5-9-2015

Increasing Bioactivity of Cancellous Bone Allografts Using Growth Factor Delivery through a Polymer Coating

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B.S., University of Connecticut, 2013

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

at the

University of Connecticut

2015

Approval Page

Masters of Science Thesis

Increasing Bioactivity of Cancellous Bone Allografts Using Growth Factor Delivery through a Polymer Coating

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Acknowledgments

I would like to thank my Major Advisor, Dr. Yusuf Khan, for his constant help and support throughout my Master's research, as well as giving me this opportunity. I would also like to thank Farzana Sharmin, the Ph.D. student who continuously helped me throughout my time on the project. I would finally like to thank Dr. Lakshmi Nair and Dr. Wendy Vanden Berg-Foels for serving as my Associate Advisors and on my defense committee.

Table of Contents

List of Figures

List of Tables

Abstract

Bone grafts are incorporated in over 2 million orthopedic surgeries worldwide every year. (Janhangir) When a defect is large enough that the body cannot properly heal on its own, a bone graft is surgically implanted into the defect site. Allografts, though commonly used, show poor bioactivity and often require the incorporation of molecules such as growth factors to achieve significant bone formation in defect sites. This study used a degradable polymer, poly(lactide-co-glycolide) (PLGA), to coat allografts and load multiple growth factors to be released. Bone morphogenetic protein-2 (BMP-2) was physically encapsulated for extended release, while vascular endothelial growth factor (VEGF) was surface adsorbed to achieve a burst release. The antibiotic gentamicin was also tested in surface adsorption as an alternative application. Results showed a thin, continuous coating was able to extend throughout the entirety of the allograft while maintaining the native porosity of the allograft. Surface adsorption of VEGF and gentamicin both showed a burst release with the majority of the release occurring in the first 24 hours. Encapsulation of BMP-2 showed an extended release, with a smaller initial burst compared to surface adsorption. This combination shows ability to create short and long term kinetics similar to that of the natural healing process in bone. It was also shown that the volume of coating, and therefore release of the growth factors, was affected by a change in the concentration of the polymer. Results show a system with potential capability to increase bioactivity of allografts in bone healing, as well as possible mitigation of infection following surgery.

Introduction

1.1 Physiology/Bone

The skeletal system consists of many bones that give the human body structural support, enabling people to move, and protecting internal organs from harm. The makeup of long bone consists of a central marrow canal surrounded by bone tissue and the periosteum, the outer layer of bone. There are two main types of bone, primary/woven bone and secondary/lamellar bone. Woven bone is the initial bone formed during embryo and fracture healing stages before being resorbed and replaced. Lamellar bone, which replaces woven bone, consists of collagen fibrils in very tightly packed and organized sheets. (Weiner)

There are 3 main types of bone cells that derive from either hematopoietic origin or differentiation of mesenchymal stem cells. Osteoblasts synthesize and distribute bone matrix to form new bone. Osteocytes are the most prevalent type of bone cell, working to maintain homeostasis of the bone mass density. Osteoclasts are bone resorptive cells, working to degrade bone and initiate phagocytosis. (Buckwalter)

1.2 Bone Tissue Type

Bones are categorized a variety of different ways, including size, shape, location, or tissue type. The most common way to classify bones is by their shape as flat bones or long/tubular bones. Long bones make up the majority of the human skeleton, while most of flat bones are contained in the facial region and ribcage. The long bones are composed of three different regions: the diaphysis, metaphyses, and epiphyses. The diaphysis is the middle region of a long bone, with a medullary canal. The epiphyses are the outer regions of the long bone containing

the articular surfaces, and the metaphysis is in between the epiphysis and the diaphysis. Flat bones do not contain any of these regions, as they are simply made up of mostly spongy bone with a thin, cortical shell. (Buck)

The structure of bone is categorized as cortical or cancellous bone. Although these 2 bone types have the same matrix composition, they are drastically different due to their porosity, metabolic activity, and structure. Cortical bone consists of dense lamellar bone that accounts for 80 percent of bone by weight in the human body. It has only 5-10 percent porosity, and provides a lot of resistance and compressive strength to the human body. Cortical bone is composed of osteons, which are concentric lamellae that surround a Haversian canal that is lined with blood vessels and endosteal cells. Osteons also make up the majority of the diaphysis in long bones. (Clarke)

Cancellous bone makes up the remaining 20 percent of bone in the body by weight. It has a porosity of 50-90 percent, giving it much greater surface area per volume than cortical bone. It is remodeled more rapidly and has a higher rate of metabolic activity compared to that of cortical bone. Cancellous bone, also known as trabecular, or spongy bone, deforms more easily than cortical bone and absorbs forces placed on the body. It is found on the outer surface of the long bones, in the metaphyses and epiphyses. It also makes up the majority of flat bones, such as bones in the facial region. Cancellous bone does not contain osteons. The lamellae of cancellous bones form semicircular “packets” that form the trabeculae. The porous structure of the trabeculae gives the bone a very high surface area. Cancellous bone also shows a greater extent of vascularization compared to that of cortical bone. Comparison of the two types of bone tissue is shown in the figure below from Buck et al.

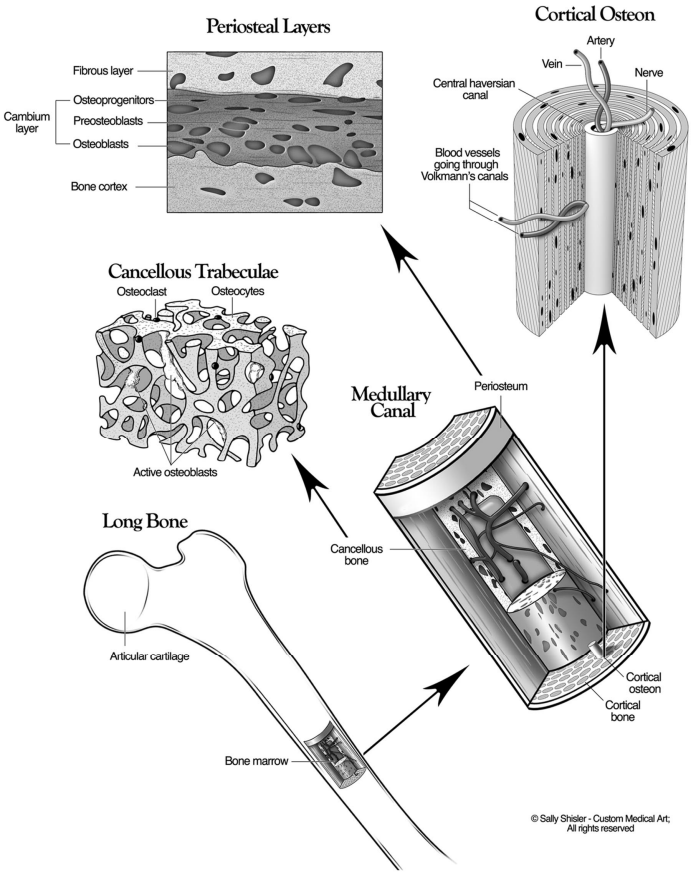


Figure 1. Bone physiology of cortical and cancellous bone (Buck)

1.3 Fracture Healing

When a skeletal defect occurs due to a bone fracture, the body must act to replace the missing bone segment and restore the native architecture of the bone. In order to do this, the body begins to enter into a coordinated cascade of events to heal the defect area of the bone. This is a very intricate and complex process that involves many components, such as different cell types, cytokines, and their receptors in various steps. The stages of healing are as follows: formation of a hematoma, inflammation, revascularization, fibrocartilage and hyaline cartilage formation, cartilage mineralization, formation of woven bone, and finally remodeling. (Mehta) Following a fracture, a blood clot is formed at the injury site when the entrapped platelets

release granules and the clot becomes a hematoma. Inflammatory cells such as neutrophils, monocytes, and lymphocytes then arrive at the hematoma. Mesenchymal stem cells (MSCs) are recruited within a couple of hours of the fracture by cytokines such as interleukins (ILs). The differentiation of MSCs into chondrocytes results in the formation of cartilage. During this step, vascularization also begins to occur due to the release of the growth factors VEGF and angiopoietin. Osteoclast progenitors are recruited and differentiate into osteoclasts in response to VEGF and RANKL stimulation. Osteoclasts synthesize proteolytic enzymes that break down organic matrix proteins. Formation of bone is caused by the differentiation of MSCs into osteoblasts, followed by transformation into osteocytes in response to growth factors such as BMP-2. With the combination of osteoblasts, osteocytes, and newly formed blood vessels being present, woven bone is able to form. Osteoblasts and osteoclasts then work in combination to create secondary bone and control remodeling by resorption and deposition to restore the bone to its original size, shape, and structure. (Kalfas)

1.4 Bone Grafting

Human bones, however, are only capable of healing on their own to a certain extent. The fracture or defect site must be less than about three millimeters wide in order for the body to be able to heal naturally. Any gap larger than this will remain in non-union unless it is treated. (Mehta) A common and trusted method of doing this is by using a bone graft. Bone grafting is a surgical procedure to fill a skeletal defect with a new bone that helps to repair the surrounding tissue and provide load bearing support at the defect site. This is done by implanting the bone graft into the defect site. New bone is then able to form between the

bone graft and the native tissue, allowing for the full restoration of the original bone's structure.

Common causes for this type of procedure include physical trauma and osteoporosis. Osteoporosis is a disease that causes a decrease in bone mass and density, as well as a reduction in the proteins located within the bone. This leads to a greater risk of fracture, along with the possible inability to heal any defects. As the number of senior citizens continues to grow, osteoporosis and other diseases related to the degradation of host bone are more prevalent than ever. As a result, the number of bone grafting surgeries is increasing each and every year. In order for full healing to occur from the bone graft surgery, the implanted bone must be able to fill the void in the defect, must be biocompatible so that the recipient's body does not reject it, and be able to initiate the steps of bone healing to integrate the host bone and bone graft. Because of these boundary conditions, the selection of what to use as a bone graft is very important. (Lauzon)

1.4.1 Autografts

The most common type of bone graft is the autograft. Autograft tissue is harvested directly from the patient. By utilizing the patient's own tissue, the bone graft is naturally biocompatible and will not be rejected by the native tissue. Autografts may be implanted without being altered from its original form. Autografts are living tissues that retain all of the cell types and proteins that would naturally be found in bone. It is extremely important to preserve these components, as they participate in new bone formation and the bridging between the bone graft and the native tissue. Autografts are able to efficiently retain their

osteogenicity, osteoinductivity, and osteoconductivity. Autografts are the gold standard of bone graft procedures and maintain a very high success rate. (Fleming)

Unfortunately, due to the way in which autografts are harvested, there are a few major drawbacks to their usage. First, because live tissue is being extracted from the patient, a second surgical location is needed, and donor site morbidity can occur at the extraction site. This prolongs the recovery period and can necessitate a second procedure. A location that is easily accessible with high bone density is normally selected for a harvest site, commonly the iliac crest in the pelvic region. However, there are very few locations in the body that have a low enough risk of donor site morbidity to be considered as a viable option for autograft harvesting. Because of this, there is a very limited supply of donor tissue available, making it difficult to supply autografts for all bone grafting needs. (Bauer)

1.4.2 Allografts

Allografts are an alternative to the use of autografts. Allografts are very similar to autografts in that they are a bone graft that utilizes human tissue as the source for extraction. The main difference is that an allograft does not come from the recipient. While it may come from the same location in the body as an autograft, an allograft is normally harvested from a cadaver, removing the risk of donor site morbidity. Along with this, supply of tissue is also less of an issue when using allografts. Because donor site morbidity is not a concern when harvesting cadaver bone, the number of viable extraction points within the body is greatly increased. (Blokhuis) However, while the use of allografts creates a solution to both of the major complications associated with autografts, they do have their own drawbacks to contend

with. One of the major concerns associated with allografts is biocompatibility between the donor and the recipient. For the surgical procedure to be a success, it must be ensured that a host immune response is not elicited by the recipient. Along with this, using cadavers as a source for harvesting bone increases the risk of disease transmission. In order to guarantee there is no contaminants or diseases within the donor tissue, the bone graft is thoroughly sterilized using treatment solutions and/or radiation that removes viral components of the tissue. However, because of this sterilization process, many of the proteins and cellular components are removed as well. The loss of these bioactive components makes it much more difficult to elicit the natural healing cascade and full union of the graft and host bone is less likely. (Laurencin) This limited osteogenicity and remodeling capacity of the allograft has led clinical studies to report complications after a 10 year period for 30-60% of allograft reconstructions. Wheeler et al. found a 50% loss in strength of allograft tissue after an in vivo period of 10 years. This was due to a decrease in the bone mineral density, as well as an increased number of micro-fractures. (Wheeler) Without new bone formation, the implanted allograft remains necrotic. While its initial mechanical properties are similar to that of native tissue, over time the lack of baseline bone remodeling causes an increase in the risk of micro-fractures occurring. (Gouin) Along with the inability to repair the allograft internally, there is also commonly incomplete osseointegration at the allograft-host junction, which causes continued nonunion. This again, will increase the chance of fracture due to fatigue when maintained for a long period of time. Due to these issues, it is imperative to increase the osteogenic capability of allografts if they are to be a suitable replacement for autografts.

