2014

BIOERODIBLE CALCIUM SULFATE BONE GRAFTING SUBSTITUTES WITH TAILORED DRUG DELIVERY CAPABILITIES

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BIOERODIBLE CALCIUM SULFATE BONE GRAFTING SUBSTITUTES WITH TAILORED DRUG DELIVERY CAPABILITIES

DISSERTATION

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

By
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2014

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ABSTRACT OF DISSERTATION

BIOERODIBLE CALCIUM SULFATE BONE GRAFTING SUBSTITUTES WITH TAILORED DRUG DELIVERY CAPABILITIES

Bone regeneration or augmentation is often required prior to or concomitant with implant placement. With the limitations of many existing technologies, a biologically compatible synthetic bone grafting substitute that is osteogenic, bioerodible, and provides spacing-making functionality while acting as a drug delivery vehicle for bioactive molecules could provide an alternative to ‘gold standard’ techniques.

In the first part of this work, calcium sulfate (CS) space-making synthetic bone grafts with uniformly embedded poly(β-amino ester) (PBAE) biodegradable hydrogel particles was developed to allow controlled release of bioactive agents. The embedded gel particles’ influence on the physical and chemical characteristics of CS was tested. Namely, the compressive strength and modulus, dissolution, and morphology, were studied. All CS samples dissolved via zero-order surface erosion consistent to one another. Compression testing concluded that the amount, but not size, of embedded gel particles significantly decreased (up to 75%) the overall mechanical strength of the composite. Release studies were conducted to explore this system’s ability to deliver a broad range of drug types and sizes. Lysozyme (model protein for larger growth factors like bone morphogenic protein [BMP]) was loaded into PBAE particles embedded in CS matrix. The release of simvastatin, a small molecule drug capable of up regulating BMP production, was also examined. The release of both lysozyme and simvastatin was governed by dissolution of CS.

The second part of this work proposed a bilayered CS implant. The physical and chemical properties were characterized similarly to the CS composites above. Release kinetics of directly loaded simvastatin in the shell, core, or both layers was investigated. A sequential release of simvastatin was witnessed giving foresight of the composite’s tunability. The sequential release
of an antibacterial, metronidazole, loaded into poly(lactic-co-glycolic acid) (PLGA) particles embedded into the shell along with directly loaded simvastatin either in the shell, core, or both layers was also observed. Through controlled release of bioactive agents, as well as a tunable layered geometry, CS-based implants have the potential to be optimized in order to help streamline the steps required for the healing and regeneration of compromised bone tissue.

KEYWORDS:

Calcium Sulfate, Bone Graft Substitutes, Multiple Drug Release, Osteogenic Agents, Antimicrobial Agents

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June 24, 2014
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BIOERODIBLE CALCIUM SULFATE COMPOSITES SCAFFOLDS WITH TAILORED DRUG DELIVERY CAPABILITIES

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Dedication

I would like to dedicate this dissertation in memory of the late Dr. Mark V. Thomas. He was a remarkable person and mentor who provided insight and vision that helped build the foundation of this work.
Acknowledgements

There are many people who have made this dissertation possible through their support and help during my time at the University of Kentucky. First, I would like to thank my mentor, Dr. David Puleo. His advice and support during my time at the University of Kentucky has been invaluable, and without his guidance this would not have been possible. I would also like to thank the rest of my committee members: Dr. Zach Hilt; Dr. Hainsworth Shin; Dr. Thomas Dziubla; and Dr. Todd Milbrandt, M.D. for serving on my committee and providing input on my research.

I would like to thank all my lab mates who have not only provided help around the lab either with my work or in general, but have also been a great support network. Thank you, Dr. Amanda Clark, Dr. Sharath Sundararaj, Dr. Matt Brown, Dr. Yuan Zou, Cheryl Rabek, Matthew Rudd, Michelle Fuentes, Paul Fisher, Sandeep Ramineni, Nick Andersen, Theodora Asafo-Adjei, Rohith Jayaram, and Amir Najarzadeh. There are several other students who have assisted me in some form or another. Thank you, Dr. Ashley Hawkins, Andrew Vasilakes, Prachi Gupta, and Dr. Dipti Biswal, Robert Wydra, and Nathanael Stocke.

I would like to give a special thanks to Brooke Clements for her emotional support and continued encouragement throughout most of my graduate school experience.

Finally, I would like to thank my family for their support during this time. They provided guidance and encouragement that helped fuel my passion and determination throughout this process.
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Chapter 1: Introduction

Bone augmentation is often required prior to, or associated with, implant placement in the oral cavity. With the limitations of many existing technologies, a biologically compatible synthetic bone grafting substitute that provides space-making functionality while acting as a drug delivery vehicle for bioactive agents could provide an alternative to ‘gold standard’ techniques. To begin, Chapter 2 discusses the background of oral and maxillofacial bone augmentation, and conditions that would merit such an operation, such as the most common and destructive inflammatory disease of humans: periodontal disease. The section discusses in further detail the current standard practices involved for treatment and bone regeneration. Next, the discussion describes current advancements in bone graft substitutes, and the use of controlled local drug delivery for better treatment. The chapter finishes by describing the three major specific aims of the research being presented in this dissertation.

The primary goal of this work was to develop a suitable synthetic bone grafting substitute that could be considered a promising alternative to the ‘gold standard’ use of autologous bone tissue. Chapter 3 illustrates the process of development and characterization of a calcium sulfate (CS) / hydrogel composite scaffold. The physical and chemical characteristics were investigated to determine if the introduction of poly(β-amino ester) hydrogel (PBAE) particles, intended for use as a drug delivery vehicle, would have an effect on the overall scaffold when embedded into CS. Degradation, compressive strength, and morphology were examined. This first segment finishes with a small pilot release study to demonstrate the controlled drug delivery potential of these CS composite implants.

To expand on the characterization of CS/PBAE composite implants presented in Chapter 3, the drug delivery potential was further investigated in Chapter 4. The concept for this chapter was to show that the composites were capable of sustaining the release of a broad range of osteogenic drug types and sizes (i.e. larger proteins vs. smaller molecule drugs). The rationale behind this
idea was to be able to tailor the device, whether a larger bone growth factor like bone morphogenetic protein or a small molecule such as simvastatin were to be needed. Various loading methods were utilized (i.e. direct loading into CS or loaded within PBAE particles embedded into CS) to investigate what method would allow for sustained or controlled release of the drug. Because directly loading drug into CS may have an impact on the structure of the composite, mechanical and degradation testing was also conducted.

Chapter 5 presents the development of a bilayered device capable of releasing multiple drugs sequentially. Due to the fact that bone regeneration is slowed in the presence of bacteria following advanced periodontal disease or in any infected bony defect, the release of an antimicrobial followed by an osteogenic drug could allow for a more streamlined treatment. This section starts out describing the similar testing methods performed in Chapter 3 to fully characterize the physical and chemical attributes of the bilayered composites as well as any effects of shell loaded poly(lactic-co-glycolic acid) (PLGA) particles. Next, the release of simvastatin was conducted with the drug loaded into the shell, the core, or both layers of the CS constructs. To allow for further tuning of the implants, the shell to core volume ratio was altered to demonstrate how the release of simvastatin could be further delayed or prolonged from a given layer during dissolution. The study concludes by investigating the sequential release of simvastatin directly loaded into calcium sulfate and metronidazole loaded PLGA particles embedded into the CS shell of the composite.

The development of CS-based composites with controlled release of bioactive molecules has the potential to greatly enhance the ability of these implants to become more effective bone graft substitutes. The introduction of a layered geometry may further allow for bone graft substitutes to be optimized in order to help streamline the multiple steps needed to regenerate tissues, thus becoming a suitable competitor or even an alternative for the use of autografts.
Chapter 2: Background and Significance

2.1. Oral and Maxillofacial Bone Augmentation

Bone augmentation, commonly employed for craniomaxillofacial applications, is the build-up of new bone on an already compromised bony surface using a grafting material to restore proper function, aesthetics, or even provide a stable platform for the insertion of a prosthetic. Currently around the world there is an increasingly high demand for functional bone grafts \[1, 2\]. Each year roughly 2.2 million bone graft procedures are performed worldwide with about half a million patients receiving bone defect repairs in the United States \[1, 3\]. For dental rehabilitation of partially or totally edentulous patients, treatment with oral implants has become common practice in the last decades, with reliable long-term results \[4\]. However, unfavorable local conditions of the alveolar ridge, due to atrophy, periodontal disease and trauma, may provide insufficient bone volume which may render implant placement impossible or unstable from a functional and esthetic viewpoint \[4-6\]. Many different techniques have been considered for augmentation; osteoconduction through the use of a grafting material, osteoinduction through the aid of growth factors, bone distraction, or guided bone regeneration (GBR) where barrier membranes maintain space for which bone to regenerate \[4, 7\]. All of which have resulted in comparable long-term implant survival \[7\].

2.2. Periodontal Disease

Periodontitis is one of the most common and destructive inflammatory diseases of humans, and is a leading cause of tooth mortality in adults \[8, 9\]. This disease is a complex inflammatory infection affecting numerous tissue types, soft and/or hard, within the oral cavity, and is caused by a buildup of bacteria. This buildup is usually due to the lack of proper personal oral hygiene administered by the patient and/or the absence of routine dental checkups. In other words, this disease can be fairly easily avoided, but has the ability to form aggressively. If left untreated the bacteria can form pockets of infection just
below the gum line allowing it to progress causing an innate immune response leading to localized inflammation [10]. This inflammatory condition can lead to a progressive destruction of periodontal tissues, namely alveolar bone, periodontal ligament, and root cementum [11, 12].

There are many different pathogens and microorganisms that play a role in periodontal infections, some of which may be more prevalent than others. For instance, the more significant pathogens associated with periodontitis are Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, and Bacteroides forsythus [9, 13-15]. These pathogens can be located in most areas of the oral cavity, but for the purpose of this research we are focusing on pathogens located in plaque. According to Slots and Jorgensen, they describe dental plaque as a form of biofilm which is defined to be a sessile community of interdependent microorganisms organized within an exopolymer that is attached to solid surfaces [9]. If untreated, plaque will continue to build acting much like a dam forming a pocket where pathogens will be allowed to flourish. This bacterial infection will cause a local inflammatory response. At early stages this inflammation of the gums is also described as gingivitis, a very common infection, affecting 75% of American Adults [16]. According to David Cochran, an amplification of this initial localized response results in the release of an array of cytokines and other mediators leading to the propagation of inflammation through the gingival tissues [10]. If still allowed to proliferate, the chronic inflammatory effect will begin to break down connective tissue and ultimately alveolar bone [10]. Although periodontitis has been found to be reversible, the extensive loss of bone tissue has the potential to lead to the loss of teeth requiring extensive and potentially very expensive regenerative surgeries [9].

Progression of this magnitude will ultimately lead to the loosening or complete loss of teeth. Periodontal disease is also associated with many different systemic effects, such as coronary artery disease, stroke, and even diabetes; all of which risking the increase of heart attack and/or other serious health problems [11, 17, 18]. The gold standard for treating periodontitis is using a regime of frequent professional cleanings, antibiotics to fight the bacterial
infection, and in severe cases, bone augmentation that usually requires multiple extensive surgical procedures to harvest autogenous donor tissue.

To counter the onset of aggressive periodontitis and attempt to control or treat the infection, there are multiple steps that need to be considered. Some of these steps involve improved oral hygiene habits, removal of all bacterial plaque, and eventually treatment with regenerative medicine [8]. The goal of periodontal therapy is to rid the site of bacteria and reduce the inflammatory response by halting cytokines that are integral to the propagation of inflammation resulting in bone resorption, thus stopping the loss of bone and thereby preserving the natural dentition [10, 12]. Depending on the severity of the infection, there are many current but different options that are available. Primarily dentists first rely on extensive debridement, such as scaling and root planning [9]. However, in a previous study described by Slots et al. where nail polish was scaled from extracted teeth, it was shown that the investigator could not fully remove all plaque and bacteria adhered on a tooth’s surface [9]. This study demonstrates the unpredictable outcome of this basic procedure and exemplifies the need for further measures to fully fight periodontal bacterial infection. Some of these other measures include the introduction of antibiotics, and even regenerative treatments using barrier membranes, autografts, demineralized freeze-dried bone allografts, bovine xenografts, and even combinations of membranes and fillers [11].

2.3. Alveolar Bone Regeneration

Currently the desire to create more biological alternatives to the permanent implantation of static synthetic materials for the management of periodontal defects has inspired the field of periodontal tissue engineering and regenerative medicine [8]. There is continuous research being performed to test existing regenerative technologies, both in human clinical trials as well as extensive animal studies to test modifications of existing devices. Many human studies look at the effects of the disease itself using clinically approved technologies. Martin et al. looked at and reported tooth loss during periodontal
treatment where patients were categorized at the inception of treatment by
disease severity and risk level [12]. They concluded that categorizing a patient by
severity may be beneficial in the management of the periodontal patient, and
could give dentists guidelines for the proper treatment from a case by case basis [12].

Augmentation procedures to allow for regeneration of alveolar bone are
extensively tested trying to find the most suitable technology to use for one to
make it easier to perform and ultimately beneficial for the patient’s well-being and
recovery. A human clinical study by Chiapasco et al. demonstrated how various
augmentation procedures can all have similar outcomes [4]. In this particular
study they looked at guided bone regeneration (GBR), onlay bone grafts, inlay
grafts, bone splitting for ridge expansion, distraction osteogenesis, and
revascularized flaps, all of which produced promising results; some slightly better
than the other [4]. In another case study, the combination of natural bone
mineral and guided tissue regeneration was investigated in eight patients with
chronic advanced periodontitis displaying intrabony defects [19]. Windisch and
his colleagues were able to conclude that the use of a guided tissue regenerative
membrane covering a defect filled with natural bone mineral may be suitable for
augmenting alveolar bone [19]. Again, many of these procedures require the use
of donor tissue; whether it was retrieved from the host or alternative sites, there
is still the requirement of a second surgery to harvest this tissue. Since many
alternative regeneration practices appear to have the same effect as donor
tissue, the properties may even be further altered to allow for drug release and
ultimately a much more rapid and healthy recovery. The concept of this project
investigates both of these scenarios.

2.4. Current Approaches

There are many different bone grafts and substitutes that could be used
for bone augmentation. Consideration in selecting a particular device includes
characteristic capabilities, availability, patient morbidity, immunogenicity,
potential disease transmission, and cost variability [20]. In addition the selected
bone graft would ideally be osteogenic, osteoinductive, osteoconductive, provide structural integrity, and have the ability to be osteointegrated to the host bone [20-22].

### 2.4.1. Autografts

Harvesting of autologous bone grafts from the patient tissue has been considered the ‘gold standard’ for augmentation procedures for alveolar ridge recovery [3, 20, 23, 24]. These bone grafts can be one of three different types; trabecular, corticotrabecular, or cortical [25]. Trabecular or cancellous bone tissue has been the most effective. Autologous bone is composed of organic and inorganic structures [25]. The organic component, which is mostly comprised of collagen, provides resilience, toughness, and also continuity [25]. The inorganic portion contributes to the stiffness, hardness, and the rigidity [25]. This component is primarily a mineral known as hydroxyapatite (HA). Within the inorganic mineral matrix osteocytes, osteoclasts, osteoblasts, and osteogenic signaling proteins and other mesenchymal tissues are found. During transplantation very few of the mature osteoblasts within the graft survive, however, osteogenic potential remains due to a sufficient number of precursor cells [22].

Bone can be harvested from a number of locations, such as the iliac crest, ribs, or even from intraoral sites [20, 23]. For oral reconstruction, bone will usually be harvested from within the oral cavity. Depending on the volume of bone needed for a given procedure, tissue can be removed from the maxillary tuberosity, ramus and symphysis on the mandible, or even debris from an implant osteotomy preparation.

Along with the many benefits of using autologous bone, there are many disadvantages. Because the tissue is taken from the patient a second surgery site is required, lengthening the overall surgical procedure. Additionally, there is a limited volume of bone that can be harvested. There are also many potential side effects when harvesting tissue. Patients can experience donor site morbidity caused by a number of factors like blood loss, wound complications,
chronic pain, infection, local sensory loss, and may even lead to cosmetic defects [20, 24, 26].

2.4.2. Allografts

Recently, allografts have become the most frequently chosen substitute accounting for about a third of the bone grafts used in the United States, annually [3, 24]. Allograft tissue is harvested tissue taken from other individuals of the same species; often cadaveric bone. This eliminates the need for a secondary surgical site which greatly reduces surgical time, blood loss, and amount of anesthesia required during the procedure [25]. It also leads to fewer complications like donor site morbidity in the patient. By using cadaveric bone grafts, there is a much higher availability allowing for the tissues to be stored and distributed through tissue banks [24]. Allografts have the same characteristics as autografts, however they lack any osteogenic cells due to processing [22]. These scaffolds come in three different forms; frozen, freeze-dried or lyophilized, and demineralized freeze-dried bone (DFDB). Processing of the grafts once they have been harvested lowers the risk of disease transmission, such as hepatitis B & C, degenerative bone diseases, and STD’s [3]. In addition, processing of the tissue can greatly reduce the biological and mechanical properties which could lead to post-operative complications including fracturing, non-union of the graft, and although the risk has been reduced, host immune response [20, 22, 24].

