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DEVELOPMENT OF A MOLDABLE COMPOSITE BONE GRAFT SUBSTITUTE RELEASING ANTIBACTERIAL AND OSTEOGENIC DRUGS

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DEVELOPMENT OF A MOLDABLE COMPOSITE BONE GRAFT SUBSTITUTE RELEASING ANTIBACTERIAL AND OSTEOGENIC DRUGS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Engineering
at the University of Kentucky

By

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Lexington, Kentucky

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ABSTRACT OF DISSERTATION

DEVELOPMENT OF A MOLDABLE COMPOSITE BONE GRAFT SUBSTITUTE RELEASING ANTIBACTERIAL AND OSTEOGENIC DRUGS

Large infected bone defects (IBD) are very complicated to treat due to their high variability; they often require multiple procedures. Bone autografts are the gold standard for treatment but have several drawbacks, such as a need for a second surgery site, limited grafting material, and donor site morbidity. The objective of this research was to develop a moldable synthetic bone grafting material capable of releasing both antimicrobial and osteogenic drugs over a clinically relevant time course for the treatment of IBDs. Current treatment methods for large IBDs require two separate procedures to treat the bone defect and the infection.

This research sought to combine these two procedures into one implantable composite bone graft substitute for the treatment IBDs. To begin, the degradation and mechanical properties of the calcium sulfate (CS) based composite material were evaluated for different compositions. Next, the controlled drug release profiles from the composite was achieved by using a shell and core system incorporating poly(lactic-co-glycolic acid) microspheres (PLGAms). The release of vancomycin from the shell began immediately and continued over the course of 6 weeks, while the release of simvastatin from the core was delayed before being released over 4 weeks. Next, an infected, critically-sized rat femoral defect model was used to test different treatment methods with and without the composite bone graft substitute. Animals treated with locally released antibiotics had survivorship rates 24% higher than those treated with systemic antibiotics, and animals that received both antibiotics and an osteogenic drug had an increased amount of bone formation at 12 weeks compared to controls.

Finally, several different anti-biofilm agents were evaluated for their ability to inhibit and/or disrupt the growth of *Staphylococcus aureus* (*S. aureus*) biofilms *in vitro*. Lysostaphin was the only drug investigated that was able to both inhibit and disrupt S. aureus biofilms. Furthermore, lysostaphin encapsulated into PLGAms maintained its bioactivity and may be useful for future incorporation into biofilm-combating materials. The bone grafting material developed here can be used to locally deliver drugs in a

temporally controlled manner to reduce the number of procedures necessary for the treatment of complex IBDs.

KEYWORDS: Biofilms, bone graft substitute, calcium sulfate, composite, sequential release.

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Chapter 1 Introduction

Unlike simple fractures, the human body cannot heal large bone defects spontaneously and requires surgical intervention in the form of bone fixation and grafting [1, 2]. This is necessary due to the tendency of fast growing scar tissue to grow within the defect, preventing the slower growing bone from being able to bridge the gap [3]. Bone grafts are used to maintain the space within the defect and stimulate bone healing without hindering growth [4, 5]. The primary material used in bone grafting is autologous bone, or bone harvested from the patient's own body [6]. Autologous bone grafting is the gold standard method of treatment due to its inclusion of osteogenic cells, low risk of infection, and no risk of rejection [3, 6]. Even with all the success of bone autografts there are still significant drawbacks, such as limited grafting material and the need for a second surgery site [3, 7]. Anytime a large bone graft is needed there has likely been significant damage to the surrounding soft tissue and, with this, a high chance of infection [8, 9]. This infection complicates the bone healing process and needs to be eliminated before grafting and fixation can be performed [2, 10, 11]. The primary aim of this research was to develop a moldable bone grafting substitute capable of delivering antibiotics and osteogenic drugs in a temporally controlled manner for the treatment of large infected bone defects (IBDs) without the need for autologous bone.

Chapter two examines the background and significance of current treatment methods for infected bone defects as well as discussing bone autografts, allografts, and many of the common synthetic substitute materials. A brief introduction to bacterial biofilms and how they relate to large bone defects is also discussed. In chapter three the ratio of calcium sulfate (CS), bio-polymer, polymer microsphere, or antibiotic was investigated for the effects on overall mechanical properties of the bone graft substitute. The effects of various setting environments such as air, incubator, or saline solution were also examined. Chapter four discusses the method and rationale for designing a temporal separation in the release of the antibiotic and osteogenic drugs. *In vitro* release profiles and bioactivity of released antibiotic against *Staphylococcus aureus* (*S. aureus*) are also presented. In chapter five the *in vivo* effectiveness of the two part composite bone graft substitute is evaluated in a critically sized rat femoral defect model. The ability of the

bone graft substitute to eliminate an acute or chronic infection and stimulate the bone healing process was evaluated at four and twelve weeks through observation, histological, and radiographic techniques. Chapter six explores alternative treatment options for bacterial biofilms which could be incorporated into future therapeutic biomaterials. Lysostaphin is an antibacterial enzyme which is capable of both inhibiting biofilm formation and disrupting established biofilms. This enzyme is loaded into poly(lactic-co-glycolic acid) (PLGA) microspheres for *in vitro* release studies and bioactivity against *S. aureus*. Finally, chapter seven will discuss the effectiveness of the moldable bone graft substitute at treating large infected bone defects and potential ideas for the material to better treat biofilm infections.

Chapter 2 Background and Significance

2.1 Infected Bone Defects

The typical causes of large IBDs are high energy traumas such as explosions, car accidents, or gunshot wounds [3, 12, 13]. Because of this a large number of IBDs are seen in military conflicts [14-16]. Approximately 80% of injuries sustained during Operations Enduring Freedom and Iraqi Freedom are the result of explosions and 50-85% of these become infected [14-17]. The majority of these injuries are sustained in the extremities where less protective equipment is worn [17]. In a prospective study on the treatment of lower extremity open fractures, the outcomes of treatment with either soap or antibiotic solution during irrigation were compared to the outcomes in terms of infection and the healing of the soft and hard tissue [9]. This study found that approximately 37-41% of patients that presented with an open lower extremity fracture showed signs of gross contamination and after treatment there was still a 13-18% chance of developing an infection [9]. A study by Harris *et al.* following lower extremity trauma found that the most common complication was infection in 34% of patients [8]. One of the most common types of organisms that occurs in trauma related infection is the Gram positive bacteria *S. aureus* [18, 19]. This is due to the prevalence of the *S. aureus* on human skin and its propensity to adhere to cartilage and bone [19-21]. Other bacterial species that are commonly found include *Streptococcus pyogenes*, *Streptococcus pneumonia*, *Escherichia coli*, and *Pseudomonas aeruginosa* [20].

2.2 Current Treatment Methods

Because of the large variability in size and location associated with IBDs, successful treatment can necessitate multiple procedures and be very challenging [13, 22-24]. In order to properly heal the bone defect, the infection must be taken care of first [2, 10, 11]. This is due to a number of different ways that the infection can negatively affect the outcome of the bone grafting procedure [18, 20, 21]. In order to implant a bone graft into a segmental defect, the free ends of bone must first be fixed, usually with implanted hardware such as plates, rods, and pins [2, 10, 11]. If the fixation hardware was implanted before the infection had been eliminated the bacteria could colonize the foreign

object within the wound site [1, 25, 26]. Once the bacteria had colonized the implant a bacterial biofilm could form, which is a sessile community of bacterial cells which secrete extracellular polymeric substances (EPS) that greatly reduce the effectiveness of most antimicrobials [27-29]. A chronic infection like a biofilm will also cause persistent local inflammation, a decrease in pH, and a decrease in oxygen levels [18, 20, 21]. These effects along with the damage that the bone has already suffered as a result of trauma can cause additional bone to be lost as the infection grows [30, 31]. In order to avoid this, treatment for IBDs begins with a series of extensive surgical debridements and irrigations to remove any infected or necrotic tissue [2, 10, 11]. The patient is also put on a regime of systemic and local antibiotics to help combat the infection [2, 10, 11]. Once the infection has been eliminated the process of fixing the bone and grafting can begin. The gold standard of grating materials has been autologous bone for a very long time due to its success at stimulating new bone growth and no risk of rejection [6, 24].

2.3 History of Bone Grafting

The first ever recorded bone graft occurred in 1668 by Job van Meek'ren, a Dutch surgeon [32-34]. In this procedure a piece of a dog skull was used as a xenograft to repair a soldier's skull [32-34]. A xenograft is a graft where the tissue comes from a different species, and unfortunately the soldier had to have the graft removed two years later after being excommunicated from the church [32-34]. Autografts, which involve harvesting the donor bone from the patient, have been used for over 100 years [6, 24]. Major reasons for their success are the incorporation of osteogenic cells, the use of an osteoconductive matrix, and no risk of rejection [3, 35]. Despite all their success, autografts still have several disadvantages such as the need for a second surgery site, limited grafting material, and donor site morbidity [35]. Allografts are a common alternative to autografts because they do not involve harvesting bone from the patient but from cadavers [6, 7]. This allows for more grafting material to be obtained and removes the need to perform an additional surgery on the patient; however there is an increased risk of diseases transmission and they do not contain the osteogenic cells that make autografts so effective [6]. In the 1960's a US surgeon named Marshall Urist discovered bone morphogenetic proteins (BMPs), growth factors belonging to the transforming

growth factor-beta (TGF-β) superfamily [36, 37]. Of particular interest to bone healing are BMP-2 and BMP-7 which are powerful osteoinductive growth factors [36, 38, 39].

2.4 Bone Graft Substitutes

Due to the limitations and drawbacks of bone autografts and allografts there has been much research into the development of bone graft substitutes [3, 6, 40]. Bone graft substitutes aim to replace the current gold standard treatment of bone autografts by stimulating bone healing and often by delivering antimicrobials, something that can't be done with traditional autografts [3, 6]. Ceramics such as calcium sulfate (CS), calcium phosphate (CaP), or hydroxyapatite (HA) are common materials used in these substitute materials due to their high biocompatibility and moderate strength [5, 41-43].
Calcium sulfate has been used as a bone grafting material for over 100 years and is biodegradable, biocompatible, and osteoconductive [22, 44, 45]. Calcium sulfate is often formed into pellets which are loaded with antimicrobials and packed into wounds to provide local delivery at the site of infection [40, 46]. Antimicrobials delivered in this manner release the majority of the loaded drug within the first 2-3 days and frequently drop below the minimum inhibitory concentration (MIC), which can lead to resistant strains of bacteria if the pellets do not degrade quickly enough or are removed [47, 48].
In a prospective study performed by Kelly *et al.*, patients with bone defects were treated with calcium sulfate pellets instead of the more traditional cancellous bone graft [44]. The CS pellets were either used alone or mixed with autografts, demineralized bone matrix, or bone marrow aspirate [44]. Patients were followed for 12 months and the defects were monitored at 1, 2, 3, 6, and 12 months radiographically [44]. The amount of grafting material left in the defect and the percentage of new bone ingrowth was quantified [44]. All groups of CS pellets showed 99% resorption and 88% of the bone void filling after six months, and only 3.6% developed complications related to the implanted material [44]. This study shows the viability of CS as a bone grafting substitute by itself but also as an expander material to increase the volume of autogenous bone grafts [44]. In another prospective study performed by McKee *et al.* antibiotic-loaded calcium sulfate beads were used to treat chronic long bone infections or an infected non-union and compared to similar cases which were treated with antibiotic

loaded poly(methyl methacrylate) (PMMA) beads [40]. The primary outcome of the study was to remove the infection and the secondary outcomes were to have new bone growth, achieve union, and reduce the number of complications/repeat operations [40]. In the calcium sulfate group, the infection was removed in 86% of the patients and seven of eight were able to achieve union [40]. In the PMMA group the infection was removed in 86% of the patients and six of eight were able to achieve union [40]. While there was no significant difference between the antimicrobial and bone forming properties of the two groups, there was a significant difference in the number of additional procedures required after treatment, with the PMMA group requiring a total of 15 further surgeries compared to seven in the calcium sulfate group [40]. This difference was accounted for by the additional procedures required to retrieve some of the PMMA beads that were interfering with the healing process since they are not biodegradable [40]. This preliminary study was able to show that calcium sulfate beads were comparable in most aspects of treating infected non-unions and even reduced the additional procedures required when compared to the more standard method of using PMMA beads [40].

Calcium phosphate is another bone graft substitute material that has been used since 1892 and is biodegradable, biocompatible, and osteoconductive [49, 50]. Calcium phosphates are typically stronger than CS based materials and degrade much more slowly [49, 50]. Similar to CS pellets, CaP can be used as a bone cement loaded with antimicrobials or growth factors and formed into pellets for packing into wounds [49, 50]. When used in pellet form a large burst release of drug is typically seen, followed by sub MIC levels for an extended period of time that can require surgical removal of the pellets even though the materials are biodegradable [51, 52]. Calcium phosphate bone cements, which are often used to fix or coat metal hardware, cannot be removed after the burst release of drug, increasing the chance of delivering sub-MIC levels of antimicrobials [51]. In a study by Field *et al.*, a femoral defect was created in sheep and treated with either autografts or tricalcium phosphate (TCP) granules [49]. The sheep were evaluated radiographically at two, four, and six months and using computed tomography after euthanasia [49]. The animals with autografts showed good bone ingrowth after two to four months while the TCP granules did not show much until six

months [49]. The lag in bone growth could be accounted for by the lack of any osteoinductive agents in the TCP group when compared to the osteoinductive autografts.

In addition to the ceramic based bone graft substitutes there is also significant interest in the development of polymer based materials such as PMMA, polyurethane, poly(lactic acid) (PLA), and PLGA [53-55]. Unlike many of the ceramic based bone graft substitutes, polymer based materials are not usually inherently osteoconductive and thus require the addition of an antibiotic or osteogenic factor to be effective in the treatment of bone defects [56]. For example, Zong *et al.* compared a PLA/PLGA and PLA/nano-hydroxyapatite (nHA) scaffold seeded with human bone marrow-derived mesenchymal stem cells ability to regrow bone within a rat calvarial defect [53]. It was found that both scaffolds showed bone formation within the defect area but that the PLA/PLGA scaffold had more mature bone formation and degraded faster than the PLA/nHA, making room for new bone formation [53]. Yoshii *et al.* showed that a polyurethane scaffold containing lovastatin, a known osteogenic drug, showed increased bone formation at four weeks compared to a polyurethane control in a rat plug defect model [54].

Moldable bone graft substitutes have several advantages over preset pellets, including being easier to handle and being better able to fill irregularly shaped bone defects [5, 57, 58]. By filling more of the space within the defect, moldable bone graft substitutes can better limit soft tissue ingrowth and minimize the space between the native tissue and implanted material [3]. A common method of creating a moldable bone graft substitute is the addition of a biocompatible polymer. Examples of such polymers include: carboxymethyl cellulose (CMC), sodium alginate, PLGA microspheres, poly(ethylene glycol) (PEG), and hyaluronan (HY) [5, 42, 57, 59, 60]. In a study by Reynolds *et al.* CS was mixed with CMC and demineralized bone matrix (DBM) and compared to CS and DBM alone in a critical sized rat calvarial defect [5]. The CMC group showed comparable amounts of bone formation, fibrous tissue, and residual material after 28 days but was found to have superior handling properties [5]. Urban *et al.* found similar results when investigating a CS and hydroxypropylmethylcellulose bone grafting putty which had similar resorption and bone formation as a CS paste alone in a critically sized canine humeri model [61]. These results show that the addition of

biocompatible polymers does not hinder bone growth and improves the handling properties of the materials making them easier to use and implant within a wound site.

In order to increase the effectiveness of bone graft substitutes, therapeutic drugs are often added in impart antimicrobial, osteogenic, or angiogenic characteristics [1, 40]. By combing both an antibiotic and growth factor, for example, one graft can theoretically treat both the infection and bone defect, something that requires several procedures and materials using traditional debridements and autografts [1]. Tang *et al.* impregnated CS with gentamicin and liposomal gentamicin, implanted them into a rat tibial defect and evaluated the effectiveness at treating a *S. aureus* infection [62]. All animals treated with locally released gentamicin showed no bacteria in their blood at the end of the study, and those treated with liposomal gentamicin CS eliminated all the bacteria from the bone as well [62]. If PMMA beads had been used, as in McKee *et al.*, they would need to be removed before bone grafting could take place so the use of a biodegradable and osteoconductive bone graft substitute could reduce the number of procedures necessary to treat IBDs [40]. Beardmore *et al.* further showed that the combination of DBM and tobramycin-impregnated CS pellets was osteoinductive, osteoconductive, and able to eradicate a *S. aureus* infection in a goat tibial model [46]. The positive control group of antibiotic-impregnated PMMA beads was also able to eliminate the infection but would require removal and additional bone grafting to heal the wound [46]. Guelcher *et al.* showed that the addition of an antibiotic to scaffolds containing BMP-2 increased the amount of regenerated bone compared to scaffolds with BMP-2 alone in a critically sized rat femoral defect model [63]. This is in agreement with knowledge previously discussed about the negative effects that bacteria can have on bone healing [18, 20, 21]. Kempen *et al.* investigated the sequential release of vascular endothelial growth factor (VEGF) and BMP-2 from a composite scaffold implanted ectopically or orthotopically in rats [64]. Scaffolds containing VEGF stimulated significantly higher blood vessel volumes after 8 weeks in subcutaneous implants than scaffolds containing no growth factors or BMP-2 alone [64]. When implanted into a critically sized rat femoral defect, scaffolds containing BMP-2 stimulated significantly higher bone volumes than control groups, but scaffolds containing VEGF and BMP-2 were not statistically different from BMP-2 only scaffolds [64].

2.4 Biofilm Infections

If left untreated, the planktonic bacteria that were implanted into the tissue can settle onto a surface and form a biofilm [1, 25, 26]. The bacterial cells can surround themselves with a thick matrix of polysaccharides, called the EPS, which hinders the diffusion of antimicrobials and host defenses from the surface to the bacterial cells within as well as facilitating nutrient intake [19, 65]. The EPS can increase the required amount of antibiotic needed to inhibit or kill the bacterial cells by as much as 1,000X which can be toxic or unachievable via systemic delivery [25, 29, 31]. Once settled, the biofilm is capable of spreading by dispersing small microcolonies that can travel to an uninfected region and start a new colony or infection [19]. This is one reason that it is so important to eliminate the infection prior to implantation of fixation hardware, since the contaminated implant could act as a nidus of infection, spreading the bacteria around the body [25, 28, 30]. A chronic infection that is not treated can have a number of negative effects on the healing process [30, 31]. An infection which the body has no method of removing will cause persistent local inflammation and an increase in host response cells at the site of infection [28, 66]. Host cells such as neutrophils, monocytes, and macrophages present in greater numbers for a prolonged period of time can cause local tissue death through the release of reactive oxygen species and proteolytic enzymes [28, 66]. Proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 can also be released by bacterial cells, further increasing the inflammatory response and negative side effects associated if prolonged [28]. There have also been a number of reports of bacterial cells attaching to and invading osteoblasts, protecting them from antimicrobial treatments and host defenses [30, 67, 68]. Once internalized the bacterial cells can trigger apoptosis in osteoblasts, potentially upsetting the balance of bone formation and resorption and negatively affecting the bone healing process [20, 30, 67]. In the United States, infections associated with joint implants range from 1-3%, 10-30% for urinary catheters, 1-7% for cardiac pacemakers, and 25-50% for cardiac assist devices [69]. Periprosthetic infections occur in approximately 1.5-2.5% of hip and knee arthroplasties and a slightly higher 3.2-5.6% of revision surgeries [70].

Since many commonly used approaches to treating infections are ineffective at eliminating biofilm infections, alternative methods are being investigated. One method

involves inhibiting microbial attachment to implant surfaces by immobilizing antimicrobial agents on the surface [28, 71-73]. Kazemzadeh-Narbat *et al.* showed that immobilizing the antimicrobial peptide HHC36 on a CaP coated titanium surface had antimicrobial activity against *S. aureus* and *Pseudomonas aeruginosa* [51]. One significant drawback to this approach is the relatively short timeframe which the coating provides antimicrobial effects due to loss of attached peptide [28]. In a study by Vester *et al.* gentamicin was released from a poly(D,L-lactide) (PDLLA) coating on a titanium Kirschner-wire (K-wire) *in vivo* and *in vitro [72]*. Release of the antibiotic occurred for 42 days *in vitro* and 7 days *in vivo* and the gentamicin loaded coatings were able to significantly reduce the adhesion of *Staphylococcus epidermidis* compared to controls [72]. Antoci *et al.* covalently attached vancomycin to a titanium surface and showed that not only did the coating prevent *S. aureus* attachment but was able to do so after storage in buffer solution for 11 months [74]. The coated titanium surfaces were also incubated with *S. aureus* for 1 or 4 weeks and no increase in MIC was seen for vancomycin [74]. Methods of extending the release timeframe or preventing release from the implant surface at all are necessary in order to eliminate the infection without fear of creating resistant bacterial strains [28]. Disrupting the biofilm EPS is another method which has potential for the treatment of biofilms since the EPS is the primary defense mechanism against the majority of antimicrobial treatments [28, 75]. Once the EPS has been disrupted, the bacterial cells should be once again susceptible to normal MIC of antibiotics [31]. Some known agents that are capable of disrupting part of the matrix structure are dispersin B, lysostaphin, and proteinase K [76-78]. Yet another method involves hijacking the biofilms' own quorum sensing system to initiate dispersal of the established biofilm back into a planktonic state, making the bacterial cells more susceptible to antimicrobials [78, 79].

2.5 Significance

Current treatment for IBDs involves several surgical procedures which increase hospital stays, patient costs, and discomfort. A bone graft substitute that could combine all these procedures would be of great benefit to the patient and much research is currently being done in this area. Many of the new methods being investigated do not

seek to combine the treatments but to improve upon the first set of procedures to treat the infection. Those that do try to combine two treatments into one procedure often attempt to treat both the infection and bone defect at the same time. The reason that the current procedures are separated is to avoid combating the infection while repairing a bone defect and has good rationale to be done this way. The aim of this research was to develop a bone graft substitute which was moldable, biodegradable, and capable of maintaining a temporal separation in the release of two different drugs for the treatment of infected bone defects. Due to the modular nature of the composite material it would also be possible to incorporate different antimicrobials, anti-biofilm agents, or osteogenic drugs to tailor the device as needed.

Chapter 3 Effects of Composition and Setting Environment on Mechanical Properties of a Composite Bone Filler

This chapter reproduced from an accepted manuscript, "Brown ME, Zou Y, Dziubla TD, Puleo DA. Effects of composition and setting environment on mechanical properties of a composite bone filler. Journal of Biomedical Materials Research Part A. 2013;101A:973-80."

3.1 Introduction

High energy trauma, resulting from events such as explosions, can cause large bony defects that cannot heal spontaneously [3, 13]. Because of the large variability in size and location associated with these wounds, treatment can be complicated and require multiple procedures [13, 22-24]. Approximately 80% of injuries in Operations Enduring Freedom and Iraqi Freedom are the result of explosions [14-16].