1.5 Growth Factors

During the cascade of bone healing, there are two main aims for the body to become healthy. New bone must be formed to fill the defect and vascularization must occur to allow the newly formed bone to be accessed by oxygen, nutrients, and cells. These two events are stimulated by the release of osteogenic and angiogenic growth factors, which cause bone formation and vascularization, respectively. (Kanczler)

Osteogenesis is the process of new bone formation. BMPs are the growth factors that are most prominent in eliciting this response. BMPs fall under the family of TGFβs and help signal and influence many different events during the bone healing process. BMPs are responsible for, among other functions, the proliferation and differentiation of osteoprogenitor cells. These osteoprogenitor cells are differentiated into osteoblasts, which are then able to transform into osteocytes. BMPs also help to stimulate chemotaxis, which recruits molecules to promote the formation of cartilage and new bone. Along with this, they are also involved in the synthesis of extracellular matrices and are present during the stages of angiogenesis. Within the group, BMP-2 and BMP-7 have become the most widely studied for bone repair due to their ability to induce bone formation in ectopic sites. (Kirker-Head)

Angiogenesis is the formation of new blood vessels, and is a critical process in fracture repair. These invading blood vessels allow nutrients, oxygen, and cells to be transported to the defect site. Without this, bone formation becomes more difficult and delayed union or non-union is much more likely. This process is brought on by a multitude of signaling growth factors. Fibroblast growth factors (FGF) stimulates proliferation and differentiation of cells that

participate in the formation of new blood vessels. These cell types include smooth muscle cells and endothelial cells. (Mehta) Based on previous research models, it is believed that the strongest stimulus for vascularization is from the growth factor VEGF. It is present throughout many steps of the fracture healing process and is believed to be not only the greatest inducer of blood vessel formation, but also a crucial component within the bone healing cascade. VEGF has four different isoforms A, B, C, and D, with VEGF-A being the most essential for inducing angiogenesis. It is the key regulator in transforming the cartilaginous matrix into a vascularized osseous tissue. It helps to stimulate the proliferation, survivability, and the migration of blood vessels throughout the defect area. Then, along with angiopoietin, another angiogenic growth factor, differentiation into mature blood vessels is achieved. (Liu) VEGF, however, appears at many other stages of the bone healing cascade, even helping to promote osteogenesis. VEGF receptors are expressed on many different types of cells such as MSCs, osteoblasts, osteoclasts, and endothelial cells. VEGF increases the blood vessel density within its vicinity, enabling a greater level of osteogenesis. It also is used in combination with RANKL to induce osteoclast progenitors to differentiate into osteoclasts during the stage of osteoclastogenesis. (Nakagawa)

Angiogenesis and bone formation are very closely related within the process of fracture healing, with growth factors such as BMPs and VEGF being released throughout the bone healing cascade. Synergistic effects of the two types of growth factor have shown that the lack of either angiogenic or osteogenic factors can lead to a higher possibility of delayed union or non-union. The dual capabilities of these growth factors lead to the idea that angiogenesis and osteogenesis are not two separate processes, but rather, simply two steps within the same process. A list of different growth factors and how they function is shown below. (Cui)

Growth Factor	Receptors	Cells producing growth factor	Target cells	Function
Bone Morphogenetic Proteins (BMPs)	Alk1, Alk2, Alk3, Alk6 (type I receptors) and ActRII, ActRIIB, BMPRII (type II receptors)	MSCs, Osteoblasts, Chondrocytes	MSCs, Osteoblasts	BMPs induce differentiation of progenitor cells into chondrocytes and osteoblasts
Growth Differentiation Factors 5 (GDF-5)	Alk6	Cartilage	MSCs	Chondrogenesis
Transforming growth factor- β (TGF-β)	Alk1, Alk5 (type I receptors) and TβRII (type II receptors)	Osteoblasts, Platelets, Immune cells, Chondrocytes	MSCs, chondrocytes, osteoblasts	Mitogenic and chemotactic for osteoprogenitor cells
Fibroblast growth factor (FGF)	FGFRs 1-4	MSCs, osteoblasts, chondrocytes, macrophages	Fibroblasts, endothelial cells, smooth muscle cells, MSCs, osteoblasts, chondrocytes	Angiogenesis, Mitogenic
Platelet derived growth factor (PDGF)	PDGFR	Platelets, smooth muscle cells, endothelial cells, macrophages	MSCs, smooth muscle cells, endothelial cells, osteoblasts	Chemotactic, angiogenesis, mitogenic
Insulin like growth factor (IGF)	IGF1R, IGF2R	Liver cells, endothelial cells, osteoblasts, chondrocytes, MSCs	Skeletal muscle cells, chondrocytes, MSCs, endothelial cells, osteoblasts	Induce protein synthesis, mitogenic, chemotactic
Vascular endothelial growth factor (VEGF)	VEGFR1, VEGFR2	Lung, kidney, heart, adrenal gland, liver, gastric mucosa, spleen, MSCs	Endothelial cells, osteoclasts, osteoblasts, MSCs	Master regulator of angiogenesis, mitogenic, survival signal for osteoclasts and osteoblasts
Angiopoietin	Tie1, Tie2	MSCs, smooth muscle cells	Endothelial cells	Chemotactic, vessel remodeling during tissue repair

Table 1. Growth Factors associated with bone healing

1.6 Growth Factor Loading for Delivery

Due to the ability of growth factors to stimulate the process of bone healing, incorporation of them into a bone graft system can cause increased bioactivity and efficacy of

the system. In order to do this, the growth factor must be bound to the system in such a way that causes the desired release kinetics. The timing and duration of release, as well as how it is administered, are very important to optimize healing. When introduced intravenously, VEGF has a half-life of less than 30 minutes, requiring many doses or massive amounts of growth factor, which can lead to detrimental vessel formation in non-target areas. (Lee) Growth factors in vivo degrade due to denaturation, oxidation, or proteolysis. These challenges must be controlled if the factors are to be delivered from an implanted construct to achieve a release profile that can mimic that of the natural healing process. One way of doing this is incorporation of the growth factor into a degradable polymer coating. The release of the growth factor is then determined by the degradation rate of the polymer. The growth factor can be integrated into the polymer coating by either chemical immobilization or physical encapsulation. Chemical immobilization can be achieved using a number of techniques, but all strive to directly tether or bind the growth factor onto the extracellular matrix of the scaffold. This allows for the growth factor to more easily interact with cells around the extracellular matrix. The most common strategies are surface adsorption due to hydrogen bonding and direct covalent immobilization of the growth factor to the matrix. This binding technique causes a burst release, with solute molecules being transported by diffusion. (Biondi) Growth factors can also be physically encapsulated into a polymer matrix to control the degradation and release of the protein. Being fully encapsulated, the release kinetics of the growth factor are controlled largely by the polymer coating, which can be designed to the parameters necessary for the specific experiment. Scaffolds used for physical encapsulation can be designed to have specific mechanical properties, degradation rates, and porosity to elicit

desired cellular responses. There are many different fabrication methods for physical encapsulation, all with different benefits and drawbacks that make them effective in certain applications. (Mourino) The table below outlines the most common techniques, along with their advantages and disadvantages.

technique	advantages	potential disadvantages
solvent casting/particulate leaching	control over porosity, pore sizes and crystallinity; high porosity	residual solvents and porogen materials; limited mechanical properties
freeze drying	high porosity and interconnectivity	limited pore sizes range (15–35 μm)
phase separation	high porosity	limited pore sizes, residual solvents (1–10 μm)
melt moulding	control over macrogeometry, porosity and pore size; free of harsh organic solvents	high temperatures
high internal-phase emulsion	control over porosity, pore size and interconnectivity	limited polymer types and mechanical properties
*in situ*polymerization	injectable; control over mechanical properties	residual monomers and cross-linking agents, limited porosity
gas foaming	free of organic solvents; control over porosity	pore interconnectivity

Table 2. Physical Encapsulation Techniques (Lee)

1.7 Bone Infection

Another main concern in all orthopedic surgeries, including bone grafting, is the possibility of osteomyelitis, or infection of the bone. This causes inflammatory destruction of the bone and patients are at a much higher risk during and after a surgery. Infection of the defect site will severely hamper or completely disrupt the healing process. The most common way of preventing or treating an infection is with the use of antibiotics. After an orthopedic surgery, the target site should receive antibiotics for up to six weeks to ensure an infection does not form. (Brady) Surgeries may include an implanted material capable of delivering antibiotics over an extended period to the defect site. This is done most commonly with the use of poly(methylmethacrylate) (PMMA) beads that can release an antibiotic over the course of a few weeks. One antibiotic used is gentamicin, an aminoglycoside antibiotic that has a very wide spectrum of bacteria that it can be used against, although it is especially effective against gram-negative microorganisms. (Quiros) However, the PMMA bone cement used is not biodegradable, allowing for the possibility of complications at a further date. The use of PMMA beads has also shown to be erratic in its delivery, as well as being related to the beginning of antibiotic resistance. (Hillbrand) With infection of bone following surgery such a high risk, the methodology of delivering antibiotics could be improved upon to ensure the success of the bone graft. Given the shortcomings outlined above, the motivation of this project was to create a system that was capable of releasing an antibiotic in a controlled way, while utilizing a biodegradable polymer to inhibit the possibility of future complications. Gentamicin, a broad spectrum antibiotic, was incorporated using surface adsorption for this purpose.

1.8 Specific Aims

This project was organized into three aims. The first aim of the study was to develop a methodology that would allow for the coating of an entire allograft, while still maintaining the native porous architecture of the bone. This would require a thin polymer coating that could penetrate throughout the trabeculae without blocking pores and diminishing the porosity of the graft, which is critical to the graft's overall healing potential. For specific aim 2, two growth factors, VEGF and BMP-2, were chosen to be incorporated within the polymeric coating to increase the overall bioactivity of the bone graft. The objective of aim 2 was to release the factors so the VEGF would release rapidly in a burst release kinetics pattern, while BMP-2 would release after the VEGF and for a prolonged period of time, to encourage the process of natural bone healing. The 3rd and final aim was to create a bone graft system that would be able to be designed with different molecules and release kinetics depending on the application needed. To this end, different polymer concentrations and growth factor loading concentrations were tested to study their effect on the release kinetics. Gentamicin was also tested as a loading molecule to show antibiotics, as well as growth factors, can be utilized within the system.

1.9 Preliminary Studies

Coating techniques were initially tested using cortical allografts harvested from the femur and tibia of Sprague-Dawley breeder rats. A polymer coating was created by dissolving 50:50 PLGA in tetrahydrofuran (THF). The solution was made with a PLGA/THF concentration of 1g/8ml. Cortical bone samples were then coated with the polymer solution before being dried of any solvent. Coated allografts were evaluated using micro-computed tomography (microCT)

and scanning electron microscopy (SEM). Results showed a thin, continuous coating on both the periosteal and endosteal surfaces. A micro-CT image below shows the coated allograft with the bone removed, allowing for a clear viewing of the two layers of coating.

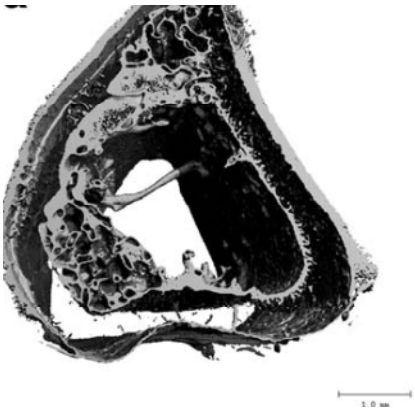


Figure 2. Micro-CT cross-sectional image of coated cortical allograft (Sharmin)

The loading of growth factors was also studied to increase the bioactivity of the sterilized allograft. VEGF and BMP-2 were the two proteins chosen to release from the bone graft for their affinity for initiating angiogenesis and osteogenesis, respectively. VEGF was loaded onto the polymer coated allograft through surface adsorption at a concentration of 5 μg/ml. This created a burst release of the growth factor with over 70 percent of the protein releasing in the first day. BMP-2 was tested by loading it through surface adsorption, as well as encapsulation within the polymer coating. It was loaded at a concentration of 500 μg/ml. Surface adsorption showed a burst release somewhat similar to the VEGF, while encapsulation showed a much more prolonged degradation, releasing significant amounts of protein for three weeks.