2.4.3. Xenografts

Similar to allografts, xenografts are also tissues that are harvested from another source. However, these particular grafts are obtained from different species [27]. Common tissue sources are bovine bone mineral and porous HA from coral skeletons [27]. BioOss® (Geistlich AG, Wolhusen, Switzerland) is considered one of the most commonly used bone graft substitute in dentistry [3]. Upon processing to remove all organic material, what remains is a porous HA chip material having good mechanical properties and a structure similar to
human bone, however, shows very little solubility [3, 6, 28]. Coralline HA derived from marine coral has a highly porous and regular skeleton structurally resembling trabecular bone [20]. Most coralline HA is treated with ammonium phosphate and sterilized [20]. This process converts the calcium carbonate skeleton to crystalline HA which can be found as granule or block form [20].

2.4.4. Alloplasts

Alloplastic bone grafts are becoming increasingly popular, however, no perfect substitute yet exists that possess the ideal qualities of an autograft [20]. These types of bone grafts can be either synthetic or deorganified biocompatible materials having a variety of shapes, sizes, and/or textures [25]. Primarily ceramics, alloplasts can be either bioinert or bioactive [25]. Bioinert ceramic grafts comprise of aluminum oxides and titanium oxides. These materials do not bond well to host tissue, therefore they are commonly used as endosteal implants, which can serve as oral abutments for dentures, or orthodontic appliances, and also used for bone fracture fixation [25]. Bioactive ceramics like hydroxyapatite (HA), tricalcium phosphate (TCP), and calcium sulfate (CS) are common bone substitutes [22, 25, 29]. Similar to bone, bioactive ceramics have been shown to have good compressive strength, however, alone they are brittle and have poor tensile strength [20, 25, 29]. When implanted, the ceramics undergo osteoconduction, although the biological response may differ from one implanted bone substitute to another due to differences in resorptive properties [25].

2.4.4.1. Hydroxyapatite and Tri-Calcium Phosphates

Hydroxyapatite is the principal mineral component of bone and very osteoconductive and osteointegrative [20, 22, 25]. It has been used in posterior lumbar fusions, coatings on implants, and external fixator pins to assist in tissue-implant interface [20]. HA has been shown to have a slow rate of resorption in vivo [20, 26].
Tri-Calcium Phosphate (TCP) is structurally similar to HA as well as the mineral phase of bone [20]. When implanted, TCP acts as a bioactive osteoconductive scaffold and integrates well with host tissue [20, 22]. Similar to HA and other phosphates, TCP has been shown to have good resistant to compressive loads, but brittle and weak under tension and shear [22, 30]. Both HA and TCP substitutes are available in paste, putty-like, solid matrix, or granule form [20].

2.4.4.2. Calcium Sulfate
Calcium sulfate (CS) has long been recognized as an osteoconductive and an osteointegrative biomaterial with an excellent reputation as a biocompatible substance [31-36]. In fact, it was one of the first bone substitutes used in orthopedics and dentistry [35]. It has been called upon for a variety of applications, such as long bone defects including osseous cavities related to tumors and cysts, and has been successfully used for craniofacial applications such as periodontal defects, alveolar bone loss recovery, and maxillary sinus augmentation [28, 37]. CS has similar mechanical strength to that of cancellous bone [38]. In vivo, CS is well tolerated, having the unique ability to become osteogenic in the presence of bone and completely absorbed by the host without inducing a significant inflammatory response [31, 33-35]. Characteristics of CS have been developed to closely match the rate of resorption by dissolution with the rate of new bone integration [36]. The rapid dissolution of CS leaves behind a calcium phosphate scaffold which helps promote osteogenic activity allowing for induce bone formation after two weeks in dogs and completely resorption after about one month [28, 39].

2.4.5. Hybrid Bone Scaffolds
Hybrid scaffolds have been given much attention because these 3-dimensional bone bioactive scaffolds can be fabricated from a variety of biomaterials such as bio-ceramics like the ones mentioned above and biodegradable polymers, natural or synthetic [40]. A number of synthetic and
natural polymers, as well as ceramics have been developed as bone tissue engineered biomaterials [1]. Because ceramics are very brittle and polymers have a weaker compressive modulus compared to native bone, combinations represent a promising alternative through the optimization of their physical and chemical properties to better mimic the tissue being replaced [40]. Many groups have developed different hybrid scaffolds; many of which have come with great success. Cao et al. developed a polyglycolic acid and beta-tricalcium phosphate scaffold [41]. They were successful in showing the scaffold had degradation rates similar to the osteogenic rate and was completely replaced by new growing bone. A hybrid system comprised of polycaprolactone and beta-tricalcium phosphate developed by Lu et al. demonstrated the tailorable physical properties of these types of scaffolds through mechanical stability throughout biodegradation [42]. In addition to the ability to produce composite formulations by utilizing advantageous properties of multiple materials, these biomaterials could be further enhanced by integrating biomolecules that would allow for the scaffold to be more osteoinductive [40].

2.5. Enhanced Bone Graft Substitutes: Bioactive Molecules

Many groups are developing synthetic grafting substitutes to be suitable alternatives to the current standard use of autografts. In doing so, several performance criteria must be met. The synthetic bone graft substitute need to be biocompatible, osteoconductive, osteointegrative to help create a secure bond with the surrounding host tissue, and mechanically stable to ensure maintenance of surgical site being augmented. Many currently used alloplasts, such as HA, TCP and CS, have demonstrated their ability to fulfill these criteria [41, 43, 44]. However, for a synthetic material to be considered a suitable replacement for autografts, the grafting material also needs to be osteoinductive, and osteogenic. Synthetic bone graft substitutes may be further improved using bioactive agents like growth factors or other drugs capable of inducing new bone formation [45]. Studies have shown enhancement of synthetic bone grafts through the release of bioactive molecules [45-47]. There are many growth factors that have been
studied for their potential use for bone regeneration. Growth factors can act as mitogens in that they enhance proliferation of certain cell types [48]. In addition, some of these factors can be considered morphogenetic because of their ability to change the phenotype of their target cells [48]. First introduced by Dr. Marshal Urist in the 1960’s, bone morphogenetic proteins (BMP) have been extensively studied for their possibility of replacing the need for autografts with an engineered synthetic material capable of delivering these proteins [49]. The bone morphogenetic protein family consists of BMP-2 through BMP-8, which are dimeric molecules belonging to the transforming growth factor-β (TGFβ) supergene family [48-50]. The TGF-β family is named for its ability to transform fibroblastic cells in monolayer culture and to stimulate colony formation [14]. BMPs are directly involved in the differentiation of cartilage and bone by controlling tissue induction and morphogenesis [49-51]. The most effective BMPs in terms of osteogenesis are BMP-2, BMP-4, and BMP-7 [48]. Introducing growth factors into grafting devices can induce rapid new bone formation, allowing for a reduction in healing time to achieve sufficient new bone volume for proper anchoring of an inserted implant [47, 52]. Other growth factors have been commonly considered due to their influence in skeletal regeneration and maintenance [48]. Insulin-like growth factor (IGF) plays an important role in general growth and maintenance of the skeleton, and has long been considered a circulating mediator of growth hormone (GH) [48]. There are two different isoforms, IGF-1 and IGF-2. IGF-1 has been known to be exclusively used in reconstructive surgery [48]. Vascular endothelial growth factor (VEGF) is another increasingly important growth factor and is considered one of the key regulators of angiogenesis during bone formation [53, 54]. Not only is the induction of new bone important, but equally important is the regeneration of the vasculature to allow for a fresh supply of nutrients to the site of tissue regeneration. Kempen et al. demonstrated the sequential release of VEGF along with BMP-2 promoted a significant increase in ectopic bone formation compared to BMP-2 alone [54].

A promising alternative to the use of growth factors is the use of smaller molecule drugs such as statins. Statins are commonly known as an inhibitor of
3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase and often prescribed to control cholesterol levels [55, 56]. Statins have been extensively studied for their ability to stimulate bone regeneration [57-60]. There are many statins that have demonstrated their ability to stimulate bone growth (i.e. simvastatin, lovastatin, fluvastatin). Simvastatin promotes bone formation \textit{in vitro} and \textit{in vivo} through the stimulation of osteoblastic activity and inhibition of osteoclastic activity [55, 56]. The successful use of simvastatin in vivo is largely controlled by the local concentration of the drug [55]. Houshmand et al. demonstrated simvastatin and lovastatin ability to promote ectopic bone formation following subcutaneous injections along or near bone [57]. However, due to its pharmacokinetic characteristics of being poorly absorbed and actively cleared in the liver, controlling the local release of relatively higher amounts of simvastatin to maintain a therapeutic level of drug suitable for accelerated bone regeneration would be ideal [56].

\subsection*{2.6. Drug Delivery of Bioactive Agents}

Being able to control the delivery of drugs to a bony defect allows for tunable dosing, as well as for extended retention time of agents being delivered [61]. However, an ongoing question relates to how bioactive molecules can be delivered to maximize their therapeutic potential [62]. A common trend to help address this issue is the use of biodegradable carrier substances incorporated into existing grafting devices or even directly incorporating the agents into scaffolds. Biodegradable polymers have been used as biomaterials in controlled-release applications [62-66]. Many of these that have been investigated for the delivery of osteoinductive factors including collagen networks, gelatin, poly(lactic acid) (PLA), polyanhydrides, and poly(DL-lactide-co-glycolide) (PLGA) [62]. PLGA is a biocompatible and biodegradable polymer that is a copolymer of poly lactic acid (PLA) and poly glycolic acid (PGA) [67, 68]. They are very popular among biodegradable polymers because of their long clinical history, favorable degradation characteristics which can be tuned by adjusting the polymer molecular weight and ratio of lactide to glycolide, and for its use as a drug
delivery apparatus for bioactive agents [68]. PLGA undergoes degradation by hydrolysis or biodegradation through cleavage. Sobe et al. developed and tested a PLGA capsule containing BMP [69]. Their controlled release system was shown to have gone through complete digestion followed by newly regenerated bone after 3 weeks [69]. PLGA has also been shown to be a good drug delivery vehicle in the form of microspheres embedded into a ceramic matrix. Yang et al. developed tri-calcium phosphate composites containing PLGA microspheres for the delivery of dexamethasone and bovine serum albumin [70]. The intended use of this device was to work not just as a scaffold, but also capable of controlled local delivery of drugs or bioactive agents to accelerate bone regeneration at the defect site [70].

Hydrogels provide specific advantages for the delivery of growth factors due to their high water content, biocompatibility, and controlled degradation [62]. There are many promising hydrogels that are capable of delivering bioactive agents [62, 65, 71]. Gelatin have been used widely in preparation of enzyme-degradable protein hydrogels [65]. It is a natural polymer derived from collagen (Young). These gels are commonly used for pharmaceutical and medical applications because of the biodegradability and biocompatibility (Young). A controlled release platform using BMP-2 loaded gelatin hydrogel developed by Sawada’s group demonstrated significant bone regeneration [72]. The gel was capable of controlling the delivery of protein resulting in a greater volume of bone formation compared to their controls (Swada). Poly(ethylene glycol) (PEG) hydrogels have also been shown to be beneficial as a material for tissue regeneration whether it be used as a drug delivery apparatus or a scaffold for osteoblasts [62, 73]. A unique family of biodegradable hydrogels, poly(β-amino esters) (PBAE), developed by Anderson et al has also been proposed for use in tissue regenerative applications [74, 75]. These gels can have a wide range of physical/chemical properties based on their macromer formulations [66, 74]. Several groups have investigated PBAE’s potential as carrier materials for controlled delivery of bioactive agents [66, 76-80].
2.7. Significance

The purpose of the work described in this document was to demonstrate how a rather simple material, calcium sulfate, with a strong successful history as a synthetic bone graft substitute could be further enhanced to become a suitable alternative to the ‘gold standard’ autograft. By allowing the material to be tailored into a customizable composite capable of controlling the local administration of bioactive agents, this device could be developed and tuned with the patient’s needs in mind based off of the prognosis of the surgical site. The outcome will not only eliminate the need for either multiple grafts, membranes, or surgeries, but will allow for the process to be as minimally invasive as possible allowing faster patient recovery and greatly having an effect on their overall health and well-being. Described below are the different specific aims that were achieved in the development of these calcium sulfate based bone graft substitutes.

2.8. Specific Aims

Specific Aim #1: Development and characterization of a calcium sulfate based space making composite bone graft substitute capable of controlled release of bioactive agents.

Calcium sulfate hemihydrate based composites embedded with poly(β-amino ester) (PBAE) biodegradable hydrogel particles were developed to act as a ‘tenting’ barrier to soft tissue infiltration, theoretically providing adequate space to enable vertical bone regeneration. Composite samples were fabricated with varying amounts (1 or 10 wt%) and sizes (53-150 or 150-250 µm) of gel particles embedded in CS. The swelling and rate of degradation of PBAE gels was explored; an important characteristic to aid in controlled release of drug. Micro-computed tomography (MicroCT) imaging was done to monitor particle distribution within the CS matrix. Destructive dissolution studies of composites was conducted to demonstrate the length of time it took for CS to complete dissolve as well as monitor how gel particles (1 wt% and 10 wt%) would influence the rate of dissolution. Compression testing was done to characterize the mechanical properties of CS composites with embedded gel particles. A pilot
release with curcumin loaded PBAE gels embedded in a CS matrix was performed to demonstrate the potential for drug release from CS-based composites.

**Specific Aim #2:** Demonstrate the capability of controlled release of a broad range of bioactive agents from CS-based composites.

CS composites developed in specific aim #1 were investigated for their drug delivery potential of a broad range of bioactive agents. Samples were produced with either directly loaded small, hydrophobic molecule (i.e., simvastatin), directly loaded hydrophilic protein (i.e., lysozyme), or 1 and 10 wt% of H6 poly(β-amino ester) (PBAE) particles containing protein. The release kinetics of the composites was evaluated for the sustained release of two very different drugs. Compression testing was also performed to monitor how the loading of drug directly into the CS matrix would affect the mechanical stability of the composites.

**Specific Aim #3:** Development of a bilayered composite with tailorable geometries and demonstrated sequential release and multiple hydrogel loaded space making calcium sulfate/hydrogel composite for tailored drug delivery of simvastatin and metronidazole to treat chronic periodontal disease.

Tailorable bilayered calcium sulfate (CS) bone graft substitutes were developed with the ability to sequentially release multiple therapeutic agents. Bilayered composite samples having a shell and core geometry were fabricated with varying amounts (1 or 10 wt%) of metronidazole-loaded poly poly(lactic-co-glycolic acid) (PLGA) particles embedded in the shell and simvastatin directly loaded into either the shell, core, or both. Microcomputed tomography (MicroCT) imaging was conducted to showed the overall layered geometry as well as determine the homogeneity of particle distribution within the shell. A destructive dissolution studies were performed to demonstrate the effect of PLGA particles (i.e., 1 vs. 10 wt%) may have on the composite during degradation.
Compressive mechanical testing was conducted to determine how the introduction of a layered geometry would influence the mechanical properties of the composite. Release studies of simvastatin directly loaded into the layers of CS were studied. By introducing a tunable layered geometry the release kinetics of simvastatin was monitored. A multiple drug release study was conducted to investigate the sequential release capacity of CS composites with metronidazole loaded PLGA particles embedded in the shell and simvastatin directly loaded into the CS matrix (either in shell, core, or both layers).
Chapter 3: Effect of Macromer Synthesis Time on the Properties of the Resulting Poly(β-amino ester) Biodegradable Hydrogels

3.1. Introduction

Dental implants have become a preferred method for replacing teeth lost to trauma or disease. Unfortunately, the disease processes which result in tooth loss may also cause the loss of supporting bone [81]. Implant placement requires sufficient bone to allow a proper anchor [81, 82]. Thus, bone regeneration or augmentation is often required prior to or concomitant with implant placement [81, 82]. Guided bone regeneration (GBR) is a common technique in which a barrier membrane is used to create a protected ‘healing chamber’ where bone growth may occur. The barrier helps stabilize the blood clot and graft material and helps exclude the ingrowth of epithelium or fibrous connective tissue, either of which may interfere with the more slowly growing immature bone tissue [83]. These membranes may be either resorbable (e.g., collagen) or non-resorbable (e.g., expanded polytetrafluoroethylene). The latter requiring a second stage surgery to remove the membrane.

GBR membranes are often used in conjunction with an autogenous bone graft because of their tendency to collapse and subsequent inability to create the growth space by themselves [84, 85]. Although autogenous bone has been widely accepted as the gold standard augmentation material, its availability from intra-oral donor sites is limited [82, 86, 87]. In the oral cavity, donor tissue is often harvested from the ramus or the symphysis of the mandible, and this can lead to undesirable donor site morbidity [31, 37, 84, 88-96]. A biologically compatible synthetic bone grafting substitute that is osteogenic, biodegradable/bioerodible, and provides spacing-making functionality could help eliminate the need for harvested bone or GBR membranes in the future.

