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INORGANIC MATERIALS FOR LOCAL DRUG DELIVERY

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INORGANIC MATERIALS FOR LOCAL DRUG DELIVERY

by

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PREFACE

This dissertation submission contains an introduction, three chapters formatted as journal articles, a discussion, conclusions, and recommendations for future work. Chapter 2 is a journal article titled "Evaluation of Antibiotic-Releasing Triphasic Bone Void Filler In-Vitro," which was published in the Journal of Functional Biomaterials in 2018. The data presented in chapter 3 has not been published or presented at the time of writing, and is being prepared for submission to the Journal of Bone and Joint Surgery. Chapter 4 is a journal article titled "Magnetic Stimuli-Responsive Chitosan-Based Drug Delivery Biocomposite for Multiple Triggered Release," which was published in the International Journal of Biological Macromolecules in 2017. The Introduction (Chapter 1), Comparison of Calcium Sulfates for the Treatment of Periprosthetic Joint Infection (Chapter 3), Discussion (Chapter 5), and Conclusions (Chapter 6) are unique to this dissertation.

ABSTRACT

Orthopedic infections, including periprosthetic joint infections and osteomyelitis, are debilitating complications that greatly increase mortality rates and expenditure of healthcare resources. Treatment protocols include surgical debridement of infected tissue, removal of any foreign implants, and several weeks of systemic antibiotic therapy, yet recurrence rates remain high. Local antibiotic delivery systems augment traditional therapy techniques by increasing antibiotic concentrations at the site of infection to levels that are unachievable by IV or oral delivery, thereby improving bacterial clearance rates. Current clinical products are commonly prepared at the surgeon's direction, with only limited data and anecdotal reports guiding antibiotic choice and loading. The purpose of this research was to evaluate several local delivery systems, including multiple calcium based bone void fillers already in use and a novel magnetically responsive microbead, to evaluate properties such as drug elution kinetics, biocompatibility, degradation, and adverse effects of drug loading on material handling characteristics. Highlights of the study results include the finding that antibiotic incorporation can affect the hardening of calcium based bone void fillers, with some commonly used antibiotics greatly increasing set time required before use compared to others. This delay in hardening or setting may negatively affect clinical use as the material must be prepared at the time of surgery. Additionally, incorporation of multiple antibiotics can alter the release kinetics compared to when each antibiotic is used alone, potentially affecting the duration of effective elution. Another local drug delivery approach to maximize efficacy of the antibiotic payload is the use of stimuli responsive materials to alter release kinetics. Incorporation of iron oxide nanoparticles into a novel chitosan-polyethylene glycol microbead system allowed drug elution to be increased on demand through the use of alternating magnetic fields to generate heat within the system. External stimulation

techniques such as this can alleviate drawbacks of current local drug delivery materials by further improving release kinetics to maximize efficacy of the drug payload.

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KEY TO SYMBOLS OR ABBREVIATIONS

Analysis of Variance: ANOVA

Antibiotic Loaded Bone Cement: ABLC

Bone Void Filler: BVF

Calcium Sulfate: CS

Dulbecco's Modified Eagle Medium: DMEM

Fetal Bovine Serum: FBS

High Performance Liquid Chromatography: HPLC

Hydroxyapatite: HA

Magnetic Nanoparticle: MNP

Methicillin-Resistant *Staphylococcus aureus*: MRSA

Microbial surface components recognizing adhesive matrix molecules: MSCRAMMs

Minimum Biofilm Eradication Concentration: MBEC

Minimum Inhibitory Concentration: MIC

Polyethylene Glycol Dimethacrylate: PEGDMA

Phosphate Buffered Saline: PBS

Periprosthetic Joint Infection: PJI

Polymethylmethacrylate: PMMA

Small Colony Variant: SCV

Staphylococcus aureus: *S. aureus*

Superparamagnetic Iron Oxide Nanoparticle: SPION

CHAPTER 1

INTRODUCTION

Orthopedic infections, including osteomyelitis and periprosthetic joint infection (PJI), are debilitating complications in terms of patient morbidity and healthcare expenditures. These infections directly result in longer hospital stays, repeat hospitalizations, up to 300% greater costs, reduced quality of life, and increased mortality rates compared to similar patients without infections (1-4). Osteomyelitis is common following trauma, with rates up to 20-30% reported for severe open fractures, and is also associated with neurovascular complications from diseases such as diabetes or smoking (5, 6). Recurrence rates remain at approximately 20% despite aggressive treatment, and there have been multiple documented cases of osteomyelitis recurring after decades (7-13). Infection rates for primary joint arthroplasty are low at 1-2%, however this number increases to 20-40% for revision surgery following PJI (14-17). Projections estimate revision hip and knee arthroplasty due to PJI will exceed $1.6 billion in the US for 2020, and it is widely believed that this cost will continue to rise exponentially as the average population age continues to increase (18-20). These infections also have a significant personal cost for the patients, who suffer pain, psychological distress, immobility, decreased quality of life, longer hospitalizations, repeat surgeries, and often require assisted living (1, 21).

Orthopedic infection treatment typically includes surgical debridement of infected tissue, removal of implants or foreign material, and 4-12 weeks of systemic antibiotic therapy (7, 22). This can be problematic as infection is often associated with underlying bone disease that may require implants for treatment, such as bone fractures or osteoarticular disease, forcing the surgeon to divert focus from treatment to combatting the infection (23, 24). Inflammation from osteomyelitis can cause the occlusion of vascular channels at the site of infection, creating a region

of devitalized bone that is inaccessible to systemic antibiotics and must be surgically excised (13). PJI is typically treated using a two stage revision procedure in which the implant and infected tissue are removed, an antibiotic loaded polymethylmethacrylate (PMMA) spacer is placed in the joint space, and the patient is treated with systemic antibiotics for 4-6 weeks. Once inflammatory markers return to normal levels, the spacer is removed and a new prosthesis is implanted (25, 26). This process was established in the 80's, and 5 year survival rates have remained stagnant around 80% in most reports (27).

Pathogenic bacteria often exist in multiple phenotypic states as a 'bet hedging' strategy to ensure survival in adverse conditions. The most common and well understood phenotype is biofilm, which has been associated with up to 80% of all infections (28, 29). The presence of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) enable *S. aureus* to readily bind to plasma and extracellular matrix proteins such as fibronectin and collagen, enabling the formation of biofilm on most tissues and implanted materials. Production of proteins, polysaccharides, and nucleic acids creates a protective shell that provides protection from immune cell attack, while altered metabolism and the presence of metabolically dormant persister cells increase the minimum inhibitory concentration (MIC) of most antibiotics by up to 1000-fold (30, 31). These protection mechanisms allow the bacteria to proliferate undisturbed, forming a community which can communicate using signaling molecules such as fatty acids to regulate growth (24). Initial attachment to implant surfaces or devitalized tissue is considered the critical event in the formation of infection, as it becomes increasingly difficult to kill the bacteria as the biofilm matures (32, 33). This attachment creates a well documented 'race to the surface' in which the implant is particularly vulnerable to bacterial attachment until integration occurs with the host tissue (34-36). Prophylactic antibiotics at the time of surgery or injury can reduce the risk of

infection, however breakthroughs still occur, especially when implants or devitalized tissue is present in the wound bed (33, 37, 38).

Small colony variants (SCVs) are also implicated in orthopedic infections, although their exact contribution is debated. Pathogenic bacteria form SCVs due to reversible mutations in metabolic pathways that greatly reduce growth rate, downregulate expression of virulence factors, and alter biochemical properties such as the cell wall charge (39, 40). Additionally, SCVs feature a significant upregulation in MSCRAMMs to facilitate rapid attachment to devitalized tissue, implants, and cells (41). The most significant mechanism of persistence is the ability to halt lysosomal maturation after phagocytosis, enabling the colonies to persist intracellularly with protection from immune defenses and antibiotics. Internal cellular defenses and apoptotic pathways are not activated due to the downregulation of virulence factors commonly associated with these bacteria, allowing the bacteria to proliferate within a cellular "Trojan Horse" (42). The metabolic and physiologic changes associated with this pathway make SCVs more resistant to antibiotics than planktonic bacteria, and the intracellular environment provides further protection as most antibiotics cannot accumulate at active concentrations within the cell (40, 43-45). While the significance of intracellular pathogens in orthopedic infections is still debated, SCVs have been isolated from chronic infections in a number of case studies (41, 46-53). Additionally, it has been shown that strains isolated from chronic osteomyelitis show increased tendencies towards SCV formation and osteoblast invasion, suggesting that *in* vivo conditions favor this phenotype (41, 54).

The anatomical location of the bacteria and condition of the patient may further complicate treatment and contribute to infection recurrence. Inflammatory reactions during osteomyelitis can cause the formation of new bone that effectively encompasses the infection,

creating a haven for bacterial persistence that must be surgically removed. Comorbidities such as diabetes or smoking cause vascular disease that limits blood perfusion in the region, and trauma or surgery can further disrupt the surrounding vasculature to limit systemic antibiotic penetration to the infection (6, 23). Recent studies have shown that *Staphylococcus aureus* can deform to submicron sizes and proliferate through the lacunar-canalicular network of bone to spread beyond the initial locus of infection (55, 56). This method of persistence was unexpected as *Staphylococcus* are generally considered to be non-motile organisms, yet they have been shown to proliferate throughout the bone using asymmetric division. It is believed that this may contribute to the finding of *S. aureus* beyond the zone of debridement despite aggressive margins into healthy tissue (12, 57).

Systemic antibiotic concentration must be kept low to prevent organ toxicity despite the ability of musculoskeletal tissue to tolerate higher doses (58). The low systemic concentrations achievable with IV or oral delivery are often insufficient to prevent or treat infections, especially as vascular insufficiencies or antibiotic properties further limit perfusion into bone. Local antibiotic delivery has been used as a method to circumvent limitations of systemic delivery and increase concentrations at the infection site (59). In its simplest form, local delivery can be performed by adding antibiotic powder directly to the wound bed prior to closure, as is common in clinical practice with vancomycin. This procedure has been used by surgeons due to evidence that it may reduce the rate of infection, including reports that infection rate for spinal fusion can be reduced by 50% (60, 61). There is skepticism about the efficacy of the procedure and evidence for its use to combat PJI is limited, with some studies even suggesting a significant increase in wound complications (62-65). However, the insignificant cost of antibiotic powder relative to infection has caused many surgeons to add vancomycin regardless of limited clinical evidence (65-

67). The primary drawback of this approach is the limited antibiotic exposure time as the drug quickly diffuses out of the wound. Incorporating the antibiotic into a biomaterial can greatly improve efficacy by slowing drug delivery into the tissue over time, thereby increasing the duration of therapeutic antibiotic levels in the tissue. This significantly improves the chances of eradicating bacteria, particularly biofilm, as the minimum biofilm eradication concentration (MBEC) is reduced with increased exposure times (68-70).

Polymethylmethacrylate (PMMA) is considered the gold standard for local antibiotic delivery and has been used for over 40 years (71, 72). PMMA can be loaded with antibiotic at low doses, around 1-4% wt., when used structurally to secure joint prostheses, but can be prepared with higher concentrations for use as a temporary drug delivery system. It was long believed that antibiotic loaded PMMA cement (ABLC) could reduce the rate of PJI (73-75), and it is still used prophylactically in a large number of arthroplasties (22). Despite its widespread usage, however, the exact efficacy of ABLC is debated (76). Few large scale randomized controlled trials have tested the use of prophylactic ABLC, and several modern reviews of have failed to find a significant reduction in the rate of PJI compared to plain cement (77-79). Furthermore, ABLC has been implicated in the rise of antibiotic resistant bacteria due to subtherapeutic antibiotic release for years after implantation (80, 81). Despite these shortcomings, ABLC has been used with success as a temporary drug delivery system that is removed after 4-6 weeks. The spacers used in two stage PJI revision are commonly made from high dose ABLC, and have been used as a successful adjunct to systemic therapy (82-84). Additionally, several studies have also shown efficacy when ABLC beads are used to treat traumatic injuries or osteomyelitis (22, 59, 85, 86).

The drawbacks associated with PMMA have driven the search for biodegradable systems which can deliver the full drug payload over a specified time interval and then naturally degrade

within the body, eliminating the need for retrieval of the delivery system. Examples of such materials include collagen, chitosan, polylactic acid, polyglycolic acid, bioactive glass, and calcium based bone void fillers typically used for bone regeneration (87, 88). Unlike PMMA, which effectively traps most of the drug within the polymer, the gradual degradation of the system ensures the complete release of encapsulated antibiotics and minimizes periods of sub-therapeutic dosing. Calcium based synthetic bone grafts, including calcium sulfate (CS), β-tricalcium phosphate (TCP), and hydroxyapatite (HA), are the most common degradable carriers. These materials are delivered in kits containing a base powder which forms a paste upon mixing with water, creating a cement that can be molded intraoperatively to form beads or fill boney defects (89). Hardening is dependent on dissolution-precipitation reactions that bind the powder together and induce phase transformations, creating a hardened structure in the desired shape (90, 91). The material properties, particularly degradation, can be tailored by altering the composition and crystallization of the graft; pure CS typically resorbs over a period of 6-12 weeks, whereas TCP degrades over 6-24 months, and HA requires 1+ years to degrade (90, 92, 93). CS alone may have limited application as a bone void filler depending on anatomical location due to its rapid resorption rate, which may outpace bone growth and limits mechanical stability (94). TCP and HA may degrade too slowly, however, so the three are commonly mixed to tailor strength and degradation properties to match functional requirements for bone healing (95-97).