The most common method of treatment begins with debridement to remove necrotic tissue followed by an autogenous bone graft to replace the missing bone [6, 22]. Autografts, which involve harvesting the donor bone from the patient, have been the gold standard for bone grafts for over 100 years [6, 24]. Major reasons for their success are the incorporation of osteogenic cells and the use of an osteoconductive matrix in the grafting material [3]. Harvesting donor bone from the patient also greatly reduces the risk of rejection when implanted [3, 35]. Even with all their success, autografts still have several drawbacks, including the need for a second surgery to obtain the donor bone and donor site morbidity that can cause pain and future complications for the patient [35]. Allografts are an alternative to autografts that removes the need for the patient to donate the bone grafting material and instead obtains it from cadavers [3]. This method does not require a second surgery site, but it increases the risk of disease transmission and infection [3, 35].

Due to the inherent limitations of traditional bone grafts, substitute bone grafting materials have been an area of intense research interest. Many of these materials are designed to be biodegradable, osteoconductive, and provide some mechanical support [3, 6, 40]. Ceramics, such as calcium sulfate (CS), calcium phosphate cements (CPC), and hydroxyapatite (HA), and various polymers, such as polyurethane and poly(lactic-co-glycolic acid), are being investigated for use as bone graft substitutes [5, 41-43]. CS has a long history of clinical use and is biodegradable, biocompatible, and osteoconductive

[22, 44, 45]. With the incorporation of antibiotics and growth factors, CS can be used to treat infections and stimulate bone formation, further increasing the material's effectiveness in bone regeneration [40, 80, 81]. Along with making more bioactive grafting substitutes, there is significant interest in developing moldable or injectable systems with the addition of biopolymers that can be more easily applied to a wound and can fill the void better than preset materials [5, 42, 45, 57, 82].

The present work focused on the development of a composite bone graft substitute, composed of CS, microspheres, and a plasticizer, that can eventually be modified to deliver biomolecules for the treatment of large bony defects, such as may result from explosions. A key factor in the effectiveness of bone graft fillers is the ability to fill the defect completely to prevent soft tissue in-growth; this moldable bone filler will be capable of filling any irregularly shaped defect and setting *in vivo*. The handling properties and moldability of CS were significantly improved by the addition of a plasticizer, such as carboxymethylcellulose (CMC) or hyaluronan (HY), which have already seen use in human patients for wound healing applications in products, such as OP-1 putty and Orthovisc®. Addition of these biomacromolecules to CS created a material that is easy to work and is still capable of setting *in vivo*. The effects of composition and environment on mechanical properties and degradation time were evaluated to determine a balance between setting time and mechanical strength.

3.2 Materials & Methods

3.2.1 General Sample Preparation

CS (hemihydrate 98%; Sigma), carboxymethylcellulose (CMC) (medium viscosity; Sigma) or hyaluronan (HY) (MW 7.46 x 10^3, 1.323 x 10^5, 3.574 x 10^5, or 2 x 10^6 Da; LifeCore Biomedical), gelatin microspheres (Gms), cellulose acetate phthalate/Pluronic F-127 microspheres (CAPPms), gentamicin (Sigma), and vancomycin (Sigma). Sample compositions used in the various studies can be seen in Table 3.1. A series of different compositions were investigated as the composite material was continuing in development, each experiment providing valuable information for the experiments to follow.

Table 3.1: Formulations of moldable bone filler used in different experiments expressed as weight percentages.

	Component (wt. %)						
Sample Set	**CS**	**CMC**	**HY**	**CAPP**	**Gms**	**Gentamicin**	**Vancomycin**
Setting Environment	90	5	0	2.5	2.5	0	0
Composition Effects on Mech. Strength over Time	85	5	0	5	5	0	0
	80	10	0	5	5	0	0
	100	0	0	0	0	0	0
Polymer Comparison	95	0	5	0	0	0	0
	95	5	0	0	0	0	0
Mass Loss	85	5	0	5	5	0	0
Set Time	87	5	0	0	5	1.5	1.5
	87	0	5	0	5	1.5	1.5
	95	0	5	0	0	0	0

Microsphere preparation is detailed in the following section. The relative amounts of each component were varied to determine the effects while maintaining a strong, moldable material. All components were dry mixed by hand prior to addition of deionized water. The amount of water required varied (usually between 300-600 µl/g) depending on the relative amounts of components in the composite, and samples were fabricated such that a non-sticky, moldable material was formed. The moldable filler was then packed into cylindrical Delrin molds (6.5 deep x 3.2 mm diameter), and samples were removed once they could be pushed out without deformation, which took between 10-20 minutes depending on the composition. Pictures demonstrating the moldable nature of the filler can be seen in Figure 3.1.

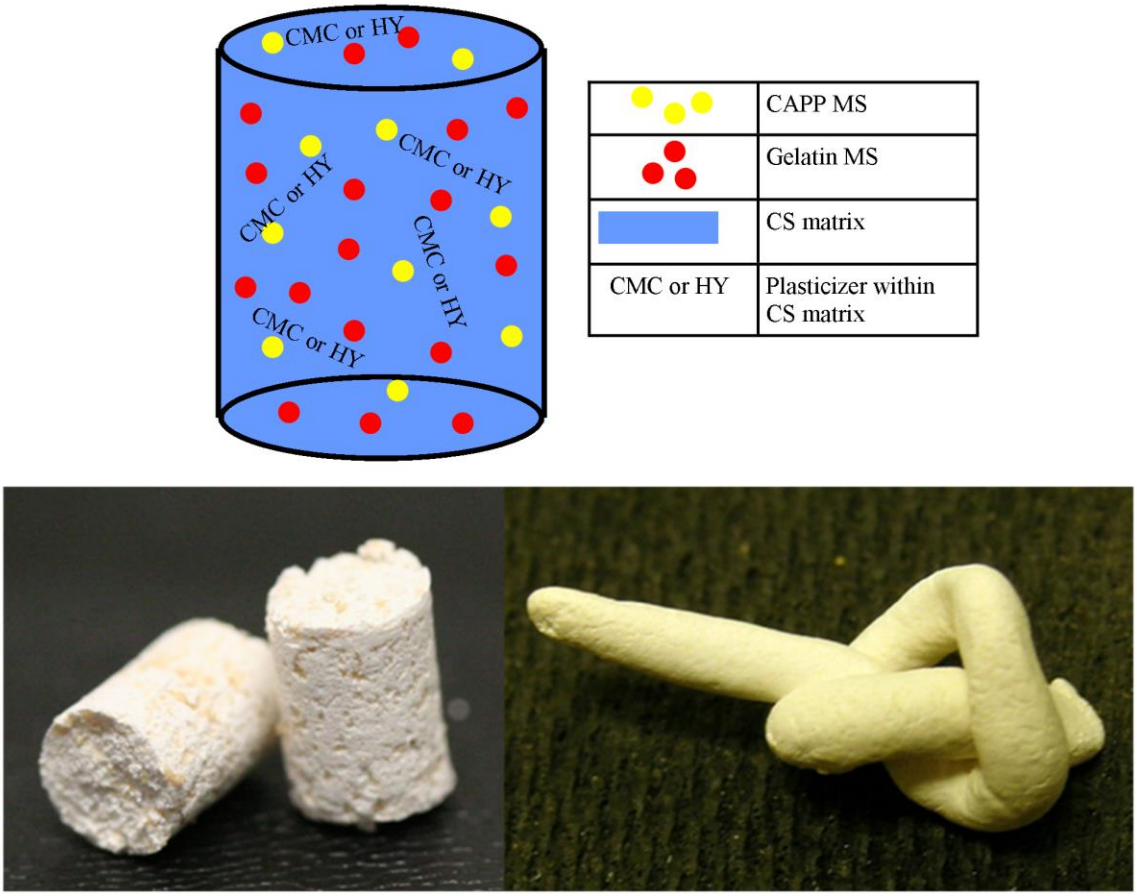


Figure 3.1 Schematic image of the cylindrical bone filler samples used for mechanical testing (top); plasticizers, 'CMC or HY', were dispersed throughout the CS matrix to improve the handling properties of the CS. A picture of actual bone filler samples (bottom left) and a demonstration of the moldable nature of the CS composite material (bottom right).

3.2.2 Microsphere Preparation

Gelatin microspheres were prepared using methods adapted from Zou et al [83, 84]. A solution of 10% gelatin in deionized of water was added to 200mL of stirring olive oil (40°C) in a drop-wise manner. The mixture was then chilled to 10 °C with stirring for 30 min before the addition of 60mL of chilled (4 °C) acetone for an hour with continuous stirring. The solution was centrifuged (123 g force for 5 minutes) before microspheres were collected by filtration (11 µm; Whatman). The microspheres were then crosslinked by being placed in stirring 20mM glutaraldehyde for 12 hours at 4°C.

The crosslinked microspheres were collected and immersed in 50mM glycine solution for 2 hours to block residual aldehyde groups.

Cellulose acetate phthalate/Pluronic F-127 microspheres were prepared by first dissolving cellulose acetate phthalate (CAP) and Pluronic in acetone at a weight ratio of a 2.33:1 (CAP: Pluronic) while shaking [85]. This emulsion was added rapidly into 4 volumes of corn oil and sonicated (25W) for 5 seconds. The CAP/oil solution was then added to 5.3 volumes deionized water along with 0.5 volume of Triton X-100 solution; the combined solution was then homogenized at 1509.2 g force for 5 minutes. The resulting suspension was centrifuged at 277 g force for 5 minutes to separate the CAPP microspheres, and the oil and water phases were removed by aspiration.

Both types of microspheres were lyophilized for 24 hours prior to use. Characterization of microspheres was reported previously, and microscopic observations showed their diameter to range from 70-150 μm and 70-110 μm for gelatin and CAP-Pluronic, respectively [84, 86]. In future work, these microspheres could be loaded with bioactive drugs for the treatment of infected bony defects.

3.2.3 Mechanical Properties of Composite Material

In order to test the properties of set composites with HY compared to CMC, mechanical testing was performed on samples 10-20min after fabrication. Samples were removed from the mold as soon as possible and allowed to air dry for 10min, at which point compression testing was performed. The aim of this part of the study was to determine how quickly the different compositions were able to set.

3.2.4 Setting Environment

Once bone filler samples were removed from the mold, they were placed in one of three environments to set: 1) oven at 40°C , 2) a fully humidified cell culture incubator at 37°C, or 3) immersion in 1.5 or 3 mL, phosphate-buffered saline (PBS), pH 7.4 at 37°C. Samples were dried in an oven for comparison against other preset calcium phosphate or calcium sulfate bone grafting materials/cements. The cell culture incubator and immersion in PBS were examined in order to simulate two extremes of the simulated *in vivo* environment. Different volumes of PBS were used in order to determine what effect

the surface area (sample) to volume (PBS) ratio had on the degradation rate of the bone filler samples.

3.2.5 Mechanical Properties and Degradation

Compression testing was performed on cylindrical samples using a Bose ELF 3300 mechanical testing system. Samples were removed from their respective setting environment, or the mold in the case of the set time experiments, and tested immediately afterwards. Load was applied at 5 N/sec until failure, and the elastic modulus (M) and ultimate compressive strength (UCS) were calculated.

Non-destructive degradation studies were performed by placing composite samples in either 1.5 or 3mL static PBS at 37°C. The solution was changed every three days, and at each time-point three samples were removed, dried for 24 hours at 40 °C, and then weighed. Volumes of 1.5 or 3 ml of PBS were used because calculations based on solubility of CS indicated the volumes would maintain sink conditions while being small enough to enable detection of released drugs in future studies. The solubility of CS is approximately 2.4mg/ml H_2O. In the case of the deg. study with 1.5ml PBS, the samples would have needed to degrade 42% over the 3 day period between samplings to reach the solubility limit of CS. Pilot study data (not shown) showed that samples reached steady state mass after 4 hours when dried at 40 °C. The initial and final weights after degradation were then used to determine the percent mass loss for each sample.

3.2.6 Statistical Analysis

One-way and two-way analysis of variance (ANOVA) was performed using Prism (GraphPad). Statistical analysis was determined at p values less than 0.05. Bonferroni's post-hoc test was performed as needed. Because certain testing groups were small, normality tests and data skew was used in order to test the validity of using ANOVA statistical methods.

3.3 Results

3.3.1 Initial Mechanical Properties of Composite Material

Bone filler samples that contained HY instead of CMC had significantly ($p < 0.05$) better mechanical properties when tested shortly after mixing (Figure 3.2). The samples containing CMC were still very moldable even after testing, which resulted in low mechanical stiffness of the cylinders. Samples that contained microspheres showed inferior mechanical strength to those containing CS and HY alone.

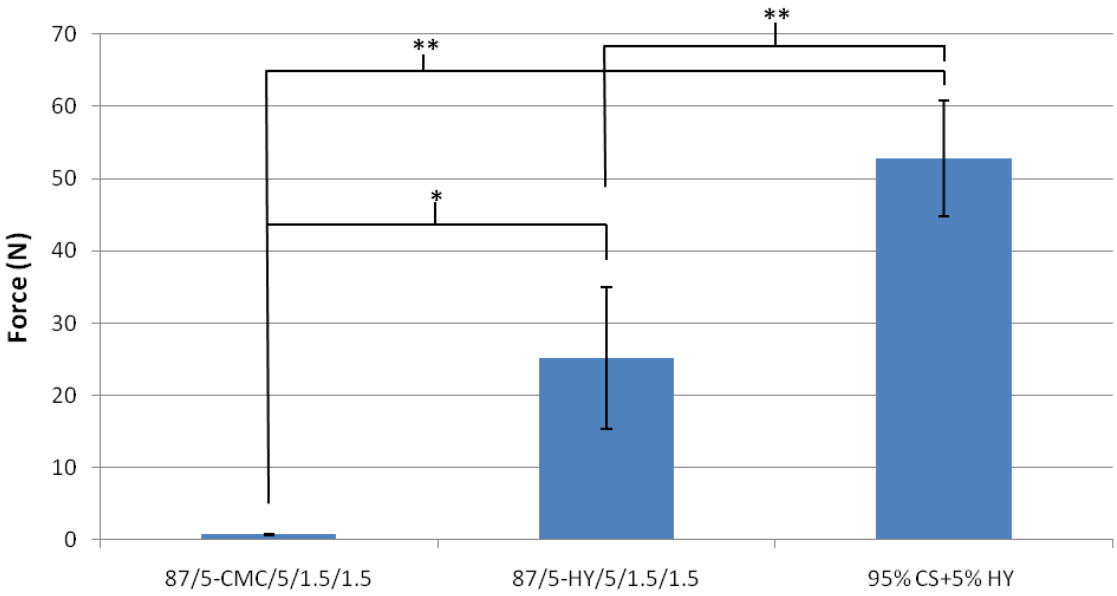


Figure 3.2: Effect of composition on initial mechanical properties of composites. All samples were evaluated within 20 minutes of fabrication, and 1.323 x10^5 Da HY was used in all samples containing HY. (Data are mean ± SD, n=6, 6, 5, or 5 for the groups evaluated, respectively; $p<0.001$, *$p<0.01$, **$p<0.001$).

The type of plasticizer used in the composite influenced the mechanical properties, but no significant difference was seen between the different MW HY samples (Figure 3.3). Samples containing Low and Med-low MW HY had compressive moduli ranging from 1400-1500 MPa, significantly higher ($p < 0.05$) than samples containing CMC with an average compressive modulus of 550 MPa.

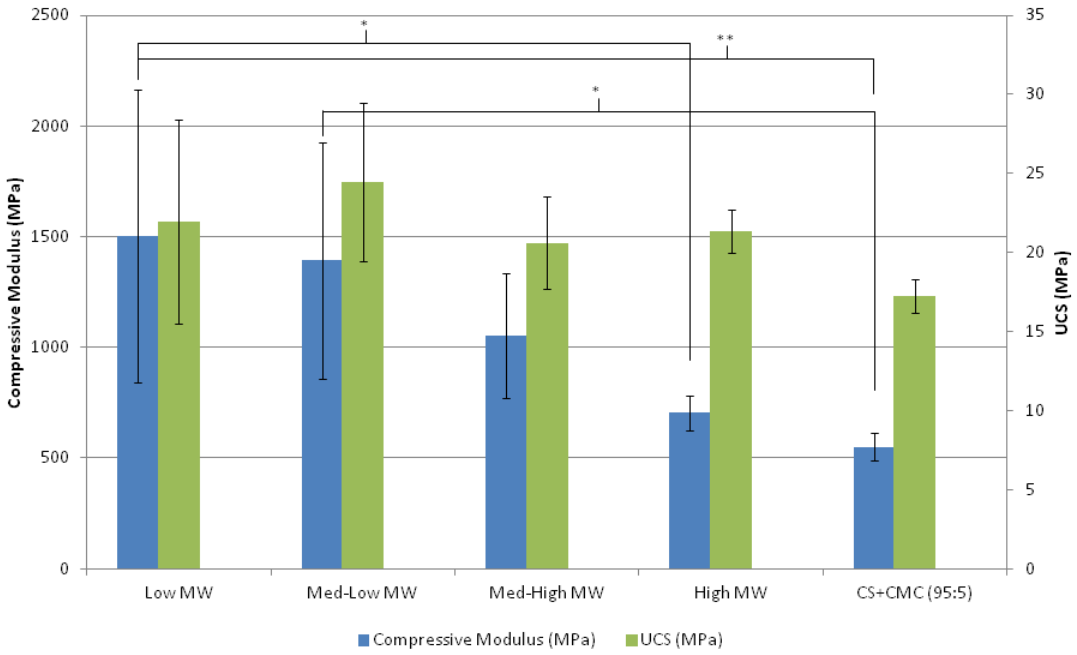
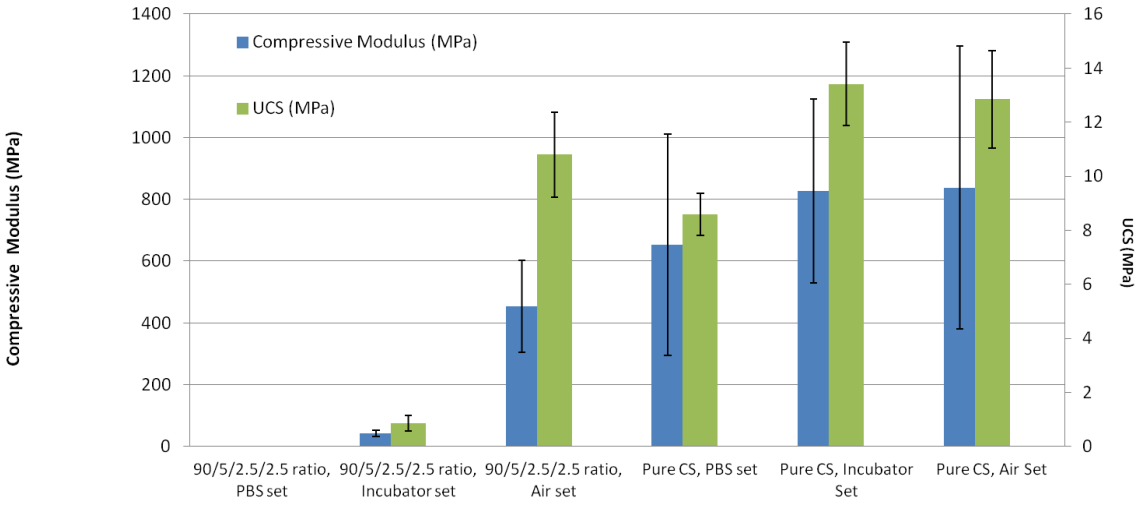


Figure 3.3: Effect of type of biopolymer on mechanical properties of CS with 5 wt% of CMC or varying MW hyaluronan. , Low=7.46 x 10^3, Med low=1.32 x 10^5, Med high=3.57 x10^5, High=2.0 x 10^6 Da. (Data are mean ± SD, n=11, 11, 5, 5, and 7 for the groups evaluated, respectively; *p<0.05, **p<0.01).

3.3.2 Setting Environment

The elastic modulus of the bone filler composites as well as CS controls under different setting conditions can be seen in Figure 3.4. Composite samples placed in wet environments, such as a cell culture incubator or submerged in PBS, showed significantly lower ($p < 0.05$) mechanical properties after 24 hours when compared to those dried in air. An UCS of 0.86 MPa and compressive modulus of 42 MPa was seen in samples placed in an incubator compared to 11 MPa and 453 MPa seen in samples air dried. Samples that were placed directly into PBS retained their cylindrical shape but did not fully set and were unable to be mechanically tested.



CS	90	90	90	100	100	100
CMC	5	5	5	0	0	0
CAPPms	2.5	2.5	2.5	0	0	0
Gms	2.5	2.5	2.5	0	0	0
Set Condition	PBS	Incubator	Air	PBS	Incubator	Air

Figure 3.4: Effect of setting environment on compressive modulus. 90/5/2.5/2.5 PBS set was not tested because the samples were too fragile to even remove from the PBS without deforming or breaking. (Data are mean ± SD, n=4, 4, 11, 8, 6, and 4 for the groups evaluated, respectively; One-way ANOVA for compressive modulus and UCS $p < 0.0001$)

3.3.3 Mechanical Properties over Time and Degradation

The relative amounts of the composite material components were shown to have a significant ($p < 0.0001$) effect on the mechanical properties (Figure 3.5). Addition of larger amounts of plasticizer or microspheres to the composites resulted in lower mechanical properties over time ($p < 0.0001$). All three compositions became stronger over the first few days as the CS was able to fully set in the humid environment.

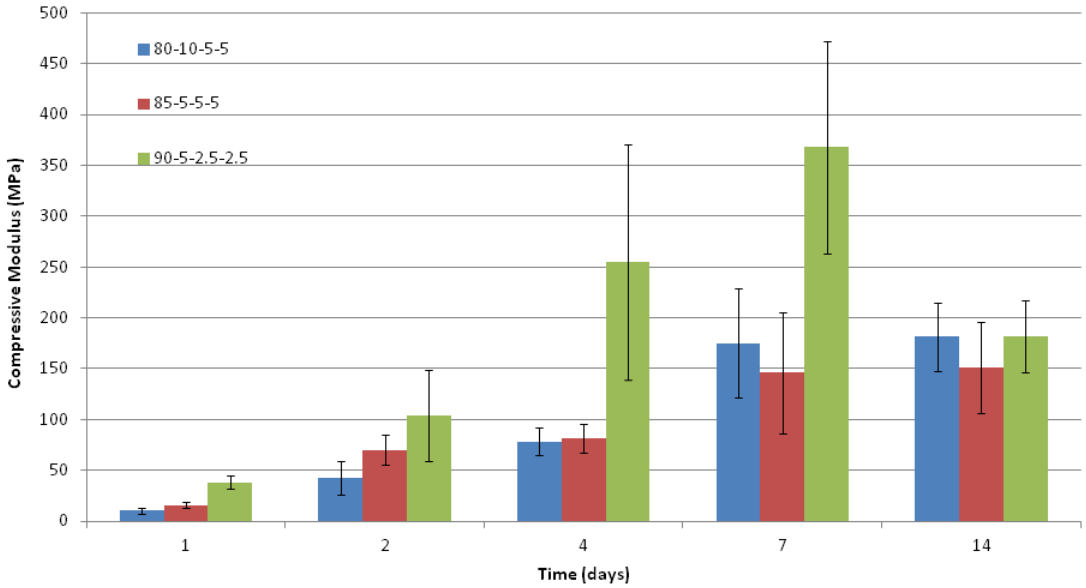


Figure 3.5: Effect of composition on modulus of the moldable bone filler dried in a humidified environment. (Data are mean ± SD, n=4; Two-Way ANOVA $p < 0.0005$).

