Optimization of Calcium Aluminate for use as a Bone Scaffold Material Through Physical and Chemical Surface Modification

Rachelle Nicole Palchesko
OPTIMIZATION OF CALCIUM ALUMINATE FOR USE AS A BONE SCAFFOLD MATERIAL THROUGH PHYSICAL AND CHEMICAL SURFACE MODIFICATION

A Dissertation
Bayer School of Natural and Environmental Science

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In partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By
Rachelle Nicole Palchesko

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ABSTRACT

OPTIMIZATION OF CALCIUM ALUMINATE FOR USE AS A BONE SCAFFOLD MATERIAL THROUGH PHYSICAL AND CHEMICAL SURFACE MODIFICATION

By
Rachelle Nicole Palchesko
February 2011

Dissertation supervised by Professor Ellen S. Gawalt

Calcium aluminates were tested as biomaterials and show promise as bone scaffolds. Initial concerns of biocompatibility led the field to focus on calcium phosphates. Here, the work focused on improving the biocompatibility and creating an optimized calcium aluminate biomaterial. To accomplish this, the physical and interfacial properties of the material were varied. The physical properties of the calcium aluminate were varied through the room temperature casting procedure and five different mixtures were created and evaluated for mechanical strength and biotolerability. The results showed that the optimal mixture had an average pore size of approximately 100µm and that autoclaving the material increased the strength and therefore was the best sterilization method for all studies. Following optimization of the physical properties, the cell adhesion peptide KRSR was immobilized onto the surface to increase specific
osteoblast adhesion and RGD was immobilized as a non-cell specific peptide control. It was determined that KRSR preferentially increased osteoblast over fibroblast adhesion and increased osteoblast adhesion compared to unmodified and RGD modified calcium aluminate which is important for a bone scaffold material. Biofilm formation and implant associated infections are still a significant problem despite advancements in sterile techniques in operating rooms. To mitigate bacterial attachment, vancomycin and ampicillin were successfully immobilized on the surface through both covalent attachment and adsorption. Vancomycin remained active after autoclaving and was extremely effective at inhibiting bacterial growth when compared to unmodified calcium aluminate and the control antibiotic dosage. Finally, both KRSR and vancomycin were simultaneously immobilized utilizing three different attachment schemes. A double immobilization scheme that allowed for antibiotic activity and increased osteoblast attachment was found producing an optimized calcium aluminate material for use as a bone scaffold.
DEDICATION

To my “Bubba”, the late Rachel A. Tuscano, the best grandmother in the world.
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Chapter 1: Background

1.1 Introduction

There are approximately 1.5 million bone-graft surgeries annually in the United States\(^1\),\(^2\) and more than 2.2 million worldwide.\(^3\) Autografts and allografts are currently the two most common methods of bone replacement making up 90% of the bone grafts used with only approximately 10% being from synthetic sources.\(^4\) The biological, chemical and mechanical requirements of a successful bone graft along with the failure of allografts and synthetic substitutes to meet these requirements are part of the reason that the autograft is still the gold standard among bone replacements. However, with the advancements in biomaterials, tissue engineering, and surgical techniques, current research gives hope that there will be a synthetic bone graft substitute that is biologically comparable to the autograft in the near future.

1.2 Requirements for Bone Substitutes

The mechanical strength, chemical and biological activity are all important factors in determining the requirements for a bone graft substitute. Synthetic biomaterials must be strong, resist wear and corrosion, be non-toxic, and favor cell attachment, proliferation and differentiation.\(^5\)\(^-\)\(^7\)

The strength of the material must have similar tensile strength, elastic modulus, and compressive strength to the cortical or cancellous bone that it is going to replace. This is very important, because materials involved in load bearing applications will have to be much stronger than those involved in craniofacial applications that are non-load

\(^1\)\(^-\)\(^2\)\(^-\)\(^3\)\(^-\)\(^4\)\(^-\)\(^5\)\(^-\)\(^7\)
Similar elastic modulus and toughness are important in avoiding stress shielding and to prevent cyclic loading fatigue fracture.  

<table>
<thead>
<tr>
<th>Testing direction</th>
<th>Compressive strength (MPa)</th>
<th>Tensile strength (MPa)</th>
<th>Young’s Modulus (GPa)</th>
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<td>133-150</td>
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<td>Transverse</td>
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<td><strong>Cancellous Bone</strong></td>
<td>Longitudinal</td>
<td>3.6-9.3</td>
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<tr>
<td><strong>Collagen</strong></td>
<td>Longitudinal</td>
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</tr>
</tbody>
</table>

*Table 1-1: Table of mechanical requirements for cancellous and cortical bone compared to synthetic materials.*

As a result, chemical reactivity of the biomaterial should be limited and include desired bioerodible or biodegradable reactions. The material provides a temporary framework allowing for the bone regeneration and repair. Therefore the material should degrade, but not too quickly in order to give the tissue time to repair.

Although the mechanical requirements and the chemical reactivity are important, the biological requirements for the material are the most important. An optimal bone graft material should be osteoconductive, osteoinductive, osteogenic, and osteointegrative. Osteoconductivity is the ability of a biomaterial to support the growth and viability of osteoblasts. Osteoblast cells are necessary for bone formation and a surface should support their growth and attachment in order to be considered osteoconductive. Osteoinductivity is the ability of the material to induce mesenchymal stem cells to differentiate into osteoblasts, osteoclasts, etc. There are few mature osteoblasts present in the human body and therefore it is advantageous for
a biomaterial to be able to induce mesenchymal stem cells that are present in bone tissue to differentiate into mature osteoblasts in order to regenerate bone tissue. Biomaterials should also allow for living cells to proliferate within the graft for tissue to regenerate and for bone to form in place of the graft, this is known as osteogenesis. Finally osteointegration is the ability of the surfaces of the bone and the graft to bind to one another. Without proper binding between the bone and graft, the implant will dislocate and bone regeneration will not occur.

The osteoinductive and osteoconductive properties of a biomaterial are directly influenced by pore size, pore connectivity and the overall porosity of the material. The porosity of natural bone varies with bone type in a range of 3-90% porosity with individual pores up to 1 mm in diameter. Pores are tunnel like holes that go from the surface to the bulk of a material either partially or completely. These pores can connect inside the bulk to the material to create a network that allows for the flow of molecules from the outside of the material in and vice versa. In a surface without pores, there is often attachment of the cells on the surface but not at the center of the implant. The center of the implant then experiences necrosis and failure. Therefore, the scaffold must allow for the transfer of nutrients and cell migration into the scaffold meaning that the material should be porous. Vascularization is important if bone is to form and grow into an implant which also requires a porous material. This vascularization requires blood supply which provides the calcium and phosphorous that are necessary for bone formation.
1.3 Current Graft/Implant Materials

1.3.1 Autografts and Allografts

The only current graft that supports osteoconductivity, osteoinductivity, osteogenicity, and osteointegration is the autograft. An autograft, a piece of bone taken from the patient’s own body, is the most desirable type of replacement because the tissue comes from the patient and therefore would not elicit an immune response. Autografts also possess all the characteristics necessary for bone growth. There are great disadvantages to autografts because they require a second surgery site for the patient which puts more strain on the body, causing possible cell death at the donor site. Additionally, the size of the graft needed could be too large for an autograft to be feasible. Allografts are bone grafts from deceased donors and can be used when the graft size exceeds that of an available autograft and possess three of the important biological requirements, osteoconductivity, osteoinductivity, and osteointegration. However, they are not osteogenic because they lack living tissue. A major disadvantage of the allograft is the introduction of possible pathogenic transmission.

1.3.2 Metal Replacements

Synthetic bone scaffold and replacement material research is receiving increasing attention although those currently available only allow for osteoconducting and osteointegration. Out of the 10% of synthetic bone substitutes used, the majority are metal replacements which are mainly used when the defect in the bone is of critical size and/or load bearing. The most common metals used are titanium and its alloy Ti-6Al-4V. It has been shown in the literature that on rough titanium surfaces osteoblasts
exhibit increased adhesion, differentiation, matrix production and calcification and there is a reduction of osteoclasts.\textsuperscript{21} Also, the INFuse Graft that is approved by the FDA for bone grafting, consists of a titanium or titanium alloy rod with a collagen sponge and bone morphogenetic protein-2 (BMP-2). Other common metal implant materials are stainless steels (usually 316L), Co-Cr alloys and more recently tantalum.\textsuperscript{5, 9, 11, 12, 22-24}

1.3.3 Ceramics, Glasses, Etc

Although metals are the most common synthetic substitutes for large bone defect filling, there are other synthetic options available such as scaffolds, when the defects are smaller and/or not load bearing in nature. Scaffolds of calcium phosphates and apatites (Vitoss) and polymer composites (Cortoss) that include tricalcium phosphate, hydroxyapatite and coralline hydroxyapatite are desirable because they mimic the real crystalline composition of bone which is calcium hydroxyl apatite.\textsuperscript{11, 12, 23, 25-28} Bioactive glass (Bioglass), demineralized bone matrix collagen, calcium sulfate (filler), calcium carbonate (filler), alumina, bovine derived bone mineral (Bio-oss) and bioactive ceramics (glass and zirconia) are less common but have all been used as bone void fillers or bone graft expanders in conjunction with autografts and allografts.\textsuperscript{3, 10, 12, 22, 29}

1.4 Failure of Synthetic Implants

1.4.1 Biological Failure

An optimal bone replacement material would be osteogenic, osteoconductive, osteoinductive, and osteointegrative; however, all replacements except for native tissue lack at least one of these four major requirements, leading to some type of implant failure
such as delayed union, nonunion, and malunions to name a few.\textsuperscript{3,30} One of the major shortcomings of synthetic replacement materials is their lack of osteogenicity and osteoinductivity.\textsuperscript{10} Current synthetic materials and allografts do not contain any living cells and therefore lack the ability to be completely osteogenic. Often synthetic materials are not osteointegrative which leads to loosening of the implant from the surrounding bone. Some materials such as bioinert metals, like titanium and cobalt/chromium alloys, and some ceramics, do not allow for osteoconductivity leading to fibrous tissue formation around the implant ultimately causing implant failure.\textsuperscript{5,9,31} Metal replacements do not contain pores which allow for cell migration, nutrient transfer, and vascularization and are necessary to eliminate necrosis at the center of the implant or tissue surrounding the implant. Titanium, for example, does not stimulate a high amount of bone formation and can lead to the formation of soft fibrous tissue that leads to complications in bone wound healing.\textsuperscript{32}

Fibrous tissue formation on any bone replacement begins with blood coagulation which leads to the production of fibrin and the migration of white blood cells.\textsuperscript{5} Macrophages then colonize the implant inducing foreign body giant cells which recruit fibroblasts that then wall off the implant.\textsuperscript{5} The release of metal debris can also lead to production of cytokines which also produce foreign body giant cells and stimulate the formation of fibrous tissue.\textsuperscript{8} These metal particles bind to specific serum proteins and induce an immunological response that leads to cell toxicity and cell lysis as macrophages phagocytose the metal particles which leads to their death.\textsuperscript{9,22} As the macrophages die, they release the metal ions into an acidic environment which causes more cells to lyse and the cycle continues.\textsuperscript{22} It has been shown that exposure of bone to
cobalt/chromium alloys causes a reduction in osteoblast proliferation due to the toxicity of the metal release.\textsuperscript{9} Hydroxyapatite and some polymers have also shown low or unpredictable resorption in the physiological environment and the implants lead to an inflammatory response and foreign body reaction like metals and lead to fibrous tissue formation. One failure that is specific to the FDA approved INFuse graft is the formation of too much bone in an inappropriate location due to the BMP-2.\textsuperscript{31, 33}

Another common cause of implant/graft failure is infection.\textsuperscript{34-42} Bacteria adhere to the surface of the implant shortly before or after implantation. The bacteria begin to form biofilms which require high systemic doses of antibiotics and are often completely resistant to antibiotics due to their extra cellular polysaccharide matrix.\textsuperscript{36, 43-49} If the infection cannot be eradicated with systemic antibiotics, the implant must be removed. This requires not only a second surgery to remove the implant, but a spacer must be put in its place. The wound is then treated with antibiotics and allowed to heal.\textsuperscript{35, 37, 42} After a prolonged healing period a third surgery is then necessary to remove the spacer and replace the implant.

\subsection*{1.4.2 Mechanical Failure}

Although the inability of synthetic implants to be osteoinductive, osteointegrative, and/or osteogenic impairs the current materials’ role in regeneration of new bone, the most common causes of bone implant failure are due to mechanical reasons. Metal corrosion within the physiological environment can lead to decreased strength and limits the fatigue life of the implant causing mechanical failure and implant loosening.\textsuperscript{7, 9, 21} Metal corrosion is caused by oxidation/reduction reactions that occur on
the surface of the implant. Some of the metals used such as titanium and its alloys, and cobalt/chromium alloys are thought to be corrosion resistant, however at the bone/implant interface, crevice corrosion and stress corrosion can occur in load-bearing implants.

A mechanism of mechanical failure common to metals, ceramics, glasses etc. is mechanical mismatch. When the mechanical properties of the materials are different than that of the bone it is replacing, stress shielding occurs. The bone surrounding the implant will be insufficiently loaded which leads to bone resorption, implant migration, aseptic loosening, and fractures. Specifically, a mismatch of Young’s Modulus can cause implant loosening. Young’s modulus for bone is between 10 and 30 GPa, whereas for metallic materials it is around 110GPa for titanium and 230GPa for cobalt chromium alloys. This type of failure is quite common and has Wolff’s Law to describe such failures; when a material is stiffer than the cortical bone, the bone is subjected to a reduced mechanical environment and the bone resorbs. Although metals exhibit stress shielding they are sufficiently strong enough to support weight in load bearing applications which reduces implant fracture.

Ceramics, polymers and glasses in load bearing applications often fracture due to the materials’ inability to sustain the mechanical forces placed on the implant. Ceramics such as hydroxyapatite and tricalcium phosphate are weak and brittle (i.e. rigid and inflexible) (Table 1-1). They are slow to resorb which can lead to detachment and fracture if too much weight is placed on them. For this reason, these ceramics are often not used on their own, but rather used in conjunction with an autograft.
1.5 Current Research Directions for Materials Design

1.5.1 Metallic Materials

Metals possess the strength necessary for many load bearing applications, but because they are not biodegradable, nor do they allow for cell ingrowth, they often lead to stress-shielding and fibrous tissue formation as mentioned earlier. Current research is focusing on the fabrication of porous metals such as titanium, stainless steel, and Co-Cr alloys to allow for cell growth into the material. By inducing pores into the metal structure, there is a possibility that blood vessels and cells would be able to grow into the implant which is necessary for nutrient transport and also bone regeneration. This could also improve the mechanical property mismatch.

Metals that are currently not clinically being used as bone replacements such as nitinol are being investigated as possible materials for bone replacements. Nitinol is desirable because of its excellent elastic and shape memory properties. Porous nitinol is also being fabricated to allow for more desirable mechanical and biological properties. “New” formulations of alloys in the titanium and Co-Cr families have been manufactured to determine their ability to allow for better biological properties.

Surface modification is being used on various metals such as nitinol to reduce corrosion and thereby reduce the amount of metal ions and metal debris that is released into the body to eliminate cytotoxic and genotoxic effects. These modifications will increase the number of metals that will be available for use by eliminating toxic effects due to ion and debris release.

Even with research to increase their biological properties, current metals still exhibit the mechanical failure associated with stress shielding. For this reason, there is a
new focus on “bioresorbable/biodegradable” metals such as magnesium.\textsuperscript{51-54} These metals provide temporary strength for support and a framework for bone regeneration with the ability to degrade within the body and allow for bone healing and regeneration while possibly eliminating the effects of stress shielding.

1.5.2 Composite Materials

Composite materials aim to combine the most desirable properties from different materials into an optimal graft whose function is superior to that of its individual components. Composites consisting of hydroxyapatite and other bioactive ceramic coatings (calcium silicate) on metals and polymers have shown promise by providing the bioactive properties of the coating with enhanced mechanical properties of the underlying core.\textsuperscript{8, 23, 55, 56}

Chen et al. fabricated a composite material consisting of a porous titanium core with a calcium ion coating.\textsuperscript{43} The coating was found to induce apatite formation on the surface of the material when immersed in simulated body fluid.\textsuperscript{43} Another group combined the biodegradable nature of the magnesium alloy with a calcium phosphate coating to try and reduce stress shielding effects while enhancing the biological properties of the scaffold.\textsuperscript{51} The data showed an increase in the bioactivity of the composite material compared to control magnesium and control titanium implants.\textsuperscript{51} Moreau et al created a calcium phosphate cement/chitosan composite and saw increased osteoconductivity and osteoinductivity of the composite material when compared to the calcium phosphate cement alone.\textsuperscript{57} These findings indicate composite materials may be more beneficial for bone grafting than single component materials.
1.5.3 Biomolecule Functionalization

Current and prospective biomaterials are being improved upon by functionalization and modification with various drugs and biomolecules.\textsuperscript{20, 31-34, 48, 58-63} The goal of drug modifications is to reduce implant failure that is caused by inflammation, infection, and other adverse tissue responses while modification with biomolecules is used to increase the bioactivity of biomaterials by increasing cell attachment and proliferation.