Antibiotics can be easily incorporated into these bone void fillers by mixing with the base powder prior to hydration, creating a degradable system that elutes high concentrations of antibiotics for weeks to months. High level prospective studies are lacking that examine the effect of calcium bone graft substitute mediated antibiotic delivery on the treatment of PJI or osteomyelitis; however, multiple small studies have demonstrated high clearance rates (63, 94, 98-

100). *In vitro* studies suggest that calcium bone void fillers can maintain high level drug elution for longer periods than PMMA, making them advantageous in treating active infections (101-104), and several small clinical trials have shown that calcium sulfate works as well as PMMA in the management of osteomyelitis (99). Drug loading is performed under surgeon direction at the time of surgery, with vancomycin, gentamicin, and tobramycin being commonly used alone or in combination. This adaptability is advantageous as drug loading can be tailored towards specific surgeon or case requirements, yet this lack of standardization makes it difficult to compare research studies. Antibiotic choice, drug loading concentrations, and even antibiotic brand can have an effect on the final properties of the bone void filler due to interference in the setting reaction (105, 106).

Despite aggressive treatment practices and local drug delivery, infection recurrence rates remain high at 20-30% for osteomyelitis and PJI (107, 108). Furthermore, inappropriate antibiotic use has greatly increased the prevalence of antibiotic resistant bacterial strains that can further complicate treatment (109-111). Infections with antibiotic-resistant organisms are known to cost significantly more than infections with sensitive organisms, and have been associated with longer hospital stays and worse outcomes (112). Methicillin resistant *Staphylococcus aureus* (MRSA), one of the most common antibiotic resistant bacteria, is typically treated with vancomycin, however the MIC for this drug is steadily rising as well (113). The reduction in available drugs, often cited as the greatest current healthcare concern, may lead to greater morbidity and mortality as treatment options disappear. These considerations warrant the further investigation and/or development of local antibiotic delivery systems that not only deliver antimicrobials in high concentrations to combat infections, but also optimize the release kinetics to ensure maximal efficacy of the delivered dose and minimize chance of resistance (71).

Traditional local drug delivery devices rely on either free diffusion or matrix degradation to release the drug payload. Novel materials are under investigation that modulate drug release in response to passive or active stimuli including temperature, pH, oxidative potential, magnetic fields, ultrasound, or light (114, 115). Stimuli-responsive delivery is advantageous as the drug elution rate can be altered to combat developing symptomology or to maintain high local elution levels for biofilm treatment. Magnetically responsive systems are particularly attractive due to the non-invasive, safe, and highly controllable manner in which magnetic fields can be applied to specific parts of the body. These systems are typically composed of a polymeric matrix containing superparamagnetic Fe_3O_4 iron oxide nanoparticles (SPIONS), which generate heat in the presence of an alternating magnetic field via Neel and Bowman relaxation (116). Temperature increases up to 50° C at the SPION surface have been reported upon stimulation with an alternating magnetic field (117, 118), with heat decaying exponentially with increasing distance from the surface. This heat can cleave nearby heat liable bonds, such as azo linkers, or induce phase changes in the surrounding polymer to improve drug diffusion out of the system (119-121). SPIONS have already seen clinical usage in the form of Feredex, a dextran coated SPION formulation that was used as an MRI contrast agent and iron replacement therapy. Furthermore, magnetic hyperthermia via external stimulation of SPIONS has already been demonstrated as safe and efficacious in small clinical trials for the treatment of various cancers (117, 122). The demonstrated *in vivo* biocompatibility and efficacy of magnetic stimulation uniquely positions SPIONS as a feasible element of next-generation local drug delivery systems.

Research Objectives:

The goal of this research project was to advance knowledge of local drug delivery systems by a) generating clinically relevant data that aids in the selection and use of currently available

systems and b) advancing knowledge of next-generation materials through the creation of a magnetically responsive antibiotic system. Towards that end, the specific research objectives were:

1. Evaluate the setting kinetics, drug elution, and degradation properties of a triphasic calcium based bone graft containing vancomycin, tobramycin, or both.
2. Evaluate the drug release, calcium ion release, and acidity of four calcium sulfate products to determine if brand or material source impact clinically relevant parameters.
3. Fabricate and characterize a chitosan-polyethylene glycol microbead containing superparamagnetic iron oxide nanoparticles for stimuli-responsive vancomycin delivery.

CHAPTER 2

Evaluation of Antibiotic-Releasing Triphasic Bone Void Filler In-Vitro

1. Introduction

Bone grafting is a well-established surgical practice in which osseous defects created by trauma, tumor resection, or arthroplasty are filled using donor-harvested bone or by using other synthetic alternatives [1–7]. Autografts, sections of bone harvested from the patient's iliac crest, are considered the gold standard of bone grafts due to their osteoconductive, osteoinductive, and osteogenic properties. These procedures, however, require a second surgical site and have limitations including pain, complications of the harvest site, and limited amounts of bone [2,4,5,8–11]. Allografts are sometimes harvested from corpses or hip prosthesis procedures and used to fill boney defects; however, these suffer from risk of disease transmission and immune-rejection, and typically lose osteogenic properties during processing [9]. Synthetic, calcium-based cements and ceramics have been available for several decades as an alternative to allografts and autografts. Three common types of calcium bone void fillers (BVF) used clinically are calcium sulfate, tricalcium phosphate, and hydroxyapatite. These synthetic BVFs serve as osteoconductive scaffolds and may exhibit osteoinductive properties depending on the macro and micro porosity of the scaffold [7,12]. Unlike calcium sulfate-based BVFs, tricalcium phosphate (TCP) and hydroxyapatite (HA) are stable at physiologic pH and must be degraded by osteoclasts or macrophages [13]. These BVFs therefore require a degree of interconnected porosity to allow vascularization and osteoblast/osteoclast infiltration to achieve rapid integration with the surrounding bone tissue [10,14]. This can be difficult to achieve in BVFs mixed within the operating room; however, porosity can be increased by adding calcium sulfate that will

naturally resorb upon implantation, creating channels in the TCP or hydroxyapatite for cellular ingrowth [13,15].

Combination BVFs have appeared on the market containing mixtures of calcium sulfate, TCP, and/or hydroxyapatite to achieve optimum dissolution rates, porosity, and mechanical properties. It is well-documented that local antibiotic delivery with polymethylmethacrylate (PMMA) greatly reduces the number musculoskeletal surgical site infections and improves clearance of musculoskeletal infections when used adjunctively with systemic antibiotics [16,17]. PMMA does have some disadvantages compared to other systems, such as the need for a second surgery to remove temporary drug delivery implants and continued low-level antibiotic elution from permanent implants that can drive the development of antibiotic-resistant bacteria. Calcium BVFs can be easily loaded with antibiotics by mixing the drugs into the calcium phosphate/sulfate powder or the liquid to form a biodegradable antibiotic delivery system, eliminating the need for follow-up surgery and reducing concerns about antibiotic resistance [18]. Antibiotics are commonly added for the treatment of active infections, such as hip arthroplasties or osteomyelitis, or for infection prophylaxis in the case of traumatic injury. Calcium-based BVFs are often prepared under surgeon direction at the time of surgery using antibiotics available in the operating room. Prior work has shown that antibiotics can affect the dissolution and precipitation-hardening reactions of calcium phosphates, thereby increasing set time at a rate dependent on antibiotic concentration within the BVF [19]. Each antibiotic/BVF combination must therefore be evaluated to ensure that the BVF set time does not unnecessarily prolong surgery.

Triphasic BVF offers potential advantages in mechanical properties and dissolution rates compared to traditional BVF. However, there is currently a lack of relevant data regarding the

handling, elution, and dissolution properties of triphasic mixtures when combined with antibiotics. In surgeon-directed use of BVF for antibiotic delivery and bone regeneration, the most commonly used antibiotics are vancomycin and tobramycin. Powdered antibiotic for reconstitution may be added to BVF powder during the mixing process prior to casting into pellets for packing into the defect. Therefore, the objectives of this study were to determine whether the addition of powdered antibiotic at clinically available quantities affects the handling, elution, or dissolution properties of triphasic BVF beads containing calcium sulfate, hydroxyapatite, and TCP.

2. Results

2.1. Set Time

Using friability as a guide, an orthopedic surgeon declared the set time for BVF without antibiotics as 7 min. This increased slightly to 8.5 min with the addition of 1 g vancomycin to the BVF powder. BVF containing tobramycin or a combination of tobramycin and vancomycin did not set within the 1.5 h study period; however, they were declared set after sitting overnight for a total of 15 h. Vicat testing, according to ASTM C472, showed that BVF set at 3.5 min without antibiotics and 5 min with vancomycin only. BVF with tobramycin required 21 h to set in accordance with the standard; however, BVF with both vancomycin and tobramycin set within 3 h.

2.2. Elution

Both tobramycin and vancomycin concentration remained above 1 mg/mL throughout the 42-day study (Figure 1). Tobramycin concentration was significantly higher in the tobramycin and vancomycin combination group than the tobramycin only group at time points 4 h, 8 h, 24 h, 7 d, and 14 d ($p < 0.05$). Conversely, vancomycin concentration was higher for the vancomycin-

only group than the vancomycin and tobramycin combination group at all time points except day 42 ($p < 0.001$).

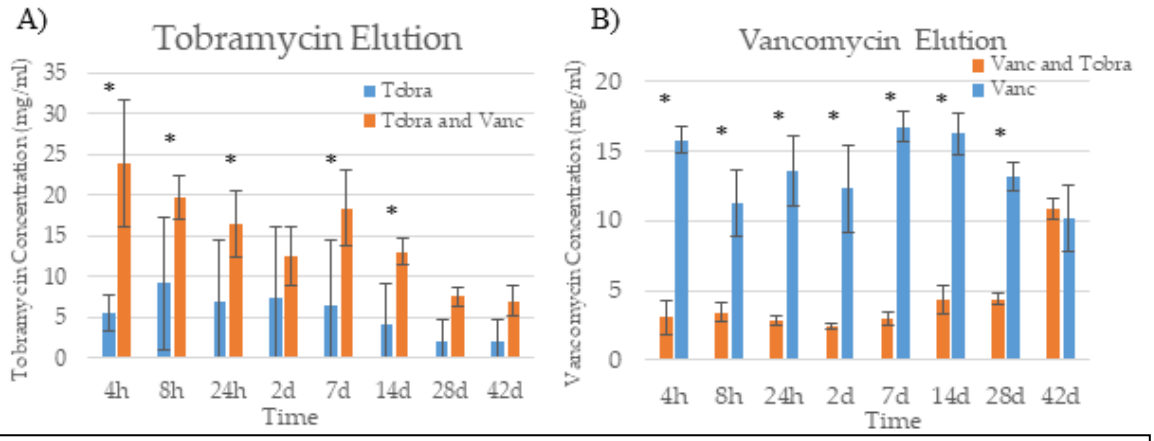

Figure 1. Tobramycin (**a**) and vancomycin (**b**) elution from calcium bone void filler (BVF) over 42 days. Mean ± standard deviation. * Signifies a statistical difference between the single and dual antibiotic loaded beads ($p < 0.05$).

2.3. Dissolution

Beads containing BVF without antibiotics were reduced to 39% of the original mass after 14 days in phosphate buffered saline (PBS) (Figure 2). Beads containing tobramycin or vancomycin alone showed similar dissolution rates to the no antibiotic control; however, the

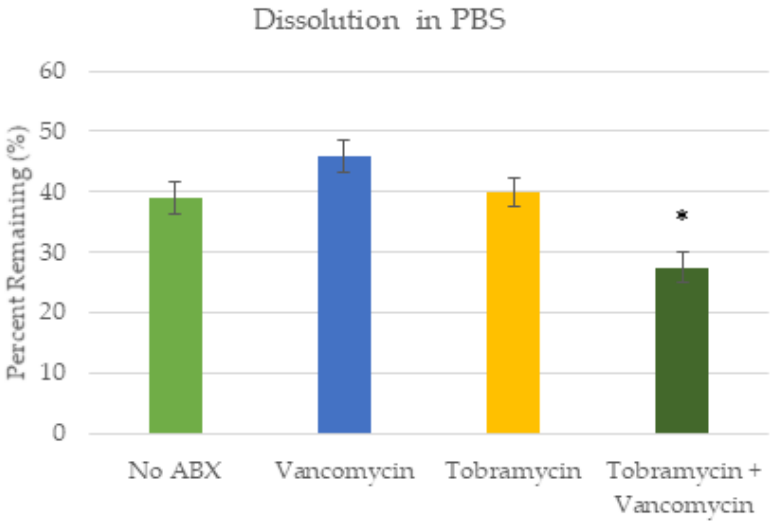

Figure 2. Calcium bone void filler (BVF) dissolution after 14 days in phosphate buffered saline (PBS). Mean ± standard deviation. * Signifies a statistical difference compared to the control ($p < 0.05$).

tobramycin and vancomycin combination showed a higher rate of dissolution than non-antibiotic controls with only 27.5% mass remaining ($p = 0.02$).