The type of plasticizer used, either CMC or HY, did not have any significant effect on the degradation rate of the bone filler composite (Figure 3.6). The time until complete degradation was approximately 18 days for both polymers examined. The samples began degrading by erosion, getting smaller throughout the 18 days; however some samples began to fragment towards the end of the degradation period. The ratio of sample surface area to volume of PBS in which they were incubated (SA/vol.), however, seemed to have a slight effect on the degradation profile by increasing the degradation rate with a higher SA/vol. ratio, but the results were not significant.

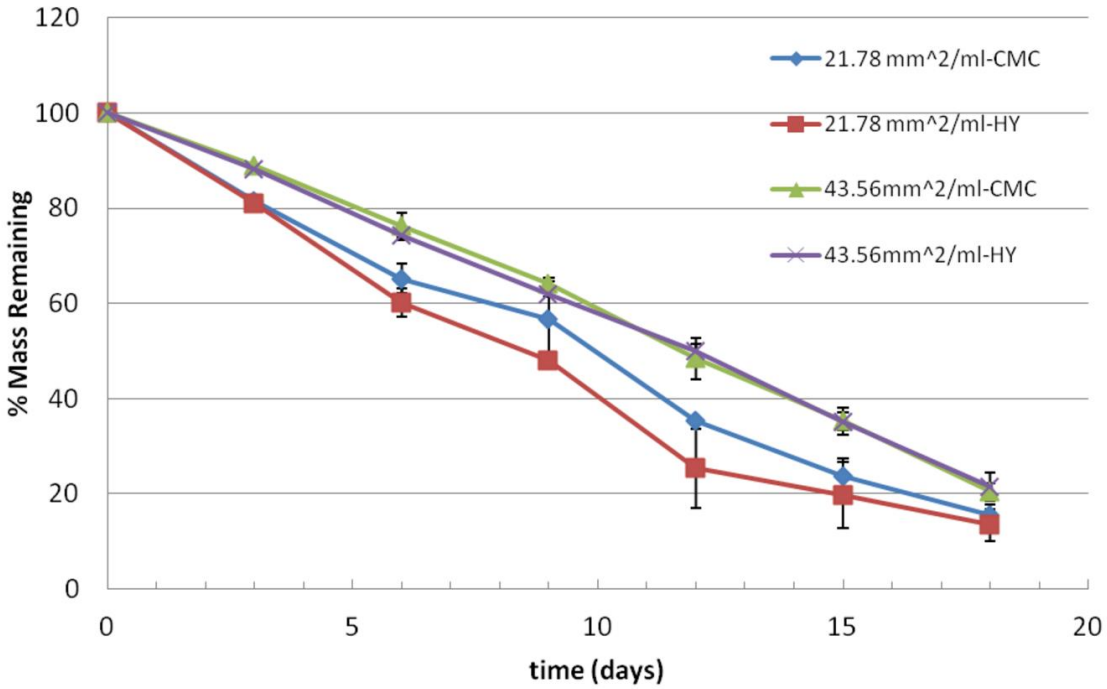


Figure 3.6: Effect of SA/Vol. ratio on degradation of composites containing either 5 wt% of CMC or HY (1.323 x 10^5 Da). Samples in 3 and 1.5 mL PBS had SA/vol. ratios of 21. 8m and 43.6 mm^2/mL, respectively; no significant difference was seen between the two SA/vol. ratios. (Data are mean ± SD, n=3).

3.4 Discussion

3.4.1 CS as a Bone Filler

CS has been used in many different forms as a bone graft substitute, such as injectable treatments, moldable putties, or as preset pellets [5, 22, 59]. CS-based materials have many useful properties for bone regeneration, such as being osteoconductive, biodegradable, and biocompatible [22, 45]. CS paste alone is very sticky and sets quickly which makes it difficult to work with in a clinical setting, the addition of a plasticizing polymer to the CS matrix makes the material injectable or moldable and generally increases the working time [5, 45, 57]. While CS has many useful natural properties, it is not osteoinductive, a key factor in the success of a bone grafting material in large defects [3]. CS based materials also do not posses any inherent

antimicrobial properties and require the same repeated debridement/irrigation procedures as traditional grafts [22, 44]. Antibiotics and osteogenic molecules can be added to CS in order to create a material that is antibacterial, osteoinductive, and osteoconductive [40, 45, 48]. In the present study, a moldable, biocompatible, and biodegradable bone filler material was created that could be loaded with different bioactive agents. The degradation and mechanical strength were shown to be tailorable depending on the ratio of components within the composite, and the properties also depended considerably on the environmental conditions around the samples as discussed below. While this composite material is not within the range needed for a fully weight-bearing material (addressed further in the next section), it is strong enough to be used in a similar manner as traditional bone grafts.

3.4.2 Compositional Effects on Mechanical Properties

Much of the recent research into bone filler substitutes has examined composite materials that are easier to handle and apply clinically [5, 45, 60]. These materials are often created by the addition of a plasticizing agent that can make the composite injectable or moldable [5, 59]. Antibiotics or growth factors are often included in these composite bone fillers in order to increase the therapeutic effects by fighting infections or actively promoting the growth of bone [47, 48, 62, 87, 88]. The ability to load both antibiotics and osteogenic molecules into the same moldable grafting material is not commonly done and creates a bone grafting substitute that has larger therapeutic applications.

The mechanical properties of bone vary considerably depending on the type of bone, the location of the bone, and the health of the patient [3]. The difference in mechanical properties between cortical and cancellous bone is very large, with the compressive modulus of cortical bone ranging from 7-25 GPa while cancellous bone ranges from 0.1-1 GPa [3, 59]. The ultimate compressive strength of cortical bone and cancellous bone ranges from 50-150 and less than 1 MPa, respectively [3]. Many bone filler materials aim to have similar mechanical properties to the bone they temporarily replace in order to reduce complications with surrounding bone and make the materials more load-bearing [3, 59] Several injectable CPC and CS systems have compressive

moduli ranging from 1-165 MPa [59, 89]. There are also other moldable systems that use either a polymer or microspheres to create a more workable putty or paste for placement into irregularly shaped defects [42, 57]. These materials had diametrical tensile strengths ranging from 6-10 MPa [42, 57]. The moldable bone filler material developed in this study had compressive moduli ranging from 10-350 MPa and UCS ranging from 5-20 MPa, depending on the formulation and setting conditions. The mechanical strength of the composite material is significantly less than that of cortical bone but near that of cancellous bone.

Using HY instead of CMC in the composite material resulted in a stronger material and a faster setting time. This was likely due to the increased number of hydroxyl groups present on the HY allowing it to imbibe large quantities of water, much like it does in cartilage [90]. The MW of the CMC used was 2.5×10^5 Da, which is slightly larger than that of the 1.3×10^5 Da HY that was chosen for continued use. Since the MW of these two polymers is similar, it likely was not much of a factor in their water retention properties. The CMC used had fewer hydroxyl groups available to bind with water in part due to substitution with carboxymethyl groups during its synthesis. By retaining more water than CMC, and thereby decreasing the amount of free water for CS dissolution, the HY biopolymer makes it easier for solution super-saturation and conversion to CS dihydrate, which resulted in faster setting. Because the present material is intended to be implanted in a moldable (*i.e.*, not set/hardened) state, the working time available to the surgeon after mixing is clinically important. A setting time of 20 minutes was used as a characterization bench mark, because setting times less than this may not provide enough time to implant the material properly. Similarly, setting times greatly exceeding 20 minutes will likely plastically deform *in situ* and degrade more quickly in the wound site.

The strength of the bone filler material was directly related to the amount of CS. More CS present within a fixed volume of water allowed the conversion from hemihydrate to dihydrate to happen more quickly because the solution can more readily become super-saturated with dihydrate. This increased amount of dihydrate form of CS caused the resulting material to possess superior mechanical properties. All three compositions became stronger over the first couple of days when stored in the cell culture

incubator at 100% humidity. This is likely due to the CS setting process being slowed down by the large amount of water in the air. Since the major structural component of the composites was not fully set during the first several testing points the bone filler samples would be weaker than fully set samples. The average compressive modulus of the 90-5-2.5-2.5 samples stored in the incubator at day 7 is approximately 360 MPa, similar to the air dried 90-5-2.5-2.5 samples which had an average compressive modulus around 400 MPa. It is likely that after 7 days the samples were fully set and the reason for the decrease in mechanical strength at day 14 was due to the excess humidity in the air weakening the CS matrix [91]. The amount of water added to the dry components to make the filler ranged from 300-600 µl/g and was chosen based on the handling properties of the resulting putty. A lower content of microspheres or plasticizer caused an increase in the mechanical properties of the composite filler due to the decreased water retention, increased amount of CS, and in the case of microspheres, a decreased number of stress concentrators. The gelatin microspheres are very hydrophilic as are the plasticizers used to create a moldable material; this added water retention would slow down the conversion of CS hemihydrate to CS dihydrate in a similar manner as described above for the setting conditions. CAPP microspheres would not contribute much to water retention since they are surface-eroding and do not absorb much water as they degrade.

3.4.3 Setting Conditions

The conditions in which the CS bone filler was set had a significant effect on the mechanical properties of the composite, which were directly related to the amount of water in the system. The additional water that the samples were exposed to in the humidified cell culture incubator or when immersed in PBS did not allow the material to ever fully set. The α-hemihydrate form of CS normally forms a stronger, denser, dihydrate material when dried [92]. During this reaction, the hemihydrate form of CS is converted into a dihydrate form; this reaction is driven by the solution becoming super-saturated with the dihydrate form followed by nucleation and crystal growth [45]. Once the solution is no longer saturated, more of the hemihydrate form can dissolve and super-saturate the solution again. With excess amounts of water in the system, it is difficult for the solution to become supersaturated and begin nucleation and crystal growth. It has

also been shown that the addition of polymers or presence of biological molecules, such as proteins, can slow down the setting time and make it difficult for the material to set *in vivo* [93]. If the material is unable to fully set *in vivo* as intended the mechanical integrity and therapeutic effects may be compromised.

In clinical applications, the actual setting environment will be likely somewhere between full immersion in solution and the cell culture incubator. Immersion in a fluid is the most common environment for investigating a bone grafting substitute's drug release and degradation profiles [47, 59, 60, 94, 95]. *In vivo* conditions would be very difficult to replicate *in vitro* since many different factors must be considered. The bone filler composite will be loosely confined within the wound site and subjected to a small but continuously exchanged fluid flow from the body along with wound healing cells and bacteria. Immediately upon implantation within the body, proteins will begin to adsorb onto the surface of the material [96]. These adsorbed proteins can become entangled in the crystal structure and slow down the set time or weaken the mechanical strength of the material [59, 89]. All of these factors will contribute to the degradation and function of the bone filler and should be considered carefully when planning *in vitro* experiments.

The degradation profiles showed the small filler samples degrading over the course of around 18 days. Although the ideal time for a grafting material to be present in a defect site is unknown, larger samples appropriate for (pre)clinical applications would take longer to degrade. Interestingly, the percentage mass remaining did not go all the way to zero. One reason for this observation was that at later time points in the experiment, the samples began to break apart considerably, and accurate mass measurements could not be taken, so the samples were considered completely degraded. Furthermore, Mamidwar et al. showed that a calcium phosphate lattice forms as calcium sulfate degrades, and this insoluble mineral phase will prevent the mass from reaching zero [97]. The degradation profiles varied with the surface area to volume ratio of the sample and the amount of solution they were immersed in; this was likely due to saturation/solubility effects of the PBS. Once the solution containing the sample became saturated, dissolution of the components would slow considerably. The sample surface area to supernatant volume ratio should affect the degradation rate by determining how quickly the solution becomes saturated, and generally the solution should be at high

enough volumes and changed frequently enough to remain at sink conditions to best understand the degradation and release mechanisms of a biomaterial.

3.4.4 Moldable Systems

Moldable bone graft substitutes have the advantages of being easier to handle during implantation than pastes as well as having the ability to conform to any irregularly shaped defect, thereby minimizing the space between the native tissue and implanted material. These systems also remove the need for a preset material and allow for a better fit of the implanted graft and host tissue. Various types of biocompatible polymers have been used in order to create moldable bone graft substitute systems, such as CMC, sodium alginate, and PLGA microspheres [5, 42, 45, 57]. When a moldable putty was created instead of a more viscous paste in these other systems, the degradation rate was comparable, while the mechanical properties were lower. In a study by Habraken et al., an injectable CPC was created using gelatin microspheres to create a workable paste and to create macropores for the ingrowth of tissue [82]. Simon et al. created a composite bone graft paste by combining poly(lactide-co-glycolide) (PLGA) microspheres with a CPC, which resulted in a material that was mechanically weaker than CPC alone but contained up to 18% micropores that could allow ingrowth of tissue [42]. This paste still suffers from the slow degradation rate of CPCs; the material showed little signs of degradation after three months, even after the outermost PLGA particles had degraded leaving micropores [42]. In a manner similar to the moldable bone filler material presented here, it would be possible to tune the degradation and mechanical properties of these moldable systems by changing the base components and ratio of components.

3.5 Conclusions

A moldable, biocompatible, and biodegradable bone grafting substitute was developed using CS, microspheres, and a plasticizer. The mechanical strength and setting time of the filler material can be tailored by altering the ratio of various components or the type of plasticizer used. Adding any components to the CS, such as biopolymer or eventually antibiotics or biomolecules, increases its functionality but decreases its mechanical strength and ability to set in harsh environments. The plasticizer HY was shown to create a stronger composite material than those using CMC while still

retaining its moldability and biocompatible nature. The samples degraded in a linear fashion over the course of 18-20 days in PBS, and samples containing HY instead of CMC showed a slightly slower initial degradation. In addition to possessing many of the desired mechanical properties of bone graft substitutes, the material can be loaded with bioactive molecules. The present composite filler will be further explored as an alternative to traditional bone grafting treatments.

Chapter 4 Release of Bioactive Molecules from a Moldable Calcium Sulfate Bone Graft Substitute

This chapter reproduced from an accepted manuscript, "Matthew E. Brown, Yuan Zou, R. Peyyala, Thomas Dziubla, and David A. Puleo. Temporal Separation in the Release of Bioactive Molecules from a Moldable Calcium Sulfate Bone Graft Substitute, Current Drug Delivery, 2014, in review."

4.1 Introduction

Open bone defects are a challenge to manage clinically because of the high variability in size, shape, and location of the wounds [13, 22-24]. The likelihood of infection and the multitude of required procedures further increases the complexity of treatment [13, 22-24]. Traditional standard of care for an infected bony defect (IBD) includes repeated debridement and 4-6 weeks of systemic antibiotics until the wound is free of infection, followed by fixation and grafting with donor bone [18, 19, 22, 98]. While autografts are the gold standard for repairing large bone defects, they have several drawbacks, such as limited supply of and the need for a second surgery, circumvented by synthetic bone graft substitutes [24, 35]. Autografts have remained the preferred treatment for bone defects, primarily due to the presence of osteogenic cells within the donor tissue, which will stimulate bone healing in the defect [3, 24]. Osteogenic drugs can be incorporated into synthetic bone grafting materials, however, to enhance their effectiveness and make them more comparable to autografts [13, 87]. These synthetic materials are often formed from calcium sulfate (CS), poly(methyl methacrylate) (PMMA), calcium phosphate (CaP), tricalcium phosphate (TCP), hydroxyapatite (HA), or biocompatible polymers [23, 40, 98-101].

A major reason why large IBDs are so difficult to treat is that the extent and type of infection can vary widely, and the presence of the bacteria delays healing [19, 23, 30]. In the worst cases, planktonic bacteria in the wound attach themselves to surfaces and form a biofilm, which is a special arrangement of bacterial cells that behave as a community and secrete a polymeric coating [19, 30]. This coating acts as a barrier to most antimicrobials, rendering them ineffective at safe systemic concentrations [28]. By delivering antibiotics locally, much higher concentrations can be achieved at the site of interest than would be possible via systemic delivery [98, 102, 103].

In clinical practice for treating IBDs, the first goal is to clear the infection before bone repair is even attempted [6, 22]. There are two primary reasons for this separation: one is to limit the foreign surfaces within the defect that could act as niduses for bacterial colonization, and the other is to avoid trying to heal bone in such a harsh environment [23, 104]. The infected milieu will cause decreases in the local oxygen content and pH, as well as promote chronic inflammation [30]. Bacteria also contribute to bone resorption, which would counteract any osteogenic treatment and result in less effective healing [30].

The present research focused on achieving a temporal separation between antimicrobial and osteogenic drugs released from a previously developed synthetic bone grafting material [105]. This separation will mimic clinical practice and allow time for the antimicrobial to treat the infection prior to the osteogenic drug stimulating bone healing. A two-part composite system composed primarily of CS was developed that utilized a moldable outer shell, which provided the prolonged release of the antimicrobial drug, around a solid core that afforded delayed release of the osteogenic drug. Hyaluronic acid (HY) was used to make the CS shell moldable and was chosen based on previous results and because it is already being used in FDA-approved wound healing applications (OrthoviscVR) [105]. Two antibiotics, vancomycin and gentamicin, were initially used to provide a material capable of combating Gram-positive and Gram-negative bacteria. This two-part method combines the advantages of moldable and pre-set bone filler systems, allowing the material to conform to irregular defects while maintaining extended release of bioactive drugs. The shell/core method was evaluated for achieving temporal separation of drug release profiles as well as bioactivity of the released antimicrobial on *Staphylococcus aureus* (*S. aureus*) bacteria.

4.2 Materials & Methods

4.2.1 Poly(lactic-co-glycolic acid) (PLGA) Microsphere Fabrication

PLGA microspheres (PLGAms) were fabricated using a double emulsion technique (W/O/W). The oil phase consisted of 13% PLGA (w/v) (50:50 L:G, 0.55-0.75 I.V.; Durect Corp.) dissolved in dichloromethane (DCM). The first emulsion was created

by adding 0.11 vol% of phosphate-buffered saline (PBS), either blank or drug-loaded (100 mg/ml vancomycin), to the PLGA-DCM solution and sonicating for 10 seconds at 25 W. This W_1/O emulsion was added to 800 ml of deionized water (containing 1% polyvinyl alcohol and 4% NaCl) in a dropwise manner and then homogenized at 2000 rpm for 3 minutes to create the second emulsion. The resulting suspension of microspheres was stirred overnight at 600 rpm to evaporate the solvent. The microspheres were collected by centrifugation and washed using deionized water before being frozen and lyophilized. The mass of drug in microspheres was obtained by first dissolving 10 mg of microspheres in 1 ml DCM, mixing with 1 ml PBS, and centrifuging at 123*g* for 5 minutes. The supernatant was analyzed by measuring the absorbance at 280 nm (Powerwave HT, Biotek), with subsequent comparison to known standards. The drug loading and encapsulation efficiency were calculated as:

$$\%\ Drug\ Loading = \left(\frac{Mass\ of\ Drug\ in\ Microspheres}{Mass\ of\ Microspheres}\right) \times 100$$

$$\%\ Encapsulation\ Efficiency = \left(\frac{\%\ Drug\ Loading}{\%\ Theoretical\ Drug\ Loading}\right) \times 100$$

4.2.2 General Sample Preparation

The following materials were used in fabricating the composite bone filler samples: CS (98% hemihydrate; Sigma), hyaluronan (HY) (MW 1.323 x 10^5 Da; LifeCore Biomedical), simvastatin (Haorui Pharma-Chem), gentamicin (Sigma), and vancomycin (Sigma-Aldrich). Compositions used for the core and shell components in the various studies can be seen in Tables 4.1 and 4.2. The "blank" formulations were used for background correction in the drug analyses and are not shown in the subsequent figures.

Table 4.1: Composition of the shells and cores of free vancomycin samples (expressed in wt%). X/Y: X=drug component of shell, Y=drug component of core. V=vancomycin, G=gentamicin, and S=simvastatin.

Sample	Layer	% CS	% HY	% Sim	% Vanc.	% Gent.
V+G/5S	**Shell**	87	10	0	1.5	1.5
	Core	90	5	5	0	0
V+G+2.5S/2.5S	**Shell**	84.5	10	2.5	1.5	1.5
	Core	92.5	5	2.5	0	0

Table 4.2: Composition of the shells and cores of PLGA microsphere samples (expressed in wt%). X/Y: X=drug component of shell, Y=drug component of core. V=vancomycin, S=simvastatin, B=blank, N=no shell present.

Sample	Layer	% CS	% HY	% Sim	% Vanc PLGAms	% Blank PLGAms
V/S	**Shell**	75	10	0	15	0
	Core	90	5	5	0	0
V/B	**Shell**	75	10	0	15	0
	Core	95	5	0	0	0
B/S	**Shell**	75	10	0	0	15
	Core	90	5	5	0	
B/B	**Shell**	75	10	0	0	15
	Core	95	5	0	0	0
N/S	**Shell**	No shell present				
	Core	90	5	5	0	0
N/B	**Shell**	No shell present				
	Core	95	5	0	0	0

The shell materials listed for each composition were mixed thoroughly before the addition of 100-125 µl deionized water; the volume of water was adjusted to keep the consistency of the moldable filler uniform. Shell consistency after mixing was similar to a moldable dough that was not sticky and could be rolled or formed into a desired shape as described previously [105]. Core pieces were prepared by loading 300 mg of materials into a cylindrical Delrin mold (6.5 mm deep x 3.2 mm diameter) followed by drying at 40 °C overnight. The moldable shells, also composed of 300 mg total material, were

wrapped around the pre-dried cores and used immediately for experiments to ensure the composite remained moldable (Figure 4.1).