Numerous papers focus on surface and bulk modification of current biomaterials with antibiotic coatings that could reduce implant failure due to infection.\textsuperscript{58, 64, 65} Silver coatings, or attachment of antibiotics to the surface of the material would kill any bacteria that attempt to attach to the surface of the implant before infection can occur and cause implant failure. This would be beneficial, but improving the biological properties of the material with biomolecules could be more important than antibiotic coatings because of the low rate of failure due to infection.

The release of drugs and various growth factors such as TGF-B, VEGF, BMP-2, FGF-2, melatonin and heparin are being investigated to determine their ability to improve current bone grafts.\textsuperscript{59, 62, 66} Sequential delivery of VEGF and BMP-2 from polymeric materials has been shown to induce bone formation and vascularization.\textsuperscript{59} The delivery was used to grow bone in a location where there was previously no bone tissue and also used to close a 5 mm defect in rat femurs.\textsuperscript{59} As mentioned earlier, BMP-2 is already used in conjunction with a collagen sponge and a titanium rod in the INFuse graft system. Modification with and addition or injection of BMP-2 with many graft materials could increase their biological success but delivery needs to be localized to reduce unwanted or
excess bone formation. Heparin modifications are being used to possibly reduce blood coagulation which would reduce the foreign body response seen with synthetic implants. Takechi et al reported newly formed bone was seen around titanium implants that had been modified with melatonin and FGF-2 whereas no new bone formation was seen around control titanium implants that had not been modified. These are some of the many growth factors that have been shown in the literature to increase bone formation. The next necessary step is narrowing down which combination of growth factors gives the best result without unwanted or out of control bone growth.

Cell adhesion peptides, specifically, RGD (arg-gly-asp), have received a lot of attention in the literature, even more so than growth factors. RGD was chosen because it is a sequence domain that is present in many extracellular matrix proteins including fibronectin whose main function is to mediate the adhesion of cells. Studies have shown that this peptide can increase the attachment of mesenchymal stem cells to different substrates (hydroxyapatite, titanium, polymer scaffolds) but not the spreading of the cells. Cell spreading is important for cell survival and cell differentiation into osteoblasts which are necessary for bone formation and regeneration. For this reason, the focus has shifted towards a different peptide—one that binds to transmembrane proteoglycans and could improve cell attachment and spreading. An example of this type of peptide is the KRSR peptide.

Sawyer et al compared the KRSR peptide to the RGD peptide modified hydroxyapatite. Although they did not find and increase the mesenchymal cell attachment, they found that cell spreading was increased. This indicated that the cells were interacting with the proteoglycan binding peptide through the cell surface
receptors. Balasundaram et al investigated the KRSR peptide on the titanium surface and found that immobilizing the peptide on the surface increased osteoblast adhesion in vitro.

Cell adhesion peptides are of increasing interest because bone formation at the interface of a bone implant occurs as a result of competition between bone regeneration and fibrous tissue formation only when bone regeneration is dominant. It is therefore necessary to try and increase the bone regeneration while limiting the fibrous tissue formation at the interface of the implant with existing bone. An optimal biomaterial would allow for the preferential attachment of osteoblasts over fibroblasts which would increase the regeneration of bone in comparison to the fibrous tissue formation.

1.5.4 Tissue Engineering

Modification of current materials and design of new materials offer promise in creating a bone graft substitute that may be comparable to the allograft, however in order to be comparable to the autograft and allow for osteogenic activity, a shift towards tissue engineering can be seen in the literature. Tissue engineering involving the implantation of cells, usually stem cells, with biomaterials, or cells encapsulated in biomaterials will provide living cells to aid in increasing osteogenic activity. For example, platelet-rich plasma is being used in concert with many biomaterials in animal models to look into its possible effects in increasing bone and vessel formation. Some of these techniques are also being employed with allografts to increase their success rate and osteogenicity.
Currently, there is a trend towards using a patient’s own cells instead of allogenic or xenogenic stem cells in tissue engineering applications. A patient’s cells are harvested and placed in bioreactors to allow for the proliferation of the cells. Once the cells have proliferated, they can then be implanted into a bone defect either alone or in combination with a biomaterial to increase the success of the graft. In the future, the tissue engineering community hopes to be able to use the patient’s body as the bioreactor environment to allow for the proliferation of cells. At this point in time, the harvesting of cells and use of a bioreactor would require more time than current biomaterials and tissue engineering approaches and therefore could only be used in non-emergency/trauma situations.

1.6 Literature Summary

The autograft remains the gold-standard graft in bone defect filling because of its osteogenic character. Current biomaterials do not meet the requirements in order to replace the autograft as the most common and desirable material. Synthetic materials fail to meet either the mechanical and/or the biological standards necessary to replace the autograft. Current research in the biomaterial and tissue engineering fields have made great progress toward finding a suitable and comparable replacement but there are still many advancements to be made before any synthetic material will take the place of the autograft.
1.7 Experimental Approach

Synthetic replacements and scaffolds strive to meet several criteria: osteoconductivity, osteoinductivity, osteogenicity, and osteointegration. Meeting all of these criteria is a daunting task and though some synthetic materials meet some of the criteria this goal has not yet been reached.\textsuperscript{3, 9, 10, 12} This project aims to improve the osteoconductive and antibacterial properties of the calcium aluminate material for use as a bone scaffold material through physical and chemical surface modification of the material. The physical properties were optimized by varying the average pore size of the material through the casting process. Different mixtures were evaluated for strength, phase composition, biodegradability and cell adhesion. The surface of the optimal mixture was then chemically modified with cell adhesion peptides to increase the osteoblast adhesion and decrease fibrous tissue formation. The surface was then modified with antibiotics to reduce bacterial adhesion and growth on the material. Finally, two biomolecules, one cell adhesion peptide (KRSR) and one antibiotic (vancomycin) were simultaneously immobilized on the surface of calcium aluminate and remain active to produce a surface that both increases osteoblast adhesion and decreases bacterial growth. This study does not address the osteogenic, osteoinductive or osteointegrative properties of the calcium aluminate at this time.
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Chapter 2: Physical Optimization of Calcium Aluminate

2.1 Introduction

Calcium aluminates were utilized as scaffolds and showed promise in initial studies\(^1\)\(^-\)\(^5\) but they have since been over-looked because of concern for their biocompatibility. However, *in vivo* studies have shown that the calcium aluminates do not elicit an inflammatory response proving biotolerability and indicating the material may be a promising bone scaffold, particularly with chemical modifications to improve its biocompatibility.\(^1\)\(^-\)\(^9\) Calcium aluminates have been re-examined in this project because the aggregates are naturally porous, non-toxic, and can be easily cast into predesigned shapes when hydrated at room temperature.

Pores are necessary for nutrient transport between an implant surface and its physiological environment as well as for cell attachment. However when pores become too large a biomaterial will lose mechanical integrity and become either brittle (i.e. rigid and inflexible) or weak, or both. It is important to optimize the pore size of a biomaterial to allow for increased nutrient transport and cell attachment, as well as strength. It is also important to optimize the process of forming desired shapes of a biomaterial. Some implants must first be cast into bulk shapes and then machined to match the size of the defect which can lead to a poor fit of the implant which will eventually cause implant loosening and failure. This is due to the high temperatures that are required to cast the material and/or the lack of necessary facilities in the hospital to be able to cast the implants. Calcium aluminate has the advantage that it is easily cast into any desired shaped through a room temperature hydration reaction which allows for custom shaped
implants that are made quickly and easily and may reduce the incidence of poor implant fit.

The hydration reaction of calcium aluminate (CA) during the casting procedure occurs in a three-step mechanism. Briefly, the surface of the calcium aluminate is hydroxylated and then begins to dissolve until the saturation limit has been reached (dissolution). After the dissolution step, the nuclei continue to grow in critical size and quantity, which is known as the nucleation phase. Finally, the precipitation phase occurs in which the hydrates precipitate out of the solution. This three-step mechanism continues until all of dissolved CA has been reprecipitated. During this hydration process the hydrated phases bind together and interlock with each other and the unhydrated phases to form the cast shape and increase the mechanical strength of the material.

The strength of the material can also be influenced by the particle size of the starting aggregates. A smaller aggregate particle size would allow for increased hydration, thus increasing the strength of the material. This would decrease the average pore size due to tighter particle packing. A larger aggregate particle size would decrease hydration, decreasing overall strength, but increase the average pore size. Combining large and small aggregate particle sizes allows for particle packing that creates a range of different pore sizes within the same material (Figure 2-1).
Figure 2-1: Schematic diagram of different types of particle packing. Aggregates are separated by size and then mixed in fixed ratios to produce mixtures with different average pore sizes.

In order to optimize the physical properties of CA for use as a biomaterial, the pore size of the calcium aluminate was varied. This was accomplished through the hydration process by changing the percent composition of different sized starting aggregates and creating five different mixtures. The different mixtures were then subjected to four different heat treatments. All mixtures were then evaluated for average pore size, strength, phases present, biodegradability and cell attachment.
2.2Materials and Methods

2.2.1 Materials

Calcium aluminate aggregates were supplied by Westmoreland Advanced Materials. NIH 3T3 Fibroblasts (CCL-92) were purchased from ATCC. Dulbecco’s Modified Eagle Media (DMEM/High glucose, Hyclone), phosphate buffered saline (PBS, - calcium and magnesium, Hyclone) fetal bovine serum and penicillin/strepavidin were purchased from Fisher. Unless otherwise noted all chemicals were used as received.

2.2.2 Pore Size Measurement

Calcium aluminate bars were prepared by a room temperature (20 °C) cast of different sized calcium aluminate aggregates with phases CaAl$_2$O$_4$ and CaAl$_4$O$_7$. Four different aggregate sizes were used in our casting process -10 + 30, -30 + 60, -60, and -325. The larger mesh numbers indicate smaller aggregates with -325 being fine powder-like cement. Five different combinations of the aggregates were cast with compositions of: A: 50% -325 and 25% each of -30 + 60 and -60; B: 25% of each of the four sizes; C: 33% each of -10 + 30, -30+60 and -60; D: 50% each of -10 + 30 and -30 + 60; and E: 100% -10 + 30. The aggregates were dry mixed at room temperature to ensure complete particle distribution. After dry mixing, doubly-distilled H$_2$O (ddH$_2$O) was mixed in and the cement paste was allowed to sit for 20 minutes at room temperature to thicken. Upon thickening, the cement was poured into a mold of a 1 X 1 X 12 inch bar and allowed to sit overnight at room temperature (20 ° C).
The cast CA bars were then heat treated in one of four ways: 20 °C (room temperature control), 120 °C, 650 °C, or autoclaved at 121 °C and 18 psi of steam (standard sterilization procedure for dry materials).

![Image]

**Figure 2-2:** Examples of pore diameter measured by optical microscopy. On the left there is an example of a larger pore, and on the right is an example of the same size area with multiple smaller pores. The scale bars show how the diameter of each pore was measured.

CA mixtures were visualized by optical microscopy on an Axioskop2 (10x magnification) and measured using the Axiovision 4 software. A two-way ANOVA analysis with Bonferroni post hoc test (p<0.05) was then used to determine any statistical differences in pore size due to mixture composition or heat treatment. Figure 2-2 shows examples of pores on the CA surface and their diameters.

### 2.2.3 Mercury Porosimetry

Ten 8 mm disks each of Mixtures A, B, and C with no heat treatment and Mixtures A\textsubscript{auto}, B\textsubscript{auto}, and C\textsubscript{auto} were sent to Delta Analytical Instruments Inc. in North Huntingdon, Pennsylvania for mercury porosimetry analysis.
2.2.4 Strength Testing

Mixtures A, B and C were cast at room temperature as described above in 1 x 1 x 12 inch bars. A total of 16 bars of each mixture were cast and 4 of each mixture were then subjected to one of the four heat treatments (a total of 4 bars of each mixture per heat treatment). The bars were then subjected to a standard four point bend test to calculate the modulus of rupture. A 1 in³ cube was then cut from each bar for a total of 4 data points for each mixture at each heat treatment to subject to a standard cold crushing test. All data was then analyzed by two-way ANOVA with Bonferroni post hoc test (p<0.05) to determine if the mixture composition or heat treatment had an effect on the strength of the material.

2.2.5 Powder X-Ray Diffraction

To determine the CA phases present in the CA Mixtures A, B and C that had been subjected to the four different heat treatments, the samples were analyzed using Powder X-ray Diffraction (PXRD) on an X’Pert PANalytical Pro. The samples were scanned from 5 to 70° 2θ with a time per step of 720.090 seconds at a scan speed of 0.002947 ° per second for a total data collection time of 6 hours. The collected patterns were analyzed using X’Pert HighScore Plus software by matching all peaks in the collected patterns to reference patterns in the database.

2.2.6 Biodegradability

Ten disks each of Mixtures A, B, and C were soaked in ddH₂O overnight and allowed to dry at room temperature to ensure they were fully hydrated. They were then
weighed and placed into 24 well plates and two milliliters of PBS was added to each well. The well-plates were placed on a shelf at room temperature. Weekly, samples were removed, dried in a 120 °C oven for 3 hours until all liquid had evaporated and then weighed to determine any change in mass. Samples were then placed back into the well plates and two milliliters of fresh PBS was added. The change in mass was observed for 26 weeks.

2.2.7 Fibroblast Attachment

NIH Swiss Albino mouse 3T3 fibroblasts were cultured until confluent and diluted to a concentration of 10,000 cells per milliliter of media (DMEM/High Glucose, 10% FBS, 1% strepavidin/penicillin). Three of each type of substrate (Mixtures A, B and C) per time point were placed in a 48-well plate and 1mL of cells was added to each well. After 1, 4, and 7 days, the number of live cells was determined using the Live/Dead/Viability/Cytotoxicity Assay Kit from Invitrogen by counting the number of live (green) versus dead (red) cells. Five spots on each sample with an area of 0.6 mm² were imaged under 10 x magnification using the Axioskop2. Three separate trials were completed with a total of 9 samples of each type with a total of 45 views for each sample type at each time point. Statistical analysis of the average number of live cells per view was done using Origin 8.0 using a one-way ANOVA with a Bonferroni post hoc test (p<0.05).
2.3 Results

2.3.1 Pore Size Control

In order to determine the optimal pore size for cell growth on this material, the calcium aluminate (CA) mixture composition was varied. This was achieved by changing the ratios of the CA aggregates during the room temperature casting process. Four different aggregate sizes were used in our casting process -10 + 30, -30 + 60, -60, and -325. The larger mesh numbers indicate smaller aggregates with -325 being fine powder-like cement. Five different combinations of the aggregates were cast with compositions of: A: 50% -325 and 25% each of -30 + 60 and -60; B: 25% of each of the four sizes; C: 33% each of -10 + 30, -30+60 and -60; D: 50% each of -10 + 30 and -30 + 60; and E: 100% -10 + 30. The CA aggregates were dry mixed and then cast into a bar mold with ddH₂O. The cast CA bars were then heat treated in one of four ways: 20 °C (room temperature control), 120 °C (for 48 hours), 650 °C (6 hours), or autoclaved at 121 °C and 18 psi of steam (1 hour). Following the heat treatments, the average diameter of the pores within the different CA mixtures was visualized by optical microscopy on an Axioskop2 (10 x magnification) and measured using the Axiovision 4 software (an example of pore sizes shown in Figure 2-2).
The average pore diameters ranged from approximately 100µm to 300µm. The average pore diameter over the four heat treatments for the CA was: Mixture A = 101.68 ± 115.60µm, Mixture B = 148.90 ± 151.05 µm, Mixture C = 210.09 ± 186.76 µm, Mixture D = 258.68 ± 174.26 µm, and finally Mixture E = 288.28 ± 176.95µm (Table 2-1). Two-way ANOVA analysis with Bonferroni post hoc test (p<0.05) determined that all mixtures had statistically significantly different pore sizes from one another, except there was no difference between Mixtures D and E. Statistical analysis also determined that the heat treatments did not affect the pore size of the material. The Mixtures D and E, with the two largest pore sizes, were determined to be unsuitable for further investigation because of their visible lack of mechanical strength.

### 2.3.2 Mercury Porosimetry

Mercury porosimetry data was used to determine the overall pore volume of Mixtures A-C with no heat treatment and that had been autoclaved. Mixture A had 27.39% porosity and Mixture A$_{auto}$ had 20.36% porosity. Mixture B had 37.25% porosity.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>-10+30</th>
<th>-30+60</th>
<th>-60</th>
<th>-325</th>
<th>Avg Pore Diameter</th>
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<tr>
<td>A</td>
<td>-</td>
<td>25%</td>
<td>25%</td>
<td>50%</td>
<td>101.68 µm</td>
</tr>
<tr>
<td>B</td>
<td>25%</td>
<td>25%</td>
<td>25%</td>
<td>25%</td>
<td>148.90 µm</td>
</tr>
<tr>
<td>C</td>
<td>33%</td>
<td>33%</td>
<td>34%</td>
<td>-</td>
<td>210.09 µm</td>
</tr>
<tr>
<td>D</td>
<td>50%</td>
<td>50%</td>
<td>-</td>
<td>-</td>
<td>258.68 µm</td>
</tr>
<tr>
<td>E</td>
<td>100%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>288.28 µm</td>
</tr>
</tbody>
</table>

**Table 2-1:** Table showing the percentage of each CA aggregate present in each mixture and the average pore size of that composition.
and Mixture B auto 28.05% porosity. Mixture C had 39.77% porosity and Mixture C auto had 31.75% porosity. These results indicated that the larger the average pore size of the material, the larger the average pore volume.