3. Discussion

Calcium sulfate, tricalcium phosphate, and hydroxyapatite have been used independently with success; however, each has disadvantages regarding dissolution and mechanical properties. Calcium sulfate has been used since the late eighteenth century to fill bony defects; however, it typically resorbs within 6–12 weeks before bone has fully filled the defect and matured [20,21]. Reports of increased inflammation, serous drainage, and questionable efficacy in vivo, likely due to the rapid resorption rate, have limited the use of pure calcium sulfate as a bone void filler [22–25]. Tricalcium phosphate (TCP)-based cements have a longer dissolution time, typically 6 months to 2 years, and have initial mechanical compression strength similar to that of cancellous bone [26,27]. TCP materials are better suited to filling large, load-bearing defects, but studies have shown that large defects still show a drop in mechanical properties at approximately 12 weeks when BVF dissolution has outpaced bone ingrowth [27]. Hydroxyapatite-based cements typically exhibit higher compression strength than TCP but take even longer to resorb, with some BVF degrading after 2–5 years and some highly crystalline scaffolds remaining permanently in the body [7,13,26,28]. These three BVF materials can form composites to achieve desired mechanical and biological properties while limiting disadvantages. Antibiotics mixed with calcium BVF reside within pores between the interlocking crystals of the calcium phosphate or calcium sulfate matrix and are released through either diffusion or BVF dissolution [19]. Drug elution from calcium phosphate-based BVF is typically described as diffusion-based, since elution outpaces dissolution; however, dissolution may result in increased porosity that improves elution at later time points [19]. When combining calcium sulfate and calcium phosphate BVF,

the calcium sulfate will resorb first and create channels or pores throughout the structure [15]. It is therefore necessary to evaluate composite BVF elution properties separately from homogeneous calcium sulfate or calcium phosphate. Results of the current study show that the addition of vancomycin had little effect on BVF set time; however, tobramycin significantly prolonged the setting reaction. Clinically, the combination of vancomycin and tobramycin released from a local drug delivery matrix may be advantageous due to reported synergistic effects of these two antibiotics, particularly against a broad spectrum of microorganisms [29,30]. Triphasic BVF beads continued to elute for up to 42 days, more than the 4–6 weeks recommended for the treatment of most osteomyelitis cases [31].

Length of set time and preparation steps is a factor for consideration for surgeons choosing to include antibiotics in triphasic BVF. Set time for plain BVF and antibiotic BVF determined using a Vicat needle and by an orthopedic surgeon using tactile feedback. Use of ASTM standardized methods provides data that can easily be compared to prior studies, while qualitative assessment by an orthopedic surgeon can corroborate set time results using tactile feedback relevant to surgical conditions. The extended set time of triphasic BVF and tobramycin-loaded groups may be due to the documented hygroscopic nature of tobramycin [32]. There is a need for future studies to evaluate direct effects of the ambient humidity and temperature levels on set time. In a study by McLaren et al., it was suggested that generic tobramycin particles occupy more volume than proprietary formulations, which could hinder their crystallization [33]. The addition of vancomycin, however, had a minimal impact on set time. Due to the lengthy set time of BVF combined with 1.2 g tobramycin, future work will include studies with lower tobramycin concentrations, a smaller volume of deionized water in the BVF mixture, and various mixing techniques to reduce set time.

Elution properties were assessed using a 42-day partial refreshment elution study. The BVF mass-to-fluid-volume ratio is based on prior studies that suggest that up to 40 calcium sulfate beads are packed into wounds with a total volume of 100 cc [34]. This was scaled back to an approximate BVF volume of 4 cc, which is comparable to the amount used in the treatment of osteomyelitis [35,36]. A partial refreshment elution study was performed to better approximate in vivo elution, which is believed to be slower than in full refreshment in vitro studies [34,37]. Concentrations of antibiotics eluted from triphasic BVF in this study suggest therapeutic efficacy over the course of bone regeneration. A similar partial refreshment elution study using calcium sulfate BVF showed that vancomycin and tobramycin concentrations dropped to less than one tenth of their peak concentration by day 42 [37]. In contrast, the vancomycin and tobramycin concentrations in this study remained at 61% and 21% of their respective peak concentrations at day 42, suggesting prolonged elution at later time points compared to plain calcium sulfate. Other studies with calcium sulfate have shown that vancomycin elution is more consistent and prolonged than that of tobramycin, as was seen in this study [38]. The reduced vancomycin elution from beads containing a combination of antibiotics is in contrast to prior research showing that vancomycin and tobramycin release from calcium sulfate was higher for combination groups than groups containing either antibiotic alone [39]. The HA or TCP components of triphasic BVF may have altered physiochemical interactions with vancomycin in the presence of tobramycin. The glycopeptide antibiotic vancomycin may show similar binding to mineral components of calcium-based BVF materials as proteins in the TGF-beta family [40,41]. The increased vancomycin elution from the combination group at day 42 also corresponds to the point at which tobramycin elution slows, suggesting that these composite beads are preferentially eluting tobramycin over vancomycin, although further work is needed to

test this finding. Together these results suggest that the triphasic mixture has an extended antibiotic release profile compared to calcium sulfate [39,42]. The difference in loading concentrations between antibiotics limits our ability to make comparisons between antibiotics, although general profile comparisons can be made. These concentrations were selected to mimic the amounts and form of antibiotics available to surgeons in the operating room, thereby providing clinically relevant data. Partial media refreshment has been shown to cause slower elution rates than full refreshment studies, making it difficult to directly compare results of this study with others [34,37,42]. Although our results describe the behavior of triphasic BVF impregnated with antibiotics in vitro, the complex in vivo environment introduces many variables that may alter elution and/or dissolution [42]. Immune cells and osteoclasts, known to degrade calcium phosphate cements in vivo [43,44], may increase the rates reported in this study.

A 14-day in vitro dissolution study was used, which compared the effects of antibiotics incorporation on dissolution in PBS. The 14-day time point was selected based on preliminary studies that suggested some of the beads may completely dissolve at longer time points. Dissolution was not affected by the addition of a single antibiotic. The BVF loaded with both antibiotics did show a significant increase in mass loss over 14 days in PBS, however. Prior studies with calcium sulfate have shown that the addition of antibiotics can affect the crystallinity of the BVF [33]. It is therefore possible that the increased dissolution rate seen in the combination group is due to reduced crystallinity of the triphasic BVF, resulting in quicker dissolution of the calcium sulfate. Future studies should include x-ray crystallography and/or mechanical testing of combination group to test this hypothesis.

In conclusion, the addition of antibiotics to Osteoboost BVF affected set time depending on the type of antibiotic used. Using combinations of antibiotics could be beneficial in using

synergistic effects of aminoglycosides and vancomycin to prevent osteomyelitis. This study aimed to guide surgeons on what to expect when using vancomycin and tobramycin with triphasic BVF. Future studies will focus on additional antimicrobials and antifungal agents.

4. Materials and Methods

4.1. Bead Preparation

Antibiotics used in the BVF mixture in this study included tobramycin sulfate (Research Products International, Prospect, IL, USA) and vancomycin hydrochloride (MP Biomedicals, Solon, OH, USA), or a combination of both). Osteoboost® triphasic BVF beads (OsteoRemedies, Memphis, TN, USA) were prepared according to manufacturer instructions by mixing BVF powder with the appropriate antibiotic powder and 7 mL of deionized water for 60 s, packing the paste into rubber molds with a spatula, and waiting until the beads hardened. A Vicat needle was used to determine set time according to ASTM C472. Briefly, the BVF powder was mixed with the appropriate antibiotic and quantity of deionized water and placed in a cylindrical mold. The Vicat needle was set to the top edge of the mixture and released. Set time was determined when the needle failed to penetrate half the depth of the BVF. Separately, the BVF was mixed by an orthopedic surgeon and packed into rubber molds provided with the BVF. The surgeon qualitatively declared set time based on the hardness of the BVF upon probing with a plastic spatula. Bead groups included powdered vancomycin (1 g), powdered tobramycin (1.2 g), or both vancomycin and tobramycin (1 g and 1.2 g, respectively). Each group consisted of 4 samples (n = 4), and the beads were mixed by an orthopedic surgeon in similar conditions to that of an operation room.

4.2. Elution

A 42-day elution study was carried out to determine the concentrations of antibiotics released from the BVF pellets. From each group, 3 g of pellets were submersed in 4 mL of phosphate buffered saline (PBS) at 37 _C with shaking (n = 4). Elution samples were collected at 4 h, 8 h, 1 day, 2 days, 7 days, 14 days, 28 days, and 42 days. At each time point, 1.5 mL elution media was removed and replaced with fresh PBS. Antibiotic concentration was then determined using high performance liquid chromatography (Dionex Ultimate 3000, Thermo Fisher Scientific,Waltham, MA, USA) with a C18 column (Hypersil Gold, Thermo Fisher Scientific, Waltham, MA, USA). A pre-column amine derivatization method was used to enable fluorescence detection of tobramycin [45].

4.3. Dissolution

Dissolution was determined for all antibiotic groups and 1 control group without antibiotics (n = 4). One pre-weighed pellet per group was placed in 4 mL PBS that was refreshed every two days for a duration of 14 days. On day 14, samples were dried in a vacuum oven, and percent mass loss was calculated.

4.4. Statistical Analysis

All statistical tests were performed using SigmaPlot software (Systat Software, Inc., San Jose, CA, USA). Two-way analysis of variance (ANOVA) with Holm-Sidak post-hoc was used to test for differences in antibiotic elution rate between groups. One-way ANOVA with Holm-Sidak post-hoc was used to test for differences in dissolution rate. A significance level of 0.05 was used for determining statistical significance between groups.

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CHAPTER 3

Comparison of Commercial Calcium Sulfates for Antibiotic Delivery in Periprosthetic Joint Infection

Introduction:

Periprosthetic joint infection (PJI) is a debilitating complication of joint arthroplasty associated with significantly higher mortality rates and healthcare costs than non-infected procedures. Typical two-stage revision procedures used to treat PJI involve the use of local drug delivery devices, typically antibiotic loaded polymethylmethacrylate (PMMA) spacers which maintain the joint space and elute antibiotics until a new implant is placed (1, 2). While PMMA mediated antibiotic delivery has been shown to reduce infection recurrence, there are several drawbacks to its use. PMMA is nondegradable and requires an additional surgery to remove the material, thereby limiting its use during the reimplantation procedure. Additionally, PMMA is a nonporous polymer with elution driven by surface diffusion, causing a rapid initial burst of drug release that quickly tapers off; this limits the period of efficacious drug delivery and has been shown to encourage bacterial resistance (3-5). Calcium sulfate (CS) based bone void fillers, traditionally used for bone grafting procedures, are advantageous for drug delivery due to their biocompatibility and resorbable nature, ensuring full drug delivery over a period of several weeks as the material completely resorbs (6, 7). The CS is commonly delivered in kits with a base powder of CS hemihydrate that, upon mixing with water, undergoes a series of dissolution and precipitation reactions that result in recrystallization to form CS dihydrate. Antibiotics can be conveniently loaded into the beads by mixing with CS hemihydrate powder prior to hydration.

CS drug choice and loading concentrations are surgeon directed, with vancomycin, tobramycin, and gentamicin being the most common. The lack of regulation or standardization

concerning antibiotic loading into CS has created a gap in clinically relevant data concerning antibiotic elution and material properties. Multiple prior studies have demonstrated that antibiotic incorporation can interfere with the setting reaction of CS, potentially altering resorption and mechanical properties (8, 9). This is concerning as a reduction in CS setting can cause increased degradation rate, which may increase inflammation through accelerated calcium ion release and further acidification of the local environment (10, 11). Additionally, multiple cases of hypercalcemia occurring several days to 2 weeks following CS treatment of infection have been reported, with many patients requiring intensive care treatment to reverse the symptoms (12-15). Previous reports and manufacturer statements have claimed that high purity synthetic CS is associated with fewer adverse reactions, either due to the absence of impurities, consistent degradation, or reduced pH of resorption products (16, 17). However, these claims are largely unsubstantiated, as most studies using CS are performed without standardization of *in vitro* or *in vivo* criteria, making it difficult to compare results as many variables affect CS performance (9, 18).

The goal of this study is to compare pH, calcium ion concentration, and antibiotic concentration of eluate samples from four brands of CS using a model of synovial fluid in a large joint. Two brands, Osteoset (Wright Medical, Memphis, TN) and Calcigen (Zimmer Biomet, Warsaw, IN), are composed of naturally sourced CS, whereas Synthecure (Austin Medical Ventures, Germantown, TN) and Stimulan (Biocomposites, Staffordshire, England) are synthetically manufactured. We hypothesize that there will be brand-specific differences in drug elution properties, calcium ion release, and local acidity when four brands of antibiotic loaded CS are compared. This study will generate clinically relevant data to aid surgeons in selecting CS bone void fillers and antibiotic combinations for use in the operating room.

Materials and Methods:

Four brands of commercial CS with and without antibiotics were used for these studies. Osteoset, Calcigen, Stimulan, and Synthecure were prepared aseptically according to manufacturer instructions and packed into a rubber mold to form identical pellets. Antibiotic loaded beads had 1 g vancomycin (Breckenridge Pharmaceuticals, Berlin, CT) and 1.2 g tobramycin (X-Gen Pharmaceuticals, Horseheads, NY) added to each 10 cc kit prior to mixing. The beads were left in the aseptic environment until hardened as determined by friability.

Elution media was prepared consisting of 25% bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA) and 75% phosphate buffered saline (PBS). Fifty grams of CS beads with antibiotics (n=4/group) or without antibiotics (n=3/group) were added to 100mL of sterile elution media. Samples were taken with complete media refreshment at days 1, 3, 7, 10, 14, 21, 28, 35, and 42. Drug concentration in each sample was determined using high performance liquid chromatography, and vancomycin concentration was measured using absorbance at 209 nm. Tobramycin was derivatized using an automated pre-column method with o-phthalaldehyde, and fluorescence was measured using an excitation of 337 nm and emission of 442 nm (19). Calcium ion concentration was determined using a colorimetric o-Cresolphthalein Complexone based Calcium Reagent Kit (Pointe Scientific, Inc, Canton, MI, USA), with absorbance measured at 600 nm. Acidity was measured using a standard benchtop pH meter.