Researchers have investigated many biocompatible ceramic materials as scaffolds for bone regeneration in attempts to replace the gold standard use of autogenous bone grafts. Calcium phosphates, such as hydroxyapatite and tricalcium phosphate, have been given much attention because of their likeness
to natural bone mineral [37, 93, 97-100]. Calcium sulfate (CS) hemihydrate has long been recognized as an osteoconductive biomaterial with an excellent reputation as a biocompatible substance [31-35]. In fact, it was one of the first bone substitutes used in orthopedics and dentistry [35]. It has been called upon for a variety of applications, such as craniofacial and long bone defects, as well as osseous cavities related to tumors and cysts [37]. Characteristically, CS has similar mechanical strength to that of cancellous bone [38]. In vivo, CS is well tolerated, having the unique ability to become osteogenic in the presence of bone and completely absorbed by the host without inducing a significant inflammatory response [31, 33-35].

In this study, calcium sulfate-based composites embedded with poly(β-amino ester) biodegradable hydrogel particles were developed to act as a ‘tenting’ barrier to soft tissue infiltration, thereby potentially enabling vertical bone regeneration ultimately leading to a suitable depth of bone for implantation of a prosthesis. The photocrosslinked biodegradable gel particles presented in this study are part of a family of materials initially developed by Anderson et al [74, 75]. These gels can have a wide range of physical/chemical properties based on their macromer formulations [74]. For the purpose of the composite system presented, a gel with a rapid degradation rate was selected as the filler that potentially may also serve as a delivery vehicle for pharmaceuticals. The introduction of these biodegradable gel particles into the composite matrix could improve the osteogenic properties of CS through a tailored, controlled release of bioactive molecules. Being able to control the delivery of drugs to a bony defect allows for tunable dosing, as well as for extended retention time of drugs being delivered [61]. For the present study, composites were embedded with unloaded poly(β-amino ester) biodegradable hydrogel particles. Their influence on the rest of the CS composites’ physical and chemical characteristics, namely mechanical strength and modulus, dissolution, and morphology, were studied. In addition, a pilot release study was conducted to demonstrate the release controllability of curcumin-loaded poly(β-amino ester) particles embedded in a CS matrix.
3.2. Materials & Methods

3.2.1. PBAE gel synthesis

A11 poly(β-amino ester) (PBAE) macromer was synthesized according to previously described methods [74, 75]. To review, step-growth polymerization was performed by combining a 1.4:1 molar ratio of diethylene glycol diacrylate (2.079 g; Polyscience, Inc., Warrington, PA) and 3-morpholinopropyl amine (1.0 g; Sigma Aldrich, St. Louis, MO). The reaction took place in a flask partially submerged in a silicon oil bath pre-heated to 85°C on a heated stir plate. After continuously stirring the reaction mixture for 16 h, it was removed from the heat and stored at 4°C until used.

Biodegradable hydrogels (HG) were subsequently made using a photo-initiated polymerization process. Two grams of cooled macromer were combined with 1 wt% of 2,2-dimethoxy-2-phenyl-acetophenone (DMPA; Sigma Aldrich, St. Louis, MO) initiator and dissolved in 1.25mL of dimethyl sulfoxide (DMSO). After vortexing for 60 sec, the homogeneous mixture was sandwiched between two glass plates using a 1.5 mm Teflon spacer to contain the viscous fluid. The macromer was exposed to UV radiation using a Lesco UV flood source (14 mW/cm²) for 5 min to initiate and propagate the photo-polymerization process. After polymerization, the gel was removed from the glass, washed for 30 m in ethanol to remove any unreacted initiator and macromer, and then freeze dried.

3.2.2. PBAE gel Swelling and Degradation

Swelling was measured as reported by Hawkins et al. [101]. Square (1 cm²) HG samples were submerged in deionized (DI) water or PBS and kept at 37°C. For the first hour, samples were removed from the fluid, carefully dabbed dry, weighed, and placed back into fluid every 10 min. During the second and third hours, samples were removed and weighed in the same fashion every 20 min. After three hours, the time intervals were increased to every hour until the sample could no longer be handled or the samples were completely degraded. The extent of swelling [S%] was determined using equation 1,
where $M_t$ was the mass at a particular time point and $M_o$ was the initial mass.

### 3.2.3. Composite Formation

The composites consisted of calcium sulfate hemihydrate (CS; Sigma Aldrich, St. Louis, MO) as the structural matrix and varying amounts of A11 HG particles. Gel particles were hand-ground from polymerized gel slabs. In order to coat the gel particles and prevent them from sticking to one another, as well as to allow grinding to be efficient, small amounts of CS powder were added during grinding. Particle sizes of 53-150 μm and 150-250 μm were obtained from grinding and sieving. To make CS control samples, 1 g of CS powder was mixed with 800 μL of deionized (DI) water for about 30 sec or until thoroughly mixed in 3 ml non-sterile syringes. The slurry was loaded into a custom-fabricated Delrin mold that could yield up to 46 samples with an average diameter of 4.75 mm and a height of about 6.5 mm. The mold was placed in a 43°C oven for 24 h to set the CS. For 1 wt% HG composite samples, 0.01 g HG was mixed with 0.99 g CS and 850 μL DI water. Samples with 10 wt% HG consisted of 0.1 g HG, 0.9 g CS, and 900 μL DI water. As with the controls, the composite mixtures were loaded into the mold and left to set in a 43°C oven for 24 h.

### 3.2.4. PBAE Particles and Composite Microarchitecture

#### 3.2.4.1. PBAE Microarchitecture

Using an Olympus IX51 light microscope, a small amount of PBAE gel particles from each size fraction, 53-150 and 150-250μm, were placed on glass slides and visually assessed. The physical structure of the particles was investigated to develop a qualitative understanding of their size distribution for a particular sieved fraction, as well as their appearance when embedded in a CS matrix. In addition to qualitative observations, the particle size was quantified using ImageJ.
3.2.4.2. Composite Microarchitecture

To monitor the distribution of HG particles within the CS matrix, microcomputed tomography (microCT) was used. Using a Scanco Medical μCT-40 scanner, specimens were evaluated with standard resolution having 250 projections with 1024 samples each. Additional parameters were set as follows: 92 μm increments, 0° angle, 70 kVp, 114 μA, 0.5 mm Al filter, and a voxel size of 12 μm. The resulting microarchitectural images were qualitatively investigated for any particle distribution trends. To qualitatively and quantitatively assess the composites, samples were evaluated with a lower threshold level of 109 before running a built-in ‘bone trabecular morphometry’ analytical tool. This tool created a three dimensional reconstruction that allowed assessment of the composite structure and its embedded gel particles as well as provided the volume percentage of embedded gel particles, average particle size, and average spacing between particles.

3.2.5. Composite Degradation

Destructive mass loss testing was performed to monitor degradation of the various composites. Samples of each type (control, 1 wt% HG, and 10 wt% HG) were weighed, measured (height and diameter), placed in separate plastic vials containing 4 mL of phosphate-buffered saline (PBS), pH 7.4, and incubated on a plate shaker at 37°C. Every four days, samples were removed and dried in a 43°C oven for a minimum of 24 hr. For the remaining samples, the PBS in vials was replaced with fresh PBS. The dried samples were weighed to determine the amount of mass lost.

3.2.5.1. Degradation: Changes in Fluid Volume

Mass loss using different fluid volumes was tested to determine the effects on composite dissolution rates. The experimental method for mass loss described above was used. Five different fluid volumes were tested: 0.5, 1.5, 3, 4, and 6 mL.
3.2.6. Composite Mechanical Testing

Compression testing was performed using a Bose ELF 3300 system. Samples without any noticeable external defects, such as cracks and/or voids, were chosen for testing. Contact surfaces were lightly sanded, if necessary, using 600 grit SiC paper to provide smooth, parallel surfaces in contact with the compression platens. Control, 1 wt% HG, and 10 wt% HG samples were tested at a rate of 0.5 N/sec until failure. Compressive modulus (M) and ultimate compressive strength (UCS) were calculated.

3.2.7. Pilot Release Study with Curcumin

A pilot study of drug release from the composite was performed to demonstrate the potential for controlled release from drug loaded PBAE gel particles embedded in a CS matrix. PBAE gels were produced with 50 wt% curcumin using a method previously described [102]. The finished gels were washed in 5 mL of acetonitrile for 1 hr. This was repeated 4 times after which the gels were lyophilized overnight. Gels were hand ground with small amounts of CS as a drying agent and sieved to obtain particles ranging from 53-150 μm or 150-250 μm. Drug loaded particles were washed with DI water for 1 minute to remove CS. The washed particles were filtered and lyophilized once more. Composites were produced using the same steps described above for both 1 wt% HG and 10 wt% HG composite formation.

Completed samples were weighed, submerged in 4 mL of PBS, and placed on a shaker plate in a 37°C oven. To allow for sink conditions, every 4 days the supernatant was collected and fresh PBS was added. This continued until all samples completely degraded. For each measurement, 1 mL of collected supernatant was treated with 50% ethanol to dissolve any suspended drug and filtered using 0.45 μm syringe filters. Supernatant from the 10 wt% HG samples was diluted to allow for accurate measurement. Gel washings and treated supernatant were measured with UV/Vis spectrophotometry at an absorbance of 420 nm.
3.2.8. Statistics

Statistical analysis of the results was performed using either a two-tailed unpaired t-test or two-way ANOVA. As appropriate, this was followed up with a Tukey-Kramer multiple comparisons post hoc test. Differences between groups were considered to be significant with p-values <0.05.

3.3. Results

3.3.1. PBAE gel Swelling and Degradation

The degree of hydrogel swelling and degradation based on the mass of liquid absorbed by the samples is displayed in Figure 3.1. During the first 60-80 minutes, the gels swelled quickly, but the rate gradually slowed and reached a plateau around 160 minutes. After this initial stage, the full extent of swelling appeared to reach a maximum for gels submerged in PBS, and the samples did not show any significant signs of further swelling. After 300 minutes, the gels submerged in DI water began to absorb liquid again at a significantly higher rate (p<0.0001) and continued until the end of the experiment. The study was concluded around 420 minutes when the samples became fragile and difficult to handle.

3.3.2. Composite Morphology

3.3.2.1. Qualitative Evaluation

Under a light microscope, gel particles were seen as large, irregularly shaped objects along with some dispersed prism-like particles considered to be residual calcium sulfate (Figure 3.2). Quantitative analysis demonstrated that the size distribution of gel particles was within the expected limits, 53-150 μm and 150-250 μm (data not shown).
Figure 3.1. Swelling of A11 PBAE gels in diH$_2$O and PBS. Data are mean ± standard deviation (n=6).
Figure 3.2. Morphology of A11(1.4) PBAE gel particles: A) 150-250 μm and B) 53-150 μm sieved size fractions. Irregularly shaped objects are gel particles (solid white arrows), and small particles are residual calcium sulfate (open white arrows).
MicroCT analysis showed the size and distribution of gel particles embedded in CS (Figure 3.3). In samples with 10 wt% loading of particles, there was a uniform distribution of particles throughout the CS matrix (Figures 3.3D, E). In Figures 3.3B and 3.3C showing composites with 1 wt% loading of gel particles, although the distribution was sparser, the particles still appeared to have been uniformly distributed throughout the matrix. Although all composite loadings showed a good distribution of particles, none of the samples were without some minor defects. For example, all samples had a few air pocket defects (black arrows in Figure 3.3) embedded along with gel particles. These defects were nearly spherical in nature and thus easily distinguishable from gel particles, which had irregular shapes as previously shown in Figure 3.2.

### 3.3.2.2. Quantitative Evaluation

Quantitative analysis was performed to develop a greater understanding of the physical structure of the composites than could be achieved by a qualitative analysis alone. Figure 3.4 shows the average porosity, average spacing between particles/voids, and average particle/void size for the different composite compositions. The porosity of about 8.31% for 10 wt% HG (150-250 μm) loading was significantly different (p<0.001) from all other loadings tested (Figure 3.4A). For all other configurations there were no significant differences in porosity among them.

For the average spacing between particles/voids, the blank samples were significantly different (p<0.001) compared to 1 wt% HG (53-150 μm), 10 wt% HG (53-150 μm), and 10 wt% HG (150-250 μm) samples (Figure 3.4B). Differences in void spacing between the 1 wt% HG loadings for both particles sizes proved to be significantly different [p<0.01, 1 wt% HG (53-150μm) vs. 1 wt% HG (150-250μm)]. When comparing the different loadings for a particular particle size, the distance between particles for the 1 wt% HG loadings were significantly larger (p<0.001) compared to the 10 wt% HG loadings except for the 1 wt% HG (53-150μm) vs. 10 wt% HG (53-150μm) relationship which was significantly different with a p<0.05.
The average particle and/or void size (Figure 3.4C) demonstrated significant differences for 1 wt% HG (53-150 μm) versus both 10 wt% HG (53-150 μm) (p<0.001) and 10 wt% HG (150-250 μm) (p<0.05).

3.3.3. Composite Degradation

During the mass loss experiments, all sample groups were tested simultaneously. Loading 1 wt% of gel particles did not have a significant effect on the dissolution rate (-3.71%/day) of the overall composite (Figure 3.5). However, increasing the gel loading to 10 wt% did significantly increase the dissolution rate -4.42%/day (p<0.05), even though complete dissolution varied by only about four days.

3.3.3.1. Degradation: Changes in Fluid Volume

Similar to the composite degradation experiment, all sample types were tested using the same PBS solution to avoid fluid dependent effects on dissolution except volume change. Shown in Figure 3.6, small fluid volumes of 0.5 or 1.5 mL proved to cause significantly slower dissolution, -0.6 or -2.7%/day, respectively (p<0.001). Increasing the fluid volumes to 3, 4, or 6 mL contributed to dissolution rates of -5.1, -6, or -6.6%/day, respectively. When compared to 3 mL, fluid volumes of 4 mL and 6 mL had significantly faster dissolution rates (p<0.05 and p<0.001, respectively).
Figure 3.3. MicroCT images of calcium sulfate/gel composites: A) Blank CS and B) 1 wt% HG 53-150 μm, C) 1 wt% HG 150-250 μm, D) 10 wt% HG 53-150 μm, E) 10 wt% HG 150-250 μm. Air pockets indicated by black arrows. All scale bars are 1.0 mm.
Figure 3.4. Quantitative morphometric results from microCT: A) Average porosity, B) Average spacing between particles for blank and composite CS samples, and C) Average size of particles/voids within CS matrix. Data are mean ± standard deviation (n=5). Symbols indicate significant differences: p<0.001 (*), p<0.001 (**), p<0.01 (#), and p<0.05 (Δ).
Figure 3.5. Dissolution profiles for blank calcium sulfate, 1 wt% HG), and 10 wt% HG composites. Data are mean ± standard deviation (n=3).
Figure 3.6. Dissolution profiles for blank calcium sulfate submerged in different volumes of PBS. Data are mean ± standard deviation (n=3).
3.3.4. Composite Mechanical Testing

Effects of HG loading on the compressive mechanical properties are shown in Figure 3.7. The ultimate stress for control blank CS samples were significantly greater than both 10 wt% HG loadings with a p<0.0001 and 1 wt% HG loadings (p<0.001). Blank CS elastic modulus, however, was only significantly greater than the 10 wt% HG loadings (p<0.0001). Composite samples with 1 wt% HG had 80-90% of the strength compared to the controls. The 1 wt% addition of gel particles had no significant effect on the modulus when compared to blank samples. Adding 10 wt% HG resulted in about 40-50% of the stress and 25-30% of the modulus of particle-free samples. Comparing samples loaded with 1 wt% HG of either 53-150 μm or 150-250 μm particles showed a significant difference (p<0.01) in their strength (3.99 ± 1.19 MPa versus 4.89 ± 0.38 MPa, respectively), however, there was no significant difference in the elastic modulus (401.35 ± 156.92 MPa and 369.1 ± 224.58 MPa, respectively). The strength of 10 wt% HG for both particle sizes were also significantly different with a p<0.01 [2.57 ± 0.24 MPa (53-150 μm) and 1.94 ± 0.36 MPa (150-250 μm)], but there wasn’t any significant difference between their elastic moduli. Comparing the two different particle sizes and their different weight percentages there were significant differences as well. For either particle size, the ultimate strength of composites containing 1 wt% HG particles was significantly greater than that for 10 wt% HG loadings [p<0.05 for 1 wt% HG (53-150μm) vs. 10 wt% HG (53-150μm), and p<0.0001 for all other composite comparisons]. The elastic modulus of 1 wt% HG (53-150 μm) vs. both 10 wt% HG loadings was significantly different with a p<0.001, whereas the differences in the elastic modulus of 1 wt% HG (150-250 μm) vs. both 10 wt% HG loadings was a little less significant with a p<0.01.
Figure 3.7. Mechanical properties of CS-HG composites: A) Ultimate compressive strength and B) compressive modulus of blank and composite calcium sulfate samples. Data are mean ± standard deviation (n=10). Symbols indicate significant differences: p<0.0001 (*), p<0.001 (**), p<0.01 (#), and p<0.05(Δ).
3.3.5. Curcumin Pilot Release

Figure 3.8 illustrates the pilot release kinetics of CS composites embedded with PBAE particles loaded with curcumin. For both 1 wt% HG loadings, the release of curcumin was steady where about 30% was released after the first 4 days and only 60-70% of the drug released during the first half (14 days) of the study. Over the course of the study there was a slow decay of the daily release percentage from the 1 wt% HG composites; 8%/day at 4 days, 6%/day at 10 days, and 4%/day at 20 days. Conversely, 10 wt% HG loadings started with a larger burst (50-60%) release of drug over the first 4 days followed by nearly 90% of the loaded drug released during the first 14 days of the 28 day study.
Figure 3.8. Release profiles for 1 wt% HG (53-150 μm and 150-250 μm) and 10 wt% HG (53-150 μm and 150-250 μm) composites. Data are mean ± standard deviation (n=5).
3.4. Discussion

The aim of this study was to understand the physical and chemical characteristics of a composite bone graft substitute that has the potential to act as a ‘tenting’ barrier to soft tissue infiltration for creating a chamber for bone regeneration, while simultaneously allowing for the controlled release of bioactive agents. The regenerated bone could provide a sufficient platform for stable placement of dental implants in periodontal applications.