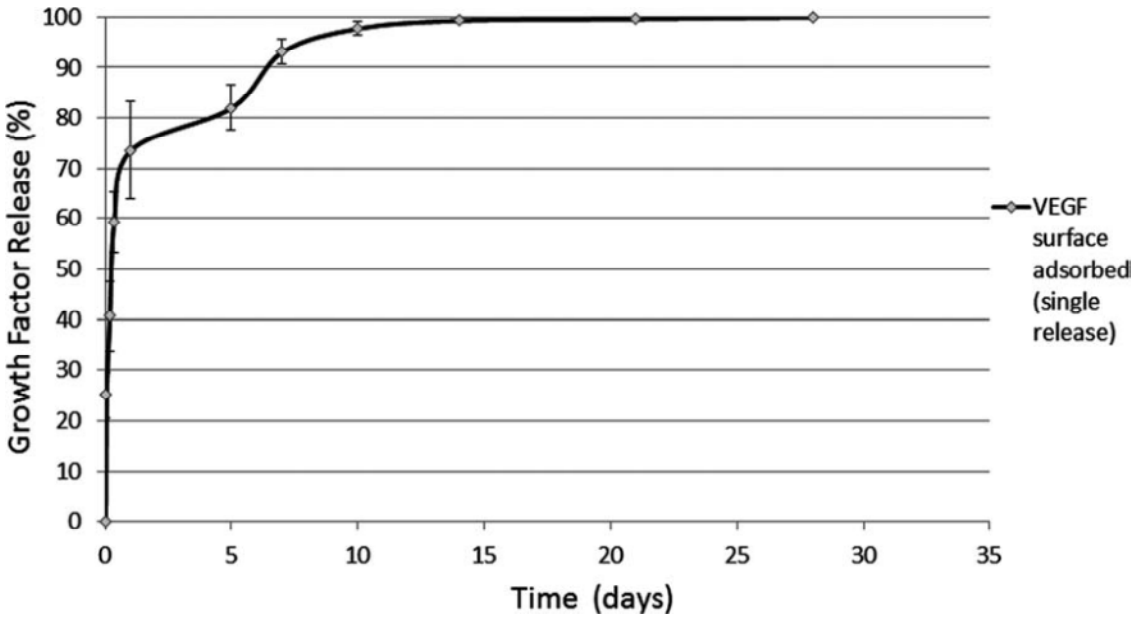


Figure 3. Cumulative release of VEGF from cortical allograft using surface adsorption (Sharmin)

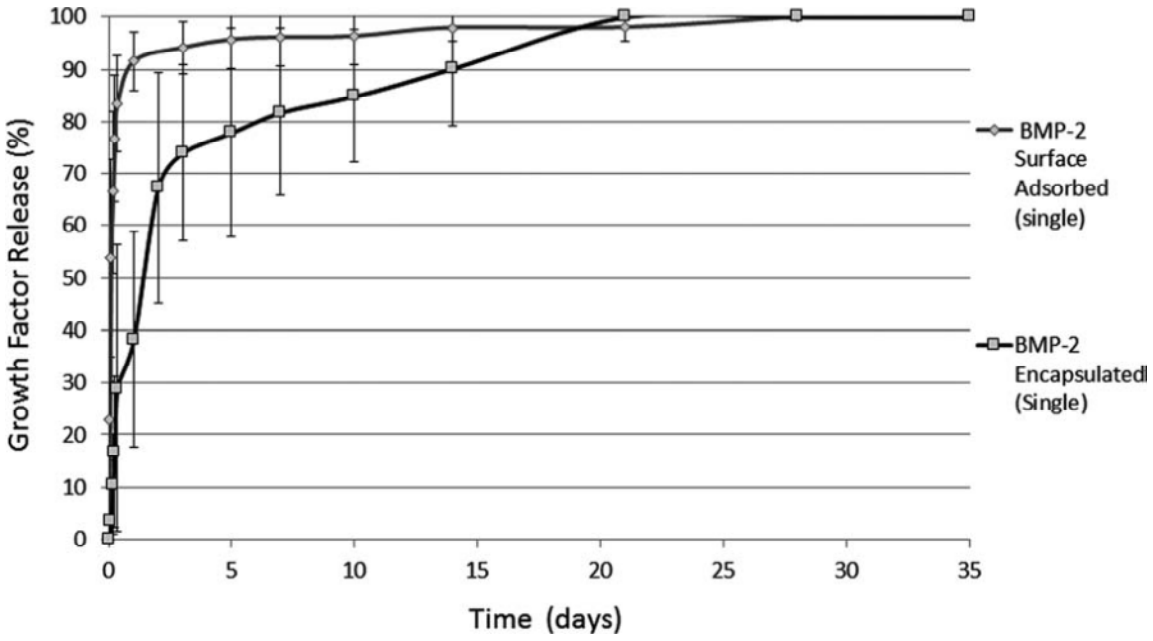


Figure 4. Cumulative release of BMP-2 from cortical bone using surface adsorption and physical encapsulation (Sharmin)

Along with testing the release kinetics of the growth factors, in vitro cell studies were done to ensure that the viability and bioactivity of the growth factors are being maintained throughout the process of being incorporated into the polymer coating and interacting with

cells during the release of the protein. A cell viability study of BMP-2 was done using human mesenchymal stem cells. An MTS assay was used to assess the proliferation of the cells due to exposure to BMP-2. It showed that the hMSCs were continuing to proliferate when interacting with the BMP-2 released from the allografts. This indicates that the growth factor maintained its bioactivity, even with its interaction with a solvent during encapsulation. Along with this MTS assay, alizarin red was used to test the mineralization of the samples over the release period. Alizarin red staining showed significant increases in cellular mineralization activity for cells that were exposed to BMP-2. This mineralization level showed that the BMP-2 released from the coating stimulated the differentiation of hMSCs into osteoblasts. Viability assay results are shown below.

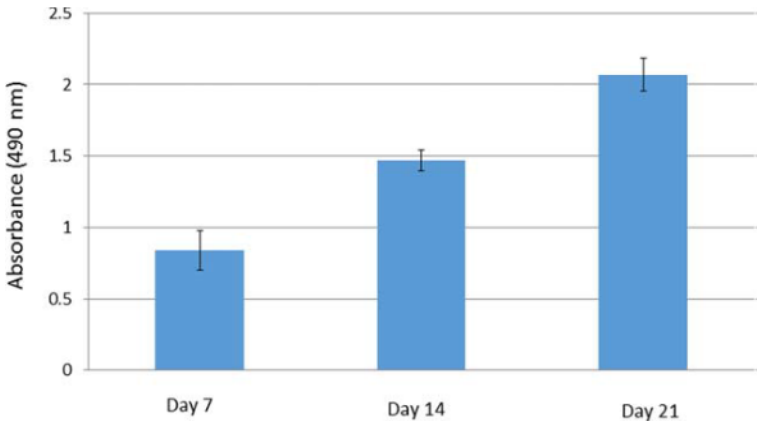


Figure 5. MTS Assay using BMP-2 to cause proliferation of hMSCs (Sharmin)

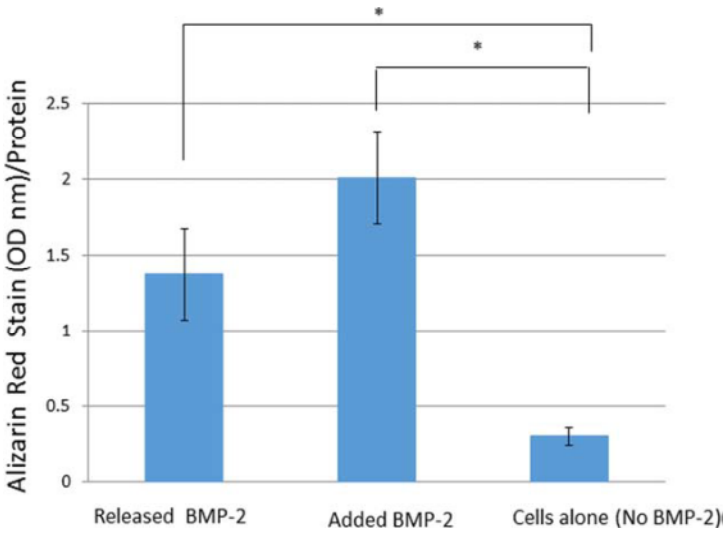


Figure 6. Alizarin Red Assay testing differentiation of osteoprogenitor cells into osteoblasts due to exposure to BMP-2 (Sharmin)

Finally, VEGF was also tested to ensure that it remained bioactive after being loaded onto the polymer-coated allograft and was released over time. A TRAP assay was conducted to test the growth factor's ability to cause cells to undergo osteoclastogenesis. VEGF released from scaffolds was compared to recombinant VEGF added directly to the samples to see the extent of osteoclast differentiation. The results are shown below.

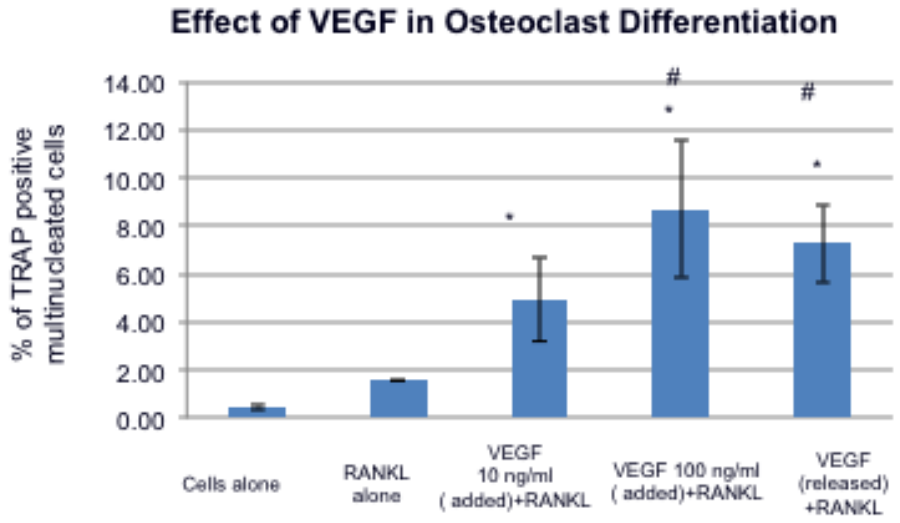


Figure 7. Osteoclastogenesis showing differentiation of osteoclast progenitor cells into osteoclasts due to exposure to VEGF and RANKL (Sharmin)

Viability assays were able to show that the bioactivity of BMP-2 and VEGF were maintained throughout the loading and release process. Surface adsorption of VEGF was able to achieve short term release kinetics with a large initial burst. Encapsulation of BMP-2 showed extended release of the protein, with the majority of the protein being released after the first week. These results using cortical bone show potential for use in bone healing applications, justifying testing of the system with cancellous bone samples. The structural differences and complexity of the native porous architecture of cancellous bone makes coating of the allograft inherently more challenging than cortical allografts, possibly changing the protocol and results of the study.

2 Methods

2.1 Polymer Coating/SEM

2.1.1 Coating of Allograft

The allografts used were 1 cm^3 canine cancellous bone graft blocks procured from Veterinary Transplant Services, Inc. and stored in a -60□ C freezer. Allografts were coated with 50:50 poly(lactic-co-glycolic acid) (PLGA). PLGA is an FDA approved polyester used for implantation procedures of orthopedic and musculoskeletal applications, and as sutures for closing wounds. PLGA was acquired from Lakeshore Biomaterials in solid, pellet form and stored in a -20□ C. PLGA was brought to room temperature before being dissolved by placing it in tetra hydro furan (THF) and agitated in a vortex until a homogeneous solution was created. Allografts were allowed to warm to room temperature. They were then placed in a 5 mm syringe and coated by pulling and pushing the polymer solution in and out of the syringe, ensuring that the allograft was always fully submerged before expelling the solution. This was repeated a total of 10 times before allowing the allograft to soak in the polymer solution for 5 minutes. After this time elapsed, the syringe process was repeated another 10 times. The allograft was then removed from the syringe and placed in a closed vial. From this point, there were two different methods used to complete the coating process.

2.1.2 Static Method

The samples were left soaking in the polymer solution at room temperature for 24 hours. The caps were then removed from the vials, allowing the solvent to evaporate. Once the solvent was completely evaporated, lyophilization of the samples was done for 24 hours to fully dry the coated allografts.

2.1.3 Dynamic Method

Samples were placed in a rotisserie rocker to continually agitate the allografts in solution throughout the coating process. The rocker used was a Labquake Rotisserie Rocker from Thermo Scientific. The samples were rotated in complete 360□ revolutions to continually invert the samples. The samples were rotated for 24 hours at room temperature. The caps were then removed from the vials to allow for solvent evaporation. The rotisserie rocker was tilted to place the samples on an angle as they were rotated. This allowed the allografts to continue to rotate without any of the polymer solution spilling out of the vial. This proceeded until solvent completely evaporated, which was followed by lyophilization of the coated allografts for 24 hours.

2.1.4 Analysis of Coated Allograft

After lyophilization of the samples was completed, the allografts were trimmed of any excess polymer coating. X-ray microtomography (Micro-CT) was then performed on the samples to evaluate the volume of polymer coating throughout the sample. The machine used was a μCT 40 from Scanco Medical. Computer rendered images showed the volume of polymer coating compared to the bone. This was done by incorporating betadine into the polymer coating to act as a contrast agent to contrast the coating from the bone. Betadine was integrated into the polymer solution after the PLGA was mixed with THF, but before the allograft was submerged. Betadine was added into the solution at a concentration of 90 μg/ml and placed in vortex until homogenized. Statistical analysis of the volume of bone and polymer coating was also calculated with micro-CT (see Statistical Analysis).