Statistical Analysis:

All statistical analysis was performed using SigmaPlot 14.0 software (Systat Software, Inc., San Jose, CA). Elution profiles, calcium ion concentrations, and pH of eluate samples were compared between groups using two way analysis of variance (ANOVA) with Holm-Sidak post hoc analysis. A level of significance $\alpha = 0.05$ was used for all statistical tests.

Results:

Elution:

Tobramycin eluted in a burst fashion, with concentrations reaching up to 20 mg/ml at the day one time point and diminishing to less than 1 mg/ml by day 14 (Figure 1). Osteoset beads released significantly more tobramycin than the other groups for the first three time points ($p < 0.01$). Calcigen beads showed a smaller initial burst release and higher concentrations later in the study, however these increases were not statistically significant due to the high variance at the earlier time points. Vancomycin elution was more consistent over time, with three out of four groups maintaining concentrations of approximately 1 ± 0.5 mg/ml from day 3 through day 42. Calcigen beads, however, eluted significantly less vancomycin than the other groups after day 7 ($p < 0.01$), with levels dropping below 10 µg/ml from day 21 through the end of the study.

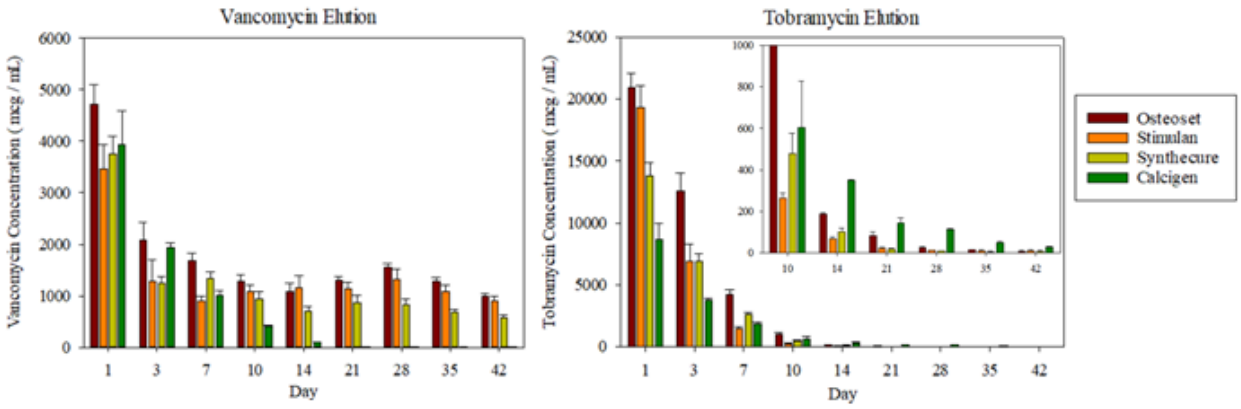

Figure 1: Vancomycin elution (left) and tobramycin elution (right). Tobramycin was eluted in a burst pattern that quickly diminished after 7 days, compared to vancomycin that had a more sustained release pattern in 3 out of 4 groups. Note the differing y-axis scale.

Calcium Release:

Cumulative calcium release was significantly different for all brands without antibiotics, with naturally sourced Osteoset and Calcigen releasing more than synthetic Stimulan and Synthecure (Figure 2, $p < 0.01$). The addition of antibiotics caused a 45% reduction in calcium release from Osteoset and a 15% reduction in calcium release from Synthecure ($p < 0.01$), with

no significant effects on release from Calcigen and Stimulan ($p > 0.05$).

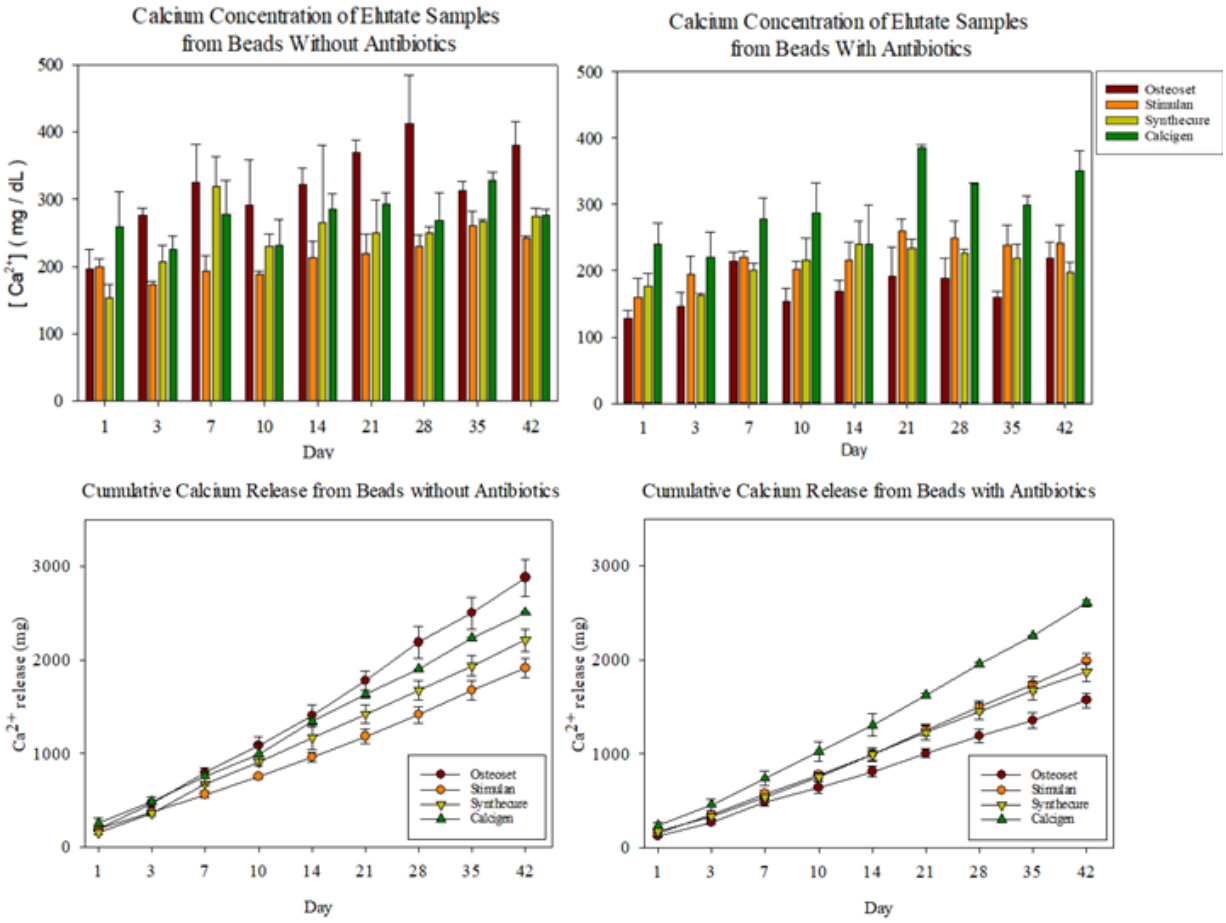

Figure 2: Calcium concentration of elution samples from beads without (A) and with (B) antibiotics. Cumulative calcium ion release without (C) and with (D) antibiotics.

pH:

The pH of eluate samples from all groups with antibiotics was approximately 6.5 ± 0.2 at the first time point followed a decreasing trend to approximately 5.6 ± 0.1 on day 42 (Figure 3). This behavior was not replicated in the beads without antibiotics, which had a relatively stable pH throughout the study with no apparent trends over time. The net result of this effect is an average pH reduction of 0.5 for antibiotic loaded samples over the day 21-42 time points. Osteoset without antibiotics had significantly lower pH than Calcigen throughout the first 21

days of the study, and lower pH than Synthecure throughout the entire study ($p<0.05$). Differences in pH among antibiotic loaded groups appear clinically insignificant after day 7.

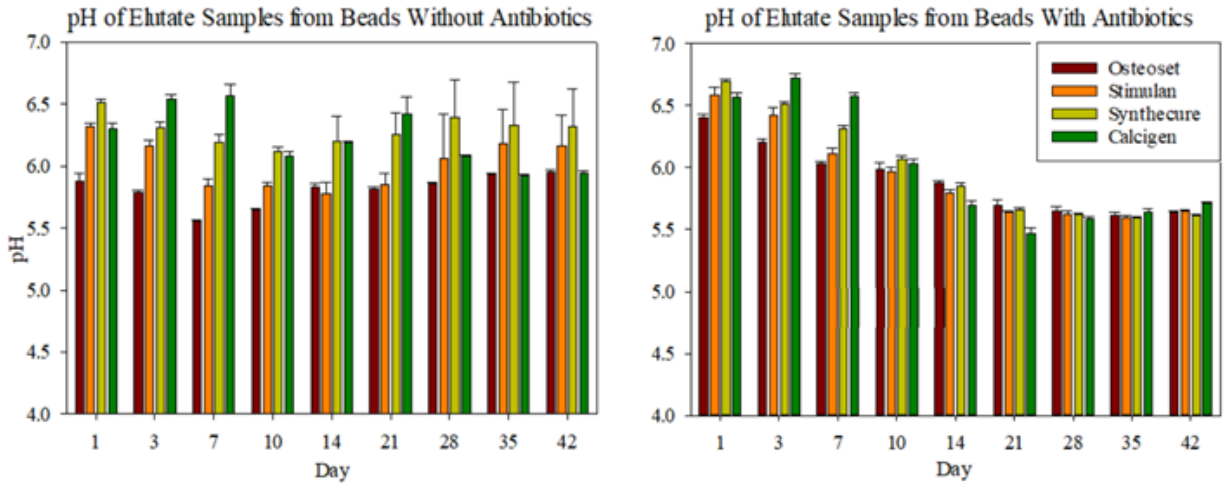

Figure 3: pH of elution samples from beads without (left) and with (right) antibiotics. Antibiotic loaded samples seem to reduce pH at later time points whereas non-loaded samples maintain a relatively constant pH.

Discussion:

CS bone void fillers have been used for antibiotic delivery for over 20 years, with many reports citing their use as beneficial in the treatment of PJI or osteomyelitis (20, 21). However, there have been some concerns over their use for surgeon directed drug delivery due to the lack of appropriate studies comparing CS products before and after antibiotic loading. The lack of standardization concerning CS use for drug delivery has led to a dearth of clinically relevant data to address these concerns, especially considering that antibiotic powder can interrupt the crystallization and setting of calcium based bone void fillers (8, 9). The purpose of this study was to analyze four commercially available brands of calcium sulfate loaded with antibiotics to determine kinetics of antibiotic release, the effect of antibiotic incorporation on calcium ion release, and the effects of antibiotic incorporation on pH. Additionally, we sought to provide drug elution data using a physiologically relevant model to the treatment of PJI by utilizing a large joint model complete with serum, as opposed to most studies that rely on DI water or PBS

(22-24). Drug release was maintained throughout the 6 week study in most groups, with vancomycin concentrations remaining elevated at day 42. Antibiotic incorporation did not increase cause an increase in calcium ion release, alleviating concerns of hypercalcemia. Antibiotic loading, however, did decrease pH throughout the course of the study, which may influence wound healing.

While the results of this study provide relevant data that can guide clinical decisions regarding CS use as a drug delivery vehicle, there are several limitations that must be considered. This study attempted to simulate the volume associated with a large joint and used elution media with serum in order to more accurately represent clinical usage, however no *in vivo* preclinical studies were performed to validate these results. Degradation, and therefore drug elution, calcium release, and acidity, may be altered under physiologic conditions, and future studies should use in vivo models to verify the findings. The addition of serum makes it more difficult to compare the results this study with others using PBS as the elution media (22-24). Additionally, 4 samples without antibiotics and 3 samples with antibiotics were lost to fungal infection due to the long study duration and use of serum, thereby reducing statistical power and potentially affecting the measured values. Future work will include larger sample sizes to minimize the effect of sample loss over that can occur after repeated sampling.

Three of the four brands continued to elute vancomycin at concentrations > 100x the minimum inhibitory concentration for most *S. aureus* strains throughout the study. Naturally sourced Osteoset maintained the highest levels of vancomycin elution with concentrations remaining above 1 mg/ml, however Synthecure and Stimulan also eluted concentrations over 500 µg/ml throughout the study. Tobramycin elution was much more rapid, with initial values up to 5x greater than those of vancomycin on day 1. Levels quickly fall to less than 5% of the

maximum concentration for each group, however, by day 14. Previous studies with calcium bone grafts have shown vancomycin elution to be slower than tobramycin, as was seen here (25, 26). The presence of therapeutic vancomycin concentrations 7 weeks into the elution study is supportive of prior evidence that CS can aid in the treatment of orthopedic infection (27, 28).