2.3.3 Strength Testing

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Average Pore Diameter (μm)</th>
<th>Heat Treatment</th>
<th>Cold Crushing (MPa)</th>
<th>Modulus of Rupture (*10⁶ N/m²)</th>
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<td>A²</td>
<td>101.68</td>
<td>None</td>
<td>59.6 ± 5.1</td>
<td>5.45 ± 2.04</td>
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<td></td>
<td></td>
<td>Autoclave</td>
<td>51.8 ± 16.1</td>
<td>9.17 ± 1.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>650°C</td>
<td>28.6 ± 2.3</td>
<td>2.81 ± 1.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120°C</td>
<td>46.4 ± 19.8</td>
<td>6.40 ± 0.97</td>
</tr>
<tr>
<td>B</td>
<td>148.90</td>
<td>None</td>
<td>45.1 ± 5.9</td>
<td>5.13 ± 2.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autoclave</td>
<td>47.4 ± 12.8</td>
<td>9.36 ± 1.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>650°C</td>
<td>20.1 ± 3.3</td>
<td>2.14 ± 0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120°C</td>
<td>38.7 ± 6.8</td>
<td>5.88 ± 2.59</td>
</tr>
<tr>
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<td>210.09</td>
<td>None</td>
<td>6.7 ± 1.3</td>
<td>1.61 ± 0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autoclave</td>
<td>6.5 ± 0.9</td>
<td>1.38 ± 0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>650°C</td>
<td>2.6 ± 0.4</td>
<td>1.09 ± 0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120°C</td>
<td>3.7 ± 1.5</td>
<td>1.27 ± 0.12</td>
</tr>
</tbody>
</table>

Table 2-2: Strength data for different mixtures and heat treatments of cast CA. None=samples which were cast at room temperature and received no additional heat treatments. Two-way ANOVA with Bonferroni post hoc test, p<0.05, i=greater modulus of rupture, ii=greater cold crushing strength, iii=decreases modulus of rupture and cold–crushing strength, iv=increases modulus of rupture).

Two-way ANOVA analysis (Bonferroni post hoc test, p<0.05) was used to analyze the cold crushing and modulus of rupture strength testing data (Table 2-2). The
results showed that heat treating the samples at 650 °C after casting, statistically significantly decreased the cold crushing strength and the modulus of rupture of all mixtures. Autoclaving the mixtures after casting statistically significantly increased the modulus of rupture of all mixtures. Mixtures A and B were statistically significantly stronger than Mixture C in the modulus of rupture test and Mixture A was stronger than Mixtures B and C in the cold crushing test.

2.3.4 Powder X-Ray Diffraction

The CA aggregates of all sizes, for example -10 + 30 and -60, used in this project were produced from the sintering of starting materials CaCO$_3$ and Al$_2$O$_3$. These aggregates were in two different phases CaAl$_2$O$_4$ and CaAl$_4$O$_7$. The two phases were then mixed with water during the hydration process and new phases of hydrated calcium aluminate were precipitated forming the bonds that make calcium aluminate castable.

To determine the new phases present in the hydrated CA, Mixtures A, B, and C that had been subjected to four different heat treatments were analyzed using Powder X-Ray Diffraction. The results are shown in Table 2-3.
Table 2-3: Phases of calcium aluminate present in each mixture after heat treatments. An “X” indicates that phase is present.

<table>
<thead>
<tr>
<th></th>
<th>A_{auto}</th>
<th>A_{120}</th>
<th>A/B_{650}</th>
<th>C_{650}</th>
<th>A/B_{room}</th>
<th>C_{room}</th>
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<tr>
<td>CaAl\textsubscript{2}O\textsubscript{4}</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CaAl\textsubscript{3}O\textsubscript{7}</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CaAl\textsubscript{2}((OH)\textsubscript{8}(H\textsubscript{2}O)\textsubscript{2})_{1.84}</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Al(OH)\textsubscript{3}</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Ca\textsubscript{2}Al\textsubscript{2}(OH)\textsubscript{12}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>3CaO\cdot Al\textsubscript{2}O\textsubscript{3}\cdot Ca(OH)\textsubscript{2}\cdot 18H\textsubscript{2}O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>5(CaO)\textsubscript{3}Al\textsubscript{2}O\textsubscript{5}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>(CaO)\textsubscript{3}Al\textsubscript{2}O\textsubscript{3}(H\textsubscript{2}O)\textsubscript{6}</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca\textsubscript{3}(Al(OH)\textsubscript{6})\textsubscript{2}</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All room temperature samples contained a hydrated phase of CaAl\textsubscript{2}((OH)\textsubscript{8}(H\textsubscript{2}O)\textsubscript{2})_{1.84}. In addition, room temperature Mixtures A\textsubscript{room} and B\textsubscript{room} contained Ca\textsubscript{3}Al\textsubscript{2}(OH)\textsubscript{12} and Mixture C\textsubscript{room} contained 3CaO\cdot Al\textsubscript{2}O\textsubscript{3}\cdot Ca(OH)\textsubscript{2}\cdot 18H\textsubscript{2}O.

All mixtures that were heat to 120 °C contained the hydrated phase (CaO)\textsubscript{3}Al\textsubscript{2}O\textsubscript{3}(H\textsubscript{2}O)\textsubscript{6}. Mixtures A\textsubscript{650} and B\textsubscript{650} contained a dehydrated phase, 5(CaO)\textsubscript{3}Al\textsubscript{2}O\textsubscript{3}. Finally all autoclaved samples contained the hydrated phase Ca\textsubscript{3}(Al(OH)\textsubscript{6})\textsubscript{2}. All mixtures at all heat treatments contained Al(OH)\textsubscript{3} and its presence indicated that Al\textsuperscript{3+} had precipitated as Al(OH)\textsubscript{3}. Additionally, no aluminum metal was present.

Figure 2-3 shows a PXRD simulated pattern for Mixture A\textsubscript{auto} and reference pattern matches for phases present. CaCO\textsubscript{3}, as mentioned above, was one of the two chemicals (the other being Al\textsubscript{2}O\textsubscript{3}) the CA aggregates were sintered from and it can sometimes been seen in the PXRD patterns as demonstrated in Figure 2-3.
Figure 2-3: PXRD simulated pattern for Mixture A autoclaved and reference pattern matches for phases present. A. Sample scan. B. Ca$_3$(Al(OH)$_6$) C. Al(OH)$_3$ D. CaAl$_4$O$_7$ E. CaCO$_3$ F. CaAl$_2$O$_4$.

2.3.5 Biodegradability

Figure 2-4 shows the average change in mass of the 10 disks of Mixtures A, B, and C versus time. The three disk types experienced similar cycles of a loss in mass followed by a gain in mass. After the 26 weeks period, there was no significant overall loss or gain in mass occurring in the disks of any of the mixtures.
2.3.6 Fibroblast Attachment

To determine which CA mixture would be optimal for biomaterial applications, disks of Mixtures A-C were cultured with NIH 3T3 fibroblasts. Three autoclaved substrates of each mixture (for each time period) were placed in a 48-well plate and 1mL of cells was added to each well. After 1, 4, and 7 days, the number of live cells was determined using the Live/Dead/Viability/Cytotoxicity Assay Kit from Invitrogen. Samples were viewed using a Zeiss Axioskop 2 under 10x magnification and five images per sample with an area of 0.6 mm² were taken using the Zeiss Axiocam. The number of live and dead cells per view was counted using the Axiovision 4 Software. Three separate trials were completed following the same procedure. The data was then

Figure 2-4: Average change in mass of Mixtures A, B, and C over 26 weeks.
analyzed using one-way ANOVA with a Bonferroni post hoc test (p<0.05) to determine if there were any statistical differences in the number of live cells on the different mixtures.

**Figure 2-5:** Day 1 Fibroblast attachment statistics. (Data labels=Mixture, average ± standard error) One-way ANOVA with Bonferroni post hoc test, of average live cells per view p<0.05, * = different from all other samples.

On Day 1 the average number of live cells per view (+/- standard deviation) was:

- Mixture A: 6.4 ± 6.0
- Mixture B: 5.3 ± 6.3
- Mixture C: 6.8 ± 8.2 (Figure 2-5).
**Figure 2-6**: Day 4 Fibroblast attachment statistics. (Data labels=Mixture, average ± standard error) One-way ANOVA with Bonferroni post hoc test, of average live cells per view p<0.05, * = different from all other samples.

On Day 4 the average number of live cells per view (± standard deviation) was:

Mixture A: 13.9 ± 20.0; Mixture B: 6.4 ± 7.7; and Mixture C: 4.9 ± 6.6 (Figure 2-6).

**Figure 2-7**: Day 7 Fibroblast attachment statistics. (Data labels=Mixture, average ± standard error) One-way ANOVA with Bonferroni post hoc test, of average live cells per view p<0.05, * = different from all other samples.
On Day 7 the average number of live cells per view (± standard deviation) was:
Mixture A: 9.6 ± 10.7; Mixture B: 9.6 ± 7.2; and Mixture C: 6.8 ± 7.8 (Figure 2-7). The statistical analysis showed that on Day 4, mixture A had significantly more live cells attached than Mixtures B and C.

2.4 Discussion

The average pore size of the CA material was easily controlled through the casting/hydration process. Five different mixtures were cast using different combinations of the four starting aggregates. Each of the five mixtures was then subjected to one of four different heat treatments. The mixtures had a range of average pore sizes from approximately 100 to 290 microns and it was found that heat treatment did not affect the average pore size of the material. However, heat treatment did affect the strength of the material and the phases of calcium aluminate present in each mixture.

The strength of the CA is due to the hydrated phases which form during the casting process and interlock the different phases of the material. This effect is seen after heat treating the cast CA to 650 °C where the hydrated phases are no longer present and the strength of the material significantly decreases. Autoclaving the CA significantly increased the strength of the mixtures. This is due to the 18 psi of steam used in the autoclaving process. The extra water from steam allows for increased dissolution of the material which then increases the precipitation of hydrated phases causing further interlocking of phases in turn increasing its strength. The temperature of autoclaving did not affect the strength, as heating the samples to 120 °C, the same temperature used in
autoclaving, in a dry environment with no steam or pressure did not have an effect on the strength of the material compared to CA with no heat treatment.

The lack of significant gain or loss in mass of the CA during a 26 week period could be due to the hydration process of CA which involves initial dissolution of the CA, followed by nucleation and finally reprecipitation.\textsuperscript{10} This would also explain the cycling of losing and gaining mass. Some of the loss is due to the slight dissolution, and the small gains are due to the precipitation of the newly hydrated CA phases. Also, the biodegradability was observed in a static environment in PBS. Any concrete observations on biodegradability of CA would have to be done during \textit{in vivo} testing due to the complex physiological environment surrounding implant materials.

2.5 Conclusions

After reviewing the results of the fibroblast adhesion, Mixture A with an average pore size of approximately 100µm was chosen for the remainder of the studies due to the increased cell adhesion on Day 4. This result is consistent with other studies that determined an average pore size of 100 to 150 microns was required for cell growth and proliferation on tricalcium phosphate, hydroxyapatite and calcium aluminate.\textsuperscript{5,11} Mixture A was also chosen due to its increased strength over Mixtures B and C and autoclaving was also chosen as the sterilization method for studies as it increases the strength of the material. This mixture contains CaAl\textsubscript{2}O\textsubscript{4}, CaAl\textsubscript{4}O\textsubscript{7}, and Ca\textsubscript{3}(Al(OH)\textsubscript{6})\textsubscript{2} phases, is stable when autoclaved and in PBS for 6 months.
2.6 References


Chapter 3: Increased Osteoblast Adhesion with Immobilization of KRSR on the Calcium Aluminate Surface

3.1 Introduction

Synthetic bone scaffolds can lead to the formation of fibrous tissue around the implant which inhibits productive cell adhesion and proliferation. This is caused by non-specific cell and protein adhesion in addition to the normal wound-healing response. These complications lead to bone resorption at the bone/implant interface causing implant loosening and ultimately implant failure.\(^1\)\(^-\)\(^4\) One way to increase cell adhesion is to combine cell adhesion peptides with scaffolds. RGD (arg-gly-asp) is the well-known sequence domain that is present in many extracellular matrix proteins, including fibronectin, whose main function is to mediate the adhesion of cells.\(^5\),\(^6\) Studies have shown that this peptide can increase the attachment of mesenchymal stem cells to different substrates (hydroxyapatite, titanium, polymer scaffolds) but does not enhance the spreading of the cells.\(^5\)-\(^7\) Cell spreading is important for cell survival and cell differentiation into osteoblasts which are necessary for bone formation and regeneration.\(^5\),\(^6\) RGD is also a non-cell specific adhesion peptide meaning it increases the adhesion of many different types of cells which can also lead to unwanted fibrous tissue formation in a bone scaffold.

Therefore, a peptide that binds transmembrane proteoglycans could improve cell attachment and spreading.\(^2\) For this application a peptide that could preferentially bind osteoblasts over other types of cells would be optimal. An example of this type of
peptide is the KRSR (lys-arg-ser-arg) peptide. KRSR-modified surfaces have been shown to bind increased numbers of osteoblasts compared to unmodified titanium surfaces and surfaces modified with RGD after four hours of incubation with cells.\textsuperscript{2, 8, 9} Additionally, Hasenbein et al found that KRSR preferentially bound osteoblasts compared to fibroblasts on micropatterned surfaces of N1[3-(trimethoxysilyl)-propyl] diethylenetriamine/ octadecyltrichlorosilane.\textsuperscript{9} Fibrous tissue formation is a common cause of implant failure and because KRSR has been shown to preferentially bind osteoblasts over fibroblasts, KRSR may be more beneficial than RGD in bone applications.

In this study, KRSR was covalently attached to the surface of cast calcium aluminate to potentially increase osteoblast adhesion on the surface and RGD was covalently attached to the surface as a control peptide for comparison purposes. Attachment was achieved through a multi-step deposition method. Each step of the attachment was characterized by Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT). Fibroblast and osteoblast attachment on modified and unmodified substrates at Days 1, 4, and 7 was then determined using a Live/Dead/Viability/Cytotoxicity assay.

3.2 Materials and Methods

3.2.1 Materials

Calcium aluminate aggregates were supplied by Westmoreland Advanced Materials. KRSRC was purchased from Genscript (96.4% purity) as lyophilized powder and reconstituted in doubly distilled H\textsubscript{2}O (ddH\textsubscript{2}O). RGDC was purchased from
American Peptide (99.3% purity) as lyophilized powder and was reconstituted in ddH$_2$O. Tetrahydrofuran (THF, 99.9% purity) and acetonitrile (ACN, 99.9% purity) were purchased from ThermoFisher. THF was distilled over sodium and benzophenone before use. 12-aminododecanoic acid (95%) was purchased from Sigma-Aldrich. 3-maleimidopropionic acid N-hydroxysuccinimide (NHS) ester (99%), 2% osmium tetroxide, and hexamethyldisilazane (HMDS) were purchased from Alfa Aesar. NIH 3T3 Fibroblasts (CCL-92) were purchased from ATCC. Dulbecco’s Modified Eagle Media (DMEM/High glucose, Hyclone), phosphate buffered saline (PBS, - calcium and magnesium, Hyclone) fetal bovine serum and penicillin/strepavidin were purchased from Fisher. Normal Human Osteoblasts (NHOsts, CC-2538), cell culture reagents, and OGM media were purchased from LONZA. Unless otherwise noted all chemicals were used as received.

### 3.2.2 Casting Calcium Aluminate

Calcium aluminate disks were prepared by a room temperature (20 °C) cast of different sized calcium aluminate aggregates with phases CaAl$_2$O$_4$ and CaAl$_4$O$_7$. The aggregates were separated by size during a sifting process in which the cement was passed through wire screens of different size meshes and “caught” on another mesh size. Three different aggregate sizes (-30 + 60 (25%), -60 (25%), and -325 (50%)) were mixed together to form the disks in the casting process. The aggregates were dry mixed at room temperature to ensure complete particle distribution. After dry mixing, doubly-distilled H$_2$O was mixed in and the cement paste was allowed to sit for 20 minutes at room temperature to thicken. Upon thickening, the cement was poured into a mold of 8 mm
diameter disks and allowed to sit overnight. The phases of the final calcium aluminate disks used in the following experiments after casting and sterilization by autoclaving were: CaAl$_2$O$_4$, CaAl$_4$O$_7$, and Ca$_3$(Al(OH)$_6$)$_2$.

3.2.3 Covalent Peptide Attachment

KRSR and RGD peptides with additional cysteine residues at the terminus were attached to the CA surface using a multi-step solution deposition. First, the substrates were placed in a 1mM 12-aminododecanoic acid in dry tetrahydrofuran (THF) solution at room temperature for one hour. The excess solvent was then removed by placement in a 120 °C oven for 24 hours. The 12-aminododecanoic acid modified CA disks were then placed in a 1mM 3-maleimidopropionic acid NHS ester in acetonitrile solution for 24 hours. The solvent was removed by vacuum (0.1 Torr) for 24 hours. The substrates were then dipped in a 1 mg/mL solution of KRSR(C) or RGD(C) in ddH$_2$O at 4°C for 24 hours, and then dried under vacuum for 24 hours (Scheme 3-1). Samples were then sterilized by autoclaving at 121 °C and 18 psi of steam for 60 minutes with fast exhaust prior to cell testing.