Samples were also analyzed using scanning electron microscopy (SEM). The allografts were first sliced in half using a razorblade. SEM was then performed to analyze the outer surface, as well as the cross section of the allograft. Allografts were positioned on the SEM sample platform and sputter coated with a gold-palladium (Au-Pd) solution before being inserted into the SEM. Images were taken to assess the volume of polymer coating within the porosity of the allografts.

Statistical Analysis

2.2 VEGF Loading and Release

2.2.1 Loading of VEGF

The growth factor VEGF was loaded onto the bone graft using surface adsorption. VEGF was purchased from R&D in 5 µg vials and stored in a -20□ C freezer. The VEGF was reconstituted using with 1 ml of deionized (DI) water to create a VEGF concentration of 5 µg/ml. Allografts were coated with three different concentrations of PLGA, being 1g/8ml, 1g/14ml, and 1g/20ml. After allografts were dynamically coated with the polymer solution and lyophilized, they were submerged in 500 µl of the VEGF solution with a concentration 5µg/ml. The allografts were allowed to soak in the VEGF solution at room temperature for 15 minutes before the samples were frozen in a -20□ C freezer. Once frozen, they were lyophilized for 24 hours. Control samples were created using the same procedure, being submerged in pure DI water instead of the reconstituted VEGF solution. Studies were completed with a sample size of N = 4.

2.2.2 VEGF Release Protocol

Samples were placed in well plates and submerged in 1.5 ml of phosphate-buffered saline (PBS). Ion concentrations and osmolarity of PBS were equivalent to that of the human body, making it more similar to the environment of the body when the allograft is implanted. The well plates were wrapped in parafilm and placed in a 37□ C room to simulate the internal temperature of the human body. Samples were taken by extracting the supernatant fluid in each well and freezing it in a -20□ C freezer until it was ready to be analyzed. The allografts were shifted to a new well at each time point and re-submerged in 1.5 ml of PBS. The growth factor was released over a 7 day period. Samples were taken at hours 1, 2, 4, 8, and 12, as well as days 1, 3, 5, and 7.

2.2.3 VEGF Analysis

VEGF samples were analyzed using the enzyme-linked immunosorbent assay (ELISA). The ELISA kit, procured from R&D Systems, was used to quantify the released VEGF in cell culture media. 50 µl of each sample was added to an individual well, followed by 50 µl of Assay Diluent. Two standard curves were also made using reconstituted VEGF diluted with PBS, as well as VEGF standards provided in the ELISA kit. The well plate was covered with an adhesive strip and placed on a Thermo Scientific rocker to incubate for 2 hours with slight agitation at room temperature. The wells were aspirated and washed with wash buffer 5 times before adding 100 µl of Mouse VEGF conjugate to each well. The well plate was then covered and incubated on the rocker for another 2 hours. The washing step was repeated, followed by 100 µl of Substrate Solution being added to each well. The plate was covered in aluminum foil to

protect from light and incubated on the rocker for 30 minutes. 100 µl of Stop Solution was then added to the Substrate Solution in each well. The optical density of each well was read using a Biotek Synergy HT plate reader at 450 nm and 540 nm. Using the optical densities of the wells, the concentration of VEGF in each sample was calculated, and cumulative release kinetics of the VEGF were calculated.

2.3 BMP-2 Loading and Release

2.3.1 Surface Adsorption Loading of BMP-2

Surface adsorption of BMP-2 was tested first to ensure the growth factor could adhere and be loaded to the polymer coating. Recombinant human BMP-2 was procured from R&D Systems as a lyophilized powder in 50 µg vials and stored at a temperature of -60° C before use. Allografts were coated with a polymer solution containing a PLGA concentration of 1g/8ml. Acetic acid was diluted with DI water to create a solution of 20 mM acetic acid. 1 ml of the solution was added to the 50 µg vial of BMP-2 to create a BMP-2 concentration of 50 µg/ml. Coated allografts were submerged in 500 µl of the BMP-2 solution and incubated for 15 minutes at room temperature. The samples were then placed in a -20° C freezer until the BMP-2 solution had completely solidified. The samples were then lyophilized for 24 hours. Control samples were submerged in 20 mM acetic acid rather than the BMP-2 solution. A sample size of N = 4 was used.

2.3.2 Encapsulation of BMP-2

Release of BMP-2 was also tested using encapsulation of the protein in the polymer coating. PLGA was dissolved in THF at a concentration of 1g/14ml. A 500 µg vial of BMP-2 from

R&D Systems was reconstituted with 500 µl of 20 mM acetic acid. This reconstituted solution was slowly mixed into the polymer solution until homogenized. Allografts were then coated and lyophilized using the dynamic coating method stated previously.

2.3.3 BMP-2 Release Protocol

Samples were placed in well plates and submerged in 1.5 ml of PBS. The well plates were wrapped in parafilm and placed in a 37□ C room to simulate the internal temperature of the human body. Samples were taken by extracting the supernatant fluid in each well and freezing it in a -20□ C freezer until it was ready to be analyzed. The allografts were shifted to a new well at each time point and re-submerged in 1.5 ml of PBS. The growth factor was released over a 7 day period. Samples were taken at hours 1, 2, 4, 8, and 12, as well as days 1, 3, 5, and 7. Encapsulation studies were done over a 21 day period to show extended release of the protein as the polymer coating degraded. Samples were taken at hours 1, 2, 4, 8, and 2, as well as days 1, 3, 5, 7, 10, 14, and 21.

2.3.4 BMP-2 Analysis

BMP-2 samples were analyzed using the ELISA assay, similarly to the VEGF samples. The ELISA kit, procured from R&D Systems, is used to quantitate the recombinant human BMP-2 in the supernatant fluid. 50 µl of each sample is added to an individual well, followed by 50 µl of Assay Diluent. Two standard curves were also made using reconstituted BMP-2 diluted with PBS, as well as BMP-2 standards provided in the ELISA kit. The well plate was covered with an adhesive strip and placed on a Thermo Scientific rocker to incubate for 2 hours with slight agitation at room temperature. The wells were aspirated and washed with wash buffer 5 times

before adding 100 µl of BMP-2 conjugate to each well. The well plate was then covered and incubated on the rocker for another 2 hours. The washing step was repeated, followed by 100 µl of Substrate Solution being added to each well. The plate was covered in aluminum foil to protect from light and incubated on the rocker for 30 minutes. 100 µl of Stop Solution was then added to the Substrate Solution in each well. The optical density of each well was read using a plate reader from Biotek Synergy HT at 450 nm and 540 nm. Using the optical densities of the wells, the concentration of BMP-2 in each sample was calculated, and cumulative release kinetics of the protein were able to be calculated.

2.4 Gentamicin Loading and Release

2.4.1 Gentamicin Loading

Surface adsorption loading of gentamicin was also tested as a possible alternative application. Gentamicin was procured from Sigma-Aldrich, packaged as a 10 ml vial and stored at a temperature of 4□ C. Gentamicin was diluted with DI water to a concentration of 100 µg/ml. A PLGA concentration of 1g/14ml was used to coat the allografts. Samples were submerged in 500 µl of the gentamicin solution and incubated at room temperature for 15 minutes. They were then stored at a temperature of 4□ C. The samples were then lyophilized for 24 hours. Control samples were submerged in pure DI water rather than the gentamicin solution.

2.4.2 Gentamicin Release Protocol

Gentamicin samples were placed in individual wells of a well plate, submerged in 1.5 ml of DI water. The well plates were wrapped in parafilm and placed in a 37□ C room to simulate

the internal temperature of the human body. Samples were taken by extracting the supernatant fluid in each well and storing it in a 4□ refrigerator until it was ready to be analyzed. The allografts were shifted to a new well at each time point and re-submerged in 1.5 ml of DI water. Studies were done over a 7 day period. Samples were taken at hours 1, 2, 4, 8, and 12, as well as days 1, 3, 5, and 7.

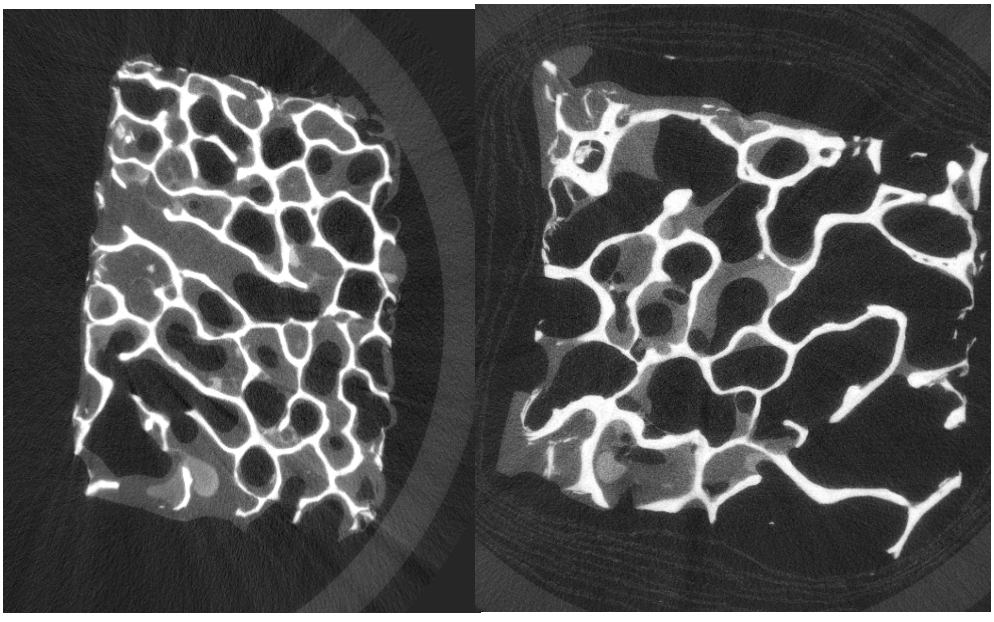
2.4.3 Gentamicin Analysis

Analysis of gentamicin samples was done using spectrophotometry. The spectrophotometer used was a Thermo Scientific Evolution 60. Gentamicin solutions were prepared by mixing the gentamicin samples, isopropanol, and an o-phthaldialdehyde reagent in equal amounts. The reagent was made using 560 ml of .04 M sodium borate, which was diluted in DI water. 2.5 g of o-phthaldialdehyde, 62.5 ml of methanol, and 3 ml of 2-mercaptoethanol were then added. The solution was then stored at room temperature, protected from light, for 24 hours. The recipe was scaled down to reflect the volume needed for samples. Once the gentamicin solution, isopropanol, and o-phthaldialdehyde were combined, the samples were incubated for 45 minutes at room temperature to allow the mixture to react. The absorbance of each sample was then read at a wavelength of 333 nm, using the spectrophotometer. A standard curve was made by diluting the gentamicin in DI water, ranging from 5-80 μg/ml. The absorbance of the samples were compared to the standard curve, providing the concentration of gentamicin for each sample. The cumulative release kinetics of gentamicin was then calculated.

3 Results

3.1 Micro-CT Analysis of Polymer Coating

Polymer-coated allografts were analyzed using micro-CT to test the extent that the polymer coating was able to penetrate the allograft trabeculae. Images of the allografts were processed showing bone as white and the polymer coating as gray. The images of allografts coated using the dynamic and static process are shown below. Both allografts were coated with identical PLGA concentrations and there were no differences in the protocol prior to the dynamic/static step. The static model shows a very poor extent of coating with the polymer solution restricted to half of the bone graft per micro-CT images. The dynamic model, however, shows a much more consistent coating with the polymer coating reaching all areas of the allograft.



Dynamic Coating **Static Coating**

Figure 8. Micro-CT cross-sectional image of allografts using dynamic vs. static coating

Different PLGA concentrations were tested to show how the viscosity of the polymer coating would affect the coating of the allograft. The volume of the coating on the allograft and the bone itself were calculated. The most dilute concentration tested showed the lowest volume of coating. In contrast, the coating volume was highest when the allograft was coated with the most viscous PLGA concentration. It was shown that an increase in coating volume occurred as the concentration of the polymer was increased, making a more viscous solution. Each concentration was tested with an n = 1 using the dynamic coating method.

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 3. Effect on Coating Volume due to change in PLGA concentration

Visual representations showing the relationship between the coating volume and the polymer concentration were also rendered using micro-CT. Micro-CT images were used to determine amount of area coated on the allograft and SEM analysis was used to qualitatively determine porosity of samples. Using a polymer concentration of 1g/14ml, the coating covers almost all areas of the allograft, distributing throughout the entirety of the bone cube. When using lower concentrations such as 1g/18ml or 1g/20ml, it is clear that there is a decrease in the volume of polymer coating, as well as a decrease in the distribution of coating throughout the allograft. Many areas appear with bone surrounded by nothing, showing the failure of the

polymer coating to bind to the bone. With all samples being prepared identically, results showed that a lower polymer concentration showed a failure to fully penetrate the entirety of the allograft.

