MicroCT imaging were used to assess the extent and volume of the coating.

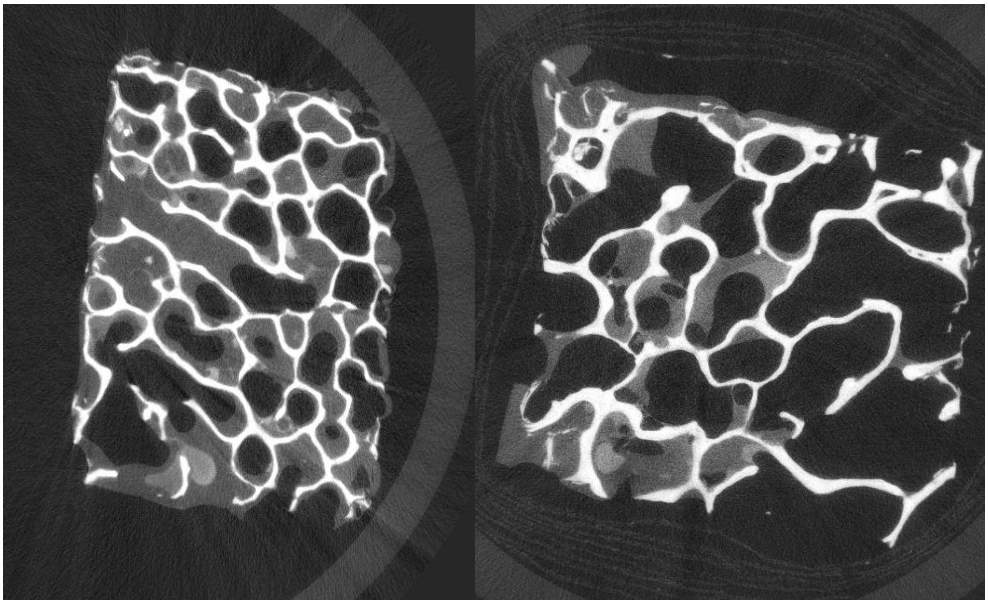

Figure 1.8.1: MicroCT imaging of PLGA coating volume of dynamically coated allografts (left), vs. a static coating (right).

MicroCT imaging in figure 1.8.1 shows the dynamic coating method proved to be superior to the static coating, with the PLGA coating being more consistent and penetrating throughout a larger extent of the porous allograft than the static coating. The static coating resulted in a much less extensive coating, with roughly half the allograft left as uncoated bone.

In addition to the coating method, SEM imaging and MicroCT were used to determine optimal polymer concentrations for the coating. A range of polymer concentrations were tested, with MicroCT quantifying the coating volume for each. There was a consistent trend of a more concentrated polymer solution resulted in more coating volume, as shown in table 1.8.1 below.

Polymer Concentration	Coating Volume
g/ml	mm^3
1:10	77.9
1:12	72.2
1:14	72
1:16	62
1:18	53
1:20	47

Table 1.8.1: Effect on Coating Volume due to change in PLGA concentration

SEM imaging was used to further analyze polymer concentrations of 1:12, 1:14, and 1:20. Figure 1.8.1 shows the SEM images for the 1:12 PLGA concentration. The coating was able to effectively extend through the allograft, however it can be seen in figure 1.8.1-B that the concentration was high enough that it began to block the natural porous architecture of the bone.

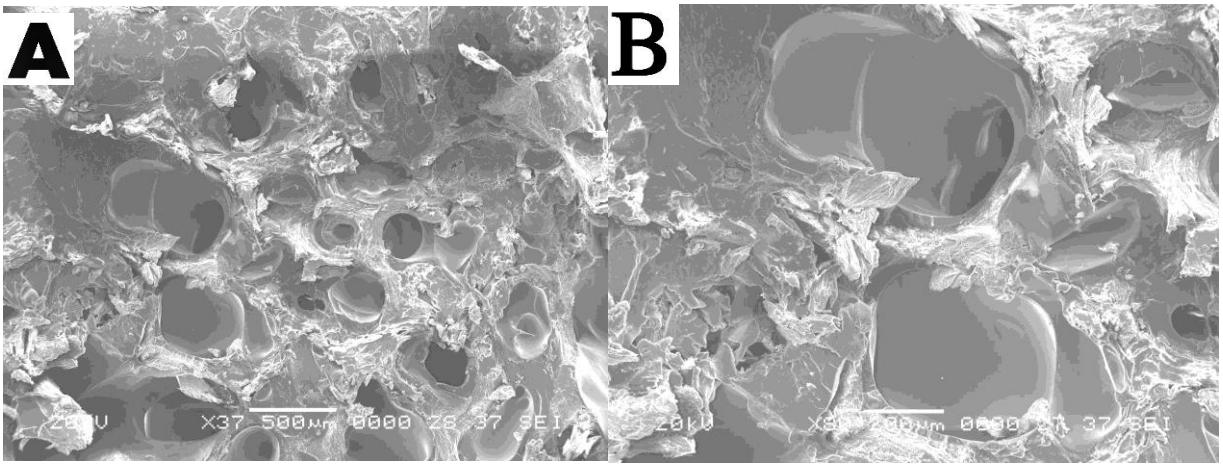

Figure 1.8.2: SEM image of dynamically coated cancellous allograft using PLGA concentration of 1:12. A) x37 magnification, B) x80 magnification

Similarly SEM imaging for concentrations of 1:14 and 1:20 PLGA were analyzed. Figure 1.8.3 shows that the 1:14 PLGA concentration was able to extend throughout the allograft while leaving the porosity of the bone largely unaffected, and was determined to be the ideal balance of

coating extension and retention of the allografts porous properties. Figure 1.8.4 displays that the polymer concentration was relatively thin, and portions of uncoated bone remain.

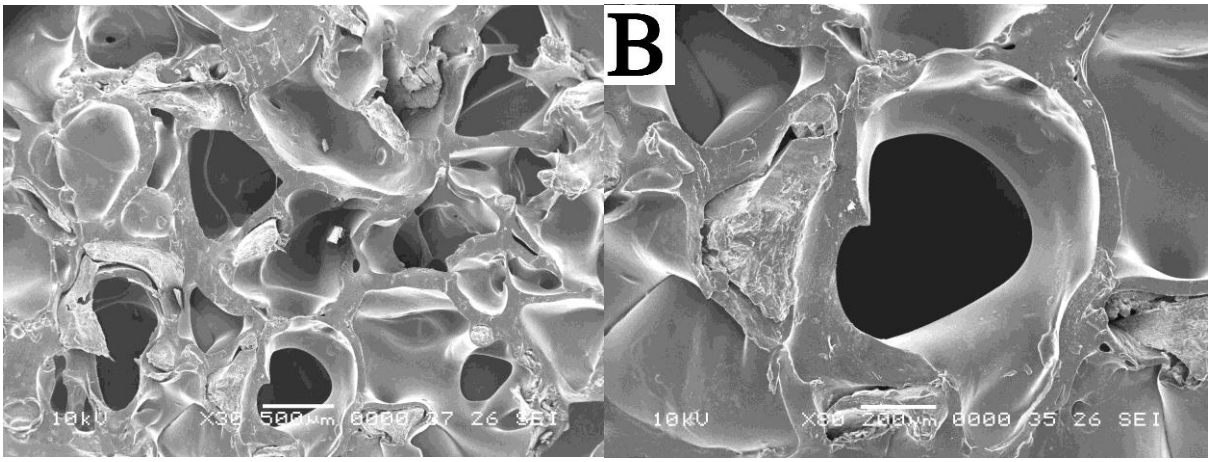

Figure 1.8.3: SEM image of dynamically coated cancellous allograft using PLGA concentration of 1:14. A) x30 magnification, B) x80 magnification

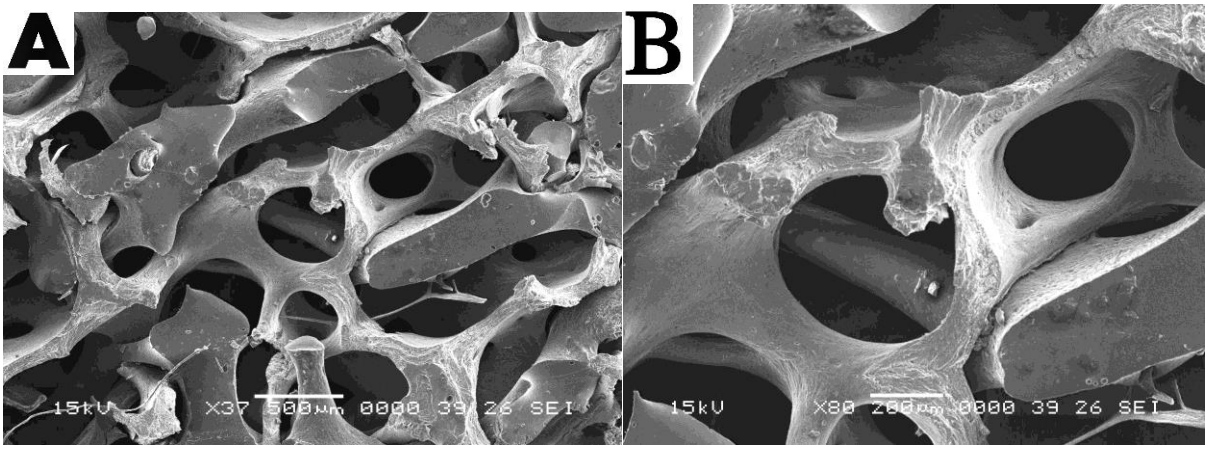

Figure 1.8.4: SEM image of dynamically coated cancellous allograft using PLGA concentration of 1:20. A) x37 magnification, B) x80 magnification

VEGF was surface adsorbed for 15 minutes at a concentration of 5 µg/ml onto the surface of the allografts at polymer concentrations (grams PLGA/ml tetrahydrofuran) of 1:8, 1:14, and 1:20, with a n = 4. The cumulative amount of VEGF released was consistent with the results of

the polymer coating volume characterization. A larger coating volume would result in more growth factor being able to attach and bind to the polymer, thus a larger release. Figure 1.8.5 depicts this relationship, as more growth factor was release with higher PLGA concentrations.

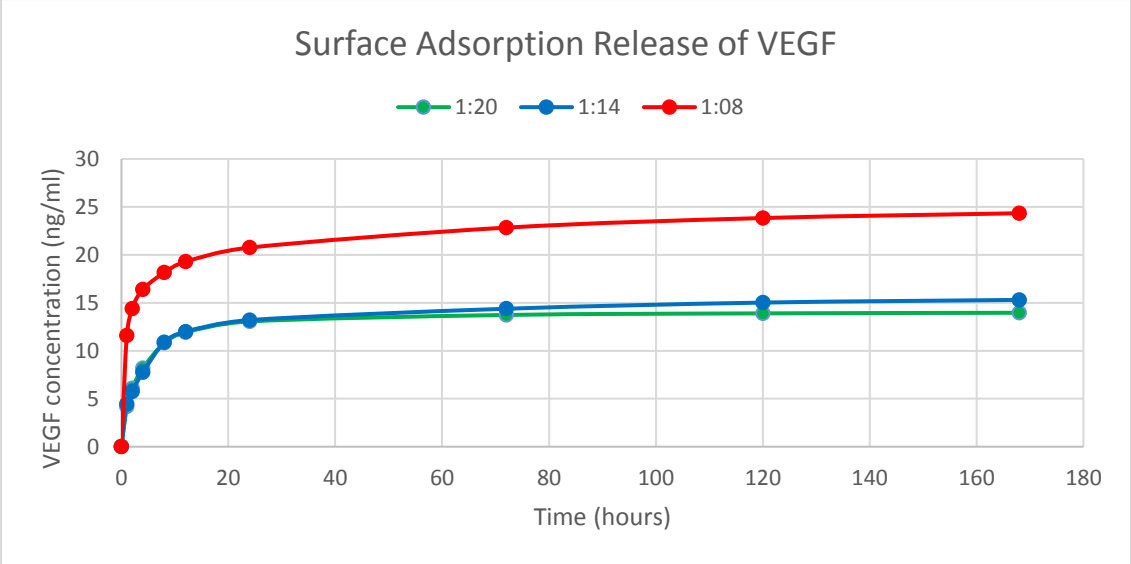

Figure 1.8.5: Cumulative release of surface adsorbed VEGF from PLGA coated allografts at concentrations of 1:8, 1:14, and 1:20.

The release kinetics of the surface adsorbed VEGF showed a large burst release, with all 3 polymer concentrations releasing greater than 85% of their total release after day 1. After day 3, little continuous release is observed for all polymers, and eventually plateaus by the end of week 1.