Scheme 3-1: Schematic representation of the three-step covalent attachment of KRSR(C) peptide to the CA surface.
3.2.4 **Diffuse Reflectance Infrared Fourier Transform Spectroscopy**

A Thermo Nicolet Nexus 470 FT-IR Spectrophotometer equipped with a diffuse reflectance attachment was used to obtain the spectra of the substrates after each deposition step. Spectra were collected with 256 scans and a resolution of 4 cm\(^{-1}\).

3.2.5 **Fibroblast Adhesion**

NIH Swiss Albino mouse 3T3 fibroblasts were cultured until confluent and diluted to a concentration of 10,000 cells per milliliter of media (DMEM/High Glucose, 10% FBS, 1% strepavidin/penicillin). Three of each type of substrate (KRSR modified, RGD modified and unmodified CA (control)) per time point were placed in a 48-well plate and 1mL of cells was added to each well. After 1, 4, and 7 days, the number of live cells was determined using the Live/Dead/Viability/Cytotoxicity Assay Kit from Invitrogen by counting the number of live (green) versus dead (red) cells. Five spots on each sample with an area of 0.6 mm\(^2\) were imaged under 10 x magnification using the Axioskop2. Three separate trials were completed with a total of 9 samples of each type with a total of 45 views for each sample type for each time point (control, RGD, KRSR). The data was then normalized to the average number of live osteoblasts on the unmodified CA at each time point and statistical analysis was done using Origin 8.0 using a one-way ANOVA with a Bonferroni post hoc test (p<0.05).
3.2.6 Osteoblast Adhesion

Normal human osteoblasts isolated from a 6 year old female were purchased from LONZA (NHOsts), cultured until confluent and diluted to a concentration of 10,000 cells per one milliliter of media (OGM media, LONZA). Three of each type of substrate per time point was placed in a 48-well plate and 1mL of cells was added to each well. After 1, 4, and 7 days, the number of live and total cells was determined following the procedure outlined in section 3.2.6.

3.2.7 Cell Proliferation

Normal human osteoblasts isolated from a 6 year old female were purchased from LONZA (NHOsts), cultured until confluent and diluted to a concentration of 10,000 cells per milliliter of media (OGM media, LONZA). Briefly, a standard curve was made by plating 0 to 10,000 cells, that had been frozen at -70 °C, in 200 µL volumes of the CyQuant cell lysis/reagent buffer (Invitrogen) in a 96-well plate and the fluorescence was read on a microplate reader with excitation/emission at 480/520 nm. Then three KRSR modified CA and three unmodified CA per time point were placed in a 48-well plate and 1mL of cells were added to each well. After 1, 4, 7, and 14 days, the number of cells was determined using the CyQuant Cell Proliferation Assay Kit from Invitrogen.

At each time point, the CA samples were removed and placed into 1mL 0.025 % trypsin in a 1.5mL microcentrifuge tube in the incubator for 30 minutes at 37 °C and 5 % CO₂ to detach the cells adhered to the samples. The samples were removed and the trypsin was centrifuged for 5 minutes at 2.2 x g. The supernatant was removed and the cell pellet was frozen overnight at -70 °C. The cell pellets were then resuspended in 200 µL of the
cell lysis/reagent buffer and placed in a 96-well plate and the fluorescence was measured. The fluorescence was then compared to the standard curve to determine the number of cells present on the samples.

3.2.8 Scanning Electron Microscopy

Normal human osteoblasts isolated from a 6 year old female were purchased from LONZA (NHOsts), cultured until confluent and diluted to a concentration of 10,000 cells per milliliter of media (OGM media, LONZA). KRSR modified CA and control CA were placed in a 48-well plate and 1mL of cells was added to each sample. After 4 days, the media was removed from the samples, and they were fixed in 2 % glutaraldehyde (in PBS) for 5 days at 4 °C. Samples were removed and washed twice with PBS and then immersed in 0.5 mL of 2 % Osmium tetroxide (OsO₄) for 1 hour at room temperature. The OsO₄ was removed and samples were washed 3 times with PBS and dehydrated in an ethanol series, by immersion for 20 minutes in each of 25, 50, 75, 90, and 100% ethanol. Samples were placed into new tubes and immersed in 1mL of HMDS for 10 minutes. Samples were placed in glass tubes and put into a dessicator for 1.5 hours before imaging. Samples were imaged on a Hitachi S-3400N-II Variable Pressure SEM under vacuum.

3.3 Results

3.3.1 Covalent Peptide Attachment

To increase osteoblast cell adhesion and therefore the biocompatibility of the CA surface, the cell adhesion peptides RGD and KRSR with terminal cysteine residues were
covalently attached to the CA surface. Covalently linking the peptide to the surface improves the adhesion strength of the peptide to the surface compared to physisorption of the peptide and it controls peptide orientation when it is placed into a physiological environment. Cysteine residues were added to the peptide sequence to facilitate immobilization. To immobilize the peptides on the CA surface, samples were immersed in a 1mM solution of 12-aminododecanoic acid (THF) for one hour at room temperature. Samples were removed from solution and placed in a 120 °C oven overnight. The samples were then analyzed using Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT) and the presence of the CH₂ asymmetric and CH₂ symmetric stretches at 2917 and 2848 cm⁻¹, respectively, in the spectrum indicate that the 12-aminododecanoic acid had been attached to the surface (Figure 3-1A).
Figure 3-1: DRIFT spectra for each step of peptide attachment. a) CH stretch region showing the CH$_2$ symmetric and asymmetric stretches from the carbon chain of 12-aminododecanoic acid to the CA surface. b) The peak at 1711 cm$^{-1}$ from the C=O stretches of the imide ring of 3-maleimidopropionic acid. c) The peak at 669 cm$^{-1}$ due to the formation of the C-S bond from the Michael addition of the cysteine residue to the imide ring.

Next, the samples were placed in a 1mM solution of 3-maleimidopropionic acid N-hydroxysuccinimide ester for 24 hours at room temperature. The ester bond of the 3-maleimidopropionic acid NHS ester was broken and an amide bond was formed between the 12-aminododecanoic acid on the surface and the 3-maleimidopropionic acid. The samples were removed from solution and placed on a vacuum line (0.1 Torr) to remove any excess solvent. DRIFT was used to once again analyze the samples. The peak in the spectrum at 1711 cm$^{-1}$ was assigned to the carbonyl bond stretches present on the imide ring and indicate that it had been successfully attached to the 12-aminododecanoic acid (Figure 3-1B). The final step of the attachment was to place the samples in a 1 mg/mL
solution of either peptide for 24 hours at 4 °C where the cysteine terminated peptide goes through a Michael addition to the imide ring. The samples were removed from solution and placed on the vacuum line overnight. The appearance of a peak at 669 cm\(^{-1}\) from the C-S bond stretch from the cysteine residue of the peptide indicated that the Michael addition had occurred through the cysteine residue and the peptide had been attached to the surface (Figure 3-1C).

### 3.3.2 Fibroblast Adhesion

To determine if the cell adhesion peptides increase the number of cells attached to the CA surface, NIH 3T3 cells were cultured until confluent and diluted to a concentration of 10,000 cells per milliliter of media. Three autoclaved substrates of each type (unmodified, KRSR modified and RGD modified) for each time period were placed in a 48-well plate and 1mL of cells was added to each well. After 1, 4, and 7 days, the number of live cells was determined using the Live/Dead/Viability/Cytotoxicity Assay Kit from Invitrogen and counted using the Axiovision 4 Software (Figure 3-2).

**Figure 3-2:** Representative live/dead images of fibroblasts attached to control unmodified CA, KRSR modified and RGD modified CA at Days 1, 4, and 7.
Three separate trials were completed following the same procedure as above.

The data was then normalized to the unmodified CA with osteoblast cell data and analyzed using one-way ANOVA with a Bonferroni post hoc test (p<0.05) to determine if there were any statistical differences in the number of cells present on the materials.

**Figure 3-3:** Day 1 Fibroblast and osteoblast attachment statistics. (Data labels=Sample type/cell line: CF=control/fibroblasts, KF=KRSR modified CA/fibroblasts, RF=RGD modified CA/ fibroblasts, CO= control/osteoblasts, KO=KRSR modified CA/osteoblasts, RO=RGD modified CA/osteoblasts) One-way ANOVA with Bonferroni post hoc test, p<0.05, * = different from CF, X= different from all other samples. Data represented as mean ± standard error.

On Day 1 the average number of normalized live fibroblasts per view (+/- standard deviation) was: control CA: 31.6 ± 5.6; KRSR modified: 104.0 ± 60.9; and RGD modified: 97.1 ± 84.6 (Figure 3-3).
Figure 3-4: Day 4 Fibroblast and osteoblast attachment statistics. (Data labels=Sample type/cell line: CF=control/fibroblasts, KF=KRSR modified CA/fibroblasts, RF=RGD modified CA/ fibroblasts, CO= control/osteoblasts, KO=KRSR modified CA/osteoblasts, RO=RGD modified CA/osteoblasts) One-way ANOVA with Bonferroni post hoc test, p<0.05, * = different from CF, X= different from all other samples. Data represented as mean ± standard error.

On Day 4: control CA: 63.7 ± 42.7; KRSR modified: 122.2 ± 83.6; and RGD modified: 122.7 ± 104.8 (Figure 3-4).
Figure 3-5: Day 7 Fibroblast and osteoblast attachment statistics. (Data labels=Sample type/cell line: CF=control/fibroblasts, KF=KRSR modified CA/fibroblasts, RF=RGD modified CA/ fibroblasts, CO= control/osteoblasts, KO=KRSR modified CA/osteoblasts, RO=RGD modified CA/osteoblasts) One-way ANOVA with Bonferroni post hoc test, p<0.05, ^ = different from CF, CO, and RO, $= different from CF and KO. Data represented as mean ± standard error.

On Day 7: control CA: 75.1 ± 78.2; KRSR modified: 224.4± 149.7; RGD modified: 201.8 ± 157.4 (Figure 3-5). Statistical analysis showed that the average number of live fibroblasts present on the samples that had been modified with cell adhesion peptides at all time points was statistically significantly higher than that of the unmodified CA control. However, there was no statistical difference between the numbers of live cells on the two peptide modified substrates (Figure 3-3 to Figure 3-5).

3.3.3 Live Osteoblast Adhesion

To determine if KRSR increased the number of osteoblasts attached to the CA surface when compared to the control and RGD modified surface, Normal Human Osteoblasts (LONZA) were cultured until confluent and diluted to a concentration of 10,000 cells per milliliter of media (OGM, LONZA). Three autoclaved substrates of
each type (control, KRSR modified and RGD modified) for each time period were placed in a 48-well plate and 1mL of cells was added to each well. After 1, 4, and 7 days, the number of live and total cells was determined using the Live/Dead/Viability/Cytotoxicity Assay Kit from Invitrogen. Samples were viewed using a Zeiss Axioskop 2 under 10x magnification and five images per sample were taken using the Zeiss Axiocam. The number of live and dead osteoblasts per view was counted using the Axiovision 4 software (Figure 3-6). Three separate trials were completed following the same procedure.

**Figure 3-6:** Representative live/dead images of osteoblasts attached to control unmodified CA, KRSR modified and RGD modified CA at Days 1, 4, and 7.

On Day 1 the average number of *live* osteoblast cells per view (+/- standard deviation) was: control CA: 100; KRSR modified: 144.9 ± 58.0; and RGD modified: 117.1 ± 56.1 (Figure 3-3). On Day 4: control CA: 100; KRSR modified: 169.1 ± 68.3; and RGD modified: 125.7 ± 55.0 (Figure 3-4). On Day 7: control CA: 100; KRSR
modified: 197.3 ± 95.9; and RGD modified: 151.6 ± 100.6 (Figure 3-5). The data was normalized to the average number of live osteoblasts on unmodified CA at each time point and then analyzed using one-way ANOVA with a Bonferroni post hoc test (p<0.05). Analysis showed that number of live osteoblasts on the KRSR peptide modified samples at Days 1, 4 and 7 was statistically significantly higher than that of the RGD modified and control samples (Figure 3-3 to Figure 3-5).

### 3.3.4 Total Osteoblast Adhesion

![Bar chart](image)

**Figure 3-7:** Day 1 Total osteoblast attachment statistics. (Data labels=Sample type/cell line: C= control/osteoblasts, K=KRSR modified CA/osteoblasts, R=RGD modified CA/osteoblasts) One-way ANOVA with Bonferroni post hoc test, p<0.05, $=$ different from all other samples. Data represented as mean ± standard error.

The total osteoblast cell data was normalized to the average number of total cells determined above on the unmodified CA at each time point. On Day 1 the average number of total osteoblast cells per view (+/- standard deviation) was: control CA: 100; KRSR modified: 121.9 ± 46.9; and RGD modified: 101.7 ± 46.7 (Figure 3-7).
**Figure 3-8**: Day 4 Total osteoblast attachment statistics. (Data labels=Sample type/cell line: C= control/osteoblasts, K=KRSR modified CA/osteoblasts, R=RGD modified CA/osteoblasts) One-way ANOVA with Bonferroni post hoc test, p<0.05, $=$ different from all other samples. Data represented as mean ± standard error.

On Day 4: control CA: 100; KRSR modified: 130.3 ± 50.7; and RGD modified: 106.7 ± 45.5 (Figure 3-8).

**Figure 3-9**: Day 7 Total osteoblast attachment statistics. (Data labels=Sample type/cell line: C= control/osteoblasts, K=KRSR modified CA/osteoblasts, R=RGD modified CA/osteoblasts) One-way ANOVA with Bonferroni post hoc test, p<0.05, *= different from C. Data represented as mean ± standard error.
On Day 7: control CA: 100; KRSR modified: 142.8 ± 61.7; and RGD modified: 124.7 ± 81.8 (Figure 3-9). The data was analyzed using one-way ANOVA with a Bonferroni post hoc test (p< 0.05). Analysis showed that the total number of osteoblasts on the KRSR peptide modified samples at Days 1, 4 and 7 was statistically significantly higher than that of the control samples and at Days 1 and 4 than that of the RGD samples (Figure 3-7 to Figure 3-9).

3.3.5 Cell Proliferation

Normal human osteoblasts (LONZA (NHOsts)) were cultured until confluent and diluted to a concentration of 10,000 cells per one milliliter of media (OGM media, LONZA). Briefly, a standard curve was made by plating 0 to 10,000 cells, that had been frozen at -70 °C, in 200 µL volumes of the CyQuant cell lysis/reagent buffer (Invitrogen) in a 96-well plate and the fluorescence was read on a microplate reader with excitation/emission at 480/520 nm. The, three of each type of substrate per time point was placed in a 48-well plate and 1mL of cells were added to each well. After 1, 4, 7, and 14 days, the number of cells was determined using the CyQuant Cell Proliferation Assay Kit. Figure 3-10 shows the standard curve for the cell proliferation assay.
**Figure 3-10:** Cell Proliferation Standard Curve. Plot of number of cells versus fluorescence intensity showing linear regression and equation.

\[ y = 24.243x + 7205.8 \]

\( R^2 = 0.9996 \)

**Figure 3-11:** Cell proliferation curve. Plot of mean number of cells (± standard deviation) vs. Days.
Figure 3-11 shows a plot of the mean number of cells (± standard deviation) vs. days of both the control CA and the KRSR modified CA. The number of cells on both samples decreased after Day 1, up until Day 7. From Day 7 to Day 14, a slight increase in the number of cells is observed. At Days 4, 7, and 14, a higher number of cells appears on the KRSR modified CA than on the control CA.

3.3.6 Scanning Electron Microscopy

To determine if cell adhesion peptide KRSR increased cell spreading on the CA surface, NH0sts (LONZA) were cultured until confluent and diluted to a concentration of 10,000 cells per milliliter of media (OGM media, LONZA). KRSR modified CA and control unmodified CA were placed in a 48-well plate and 1 mL of cells was added to each well. After 4 days, the media was removed from the samples, and were fixed to maintain cell morphology.
Figure 3-12: Scanning Electron Micrographs. A and B show images of rounded cells on control unmodified CA. C and D show images of spread cells on KRSR modified CA.

Samples were imaged on a Hitachi S-3400N-II Variable Pressure SEM under vacuum. All osteoblasts present on the unmodified CA were small and rounded (Figure 3-12A and B), whereas the osteoblasts present on the KRSR modified CA were spread and elongated (C and D).

3.4 Discussion

In vitro testing with fibroblasts showed that the cell adhesion peptides did increase cell attachment to the CA surface when compared to the control. This is in agreement with the current literature that has shown that these peptides increase cell attachment to a variety of surfaces.\textsuperscript{2,5-18} These results showed that the peptides were still
active on the surface and improved the biocompatibility of the CA surface. However, there was no statistical difference in the number of fibroblasts attached to the KRSR and RGD modified surfaces.

Previous studies have shown that initially osteoblasts preferentially adhere to KRSR over other cell types and compared to other cell adhesion peptides. Dee et al showed that borosilicate surfaces modified with KRSR bound more osteoblasts than those modified with RGD and bound more osteoblasts than other cell types when incubating the surfaces with either osteoblast, endothelial cells, or fibroblasts at the 4 hour time point. However, studies longer than 24 hours showed that RGD bound more osteoblasts than KRSR. Schuler et al observed that RGD modified titanium and control titanium surfaces bound more osteoblasts than KRSR modified titanium after seven days. Our results indicate that on Days 1, 4, and 7, KRSR modified CA binds a higher number of osteoblasts compared to the RGD modified and control samples. Therefore on the CA surface, the KRSR peptide interacts more favorably with osteoblasts than the RGD cell adhesion peptide.

