Implantable Medical Devices for Local Drug Delivery and Tissue Regeneration to Combat Chronic Bacterial Infection

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(Original signatures are on file with official student records.)
Implantable Medical Devices for Local Drug Delivery and Tissue Regeneration to Combat Chronic Bacterial Infection

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ABSTRACT

Local drug delivery has been an area of exceptional interest in the treatment of chronic bacterial infections, particularly in areas where there are medical implants placed. Medical implants are widely utilized and are becoming increasingly popular with time. With the increasing use of medical implants, concomitant bacterial infection is also increasing, and this type of bacterial infection can be exceedingly difficult to clear. The following body of work focuses on implant-associated bacterial infection, with an emphasis on chronic Staphylococcus aureus (S. aureus) infection, particularly osteomyelitis. Specifically, this work is focused within the scope of utilizing locally implantable medical devices with dual platform capabilities to deliver drugs and, when applicable, regenerate tissues that are lost due to injury, device placement or resulting from treatment regimen. This work provides a thorough background in local drug delivery devices that are significant in the treatment of osteomyelitis, a comprehensive review of animal models to advance research of bacterial osteomyelitis, a critical review and proposed experimental design to improve data interpretation from in vitro drug elution experiments, describes the use of a commercially available collagen matrix as a local drug delivery device with emphasis on tissue integration, and lastly, describes the in vitro and in vivo investigation of S. aureus sequence type 398 as a pathogen of osteomyelitis.
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CHAPTER I: INTRODUCTION
A version of this introduction was previously published by Caroline Billings and David E. Anderson:


This manuscript, aside from formatting, has not been edited for inclusion in this thesis. David E. Anderson is a co-author on this manuscript and was involved in conceptualization, editing, and supervision.

**Abstract**

As medicine advances and physicians are able to provide patients with innovative solutions, including placement of temporary or permanent medical devices that drastically improve quality of life of the patient, there is the persistent, recurring problem of chronic bacterial infection, including osteomyelitis. Osteomyelitis can manifest as a result of traumatic or contaminated wounds or implant-associated infections. This bacterial infection can persist as a result of inadequate treatment regimens or the presence of biofilm on implanted medical devices. One strategy to mitigate these concerns is the use of implantable medical devices that simultaneously act as local drug delivery devices (DDDs). This classification of device has the potential to prevent or aid in clearing chronic bacterial infection by delivering effective doses of antibiotics to the area of interest and can be engineered to simultaneously aid in tissue regeneration. This review will provide a background on bacterial infection and current therapies as well as current and prospective implantable DDDs, with a particular emphasis on local DDDs to combat bacterial osteomyelitis.

**Implantable Medical Devices—Benefits and Challenges**

There are continual advances made in the fields of medicine and science. These advances include the creation of a wide array of implantable medical devices from indwelling vascular or urinary catheters to total hip replacements and cardiac pacemakers. Each of these devices serves a unique purpose, and despite significant differences in form and function, implantable medical devices are uniformly considered to improve the quality of life of the patients in which they are utilized [1]. Approximately 8–10% of Americans, or 5–6% of people in industrialized countries, are estimated to have received an implantable medical device [1,2]. In the United States alone, there are more than five million medical devices or implants used annually [3]. Implantable medical devices serve a wide variety of indications and allow physicians to improve patients’ lives by stabilizing complicated fractures using metal rods, pins, screws, and plates [4,5]; providing children suffering from sensory deafness with practically normal speech and language development by utilizing cochlear implants [1,6]; and providing high quality of life to patients suffering from cardiac disease by implantation of artificial valves [7], pacemakers [8], and cardiac defibrillators [1,2], among many other procedures. Despite incredible advances in the impressive arena of implantable medical devices, there remain persistent challenges.
These challenges include insufficient biocompatibility of devices, which can be associated with foreign body responses [9–11], biofilm formation on devices [3], and chronic bacterial infection associated with the site of implantation or device [11–14]. Each of these challenges may lead to implant failure at any point during the in situ lifetime of the device [15].

**Bacterial Infection—Risks and Current Therapies**

Bacterial infection is a common, yet catastrophic, complication [16] that can occur following the implantation of a medical device, regardless of body site [11,13,17]. Bacterial infection falls under the umbrella of healthcare-associated infections (HAIs) [18]. The Centers for Disease Control (CDC) report that surgical site infections (SSIs) comprise 20% of HAIs, with an estimated 110,800 SSIs reported in 2015 [19] and an estimated one million implant-associated infections occurring each year [20]. Average rates of infection for initially inserted implants range from 2 to 40% depending on implant type, with orthopedic implants in the range of 2–5% [21]. Bacterial infection can be caused by traumatic or contaminated wounds, such as open fractures [12,16,22], as well as hematogenous or perioperative bacterial seeding [23]. The presence of an implant increases the risk of infection [24]. The risk of infection is multifactorial and thought to be due in part to lowered local host defenses [12,13,25] as a result of tissue trauma, presence of foreign material [13], and alterations in fluid dynamics [26]. These described alterations, in combination with the trauma of disease and device placement, can create a local environment that is susceptible to infection with a lower infective dose of bacteria [12,13,25,27]. Additionally, bacteria may be able to adhere to indwelling devices and form three-dimensional communities of bacterial cells and exopolysaccharide matrix (biofilms). These biofilms can cause persistent, recurrent bacterial infection and are typically resistant to traditional antimicrobial therapy [3,13,17,20,21,28–31]. Separately from implant-associated complications, host factors such as systemic health and lifestyle play a role in the manifestation of bacterial infection. Examples include patients with comorbidities such as diabetes mellitus, obesity, immunosuppression, and patients with certain lifestyle choices such as smoking [12,32–36].

Current treatment for implant-associated bacterial infection is comprised of prolonged systemic antimicrobial therapy (often lasting weeks to months), revision surgeries, and implant removal. Revision surgeries include local debridement of compromised tissues and, typically, removal of the associated implant followed by re-implantation of a new device after resolution of the infection [21,25,37]. Re-implantation of a new device prior to complete clearance of infection can reinitiate the process and add to a prolonged and difficult recovery. These cases often require multiple revision procedures and are accompanied by risk of failure at each step [25,38,39], as well as a higher risk of infection than initial implantation [40]. These treatments can increase the costs of healthcare and may require hospitalization of the patient. Another area of concern is prolonged systemic antimicrobial therapy, which can be accompanied by adverse systemic side effects or toxicity, as well as increased risk of antimicrobial resistance.
These cases are challenging for clinicians to successfully diagnose and manage. They also place a heavy burden upon the healthcare system, as management often extends the duration of hospital stay and is expensive, with cost estimated to range from $10,000 to $25,000 per case, depending on the type of implant, degree of infection, and treatment protocol. Costs may increase by $20,000 per admission into the hospital, may exceed $90,000 when a prosthetic joint is involved, and may exceed $150,000 if following orthopedic trauma. Important to note is the physical and psychological impact of implant failure and treatment protocols on the patient. Undoubtedly, the treatment process has a significant impact on the patient’s quality of life, and this should be kept in consideration when undergoing diagnosis, surgical planning, and management of implant-associated bacterial infection. The focus of this review is to describe the current approach to bacterial osteomyelitis and the use of drug delivery devices in the management of this disease.

**Bacterial Osteomyelitis**

Osteomyelitis is an inflammatory disease caused by infecting microorganisms that leads to bone destruction and, ultimately, progressive bone loss. Typically, osteomyelitis is caused by bacteria, most commonly by Gram-positive Staphylococci species including *Staphylococcus aureus* and *Staphylococcus epidermidis*, although fungal osteomyelitis occurs as well. There are three main categories of osteomyelitis, listed here in order of decreasing frequency: (1) secondary to contiguous focus of infection, often resultant from trauma, surgery, or implanted prosthetic material; (2) secondary to vascular insufficiency, often a result of diabetic foot ulcers; and (3) hematogenous. There is considerable variation in the etiology and presentation of osteomyelitis. In this review, the focus will be on bacterial osteomyelitis that occurs following trauma, surgical procedures, or implanted materials, which is reported to account for 47 to 50% of osteomyelitis cases in adults. These cases are most commonly caused by *S. aureus*, including methicillin-resistant *S. aureus* (MRSA).

Osteomyelitis may present acutely with fever, pain, abscess formation, signs of local inflammation, and a draining tract. These symptoms of osteomyelitis may present shortly after reduction of a traumatic, open fracture, or other surgical procedure. While this presentation is certainly compelling for active bacterial osteomyelitis, confirmatory evidence may not be present on diagnostic imaging such as radiographs for 2–3 weeks following onset of infection. In contrast, patients suffering from chronic osteomyelitis resulting from presence of avascular, necrotic bone, biofilm, or prosthetic material may present years after the initial insult. These cases are more likely to present with a subtle constellation of symptoms and may be recognized solely by focal tenderness during physical exam, with suspicions increasingly raised on diagnostic testing. The distinction between acute or chronic osteomyelitis is often challenging and may require histopathological examination of bone biopsies to delineate disease chronicity at the cellular level. Histology may
also demonstrate the presence of both chronic and acute changes in a single specimen [65], highlighting the progressive nature of osteomyelitis. These variations contribute to the challenge of obtaining a swift and specific diagnosis of osteomyelitis. Diagnosis typically involves physical examination, radiographic imaging, hematology and serum biochemistry, culture of blood, and wound tissues, and often includes advanced imaging such as computed tomography (CT) or magnetic resonance imaging (MRI) [51]. The gold standard for diagnosis involves bone biopsy and culture [36,44,52,53], as well as histopathological examination of the bone [34,36].

Once diagnosed, bacterial osteomyelitis should be treated intensively, as osteomyelitis increases patient mortality by an estimated 8% [45]. Treatment for this type of osteomyelitis is similar to that described above for general bacterial infection, as it is constituted by surgical debridement of affected tissues, removal of affected implants or material, preservation of vascular supply, and systemic antimicrobial therapy guided by culture and sensitivity [25,32,34,44,51–53,65–67]. The success of osteomyelitis therapy lies within the quality of surgical debridement [50,68], which is challenging because of the need to debride all affected tissues while also preserving as much form and function for the patient as possible [25,32,44]. Inadequate debridement is one of the most common reasons for reoccurrence of chronic osteomyelitis [44]. In addition to thorough debridement, antimicrobial therapy is required and is accompanied by challenges such as inadequate penetration, antimicrobial resistance, presence of biofilms on devices or necrotic bone, and side effects of protracted antimicrobial therapy [44,45,56,66,69]. When an implant is associated with osteomyelitis, the decision to leave or remove the implant [17,65,69] is crucial and can impact treatment and patient quality of life. Additionally, replacing an implant following debridement and antimicrobial therapy poses a challenge, as locally compromised host defenses will render that site more highly susceptible to bacterial infection [13,25,70].

Major challenges in osteomyelitis therapy include antimicrobial therapy and inadequate penetration into bone, risk of chronic or recurrent bacterial infection, and extensive tissue destruction [34,60,65,66]. These challenges can be attributed in part to the abilities of S. aureus as a pathogenic organism [55,61]. S. aureus is not only a common commensal species [71,72], but also a versatile competitor and dangerous pathogen, with virulence factors that lend themselves towards causation of a diverse range of diseases [73–75]. The ability of S. aureus to evade the immune system adds to the difficulty of effectively treating osteomyelitis. Immune system evasion is accomplished with four main mechanisms, including the following: (1) Abscess formation [65,76]. This is a process controlled by the host and pathogen. This process ultimately offers protection to S. aureus by sequestering a focus of infection away from easy immune system access [65,77]. (2) Biofilm formation [36,76,78–80]. Biofilms offer protection by providing a physical barrier between immune cells and bacterial cells, while providing immense phenotypic diversity, which lends itself towards antimicrobial resistance. Biofilms also allow for horizontal gene transfer and acquisition of virulence mechanisms [65,76,81]. (3) Osteocyte-lacuno canalicular network (OLCN) invasion [65,76,82]. The ability of S.
S. aureus to gain access to the canalicular system of bone is thought to provide nutrients to bacterial cells while simultaneously protecting these cells from immune attack. OLCN invasion is proposed as an important mechanism in the persistence and recurrence of osteomyelitis [82]. (4) Intracellular persistence of S. aureus [36,49,65,80,83]. S. aureus has proven to be capable of internalization in a variety of cell types including osteoblasts [83–87] and, although the mechanisms are not fully understood, any period of intracellular persistence is thought to protect S. aureus from the immune system as well as antibiotic therapies [76,83].

With these challenges in mind, as well as the desire to reduce financial burden, length of hospital stay, and patient morbidity and mortality [45,65,88], the need for effective, practical strategies to improve the therapy of osteomyelitis is clear and leads investigators to study local drug delivery devices (DDDs), especially those that can serve as tissue regeneration platforms [44,56]. Local administration of antimicrobials can be accomplished via biodegradable or non-biodegradable DDDs. Degradable devices are desirable to eliminate the need for future removal of the device. Local administration is intended to mitigate the side effects of systemic antimicrobials while providing greater local concentrations of antimicrobials [56,66,89–92] and improved penetration to target tissues [41] to more effectively clear bacterial infection [93]. Recently, drug delivery systems have been designed to possess dual platform capabilities to aid in bone regeneration [56]. This class of multifunctional devices holds immense promise in the treatment and clearance of bacterial osteomyelitis and offers to improve the lives of patients suffering from this life-altering disease.

**Desirable Characteristics of Local Drug Delivery Devices**

There are many local drug delivery systems that have been investigated [9], and through these investigations, certain characteristics have emerged as most beneficial to success of the system. Ideal characteristics include the following:

1. Biocompatibility [2,56,94–96];
2. Predictable, inert degradation [22,56,89,97];
3. Sustained, clinically significant drug release [45,57,68];
4. Appropriate mechanical strength to support surrounding tissue [68];
5. Appropriate architecture to facilitate tissue ingrowth, when applicable [98–100].

In combination, these characteristics provide the conceptual ideal local drug delivery system. This system can load and elute antimicrobials at a clinically significant concentration, and for a suitable length of time, be safely implanted into the area of interest without causing a foreign body response [10]. This system will also degrade over a predictable length of time without generating harmful byproducts. The additional benefit of appropriate mechanical support is not provided by most of the currently investigated DDDs, but when available, can reduce additional surgical manipulations.
with hardware placement and lessen the risk of infection and device failure. Although these characteristics are universal to local DDDs designed to clear bacterial infections in multiple tissues, the examples in this review are specific to bone and the treatment of bacterial osteomyelitis.

The above description of sustained, clinically significant drug release is easily stated, yet difficult to fully understand and achieve [101]. In fact, drug elution kinetics in local DDDs have considerable variation [92] based on the size, shape, and composition of the delivery device [57,92]; the drug that is loaded [102,103]; the manner in which the drug is loaded [104]; and the local environment into which the device is placed [57,102,105]. To this end, in vitro testing of each device and drug combination must be completed prior to consideration for in vivo use. While an imperfect predictor of in vivo performance, in vitro testing is useful and may bring significant hurdles or advantages to light. Drug elution kinetics in traditional drug delivery systems rely heavily upon local flow rates, which often result in initial burst-release of drugs [57,93,103,104,106] from the material surface, followed by sustained, gradually reducing drug release [93,103] as the porosity of the device is exploited [101]. This drug release profile can be undesirable in vivo if drug release cannot be sustained over the minimum inhibitory concentration (MIC) and the minimum biofilm eradication concentration (MBEC) of the bacteria of interest [107], and if release cannot be sustained for a period of 3–4 weeks [103,104]. An attraction to stimuli-responsive materials is the controllability of drug elution kinetics [104], which may provide drug release over MIC for longer periods of time compared with traditional systems.

Local Drug Delivery Devices for Bacterial Osteomyelitis

Bone Cement

Historically, the most widely utilized local drug delivery device for combatting bacterial osteomyelitis has been antibiotic-loaded polymethylmethacrylate (PMMA), or bone cement [34,65,89,108–111]. In orthopedic procedures, antibiotic-loaded PMMA is often utilized to create beads or bead chains (demonstrated in Figure 1.1, located in Appendix I) to pack infected sites or as a cement applied prophylactically to prostheses [57,101,112]. This system is considered the current gold standard [66,113,114], has multiple commercially available formulations [107,115,116], and is often used to complement parenteral antimicrobial therapy [117]. This system has clear benefits including mechanical stability, suitability for use with numerous heat-stable antibiotics [111,112], release of metabolically active [114] antimicrobial compounds above the MIC of most common pathogens over a period of hours to days following implantation [112], and elimination of dead space from debridement [34] or wounds. PMMA has recognized shortcomings including the exothermic polymerization reaction during PMMA formation, which limits the use of heat-labile antibiotics [17,112,115] and creates concerns of tissue damage or necrosis [98,115]; wide variation in elution kinetics based on the type of bone cement; antimicrobial compound and mixing method chosen [101,114]; and sand incomplete release of antibiotics, which raises concerns for persistent low level antimicrobial release and subsequent antimicrobial resistance.
[14,70,107,109,118,119]. Finally, the lack of degradation of PMMA beads is a recognized shortcoming, as persistent foreign material may create wear particles and is an excellent substrate for biofilm formation [17,107,109,115,120], which can be a nidus of inflammation or initiate bacterial seeding to other sites [121]. Additionally, the body may mount a foreign body response to the indwelling material, and additional surgeries for removal of persistent beads are typically required [41,108,112,118,119].

To improve PMMA as a local drug delivery system, there have been many investigations into the properties of bone cements, including antibiotic elution, bone ingrowth, and mechanical properties [115], as shown in the flow diagram in Figure 1.2. Porosity has come to light as an important factor [70] for both elution characteristics and bone ingrowth [98,122]. However, increased porosity can decrease the mechanical strength of the cement [31]. This highlights the need for balance in providing the desired porosity while maintaining sufficient mechanical strength. Frew et al. [123] investigated differences between in vitro elution characteristics of gentamicin and vancomycin from commercially prepared cement versus hand-mixed cement. They found that hand-mixing vancomycin powder into PMMA/gentamicin cement provided greater than a fivefold and twofold increase in cumulative elution of vancomycin and gentamicin, respectively. These results were accompanied by greater variation in elution as compared with commercially available prepared cement. Greater cumulative elution and wider variation in elution in this situation were attributed mainly to variation in porosity, as hand mixing is thought to create a more heterogenous mixture, which provides greater porosity and slightly poorer mechanical characteristics. These results align with the available literature, which cites mixing technique as an important determinant of porosity [70,124]. Nugent et al. [125] found that elution of tobramycin from PMMA increased with increased porosity, as caused by increased fraction of the poragen xylitol. They also discovered that the compressive strength of the cement decreased with increased porosity and prolonged elution time in vitro. Similar results are reported by other studies [110,126,127], and the loss of mechanical strength with additives is commented on by Arora et al. [128].

There have been investigations into the incorporation of bioresorbable, osteoconductive, or osteoinductive components such as calcium phosphate (CaP), tricalcium phosphate (TCP, α-TCP, β-TCP), and hydroxyapatite (HA) into bone cements with the goal of controlling antibiotic release and simultaneously encouraging bone ingrowth [98,115,129,130]. It has been determined that the optimal pore size for bone ingrowth is within the range of 150–400 µm [98,115], and CaP materials should be selected with that in mind [115]. Fini et al. [130] compared a PMMA and α-TCP composite with PMMA and found that the porous architecture of the composite increased osteoblast viability in vitro and had a significantly greater rate of new bone mineralization in rabbit bone in vivo. These results speak to the increased biocompatibility of the composite. Vazquez et al. [131] found that incorporating β-TCP particles into PMMA extended the curing time of the cement. This finding agrees with those of Beruto et al. [132] and Lin et al. [98], who found that the addition of chitosan/β-TCP microspheres to PMMA cement increased the curing time and decreased the curing temperature of the cement composites. These findings are helpful, as they offer the surgeon additional time to form the cement and provide a lower polymerization reaction temperature, which lessens potential tissue
damage and may improve biocompatibility [98]. The option for a bioresorbable component to bone cement is intriguing and may eliminate the need to remove PMMA/CaP composites, as increased biocompatibility, osteoconduction, and osteoinduction may lead to device integration into bone [115].

Despite these investigations and advances with regard to antibiotic-loaded PMMA, challenges remain that raise concerns for the long-term suitability of this system for use in the clearance of bacterial osteomyelitis. In fact, leaders in the treatment of musculoskeletal infections met in 2019 for review and discussion of the available literature regarding antibiotic-loaded bone cement. They concluded that, although it is used frequently and supported anecdotally, there is a lack of strong evidence supporting the clinical benefit of this drug delivery system [107]. Persistent challenges include the precise tunability of elution kinetics, as this system is affected by many manufacturing variables and raises concerns regarding the predictable in vivo performance of PMMA [107]. Additionally, the risks of biofilm formation on indwelling PMMA, antimicrobial resistance from extended low level antimicrobial elution, and the need for surgical removal of this system are major [107]. These risks are unlikely to be completely overcome without severely compromising the mechanical properties or drug delivering capacity of this material. With these challenges in mind, there has been increased interest in biocompatible, biodegradable devices [109], which will be discussed further.

**Bone Grafts**

Autologous or allogenic bone grafts are sometimes utilized in the treatment of osteomyelitis, particularly when there is substantial bone loss, whether that is the result of trauma or extensive debridement of compromised bone [36,50,133,134]. While autologous bone grafts provide excellent osteoinductive, osteogenic, and osteoconductive properties, they require an additional harvest procedure, which is painful and can create donor site morbidity [66,90,135–137]. Recently, Kim et al. [135] proposed that the proximal tibia be utilized as a harvest site for cancellous bone, suggesting that this procedure is less painful than the traditional anterior iliac crest harvest site. Owing to challenges in harvest procedures and concerns of patient morbidity when large amounts of bone are required, allogenic bone grafting is sometimes elected instead of autologous grafts [66,137,138]. Allogenic bone grafts are typically used either frozen or freeze-dried, rather than fresh, to reduce the risks of immunogenicity or disease transmission [138,139]. While these methods are helpful to prevent rejection of the graft, the mechanical, osteogenic, and osteoinductive properties may be adversely affected by the processing [137,140,141]. In 2010, Ketonis et al. [142] reported that bone allografts are utilized for more than 800,000 musculoskeletal procedures in the United States each year. Unfortunately, over 11% of implanted bone grafts develop infection, which is thought to be due in part to biofilm formation on the implanted graft [142].

Regardless, autologous and allogenic morselized cancellous bone is used in various orthopedic applications and is proposed as a drug delivery device for the treatment of chronic osteomyelitis [56,136,142]. Cancellous bone can be impregnated with antibiotics before implantation either by mixing powdered antibiotics with the graft or direct soaking
Lewis et al. [136] demonstrated rapid in vitro release of antibiotics (gentamicin) from bovine cancellous chips within the first two days after impregnation, followed by a consistent rate of release for the remainder of the 14-day study. Lewis et al. [90] also reported that demineralized bone matrix (DBM) could be loaded with, and deliver, gentamicin locally without diminishing the osteoinductive properties of DBM in an in vivo rat ectopic pouch model. Ketonis et al. [142] investigated the feasibility of bonding vancomycin to morselized allograft bone to mitigate bacterial colonization of the allograft. They found that vancomycin could be covalently modified and that S. aureus colonization was prevented in vitro. Covalent modification of antimicrobials presents an intriguing option to prevent biofilm formation on allografts.

Despite the intriguing experimental discoveries centered around bone allografts as multifunctional systems, it is unlikely that autologous or allogenic bone grafts will serve the need for a single system to achieve simultaneous treatment of bacterial osteomyelitis and bone regeneration. The described limitation is a reflection of the trade-off between desirable osteogenic capabilities and the risk of immunogenicity and infection that accompanies allogenic grafts [137,143], as well as the serious risks of patient morbidity that accompany autologous grafting [66]. Recognizing limitations with bone grafts as stand-alone local DDDs, the most promising option remains to incorporate these components into other systems. There have been extensive investigations into alternative materials to deliver antimicrobial compounds and provide the osteogenic properties necessary to regenerate bone tissue.

**Synthetic Bone Graft Substitutes**

Synthetic bone graft substitutes are of interest for local drug delivery in the management of osteomyelitis, especially because they hold potential for dual-platform functionalities. This classification of material includes ceramics such as calcium sulfate [108], calcium phosphate, and porous alumina [17,68,120,144], as well as bioactive glass [17,145].

**Calcium Sulfate**

Biodegradable ceramics, such as calcium sulfate and calcium phosphate, are of strong interest for simultaneous use as bone void fillers and drug delivery vehicles in the clearance of bacterial osteomyelitis [68]. Calcium sulfate has been used in bone grafting since 1892 [68,146], and has a compressive strength equal to that of cancellous bone [68]. It is relatively inexpensive and is commercially available as hard pellets and liquid grafts [147]. Additionally, calcium sulfate possesses a quick resorption time, with a range of 3–12 weeks, depending on application (Figure 1.3) [68,146,147]. Jackson et al. [41] reported on calcium sulfate pellets loaded with amikacin, gentamicin, or vancomycin in vitro and found that the pellets eluted the antibiotics and dissolved completely within 16 h. These rapidly dissolving pellets loaded with amikacin were studied in an in vivo goat model by Bransetter et al. [89]. In that model, the pellets dissolved completely within 12 h and eluted amikacin above the MIC of *Pseudomonas aeruginosa* in 4–8 h. This type of pellet may be useful as part of a multimodal management plan for traumatic or contaminated wounds to provide rapid bacterial decontamination of an area, but this
treatment is unlikely to be able to provide sustained antibiotic delivery. McKee et al. [148] demonstrated the use of calcium sulfate pellets as a dual platform device, delivering tobramycin and promoting bony union in cases of infected long bone non-unions in a prospective clinical trial. The experimental device was found to be particularly helpful to eliminate dead space and deliver antimicrobials while biodegrading. This study reported a 92% success rate (determined by clearance of osteomyelitis and creation of bony union), and a rate of 8% infection recurrence and drainage of antibiotic-rich fluid after pellet dissolution. Maale et al. [29] investigated the ability of a purified calcium sulfate preparation loaded with tobramycin and vancomycin to inhibit biofilm formation without stimulating systemic toxicity in 50 patients undergoing revision arthroplasty for infected total joints or after multiple major revisions. The patients demonstrated significant local antibiotic concentrations in the first five post-operative days and noted no persistent wound drainage, as has previously been described [148].