BMP-2 was both surface adsorbed at 50 µg/ml and encapsulated at 67 µg/ml in polymer concentrations of 1:8 and 1:14 respectively. Surface adsorbed BMP-2 followed similar release kinetics as VEGF, though to a much more drastic extent.

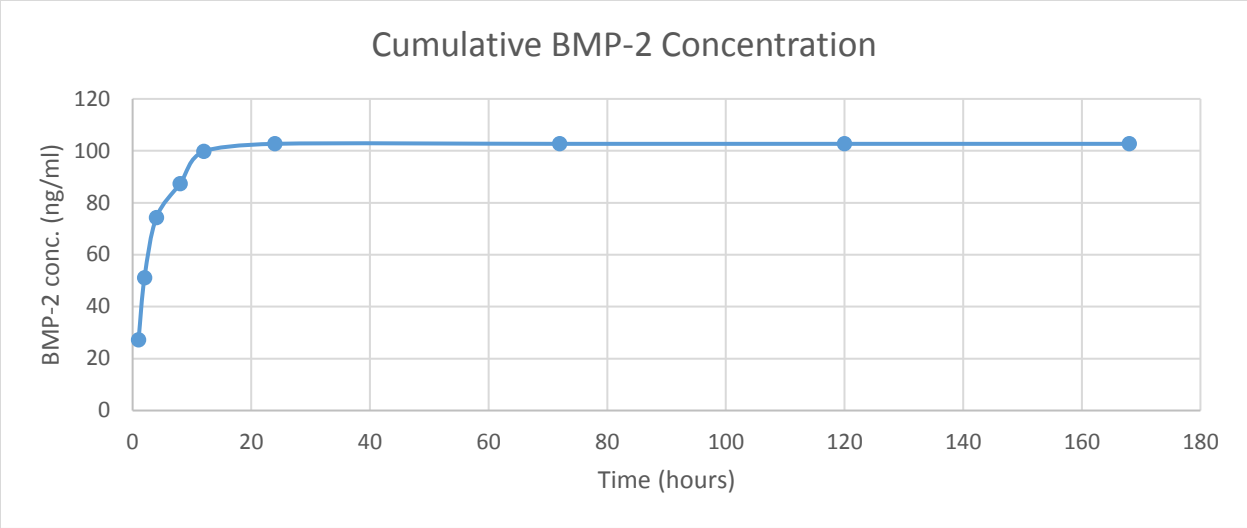

Figure 1.8.6: Cumulative release of surface adsorbed BMP-2 from PLGA coated allografts at a concentration of 1:8

As the image above shows, 100% of BMP-2 was released by the end of the first day, followed by an immediate plateau with no observed gradual release afterwards. Surface adsorbed release is dictated through diffusion, and BMP-2's smaller molecular weight compared to VEGF caused a much more significant burst release. A release of encapsulated BMP-2 was measured throughout the duration of 3 weeks. A burst release was observed within the first day, but to a lesser extent than the surface adsorbed loading. The BMP-2 was continuing to demonstrate sustained release, even by the end of the 3 week period.

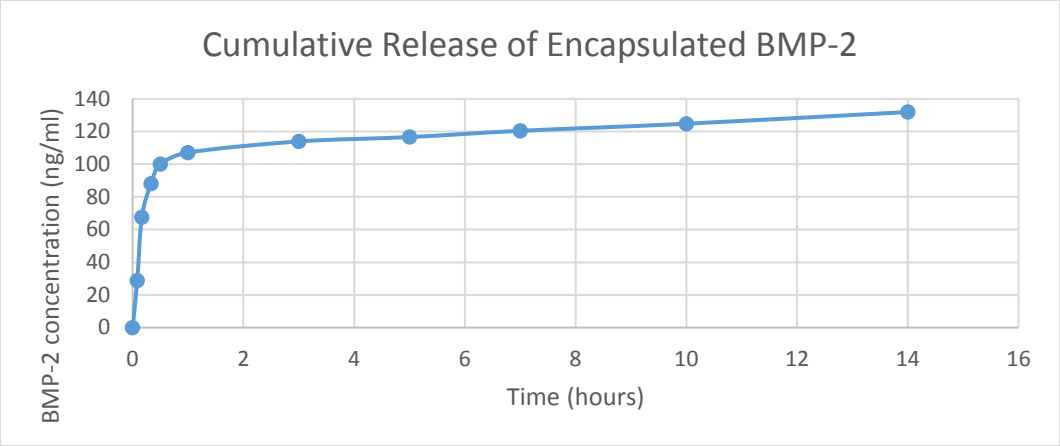

Figure 1.8.7: Cumulative release of encapsulated BMP-2 from PLGA coated allografts at a concentration of 1:14

Finally, gentamicin was surface adsorbed on allografts with a PLGA concentration of 1:14. Following the trend of surface adsorbed release, over 70% of gentamicin was released within the first day, half of that release occurring within the first few hours. The gentamicin continued to release for the duration of the first week, similar to the surface adsorbed VEGF, and did not appear to be at a plateau by the end of the study.

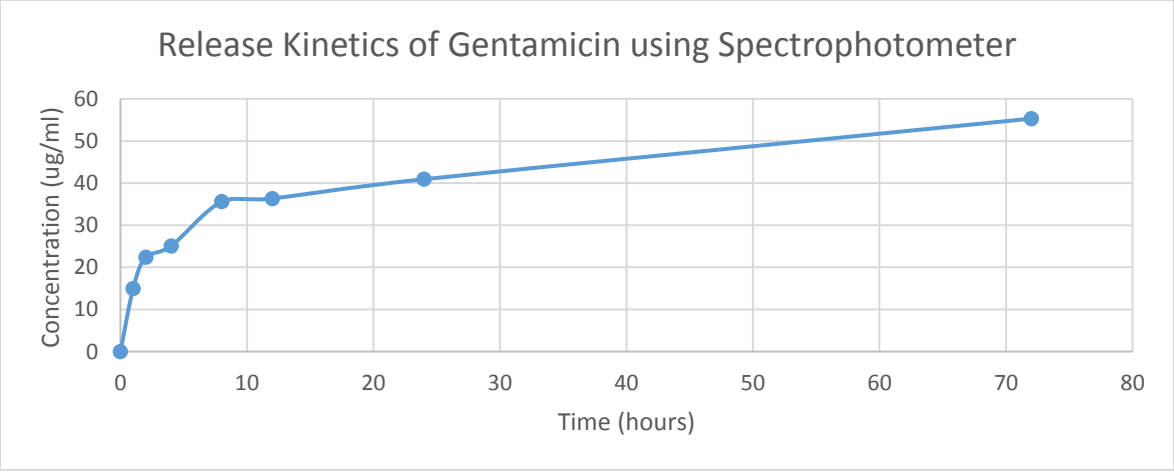

Figure 1.8.8: Cumulative release of surface adsorbed gentamicin from PLGA coated allografts at a concentration of 1:14

Materials and Methods

2.1. Polymer Coating

The entirety of the allograft coatings were completed using the following procedure, referred to as the dynamic coating procedure. Cancellous canine allografts were obtained from Veterinary Transplant Services, Inc. with dimensions of 1.0 cm x 1.0 cm x 0.5 cm ± 0.2 cm. The samples were cut in half width-wise for final estimated dimensions of 1.0 cm x 0.5 cm x 0.5 cm. The allografts were stored in a -20° C freezer. Poly(lactic-co-glycolic acid), PLGA, was purchased from Lakeshore Biomaterials, in a solid, crystal pellet form, and stored at -20° C.

To coat the allografts, both the PLGA and allografts were brought to room temperature prior to coating. The PLGA was dissolved in tetrahydrofuran (THF) at different amounts to obtain a desired concentration of PLGA solution in g PLGA/ml THF. The PLGA was dissolved in a vortex machine until no solid pellets remained and the solution was homogeneous. Allografts were weighed prior to coating, and then placed in a 5 mm syringe. The polymer solution was then pushed and expelled through the syringe containing the allograft a total of 10 times, repeated twice. The allografts were then submerged in approximately 2 ml of polymer solution inside a closed vial, and placed in a Labquake Rotisserie Rocker from Thermo Scientific, allowing the allografts to be continuously rotated, as well as constantly agitated in the polymer solution throughout the coating process. The goal of this design was to achieve continuous coating throughout the allograft, specifically a coating that extended throughout the porous nature of the cancellous allograft. After 24 hours in the rocker, the caps were removed from the vial to allow the solvent to evaporate from the polymer solution, and the rocker was placed at an angle to allow rotation without spilling any solution. After at least 24 hours to ensure all the solvent had evaporated, the samples were lyophilized for an additional 24 hours to

ensure the complete drying of the samples, upon which the samples were weighted again to calculate the total mass of the polymer coating.

2.2. Polymer Degradation Characterization

Allografts were characterized qualitatively using SEM imaging to observe and analyze the degradation kinetics of the PLGA coating over time. 10 cortical allografts were harvested from the long bones of retired breeder rats, and dynamically coated with a PLGA concentration of 1:8, with one bone serving as a control allograft with no PLGA coating. No growth factor or other proteins were added to the polymer solution. After lyophilization, the allografts were submerged in PBS at 37° C for the following time points; 1 day, 3 days, 5 days, 7 days, 2 weeks, 3 weeks, 4 weeks, 5 weeks, and 6 weeks. At the corresponding time point, the allograft was removed from the PBS solution and lyophilized to ensure the complete drying of the sample prior to SEM imaging.

The allografts were cut in half with a blade, and placed on the SEM sample stage platform using an adhesive strip, in a manner that allowed viewing of the surface and cross-sectional views of the allograft. The allografts were sputter coated using gold-palladium to prevent the charging of the samples, and placed in the SEM. Multiple views were captured from each sample in order to view the entire allograft. The amount of PLGA coating on the allografts was compared to control images to qualitatively assess the PLGA degradation over time *in vitro*.

2.3. BMP-2 Release Study

2.3.1. Loading of BMP-2

The release of the growth factor protein BMP-2 was tested via encapsulation. A 500 μg vial of recombinant human BMP-2 powder was purchased from Fisher Scientific, and stored at

-60° C prior to use. Glacial acetic acid was diluted with DI water to a solution of 20 mM acetic acid, 500 µl of which was used to reconstitute the BMP-2 powder. PLGA was dissolved in THF at three different concentrations; 1:8, 1:14, and 1:20 g PLGA/ml THF. Once the polymer solutions were fully homogeneous, 500 µl of reconstituted BMP-2 solution was slowly added to each individual polymer solution, 100 µl at a time, to avoid any precipitation. The theoretical loading concentration was 67 µg/ µl. Control allografts were additionally coated by adding 500 µl of just 20 mM acetic acid at the same rate, to separate polymer solutions of 1:8, 1:14, and 1:20. Upon fully adding the BMP-2 solution, allografts were coated using the dynamic procedure. A total of n = 4 allografts were tested for each polymer solution.

2.3.2. BMP-2 Release

The allografts were placed in a 4x6 well plate, with one allograft per well. The allografts were immersed in 1.5 ml of PBS and the well plate was wrapped in parafilm to reduce PBS evaporation. The allografts were placed in a 37° C room, to simulate the temperature, pH, and ionic concentrations of the human body. At specific time point intervals, the supernatant fluid was collected and stored at -20 ° C, and the allografts were moved to a new well and re-submerged in PBS. The time points taken were hours 1, 2, 4, 8, 12, and days 1, 3, 5, 7, 10, 14, 21, 28, 35, and 42 for the 1:14 and 1:20 polymer concentrations, while the 1:8 polymer concentration had time points taken up to the 28 days.

2.3.4. BMP-2 Analysis

BMP-2 samples were quantified via an enzyme-linked immunosorbent assay (ELISA), purchased from R&D Systems. The ELISA kit measures the concentration of BMP-2 present in each sample collected from the release study. Using the BMP-2 standard from the kit, standard

BMP-2 concentrations of 4000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, and 0 pg/ml were created. 100 µl of Assay Diluent was added to each well of the 96 well microplate, followed by 50 µl of either the sample, control, or standard. The plate was covered with an adhesive strip and placed on a shaker for 2 hours at room temperature. 400 µl of Wash Buffer was added to each well and then aspirated, for a total of 4 washes. 200 µl of BMP-2 Conjugate was added to each well, incubated for an additional 2 hours, and then the wash process was repeated. 200 µl of Substrate Solution was added and covered from light at room temperature for 30 minutes, followed by the addition of 50 µl of stop solution. The plates were read using a BioTek Synergy HT plate reader at wavelengths of 450 nm and 540 nm.