A statistical comparison (one-way ANOVA, Bonferroni post hoc test, p<0.05) was done between the fibroblast and osteoblast cell trials to determine if the KRSR peptide preferentially bound osteoblasts over fibroblasts. On Days 1, 4, and 7, KRSR was shown to bind more osteoblasts than RGD and unmodified CA, and on Days 1 and 4 more osteoblasts than fibroblasts. On Day 7, there was no statistical difference between the number of fibroblasts or osteoblasts bound by the KRSR modified samples. It was also observed that the control CA samples bound more osteoblasts than fibroblasts on Day 1.
This was also evident in the cell proliferation assay. Both the unmodified and KRSR modified CA samples had a decrease in the number of cells from Day 1 to Day 7, and then a slight increase from Day 7 to Day 14. The initial decrease is expected as the cells that were not adhered well are no longer present on the samples after Day 1 and the NHOsts are a primary cell line which proliferates rarely and at a much slower rate than the NIH 3T3 fibroblasts. Also it is not known if all of the cells attached to the samples were removed by incubation in trypsin.

Another method of evaluating material suitability for biological purposes is to study the morphology of the cells on the surface. Cell spreading is necessary for cell viability and proliferation. If cells are rounded as opposed to spread on the surface, they are unable to survive and proliferate. One way to examine the morphology of cells on a surface is through Scanning Electron Microscopy (SEM). Control unmodified and KRSR modified CA were incubated with osteoblasts for 4 days to allow for complete adherence of the cells to the surface. The samples were then fixed and analyzed using SEM. SEM images indicate that attaching the KRSR peptide to the calcium aluminate surface allowed for osteoblast spreading, whereas the osteoblasts present on the unmodified CA were rounded.

3.5 Conclusions

Porous calcium aluminate scaffolds were cast at room and two cell adhesion peptides, KRSR and RGD were covalently linked to the CA surface using a multi-step solution deposition method. Both peptides increased the fibroblast adhesion compared to the control surface. KRSR increased osteoblast adhesion when compared to the control
and RGD modified surface. KRSR also allowed spreading of osteoblasts on the CA surface, whereas unmodified CA did not. The CA scaffold modified with KRSR would offer a surface that attracts more osteoblasts than fibroblasts which is advantageous when trying to increase bone cell growth.
3.6 References


Chapter 4: Antibacterial Activity of Vancomycin and Ampicillin Immobilized on the Calcium Aluminate Surface

4.1 Introduction

The risk of implant associated infections is significant even with the advancements in sterile techniques and facilities in operating rooms. Infection can be as high as 4% in primary and 30% in revision arthroplasties alone.\textsuperscript{1-9} These bacterial infections can lead to painful revision surgeries and implant failure. Current antibiotic therapies are not effective against these infections that often stem from resistant biofilms. The biofilms are mainly comprised of biofilm anchored bacteria, a polysaccharide extracellular matrix and some free-floating planktonic bacteria that could infect other areas of the body. The extracellular polysaccharide matrix protects the biofilm from any harsh environment or chemicals including antibiotics.\textsuperscript{8-13} This makes it almost impossible for oral or injected antibiotics to eradicate the entire infection and re-infection often occurs with the new implant. Biofilms also increase antibiotic resistance as the antibiotics are introduced to fight the infection are unable to penetrate into the biofilms protective extracellular polysaccharide matrix and are only able to kill some of the planktonic bacteria.\textsuperscript{6, 10, 11, 14, 15}

One of the most common bacteria found in implant associated infections is \textit{Staphylococcus aureus (S. aureus)}. 

One way to combat these infections would be to attach low doses of antibiotics to the surface of the implants. Attaching the antibiotics to the surface would allow them to be active locally and immediately after implantation of the device. This would inhibit initial bacteria growth and attachment to the implant. Two different methods of attaching antibiotics to the surface are adsorption or covalent linkage. Both of these attachment
methods to biomaterials would make the antibiotics available immediately after implantation reducing the risk of infection and would allow for the use of smaller dosages of antibiotics. These smaller dosages and the use of a local delivery system (vs. systemic delivery) will reduce the risk of antibiotic resistance.

In this study, ampicillin and vancomycin were either adsorbed or covalently attached to the calcium aluminate surface and evaluated for their biological activity against a *Staphylococcus aureus* strain of bacteria (UAMS1) and/or *Escherichia coli*. Adsorption of molecules to a surface is achieved through a physisorption of the molecule through interactions such as hydrogen bonding, van der Waals forces, and dipole interactions, between functional groups on the surface and on the molecule. These adsorption interactions are generally not as strong as covalent or ionic interactions. Antibiotics adsorbed to the surface could be more easily removed by shear forces of blood flow. Additionally, another biomolecule in the surrounding tissue may provide a more favorable interaction, thus removing the antibiotic from the implant.

Covalently linking molecules to a surface allows them to be anchored to the surface for more control over their presentation to the biological environment. If the site of attachment is chosen appropriately the molecule will remain bioactive. However, some attachment methods may lead to inactivity. Therefore, careful surface synthesis, characterization and activity analysis is required. Covalent attachment may also lead to longer retention times in physiological environments which would lead to longer effectiveness.

Two common antibiotics prescribed for implant associated infections are ampicillin and vancomycin. Ampicillin is a common antibiotic used to fight infections in
a systemic manner and is one of the first lines of defense. It is a penicillin beta lactam type antibiotic that is effective against gram positive and some gram negative bacteria by inhibiting the third stage of cell wall synthesis by binding to and acylating the transpeptidases that catalyze the cross-linking of the peptidoglycan cell wall leaving the enzyme inactive.\textsuperscript{15-17} Vancomycin is a glycopeptide antibiotic that is effective against gram positive bacteria by inhibiting proper cell wall synthesis. Vancomycin binds to the D-ala-D-ala termini of the growing peptidoglycan wall and prevents the transpeptidases from accessing the pentapeptide and ultimately prevents its crosslinking.\textsuperscript{14, 15, 18-21} Vancomycin forms five hydrogen bonds with the peptidoglycan terminal residues through four hydrogens and one oxygen from five secondary amides (Scheme 4-1). Both ampicillin and vancomycin leave the bacteria cells unable to respond to changes in osmotic pressure with improperly linked cell walls and the cells lyse.

\textbf{Scheme 4-1:} Schematic representation of the interaction between vancomycin and the D-ala-D-ala residues on the bacterial wall peptidoglycan.
The goal of this project was to adsorb and covalently attach ampicillin and vancomycin to the calcium aluminate surface to add antibacterial properties to the material. Each step of the attachment was verified using Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT). Antibacterial effectiveness was determined by a bacterial turbidity test in liquid culture and a zone inhibition assay on Mueller-Hinton 2 agar using *Staphylococcus aureus* (UAMS1) for both antibiotics and *Escherichia coli* for ampicillin.

### 4.2 Materials and Methods

#### 4.2.1 Materials

Calcium aluminate aggregates were supplied by Westmoreland Advanced Materials. Tetrahydrofuran (THF, 99.9% purity), methanol (MeOH, Certified ACS, >99.8% pure), Molecular Biology Grade Water (Hyclone), and Difco Tryptic Soy Broth were purchased from Thermo Fisher. THF was distilled over sodium and benzophenone before use. Vancomycin hydrogen chloride from *Streptomyces orientalis*, ampicillin sodium salt, 1,12-dodecanedicarboxylic acid, *N*-hydroxysuccinimde, and Mueller-Hinton 2 agar were purchased from Sigma-Aldrich. *N’N’*-dicyclohexylcarbodiimide was purchased from Fluka. *S. aureus* strain UAMS1 was provided by Allegheny General Singer Center for Genomic Sciences. *E.coli* (DH5a) was provided by Dr. Michael Cascio at Duquesne University. Normal Human Osteoblasts (NHOsts, CC-2538), osteoblast culturing reagents, and OGM media were purchased from LONZA. All materials were used as received unless otherwise noted.
4.2.2 Casting Calcium Aluminate

Calcium aluminate disks were prepared by a room temperature (20 °C) cast of different sized calcium aluminate aggregates with phases CaAl$_2$O$_4$ and CaAl$_4$O$_7$. The aggregates are separated by size during a sifting process in which the cement is passed through wire screens of different size meshes and “caught” on another mesh size. Three different aggregate sizes (-30 + 60 (25%), -60 (25%), and -325 (50%)) were mixed together to form the disks in the casting process. The aggregates were dry mixed at room temperature to ensure complete particle distribution. After dry mixing, doubly-distilled H$_2$O was mixed in and the cement paste was allowed to sit for 20 minutes at room temperature to thicken. Upon thickening, the cement was poured into a mold of 8 mm diameter disks and allowed to sit overnight. The phases of the final calcium aluminate disks used in the following experiments present in the disks after casting and sterilization by autoclaving are: CaAl$_2$O$_4$, CaAl$_4$O$_7$, and Ca$_3$(Al(OH)$_6$)$_2$.

4.2.3 Covalent Antibiotic Attachment

Ampicillin and vancomycin were covalently attached to the calcium aluminate surface using a multi-step solution deposition. First, the calcium aluminate disks were placed in a 1mM 1,12-dodecanedicarboxylic acid in dry tetrahydrofuran (THF) solution at room temperature for one hour. The excess solvent was removed by placement in a 120 °C oven for 24 hours. The 1,12-dodecanedicarboxylic acid modified disks were placed in a solution that was 2mM $N'N'$-dicyclohexylcarbodiimide (DCC) and 2mM $N$-hydroxysuccinimide (NHS) in dry THF for 24 hours. The solvent was removed by vacuum (0.1 Torr) for 24 hours. The disks were then dipped in a 1 mg/mL solution of
either ampicillin or vancomycin in methanol (MeOH) at 30 °C for 24 hours, and dried under vacuum for 24 hours. (Scheme 4-2) Samples were sterilized by autoclaving at 121 °C and 18 psi of steam for 60 min with fast exhaust prior to antibiotic testing.

Scheme 4-2: Schematic representation of the three-step covalent attachment of vancomycin to the calcium aluminate surface.

4.2.4 Adsorption of Antibiotics

Calcium aluminate disks were immersed in a 1 mg/mL solution of either ampicillin or vancomycin (MeOH) at room temperature for 24 hours followed by excess solvent removal by vacuum for 24 hours. Samples were sterilized by autoclaving as described above.

4.2.5 Diffuse Reflectance Infrared Fourier Transform Spectroscopy

A Thermo Nicolet Nexus 470 FT-IR Spectrophotometer equipped with a diffuse reflectance attachment was used to obtain the spectra of the substrates after each deposition step. Spectra were collected with 256 scans and a resolution of 4 cm⁻¹.
4.2.6 *Staphylococcus aureus* Bacterial Turbidity Tests

A standard optical density analysis of bacterial growth was used to determine the antibiotic activity of ampicillin and vancomycin attached on the calcium aluminate surface against gram positive bacteria *S. aureus* (UAMS1). The bacteria was diluted to 0.1 optical density at 600 nm (OD$_{600nm}$) in Tryptic soy broth with 0.1 M NaCl and 0.08 % glucose. Two milliliters of the bacterial suspension was placed in with each type of sample. There were five samples of each type in each trial for three separate trials. Samples were incubated at 37 ºC and 5 % CO$_2$ for 3.5 hours. The disks were then removed from the bacterial solution and the optical density at 600 nm was recorded. Statistical analysis of the average optical density at 600 nm was done using a one-way ANOVA with a Bonferroni post hoc test (Origin 8.0) (p<0.05). The sample groups were: no disk (B, bacteria only), control unmodified calcium aluminate disk (C), ampicillin adsorbed to the disk (A), ampicillin linked to the disk (AL), vancomycin adsorbed to disk (V), and vancomycin linked to the disk (VL). A set of control vancomycin 30µg disks (VS) was also tested to compare to the vancomycin modified samples.

4.2.7 *Staphylococcus aureus* Zone Inhibition Assay

A standard zone inhibition assay was used to determine the activity of the antibiotics attached to the calcium aluminate surface compared to the standard antibiotic. *S. aureus* (UAMS1) was diluted to 0.1 OD$_{600nm}$ in Tryptic soy broth with 0.1 M NaCl and 0.08 % glucose and spread onto Mueller-Hinton 2 Agar plates with cotton swabs. Five plates for each antibiotic were used for each trial with a total of three trials. The plate set up for the vancomycin assay was: a standard disk of 30 µg of vancomycin (VS) was
placed in the upper left quadrant of the plate, C-upper right, V-lower left, and VL-lower right. The plate set up for the ampicillin assay was: a standard disk of 10 µg of ampicillin (AS) was placed in the upper left quadrant of the plate, C-upper right, A-lower left, and AL-lower right. Plates were incubated agar side down (to ensure samples did not displace) for 24 hours at 37 ºC and 5 % CO₂. The diameter of the zone with the absence of bacterial growth was then measured in millimeters. Statistical analysis using a one-way ANOVA with Bonferroni post hoc test (p<0.05) was used to determine any differences in the diameters of the zones produced.

4.2.8 *Escherichia coli* Bacterial Turbidity Tests

A standard optical density analysis of bacterial growth was used to determine the antibiotic activity of ampicillin attached to the calcium aluminate surface against gram negative bacteria. *E.coli* (DH5α) was diluted to 0.1 optical density at 600 nm (OD_{600nm}) in Lysogeny Broth. Two milliliters of the bacterial suspension was placed in with each type of sample. There were five samples of each type in each trial for three separate trials. Samples were incubated at 37 ºC and 5 % CO₂ for 3.5 hours. The disks were then removed from the bacterial solution and the optical density at 600 nm was recorded. Statistical analysis of the average optical density at 600 nm was done using Origin 8.0 using a one-way ANOVA with a Bonferroni post hoc test (p<0.05). The sample groups were: no disk (B, bacteria only), control unmodified calcium aluminate disk (C), ampicillin adsorbed to the disk (A), and ampicillin linked to the disk (AL).
4.2.9 *Escherichia coli* Zone Inhibition Assay

A standard zone inhibition assay was used to determine the activity of the antibiotics attached to the calcium aluminate surface compared to the standard antibiotic. *E.coli* (DH5α) was diluted to 0.1 OD<sub>600nm</sub> in Lysogeny Broth and spread onto Mueller-Hinton 2 Agar plates with cotton swabs. Five plates for each antibiotic were used for each trial with a total of three trials. The plate set up for the ampicillin assay was: a standard disk of 10 µg of vancomycin (AS) was placed in the upper left quadrant of the plate, C-upper right, A-lower left, and AL-lower right. Plates were incubated agar side down (to ensure samples did not displace) for 24 hours at 37 °C and 5 % CO<sub>2</sub>. The diameter of the zone with the absence of bacterial growth was then measured in millimeters.

4.2.10 Release of Vancomycin

To determine the difference in drug release between the vancomycin adsorbed and linked samples, calcium aluminate with adsorbed vancomycin and with linked vancomycin was soaked in Molecular Biology Grade water for 1, 3.5, and 24 hours. At each time point, the disks were removed and the water was analyzed using Electrospray Ionization Time of Flight Mass Spectroscopy on a Bruker microTOF. Samples were analyzed in positive mode. The following settings were used: endplate set off = -500 V; capillary voltage = -4500 V; nebulizer = 0.4 Bar; nitrogen dry gas = 4.0 l/min; dry temperature = 200 °C; capillary exit voltage = 139.0 V; skimmer 1 = 40.0 V; Hexapole 1 = 23.0 V; Hexapole RF = 500.0 V; and skimmer 2 = 22.0 V.
4.2.11 Osteoblast Adhesion to Antibiotic Modified Calcium Aluminate

Normal human osteoblasts isolated from a 6 and 11 year old female, respectively, and purchased from LONZA (NHOsts) were cultured until confluent and diluted to a concentration of 10,000 cells per one milliliter of media (OGM media, LONZA). Three of each type of substrate per time point were placed in a 48-well plate and 1mL of cells was added to each well. After 1, 4, and 7 days, the number of cells were determined using the Live/Dead/Viability/Cytotoxicity Assay Kit from Invitrogen by counting the number of live (green) versus dead (red) cells. Five spots on each sample with an area of 0.6 mm² were imaged under 10x magnification using the Axioskop2. Three separate trials were completed with a total of 9 samples of each type with a total of 45 views for each sample type for each time point. The data was then normalized to the average number of live or total osteoblasts on the unmodified CA at each time point and statistical analysis was done using Origin 8.0 using a one-way ANOVA with a Bonferroni post hoc test (p<0.05).