3.4.1. PBAE gel Swelling and Degradation

Degradable PBAE gel particles were synthesized to serve as a protective delivery vehicle for osteogenic agents and/or other drug(s) when embedded in a calcium sulfate matrix. Initial studies were performed to determine whether swelling of the particles following exposure to a physiological environment would be extensive enough to adversely affect the overall composite structure. In vitro experiments showed three distinct stages in the swelling profiles. After the initial swelling stage, absorption of liquid slowed, eventually reaching a plateau. It has been well established that swelling of hydrogel occurs due to the electrostatic repulsion of ionic charges within the polymer due to the presence of absorbing liquid [103, 104]. During the plateau phase, osmotic forces driving fluid into the hydrophilic gels equaled the opposing elastic force created by the stretching of the crosslinked polymer. After the plateau, the polymer backbone began failing at a faster rate through hydrolytic scission of the ester bonds, yielding small molecule bis(β-amino acids), diol products, and poly(acrylic acid) kinetic chains [74]. This gradual breakdown of the polymer matrix allowed for further expansion of the mesh and continued absorption of liquid into the gel. Anderson et al. had previously demonstrated that A11 PBAE gels degrade very rapidly (<24 hr), which is similar to the results seen in the present study where gels began to rapidly breakdown through hydrolysis beginning around 4-5hr resulting in complete degradation in less than 24 hr [74].

An advantage with the PBAE family of hydrogels is that there are a variety of possible combinations of diacylates and amines that can produce similar
range of properties. Hawkins *et al.* studied a PBAE gel comprising poly(ethylene glycol) diacrylate (H) and isobutylamine (6) in a 1.2 molar ratio [101]. The components are different from those used for the present A11 gel, however, the H6 system studied by Hawkins also degraded in less than 24 hours. Although the H6 gel swelled about 50% more during the first 4hr compared to the A11 gels, they both reached a plateau followed by a rapid degradation of the polymer starting around 4-5hr [101]. The similarities between these two PBAE’s demonstrate the versatility of this gel family and how the gel component in this system may be altered to accommodate different formulations if needed.

### 3.4.2. Composite Morphology

When creating the gel particles to be embedded in calcium sulfate, gel slabs were ground with a small amount of calcium sulfate to act as a ‘drying agent’ similar to talcum powder to prevent particles from sticking to one another. Images showed a small amount of calcium sulfate crystals remaining with the gel particles after sieving. The smaller 53-150 μm gel particles had more residual calcium sulfate compared to the larger 150-250 μm particles. Calcium sulfate is inherently denser than the gels. Therefore, the greater amount of calcium sulfate present with the 53-150 μm size particles must be taken into account in future studies investigating drug loading and release. The larger particles have a smaller surface area to volume ratio compared to the smaller particles, thus requiring a smaller amount of calcium sulfate to coat and process the particles.

Qualitative analysis of microCT images indicated a uniform distribution of gel particles at both 1 and 10 wt% loadings. The composite slurry, when mixed well, allows for a short working window for easy filling of the molds because of the rapidly setting nature of the calcium sulfate. Due to the viscosity of this slurry, gel particles were suspended and prevented from aggregating with one another. Also, any buoyancy effects that could cause particles to collect at one end of the samples and cause inhomogeneity were prevented. Once set, the gel particles were locked in place, and any swelling of particles that may have occurred while mixing the slurry was reversed resulting in contraction of these particles. This in
turn creates protective voids wherein dried gel particles reside. An important feature of this process was the creation of a tolerance ‘gap’ between the wall of the voids and the HG particles preventing composite failure due to swelling of gel particles during CS dissolution. In addition, because these irregularly shaped voids are stress concentrators themselves, they may also affect structural integrity under an applied load.

Quantitative evaluation of the composites’ porosities showed a dramatic decrease in porosity for the 10 wt% HG loading of 53-150 μm particles (2.67%) compared to the 10 wt% HG of 150-250 μm particles (8.31%). The presence of residual calcium sulfate, especially with the 53-150 μm size fraction, could affect loading because the CS will incorporate with the rest of the matrix, leaving behind a smaller quantity of gel particles. Interestingly, residual calcium sulfate did not play a significant role when comparing the 1 wt% HG loadings for both particle sizes. The contribution of air pockets to the porosity, particle/void spacing and size calculations for 1 wt% HG specimens may have counteracted the effects of residual CS described above. However, the number of possible air pockets within the 10 wt% HG loadings may not have significantly contributed to these calculations due to the much larger quantity of gel particles present. This trend can be exemplified using the particle size measurements for 1 wt% HG (53-150 μm) and 10 wt% HG (53-150 μm) where the resulting porosity values were significantly different, although the particles used for these composites were the same.

3.4.3. Composite Degradation

CS composites degrade by means of dissolution and subsequent surface erosion. Bulk degradation was not observed because of the dense nature of the ceramic and lack of an open-celled porous network. Although the gel particles created a porous structure within the matrix, the pores were ‘closed’. This structure should enable the composites to achieve zero-order release, whereby only the gel particles exposed on the surface during CS erosion will be permitted to swell, degrade, and release their active molecules. The HG loading-
independence of composite degradation rate may allow for easy tuning to provide long enough mechanical stability or protection from soft tissue infiltration while also allowing the sustained delivery of a sufficient amount of drug-loaded HG particles to stimulate bone formation.

Lewis et al. investigated a calcium sulfate composite containing carboxymethylcellulose (CMC) or hyaluronan (HY) to improve CS’s handling or workability, mechanical, and degradation properties [31]. They showed that the introduction of these biopolymers allowed for better handling properties as well as increased compressive strength. Introduction of CMC and HY, however, had a drastic effect on the degradation rate of the composite. As the mass of CMC increased, the degradation rate also increased (12%/day for a loading of 10% CMC compared to 1.6%/day for plain CS) [31]. The purpose of the study was to develop a composite material with better handling abilities for insertion into a defect, however, the introduction of CMC and HY significantly increased the rate of dissolution, compromising the original absorption rate of calcium sulfate [31]. For the current study, the addition of 1 wt% PBAE gel particles did not have a significant effect on the dissolution rate of calcium sulfate (3.71%/day), and after increasing the amount of particles to 10 wt%, the rate of dissolution increased to 4.42%/day. Gao et al. investigated the use of gelatin with calcium sulfate. In their study, the resulting degradation rates for calcium sulfate and their composites, 4.3%/day to 5.2%/day, were similar to the rates observed in the present study for CS composites with embedded PBAE gel particles [91]. Another group investigated the use of calcium phosphate (CaP) microspheres within a CS matrix [105]. They were able to increase the retention time of the composite compared to blank CS, prolonging the absorption of CS in vivo. Urban et al. were also able to demonstrate a greater amount of bone formation over the course of 26 weeks from the CS/CaP composite compared to the CS pellets alone [105]. In the present study, CS’s dissolution rate was not compromised by the incorporation of embedded gel particles. Using the original dissolution rate for CS, these embedded PBAE gel particles could perform as delivery vehicles for
bioactive drugs helping to increase the rate of tissue regeneration, thus possibly reducing the length of time needed for healthy bone to regenerate.

3.4.3.1. Fluid Volume Effects

The volume of fluid present during composite degradation in vivo is unknown, and the rate of fluid turnover is likely to have a notable effect on the rate of degradation. A degradation study utilizing multiple fluid volumes was performed to simulate the effects of different sink conditions that would result from different fluid volumes and/or turnover rates. The idea behind this experiment was to show the diversity of dissolution rates based on the volume of fluid possibly present in a particular wound site. In the case of implanting a CS composite in vivo, the rate at which the implant degrades will be key for proper resorption and replacement with healthy bone. As would be expected, smaller fluid volumes of 0.5 mL or 1.5 mL significantly reduced the rate of dissolution, 0.6%/day or 2.7%/day, respectively. Conversely, increasing the fluid volume above 4 mL increased the rate of dissolution to about 6%/day. The similarity of the faster dissolution rates (6%/day and 6.6%/day) for fluid volumes ≥4 mL suggested a threshold for the sink conditions was reached that caused dissolution to become constant. Considering the surface area of the sample versus the volume of fluid used, it is fair to conclude that dissolution of cylindrical calcium sulfate samples with an estimated surface area of 114mm² (assuming sample diameter of 4.66mm and height of 6.7mm) will be constant regardless of the volume used above the 3-4 mL threshold because of the limited amount of surface exposed.

3.4.4. Composite Mechanical Properties

Mechanical stability is important when developing a bone graft substitute material that will provide support for bone formation. Depending on the procedure performed, the majority of the graft could reside within a defect site where surrounding bony tissue would act as a protective shelter. The remaining portion, or cap, would protrude from the defect to provide both an aid for the vertical augmentation of bone, and to act as a ‘tenting apparatus’ distributing any load
from overlaid soft tissue while simultaneously preventing any ingrowth of soft tissue. An example of such a space-making site is the socket remaining after removal of a tooth. A graft placed into such a site (or a blood clot which fills the socket) will be protected and stabilized by the surrounding bony walls.

A more challenging application would be sites that are not ‘space-making’, i.e., sites that lack topography to contain and protect the ‘regeneration chamber’. This would require development of implant materials that are strong enough to create and maintain space in the absence of any surrounding bone, and prevent soft tissue ingrowth while permitting new bone formation. The best example would be vertical bone growth in a badly resorbed mandible or maxilla. In the present study, CS composites were developed for this purpose.

Gel loading of 1 wt% had a 10-20% decrease in the mechanical strength of the calcium sulfate composites. Because the 1 wt% HG loading ratio did not affect the dissolution rate, these composites may be preferable to blank calcium sulfate in clinical applications because of the added potential as a drug delivery vehicle. Addition of 10 wt% HG decreased calcium sulfate’s strength and stiffness by 50-60% and 70-75%, respectively, creating a much weaker composite. In this study we considered 10 wt% HG an upper threshold for particle loading because of the potential adverse effects on the mechanical integrity. Any differences among the composite samples can be explained by the differences in their microstructure, e.g., stress concentrators formed by voids housing gel particles, minor microstructural defects, and growth and propagation of crystals as CS sets. In a study conducted by Kim et al., injectable calcium phosphate cements were developed [106]. Their compressive strength results for samples that contained 0-15 wt% of hydroxyapatite ranged between 2.5-4.5 MPa [106]. Interestingly, these results are similar to the compressive stresses witnessed in the present study. Blank CS, 1 wt%, and 10 wt% gel samples resulted in compressive stresses around 5.5, 4, and 2 MPa, respectively.

Lewis et al investigated the compressive strength of their biopolymer/CS composites material [31]. Their blank calcium sulfate samples had a 10 MPa compressive strength that was stronger than the present results. An interesting
aspect of biopolymer/CS composites compared to HG/CS was the similarity in the amount of fluid that was used for production compared to the differences found in the resulting mechanical strengths. The fluid to powder ratio of Lewis et al. ranged between 0.8-1.33 mL/g compared to 0.8-0.9mL/g found in the present study [31]. Another group investigated the use of an α-hemihydrate CS for a composite structure incorporating biomimetic apatite nanoparticles for controlled drug delivery [99]. That study utilized two liquid to powder ratio, 0.6 and 0.4 mL/g. Although these ratios were lower than those used in Lewis’ study or the present study, the compressive strength of CS (2-4MPa) was comparable [99]. The similarities in compressive strength help demonstrate that the liquid to powder ratio does not appear to have a significant effect on the strength of the material within the ranges presented. An attempt to optimize liquid content and/or loading of gel particles to achieve a desired grafting device will be investigated in the future.

3.4.5. Curcumin Pilot Release

As a naturally occurring drug originating from the dietary spice turmeric, curcumin has a broad range of therapeutic functions which include anti-inflammatory, antioxidant, anti-bacterial, and more recently, anti-resorptive [52, 102, 107]. Groups have investigated curcumin’s ability to reduce or prevent the resorption of bone. Ozaki et al. studied curcumin’s effects on osteoclast apoptosis in rabbits [107]. Their findings concluded that curcumin does play a significant role in osteoclast apoptosis, and furthermore, were able to demonstrate the inhibitory effects of osteoclastic bone formation [107]. Early exposure of a multi-functional drug that was controllably released from the proposed CS composites could significantly reduce alveolar bone regeneration time due to the lack of other complications produced by inflammation and/or bone resorption. This would in turn allow for a much earlier application of a dental prosthesis restoring normal function.

A pilot study to demonstrate the controlled or sustained release of a drug from CS composites was conducted by releasing curcumin from curcumin-loaded
PBAE gel particles embedded within a CS matrix. Over 28 days, the release of curcumin was monitored. As per the results of this experiment 1 wt% HG loaded composites showed a sustained release of drug with a slow decay in the daily rate of release. 10 wt% HG had a much greater burst of drug during the first 4 days (~50%), but over the course of the final 24 days the release was sustained. Continuous dissolution of CS permits only the PBAE particles that are exposed to the surface of the composite to break down, and therefore, allowing the drug to be released. According to the curcumin release investigated in Wattamwar's study, the antioxidant was directly incorporated into the PBAE macromer prior to polymerization [102]. This process helps prevent the premature release or diffusion of drug from the gel matrix prior to hydrolysis allowing for further controlled release of curcumin [102]. Additionally, during the dissolution process the total exposed surface area slowly decreases which in turn only allows a smaller volume of exposed particles overtime to breakdown and release the drug. This trend helps explain the slow decay in the daily rate of curcumin release, and furthermore helps establish the fact that the release of curcumin from HG particles is controlled by the way HG is released during dissolution of CS composites.

3.5. Conclusion

Calcium sulfate-based composites with embedded poly(β-amino ester) biodegradable gel particles were developed to potentially serve as a bone graft substitute for vertical bone regeneration. The gel particles are intended to provide the eventual delivery of bioactive molecules to enrich the bone augmentation process without physically compromising the capacity of the composite to be used as a grafting substitute. It was determined that the PBAE gel particles were uniformly distributed within the CS matrix, and the embedded particles had minimal impact on the zero-order dissolution rate. However, from a destructive in vitro dissolution study, it was shown that fluid volume turnover will have a large effect on the rate at which these composites will degrade. It was also determined the PBAE particles did have a significantly negative effect on the mechanical
strength of the composites. As much as a 75% drop in its strength was seen with a 10 wt% loading of particles. On the other hand, since the dissolution rate was minimally affected with the increased loading of gel particles, and the composites are designed to have an effective osteogenic drug delivery capability to help enhance tissue regeneration. Additionally, the decrease in strength does not rule out their potential to be a useful synthetic bone graft substitute for vertical ridge augmentation. A pilot release study of curcumin from particles embedded within the composites demonstrated a sustained release of the drug that was governed by the degradation of embedded particles during the dissolution of CS over time. Further developments will investigate the composite bone graft substitute's capacity to deliver a broad range of drugs and their capability to be tailored on a case by case basis.
Chapter 4: Drug Release from Calcium Sulfate-Based Composites

4.1. Introduction

Bone augmentation through the application of onlay autogenous bone grafts is a suitable method for vertically restoring maxillofacial bone defects [43, 44, 89, 108-112]. Although autografts are considered the ‘gold standard’ for augmentation procedures, they have multiple disadvantages as well. Their availability from intra-oral donor sites is limited, and harvesting tissue, which requires a secondary surgery, can lead to undesirable donor site morbidity and chronic discomfort [31, 37, 84, 88-96]. Many groups are developing synthetic grafting substitutes to replace the current standard. In doing so, several performance criteria must be met. The synthetic bone graft substitute needs to be biocompatible, osteoconductive to help create a secure bond with the surrounding host tissue, and mechanically stable to ensure that vertical augmentation can be achieved without collapse [22, 51, 113]. Calcium sulfate (CS) hemihydrate has an excellent reputation as a biocompatible and osteoconductive substance [31-35, 46]. In vivo, calcium sulfate becomes osteogenic in the presence of bone and is completely absorbed without inducing a significant inflammatory response [31, 33-35, 46]. In addition, CS is mechanically similar to cancellous bone [38].