2.4. Gentamicin Release Study

2.4.1. Surface Adsorption of Gentamicin

Two separate PLGA solutions were created to test the release of surface adsorbed gentamicin, 1:14 and 1:20. PLGA was dissolved in THF to obtain the desired concentration, and coated following the previously mentioned dynamic coating procedure, for a total of n = 4 allografts and 4 controls for each polymer solution. Gentamicin was loaded onto the allografts via surface adsorption. Gentamicin was purchased from Sigma-Aldrich, as a stock solution of 10 mg/ml and was stored at a temperature of 4° C. The gentamicin solution was diluted with deionized water to a loading concentration of 100 µg/ml. The coated allografts were removed from the lyophilizer, and organized such that 4 bones of each polymer solution would be loaded with gentamicin, and the remaining 4 would serve as control groups. The experimental allografts were completely submerged in 1 ml of the gentamicin solution for 15 minutes at room temperature, while the control allografts were submerged in DI water following the same

parameters. The allografts were stored at a temperature of -20° C for 24 hours, followed by 24 hours of lyophilization.

2.4.2. Encapsulation of Gentamicin

Gentamicin was additionally encapsulated in a 1:14 polymer solution, to compare the release kinetics with that of the surface adsorbed gentamicin, as well as determine if encapsulated gentamicin and BMP-2 showed similar kinetics. PLGA was dissolved in THF to obtain the two distinct polymer concentrations and an n = 4. The stock solution of gentamicin was diluted with DI water to a concentration of 800 μg/ml, and 500 μl was added to the polymer solution 100 μl at a time. A control group was created by adding 500 μl of DI water at the same rate to a separate vial also containing a 1:14 polymer concentration. The allografts were coated using the dynamic method, with an approximate loading concentration of 50 μg/ μl.

2.4.3. Gentamicin Release

Gentamicin was released with the same procedure as BMP-2. Allografts were placed in the wells of a 24 well plate, submerged in 1.5 ml of PBS, and stored in the 37° C room. The supernatant fluid was collected and stored at -20° C prior to analysis, and time points were taken at hours 1, 2, 4, 8, 12, and days 1, 3, 5, 7, 10, 14, 21, 28, 35, and 42.

2.4.4. Gentamicin Analysis

The samples were removed from the -20° C freezer, and set at room temperature to gradually thaw. The gentamicin stock solution was diluted with PBS to obtain .5 ml of concentrations of 320 μg/ml, 160 μg/ml, 80 μg/ml, 40 μg/ml, 20 μg/ml, 10 μg/ml, 5 μg/ml, and pure PBS, to be used later to establish a standard curve for gentamicin. A gentamicin reagent was made to bind to the amino groups of the gentamicin samples, allowing them to be quantified via

spectrophotometry. 2-propanol, o-phthaldialdehyde, and 2-mercaptoethanol were purchased from Sigma-Aldrich. 0.25 grams of o-phthaldialdehyde, 6.25 ml of methanol, and 300 µl of 2-mercaptoethanol were added to 56 ml of a .04 sodium borate solution. The resulting solution was protected from light and stored for 24 hours prior to use. When the gentamicin samples were thawed, 100 µl of each the sample, reagent, and 2-propanol were added to an individual well of a 96 well plate, including the 8 standard samples. The propanol was added to prevent the precipitation of products being formed. The well plates were then stored for 45 minutes, and then read using a BioTek Synergy HT plate reader at a wavelength of 332 nm. Using the absorbance readings for the samples with known concentration, a standard curve was created and used to determine the concentration of the samples.

The data figures for both gentamicin as well as BMP-2 are presented as the mean cumulative release from a sample size of n = 4, and all error bars represent the standard deviation of the data.

2.5. *In Vivo* Evaluation of Dually Loaded Allografts

2.5.1. Allograft Preparation

Cortical allografts approximately 6 mm in length were coated with PLGA at a ratio of 1:8. 3 different experimental groups with an n= 4 were organized; control allografts containing no coating, BMP-2 only allografts containing encapsulated BMP-2, and dually loaded allografts containing encapsulated BMP-2 and surface adsorbed VEGF. The loading concentration of the BMP-2 allografts was approximately 300 ng/ml, while the loading concentration of VEGF was approximately 180 ng/ml. BMP-2 was reconstituted using 20 mM acetic acid, while VEGF was reconstituted using DI water. The coating and encapsulation only procedures followed the

similar steps as previously described. The dually coated group was first encapsulated with the BMP-2 solution. After lyophilization, samples were placed in an ice bath, from which they were surface adsorbed in the VEGF solution as previously described.

2.5.2. *In vivo* Defect and Allograft Implantation

10 week old male Sprague-Dawley rats weighing in a range of 300-325 grams were used in the *in vivo* implantation of the allografts. All procedures were performed in accordance with approved Institutional Animal Care and Use Committee guidelines. The right forelimb of the rat was shaved and sterilized with betadine and 70% ethanol. An incision was made through the skin and periosteum of the rat, and a 6 mm critical sized defect was created in the femur. A small plate was attached to the femur via Kirchner wires and steel cerclage wires to stabilize the femur during the procedure. The allografts were then fit into the defect site, and fixed in place with the use of a single 4-0 Vicryl (Ehticon, Somerville, NJ) cerclage stich. The 4-0 sutures were used to close the muscle, subcutaneous tissue, and skin. The rats were sacrificed at 4 and 8 weeks post-surgery, upon which the femurs were harvested and stripped of the metal pins and sutures, and fixed in 70% ethanol at 4° C prior to analysis. Figure 2.5.2.1 below visualizes this process.

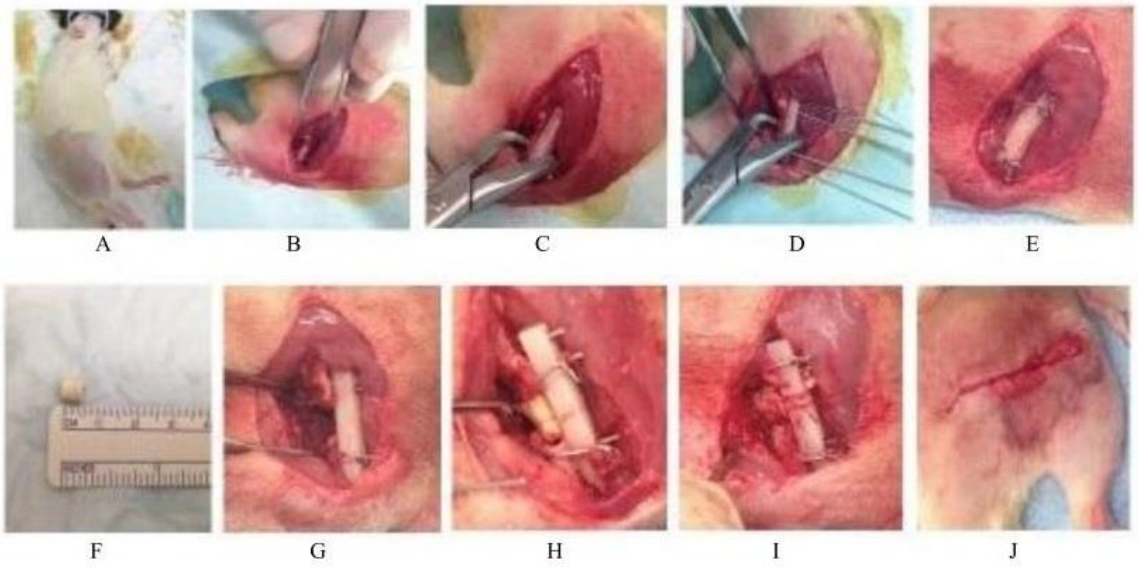

Figure 2.5.2.1: Surgical procedure of allograft implantation. The rat leg is prepped by shaving and washing with betadine and 70% ethanol (A). The rat leg and muscle is incised (B). A polyethylene plate is pressed up to the femur (C). Kirschner wires are used to fix the plate to the femur (D). Surgical wires are used to further secure the plate to the bone (E). Allografts cut to approximately 6 mm are measured to approximate the desired defect site size (F). A defect is created in the femur using a dremel (G). The allograft is press fit in the defect site (H). 4-0 sutures are used to secure the allograft to the plate and in the defect site (I). The muscle, tissue, and skin are stitched back together (J).

2.5.3. X-Ray Analysis

Femurs were analyzed using a Faxitron X-ray machine with its corresponding Faxitron DC-Beta SR v1.4 software. Images of each femur were taken at 26 kV for 6 seconds.

2.5.4. MicroCT Analysis

Three-dimensional views of the defect site were recreated using micro-focus X-ray computed tomography (μCT40, Scanco Medical AG, Basserdorf, Switzerland.) The defect site

was viewed between the two pin holes, at 55 kV and 145 μA with a 300 millisecond integration time.

3 Results

3.1. SEM Characterization of Polymer Degradation

Polymer coated cortical allografts were analyzed qualitatively, using topographical images from an SEM scan. Cortical allografts were coated with a PLGA solution of 1:8, and submerged in PBS at 37 °C. The allografts were cut in half to allowing viewing of surface and cross-section of the bone. Images were taken from allografts submerged in PBS for varying time points, to assess the degradation process of the polymer coating over time, shown in figure 3.1.1.

In the SEM scans, uncoated bone is seen as slightly brighter in the image, and has a jagged appearance, seen in figure 3.1.1 (a). The PLGA coating appears smoother, and a slightly darker color, most evident in figure 3.1.1 (b). Allografts submerged for three days showed a very large percentage of the surface bone coated with PLGA, while images of subsequent time points showed gradually less polymer coating, and more uncoated bone as the polymer began to degrade in the PBS. By day 42, very little polymer is seen even in the more magnified image, indicating almost all PLGA had degraded in the PBS. For each time point, there was a consistent trend showing the surface of the allograft contained slightly more coating than the cross section.

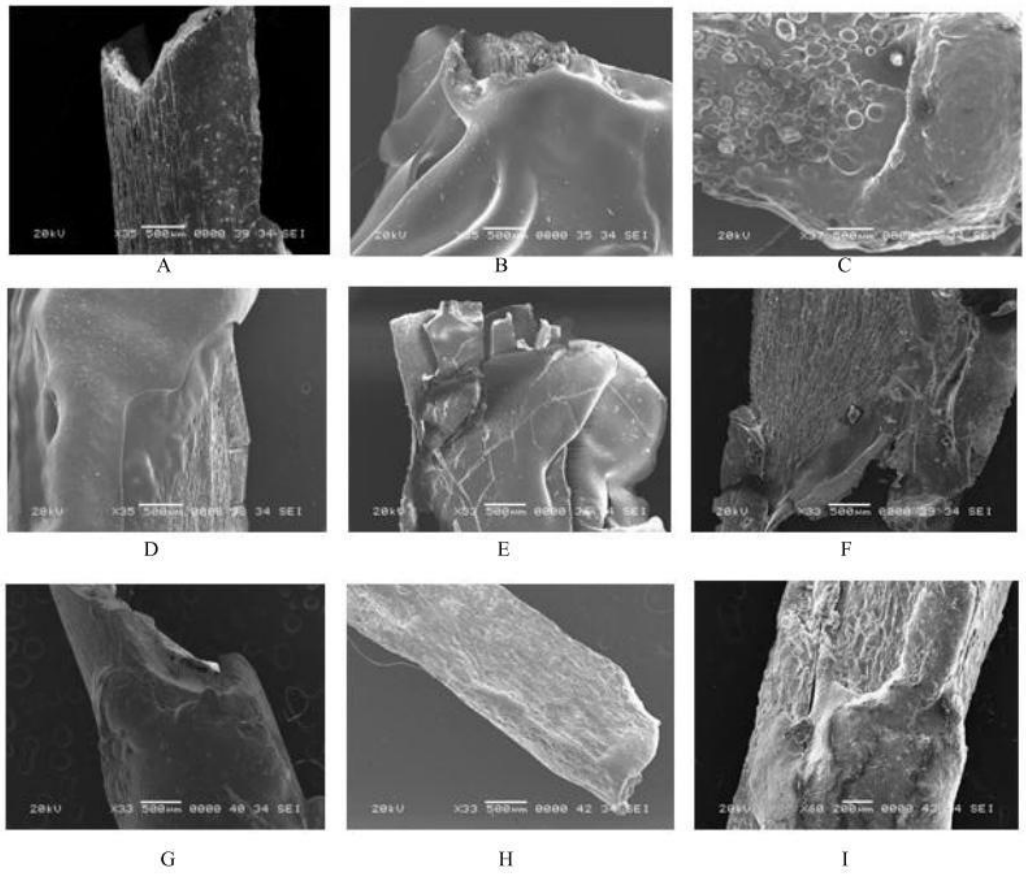

Figure 3.1.1: Surface views of PLGA coated allografts. Uncoated bone is shown in (A). B through I represent days 3, 5, 7, 14, 21, 28, 35, and 42 respectively. Throughout the duration of the study, SEM imaging shows less polymer coating present, and more uncoated bone, displaying the degradation kinetics of the coated allografts.