Clinically, calcium sulfate is not used as frequently as calcium phosphates for bone regeneration because of its rapid absorption, rapid loss of mechanical strength [149], and propensity to produce a serous discharge, which is thought to be a result of acidic byproducts during material degradation [68]. Based on the available literature, calcium sulfate may have a place in the treatment of bacterial osteomyelitis when rapid antimicrobial delivery, the ability to encourage bone development, and rapid degradation of the DDD are appropriate. As such, calcium sulfate is not an ideal single-system local DDD.

**Calcium Phosphate**

Calcium phosphates have been used since the 1980s to enhance the osseointegration of metal implants [150] and are currently a popular synthetic graft substitute, as their chemical structure is similar to the mineral stage of bone [17,151]. These characteristics open a window of possibility into enhanced tissue regeneration, as they provide biocompatibility, bioactivity, and strong osteoconductive properties [95,150,152]. Calcium phosphates have a range of biodegradation profiles and mechanical properties, which are dictated by the calcium to phosphate ratio of the material [17,150]. The most commonly investigated CaP ceramics include HA, TCP, β-TCP, and dicalcium phosphate [17,68,152]. These CaP ceramics are typically in the forms of scaffolds, granules, cements, and coatings [95,150,152]. Additional benefits when using a CaP cement include an isothermic setting reaction, which allows a wider antimicrobial selection [17,149] than that of PMMA cement, and increased stimulation of angiogenesis when incorporating HA [17].

While there are strong benefits to CaP ceramics, there are also challenges. Challenges include inadequate mechanical strength, especially if needed in load-bearing portions of the skeleton [95,152]; small pore size and limited interconnectivity of pores, which can limit bone ingrowth and impact drug loading and release [95,150,153]; incongruities in material degradation and bone regeneration rates [95,154]; as well as the risk of bacterial colonization of slowly degrading ceramics [17]. Current investigations in the field of CaP
ceramics have been centered around improving porosity [95,154–156], improving mechanical strength [95,156], and encapsulating drugs and growth factors into the CaP carrier [95]. Investigations into porosity of CaP ceramics have concluded that biodegradable poly (lactic-co-glycolic) acid (PLGA) microspheres are suitable for incorporation into CaP cements to increase porosity. PLGA microspheres typically degrade prior to the rest of the matrix, creating macropores that facilitate bone ingrowth and implant fixation [155,156], and may make the ceramic less brittle and, consequently, more appropriate for clinical use [157]. Duan et al. [154] found that the incorporation of PLGA microspheres into a CaP cement had appropriate osteoconductive properties, biodegradation, and good biomechanical properties in an in vivo rabbit model. An alternative approach to modifying porosity resulted in an injectable CaP drug delivery foam, which was produced and evaluated in vitro [153]. This drug delivery foam was capable of providing consistent release of bioactive doxycycline with potential for 3–4 weeks of sustained delivery in an in vitro system. This original investigation is an intriguing option for drug delivery in non-load bearing situations and warrants in vivo investigation. The works of Gonzalez-Sanchez et al. [158] and Bastari et al. [159] highlight the role of CaP films and coatings in nanomedicine and encapsulation of drugs for the clearance of osteomyelitis and enhancement of bone regeneration.

Ultimately, CaP materials provide many attractive qualities, including isothermic setting reaction, suitability for use with a wide range of antimicrobial compounds, and the ability to form strong bone–material interfaces. These characteristics certainly lend themselves towards use as a dual platform device for the management of bacterial osteomyelitis. Limitations are currently focused on variation and unpredictability in release kinetics, risk of suboptimal biocompatibility, and difficulty in large-scale production of a universally appropriate system [149]. With this in mind, CaP materials are of interest for use in individual situations, but each system deserves careful in vitro and in vivo investigation before commonplace clinical usage for local drug delivery and bone regeneration.

Alumina
Alumina is an inert substance with good wear properties and is used frequently in artificial joint replacements and dental applications [160] owing to good biocompatibility and compressive strength [161]. Porous alumina is also clinically utilized in the fabrication of orbital implants following enucleation with the goal of allowing a fibrovascular network ingrowth through the device [152]. However, porous alumina has also been investigated as a drug delivery scaffold for clearance of bacteria and prevention of bacterial colonization [144,161,162]. Alumina-based ceramics are described to completely release loaded antibiotics and successfully resist bacterial colonization and biofilm formation in vivo [161]. Fiorenza et al. [144] describe a case of chronic osteomyelitis caused by S. aureus that underwent a successful one-stage surgical procedure using gentamicin-loaded porous alumina ceramic. The precise size and shape of the ceramic were determined via pre-operative CT scan (Figure 1.4), and the customized ceramic was loaded with gentamicin, as selected by culture and sensitivity.
Follow up of greater than 14 months post-operatively indicated resolution. Similar positive results were reported by Denes et al. [162], who reported two patients affected by mediastinitis resulting in destruction of the sternum, and one patient with an infected ankle arthroplasty. These patients were treated with antibiotic loaded porous alumina in a one-step surgical procedure made possible by the compressive strength of alumina and were reported to remain infection-free at follow-up over 12 months post-operatively. This material and procedure represent a promising option for treatment of osteomyelitis that involves bone loss and allows for pre-operative imaging. Particular strengths of this system include the surface resistance to bacterial colonization, which could greatly reduce the risk of persistent bacterial infection, and the capability for a single surgical intervention, as opposed to multiple revision procedures.

**Bioactive Glass**

Bioactive glass is a synthetic silica-based material [163] that was initially developed in the 1960s [145,164] and has been used clinically since 1985 [152,165]. The main benefits of bioactive glasses include bioactivity, osteoconductive and osteoinductive properties, biodegradation [152,166], load bearing capabilities [163], and the ability to create a local environment that is hostile to microbial growth [113,163]. In fact, bioactive glass is said to produce higher quantity and quality of bone when compared with synthetic HA [167] and newer hybrid materials have tailorable degradation, which is attractive [168]. Bioactive glasses have become a focus of investigations for drug delivery within the last one to two decades [166,169,170] because of their strong regenerative qualities, reported biocompatibility [171], and the initial experimental use of bioactive glasses in the treatment of chronic osteomyelitis [172]. Hasan et al. [167] created a biodegradable, bioactive glass-based antibiotic-releasing putty designed to be press-fitted into bone defects to provide support for bone growth while delivering antimicrobials (vancomycin) for 4–6 weeks to combat bacterial osteomyelitis. This material demonstrated vancomycin elution above the MIC of *S. aureus* for over 6 weeks *in vitro*, and as a putty, is attractive to surgeons because it can be formed into various dimensions [168]. Similarly, Soundrapandian et al. [166] found that a gatifloxacin-loaded bioactive glass loaded released gatifloxacin for up to 6 weeks *in vitro*. Drug release was influenced by scaffold size, concentration of drug solution, polymer coat, and dissolution medium. These results, along with the advantageous bioactive properties of bioactive glasses, leave them as an interesting multifunctional device for treatment of bacterial osteomyelitis. The current challenges lie within the optimal composition and fabrication process of bioactive glasses [171]. However, with a scalable process, it is likely that these materials will be further investigated for drug delivery in the treatment of bacterial osteomyelitis.

**Polymers**

**Natural Polymers**

Natural polymers are of interest in tissue engineering and drug delivery as a result of their bioactivity, biocompatibility, and biodegradation. These qualities are accompanied by
risks of immunogenicity, as well as poor mechanical properties, which limit their use in load-bearing situations [113,118]. Natural polymers, such as collagen, fibrin, and chitosan, are commonly utilized in a wide array of applications including attempted creation of blood vessels [173] and local delivery of antibiotics in many soft tissue and orthopedic applications [69,174]. Collagen is of particular interest, as it is a key component of the extracellular matrix [69,113]. Investigations into collagen and fibrin gels as DDDs have typically reported a rapid burst release \textit{in vitro}, with approximately 90\% of the antibiotic released during the first day and complete elution occurring by the fourth day [69]. Chitosan gels and sponges have been reported \textit{in vitro} to produce a sustained antimicrobial release sufficient to inhibit \textit{S. aureus} growth over the course of three weeks [175]. These gels have potential to be used as coatings to minimize the initial burst release of antibiotics [69]. Chitosan carries the additional intrigue of intrinsic antibacterial properties [176]. The role of these natural materials in the clearance of bacterial osteomyelitis is most likely strongest as adjunctive therapy either in acute infection, situations where mechanical stabilization is provided by other materials, or as coatings upon other materials to prolong antimicrobial delivery.

**Synthetic Polymers**

Synthetic polymers are an area of extreme interest for use in local DDDs [113] for the clearance of chronic bacterial infection and bacterial osteomyelitis [69]. These materials are attractive because of their general biocompatibility, biodegradation, and versatility [113,118], which includes tunable drug release kinetics and degradation rates [69]. Synthetic polymers also have more controlled manufacturing methods, which lends confidence to quality control and lessens concerns of immunogenicity and imperfections [69,113,177,178]. Similar to natural polymers, synthetic polymers possess less than ideal mechanical properties, which leaves them inadequate for independent use in load-bearing areas [113,118,177]. Additionally, the degradation of synthetic polymers most often occurs via hydrolysis, which can create an acidic pH in tissue adjacent to the implant, resulting in inflammation, local tissue damage, and potential alteration of local antimicrobial efficacy [69,113,169]. There are many polymers that are of interest, including PLGA, one of the most well investigated polymers [169], as well as polyurethane (PUR), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(caprolactones) (PCL) [22,69,113]. McLaren et al. [22] investigated the ability of an injectable PLGA modified with plasticizer polyethylene glycol (PEG) and antibiotics (gentamicin and clindamycin) to prevent bacterial infection and facilitate new bone growth in an \textit{in vivo} ovine contaminated bone defect. This material was found to release about 50\% of its antibiotics within the first seven days of elution and was effective at preventing persistent bacterial infection in this model. Additionally, less than 1\% of the loaded antibiotics were present three weeks after implantation, which suggests this system may offer a lower risk of antimicrobial resistance than PMMA, which has been found to release subtherapeutic levels of antibiotics even five years after implantation [14]. Alongside investigations of pure polymers, there are innumerable options for composite polymers, which are an area of interest and promise in drug delivery and tissue regeneration [113]. Recently, a composite polymeric scaffold was reported to be an
effective delivery platform for delivery of antibiotics for the elimination of *S. aureus* from contaminated bone defects in rabbits. This scaffold was purported to serve the dual role of tissue regeneration scaffold and antibiotic delivery device [105].

Synthetic polymers provide a seemingly endless supply of materials and composites to engineer biocompatible, biodegradable devices that deliver drugs and regenerate bone. Extensive options are, of course, accompanied by the need for extensive investigation and characterization before commonplace clinical usage of any individual polymer system. It is highly likely that synthetic polymers will continue to provide clinically relevant results in the management of bacterial osteomyelitis.

**Emerging Nanotechnology for Combatting Bacterial Infection**

Nanotechnology, with an emphasis on nanopatterning and nanoparticles, has emerged as an area of incredible interest in simultaneous stimulation of tissue regeneration, prevention of bacterial infection, and mitigation of antimicrobial resistance via intrinsic device properties and drug delivery [42,179–182].

**Nanopatterns**

Nanopatterning refers to the micro and nano-scale surface features that are either inherent to surfaces, such as nanopillars on cicada wings [183], various nanotextures on plants, lizards, and sharks [180], or inspired by nature and carefully engineered in a laboratory [54,184]. Nanopatterns provide a multitude of applications, from the ability to kill adherent bacteria [184] to the ability to determine stem cell fate [181]. While mechanisms of bactericidal action are not completely elucidated, proposed hypotheses are typically centered around mechanical forces [54], including the stretching, puncturing, and eventual rupture of bacterial cells [54,96,185]. These hypotheses acknowledge the complex dynamics between various bacterial cells and nano-patterned surfaces and have recognized factors such as the presence of cellular motility [96], cellular morphology (e.g. rod-shaped vs. coccoid) [186], cell wall components and structure (Gram-positive vs. Gram-negative) [187], and extracellular polymeric substance (EPS) [185], as important in nanopattern–bacterial cell interactions. The main benefits of utilizing nanopatterns for intrinsic antibacterial activity include elimination of antimicrobial agents from surface coatings and delivery vehicles. Nanopatterns can mitigate the risks of subtherapeutic levels of antimicrobials and propagation of antimicrobial resistance, especially in the face of biofilms, which can harbor bacterial cells that are 10–1000-fold less susceptible to antimicrobials than planktonic bacteria [184,188–190]. Additionally, nanopatterns may enhance biocompatibility by bypassing the need for chemical surface modifications [184].

Dickson et al. [184] utilized a scalable process of soft and nanoimprint lithography to imprint nanopillars onto PMMA films to investigate a potentially broadly bactericidal surface pattern targeted against *Escherichia coli (E.coli)*, which is a leading cause of Gram-negative orthopedic implant infections [191]. Smaller, more closely spaced pillars were more effective, possibly owing to greater stresses as a result of the bacteria
contacting more pillars simultaneously. Michalska et al. [180] systematically investigated bactericidal activities of a variety of surfaces with three Gram-negative bacterial species (Escherichia, Pseudomonas, and Rhodobacter) and a Gram-positive Bacillus. They also observed two obvious mechanisms; one being that longer, sharp pillars were able to directly pierce microbial cells, nonselective of species. The second mechanism being that shorter, blunt pillars required multifaceted cellular interactions to eventually stretch and tear membrane envelopes. The effects of interspacing and controlled disorder on the functionality of a specific bactericidal nanopattern were investigated by Modaresifar et al. [54] utilizing S. aureus, as the most common pathogen in implant-associated infections. This study quantified numbers and characterized the morphology of S. aureus cells on nanopatterns via scanning electron microscopy (SEM) (Figure 1.5) and determined, similarly to Dickson et al. [184], that decreased interspacing (100 nm) demonstrated the greatest bactericidal efficiency and that controlled disorder did not enhance bactericidal efficiency. Similar to other reports, the main bacterial killing mechanism was direct penetration of the cell wall and eventual rupture. Widyaratih et al. [181] investigated the antibacterial behavior of multiple types of osteogenic nanopatterns using a strain of E.coli. Nanopillars were created on silicon wafers using an electron beam-induced deposition (EBID) system. The results confirmed previous work that controlled nanopatterns can be produced by EBID and indicated that nanopatterns containing features of interspace and controlled disorder, which are derived from osteogenic nanopatterns, could exhibit bactericidal properties against E. coli.

These investigations spark interest into the creation of controlled, reproducible nanopatterns to serve in a dual platform functionality, for tissue regeneration and prevention of bacterial infection, by instructing mesenchymal stem cells to commit to osteogenic lineage and exerting bactericidal effects [181]. Dual platform functionality could result in profound nanomanufacturing to prevent biofilm formation on the surfaces of a wide range of implantable devices. The potential to create nanopatterns on materials appropriate for orthopedic use, such as titanium and polymers [181], is fascinating and unlocks an incredibly promising area of exploration. This area holds exceptional promise in orthopedic procedures and the prevention and treatment of bacterial osteomyelitis.

**Nanoparticles**

Nanoparticle delivery systems are based on magnetic nanoparticles (MNPs), which are described as a class of <100 nm engineered materials, typically composed of iron, nickel, cobalt, and their oxides, that can be manipulated by an applied external magnetic field [192]. Nanoparticle delivery systems offer many advantages, including immune system evasion, the ability to modulate drug release kinetics and target drugs to specific sites, improved multi-drug delivery [42], and potential bacterial detection [193]. Various combinations of MNPs and antibiotics have been investigated for the ability to penetrate bacterial cells and biofilms as a method to render bacteria inactive [193], and there are many investigations into general antibacterial strategies, as well as strategies specifically geared towards bacterial osteomyelitis.
Geilich et al. [189] developed a multi-compartment polymersome formulation that contains hydrophobic superparamagnetic iron oxide nanoparticles (SPIONs) and hydrophilic methicillin that is biocompatible and intended for the treatment of medical device-associated infections. The efficacy of this system was assessed in an in vitro methicillin-resistant Staphylococcus epidermidis biofilm. The results indicated that this system of SPIONs, co-encapsulated with antibiotics, was able to eliminate the biofilm in vitro via direct application and external magnetic stimulation. Akram et al. [194] investigated a triple combination therapy of silver magnetite nanoparticles (AgNPs) with blue light and either amoxicillin, azithromycin, clarithromycin, linezolid, or vancomycin against 10 clinical isolates of MRSA in vitro. This work is interesting, as it combines the antibacterial activity that AgNPs have been said to promise [195,196] with the efficacy of blue light against MRSA and the properties of conventional antibiotic therapy. The results indicated enhanced bactericidal activity of AgNPs applied in combination with blue light and found that bactericidal activities were greatest when either clarithromycin or azithromycin was included in the triple therapy. This triple combination therapy presents an intriguing novel approach to combatting MRSA infections while reserving last-line therapies such as vancomycin, although honing for clinical use is definitely warranted.

There are multiple investigations into nanoparticle systems for the treatment of bacterial osteomyelitis designed to overcome the shortcomings of current therapies [158,190,197–199]. Posadowska et al. [197] suggested the use of a vancomycin-enriched injectable gellan gum (hydrogel) matrix. Enrichment was accomplished by both dissolved vancomycin and vancomycin encapsulated in PLGA nanoparticles. The results from in vitro studies indicated relatively simple and precise dosing of the hydrogel, a prominent burst release of vancomycin followed by a prolonged, sustained delivery that was thought to be due to a combination of erosion-diffusion release, appropriate antimicrobial activity against Staphylococcus sp., and cytocompatibility with osteoblast-like cells. Similarly, Gonzalez-Sanchez et al. [158] developed an acrylate multifunctional orthopedic hydrogel that was reported in vitro to be osteoconductive, and possessed antibacterial effects as a result of silver nanoparticle adsorption.

Qiao et al. [198] report on the use of Fe₃O₄ nanospheres combined with functionalized carbon nanotubes (CNTs) and gentamicin in conjunction with a combined microwave calorific-chemotherapy (MCCT) system for dual-targeting and microwave (MV)-excited drug release for the clearance of MRSA-induced osteomyelitis. This study found that Fe₃O₄/CNT/gentamicin had excellent bacteria-capturing capabilities in vitro, and demonstrated bacterial load reduction in an in vivo rabbit model of osteomyelitis. The proposed mechanism of action of this system is complex. It is thought to be initiated with a synergistic reaction of the synthesized nanocaptor binding bacteria, which then produces heat under MV stimulation and triggers the release of gentamicin. In a fairly similar fashion, Fang et al. [190] investigated the heating effect of MNP-induced hyperthermia to both destroy biofilm and promote antibiotic efficacy to improve the treatment of peri-implant osteomyelitis. An in vivo rat model was created by implanting a
metallic needle with or without bacterial contamination into the intramedullary canal of the femur. Fe$_3$O$_4$ nanoparticles were utilized in combination with intramuscular injection of vancomycin +/- magnetic hyperthermia. Colony forming units (CFUs) and histology indicated that the combination of MNPs and hyperthermia could destroy the experimental biofilm and enhance the overall therapeutic effect of systemic and local therapy.

Lastly, Ak et al. [199] developed a novel biodegradable, biocompatible, physically targeted gentamicin-loaded gelatin nanoparticle system for the local treatment of bacterial osteomyelitis. Through an in vivo rat model of proximal tibial S. aureus osteomyelitis, the drug delivery system was shown to hasten the recovery time of infected rats in comparison with free gentamicin or placebo therapy. Additionally, this system demonstrated controlled drug release in vitro and warrants further investigation into use for the treatment of osteomyelitis.

Ultimately, the works described above represent novel endeavors into the treatment of implant-associated biofilms, bacterial infection, and osteomyelitis. These investigations have the potential to mitigate many of the challenges that face current local drug delivery and tissue regeneration systems. As is common with nanotechnologies, there may be challenges associated with upscaling the proposed systems. Additionally, detailed investigations into safety are necessary prior to clinical translation, as it is well understood that MNP systems carry risks of in vivo toxicity resultant from nanoparticle composition [200] as well as accumulation within the body [201].

Antimicrobial Strategies

When pursuing systemic antimicrobial therapy, as well as local antimicrobial delivery, there are many considerations, including bactericidal versus bacteriostatic and time-versus concentration-dependent antibiotics [202]; the choice to use single-agent or combination therapy [101]; along with concerns of patient sensitivity, compliance, and adverse effects [203]. Local drug delivery can mitigate the severity of systemic toxicity and adverse effects, but local drug delivery is accompanied by considerations of the feasibility of drug incorporation into the DDD. While this review does not focus on specific antimicrobial strategies, the authors recommend the following references for those readers interested in learning more about antibiotics for local drug delivery: [101,203–205].

Conclusions and Future Perspectives

Bacterial infection is a serious complication following surgical implantation of medical devices and may occur shortly post-operatively or months to years later. Current therapies of systemic antimicrobials, surgical debridement, and local drug delivery are imperfect. Chronic, persistent, and recurrent bacterial infection still plague this patient population. Certainly, there are steps being taken to reduce the risk of post-operative bacterial infections including osteomyelitis. These steps include remaining cognizant of
the risk for multidrug-resistant bacterial organisms; providing patient education and encouraging patients to cease smoking in the weeks leading up to scheduled procedures; maintaining appropriate and stable blood glucose and body temperature; and maintaining exceptional skin, body, and wound hygiene peri-operatively [33,35,47]. Despite these measures, post-operative bacterial infection will still occur and, therefore, the needs for effective therapies remain. While there is significant progress being made in the field of multifunctional devices for tissue regeneration and drug delivery, progress is slow, as these devices are quite specific to their intended application, and persistent challenges remain. Challenges include adverse host reaction to the material, insufficient antimicrobial delivery, biofilm formation on the device, as well as insufficient mechanical properties and mismatched degradation and regeneration profiles.

Further investigations into engineering materials that possess the ideal characteristics for a drug delivery device, including biocompatibility, clinically significant and controlled release of drugs, predictable and inert degradation, and appropriate mechanical characteristics, will continue to provide new and improved therapies. During these pursuits, it is important to keep cost in mind. There are many challenges in scaling the production of devices from small batches to more readily available products, and product cost may influence the provider’s decisions to utilize certain products. Recent novel discoveries and approaches, such as porous alumina for simultaneous drug delivery and mechanical support, and modifications and novel combinations within the class of synthetic polymers offer tremendous potential for future cures to the prevention and treatment of these debilitating infections. Perhaps one of the most intriguing and promising opportunities is the field of nanotechnology. Nanotechnology offers promise for minimally invasive and local therapies that are bactericidal, regenerative, and stimuli-responsive and may minimize the risks of developing antimicrobial resistance, biofilm formation, and device failure. Lastly, there are many promising devices and investigations. Perhaps an ideal way to approach the persistent, significant problem of bacterial osteomyelitis is to acknowledge that there may not be one single ideal device. Instead, there are multiple appropriate options for various clinical scenarios.
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APPENDIX I

Figure 1.1. Antibiotic-loaded PMMA beads utilized in orthopedic procedures. In the left-hand image, PMMA beads are used in treatment of total knee arthroplasty, in the right-hand image, in the treatment of chronic osteomyelitis. Reprinted from van Vugt et al. [112] following the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/) (Accessed July 11, 2021).

Figure 1.2. Flow chart describing ideal properties of antibiotic-loaded bone cements. Reprinted from Wall et al. [115] following the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/) (Accessed July 11, 2021).

Figure 1.3. Radiographs demonstrating use of calcium sulphate pellets. (a) MRI image demonstrating extensive medullary edema, intramedullary abscess and cortical involucrum. (b) infection treated by excision via medullary reaming. Dead space filled with calcium sulphate pellets loaded with gentamicin. Shown via radiograph (c) follow up radiograph at 4 months post-operatively. Calcium sulphate pellets have dissolved completely. Reprinted from Ferguson et al. [68] following the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/) (Accessed on September 16, 2021).
Figure 1.4. CT scan images demonstrating use of porous alumina ceramic. Image A: pre-operative axial CT scan of the femur. Bone loss and bone remodeling as a result of chronic infection (osteomyelitis) is seen. Image B: axial CT scan after follow-up of 11 months. Tight contact between bone and porous alumina ceramic is seen and demonstrates appropriate biocompatibility and osseointegration. Reprinted from Fiorenza et al. [144] following the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/) (Accessed September 16, 2021).

Figure 1.5. SEM images of S. aureus. Images demonstrate S. aureus on (b) control surface, (c-i) various experimental surfaces. Damaged bacterial cells can be identified by an irregular morphology (c-i) compared to healthy cells in typical coccoid morphology (b). (f) nanopillars with interspace of 100nm and (g) nanopillars with interspace of 170nm demonstrated most efficient bactericidal properties. Reprinted from Modaresifar et al. [54] following the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/) (Accessed September 16, 2021).
CHAPTER II: ROLE OF ANIMAL MODELS TO ADVANCE RESEARCH OF BACTERIAL OSTEOMYELITIS
A version of this introduction was previously published by Caroline Billings and David E. Anderson:


This manuscript, aside from formatting, has not been edited for inclusion in this dissertation. David E. Anderson is a co-author on this manuscript and was involved in conceptualization, editing, and supervision.