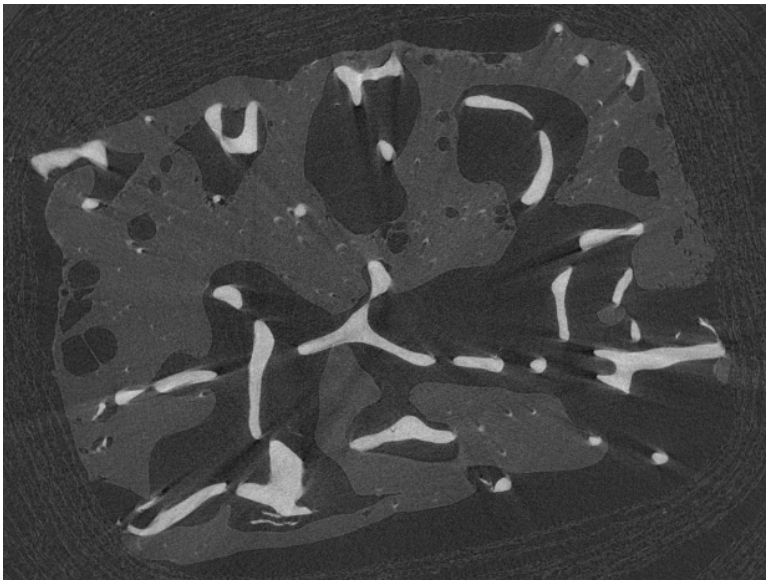


Figure 9. Micro-CT image using dynamically coated allograft with PLGA concentration of 1:14

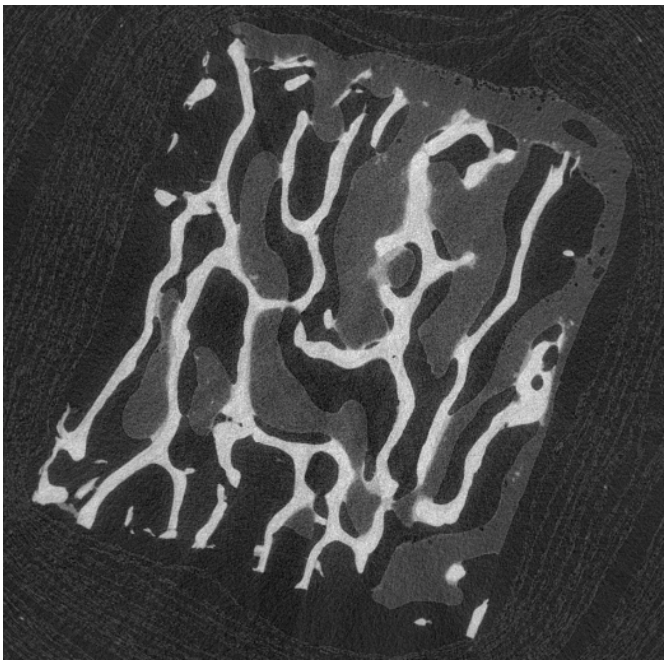


Figure 10. Micro-CT image using dynamically coated allograft with PLGA concentration of 1:18

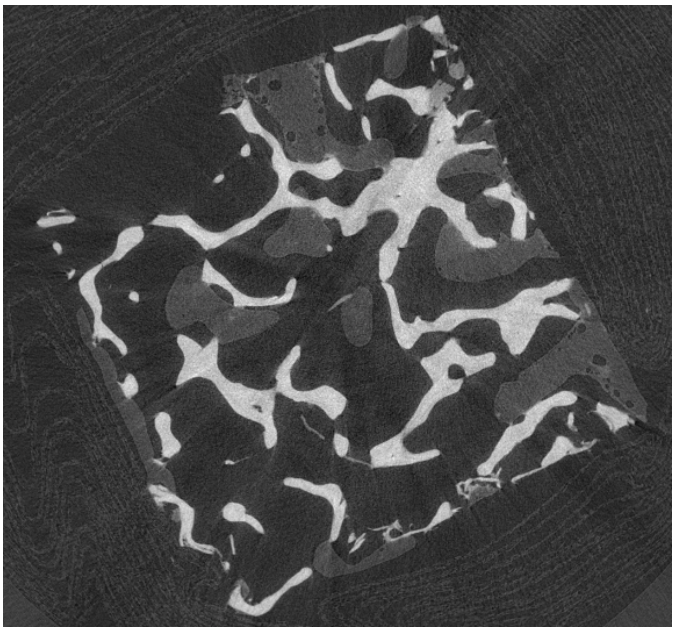


Figure 11. Micro-CT image using dynamically coated allograft with PLGA concentration of 1:20

3.2 SEM Analysis of Polymer Coating

SEM was used to take topographical images of the different polymer-coated allografts. Bone cubes were sliced in half and prepared to allow for viewing of the sample surface, as well as the cross section. Multiple polymer concentrations were tested and analyzed using SEM to examine the extent of coating throughout the sample, as well as the porosity of the allograft. An analysis of an allograft coated with a PLGA concentration of 1g/12ml is shown below.

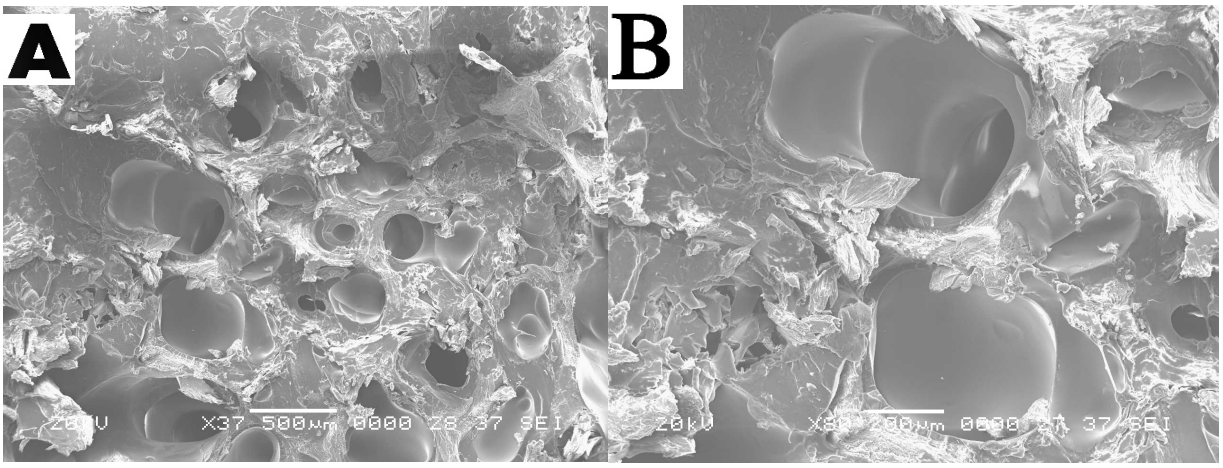


Figure 12. SEM image of dynamically coated cancellous allograft using PLGA concentration of 1:12. A) x37 magnification, B) x80 magnification

This depicts a cross section of the allograft with the bone showing in the bright and jagged areas, while the coating is shown in the smooth, darker areas of the sample. In Figure 12-A, it is seen that the coating has effectively penetrated through the sample, showing areas of coating in almost all pores, while still seeing bone in between due to the cross-sectioning. Figure 12-B, however, shows an area where the polymer solution has completely clogged one of the pores, negatively affecting the natural architecture of the bone.

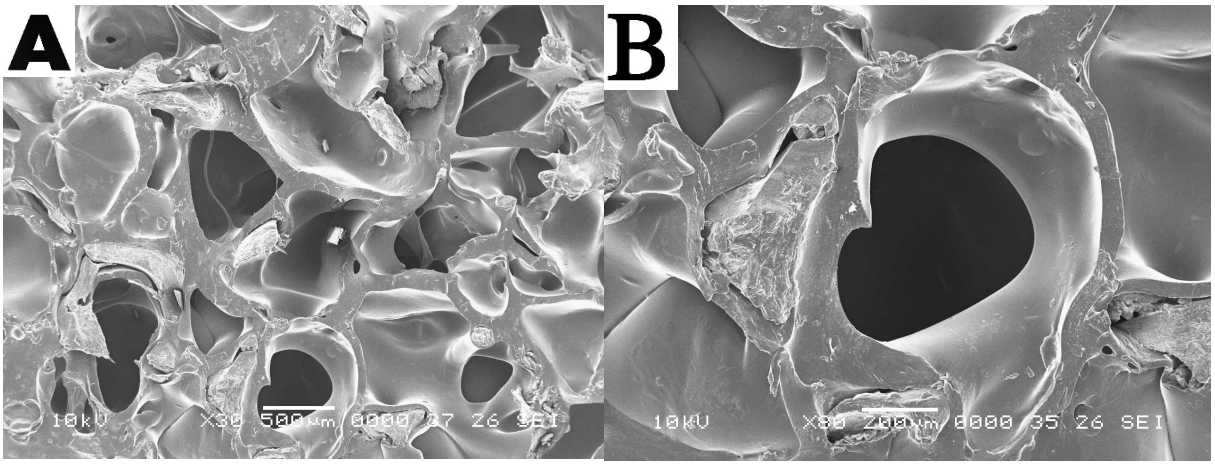


Figure 13. SEM image of dynamically coated cancellous allograft using PLGA concentration of 1:14. A) x30 magnification, B) x80 magnification

When decreasing the polymer concentration to a level of 1g/14ml, a significant difference is already noticed. In the low-magnification image on the left, there is still extensive polymer coating throughout the cross section of the allograft, seeing a layer of polymer coating along the edges of the pores, while native bone remains in between. The change, however, is seen in porosity of the allograft, showing very large pores throughout the entirety of the sample, with no real clogging to be noted. This polymer concentration shows the ability to create a thin layer of coating throughout the allograft that minimally affects the porosity of the bone. When decreasing to even lower concentrations, other issues begin to arise.

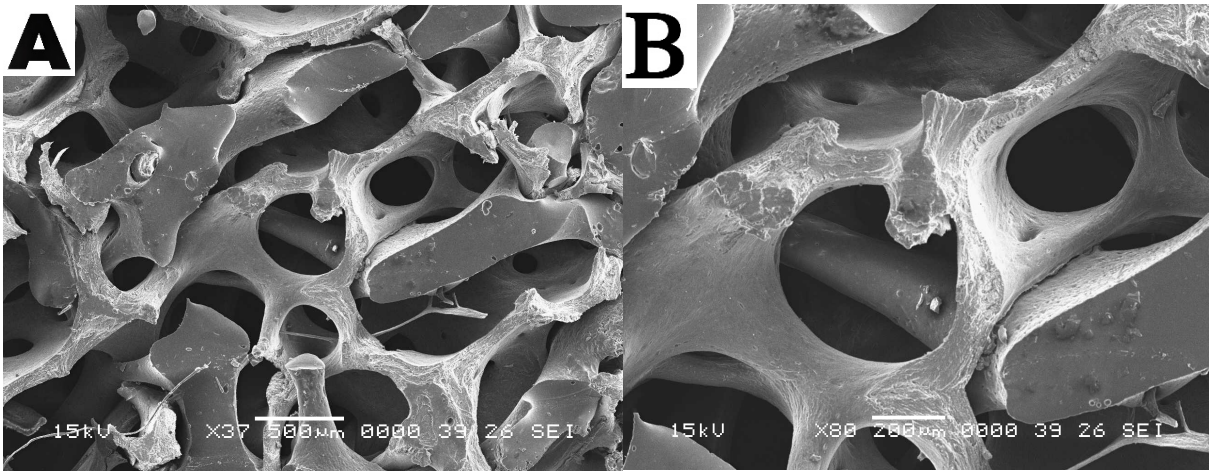


Figure 14. SEM image of dynamically coated cancellous allograft using PLGA concentration of 1:20. A) x37 magnification, B) x80 magnification

These are low and high magnification SEM images taken using a polymer concentration of 1g/20ml. As shown in the images, there is a very high amount of bone shown without having very much polymer coating to be seen. The entire area seen in the image is essentially devoid of polymer coating. When using a lower polymer concentration, a drop-off of polymer coating volume is noted, with some areas of the allograft containing only native bone.

3.3 Analysis of Surface Adsorbed VEGF Release

The growth factor VEGF was surface adsorbed and released over a 7 day period. Three groups of polymer concentration were tested to see its effect on the release kinetics of the growth factor. The three concentrations tested were 1g/8ml, 1g/14ml, and 1g/20ml, each with an n=4.