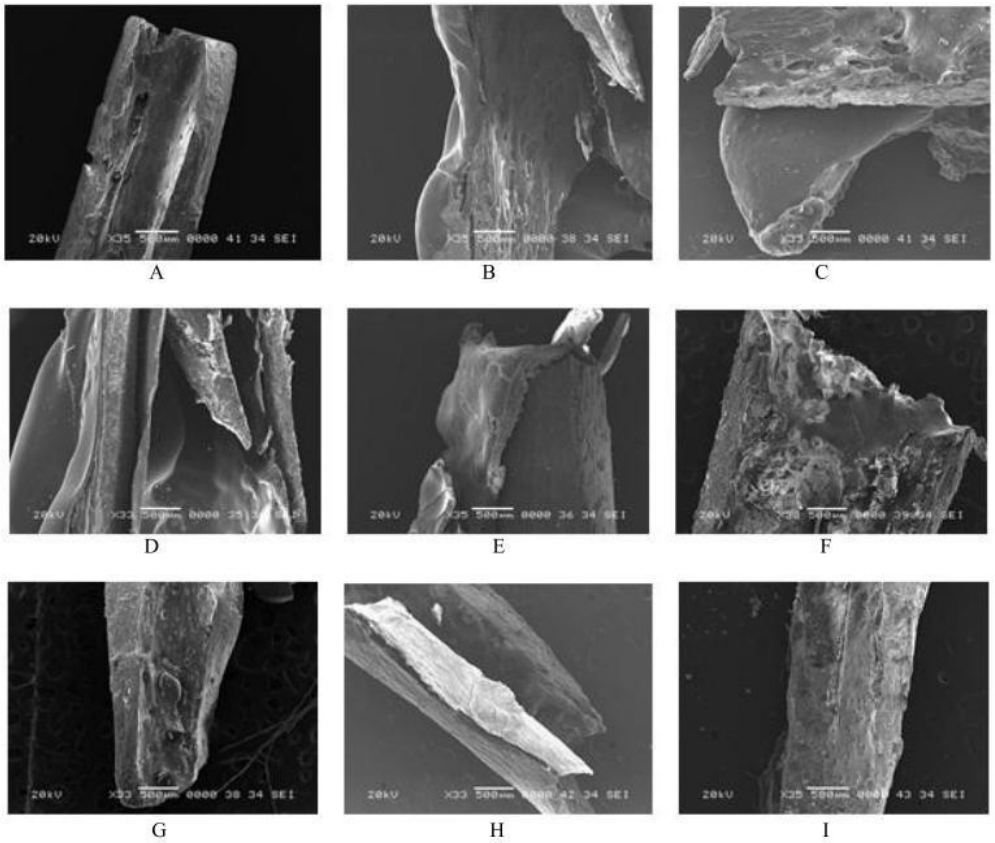

Figure 3.1.2: Cross sectional views of PLGA coated allografts. Uncoated cross sectional bone is shown in (A), while B through I represent days 3, 5, 7, 14, 21, 28, 35, 42 respectively. The PLGA degrades in a similar manner over time as the surface view, with more uncoated bone present the later the time point is. However, less PLGA coating is seen in the cross sectional views in general compared to the surface coating.

3.2. Analysis of Encapsulated BMP-2

Cancellous allografts with an n = 4 were coated with 1:8, 1:14 and 1:20 PLGA coatings, with BMP-2 physically encapsulated in the polymer solution. The growth factor exhibited burst release, with 82%, 67%, and 53% of the BMP-2 being released in the first 24 hours for the 1:8,

1:14 and 1:20 concentrations respectively. However, the allografts maintained an extended release of the protein as well, with steady release shown over 4 weeks, with an eventual plateau of growth factor released by day 42. The PLGA concentration of 1:8 is currently at the 4 week time point, thus 42 full days of release had not been calculated to determine the extent of sustained release. The total cumulative release concentration of BMP-2 from the 1:14 coated allografts was 92 ng/ml, and 33 ng/ml from the 1:20. The current 14 day cumulative release of BMP-2 from the 1:8 PLGA concentration is 130 ng/ml.

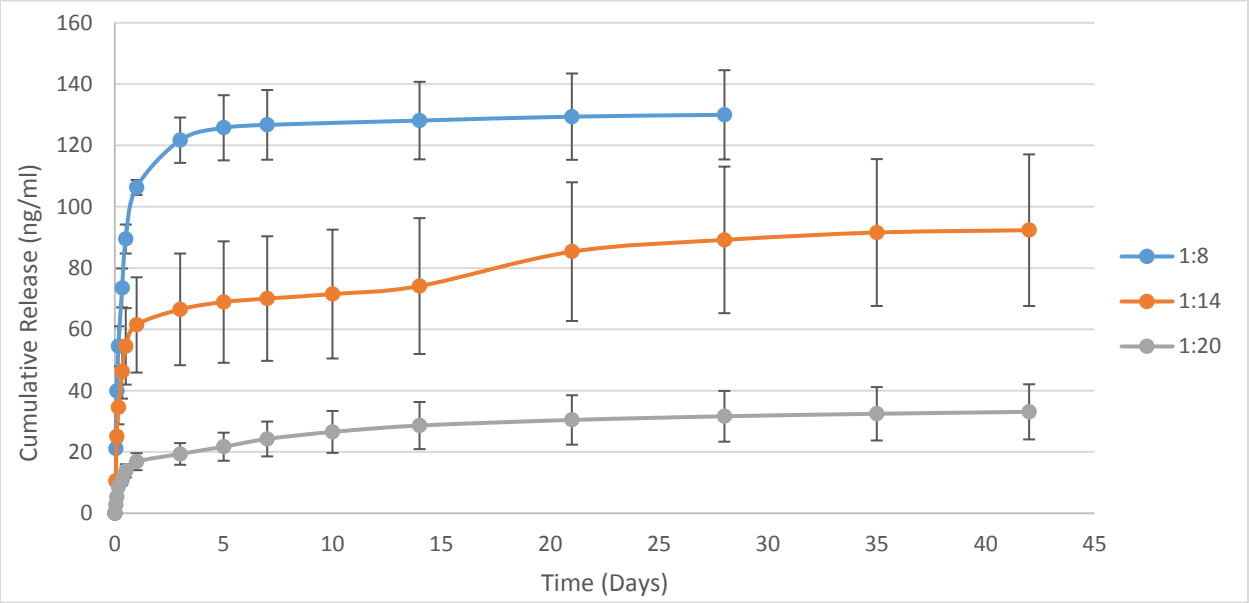

Figure 3.2.2: Release of encapsulated BMP-2 from 1:8, 1:14, and 1:20 PLGA concentrations. Release is sustained and continues until 42 days in the case of the two later polymer concentrations, after an initial burst release

3.3. Analysis of Encapsulated Gentamicin

Gentamicin was encapsulated in PLGA with a 1:14 ratio, and used to coat cancellous allografts with an n = 4. Gentamicin was diluted down from its stock solution, and added to the PLGA solution prior to coating. The antibiotic was released in PBS at 37°C for 6 weeks. By the

end of the first day, 63% of the gentamicin was released, however there was a steady release of gentamicin up until week 4. The cumulative release concentration was 118 µg/ml. Figure 3.3.1 depicts the encapsulated gentamicin release kinetics, and figure 3.3.2 compares the percent release of encapsulated gentamicin and BMP-2 from 1:14 PLGA. The two curves show very similar release kinetics of the two encapsulated molecules. BMP-2 and gentamicin both showed a burst release of 67% and 63% in the first day respectively, and both achieved 100% release by day 35.

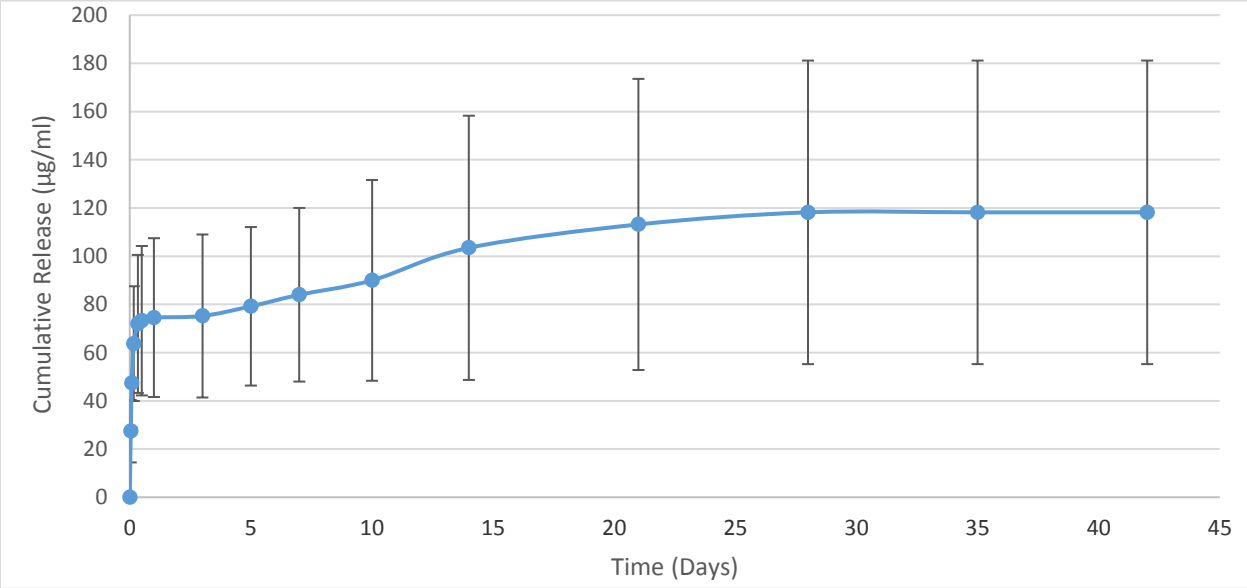

Figure 3.3.1: Release of encapsulated gentamicin from 1:14 PLGA coated allografts. Release appears to be sustained for up to 21 days, after which release begins to plateau.

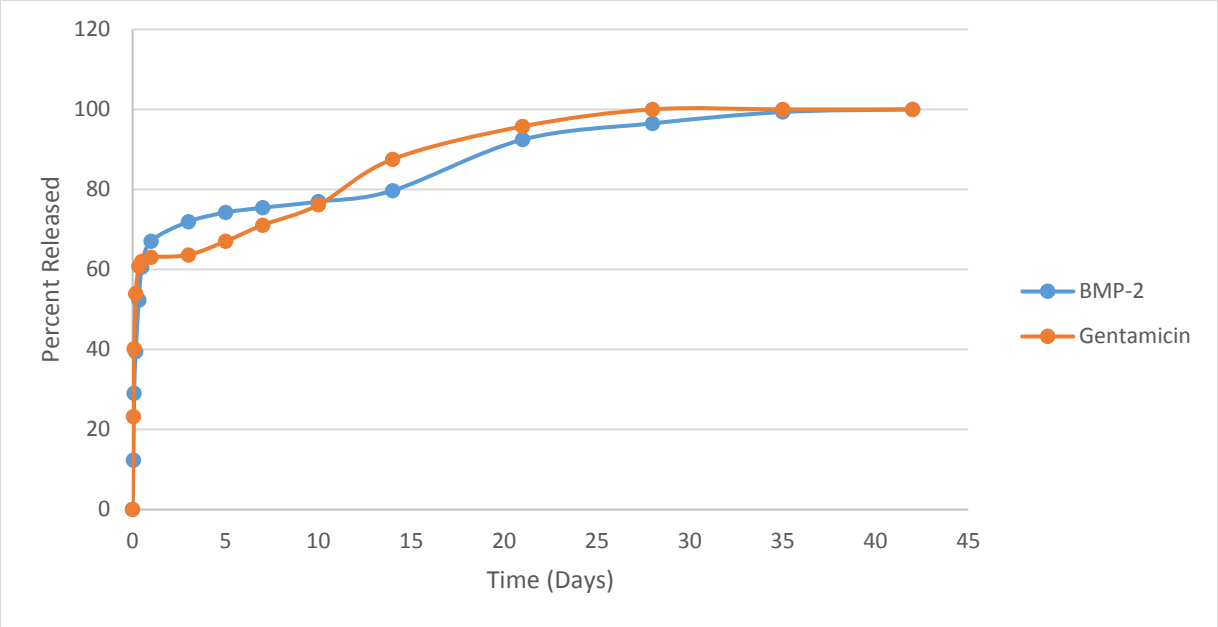

Figure 3.3.2: Percentage released of BMP-2 and gentamicin from 1:14 PLGA coated allografts. The release kinetics appear to be comparable in terms of burst release and total release time.