Although synthetic grafting alternatives, such as CS, can be effective, their osteoconductive properties may be improved through the aid of bioactive agents like growth factors or other osteogenic agents capable of inducing new bone formation [45]. Studies have shown synthetic bone grafts enhanced through the release of bioactive molecules [45-47]. An ongoing question relates to how these molecules can be delivered to maximize their therapeutic potential [62]. One possible approach is to load drug directly into a CS construct. If uniformly distributed throughout the matrix, dissolution of CS will result in release of the drug. Based on release of fibroblast growth factor, Rosenblum et al. concluded that CS could be a suitable carrier for sustained drug delivery [46]. Another way to load drug is through the aid of carrier particles. Biodegradable hydrogel
networks provide specific advantages for drug delivery due to their high water content, general biocompatibility, and controlled degradation via hydrolysis or by enzymatic degradation leading to controlled drug delivery [62, 65, 78]. In particular, the poly(β-amino ester) (PBAE) family of hydrogels has properties that are easily tuned to achieve great control as a drug delivery vehicle [66, 71, 78]. Previous research has shown that PBAE particles can be uniformly embedded into a CS matrix, which may allow further control over the release of drug from these particles as the CS matrix erodes [113].

Several osteogenic drugs are available. These range from larger proteins, such as bone morphogenetic proteins (BMPs; 30-38 kDa) down to small molecules, such as the statin family of drugs (<1 kDa). BMPs control tissue induction and morphogenesis and are directly involved in the differentiation of cartilage and bone [49-51]. Introducing BMPs into grafting devices can stimulate rapid new bone formation, allowing for a reduction in healing time to achieve sufficient new bone volume for proper anchoring of an inserted implant [47, 52]. Like all statins, simvastatin (419 Da) is commonly known as an inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase and often prescribed to control cholesterol levels [55, 56]. Importantly, however, simvastatin promotes bone formation \textit{in vitro} and \textit{in vivo} through the stimulation of osteoblastic activity and inhibition of osteoclastic activity [55, 56]. Due to its pharmacokinetic characteristics of being actively cleared in the liver, controlling the local release of relatively higher amounts of simvastatin from a synthetic bone graft can maintain a therapeutic level of drug suitable for accelerated bone regeneration [56].

In the present study, previously developed CS-based composites were investigated for their ability to control the release of drugs [113]. A larger hydrophilic protein and a smaller hydrophobic molecule were evaluated to demonstrate that a range of osteogenic drugs could be released from the composites, either loaded in hydrogel particles or directly loaded into the CS matrix. The study will investigate how the solubility and other characteristics will affect the way the agents are loaded into CS to achieve a controlled and
consistent release. Lysozyme was chosen as a model protein to determine the potential to adjust protein release, and simvastatin was used as a representative small molecule drug.

4.2. Materials and Methods

4.2.1. Hydrogel synthesis

H6 PBAE macromer was synthesized according to previously described methods [78, 113]. To review, polyethylene glycol (PEG) 400 diacrylate (Polysciences, Inc., Warrington, PA) and isobutylamine (Sigma-Aldrich, St. Louis, MO) were combined in a 1.2:1 molar ratio and placed in a flask partially submerged in a silicon oil bath pre-heated to 85°C. The reaction mixture was continuously stirred for 48 hr and then removed from the heat. The resulting macromer was stored at 4°C until used.

Biodegradable hydrogels (HG) were made by photopolymerization. Two grams of cooled macromer and 1 wt% of 2,2-dimethoxy-2-phenyl-acetophenone (DMPA; Sigma Aldrich, St. Louis, MO) initiator were dissolved in 1.25 mL of dimethyl sulfoxide (DMSO). H6 loaded with lysozyme was made the same way except that 100 mg of lysozyme (14 kDa; Sigma-Aldrich; solubility: ~300 mg/mL in H2O) was added along with the initiator. After vortexing for 60 sec, the homogeneous mixture was pipetted in between glass plates separated by a 1.5 mm Teflon spacer. The macromer was exposed to UV radiation from a Lesco UV flood source with an intensity of 14 mW/cm² for 5 min starting at room temperature. After polymerization, the gel was washed with ethanol for 30 minutes to remove any unreacted initiator and macromer and then lyophilized overnight.

4.2.2. Protein release from H6 PBAE gels

To assess release kinetics from the gel alone, circular (diameter: 9 mm) samples of protein-loaded H6 PBAE were weighed, submerged in 4 mL of PBS, and incubated at 37°C on a plate shaker. For the first hr, supernatant was collected for measurement and replenished every 10 min. During the second and
third hr, supernatant was collected and replenished every 20 min. After 3 hr, the time intervals were increased to every hr until the samples completely degraded. Collected supernatant was measured using the MicroBCA Protein Assay (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer’s protocol.

4.2.3. Formation of drug-loaded calcium sulfate composites

The composites consisted of calcium sulfate hemihydrate (Sigma-Aldrich) as the structural matrix and different amounts of lysozyme-loaded HG particles. Hydrogel particles were hand-ground from polymerized gel slabs using a mortar and pestle at room temperature. In order to coat the gel particles and prevent them from sticking to one another, as well as to allow grinding to be efficient, small amounts of CS powder were added during grinding. Particle sizes of 53-150 μm and 150-250 μm were obtained from grinding and sieving. After grinding, gel particles were washed with 100% ethanol for 1 min to remove residual CS, filtered, and air-dried.

CS samples were fabricated as previously described.[113] Briefly, control (drug- and HG-free) CS samples were made by thoroughly mixing 1 g of CS powder and 800 µL of deionized (DI) water in 3 mL syringes fitted with blunt-tipped needles. The slurry was loaded into a custom Teflon mold having a diameter of 4.7 mm and a height of 6.5 mm and allowed to set at 43°C for 24 hr. Similar to the CS control samples, 1 wt% composite samples were created by mixing 0.01 g HG particles with 0.99 g CS and 850 µL DI water. Samples with 10 wt% protein-loaded particles were made by mixing 0.1 g HG, 0.9 g CS, and 900 µL DI water. As for the controls, the composite mixtures were also allowed to set at 43°C for 24 hr. CS samples directly loaded with lysozyme were also made for comparing with protein release from CS-HG composites. Two different loadings were selected to match the amounts in hydrogel composite samples. Briefly, 500 µg or 5 mg of lysozyme were mixed with 1 g CS powder and 800 µL DI water to create the low and high loadings, respectively. The slurries were loaded into a mold and allowed to set at 43°C for 24 hr.
Simvastatin-loaded samples were created by adding 20 mg of simvastatin (Haouri Pharma-Chem, Inc., Edison, NJ; 419 Da; solubility: 12.2 μg/mL in H₂O) to 1 g of CS powder prior to mixing with 850 μL of DI water. The slurry was injected into a custom-fabricated Teflon mold and placed in a 43°C oven for 24 hr to set the CS.

4.2.4. Surface exposure of HG particles on CS surface

To visually demonstrate the surface exposure of protein containing HG particles at the surface of CS during dissolution, samples were produced containing gel particles dyed with blue food coloring. HG particles (150-250 μm) were fabricated using the same methods described above for gel polymerization and grinding. The finished gel particles were soaked for 5 mins in a 1 mL solution of DI and 3 drops of blue food coloring. After 5 mins, the particles were washed and vacuum filtered. The washed particles were quickly frozen at -80°C and lyophilized overnight. To produce the CS composites, methods for composite fabrication described above for a 10 wt% HG loaded samples were used. Photographs were taken using a Canon DS126071 camera fitted with a 60 mm macro lens prior to exposure to PBS and after 24 hr of dissolution in 4 mL PBS.

4.2.5. Drug release from CS samples

Protein release from CS composites was conducted with samples for each treatment (blank, 1%HG 53-150 μm, 10%HG 53-150 μm, 1%HG 150-250 μm, 10%HG 150-250 μm, direct low, and direct high) initially weighed, submerged in 4 mL of PBS, and incubated at 37°C on a plate shaker. Every 4 days, supernatant was collected for measurement and replaced with 4 mL of fresh PBS until all samples had completely degraded. Collected supernatant was measured using the MicroBCA Protein Assay slightly modified from the manufacturer's protocol due to CS precipitation observed when the working reagent was added. Briefly, 200 μL of supernatant and 200 μL of working reagent were combined and incubated at 37°C for 2 hr. Vials were centrifuged at 10,000 G for 2 min to
separate any CS precipitate that formed during incubation, and absorbance of the cleared reagent was measured at 562 nm. Lysozyme standards were also assayed using this process to allow for consistent results.

Simvastatin release was conducted by pre-weighing both blank and simvastatin-loaded CS samples, submerging them in 4 mL of PBS, and incubating at 37°C on a plate shaker. Every 4 days, supernatant was collected for measurement and then replenished with 4mL of fresh PBS. The collected supernatant was treated with 100% ethanol in a 50:50 volume ratio of supernatant to solvent. After filtration (0.45 μm), absorbances were measured at 240 nm and compared to a series of standards.

4.2.6. Mechanical properties

Compression testing was performed to determine whether incorporation of directly loaded drug (simvastatin and lysozyme) or protein-loaded H6-PBAE particles into CS would affect the mechanical properties. Testing was conducted using a Bose ELF 3300 system. Contact surfaces were lightly sanded, if necessary, using 600 grit SiC paper to provide smooth, parallel surfaces in contact with the compression platens. Control (blank, drug-free), directly loaded simvastatin, low and high dose of directly loaded lysozyme, 1 wt%HG, and 10 wt%HG samples were tested at a rate of 0.5 N/sec until failure. Compressive modulus (M) and ultimate compressive strength (UCS) were calculated.

4.2.7. Statistics

Statistical analysis of the mechanical compression results was performed using two-way ANOVA. As appropriate, the analysis was followed with a Tukey-Kramer multiple comparisons post hoc test. Differences between groups were considered to be significant with p-values < 0.05.
4.3. Results

4.3.1. Protein release from H6 PBAE gels

Figure 4.1 displays the kinetics of lysozyme release from H6 PBAE discs. During the first hr, the gels swelled steadily with little protein (~4.5%) being released. For the next two hr, the rate of protein release continued to increase from 13% per hr to a steady state of 22.8% per hr, which was sustained for the remainder of the experiment. After four hr, the gels became too difficult to handle, and consequently the degree of swelling could not be determined.

4.3.2. Surface exposure of HG particles on CS surface

Figure 4.2 shows a couple photographs displaying hydrogel particles exposed at the surface of CS composites. The left photo shows a freshly made composite. HG particles appeared to be uniformly distributed at the surface. After a day of dissolution (right image), the top layer of gel had degraded leaving behind pockets in the CS (right photo insert).

4.3.3. Protein release from composites

Displayed in Figure 4.3 are the results for the release of lysozyme directly loaded into CS. For the lower loading, no protein was released during the first 4 days (Figure 4.3A). From 4-16 days, release was fairly steady at a rate of 4.5%/day, and at day 16, the rate decreased to about 1.4 %/day. During the final 4 days, the rest of protein was released at a rate of 8.7%/day. However, large variations in release were observed; fluctuations as high as ±30%/day were seen. The instantaneous release pattern was erratic and unpredictable. Figures 4.3B show results for samples with the high direct loading of protein. A large burst (~65%) of protein was observed, after which release was fairly steady at a rate of 1.15%/day with some fluctuations. The high direct loading also showed large deviations (±15-20%/day) among the samples.

Figure 4.4 shows the release kinetics for CS composites containing embedded PBAE particles loaded with lysozyme. A steady release of lysozyme from 1 wt% HG composite loadings (both particle sizes) was observed, where a
30-40% burst of protein was measured during the first 4 days, and 70-80% of the protein was released after 14 days (Figure 4.4A). The rate of protein release from 1 wt% HG samples slowly decayed over the duration of the experiment. At the beginning, the average rate of release was as much as 7-10%/day at 4 days, while at later time points, the average rate decreased to 5-7%/day at 12 days and 4%/day at 24 days. On the other hand, for composites loaded with 10 wt% HG, release started with a much larger burst of protein (55-70%) at an average rate of about 17%/day during the first 4 days followed by 80-90% being released during the first 12 days of the 24 day study (Figure 4.4B). After about 8 days, release remained fairly steady at an average rate between 0.5% and 3% per day.

### 4.3.4. Simvastatin release from calcium sulfate

Figure 4.5 illustrates the release kinetics for CS composites directly loaded with simvastatin. There appeared to be a short delay in the initial release of simvastatin, and over the first 8 days, roughly 12% of the drug was released. Following this lag period, the average rate shifted upward to about 5.5% of simvastatin per day and remained steady between days 8 and 20. After 20 days, release slowed to 3%/day and remained close to this rate for the final 8 days of the 28 day study.

### 4.3.5. Mechanical properties

For the ultimate compressive strength, Figure 4.6A, CS blanks were significantly stronger than samples with the high dose of directly loaded lysozyme (p<0.01), 1 wt% HG composites (p<0.001), and 10 wt% HG composites (p<0.001). The strength of 10 wt% HG (1.0 ± 0.3 MPa) compared to CS blanks (5.5 ± 0.6 MPa) was about 80% weaker. Compared to all drug loaded samples, the strength of 10 wt% HG was also significantly lower than all other samples (p<0.001). In addition, the decrease in strength for the high direct loading of lysozyme (4.3 ± 0.5 MPa) and 1 wt% HG composites (4.1 ± 0.4 MPa) was only about 20-25% compared to the CS blanks.
The elastic modulus, Figure 4.6B, for CS blanks (406 ± 126 MPa) was significantly higher than that for directly loaded simvastatin samples (267±68 MPa) and 10 wt% HG composites (189 ± 89MPa) (p<0.01). There were no significant differences between the controls and low direct lysozyme, high direct lysozyme, and 1 wt% HG composite samples. Elastic modulus for 10 wt% HG composites was also significantly less than that for low direct lysozyme (430 ± 154 MPa; p<0.001), high direct lysozyme (349 ± 110 MPa; p<0.05), and 1 wt% HG composites (383 ± 126 MPa; p<0.01).
FIGURE 4.1. Lysozyme release from PBAE gels along with the swelling profile of H6 PBAE. Data are mean ± standard deviation (n=3).
FIGURE 4.2. Photograph depicting HG surface exposure on CS composites. HG particles dyed blue for contrast. (Left) Sample before 24 hr dissolution in PBS. (Right) Sample after 24 hr in PBS.
FIGURE 4.3. Release of lysozyme directly loaded into CS. (a) Cumulative profile for the low protein loading. (b) Cumulative profile for the high protein loading. Data are mean ± standard deviations (n=5).
FIGURE 4.4. Cumulative release of lysozyme from CS composites having two different HG particle sizes (53-150 μm and 150-250 μm). (a) 1 wt% HG and (b) 10 wt% HG. Data are mean ± standard deviations (n=5).
FIGURE 4.5. Cumulative release of simvastatin directly loaded into CS. Data are mean ± standard deviation (n=5).
FIGURE 4.6. Mechanical properties of simvastatin- and protein-loaded CS. (a) Compressive strength; (b) Compressive elastic modulus. Data are mean ± standard deviation (n=10). Symbols (*) and (#) indicates significant difference: p<0.01 and p<0.05, respectively.
4.4. Discussion

The aim of the present study was to demonstrate that previously developed CS composites[113] are capable of controlling the release of different bioactive molecules having a broad range of physical and chemical properties such as size, function, and solubility by utilizing either direct loading of drug into the CS matrix or with the aid of PBAE hydrogel particles.

4.4.1. Protein release from H6 PBAE gels

H6 PBAE particles were investigated as a drug delivery vehicle embedded in a CS matrix. For this study, gel particles were loaded with the protein lysozyme. Lysozyme, an extensively studied and characterized protein, has become a reliable substitute for larger osteogenic molecules, such as bone morphogenetic proteins [114, 115]. A study of protein release directly from the gel was conducted first. After a slow initial release of lysozyme, a higher steady state was sustained for the remainder of the experiment. The overall trend suggested that lysozyme was trapped within the gel allowing only a slow diffusion of protein from the matrix during swelling followed by protein being released as the gel degraded. This was desired for allowing release of protein from the composites to occur only when the gel particles are exposed and released themselves.