4.3 Results

4.3.1 Covalent Antibiotic Attachment

To add antibacterial activity to the calcium aluminate material, ampicillin and vancomycin were attached to the surface, separately. Covalently linking the antibiotic to the surface controls antibiotic orientation at the surface of the implant material and its stability on the surface. This would allow for a strictly local dose aimed at reducing implant infection by bacterial attachment and subsequent biofilm formation. To immobilize the antibiotic on the surface, samples were immersed in a 1mM solution of
1,12-dodecanedicarboxylic acid (THF) for one hour at room temperature. Samples were removed from solution and placed in a 120 °C oven overnight. The samples were then analyzed using Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT) and the presence of the CH$_2$ asymmetric and CH$_2$ symmetric stretches at 2916 and 2848 cm$^{-1}$, respectively, in the spectrum indicated that the 1,12-dodecanedicarboxylic acid had been attached to the surface (Figure 4-1A).$^{22,23}$ The COOH region of the infrared spectrum contained several peaks that were due to both the binding of the dicarboxylic acid molecule to the surface through one of the carboxylic acid end groups and the presentation of the other carboxylic acid tail group at the interface for use in further reactions (Figure 4-1B). The peaks at 1707 cm$^{-1}$ (C=O stretching) and 1377 cm$^{-1}$ (C-O-H stretching) were due to the tail carboxylic acid group which can be used in further reactions. The large peak at 1566 cm$^{-1}$ was consistent with bidentate bonding of the acid to the surface in a carboxylate-like motif.$^{24}$ Next, the samples were placed in a 2mM \textit{N’N’}-dicyclohexylcarbodiimide (DCC)/ 2mM \textit{N}-hydroxysuccinimide (NHS) solution in dry THF for 24 hours at room temperature. The samples were removed from solution and placed on a vacuum line (0.1 Torr) to remove any excess solvent. The DCC reacted first with the COOH tail group and then was displaced by the NHS. DRIFT was used to once again analyze the samples and the peak in the spectrum at 1701 cm$^{-1}$ was assigned to the stretching of the carbonyl bond on the succinimide ring. This indicated that the succinimide ring had been successfully attached to the carbon chain (Figure 4-1C). The final step of the attachment was to place the samples in a 1 mg/mL solution of the antibiotic for 24 hours at 4 °C. The NHS was displaced and an amine group on the antibiotic reacted with O’forming an amide bond. The samples were removed from
solution and placed on the vacuum line overnight. Vancomycin immobilization was confirmed by the appearance of a peak at 1235 cm\(^{-1}\) which is attributed to the amine groups present in the vancomycin structure (Figure 4-1D). Ampicillin immobilization was confirmed by the appearance of peaks in the spectrum from 1792 to 1733 cm\(^{-1}\) due to different carbonyl groups present in the ampicillin structure (Figure 4-1E).

**Figure 4-1:** DRIFT spectra characterizing the attachment of the antibiotic to the surface. A) CH\(_2\) asymmetric and symmetric stretches at 2916 and 2848 cm\(^{-1}\) due to the alkyl chain in 1,12-dodecanedicarboxylic acid. B) Peak at 1701 and 1377 cm\(^{-1}\) due to the “free” carboxylic groups from the C=O and C-O-H stretching respectively. Peak at 1566 cm\(^{-1}\) consistent with the bidentate bonding of the acid to the surface. C) Peak at 1701 cm\(^{-1}\) assigned to the carbonyl bond stretches present on the succinimide ring. D) Peak at 1235 cm\(^{-1}\) in the vancomycin attachment from amine groups present in the vancomycin structure. E) Peaks between 1792 and 1733 cm\(^{-1}\) in the ampicillin attachment from the carbonyl groups present in the ampicillin structure.

4.3.2 Adsorption of Antibiotics

Covalent linkage of biomolecules to a surface could render them inactive; for this reason a second attachment method was investigated. Ampicillin or vancomycin was
adsorbed by immersing the disks in a 1 mg/mL solution of the antibiotic in methanol for 24 hours at room temperature. The samples were then placed on a vacuum line and excess solvent and antibiotic were removed for 24 hours at 0.1 Torr. DRIFT was used to determine the presence of the antibiotic on the surface after adsorption. Figure 4-2A, is the DRIFT of solid vancomycin powder. The peaks present at 2960, 2935, and 2876 cm\(^{-1}\) are due to CH\(_3\) and secondary amide groups, respectively. Figure 4-2B shows the vancomycin adsorbed to the surface. The peaks present at 2954, 2932, and 2871 cm\(^{-1}\), were consistent with those in the vancomycin solid.

Figure 4-2: Adsorption of vancomycin to the surface. A) DRIFT of solid vancomycin powder. Peaks at 2960, 2935, and 2876 cm\(^{-1}\) are due to the CH\(_3\) groups and secondary amides respectively. B) Vancomycin adsorbed to the surface. The peaks present at 2954, 2932, and 2871 cm\(^{-1}\) are consistent with those seen in the solid vancomycin powder.

Figure 4-3A shows the DRIFT of solid ampicillin powder. The peaks present at 2980, 2923, and 2873 cm\(^{-1}\) are due to the CH\(_3\), CH\(_2\), and secondary amide groups, respectively. Figure 4-3B shows the ampicillin adsorbed to the calcium aluminate surface. The peaks at 2977, 2923, and 2862 were consistent with that of solid ampicillin.
Figure 4-3: Adsorption of ampicillin to the surface. A) DRIFT of solid ampicillin powder. Peaks at 2980, 2923, and 2873 cm⁻¹ are due to the CH₃, CH₂ groups and secondary amides respectively. B) Ampicillin adsorbed to the surface. The peaks present at 2977, 2923, and 2863 cm⁻¹ are consistent with those seen in the solid ampicillin powder.

4.3.3 *Staphylococcus aureus* Bacterial Turbidity Test

Antibiotic activity in culture was determined by a standard optical density test for bacteria. *S. aureus* (UAMS1) was diluted to an optical density of 0.1 (at 600 nm). Two milliliters of the bacterial solution was placed in a 15 mL centrifuge tube with each of the samples. Five samples each of the bacteria solution with no disk (B), control unmodified calcium aluminate disks (C), adsorbed ampicillin (A), linked ampicillin (AL), adsorbed vancomycin (V), linked vancomycin (VL), and a 30µg vancomycin standard (VS) were used in each trial. Samples were incubated for 3.5 hours at 37 ºC with 5% CO₂. The samples were then removed from the incubator and the optical density at 600 nm was recorded. A one-way ANOVA (p < 0.05) with Bonferroni post hoc test (p < 0.05, or 0.01, or 0.001) was used to determine the statistical significance in bacterial growth inhibition (Figure 4-4). The OD₆₀₀nm for the samples were: B = 0.564 ± 0.137; C = 0.406 ± 0.145; A = 0.412 ± 0.183; AL = 0.401 ± 0.197; V = 0.186 ± 0.062; VL = 0.185 ± 0.067; VS = 0.269 ± 0.006.
Figure 4-4: One-way ANOVA with Bonferroni post hoc test of bacterial turbidity test data. Data is represented as the mean OD600nm ± standard error. B=bacterial solution no sample, C=unmodified CA, A=adsorbed ampicillin/CA, AL=ampicillin linked/CA, V=adsorbed vancomycin/CA, and VL=vancomycin linked/CA. (X, p<0.05 with respect to B; #, p<0.001 with respect to B, C, and A, and p<0.01 with respect to AL; *, p<0.001 with respect to all samples except V).

Unmodified disks (C) and ampicillin linked disks (AL) significantly inhibited bacterial growth compared to the bacterial solution (B) with no sample (p < 0.05). Vancomycin adsorbed disks (V) inhibited bacterial growth significantly compared to the bacteria solution, adsorbed ampicillin disks (A), ampicillin linked disks (AL), and the unmodified disks. Vancomycin linked disks (VL) inhibited bacterial growth significantly compared to all samples except V (p < 0.001). There was no statistical significance found between vancomycin adsorbed or linked to the disks in their ability to inhibit
bacterial growth in culture (Figure 4-4). Both V and VL were significantly better at inhibiting bacteria growth when compared to VS, indicating that our surfaces are just as, if not more effective at inhibiting bacteria growth than a dosage of 30 µg of vancomycin (VS) (p < 0.05) (Figure 4-5).

Figure 4-5: One-way ANOVA with Bonferroni post hoc test of vancomycin data. Data is represented as the mean OD<sub>600nm</sub> ± standard error. V = adsorbed vancomycin, VL = vancomycin linked, VS = 30µg vancomycin standard. (^, p < 0.05 with respect to VS).

4.3.4 *Staphylococcus aureus* Zone Inhibition Assays

To determine the effectiveness of the antibiotics on the disk surface when compared to a control dosage (30 µg of vancomycin, or 10 µg of ampicillin) of the antibiotic, a zone inhibition assay was used. *S. aureus* (UAMS1) was diluted to 0.1 OD<sub>600nm</sub> in Tryptic soy broth with 0.1 M NaCl and 0.08 % glucose and spread onto Mueller-Hinton 2 Agar plates with cotton swabs. For the vancomycin assay, one sample each of the standard antibiotic 30 µg of vancomycin (VS), C, V, and VL was placed on each plate. For the ampicillin assay, one sample each of the standard antibiotic 10 µg of
ampicillin (AS), C, A, and AL was placed on each plate. Five plates for each antibiotic were used for each trial with a total of three trials. Plates were then incubated agar side down (to ensure no displacement of the disks) for 24 hours at 37 ºC and 5 % CO$_2$. After 24 hours the zone of inhibition around the disk was measured.

Figure 4-6: Photographs of sample zone inhibition assay plates. A) Sample plate of vancomycin zone inhibition assay. Top left: standard 30μg vancomycin disk; top right: unmodified CA disk; bottom left: vancomycin adsorbed on CA; bottom right: vancomycin linked to CA. B) Sample plate of ampicillin inhibition assay. Top left: standard 10μg ampicillin disk; top right: unmodified CA disk; bottom left: ampicillin adsorbed on CA; bottom right: ampicillin linked to CA.

A one-way ANOVA with a Bonferroni post hoc test (p<0.05) was used to determine the statistical difference in the zones of inhibition of bacterial growth. For vancomycin, the average zones were: 30 μg vancomycin (VS) = 17± 1 mm; unmodified (C) = 0 mm; adsorbed vancomycin (V) = 16 ± 6 mm; linked vancomycin (VL) = 14 ± 2 mm (Figure 4-6A). For ampicillin, only the control 10 µg disk of ampicillin produced a zone of 19 ± 2 mm (Figure 4-6B). There was no statistical difference between the sizes of the zones of inhibition of the vancomycin modified CA and the control dosage (Figure 4-7).
Figure 4-7: One-way ANOVA with Bonferroni post hoc test of vancomycin data. Data is represented as the mean zone(mm) ± standard error. V = adsorbed vancomycin, VL = vancomycin linked, VS = 30µg vancomycin standard. (p<0.05)

4.3.5 *Escherichia coli* Bacterial Turbidity Test

Antibiotic activity of ampicillin against gram-negative bacteria in culture was determined by a standard optical density test for bacteria. *E.coli* (DH5α) was diluted to an optical density of 0.1 (at 600 nm). Two milliliters of the bacterial solution was placed in a 15mL centrifuge tube with each of the samples. Five samples each of the bacteria solution with no disk (B), control unmodified calcium aluminate disks (C), adsorbed ampicillin (A), and linked ampicillin (AL) were used in each trial with a total of 3 trials. Samples were incubated for 3.5 hours at 37 °C with 5 % CO₂. The samples were then removed from the incubator and the optical density at 600 nm was recorded. A one-way ANOVA (p < 0.05) with Bonferroni post hoc test was used to determine the statistical significance in bacterial growth inhibition. The OD₆₀₀nm for the samples were: B= 1.22 ±
0.25; C= 1.29 ± 0.23; A= 1.22 ± 0.15; and AL= 1.26 ± 0.24. (Figure 4-8) No statistical difference was observed between sample types.

Figure 4-8: One-way ANOVA with Bonferroni post hoc test of *E.coli* bacterial turbidity test data. Data is represented as the mean OD600nm ± standard error. B=bacterial solution no sample, C=unmodified CA, A=adsorbed ampicillin/CA, AL=ampicillin linked/CA. (p<0.05)

4.3.6 *Escherichia coli* Zone Inhibition Assays

To determine the effectiveness of the ampicillin on the disk surface when compared to a control dosage (10µg of ampicillin) of the antibiotic, a zone inhibition assay was used. *E.coli* (DH5α) was diluted to 0.1 OD₆₀₀nm in Lysogeny Broth and spread onto Mueller-Hinton 2 Agar plates with cotton swabs. For the assay, one sample each of the standard antibiotic 10µg of ampicillin (AS), C, A, and AL was placed on each plate. Five plates for each antibiotic were used for each trial with a total of three trials. Plates were then incubated agar side down (to ensure no displacement of the disks) for 24 hours at 37 °C and 5 % CO₂. After 24 hours the zone of inhibition around the disk was measured. Only the control 10µg disk of ampicillin produced a zone of 25 ± 4 mm.
4.3.7 Release of Vancomycin

To determine the difference in drug release between the vancomycin modified samples, calcium aluminate with adsorbed vancomycin and with linked vancomycin were soaked in Molecular Biology Grade Water for 1, 3.5, and 24 hours. At each time point, the disks were removed and the water was analyzed using Electrospray Ionization Time of Flight Mass Spectroscopy on a Bruker microTOF. For all samples a peak at 471.3 m/z was observed. This corresponds to the vancomycin molecule with the loss of one chlorine atom, with a total charge of + 3. For the calcium aluminate samples with adsorbed vancomycin, the counts for the peak observed at 471.3 were after 1 hour 5806 counts (Figure 4-9A), after 3.5 hours 421 counts (Figure 4-9B), and after 24 hours 12,090 counts (Figure 4-9C).
**Figure 4-9:** Release of adsorbed vancomycin. A) Mass spectra after 1 hour soak in molecular biology grade water. B) Mass spectra after 3.5 hour soak. C) Mass spectra after 24 hour soak. Peak at 471.3 corresponds to vancomycin with the loss of a chlorine and an overall charge to +3.
Figure 4-10: Release of linked vancomycin. A) Mass spectra after 1 hour soak in molecular biology grade water. B) Mass spectra after 3.5 hour soak C) Mass spectra after 24 hour soak. Peak at 471.3 corresponds to vancomycin with the loss of a chlorine and an overall charge to +3.
For calcium aluminate with linked vancomycin, the counts observed at 471.3 were, after 1 hour 370 counts (Figure 4-10A), after 3.5 hours 492 counts (Figure 4-10B), and after 24 hours 1132 counts (Figure 4-10C). Indicating that more vancomycin is being released from the sample in which the vancomycin is adsorbed to the substrate compared to the linked vancomycin substrates.

4.3.8 Osteoblast Adhesion to Antibiotic Modified Calcium Aluminate

To determine if any of the modifications increased the number of osteoblasts attached to the CA surface when compared to the control, Normal Human Osteoblasts (LONZA) were cultured until confluent and diluted to a concentration of 10,000 cells per milliliter of media (OGM, LONZA). Three autoclaved substrates of each type for each time period were placed in a 48-well plate and 1mL of cells was added to each well. After 1, 4, and 7 days, the number of the cells was determined using the Live/Dead/Viability/Cytotoxicity Assay Kit from Invitrogen. Samples were viewed using a Zeiss Axioskop 2 under 10x magnification and five images per sample were taken using the Zeiss Axiocam. The number of live and dead cells per view was counted using the Axiovision 4 Software and the data was normalized to the average number of live or total osteoblasts on the unmodified CA at each time point. Three separate trials were completed following the same procedure. The data was then analyzed using one-way ANOVA with a Bonferroni post hoc test (p< 0.05) to determine if there were any statistical differences between the materials.
**Figure 4-11:** Day 1 one-way ANOVA with Bonferroni post hoc test (p<0.05) live and total osteoblast data. C=unmodified CA, A= CA with adsorbed ampicillin, AL= CA with ampicillin linked, V= CA with vancomycin adsorbed, VL=CA with vancomycin linked. Data represented as mean ± standard error. A) Normalized number of live osteoblasts, * =different from C B) Normalized number of total osteoblasts.

On Day 1 the average number of *live* cells per view (+/- standard deviation) was: control CA: 100 ; A: 111.1 ± 68.0; AL: 140.4 ± 81.8; V: 120.7 ± 66.0; VL: 142.6 ± 81.4 (Figure 4-11A) and the average of *total* cells per view was: control CA: 100 ; A: 100.2 ± 55.0; AL: 116.5 ± 63.6; V: 101.0 ± 56.0; VL: 114.1 ± 63.8.(Figure 4-11B) AL and VL had higher numbers of *live* cells compared to control CA, however no difference was seen in the *total* number of cells.
Figure 4-12: Day 4 one-way ANOVA with Bonferroni post hoc test (p<0.05) live and total osteoblast data. C=unmodified CA, A= CA with adsorbed ampicillin, AL= CA with ampicillin linked, V= CA with vancomycin adsorbed, VL=CA with vancomycin linked. Data represented as mean ± standard error. A) Normalized number of live osteoblasts, $ =different from C and AL B) Normalized number of total osteoblasts, #=different from AL.

On Day 4 the average number of live cells per view was: control CA: 100; A: 146.7 ± 102.6; AL: 100.0 ± 128.3; V: 181.5 ± 114.1; VL: 171.9 ± 128.3 (Figure 4-12A) and the average of total cells per view was: control CA: 100; A: 126.7 ± 80.7; AL: 84.1 ± 71.6; V: 120.4 ± 63.4; VL: 117.8 ± 83.1. (Figure 4-12B) V and VL had higher numbers of live cells compared to control CA and AL. A had higher total number of cells compared to AL.
Figure 4-13: Day 7 one-way ANOVA with Bonferroni post hoc test (p<0.05) live and total osteoblast data. C=unmodified CA, A= CA with adsorbed ampicillin, AL= CA with ampicillin linked, V= CA with vancomycin adsorbed, VL=CA with vancomycin linked. Data represented as mean ± standard error. A) Normalized number of live osteoblasts, # =different from C and VL B) Normalized number of total osteoblasts, ^=different from AL, & =different from C, A, and V.