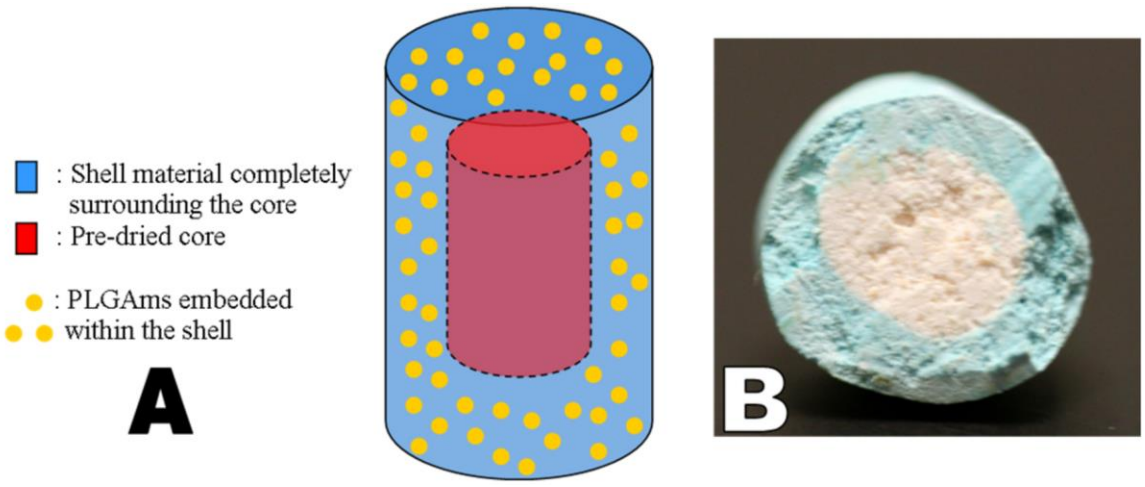


Figure 4.1: A) Schematic representation of the shell-core structure of bone filler samples. Note: illustration not to scale. B) Image showing cross-section of a bone filler sample in which the shell material had been stained blue.

4.2.3 Release Profiles

Release studies were performed by incubating the two-layered samples in 4 ml of PBS at 37°C with gentle shaking. The solution was changed every day for the free antibiotic samples and every three days for samples containing PLGAms. For comparison, 30 mg of vancomycin-loaded PLGA microspheres alone were shaken in 4 ml of PBS at 37°C, and the solution was changed every day for four days and every third day afterwards. The collected supernatants were frozen until analysis. Groups with comparable release results not shown to enhance readability of figures.

Vancomycin concentrations were determined by measuring absorbance at 280 nm, and gentamicin concentrations were determined by reaction of *o*-phthaldialdehyde and measuring absorbance at 333 nm [106]. Simvastatin was measured by high-performance liquid chromatography (HPLC; Hitachi Primaide, C18 column, 5 µm). The mobile phase consisted of 70% acetonitrile and 30% water (containing 1% trifluoroacetic acid), and absorbance was read at 240 nm. Before analysis, all supernatants were filtered (0.45 µm) before the addition of 0.25 mg/ml ethylenediaminetetraacetic acid (EDTA) to prevent precipitation of calcium.

4.2.4 Antibacterial Bioactivity

To test the effectiveness of antibiotic released from the bone filler samples, a traditional Kirby-Bauer (KB) study was performed as well as a modified KB study in which the entire samples were used. For the conventional KB study, 5 µl of release supernatant were soaked into a filter paper disc and placed on a blood agar plate seeded with *S. aureus* (ATCC 25923; McFarland standard 0.5). The resulting zone of inhibition (ZOI) was measured after incubating for 24 hours. In the modified KB study, the plate was seeded with the same amount of *S. aureus*, but the entire core-shell bone filler sample was placed directly on the agar (Figure 4.2) for 24 hours before the ZOI was measured. The sample was then transferred to a newly seeded agar plate and again incubated for 24 hours, after which the ZOI was measured and the process repeated until no inhibition of bacterial growth was seen. The total area of inhibition was measured using NIH ImageJ.

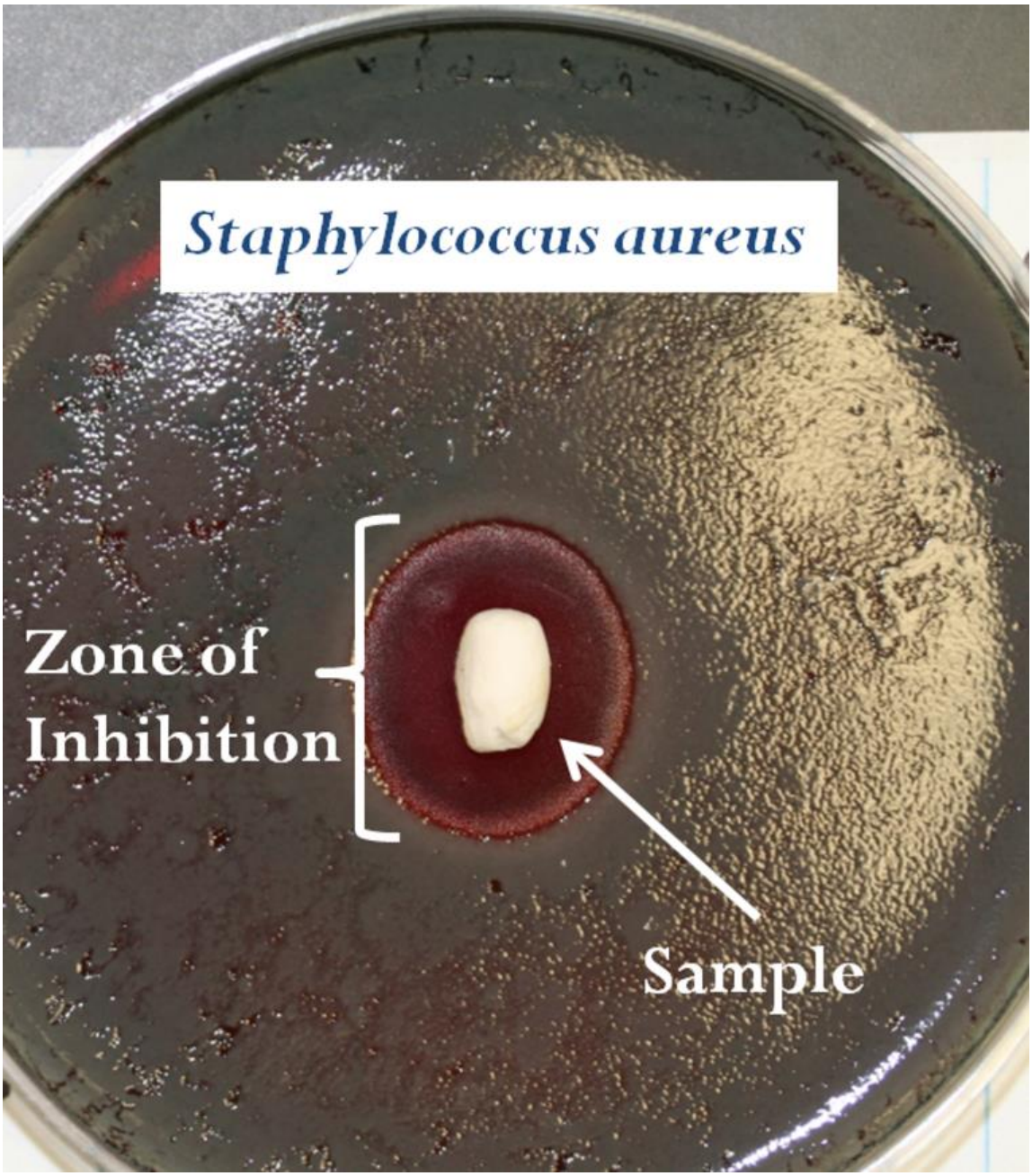


Figure 4.2: Setup of the modified Kirby-Bauer study in which an entire shell/core bone filler sample was tested instead of filter paper loaded with release supernatant.

4.2.5 Statistical Analysis

One- and two-way analysis of variance (ANOVA) was performed using Prism software (GraphPad). Statistical significance was determined at p values less than 0.05. Tukey's post-hoc test was performed as needed.

4.3 Results

4.3.1 Release Profiles

Release profiles for vancomycin and gentamicin loaded directly into the CS matrix can be seen in Figure 4.3. Around 80-90% of the drug was released within the first day, and the remaining drug was slowly released over the next two to three days. Instantaneous concentrations were as high as 330 µg/ml at day 1 but dropped to near 0 µg/ml by day 3. There was no statistical difference in release concentrations for the two antibiotics, but a significant effect with respect to concentration and time was shown ($p < 0.0001$). Full release results can be seen in Supplemental 1. Vancomycin release profiles from PLGA microspheres alone and from PLGA microspheres within the composite bone filler material can be seen in Figure 4.4. PLGA microspheres had a 6% vancomycin loading and a 17% encapsulation efficiency. A 33% smaller burst release compared to free loaded vancomycin was seen during the first day, and drug was slowly released from the microspheres embedded within the bone filler material over the course of the next six weeks. The concentration of vancomycin stayed above 10 µg/ml for the first 30 days and above 1 µg/ml throughout the full course of the material's degradation. The profile for release of simvastatin from the bone filler can be seen in Figure 4.4. When simvastatin was loaded into the pre-dried core with the shell acting as a barrier, release was delayed for around 12 days, after which the drug was slowly released for the next four weeks. Cumulative release profiles for vancomycin loaded into PLGAms alone, vancomycin loaded into the bone filler samples, and simvastatin can be seen in Figure 4.4B. Statistical analysis of both the instantaneous and cumulative release profiles showed significant effects ($p < 0.0001$) for the drug type, time interval, and interaction between drug and time.

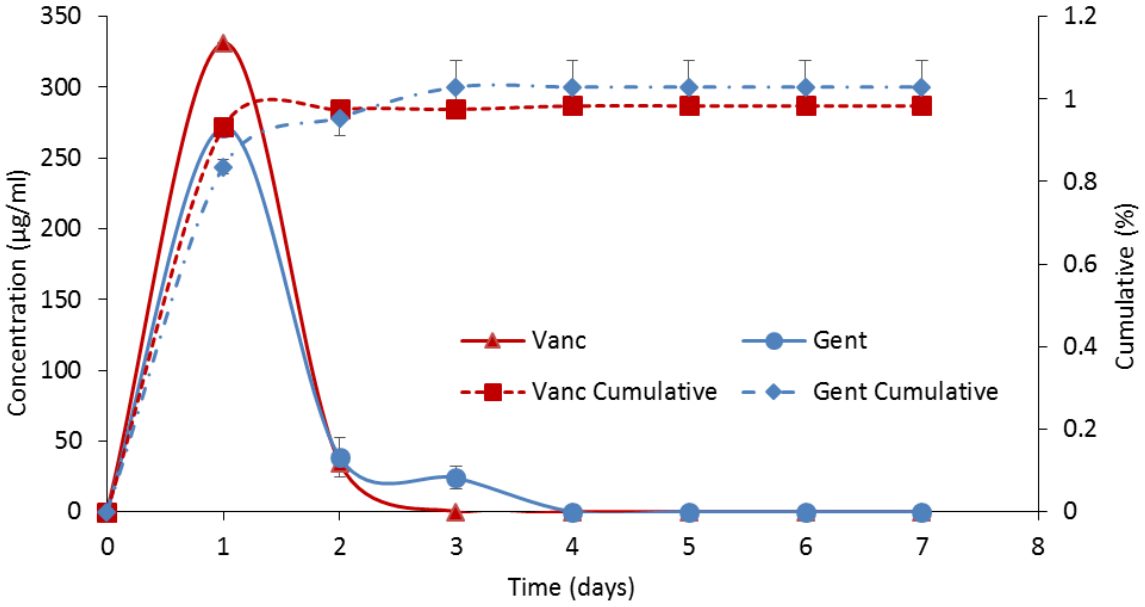


Figure 4.3: Release profiles for gentamicin and vancomycin loaded directly into the CS matrix (V+G/5S from Table 1). Data are mean ±SEM, n=4.

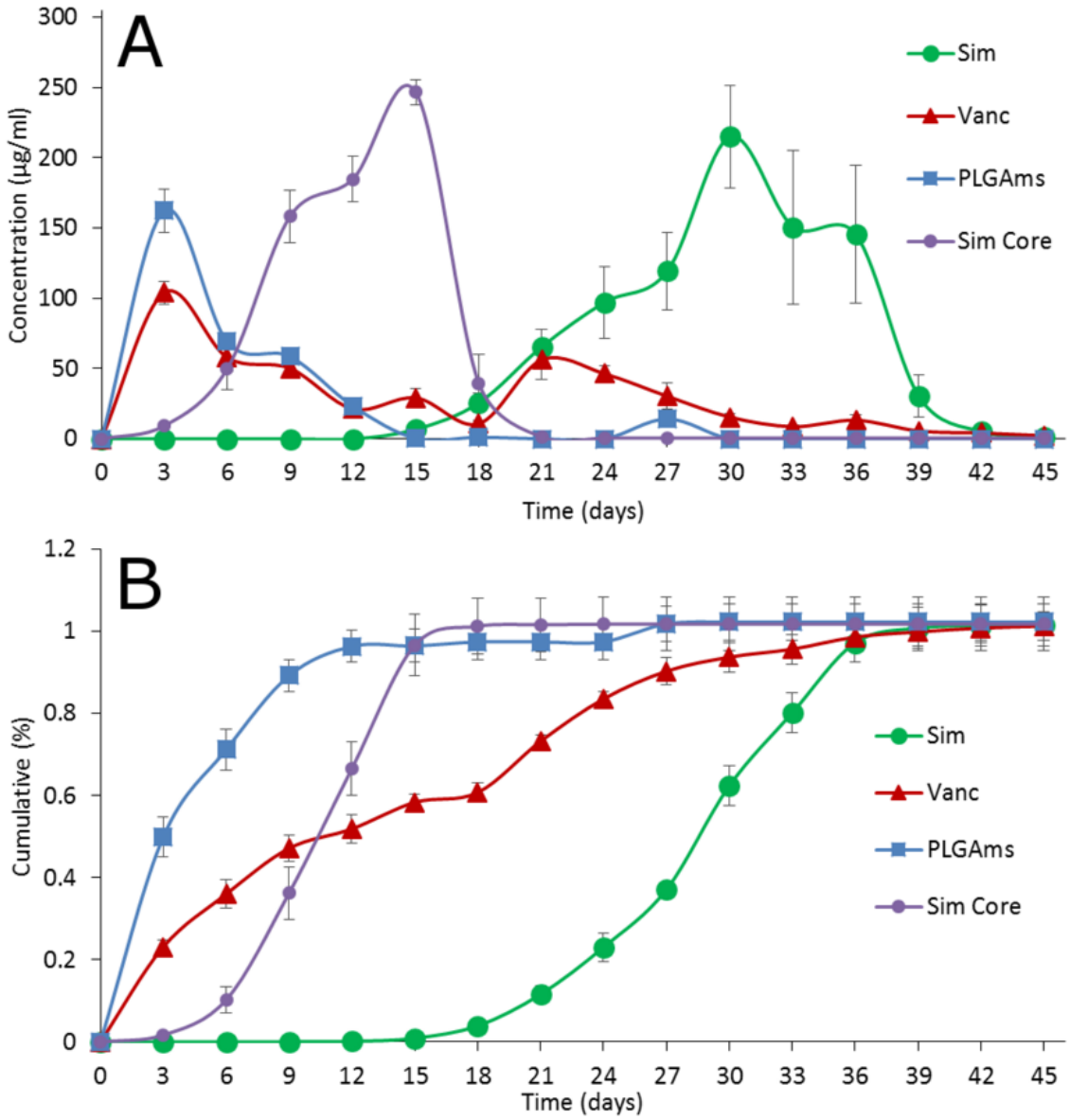


Figure 4.4: A) Instantaneous and B) cumulative release profiles for PLGA microspheres alone (PLGAms) and the two-layered bone filler. Sample composition was V/S as seen in Table 2. Data are mean ± SEM n=5.

4.3.2 Antibacterial Bioactivity

Bioactivity of the released antibiotic against *S. aureus* can be seen in Figure 4.5. The traditional Kirby-Bauer results showed inhibition of bacterial growth from the supernatant for almost 1 week. When composite bone filler samples were used instead of supernatant-soaked filter paper, the total area of inhibition was approximately six times larger. The area of inhibition for complete samples was not only significantly larger than

that for the supernatant KB, but antimicrobial activity lasted for at least two weeks. There was no statistical difference seen between the V+G/2.5S and V+G+2.5S/2.5S groups through 12 days. The solid line in Figure 4.5 represents the ZOI measured in a traditional KB study with 100 µg/ml vancomycin for comparison. Supernatants alone maintained the same ZOI for one day while the composite bone fillers maintained a comparable ZOI to the 100 µg/ml vancomycin control for around 12 days. Two-way ANOVA of the area of inhibition results for V+G/2.5S, V+G+2.5S/2.5S, and the supernatant KB showed statistically significant differences with respect to sample type, time, and the interaction between the sample type and time ($p < 0.0001$).

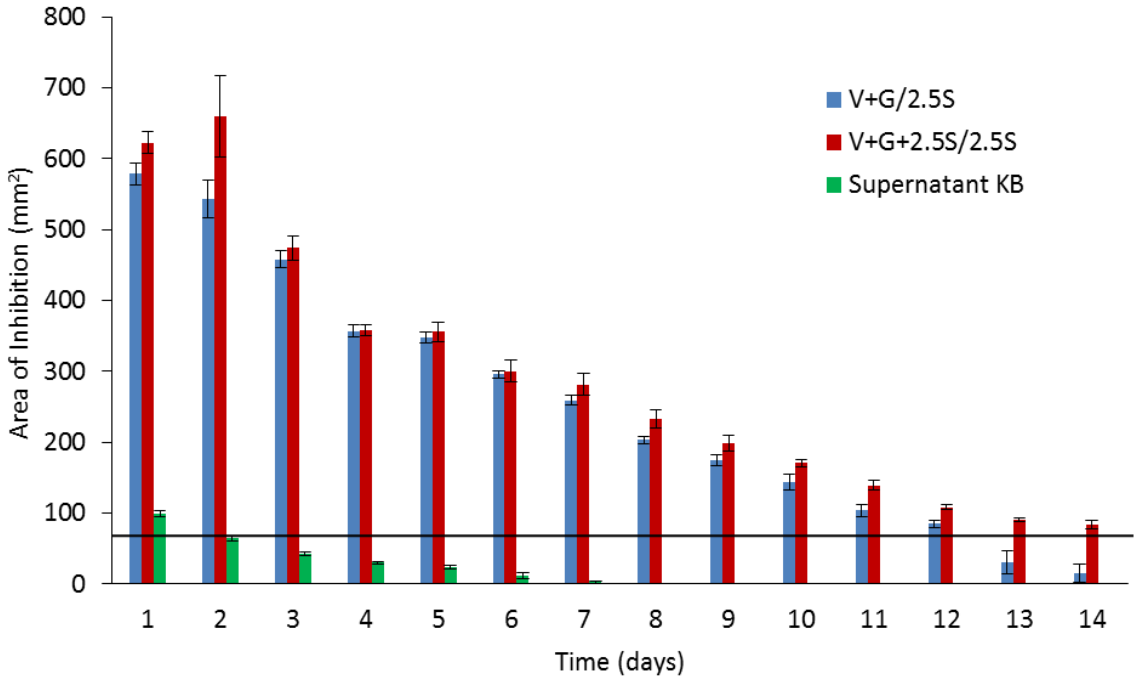


Figure 4.5: Results from the traditional Kirby-Bauer experiment (supernatant KB, V+G+2.5S/2.5S) and the modified KB study (V+G/2.5S and V+G+2.5S/2.5S). Solid line represents the area of inhibition for 100 µg/ml vancomycin for reference. Data are mean ±SEM, n=4-12.

4.4 Discussion

The current clinical treatment for IBDs requires extensive debridement until the infection has been eliminated followed by bone grafting [6, 22]. This approach, which requires multiple procedures, is needed because implanting the bone graft in the presence

of an infection greatly reduces its potential for success, and the graft itself can become a site for bacterial attachment [26, 107]. Along with debridement, it is common for systemic antibiotics to be administered for 4-6 weeks to ensure the infection has been eliminated [10, 44, 66, 98]. This long treatment time is necessary due to the inefficiency of the delivery method at achieving effective concentrations at the infected site [108]. With localized delivery, much higher doses of the required antibiotics can be obtained without the danger of toxicity that can be associated with systemic delivery [98, 108]. Local delivery of antibiotics also avoids first pass metabolism, which is a major problem for oral delivery, and can result in a larger amount of the administered drug reaching the infected site than does IV administration [108]. While no standard timeframe has been established for localized treatment of infections, it is generally agreed upon that antibiotics should remain above minimum inhibitory concentration (MIC) values for as long as possible, usually 4-6 weeks, to increase the likelihood of eliminating the infection and reducing resistance development [10, 44, 63, 66, 98].

Vancomycin loaded directly into the CS shell of the composite bone filler diffused into solution very quickly, with the majority of the payload being released over the course of the first two days. This burst release is consistent with previous literature in which CS, CaP, or PMMA beads impregnated with antibiotics released 80-90% of the loaded drug within the first few days [11, 40]. This direct loading method, which can achieve very high local concentrations for very short periods of time, is not practical for a device intended for implantation without removal, because the biomaterial could act as a substrate for bacterial colonization after the majority of the drug has been released. In contrast, vancomycin loaded into PLGA microspheres was released more slowly, over the course of 6 weeks, when the microspheres were loaded into the composite bone filler, achieving the target timeframe of 4-6 weeks. The concentrations released from the bone filler samples remained above the MIC_{90} for *S. aureus* (approximately 1 μg/ml for vancomycin) for several weeks, but the duration could potentially be extended by using slower degrading microspheres or incorporation of antibiotic-loaded microspheres into the core of the samples as well as the shell [109].

An infected wound site is an inhospitable environment for drugs to be released into or for bone to heal properly [19, 23, 30]. This site will have numerous types of host

cells, including neutrophils, monocytes, and macrophages [30]. While these cells are part of the body's natural defense against bacterial infections, the resulting inflammation, such as would be the case with a persistent biofilm that the cells cannot remove by themselves, can cause more damage than good [30]. Prolonged inflammation will cause host response cells to be present in greater numbers and for a longer time period than usual and can lead to tissue death and accelerated degradation of implanted materials through the release of reactive oxygen species and proteolytic enzymes [30, 110-113]. Along with the host phagocytic cells, bacterial colonies will release proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 [20, 30]. These inflammatory cytokines activate osteoclasts to resorb bone and/or inhibit osteoblastic activity to disrupt the balance of bone removal/formation [30].

To avoid the destruction of drug or the inefficiency of healing bone in a hostile environment, release of the osteogenic molecule simvastatin was delayed from the present bone filler material. This delay gives the antimicrobial drugs time to act on the infection before bone healing would be stimulated, potentially increasing the effectiveness of the drug and quality of bone healing [114, 115]. Previous studies by Chen et al. investigated the use of BMP-2 and BMP-7 in infected femoral defects with and without systemic antibiotic treatment [114]. The results showed that, while high doses of the osteoinductive proteins were able to stimulate some bone formation without antibiotics, there was a significant increase in bridging of the defects when the treatment was combined with antibiotics, which led the authors to conclude that the timing for administering each drug should be investigated [114]. Thus, in a similar manner to current clinical practice, the two required treatments for treating IBDs could be released at different times from the same device.