Calcium ion release is a natural result of CS degradation and resorption, creating the potential for hypercalcemia if the material degradation outpaces calcium removal (12-15). Furthermore, as illustrated in this study and others, CS degradation does create a local decrease in pH and this acidity may increase inflammation at the local wound bed. These factors may contribute to serous drainage complications which have been associated with CS use for decades, and raise concerns about increased inflammation affecting wound healing (17, 29-33). Theoretically, these concerns are validated as it is known that antibiotic powder can interfere with the dissolution and precipitation reactions required for the hardening of CS, potentially causing incomplete setting and rapid degradation upon implantation (8, 9, 34). Clinical findings reinforce these concerns as there have been multiple recent reports of hypercalcemia in patients treated with CS, and no specific cause or risk factor has been identified to date (12-15, 35-37). The data from this study suggests that antibiotic loading does not trigger increased calcium release, with Osteoset and Synthecure actually releasing less calcium after antibiotics are added. Factors that may affect the release of calcium ions from antibiotic loaded beads include the decreased percentage of calcium by weight, increased porosity after the antibiotic powder has been released, reduced pH at later time points, or interference between the high concentrations of antibiotic and calcium resorption equilibrium. The data in this study does not show a clear correlation between antibiotic elution or pH and calcium ion release. Similarly, there was no clear trend between pH and CS source. The pH of all groups with antibiotics was within ± 0.1

between days 21-42, suggesting that the effects of antibiotics completely overshadow any differences between CS source. The reduction in pH compared to samples without antibiotics may be due to the acidic nature of vancomycin itself, and further investigation is needed to determine if other antibiotics cause similar reductions in pH. This long term increase in acidity may contribute to wound inflammation and warrant the use of lower doses of CS containing vancomycin and tobramycin.

In conclusion, this study showed that CS source does not appear to negatively impact material properties regarding drug elution, calcium ion release, and acidification of the local environment when used to deliver antibiotics. Drug elution was shown to continue for up to 7 weeks for vancomycin, but tobramycin elution is greatly reduced after 2 weeks. Therapeutic drug concentrations were maintained much longer than prior studies with PMMA, suggesting increased efficacy when using CS for drug delivery. Antibiotic incorporation does not contribute to increased calcium ion release, suggesting surgeons concerned about hypercalcemia should instead examine CS volume, anatomical location, and local environment perfusion as potential causes.

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Chapter 4

Magnetic Stimuli-Responsive Chitosan-Based Drug Delivery Biocomposite for Multiple Triggered Release

1. Introduction:

Local drug delivery devices are designed to release therapeutics at a site of injury or disease, and maintain high concentrations of drug at desired locations while limiting systemic concentrations and toxic side effects [1-3]. While superior to conventional intravenous or intramuscular drug delivery methods, these local systems are limited in that they typically follow first order release patterns that release a set amount of drug despite changing physiological conditions [4]. Elution or release rates will eventually drop below therapeutic levels, which is especially problematic for antibiotic delivery systems, as sub-minimum inhibitory concentrations of antibiotics can lead to antibiotic resistant strains of bacteria [5-7]. Stimuli responsive or "smart" delivery systems offer additional control over the spatial and temporal release of encapsulated drugs through use of passive or active stimuli to alter the delivery matrix characteristics [8-10]. Active stimuli include magnetic fields, electric fields, ultrasonic waves, light, and externally generated heat, all of which can be applied to supply an on-demand increase in drug concentration at the target site due to often reversible changes in material conformations, phases, or chemical states [11-16]. The ability to externally modulate elution kinetics makes active-stimuli responsive systems advantageous, as it gives clinicians additional control over local drug concentrations throughout the treatment period. The spatiotemporal control offered by stimuli responsive biomaterials is particularly advantageous in the treatment of cancer and infection, in which high levels of therapeutic drug are needed at the target site but can induce toxic effects if administered systemically [17-20].

Of the active stimuli, magnetic stimulation is advantageous due to ease of use and the ability to target carriers in deeper tissues [23]. Magnetically responsive delivery systems are loaded with superparamagnetic nanoparticles (MNP) < 15 nm, typically iron oxide Fe_3O_4, that become magnetized under the influence of an externally applied magnet [23, 24]. MNP have effectively been functionalized and coated with biocompatible polymers to yield nanoscale core-shell structures [10], loaded into the center of liposomes [25], and incorporated into hydrogels [26]. Under magnetic stimulation, the incorporated MNP generate local hyperthermic conditions which can either be used to treat tumors or induce drug release by way of improved diffusion, increased polymer matrix permeability, or breaking of temperature-labile drug linkers [23]. Stimulation response magnitude may be dependent on the frequency and strength of magnetic stimulation, providing a means of fine-tuning drug release [24]. MNP have been used to induce hyperthermia for tumor therapy in several clinical studies, with stimulations tolerated for durations of 1 hour or more at field strengths of up to 6 mT in the pelvic region [27, 28] and 17 mT intracranially [29]. Many of these studies use the MFH300F AC magnetic field applicator (MagForce Nanotechnologies GmbH, Berlin) which is large enough to accommodate humans and can produce magnetic fields from 0-23 mT at 100 kHz. MNP loaded polymer matrices have also been used in stimuli responsive drug delivery studies, with high frequency alternating magnetic stimulation strengths ranging from as low as 4 mT [30] to as high as 100 mT [31].

We hypothesized that Fe_3O_4 MNP could be loaded into chitosan microbeads cross linked with polyethylene glycol dimethacrylate (PEGDMA) to create a magnetically responsive local drug delivery system for treatment of musculoskeletal infections secondary to orthopedic surgery or trauma. Microbeads were loaded with vancomycin, an antibiotic with strong activity against genus *Staphylococcus* bacteria that are highly prevalent in musculoskeletal infections.

PEGDMA is available in molecular weights ranging from M_n = 198 g/mol to over M_n = 20, 000 g/mol. We further hypothesize that the chosen length of cross linker will have a direct effect on polymeric structure of the beads, with potential implications drug elution properties and effects on cells. In the present study, chitosan microbeads cross linked with various lengths of PEGDMA were compared to determine what effects, if any, the length of polymer cross linker had on bead swelling, drug elution rate, responsiveness to magnetic stimulation, degradation properties, and cytocompatibility. The effects of various magnetic field strengths and field frequencies on drug elution rate were also assessed for beads made with M_n = 550 PEGDMA.

2. **Materials and Methods:**

2.1 Materials

Chitopharm S chitosan powder with an 82.5 ± 1.7 degree of deacetylation and an average molecular weight of 250.6 kDA was purchased from Chitopharm (Tromsø, Norway). NIH 3T3 fibroblast cells were acquired from ATCC. Cellgro Dulbecco's modified eagle medium with L glutamine and sodium pyruvate (DMEM), fetal bovine serum, Normocin (Corning, Manassas VA) were purchased from Fisher Scientific. Concentrated phosphate buffered saline (PBS), mineral oil, and high performance liquid chromatography (HPLC) reagents (Fisher Scientific, Hampton, NH) were purchased from Fisher Scientific. CellTiter-Glo viability assays were purchased from Promega (Madison, WI). Lysozyme Type VI from chicken egg white was purchased from MP Biomedicals LLC (Solon, OH). Vancomycin and polyethylene glycol dimethacrylate (PEGDMA) M_n= 550 and M_n = 750 were purchased from Sigma Aldrich (St. Louis, MO). PEGDMA M_n = 200 and M_m = 600 were purchased from Polysciences (Warminster, PA).

2.2 Fabrication of microbeads

Fe_3O_4 MNP were made according to a previously developed protocol by Kang et al. [32]. Iron oxide (Fe_3O_4) and Iron chloride ($FeCl_3$) were dissolved in HCl at concentrations of 0.5M and slowly dropped into dilute sodium hydroxide at pH 11-12. MNP were washed several times with HCL and deionized water prior to use. Previous imaging has shown the MNP average diameter to be approximately 10.89±2.67 nm. Each batch of MNP was characterized using x-ray diffraction (D8 Advance, Bruker Corporation, Billerica, MA) to ensure consistency. One gram of MNP was sonicated in 47 mL DI water for 1 hour to ensure even MNP distribution. There was no significant temperature increase. Two grams of chitosan powder, 400 mg vancomycin and 0.5 mL glacial acetic acid was then added and mixed by hand. The solution was stirred using a non-magnetic overhead impeller for 24 hours. The microbeads were then fabricated using a water in oil emulsion process. The oil solution consisted of 75 mL light mineral oil, 75 mL heavy mineral oil, 15 mL of the appropriate PEGDMA cross-linker and 2g Span 80 surfactant. The oil solution was stirred using a separate impeller and heated to 37° C. Fifteen mL of the chitosan-MNP-vancomycin solution was slowly added to the stirring oil using a 30-mL syringe. The solution was slowly heated to 60° C and allowed to stir for 24 hours. The beads were then washed to remove oil, surfactant and excess PEGDMA using a vacuum flask and successive treatment with hexanes, methanol, and acetone. The beads were then collected, weighed, and put into vials for the separate experiments.

2.3 Imaging

Beads were attached to stubs by adhesive carbon backing and then sputter coated with a Gold/Palladium (80:20) thin film approximately 20nm thick. A field emission scanning electron microscope (Nova NanoSEM 650, FEI, Hillsboro, Oregon) was used to image chitosan-

PEGDMA M_n = 550 beads with MNP. ImageJ (National Institute of Health, Bethesda, Maryland) was used to determine bead diameter.

2.4 Swelling ratio

50 mg of chitosan microbeads with each length of PEGDMA cross-linker were placed in 5 mL tubes with 2 mL of PBS at room temperature (n=3). After 24 hours, PBS was drained and excess liquid was removed using Kimwipes™ (Kimberly-Clark Kimetch, Irving, Texas). Samples were then immediately reweighed [33, 34]. Swelling ratio was determined as a percent increase in mass after immersion in PBS.

$$\text{Swelling ratio} = \frac{\text{weight after soaking}}{\text{initial dry weight}} \times 100\%$$

2.5 Elution and stimulation

To determine *in vitro* elution patterns, 2 mL of PBS were added to 50 mg of beads (n=4) at room temperature, with groups for each length of cross-linker and four additional groups with only PEGDMA M_n=550 to test the effects of field strength and field frequency (Table 1). Sampling occurred every 24 hours with complete media refreshment for 8 days. Each group was subjected to 30 minutes of magnetic stimulation or sham stimulation on days 3, 5 and 7 using a MagneTherm™ magnetic field generator (NanoTherics, Newcastle, UK). All lengths of PEGDMA beads were stimulated at 23 mT and 109.9 kHz. To assess the effect of field strength, M_n = 550 beads were tested at 75% and 50% of the original 23 mT field strength (17 mT and 11mT respectively) at a field frequency of 109.9 kHz. Field frequency was also tested on M_n = 550 beads at frequencies of 109.9 kHz, 330.4 kHz and 524.8 kHz with a field strength of 17 mT. Small 25 µL samples were taken before and after stimulation to determine if vancomycin concentration increased during this period. Total vancomycin release was estimated as the total amount of vancomycin eluted into PBS once the daily elution rate had dropped below 1% of the

cumulative elution for that group. To estimate theoretical vancomycin loading, the estimated cumulative release value was divided by bead mass. Vancomycin concentration in eluate samples was determined using high performance liquid chromatography (Dionex UltiMate 3000 HPLC, Thermo Scientific, Waltham, MA) with a C18 150x4.6 mm column (Hypersil Gold, Thermo Scientific). HPLC mobile phase consisted of 70% (0.124M KH_2PO_4 and 0.08M K_2HPO_4) and 30% acetonitrile pumped at 1 mL/min [35]. Retention time was approximately 1.6 minutes.

Table 1: Stimulation Parameters

PEGDMA molecular weight	Magnetic Field Strength	Magnetic Field Frequency
200	23 mT	109.9 kHz
550	23 mT	109.9 kHz
600	23 mT	109.9 kHz
750	23 mT	109.9 kHz
550	17 mT	109.9 kHz
550	11 mT	109.9 kHz
550	17 mT	330.4 kHz
550	17 mT	524.8 kHz
200	0 mT	N/A
550	0 mT	N/A
600	0 mT	N/A
750	0 mT	N/A

2.6 Degradation

To determine the degradation profile of Chitosan-MNP beads, a 21-day degradation study was performed using beads cross-linked with M_n = 550 PEGDMA with time points at 3, 5, 7, 14 and 21 days. A 3-day elution study was performed using PEGDMA M_n = 200, M_n = 600 and M_n = 750 cross-linked beads to compare initial degradation rates. Each group included 3 bead samples per time point weighing 50 mg per sample. Ten mL of 1 mg/mL lysozyme in PBS were added to each test tube and replaced during every 48-hour interval to replenish the enzymes that promote degradation. Beads were incubated at 37° C under constant orbital shaking throughout the experiment. At the end of every time point, the lysozyme solution was removed, and the beads were washed twice with 20 mL of deionized water. The beads were placed in a vacuum oven at 50 °C and -20 PSI until no changes in weight occurred during daily re-weighing. At the end of each drying cycle the weight was recorded and the percentage remaining was calculated using the equation:

Percent Remaining (%) = (Final Sample Weight (mg))/ (Initial Sample Weight (mg)) × 100

2.7 Cytocompatibility

NIH3T3 fibroblast cells were seeded into 24 well flat bottom plates at a density of 1 x 10^4 cells/cm^2 in DMEM media supplemented with 10% fetal bovine serum (FBS) and 100 µg/mL Normocin™ antibiotic/antimycotic. All cells were stored in a humid incubator at 37° C and 5% CO_2. After overnight attachment and observation, beads from each group were added to wells at concentrations of 5 and 10 mg/cm^2 of well plate surface area (n=4). Silicone beads were added at a density of 10 mg/cm^2 to serve as bioinert control beads. Four wells did not receive any beads and were used as positive cell growth controls. On day three, wells were imaged using an EVOS microscope (AMG, Washington) and ATP concentration, which is proportional to the number of

metabolically active cells, was quantified compared to controls using CellTiter-Glo®. CellTiter-Glo was added to each well and the luminescence of the media/CellTiter-Glo solution was used to quantify ATP concentration [36, 37]. Measured values were normalized to the control group that did not receive beads.