On Day 7 the average number of live cells per view was: control CA: 100; A: 124.4 ± 140.7; AL: 129.4 ± 110.4; V: 176.7 ± 98.6; VL: 86.7 ± 87.7 (Figure 4-13A) and the average of total cells per view was: control CA: 100; A: 105.0 ± 84.3; AL: 81.4 ± 58.4; V: 119.7 ± 64.8; VL: 58.8 ± 48.1 (Figure 4-13B). V had a higher number of live cells compared to VL and control CA, and a higher number of total cells compared to AL. VL had a lower number of total cells compared to V, A, and control CA.

4.4 Discussion

Implant associated infections are difficult to eradicate due to their antibiotic resistant characteristics. Often the infection is noticed only in very advanced stages where removal of the implant is the only therapy available. For this reason, an antibiotic that would be delivered only locally and present immediately after implantation could be
advantageous. In this study, ampicillin and vancomycin were attached to the calcium aluminate surface through two different mild methods: adsorption and a three-step covalent linkage. DRIFT confirmed that the antibiotics were attached successfully using both methods. All samples were sterilized by autoclaving before use in any bacteria tests. To confirm that autoclaving would not affect the integrity of the modification, DRIFT was used to analyze the samples after sterilization and the spectra remain unchanged (Data not shown). Ampicillin is often temperature sensitive so ethanol sterilization and no sterilization methods were tested in a zone inhibition assay alongside the standard antibiotic and autoclaved samples. No difference was seen between the sterilization methods in antibacterial activity.

Bacterial turbidity tests showed that both vancomycin modified samples were the most effective at inhibiting *S. aureus* bacterial growth in culture compared to ampicillin modified samples (*p*<0.001). For the two different antibiotic attachment methods utilized for vancomycin, no statistical difference in antibacterial activity was found. This indicates that the covalent linkage does not affect the antibacterial properties of the vancomycin. The vancomycin is linked through one of the two primary amines (NH$_2$ groups) that are not involved in the hydrogen bonding of vancomycin to the substrate D-ala-D-ala. Additionally, both the unmodified disks and ampicillin linked disks inhibited bacteria growth in culture. This indicates that the calcium aluminate itself has some antibacterial properties. The linkage of the ampicillin through its NH$_2$ group orients it actively at the interface in order to inhibit bacterial growth. While the amine group isn’t necessary for the antibacterial activity, it does aid in ampicillin’s ability to penetrate the cell wall more effectively, thereby likely reducing ampicillin’s effectiveness.$^{17}$ Ampicillin
adsorbed to the surface did not inhibit bacterial growth; this may be a result of the orientation of the molecule on the surface. For example, the carboxylic acid group or the C=O in the four membered ring could be interacting with the oxide surface. This would inhibit ampicillin from interacting with the transpeptidases rendering the ampicillin inactive. 

To compare the linked and adsorbed antibiotics to the standard antibiotic dosages, a standard zone inhibition assay was done. The results show that vancomycin linked and adsorbed to the disks were as effective as the control dose of the antibiotic. The adsorbed antibiotic was able to travel farther from the disk as evidenced by the larger zone of inhibition indicating that it may release faster than the linked counterpart in physiological environments. Unmodified disks and both ampicillin modified disks did not produce any zones of inhibition. This was expected as their inhibition of bacteria growth in culture was not as significant as vancomycin.

*S. aureus* strains often become antibiotic resistant to penicillin-type antibiotics. To determine if ampicillin’s inactivity was due to resistant bacteria, the ampicillin modified surfaces were tested for antibacterial activity using the gram negative strain *E. coli*. It was shown that the ampicillin modified surfaces did not inhibit bacterial growth in culture or in the zone inhibition assay, indicating that the ampicillin modified surfaces are ineffective at inhibiting all bacterial growth.

Covalent linkage could present a more favorable method of attachment when compared to adsorption for biomedical applications. The linkage of the antibiotic may ensure a more local delivery of the antibiotic in physiological environments compared to adsorption where the antibiotic could be more easily released into the surrounding tissue.
or into the bloodstream. Covalent linkage should also allow for the antibiotic to remain at the site of implantation for a longer period of time than adsorption. ESI-TOF mass spectrometry indicated that samples in which the antibiotic was adsorbed released more vancomycin at 1 and 24 hours than the samples in which the antibiotic was linked. After 3.5 hours, the amount of vancomycin that was released decreased indicating equilibrium between the vancomycin on the sample and in the water. This would not occur in a physiological environment, as the antibiotic that was released would be removed from the site of implantation through blood flow and would not be in equilibrium on the surface. Therefore, covalent linkage of the antibiotic would be more beneficial in physiological environments because the activity of the antibiotic did not vary with attachment method.

Cell adhesion tests indicate that some of the antibiotic modifications increase live cells numbers on the CA surface compared to the control but none of the modifications increased the total number of cells compared to the control. Also, none of the modifications consistently increase cell adhesion at the different time points. This indicates that although overall cell adhesion is not increased by the modifications, they increase the viability of the cells on the CA surface and the modifications are therefore non-toxic.

Vancomycin was previously attached to a titanium alloy and was shown to be still active in preventing bacterial growth and adhesion to the alloy.\textsuperscript{1, 14, 25, 26} The previously reported method involved passivation of the surface with sulfuric acid, multiple wash steps in dimethylformamide and three coupling steps.\textsuperscript{1, 14, 25, 26} The method presented here is simple and mild. It involves three-steps that can all be done at low temperatures with common solvents and does not require passivation or multiple wash steps. This
method is also applicable to any surface that contains hydroxyl or \( \mu \)-oxo groups which would include the metal-oxide surfaces of common alloys such as SS316L and Ti6Al4V. Additionally, any antibiotic or molecule with an NH\(_2\) functional group that is not involved in its function could be attached through this linker system.

4.5 Conclusions

Porous calcium aluminate scaffolds were cast at room temperature and ampicillin and vancomycin were either adsorbed or covalently linked to the calcium aluminate surface using a multi-step solution deposition method. The covalent linkage system is gentle and applicable to any surface that contains reactive hydroxyl or \( \mu \)-oxo groups. Additionally, it can be used with any biomolecule containing an amine group. This method is also more beneficial than the other current method to covalently attach vancomycin to titanium because of its mild nature.\(^1\)\(^,\)\(^{14}\)\(^,\)\(^{25}\)\(^,\)\(^{26}\) Unmodified calcium aluminate disks and disks with ampicillin linked were able to inhibit *S. aureus* bacterial growth in culture, however, vancomycin either linked or adsorbed to the surface was more effective at inhibiting bacterial growth. It was determined that covalently linking the antibiotic to the surface allowed for a smaller more controlled release and would be more beneficial than adsorption in a physiological environment. The antibiotic modifications are also non-toxic to osteoblast cells and can increase viability of cells on the CA surface.
4.6 References


Chapter 5: Immobilization of Two Bioactive Molecules

5.1 Introduction

Current biomaterials are unable to meet the vast demands that a physiological environment places on them. For that reason, many different materials are modified with growth factors, antibiotics, and other biomolecules or functionalities to increase the biocompatibility and bioactivity of the materials. These modifications can add beneficial properties to the biomaterials, but usually strive to meet one physiological demand that is placed on the material and are limited in the amount or type of biomolecules that can be attached to the surface.

For example, antibacterial coatings are added to decrease biofilm formation on the surface of implanted biomaterials.\textsuperscript{1-4} VEGF is attached to increase vascularization around or into the implant surface and BMP-2 is used to increase bone formation. Surface modifications using cell adhesion peptides are utilized to increase initial cell adhesion.\textsuperscript{5-9} Most of these studies used adsorption of the biomolecules to the surface and not covalent attachment.\textsuperscript{9-14} This choice may be due to the lack of available functional groups on the biomaterial surface for covalent attachment or to the loss of activity of biomolecules that can occur if the site of covalent attachment is improperly chosen.

Most surface modification methods are also only able to attach one specific molecule or one class of molecule, unless patterning of the surface is involved.\textsuperscript{1-4, 11, 15-20} Patterning requires multiple complex steps and only allows for different molecules to be immobilized and segregated on the surface.\textsuperscript{21} Some research groups have been able to attach two different growth factors, peptides or proteins\textsuperscript{5, 7, 8}, however the molecules are attached in the exact same manner, or through patterning or gradients which can be
difficult to create.\textsuperscript{8,20} Many immobilization methods require harsh conditions that also modify the bulk properties of the material or can leave small amounts of toxic chemicals after the biomolecule has been attached. The methods can also take multiple repeats of each step which can result in lengthy modification times.

The goal of this project was to develop an attachment scheme to immobilize both an antibiotic (vancomycin) and a cell adhesion peptide (KRSR) that would allow for a calcium aluminate surface that both increases osteoblast attachment and decreases bacterial growth. The optimal scheme would have a minimal amount of mild steps involved, utilize different functional groups to selectively attach the two different biomolecules and allow the biomolecules to retain their activity.

5.2 Materials and Methods

5.2.1 Materials

Calcium aluminate aggregates were supplied by Westmoreland Advanced Materials. KRSRC was purchased from Genscript (96.4\% purity) as lyophilized powder and reconstituted in doubly distilled H\textsubscript{2}O (ddH\textsubscript{2}O). Tetrahydrofuran (THF, 99.9\% purity), methanol (MeOH, Certified ACS, >99.8\% pure), Molecular Biology Grade Water (Hyclone), and Difco Tryptic Soy Broth were purchased from Thermo Fisher. THF was distilled over sodium and benzophenone before use. Vancomycin hydrogen chloride from \textit{Streptomyces orientalis}, 1,12-dodecanedicarboxylic acid, \textit{N}-hydroxysuccinimide, 12-aminododecanoic acid and Mueller-Hinton 2 agar were purchased from Sigma-Aldrich. \textit{N’N’}-dicyclohexylcarbodiimide was purchased from Fluka. \textit{S. aureus} strain UAMS1 was provided by Allegheny General Singer Center for Genomic Sciences.
Normal Human Osteoblasts (NHOsts, CC-2538), osteoblast culturing reagents, and OGM media were purchased from LONZA. All materials were used as received unless otherwise noted. 3-maleimidopropionic acid N-hydroxysuccinimide (NHS) ester (99%) was purchased from Alfa Aesar.

5.2.2 Casting Calcium Aluminate

Calcium aluminate disks were prepared by a room temperature (20 °C) cast of different sized calcium aluminate aggregates with phases CaAl$_2$O$_4$ and CaAl$_4$O$_7$. The aggregates were separated by size during a sifting process in which the cement was passed through wire screens of different size meshes and “caught” on another mesh size. Three different aggregate sizes (-30 + 60 (25%), -60 (25%), and -325 (50%)) were mixed together to form the disks in the casting process. The aggregates were dry mixed at room temperature to ensure complete particle distribution. After dry mixing, doubly-distilled H$_2$O was mixed in and the cement paste was allowed to sit for 20 minutes at room temperature to thicken. Upon thickening, the cement was poured into a mold of 8 mm diameter disks and allowed to sit overnight. The phases of the final calcium aluminate disks used in the following experiments present in the disks after casting and sterilization by autoclaving are: CaAl$_2$O$_4$, CaAl$_4$O$_7$, and Ca$_3$(Al(OH)$_6$)$_2$.

5.2.3 Double Covalent Attachment of Vancomycin and KRSR (VLKL)

Both vancomycin and KRSR were covalently attached to the calcium aluminate surface through a multi-step solution deposition method. First, the calcium aluminate disks were immersed in a solution that was 1mM 12-aminododecanoic acid and 1mM
1,12-dodecanedicarboxylic acid (THF) for 1 hour at room temperature. Excess solvent was removed by placement in a 120°C oven for 24 hours. The modified disks were placed in a 1mM 3-maleimidopropionic acid NHS ester in acetonitrile solution for 24 hours. The solvent was removed by vacuum (0.1 Torr) for 24 hours. The substrates were then placed in a solution that was 2mM N’N’-dicyclohexylcarbodiimide (DCC) and 2mM N-hydroxysuccinimide (NHS) in dry THF for 24 hours. The solvent was removed by vacuum (0.1 Torr) for 24 hours. The disks were dipped in a 1 mg/mL solution of vancomycin in methanol (MeOH) at 30 °C for 24 hours, and dried under vacuum for 24 hours. Finally the samples were dipped in a 1 mg/mL solution of KRSR(C) in ddH₂O at 4°C for 24 hours, and dried under vacuum for 24 hours. Scheme 5-1 shows a schematic representation of the VLKL attachment method. Samples were sterilized by autoclaving at 121 °C and 18 psi of steam for 60 min with fast exhaust prior to antibiotic and cell adhesion testing.

Scheme 5-1: Schematic representation of the five-step covalent VLKL attachment of both vancomycin and KRSR. 1. 3-maleimidopropionic acid NHS ester (ACN) 2. DCC/NHS (THF) 3. Vancomycin (MeOH) 4. KRSR(C) (ddH₂O).
5.2.4 Double Covalent Attachment of KRSR and Vancomycin (KLVL)

A second method for covalently attaching both KRSR and vancomycin to the surface was utilized to investigate any loss of activity due to the order of biomolecule attachment. First, the calcium aluminate disks were immersed in a solution that was 1mM 12-aminododecanoic acid and 1mM 1,12-dodecanedicarboxylic acid (THF) for 1 hour at room temperature. Excess solvent was removed by placement in a 120º C oven for 24 hours. The modified disks were placed in 1mM 3-maleimidopropionic acid NHS ester in acetonitrile solution for 24 hours. The solvent was removed by vacuum (0.1 Torr) for 24 hours. The substrates were placed in a solution that was 2mM N,N'-dicyclohexylcarbodiimide (DCC) and 2mM N-hydroxysuccinimide (NHS) in dry THF for 24 hours. The solvent was removed by vacuum (0.1 Torr) for 24 hours. The samples were then dipped in a 1 mg/mL solution of KRSR(C) in ddH₂O at 4ºC for 24 hours, and dried under vacuum for 24 hours. Finally, the disks were dipped in a 1 mg/mL solution of vancomycin in methanol (MeOH) at 30 ºC for 24 hours, and dried under vacuum for 24 hours. Scheme 5-2 shows a schematic representation of the KLVL attachment method. Samples were sterilized by autoclaving at 121 ºC and 18 psi of steam for 60 min with fast exhaust prior to antibiotic and cell adhesion testing.
Scheme 5-2: Schematic representation of the five-step covalent attachment of both vancomycin and KRSR. 1. 3-maleimidopropionic acid NHS ester (ACN) 2. DCC/NHS (THF) 3. KRSR(C)(ddH2O) 4. Vancomycin (MeOH).

5.2.5 Covalent Attachment of KRSR and Adsorption of Vancomycin (KLVA)

A third method of attachment in which the peptide KRSR was covalently attached and the antibiotic vancomycin was adsorbed was used to ensure that the addition of the antibiotic to the peptide modified surface did not cause loss of activity of the peptide. First, the substrates were placed in a 1mM 12-aminododecanoic acid in dry tetrahydrofuran (THF) solution at room temperature for one hour. The excess solvent was removed by placement in a 120 °C oven for 24 hours. The 12-aminododecanoic acid modified CA disks were placed in a 1mM 3-maleimidopropionic acid NHS ester in acetonitrile solution for 24 hours. The solvent was removed by vacuum (0.1 Torr) for 24 hours. The substrates were dipped in a 1 mg/mL solution of KRSR(C) in ddH2O at 4 °C for 24 hours and dried under vacuum for 24 hours. Finally, the disks were dipped in a 1 mg/mL solution vancomycin (MeOH) at room temperature for 24 hours, and dried under vacuum for 24 hours. Scheme 5-3 shows a schematic representation of the KLVA
double modification method. Samples were sterilized by autoclaving at 121 °C and 18 psi of steam for 60 min with fast exhaust prior to antibiotic and cell adhesion testing.

**Scheme 5-3**: Schematic representation of the four-step covalent attachment of KRSR followed by vancomycin adsorbed to the surface in the 4th step.

### 5.2.6 Diffuse Reflectance Infrared Fourier Transform Spectroscopy

A Thermo Nicolet Nexus 470 FT-IR Spectrophotometer equipped with a diffuse reflectance attachment was used to obtain the spectra of the substrates after each deposition step. Spectra were collected with 256 scans and a resolution of 4 cm⁻¹.

### 5.2.7 *Staphylococcus aureus* Bacterial Turbidity Tests

A standard optical density analysis of bacterial growth was used to determine the antibiotic activity of vancomycin attached on the calcium aluminate surface against gram positive bacteria. *S. aureus* (UAMS1) was diluted to 0.1 optical density at 600 nm (OD⁶₀₀nm) in Tryptic soy broth with 0.1 M NaCl and 0.08 % glucose. Two milliliters of the bacterial suspension was placed in with each type of sample. There were five samples of each type in each trial for three separate trials. Samples were incubated at 37 °C and 5 % CO₂ for 3.5 hours. The disks were then removed from the bacterial solution and the optical density at 600 nm was recorded. Statistical analysis of the average optical density at 600 nm was done using Origin 8.0 using a one-way ANOVA with a Bonferroni post
hoc test (p<0.05 and p<0.001). The sample groups were: no disk (B, bacteria only), VLKL, KLVL and KLVA. A set of control vancomycin 30µg disks (VS) was also tested to compare to the vancomycin modified samples.