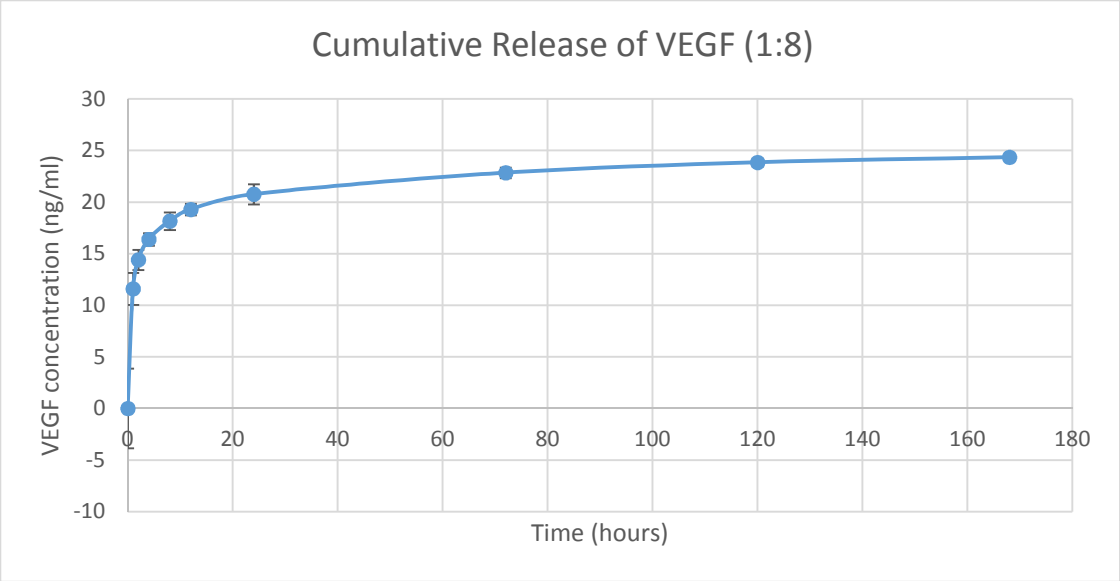


Figure 15. Surface Adsorption Release of VEGF from dynamically coated cancellous allograft with PLGA concentration of 1:8

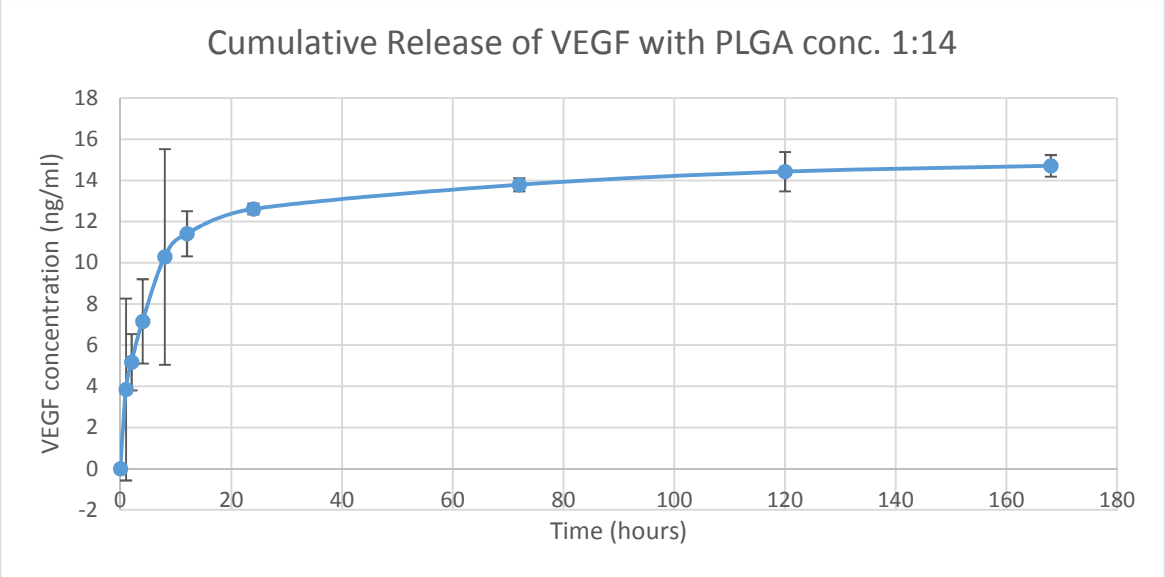


Figure 16. Surface Adsorption Release of VEGF from dynamically coated cancellous allograft with PLGA concentration of 1:14

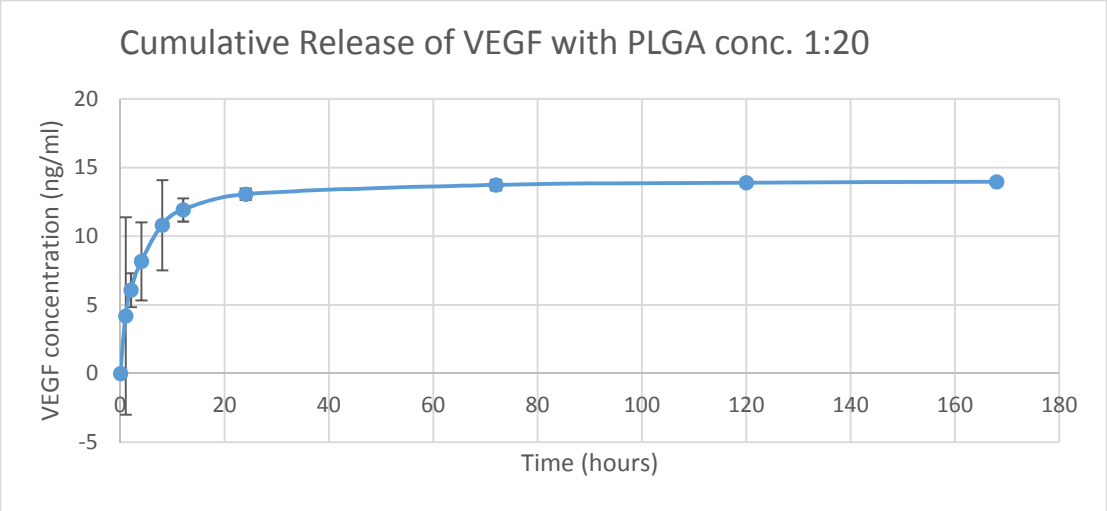


Figure 17. Surface Adsorption Release of VEGF from dynamically coated cancellous allograft with PLGA concentration of 1:20

All groups showed a large burst effect within the first 24 hours, releasing the majority of protein that was adsorbed. After this initial burst, there is a small amount of sustained release over the rest of the week. Results also show that a greater concentration of polymer leads to a higher magnitude of release. The most viscous polymer concentration tested showed the largest release of VEGF, while the lowest concentration released the least VEGF over the 7 day period.

3.4 Analysis of BMP-2 Release

3.4.1 Analysis of Surface Adsorbed BMP-2 Release

BMP-2 was loaded onto the polymer-coated allograft through surface adsorption using an n=4. The protein was released over a 7 day period, showing an extremely high burst release. All BMP-2 that was surface adsorbed to the polymer-coated allograft was released within the first 24 hours, with no protein being released for the remainder of the week. The release kinetics of the BMP-2 shows a much more drastic burst release than either the VEGF or

gentamicin, which both showed a smaller initial burst that allowed for a gradual release in the following days.

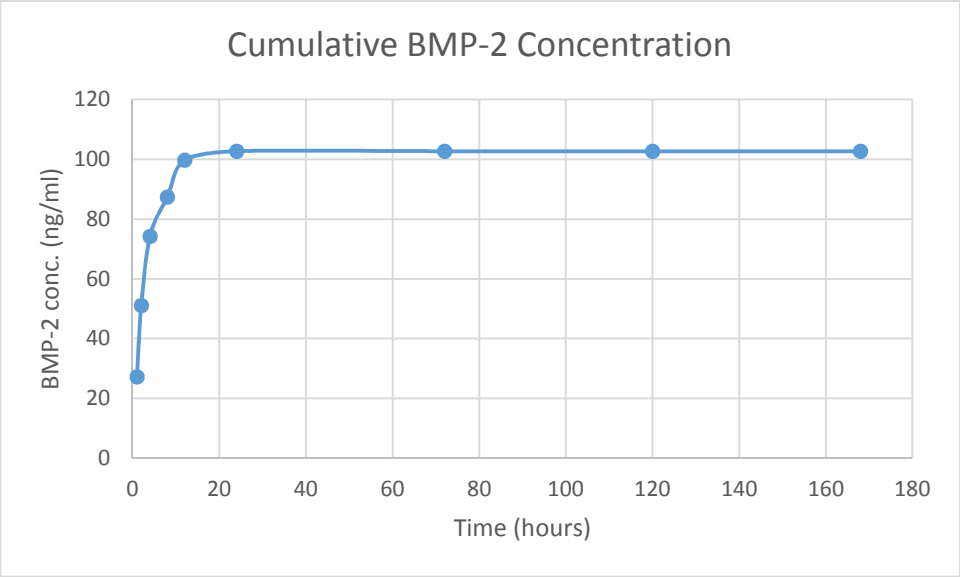


Figure 18. Surface Adsorption Release of BMP-2 from dynamically coated allograft using PLGA concentration of 1:8

3.4.2 Analysis of Encapsulated BMP-2 Release

BMP-2 was loaded into the allograft system using physical encapsulation using an n=4. The reconstituted growth factor was mixed into the polymer solution until homogenized before coating the allograft. The allograft was loaded with a BMP-2 concentration of 67µg/ml and was released over a period of 3 weeks. The protein still showed an initial burst release in the first 24 hours, but to a lesser extent than seen when using surface adsorption. A sustained release was seen after the initial burst for the remainder of the 3 week period. It is also seen that the second and third weeks show a higher level of release compared to the first week after the initial burst.

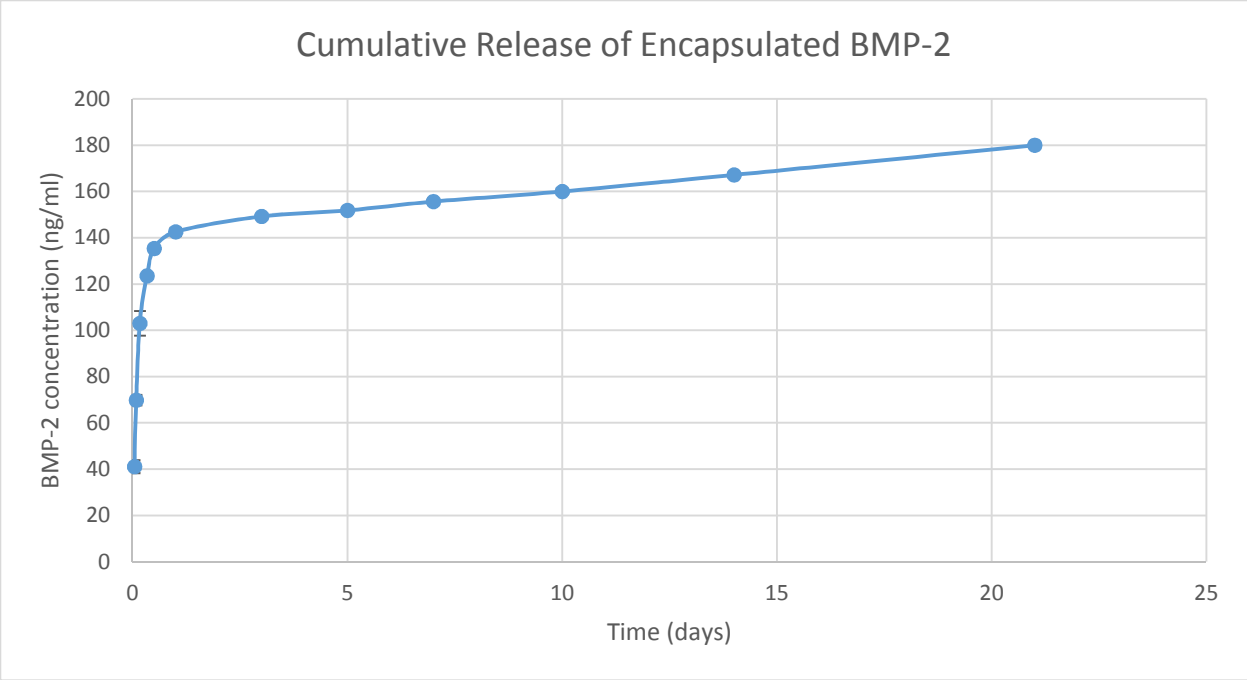


Figure 19. Encapsulated Release of BMP-2 from dynamically coated allograft using PLGA concentration of 1:14

3.5 Spectophotometry Analysis of Gentamicin

Gentamicin was loaded onto the polymer-coated allograft through surface adsorption and analyzed to find the cumulative release concentration. The release kinetics show a large burst release at the beginning of the study. About 70 percent of the total release occurred within the first 24 hours, with almost half of it releasing in the first couple of hours. However, it does still show a gradual release after the first day, continuing to release the antibiotic throughout the week, similarly to VEGF.