In research conducted by Guelcher et al., a polyurethane scaffold was loaded with BMP-2 and vancomycin for the treatment of infected, critically-sized femoral defects in rats [63]. These injectable scaffolds showed an initial burst release of BMP-2 followed by sustained release over the course of 3 weeks; vancomycin had a much smaller burst release with sustained release over the course of the 3 weeks evaluated. The simultaneous release of an osteogenic molecule and antimicrobial stimulated significantly more bone formation than did release of BMP-2 alone. This positive result was

attributed to the dual release of drugs, which allowed the infection to be controlled while bone healing was taking place, a critical step in achieving bone healing in an infected site [1, 63]. Pauly et al. compared two different doses of simvastatin and one dose of BMP-2 in a rat tibial fracture model [116]. The different treatments were released locally from a polymer-coated implant and the bones evaluated mechanically [116]. The low and high doses of locally released simvastatin resulted in comparable maximum loads and torsional stiffness of the evaluated tibias [116]. The time course for testing the healing bones in the study was 28 and 42 days but would need to be longer if there was a critical-sized bone defect. To be most effective in a large defect, simvastatin would need to be delivered for several weeks over the course of bone healing [54, 117]. The ideal time delay before the stimulation of bone healing is unknown and would likely depend on the type and severity of the infection as well as the antimicrobial treatment. A main consideration would be to allow enough time for the wound site to return to baseline physiological levels, *e.g.*, pH, cell populations, and vascularity, so the drug is not wasted and bone healing can occur effectively.

In work by Strobel et al., a polymeric coating was developed to release gentamicin, BMP-2, and insulin-like growth factor I (IGF-I) in a time-dependent manner [2]. Gentamicin and IGF-I were released quickly at first and followed by slow release of BMP-2 over several weeks [2]. An additive effect was seen on the metabolic activity of primary osteoblasts from the multi-drug releasing implant compared to single drug releasing implants [2]. Both the antimicrobial and growth factors were released simultaneously *in vitro*, although at different rates. If these drugs were to be released into a contaminated bone defect, as is often the case in large bony defects, it is likely that the same effects on local cells would not occur due to the physiologically altered environment [20, 30].

In the present study, the delayed release of simvastatin was achieved in two distinct ways: 1) the physical barrier of the outer shell around the simvastatin-loaded core and 2) the delayed physiological effect on bone from simvastatin. The shell and core design of the bone filler utilized a physical barrier to diffusion of simvastatin into solution that was capable of delaying release of the drug for around 12 days. The other cause of delay is innate to the function of the simvastatin *in vivo*. From the time that

simvastatin begins to act on cells, there is a 1-2 week delay before increased levels of BMPs are seen [118]. The physical shell method is necessary because simvastatin released into an infected site would still encounter a harsh environment, even if its effects would not be seen for 1-2 weeks. Use of the shell and core method has another advantage in that the system can have both moldable and non-moldable components. This allows the outer shell to be moldable and conform to irregularly shaped defects, while the core can be pre-dried and remain in the defect site for a longer period of time.

Bioactivity of vancomycin released *in vitro* was shown in two different types of Kirby-Bauer experiments, confirming that the ability to kill bacteria was retained when released for several weeks *in vitro*. The traditional KB assay showed inhibition of *S. aureus* for almost a week, while the modified version in which entire bone filler samples were used showed inhibition for around two weeks and at significantly larger areas of inhibition. The increased area of inhibition seen in the modified KB, as high as six times that of the traditional KB, can be attributed to several factors. The first is that there was a significantly larger loading of antimicrobial drug in the entire sample than in the filter paper discs used in the traditional KB study. The traditional KB study involved loading filter paper discs with 5 µl of release supernatant, which at its highest concentration would result in less than 1 µg of vancomycin being in the disc. Even at high concentrations, the actual amount of antimicrobial drug being loaded into each disc and diffused through the relatively large agar plate will be small. The full samples, however, were initially loaded with 3.75 mg of vancomycin that could diffuse into the blood agar plates. It is unlikely that all of the drug would diffuse out of the sample into the agar because a new agar plate was used for each time point and significant inhibition was seen for two weeks. The modified KB study was conducted in order to compare the release profiles and effectiveness of vancomycin release in different environmental conditions. When placed in sink conditions, vancomycin, being a hydrophilic drug, will diffuse out of the CS matrix quickly. While this is a commonly used *in vitro* test condition, it does not capture the conditions of an *in vivo* environment. Because it would be extremely difficult to replicate all of the *in vivo* conditions, an alternate test was used, in which the samples, as could be implanted into wound sites, would be releasing into a agar matrix under warm and humidified conditions.

4.5 Conclusions

A moldable composite bone filler material was shown to release a bioactive antimicrobial agent in a controllable manner for six weeks while postponing the release of an osteogenic drug for 12 days. A delay was intentionally designed into the system to avoid drug loss and inefficient healing associated with attempting to repair a bone defect in the presence of an infection. The temporal separation in the release of simvastatin was achieved by using a two part system comprising a moldable outer shell that also acted as a barrier and a pre-formed core. The promising results seen from this material warrant further investigation of the bone filler in a rigorous infected segmental defect model to verify the effectiveness of the treatment *in vivo*.

Chapter 5 Testing of a Bioactive, Moldable Bone Graft Substitute in an Infected, Critically-Sized Defect Model

5.1 Introduction

Large infected bone defects (IBDs) are complicated to treat and require multiple procedures for a successful outcome [1, 2, 112, 113]. These wounds are typically caused by high energy trauma, which can damage the surrounding soft tissue, underlying hard tissue, and often lead to infection [1, 2, 112, 113]. IBDs thus have a high military significance, with approximately 80% of injuries sustained during Operations Enduring Freedom and Iraqi Freedom being the result of explosions and 50-85% of these becoming infected [14-16]. Although not as common, infections occur in approximately 13-18% of open fractures in civilians [9].

Current treatment methods begin with extensive surgical debridement and irrigation combined with systemic antibiotic delivery [2, 10, 11, 105]. Once the infection has been eliminated, the process of healing the tissue can begin, typically with bone grafting [2, 10, 11, 105]. The most common and successful bone graft is the autograft, which has been the gold standard for over 100 years [6, 24]. The success of autografts is due largely to the inclusion of living osteogenic cells that have no risk of rejection because they come from the patient's own body [3]. Even with the success of autografts, however, there are still several drawbacks, such as the need for a secondary surgical site to obtain the donor bone, limited grafting material, and donor site morbidity [35].

To improve upon current treatment methods, materials that can replace bone autografts are being developed [3, 6, 40]. The most common materials for these bone graft substitutes are calcium sulfate (CS), calcium phosphate cements (CPC), hydroxyapatite (HA), polyurethane (PUR), and poly(lactic-co-glycolic acid) (PLGA) [41, 42, 119, 120]. These materials are often loaded with antimicrobial or osteogenic drugs to increase their effectiveness at treating large IBDs [41, 121]. Newer treatments have focused on delivering the antibiotics locally at the site of infection to decrease the amount of drug needed and more effectively combat the infection [116, 122]. One common method of local delivery involves loading antimicrobial drugs into pellets or soaking pellets in drug solutions that can then be packed into the wound site for local delivery

[55, 123, 124]. The problem with this method is that there is little control over drug release, and there is often a large burst of drug within the first day or two followed by an extended release of sub-therapeutic concentrations [23, 40, 55, 102]. Nondegradable pellets and those degrading too slowly must eventually be removed so they do not create drug-resistant bacteria or act at niduses for further infection [23, 40, 55, 102].
The current work focused on the *in vivo* testing of a drug-releasing, CS-based, moldable composite bone graft substitute that was previously developed. This composite material was designed to release antimicrobial and osteogenic drugs in a temporally separated manner to allow time for the infection to be treated prior to stimulating bone healing. The effectiveness of the composite material was evaluated in an infected, critically sized rat femoral defect model by analyzing the survival of animals, radiography, microcomputed tomography, and histology.

5.2 Materials & Methods

5.2.1 Moldable Bone Filler

The moldable bone filler was previously developed as a two part system consisting of a pre-formed core surrounded by a moldable shell [105]. The filler consisted of calcium sulfate (CS) (98% hemihydrate; Sigma), hyaluronan (HY) (MW 1.323×10^5 Da; LifeCore Biomedical), simvastatin (Harorui), and PLGA microspheres containing vancomycin (Sigma). Vancomycin was encapsulated into PLGA (50:50 L:G, acid-terminated, 0.55-0.75 dl/g I.V.; Durect Corp.) using a double emulsion method as previously published. Blank (drug-free) microspheres were prepared the same way except for the omission of antibiotic from the first water phase. The different core and shell compositions used can be seen in Table 5.1.

Core pieces were prepared in batches by mixing 3 g dry materials with 1.4 ml deionized (DI) water and placing the putty into cylindrical Delrin molds (6.5 mm deep x 3.2 mm diameter) where they were allowed to dry overnight at 40 °C overnight. The shells were composed of 200 mg total material (CS, HY, and PLGA). The dry materials were mixed thoroughly before the addition of 100-125 µl DI water, depending on the

composition. The now moldable shell material was wrapped around the pre-dried core immediately prior to implantation.

Table 5.1: Composition of bone filler composites evaluated.

		weight % component				
		CS	**HY**	**Blank-PLGAms**	**Vanc-PLGAms**	**Simvastatin**
Blank	Shell	75	10	15	0	0
	Core	100	0	0	0	0
Antimicrobial	Shell	75	10	0	15	0
	Core	100	0	0	0	0
Osteogenic	Shell	75	10	15	0	0
	Core	94	0	0	0	6
Antimicrobial + Osteogenic	Shell	75	10	0	15	0
	Core	94	0	0	0	6

5.2.2 Infected Femoral Defect Model

All animal studies were conducted at the University of Kentucky in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC). Effectiveness of the composite bone filler *in vivo* was investigated using an established model of critically sized infected femoral defects in adult male Sprague-Dawley rats (375-400 g) [114, 125]. The diaphysis of one femur was exposed using a lateral approach and fixed using a polyacetyl plate (25 x 5 x 2.5 mm) and 6 Kirschner wires (K-wires; 1.6 mm, threaded; Synthes). Using guide marks on the fixation plate, a 6 mm defect was then made using a surgical bur as seen in Figure 5.1A. A bovine type I collagen sponge (Stryker Biotech) containing 10^4 CFU of *S. aureus* (ATCC 25923) was placed into the defect and the wound closed in layers. After either 6 hours (acute infection) or 2 weeks (chronic infection), the wounds were surgically debrided and the respective treatment applied, meaning that either a moldable bone filler was implanted or left empty as a control (Figure 5.1B). Animals were censored from the study before the clinical endpoint if loss of fixation, dehiscence, or tissue necrosis around wound site was

observed. The test groups evaluated can be seen in Table 5.2. Systemic administration of antibiotics involved daily subcutaneous injections of ceftriaxone (50 mg/kg) for 1 month.

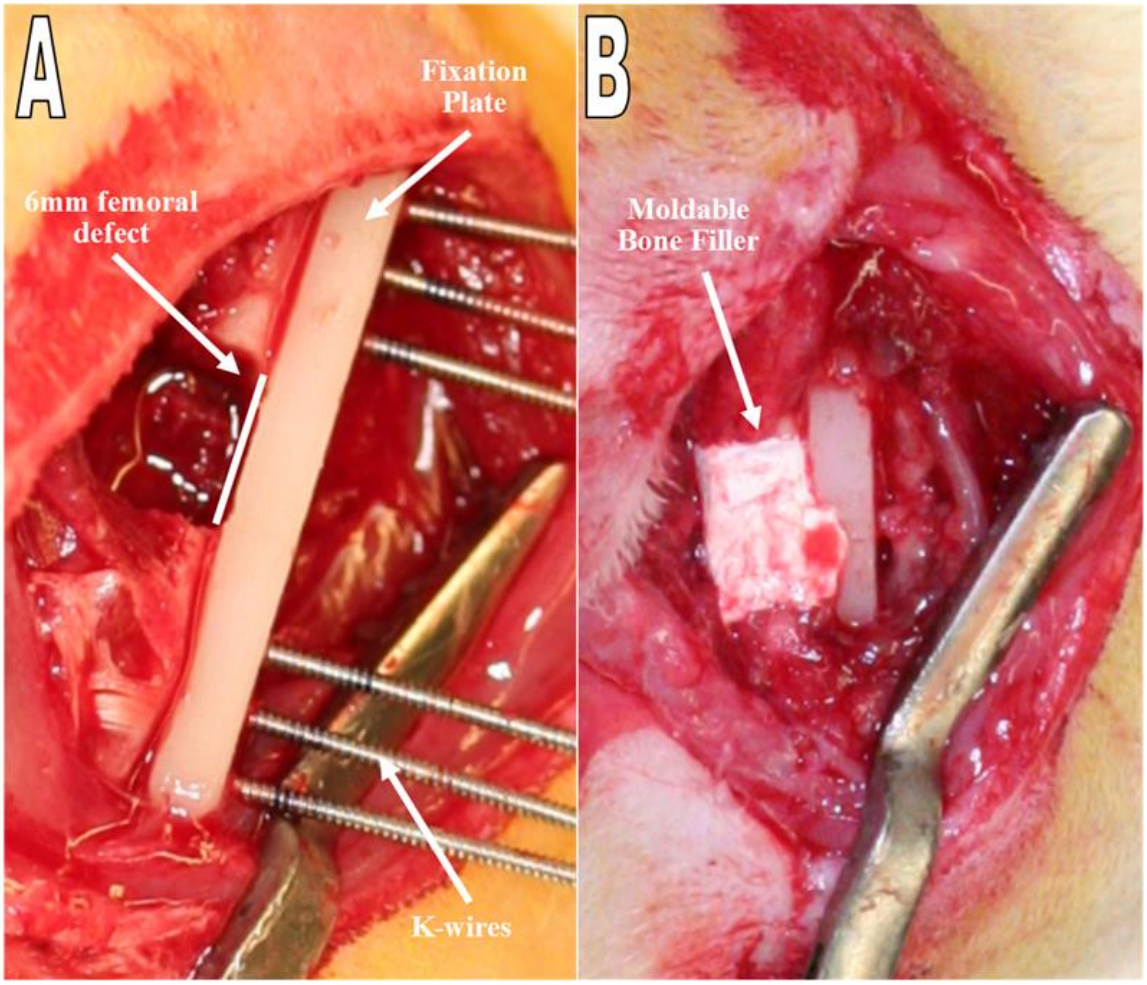


Figure 5.1: Segmental femoral model used. A) Critical-sized defect with fixation plate and K-wires. B) Plated rat femur showing moldable bone filler implant after debridement.

Table 5.2: Study design for the chronic and acute infection models. Key codes: NI - non-infected, CI - chronically infected, AI - acutely infected.

	Endpoint	
Group Description	**4 wk**	**12 wk**
Empty	NI, CI, AI	NI, CI, AI
Blank	NI, CI, AI	NI, CI, AI
Osteogenic	NI, CI, AI	NI, CI, AI
Antimicrobial	CI, AI	CI, AI
Antimicrobial + Osteogenic	CI, AI	CI, AI
Osteogenic + Systemic Antibiotics	CI, AI	CI, AI

5.2.3 Analysis

Animals were observed daily for the first 7 days post-operatively and weekly until the primary endpoint at either 4 or 12 weeks post-debridement. Femurs were then removed by disarticulation, leaving the muscle in place to minimize disruption of the defect site, radiographed, and fixed in 10% buffered formalin for 2 weeks. The femora were scanned by microcomputed tomography (microCT; Scanco μCT40) using a voxel size of 30 μm and X-ray settings of 70 kVp and 114 μA. ImageJ was used to evaluate bone area within the defect site from radiographs using the K-wires as guides and tracing radiopaque material within the region of interest.

Wax-it Histological Services embedded the samples from all the groups in poly(methyl methacrylate) (PMMA) and sectioned along the longitudinal axis of the femur to allow visualization of the bone defect. Sections were cut at 10 μm, deplastinated, and stained using hematoxylin and eosin (H&E) or Goldner's trichrome. Undecalcified specimens were fixed in 10% neutral buffered formalin, dehydrated in graded alcohols, and embedded in methyl methacrylate. Sections were obtained with a band saw and ground to approximately 60-80 μm in thickness using an Exakt cutting-grinding system (Exact 310 CP, Exact Technologies, Oklahoma City, OK). Stained sections were imaged using a Nikon Eclipse E600 microscope (Nikon) with an attached Olympus DP71 camera (Olympus).

5.2.4 Statistics

One-way analysis of variance (ANOVA) was performed using Prism software (GraphPad) for analysis of bone area within the defect sites. Statistical analysis was determined at p values less than 0.05 and Bonferroni's post-hoc test was performed when needed. Survival was assessed using Kaplan-Meier curves and compared using log-rank (Mantel-Cox) test. Survival in this case refers to the rate of censoring animals from the study using criteria defined in 5.2.2.

5.3 Results

5.3.1 Survival

The effect of local antibiotic treatment on survival of the animals compared to those without any antibiotics can be seen in Figure 5.2A. Note that while survival curves are shown, no animals actually died from the procedures or infection, but the figures show the percentage of animals that were euthanized for humane reasons or at the request of the attending veterinarian. Locally released vancomycin increased survivorship by 58% over the course of 12 weeks compared to the control groups ($p=0.001$). A comparison of survival between chronically infected animals treated with the locally released antibiotics and those treated with one month of systemic antibiotics showed that there was no statistical difference between the rates of survival, although the local antibiotic groups had a final survival percentage approximately 24% higher than those treated with systemic antibiotics (Figure 5.2B). Animals treated with systemic antibiotics did have a slightly higher survival rate than those not receiving any type of antimicrobial ($p < 0.05$, data not shown). For the acute (6 hr) infection model, no significant difference was seen between animals that received no antibiotics and those treated with locally delivered antibiotics, although animals that received local antibiotics had a final survival percentage approximately 22% higher (Figure 5.2C). Through the course of 12 weeks, chronically infected animals receiving no antibiotics fared worse than did those in the acute infection groups (Figure 5.2D). Survivorship among acutely infected animals was approximately 40% higher compared to chronically infected animals ($p < 0.05$).

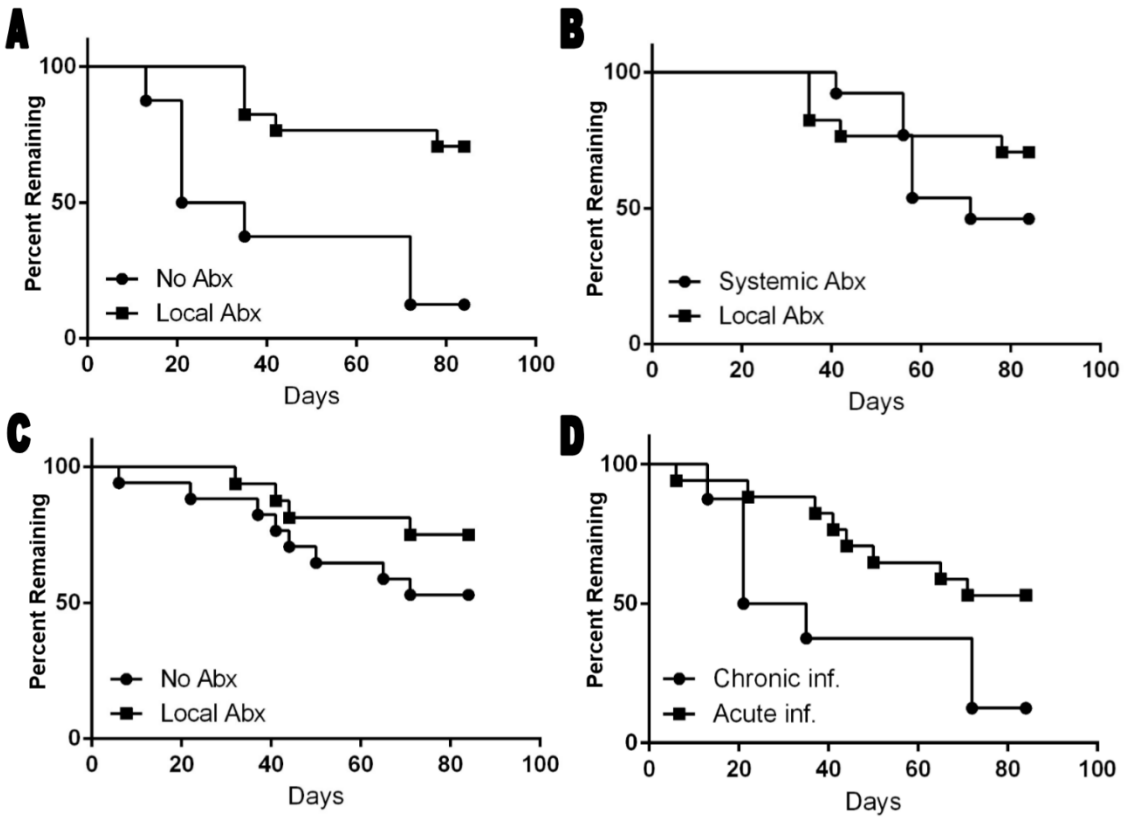


Figure 5.2: Survival curves over 12 wk. A) Effect of locally released antibiotics (Abx) in chronically infected animals. B) Effect of local vs. systemic administration of antibiotics in chronically infected animals. C) Effect of locally released antibiotics in acutely infected animals. D) Comparison of acutely (6 hr) and chronically (2 wk) infected animals with no antibiotic treatment.

5.3.2 MicroCT and Histology

Representative microCT images for empty, blank, osteogenic, antimicrobial, and osteogenic + antimicrobial treatment groups for the acute and chronic infection models can be seen in Figures 5.3 and 5.4, respectively. Acutely infected animals that received only antimicrobial treatment had little bone formation but did show signs of maintaining the cortical bone adjacent to the defect in the presence of the infection. Animals treated with antimicrobial and osteogenic drugs showed the most bone formation, but it was still unable to bridge the defect in the presence of the infection.