3.4. Analysis of Surface Adsorbed Gentamicin

Gentamicin was also loaded onto the cancellous allografts via surface adsorption, at polymer concentrations of 1:14 and 1:20. Both polymer concentrations showed similar release kinetics, seen in figure 3.4.1. The kinetics began as a burst release, followed by a plateau in release – the expected trend observed for surface adsorbed agents. However at day 10 and 14, there was additional release from the 1:20 and 1:14 PLGA respectively, followed by another plateau. Because of this additional release, the relative burst release was not as significant as seen in the surface adsorbed VEGF from the preliminary studies, in this case only 56% and 62% of gentamicin was delivered from the 1:14 and 1:20. More gentamicin was available from the higher polymer concentration, with cumulative release values of 292 μg/ml and 44 μg/ml for 1:14 and 1:20 PLGA respectively.

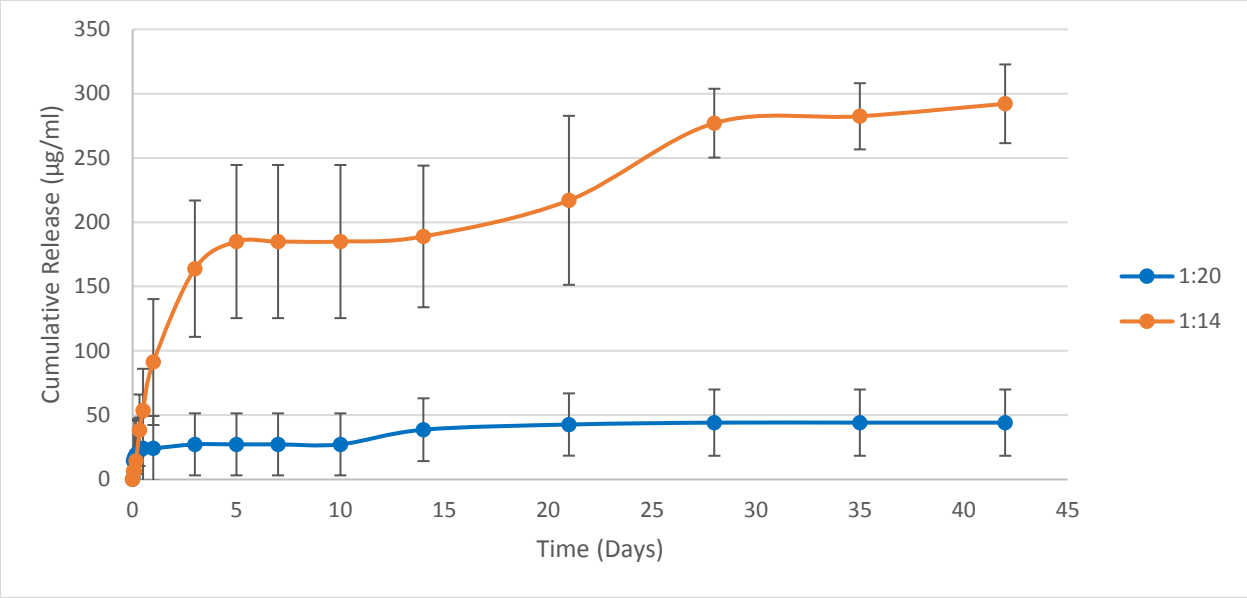

Figure 3.4.1. Release kinetics of surface adsorbed gentamicin from 1:14 and 1:20 PLGA. Both release curves showed burst release followed by a plateau, as well as a period of additional release in the middle of the release time period.

Figure 3.4.2 additionally shows the relationship between both encapsulated and surface adsorbed gentamicin released from 1:14 PLGA, portrayed non-cumulatively. As the figure shows, surface adsorbed release allows for a greater availability of the drug within the first 1 – 7 days, however after 1 week there is no more gentamicin to be released. On the other hand, encapsulated gentamicin has a much lower amount available within the first week, however as the surface adsorbed gentamicin completed its release, encapsulated gentamicin began to sustainable release for a longer period of time.

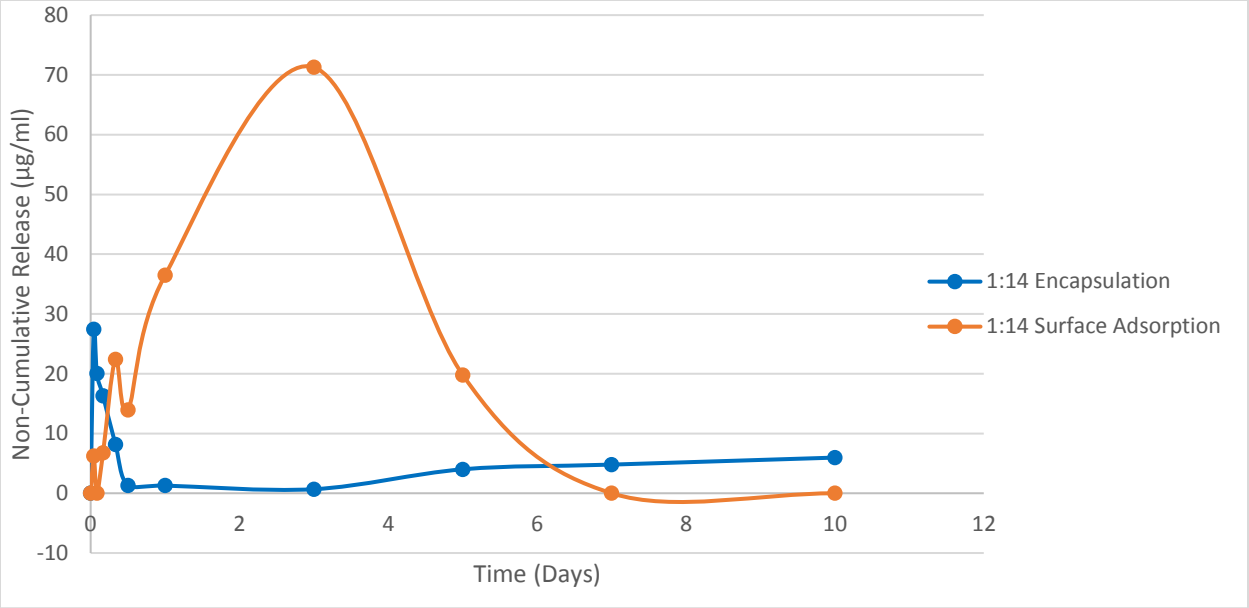

Figure 3.4.2: Release kinetics of both surface adsorbed and encapsulated gentamicin from 1:14 PLGA. Surface adsorbed gentamicin showed more gentamicin available in the first week, while encapsulated gentamicin showed less available, however a more long term sustained release.

3.5. *In Vivo* X-ray Analysis

X-ray radiographs were taken throughout the healing process to assess the new bone formation in the control groups, BMP-2 alone, and BMP-2 and VEGF groups. Figure 3.5.1 shows the control allograft after 4 weeks of healing. The image shows no indication of callus formation or mineralization formed at the defect site, indicating the allograft implantation was non-union at the defect site. Radiograph images were also taken and analyzed every 2 weeks post-implantation. Figure 3.5.2 displays the bone formation observed throughout the course of the healing process.

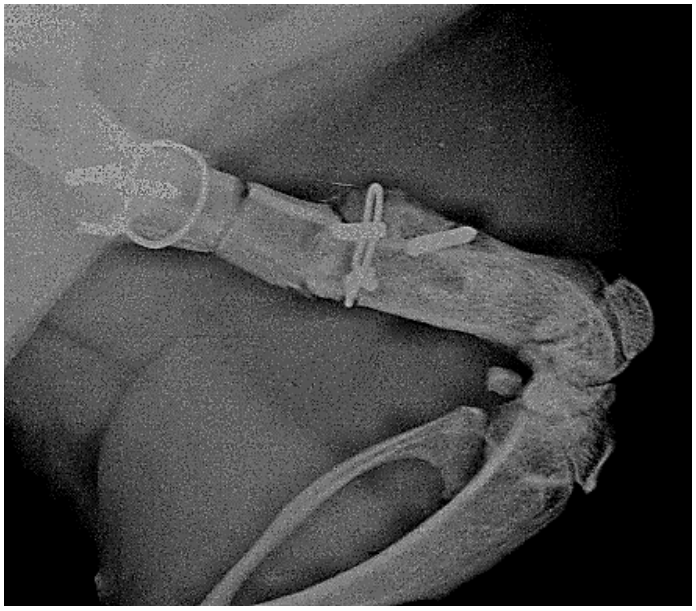

Figure 3.5.1: X-ray radiograph of the control allograft after 4 weeks of implantation. No healing or bone bridging is observed.

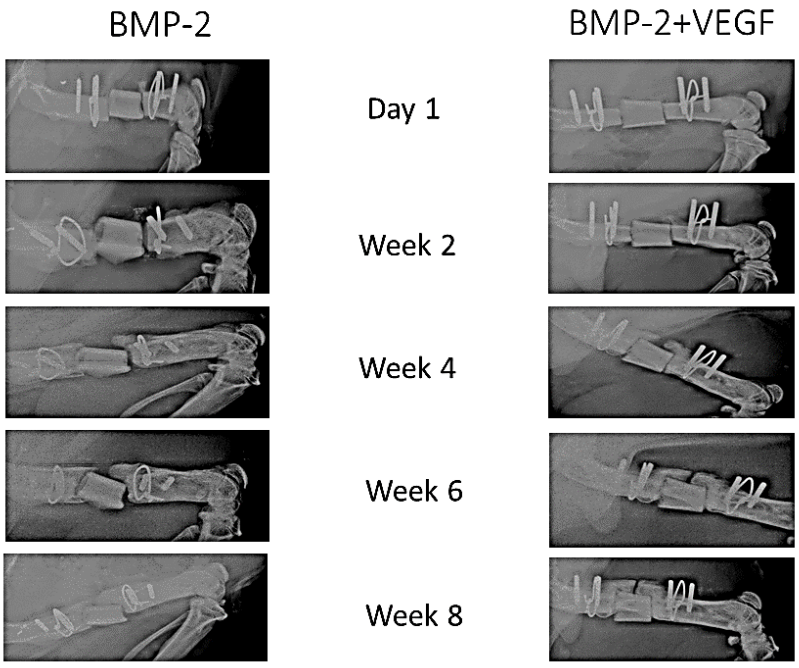

Figure 3.5.2: Radiograph images taken every 2 weeks post-implantation, to compare BMP-2 alone to a combination of BMP-2 and VEGF.

Bone bridging is seen in the image as the new opaque formation above the allograft in the defect site, indicating potential mineralization and bone formation is occurring due to bridging. The dually loaded allograft shows nearly complete bridging of the ends of the allograft and host bone due to new bone formation by week 8, suggesting the allograft forms a near complete union at the defect site. In the BMP-2 and VEGF group, callus formation and bridging are first observed in the radiographs at week 4. At week 4, 6, and 8, increased mineralization and bridging are seen throughout the increasing healing time in both groups, however the dually loaded allograft shows slightly augmented bridging compared to BMP-2 alone. The BMP-2 alone group shows enhanced callus formation compared to the control bone by the week 4 time point.

3.6. MicroCT Analysis

MicroCT imaging was done at 4 and 8 weeks post-implantation. MicroCT images and radiographs were taken to further verify callus and bone formation at the defect site. MicroCT radiographs confirmed the X-ray images, demonstrating that bone bridging and mineralized callus formation was enhanced for the dually loaded allograft compared to both the BMP-2 alone at week 4, and both groups showed callus formation and some bone bridging. Compared to the control group, both growth factor loaded allografts show enhanced callus formation, as seen in figure 3.6.1. 3D images of the allograft implantation at the defect site were rendered using MicroCT, to allow the viewing of the bone formation after 4 and 8 weeks. Figure 3.6.2 shows at week 4, minimal bridging and bone formation occurred at week 4 in the BMP-2 alone and combined growth factor groups, while the control bone remained as a non-union, with little to no healing observed. By week 8, the BMP-2 alone group displayed callus formation and bridging around the allograft, while the combined group showed an increase of bone healing and a nearly complete bony union was formed at the defect site.