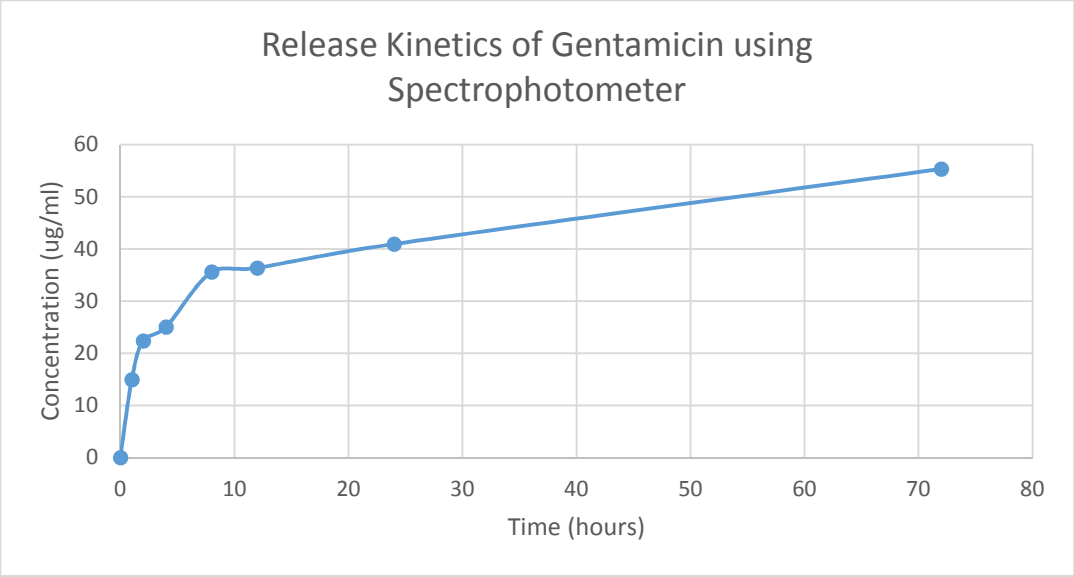


Figure 20. Surface Adsorption Release of Gentamicin from dynamically coated allograft using PLGA concentration of 1:14

4 Discussion

4.1 Polymer Coating

The polymer coating used to coat the allografts is the most important aspect of the system developed here to ensure proper growth factor delivery. The coating must be able to cover the entirety of the sample while keeping its native porous architecture, as well as being suitable to bind and release the bioactive molecules. PLGA was chosen to create the coating because it is a polymer that is FDA approved and has been widely studied for delivering growth factors, due to its affinity to bind proteins to its surface, as well as having the capability of releasing them in a controlled way. A study by Schrier et al. showed that the binding capacity

of PLGA is affected by the molecular weight of the polymer, as well as the acid number of the polymer, which correlates to the hydrophilic/hydrophobic nature of the polymer. It also showed that molecular weight linearly related to binding capacity, and hydrophobic PLGA showed a greater affinity for surface adsorption binding of proteins. The release of proteins from PLGA is biphasic, with an initial burst, as well as an extended release as the polymer degrades. The burst release is caused by protein on the surface of the coating interacting with the medium surrounding it. The extended release occurs through hydrolysis of the polymer to allow for drug release through diffusion and erosion. The drug release is affected by the drug type and concentration, as well as the hydrophobicity and molecular weight of the polymer. (Schrier) PLGA is very useful when creating drug delivery systems due to the way in which it is able to load and release growth factors. Its biphasic release makes it an optimal polymer to deliver multiple growth factors due to the fact that it is capable of causing a burst effect or an extended release. Using surface adsorption and encapsulation, it is able to exhibit a wide range of release kinetics. Properties of PLGA can also be changed in order to optimize a system for specific applications. PLGA, being a copolymer containing PLA and PGA, can be procured in many different variations, including molecular weight and ratio of the two polymers. PGA has been shown to be more hydrophobic than PLA, and a higher level of PGA also correlates to a faster degradation rate of the polymer. (Athanasiou) Therefore, PLGA can be procured in different forms depending on the specific binding and release properties that are needed.

Two different methods of coating the allograft samples were tested in this study. The static coating process was done to mimic the preliminary study using cortical allografts. However, due to the intricate architecture of trabeculae within cancellous bone, coating of a

cancellous allograft is a more complex process. The cortical bones used were mostly femur bones from rats cut down to only the diaphysis of the long bone. This singular hollow tube makes it easy to cover the entirety of the sample by simply submerging the allograft in the polymer solution. The single tube sample can also be easily coated evenly during solvent evaporation, due to gravity pulling the solution downward and causing a uniform layer of polymer to form around the bone as it evaporates. Due to the porous nature of the trabeculae in cancellous bone, it is very difficult to attain a continuous coating throughout the sample. The static coating process showed a high level of inconsistency, with varying levels of polymer solution throughout the allograft. This is most likely due to large amounts of polymer settling on trabeculae during the coating process. Due to the static process, larger layers of polymer can form in certain areas of the sample, even clogging entire pores, while areas of pure bone remain with no polymer solution coating them. The dynamic method was designed to keep the allograft and the polymer solution constantly moving throughout the coating of the allograft and the evaporation of the solvent. The constant displacement of the fluid through the allograft reduces likelihood of the polymer solution settling in certain areas of the allograft. It also increases the chances that the polymer solution manages to reach all areas of the bone graft. This is shown by the results, as the dynamic method created a much more continuous coating throughout the sample. SEM analysis also showed a greater level of porosity in the dynamic samples, while the static counterparts showed more blockage of pores.

Dynamically or statically coating the allografts, however, was not the only factor that affected the porosity of the allografts. The polymer concentration was also found to have a large impact on the coating volume, and therefore, the porosity of the sample. Micro-CT

analysis showed that an increase in the concentration of PLGA directly related to an increase in the volume of polymer that coated the allograft. This is most likely due to the viscosity of the polymer solution increasing as the concentration of PLGA is increased. When the polymer solution has a lower polymer concentration, the viscosity decreases, causing the solution to easily flow through the trabeculae of the allograft. At a higher polymer concentration, the viscosity is much greater and has a greater capability to bind to the allograft. As the overall polymer concentration, and therefore, volume is increased, the thickness of the polymer coating naturally increases and causes a greater risk of reducing the porosity of the bone graft. SEM analysis confirmed the micro-CT data, showing a thicker coating with porosity decreasing as the polymer concentration of PLA increased. The lowest concentration of PLGA tested was 1g/20ml, where the sample exhibited areas of uncoated bone. The polymer concentration, and therefore, coating volume, was too low for the polymer solution to be able to reach all areas of the allograft. Based on visual inspection, the most optimal concentration of PLGA for the allografts appears to be 1g/14ml. This concentration exhibited the greatest coating volume without negatively affecting the porosity of the native bone. Optimal polymer concentration can vary based on the specific sample, but it must achieve a continuous coating without affecting the native porosity of the bone.

4.2 VEGF Loading and Release

Coating of the allograft with a polymer allows for the incorporation of growth factors in order to increase the bioactivity of the bone graft and cause greater bone formation at the defect site. BMP-2 and VEGF were the two growth factors that were chosen to accomplish this in the study. While BMP-2 is used very commonly, VEGF and other angiogenic growth factors in

general, are utilized much less so. While bone growth and formation is the overall goal when implanting a bone graft, angiogenesis is a crucial part of the healing process. VEGF was selected because it is seen as the main controlling factor for angiogenesis to occur in naturally healing bone, causing the formation and maturation of blood vessels throughout the target site. (Nakagawa) Because a bone graft is only able to incorporate a certain number bioactive molecules, it is very important to select growth factors that will be able to initiate all of the stages of bone healing. VEGF is the most widely used angiogenic growth factor due to its continued use throughout the healing process. During natural healing, it is expressed to vascularize the osseous tissue as well as being present during the differentiation of bone forming cells. (Liu, Berendson) This continued usage throughout the healing process shows that VEGF affects many of the different stages of healing, making it ideal for a system with a limited number of bioactive components.

VEGF was bound to the polymer coating through surface adsorption, allowing the VEGF molecules to interact with molecules from the surface of the polymer. This was done to allow for a burst release of VEGF, before the extended release of the BMP-2. It is also useful to surface adsorb VEGF, as it has been shown to be influenced by certain receptors in the ECM, such as integrins. Allowing VEGF to freely interact with these receptors at the surface can cause an increase in the activation of growth factor receptors in the area, and overall greater functionality within the defect site. (Lee) VEGF was surface adsorbed using three different concentrations of PLGA in order to test its effect on the release kinetics of the growth factor. All allografts were loaded with a VEGF concentration of 5ug/ml. This concentration was chosen to cause a release concentration on the nano-scale, as this is enough to cause a therapeutic

effect without being above the threshold to negatively affect the patient. As the VEGF was always loaded through surface adsorption and the loading concentration was never changed, the three VEGF release studies all exhibited similar release kinetics. There was a large burst release of the growth factor during the first 24 hours, with over 80 percent of the growth factor being released during this time. After this time, there was a continual release of small magnitude for the remainder of the first week. This showed the ability of the surface adsorption technique to cause a burst release of the growth factor. Using these release kinetics with VEGF, it will allow for early vascularization of the defect site before the encapsulated growth factor is released. This is done to mimic the cascade of events in the natural healing process of bone. The difference in polymer concentration also caused a change in the amount of VEGF that was able to be released by the allografts. At a higher polymer concentration, more VEGF was released over the 7 day period, while a lower polymer concentration correlated to a smaller magnitude of release. This indicates that the loading volume of the growth factor may increase as the concentration of PLGA is increased. A comparison of the release of the three different PLGA concentrations is shown below.

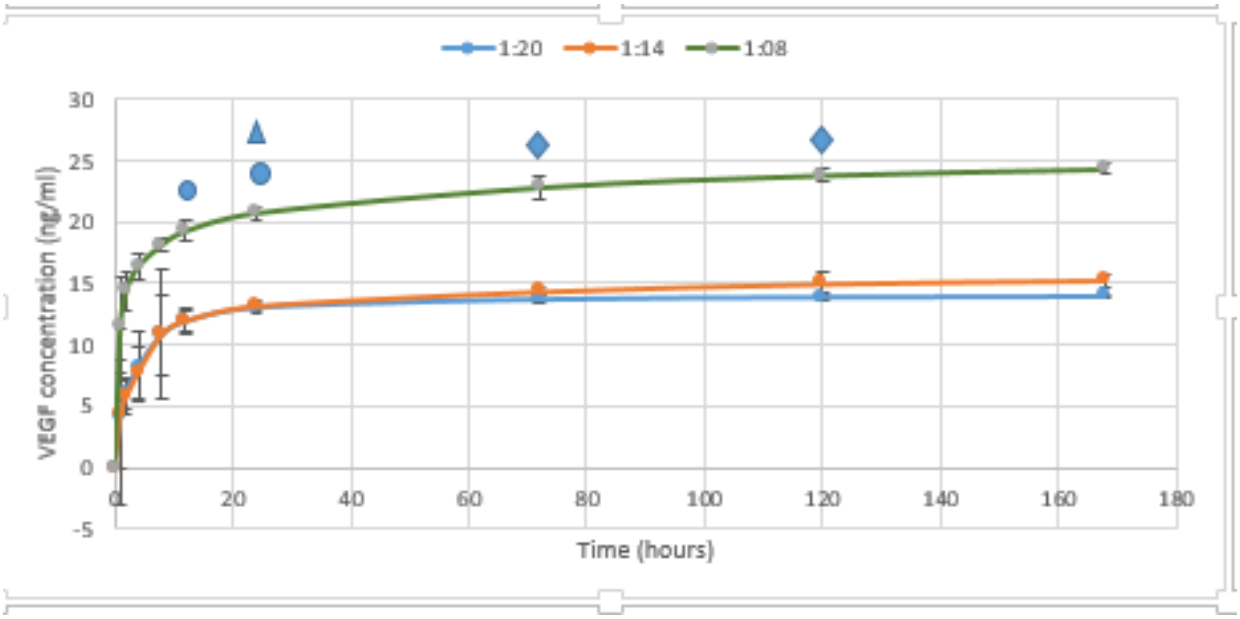


Figure 21. Release of VEGF through surface adsorption comparing PLGA concentrations of 1:8, 1:14, and 1:20. Variance analysis and Tukey post test done for statistical analysis. Statistical differences between groups shown by: □: 1:8 and 1:14, Δ: 1:8 and 1:20, ◊:1:14 and 1:20

4.3 BMP-2 Loading and Release

The release of BMP-2 was studied both through surface adsorption and physical encapsulation. Magnitude of protein release from surface adsorption was similar between BMP-2 and VEGF when compared to their initial loading amounts and concentrations. BMP-2 was loaded at a concentration one order of magnitude greater than VEGF, which is reflected in the release kinetics. VEGF, however, showed a slightly greater recovery rate than BMP-2. This indicates that VEGF may have a slightly greater binding efficiency to the PLGA polymer coating than the BMP-2 does, most likely due to a larger molecular weight for VEGF. The main difference between the releases, however, is the nature of the burst release seen in the first day. Within the first 24 hours, almost the entire 100 percent of the BMP-2 was released,

leaving no extended release. This same trend of initial burst is seen with surface adsorption of either VEGF or gentamicin, but the release kinetics of BMP-2 showed a much more aggressive profile with a much larger release percentage in the first 24 hours. In the same time period, VEGF had released only 70 percent of the total amount of protein released during the study. Factors that tend to affect these release kinetics include structure and hydrophilicity of the scaffold, pKa and isoelectric points of the molecules, and molecular weights of the components. When compared to VEGF, the structure and hydrophilicity of the scaffold are identical, as the same allografts and polymer concentrations were used. Similarly, VEGF and BMP-2 both have isoelectric points of 8.5. The only factor in which they differ is molecular weight, with BMP-2 and VEGF having molecular weights of 26 kDA and 45 kDA, respectively. This difference in molecular weight causes the change in release kinetics, with a lower molecular weight causing more of a burst release.