2.8 Statistical Analysis

All statistical tests were performed using SigmaPlot software (Systat Software, San Jose, California). One-way ANOVA with a Holm-Sidak post-hoc analysis was used to detect differences in swelling ratio, degradation rate, stimulation response and cytocompatibility between groups with different cross-linkers. The three stimulations were treated as separate events due to expected decreases in elution rate and stimulation response magnitude with time. The level of significance was taken as $\alpha = 0.05$.

3. Results and Discussion

3.1. Characterization

SEM images show spherical beads with slightly porous outer surfaces (Fig. 1). The beads embedded with MNP were 210 ± 40 microns in diameter with dimples covering the entire area of the beads. Chitosan beads have been manufactured using a wide variety of fabrication techniques with bead sizes ranging from the nanoscale to several millimeters [38, 39]. Previous research on microbeads has shown that elution rate increases for beads with smaller diameters, likely as a result of a higher surface area to volume ratio [40]. This increased elution rate reduces the effective release duration from small beads, limiting their potential for infection treatment or

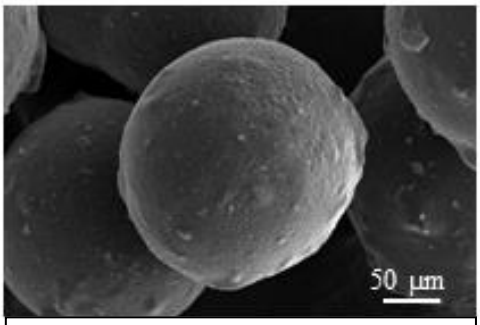

Figure 1: SEM micrograph showing chitosan-PEGDMA M_n= 550 microbeads with MNP at 1000x magnification.

prevention. The 200 µm diameter of the beads used in this study is a compromise between achieving prolonged elution duration while still being small enough to fill irregularly shaped wound beds.

3.2 Swelling Ratio

Beads from all groups swelled to at least twice their original size (Fig. 2), with no clear trend between cross-linker size and swelling ratio. Beads cross-linked with Mn = 550 PEGDMA swelled to 300% of their original size, which is statistically higher than beads cross linked with Mn = 200, Mn = 600 or Mn = 750 PEGDMA ($p < 0.001$). Beads cross-linked with Mn = 200 PEGDMA swelled significantly more than Mn = 600 and Mn = 750 beads ($p = 0.01$). However, there were no significant differences between the 600 and 750 groups ($p > 0.5$). It was anticipated that the shortest cross linker in the study, Mn = 200 PEGDMA, would produce highly cross linked beads with low swelling ratios, while higher molecular weights would result in fewer chitosan-PEGDMA bonds and a more flexible polymer matrix. The increase in swelling ratio for Mn = 550 cross-linker compared to Mn = 200 is comparable to the behavior of other cross-linked PEG hydrogels [44] but the larger Mn = 600 and Mn = 750 PEGDMA cross linked beads allowed less water influx than expected. Results suggest that the size of PEGDMA molecules with Mn above 550

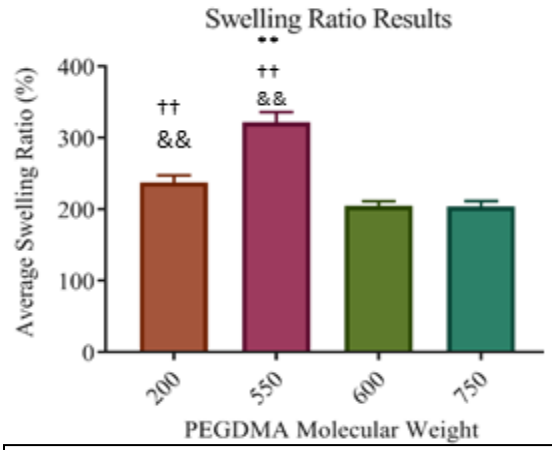

Figure 2: Graph shows swelling ratio of chitosan beads with various lengths of PEGDMA after 24 hours (n = 3). Mean ± standard deviation. * Represents statistical difference compared to 200 (* $p < 0.05$, ** $p < 0.01$). # Represents statistical difference compared to 550 (# $p < 0.05$, ## $p < 0.01$). & Represents statistical difference compared to 600 (& $p < 0.05$, && $p < 0.01$). † Represents statistical difference compared to 750 († $p < 0.05$, †† $p < 0.01$).

may limit the number of PEG molecules that are incorporated within a bead, which may result in varying degrees of cross-linking densities, primary chain cyclization (PEGDMA chains crosslinking within the molecule), or drug molecule binding [45, 46].

3.3 Elution and Stimulation

Data shows that all groups eluted vancomycin in a burst-release pattern over the first 2-3 days, with exponential decrease in release over the remainder of the 8-day elution study (Fig. 3). Beads cross-linked with Mn = 600 PEGDMA eluted 33% more vancomycin on day 1 and 81% more vancomycin on day 2 compared to the cross-linker group with the next highest elution rate ($p < 0.001$). Beads cross-linked with Mn = 550 PEGDMA had higher mean elution rates on days 3-8 in the stimulated group and days 5-8 in the control group, however differences were not statistically significant ($p > 0.05$). Cumulative vancomycin release per 50 mg of beads ranged from 1.7 mg to 3.5 mg, with Mn = 600 beads releasing more vancomycin than the other cross linker groups (Table 2, $p < 0.001$). Beads cross linked with Mn = 550 PEGDMA eluted significantly more vancomycin than Mn = 750 beads ($p < 0.001$), although the increase over Mn = 200 beads was not significant ($p = 0.31$).

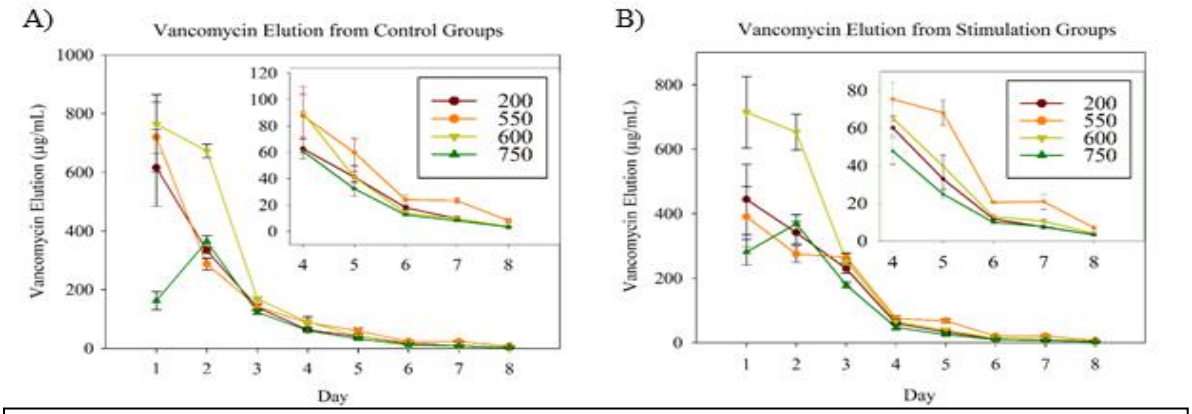

Figure 3: Graph shows the complete 8-day elution of (A) control and (B) stimulated groups for all lengths of PEGDMA cross-linker used in this study (n=4). Stimulations were performed on days 3, 5 and 7 with a field strength of 23 mT and frequency of 109.9 kHz. Mean ± standard

Table 2: Cumulative Vancomycin Release (mg)

PEGDMA Mn	Average Vancomycin release (mg)	Standard Deviation (mg)
200	2.36	0.25
550	2.48	0.31
600	3.52	0.14
750	1.69	0.2

The Mn = 550 PEGDMA group had a significantly higher stimulus response than other crosslinker groups, with a 94 µg/ml increase during the first stimulation period that resulted in an overall 45% increase in day 3 elution compared to nonstimulated control samples ($p < 0.01$)., This is not surprising given the swelling ratio data, which shows that the Mn = 550 beads undergo larger changes in volume than beads cross-linked with other lengths of PEGDMA. It can be reasoned that any stimulation induced hyperthermia or matrix permeability increases would cause a greater effect in these beads due to increased matrix flexibility. One way ANOVA was unable to detect significant differences between control and stimulated groups during the third stimulation period, but eluate samples from the Mn = 550 beads showed a 20% increase in vancomycin concentration during this period (Fig. 4A). Responsiveness to three separate stimulation periods suggests that any MNP-hyperthermia induced changes are temporary and dissipate when the magnetic field is removed.

There was a statistically significant increase of approximately 100 µg/mL vancomycin from beads stimulated at 17 mT and 23 mT compared to 11 mT at day 3 ($p < 0.001$, Fig. 4B).

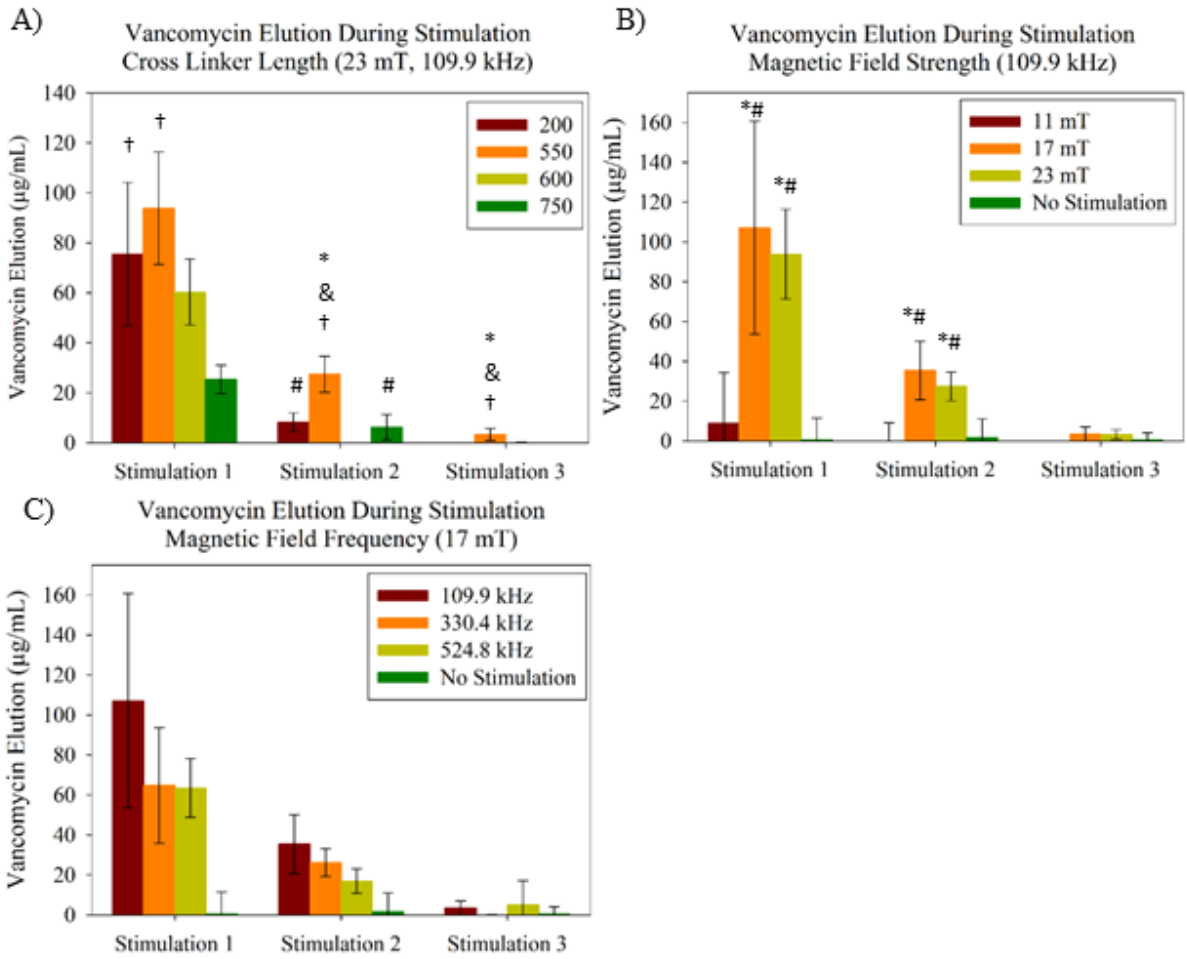

Figure 4: Bar graphs compare the increase in vancomycin concentration during the three stimulation periods for A) various lengths of PEGDMA cross-linker (* Represents statistical difference compared to 200, # represents statistical difference compared to 550, & represents statistical difference compared to 600, † represents statistical difference compared to 750, $p < 0.05$), B) three magnetic field strengths (* Represents statistical difference compared to control, # represents statistical difference compared to 11 mT, & represents statistical difference compared to 17 mT, † represents statistical difference compared to 23 mT, $p < 0.05$), and C) three magnetic field frequencies. Mean ± standard deviation (n = 4 in all graphs).

There was also an increase of approximately 30 µg/ml from beads stimulated at 17 mT and 23 mT on day 5, which was statistically higher than controls and beads stimulated at 11 mT ($p = 0.01$). These findings agree with studies showing diclofenac release from MNP-loaded chitosan carrageenan cross-linked with iron salts increase in response to higher magnetic fields [47], although not in a linear fashion for the tested range of field strengths. Beads subjected to 109.9

kHz stimulation eluted more vancomycin during the first stimulation at 107 µg/mL, compared to 65 µg/mL and 64 µg/mL for beads stimulated at 330.4 kHz and 524.8 kHz, respectively, however the number of samples was not great enough to detect statistical significance (Figure 4C, $p = 0.2$).