Hawkins et al. demonstrated sustained release of lysozyme from A11 poly(β-amino ester) [116]. The method for loading lysozyme in the A11 PBAE gels was similar to that used with H6 PBAE gels in this study. Although the two polymers have different compositions, they also have some similarities as members of the PBAE family of hydrogels [66, 71, 77, 78, 116]. Among this family of materials, a variety of possible combinations of diacylates and amines can produce similar range of properties [66, 71, 77, 78, 116]. The similarities between these two PBAEs demonstrate the adaptability of this gel family to accommodate different formulations or drug types if needed.
4.4.2. Protein release from CS

To determine the effectiveness of controlling release of protein from CS, a series of release experiments compared release of lysozyme from composites to release of protein directly loaded into the CS matrix. To allow for an accurate comparison, the lysozyme amount for 1 wt% HG was matched with the low direct loading, and the loading for 10 wt% HG was the same as that for high direct loading. Two different release trends were seen for direct loading. During the 28 day study for the lower direct loading of lysozyme, the amount of protein released fluctuated with no noticeable consistencies. In addition to this variability, there was a delay in the release of protein during the first 4 days. Although the cumulative release for the lower direct loading showed an overall linear trend, there were large deviations from the means, which may indicate inhomogeneous distribution of protein within and between samples. When comparing the low direct loading to the 1 wt% HG samples, release of lysozyme from the composites was more consistent. Previous research demonstrated good gel particle distribution in the composites [113]. The slow decay in the release of protein from the 1 wt% HG composites was similar to that observed for a pilot study with the antioxidant curcumin [113]. An important difference from the curcumin release study was that the drug was previously added during PBAE macromer synthesis, which allowed it to covalently bind with the diacylate [102]. This process helped prevent premature release or diffusion of drug from the gel matrix prior to hydrolysis, providing greater control over curcumin release [102]. Instead of being covalently incorporated during macromer synthesis, the macromer was polymerized around the protein in the present studies. Because lysozyme is a much larger molecule, the protein could be entrapped in the polymer mesh structure. This trapping of protein allows release from the gel matrix only after particles near the surface of the dissolving CS have been freed and begun to degrade. As discussed in section for protein release from H6 PBAE gels, protein was prevented from leaving the gel matrix until the gel showed signs of hydrolysis. This illustrates that protein was not prematurely released prior to exposure of HG particles during composite erosion. Based on
the images displaying HG particles (dyed blue) embedded into CS, the gel particles that were at or very near the surface had degraded leaving behind small pockets in the CS, which in turn began to expose more blue colored gels below the surface. This observation helps to demonstrate how the release of protein from embedded HG particles is governed by CS dissolution. In addition, in a previous study, MicroCT images showed a uniform distribution of HG particles throughout a CS matrix which could allow for a sustained release as CS dissolved [113]. The slow decay in the release of protein from the 1 wt% HG composites can be attributed to the slow decrease in surface area of CS over the duration of the release study, which leads to a smaller amount of gel particles exposed over time and thus a slowly decreasing mass of protein being released.

The two different higher loadings (high direct and 10 wt% HG) demonstrated similar release kinetics. However, the advantage for the 10 wt% composites was the smaller variability among samples and therefore better control of protein release. During the first several days of the release, a large surface area of CS was exposed, allowing for a much larger volume of gel particles to be released. This could lead to an accumulation of gel particles releasing protein early on and thus contribute to the burst witnessed from the 10 wt% HG composites. As for the high direct loading of lysozyme in CS, greater early protein release could result from a segregation effect due to solubilized protein accumulating at the surfaces in excess water as the CS slurry sets. Furthermore, as water was consumed by reaction from the hemihydrate to dehydrate forms of CS, some solubilized protein may be precipitated and trapped within the setting matrix. The more variable release of protein could be attributed to a heterogeneous distribution of lysozyme among the CS matrix potentially caused by protein aggregation, CS crystal formation causing closure of small pockets containing larger amounts of protein, and/or poor mixing of protein and CS before and after addition of water. With the addition of carrier particles, such as protein-loaded PBAE, the variations can be reduced or eliminated altogether.
4.4.3. Simvastatin release from CS

To demonstrate the release of small molecule osteogenic agents, simvastatin was directly loaded into CS. Ginebra et al. described how drug may be trapped between crystals in a ceramic matrix; dissolved in the liquid within pores, adsorbed to the crystal surface, or in solid form as drug crystals [117]. Because simvastatin has low water solubility, the drug was likely to be suspended in the CS slurry and not dissolved in the water. With this in mind, as water was consumed during setting or excess water was driven to the exterior of the samples, simvastatin remained in the CS matrix. As a result, the drug was released as the material dissolves with consistent results. Based on the ability to control release by directly loading simvastatin in CS, incorporating the drug in hydrogel particles is unnecessary and would introduce unneeded material in the system.

Nyan et al. also investigated simvastatin directly loaded into CS. They conducted an in vivo study in critically-sized rat calvarial defects [45]. A dramatic increase in bone volume was observed after 8 weeks, which was significantly greater than for any other group [45]. However, substantial soft tissue inflammation was also observed during the first several weeks, and the authors concluded that this was most likely caused by a burst release of simvastatin following early resorption of CS [45]. Although dissolution of the implants was not assessed, the present results support the hypothesis that rapid erosion of CS would release a burst of drug.

4.4.4. Mechanical properties

With the incorporation of drug either directly loaded or in PBAE particles embedded in CS, compromise of mechanical stability can become a concern. The present drug-loaded composites are being developed as bone graft substitutes with tunable drug delivery capabilities. With this in mind, it is important to note that much of the strength of the grafting device must be preserved to allow for a stable implant capable of providing a suitable ‘healing chamber’ for tissue regeneration as well as allow for controlled release of drug as
the implant dissolves. A previous study demonstrated that the addition of additives did not have a dramatic effect on the dissolution of the composite, therefore the attention is drawn to the preservation of the mechanical strength of the composites [113]. In the present study, the comparison of compressive properties for CS with directly loaded simvastatin to those for blank CS showed that the drug did not have a significant effect on the overall strength. However, incorporation of simvastatin reduced the modulus about 30% compared to the CS control samples. Although a small molecule drug, simvastatin may be creating small disruptions in the CS crystal interactions that could lead to greater shearing along crystal surfaces resulting in more deformation prior to failure. Yin et al. reported that a small amount (10%) of directly loaded simvastatin did not significantly compromise the compressive strength of calcium phosphate [118]. In the current study, 20 mg, or 20 wt%, was loaded into CS prior to sample production. Although the materials were different, this finding demonstrates that the incorporation of a small amount of simvastatin does not appear to affect the supporting material, thus preserving its overall strength.

The effect of loading a much larger drug molecule compared to simvastatin on the mechanical stability of CS was also tested. Protein, i.e., lysozyme, was either directly loaded into the CS matrix or it was polymerized into H6-PBAE hydrogel particles that were then incorporated into the CS. Directly loading lysozyme into the CS matrix at the amounts tested did not have much of an effect on the overall mechanical properties. Addition of HG particles, however, did affect the mechanical stability of CS. The significant decrease in strength and modulus for 10 wt% HG particle loading demonstrates a possible limit to the amount of drug-loaded particles that can be added. In a previous study, Orellana et al. showed that the addition of 10 wt% of A11 PBAE particles decreased the strength and modulus of CS by 50% and 70%, respectively [113], which suggested a comparable upper threshold of particle loading. A potential explanation for the particle loading effect could be contributed to the altered microstructure due to stress concentrators created by the gel particles or even minor bubbles/pores [113]. For a drug delivery platform that not only preserves
as much of the mechanical stability of CS as possible but also allows controlled release of drug as the material erodes, a loading of 1 wt% HG may be considered the better option based on the results observed in the mechanical tests and the consistency witnessed for the release of a larger hydrophilic molecule.

4.5. Conclusion

Controlled release of bioactive molecules has the potential to greatly enhance the ability of calcium sulfate-based composites to become more effective bone graft substitutes. In the present study, release kinetics were investigated for two different agents, a small, hydrophobic drug and a much larger, hydrophilic protein, incorporated into CS. Controlled release of both molecules could be achieved but by different means. Whereas direct loading was appropriate for simvastatin, control of lysozyme release required incorporation of the protein in degradable polymeric particles prior to embedding in CS. Adding a drug, either directly into CS or via PBAE particles, however, can negatively affect mechanical properties. In particular, greater amounts of gel particles significantly reduced compressive strength and modulus. Therefore, the balance between drug content and mechanical properties must be determined for different applications. Based on the current findings, CS-based synthetic bone graft substitutes demonstrated the ability to sustainably release both large and small bioactive molecules, opening up potential opportunities to deliver a broad range of therapeutic agents.
5.1. Introduction

Healing of infected bony defects, such as those resulting from periodontal disease, has become a major focus in the field of bone tissue engineering [119]. Onset of periodontitis, a bacterial infection affecting the gingiva, alveolar bone, periodontal ligament, and root cementum, leads to the initiation and propagation of chronic inflammation, eventually causing the destruction of surrounding connective tissue and bone [8, 10, 14, 120-122]. Periodontal infections are first treated with extensive debridement and scaling of plaque [8, 14, 121, 123]. Some pathogens may not be susceptible to mechanical removal, however, because they often ‘hide’ deep in gingival pockets around compromised bony tissue [14, 124]. These bacteria can frequently trigger reoccurrence of the initial infection, which greatly affects restoration or preservation of lost bone [123]. Antibiotics systemically delivered orally or locally administered using topical gels, creams, or films are then used to eliminate the pathogens prior to implantation of grafting material [14, 119, 123].

An infected periodontal pocket contains an abundance of microflora, such as Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis [14, 15]. These pathogens are often protected within a biofilm, which allows them to flourish and requires a substantially higher dose of antibiotic to fully eradicate the bacteria [14, 15]. Due to clearance in the blood during systemic administration, the resulting low local concentrations of antimicrobial agents at the infected site often do not meet the levels required to kill the microbes [120-122, 125]. As a result, long-term therapy with high systemic doses of antimicrobial agents may be needed to fully eliminate the infection. However, this treatment could potentially cause adverse effects, such as liver and kidney damage, or lead drug resistance [126]. Consequently, local administration of drug directly to the site of infection may prove more effective by providing a higher concentration while using a smaller dose [14, 121]. Of the different antibiotics used for dental
applications, metronidazole significantly reduces periodontal infection when compared to others [63, 125]. In addition, using a biodegradable material capable of controlling the amount of metronidazole released may allow for higher sustained and more effective concentrations to be obtained at the site.

Following treatment of periodontal infections, regeneration of bony tissue can begin. Bone regeneration using autografts is considered the 'gold standard'. Intra-oral donor sites are limited, however, and harvesting can lead to undesirable donor site morbidity and chronic discomfort [31, 37, 45, 54, 64, 84, 88-96]. Another option is the use of allografts harvested from cadaveric bone tissue. Although frozen, freeze-dried, and/or demineralized, patients receiving the grafts are still at risk of immunologic rejection or disease transmission [56, 64, 127]. Allografts have been combined with osteoinductive growth factors to make them more effective [45]. Because of the limitations, and even risks, involved with autogenous and allogeneic materials, much attention has turned to the development of innovative bone graft substitutes. Calcium sulfate (CS) represents a promising alternative [45]. CS becomes osteogenic in the presence of bone, and through dissolution, the material is completely absorbed without inducing a significant inflammatory response [31, 33-35, 46]. Like many other synthetic grafting alternatives, however, the efficacy of CS can be further enhanced with the aid of bioactive agents.

A beneficial addition would be the incorporation of statin drugs. Statins are widely known as inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase that help control cholesterol levels, but these pleiotropic drugs also have osteogenic activity [55-57, 59, 60, 128, 129]. In vitro and in vivo studies have demonstrated the positive effects of simvastatin through stimulation of osteoblastic activity and inhibition of osteoclastic activity [55-59, 128, 129]. Simvastatin was shown to be effective when released from a CS matrix in vivo [45].

A bone grafting device capable of sequentially releasing an antibiotic followed by an osteogenic agent may be able to treat infection while still being able to regenerate bone. Little research has investigated the release kinetics of
antimicrobial and osteogenic drugs from the same grafting device [119]. In the present study, dual drug-loaded, bilayered CS composites comprising a shell and core geometry with embedded poly(lactic-co-glycolic acid) (PLGA) particles were developed. After evaluating the compressive strength and modulus, dissolution, and morphology of bilayered composites, the tunable sequential release kinetics of metronidazole and simvastatin from the composites were explored.

5.2. Materials and Methods

5.2.1. Metronidazole-loaded PLGA Particles

Poly(lactic-co-glycolic acid) (Durect Corp., Birmingham, AL; 50:50; inherent viscosity: 0.55-0.75 dL/g; carboxylate end group) particles loaded with metronidazole (Sigma-Aldrich, St. Louis, MO) were created by film-casting and hand-grinding. Initially, 25 mg of metronidazole were combined with 200 mg of PLGA and dissolved in 1 mL of dimethyl sulfoxide (DMSO; an FDA Q3C Class 3 solvent). The solution was poured into a circular Teflon mold, frozen quickly at -80°C, and lyophilized to remove the DMSO. The dried film was hand ground to obtain particle sizes between 150-250 µm. A small amount of CS was used to prevent the polymer from sticking during grinding. The particles were washed with ethanol to remove residual CS powder on the surface of the polymer. Ethanol was chosen for washing to prevent drug loss because the low solubility of metronidazole in this solvent. Washed particles were vacuum-filtered, rapidly air-dried, and stored at -20°C until used.

A short-term study of metronidazole-loaded microparticles was conducted to determine how much drug may be released during the setting phase of composite formation. For this purpose, 10 mg of washed PLGA particles were incubated at 37°C in 1 mL of phosphate-buffered saline (PBS), pH 7.4. Supernatant was collected and replaced with fresh PBS every 15 min for the first hr, every 30 min for the 2nd hr, every hr for the 3rd and 4th hr, and finally increased to every 2 hr for the 6th and 8th hr time points. Supernatants were filtered (0.45 µm) and the absorbance measured at 318 nm.
5.2.2. Bilayered Calcium Sulfate Composites

Fabrication of the bilayered composites is illustrated in Figure 5.1. The composites consisted of calcium sulfate hemihydrate (Sigma-Aldrich) as the structural matrix. First, blank CS samples without layers were produced by combining 1 g of CS with 800 μL of deionized (DI) water. The slurry was injected into a mold having a diameter of 6.3 mm and a height of 12.6 mm. The loaded mold was placed in a 43°C oven for 24 hr to allow for the CS to completely set.

To begin formation of bilayered composites, cylindrical cores were produced in Teflon molds having a diameter of 4.7 mm and a height of 10 mm. A small, 8 mm long metal peg with a 0.63 mm diameter was fitted precisely in the center of the mold, with about 2.5 mm of the peg embedded into the core. The pegs suspended and centered the cores for shell production later. To make blank CS cores, 800 µL of DI water was added to 1 g of CS powder and mixed thoroughly in 3 mL non-sterile syringes fitted with a 16 gauge blunt needle. The slurry was loaded into the custom-fabricated Teflon mold and placed in a 43°C oven for 24 hr to set the CS. For cores loaded with simvastatin, the same process was used, however 20 mg of simvastatin (Haouri Pharma-Chem, Inc., Edison, NJ) were mixed along with the CS and DI water. Pegs were removed from the cores when they were dried, and the cores were stored at room temperature with desiccant until used.

To form the shell around the cores, another Teflon mold was created with cylindrical holes having the diameter of 6.3 mm and a height of 12.6 mm. The base plate was fabricated with 3.5 mm deep holes into which metal pegs were securely inserted. This depth allowed the cores to be positioned precisely in the center of the mold, thus allowing the shell to surround the core. Blank and simvastatin-loaded shells were created using the same method described above for the cores, where 1 g of CS was mixed with 800 μL of DI water, and in the case of simvastatin-loaded shells, 20 mg of drug were directly added. For samples containing metronidazole-loaded PLGA, the particles were added to both the blank and simvastatin-loaded shells at either 1 or 10 wt% and then mixed with 850 μL DI water in 3 mL syringes fitted with a blunt-tipped needle for
easy, consistent filling of the mold. Using these formulations for the shells, bilayered composites were formed by filling the molds about half full. Next, prefabricated cores were quickly dipped in DI water to wet the surface, which allowed for smooth coverage of the shell slurry around the core, inserted into the mold, and pressed down onto the metal pegs until they stopped. The pegs positioned the cores and held them in place during setting of the shell slurry. The filled mold was placed into a 37°C oven and allowed to dry overnight. For simplification, the different types of samples were given abbreviated names (Table 1).

The shells and cores described had a volume ratio of 50:50. Two other ratios were tested with simvastatin loaded into the shell only (SSBC), core only (BSSC), or both layers (SSSC). These samples were used to demonstrate how a change in the volume ratio would affect drug release from the composites. Table 2 lists the volume ratios used and the dimensions of the respective core and shell components. Custom molds were created to accommodate the different sizes, but the rest of the fabrication process was the same as described previously.
Figure 5.1. Schematic depicting the process of forming a bilayered CS composite. From left to right: core formation; insertion of core into shell mold; final composite showing core encased within a CS shell. Images are to scale.
Table 5.1. Abbreviations for the different sample types fabricated. Codes are read: BSBC » (B)(S)C » (Loading) in SHELL, (Loading) in CORE.