BMP-2 was also bound to the polymer coating through physical encapsulation to achieve an extended release of the protein. This encapsulation of the protein showed to decrease the initial burst release seen when using surface adsorption. This decrease in the burst release is seen in conjunction with a sustained release over the 3 week period. While surface adsorption of BMP-2 led to 100 percent of release in the first day, encapsulation shows a significant amount of release even in the third week. This extended release allows for BMP-2 to continually be present within the defect site following surgery. This allows for greater levels of bone formation that will continue to grow and proliferate.

4.4 Gentamicin Loading and Release

With so many different possibilities of applications within a drug delivery system, it is important that a system shows not only a high level of efficacy, but also a large amount of flexibility. For this purpose, gentamicin was also tested as a release molecule through surface adsorption to the PLGA polymer coating. Gentamicin is an antibiotic that is very commonly used, even making the World Health Organization's List of Essential Medicines. During bone graft surgery, factors such as implantation of a foreign substance and exposure of the tissue to environmental factors inherently leave the tissue at a greater risk for osteomyelitis, or bone infection, to occur. However, systemic administration of broad-spectrum antibiotics has proven to be insufficient in an attempt to remove the risk of infection. (Zhang) Gentamicin is often used during orthopedic bone surgery to administer antibiotic to the defect site and combat any infection that may occur. It is commonly used due to its wide antibacterial spectrum, but is not systemically active when taken orally. It must be administered intravenously or by some method of drug delivery to have an effect. (Quiros) Gentamicin is commonly incorporated into delivery systems utilizing PMMA cements as the loading scaffold. These cements, however, are not biodegradable and require a second procedure for removal. Along with this, they have shown poor control over the degradation of the antibiotic, leading to erratic release kinetics. Use of biodegradable polymers as a loading scaffold for gentamicin has shown greater control of the release of antibiotic, as well as removing the need for a second surgery. Zhang et al. used poly-DL-lactide (PDLLA) scaffolds to release gentamicin in a controlled manner. They also showed that the release kinetics could be affected by changing the molecular weight of the molecules, as well as by changing the polymer volume. (Zhang) Similarly, Virto et al. showed sustained release for over 30 days by encapsulating gentamicin within a PLGA scaffold. (Virto)

Release of gentamicin was studied through surface adsorption, as a possible replacement molecule for VEGF. Drug concentrations were measured using spectrophotometry, with a procedure based off of a protocol designed by Sampath and Robinson. (Sampath) Release of gentamicin showed a large initial burst, with around 80 percent of the gentamicin loaded onto the scaffold being released within the first 24 hours. A low level of steady release was observed in the following days. To reduce risk of infection to a minimum, it is generally believed that antibiotic administration needs to last for 4-6 weeks after surgery, with a large initial burst as the risk of infection is greatest immediately after surgery. (Zhang) The high level of burst exhibited in the study shows the ability to manage the risk of infection during the period immediately following surgery. However, with such a high percentage of the antibiotic being released during the first 24 hours, the risk of having an insufficient amount of antibiotic for extended release increases. Based on the release kinetics exhibited, it is unlikely that a steady release of gentamicin would be able to be sustained for a 4-6 week period. This is to be expected when looking at a molecule released through surface adsorption. If sustained release of gentamicin is valued higher than an initial burst, physical encapsulation of the antibiotic could be utilized to ensure the extended release is maintained for the 4-6 week period after surgery. Gentamicin could also be used alone with both types of loading being used. The combination of the short and long term kinetics should effectively cause a continuous release of gentamicin for a sufficiently long period. The release study of gentamicin shows that the polymer coated allografts are able to incorporate different types of molecules extending beyond growth factors, as well as showing that the release kinetics are similar, regardless of what is used as a release molecule.

5 Conclusion

The polymer solution was shown to effectively coat a cancellous allograft while maintaining the native architecture of the bone. In optimizing the polymer concentration along with the use of a dynamic coating method, polymer coating was able to penetrate through the entirety of the sample, creating a thin, continuous polymer coating that did not negatively affect the porosity of the cancellous bone. Polymer concentration was shown to have an effect on the volume of the coating, with the coating volume increasing as the polymer concentration became greater and the solution became more viscous. The growth factors BMP-2 and VEGF were able to be loaded onto the polymer-coated allografts and released in a controlled and desired manner. Using surface adsorption and physical encapsulation of the growth factors, releases of initial burst and gradual release were able to be achieved. Surface adsorption of VEGF resulted in a burst release with almost all of the protein being released in the first few days. Physical encapsulation of BMP-2 showed an initial burst, followed by sustained release throughout the second and third weeks. This gradual release can be seen after the VEGF had essentially completed its release. This combination and timing of growth factor release has been shown to have very good success in previous studies. Kempen et al. incorporated VEGF and BMP-2 in PLGA microspheres with similar release kinetics. Findings showed that synergistic effects of the two growth factors caused a greater amount of bone formation, along with the presence of VEGF leading to greater levels of vascularization as well. (Kempen) Kanczler et al. showed similar results loading BMP-2 and VEGF onto alginate scaffolds, gaining enhanced bone formation when using the two growth factors in combination. (Kanczler) Growth factors were proven to stay bioactive after being introduced to the polymer solution and throughout its

release. These findings show the ability to deliver growth factors in a way to mimic the natural healing process of bone in the human body. Multiple growth factors are able to be released in a controlled manner with the ability to load any desired concentration while still maintaining the physical structure of the allograft. Gentamicin was also surface adsorbed, and was shown to release very similarly to VEGF, having a strong initial burst in the first day with a gradual release over the rest of the first week. This shows the ability for other types of molecules to be delivered using the system, allowing for use in many different applications. The release kinetics shown by these three molecules show the ability to help stimulate the bone healing process as well as help to mitigate post-surgical infection.

Future Directions

There are many steps that still needs to be taken before this system could possibly be ready for any sort of clinical use. The coating technique was optimized, finding greater success using a dynamic coating technique, as well as using a PLGA concentration of 1g/14ml. Moving forward, a greater focus needs to be placed on the release of the growth factors and the factors that affect their release. More studies need to be done with different polymer concentrations to better understand the extent of its effect on the release of loaded growth factors. The combined release of BMP-2 and VEGF also needs to be studied in order to show how their incorporation together affects the release kinetics. The combination of the burst release and the extended release should combine to cause a continuous release of protein from the scaffold. This needs to be tested to ensure it will properly work, as well as testing its variability.

This continuous release has great potential with antibiotics like gentamicin, which should be released over a 4-6 week period. In vivo studies also need to be conducted using the cancellous allografts to test their effectiveness when actually used on a test subject.

Resources

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

Weiner, Steve, Wolfie Traub, and H.daniel Wagner. "Lamellar Bone: Structure–Function Relations." *Journal of Structural Biology* 126.3 (1999): 241-55. Web.

Buckwalter, J. A., and R. R. Cooper. "Bone Structure and Function." *Instructional Course Lectures* 36 (1987): 27-48. Web.

Clarke, B. "Normal Bone Anatomy and Physiology." *Clinical Journal of the American School of Nephrology: CJASN* 3 Suppl 3 (2008): 131-39. Web.

Buck, D., and G. Dumanian. "Bone Biology and Physiology: Part II. Clinical Correlates." *Plastic Reconstructive Surgery*129.6 (2012): 950e-56e. Web.

Mehta, Manav, Katharina Schmidt-Bleek, Georg N. Duda, and David J. Mooney. "Biomaterial Delivery of Morphogens to Mimic the Natural Healing Cascade in Bone." *Advanced Drug Delivery Reviews* 64.12 (2012): 1257-276. Web.

Kalfas, Iain H. "Principles of Bone Healing." *Neurosurgical FOCUS* 10.4 (2001): 1-4. Web.

Lauzon, Marc-Antoine, Éric Bergeron, Bernard Marcos, and Nathalie Faucheux. "Bone Repair: New Developments in Growth Factor Delivery Systems and Their Mathematical Modeling." *Journal of Controlled Release* 162.3 (2012): 502-20. Web.

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.

Bauer, Thomas W., and George F. Muschler. "Bone Graft Materials: An Overview of the Basic Science." Clinical Orthopaedics & Related Research 371 (2000): 10-27. Web.

Blokhuis, T.j., and T. Lindner. "Allograft and Bone Morphogenetic Proteins: An Overview." Injury 39 (2008): S33-36. Web.

Laurencin, C. T., A. M. A. Ambrosio, M. D. Borden, and J. A. Cooper. "Tissue Engineering: Orthopedic Applications." Annual Review of Biomedical Engineering 1.1 (1999): 19-46. Web.

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. Web.

Gouin F, Passuti N, Verriele V, Delecrin J, Bainvel JV. Histological features of large bone allografts. J Bone Joint Surg Br 1996, Jan;78(1):38-41.

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

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

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

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. Web.

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. Web.

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. Web.

Biondi, Marco, Francesca Ungaro, Fabiana Quaglia, and Paolo Antonio Netti. "Controlled Drug Delivery in Tissue Engineering." Advanced Drug Delivery Reviews 60.2 (2008): 229-42. Web.

Mourino, V., and A. R. Boccaccini. "Bone Tissue Engineering Therapeutics: Controlled Drug Delivery in Three-dimensional Scaffolds." Journal of The Royal Society Interface 7.43 (2009): 209-27. Web.

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. Web.

Quiros, Y., L. Vicente-Vicente, A. I. Morales, J. M. Lopez-Novoa, and F. J. Lopez-Hernandez. "An Integrative Overview on the Mechanisms Underlying the Renal Tubular Cytotoxicity of Gentamicin." Toxicological Sciences 119.2 (2010): 245-56. Web.

Belt, Hilbrand Van De, Daniëlle Neut, Willem Schenk, Jim R Van Horn, Henny C Van Der Mei, and Henk J. Busscher. "Infection of Orthopedic Implants and the Use of Antibiotic-loaded Bone Cements: A Review." Acta Orthop Acta Orthopaedica 72.6 (2001): 557-71. Web.

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.

Schrier, Jay A., and Patrick P. Deluca. "Recombinant Human Bone Morphogenetic Protein-2 Binding and Incorporation in PLGA Microsphere Delivery Systems." Pharmaceutical Development and Technology 4.4 (1999): 611-21. Web.

Athanasiou, Ka, Cm Agrawal, Fa Barber, and Ss Burkhart. "Orthopaedic Applications for PLA-PGA Biodegradable Polymers." Arthroscopy: The Journal of Arthroscopic & Related Surgery 14.7 (1998): 726-37. Web.

Liu, Yanqiu, Agnes D. Berendsen, Shidong Jia, Sutada Lotinun, Roland Baron, Napoleone Ferrara, and Bjorn R. Olsen. "Intracellular VEGF Regulates the Balance between Osteoblast and Adipocyte Differentiation." Journal of Clinical Investigation J. Clin. Invest. 122.9 (2012): 3101-113. Web.

Zhang, Xichen, Urs P. Wyss, David Pichora, and Mattheus F. A. Goosen. "Biodegradable Controlled Antibiotic Release Devices for Osteomyelitis: Optimization of Release Properties." Journal of Pharmacy and Pharmacology 46.9 (1994): 718-24. Web.

Virto, M., B. Elorza, S. Torrado, M. Elorza, and G. Frutos. "Improvement of Gentamicin Poly(d,l-lactic-co-glycolic Acid) Microspheres for Treatment of Osteomyelitis Induced by Orthopedic Procedures." Biomaterials 28.5 (2007): 877-85. Web.

Sampath, Suchitra S., and Dennis H. Robinson. "Comparison of New and Existing Spectrophotometric Methods for the Analysis of Tobramycin and Other Aminoglycosides." Journal of Pharmaceutical Sciences 79.5 (1990): 428-31. Web.

Kempen, Diederik H.r., Lichun Lu, Andras Heijink, Theresa E. Hefferan, Laura B. Creemers, Avudaiappan Maran, Michael J. Yaszemski, and Wouter J.a. Dhert. "Effect of Local Sequential VEGF and BMP-2 Delivery on Ectopic and Orthotopic Bone Regeneration." Biomaterials 30.14 (2009): 2816-825. Web.