Abstract

Osteomyelitis is an inflammatory bone disease typically caused by infectious microorganisms, often bacteria, which causes progressive bone destruction and loss. The most common bacteria associated with chronic osteomyelitis is Staphylococcus aureus. The incidence of osteomyelitis in the United States is estimated to be upwards of 50,000 cases annually and places a significant burden upon the healthcare system. There are three general categories of osteomyelitis: hematogenous; secondary to spread from a contiguous focus of infection, often from trauma or implanted medical devices and materials; and secondary to vascular disease, often a result of diabetic foot ulcers. Independent of the route of infection, osteomyelitis is often challenging to diagnose and treat, and the effect on the patient’s quality of life is significant. Therapy for osteomyelitis varies based on category and clinical variables in each case. Therapeutic strategies are typically reliant upon protracted antimicrobial therapy and surgical interventions. Therapy is most successful when intensive and initiated early, although infection may recur months to years later. Also, treatment is accompanied by risks such as systemic toxicity, selection for antimicrobial drug resistance from prolonged antimicrobial use, and loss of form or function of the affected area due to radical surgical debridement or implant removal. The challenges of diagnosis and successful treatment, as well as the negative impacts on patient’s quality of life, exemplify the need for improved strategies to combat bacterial osteomyelitis. There are many in vitro and in vivo investigations aimed toward better understanding of the pathophysiology of bacterial osteomyelitis, as well as improved diagnostic and therapeutic strategies. Here, we review the role of animal models utilized for the study of bacterial osteomyelitis and their critically important role in understanding and improving the management of bacterial osteomyelitis.

Clinical Disease and Patient Impact

Osteomyelitis is an inflammatory bone disease that results in progressive bone destruction and bone loss and is typically caused by infectious microorganisms [1–4]. The most common causative organisms are bacteria [1], specifically Gram-positive Staphyloccoci such as Staphylococcus aureus (S. aureus) [1–3, 5–8]. There are three main etiologies of osteomyelitis: hematogenous, trauma or surgery associated, and
secondary to vascular disease. Hematogenous osteomyelitis is most common among pediatric patients [5, 7, 8]. Injury associated osteomyelitis may be spread from a contiguous focus of infection, may be secondary to trauma, or may be associated with surgery, especially where implanted medical devices are used. This may occur in individuals of any age [2, 3, 5–7]. Osteomyelitis also commonly occurs secondary to vascular insufficiency and is often a result of diabetic foot ulcers (DFU) [2, 6, 7]. The annual incidence rate of osteomyelitis in the United States is not precisely known. In 1999, the incidence was reported to be as high as one out of every 675 hospital admissions, which translates to approximately 50,000 cases annually [9]. Since that time, the incidence of osteomyelitis cases of all categories has been increasing (8). The rise in caseload is partially due to increases in cases of diabetes [8], trauma [10], numbers of reconstructive orthopedic procedures and implanted prosthetic materials [6, 11–13], and also may be associated with improvements in diagnosis [2].

Clinical presentation of patients suffering from osteomyelitis is variable. Acute osteomyelitis may present with fever, redness, pain and draining lesions. Symptoms of chronic osteomyelitis may be vague, with a wide array of clinical features which may be as subtle as simple focal swelling and tenderness on physical examination [2, 6, 7, 11, 14, 15]. Nonspecific clinical presentation necessitates a thorough patient workup for successful diagnosis [16]. Diagnostic testing often includes physical examination, hematology and biochemistry panels, measurement of C-reactive protein (CRP), culture and sensitivity testing of bone and wound samples, and imaging such as radiographs and ultrasound. Radiographic evidence of bone changes lag behind pathologic changes, so early disease may not be apparent on standard radiographs [7, 11, 17]. Advanced imaging can be helpful, and magnetic resonance imaging (MRI) or computed tomography (CT) [1, 11, 16] may be required. Despite the abundance of available tests that may be employed, there are few early pathognomonic findings for osteomyelitis [7, 18, 19]. Therefore, while osteomyelitis may be suspected, the gold standard of diagnosis requires a bone biopsy for culture [2, 5, 11, 18–20] and histopathologic examination [7, 11, 18–21].

Osteomyelitis results in significant morbidity and mortality to the patient [6, 10, 14, 22, 23], and expedient, intensive treatment is indicated. The most common clinical approach to treatment of bacterial osteomyelitis involves a combination of medical and surgical management [4, 6, 11, 18, 24, 25]. Systemic antibiotic therapy should be guided by microbial cultures whenever possible [11]. In the absence of culture and sensitivity results, empirical, broad-spectrum antibiotics are usually administered [4, 5, 18]. Antimicrobial therapy is typically administered for a minimum of 4–6 weeks [2, 19, 20, 24, 26] and is often continued for longer periods of time in an attempt to mitigate risks of chronic osteomyelitis [20]. Some clinicians advocate treatment for up to six months after diagnosis [19, 20, 26]. Local antibiotic therapy may be instituted to complement systemic antimicrobial therapy [27]. Surgical debridement of affected tissue is routine treatment in conjunction with medical management [2, 15, 18, 19]. A hallmark of osteomyelitis is the presence of necrotic bone [2, 6, 15], which is readily colonized and surrounded by biofilm [11, 28]. Biofilms often result in persistence of bacterial infection. Persistence is
multifactorial and is partially due to the protective slime matrix that provides a physical barrier between immune cells and bacterial cells [6, 11, 14] and can impair diffusion of antibacterial substances [29]. Persistence also results from the physiologic environment of biofilms, which allows for enhanced antimicrobial resistance through creation and persistence of immense phenotypic diversity, including metabolically inactive bacteria and subpopulations of “persisters” or phenotypically resistant bacteria [30–33]. Debridement of necrotic bone should be thorough, with the goal of reaching healthy, viable tissue and removing sources of biofilm. This often includes removing implanted hardware [2, 5]. While this approach sounds straightforward and reasonable to accomplish, there are many challenges in the treatment of osteomyelitis which often leave patients suffering relapses or struggling with chronic infections [11, 34–36]. Particular challenges include inadequate debridement [2, 15, 19, 30], metabolically inactive bacteria or bacteria embedded in biofilm [2, 12, 30], inadequate antimicrobial penetration to infected tissues [37], antimicrobial resistant bacterial species [2, 25], and loss of tissue or organ function to the patient during treatment [12, 38]. Challenges are augmented by the negative impact of treatment on patient quality of life [25], increased risk of bacterial infection upon hardware reimplantation [39], and the ability of S. aureus to evade the host immune system [30, 40].

Bacterial osteomyelitis has a progressively increasing incidence, and it is important to reduce morbidity and mortality to patients while concurrently reducing the burden on the healthcare system [11]. Continued improvements in the understanding, diagnosis, and therapy of bacterial osteomyelitis are necessary to accomplish these goals. As a result of variable patient population, case presentation and disease management, clinical osteomyelitis research has proven difficult [38]. A major step in achieving improved diagnostic and therapeutic methods lies within animal modeling of this disease. In vivo models facilitate groundbreaking research by allowing scientists to expand upon promising in vitro discoveries and utilize research findings to improve the lives of patients suffering from osteomyelitis. Ultimately, animal models promise to speed advances in modern medicine. The purpose of this review is to highlight a range of animal models used to study bacterial osteomyelitis. While it is not possible to present all of the features for each individual model, this review will emphasize the limitations and benefits of the most common animal models used to investigate the pathogenesis, diagnostic methods, and therapeutic strategies to better understand and combat bacterial osteomyelitis.

Model Development

There are many approaches to inducing bacterial osteomyelitis in animal models. This review will focus on two main categories of bacterial osteomyelitis induction: surgical and hematogenous. Authors have chosen to exclude detailed discussion of in vivo modeling of osteomyelitis secondary to DFU. There are reports of modeling bacterial infection with diabetic rodent strains, however, osteomyelitis resulting from DFU is a multifactorial, chronic condition and the complexity of modeling and translational
healing differences raise concerns regarding reliable in vivo models [41–44]. To surgically induce bacterial osteomyelitis in any species, there are a few necessary components. An injury to bone tissue [45] is required, and typically stems from mechanical trauma with or without the addition of a sclerosing agent [22, 46]. A foreign body or medical device may be used to serve as a nidus for bacterial colonization [47]. Bacterial inoculation is necessary and may be accomplished via direct administration of a bacterial inoculum [48], soaking of a foreign object or hardware in a bacterial suspension, creating a biofilm on a piece of hardware for implantation [49], or by intravenous (IV) administration of bacterial suspension (hematogenous seeding) [50]. Many investigators choose to seal the bone defect, e.g. using sterile bone wax to ensure local containment of the bacteria and minimize undesired concomitant soft tissue infections [51]. Induction of hematogenous bacterial osteomyelitis typically carries the advantage no required surgical manipulations or placement of foreign materials [52, 53]. Hematogenous models are designed to closely mimic the acute hematogenous osteomyelitis that most commonly occurs in pediatric patients [52, 54].

Within these two categories, many differences exist in model design. Differences include the type of bone injury and surgical approach, bacterial strain and colony forming unit (CFU) count, administration vehicle and quantity of bacterial inoculum, as well as length of study and monitoring techniques. It is crucial to consider the bacterial species and strain that will be utilized in animal modeling. During initial model establishment, it is recommended to utilize a bacterial strain with well documented behavior within the chosen animal species. After confirming that osteomyelitis can be established in the selected model, the bacterial species, strain, dose, and even delivery vehicle may be altered to best accomplish the research objectives. On that token, investigators should consider the species-specificity, antimicrobial sensitivity profile, and clinical relevance of the chosen pathogen. These pillars of model development are highlighted by Laratta et al. [55] and commented on by Johansen et al. [56]. Markers of success within model development typically include clinical manifestation of disease, evidence of osteomyelitis on histopathology, and positive bone cultures upon study completion. Most investigators elect to pulverize bone samples and perform bacterial culture from the pulverized samples. Confirmation of bacterial cultures using polymerase chain reaction (PCR) has become routine since the method was described in 1999 [54].

**Small Animal Models**

**Mouse Models**

**Model Development**

There are many surgical models of bacterial osteomyelitis performed in murine models. Models typically utilize long bones, although alternatives such as vertebral models are also reported [57]. An extensive review of murine models, including the goal, method, and bacterial inoculum used in each study, was recently published by Guarch-Pérez et al. [58]. One approach used by multiple investigators was described in 2008 as a model to assess intramedullary response to titanium particles [59]. This surgical approach is
accomplished by creating a medial parapatellar arthrotomy to access the femur. Once accessed, a defect extending to the medullary cavity of the femur is created. Kirschner wire (K-wire) is inserted into the femoral medullary canal and penetrated into the patellofemoral joint space. Bacterial inoculation occurs via direct application of a bacterial suspension and the surgical site is closed \[48, 60, 61\]. This model was recently adapted and modified to model shoulder implant infections \[62\]. In this study, investigators were able to reliably induce bacterial osteomyelitis using a bioluminescent strain of \textit{S. aureus} and were able to track infection with radiographs and bioluminescent imaging (BLI). Another surgical approach that is utilized in various forms by many investigators is described well by Funao et al. \[63\]. Much of this approach is similar to that described above; the distal portion of the femur is exposed surgically, and a 0.5 mm drill hole is created to expose the medullary canal of the femur. Rather than placing an implant, bioluminescent \textit{S. aureus} is inoculated directly into the defect. The defect is then sealed with bone wax and the surgical site is closed. Another unique model of murine bacterial osteomyelitis is the hematogenous model described by Horst et al. \[52\]. This model does not involve surgical manipulation or placement of foreign material. Instead, mice received one injection of \textit{S. aureus} in phosphate-buffered saline (PBS) via the lateral tail vein. This model was created to closely mimic both acute and chronic hematogenous bacterial osteomyelitis and is unique in that it does not require additional bone injury. These approaches highlight the various methods available to induce bacterial osteomyelitis and the subtleties between the various models.

\textbf{Insights Into Pathogenesis}

While arguably each investigation into bacterial osteomyelitis provides information on pathogenesis, there are experiments designed to evaluate specific questions regarding the pathogenesis of bacterial osteomyelitis \[64\]. One such experiment, described by De Mesy Bentley et al. \[65\], utilized two murine long bone infection models and captured groundbreaking transmission electron microscopy (TEM) images of \textit{S. aureus} invading and residing within the osteocyte lacuno-canalicular network (OLCN) of live bone. \textit{Staphylococcus aureus} cells are thought to be protected while within the canaliculi system, as immune cells are likely too large to successfully access this area of the body. Therefore, these findings offer insight into the ability of \textit{S. aureus} to evade the host immune system and cause latent and recurrent osteomyelitis. Zoller et al. \[40\], established and utilized a murine model of bulk allograft infection to expand upon the findings of de Mesy Bentley et al. by investigating the mechanisms of immune system evasion by \textit{S. aureus}, specifically microarchitecture of implant surfaces as a potential factor in increased bacterial colonization. \textit{Staphylococcus aureus} was discovered within allograft cortical haversian canals and submicron canaliculi within the native mouse femur. Results indicated that bulk allograft implant material was more susceptible to bacterial infection even at low bacterial inoculums compared to stainless steel implants. This finding suggests that implant microarchitecture is incredibly important and may offer bacteria a submicron reservoir to evade clearance by the immune system. The work of Masters et al. \[66\] expanded upon these findings by investigating the role of \textit{S. aureus} cell wall synthesis machinery and surface adhesins in OLCN invasion. The authors
established a model of bacterial osteomyelitis by placing stainless steel pins inoculated with various mutant strains of *S. aureus* into the medial tibia of mice. Results showed significant changes in OLCN invasion, abscess formation and pathogenic bone loss with the deletions of penicillin binding protein 3 and 4 (PBP3, PBP4) and autolysin (Atl), indicating that cell wall synthesis machinery can modulate *S. aureus*’ pathogenesis in osteomyelitis.

**Improvements in Diagnostic Capabilities**

While there are multiple reports of utilizing BLI and *in vivo* micro-CT in murine models [63, 67, 68], these reports are often geared toward improving the *in vivo* modeling system rather than improving diagnostic capabilities for clinical patients [63]. Recently, however, Isogai et al. [69] performed plasma metabolome analysis in a model of murine osteomyelitis caused by *S. aureus* and identified 12 metabolites as candidate positive biomarkers and two candidate negative biomarkers for osteomyelitis. Novel plasma biomarkers are aimed to improve the early diagnosis of osteomyelitis. Improvement in the early diagnosis of osteomyelitis is of great interest, as there are currently many challenges in obtaining a swift and specific diagnosis in clinical patients.

**Investigations Into Therapeutic Strategies**

A major goal of *in vivo* osteomyelitis work is to evaluate novel treatment strategies and investigate potential efficacy for clinical use. There are many investigations focused on various combinations or applications of antibiotics for clearance of osteomyelitis [48, 70–72]. Jørgensen et al. modeled the particularly challenging situation of biofilm presence upon orthopedic implants. They investigated the efficacy of rifampicin-containing combinations of antimicrobials compared with non-rifampicin-containing combinations of antimicrobials in reducing bacterial counts or clearing infection. Results indicated that combinations of antimicrobials that included rifampicin, as well as the combination of daptomycin and linezolid, were more effective in reducing bacterial burden than combinations not containing rifampicin [70]. There are also many investigations into novel therapeutics [73–75]. Wang et al. utilized a model of *S. aureus* hematogenous orthopedic implant infection to identify specific virulence factors to be translated into therapeutic targets. This work identified two key pathogenic factors, anti-α-toxin (AT) and anti-clumping factor A (ClfA) and demonstrated markedly improved efficacy in infection treatment utilizing human anti-AT/anti-ClfA combination therapy [50]. Similarly, Yokogawa et al. [76] created a novel murine one-stage revision model of methicillin-resistant *S. aureus* (MRSA) implant-associated osteomyelitis. This model facilitated discovery of synergistic activity of vancomycin and anti-glucosaminidase (Gmd). Identification of alternative therapeutics is important, as medical device implantation continues to increase and antimicrobial resistance (AMR) is becoming increasingly prevalent.

**Conclusion**

Murine models are particularly helpful to researchers investigating bacterial osteomyelitis. Main attractions of the mouse model include the small size, economics,
and genetic and molecular tools that are available to tailor murine strains and facilitate a wide array of investigations. Indeed, mouse strain selection is of paramount importance as strains contain significant differences from one another. Investigators should consider the primary research objective of the model to guide strain selection and ensure research objectives can be accomplished appropriately. This pillar of model development is highlighted nicely by Dworsky et al. [57]. These advantages make mice attractive for investigations into pathogenesis and proof of concept models [58, 72]. Also, mice allow for certain longitudinal monitoring techniques, such as BLI and in vivo microCT. Longitudinal monitoring is an asset that adds strength and clarity to data collection as individuals can be compared to themselves over multiple timepoints. While mice can mimic the human inflammatory response of osteomyelitis [58], their bone structure and bone remodeling process are less similar to humans than other animal models provide [77]. As a result of the mouse’s small size, complex and multi-stage surgical procedures are not impossible, but are challenging to perform. This small size also prohibits the investigation and translation of implants intended for human use. Additionally, serial blood collection is limited by volume and frequency. When considering the benefits and limitations of murine models, it can be concluded that mice are an excellent tool for early investigations from in vitro to in vivo modeling and proof of concept work.

**Rat Models**

**Model Development**

Rats provide a variety of models that produce well-characterized and reliable bacterial osteomyelitis. Significant historical developments have previously been described [36, 64, 78, 79]. Currently, the most popular rat models are of long bone osteomyelitis and most often utilize the tibia [17, 22, 80–83] or femur [84–90]. Long bone models rely on mechanical trauma, placement of foreign bodies, or creation of fractures, all typically with concurrent sealing of the defect area with bone wax to contain bacterial inoculums and prevent concomitant soft tissue infection. Alternative models include mandibular models [91], vertebral models [92], joint prosthesis models [93], and hematogenous models [94, 95]. Hematogenous models required additional surgical manipulations to successfully establish osteomyelitis. This may be a result of the rat’s ability to respond to acute infection, which can rapidly clear peripheral infection and may complicate infection models [78]. Although reports of rat osteomyelitis models exist, a comprehensive review of these models is lacking. In this review, we present a detailed summary of rat osteomyelitis models that were utilized to inform this review (Attachment 2.1).

**Insights Into Pathogenesis**

Similar to murine models, rat models can be utilized for investigations into pathogenesis. Rat models have facilitated valuable discoveries, including investigations of virulence factors associated with *S. aureus* biofilms and the ability of *S. aureus* to function as an intracellular pathogen. Biofilms are well recognized as a source of recalcitrant bacteria that can impair antibiotic treatment of osteomyelitis and cause persistent or recurrent osteomyelitis, particularly when orthopedic implants are in place [13, 31, 111]. Two studies that have pursued the in vivo investigation of biofilm virulence factors and genetic
components in rats include the investigation by Wu et al. [107], which demonstrated that overexpression of ASyyG led to a reduction in biofilm formation and in vivo pathogenicity of MRSA in a model of rat tibial osteomyelitis; as well as the investigation by Sahukhal et al. [110] who utilized a model of implant-associated osteomyelitis. This investigation demonstrated that deletion of the msaABCR operon of S. aureus (USA300 LAC) resulted in defective biofilm production and reduced severity of bacterial osteomyelitis. The capability of S. aureus to function as an intracellular pathogen is considered to be a mechanism of immune system evasion and a source of recurrent, persistent osteomyelitis [31] and is supported by in vitro evidence [112, 113]. Based on that in vitro evidence, Hamza et al. investigated and confirmed the ability of purely intracellular S. aureus to induce osteomyelitis in a rat model [10].

**Improvements in Diagnostic Capabilities**

Similar to murine models, rat models have allowed for improvements in diagnostic or longitudinal monitoring capabilities in experimental models. Examples of these improvements include the findings of Stadelmann et al. [99], who demonstrated the use of in vivo microCT to longitudinally monitor bacterial osteomyelitis in a rat tibial model, thus offering a method to limit numbers of animals needed for experiments and to add strength to collected data. Also, Aktekin et al. evaluated the utility of available scoring systems for the radiographic evaluation of experimental osteomyelitis. Authors utilized a tibial model of osteomyelitis and evaluated serial radiographs throughout their study period, ultimately concluding that it is best to evaluate and report each radiograph individually, rather than appointing a numerical grade from a previously published grading scale [17]. This is a valuable report for experimental studies, and with appropriate radiographic interpretation, is likely to add strength to radiograph assessments. An improvement to in vivo studies that holds potential to translate into human medicine is the investigation into various tracers for positron emission tomography (PET) to successfully image osteomyelitis and differentiate between bone infection and bone healing [51]. The work investigating PET tracers indicated that Gallium-68 (68Ga), did not accumulate in healing bone, only infected bone. This work brings interest to the use of 68Ga and PET for clinical patients, although further work is needed to clarify use and safety concerns. Another interesting foray into improving diagnostics for clinical patients was completed by Deng et al. who described the potential use of extracellular vesicles (EVs) as a diagnostic marker for acute osteomyelitis [109].

**Investigations Into Therapeutic Strategies**

Rats are recognized to be more resilient than mice and therefore are well suited to investigations into therapeutic strategies, such as antibiotic trials. Indeed, there are many investigations into antibiotic therapies. These include therapeutic efficacy assessments of systemic antibiotics administered solo or in combination [98, 100, 103, 105], investigations of local antibiotic delivery systems [80, 83, 88] and antibiotics in combination with alternative therapies such as omega-3 fatty acid supplementation [108]. There also are investigations into novel therapeutic strategies such as the use of photodynamic therapy (PDT) to treat contaminated orthopedic implants and minimize
reliance on antibiotic therapy to clear implant associated bacterial osteomyelitis [13]. Recently, Cobb et al. [106] investigated the feasibility of utilizing a bacteriophage to mitigate bacterial osteomyelitis, biofilm, and soft tissue infection.

Conclusion
Rat models are a valuable animal resource in the study of osteomyelitis. They provide similar benefits to mice, including small size, economics, ease of housing and handling, and well-characterized strains that provide appropriate uniformity and enable study of disease pathophysiology relevant to that seen in people [97]. Rats have the ability to tolerate sustained, high dose antibiotic therapy [97]. While larger than mice, rats remain too small for assessment of orthopedic hardware for human use, and multi-step revision procedures, although not impossible, remain challenging. Uniquely, the rat is one of few ideal species for modeling of mandibular osteomyelitis [91, 95] because of their size, anatomy, and general hardiness. Therefore, the strength of rat models lies within the ability to investigate pathogenesis and pursue initial investigations into therapeutic strategies to further understand in vitro data and gain in vivo knowledge prior to utilizing a larger animal model.

Rabbit Models
Model Development
Rabbits provide many useful and reliable models of bacterial osteomyelitis. The systematic review by Reizner et al. [78] details significant historical developments and the review by Bottagisio et al. provides a thorough overview of model development and utility [114]. Historically and currently, the most utilized models are long bone models, including tibial [115–121], femoral [49, 122–124], and radial [125–129]. Alternative models such as joint prostheses [130, 131], mandibular defects [132], vertebral models [133–136] and implant infection via hematogenous seeding [137] exist. Induction of osteomyelitis among these various models can be accomplished via mechanical trauma, either defect [117] or fracture [49] creation and bacterial contamination with or without application of a sclerosing agent or foreign body placement [115], or through placement of contaminated implants [49, 129]. Bone wax may be used to seal defect areas and prevent bacterial leakage and concomitant soft tissue infection [116]. A benefit of rabbits compared to smaller models such as mice and rats is the improved ability to model chronic osteomyelitis [138] and perform revision procedures such as debridement [23, 116], which improves the capabilities of modeling human osteomyelitis and therapy. Rabbits offer a distinct advantage in studying bone disease because full segmental defects of the radius can be created without the need to stabilize the bone using orthopedic implants.

Insights into Pathogenesis
Majority of reports into pathogenesis utilize well-characterized and reproducible rabbit models and are related to the capabilities of various bacterial species and strains [117, 121, 128] to induce osteomyelitis, as opposed to mechanistic work that more often is performed in murine and rat models. For example, Gahukamble et al. [117] describe an
investigation into the abilities of *Staphylococcus lugdunensis* (*S. lugdunensis*) and *Propionibacterium acnes* (*P. acnes*) to establish osteomyelitis in a model that was previously characterized with a strain of *S. aureus* isolated from an infected human hip prosthesis [139]. Results indicated that both organisms could induce osteomyelitis and described varying severity and clinical presentation. This work again emphasizes the importance of considering model development and bacterial strain selection during experimental design.

**Improvements in Diagnostic Capabilities**

Similar to murine and rodent models, there are studies aimed to improve the longitudinal monitoring of experimental osteomyelitis in rabbit models [21, 115] to improve utility of animal modeling and reduce required animal numbers. Odekerken et al., demonstrated that 18F-FDG micro-PET is a sensitive diagnostic tool for detecting early bone pathology, including early osteomyelitis [21], even in the presence of titanium implants [118]. This method of imaging could differentiate between aseptic and infected bone as early as three weeks post-operatively and post-infection. Authors suggest that 18F-FDG PET carries potential as an early detector of clinical osteomyelitis cases, which is further confirmed by a retrospective analysis of clinical osteomyelitis cases performed by Wenter et al. [140]. An important investigation geared toward improving available diagnostics was performed in a rabbit model of chronic osteomyelitis. In this study, the capability of PCR to return positive results was compared with traditional osteomyelitis diagnosis via radiographs and bacterial cultures of bone biopsies taken via different methods. Results indicated that PCR was a sensitive diagnostic tool and described techniques to determine species identification [23]. It deserves recognition that while PCR is a strong tool to detect low bacterial burdens or metabolically inactive bacteria that may not yield positive bacterial culture, PCR results will not provide antibiotic susceptibility data. The described PCR techniques are useful for experimental models and also offer utility for clinical cases.