<table>
<thead>
<tr>
<th>Shell Composition</th>
<th>Core Composition</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>Blank</td>
<td>BSBC</td>
</tr>
<tr>
<td>Blank</td>
<td>Simvastatin</td>
<td>BSSC</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>Blank</td>
<td>SSBC</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>Simvastatin</td>
<td>SSSC</td>
</tr>
<tr>
<td>1 wt% PLGA</td>
<td>Blank</td>
<td>1-BSBC</td>
</tr>
<tr>
<td>1 wt% PLGA</td>
<td>Simvastatin</td>
<td>1-BSSC</td>
</tr>
<tr>
<td>1 wt% PLGA &amp; Simvastatin</td>
<td>Blank</td>
<td>1-SSBC</td>
</tr>
<tr>
<td>1 wt% PLGA &amp; Simvastatin</td>
<td>Simvastatin</td>
<td>1-SSSC</td>
</tr>
<tr>
<td>10 wt% PLGA</td>
<td>Blank</td>
<td>10-BSBC</td>
</tr>
<tr>
<td>10 wt% PLGA</td>
<td>Simvastatin</td>
<td>10-BSSC</td>
</tr>
<tr>
<td>10 wt% PLGA &amp; Simvastatin</td>
<td>Blank</td>
<td>10-SSBC</td>
</tr>
<tr>
<td>10 wt% PLGA &amp; Simvastatin</td>
<td>Simvastatin</td>
<td>10-SSSC</td>
</tr>
<tr>
<td>No Layer Blank (No Drug)</td>
<td></td>
<td>NL</td>
</tr>
</tbody>
</table>

Note: PLGA particles contained metronidazole.
Table 5.2. Shell to core volume ratios and dimensions of the bilayered composites

<table>
<thead>
<tr>
<th>Volume Ratio</th>
<th>Shell Height</th>
<th>Shell Diameter</th>
<th>Core Height</th>
<th>Core Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:50</td>
<td>12.6 mm</td>
<td>6.3 mm</td>
<td>10 mm</td>
<td>4.7 mm</td>
</tr>
<tr>
<td>70:30</td>
<td>9.6 mm</td>
<td>4.7 mm</td>
<td>6.2 mm</td>
<td>3.2 mm</td>
</tr>
<tr>
<td>85:15</td>
<td>9.6 mm</td>
<td>4.7 mm</td>
<td>6.2 mm</td>
<td>2.4 mm</td>
</tr>
</tbody>
</table>
5.2.3. Composite Microarchitecture

To monitor the distribution of PLGA particles within the CS shell matrix as well as the interface between shell and core, microcomputed tomography (microCT) was employed. Using a Scanco Medical μCT-40 scanner, specimens were evaluated at high resolution. Other parameters were set as follows: 156 μm increments, 0° angle, 70 kVp, 114 μA, 0.5 mm Al filter, and a voxel size of 8 μm. The raw images were qualitatively investigated for particle distribution trends, core orientation, and shell-core interaction. In addition, qualitative and quantitative assessment of the composites was conducted using a built-in ‘bone trabecular morphometry’ analytical tool with a lower threshold level of 130, gauss sigma of 3.0, and gauss support of 9. This script created a three-dimensional reconstruction that allowed visual assessment of cross-sections through the composite and provided the volume percentage of embedded polymeric particles and internal voids.

5.2.4. Composite Dissolution

Destructive testing was used to monitor dissolution of the bilayered composites. BSBC, 1-BSBC, and 10-BSBC samples were weighed, placed in separate 20 mL scintillation vials containing 12 mL of PBS and incubated on a plate shaker at 37°C. Every 4 d, replicate samples of each type were removed and dried at 43°C overnight. For the remaining samples, the PBS was replaced with fresh solution. The dried samples were weighed to determine the amount of material remaining, which was then used to calculate the percentage of residual mass.

5.2.5. Mechanical Properties

Compression testing was performed to investigate any effects on mechanical properties caused by the layered geometry, simvastatin directly loaded into CS as well as the introduction of PLGA particles into the shell of the composites. All samples types listed in Table 1 were evaluated. Testing was accomplished using a Bose ELF 3300 system. Contact surfaces were lightly
sanded, if necessary, to create parallel surfaces in contact with the compression platens. All samples were loaded at a rate of 0.5 N/sec until failure. Compressive modulus (M) and ultimate compressive strength (UCS) were calculated [130].

5.2.6. Drug Release from Bilayered Composites

5.2.6.1. Simvastatin Release

Release of simvastatin was measured for composites having shell to core volume ratios of 50:50, 70:30, and 85:15. BSBC, BSSC, SSBC, and SSSC samples for all volume ratios were prepared using the same simvastatin loading described in section 2.2. Samples were pre-weighed and submerged in PBS. Considering the overall size differences between the sample types, different volumes of PBS were used to maintain similar fluid volume to composite surface area ratios. To determine suitable sink conditions, sample surface area to solution volume ratios of 50:50 samples was compared to those used in previous research [113]. A small pilot study (data not shown) showed that the dissolution rate of 50:50 samples remained constant for volumes above 10 mL. To avoid saturation of CS or drug in PBS, a larger volume (12 mL) was used for the 50:50 samples. All other samples were placed in 4 mL, similar to previous release studies [131]. To determine how simvastatin would be released from bilayered composites dissolving in non-sink conditions, 50:50 samples were also immersed in 8 mL of PBS. All samples were incubated at 37°C on a plate shaker. Every 4 d, supernatant was collected and replenished with fresh PBS. Collected supernatant was treated with 100% ethanol in a 50:50 volume ratio to make sure all simvastatin was in solution. The mixture was then 0.45 µm-filtered and absorbance measured at 240 nm.

5.2.6.2. Multiple Drug Release

To investigate the kinetics of metronidazole and simvastatin release from bilayered CS composites, samples were pre-weighed, submerged in 12 mL of PBS, and incubated at 37°C while on a plate shaker. Supernatant was collected and replenished every 4 d. Two aliquots from each sample were kept for
measurement of metronidazole and simvastatin separately. Metronidazole in syringe-filtered (0.45 µm) supernatant was assayed directly using UV spectroscopy at 318 nm. Simvastatin was measured using high performance liquid chromatography (HPLC) on a Hitachi Primaide system fitted with a Kinetix C18 column (5 µm, 4.6 x 150 mm). Prior to measurement, supernatant was mixed with 5 mM EDTA (pH 8.0) at a 50:50 volume ratio and allowed to sit overnight. EDTA, a common chelating agent, was used to remove calcium ions that could precipitate during HPLC analysis. Next, this mixture was mixed with 100% ethanol in a 50:50 volume ratio to ensure complete dissolution of the poorly soluble simvastatin. The final sample composition was 25% supernatant, 25% EDTA, and 50% ethanol. A 70:30 (acetonitrile : DI water + 0.01% trifluoroacetic acid) isocratic mobile phase at a flow rate of 1 mL per minute was used. Simvastatin was detected at 240 nm.

5.2.7. Statistics

Statistical analysis of the results was conducted using either a two-tailed unpaired t-test or one-way ANOVA. As appropriate, a Tukey-Kramer multiple comparison post hoc test was implemented. Linear regression was performed on sustained release profiles and the calculated slopes compared for significant differences using a two-tailed t-test. Differences between groups were considered to be significant with p-values <0.05.

5.3. Results

5.3.1. Metronidazole-loaded PLGA Particles

Metronidazole-loaded PLGA particles alone were first evaluated to determine how well the polymer controlled drug release. According to the results in Figure 5.2, metronidazole was released steadily during the first 3 hr at a rate of about 4 µg per minute. Only about 8% of the drug was released during the first hr. After 3 hr, 30% of the total loading of metronidazole had been released. From this point forward, the release of drug from the particles slowed, with roughly 35% of the loaded metronidazole released after 6 hr.
5.3.2. Composite Microarchitecture

5.3.2.1. Qualitative Evaluation

MicroCT images showed the CS cores embedded within the layered composites and their interaction with the CS shells, as well as the distribution of PLGA particles embedded in the shells (Figure 5.3). For comparison, results for a CS sample without layers (NL) are included. Minor defects (bubbles and other discontinuities) were found throughout the CS matrix in all samples. These defects were nearly spherical in nature and, thus, easily distinguishable from PLGA particles, which were irregularly shaped. The blank (drug-free) layered samples, BSBC, showed the CS core embedded within the CS shell and oriented parallel to the outer walls. The distribution of 1 wt% PLGA particles in the shells of 1-BSBC samples appeared to be uniform but sparse (Figure 5.3, top right). In 10-BSBC samples with 10 wt% loading of particles in the shells, there was a uniform but more frequent distribution of particles throughout the CS shell (Figure 5.3, bottom right). Similar to BSBC, both 1-BSBC and 10-BSBC had cores that were embedded in the center of the composite with a parallel orientation to the outer surface. Additionally, all layered samples had some minor defects located at the shell/core interface, with most of these discontinuities occurring near the top and bottom of the samples.

5.3.2.2. Quantitative Evaluation

Morphometric analysis was conducted to better assess the overall microarchitecture of bilayered CS samples. As shown in Figure 5.4, the average volume percentage of voids in NL samples (0.96%) was significantly lower than that for all others (p<0.001). Furthermore, the particle content of 10-BSBC samples, 7.59%, was significantly higher (p<0.001) than that of both BSBC and 1-BSBC. Percentages for BSBC (3.30%) and 1-BSBC (4.52%) were not significantly different.
5.3.3. Composite Dissolution

Loading 1 wt% of PLGA particles into the shells of bilayered composites did not have a significant effect on the dissolution rate (-3.1%/d) (Figure 5.5). Increasing the loading to 10 wt% PLGA, however, significantly increased the dissolution rate to -3.43%/d (p<0.001), even though the time for complete dissolution differed by only about 4 d.

5.3.4. Mechanical Properties of Layered CS

Fabrication of layered structures significantly affected the mechanical properties of CS composites (Figure 5.6). The ultimate compressive strength of NL (5.40±0.38 MPa) samples was not significantly higher than that of BSSC (4.14±0.75 MPa) and 1-BSBC (4.33±0.72 MPa), but it was significantly greater than that for BSBC (3.80±0.46 MPa, p<0.01) and all other bilayered samples (p<0.001) (Figure 5.6A). Within subgroups for PLGA particle loading, the strength of 1-BSBC (4.33±0.72 MPa) was significantly higher than both 1-SSSC (3.03±0.2 MPa) and 10-BSSC (3.03±0.4 MPa) (p<0.05). There were no other significant differences seen for layered samples, both loaded with PLGA and without.

The compressive elastic modulus of NL (712±139.6 MPa) was significantly higher (p<0.001) than that for SSBC (250±37.6 MPa), 1-BSBC (172±96.9 MPa), 1-SSSC (162±70.4 MPa), 10-BSBC (237±84.2 MPa), 10-BSSC (154±58.4 MPa), 10-SSBC (216±87.8 MPa), and 10-SSSC (184±29.6 MPa) bilayered samples (Figure 5.6B). The average modulus of the blanks was also significantly greater than those of SSSC (306±157.6 MPa, p<0.01) and 1-SSBC (391±282 MPa, p<0.05). BSSC (495±184 MPa) samples had a significantly higher (p<0.05) modulus than did 1-BSBC, 1-SSSC, and 10-BSSC. There were no other significant differences between layered samples and their subgroups.
Figure 5.2. Cumulative release of metronidazole from 150-250 μm PLGA particles. Data are mean ± standard deviation (n=3).
Figure 5.3. Representative microCT images of CS/PLGA composites: raw X-ray slices and cross-sections of 3D reconstructions. Closed arrows mark bubbles, and open arrows with circles indicate PLGA particles. Scale bars denote 1 mm.
Figure 5.4. Volume percentage of voids/particles in CS/PLGA composites determined by microCT. Data are mean ± standard deviation (n=5). Symbols (*) indicates significant differences (p<0.001).
Figure 5.5. Mass loss profiles for bilayered blank CS and composites with 1 and 10 wt% PLGA loaded into the shells. Data are mean ± standard deviation (n=3).
Figure 5.6. Mechanical properties of CS/PLGA composites with directly loaded simvastatin: A) ultimate compressive strength and B) compressive modulus. Data are mean ± standard deviation (n=5). Symbols indicate significant differences: p<0.001 (*), p<0.01 (#), and p<0.05 (Δ).
5.3.5. Drug Release from Bilayered Composites

5.3.5.1. Simvastatin Release

To demonstrate temporally controlled release of simvastatin using bilayered composites, several experiments were conducted using different shell to core volume ratios. Figure 5.7A shows the cumulative release of simvastatin from composites that had an 85:15 shell to core volume ratio. The sustained release of drug from samples with simvastatin loaded into both shell and core (SSSC), 0.055 mg/d, was significantly faster (p<0.001) than for SSBC (0.043 mg/d) and SSBC+BSSC (0.046 mg/d). Minimal drug loaded into the core only (BSSC) was released during the first 24 d. From that point until the samples dissolved, however, release of simvastatin from CS cores increased to 18 μg/d, whereas release from SSBC samples was finished. The total amount of simvastatin released from BSSC (shell) and SSBC (core) samples was 0.24±0.05 mg and 1.19±0.01 mg, respectively. The rate of release from BSSC was significantly slower than that for both SSSC (p<0.05) and SSBC+BSSC (p<0.01).

Figure 5.7B shows the results for simvastatin released from composites consisting of 70% shell volume and 30% core volume. Over the first 20 d, the drug release rate from SSBC (0.046 mg/d) was significantly faster (p<0.01) compared to 0.041 mg/d for SSSC. Little to no drug was released from BSSC samples during the first 20 d, followed by an upward shift to a rate of 0.022 mg/d, which was significantly slower than the rates for both SSSC and SSBC+BSSC (p<0.01). For BSSC samples, 0.32±0.03 mg of drug was released. After 20 days of simvastatin release from SSBC samples, the shells had completely dissolved and released 1.30±0.07 mg of simvastatin.

The results for release of simvastatin from composites with a 50:50 shell to core volume ratio are depicted in Figure 5.7C. For the first 16 d of the experiment, the rate of release from SSBC composites, 0.095 mg/d, was significantly slower (p<0.05) than that for SSSC (0.13 mg/d) and SSBC+BSSC (0.12 mg/d). During the same period, a small amount of drug was released from BSSC at a slow rate of 0.025 mg/d. After 16 d and until the composites dissolved, the release rate from BSSC decreased to 0.022 mg/d, which was significantly slower than the rate for SSBC (p<0.01). The total amount of simvastatin released from BSSC (shell) and SSBC (core) samples was 0.32±0.03 mg and 1.30±0.07 mg, respectively. The rate of release from BSSC was significantly slower than that for both SSSC (p<0.05) and SSBC+BSSC (p<0.01).
dissolved, the rate of drug release from SSBC samples gradually slowed as the shell finished dissolving, a trend similar to what was observed in both Figures 5.7A and 5.7B. A total of 1.52±0.3 mg of drug was released from the shell. At its peak, the rate of release from BSSC samples, 0.10 mg/d, was not significantly different from the rates observed for SSSC and SSBC+BSSC samples, ultimately releasing a total 1.44±0.24 mg of simvastatin.

The total amount of simvastatin released from the cores (BSSC) for 50:50 (1.44±0.24 mg) samples was significantly different (p<0.001) than that for samples with either a 70:30 (0.32±0.03 mg) or 85:15 (0.24±0.05 mg) shell to core volume ratio. There was no significant difference in the total drug release between the 70:30 and 85:15 BSSC samples. For 50:50 SSBC samples, the total amount released (1.52±0.3 mg) from the shells was significantly different (p<0.001) than for 70:30 SSBC (1.30±0.07 mg) and 85:15 SSBC (1.19±0.1 mg) samples. In addition, the total amount of drug released from 70:30 SSBC samples was significantly different than from 85:15 SSBC samples (p<0.01). When comparing the total amount of drug released when SSBC and BSSC results are combined (SSBC+BSSC) to the total amount of drug released from SSSC samples, SSBC+BSSC specimens with an 85:15 shell to core volume ratio had a total combined release that was significantly lower (p<0.01) than the total amount of simvastatin released from the SSSC with the same volume ratio.