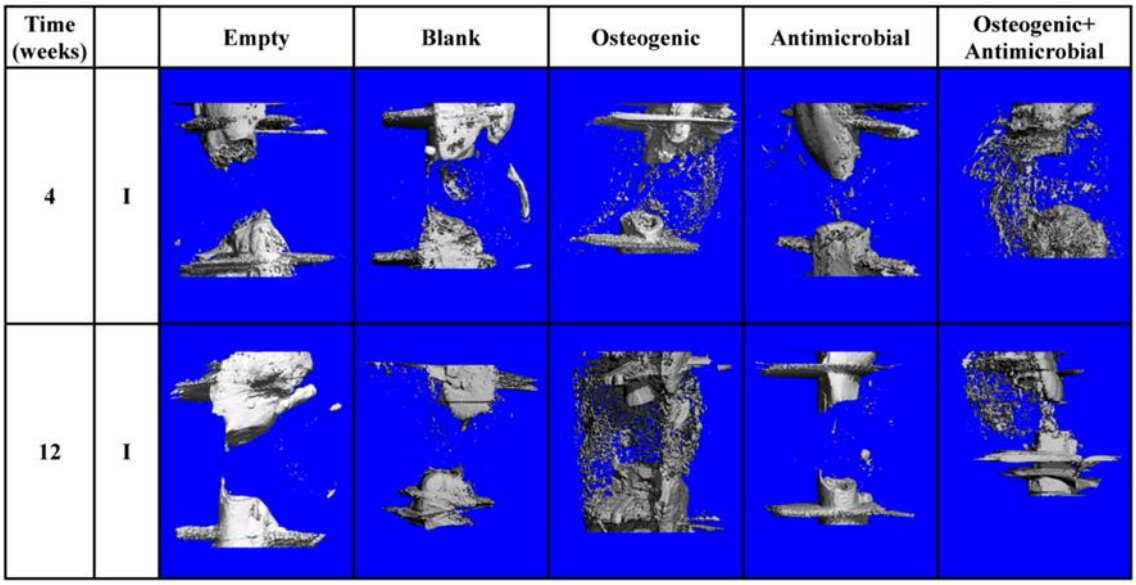


Figure 5.3: MicroCT images of defect sites evaluated for acute infection model.

For chronically infected animals, the most bone formation within the defect was seen in animals that were not infected or were treated with antimicrobials, either locally delivered vancomycin or systemically delivered ceftriaxone. Animals that received no antimicrobial treatment did not survive to be evaluated at 12 wk.

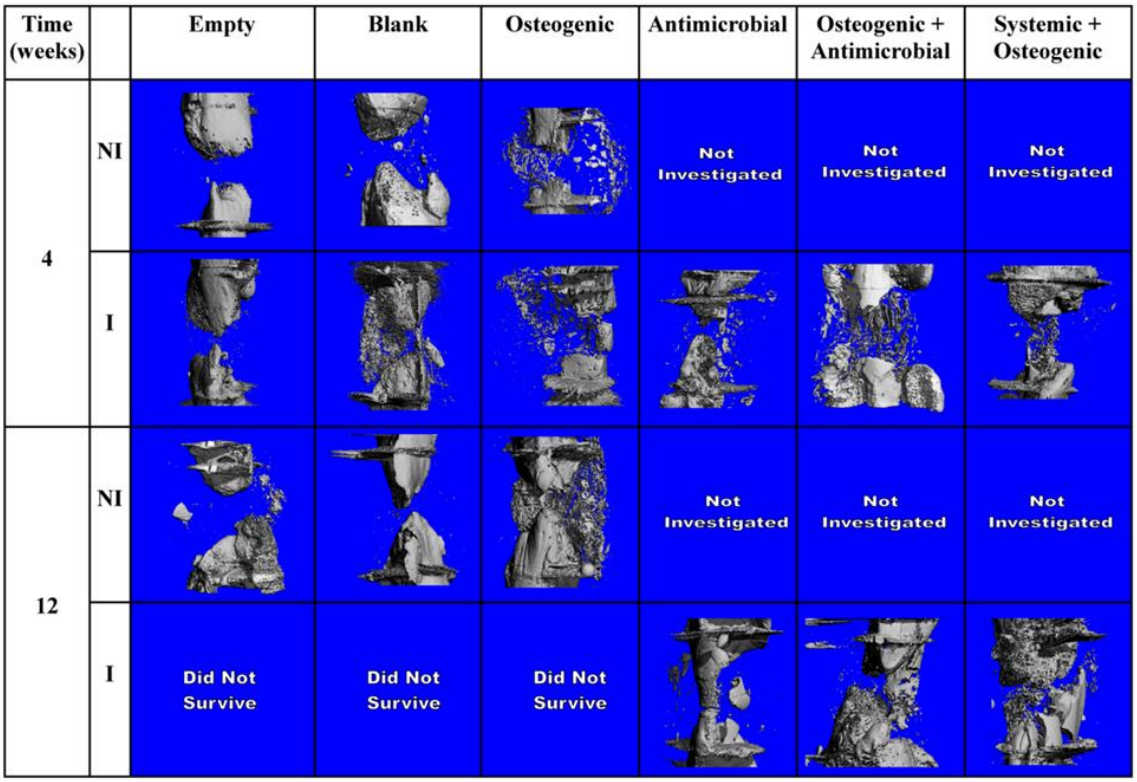


Figure 5.4: MicroCT images of defect sites evaluated for chronic infection model.

Bone growth into the defect site after 12 weeks was analyzed for chronically infected and non-infected animals and can be seen in Figure 5.5. Non-infected animals treated with an osteogenic drug showed significantly larger bone areas within the defect site than infected animals receiving no antimicrobial treatment of any kind ($p < 0.05$). Infected groups receiving antimicrobials were insignificantly different from all non-infected groups.

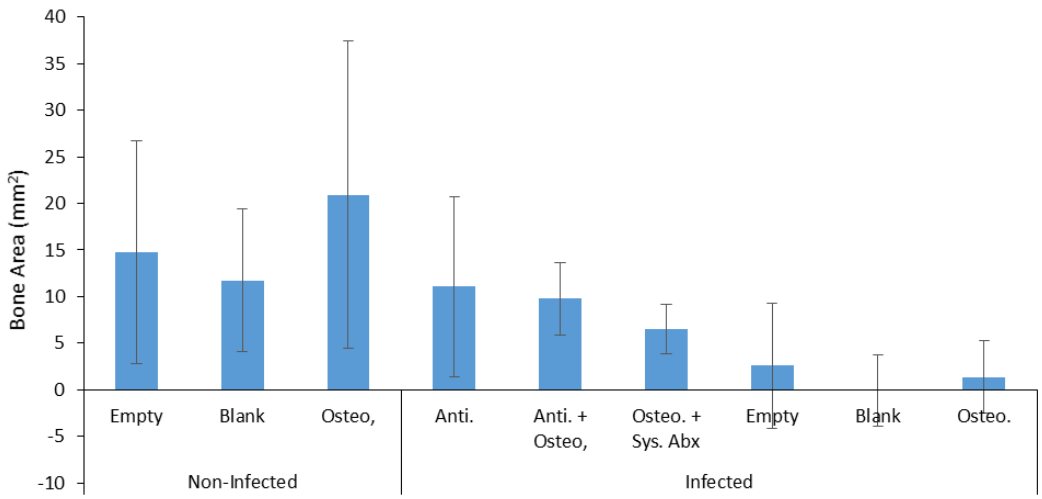


Figure 5.5: Bone areas for treatment groups at 12 weeks. Osteo = osteogenic, Anti. = antimicrobial, Sys. Abx. = systemic antibiotics (Data are mean ±SD, n=4-6).

Figure 5.6 shows sections of entire femurs from non-infected and chronically infected animals at 12 weeks. Bone healing can be seen in the non-infected groups, particularly in the osteogenic group, but little to no bone healing was present in the infected groups. These findings were supported by the bone area within the defect results from Figure 5.5.

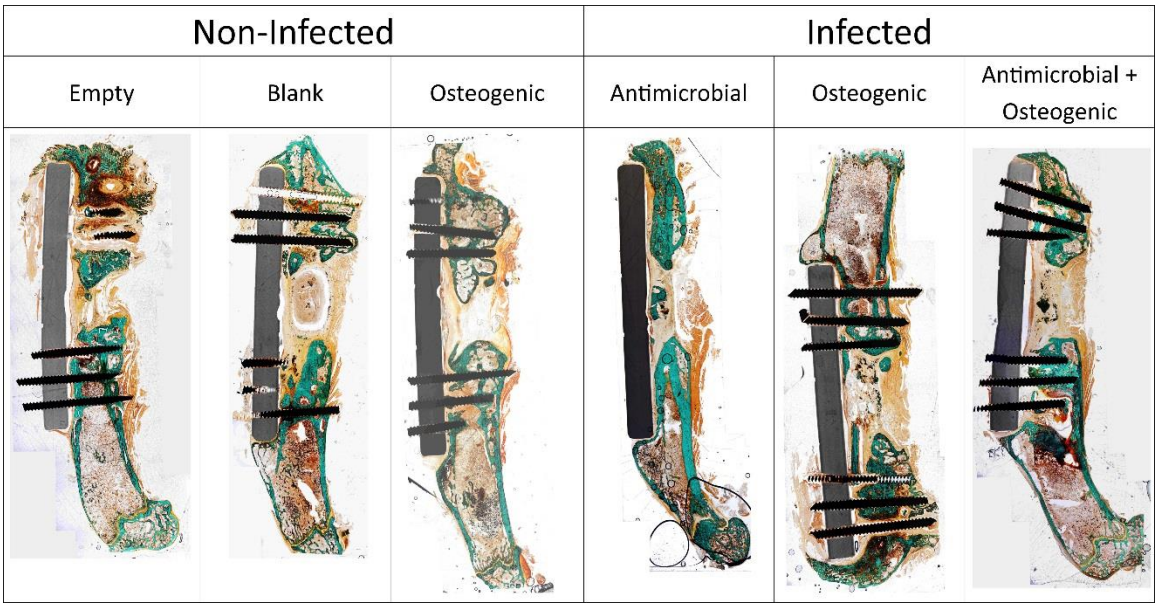


Figure 5.6: Whole femur sections stained with Goldner's trichrome for non-infected and chronically infected animals at 12 weeks.

Representative histological thin sections stained with H&E from acutely and chronically infected defect sites can be seen in the Supplementary 2 and 3. For acutely infected animals, empty defects showed some signs of bone healing at 4 wk, although it was disorganized, and a greater amount of bone healing at 12 wk. Blank implants resulted in defect maintenance at 4 wk and signs of bone formation at 12 wk. Antimicrobials alone resulted in minor bone formation at 12 weeks, but not as much as the non-infected controls. Animals receiving osteogenic and antimicrobial drugs showed what looks like a shell of bone formation around the site of infection.

5.4 Discussion

IBDs are difficult to treat clinically because they present two distinct challenges within the same wound site, an aggressive infection and a large bone defect. The treatment of IBDs is often separated into two phases, the first treating the infection and the second repairing the bone [6, 22]. By controlling the release of antimicrobial and osteogenic agents in a temporal manner, the two phases of treatment can be combined into one device. This is accomplished by first releasing vancomycin from the outer portion of the moldable bone filler followed by a delayed release of simvastatin from the inner core portion. This separation is necessary due to the difficulty of eliminating

bacteria, which attach to damaged and necrotic tissue and even invade living bone cells, making removal difficult [30, 126][25, 126]. Furthermore, the bacteria can colonize and form a biofilm on implanted fixation hardware required to treat large bone defects [25, 26][25, 26, 31]. Antibiotic concentrations up to 1,000X higher can be needed to treat biofilms compared to planktonic bacteria [25, 29, 31].

The incomplete bridging within the non-infected simvastatin treated groups could be due to the delayed action of simvastatin. The release of simvastatin was intentionally delayed by almost 2 weeks to allow time for the infection to be treated first. Once released from the bone filler material, it can take 3-7 days for simvastatin to upregulate bone morphogenetic protein 2 (BMP-2) and bone sialoprotein [118]. The total time from filler implantation to simvastatin having significant effects on osteoblasts is as much as 3 weeks, meaning the 4 week time point analyzed was insufficient for bone formation. The 12 week time point then was effectively only 9 or 10 weeks from the start of simvastatin activity, which may be insufficient for a bone defect this large to significantly heal [41, 125]. Since little bone formation was seen in the non-infected groups, it was not surprising that there was not much bone within the defects of infected animals. In addition to the delayed action of simvastatin, the negative effects of a persistent infection would hinder bone healing and can even cause bone loss [30, 31].

The negative effects of the infection that was never eliminated made bone healing within the defect difficult, but it appears that the most bone formation was seen in infected animals receiving antimicrobials and osteogenic drugs. These animals showed what appears to be a shell of bone formation around the site of infection, indicating that healing was occurring but was unable to proceed properly due to the uncontrolled infection. This observation was confirmed through histological analysis of the defect sites in which the most bone formation was seen in the defects of animals treated with simvastatin. A small amount of bone healing without the effects of infection was seen in the non-infected blank and osteogenic controls, likely due to the osteoconductive nature of the CS based material and the osteogenic effects of simvastatin. Chronically infected animals that did not receive any antimicrobial treatment did not survive to be evaluated at the 12 week time point where the most bone formation would have been expected.

In the present studies, the fixation hardware used to stabilize the femur was not removed during the irrigation and debridement procedures but instead left in place [114, 125]. This created a biofilm infection on the plate and wires that was difficult to treat. None of the infections were truly eliminated, although animals treated with locally released antibiotics had a significantly higher survival rate compared to the control groups. This finding was likely due to the released antibiotics having high enough concentrations to inhibit planktonic *S. aureus* bacteria, but never reaching concentrations high enough to fully disrupt and kill the biofilm. The higher survival rates among animals treated with antibiotics indicates that the systemic antibiotics had a similar positive effect on the infected tissue as did the locally released antibiotics but were unable to completely eliminate the biofilm before the course of treatment was concluded. Survival rates among locally released antibiotic groups were statistically the same as those receiving systemic antibiotics, indicating that the use of local antibiotics was comparable to the more commonly prescribed systemic antibiotics. A comparison between the acutely and chronically infected animals at 12 weeks showed that the acute infection was less severe in the long term, which was likely due to the chronic infection having two additional weeks of unhindered growth in the defect site. The lack of difference at 12 weeks between animals receiving no antibiotics and those receiving local antibiotics in the acutely infection model can likely be attributed to the less severe nature of the infection. Even though the infection was considered less severe in terms of survival and initial bacterial incubation time, however, the infection still presented a difficult challenge to address without removal of contaminated hardware.

Kazemzadeh-Narbat *et al.* coated titanium with a multilayered coating that provided sustained release of a broad spectrum antimicrobial peptide (HHC-36) that was active against both Gram-positive and Gram-negative bacteria [127]. Antoci *et al.* covalently attached vancomycin to titanium intramedullary implants and demonstrated that the modified implants were able to significantly inhibit bacterial adhesion for several weeks in a periprosthetic infection model in rat femurs [128]. Rodríguez-Évora *et al.* delivered two different doses of BMP-2 from a segmented polyurethane/PLGA/β-tricalcium phosphate composite into a critically sized rat calvarial defect [129]. Histomorphometry showed that after 8 weeks the low and high dose groups had repaired

approximately 10 and 30% of the defects, respectively [129]. In comparison, after 12 weeks the amount of defect repair was approximately 20% for the low dose and 60% for the high dose of BMP-2 [129]. In a study by Beardmore *et al.*, the effectiveness of local release of tobramycin from CS pellets mixed with demineralized bone matrix was evaluated in an infected tibial model in sheep [46]. After three weeks, all of the control groups remained infected, while the locally released antibiotics were able to eliminate the *S. aureus* bacteria within the wound site [46]. Chen *et al*. investigated the use of BMP-2 with and without systemic antibiotics in an infected rat femoral defect model and found that, while bone formation occurred in animals that received no antibiotics, there was significantly more bone healing in groups including antibiotics [130]. Guelcher *et al*. saw similar results using BMP-2 loaded polyurethane scaffolds [63]. Both groups showed that bacteria were still present in the defect site at the end of the study period, even in animals that received systemic or locally released antibiotics [63, 114, 130]. The present findings are in agreement with the previous reports that even locally released antibiotics well above the MIC were not enough to eliminate all the bacteria when the contaminated hardware was left in place. Both of these studies showed that partial bone healing was possible within an infected defect site without fully eliminating the present bacteria [63, 130]. To better address the full extent of the problem, methods for treating contaminated hardware that do not rely on removal should be explored. One such method is a new class of drugs that can specifically target biofilms [28, 31]. These drugs could be used alone or in conjunction with antibiotics in order to disrupt the biofilm present on the contaminated hardware and prevent new bacteria from colonizing while bone healing takes place [131].

5.4 Conclusions

A composite bone graft substitute that is capable of sequentially releasing antimicrobial and osteogenic drugs was evaluated in a critically sized infected femoral defect model. Releasing antibiotics locally at the site of infection increased survivorship by 58% compared to control animals and by 24% when compared to animals receiving systemic antibiotics. Incomplete bone bridging was seen in treatment groups and was attributed to not fully eliminating the bacterial biofilm present in the bone defect. In

order to fully treat both the bone defect and infection, new treatments capable of treating established biofilms should be investigated.

Chapter 6 Effectiveness of Anti-Biofilm Agents against Staphylococcus Aureus Biofilms and In Vitro Release from Polymeric Microspheres

6.1 Introduction

Biofilms are sessile communities of bacterial cells that differ from their planktonic form in a number of ways, including secreting protective extracellular polymeric substances (EPS) and altering their normal gene expression [27-29]. These infections are difficult to treat clinically and can cause increased hospital stays, costs, or failure of implanted devices [19, 25, 132]. The EPS makes many traditional antimicrobials, such as antibiotics, ineffective and prevents host defenses from reaching the bacterial cells [19, 25, 27, 132]. It can take 1,000X the minimum inhibitory concentration (MIC) of traditional antibiotics to eradicate established biofilms, amounts that may be unachievable via systemic delivery or potentially toxic [25, 29, 31].

Current clinical practice for the treatment of a biofilm is to physically remove the source of the infection by removing any infected hardware, extensive debridement of infected tissue, and a lengthy regime of systemic antibiotics [6, 22, 25]. This process is both painful and costly for the patients, and success is dependent on elimination of all bacterial cells to prevent resurgence of the biofilm [25]. New methods of combating biofilms have focused largely on releasing traditional antibiotics locally at the source of the infection, achieving much higher concentrations than would be possible otherwise [122, 133]. These methods work fairly well at inhibiting the growth of biofilms but require large amounts of drugs to treat established biofilms and thus are not efficient [122, 133].

Recently, interest has been growing in identifying drugs that specifically target biofilms, either inhibiting them from attaching or growing, or by disrupting existing biofilms [19, 27, 28]. A commonly used antibiotic for treating *Staphylococcus aureus* infections is the glycopeptide vancomycin [19, 27]. New methods of using vancomycin and other powerful antibiotics have focused on releasing them locally at the source of the infection [27, 41, 134, 135]. The MIC for MRSA when using vancomycin has been increasing over the years, indicating that some strains may be developing resistance [27, 136]. The D-amino acids are a new class of anti-biofilm (ABF) agent being investigated

for their ability to inhibit the growth and trigger the disassembly of bacterial biofilms [137-139]. Certain D-amino acids are thought to work by preventing the bacteria from aggregating and forming a complete biofilm, which makes them much easier to treat [137, 138]. Lactoferrin is a protein found in the innate immune system that can act as an antimicrobial by iron chelation, which destabilizes the biofilm membrane [140, 141]. It is often used in conjunction with the rare alcohol sugar xylitol, which is commonly used as an oral biofilm inhibitor [140-142]. Fatty acids have also been shown to work synergistically with common antibiotics, such as daptomycin or vancomycin, to inhibit the growth of methicillin-resistant *S. aureus* (MRSA) infections [143, 144]. Lysostaphin is an endopeptidase that both inhibits and disrupts bacterial biofilms by cleaving the crosslinks in the cell walls of *Staphlococcus spp.*, killing bacterial cells and preventing biofilm formation [76, 131].

The present studies focused on screening drugs shown to inhibit biofilm formation at low concentrations and identify one capable of disrupting an existing biofilm. This ability to inhibit as well as disrupt is crucial for future use in treating infected hardware or tissue. Lysostaphin was identified as an ABF drug that could both inhibit and disrupt *S. aureus* biofilms and was chosen for additional studies on loading the protein into a drug delivery vehicle.

6.2 Materials & Methods

The following drugs were investigated for their ability to inhibit or disrupt *S. aureus* biofilms:, lysostaphin (lyso) (AMBI Products), xylitol (ACROS Products), lactoferrin (Sigma), D-phenylalanine (D-Phe) (ACROS Products), D-proline (D-Pro) (ACROS Products), D-tyrosine (D-Tyr) (ACROS Products), L-phenylalanine (L-Phe) (Sigma), L-proline (L-Pro) (Sigma), L-tyrosine (L-Tyr) (Sigma), and vancomycin (vanc) (Sigma).

6.2.1 Anti-Biofilm Assays

The ABF assays used to evaluate the different drugs were adapted from those described by Hochbaum *et al.* [137]. A schematic for each type of assay can be seen in Figure 6.1. For inhibition studies, 215 µl brain heart infusion (BHI) supplemented with

1% glucose and 2% NaCl were added to 96-well plates with 25 µl of the drug of interest and 10 µl of a 1/100 dilution of *S. aureus* (ATCC 25923) from an overnight culture. For disruption studies, the bacteria were added to the supplemented BHI and allowed to grow in 96-well plates for 24 hours prior to a refresh of the media and addition of the treatment. After 24 hours of exposure, the media was carefully removed and each well rinsed once with phosphate-buffered saline (PBS). Next, 250 µl of a 0.1% crystal violet solution was added to each well and allowed to sit for 15 minutes. The stain was then removed and each well rinsed twice with deionized water. To quantify the amount of stained bacteria, 250 µl of ethanol was added to each well and allowed to shake for 2 hours. Absorbances were measured at 595 nm after diluting the eluted stain. Data are reported as optical density (OD). For dual treatment studies, after the first 24 hours of exposure to the drug, the media was refreshed and another treatment (or none) added.