The ability to modulate vancomycin release with separate stimulations is advantageous compared to other smart delivery systems. Magnetoliposomes are another externally tunable drug delivery system, but rapidly release the complete drug payload as the MNP heats the surrounding lipids beyond its phase transition temperature [48]. Drug release from pH responsive nanogels follows a similar pattern, eluting most of the drug payload within 24 hours of exposure to acidic solutions [49]. Drug elution from chitosan-PEGDMA microbeads can be controllably increased using external magnetic field stimulation but, unlike many smart delivery systems, the beads will still retain enough drug to continue delivering therapeutic doses for extended periods. This is especially beneficial in the treatment of bacterial infections, which require prolonged treatment with high concentrations of antibiotics.

3.4 Degradation

Local drug delivery devices should be consistently and functionally biodegradable to avoid issues such as buildup of degradation byproducts, rapid elution of drug payload, or future bacterial seeding after drug delivery is complete. According to results, 70 % (±1%) of degradation in the Mn = 550 PEGDMA group takes place within the first three days of the degradation period (Fig. 5A). After the first three days of the degradation period, there seemed to be no significant change in the degradation profile of Chitosan-PEGDMA MNP-loaded chitosan microbeads. When the microbeads were compared to cross-linkers with different molecular weights, there was a trend towards increased degradation rate for increasing molecular weight of

PEGDMA cross-linker (Fig. 5B), with statistically significant differences in degradation rate between all groups ($p < 0.01$) with the exception of Mn = 600 and Mn = 750 beads ($p = 0.17$).

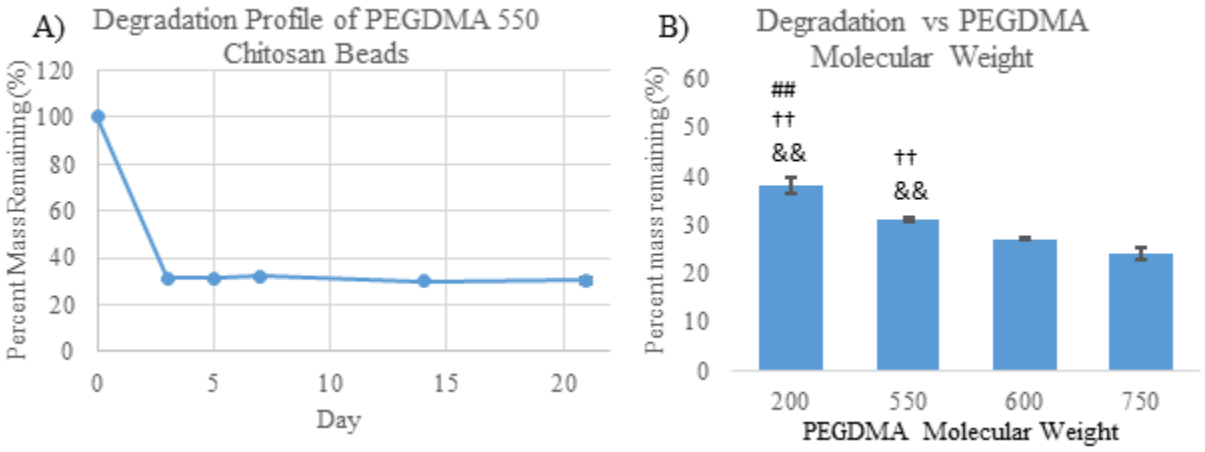

Figure 5: A) Graph shows the 21-day degradation profile for beads cross-linked with Mn = 550 PEGDMA (n=3). B) A bar graph compares the three-day degradation of various chitosan beads cross-linked with various lengths of PEGDMA (n = 3). Mean ± standard deviation. * Represents statistical difference compared to 200 (* $p < 0.05$, ** $p < 0.01$). # Represents statistical difference compared to 550 (# $p < 0.05$, ## $p < 0.01$). & Represents statistical difference compared to 600 (& $p < 0.05$, && $p < 0.01$). † Represents statistical difference compared to 750 († $p < 0.05$, †† $p < 0.01$).

Lysozyme is known to be found in various bodily tissues, including serum in concentrations ranging from 1 to 14 µg/mL [50, 51], in contrast to the 1 mg/mL concentration used in this study. An elevated concentration was chosen for comparison with previous studies [52-54], although this likely results in accelerated degradation times compared to in vivo use. Lysozyme breaks chitosan down by cleaving the (β 1→4) glycosidic bonds between polysaccharide units in the polymer leaving glucosamine byproducts that are incorporated into proteoglycans or metabolized by the body [55]. Approximately 30% of the microbead composition consisted of MNP therefore, the degradation profile suggests that the majority of the chitosan-PEGDMA composite had degraded entirely by day 3 in this accelerated degradation study. Up to 80% of initial weight was retained after elution studies in PBS without lysozyme, suggesting that enzymatic degradation by lysozyme is the predominant mechanism measured in

this evaluation. This would likely mean a prolonged degradation time when implants are placed in vivo since elevated lysozyme concentrations were used in this accelerated test. In vivo degradation studies are needed to adequately determine timing of degradation response.

PEG hydrogels typically have similar or slower degradation rates to those in our study [45, 56, 57]. The enzyme-cleavable chitosan backbone for PEG cross-linked beads may provide a means to tailor degradation to match needs based on drug elution or tissue in-growth rates [58]. In studies of other forms of cross-linked chitosan microbeads, little or no degradation of composites is observed over the course of weeks, both in vitro and in vivo [34, 59, 60], which may limit the utility for a local drug delivery system. Systems that undergo no or little degradation can serve as a scaffold for hematogenous bacterial attachment after drug delivery is completed, increasing the risk of infection compared to the degradable beads presented in this study.

3.5 Cytocompatibility

Cells exhibited normal spindle shaped morphologies when exposed to either silicone or vancomycin loaded chitosan-PEGDMA beads (Fig. 6). Cellular metabolism ranged from 97% to 152% for groups with MNP-loaded microbeads added (Fig. 7). There was a significant increase in ATP concentration for samples treated with MNP beads at a concentration of 5 mg/mL ($p<0.001$), but no increase in ATP concentration for samples that received non-MNP beads ($p = 0.77$). The results from the metabolism assay confirm visual microscopic observations that both Si and MNP beads showed high compatibility with cells.

Both chitosan and PEG have favorable biocompatibility properties and should not have a negative effect on cellular proliferation or metabolism [61-64]. Vancomycin is a commonly used antibiotic with no reported effects on fibroblast growth at concentrations up to 1 mg/ml [65]. The iron oxide within microbeads may play a role in the increased viability for the lower concentration of MNP-loaded microbeads, showing similar findings to a study by Berry et al. in which bare iron oxide nanoparticles caused decreased viability but showed a 33% increases when modified with the protein albumin [66]. Hydrogels of PEG and PEG blended with chitosan that use photoinitiated PEGDMA or PEG diacrylate have been used to successfully entrap cells and support cell viability [67, 68]. High percent cell viability may also indicate that residual unbound PEGDMA is removed during the wash steps, as unreacted methacrylates have known toxicity to fibroblasts and other cells [69-71]. Further evaluation of tissue response to MNP-loaded chitosan-PEG microspheres should

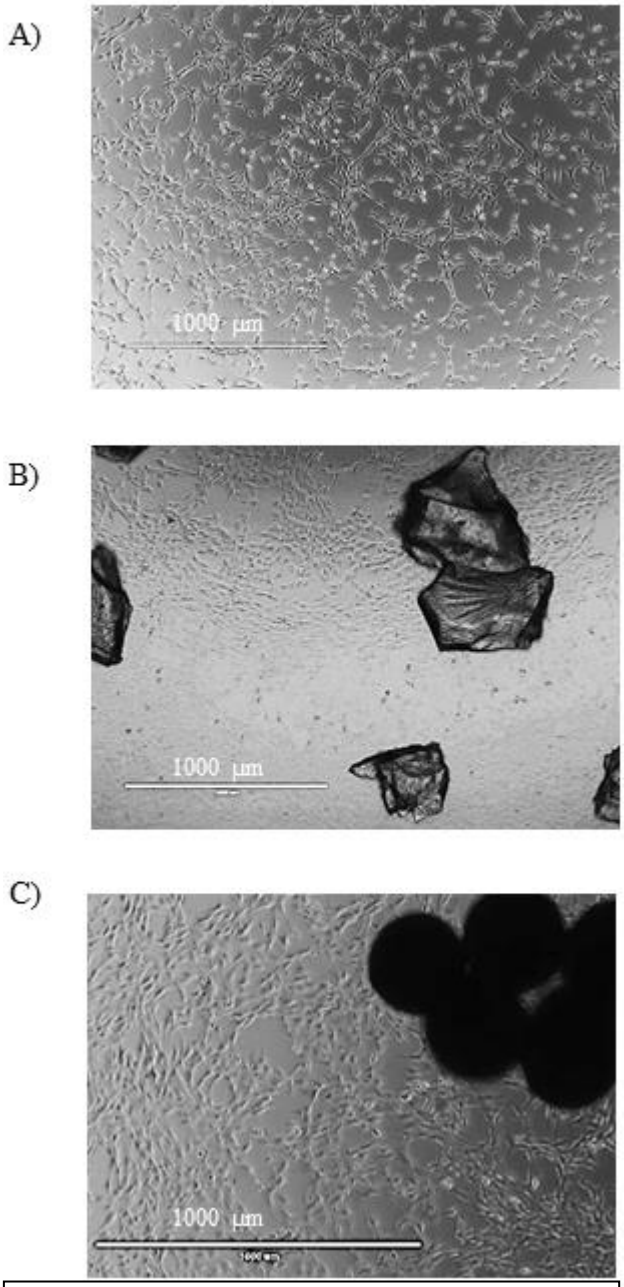

Figure 6: Photomicrographs of NIH-3T3 cells growing (A) on control tissue culture plastic, (B) near silicone microparticles and C) near chitosan-PEGDMA M_n = 550 microbeads from the MNP Vanc 5 mg/mL group.

include evaluation of immune cell activation as well as in vivo implantation.

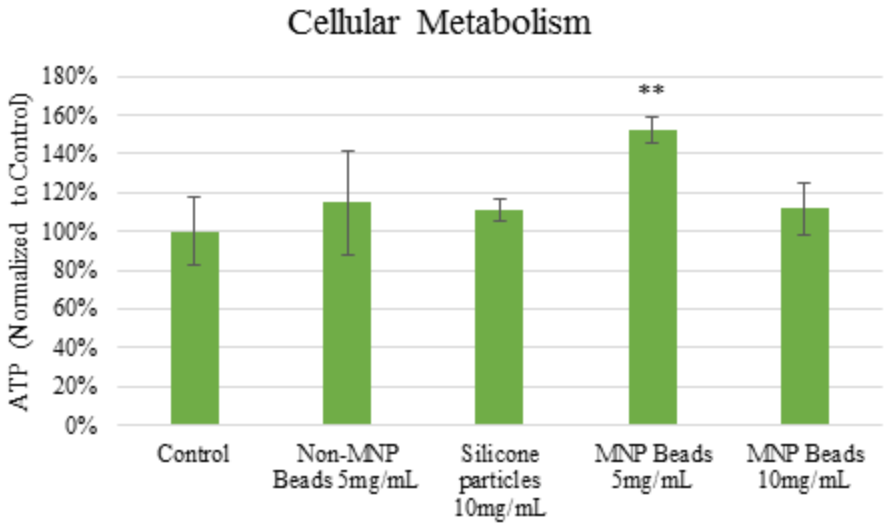

Figure 7: NIH-3T3 cell metabolism after 3 days exposure to chitosan-PEGDMA M_n = 550 microbeads with and without MNP and silicone microparticles (n = 4). Cell counts are normalized to control samples. Mean ± standard deviation. * Represents statistical difference in ATP concentration compared to control (* $p < 0.05$, ** $p < 0.01$).

4. Conclusion

In this preliminary study, it was shown that MNP loaded chitosan microbeads cross-linked with PEGDMA are capable of releasing vancomycin for up to 8 days. Short 30-minute magnetic stimulations can significantly increase daily drug elution rate up to 45% in beads cross linked with M_n = 550 PEGDMA ($p < 0.01$), likely via a temporary increase in permeability due to MNP generated hyperthermia. The polymer matrix is rapidly biodegradable and shows no signs of significant cytotoxicity against fibroblast cells *in vitro*. The ability to increase drug elution on demand makes these beads appealing as a potential infection prevention and treatment device, as magnetic stimulation can be used to either increase drug delivery post-implantation for maximum drug concentration or maintain therapeutic drug levels after traditional delivery systems would have fallen below the desired elution rate. Further extension of these drug

delivery matrices to release of proteins, small molecules, or other chemotherapeutics may prove useful in a wide-range of clinical applications, such as tissue regeneration, pain relief, and cancer treatment. Ongoing studies will continue to evaluate the effect of general hyperthermia on vancomycin release, effect of MNP loading concentration on bead responsiveness, effects of additional stimulation parameters such as duration and number of stimulations, characterize the bead polymer matrix, and test *in vivo* efficacy.