By reducing the volume of PBS from 12 mL to 8 mL, the dissolution time of samples having a 50:50 shell to core ratio was doubled (Figure 5.8). During the first 32-36 d of incubation, simvastatin was released from both SSSC and SSBC samples at a rate of 0.074 mg/d and 0.077 mg/d, respectively. Also, a small amount of drug from BSSC samples was released during the same time frame at a rate of 0.009 mg/d. From day 36 until the end of the experiment (68 d), the rate for the SSSC samples continued steadily, however the rate of release from SSBC decreased to zero around 48 d. Additionally, the rate of release from BSSC samples increased to 0.051 mg/d and remained at this rate until the samples finished eroding.
Figure 5.7. Cumulative release of simvastatin from bilayered samples having different shell to core volume ratios: (A) 85:15; (B) 75:25; and (C) 50:50. Data are mean ± standard deviation (n=5).
Figure 5.8. Cumulative release of simvastatin from bilayered samples incubated below sink conditions. The samples tested had a 50:50 shell to core volume ratio. Data are mean ± standard deviation (n=5).
5.3.5.2. Multiple Drug Release

Figure 5.9 shows results for release of metronidazole from PLGA particles embedded in CS shells as well as the release of simvastatin directly loaded in the shell or core of bilayered composites. The samples used for this experiment had a 50:50 shell to core volume ratio. Based on this ratio and the dissolution results presented in Figure 5.5, the shell and core portions of the composites were predicted to dissolve completely in 14-16 d of the 28-32 d dissolution period for the complete composite. For composites with 1 wt% PLGA particles embedded in CS, a large amount of metronidazole (65%) was initially released from the shells during the first 4 d at a rate of 16.3%/d (Figure 5.9A). After the initial burst of metronidazole, the release of drug slowed to a rate of 4.0%/d and continued to slowly decay to zero until the shells completely dissolved. Composites with 10 wt% PLGA particles showed a similar burst of metronidazole during the first 4 d, with as much as 55% of the total drug released (Figure 5.9B). The rate of release of metronidazole decayed from 13.7%/d through the first 4 d, to 6.7%/d from days 4-8, and finally down to 0% by d 12 of the release. The results in Figure 5.9 were normalized based on the amount of drug loaded into the respective layer rather than the complete composite. This allowed for direct comparison of the temporal release observed between metronidazole and simvastatin. When simvastatin was loaded into only the shell for 1 wt% and 10 wt% PLGA composites, metronidazole was initially released at a higher rate than simvastatin (2-6.3%/d). Because of the slow initial rate of simvastatin release, a short lag in the release profile developed, creating separation from the metronidazole profile and allowing for simvastatin to be released for up to 8 d longer. When the drugs were separated by keeping metronidazole-containing PLGA particles in the shell and loading simvastatin in only the core, 80-90% of the metronidazole was released over approximately 12 d before trace amounts of simvastatin were detected. After 16 d, the shells had completely dissolved, and metronidazole was no longer detected. In addition, the majority of simvastatin, isolated to only the core, was released starting after 12 d. Due to the layers
separating the two drugs, a sequential release was observed with all metronidazole drug released prior to simvastatin.
Figure 5.9. Cumulative release of simvastatin and metronidazole from bilayered composites. Normalized profiles of directly loaded simvastatin and metronidazole loaded into PLGA particles released from composites with (A) 1 wt% and (B) 10 wt% PLGA particles loaded in shells. Data are mean ± standard deviation (n=5).
5.4. Discussion

5.4.1. Metronidazole-loaded PLGA Particles

In previous studies, two types of PBAE hydrogel particles were used as drug delivery vehicles [113, 131]. Because bilayered CS composites are being developed as tunable implants with the idea of being a ‘plug and play’ system, PLGA was introduced to help demonstrate this versatility. The use of different polymers could allow for carrier particles to be matched with different drug properties, such as size and solubility. A previous study showed that loading a small hydrophilic drug into carrier particles prior to embedding into a CS matrix significantly reduced the burst release witnessed when the drug was directly loaded into CS [131]. Biodegradable PLGA microparticles were used in the present study to assist with the sustained release of metronidazole from bilayered composites. Because the particles were exposed to water during the setting phase of CS, a release study was conducted using a larger volume of water than present during composite formulation to monitor the potential for premature release of drug from PLGA. No initial burst of drug was observed, indicating that the majority of the drug was contained within the PLGA microparticles during the formation of the composites.

5.4.2. Composite Microarchitecture

Qualitative assessment of the morphology of bilayered composites showed good distribution of PLGA microparticles embedded into CS shells at 1 and 10 wt%. The initial CS slurry was kept sufficiently fluid to prolong the working phase and allow for easy filling of the molds yet viscous enough to suspend PLGA particles during the setting phase. This trend has been shown in previous research in which hydrogel particles were uniformly distributed throughout a CS matrix using similar powder to liquid volume ratios [113]. Another study, showed the exposure of particles at or near the surface before dissolution followed the pitting of CS resulting from the release of particles from those locations after a short duration submerged in PBS [131]. With the aid of small, metallic pegs, which were removed after fabrication, preformed blank and
Simvastatin-loaded cores were positioned in the center of the composites. Small defects were observed along the shell/core boundary. Many of these were air bubbles trapped as the CS set. Larger bubbles that appeared to accumulate near one end of the samples along the shell/core interface were most likely caused by an air pocket created when the cores were pressed into CS slurry.

Quantitative measurements assessed the particle volume fraction of bilayered CS composites. Although a significant increase in porosity was seen between samples containing 1 wt% and 10 wt% PLGA, the difference was not 10-fold. This lack of separation could be due to other defects, such as air pockets found along the shell/core interface. The script used to calculate the porosity is limited to only distinguishing between what is solid and what is not. The introduction of bubbles adds error to the calculations because these imperfections show up as radiolucent spaces similar to PLGA particles. Comparing the solid and layered samples demonstrates that defects strongly influenced on the porosity calculations.

5.4.3. Composite Dissolution

Calcium sulfate is a dense material that dissolves via surface erosion [33, 113]. Although the embedded PLGA particles were distributed throughout the CS matrix, they did not appear to be interconnected, which otherwise would have allowed for fluid to seep into the composite. During erosion, closed pockets with PLGA particles near the surface were exposed, releasing the polymer particles and increasing the surface area for further dissolution. Consequently, as the composites dissolved and PLGA particles were released, the surface area to volume ratio increased, allowing for faster dissolution of CS. Furthermore, because embedded PLGA particles did not change the dissolution characteristics of CS, the shorter lifespan when 10 wt% particles were added was related to the smaller overall volume of CS per sample that needed to dissolve. These trends have also been witnessed in a previous study in which dissolution of CS was observed after hydrogel particles were uniformly distributed throughout the composite [113]. Thus, the presence of polymer particles, even having different
5.4.4. Mechanical Properties of Layered CS

To be a suitable alternative to autologous bone, the ideal synthetic material would have characteristics similar to those of tissue at the implantation site. CS has been described as having properties being similar to cancellous or trabecular bone [38]. Investigating the mechanical properties of human trabecular bone from the mandible, Misch et al. measured an ultimate compressive strength of $3.9 \pm 2.7$ MPa and elastic modulus of $96.2 \pm 40.6$ MPa [132]. The introduction of a bilayered geometry significantly affected properties compared to solid CS samples, with up to a 44% and 78% reduction in the strength and elastic modulus, respectively. Note, however, that the properties of the layered composites were comparable to those of trabecular bone. The decrease in strength compared to the samples without layers could be due to the small air pockets along the shell/core interface acting as discontinuities within the composite, both at the interface parallel to the central axis of the cylinder as well as at the ends of the core. These stress concentrators contributed to a 17-44% decrease in the overall strength of the composites. The addition of either 1 or 10 wt% PLGA particles to CS shells did not have an effect when compared to blank bilayered samples. In previous research, as much as a 50-60% reduction in strength was seen following the addition of 10 wt% of gel particles to the monolithic CS matrix [113]. In the present study, the PLGA particles were isolated to only the shell of the composites. Also, the presence of a solid blank core may have provided reinforcement for the composite, which may allow a greater range of PLGA particle loading that would provide greater control over the drug dose within the composites.

The loading of simvastatin directly into the shell, core, or both layers generally did not significantly affect the strength of the bilayered composites, even though isolated differences were observed (1-BSBC versus 1-SSSC and 10-BSSC). In another experiment conducted by Orellana et al., loading of
simvastatin directly into monolithic CS did not have a significant effect on the strength of the samples [131]. Another group determined that up to 10% loading of simvastatin into calcium phosphate samples did not significantly affect the compressive strength [118]. The present study had a lower loading of simvastatin (i.e., 2 wt%), however. Thus, direct loading of simvastatin does not affect the overall strength of different materials.

5.4.5. Drug Release from Bilayered Composites

The layered geometry used for the CS samples provided a unique platform for achieving a customizable sequential release of therapeutic agents. Loading of PLGA microparticles into the CS matrix allows for further tailoring of drug release.

5.4.5.1. Simvastatin Release

The present experiments were designed to demonstrate how the release of simvastatin can be tailored depending on which layer the drug was loaded in, whether it was the shell only, core only, or both. Furthermore, to illustrate the ability to adjust the duration or even the delay of drug release from either the shell or core, the shell to core volume ratio was altered. However, there were limitations to how much the volume ratio could be adjusted. For instance, the 50:50 shell to core ratio was considered the maximum. With the present dimensions, increasing the core volume beyond this point would create a thin and unstable shell. On the other hand, if the ratio was made so the shell would be greater than 85% of the total volume, the local concentration of drug released from the core could be too low to be therapeutically relevant.

For all of the release profiles that had simvastatin loaded in only the cores, there was a small amount of drug released from the start of composite dissolution up to when the core was completely exposed. Because the cores were suspended using a small metal peg that was later removed after the samples were fabricated, the hole that remained may have been large enough to allow a noticeable, but statistically insignificant, amount of simvastatin to be released.
In addition to investigating the effects of adjusting the shell to core volume ratio, the volume of PBS used for the release study was reduced from 12 mL to 8 mL. Because of the possible diverse environments in various implantation sites, it is likely for the implant to encounter different fluid volumes and/or turnover rates that may not allow for sink conditions. A small study demonstrated how release of simvastatin would change under non-sink conditions. To conduct the comparison, the 50:50 volume ratio was used. Interestingly, even with the reduction in the volume of PBS to 8 mL, the transition after shell depletion to core only erosion occurred around the halfway point, similar to the results seen under sink conditions. In previous work, the effects of fluid volume on the dissolution of CS were investigated [113]. It was determined that the change in volume of fluid or even the turnover rate could have a large effect on the dissolution of CS [113]. The duration of drug release can be greatly prolonged (doubled in the present study) using different fluid volumes. McLaren et al. also observed a large difference in the rate of drug release from calcium sulfate pellets when the fluid was completely refreshed at each time point versus exchanging only a fraction of the fluid volume [133]. In the present study, although the rate of release slowed, the mechanism of drug release remained dependent on dissolution of CS. This could allow for tailoring of drug loading and/or the sample geometry according to the physiological conditions expected at the implant site.

5.4.5.2. Multiple Drug Release

A multiple drug release study was conducted to investigate the release kinetics of bilayered composites loaded with an antimicrobial agent and an osteogenic agent. Findings for samples with metronidazole in PLGA particles and simvastatin directly loaded into CS demonstrated sequential release. Polymeric particles loaded with metronidazole and embedded into the shell were exposed as CS experienced surface erosion, and sustained release of the drug occurred as the PLGA particles subsequently degraded. Release from both 1 and 10 wt% particles embedded into the CS shell was sustained until the shell portion of the bilayered composites completely dissolved. Controlled release of metronidazole from PLGA microspheres after an initial burst has been described
as a possible treatment for periodontal disease [134]. By embedding particles into a CS matrix, the release of metronidazole was controlled throughout the first 16 d of the present study. In addition, previous research has demonstrated that drug-loaded hydrogel particles uniformly distributed throughout a CS matrix allowed for a controlled release of drug, which only occurred due to the breakdown of particles exposed at the surface of the dissolving composite [113, 131].

With the intended use of these composites as a grafting substitute for alveolar bone augmentation, the oral cavity presents challenges for proper tissue regeneration due to the environment being rich with bacteria that can colonize natural and synthetic substrates. Thomas and Puleo described the implications of infection and inflammation in periodontal disease and tissue regeneration [135]. Currently, the standard treatment for infected periodontal defects has antimicrobial agents being administered, either systemically or locally, prior to implantation of grafting material, which only delays the overall recovery of lost tissue [14, 119, 123]. Administration of antimicrobial agents allows for better bone formation. Chen et al. investigated the effects of two different growth factors in a chronically infected bony defect in rat femurs [136]. Although some healing occurred in the infected sites, the extent of bone formation was greater with the systemic administration of antibiotics [136]. To further enhance the process of fighting infection and then regenerating lost tissue, release of an antibacterial agent from the graft starting at the time of implantation may prove beneficial to help reduce the overall healing time.

Many studies have investigated dual purpose implantable scaffolds, but none have employed a concentric cylindrical CS system as described in the present studies. Reis et al. developed drug-free, bilayered membranes comprising a continuous outer layer of PLGA with a porous calcium phosphate inner layer for the regeneration of lost periodontal tissue [137]. For infected sites, Nguyen et al. developed a co-culture model using methicillin-sensitive Staphylococcus aureus and mouse bone marrow stromal cells to investigate the dual effects of an antibiotic, vancomycin, along with bone morphogenetic protein-
Separately, the two agents were not effective, but when delivered together, the needed concentration of vancomycin was significantly reduced, suggesting that lower, non-toxic doses could be used [119]. An *in vivo* study in which vancomycin and BMP-2 were delivered simultaneously from a biodegradable polyurethane scaffold demonstrated that bone formation could be regenerated within an infected defect [138]. However, these systems release the drugs simultaneously. Considering the intent for the current device to help streamline the existing treatment process, it was encouraging to see metronidazole released before simvastatin, even when loaded into the shell together. The difference in the release kinetics can be explained primarily by the way the two drugs were loaded. Previous work has shown that release of drug from polymer particles embedded into a CS matrix had a rapid, initial burst followed by decay in the rate of release [113, 131]. The lower rate is attributed to the decrease in surface area as CS degrades, leading to a smaller volume of particles exposed at the surface over time [113, 131]. Simvastatin, on the other hand, is directly mixed with CS during sample formation, and due to the hydrophobic nature of the drug, it does not become segregated to the surface during the setting of CS. The release of simvastatin is, therefore, governed by the surface erosion characteristics of CS, which were shown to be linear. This allowed for a near constant rate of release of simvastatin. These differences in release kinetics between the two drugs and their means of loading allowed for enough separation for all the metronidazole to be released 4 d sooner than simvastatin. When simvastatin was loaded into only the core while PLGA particles loaded with metronidazole remained in the shell, there was a much greater lapse in time for a fully separated sequential release to occur, which may be useful for mimicking the clinical sequence of events for treating infection and subsequently restoring lost or damaged tissue.

### 5.5. Conclusion

In the present study, novel bilayered CS composites were investigated for their ability to provide tailored release of therapeutic agents as well as a
sequential release of different drugs. Such a system may be useful as a bone graft substitute for treating infected bony defects, e.g., periodontal pockets. Although the shell and core geometry reduced mechanical strength of the composites, the properties were similar to those for mandibular trabecular bone. This may be an important trait that could allow for these implants to better mimic the surrounding target tissue being treated. Changing the shell to core volume ratio dictates the duration of drug release from each layer. When metronidazole and simvastatin were loaded together in the shell or in separate layers, temporal separation of the two drugs was achieved. Being able to tune such a system may help streamline the multiple steps needed to regenerate tissues more efficiently.
Chapter 6: Summary and Conclusions

Calcium sulfate based bone graft substitutes were developed not only as space-making implants for proper bone regeneration, but also to have the ability to control the release of multiple agents to aid in the healing process. CS composite samples fabricated with varying amounts (1 or 10 wt%) and sizes (53-150 or 150-250 µm) of embedded A11 PBAE gel particles was shown, using MicroCT, to be uniformly distributed throughout CS. During dissolution CS samples degraded via surface erosion, with the amount of gel particles (i.e. 10 wt% gel particles) having only a small but significant effect on the dissolution rate (4% vs. 5% per day). Compression analysis determined that the amount, but not the size, of gel particles had a significant effect on the overall mechanical strength of the composite. The swelling and rate of degradation of A11 PBAE gels alone occurred in less than 24 hours. This turned out to be an important characteristic that aided in the controlled release of curcumin from A11 PBAE gels embedded in a CS matrix.

The versatility of these implants to release a broad range of therapeutic agents was examined with the intention that drugs could be chosen for patient specific cases. Whereas sustained release of directly loaded simvastatin was achieved, direct loading of small amounts of lysozyme resulted in highly variable release. Direct loading of a larger amount of protein generated a large burst, 65% of total loading, followed by sustained release of that protein. Release of lysozyme from 1 wt% H6 PBAE particles embedded into CS was more controllable than when directly loaded, and for 10 wt% of protein-loaded H6 PBAE particles, a higher burst was followed by sustained release, comparable to the results for the high direct loading. Compression testing determined that incorporation of directly loaded drug or drug-loaded H6 PBAE particles weakened CS. In particular, H6 PBAE particles had a significant effect on the strength of the composites, with a 25% and 80% decrease in strength for 1 wt% and 10 wt% particle loadings, respectively. These results were similar to those witnessed for the composites loaded with A11 PBAE particles. These CS-based
composites demonstrated the ability to sustainably release both macromolecules and small molecules, supporting the potential for these implants to release a range of bioactive agents.

The concept of further tailoring the drug release was examined by creating a bilayered system that could allow for more complex release kinetics, as well as the release of multiple therapeutic agents. Bilayered composite samples having a shell and core geometry were fabricated with varying amounts (1 or 10 wt%) of metronidazole-loaded PLGA particles embedded in the shell and simvastatin directly loaded into either the shell, core, or both. MicroCT images showed the overall layered geometry as well as homogenous distribution of PLGA within the shells. Dissolution studies, similar to those conducted for the composites with A11 PBAE, demonstrated that the amount of PLGA particles (i.e., 1 vs. 10 wt%) had a small but significant effect on the erosion rate (3% vs. 3.4% per day). Mechanical testing determined that introducing a layered geometry had a significant effect on the compressive strength, with an average reduction of 35%. Sustained release of simvastatin directly loaded into CS demonstrated that changing the shell to core volume ratio dictated the duration of drug release from each layer. When loaded together in the shell or in separate layers, sequential release of metronidazole and simvastatin was achieved. By introducing a tunable layered geometry capable of releasing multiple drugs, CS-based bone graft substitutes could be tailored in order to help streamline multiple steps needed to regenerate tissue in infected defects.

Overall, this dissertation illustrated the development of CS-based synthetic bone grafts that could be suitable alternatives to ‘gold standard’ approaches. Through controlled release of bioactive agents, CS-based implants have the potential to greatly enhance their effectiveness as bone grafts. In addition, the introduction of a tailorable layered geometry would allow for CS-based bone graft substitutes to be further optimized in order to help streamline the steps during the healing and regenerative process of compromised bone tissue within the oral cavity.

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References


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