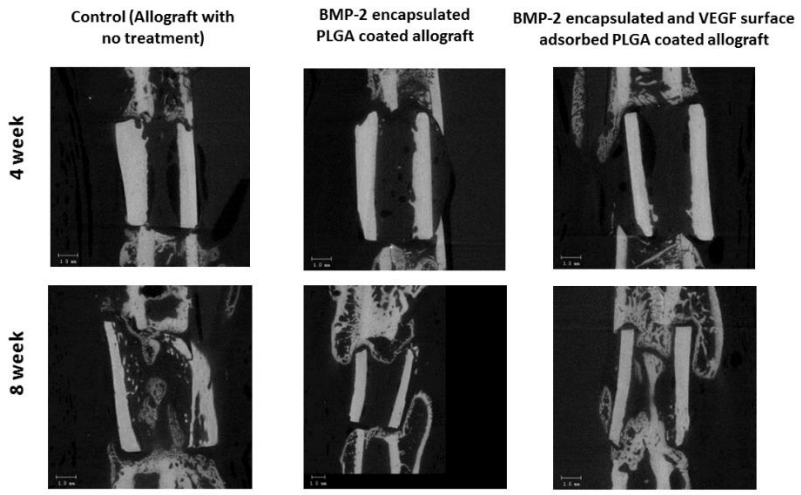

Figure 3.6.1: MicroCT radiographs of control, BMP-2, and combined growth factor groups after 4 and 8 weeks of allograft implantation. No callus formation or bridging was seen in the control group. Callused bridging was seen in week 8 in the BMP-2 group, and at both week 4 and 8 for the combined group.

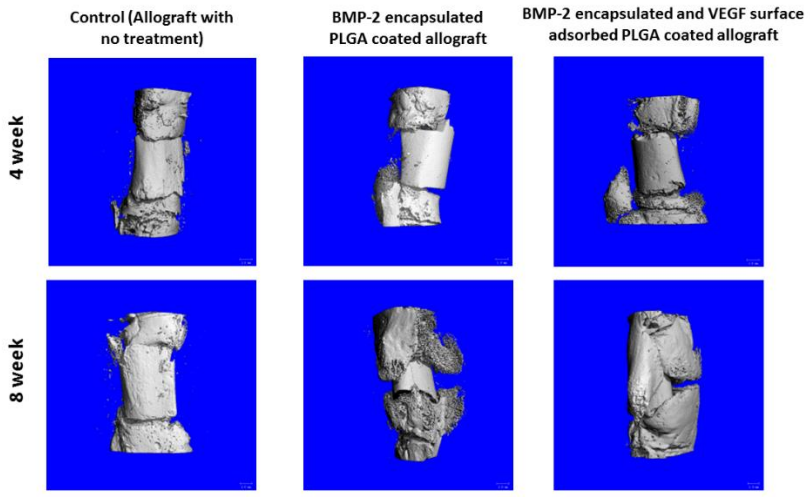

Figure 3.6.2: 3D rendered images of the defect/healing site. Control groups showed very little bone formation. A partial bony union was observed by 8 weeks in the BMP-2 alone group, while increased bone bridging was seen by 8 weeks in the BMP-2 and VEGF group, with partial bridging seen at week 4 for the same group.

4 Discussion

4.1. Polymer Degradation

The polymer coating is the most significant aspect of the delivery of bioactive agents from coated allografts, as the polymer coating is the means behind growth factor and antibiotic loading, as well as the driving force for delivery due to degradation. Preliminary studies of this project characterized the most efficient procedure for coating allografts with PLGA. Using MicroCT to quantify the coating volume, and SEM imaging to qualify the extent of the coating, it was determined that a polymer concentration of 1:14 was the most ideal in terms of the coating extent and porosity. The polymer solution was viscous enough that it covered the entire allograft and infiltrated the porous matrix of the bone. Concentrations of 1:12 were too viscous, and clogged some of the allograft pores, effectively eliminating the natural porous nature of the bone, while polymer concentrations of 1:20 produced a minimal coating volume that left small regions of uncoated bone.

SEM images were taken to assess and characterize the PLGA degradation from being submerged in PBS for up to 42 days, in order to establish a proper time period for encapsulated *in vitro* release studies to take place. PLGA degradation is the mechanism behind drug delivery in this case, and the SEM images were used to verify that the polymer degradation was occurring in a pattern that correlated to the *in vitro* release. Bones submerged in PBS for 3 days showed a fully coated allograft with very little uncoated bone, however the majority of growth factor is released during this first day. This can be explained because the burst release is not due to hydrolytic degradation of PLGA, but rather the interaction of PBS and the proteins bound to the surface of the polymer coated allograft, as well as their solubility. SEM images were taken for bones submerged for 3, 5, and 7 days, and every week after that for 6 weeks. Each successive

time point showed a slightly larger region of uncoated bone, represented by a lighter, jagged appearance, indicating that the polymer had begun to degrade. This slow process of degradation represents the gradual release of encapsulated BMP-2 and gentamicin after the initial burst, corresponding the polymer degradation to the drug release. However despite gradually degrading, a PLGA coating was still present on the allograft, indicating the graft's ability to encapsulate and release proteins in a sustained and long term manner. By time points even as late as 4 weeks, PLGA coated still was visible on the allograft, however by the time the allograft submerged for 42 days was imaged, it was noted that it was nearly all uncoated bone, suggesting nearly complete polymer degradation. This serves as a suggestion and verification of how long *in vitro* release studies should occur, based on the premise that the therapeutic agents will have been completely released by the time the PLGA has degraded. This study did not research the possibility of growth factors or antibiotics binding to the graft itself, and the qualitative results of this study were analyzed under the principle that the therapeutic agents will be completely released when there is no polymer left. Because no PLGA was seen by week 6, it is an indication that there is no more growth factors or antibiotics bound to the system yet to be delivered, thus a 6 week time point is sufficient to gather release data. This is additionally seen in the corresponding plateaus by 6 weeks, and limited release after 4 weeks in the BMP-2 and gentamicin encapsulation studies.

It is important to note that the polymer degradation study was performed using cortical allografts and a 1:8 polymer coating, while the *in vitro* studies were using cancellous allografts, and most were focused on lower PLGA concentrations. While the pattern of gradual polymer degradation does correlate to the release profiles observed, there are significant differences that exist between the two studies. One of the major differences in the lack of pores present in

cortical allografts. The presence of pores could introduce variability in the degradation and coating process, and may have altered how the PLGA degrades compared to the cortical allografts. The other main difference was the use of a 1:8 PLGA concentration, which was only used for one *in vitro* release study in this present work. The preliminary studies determined that even a 1:12 polymer concentration clogged the native pores of the allograft, and the lower concentrations of PLGA that were used in the *in vitro* studies resulted in a lower coating volume, and potentially a difference in degradation time. Despite the differences in the SEM study and the *in vitro* studies, the release kinetics appear to show correlating patterns as seen in the SEM characterization, and thus the polymer degradation is thought to be similar between the two.

4.2. Release of Encapsulated of BMP-2

BMP-2 encapsulation followed the trend established from the SEM characterization study. An initial burst release of 82%, 67%, and 53% for 1:8, 1:14 and 1:20 was observed, and is likely caused by the contact between the PBS and surface of the polymer coating. The burst release for the 1:8 PLGA concentration is significantly larger than the other two concentrations, however it's possible that since time points have only been taken up to day 28, there is still potential BMP-2 to be released, which would result in a smaller burst release relative to the cumulative concentration. However, at the 28 day time point, 97% and 96% of BMP-2 was released from the 1:14 and 1:20 PLGA. If a similar trend is followed in the remaining 2 weeks of release from the 1:8 concentration, there will not be significant increases in the cumulative release. After burst release, sustained release was achieved up until around 21 days and eventually stopped releasing by day 42, again following the trend of the polymer slowly bulk degrading during this time period. The plateau seen by the end of the study indicates no further release is occurring, a trend that is related to very little polymer coating left on the allograft as

seen in the SEM degradation study images. Encapsulating BMP-2 within the polymer matrix will provide protection from the physiological environment, and prolong its very short half-life [19]. This, in combination with the prolonged release, will allow BMP-2 to remain at the defect site for up to 6 weeks, an occurrence that is typically achieved by frequent or supraphysiological dosage amounts [31]. The continuous, prolonged presence of BMP-2 will continue to stimulate osteoprogenitor cells, such as hMSCs, to proliferate and differentiate into bone forming osteoblast cells, a process that mimics the natural bone healing process. Preliminary studies assessed the burst release of VEGF, attempting to mimic natural bone healing as initial release of VEGF begins vessel formation. This study targets the natural healing process by continuously delivering BMP-2, which is naturally found at the defect site for a longer period of time, observed by bone callus formation occurring for 4 to 6 weeks after injury. By achieving a slight release, BMP-2 can be introduced in the system and immediately begin to differentiate bone forming cells, but the true significance lies in the ability for the allograft to maintain the delivery of BMP-2, biomimetically stimulating new bone growth for a duration of 6 weeks. This is hypothesized to result in an allograft that has enhanced osteoinductive properties and bone formation, due to the extended delivery of osteogenic growth factors, as well as an initial burst release to begin the process of new bone formation upon implantation.

It is also significant to observe that the cumulative release of BMP-2 was larger with higher PLGA coating concentrations. This correlates to the preliminary studies, demonstrating that higher concentrations had a higher coating volume. The 1:20 concentration insufficiently infiltrated the entire porous nature of allograft, and led to a decreased coating volume. With a decrease in coating volume, the 1:20 PLGA concentration was unable to attach and bind to as much of the protein as the higher concentrations. The 1:14 concentration showed extended and

continuous coating throughout the allograft, and the 1:8 concentration was not imaged, though MicroCT verified it had the highest coating volume of all 3 polymer concentrations. This results in larger coating volumes having a larger surface area to bind proteins. These results were verified by quantification, showing that 1:14 PLGA released 92 ng/ml, almost three times as much as the 33 ng/ml released by the 1:20 PLGA concentration. By day 28 the BMP-2 released from 1:8 PLGA already had a cumulative release concentration of 130 ng/ml. Additionally, the results provide evidence to the hypothesis that varying polymer concentration will subsequently vary the release kinetics, and demonstrate the flexibility the coated allografts can achieve by modifying both polymer and factor loading concentrations. The significance of a flexible, variable allograft is beneficial for clinical applications. Depending on the type, size, and location of the bone defect site, different polymer concentrations can be synthesized depending on the amount of growth factor delivery required, while not requiring larger systemic deliveries.

4.3. Release of Encapsulated Gentamicin

The release of encapsulated gentamicin from 1:14 PLGA follows similar trends to that of encapsulated BMP-2, a burst release following by prolonged delivery. The burst effect of gentamicin was 63% of the total release, compared to 67% of the total burst release from encapsulated BMP-2, demonstrating comparable burst release kinetics. The burst release of gentamicin occurred slightly quicker, with the burst release seen in the first 12 hours compared to the first day, but this may have potentially been caused by gentamicin's lower molecular weight or higher solubility compared to BMP-2. The similar release kinetics serve as verification that loading and releasing antibiotics can be accomplished in a similar manner to growth factors, allowing the potential use of the allograft to release a combination of growth factors and

antibiotics, to fight off bone infection that may arise from the implantation, as well as promote new bone growth to the defect site.

Following the burst release, gentamicin continued to release from the allografts up until day 28, and following that began to plateau. This is imperative to the function of antibiotic to fight bone infection. Antibiotic treatment should continue for 6 weeks post-surgery to properly prevent osteomyelitis from forming [21]. The slight burst release of gentamicin allows the antibiotic to be present in physiological amounts locally within a day of allograft implantation, for immediately defense against infection, and not allow an infection to form to the point where it becomes difficult to treat. The subsequent sustained release is critical in maintaining antibiotic presence at the implantation site, to enable the gentamicin to continue to fight off infection for the recommended six weeks, and ensures that not all gentamicin is released prior to an infection being fully eliminated. The release kinetics observed in this study are an attempt to mimic antibiotic treatment of administration in the operating room or even prior to surgery occurring, as well as for up to 4 to 6 weeks post-surgery. The burst release followed by 28 days of prolonged release demonstrated in this study suggests the potential the allografts have to release gentamicin in accordance with standard systemic administration, while being delivered locally to potentially reduce side effects.