**Investigations Into Therapeutic Strategies**

Rabbits are widely utilized to test therapeutic strategies for the clearance of bacterial osteomyelitis. Rabbits are hindgut fermenters, which means that they may process oral antibiotics differently than humans [64]. Nonetheless, rabbits have been widely utilized for evaluation of systemic and locally delivered antibiotic therapies [114, 123, 141]. Rabbits also are a useful modeling system for evaluation of antibacterial coatings upon implants and local drug delivery systems [114, 129], as demonstrated by the use of silver ion doped calcium phosphate beads [120]. There have also been investigations into alternative therapies for osteomyelitis, including the work performed by Kishor et al., investigating the use of bacteriophages to clear chronic osteomyelitis [142]. In this study, *S. aureus* specific phages were purified, characterized, and utilized as a therapeutic in a model of acute and chronic femoral osteomyelitis. High doses of phage cocktail were found to be effective to clear *S. aureus* infection. This work presents an intriguing consideration for specific therapy of bacterial osteomyelitis. Another interesting study investigated the use of locally applied ozonated oxygen in a rabbit femoral model. While this treatment did not
eliminate osteomyelitis, it did seem to lessen the clinical and radiographic markers of disease [122].

**Conclusion**

Rabbits fill a unique niche in in vivo osteomyelitis research. They are often utilized when the research goal involves assessment of orthopedic hardware or locally applied therapeutics and a small animal is needed, whether that need is dictated by animal housing limitations or by stage of research development. Rabbits provide a more relevant size to evaluate some human orthopedic implants, as well as an appropriate size to be maintained long-term so that revision procedures can be performed. Rabbits also provide a more similar immune system and long bone density to humans than mice and rats provide [143, 144]. Despite these benefits, rabbit models are accompanied by more complex challenges including respiratory depression under anesthesia, hindgut fermentation, which impacts the ability to assess oral antibiotic therapies, and variation in bone healing response of young rabbits compared to humans. Most rabbit modeling should be performed in mature rabbits to maximize translation of results to clinical patients.

**Large Animal Models**

**Pig Models**

**Model Development**

Pigs are not as widely utilized to model bacterial osteomyelitis, but the models that are available are effective, well-characterized, and have seen logical progression. Studies may utilize either mini-pigs or commercial pigs. Perhaps the most widely utilized model of porcine osteomyelitis is a hematogenous model [56, 145–149]. Alternative models include mandibular osteomyelitis [46, 150], tibial implant-related osteomyelitis [151–154], and traumatic tibial osteomyelitis [155]. When the hematogenous model of osteomyelitis was initially introduced, an inoculum of *S. aureus* (S54F9) was administered IV through a lateral ear vein without any additional trauma. This IV inoculation resulted in acute, suppurative pneumonic and osteomyelitic lesions. Lesions of osteomyelitis were found primarily in the long bones, but also in the costochondral junctions of ribs [147]. This model has been modified and is most frequently used by administering bacterial inoculums into the femoral artery [145, 146, 148, 149]. Femoral artery inoculation is reliable in inducing osteomyelitis localized to the injected limb. This technique may produce concurrent soft tissue infections, injection site abscesses, and the degree of disease during the study may be variable [148, 156]. However, this remains a strong technique for modeling acute hematogenous (juvenile) osteomyelitis.

**Insights Into Pathogenesis**

Pigs have not been utilized as widely as mice and rats to investigate pathogenesis of osteomyelitis, but there are a few interesting reports. One such study was carried out in a hematogenous model of osteomyelitis to determine the infection potential and disease characterization of three different strains of *S. aureus* [56]. This work compared the typically utilized strain of porcine *S. aureus* (SF549) with two human strains of *S. aureus*
(UAMS-1 and NCTC-8325-4). Results indicated that UAMS-1 and NCTC-8325-4 were less successful in establishing osteomyelitis than the porcine specific strain. Authors hypothesize that this may be due to increased host specificity, in contrast to rodent models, and that inoculation dose may play a role, which again brings attention to the importance of model and bacterial strain selection during experimental design. Additionally, an interesting discovery of biofilm within bone lesions shortly after infection was made and raises the concern that biofilms may form quite early on in disease. Jødal et al. investigated blood perfusion using $^{[15O]}$water PET, and confirmed their hypothesis that blood perfusion would be increased in osteomyelitis-diseased bone as compared to healthy bone. While blood perfusion was increased in diseased bone as compared to healthy bone, blood perfusion was four-fold greater in areas of soft tissue infection than diseased bone [156].

**Improvements in Diagnostic Capabilities**

Afzelius et al. [149, 157] have made multiple investigations involving ideal tracing agents for diagnosing osteomyelitis. They investigated the use of more specific radiotracers, including: $^{68}$Ga-labeled DOTA-K-A9, DOTA-GSGK-A11, $^{[18F]}$NaF, $^{68}$GaGa Ubiquicidin, and $^{68}$GaGa-DOTA-Siglec-9, and compared them to the use of $^{[18F]}$FDG. This study demonstrated no accumulation of the more specific radiotracers, but positive accumulation of $^{[18F]}$FDG [149]. Investigators also compared $^{[99mTc]}$Interleukin-8 (IL-8) scintigraphy with $^{[18F]}$FDG PET/CT in a hematogenous porcine model of osteomyelitis and found that $^{[99mTc]}$IL-8 was simple to prepare and use, and that it was capable of detecting 70% of lesions compared with 100% sensitivity of $^{[18F]}$FDG PET/CT. This makes $^{[99mTc]}$IL-8 scintigraphy a promising candidate for further investigation for use in children, to decrease the radiation exposure, as compared to utilizing $^{[18F]}$FDG PET/CT [157]. Another interesting study was performed by Lüthje et al., who investigated the regulation of various acute phase proteins during osteomyelitis and found a significant pro-inflammatory local response to osteomyelitis, with limited systemic response. These findings confirm that osteomyelitis remains challenging to diagnose based on systemic findings and adds to the understanding that local investigation is necessary [153].

**Investigations Into Therapeutic Strategies**

Most porcine studies thus far have been accomplishing model development, pharmacokinetic work [152] and diagnostic methods. There is even one investigation into bone regeneration techniques in the face of osteomyelitis [46]. Hill et al. [155] completed a study utilizing tibial implant-associated osteomyelitis and found that they could prevent osteomyelitis by administering combination antibiotic therapy every 6h for 7 days. Jensen et al. [154] comment that pigs provide an ideal model for investigation into implant surface coatings, medical and surgical treatment regimes, and vaccination against *S. aureus*.

**Conclusion**

Pigs, particularly mini-pigs, offer many benefits, including size that is appropriate for complex or multi-stage procedures and for assessments of orthopedic hardware for human
use. Porcine bone possesses similar fracture stress to human bone [158], hematogenous modeling creates a very similar situation to juvenile hematogenous osteomyelitis, and the gastrointestinal system of pigs is appropriate to receive oral antibiotics. There are many challenges when using pig models, including rapid growth and excessive mature body weight when utilizing commercial pigs [159], shorter long bones than found in people [154], the greater expense associated with a large animal model, variation in degree of disease manifestation, as well as a generally fractious demeanor. Porcine models are not currently as widely utilized as small animal models of osteomyelitis but provide an ideal model for the study of hematogenous osteomyelitis, offer great capabilities into investigation of imaging techniques, and are an area of interest for further development in the modeling of osteomyelitis. In general, commercial pigs are suitable for proof of concept and model development work, as they are less expensive than mini-pigs, but for longer-term studies and more appropriate translational work, mini-pigs should be utilized.

**Sheep Models**

**Model Development**

Kaarsemaker et al. [160] initiated development of ovine models of osteomyelitis via creation of a tibial defect and subsequent bacterial inoculum injection into the medullary cavity of adult sheep. This study provided valuable information, including the ability to establish osteomyelitis in sheep and also the requirement for peri-operative systemic antibiotics to lessen the risk of fatal sepsis. Since then, a variety of long bone models have been developed, focused on the tibia [161, 162] or the femur [163], and often involving hardware infected with biofilm or planktonic bacteria [164] with or without revision procedures [162, 165]. There remain a variety of techniques of creating bone injury, from unicortical defects and medullary canal inoculation [165] to osteotomies stabilized with experimental hardware [166]. Recently, Moriarty et al. [162] established a model to replicate a failed two-stage revision procedure utilizing a MRSA infected intramedullary nail. This will likely be a valuable model to evaluate therapeutic strategies moving forward.

**Investigations Into Therapeutic Strategies**

Most investigations into therapeutics in ovine models have been centered upon experimental implants, systemic or local antibiotic therapies, and the ability to replicate the multi-stage revision procedures utilized in human medicine. There have been multiple investigations into local drug delivery devices to clear osteomyelitis. Boot et al. performed a multi-stage revision procedure and compared an injectable hydrogel impregnated with gentamicin and vancomycin to an antibiotic-loaded bone cement impregnated with gentamicin and vancomycin. Investigators were able to clear significantly more cases of osteomyelitis in the experimental hydrogel group, compared to the bone cement group, thereby presenting this material as a promising candidate for further exploration [165]. Stewart et al. investigated another concept in local drug delivery by creating a vancomycin-modified titanium plate that demonstrated decreased clinical signs of infection, prevented biofilm formation and promoted bone healing in an infected tibial osteotomy model [166].
Conclusion
Currently, sheep are most often utilized for investigations into therapeutics utilizing long bone models. As such, sections regarding pathogenesis and diagnostic innovations were not included. Regardless, sheep are a valuable animal resource for the modeling of bacterial osteomyelitis, particularly focused on long bones. Sheep provide an ideal long bone size to perform complex procedures, replicate the treatment strategies utilized in clinical cases such as multiple revision procedures, and assess orthopedic hardware and devices for human use. Many characteristics of ovine bone are similar to that of humans, including torsional stiffness and osteogenesis [64], which adds to the strength of ovine modeling. Challenges associated with ovine modeling include the risk of sepsis, which may require peri-operative antibiotics, as well as the cost of housing and maintaining a large animal.

Goat Models
Model Development
Most caprine models of osteomyelitis utilize the tibia, although models have variable approaches. Salgado et al. described a unicortical tibial defect with concurrent application of a sclerosing agent. *Staphylococcus aureus* was inoculated into the medullary canal and the defect was sealed with bone wax. In this model, goats received a perioperative dose of IV antibiotics. Induction of osteomyelitis was successful and no goats suffered from fatal sepsis [167]. In an adaptation of this model, the sclerosing agent and perioperative antibiotics were omitted, and osteomyelitis was successfully induced, again with no reported sepsis [168]. Other tibial models include the internal fixation of a tibial osteotomy [169] and percutaneous pin placement throughout the tibia [170]. Through these investigations, researchers have also proposed histology scoring systems, to aid in the evaluation of model development [169].

Investigations Into Therapeutic Strategies
Similar to sheep, goats serve as viable translational models for investigations into therapeutic strategies. Wenke et al. utilized a similar model to that of Salgado et al. to investigate the efficacy of tobramycin-loaded calcium sulfate pellets compared to the efficacy of tobramycin-loaded antibiotic beads to treat bacterial osteomyelitis. Calcium sulfate and bone cement formulations loaded with tobramycin performed well, raising interest into the use of calcium sulfates for local drug delivery, as they do not require an additional procedure for removal [168]. Tran et al. [169] investigated a silver-based antibacterial coating on intramedullary nails. In an experiment utilizing two goats, the goat that received the experimental implant displayed less severe signs of osteomyelitis than the control goat. An interesting experiment was performed to investigate the utility of a directly applied electric current to eliminate osteomyelitis over the course of 3 weeks. Authors found that electric currents were able to prevent signs of infection and suggest that this would be effective in clinical situations [170]. Salgado et al. also reported an investigation of muscle vs. non-muscle flaps for reconstruction of defects and effective clearance of osteomyelitis. This study was designed as a result of discrepancies
in the literature, with some reports of muscle flaps being superior and vice versa. This study found no difference between muscle and non-muscle flaps and re-emphasized that the most critical factor in treatment of bacterial osteomyelitis is thorough debridement [38].

**Conclusion**
Similar to sheep, goats possess great utility in modeling bacterial osteomyelitis, and this utility lies primarily within the size and composition of the caprine long bones, specifically the tibia. Long bone size and composition makes goats ideal for complex procedures and multi-stage surgeries. Goats provide an excellent model for assessment of orthopedic hardware intended for human use, as well as examination of local drug delivery devices and experimental coatings. Goats have not suffered from the reported sepsis that affected sheep when receiving intramedullary bacterial inoculation, which may aid researchers when selecting either sheep or goats as a model. Similar to any large animal model, goats are accompanied by greater costs than small animal models. As the majority of caprine modeling has been performed to either establish a reliable model or assess treatment options, the sections for pathogenesis and diagnostic investigations were omitted.

**Dog Models**

**Model Development**
Canine models have been used in the past to model osteomyelitis, although today they are not widely utilized. Similar to caprine and ovine models, canine models have primarily utilized long bones, specifically the tibia [53, 171, 172] and the femur [47, 173, 174], although a vertebral model has also been described [175]. Models vary in approach. Deyesine et al. described an injection of bacterial inoculum into the tibial nutrient artery without any additional trauma. This approach was effective in establishing osteomyelitis, but also resulted in the loss of three dogs from septicemia [53]. Most other models report bone trauma and bacterial inoculation of the medullary canal, whether that is by direct inoculation or placement of an infected implant [47, 171, 174]. Khodaparast et al. had success in establishing osteomyelitis via application of a penetrating captive bolt device to the tibia of dogs to create an open fracture. This approach was selected in order to mimic traumatic osteomyelitis. This model involved the placement of microdialysis probes for sample collection, which is a valuable tool [172] when investigating the dynamics of local environments, whether that is physiologic dynamics or drug delivery profiles.

**Investigations Into Pathogenesis**
As described above, Khodaparast et al. [172] established a tibial fracture model of canine osteomyelitis and placed microdialysis probes with the goal of exploring the role of vascular endothelial growth factor (VEGF) as a rate-limiting step in wound healing. This was investigated by measuring VEGF mRNA levels in response to *S. aureus* osteomyelitis and *S. aureus* osteomyelitis treated with a rotational gastrocnemius muscle flap. The muscle flap was investigated because wound healing is accelerated in the presence of well-vascularized tissue. VEGF mRNA levels were found to be greater in the animals with
osteomyelitis that received the rotational muscle flap as compared to those who did not. This finding suggests that type of surgical closure impacts specific biological signals and cellular pathways, and may add strength to the recommendation for utilizing muscle flaps for improved wound healing in reconstructive surgeries. Another investigation into pathogenesis was performed by Chen et al. [175] who aimed to investigate the presence, type, and origin of bacteria adjacent to metal implants utilized in the surgical management of pyogenic vertebral osteomyelitis. Investigators found that bacteria were retrieved not only from metal implants, but also from surrounding bone, despite the lack of radiographic signs of infection. These findings suggest that metallic implants are not necessarily the source of persistent or recurrent bacterial infection in vertebral osteomyelitis.

Conclusion
Dogs provide strong models for long bone osteomyelitis. Benefits include appropriate size to perform complex and multi-stage procedures, bone composition and density that is most similar to humans out of the available species [158], temperament that is amenable to handling, as well as well-characterized anesthetic and imaging protocols. Despite these strengths, canine models are no longer frequently utilized for osteomyelitis research. Osteomyelitis research is terminal, and ethical concerns are raised when considering these companion animals as research models. Therefore, despite the provided benefits, it is unlikely that dogs will have a resurgence in popularity for osteomyelitis modeling.

Conclusions and Future Directions
Through the currently available reports of advancements in the management and understanding of osteomyelitis that animal models have facilitated, it is clear that animal models are vital in osteomyelitis research. With the plethora of available species and approaches to model bacterial osteomyelitis, it is also clear that each species provides specific strengths and certain shortcomings, as is highlighted in this review. Based on current information, we suggest an approach where proof of concept work is performed in small mammal models, either a mouse or a rat model. Advanced pathogenesis investigations can also be carried out in small mammal models, either a mouse, rat, or rabbit model. Complex treatment strategies, whether local or systemic, are best suited for large animal models, either mini-pigs, sheep, or goats, to mimic the human response as closely as possible. Improvements to diagnostic procedures may be performed in a variety of models; initial investigations, especially into novel imaging techniques, are best suited for rodent models. Ideally, imaging techniques would be validated in large animal models before preclinical testing. Regardless of the specific indication and utility, the knowledge we gain from animal models of osteomyelitis is an essential asset to the understanding, diagnosis and treatment of bacterial osteomyelitis, and animal modeling is a crucial step toward improving the lives of patients suffering from this life-altering disease.
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CHAPTER III: COMPARISON OF IN VITRO METHODS FOR ASSESSMENT OF DRUG ELUTION FROM A COLLAGEN-BASED SCAFFOLD USED AS A DRUG DELIVERY DEVICE
Abstract

In vitro drug elution experiments are commonly performed when evaluating fitness of drug delivery devices for in vivo use. Evaluation of drug elution characteristics spans many drug delivery applications including local delivery of antimicrobials and chemotherapeutics; and is of particular interest for prevention and treatment of orthopedic infections. Despite widespread utility, there is little agreement in methodology to perform such studies, and there are recognized limitations in published works. We evaluated three of the most commonly reported in vitro drug elution methods. We utilized a commercially available collagen matrix (Fibro-Gide®, Geistlich) and an antibiotic that is widely used for local antimicrobial therapy (gentamicin). The protocols used were: 1. complete replacement of media and washing of device, 2. complete replacement of media without washing or 3. partial replacement of media. Results show statistically significant differences in elution characteristics among the three methods utilizing this delivery vehicle and drug. These results may provide the framework for moving towards more consistent methodology for in vitro elution experiments and address certain acknowledged limitations in the literature.

Introduction

Local drug delivery has become a common tool in clinical practice as a way for physicians to deliver medications to patients while minimizing risks of systemic side effects and toxicity that can occur with systemic administration, circulation, and potential accumulation of drugs [1-3]. Local drug delivery encompasses many applications, including delivery of antimicrobials, growth factors, chemotherapeutic agents or anesthetic compounds [1,2,4-6]. With a wide range of applications, the benefits of local drug delivery are considerable, ranging from improving patient’s post-operative comfort [5], increasing drug penetration to target sites to improve therapeutic success [7,8], and delivering high local doses of chemotherapy to shrink tumors while avoiding the significant morbidity associated with systemic chemotherapy [4,9]. One benefit in particular is for the treatment of implant-associated bacterial infection [10,11].

Implantable medical devices are immensely important in today’s healthcare system. There is an extensive array of devices, from urinary catheters to prosthetic heart valves and artificial joints. These devices improve the quality of life of tens of millions of patients around the world [10,12,13]. While the lifespans and capabilities of these devices have improved over the years, the risks of bacterial colonization, biofilm formation and persistent or recurrent bacterial infection remain and constitute a leading cause of device failure [10,14,15]. Risk of infection is multifactorial and combines systemic host factors, local tissue environments, and device characteristics [11]. Treatment of device-associated bacterial infection traditionally involves systemic antimicrobial therapy, local debridement of affected tissues, and often requires device removal and replacement [10,16]. The use of local drug delivery devices can be used to strengthen the treatment protocol or can be placed initially, in cases where bacterial contamination is known or
suspected [10], with the goals of clearing bacterial contamination and preventing persistent bacterial colonization.

Local drug delivery devices serve a broad range of applications. To accommodate these functions, there are many different forms for these devices; including but not limited to: poly(methyl methacrylate) (PMMA) cement [10,11], calcium sulfates [3,4], natural and synthetic polymers such as collagen, chitosan, fibrin, polyurethanes, and poly(lactic-co-glycolic acid) (PLGA) [17], and metal alloys [18]. Within this broad range of materials and applications, there are multiple consistencies in the development of experimental devices for in vivo use. Prior to moving an experimental drug delivery device to an in vivo investigation, the device’s material characteristics should be thoroughly characterized [19]. Devices will typically be assessed for biocompatibility, to reduce the risk of a material-induced foreign body response, which is non-conducive to healing [20].

Devices will be loaded with the drug they are intended to deliver, to assess in vitro drug elution characteristics. The goal of in vitro elution testing is to gain a sense of how the chosen drug, drug loading strategy, and device characteristics will contribute to release kinetics [15,21]. These experiments may aid in determining expected bioactivity of the released drug throughout the experimental period [22-24]. These experiments do not perfectly simulate an in vivo environment, but possess substantial utility in elucidating effects of material formation [25,26], porosity [27-29], and drug variation [30] on elution profile. Such experiments are useful as predictors of likely in vivo behavior of local drug delivery devices and can guide the decision to pursue in vivo investigations of experimental devices.

While in vitro drug elution experiments are frequently performed and possess utility in determining device fitness for in vivo drug delivery, currently there is no uniform approach to performing the experiments. This leads to difficulty in interpreting methodology and results, and limitations in comparing devices and drugs. Our aim was to investigate the effects of methodology on drug elution characteristics utilizing one device and drug combination. We utilized a commercially available collagen matrix (Fibro-Gide®, Geistlich Pharma AG, Wolhusen, Switzerland) and gentamicin, an antibiotic frequently used for local or systemic treatment of bacterial infections [3,31-34]. We utilized three of the most commonly reported protocols which involved either: 1. complete replacement of media and washing of device [35-37], 2. complete replacement of media without washing [7,26,38] or 3. partial replacement of media [4,39,40]. We hypothesized that the protocol utilized would have a significant effect on the elution characteristics, including the rate of release and amount of recovered gentamicin.

**Materials and Methods**

**Material and Drug Loading**
Commercially available collagen matrix (Fibro-Gide®, Geistlich Pharma AG, Wolhusen, Switzerland) was utilized. Fibro-Gide® is provided as a sterilized block that varies in length and width but has a fixed height of 6mm. For the purpose of these experiments,
Fibro-Gide® (15x20x6mm) was handled in sterile conditions and sectioned into 5x6mm cylinders utilizing a sterile 5mm biopsy punch. Experimental cylinders (5x6mm) were created from the material and hydrophilicity was determined by calculating the equilibrium water content (EWC), as listed in Equation I.

Equation I: \[ \text{EWC} (\%) = \left( \frac{\text{Weight hydrated sample} - \text{weight dry sample}}{\text{Weight hydrated sample}} \right) \times 100 \]

Dry weights of cylinders (n=3) were obtained, the cylinders were loaded with phosphate buffered saline (PBS) and allowed to soak for 24 hours, at which point the wet weight was obtained. This process determined the amount of drug loaded into experimental cylinders.

Cylinders were loaded by applying the pre-determined volume of 100μl gentamicin sulfate solution at a concentration of either 100mg/mL or 50mg/mL. The 50mg/mL concentration was created by diluting gentamicin (100mg/mL) 1:1 with sterile water (for injection). Total amount of gentamicin loaded into the cylinders was either 10mg or 5mg, based on concentration of the solution. Once gentamicin was applied, the cylinders were allowed to incubate at room temperature for 12 hours and were then moved to new wells to begin elution sampling protocols.

**Sampling Protocols**

After loading, all samples were individually incubated in PBS (2mL/well) at 37°C. At pre-determined timepoints, (3, 24, and 48 hours and days: 3, 4, 6, 8, 10, 12 and 14) samples were removed from incubation for eluant collection. Sample collection occurred under sterile conditions with one of three protocols (n=3/dose/protocol). Eluant samples were saved in cryovials (in duplicates) at -80°C for analysis.

1. Washing protocol: Indwelling media removed from wells and saved. Samples washed five times with 2mL of sterile water (total = 10mL/well). Fresh PBS replaced to each well (2mL/well).

2. Complete turnover protocol: Indwelling media removed from wells and saved. Fresh PBS replaced to each well (2mL/well).

3. Partial turnover protocol: Portion of indwelling media (total = 200μL/well) removed from wells and saved. Equivalent volume of fresh PBS replaced to each well (200μL/well), resulting in a 10% volume dilution of remaining eluant media.

**Drug Concentration**

The concentration of gentamicin in eluant samples from gentamicin impregnated Fibro-Gide® was determined using ultra high-pressure liquid chromatography (UHPLC) with mass spectrometry detection after dilution of the PBS samples with an internal standard solution (Analytical Chemistry Service, College of Veterinary Medicine, Iowa State University, Ames, IA). The UHPLC consisted of an UltiMate 3000 Pump, Column...
Compartment and Autosampler (Thermo Scientific, San Jose, CA, USA) coupled to an Orbitrap mass spectrometer (Q Exactive Focus, Thermo Scientific, San Jose, CA, USA). The analysis was performed by hydrophilic interaction chromatography (HILIC) with a ZIC HILIC column, 150 mm x 2.1 mm, 5 µm particles (Merck KGaA, Darmstadt, Germany through EMD Millipore, MA, USA). Gentamicin consists of a mixture of four components: Gentamicin C1 (0.767 fraction of total; gentamicin C2/2a 0.175 fraction; gentamicin C1a 0.058 fraction). Calibration curves for gentamicin C1 and gentamicin C2/2a exhibited a correlation coefficient ($r^2$) exceeding 0.995 across the concentration range. One of three calibration curves for gentamicin C1a had a correlation coefficient ($r^2$) in the 0.985 range, while others exhibited $r^2$ exceeding 0.991. The limit of quantitation (LOQ) was 0.04 µg/mL for gentamicin C1 and 0.01 µg/mL for the other two gentamicin components. The limit of detection (LOD) was 0.01 µg/mL for gentamicin C1 and 0.005 µg/mL for the other two gentamicin components.