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CHAPTER 5

Discussion

The purpose of this research was to advance the use of local antibiotic therapy to combat orthopedic infections by a) providing data that enables surgeons to make better choices when selecting from devices currently available and b) investigating a new technology that enables non-invasive modulation of drug release after implantation. Orthopedic infections, including PJI and osteomyelitis, are serious complications which cost billions of dollars annually and are debilitating for the patients themselves. Systemic antibiotic therapy is useful and required in the management of these infections, however disruptions in vasculature and the presence of biofilm limit the effectiveness of the low concentrations achievable with IV or oral delivery. Local antibiotic therapy serves as a powerful adjunct to systemic therapy by increasing concentrations of antibiotic in the wound bed to levels which may be effective against biofilm, thereby reducing the incidence or recurrence of infection.

Calcium based synthetic bone grafts are commonly adopted for local antibiotic delivery due to the ease of drug loading, increased duration of drug elution compared to PMMA, and degradability that precludes the need for follow up surgery or risk of infection (99, 101). These grafts are composed of calcium sulfate or various forms of calcium phosphate, such as β-tricalcium phosphate (TCP) and hydroxyapatite (HA), which can be mixed to tune degradation and elution rates for the intended purpose. The ease of drug loading and wide variety of antibiotics that are compatible with calcium bone void fillers is advantageous in that drug choice and concentration can be tailored for each intended usage, however this does create concerns in that antibiotic loading may affect material properties. Calcium sulfate and phosphate bone void fillers are prepared by mixing a base powder with water, initiating a series of dissolution and

precipitation reactions that results in the recrystallization of the material (106). Antibiotic powders act as impurities that create voids or slow crystal growth, potentially affecting the hardening and final characteristics of the material (105). Adverse effects, if any, may differ for each antibiotic, as even the brand of antibiotic has been shown to have an effect on drug elution (123). It is therefore imperative that all carrier and antibiotic combinations are properly tested to ensure that properties such as material handling characteristics, degradation rate, acidity, and drug elution are evaluated prior to use to ensure that the bone void filler performs as expected. Unfortunately, the lack standardization between antibiotic choices, loading concentrations, *in vitro* elution protocols, and *in vivo* usage makes it difficult to compare results between studies (124, 125).

The triphasic bone void filler tested in chapter two contained a proprietary mixture of CS, TCP, and HA designed to improve bone regeneration by optimizing material degradation. This study sought to evaluate the triphasic bone void filler as a delivery vehicle for tobramycin and vancomycin, which are commonly used in calcium bone grafts for broad spectrum coverage of most orthopedic pathogens. Set time, or the time it takes for the material to harden after mixing, is an often overlooked but important parameter as the material must be typically prepared during surgery. This study demonstrated that the addition of tobramycin increased the set time of the material by several hours despite preparing the material in a controlled environment according to manufacturer instructions. Such an increase may be unacceptable clinically as it may increase operating time, negating any advantages from local antibiotic delivery (126). While it is known that antibiotics increase the set time of calcium bone void fillers, no other reports have noted such lengthy increases, highlighting the need to evaluate each material and drug combination prior to use. Additional testing demonstrated that the set time could be decreased to

approximately 30 minutes by reducing the volume of water used to hydrate the material, however this may adversely affect crystallization. Several alternative methods are known to reduce set time but were not evaluated in this study. Fine particles of calcium sulfate dihydrate and/or hydroxyapatite can be added during mixing to accelerate the reaction by alleviating the need for new crystal nucleation. The hydration solution can be modified by the addition of acids or salts, particularly phosphates, which accelerate set time by increasing initial solubilization or promoting crystallization via ion oversaturation (127-129). Further testing would be needed to assess the crystallinity, mechanical properties, and degradation rate before recommending changes to lower set time.

The triphasic material was shown to elute both vancomycin and tobramycin at concentrations three orders of magnitude greater than the minimum inhibitory concentration for most strains of *S. aureus* and *P. aeruginosa*. These high levels were reached within 4 hours of submersion in PBS and maintained throughout the 7 week study, suggesting that a single dose of the material can supplement systemic antibiotic therapy throughout the course of treatment for osteomyelitis or two stage PJI. The high initial levels may be advantageous as the wound may become contaminated at the time of surgery, and bacteria can attach to any implants or devitalized tissue in the wound to survive despite prophylactic antibiotics (130-133). Early and prolonged treatment increases the probability of successfully eradicating these bacteria. This study utilized a partial refreshment elution strategy in which 37.5% of the total elution media was exchanged at each time point. While less common that full refreshment strategies, it has been suggested that this method is a better approximation of drug release *in vivo* (125, 134). Furthermore, this elution method was adopted from a previous study and allowed for direct comparison with a pure calcium sulfate graft, which is typically not possible due to differences in

time points, elution volume, volume and surface area of material used, etc (125). Compared to pure calcium sulfate, the triphasic material has a prolonged elution profile with increased concentrations at later time points. This data allows surgeons already familiar with calcium sulfate to make direct comparisons between the two materials and set expectations accordingly.

The studies in chapter 3 were designed to compare 4 brands of calcium sulfate in regards to calcium ion release, acidity, and drug elution. CS has long been associated with sterile wound drainage which typically resolves without treatment or complications, yet is concerning for patients and physicians (135, 136). Furthermore, reports of hypercalcemia following CS mediated local drug delivery merit further investigation into the effects of antibiotics on degradation. Osteoset and Calcigen used are sourced from gypsum that is mined from the ground, whereas Stimulan and Synthecure are synthetically produced from high purity starting materials. The manufacturer's literature for Stimulan suggests that the highly controlled nature of production ensures a controlled degradation and elution rate, results in fewer impurities, causes the material to degrade at physiologic pH, and is associated with approximately 90% fewer drainage complications than naturally sourced products. These statements have been found in other published articles, however few studies have directly compared natural and synthetic CS, and it appears most evidence is based on small retrospective reports (137-139).

Three of the four brands of CS continued to elute vancomycin at concentrations > 100x the MIC for *S. aureus* throughout the 42 day study. Naturally sourced Osteoset eluted significantly more vancomycin than both synthetic Stimulan and Synthecure at time points 1, 3, and 7 ($p < 0.01$), and maintained nonsignificant increases over both at all time points. Tobramycin elution was much more rapid in all groups, with maximum concentrations 4x higher than that of vancomycin, and elution dropped to less than 10x of this maximum concentration by

day 14. Osteoset again performed well, with significantly more tobramycin released at day 1, 3, and 7 compared to the other brands ($P < 0.01$). Calcigen eluted a smaller initial burst and maintained higher levels of tobramycin at later time points, however the increase is not significant. The rapid drop in tobramycin elution at such early time may not be ideal as most reports identify at least 4-6 weeks of antibiotic therapy as required for treatment of PJI and osteomyelitis (7). Reduced broad spectrum coverage past day 14 may limit performance against common gram negative pathogens such as *Pseudomonas aeruginosa* (140, 141). Unfortunately, comparisons with the triphasic material in Ch.3 is complicated by the different elution time points, ratio of bone void filler to volume of elution media, presence of serum in the media, and partial vs full media refreshment. This lack of standardization is relatively common with *in vitro* drug delivery studies and greatly limits the ability to compare materials (124, 125).

Recent reports of hypercalcemia have raised concerns about the use of CS for drug delivery, particularly as antibiotics can alter the crystallization of the graft. It has been suspected that excess calcium ion concentration can induce an osmotic effect responsible for wound drainage in up to 30% of cases (135, 142-146). Additionally, CS resorption is known to increase acidity in the wound bed, which may worsen with the addition of acidic antibiotics such as vancomycin. Synthetic CS without antibiotics released fewer calcium ions than naturally sourced Osteoset and Calcigen, however there were no other obvious differences attributable to the material source. Likewise, no increase in calcium ion release was detected after the addition of antibiotics. The antibiotics did cause a gradual reduction in pH over the first 3 weeks of the study that resulted in an average drop in pH of 0.5 at later time points. This change did not correlate directly with any changes in antibiotic concentration or calcium ion release, and further investigation may be needed. This study utilized a large joint model with large sample volumes

but small sample size. This approach was conducted at the request of the surgeons consultants for these studies, however does limit statistical power and may be a possible cause for increased variance in the data. Future studies with an emphasis on increased sample size and replication may find additional differences.

Traditional delivery systems, including CS as discussed above, are limited in that drug elution cannot be altered after implantation. Tobramycin elution from the CS beads in Ch. 3 dropped to less than 5% of maximal values by day 14, however low concentrations of drug release were detected throughout the study. Prolonged sub-therapeutic elution such as this is common in most drug delivery systems. Novel stimuli responsive "smart" delivery systems offer additional control over the spatiotemporal release of drug through the use of passive or active stimulation techniques, including pH, bacterial enzymes, light, ultrasound, and magnetic fields (147-148). The system can therefore modulate drug release in response to environmental changes or external stimulation by the caregiver, ensuring maximal efficacy of the encapsulated drug. Magnetically responsive systems are particularly attractive due to ease and safety of targeting deep tissue, as has been demonstrated in clinical trials (117, 122). The study in chapter 4 details the addition of SPIONS to a chitosan-polyethylene glycol microbead to create a stimuli responsive vancomycin delivery system. Many of the magnetically responsive systems reported in the literature are nanoparticles, which are of great interest due to the potential for IV delivery. The drug payload of these systems is rapidly exhausted due to the high surface area to volume ratio, however, and the need for frequent dosing may reduce patient compliance. This study instead sought to create microbeads, deliverable through a syringe, which can maintain drug elution for at least one week. Preliminary studies indicated that elution levels dropped below therapeutic levels after approximately 12 days, however subsequent magnetic stimulation could

increase antibiotic concentrations above the MIC of *S. aureus* (149). The ability to maintain therapeutic release as the carrier nears exhaustion may be advantageous as subtherapeutic release from PMMA is known to encourage bacterial resistance, and the increased duration of release may help eradicate biofilm (68, 80, 81). This study was performed to further characterize the beads and determine parameters that improved drug elution or stimuli responsiveness. Increases up to 45% were seen in the daily elution of vancomycin using 30 minute magnetic stimulation periods, suggesting that short stimulation can trigger clinically relevant changes in drug release. The magnetic field strength was shown to have an effect on stimulation efficacy, however the effect was nonlinear over the tested field strengths and suggests saturation. Magnetic field strength would be an important consideration in the usage of such as system as the required field strength will be dependent on distance to the drug carrier.

CHAPTER 6

Conclusions

Orthopedic infections are a major burden both in terms of cost and patient morbidity. Treatment often requires multiple surgeries, long hospitalization periods, and prolonged systemic antibiotic therapy. Local antibiotic delivery systems are believed to increase the efficacy of systemic antibiotic treatment by increasing drug concentrations at the wound site to levels that are unsustainable with IV delivery. The variety of systems, including polymethylmethacrylate and numerous combinations of synthetic bone grafts, and antibiotic choices available for use allows surgeons to tailor treatment as needed, but carrier and antibiotic combinations should be tested prior to use in humans.

It was demonstrated that calcium sulfate and calcium phosphate bone graft substitutes can elute therapeutic doses of antibiotics for up to 7 weeks *in vitro*. The addition of antibiotics was found to affect material properties in ways that may be product specific. Tobramycin was found to slow the hardening of a triphasic-calcium bone graft mixture sufficiently to question its use in the operating room, however this was not noted during incorporation into calcium sulfate. Furthermore, antibiotic loading was found to influence factors such as degradation and pH of the resorption productions, which may have an adverse effect on clinical performance.

Stimuli responsive materials may improve the efficacy of future local drug delivery systems through fine control of spatiotemporal release. The addition of Fe_3O_4 nanoparticles to a polymeric microbead enabled external modulation of drug release through the use of alternating magnetic fields. This additional level of control may improve the efficacy of future delivery systems by improving the kinetics of drug release, maximizing the duration of therapeutic elution while minimizing the risk of generating antibiotic resistant bacteria.

CHAPTER 7

Recommendations for Future Work

The studies in Ch. 2 and Ch. 3 provide clinically relevant data concerning the use of calcium sulfate and calcium phosphate based bone void fillers for antibiotic delivery. It was found that tobramycin interfered with the setting of the triphasic material in chapter 2. Future studies should evaluate methods that surgeons can use to improve set time, such as using a heat lamp to increase temperature, replacing the hydration solution with saline or other salt solutions readily available in the operating room, or adding accelerator particles in the form of crushed beads prepared without antibiotics. Furthermore, the material should be evaluated using mechanical testing, x-ray diffraction, electron microscopy, and density measurements to ensure the materials characteristics as a bone graft substitute are not being adversely affected. The calcium sulfate products studied in Ch. 3 were only tested after loading with a combination of tobramycin and vancomycin. Additional antibiotics should be tested including gentamycin. Additionally, we used serum in the elution media at the request of surgeon consultants. A future study should be done comparing regular PBS and PBS containing serum at various concentrations to determine there are any significant differences in elution.

The stimuli responsive system in Ch. 4 represent the next generation of local drug delivery devices. The microbeads used in the study maintained elution for approximately 1 week before antibiotic concentrations began to approach subtherapeutic levels. This duration is likely to be insufficient for the treatment of orthopedic infections. Future studies can improve efficacy by reducing the size to facilitate reapplication or utilizing covalent drug linkers to provide additional control over drug release. Furthermore, magnetic stimulation was provided using a small benchtop electromagnet. Clinical usage of magnetically responsive systems is not viable

unless convenient and reasonably priced systems can be created for at home use. Future studies should include the creation of an easy-to-use stimulator and *in vitro* models of stimulation through tissue as additional proof of concept.

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