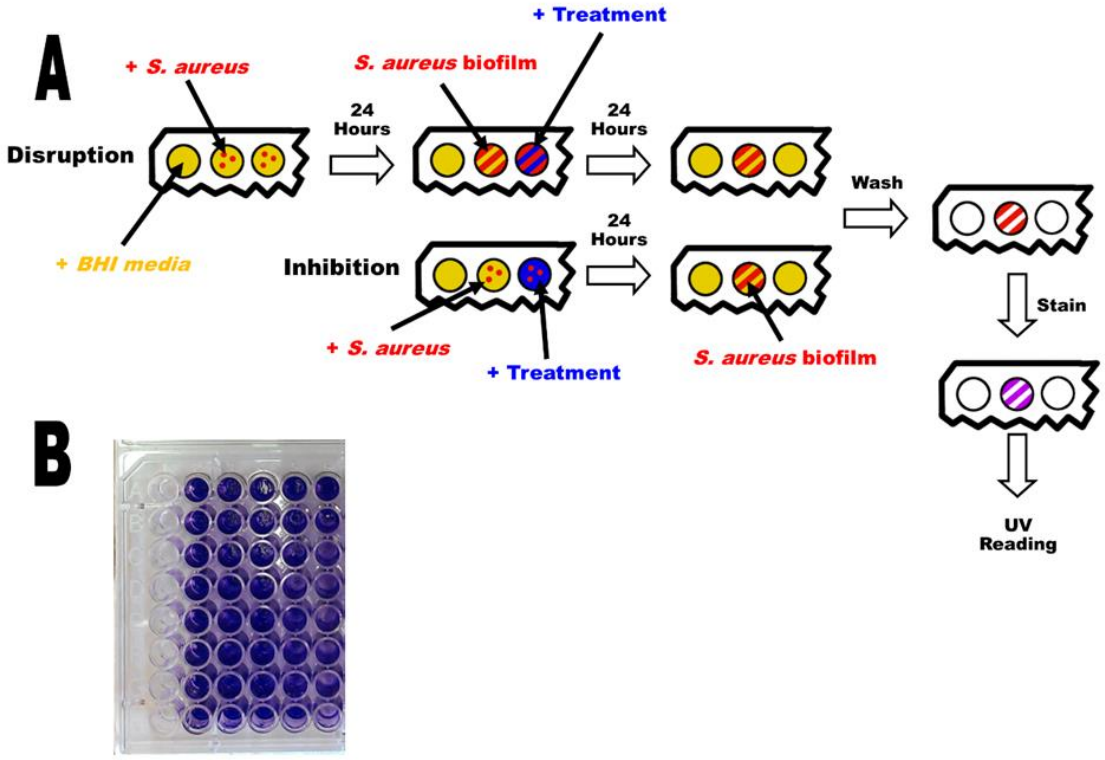


Figure 6.1: Schematic of ABF assays. A: Yellow indicates BHI media, and red dots are *S. aureus* cells, while red line patterns are *S. aureus* biofilms; blue indicates added treatments; purple indicates cells stained with crystal violet. B: Picture of stained plate; left column was not inoculated with bacteria.

6.2.2 Radio-labeling

Lysostaphin was labeled with ^{125}I using the IODO-GEN Iodination Reagent (Pierce). First, a 1 mg/ml stock solution of IODO-GEN was made by dissolving in chloroform, and then 250 µl of this solution was transferred to another tube and evaporated with nitrogen. Next, 100 µl of a 1 mg/ml stock solution of lysostaphin was added along with 16 µl of ^{125}I and allowed to react at room temperature for 10 minutes. A protein desalting spin column (Pierce) was used to separate free ^{125}I from the labeled protein by spinning at 1500*g* for 2 minutes and precipitating the protein into a solution of 20 wt% trichloroacetic acid (TCA) and PBS with 10% bovine serum albumin (BSA). The radioactivity of the labeled protein, labeled drug-loaded microspheres (see next section), and labeled release supernatants was measured using a WIZARD^2 Automatic Gamma Counter (Perkin Elmer 2470).

6.2.3 PLGA Microsphere Fabrication

PLGA microspheres (PLGAms) were fabricated using a double emulsion technique ($W_1/O/W_2$). The oil phase consisted of 13% w/v PLGA (75:25 L:G, acid-terminated, I.V. 0.55-0.75 dl/g; Durect Corp.) dissolved in dichloromethane (DCM). The first emulsion was created by adding 0.11% v/v of PBS, either blank or drug-loaded (65 mg/ml lysostaphin), to the PLGA-DCM solution and sonicating for 10 seconds at 25 W. For the radiolabeled lysostaphin microspheres, labeled protein was added to the stock protein immediately before creating the first emulsion. This W_1/O emulsion was then added to 300 ml of deionized water (containing 1% methylcellulose and 4% NaCl) in a dropwise manner and homogenized (2000 rpm) for 3 minutes to create the second emulsion. The resulting suspension of microspheres was stirred overnight at 600 rpm to evaporate the solvent. The microspheres were then collected by centrifugation, washed using deionized water, and lyophilized.

6.2.4 Release Profiles and Bioactivity

For release profile studies, 30 mg of PLGA microspheres loaded lysostaphin (labeled or non-labeled) were placed in 2 ml of PBS and shaken at 37°C in a water bath. At predetermined time points, the samples were centrifuged at 327*g* force for 3 minutes

and the supernatant completely changed. The collected supernatants were frozen until analysis. Labeled lysostaphin microspheres were used made to analyze the loading and release characteristics of lysostaphin while the non-labeled microspheres were used in the bioactivity studies. The release profiles for the microspheres were analyzed by measuring 1 ml of release supernatant using the WIZARD^2 Automatic Gamma Counter (Perkin Elmer 2470). Bioactivity was determined by comparing the effectiveness of release supernatants at inhibiting or disrupting biofilm formation compared to known lysostaphin concentrations.

6.2.5 Statistical Analysis

One-way analysis of variance (ANOVA) was performed using Prism software (GraphPad). Statistical analysis was determined at p values less than 0.05. Tukey's post-hoc test was performed as needed.

6.3 Results

6.3.1 ABF Drug Screening

Results from the biofilm inhibition study can be seen in Figure 6.2. All of the lysostaphin and vancomycin concentrations, down to 0.5 and 5 µg/ml, respectively, were able to inhibit biofilm formation comparable to the negative control ($p < 0.0001$ compared to negative control). None of the other treatments, i.e., xylitol, lactoferrin, xylitol+lactoferrin, or D-amino acids, inhibited the biofilm from growing.

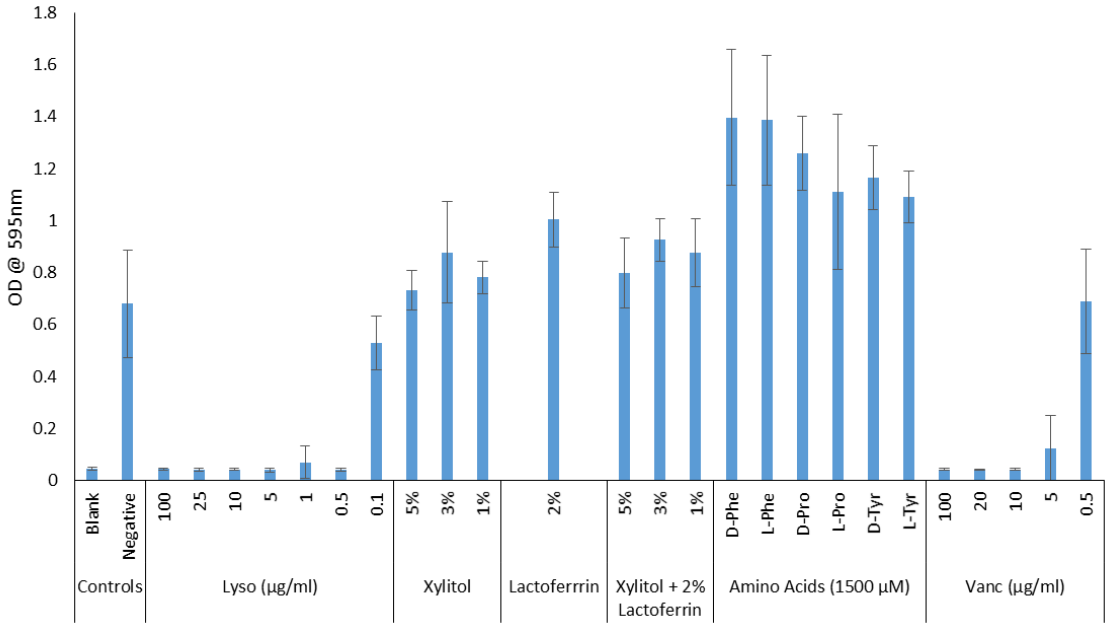


Figure 6.2: Biofilm inhibition results for the drugs investigated. Data are mean ± SD, n=8.

Results from the biofilm disruption studies can be seen in Figure 6.3. Lysostaphin was able to disrupt the existing biofilm at concentrations as low as 5 µg/ml ($p < 0.0001$), but the xylitol, lactoferrin, or amino acids did not have any measureable effect. None of the vancomycin concentrations investigated showed any significant difference from the negative control.

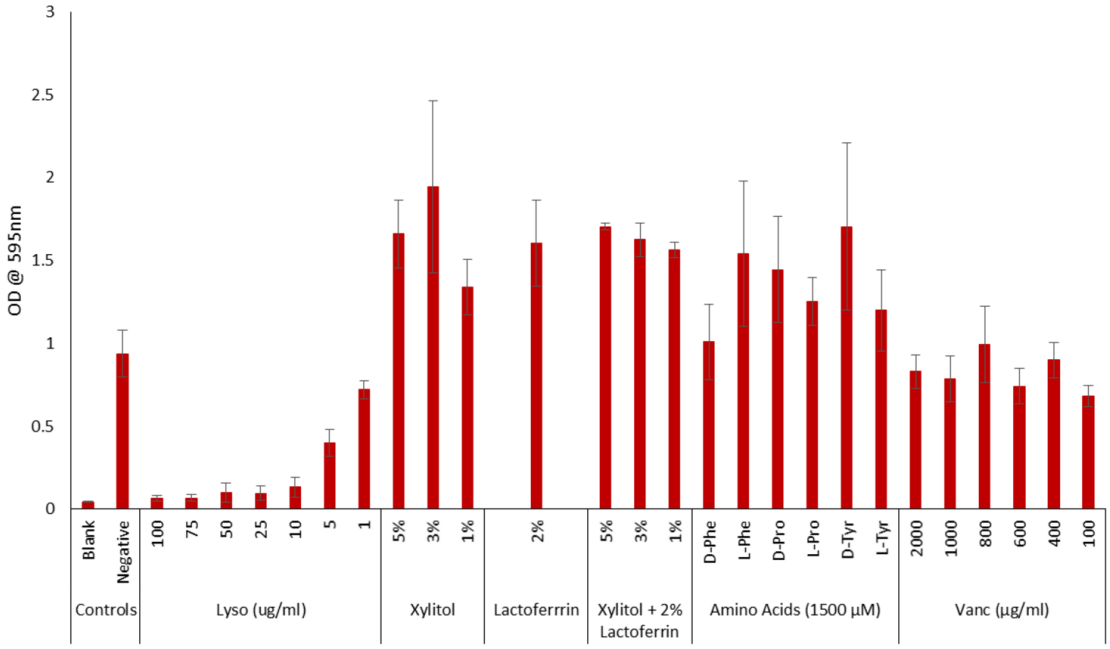


Figure 6.3: Biofilm disruption results for the drugs investigated. Data are mean ± SD, n=8.

Based on the previous inhibition and disruption results, additional studies on lysostaphin were performed to determine at what lower concentrations it was effective (Figure 6.4). Lysostaphin was able to inhibit biofilm formation at concentrations as low as 0.1 µg/ml ($p < 0.05$) and disrupt existing biofilms starting at 1 µg/ml ($p < 0.001$) and up.

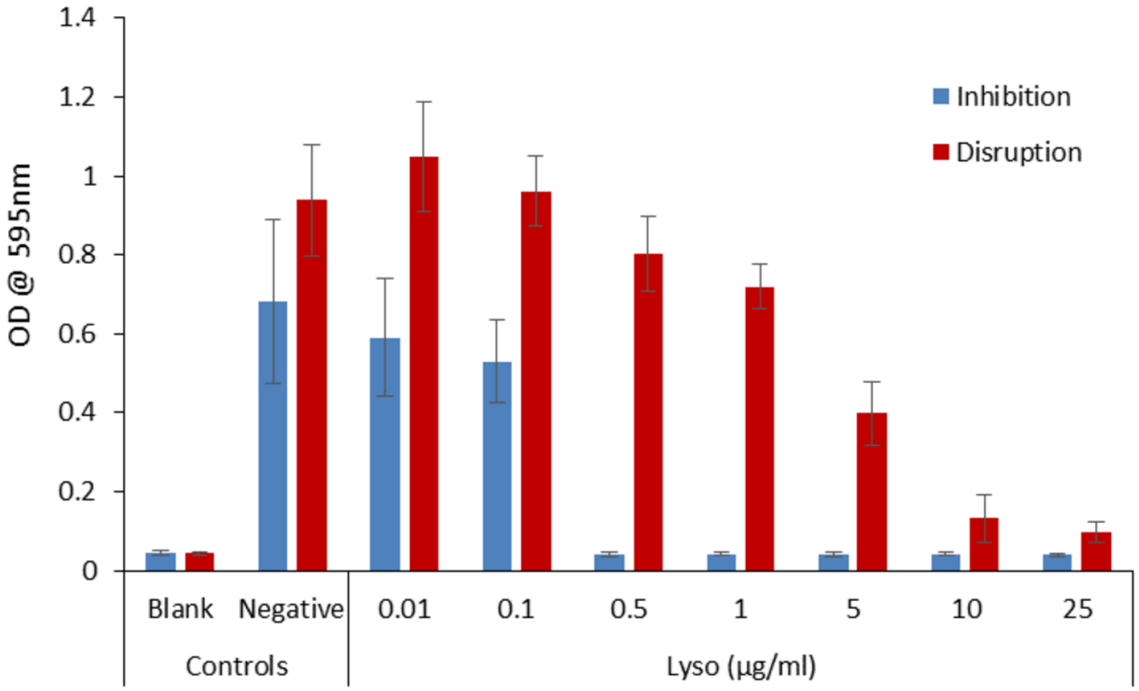


Figure 6.4: Biofilm inhibition and disruption results for lysostaphin at decreasing concentrations. Data are mean ± SD, n=8.

Dual treatment results can be seen in Figure 6.5. Treatment once with lysostaphin at concentrations as low as 10 µg/ml resulted in dispersion of biofilm that was not able to re-grow after 24 hours with new BHI media ($p < 0.0001$). All dual treatments with lysostaphin resulted in biofilm disruption, but vancomycin alone had little effect.

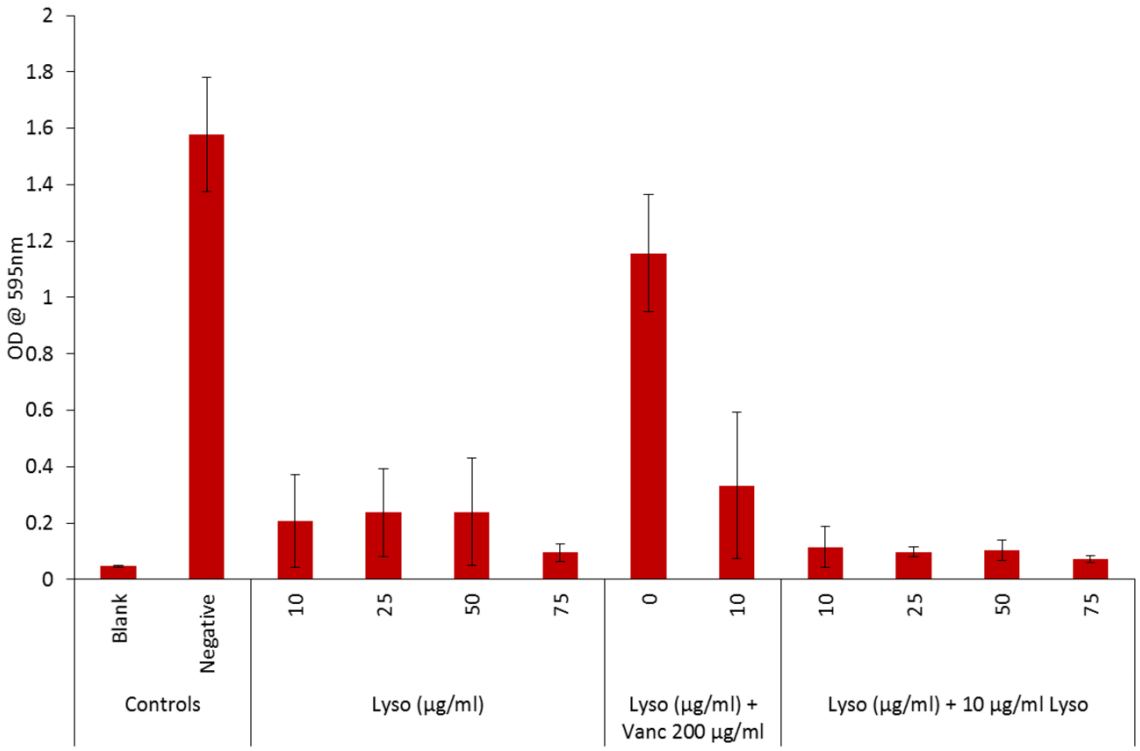


Figure 6.5: Biofilm disruption with lysostaphin dual treatment. Data are mean ±SD, n=8.

6.3.2 In vitro release and activity of lysostaphin

Encapsulation of lysostaphin into PLGAms resulted in a final loading of 8.5wt%, encapsulation efficiency (EE) of 65%, and average size of 40±15 µm. Profiles for release of lysostaphin encapsulated in PLGAms can be seen in Figure 6.6. When released from PLGA microspheres, lysostaphin reached a maximum instantaneous concentration of 57 µg/ml at day one and remained above 5 µg/ml for 19 days.

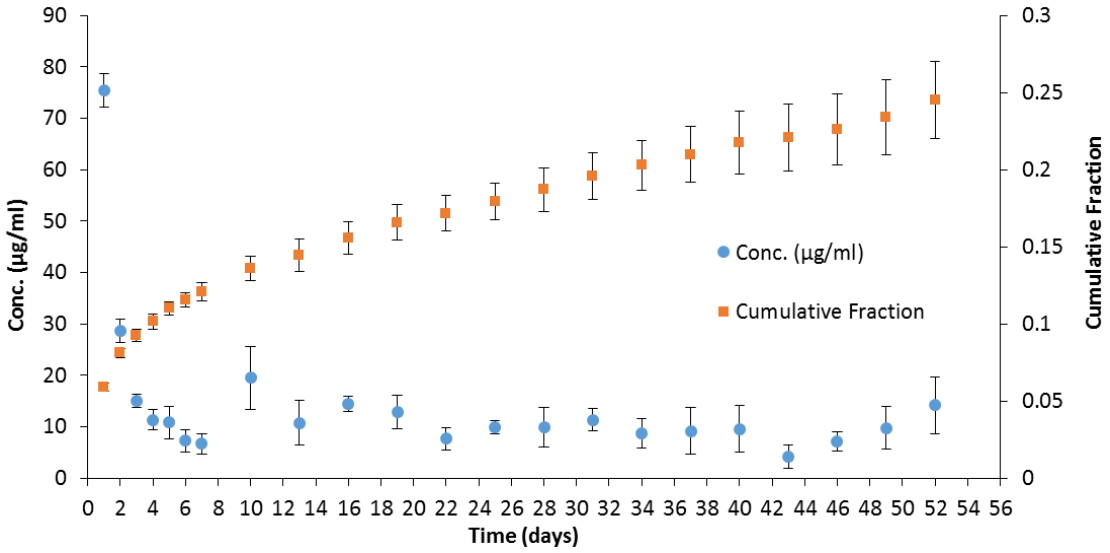


Figure 6.6: Release profile for lysostaphin-loaded PLGAms. Data are mean ±SD, n=5.

The bioactivity of lysostaphin in release supernatants can be seen in Figures 6.7 and 6.8. Release supernatants were able to inhibit biofilm formation and disrupt existing biofilms for 4 days when compared to the negative control and their corresponding blanks (bPLGAms) ($p < 0.05$). Compared to the negative control, release supernatants inhibited biofilm formation by 43% on day 1 and 81, 86, 86, 41, and 22% for days 2, 3, 4, 5, and 6, respectively (Figure 6.7). When evaluated in the disruption study, release supernatants decreased the amount of biofilm by 33% on day 1 and 52, 52, 61, 16, and 0 % for days 2, 3, 4, 5, and 6, respectively (Figure 6.8). After day 4 of release, there were no statistically significant differences between the drug-loaded PLGAms release supernatants, blank PLGAms release supernatants, and negative control.

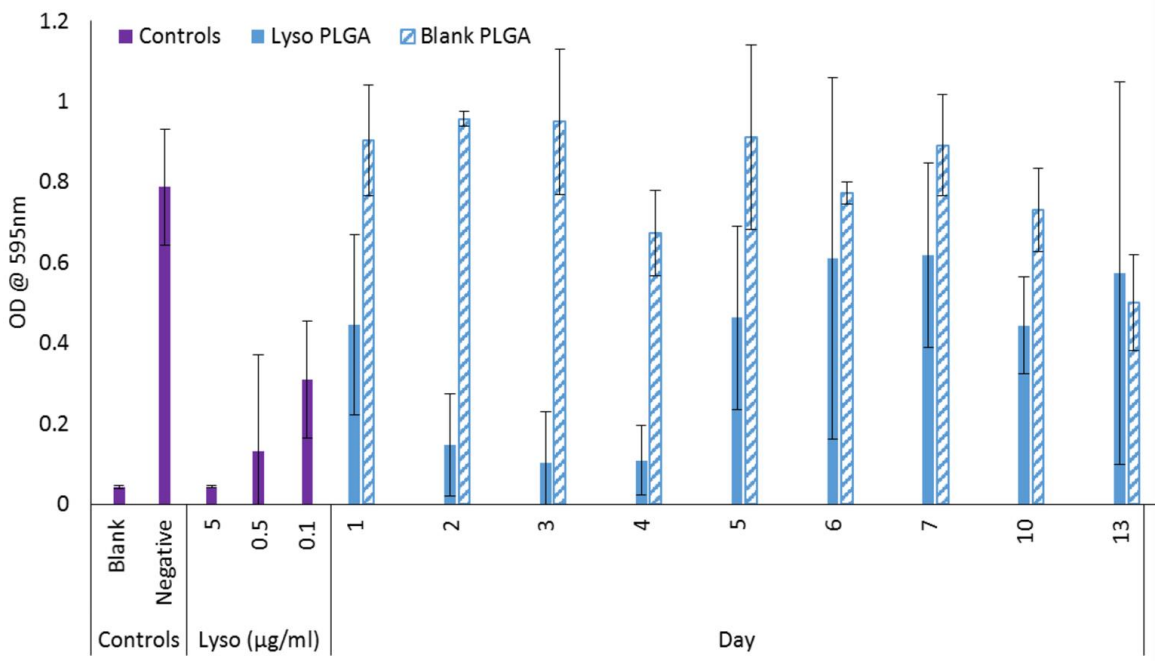


Figure 6.7: Ability of lysostaphin released from PLGAms to inhibit biofilms. Data are mean ±SD, n=3-8.