**Statistical Analysis**

The effects of method, dose and time on response variable total gentamicin were examined using mixed model analysis for repeated measures. Ranked transformation was applied when diagnostic analysis on residuals exhibited violation of normality and equal variance assumptions using Shapiro–Wilk test and Levene’s test. Post hoc multiple comparisons were performed with Tukey’s adjustment. Statistical significance was identified at the level of 0.05. Analyses were conducted in SAS 9.4 TS1M4 (SAS institute Inc., Cary, NC).

**Results**

Samples loaded with 10mg of gentamicin that underwent the washing (Wash) protocol had an elution of 6.88 ± 0.49mg (68.8 ± 0.49% total drug recovery) during the 14-day study period, with a peak reported elution of 6.84 ± 0.53mg at three hours. This peak elution is equivalent to eluting approximately 68% of loaded gentamicin within the first three hours. Samples loaded with 10mg of gentamicin that underwent the complete turnover (CT) protocol (n=3), had an elution of 9.97 ± 1.5mg (99.7 ± 1.5% total drug recovery) during the 14-day study period, with a peak reported elution of 9.14 ± 0.36mg at three hours. This peak elution is equivalent to eluting 91.4 ± 0.36% of loaded gentamicin within the first three hours. Samples loaded with 10mg of gentamicin that underwent the partial turnover (PT) protocol (n=3), had a peak reported elution of 9.5 ± 2.8mg at three hours. This peak elution is equivalent to eluting 95 ± 28% of the loaded gentamicin within the first three hours. The as-measured concentration of gentamicin in the eluant for the PT method represents an accumulation of gentamicin in the media over time and must be corrected for change in volume and new drug eluted per sampling interval (Table 3.1). PT protocol sample analysis was adjusted to account for repeated sampling and dilution throughout the study period and additional drug eluted during each sampling period (partial turnover calculated and adjusted for volume; PT VA). When adjusted for repeated sampling of accumulated gentamicin, the reported elution
(percentage of gentamicin recovered throughout study period) was 63.2 ± 10.9% for the PT VA (Table 3.1).

Samples loaded with 5mg of gentamicin that underwent the Wash protocol had an elution of 3.68 ± 0.19mg (73.6 ± 3.8% total drug recovery) during the 14-day study period, with a peak reported elution of 3.62 ± 0.09mg at three hours. This peak elution is equivalent to eluting 72.4 ± 1.8% of loaded gentamicin within the first three hours. Samples loaded with 5mg of gentamicin that underwent the CT protocol (n=3), had an elution of 4.95 ± 0.57mg (99 ± 11.4% total drug recovery) during the 14-day study period, with a peak reported elution of 4.34 ± 0.31mg at three hours. This peak elution is equivalent to eluting 86.8 ± 6.2% of loaded gentamicin within the first three hours. Samples loaded with 5mg of gentamicin that underwent the PT protocol (n=3), had a peak reported elution of 3.69 ± 0.09mg at three hours. This peak elution is equivalent to eluting 73.8 ± 1.8% of loaded gentamicin within the first three hours. PT protocol sample analysis was adjusted to account for repeated sampling and dilution throughout the study period and additional drug eluted during each sampling period (partial turnover calculated and adjusted for volume; PT VA). When adjusted for repeated sampling of accumulated gentamicin, the reported elution (percentage of gentamicin recovered throughout study period) was 71.4 ± 0.8% for the PT VA (Table 3.1).

There were significant differences found between doses of gentamicin loaded, between sampling protocols, and as a factor of time. The PT VA protocol resulted in elution of similar amounts of drug compared with the wash protocol. The CT protocol eluted statistically significantly greater concentrations of gentamicin (regardless of loaded dose) than the Wash protocol (p value=0.017). Samples loaded with 10mg of gentamicin eluted statistically more gentamicin than samples loaded with 5mg (p value <0.0001). For the CT and Wash protocols, there were significant differences found between gentamicin concentrations at consecutive timepoints; CT protocol eluted statistically greater amounts of gentamicin at 3 hours compared to 24 hours (p <0.0001), as well as at day 4 compared to day 6 (p <0.0001), and day 12 compared to day 14 (p = 0.01). Throughout the Wash protocol, statistically greater amounts of gentamicin were found at 3 hours compared to 24 hours (p < 0.0001) and remaining consecutive timepoints did not differ significantly. Within the PT VA protocol, there were no statistically significant differences in gentamicin concentrations between consecutive timepoints. Each protocol and dose with the exception of the PT VA protocol demonstrated similar elution profiles, characterized by an initial burst release of the majority of drug within a 3-hour period, followed by a sustained, low-level drug release. PT protocol without adjustments for drug accumulation and repeated sampling through time demonstrated a gradual, sustained elution curve with little change over time (Figure 3.1). PT protocol when adjusted for drug accumulation and sampling over time (PT VA) demonstrated an initial burst release, followed by sustained, low-level release (Figure 3.2).
Discussion

Results of this method comparison demonstrated significant differences between protocols and also demonstrated differences in the generated elution curves. These results were anticipated, and are relevant within the realm of local drug delivery devices. This study confirms that varying elution protocols within an otherwise controlled experimental design may result in significantly different elution profiles and amounts of recovered drug. It can be extrapolated from these results that the sampling protocol has significant effects on the concentration of drug in the eluant, amount of drug recovered, and the elution curve generated from the data. An important portion of the above results is the partial turnover (PT) and adjusted partial turnover (PT VA) protocol results. Without adjusting the PT protocol for volume of media removed at each sampling point and calculating the amount of additional drug eluted during the associated sampling period, the percentage gentamicin recovery would erroneously appear to be much greater than the amount of drug loaded. This can be attributed to the nature of the PT protocol. While it demonstrates the theoretical ideal drug elution profile, which is a gradual and sustained curve [19], the protocol removes so little of the media that surrounds the drug delivery device that the results cannot be interpreted with respect to the amount of drug concentration measured at each time point. Without fully removing the surrounding media, the amount of new drug eluted per sampling period can only be estimated based on changes in concentration. This can lead to erroneous interpretation of results when there is low-level drug release, as variations exist in the precision of drug concentration analysis. This elution protocol would likely only be relevant to model a device utilized to elute drug in a confined space or severely compromised tissue where eluted drug could not be eliminated.

These findings, while not unexpected, are important, as they suggest that when performing in vitro drug elution experiments, it would be valuable to closely consider the protocol to be used in context with the literature relative to the most commonly utilized protocols in the investigator’s specific area of interest, and then to also consider the goal of the elution experiment. Most often, investigators consider the goal of in vitro elution experiments to be the generation of a predictable in vivo elution profile. This goal is often unattainable, as the in vivo environment is difficult to simulate, and even a well-designed in vitro environment will differ in certain aspects. When the everchanging fluid dynamics of the body are considered, it can be reasonably argued that a washing protocol or a continuous flow protocol [41] would most accurately simulate an in vivo environment. However, most recent reports of in vitro elution experiments for local drug delivery devices have focused on either a partial or complete turnover protocol [4,26,42]. This may reflect the desire to model areas of tissue injury when vascular and lymphatic supplies, and therefore fluid dynamics, have been altered and fluid may be accumulating. Such experimental protocols may demonstrate more favorable elution curves, as less drug will be lost during the washing process. In such protocols, it is possible that greater drug concentrations may be present throughout the study period. If the same device, drug, and dose combinations were moved to an in vivo environment, it is likely that a far lower concentration of drug would be recovered.
It is important to acknowledge that this study focused on one device and drug combination and one loading mechanism; and it is important to note the loading mechanism. The collagen matrix cylinders in this experiment were loaded by impregnation, which facilitates drug incorporation by formation of weak bonds such as Van der Waals forces. This simulated an intra-operative technique for adding antimicrobial drugs to tissue scaffolds at the time of surgery. Under the conditions of this experiment, and using a collagen scaffold, there was no significant benefit to loading a greater amount of drug relative to the elution curve as the vast majority of drug was eluted within a short period of time. Local drug delivery devices that are loaded via impregnation typically exhibit an initial burst release of drug, followed by lower-level release, for variable amounts of time. This is in contrast to materials that are loaded via specific molecular-linkages or stimuli-responsive systems, which may exhibit more finely-tunable controlled-release profiles [17,43].

**Conclusion and Future Directions**

This study demonstrates that sampling protocol may have significant effects on *in vitro* drug elution profiles. This is an expected but relevant phenomenon within *in vitro* drug elution experiments. When investigating the *in vitro* drug elution characteristics of local drug delivery devices, the experimental design is important, and the sampling parameters should be selected with careful consideration to the experimental question. As the overall goal of determining the *in vitro* drug elution profile of local drug delivery devices is to determine suitability for *in vivo* usage and predict *in vivo* drug elution profiles, it would be interesting to compare the *in vitro* profiles generated by these devices, drug and dose combinations with *in vivo* profiles generated by the same device, drug, and dose combinations. This may elucidate an experimental design that is closest to simulating an *in vivo* environment and aid in experimental set-up. It may also be of value to pursue a similar methodology comparison utilizing media within a range of pHs. PBS (pH 7.4) is the most commonly utilized media for *in vitro* elution experiments [4,38,44], but *in vivo* environments may have pH variation, particularly in areas of tissue damage or implant-associated infection, where the pH may be more acidic.
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APPENDIX III

Table 3.1. Percent of Gentamicin Recovery

<table>
<thead>
<tr>
<th>Protocol, 5mg Dose</th>
<th>Gentamicin Recovered (%)</th>
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<tbody>
<tr>
<td>Washing (Wash)</td>
<td>73.6 ± 3.8</td>
</tr>
<tr>
<td>Complete Turnover (CT)</td>
<td>99 ± 11.4</td>
</tr>
<tr>
<td>PT Volume Adjustment (PT VA)</td>
<td>71.4 ± 0.8</td>
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</table>

<table>
<thead>
<tr>
<th>Protocol, 10mg Dose</th>
<th>Gentamicin Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washing (Wash)</td>
<td>68.8 ± 0.49</td>
</tr>
<tr>
<td>Complete Turnover (CT)</td>
<td>99.7 ± 1.5</td>
</tr>
<tr>
<td>PT Volume Adjustment (PT VA)</td>
<td>63.20 ± 10.9</td>
</tr>
</tbody>
</table>

Complete turnover protocols recovered the most gentamicin regardless of dose (p < 0.0001). For 5mg dose, CT protocol recovered statistically greater concentrations of gentamicin compared to wash and PT VA protocols (p = 0.0042, and p < 0.0001, respectively). For 10mg dose, CT protocol recovered statistically greater concentrations of gentamicin compared to wash and PT VA protocols (p = 0.01 and p <0.0001, respectively).
Figure 3.1. Gentamicin elution over time (A): Median eluted gentamicin generated by each method with a loaded dose of 5mg gentamicin/device. (B): Log transformation applied to elution curves generated each method with a loaded dose of 5mg gentamicin/device. (C): Median eluted gentamicin generated by each method with a loaded dose of 10mg gentamicin/device. (D): Log transformation applied to elution curves generated each method with a loaded dose of 10mg gentamicin/device. Due to wide concentration range, log transformation facilitates most clear data visualization [45]. Wash and CT elution curves demonstrate anticipated initial burst release of drug followed by sustained lower-level drug release. PT VA protocols demonstrate gradual, sustained elution curves, which is characteristic of a desirable elution profile [19]. Protocols represented as: PT VA= partial turnover adjusted for volume and accumulated gentamicin over time, Wash = washing, CT = complete turnover.

Figure 3.2. Gentamicin elution over time generated by PT protocol. A and B: Elution curves of median gentamicin eluted under the PT protocol with no adjustments (PT), adjusted for volume and accumulated gentamicin over time (PT VA) and adjusted to reflect removal of accumulated gentamicin “partial turnover calculated to reflect newly eluted drug” (PT ND). (A): devices loaded with 5mg gentamicin. (B): devices loaded with 10mg gentamicin. PT and PT VA demonstrate gradual, sustained elution over time compared to PT ND that demonstrates the characteristic burst release followed by sustained lower-level release.
CHAPTER IV: EFFECTS ON TISSUE INTEGRATION OF COLLAGEN SCAFFOLDS USED FOR LOCAL DELIVERY OF GENTAMICIN IN A RAT MANDIBLE DEFECT MODEL
Abstract

Surgical site infections (SSIs) are a common complication following orthopedic surgery. SSIs may occur secondary to traumatic or contaminated wounds or may result from invasive procedures. Development of biofilms are often associated with implanted materials used to stabilize injuries and facilitate healing. Regardless of the source, SSIs can be challenging to treat. This has led to the development of devices that act simultaneously as local antibiotic delivery vehicles and as scaffolds for tissue regeneration. The goal for the aforementioned devices is to increase local drug concentration in order to enhance bactericidal activity while reducing the risk of systemic side effects and toxicity from the administered drug. The aims of this study were to assess the effect of antibiotic loading of a collagen matrix on the tissue integration of the matrix using a rat mandibular defect model. We hypothesized that the collagen matrix could load and elute gentamicin, that the collagen matrix would be cytocompatible in vitro, and that the local delivery of a high dose of gentamicin via loaded collagen matrix would negatively impact the tissue-scaffold interface. Results indicate that the collagen matrix could load and elute the antimicrobial gentamicin, that it was cytocompatible in vitro with or without the presence of gentamicin, and found no significant impact on the tissue-scaffold interface when the device was loaded with a high dose of gentamicin.

Introduction

Surgical site infections (SSIs) are a common yet potentially devastating complication following surgery [1]. SSIs are most frequently caused by bacterial organisms, typically gram-positive Staphylococcal bacterial species, such as Staphylococcus aureus (S. aureus) [2-4], although gram-negative organisms such as Pseudomonas, Enterococcus, and Escherichia coli also occur [2,3]. Orthopedic surgery is accompanied by a significant risk of SSI, with an estimated 31,000-35,000 cases annually in the United States [2]. Infection risk is multifactorial. Risk is partially due to the nature of orthopedic surgery, as orthopedic procedures often require indwelling hardware to stabilize bones, repair fractures or replace joints [2]. Indwelling hardware is known to be at risk of microbial contamination and subsequent biofilm formation or chronic infection for the lifetime of the indwelling device [5]. Infection risk is also a result of the patient population and
presentation. Individuals requiring joint replacements may possess comorbidities increasing their likelihood of SSI, and presentation may involve trauma or contaminated injuries, also increasing the risk of bacterial infection [1,6].

SSIs can cause significant morbidity and mortality to the patient [3,7], particularly because treatment relies heavily upon systemic antimicrobial therapy and surgical revision procedures [8,9], which may leave patients suffering adverse side effects from systemic antibiotics [9] or with impaired function from tissue loss or implant removal during surgical revisions. Traditional treatment strategies often fall short of a cure, which leads to persistent bacterial infection. This may be due to antimicrobial resistant bacterial species, such as methicillin-resistant *S. aureus* (MRSA) [4], inadequate antimicrobial penetration, whether secondary to inadequate tissue penetration [9,10] or metabolically inactive bacteria safely sequestered in biofilms [2,9], as well as recurrent bacterial infection that can occur due to indwelling devices [11-13], which pose a risk of bacterial infection for the lifetime of the device. For these same reasons, SSIs also place a substantial burden on the healthcare system [3,7]. The estimated annual cost of managing SSIs is reported by the Centers of Disease Control (CDC) to be $3.3 billion [7] and significant SSIs are recognized to increase duration of hospitalization by an average of 9.7 days [7].

New strategies to overcome current limitations in treatment of SSI are needed, and locally implantable medical devices used to delivery antimicrobials may help to prevent bacterial colonization of tissues. Recently, interest has increased for use of scaffolds that can simultaneously aid in tissue regeneration and serve as local drug delivery devices [9,14,15]. Devices that are biocompatible and bioresorbable are of particular interest in order to reduce concerns of a foreign body response and eliminate the need for revision procedures to remove the implanted device [13,16]. An ideal device within this class is able to deliver sufficiently high concentrations of antimicrobials to the surrounding tissues to overcome the hurdle of inadequate tissue penetration and also to impede biofilm formation [16,17], all while utilizing a dose of antimicrobials that will not injure the surrounding tissues or impair tissue healing [15,18,19].

Collagen is used frequently in biomedical applications and is of extreme interest for use as a tissue regeneration and drug delivery device [20,21]. Collagen is one of the most abundant proteins in the body and is a major component of the extracellular matrix (ECM) [22]. The ECM serves to organize cells in 3D space and provide attachment points and environmental signals for tissue development. Functionally, natural collagen within the body provides extensive mechanical support. Collagen is also recognized to be involved in many other tissue functions, including tissue repair [21]. When utilizing collagen as a biomaterial, there are many variations to choose from, and it is necessary to process the collagen to ensure that it is safe and non-immunogenic to the recipient. Additionally, the collagen will most likely need to be modified in some way, such as cross-linked with elastin, to slow degradation rates and add elasticity to complement the stiffness provided by collagen [23]. One of the most common forms of collagen used in commercial biomaterial scaffolds is xenogenic collagen of porcine origin [24]. There are many variations of porcine collagen matrices developed for various indications, but the primary goals of collagen matrices are to provide: excellent biocompatibility, a highly porous structure to allow for tissue ingrowth and matrix incorporation, mechanical properties
similar to tissue of interest, and degradation properties that match the speed of tissue regeneration [23].

Within this work, our goal was to evaluate the utility of a commercially available collagen matrix, (Fibro-Gide®, Geistlich), typically used for soft-tissue regeneration, as a cytocompatible, therapeutically effective drug delivery device, with a special emphasis on effects of a high dose delivery of gentamicin on the tissue-scaffold interface. We hypothesized that loading tissue regeneration scaffolds with high doses of antibiotics, known to be cytotoxic, would result in decreased tissue integration of the scaffold. Our objectives of these experiments using the chosen collagen matrix were: (1) to assess loading and elution of gentamicin from the matrix, (2) to evaluate cytocompatibility, in vitro, of the matrices in the presence of antibiotics, and (3) to determine if loading with a high dose of the antibiotic within the device would negatively impact the tissue-scaffold interface and subsequent tissue integration, in vivo.

**Materials and Methods**

**Material Description**
Commercially available porcine collagen matrix (Geistlich Fibro-Gide®, Geistlich Pharma AG, Wolhusen, Switzerland) was utilized for in vitro and in vivo experiments. This material is described by the manufacturer as a porous, resorbable, volume-stable matrix composed of reconstituted, chemically cross-linked collagen that is intended for soft tissue augmentation procedures [25]. Fibro-Gide® has one porous layer that consists of 60-96% (w/w) porcine collagen (types I and III) and 4-40% (w/w) elastin. Average pore diameter is 92μm and the material possesses 93% volume porosity with interconnected pores [24]. Fibro-Gide is provided as a sterilized block that varies in length and width but has a fixed height of 6mm. For the purpose of these experiments, Fibro-Gide® (15x20x6mm) was handled in sterile conditions and sectioned into 5mm diameter x 6-mm height cylinders ($V = \pi r^2 h = \pi \cdot 2.5^2 \cdot 6 \approx 117.8$) utilizing a sterile 5mm biopsy punch. Scanning electron microscopy (SEM) images of Fibro-Gide® cylinders were obtained utilizing a Zeiss EVO MA15 scanning electron microscope (UT Institute for Advanced Material Manufacturing).

**Antimicrobial Selection**
Gentamicin sulfate solution (100mg/mL, VetOne, Boise, ID, USA) was utilized for these experiments. Gentamicin is an aminoglycoside antibiotic with bactericidal activity against a range of gram-negative bacterial species and some methicillin-susceptible *S. aureus* species [26]. This antibiotic was largely chosen due to the use of gentamicin to prevent and treat surgical site infections, whether administration is systemic or accomplished via a local drug delivery device [17,27-30]. Gentamicin also was selected for the well-characterized toxicity profile in rats [31,32], possession of cytotoxic behavior at high concentrations in vitro [15], as well as the highly concentrated drug formulation, which facilitated higher loading doses onto the Fibro-Gide® collagen matrix.
**In vitro Drug Loading and Elution**

**Hydrophilicity**

Hydrophilic properties of the material were determined by calculating the percent equilibrium water content (EWC) (equation 1). Phosphate buffered saline (PBS) was added in 10μL increments until the devices were saturated and wet weights were recorded.

Equation 1: \[ EWC (\%) = \frac{\text{Weight hydrated sample} - \text{weight dry sample}}{\text{Weight hydrated sample}} \times 100 \]

Based on the determined hydrophilic properties, the loading volume of gentamicin (100mg/mL) was calculated. The optimal loading volume calculated and utilized was 100μL/5mm diameter, 6mm height cylinder.

**Drug Loading**

Experimental Fibro-Gide® cylinders were loaded with either a high (10mg) or low (5mg) dose of gentamicin, (n=3 cylinders/dose). To load 10mg of gentamicin, 100μL of 100mg/mL gentamicin solution was applied to experimental cylinders (n=3) under sterile conditions. To ensure an equal volume across each cylinder, gentamicin (100mg/mL) was diluted 1:1 with sterile water for injection to create a 50mg/mL solution. 100μL of the resulting 50mg/mL solution was then applied to experimental cylinders (n=3) to load 5mg of gentamicin. Once loaded with gentamicin, cylinders were incubated at room temperature for four hours.

**Drug Elution Protocol**

Once loaded, cylinders were completely submerged in PBS (2 mL) and incubated in a water-jacketed incubator at 37°C to mimic physiologic temperature. Using the complete turnover protocol discussed in chapter III, supernatant was collected via complete media removal at pre-determined timepoints (3, 24, and 48 hours and on days: 3, 4, 6, 8, 10, 12 and 14). At each timepoint, 2mL of fresh PBS was replaced and devices were returned to incubation.

**Drug Concentration**

The concentration of gentamicin in eluent samples from gentamicin impregnated Fibro-Gide® was determined using ultra high-pressure liquid chromatography (UHPLC) with mass spectrometry detection after dilution of the PBS samples with an internal standard solution (Analytical Chemistry Services, College of Veterinary Medicine, Service, Iowa State University, Ames, IA). The UHPLC consisted of an UltiMate 3000 Pump, Column Compartment and Autosampler (Thermo Scientific, San Jose, CA, USA) coupled to an Orbitrap mass spectrometer (Q Exactive Focus, Thermo Scientific, San Jose, CA, USA). The analysis was performed by hydrophilic interaction chromatography (HILIC) with a ZIC HILIC column, 150 mm x 2.1 mm, 5 μm particles (Merck KGaA, Darmstadt, Germany through EMD Millipore, MA, USA). Gentamicin consists of a mixture of four components: Gentamicin C1 (0.767 fraction of total; gentamicin C2/2a 0.175 fraction; gentamicin C1a 0.058 fraction). Calibration curves for gentamicin C1 and gentamicin C2/2a exhibited a correlation coefficient (r2) exceeding 0.995 across the concentration
One of three calibration curves for gentamicin C1a had a correlation coefficient (r²) in the 0.985 range, while others exhibited r² exceeding 0.991. The limit of quantitation (LOQ) was 0.04 µg/mL for gentamicin C1 and 0.01 µg/mL for the other two gentamicin components. The limit of detection (LOD) was 0.01 µg/mL for gentamicin C1 and 0.005 µg/mL for the other two gentamicin components.

In Vitro Cytocompatibility
Cell Culture Conditions
Commercially obtained MC3T3-E1 cells (ATCC) were utilized for all in vitro assays, as previously described by Jackson et al. [33] and Bow et al. [34]. Cells were expanded in tissue culture polystyrene flasks at 37°C and 5% CO₂ in αMEM media with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (pen-strep). Media was changed every 2 to 3 days. Once cell cultures had reached approximately 90% confluency, cells underwent enzymatic release from the growth substrate utilizing 0.25% Trypsin-EDTA solution for 2 minutes at 37°C. Cells were collected and allocated to experimental set-up.

Cell Seeding to Scaffolds
Fibro-Gide® cylinders were sharply sectioned into wafers (5x1.5mm) under sterile conditions. Each wafer was then placed into an individual well of a non-treated polystyrene plate and seeded individually with 5.0x10⁴ of MC3T3-E1 cells/15µL of growth media. Seeded wafers were allowed to incubate at room temperature for 30 minutes to allow for cells to infiltrate the wafer. After 30 minutes, 0.5mL of one of three variations of cell culture media was added. Media variations are as follows: (1) αMEM with 10% FBS and 1% pen-strep, (2) αMEM with 10% FBS and 20µg/mL of gentamicin, and (3) αMEM with 10% FBS and 200µg/mL of gentamicin. After media addition, plates were incubated at 37°C and 5% CO₂. Control parameters were provided by plating 1.0x10⁴ MC3T3-E1 cells without Fibro-Gide® wafers in tissue culture-treated plates with each of the three described media variations (positive control). Negative controls were provided by Fibro-Gide® wafers and tissue culture-treated plates without any cells seeded. Plates were incubated for 3, 5 and 7 days to facilitate in vitro assays for cellular adhesion and proliferation.

Cell Adhesion and Proliferation
Calcein-AM staining was performed to determine cellular adhesion and viability on Fibro-Gide® wafers. At 3, 5 and 7 days, samples (n=3/variation/timepoint) were incubated with 2µg/mL calcein-AM staining solution at 37°C for five minutes. Fluorescent images of all samples at each time point were taken to verify the presence and viability of cells [33]. Interestingly, the vast majority of cells attached to the underside of the Fibro-Gide® wafers and were visualized after the wafers were gently turned over (180°) utilizing sterile forceps.

MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) [34] was performed to determine cell proliferation on Fibro-Gide® wafers. At 3, 5 and 7 days, 100µL of MTS reagent was added to samples
(n=3/variation/timepoint) within 0.5mL indwelling media. Samples were incubated for three hours at 37°C and 5% CO₂. Absorbance of the formazan complex formed through this assay was measured at 490nm [35]. As described above, positive controls for both calcein-AM staining and the MTS assay were provided by MC3T3-E1 cells seeded on polystyrene tissue culture-treated plates and negative control parameters were provided by Fibro-Gide® wafers and polystyrene tissue culture-treated plates without any cell seeding. Negative controls were also utilized in data interpretation, to account for background signal caused by Fibro-Gide® wafers.

Cell proliferation and ingrowth on Fibro-Gide® wafers on days 3, 5 and 7 was also assessed via histology with hematoxylin and eosin (H&E) staining.

**In Vivo Evaluation**

**Rodent Model**

Female Sprague-Dawley rats (n=12) ranging from 190 – 210 grams were utilized. Animals were housed, cared for, and handled under standard conditions and in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines for the duration of the study. The model utilized in this experiment is a variation of a previously described critical size mandibular defect model [36-38].

**Surgical Preparation and Surgical Procedure**

Rats were anesthetized with isoflurane and received a pre-operative dose of buprenorphine (0.05mg/kg) subcutaneously (SQ). Surgical preparation included shaving fur from the left lateral neck to the left ear pinna. Aseptic skin preparation was accomplished with chlorhexidine and isopropyl alcohol. Eyes were lubricated with sterile lubricant and rats were maintained on isoflurane inhalant anesthetic via nose cone throughout the surgical procedure.

During surgery, skin and muscle layers were sharply dissected to expose the left mandible, and a critical-sized (5mm) circular defect was created in the ramus of the mandible utilizing a handheld microdrill (Ideal Microdrill, CellPoint Scientific, Inc. Gaithersburg, MD. USA) with 5mm circular bit. Defects were filled with sterile collagen matrix cylinder (Fibro-Gide®) in either the native form or impregnated with 10mg (100μL) of gentamicin (100mg/mL; approximately 40mg/kg dose of gentamicin). Native or impregnated collagen matrix relegated animals into either control (n=6) or experimental group (n=6), respectively.

No post-operative antibiotics were provided systemically or parenterally. Following surgery, animals were housed individually, provided free choice water, and maintained on a soft gel diet to minimize mechanical trauma from gnawing or chewing. These conditions were maintained for the remainder of the study. Animals were monitored every 12 hours for the first five days following surgery and additional doses of 0.05mg/kg buprenorphine were administered SQ every 12 hours for the first three days post-operatively. Four weeks post-operatively, rats were humanely euthanized via anesthetic (isoflurane) overdose and thoracotomy for sample collection.
Computed Tomography Analysis
After sacrifice, animals were scanned using computed tomography (CT) to evaluate the defect sites. Animals were positioned in sternal recumbency on the CT table and scanning parameters were limited to the skull of each animal. Sectional scans and 3D renderings of the regions of interest (ROIs) were collected and reviewed by a board-certified radiologist. Qualitative analysis of the defect area and surrounding bone was compiled.

Histological Analysis
Following CT scanning, left hemimandibles were harvested, formalin fixed, and decalcified in Formical-2000 for 48 hours until they could be sharply dissected with minimal resistance. After decalcification, hemimandibles were transferred to 10% neutral buffered formalin and were submitted for histology (University of Tennessee College of Veterinary Medicine, Veterinary Diagnostic Laboratory, Histopathology Service). Hemimandibles were embedded within paraffin, 4μm decalcified sections were obtained and stained with H&E. Slides were assessed qualitatively and semi-quantitatively by investigators as well as a board-certified veterinary pathologist who was blinded to treatment groups. Evaluation parameters of the tissue-scaffold interface were based on a modified-ordinal grading scale [39,40] to evaluate degree of tissue-scaffold integration and severity of inflammatory response. Tissue-scaffold integration was evaluated based on angiogenesis throughout the scaffold, connective tissue infiltrate into the scaffold, cellular and connective tissue infiltration into surrounding tissues and fibrous tissue encapsulation. The aforementioned categories were graded based on a point system from 0 to 3 with the following categories: (0) absent, (1) mild, (2) moderate, and (3) marked. Tissue-scaffold integration had a maximum positive score of 9 points [(angiogenesis + connective tissue infiltration into scaffold + cellular and connective tissue infiltration to surrounding tissues) – fibrous tissue encapsulation]. Degree of inflammatory response was evaluated based on inflammatory reaction, degree of fibrous tissue encapsulation, suppurative vs. non-suppurative cellular response and presence or absence of necrotic material. Inflammatory reaction and degree of fibrous tissue encapsulation were graded based on a point system from 0 to 3 with the following categories: (0) absent, (1) mild, (2) moderate, and (3) marked. Suppurative cellular response and presence of necrotic material were reported as nominal data, either (0): absent or (1): present. Degree of inflammatory response had a maximum severity score of 8 points (inflammatory response + fibrous tissue encapsulation + suppurative cellular response + necrosis).

Statistical Analysis
The effects of treatment, dose and time on response variable total gentamicin were examined using mixed model analysis for repeated measures. Ranked transformation was applied when diagnostic analysis on residuals exhibited violation of normality and equal variance assumptions using Shapiro–Wilk test and Levene's test. Post hoc multiple comparisons were performed with Tukey’s adjustment. Statistical significance was identified as p-values (alpha-error) at < 0.05. Analyses were conducted in SAS 9.4 TS1M4 (SAS institute Inc., Cary, NC). Effects of treatment, concentration and time on
MTS were analyzed using repeated measures ANOVA, with treatment and concentration as the between-subject effects while time as the repeated factor. Diagnostic analysis was conducted to exam model assumptions. Ranked transformation was applied if diagnostic analysis exhibited violation of normality and equal variance assumptions. Post hoc multiple comparisons were performed with Tukey’s adjustment. Statistical significance was identified at the level of 0.05. Analyses were conducted in SAS 9.4 TS1M7 for Windows 64x (SAS Institute Inc., Cary, NC). A student’s T-test (two-tailed, assuming homoscedasticity) was performed to evaluate for any significant differences between histological scores of control and experimental groups.

Results

Hydrophilicity
The 5-mm diameter biopsy punch effectively cut the Fibro-Gide® material into cylinders (n=6) with average height and width of 5.53 ± 0.22mm and 5.22 ± 0.12mm, respectively. Cylinders could load 100μL of PBS or gentamicin (100mg/mL or 50mg/mL) without leaving excess residue on loading platform. This was considered to be the maximum saturation and maximum loading dose for this material size and type. Initial average dry weight of Fibro-Gide® cylinders was 9.84 ± 0.57mg. Addition of 100μL of 50 or 100mg/mL gentamicin resulted in a 1211.20 ± 346.11% increase in weight of material, with average post-loading weight of Fibro-Gide® cylinders equal to 128.96 ± 2.54mg.

Gentamicin Elution
Fibro-Gide® samples loaded with 5mg gentamicin (n=3) eluted an average of 4.95 ± 0.57mg gentamicin throughout the 14-day period with peak elution of 4.47 ± 0.31mg at three hours. This is equivalent to eluting 89.4 ± 6.2% of the loaded gentamicin within the first three hours. Fibro-Gide® samples loaded with 10mg gentamicin (n=3) eluted an average of 9.97 ± 1.5mg throughout the 14-day period with peak elution of 9.14 ± 0.36mg at three hours. This is equivalent to eluting 91.4 ± 3.6% of the loaded gentamicin within the first three hours. Elution curve (Figure 4.1) demonstrates an initial burst release followed by a gradual lower-level release over the 14-day study period.

In Vitro Cytocompatibility
MTS assay results (Figure 4.2) showed that MC3T3-E1 cells were able to proliferate on Fibro-Gide® wafers when exposed to standard αMEM growth media as well as αMEM growth media containing a low (20μg/mL) or a high (200μg/mL) dose of gentamicin. As expected, proliferation of cells in standard cell culture treated-plates was significantly greater than proliferation of cells on Fibro-Gide® wafers (p<0.0001). For cells in cell culture and on scaffolds, proliferation of cells in the low and high dose gentamicin media was significantly greater than proliferation of cells in the standard cell culture media (p=0.0002 and p=0.0175, respectively). Cells in low-dose gentamicin media outperformed cells in high-dose gentamicin media (p=0.0275). There were differences seen through time, with day 3 having significantly less cellular proliferation regardless of substrate than days 5 or 7 (p<0.0001 and p<0.0001, respectively). There were no
significant differences between the cellular proliferation between days 5 and 7 (p=0.1985).

Calcein-AM staining showed that MC3T3-E1 cells were able to proliferate and remain viable on Fibro-Gide® wafers when exposed to standard αMEM growth media as well as αMEM growth media containing a low or high dose of gentamicin. Positive controls as well as experimental samples all demonstrated an increase in numbers of fluorescent cells between days 3, 5 and 7. Images of fluorescently tagged cells highlight the three-dimensional nature and porous texture of the Fibro-Gide® wafers (Figure 4.3).

Histopathology with H&E staining (Figure 4.4) demonstrated the porous nature of the collagen matrix and showed individual cells in various orientations throughout the matrix, confirming the hypothesis that cells can proliferate on Fibro-Gide® and also grow into the porous channels.

**In Vivo Biocompatibility**

**Animal Observation and Care**

No anesthetic complications occurred. Animals subjectively recovered well and appeared comfortable. Animals did not develop significant facial swelling or discomfort that impeded their ability to eat, drink or interact with their environment. Objectively, incisions healed without any surgical site infections, and animals gained weight throughout the study. As a result of eating a soft gel diet instead of a regular rodent chow pellet, one rat developed a malocclusion, or overgrowth of the incisor teeth, which resulted in secondary side effects and euthanasia prior to the intended endpoint. This animal was not included in data analysis, resulting n=5 control animals and n=6 experimental animals. Otherwise, all remaining rats completed the study uneventfully but most required corrective tooth trimming once weekly to prevent additional instances of dental malocclusion.

**CT Analysis**

One month after surgery, qualitative and semi-quantitative CT analysis of the control group demonstrated mild to moderate periosteal reaction, absent to mild medullary sclerosis, absent to mild soft tissue swelling, and no evidence of bone lysis or bone healing. CT analysis of the experimental group demonstrated mild to moderate periosteal reaction, mild medullary sclerosis, absent to mild soft tissue swelling, and no evidence of bone lysis or healing. Qualitative CT analysis also demonstrated variation in defect placement (Figure 4.5), including defect placement that extended off the caudal border of the mandible and placement that extended into the oral cavity.

**Histological Analysis**

Control group: Control animals (those that received a defect and non-impregnated collagen matrix) had an average tissue-scaffold integration score of 4.2 ± 2.77 (maximum score of 9) and an average inflammatory score of 4.8 ± 1.92 (maximum score of 8, denoting most inflammatory process). All but one of these sections demonstrated similar qualities and degrees of angiogenesis, fibrous tissue encapsulation, connective tissue infiltration into the collagen matrix, and mononuclear cellular population. One specimen, (rat #9), differed
greatly from the other control samples, with a tissue-scaffold integration score of 0 and a degree of inflammation score of 8. This animal displayed severe, suppurative inflammation with degenerative neutrophils and areas of necrosis within the collagen matrix. On CT analysis, this animal also suffered a pathologic fracture ± osteomyelitis (bone infection). One animal (rat #11) had the presence of few multi-nucleated giant cells (MNGCs) distributed within the periphery of the collagen matrix. This animal had no surgical complications or abnormalities on CT analysis.

Treatment Group: Experimental animals (those that received a defect and collagen matrix impregnated with 40mg/kg gentamicin) had an average tissue-scaffold integration score of 4.0 ± 2.0 (maximum score of 9) and an average inflammatory score of 5.5 ± 2.16 (maximum score of 8, denoting most inflammatory process). Similar to control specimens, these sections demonstrated similar qualities, extent of angiogenesis, fibrous tissue encapsulation, connective tissue infiltration into the collagen matrix, and mononuclear cellular population. Two specimens (rats #2, 10) differed greatly from other experimental samples, with tissue-scaffold integration scores of 1 and 2, respectively, and degree of inflammation scores of 8. These animals displayed severe, suppurative inflammation with degenerative neutrophils and areas of necrosis within the collagen matrix. Neither of these animals had evidence of osteomyelitis on CT analysis and neither animal suffered a pathologic surgical complication. Three animals (rats #8, 10 & 12) had one to few MNGCs present around the periphery of the collagen matrix. Both rats #8 and 12 had some extension of the surgical defect into the caudal mandibular border.

Between both groups, all specimens displayed mononuclear cellular infiltrate into the collagen matrix. Specimens varied in quantity of fibrous tissue surrounding the collagen matrix as well as infiltration of blood vessels into center of the collagen matrix. Four specimens (36% of animals) displayed evidence of MNGCs (one control specimen, three experimental specimens). Three specimens (27% of animals) displayed severe, degenerative, suppurative inflammation (one control specimen, two experimental specimens). There were no statistically significant differences detected between tissue-scaffold integration scores of control and experimental animals (p=0.89) or between degrees of inflammation between the two groups (p=0.58). Representative images of the described findings are displayed in Figure 4.6.

Discussion

Results from this work indicate that commercially available collagen matrix Fibro-Gide® can support the proliferation and viability of MC3T3-E1 cells in vitro, even in the presence of gentamicin. Gentamicin is an aminoglycoside antibiotic that is known to possess an acidic pH, which can alter local in vitro or tissue environments, and that can also alter mitochondrial respiration, enhancing the generation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide [41]. ROS are recognized to cause DNA damage which can lead to cell death. ROS also can cause decreased cellular proliferation, decreased angiogenesis, and stimulate inflammation [42]. Therefore, the ability of cells to proliferate in the presence of gentamicin in vitro, is important. Histology of a cell-loaded collagen matrix demonstrated the ability of MC3T3-E1 cells to proliferate and grow within the porous channel system provided by Fibro-Gide®. This
finding supports the ability of the collagen matrix to support tissue ingrowth, which supports its biocompatibility and subsequent biodegradation. On the basis of these studies, Fibro-Gide® can be loaded with and elute the antimicrobial gentamicin. *In vitro* elution was characterized by an initial burst release followed by sustained lesser release for the 14-day study period. The described release kinetics fall into a pattern typical of devices loaded by impregnation rather than specific molecule-linkages or stimuli-responsive systems [9,20]. It should be noted that impregnation via material soaking in antibiotic solutions is the most common method used for loading of collagen materials [20].

These *in vitro* data support an *in vivo* investigation to determine biocompatibility of the material when impregnated with a high dose of antimicrobial. *In vivo* investigation in a rat mandible defect indicated satisfactory tissue-scaffold integration and mild degrees of inflammation given the clinical scenario of critical-sized mandibular defect, which inherently induces tissue trauma and associated inflammation. Histology demonstrated a mixture of connective tissue infiltrating throughout the collagen matrix along with the presence of mononuclear cellular infiltrate. Mononuclear cells, such as lymphocytes, are components of the host immune system and play important, multifaceted roles in inflammation and tissue healing [24]. Lymphocytes have been recognized to not only respond to acute inflammation and be associated with chronic inflammation, but also to regulate angiogenesis, tissue healing and regeneration [43,44]. Finding formed connective tissue throughout the collagen matrix instead of solely fibrous connective tissue surrounding the collagen matrix supports the *in vivo* biocompatibility of the device [45,46], even while loaded with gentamicin. The presence of MNGCs is controversial [47,48]. When there is a significant or overwhelming population of MNGCs in conjunction with a fibrotic ring of tissue and/or dense neutrophilic inflammation surrounding an implanted device, it can be deduced that the body is mounting a foreign body response to that material [45]. However, there are also situations in which material degradation can induce the formation of a smaller number of MNGCs [23,49]. Recognizing that Fibro-Gide® is a resorbable collagen matrix, it is reasonable to believe that the limited number of MNGCs noted along the periphery of four scaffolds is reflective of the intended degradation process and not an adverse foreign body response.

While the majority of specimens indicated satisfactory tissue-scaffold integration and degrees of inflammation, it is important to consider that three specimens demonstrated marked suppurative inflammation and areas of necrosis throughout the collagen matrix. One of these specimens was associated with a pathologic fracture and evidence of osteomyelitis on CT scan. It is likely that the pathologic fracture occurred peri-operatively and resulted in a local inflammatory environment that encouraged the development of a suppurative response. This may have been exacerbated by mobility of implant within the defect site due to the pathologic fracture or exposure to the oral cavity, which can rapidly increase the degradation rate of collagen [50,51]. In the other two specimens, the animals had an unsatisfactory tissue-scaffold reaction. Suppurative tissue-scaffold reactions in these two experimental specimens, may be due to individual immune response, mobility of implant within defect site, bacterial contamination, or exposure to the oral cavity, which can increase the degradation rate of collagen [50,51]. It should also be taken into consideration that gentamicin, although it carries many benefits,
also has the risk of altering local tissue environments by promoting an acidic pH and by enhancing the generation of ROS [41]. It is possible that the enhanced presence of ROS by action of gentamicin may have stimulated an increased rate of apoptosis, leading to the necrotic foci throughout the collagen matrix and stimulating the intense inflammatory tissue response. It is reasonable to consider the possibility that other antibiotics may impact the tissue-scaffold interface and degrees of inflammation differently. Another consideration when interpreting in vivo biocompatibility data is the variation in defect placement and the potential contribution of that variation ± pathologic fractures on the tissue-scaffold interface visualized on histopathology.

SSIs can be superficial or deep, and may involve medical implants or areas of tissue loss, whether that tissue loss is due to trauma, resultant from surgery, or secondary to revision procedures such as debridement. When dealing with SSIs that extend to deeper tissue planes, a locally implantable drug delivery device may add strength to the treatment regimen. Similarly, when SSIs involve any aspect of tissue loss or destruction, the ability to treat infection while aiding in tissue regeneration is likely to restore form and function to the patient more rapidly and with fewer interventions than traditional treatment strategies. Additionally, the potential to utilize an FDA approved tissue regeneration product as a dual-platform device for drug delivery holds exceptional opportunities to prevent bacterial infection in local tissue environments. The strength in this transition comes not only in the consistency and availability of the product, but also in healthcare provider familiarity with the product. When considering consistency and availability, a major advantage of utilizing a currently available material is the production process. Many investigations into biomaterials for drug delivery and tissue regeneration demonstrate positive results when the materials are fabricated in small batches but encounter significant technical and performance challenges once scaling up material production is attempted. Therefore, the investigation of a commercially available collagen matrix that has previously demonstrated appropriate biocompatibility [23,24,46] for utilization as a dual-platform device is a reasonable undertaking and provides valuable information.

**Limitations**

Limitations within this work include small sample size, variability in defect placement and lack of concurrent in vivo drug elution kinetic characteristics. Greater consistency in defect placement in a larger number of animals is ideal to maintain a uniform population and would remove the consideration of defect placement from histological analysis. Another limitation of this study was our imaging modality. While CT provided useful information and valuable 3D renderings of mandibular defects, microCT would have provided finer detail and allowed for quantitative analysis of bone defects, rather than solely qualitative and semi-quantitative measurements. While there is always variation in histologic appearance based on inter-animal variation, exact specimen positioning within paraffin block, and particular 4μm sections obtained.

**Conclusions and Future Directions**

This work was performed to assess the overall fitness of commercially available collagen matrix Fibro-Gide® for utilization as a locally implantable drug delivery device, in
conjunction with the products labeled usage of soft tissue regeneration device. To assess overall fitness, we began with the following three hypotheses: the device (1) would be able to load and elute antimicrobials, (2) would be cytocompatible in vitro, and lastly, (3) a high dose of antimicrobials loaded within the device would negatively impact the tissue-scaffold interface in vivo. We demonstrated that Fibro-Gide® is able to load and elute the antimicrobial gentamicin, with the expected elution kinetics, confirming the first hypothesis. We demonstrated cytocompatibility in vitro with or without the presence of gentamicin, confirming the second hypothesis. Lastly, we found no significant differences between the tissue-scaffold interface of animals that received either the native device or device loaded with a high dose (40mg/kg) of the antimicrobial gentamicin, therefore rejecting the third hypothesis. Based on these parameters, we conclude that this commercially available collagen matrix holds value for further consideration to be utilized as a local drug delivery device, especially in situations where soft-tissue regeneration or augmentation is desired.

To further investigate the value of this collagen matrix for use as a dual-platform device, a reasonable initial step is to perform a similar experiment utilizing a larger number of animals and a slightly smaller defect size. This approach would likely result in more uniform defect placement, and would more clearly elucidate the impact of a high dose of gentamicin on the tissue-scaffold interface, eliminating the concern of pathologic fractures leading to suppurative tissue responses. Further investigations may focus on a wider array of drugs to evaluate drug elution characteristics and subsequent device compatibility, or may focus on fine-tuning of drug elution kinetics, either by assessing in vivo elution [20] or by alternative drug loading strategies. Situations of particular value for utilization of this collagen matrix as a dual-platform device include surgical site infections that involve soft tissue loss, and cases where systemic antimicrobial usage needs to be minimized.
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Randomized Trial.


Figure 4.1. Median gentamicin elution from Fibro-Gide® cylinders. Cylinders were loaded with either 5mg (n=3) or 10mg (n=3) gentamicin. Log transformation applied to best visualize elution curve, including both the initial burst release and sustained lower-level release of gentamicin.

Figure 4.2. MTS assay to measure cellular proliferation. Left-side panel (Cells) demonstrates mean cellular proliferation, measured through absorbance, of MC3T3-E1 cells in cell culture exposed to either standard media, a low dose of gentamicin or a high dose of gentamicin, through time. Right-side panel (Sc. Cells) demonstrates mean cellular proliferation, measured through absorbance, of MC3T3-E1 cells on Fibro-Gide® wafers exposed to either standard media, a low dose of gentamicin or a high dose of gentamicin, through time. Cells in cell culture had a significantly higher proliferation than cells on collagen matrix (p<0.0001). Sc. Cells = cells on Fibro-Gide® wafers.
Figure 4.3. Calcein-AM staining. Images of calcein-AM staining of MC3T3-E1 cells on Fibro-Gide® wafers with inset positive control images of MC3T3-E1 cells in cell culture. 1A-C: cells on matrix on days 3 (A), 5 (B), 7 (C) in standard media. 1a-c: cells in culture on days 3 (a), 5 (b), 7 (c), standard media. 2A-C: cells on matrix on days 3 (A), 5 (B), 7 (C) exposed to low dose gentamicin. 2a-c: cells in culture on days 3 (a), 5 (b), 7 (c), low dose gentamicin. 3A-C: cells on matrix on days 3 (A), 5 (B), 7 (C), exposed to high dose gentamicin. 3a-c: cells in culture on days 3 (a), 5 (b), 7 (c), high dose gentamicin.

Figure 4.4. SEM and H&E-stained histology images of Fibro-Gide® collagen matrix. Panel A: SEM image of Fibro-Gide® cylinder. Panels B-D: histology images taken at 40X magnification of Fibro-Gide® seeded with MC3T3-E1 cells on days 3 (B), 5 (C), 7 (D) of cell culture. Dark pink material is collagen matrix, dark purple material = cell nuclei, denoted by black arrows.
Figure 4.5. 3D CT scan renderings of mandibular defect. Image A displays the ideal placement of the critical-sized (5mm diameter), full-thickness bone defect on rodent hemimandible. Image B demonstrates defect placement that is too far caudal on the hemimandible. Both images provide appreciation for the difficulty of completing quantitative analysis due to small defect and animal size.

Figure 4.6. Histology images from control and experimental animals. 1A. 5X magnification, demonstrating cellular and connective tissue infiltration into native collagen matrix (control animal, rat #7). 1B. 20X magnification, demonstrating cellular and connective tissue infiltration into native collagen matrix (control animal, rat #7). 2A. 5X magnification, demonstrating cellular and connective tissue infiltration into antibiotic-loaded collagen matrix (experimental animal, rat #4). 2B. 20X magnification, demonstrating cellular and connective tissue infiltration into antibiotic-loaded collagen matrix (experimental animal, rat #4). 3A. 5X magnification, demonstrating thick ring of fibrous tissue separating bone from native collagen matrix and surrounding suppurative inflammation (control animal, rat #9). 3B. 40X magnification, demonstrating dense population of neutrophils (left side of image), and necrotic cellular infiltration into native collagen matrix (control animal, rat #9). 4A. 10X magnification, demonstrating cellular and connective tissue infiltration into antibiotic-loaded collagen matrix (experimental animal, rat #12). 4B. 40X magnification, demonstrating presence of multinucleated giant cells, as well as blood vessels, throughout antibiotic-loaded collagen matrix (experimental animal, rat #12). Boxes are surrounding select blood vessels to highlight angiogenesis throughout collagen matrix. Stars denote bone. Double-sided arrow highlights thick rim of fibrous connective tissue. Thick arrows point at select multi-nucleated giant cells.
CHAPTER V: IN VITRO AND IN VIVO ASSESSMENT OF CAPRINE ORIGIN STAPHYLOCOCCUS AUREUS STRAIN ST398 AS AN OSTEOMYELITIS PATHOGEN
Abstract

Osteomyelitis is an inflammatory bone disease characterized by progressive bone destruction and loss. This disease causes significant morbidity and mortality to the patient and poses therapeutic challenges for clinicians. *Staphylococcus aureus* (SA) is a significant and well-recognized causative organism in the pathogenesis of bacterial osteomyelitis. To improve the efficacy of therapeutic strategies to combat bacterial osteomyelitis, there is a need to define the molecular epidemiology of bacterial organisms more clearly and further the understanding of the pathogenesis of *S. aureus* osteomyelitis. We conducted *in vitro* characterization of the pathogenic capabilities of a clinical isolate of *S. aureus* ST398 derived from a clinical case of osteomyelitis in a goat. We also report a rodent mandibular defect model to determine the ability of ST398 to cause osteomyelitis. Our results indicate that ST398 can invade and distort pre-osteoblastic cells in cell culture and induce significant inflammation as an intracellular organism. We also demonstrate the ability of ST398 to induce osteomyelitis in a rat mandibular model.