4.4. Release of Surface Adsorbed Gentamicin

Surface adsorbed gentamicin followed a different trend than that of the encapsulated data. While encapsulated gentamicin had a burst release followed by a sustained release, surface adsorbed gentamicin showed a much larger burst release, followed by an immediate plateau. However, both polymer concentrations for this release study showed an additional time point of release in the middle of the study. This additional release caused the burst release to be less

pronounced relative to the whole cumulative release of gentamicin. Similar to the encapsulated BMP-2, the trend of a higher PLGA concentration resulting in more release is also seen in this case. This aspect is again important in terms of the controllability of the construct. By simply altering the polymer concentration prior to coating, more or less gentamicin can be theoretically loaded and released from the allograft. In terms of a clinical application, controllability is a beneficial, and allows the potential of altering the coating parameters based on surgeon or medical staff advice, for a more patient customized allograft based on their specific defect site.

It is also worth noting that the surface adsorption does not follow the trend of prolonged release as seen in the encapsulation studies as well as SEM characterization. This trend is reflected in figure 3.4.2. Surface adsorption delivers a much larger amount of gentamicin within the first week than encapsulation, however it shows no long term release compared to the sustainability of encapsulated release. This is due to the mechanism of release of surface adsorption is not tied to bulk degradation of PLGA, but rather surface diffusion of the proteins upon placed in PBS, which happens rapidly. Upon the diffusion of the entirety of antibiotic, the PLGA will continue to degrade. However, no antibiotic was encapsulated within the PLGA matrix but rather just on the surface, thus the PLGA will degrade but release no further amounts of antibiotic. The goal of surface adsorption of gentamicin is to use the short term, extreme burst release in combination with a long term delivery of additional therapeutic agents. An allograft with surface adsorbed gentamicin would allow a substantial amount of antibiotic to immediately begin fighting and preventing infection, more so than encapsulated gentamicin. Using biphasic drug loading and release, this would be combined with encapsulated growth factors or additional antibiotics, to allow for long term delivery of antibiotics or osteogenic agents, while significantly

administering a dose of antibiotics immediately following implantation, when the wound site is most exposed to the environment that will cause infection.

It is worth noting that the gentamicin release studies all showed large standard deviation errors, as well as large cumulative releases compared to their loading concentrations. In addition, the surface adsorbed kinetics displayed the irregular increase midway through their release period. It is thought that the chemical assay synthesized, based on [39] played a role in how the absorbance values were subsequently calculated into concentration readings. It is thought that the o-phthalaldehyde in particular is a sensitive chemical, and may be affected by air oxidation, light, or time since use [40], all factors that could have potentially altered gentamicin readings. One piece of evidence behind this thought is that based on table 4.3.1 below. This table shows the absorbance of PBS, the assay, and propanol with no gentamicin loaded, from multiple standard curves ran throughout the year. In theory, since the 0 μg/ml concentration of the standard curves are identical solutions, the values would be similar during each run. However, as the table shows, these values varied greatly between multiple standard curves developed using identical protocols. This trend is additionally seen in figure 4.3.1 as well. This figure shows all the standard curves, which in theory should follow the same trend, however the graphs are shifted up along the Y axis based on what value the PBS and reagent alone had. When standard curves were corrected by subtracting the 0 μg/ml concentration reading from all values, the resulting standard curves showed more consistency in terms of slope as well as absorbance values. This led us to believe that the release profiles for gentamicin are correct in terms of trends and patterns, but the translation from optical density to a concentration value themselves might be skewed in the Y axis direction, potentially explaining the large cumulative release, or

even the bump in surface adsorbed release, as all of the samples of a specific release study were not quantified on the same day.

Trial	0 µg/ml Absorbance
1	0.979
2	0.276
3	0.649
5	0.953
6	0.293
Standard Deviation	0.341092363

Table 4.3.1: Variability in absorbance readings for PBS and the assay kit alone

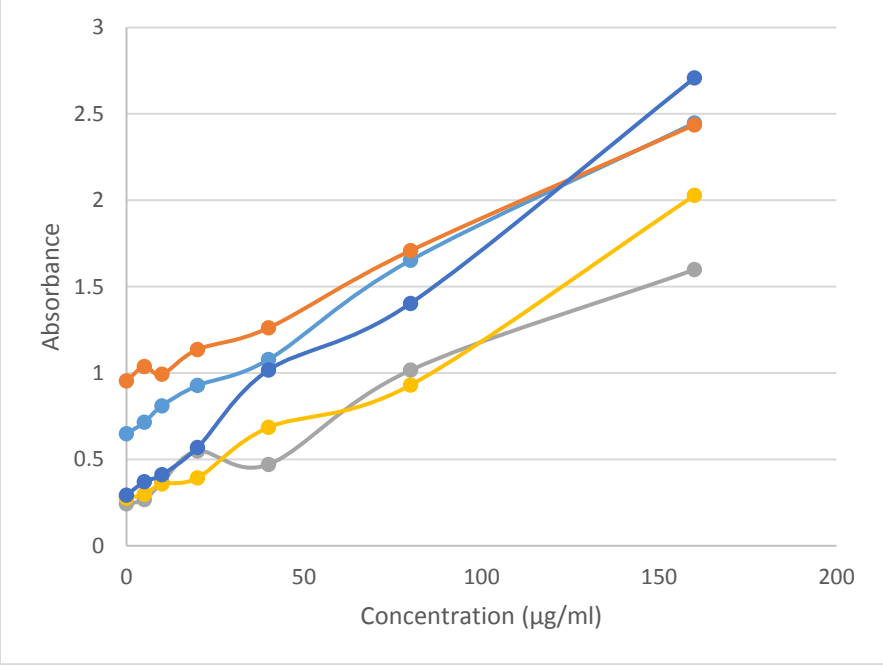

Figure 4.3.1: The varying gentamicin standard curve ran multiple times throughout the year. The variability of the standard curves is shown with the shifts up the Y axis.

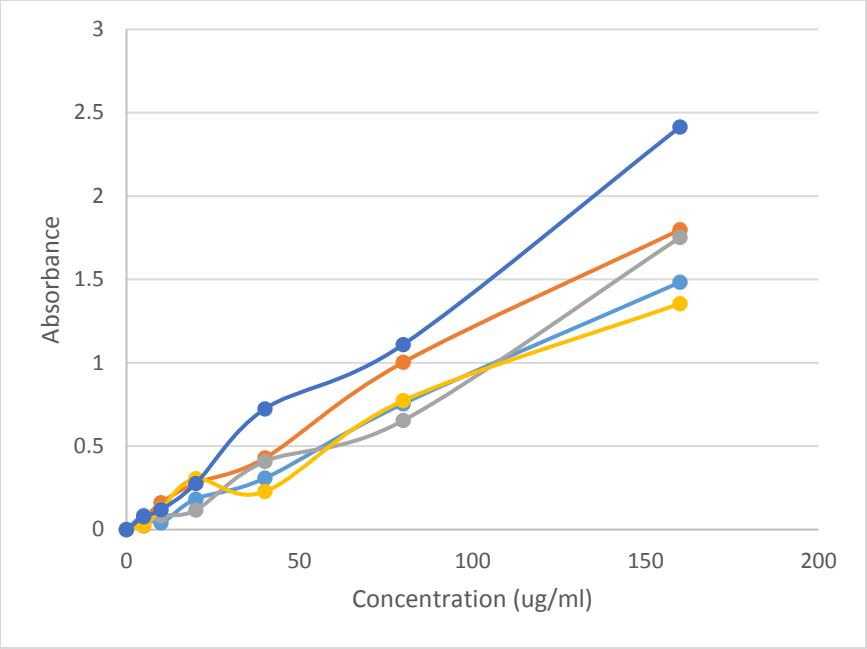

Figure 4.3.2: Corrected gentamicin standard curves appear to be more consistent and show similar trend lines.

4.4 *In Vivo* Healing of a Critical Size Defect

The aim of the *in vivo* study was to demonstrate that the combined delivery of BMP-2 and VEGF would ultimately lead to an increased amount of new bone formation in the defect site after 8 weeks. This hypothesis is based on related literature that dictates a combined delivery of BMP-2 and VEGF leads to more bone formation than either of the growth factors alone [26-28]. For instance, the sequential release of both VEGF and BMP-2 from poly(propylene fumarate) scaffolds with gelatin microparticles was shown to accelerate the healing process in a critical size defect in a rat model [26]. Collagen type I and osteopontin expressions were all found to be enhanced from the combined delivery of VEGF and BMP-2, indicating increased chemotactic migration and bone remodeling activity respectively. These results are thought to be due to VEGF and BMP-2 respective roles of angiogenesis and osteogenesis in bone healing. VEGF is a

widely used growth factor responsible for the formation of new blood vessels, a key step in bone or any wound healing process. BMP-2 stimulates osteogenesis due to the proliferation and differentiation of osteoprogenitor cells into osteoblasts, which form woven bone, and the two have displayed synergistic effects when delivered together.

The design of the allograft involved encapsulated BMP-2, and surface adsorbed VEGF. Based on the preliminary studies, the release kinetics should display a burst release of VEGF, followed by a sustained release of BMP-2 [29]. The proposed design was sought to have an immediate burst release of VEGF to the defect site to begin the process of angiogenesis. The accelerated formation of new blood vessels may potentially enhance the formation of new bone, due to the immediate access of nutrients, cells, oxygen, and waste removal of the defect site. In addition to angiogenesis, VEGF in combination with the RANK ligand stimulates the process of osteoclastogenesis [16]. This process breaks down bone to remodel, allowing osteoblasts too lay down the formation of new bone. This process is targeted in this study from the prolonged release of the encapsulated BMP-2. BMP-2 stimulates osteoblast differentiation, to form new bone out of the resorbed mineral substance from osteoclastogenesis. A prolonged release of BMP-2 could potentially continue to recruit and differentiate osteoblast progenitor cells to continue to form new bone for a longer period of time.

The radiograph and MicroCT results suggest overall that the combination of growth factors led to more new bone formation than the control allograft and just BMP-2 alone. A significant amount of bone formation occurred after 4 weeks in both the experimental groups compared to the control group, indicating the delivery and viability of the growth factors was successful. At 4 weeks, more bone was seen in the combined group than the BMP-2 alone group, as evident from the increased bone bridging and callus formation seen on the radiograph and 3D

rendered images. This potentially suggests that the synergistic effects of the combined growth factors was more prominent and effective at earlier time points, a trend that has also been observed in additional literature [26, 28, 30]. The seemingly accelerated bone healing compared to just BMP-2 can potentially may be explained by the surface adsorbed burst of VEGF, forming early blood vessels and resorbing bone for enhanced bone healing. By 8 weeks, the radiographs and MicroCT showed increases in bone formation between both experimental groups, with bridging appearing to get closer to approaching complete integration of the graft. The 3D MicroCT image showed that the control allograft was still unhealed at the defect site, with a visible gap between the allograft and the host bone. The BMP-2 alone group showed partial bridging and integration with the host bone by 8 weeks, however there was still partial non-union. The combined group showed an almost complete bridging and union of the host bone and allograft. These results indicate that similar to the 4 week time period, the combined synergy of BMP-2 and VEGF resulting in more bridging and integration with the host bone, and ultimately enhanced bone healing.

A critical aspect that this study addresses is the use of a controlled and combined delivery of growth factors to reduce the therapeutic dosage of BMP-2. In additional to large doses of BMP-2 being expensive, supraphysiological doses can lead to ectopic or cyst-like bone formation [31, 32]. One of the most popular clinical treatments involved BMP-2, INFUSE bone graft, contains 1.5 ml/mg rhBMP-2, exceeding the physiological levels of natural BMP-2 [33, 34]. Compared to the 1.5 ml/mg, the allograft used in these study contained 300 ng/ml. Despite containing orders of magnitude less BMP-2 than 1.5 ml/mg, BMP-2 alone showed enhanced bone formation compared to control allografts, though not sufficient enough for complete bridging after only 8 weeks. However, the low dose of BMP-2 combined with VEGF resulted in

the synergistic effect and almost complete bridging. Additionally, the amount of BMP-2 used in these allografts were considered sub-optimal therapeutic amounts in rodents [35], however used in combination with VEGF still achieved the nearly complete bony union. This suggests that the loading and controlled release of BMP-2, in addition with the combined delivery of BMP-2 and VEGF, ultimately leads to greater bone formation compared to control allografts, while requiring magnitudes less growth factor than is currently used clinically.