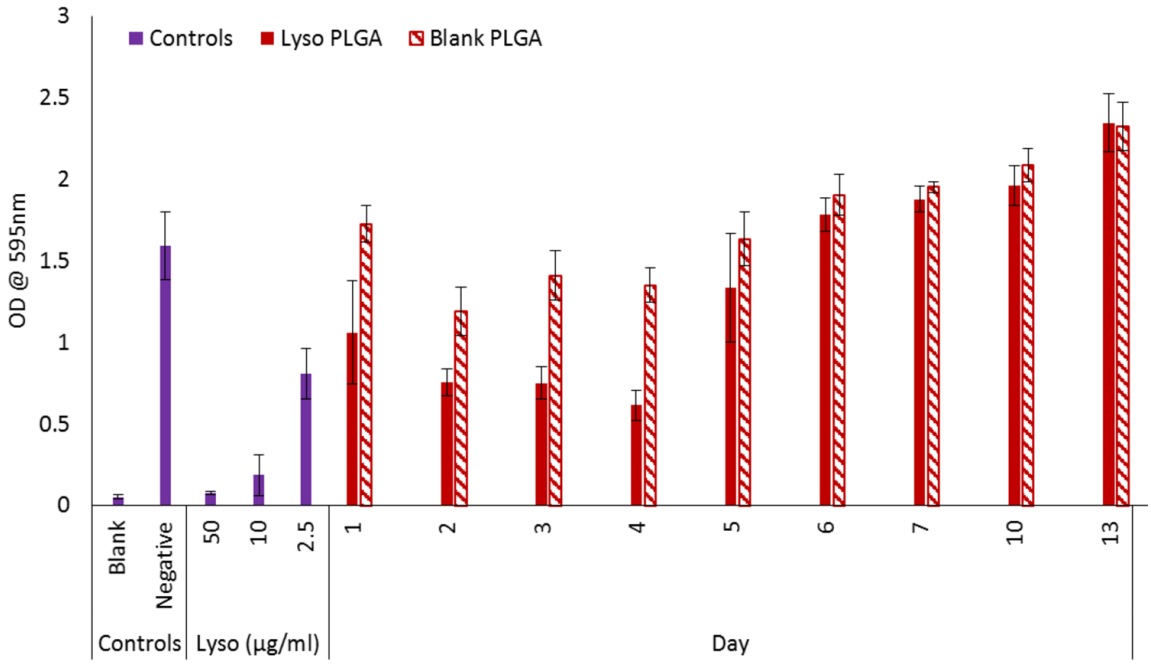


Figure 6.8: Ability of lysostaphin released from PLGAms to disrupt biofilms. Data are mean ±SD, n=3-8.

6.4 Discussion

One of the more commonly used antibiotics for *S. aureus* infections is vancomycin, which works by interfering with cell wall formation in Gram-positive bacteria [115, 145-147]. Vancomycin works well at inhibiting biofilm formation because it acts on the cell walls before the protective EPS can be formed [27, 29]. However, once the biofilm has formed, vancomycin is effective at only concentrations 1,000X higher than the MIC [25, 29, 31]. This is due to the protective nature of the EPS which acts as a physical barrier between antimicrobials and the bacterial cells within the biofilm [25, 28, 29, 31]. One common method of circumventing this problem is to deliver the antibiotic locally at the site of infection to avoid systemic toxicity and achieve high concentrations with little drug waste [27, 115, 148]. Local delivery methods can often achieve high concentrations initially followed by low levels for days, weeks, or even months [27, 146, 148]. Gálvez-López *et al.* evaluated the release kinetics of 11 different types of antibiotic loaded bone cements and found that while effective MIC were seen for around 30 days in some cases, none of the concentrations reached would be effective at disrupting a biofilm [149]. In clinical practice, antibiotic loaded beads are an accepted osteomyelitis

treatment option. When combined with systemic antibiotics and debridements these antibiotic loaded beads are effective in eliminating chronic infections even when only releasing effective concentrations for a few days [40, 102]. Despite the promising release kinetics of many of these systems, there is still great difficulty in treating an established biofilm with local antibiotics alone due to the inability of the antibiotics to penetrate the EPS and act on the bacteria [25, 27]. The results from the present study show trends consistent with the literature [31, 150], with vancomycin inhibiting biofilm formation at concentrations as low as 5 µg/ml but being unable to disrupt an existing biofilm at concentrations as high as 2,000 µg/ml. Consequently, although vancomycin may not be the best choice to treat an existing biofilm, it can act as a preventative agent to keep bacteria from growing initially or re-growing (persisting) after treatment. Because of the difficulty of using traditional treatments, drugs that specifically target biofilms are of great interest [28, 31].

A class of agents that has shown potential for use as ABF agents are the D-amino acids [137-139]. Hochbaum *et al.* compared the effectiveness of D and L isomers of proline, tyrosine, phenylalanine, tryptophan (Trp), and leucine (Leu) at inhibiting *S. aureus* biofilms *in vitro* at concentrations ranging from 0-500 µM [137]. It was found that the D-Phe and D-Pro were effective at only the highest concentration of 500 µM, while the D-Tyr was effective at 100 and 500 µM [137]. A cocktail of all three D-amino acids was found to be effective at inhibiting biofilm formation at concentrations as low as 10 µM [137]. None of the D-tryptophan, D-leucine, or L isomer treatments had any effect on the biofilms [137]. Kolodkin-Gal *et al.* reported activities for D-Tyr at 3 µM, D-methionine (D-Met) at 2 mM, D-tryptophan at 5 mM, and D-leucine at 8.5 mM for *Bacillus subtilis* [138]. A combination of all four amino acids was found to have a MIC of approximately 10 nM [138]. Against *S. aureus*, D-Tyr was effective at inhibiting biofilms at 50 µM, and a mixture of D-Tyr, D-Leu, D-Met, and D-Trp was effective at 15 nM of each amino acid. None of the D-amino acids investigated in the present study had any effect on inhibiting or disrupting biofilms when compared to their inactive L-isomers. It may be that higher concentrations were needed to see the desired effect, but higher dose requirements make incorporation into a device much more challenging. In a recent study by Sanchez *et al.*, D-amino acids were evaluated *in vitro* for their effectiveness

before being delivered locally from a scaffold in an infected segmental defect model [151]. D-Phe, D-Met, D-Pro, and D-Trp were shown to be effective at reducing the biomass of *S. aureus* biofilms at concentrations greater than 1 mM [151]. When tested *in vivo*, a cocktail of D-Met, D-Pro, and D-Trp at concentrations of 5 and 10 mM significantly reduced the bacterial counts and number of infected bone samples harvested [151]. The D-amino acids are being investigated for use as an ABF agent due to their wide spectrum of use and ability to work synergistically with each other to inhibit biofilm formation. However, the varied effective concentrations and high amounts usually needed to disrupt existing biofilms may limit their use without being combined with another agent to increase activity.

Lactoferrin and xylitol are two ABF agents that are under investigation for their ability to inhibit biofilm growth when used synergistically [140, 142]. Lactoferrin is an iron-chelating agent that is able to destabilize the bacterial membrane, and xylitol is a rare sugar alcohol that inhibits the ability of the bacteria to respond to the iron chelation caused by lactoferrin [140]. In a study by Ammon *et al.*, lactoferrin and xylitol together showed a 1-2 log reduction in the bacterial load of MRSA [142]. This effect was increased to a 6-log reduction when combined with a silver-eluting wound dressing [142]. In the current study, neither lactoferrin nor xylitol by themselves or together had any effect at inhibiting or disrupting biofilms. Because their effectiveness can be increased significantly with the addition of other antimicrobials, it may be worth investigating their potential use in combined ABF treatments.

Lysostaphin is an antibacterial enzyme that acts on the pentaglycine crosslinks in *S. aureus* cell walls [76]. Wu *et al.* reported that lysostaphin was able to kill planktonic *S. aureus* at concentrations as low as 0.001 µg/ml (MIC_{90}), and it disrupted existing biofilms at concentrations as low as 12 µg/ml even when 800 µg/ml vancomycin or clindamycin was not effective [76]. Lysostaphin was also shown to have synergistic effects with common antibiotics, such as clarithromycin and doxycycline [131]. In the present studies, lysostaphin was shown to disrupt biofilm formation at concentrations as low as 10 µg/ml and inhibit biofilm formation starting at 0.1 µg/ml. The low dose requirements for lysostaphin to inhibit biofilm formation make it a good potential candidate for prolonged delivery from a biomaterial where a moderate burst would be

effective enough to disrupt the existing biofilm and the subsequent low levels of release concentrations would be enough to inhibit future biofilm development.

When released from PLGA microspheres, lysostaphin reached a maximum concentration of 57 µg/ml at day one, and was released in a controlled manner capable of not only inhibiting biofilm formation but also disrupting existing *S. aureus* biofilms for 4 days. From the initial ABF assays characterizing the effectiveness of lysostaphin, it was expected that concentrations above 0.5 µg/ml would inhibit biofilm formation and that 10 µg/ml would disrupt existing biofilms. Due to the nature of the ABF assay, release supernatants must be diluted by 2X to be evaluated. This means that release supernatants would need to have initial concentrations around 1 µg/ml or 10 µg/ml, respectively, to inhibit or disrupt biofilms. Inhibition would, therefore, be expected for days 1-13 of release and disruption for the first 4-5 days. It is likely that fabrication of the lysostaphin-loaded PLGAms resulted in some inactivation of the protein, reducing the effectiveness at combating biofilms [152-154]. Addition of a sugar, such as sucrose or trehalose, during the encapsulation process can protect the protein and increase activity [155, 156].

6.5 Conclusions

Lysostaphin was confirmed as an ABF agent capable of both inhibiting biofilm growth and disrupting existing biofilms. Furthermore, lysostaphin could be encapsulated into PLGAms and released over time *in vitro*, maintaining its ability to inhibit and disrupt biofilms for 4 days. The observed antibiofilm activity at concentrations easily obtained from drug delivery vehicles or other biomaterials gives lysostaphin interesting potential as an antimicrobial treatment. The promising results seen from this study warrant further investigation of lysostaphin and its potential to be incorporated into an ABF biomaterial.

Chapter 7 Conclusions

A moldable, biocompatible, and biodegradable bone graft substitute has been developed which is capable of being loaded with therapeutic drugs for the treatment of infected bone defects. Initial studies involved evaluating the mechanical and degradation properties of composites with different ratios of components. The ability to interchange the type of plasticizer used or microspheres embedded enable the degradation time and drug release profiles to be tailored to specific applications.

Typical treatment for infected bone defects involves two separate sets of procedures, the initial treatment for the infected tissue followed by bone grafting. In order to combine these two treatment steps into one procedure, the moldable bone grafting material developed previously was modified to deliver drugs in a temporally separated manner. This two-layered system is capable of delivering antibiotics *in vitro* for clinically relevant periods of time and delaying the release of osteogenic drugs to mimic a two-step procedure.

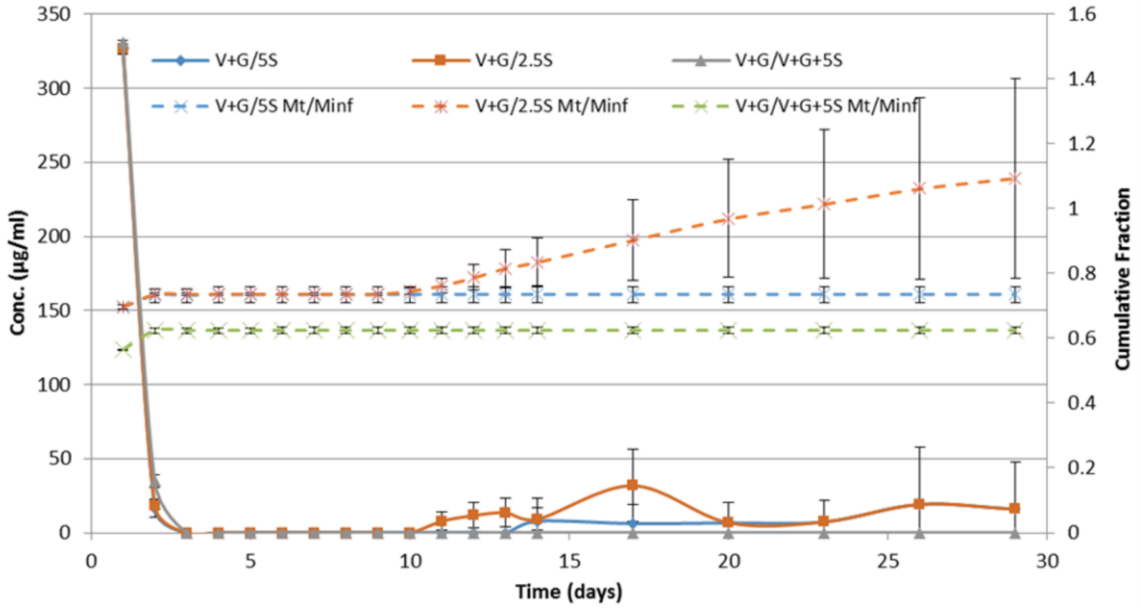
In order to test the effectiveness of the developed bone graft substitute at treating an IBD, an established critically sized infected rat femoral defect model was used. Composites that released antibiotics locally increased survivorship of animals when compared to those receiving systemic antibiotics. This indicates that the developed material was as effective as more commonly used clinical methods at treating biofilm infections. There was no difference in the new bone formation of the treatment groups and non-infected control groups, indicating that the timeframe investigated was not long enough and that the infection, which was never fully eliminated, had a negative effect on the newly forming bone.

When investigating the ability of different drugs to treat biofilms, even very high doses of antibiotic were unable to disrupt an existing biofilm. Lysostaphin was identified as a potential anti-biofilm agent which was capable of both disrupting and inhibiting biofilms at low concentrations. The loading of lysostaphin into polymeric microspheres was able to extend the release and increase the therapeutic potential of the drug. Lysostaphin shows great promise as an anti-biofilm drug capable of being incorporated into antimicrobial biomaterials or devices.

Infected bone defects as a result of high energy trauma are often contaminated with multiple species of bacteria and can be irregular in shape. Since the drug-releasing components are swappable, without significant effect on the degradation time or strength of the material, it would be possible to incorporate species-specific antibiotics alone or in conjunction with anti-biofilm agents for treatment of challenging biofilm infections. The inclusion of osteogenic or angiogenic growth factors in addition to the antimicrobials could further increase the therapeutic potential of the bone graft substitute to regrow bone in complex defects.

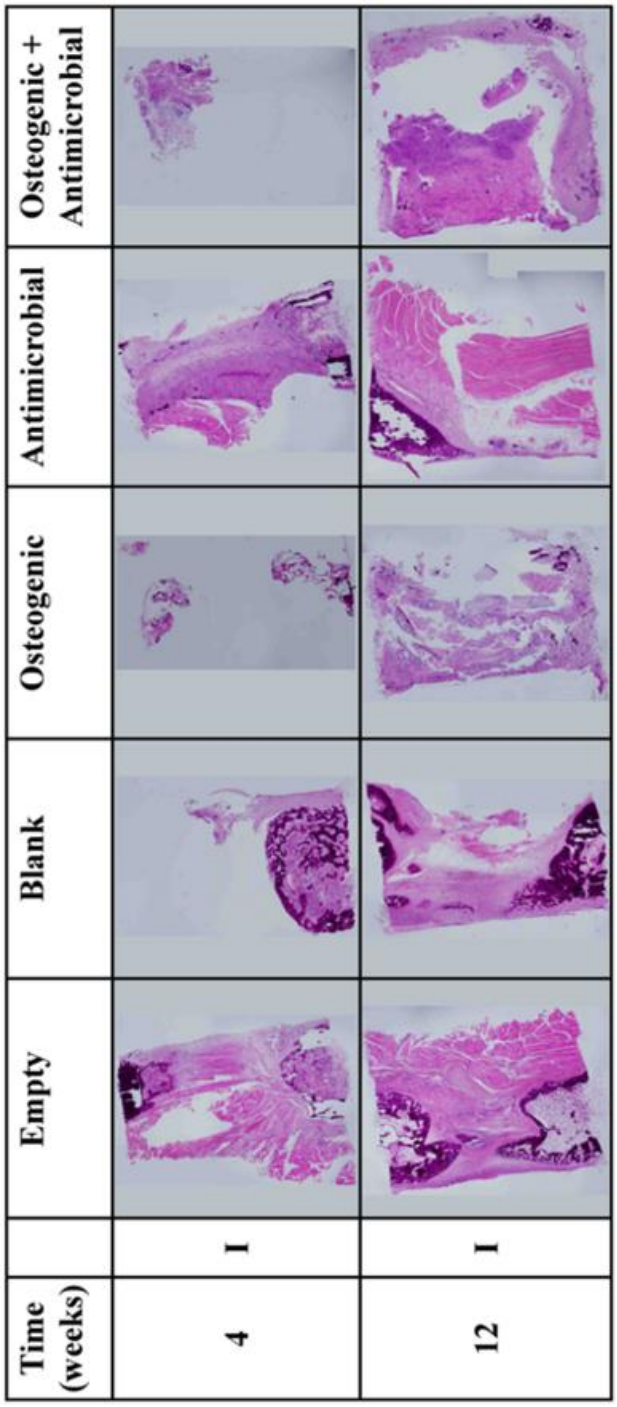
This work shows that a moldable and biodegradable bone graft substitute can be modified to release different drugs in a controllable manner. The composite material developed is capable of treating a local infection with the same efficacy as systemic antibiotics while providing additional therapeutic benefits, such as space maintenance and the delivery of an osteogenic drug and osteoconductive materials.

Appendix A Supplemental Figures

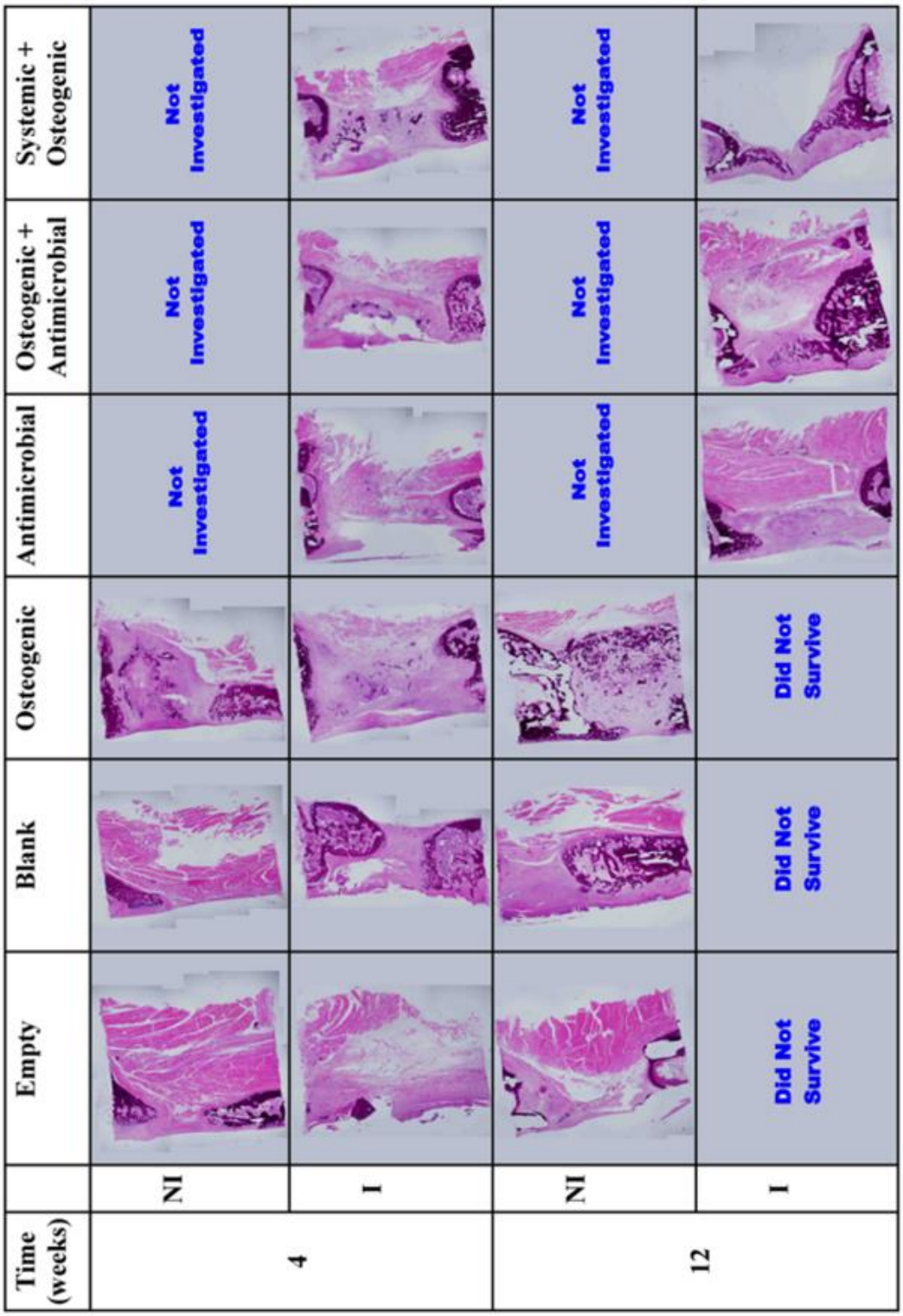


Supplemental 1: Full results from free antibiotic release studies. Data are mean ±SD, n=5.

Appendix B Supplemental Figures



Supplemental 2: Micrographs of defect sites for paired sections stained with H&E at 4 and 12 wk for acutely infected animals.



Supplemental 3: Micrographs of defect sites stained with H&E (left) and Goldner's trichrome (right) at 4 and 12 wk for chronically infected animals.

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Publications:

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- Matthew E. Brown, Yuan Zou, R. Peyyala, Thomas Dziubla, and David A. Puleo. Temporal Separation in the Release of Bioactive Molecules from a Moldable Calcium Sulfate Bone Graft Substitute, Current Drug Delivery, in review 2014.
- Matthew E. Brown, Gus Huerta, Rebecca Peyyala, Todd Milbrandt, Thomas Dziubla, and David A. Puleo. Effectiveness of Anti-Biofilm Agents against Staphylococcus Aureus Biofilms and In Vitro Release from Polymeric Microspheres. In preparation for submission.
- Matthew E. Brown, Yuan Zou, Rebecca Peyyala, Larry Sunningham, Todd Milbrandt, Thomas Dziubla, and David A. Puleo. Testing of a Bioactive, Moldable Bone Graft Substitute in an Infected, Critically-Sized Defect Model. In preparation for submission.

Professional Conference Presentations (Poster/Oral):

- Matthew E. Brown, Gus Huerta, Rebecca Peyyala, Todd Milbrandt, Thomas Dziubla, and David A. Puleo. Lysostaphin as an effective anti-biofilm agent against *Staphylococcus Aureus* biofilms and its potential biomaterial applications, Presentation, The Society for Biomaterials Conference at Case Western Reserve University in Cleveland, OH. October 2013.
- Matthew E. Brown, Gus Huerta, Rebecca Peyyala, Todd Milbrandt, Thomas Dziubla, and David A. Puleo. Effectiveness of Anti-biofilm Agents against Staphylococcus aureus biofilms, Poster, Society for Biomaterials Annual Meeting, Boston, MA. April 2013.
- Matthew E. Brown, Yuan Zou, Rebecca Peyyala, Larry Sunningham, Todd Milbrandt, Thomas Dziubla, and David A. Puleo. Testing of a Bioactive, Moldable Bone Graft Substitute in an Infected, Critically-Sized Defect Model, Poster, Society for Biomaterials Annual Meeting, Boston, MA. April 2013.
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