Introduction

Osteomyelitis is an inflammatory bone disease, typically caused by bacteria, that causes progressive bone destruction and loss [1]. This destructive process results in significant morbidity and mortality to affected individuals [2]. *Staphylococcus aureus* (SA), a common commensal organism [3,4], is also described as a versatile and dangerous human pathogen [4,5], and is responsible for the majority of bacterial osteomyelitis cases [1,2,6,7]. Various etiologies of osteomyelitis are recognized, including 1. hematogenous, 2. secondary to trauma or invasive procedures such as surgery and implantation of indwelling medical implants, and 3. secondary to vascular insufficiency, such as diabetic foot ulcers [8,9]. Regardless of etiology, osteomyelitis is a serious condition that warrants swift and intensive therapy. Therapeutic strategies for acute and chronic osteomyelitis are heavily reliant upon systemic antimicrobial therapy which may be complemented by local antimicrobial therapy [9,10]. Standards of practice for treatment of osteomyelitis, especially in chronic cases, also include surgical debridement of affected and devitalized tissues, which may involve removal of implanted hardware [2]. Despite rapid and appropriate intervention [11], osteomyelitis often becomes chronic, a phenomenon due in large part to the pathogenic capabilities of *S. aureus* [12].

The ability of SA to create chronic, recalcitrant, or recurrent osteomyelitis [13] through virulence factors and immune system evasion is multifactorial [12,14-18] and incompletely understood. Mechanisms of immune system evasion include *Staphylococcal* abscess formation, biofilm formation, osteocyte-lacuno canalicular network (OLCN) invasion, as well as intracellular invasion and persistence [12,15,19]. In addition to greater understanding of the mechanisms of immune system evasion and the contributions to infection persistence and recurrence, there is also a need to characterize and understand the molecular epidemiology of SA strains causing bacterial osteomyelitis. While SA and
methicillin-resistant *S. aureus* (MRSA) are recognized as common etiologic agents of osteomyelitis [10], there remains work to be done in identifying specific sequence types (ST) and clonal complexes (CC) that cause osteomyelitis [6]. Thorough characterization of the molecular epidemiology of SA strains involved in osteomyelitis may contribute to understanding of pathogenesis [16] and strengthen therapeutic strategies, whether that applies to pre-existing strategies, novel techniques, or combinations thereof.

Along this vein of investigation, SA ST398 has been increasingly recognized as an important human pathogen [20-23]. Previously thought to be primarily a colonizer and occasional pathogen of livestock [24,25], ST398 is now a known causative organism of serious human infections, sometimes carrying methicillin resistance [25-28] and occurring in the absence of contact with livestock [29]. Human illnesses caused by ST398 include soft tissue infections [29], bone and joint infections and osteomyelitis [16,30,31], bloodstream infections (BSI) [21], as well as pneumonia including ventilator-associated pneumonia and lethal, necrotizing pneumonia [20]. Currently, virulence factors and pathogenesis of ST398 are incompletely understood [32]. Further investigations of CC398 and ST398 are imperative to continue advancing the understanding of *Staphylococcus* illness, particularly osteomyelitis, with the goals of ultimately enhancing therapeutic strategies, minimizing negative impact to the patient, and reducing overall burden on the healthcare system.

In this work, we describe an investigation into the patho-mechanisms of methicillin-sensitive *S. aureus* ST398, clinically isolated from a goat suffering hypertrophic osteomyelitis. It has been recently reported that the ability of SA to gain intracellular access to osteoblasts may not only contribute to immune system evasion but also may play a role in shifting osteoblastic activity to induce inflammatory bone pathology [33,34]. Our goal was to investigate for strain-dependent differences in pathogenesis and virulence of SA and assess interspecies capabilities of ST398 as an etiologic agent of bacterial osteomyelitis. Capabilities of ST398 to invade pre-osteoblastic cells and trigger inflammatory responses including apoptosis were investigated *in vitro*, as compared with capabilities of strains USA300 and Cowan1. Also, *in vivo* studies were conducted to determine the capability of ST398 to induce osteomyelitis in a rodent mandibular defect model. We hypothesized that there would be strain dependent differences in pathogenesis and virulence of SA, and that ST398 would be capable of inducing osteomyelitis cross-species.

**Materials and Methods**

**In Vitro Methodology**

**Bacterial Strain Selection and Preparation**

SA strain ST398 was selected for these experiments as a clinical isolate from a goat suffering hypertrophic osteomyelitis following orthopedic surgery [35]. ST398, as described above, is recognized as a colonizer and pathogen of livestock as well as people. This isolate was identified as methicillin-sensitive despite resistance to other classes of antimicrobials (Table 5.1). SA USA300 is a human origin osteomyelitis isolate that
produces severe, invasive osteomyelitis in pediatric and adult patients [36]. USA300 is recognized as a highly virulent *Staphylococcal* strain [37] and is often utilized in osteomyelitis research, both *in vitro* [38,39] and *in vivo* [39-41]. SA Cowan1 (ATCC 12598) is a non-cytotoxic human origin septic arthritis isolate [38]. Cowan1 is often utilized experimentally as a minimally virulent strain of SA [42].

Bacterial inoculums were prepared for cell culture by growing each of the three bacterial strains overnight in 5mL of tryptic soy broth (TSB) at 35°C with aeration. Bacteria were harvested by centrifugation (10 minutes at 4300Xg), washed twice in 5mL of Hank’s balanced salt solution (HBSS) and resuspended in Minimum Essential Media α (α-MEM) (Thermo Fisher Scientific) with 10% fetal bovine serum (FBS) as described by Tucker et al. [18].

**Cell Culture and Bacterial Inoculation**
Commercially obtained MC3T3-E1 cells (ATCC) were utilized for *in vitro* experiments. MC3T3-E1 cells are immature osteoblastic cells of murine origin. Cells were expanded in tissue culture treated polystyrene flasks and incubated under standard conditions (37°C and 5% CO₂) in α-MEM media with 10% (FBS), 1% penicillin streptomycin (pen-strep) and amphotericin B. Media was changed every 2 to 3 days. Once cell cultures had reached approximately 90% confluency, cells underwent enzymatic release from the growth substrate utilizing 0.25% Trypsin-EDTA solution for 2 minutes at 37°C. Cells were collected and allocated to experimental set-up.

Expanded MC3T3-E1 cells were plated at a density of 1x10⁵ in 12-well tissue culture-treated plates or 12-well transwell plates (Thermo Fisher Scientific). Cells were maintained in α-MEM media under standard conditions as described above. Media was changed every 2-3 days until cells reached 80% confluence. After reaching 80% confluence, three wells of each plate (biologic triplicates) were allocated as uninfected controls, and remaining wells were allocated to either ST398, USA300 or Cowan1, all in triplicate. Experimental wells were then infected as previously described by Tucker et al. [18], with a bacterial inoculum of 10⁸ colony forming units (CFU) per well, which provided a multiplicity of infection (MOI) of 250:1. Post-inoculation, control and experimental wells were incubated under standard conditions for 45 minutes to allow for bacterial invasion of plated cells. Post-invasion and incubation, supernatant was removed, cells were washed twice with 3mL of HBSS (total=6mL), and α-MEM media containing 25μg gentamicin (gentamicin sulfate, 100mg/mL) was replaced in wells to eliminate remaining extracellular bacteria.

**Apoptosis Assay**
Following bacterial invasion, control and experimental samples in biologic triplicates were dedicated to apoptosis detection utilizing the Annexin V-PI Apoptosis Detection Kit (Clonetech Laboratories Inc.). After 4 and 20 hours of incubation, supernatants were removed and saved from each well. Cells were washed once with 3mL of HBSS, and wash collection was saved with the collected supernatant, as previously described [18].
Cells were analyzed by flow cytometry utilizing an Attune flow cytometer. Cells undergoing apoptosis were positive for fluorescein isothiocyanate (FITC) and dead cells were stained with Sytox green. Cells were gated based on size (SSC) and forward scatter (FSC).

**Inflammatory Cytokine Analysis**

Control and experimental samples in biologic triplicates were dedicated to inflammatory cytokine analysis. These samples underwent supernatant collection, washing, and media replacement at 8, 24 and 48 hours post-bacterial invasion. Supernatant was saved at -80°C. Concentration of interleukin-6 (IL-6) was determined by enzyme-linked immunosorbent assay (ELISA) analysis per manufacturer instruction (R&D Systems, Minneapolis, MN. USA). Optical densities of samples and standards were measured at 450nm with an iMark microplate reader (BioRad).

**Osteoregulatory Cytokine Analysis**

Control and experimental samples in tissue-culture treated plates in biologic triplicates were dedicated to cytokine analysis to evaluate bone signaling and remodeling. Samples underwent supernatant collection, washing and media replacement immediately following bacterial invasion, (time 0). Following this sample collection, media was replaced with osteoinductive media (α-MEM media supplemented with 10mM beta glycerophosphate, 10nM dexamethasone and 155µM ascorbic acid) [43] that was changed every 2-3 days. Sampling procedure was repeated at days 7, 14 and 21. Supernatant was saved at -80°C. Concentrations of osteoprotegerin (OPG) and osteopontin (OPN) were determined by ELISA analysis per manufacturer instruction (R&D Systems, Minneapolis, MN. USA). Optical densities of samples and standards were measured at 450nm with an iMark microplate reader (BioRad).

**Transmission Electron Microscopy**

Samples seeded on the transwell plate were dedicated to transmission electron microscopy (TEM) following bacterial invasion. Samples were fixed in cacodylate buffered (0.5M) glutaraldehyde (3%) for one hour, washed three times in buffer and then post-fixed in 2% buffered osmium tetroxide for one hour. After post-fixation, samples were washed in water three times over 30 minutes and then dehydrated in an ethanol gradient (10%, 25%, 50%, 75%, 95%, 100%) 15 minutes per step, with two final steps in 100% propylene oxide. Following dehydration, samples were embedded in epoxy (EMBed 812) using the following routine:

- EMBed 812: Propylene oxide 1:3 (two hours)
- EMBed 812: Propylene oxide 3:1 (overnight)
- EMBed 812 100% (three changes over 8 hours)

Samples were then placed in fresh EMBed 812 in flat embedding molds and the epoxy was cured overnight in an oven at 68° C. Ultrathin sections (~100nm) were cut on a Leica UC7 ultra-microtome using a diamond knife (Diatome). Sections were mounted on copper grids and stained with 50% methanolic uranyl acetate (45min) washed in water and post-stained with Reynolds lead citrate (5minutes), washed and completely dried.
before imaging. Sections were imaged with a JEOL 1400-Flash (JEOL, USA) operating at 120kV. Images were recorded with a GATAN One View camera.

**In Vivo Challenge**

**Rat Mandibular Defect Model**

Twenty-four, 8-week-old, male, outbred Wistar rats (Charles River Laboratories, USA) weighing 265 ± 23.45g were used in this study. Animals were housed and cared for according to Institutional Animal Care and Use Committee (IACUC) guidelines. All procedures were approved by IACUC as well as the Institutional Biosafety Committee (IBC). Animals were maintained in laboratory space approved for biosafety level 2 (BSL-2) procedures under standard conditions with free choice rodent chow and water.

SA ST398 (caprine clinical isolate) was utilized as a sole bacterial organism to induce osteomyelitis in a rat mandibular defect model. ST398 was maintained on blood agar at 4°C throughout study period. Thirty-six hours prior to surgical inoculation, one bacterial colony was removed from the maintained plate, streaked onto a blood agar plate and placed in an aerobic incubator at 35°C. Four hours prior to surgical inoculation, the freshly streaked plate was removed from incubation, 1-3 colonies were isolated, placed in trypticase soy broth (TSB) and incubated for three hours at 35°C with aeration. After three-hour incubation, bacterial broths were diluted to $10^8$ CFU/mL utilizing sterile saline. This CFU count was equivalent to a McFarland standard of 0.5-0.52.

Bacterial osteomyelitis was induced using a procedure adapted from Sodnom-Ish et al. [44]. Briefly, the procedure operated under the premise of creating bone trauma, introducing a high dose of planktonic bacteria locally, and placing foreign material within traumatized bone to provide a nidus for bacterial colonization. This procedure fulfills the main tenants of osteomyelitis induction in animal models [45,46]. All procedures were performed in a biosafety cabinet. Rats were anesthetized using anesthetic gas (isoflurane) and administered a 0.03mg/kg dose of buprenorphine subcutaneously (SQ). Animals were maintained on isoflurane vaporized into 100% oxygen via nose cone throughout the surgical preparation and procedure. Surgical preparation consisted of shaving the fur from the right hemimandible, and skin was aseptically prepared utilizing povidone iodine and isopropyl alcohol. During surgery, skin and muscle layers were sharply incised to expose the right hemimandible. A 3mm circular defect was created utilizing a 3mm diameter carbide round drill bit (Stryker Instruments, Kalamazoo, MI. USA) attached to a handheld microdrill (Ideal Microdrill, CellPoint Scientific, Inc. Gaithersburg, MD. USA). Defect placement was on the right mandibular ramus, caudal to tooth roots and ventral to pulp cavity. Once the defect was created, a 3x2mm circular wafer of sterile, commercially available bovine-origin bone mineral replacement material (Geistlich Bio-Oss® Collagen, Geistlich Pharma AG, Wolhusen, Switzerland) was placed into the defect site. Rats were allocated into one of four groups;
1. Group I (negative control for model effect): received bone defect and Bio-Oss® Collagen pre-loaded with 2.25mg of vancomycin (vancomycin hydrochloride, 50mg/mL, Hospira, Lake Forest, IL. USA)
2. Group II (SA + high dose vancomycin): received bone defect, Bio-Oss® Collagen pre-loaded with 2.25mg of vancomycin and local application of 50μL of ST398 bacterial suspension (10^8 CFU)
3. Group III (SA + low dose vancomycin): received bone defect, Bio-Oss® Collagen pre-loaded with 1.12mg of vancomycin and local application of 50μL of ST398 bacterial suspension (10^8 CFU)
4. Group IV (SA infection): received bone defect, Bio-Oss® Collagen without antimicrobial, and local application of 50μL of ST398 bacterial suspension (10^8 CFU)

Muscle and skin incisions were closed with 4-0 poliglecaprone 25 (Monocryl, Ethicon, Inc. Raritan, NJ. USA) suture in an interrupted cruciate pattern.

Once recovered from anesthesia, animals were provided free choice water and soft gel diet. Animals were administered 0.03mg/kg buprenorphine SQ every 12 hours for the first three days following surgery. At day five post-operatively, animals were transitioned from the soft gel diet to regular rodent chow. Animals were maintained for four weeks with once weekly venipuncture, weighing, and surgical site inspection. At four weeks, animals were sacrificed via anesthetic overdose (isoflurane) and thoracotomy. At the time of sacrifice, gross necropsies were performed. Right hemimandibles were harvested under aseptic conditions. During harvest, cultures were obtained from bone defects, bordering soft tissues, and any other apparent lesion present. Hemimandibles were formalin fixed in 10% neutral buffered formalin (NBF) and micro-computed tomography (micro-CT) was performed, followed by histological analysis.

**Material Description**
Bio-Oss® Collagen was utilized as dual drug delivery device and scaffold material to fill space and serve as a potential nidus for bacterial colonization within the defect site. Bio-Oss® is described by the manufacturer as a biocompatible bone mineral matrix composed of 90% purified, bovine-derived small bone particles and 10% porcine collagen [47]. Bio-Oss® Collagen is provided as a sterilized block of varying sizes that can be trimmed into desired size and shape either dry or moistened. There are interconnected pores that extend throughout the material which contribute to hydrophilicity of the device, and allow for simple hydration of the matrix [47]. This material is labeled and utilized for defect-filling and guided bone regeneration (GBR) [47,48].

**In Vitro Drug Loading and Elution**
To determine the loading capacity of Bio-Oss® Collagen for vancomycin, in vitro hydrophilicity and drug elution experiments were performed. Material was trimmed into 3x2mm circular wafers utilizing a 3mm biopsy punch and sharp dissection with a #10 blade. Hydrophilic properties of the material were determined by calculating the percent...
equilibrium water content (EWC) (equation 1). Phosphate buffered saline (PBS) was added in 5μL increments until the devices (n=3) were saturated and wet weights were recorded.

Equation 1: \( EWC \ (\%) = \frac{(Weight \ hydrated \ sample - weight \ dry \ sample)}{weight \ hydrated \ sample} \times 100 \)

Based on the determined hydrophilic properties and the size of 3x2mm, the optimal loading volume of vancomycin (50mg/mL) was determined to be 45μL.

Bio-Oss® Collagen wafers were loaded with either 2.25mg (high dose, n=3) or 1.12mg (low-dose, n=3) of vancomycin. Vancomycin dose of 2.25mg was accomplished by loading 45μL of 50mg/mL vancomycin solution. To ensure equal loading volume across devices, the 1.12mg dose was accomplished by loading 45μL of 25mg/mL vancomycin. The lower concentration was created by diluting 50mg/mL vancomycin 1:1 with sterile water for injection. Devices were loaded and allowed to incubate at room temperature for four hours prior to use in the elution experiment.

At four hours post-loading, devices were placed into sterile polystyrene plates in individual wells with 2mL PBS/well. Devices were incubated at 37°C to mimic physiologic temperature. At pre-determined timepoints (3, 12, 24 and 48 hours, and on days 4, 6, 8, 10, 12 and 14), supernatant was removed, saved at -80°C and 2mL of PBS was replenished in each well prior to returning to incubation.

**Analytical Chemistry**

Analysis of vancomycin in phosphate buffered saline (PBS) samples was conducted using reversed phase HPLC (University of Tennessee College of Veterinary Medicine Pharmacology Laboratory). The system consisted of a 2695 separations module and a 2487 UV detector (Waters, Milford, MA, USA.). Separation was attained on a Waters XBridge C\textsubscript{18} 4.6 x 250 mm (5 μm) column protected by a 5 μm XBridge C\textsubscript{18} guard column. The mobile phase was an isocratic mixture of 0.1% formic acid in water pH 3.0 with 2 M NaOH and acetonitrile (90:10). It was prepared fresh daily using double-distilled, deionized water filtered (0.22 μm) and degassed before use. The flow rate was 1.0 ml/min and UV absorbance was measured at 240 nm.

Vancomycin was extracted from PBS samples using a precipitation method. Briefly, previously frozen PBS samples were thawed and vortexed and 100 μL was transferred to a screw-top test tube followed by 15 μL internal standard (100 μg/mL caffeine). Five hundred microliters of acetonitrile was added and the tubes were vortexed for 30 seconds and then centrifuged for 10 minutes at 1000 X g. The organic layer was transferred to a 16x100 mm tube and evaporated to dryness with nitrogen gas. Samples were reconstituted in 250 μL of mobile phase and 100 μL was analyzed.

Standard curves for PBS analysis were prepared by fortifying PBS with vancomycin to produce a linear concentration range of 0.1–2000 μg/mL. Calibration samples were
prepared exactly as PBS samples. Average recovery for vancomycin was 95% while intra and inter-assay variability ranged from 1.6 to 4.5% and 2.2 to 7.9%, respectively. The lower limit of quantification was 0.1 µg/mL.

Cytokine Expression Analysis
RNA was extracted whole blood samples collected at predetermined timepoints (day 0, weeks 1, 2, 3 and 4) utilizing RNeasy mini kits (Qiagen, Hilden, Germany). Quantity and quality of extracted RNA was assessed via NanoDrop Spectrophotometer and samples were saved at -80°C until polymerase chain reaction (PCR) analysis. Real-time PCR was performed to assess IL-6, IL-1a, RANKL and sost gene expression utilizing TaqMan™ Gene Expression Assays (Thermo Fisher Scientific) for IL-6, IL-1a, RANKL, and sost, respectively, with GAPDH (Thermo Fisher Scientific) as a housekeeping gene. Assays were run on QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific). Samples were run in technical duplicates. Cycle threshold (CT) values from technical duplicates were averaged together. Ct values from day 0 were utilized as a baseline for each animal and gene, and the delta-delta Ct method was used to determine relative fold gene expression through time.

SA Recovery and Analysis
Cultures obtained from bone and bordering soft tissues were submitted for aerobic and anaerobic culture and observed each day for five total days. The following agar plates were inoculated: Columbia Blood agar with 5% sheep blood and Columbia Naladixic Acid (CNA) with 5% sheep blood agar (35°C, 5% CO₂), MacConkey II (35°C, ambient), CDC and Phenylethyl alcohol (PEA) (35°C, anaerobic). Isolated colonies were then Gram stained and identified via matrix-assisted laser desorption/ionization coupled with time-of-flight mass spectrometry (MALDI-TOF) (Bruker). ASTs for SA isolates were performed via Kirby-Bauer (KB) and repeated via minimum inhibitory concentration (MIC) (ThermoFisher Scientific). KB: Overnight subculture was emulsified in 0.85 saline to reach a 0.5 McFarland. The dilution was swabbed on a Mueller-Hinton (MH) plate, allowed to dry for 10-15 minutes, and had discs added, all per Clinical and Laboratory Standards Institute guidelines (CLSI Vet 01). Plates were incubated for 24 hours at 35°C ambient. Zones of inhibition (ZOI) were measured (mm) and documented as susceptible, intermediate or resistant (S/I/R). MIC: Following CLSI Vet 01, overnight subculture was emulsified in sterile water to reach 0.5 McFarland; 30µL added to 11.0 ml MH broth; 50µL added to each well of COMPGP plate and incubated for 24 hours at 35°C ambient. MIC plates were read using BioMic. Additionally, whole genome sequencing (WGS) was performed on recovered SA isolates to determine individual isolate characteristics.

Following culture, DNA extraction was performed on culturette swabs utilizing DNeasy kits (Qiagen, Hilden, Germany). Quantity and quality of DNA was assessed via NanoDrop Spectrophotometer and samples were saved at -20°C. PCR analysis (real-time PCR) was performed to investigate presence of SA according to the University of Tennessee College of Veterinary Medicine Immunology and Virology Laboratory
procedure. Samples were run in technical duplicates on the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific). Ct values from technical duplicates were averaged together and values were interpreted to be positive or negative for the presence of SA DNA from the culturette swab.

**Micro-Computed Tomography**
Right hemimandibles were imaged using a micro-CT specimen scanner (μCT 35, Scanco Medical; Bassersdorf, Switzerland). Scan parameters were 55kVp, 145μA, 300msec exposure time, average of 3 exposures per projection, 0.5 mm aluminum filter, 500 projections per 180 degrees and a 10 micron voxel size. The raw images were calibrated using a hydroxyapatite (HA) phantom of varying HA concentrations. Noise in the images was reduced by use of a low-pass Gaussian filter. A threshold of 380mgHA/cc was used to partition mineralized tissue from other less-dense tissues. Bone volume fraction (BV/TV) was determined by dividing the number of voxels more dense than the threshold representing mineralized tissue (BV: bone volume) by the total number of pixels in the region (TV: total volume). The mean density of all material in the volume is apparent bone mineral density (aBMD). The mean density of only the mineralized material is the tissue mineral density (TMD). μCT was performed by the University of California, Davis Veterinary Orthopedic Research Laboratory.

**Histological Analysis**
Excess soft tissue was trimmed from formalin-fixed hemimandibles (if applicable). Hemimandibles were decalcified in Formical-2000 for 48 hours until they could be sharply dissected with minimal resistance. After decalcification, hemimandibles were transferred to 10% NBF and submitted for histology along with recovered soft tissue (University of Tennessee College of Veterinary Medicine, Veterinary Diagnostic Laboratory, Histopathology Service). Tissues were embedded within paraffin, 4μm decalcified sections were obtained and stained with Hematoxylin and Eosin (H&E). Slides were evaluated qualitatively and scored in a binary fashion, recording the presence or absence of osteomyelitis. Osteomyelitis was characterized by suppurative or degenerative neutrophilic inflammation, inflammatory infiltrates into bone tissue, and bone destruction or necrosis.