5 Conclusion

The polymer coating was optimized in the previous study, and this study characterized and assessed its degradation *in* vitro, as encapsulated molecules are released through the degradation process. Preliminary studies, as well our recent BMP-2 release studies performed show a sustained release up until 6 weeks. This is consistent with the findings of the SEM degradation, showing qualitatively that polymer coating still remained on the bone throughout the course of 6 weeks, though less remained at each time point, indicating long term sustained release of protein. Additionally, at the 6 week time point, very little polymer coating is observed to remain on the allograft, consistent with the gradual decrease and plateau of cumulative release. Each release study indicated similar kinetics; surface adsorbed molecules in general showed significant burst release followed by a very gradual release and plateau, while encapsulated release showed moderate burst release with prolonged release afterwards. These release kinetics are important depending on the function of the loaded protein. Long term BMP-2 delivery has been thought to be beneficial for the continuous differentiation of osteoblasts to form new bone. Gentamicin would benefit from both immediately release as well as long term delivery, to mimic the antibiotic protocol used in bone surgery. Lastly, the *in vivo* implantation of cortical allografts implanted in a femoral segmental defect indicated that the synergy of angiogenic and osteogenic

growth factors is more effective than either alone, corroborating with many previous studies. The combined group showed a more extensive callus formation and bone bridging at both 4 weeks and 8 weeks. The combined loading resulted in a near bony union after 8 weeks, as well as enhanced results at 4 weeks compared to BMP-2 alone, potentially indicating the combined angiogenic and osteogenic growth factor release leads to more accelerated bone healing.

6 Future Directions

There are still many steps, studies, and trials to complete before this bone graft system would be approved for any clinical applications. We have loaded a variety of therapeutic agents to allografts, the next likely step will be to show the release kinetics of dually loaded allograft. This could be performed by encapsulating one protein and surface adsorbing another, such as the BMP-2 and VEGF combination used in the *in vivo* studies with positive results. It could also entail loading the same agent or growth factor both encapsulated and surface adsorbed such as gentamicin, to achieve significant burst release as well as sustained release for 6 weeks after. Release kinetics for dually coated allografts need to be measured to ensure dually coated allografts results in similar trends of burst release of the surface adsorbed protein, and prolonged release of the encapsulated one, as seen with a single loaded protein. Additional *in vitro* cell studies should be performed, such as gentamicin functionality, MTS assays to ensure cell viability upon protein release, TRAP assay to test osteoclast differentiation, or assays to test mineralization follow BMP-2 release. The final step to perform will be a similar *in vivo* study on an animal model to test new bone formation upon implantation with various growth factors or gentamicin loaded onto the allograft.

References

[1] Clarke B. Normal Bone Anatomy and Physiology. Clinical Journal of the American Society of Nephrology : CJASN. 2008;3(Suppl 3):S131-S139. doi:10.2215/CJN.04151206.

[2] U. Kini and B. N. Nandeesh, "Physiology of bone formation, remodeling, and metabolism," in Radionuclide and Hybrid Bone Imaging, I. Fogelman, G. Gnanasegaran, and H. Wall, Eds., pp. 29–57, Springer, 2012.

[3] Feng X. Chemical and Biochemical Basis of Cell-Bone Matrix Interaction in Health and Disease. Current chemical biology. 2009;3(2):189-196. doi:10.2174/187231309788166398.

[4] Chapter 6: Bones and Skeletal Tissues. (n.d.). Retrieved April 13, 2016, from http://classes.midlandstech.edu/carterp/Courses/bio210/chap06/lecture1.html

[5] Iain H. Kalfas, MD (2001). "Principles of Bone Healing". WebMD LLC.

[6] Gugla Z, Lindsay RW, Gogolewski S. New Approaches in the Treatment of Critical-Size Segmental Defects in Long Bones. Macromol Symp 2007;253: 147-161.

[7] Lindsey RW, Gugala Z, Milne E, Sun M, Hannon FH, Latta LL. The efficacy of cylindrical titanium mesh cage for the reconstitution of a critical-size canine segmental femoral diaphyseal defect. J Orthrop Res 2006;24(7): 1438-1453.

[8] Klokkevold, PR, Jovanovic, SA (2002). "Advanced Implant Surgery and Bone Grafting Techniques". In Newman, Takei, Carranza. Carranza's Clinical Periodontology (9th ed.). Philadelphia: W.B. Saunders. pp. 907–8.

[9] Fleming, James E., Charles N. Cornell, and George F. Muschler. "Bone Cells And Matrices In Orthopedic Tissue Engineering." Orthopedic Clinics of North America 31.3 (2000): 357-74. Web.

[10] Vangsness CT, Jr, Garcia IA, Mills CR, Kainer MA, Roberts MR, Moore TM. Allograft transplantation in the knee: tissue regulation, procurement, processing, and sterilization. Am J Sports Med. 2003;31(3):474–81.

[11] Almeida, Otavio Machado de, Jorgetti, Wanda, Oksman, Denis, Jorgetti, Camilo, Rocha, Diógenes Laércio, & Gemperli, Rolf. (2013). Comparative study and histomorphometric analysis of bone allografts lyophilized and sterilized by autoclaving, gamma irradiation and ethylene oxide in rats. Acta Cirurgica Brasileira, 28(1), 66-71.

[12] Wheeler, Donna L., and William F. Enneking. "Allograft Bone Decreases in Strength In Vivo over Time." Clinical Orthopaedics and Related Research &NA;.435 (2005): 36-42.

[13] Kanczler, J. M., and R. O.C Oreffo. "Osteogenesis and Angiogenesis: The Potentil for Engineering Bone." European Cells and Materials 15 (2008): 100-14.

[14] Kirker-Head, C. "Potential Applications and Delivery Strategies for Bone Morphogenetic Proteins." Advanced Drug Delivery Reviews 43.1 (2000): 65-92.

[15] Liu, Yanqiu, and Bjorn R. Olsen. "Distinct VEGF Functions During Bone Development and Homeostasis." Archivum Immunologiae Et Therapiae Experimentalis 62.5 (2014): 363-68.

[16] Nakagawa, Mari, Toshio Kaneda, Toshiya Arakawa, Shuichi Morita, Takuya Sato, Takeo Yomada, Koji Hanada, Masayoshi Kumegawa, and Yoshiyuki Hakeda. "Vascular Endothelial Growth Factor (VEGF) Directly Enhances Osteoclastic Bone Resorption and Survival of Mature Osteoclasts." FEBS Letters 473.2 (2000): 161-64.

[17] Mehta M, Schmidt-Bleek K, Duda GN, Mooney DJ. Biomaterial delivery of morphogens to mimic the natural healing cascade in bone. Adv Drug Deliv Rev 2012;64(12):1257-76.

[18] Lee, K., E. A. Silva, and D. J. Mooney. "Growth Factor Delivery-based Tissue Engineering: General Approaches and a Review of Recent Developments." Journal of The Royal Society Interface 8.55 (2010): 153-70.

[19] F. Wegman, A. Bijenhof, L. Schuijff, F. C. Öner, W. J. A. Dhert, and J. Alblas, "Osteogenic differentiation as a result of BMP-2 plasmid DNA based gene therapy in vitro and in vivo," European Cells and Materials, vol. 21, pp. 230–242, 2011.

[20] Hirenkumar K. Makadia and Steven J. Siegel. Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. Polymers (Basel). 2011 Sep 1; 3(3): 1377–1397.

[21] Brady, R. A., J. G. Leid, J. W. Costerton, and M. E. Shirtliff. "Osteomyelitis: Clinical Overview and Mechanisms of Infection Persistence." Clinicla Microbiology Newsletter 28.9 (2006): 65-72.

[22] J.T. mader, M.E. Shirtliff, S.C. Berquist, J. Calhoun. Antimicrobial treatment of chronic osteomyelitis. Clin Orthop RelatRes, 360 (1999), pp. 47-65

[23] S.L. Henry, D. Seligson, P. Mangino, G.J. Popham. Antibiotic-impregnated beads. Part I: bead implantation versus systemic therapy. Orthop Rev, 20 (1991), pp. 242–247

[24] Infections After Fracture-OrthoInfo - AAOS. (n.d.). Retrieved April 16, 2016, from http://orthoinfo.aaos.org/topic.cfm?topic=A00580

[25] "Gentamicin Sulfate". The American Society of Health-System Pharmacists. Retrieved Aug 15,2015.

[26] Patel ZS, Young S, Tabata Y, Jansen JA, Wong ME, Mikos AG. 2008. Dual delivery of an angiogenic and an osteogenic growth factor for bone regeneration in a critical size defect model. Bone 43:931-940.

[27] Ramazanoglu, Mustafa, Rainer Lutz, Philipp Rusche, Levent Trabzon, Gamze Torun Kose, Christopher Prechtl, and Karl Andreas Schlegel. "Bone Response to Biomimetic Implants Delivering BMP-2 and VEGF: An Immunohistochemical Study." Journal of Cranio-Maxillofacial Surgery (2013).

[28] Diederik H.R. Kempen, Lichun Lu, Andras Heijink, Theresa E. Hefferan, Laura B. Creemers, Avudaiappan Maran, Michael J. Yaszemski, Wouter J.A. Dhert, Effect of local sequential VEGF and BMP-2 delivery on ectopic and orthotopic bone regeneration, Biomaterials, Volume 30, Issue 14, May 2009, Pages 2816-2825, ISSN 0142-9612, http://dx.doi.org/10.1016/j.biomaterials.2009.01.031.

[29] Sharmin, Farzana, Douglas Adams, Michael Pensak, Alexander Dukas, Jay Lieberman, and Yusuf Khan. "Biofunctionalizing Devitalized Bone Allografts through Polymer-mediated Short and

Long Term Growth Factor Delivery." Journal of Biomedical Materials Research Part A (2015): n. pag. Web.

[30] Young S, Patel ZS, Kretlow JD, Murphy MB, Mountziaris PM, Baggett LS, Ueda H, Tabata Y, Jansen JA, Wong M, Mikos AG. Dose effect of dual delivery of vascular endothelial growth factor and bone morphogenetic protein-2 on bone regeneration in a rat critical-size defect model. Tissue Eng A 2009;15:2347–2362.

[31] Gottfried ON, Dailey AT. Mesenchymal stem cell and gene therapies for spinal fusion. Neurosurgery 2008; 63:380-91.

[32] Villavicencio AT, Burneikiene S, Nelson EL, Bulsara KR, Favors M, Thramann J. Safety of transforaminal lumbar interbody fusion and intervertebral recombinant human bone morphogenetic protein-2. J Neurosurg Spine 2005; 3:436-43.

[33] Schmidmaier G, Schwabe P, Strobel C, Wildemann B. Carrier systems and application of growth factors in orthopaedics. Injury 2008;39(Suppl. 2): S37e43.

[34] Srouji S, Ben-David D, Lotan R, Livne E, Avrahami R, Zussman E. Slow-release human recombinant bone morphogenetic protein-2 embedded within electrospun scaffolds for regeneration of bone defect: in vitro and in vivo evaluation. Tissue Eng Part A 2010;17:269e77.

[35] Zara, N. Janette et al., High Doses of Bone Morphogenetic Protein 2 Induce Structurally Abnormal Bone and Inflammation In Vivo. TISSUE ENGINEERING: Part A Volume 17, Numbers 9 and 10, 2011.

[36] Jahangir, Alex, Ryan M. Nunley, Samir Mehta, and Alok Sharan. "Bone-graft Substitutes in Orthopaedic Surgery." American Academy of Orthopaedic Surgeons January (2008)

[37] Inmaculada Ortega-Oller, Miguel Padial-Molina, Pablo Galindo-Moreno, Francisco O'Valle, Ana Belén Jódar-Reyes, and Jose Manuel Peula-García, "Bone Regeneration from PLGA Micro-Nanoparticles," BioMed Research International, vol. 2015, Article ID 415289, 18 pages, 2015. doi:10.1155/2015/415289

[38] Cui, Quanjun, Abhijit S. Dighe, and James N. Irvine Jr. "Combined Angiogenic and Osteogenic Factor Delivery for Bone Regenerative Engineering." Current Pharmaceutical Design 19.19 (2013): 3374-383.

[39] Baro, M., Sánchez, E., Delgado, A., Perera, A., & Évora, C. (2002). In vitro–in vivo characterization of gentamicin bone implants. Journal of Controlled Release,83(3), 353-364. doi:10.1016/s0168-3659(02)00179-7

[40] Protocol by Uptima

[41] Liu, Y., & Olsen, B. R. (2014). Distinct VEGF Functions During Bone Development and Homeostasis. Arch. Immunol. Ther. Exp. Archivum Immunologiae Et Therapiae Experimentalis, 62(5), 363-368. doi:10.1007/s00005-014-0285-y

[42] Nakagawa, M., Kaneda, T., Arakawa, T., Morita, S., Sato, T., Yomada, T., . . . Hakeda, Y. (2000). Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. FEBS Letters,473(2), 161-164. doi:10.1016/s0014-5793(00)01520-9