**Statistical Analysis**
IL-6 ELISA Data: The effects of strains, time and their interaction on IL-6 concentration were evaluated using mixed model analysis for repeated measures with time as the repeated factor. Rank data transformation was applied when diagnostic analysis on residuals exhibited violation of normality and equal variance assumptions using Shapiro–Wilk test and Levene's test. Post hoc multiple comparisons were performed with Tukey’s HSD. Statistical significance was identified as p-values (alpha-error) at < 0.05. Analyses were conducted in SAS 9.4 TS1M7 (SAS institute Inc., Cary, NC). Vancomycin elution data: The effects of treatment, dose and time on response variable total vancomycin were examined using mixed model analysis for repeated measures. Ranked transformation was
applied when diagnostic analysis on residuals exhibited violation of normality and equal variance assumptions using Shapiro–Wilk test and Levene’s test. Post hoc multiple comparisons were performed with Tukey’s adjustment. Statistical significance was identified as p-values (alpha-error) at < 0.05. Analyses were conducted in SAS 9.4 TS1M4 (SAS institute Inc., Cary, NC). PCR cytokine data: effects of group and week on delta delta CT of the genes were analyzed using repeated measures ANOVA respectively, with group as the between-subject factor while week as the repeated factor. Diagnostic analysis was conducted to exam model assumptions. Ranked transformation was applied if diagnostic analysis exhibited violation of normality and equal variance assumptions. Post hoc multiple comparisons were performed with Tukey’s adjustment. Statistical significance was identified at the level of 0.05. Analyses were conducted in SAS 9.4 TS1M7 for Windows 64x (SAS institute Inc., Cary, NC). Micro-CT data: Two-way mixed effect ANOVA model was utilized to analyze the effect of treatment, ROI and the treatment by ROI interaction on TV, BV, BV/TV ratio, apparent bone mineral density, and tissue bone mineral density, with treatment as the between subject factor and ROI as the within subject factor (SAS 9.4, PROC GLIMMIX). The least square means computed and separated using the Fisher Least Significant Difference (LSD) Method. A Shapiro-Wilk W and QQ normal plot were used to evaluate the normality of the ANOVA residuals. The variable TV was taken rank transformation because of non-normality of TV. P<0.05 was considered as significant. SAS Institute Inc., Cary, NC. version 9.4, release TS1M7 was used for all analysis.

Results

In Vitro Characterization
Apoptosis Assay
Results (Figure 5.1) are reported as mean percent of cells undergoing apoptosis when counting 10,000 cell events. USA300 appeared to induce a more severe apoptotic effect (22.1% at 4hr, 80.6% at 20hr) compared to ST398 (30% at 4hr, 64.2% at 20hr). Cells infected with Cowan1 underwent apoptosis to a lesser extent (15% at 4hr, 44.1% at 20hr). Uninfected cells acted as a control (9% at 4hr, 35% at 20hr).

Inflammatory Cytokine Analysis
Results (Figure 5.2) are reported as average IL-6 concentration (pg/mL⁻¹). Cowan1 infection initiated a strong initial inflammatory response that ebbed by 48 hours (108.84 at 8hr, 206.89 at 24hr, and 108.84 at 48hr). USA300 showed an initial increase in inflammation that slightly decreased (71.75 at 8hr, 136.6 at 24hr, 104.31 at 48hr). ST398 showed inflammation that initially decreased followed by a marked increase by the end of the study period (105.17 at 8hr, 85.89 at 20hr, and 125.98 at 48hr). There was a significant difference between Cowan1 and ST398 at 24 hours, with Cowan1 demonstrating greater concentrations of IL-6 (p=0.0342). Otherwise, no significant differences were detected.
Osteoregulatory Cytokine Analysis
Results are reported as average OPN and OPG concentration (pg/mL\textsuperscript{-1}). Control samples expressed OPN and OPG at consistent levels throughout the study period (OPN: 3851.96 at 0d, 3906.41 at 7d, 2643.44 at 14d, and 3920.3 at 21d, OPG: 2069.09 at d0, 2832.12 at d7, 2093.18 at d14, and 2592.58 at d21). Cowan1 infection expressed OPN and OPG only on day 0 (3887.7, 2166.21). USA300 infection expressed OPN and OPG only on day 0 (3898.81, 2107.42). ST398 infection expressed OPN and OPG only on day 0 (3878.63, 2185).

Transmission Electron Microscopy
Proportions of intracellular and extracellular SA differed between strains (Figure 5.3). Additionally, morphologic differences were observed between SA strains within infected pre-osteoblastic cells. Cowan1 appeared lowly infective, with few intracellular bacteria and no observed extracellular bacteria. Intracellular Cowan1 displayed distorted shapes. USA300 appeared to be highly infective, with many infected pre-osteoblastic cells. Many infected cells contained more than one bacterial organism. Extracellular USA300 was observed. There was also evidence of USA300 bacterial organisms along interrupted pre-osteoblastic cellular membranes. These likely represent extension of filapodia to incorporate USA300 into the cell [49] as well as cell rupture. ST398 bacterial organisms with undisturbed morphology were observed within pre-osteoblastic cells, confirming the ability of ST398 to establish intracellular infection. No infected cell contained more than one bacterial organism. Extracellular ST398 was observed. Many extracellular ST398 were in contact with pre-osteoblastic cellular membrane and were encircled by membranous material.

Vancomycin Elution from Bio-Oss® Collagen, In Vitro
Bio-Oss® Collagen (3x2mm wafers) were able to load 45μL of vancomycin, (25 or 50mg/mL). Wafers loaded with 1.12mg and 2.25mg of vancomycin eluted vancomycin at concentrations above 0.1μg/mL for 24 hours, at which point concentrations dropped below the limit of quantification (0.1μg/mL) and were not detectable from day 4 (1.12mg) and day 6 (2.25mg) onwards (Figure 5.4). Bio-Oss® Collagen loaded with 1.12mg of vancomycin (n=3) eluted an average of 0.27 ± 0.02mg of vancomycin over a 24-hour period with peak elution of 0.22 ± 0.04mg occurring at three hours. This peak elution is equivalent to eluting 19.77 ± 0.004% of the loaded vancomycin within the first three hours. Bio-Oss® Collagen loaded with 2.25mg of vancomycin (n=3) eluted an average of 0.38 ± 0.02mg of vancomycin over a 24-hour period with peak elution of 0.29 ± 0.01mg occurring at three hours. This peak elution is equivalent to eluting 13.32 ± 0.47% of the loaded vancomycin within the first three hours.

In Vivo Challenge
Animal Observation and Care
One anesthetic complication occurred, resulting in the death of one rat. Remaining animals (n=23) recovered well from surgery. Adjusted for the loss of one animal, group sizes were as follows: Groups I, II and IV, n=6, Group III, n=5. In the first four days
following surgery, animals experienced variable degrees of soft tissue swelling localized to the right mandible. Animals in Group I experienced mild to moderate focal swelling while animals in Group IV experienced moderate to severe swelling generalized to the right mandible. In two animals (rats 21 and 23), the severe, generalized soft tissue swelling to the right mandible caused substantial blepharospasm of the right eye. These animals had mild soft tissue swelling that persisted throughout the study period. They also developed circular areas (2-3mm diameter) of erythema, alopecia and moisture on the skin overlying the surgical site. Throughout the study period, animals ate and drank well. With the exception of animals experiencing severe and generalized swelling, animals appeared subjectively comfortable post-operatively. Once the severe swelling resolved, those animals appeared comfortable as well. Objectively, animals gained weight throughout the study and interacted appropriately with their environment.

**SA Recovery and Characterization**

SA was identified in four rats (Group IV, n=3; Group III, n=1) by in vitro culture or PCR analysis. Microbiologic cultures yielded positive SA cultures from the bone of two animals from Group IV (rats 18 and 21), along with incidental organisms, including: *Escherichia coli, Staphylococcus xylosus, and α-Streptococcus*. PCR analysis demonstrated the presence of SA in the bone of two animals from Group IV (rats 21 and 23) and one from Group III (rat 14), and the soft tissues of two animals from Group IV (rats 21 and 23). This work used the *S. aureus* MLST database at the University of Oxford (https://pubmlst.org/saureus) for whole genome multilocus sequence typing [50]. All isolates were identified as ST398. Ultimately, four (23.5%) out of 17 inoculated rats developed clinical, histologic or microbiologic evidence of SA ST398 osteomyelitis. Of the four SA+ rats, one was in Group III, having been inoculated with SA in the presence of low dose vancomycin (one out of five Group III rats; 20%); three were in Group IV, having been inoculated with SA without antibiotics (three out of six Group IV rats; 50%). The recovered SA had identical antimicrobial susceptibility profiles to the inoculated SA (Table 5.1).

**Cytokine Analysis**

Delta delta C_t values were utilized to determine relative fold gene expression through time. Sclerostin (Sost) was poorly expressed across groups and throughout time. RANKL expression (Figure 5.5) appeared to increase at weeks two and three and decrease at week four in all groups except the Group I, where RANKL expression did not appear to vary through time. IL-6 expression (Figure 5.6) appeared to follow a similar pattern to RANKL expression, increasing at weeks two and three and decreasing again at week four although there were no significant differences noted between groups, through time. IL-1a expression (Figure 5.7) in Group IV was significantly greater at week three compared to week one (p=0.0003) and significantly greater at week three than week four (p=0.0228).

**Micro-CT Analysis**

Micro-CT images, 30 days after creation of the bone defect, demonstrated consistent defect placement with variations in defect size and bone erosion, most prominent within
Groups II-IV. Group III had significantly greater apparent bone mineral density (aBMD) compared to Groups I and IV (p=0.009 and p=0.0467). For remaining parameters of total bone mineral density (tBMD), bone volume (BV) and total volume (TV) there were significant differences between intact and defect bone (p=0.0002, p<0.0001, and p<0.0001, respectively), but no significant differences between treatment groups (Table 5.2).

**Histological Analysis**

Severe, necrotizing osteomyelitis was detected in one sample from a rat in Group III that was SA culture negative and PCR positive for SA in bone. Osteomyelitis in this sample was characterized by widespread suppurative inflammation, with areas of necrosis, bone destruction and extension of suppurative inflammation into the root of the right mandibular incisor (tooth 401). Osteomyelitis was not detected in the histopathology slides from other samples (Figure 5.8).

**Discussion**

Investigating the patho-mechanisms and pathogenic capabilities of SA sequence types as they emerge and cause disease will aid in trend recognition and contribute valuable information to our understanding of disease pathogenesis, potentially even guiding therapeutic strategies. ST398 has now been recognized as an important human pathogen, despite originating in livestock such as cattle and sheep [32]. ST398 was isolated by our laboratory from goats suffering hypertrophic osteomyelitis following orthopedic surgery. This finding alone is significant, as there are few reports of ST398 affecting goats [35,51]. This finding in conjunction with the knowledge that SA osteomyelitis is a devastating, progressive disease that persists despite modern therapeutics [12,15], warrants an investigation into strain-related disease characteristics. In this study, we were able to assess pathogenic mechanisms for ST398 interaction with immature osteoblasts, establish a rat mandible bone defect model for the study of ST398 osteomyelitis using a commercially available bone particle/collagen implant, demonstrate that ST398 could establish infection in the presence of low dose vancomycin, and suggest that delivery of higher concentrations of vancomycin using the commercially available implant was effective in preventing bacterial colonization of the bone and soft tissues.

*In vitro* characterization demonstrated the ability of ST398 to invade immature osteoblast cells in cell culture and establish intracellular reservoirs of bacterial organisms. Intracellular sequestration of SA is recognized as a potential contributor to recurrent or chronic osteomyelitis [18], although specific mechanisms and duration of intracellular persistence are currently poorly understood. It is possible that intracellular invasion of osteoblastic cells may occur only briefly, as a mechanism to evade initial immune system detection. It is hypothesized that invaded cells may then be triggered to undergo apoptosis, ultimately contributing to a pro-inflammatory state and dysregulation of bone homeostasis through altered cell signaling [18,52]. To this effect, our *in vitro* investigation demonstrates the ability of SA to induce apoptosis of MC3T3-E1 cells in cell culture, and
stimulate production of IL-6, with subtle variation between strains, suggesting that there may be strain dependent differences in virulence of SA causing osteomyelitis. IL-6 has been found to be similarly useful in diagnosis of chronic osteomyelitis as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) [53]. There remains a question of the specific role of IL-6 in SA infection and clinical osteomyelitis; whether IL-6 production is protective of the affected bone tissue, recruiting the innate immune system to respond to infectious and inflammatory stimuli, or if IL-6 production may contribute to inflammatory stimuli that trigger progressive bone destruction [54]. Our in vitro results demonstrate IL-6 production from SA infected cells throughout a 48-hour period, paralleled by initial production of OPN and OPG, regulators of bone destruction and formation, respectively. These results suggest that an initial production of IL-6 may reflect a protective effect on osteoregulatory mechanisms. It is also possible that the infective dose of SA within the experimental design did not induce the degree of intense inflammation required to induce IL-6 mediated osteodysregulation. A particularly intriguing discovery on TEM imaging was extracellular USA300 and ST398. There were multiple USA300 bacteria that appeared alongside a discontinuous MC3T3-E1 cellular membrane. These findings may represent the previously documented phenomena of extended filipodia to engulf the bacteria [49]. Discontinuous cellular membranes may also represent apoptotic cells releasing bacteria, or impairment of cell membrane integrity secondary to fixation and sample processing. Artifact secondary to fixation and processing is considered to be less likely, due to the low frequency with which discontinuous cell membranes were observed. Numerous ST398 cells were located extracellularly. Those that were located in closer proximity to MC3T3-E1 cells were observed to have halos of material matching the appearance of cell membrane surrounding them. This likely represents a component of the engulfment process, although a specific mechanism cannot be identified and described solely via TEM. Taken together, in vitro results confirm the ability of ST398 to invade osteoblastic cells and induce apoptosis and IL-6 expression similar to USA300, a well-recognized human bone pathogen.

An in vivo challenge was performed to investigate the capability of ST398 as an interspecies bone pathogen. To accomplish this, we evaluated the ability of ST398 to induce osteomyelitis in a rat mandible defect model. This model, based upon previously published literature, and fulfilling the main tenants of an animal model of osteomyelitis, had a success rate of 50% in rats inoculated with SA but not treated with any antibiotic and 20% of rats challenged with SA and a low dose of antibiotic. The recovered SA had identical antimicrobial susceptibility profiles to the inoculated SA. Affected animals expressed IL-6 and RANKL over the study period, demonstrating increases in IL-6 expression at weeks two and three, and RANKL expression that increased, followed by a return closer to baseline by week four. Expression of IL-6 and RANKL, although demonstrating no statistically significant differences between experimental groups, again highlight the intersection of inflammation and bone remodeling. When observed, histological evidence of osteomyelitis was quite pronounced and confirmed the ability of ST398 to induce inflammation as well as bone remodeling and destruction. The chosen
model, while fulfilling the main tenants of animal modeling, i.e. bone trauma, local inoculation of a high dose of planktonic bacteria and placement of foreign material, was inconsistent in establishing clinical, microbiologic, and histological osteomyelitis. This may reflect the need to alter the experimental protocol, either by including a sclerosing agent [55], utilizing a higher bacterial inoculation dose to overcome the impressive ability of the rat’s immune system to clear acute peripheral infections [56], or may accurately reflect an inconsistent ability of ST398 to induce osteomyelitis in the rat.

Limitations

Histological examination was minimally rewarding, which is thought to be due in part to tissue sectioning. Authors suggest sectioning hemimandibles coronally to separate incisor teeth from caudal bone tissue. This may improve sectioning of tissues and provide more accurate slices of bone tissue within the ROI. Recovery of SA from the animals required a combination of culture and PCR and samples from bone and soft tissues.

Conclusions and Future Directions

Understanding *Staphylococcal* strain differences is incredibly valuable. SA is a dangerous, versatile human pathogen that may transition from commensal organism [3] to disease-causing agent, either within the same individual, between individuals [57], or cross-species [32]. This work demonstrates the ability of ST398 to establish intracellular infection, drive apoptosis and create a pro-inflammatory state *in vitro*. TEM imaging displays intriguing cell membrane activity that may be indicative of cellular internalization mechanisms. In an *in vivo* rat model, ST398 is an inconsistent driver of osteomyelitis, although when successfully established, is capable of causing clinical, microbiologic and histologic evidence of osteomyelitis. Given the emergence of ST398 as a dangerous, sometimes lethal, human pathogen, further investigation of pathogenesis is warranted. This may include an *in vivo* dose curve of ST398 in comparison to a more widely characterized strain, such as USA300 or UAMS1 to establish more clearly the interspecies potential of ST398 to establish osteomyelitis. Once established, there may be value in investigating for intracellular bacteria from harvested bone, whether that is through TEM or *in vivo* monitoring techniques to evaluate viability of bone cells. Additionally, authors suggest utilizing microbiology techniques in conjunction with PCR, to improve sensitivity of SA detection.
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APPENDIX V

Table 5.1. Antimicrobial Susceptibility of SA ST398 Isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ZOI (mm)</th>
<th>Category</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>N/A</td>
<td>R</td>
<td>4</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>6</td>
<td>R</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>6</td>
<td>R</td>
<td>&gt;54</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>22</td>
<td>S</td>
<td>0.87</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>27</td>
<td>S</td>
<td>0.3</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10</td>
<td>R</td>
<td>&gt;33</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>N/A</td>
<td>S</td>
<td>&lt;=1</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>N/A</td>
<td>S</td>
<td>&lt;=1</td>
</tr>
</tbody>
</table>

Antimicrobial susceptibility test (AST) results for ST398 isolate utilized for in vitro and in vivo experiments. AST values of recovered ST398 isolates (from rats 18 and 21) and inoculated ST398 did not differ.
Figure 5.1. Apoptosis assay. Flow cytometry annexin staining for early (4hr) and late (20hr) apoptosis. USA300 appeared to induce a stronger apoptotic affect compared to ST398. Cells infected with Cowan1 underwent apoptosis to a lesser extent. Uninfected cells served as the control.
**Figure 5.2.** Average IL-6 expression. MC3T3-E1 cells were inoculated with SA strains ST398, USA300 or Cowan1. Cowan1 showed an initial strong inflammatory response that lessened at 48 hours. USA300 became stronger over time. ST398 showed an ebb followed by an increase in inflammatory response. IL-6 expression by Cowan1 at 24 hours was significantly greater than IL-6 expression at 24 hours by ST398. No other significant differences were detected.
Figure 5.3. TEM images. Images obtained from MC3T3-E1 cells infected with SA strains Cowan1 (A), USA300 (B-D), and ST398 (E, F). (A): single intracellular Cowan1 organism (black arrow), distorted in shape. (B): one osteoblastic cell infected with multiple USA300 organisms (black arrows). (C, D): USA300 organisms bordering osteoblastic cells. Thick black arrows denote discontinuous cellular membranous material which may indicate incorporation of USA300 into the cell by filipodia or may indicate disrupted cellular membranes. (E): one intracellular and one extracellular ST398 organism. (F): Extracellular ST398 organisms. One ST398 bordering an osteoblastic cell, surrounded by a halo of membranous material. Scale bar = 0.7μm.
Figure 5.4. Vancomycin elution from Bio-Oss® Collagen, *in vitro*, over time. Devices (n=3) were loaded with either 1.12mg (low dose) or 2.25mg (high dose). Devices demonstrate a release profile characterized by an initial burst release occurring up to 12 hours. This profile can be translated to a similarly rapid *in vivo* release.

Figure 5.5. RANKL expression. RANKL expression through time expressed as relative fold gene expression by experimental group. RANKL expression trends higher at weeks two and three and declines at week four. No significant differences were detected between groups through time.
Figure 5.6. IL-6 expression. IL-6 expression through time expressed as relative fold gene expression by experimental group. IL-6 expression follows a similar trend to RANKL expression, trending higher at weeks two and three and declining at week four. Data appears skewed as a result of Group III (rat 14) that had greater IL-6 expression at all timepoints although between groups there were no significant differences detected.

Figure 5.7. IL-1a expression. IL-1a expression through time expressed as relative fold gene expression by experimental group. IL-1a expression in Group IV was significantly greater at week three compared to week one (p=0.0003) and significantly greater at week three than week four (p=0.0228).
Table 5.2. Micro-CT Comparison of Intact and Defect Bone

<table>
<thead>
<tr>
<th>Defect Bone</th>
<th>aBMD [mg HA/ccm]</th>
<th>tBMD [mg HA/ccm]</th>
<th>BV (mm$^3$)</th>
<th>BV/TV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>2372.40 ± 610.0</td>
<td>6677.54 ± 267.9</td>
<td>4.52 ± 1.47</td>
<td>0.32 ± 0.10</td>
</tr>
<tr>
<td>Group II</td>
<td>1847.43 ± 735.03</td>
<td>6819.92 ± 626.35</td>
<td>3.36 ± 1.78</td>
<td>0.24 ± 0.12</td>
</tr>
<tr>
<td>Group III</td>
<td>2408.62 ± 821.36</td>
<td>6779.74 ± 741.55</td>
<td>4.54 ± 1.88</td>
<td>0.32 ± 0.13</td>
</tr>
<tr>
<td>Group IV</td>
<td>2022.78 ± 728.32</td>
<td>6478.24 ± 839.95</td>
<td>3.93 ± 1.71</td>
<td>0.28 ± 0.12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intact Bone</th>
<th>aBMD [mg HA/ccm]</th>
<th>tBMD [mg HA/ccm]</th>
<th>BV (mm$^3$)</th>
<th>BV/TV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>2267.36 ± 389.49</td>
<td>5926.12 ± 199.04</td>
<td>1.32 ± 0.27</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td>Group II</td>
<td>3070.02 ± 1519.49</td>
<td>6290.22 ± 656.75</td>
<td>1.73 ± 0.80</td>
<td>0.43 ± 0.20</td>
</tr>
<tr>
<td>Group III</td>
<td>3459.6 ± 1151.21</td>
<td>6448.25 ± 422.55</td>
<td>1.98 ± 0.16</td>
<td>0.49 ± 0.16</td>
</tr>
<tr>
<td>Group IV</td>
<td>2637.06 ± 620.20</td>
<td>5844.45 ± 325.13</td>
<td>1.59 ± 0.38</td>
<td>0.39 ± 0.09</td>
</tr>
</tbody>
</table>

Group III had significantly greater apparent bone mineral density (aBMD) compared to Groups I and IV (p=0.009 and p=0.0467). For remaining parameters of total bone mineral density (tBMD), bone volume (BV) and total volume (TV) there were significant differences between intact and defect bone (p=0.0002, p<0.0001, and p<0.0001, respectively), but no significant differences between treatment groups.
Figure 5.8. Micro-CT and histopathology images. (A–E): Images (A – Micro-CT, B–E – Histology) collected from right hemimandible that tested positive for SA (Group III; rat 14). (A): Micro-CT reconstructed image of defect area within mandible. Bone particles within irregularly shaped defect indicate presence of Bio-Oss® Collagen. (B): H&E-stained section of hemimandible, demonstrating mandibular bone (pink tissue) with severe, infiltrative inflammation (black arrow). Double-headed arrow highlights areas of necrotic bone. Findings are supportive of osteomyelitis. 5x magnification. (C): 10x magnification of identical bone section. Double-headed arrow highlights areas of necrotic bone. (D): 20x magnification of identical bone section. Demonstrates areas of necrotic bone (dark purple), amid bone tissue (pink) that is infiltrated and distorted by inflammatory infiltrates. (E): 40x magnification of identical bone section, demonstrating infiltration and distortion of bone by inflammatory infiltrates amid areas of necrosis (light pink). (F): H&E-stained histology section, 5x magnification, of right hemimandible that was culture negative for SA (Group II, rat 10). Healthy mandibular bone (pink) surrounds a defect area containing Bio-Oss® Collagen (thick arrow = bone particles) that has been infiltrated by blood vessels.
CHAPTER VI: CONCLUSION
Chronic bacterial infection, particularly *S. aureus* osteomyelitis, is a significant cause of patient morbidity and mortality. Despite intensive interventions aligned with current medical standards, bacterial infection is often persistent, and plagues affected individuals. The work described in the preceding chapters is a thorough compilation of literature and scientific exploration focused on the utilization of locally implantable drug delivery devices to delivery antimicrobials and regenerate tissues to improve treatment strategies for chronic bacterial infection, specifically *S. aureus* osteomyelitis.

Chapter I provides a thorough synthesis of historic, current, and prospective materials and systems utilized as local drug delivery and tissue regeneration (dual platform) devices. This chapter highlights shortcomings in current devices and discusses potential of emerging systems such as nanotechnology to overcome current limitations by improving tunability and specificity of *in vivo* device action. Chapter II provides a comprehensive overview of animal models to advance osteomyelitis research, fills a void in synthesis of rodent literature through creation of a detailed table of rat osteomyelitis models, and discusses principles of animal osteomyelitis model development. Chapter III compares three different *in vitro* drug elution protocols utilizing controlled device and drug parameters and provides framework to select *in vitro* drug elution protocols and interpret the generated data.

Chapter IV details a thorough investigation of a commercially available collagen matrix for utilization as a local drug delivery device in an effort to bypass a major hurdle of device production, which is efficient production of effective, consistent material. This investigation demonstrated *in vitro* cytocompatibility, adequate drug release kinetics utilizing gentamicin, and no significant differences in tissue integration scores between native and gentamicin-loaded devices in an *in vivo* rat mandible defect model. Chapter V built upon the previous chapters and translated the rat mandible defect to model bacterial osteomyelitis utilizing *S. aureus* ST398. This chapter provides further understanding of ST398, an emerging human pathogen, as a pathogen of osteomyelitis.

Ultimately, this work synthesized the historical advances, current practices, and emerging strategies to study and combat chronic bacterial infection, specifically *S. aureus* osteomyelitis; and made significant contributions, including a framework to develop *in vitro* drug elution protocols, a consistent surgical mandibular defect model in the rat, and demonstrated the ability of *S. aureus* ST398 to establish inflammation and intracellular infection *in vitro* and to establish osteomyelitis cross-species in a rat model.
VITA

Caroline Billings earned her Doctor of Veterinary Medicine degree from the University of Tennessee College of Veterinary Medicine in 2020 where she was enrolled in the dual DVM-PhD program. After graduating with her DVM, she became a full-time graduate student under the mentorship of David E. Anderson. Her work in graduate school has focused on the clearance of chronic bacterial infection utilizing locally implantable drug delivery devices, with a special focus on infections caused by *Staphylococcus aureus*. During her time in graduate school, Caroline has generated three first author publications, has additional first author and co-author manuscripts in progress, and has presented a collection of posters and oral presentations at local and regional conferences.