5.2.8 *Staphylococcus aureus* Zone Inhibition Assay

A standard zone inhibition assay was used to determine the activity of the antibiotic attached to the calcium aluminate surface compared to the standard antibiotic. *S. aureus* (UAMS1) was diluted to 0.1 OD₆₀₀nm in Tryptic soy broth with 0.1 M NaCl and 0.08 % glucose and spread onto Mueller-Hinton 2 Agar plates with cotton swabs. The plate set up for the vancomycin assay was: a standard disk of 30 µg of vancomycin (VS) was placed in the upper left quadrant of the plate, and then one each of the following 3 samples types VLKL, KLVL, KLVA was placed in the remaining quadrants. Plates were incubated agar side down (to ensure samples did not displace) for 24 hours at 37 ºC and 5 % CO₂. The diameter of the zone with the absence of bacterial growth was then measured in millimeters. Statistical analysis was done using a one-way ANOVA with Bonferroni post hoc test (p<0.05) to determine any differences in the diameters of the zones of inhibition.

5.2.9 Osteoblast Adhesion to Double Modified Calcium Aluminate

Normal human osteoblasts isolated from a 6 and 11 year old female and purchased from LONZA (NHOsts) were cultured until confluent and diluted to a concentration of 10,000 cells per one milliliter of media (OGM media, LONZA). Three of each type of substrate per time point was placed in a 48-well plate and 1mL of cells
was added to each well. After 1, 4, and 7 days, the number of cells was determined using the Live/Dead/Viability/Cytotoxicity Assay Kit from Invitrogen by counting the number of live (green) versus dead (red) cells. Five spots on each sample with an area of 0.6 mm² were imaged under 10x magnification using the Axioskop2. Three separate trials were completed with a total of 9 samples of each type with a total of 45 views for each sample type at each time point. The data was then normalized to the number of live or total cells on the unmodified CA at each time point and statistical analysis was done using Origin 8.0 using a one-way ANOVA with a Bonferroni post hoc test (p<0.05).

5.3 Results

5.3.1 DRIFT Spectroscopy of KRSR and Vancomycin Attachment

To increase cell adhesion and the antibacterial properties of the calcium aluminate surface simultaneously, the cell adhesion peptide KRSR and the antibiotic vancomycin were covalently attached to the CA surface. To immobilize the biomolecules on the CA surface, samples VLKL and KLVL were immersed in a 1mM 12-aminododecanoic acid/1mM 1,12-dodecanedicarboxylic acid solution (THF) for one hour at room temperature. Samples were removed from solution and placed in a 120°C oven overnight. The samples were analyzed using Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT) and the presence of the CH₂ asymmetric and CH₂ symmetric stretches at 2922 and 2851 cm⁻¹, respectively, in the spectrum indicated that the acids had been attached to the surface (Figure 5-1A). For KLVA, these peaks were also indicative of the 12-aminododecanoic acid being present on the surface. Next, all three types of samples (KLVA, VLKL, and KLVL) were placed in a 1mM solution of 3-
maleimidopropionic acid N-hydroxysuccinimide ester for 24 hours at room temperature. The ester bond of the 3-maleimidopropionic acid N-hydroxysuccinimide was broken and an amide bond was formed between the 12-aminododecanoic acid on the surface and the 3-maleimidopropionic acid while the COOH tail group of the 1,12-dodecanedicarboxylic acid remains unreacted in the VLKL and KLVL samples.

Figure 5-1: DRIFT spectra for each step of peptide attachment. A) CH stretch region showing the CH$_2$ symmetric and asymmetric stretches from the carbon chains of the organic acids to the CA surface. B) The peak at 1711 cm$^{-1}$ from the C=O stretches of the imide ring of 3-maleimidopropionic acid NHS ester. C) Peak changes from 1800 to 1000 cm$^{-1}$ are assigned to the carbonyl bond stretches present on the succinimide ring. D) The appearance of a peak at 1230 cm$^{-1}$ in the vancomycin attachment is from the amine groups present in the vancomycin structure. E) The peaks at 760 and 714 cm$^{-1}$ due to the formation of the C-S bond from the Michael addition of the cysteine residue to the imide ring.

The samples were removed from solution and placed on a vacuum line (0.1 Torr) to remove any excess solvent. DRIFT was used to analyze the samples and the peaks in the spectrum at 1792 and 1714 cm$^{-1}$ were assigned to the carbonyl bond stretches present
on the imide ring and indicated that it had been successfully attached to the carbon chain (Figure 5-1B). The KLVL and VLKL samples were placed in a 2mM \( N'N' \)-dicyclohexylcarbodiimide (DCC)/ 2mM \( N \)-hydroxysuccinimide (NHS) solution in dry THF for 24 hours at room temperature. The samples were removed from solution and placed on a vacuum line (0.1 Torr) to remove any excess solvent. The DCC reacted first with the COOH tail group and was displaced by the NHS. The DRIFT results for this step of the attachment (red) showed a change from the previous modification step’s spectrum (in blue) from 1800 to 1000 cm\(^{-1}\) due to the changes in the carbonyl stretching region of the spectrum that are a result of the carbonyl groups present on the succinimide ring indicating it had been successfully attached (Figure 5-1C).

For VLKL, vancomycin was attached in the fourth step by immersing the samples in a 1 mg/ mL solution the antibiotic for 24 hours at 4°C. The NHS was displaced and an amine group on the antibiotic reacted with \( O \) forming an amide bond. The samples were removed from solution and placed on the vacuum line overnight. Vancomycin immobilization was confirmed by the appearance of a peak at 1230 cm\(^{-1}\) which was attributed to the amine groups present in the vancomycin structure (Figure 5-1D). KRSR(C) was immobilized in the fifth step by immersing the samples in a 1mg/mL solution of the peptide for 24 hours at 4°C where the cysteine terminated peptide went through a Michael addition to the imide ring. The samples were removed from solution and placed on the vacuum line overnight. The appearance of peaks at 760 and 714 cm\(^{-1}\) were due to the C-S bond stretch indicating that the Michael addition had occurred through the cysteine residue and the peptide had been attached to the surface (Figure 5-1E).
For the KLVL samples, KRSR(C) was attached in the fourth step by immersing the samples in a 1 mg/mL solution of KRSR(C) for 24 hours at 4 °C where the cysteine terminated peptide went through a Michael addition to the imide ring. The samples were removed from solution and placed on the vacuum line overnight. The appearance of peaks at 760 and 714 cm⁻¹ were due to the C-S bond stretch from which indicated that the Michael addition had occurred through the cysteine residue and the peptide had been attached to the surface (Figure 5-1E). Vancomycin was attached in the fifth step by immersing the samples in a in a 1 mg/ mL solution the antibiotic for 24 hours at 4 °C. The NHS was displaced and an amine group on the antibiotic reacted with O’forming an amide bond. The samples were removed from solution and placed on the vacuum line overnight. Vancomycin immobilization was confirmed by the appearance of a peak at 1230 cm⁻¹ which was attributed to the amine groups present in the vancomycin structure (Figure 5-1D).

For KLVA, the KRSR(C) was attached in the third step by placing the samples in a 1 mg/mL solution of the peptide in ddH₂O for 24 hours at 4 °C. The excess solvent was removed by vacuum for 24 hours. The peaks due to the C-S bond stretch are used to determine the KRSR(C) peptide has been successfully immobilized (Figure 5-1E). The samples are then immersed in a 1mg/mL solution of vancomycin in methanol at room temperature for 24 hours and the excess solvent is removed by placement on a vacuum line at 0.1 Torr. The peak at 1230 cm⁻¹ is used to determine the adsorption of vancomycin on the surface and is assigned to the amine groups present in the vancomycin structure (Figure 5-1D).
5.3.2 *Staphylococcus aureus* Bacterial Turbidity Test

Antibiotic activity in culture was determined by a standard optical density test for bacteria. *S. aureus* (UAMS1) was diluted to an optical density of 0.1 (at 600 nm). Two milliliters of the bacterial solution was placed in a 15mL centrifuge tube with each of the samples. Three trials with 5 samples each of a bacteria solution with no sample (B), KLVA, VLKL, and KLVL, and one trial of 5 samples of the 30µg standard vancomycin disk (VS) were incubated for 3.5 hours with a 0.1 OD$_{600nm}$ *S. aureus* (UAMS1) bacteria solution Tryptic soy broth with 0.1 M NaCl and 0.08 % glucose at 37°C.

![Figure 5-2: Bacteria Turbidity Trials with *S. Aureus*. At p<0.05, * is different from B. At p<0.001 $ is different from B and VLKL; ^ is different than B, VS, and VLKL. In this case “different from” indicates it has a statistically lower optical density or less bacteria in solution. (Represented as mean ± standard error).](image)

After 3.5 hours, the sample disks were removed and the optical densities were (mean ± standard deviation): VS = 0.269 ± 0.006; B = 0.459 ± 0.032; KLVA = 0.186 ±
0.009; VLKL = 0.430 ± 0.037; KLVL = 0.186 ± 0.012. One-way ANOVA with Bonferroni post hoc test was used to determine statistical significance (Figure 5-2). At p < 0.05, VLKL decreased bacteria growth compared to B. At p < 0.001 VS decreased bacteria growth compared to B and VLKL; and KLVL and KLVA significantly decreased bacteria growth compared to B, VS, and VLKL. There was no statistical difference between KLVA and KLVL.

5.3.3 *Staphylococcus aureus* Zone Inhibition Assays

To determine the effectiveness of the antibiotic on the CA surface when compared to a control dosage (30 µg of vancomycin) of the antibiotic, a zone inhibition assay was used. *S. aureus* (UAMS1) was diluted to 0.1 OD$_{600nm}$ in Tryptic soy broth with 0.1 M NaCl and 0.08 % glucose and spread onto Mueller-Hinton 2 Agar plates with cotton swabs. Five plates for each antibiotic were used for each trial with a total of four trials for VS, KLVA and VLKL and three trials of KLVL. Each plate contained one of each type of disk. Plates were incubated agar side down (to ensure samples did not displace) for 24 hours at 37 °C and 5 % CO2. The diameter of the zone with the absence of bacterial growth was then measured in millimeters.
Figure 5-3: Zone Inhibition Assay. * At p<0.05, VLKL had a statistically smaller zone of inhibition than all other samples. At p<0.05 KLVA and KLVL were as effective at killing bacteria as the vancomycin standard (VS). (Represented as mean ± standard error).

The zones of inhibition were (mean ± standard deviation): VS = 18.4 ± 1.0 mm; KLVA = 17.9 ± 1.60 mm; VLKL = 14.4 ± 2.50; and KLVL = 18 ± 3.1 mm. One-way ANOVA with Bonferroni post hoc test was used to determine statistical significance (Figure 5-3). At p < 0.05 VLKL had a statistically significantly smaller zone of inhibition compared to all other samples, however KLVA and KLVL were as effective at inhibiting bacteria growth as the standard VS disk.

5.3.4 Osteoblast Adhesion to Double Modified Calcium Aluminate

To determine if any of the modifications increased the number of osteoblasts attached to the CA surface when compared to the control, Normal Human Osteoblasts
(LONZA) were cultured until confluent and diluted to a concentration of 10,000 cells per milliliter of media (OGM, LONZA). Three autoclaved substrates of each type for each time period were placed in a 48-well plate and 1mL of cells was added to each well. After 1, 4, and 7 days, the number of the cells was determined using the Live/Dead/Viability/Cytotoxicity Assay Kit from Invitrogen. Samples were viewed using a Zeiss Axioskop 2 under 10x magnification and five images per sample were taken using the Zeiss Axiocam. The number of live and dead cells per view was counted using the Axiovision 4 Software. Three separate trials were completed following the same procedure. The data at each time point was then normalized to the average number of live or total osteoblasts on the unmodified CA and analyzed using one-way ANOVA with a Bonferroni post hoc test (p < 0.05) to determine if there were any statistical differences between the materials.

On Day 1 the normalized average number of live cells per view (+/- standard deviation) was: control CA: 100; KLVA: 196.1 ± 76.3; VLKL: 253.3 ± 128.1; KLVL: 321.1 ± 132.9 (Figure 5-4) and the average of total cells per view was: control CA: 100; KLVA: 128.9 ± 48.9; VLKL: 159.0 ± 79.6; KLVL: 200.3 ± 77.4 (Figure 5-5). Statistical analysis indicated that KLVA had a higher number of live cells than the control; VLKL a higher number of live cells than the control and KLVA and more total cells than the control; and KLVL had more live cells and total cells than all other samples.
Figure 5-4: Live Day 1 Osteoblast Attachment. One-way ANOVA with Bonferroni post hoc test at p<0.05, * is different from C; $ is different from KLVA and C; # is different from all other samples. Data represented as mean ± standard error. C = unmodified CA; KLVA = KRSR(C) attached first then vancomycin adsorbed; VLKL = attachment with vancomycin attached first followed by KRSR(C); KLVL = attachment with KRSR(C) attached first followed by vancomycin.

Figure 5-5: Total Day 1 Osteoblast Attachment. One-way ANOVA with Bonferroni post hoc test at p<0.05, * is different from C; $ is different from KLVA and C; # is different from all other samples. Data represented as mean ± standard error. C = unmodified CA; KLVA = KRSR(C) attached first then vancomycin adsorbed; VLKL = attachment with vancomycin attached first followed by KRSR(C); KLVL = attachment with KRSR(C) attached first followed by vancomycin.
Figure 5-6: Live Day 4 Osteoblast Attachment. One-way ANOVA with Bonferroni post hoc test at p<0.05, * is different from C; $ is different from KLVA and C; # is different from all other samples; ^ is different from KLVA. Data represented as mean ± standard error. C = unmodified CA; KLVA = KRSR(C) attached first then vancomycin adsorbed; VLKL = attachment with vancomycin attached first followed by KRSR(C); KLVL = attachment with KRSR(C) attached first followed by vancomycin.

On Day 4 the average number of *live* cells per view (+/- standard deviation) was: control CA: 100; KLVA: 99.6 ± 65.3; VLKL: 139.1 ± 68.0; KLVL: 189.8 ± 83.6 (Figure 5-6) and the average of *total* cells per view was: control CA: 100; KLVA: 74.2 ± 47.0; VLKL: 103.1 ± 48.4; KLVL: 127.2 ± 54.8 (Figure 5-7).
Figure 5-7: Total Day 4 Osteoblast Attachment. One-way ANOVA with Bonferroni post hoc test at p<0.05, * is different from C; $ is different from KLVA and C; # is different from all other samples; ^ is different from KLVA. Data represented as mean ± standard error. C = unmodified CA; KLVA = KRSR(C) attached first then vancomycin adsorbed; VLKL = attachment with vancomycin attached first followed by KRSR(C); KLVL = attachment with KRSR(C) attached first followed by vancomycin.

Statistical analysis indicated that KLVA had significantly less live cells than all other samples; VLKL had a higher number of live cells than the control and KLVA and more total cells than KLVA; and KLVL had a higher number of live cell than all other samples and a higher number of total cells than KLVA and the control.
Figure 5-8: Live Day 7 Osteoblast Attachment. One-way ANOVA with Bonferroni post hoc test at p<0.05, * is different from C; $ is different from KLVA and C; # is different from all other samples; ^ is different from KLVA. Data represented as mean ± standard error. C = unmodified CA; KLVA = KRSR(C) attached first then vancomycin adsorbed; VLKL = attachment with vancomycin attached first followed by KRSR(C); KLVL = attachment with KRSR(C) attached first followed by vancomycin.

On Day 7 the average number of live cells per view (+/- standard deviation) was: control CA: 100; KLVA: 78.7 ± 52.5; VLKL: 147.6 ± 149.0; KLVL: 245.3 ± 123.9 (Figure 5-8) and the average of total cells per view was: control CA: 100; KLVA: 64.2 ± 35.8; VLKL: 96.5 ± 93.1; KLVL: 146.7 ± 67.6 (Figure 5-9). Statistical analysis indicated that KLVA had significantly less total cells than the control; VLKL had significantly more live cells than KLVA; and KLVL had significantly more live and total cells than all other samples.
Figure 5-9: Total Day 7 Osteoblast Attachment. One-way ANOVA with Bonferroni post hoc test at p<0.05, * is different from C; $ is different from KLVA and C; # is different from all other samples; ^ is different from KLVA. Data represented as mean ± standard error. C = unmodified CA; KLVA = KRSR(C) attached first then vancomycin adsorbed; VLKL = attachment with vancomycin attached first followed by KRSR(C); KLVL = attachment with KRSR(C) attached first followed by vancomycin.

5.4 Discussion

To increase the osteoblast adhesion on the calcium aluminate surface while simultaneously decreasing the bacterial attachment, both KRSR and vancomycin were immobilized on the surface. Three different attachment schemes were utilized to ensure that both biomolecules remained active and to determine the best order of attachment for optimal osteoblast adhesion and antibacterial activity.

The first attachment scheme used was the VLKL method. This scheme was designed to have the fewest possible deposition steps, and to attach the peptide in the final step in case the vancomycin attachment conditions were too harsh for the peptide and the activity of the peptide was lost. The KLVL method was then developed to evaluate the activity of both biomolecules when the peptide was attached first and the
final step of attachment was the addition of vancomycin. This scheme was designed to also have the minimal amount of deposition steps, but the reverse of the last two steps of the VLKL method was to evaluate the effect immobilization order had on the activity of the biomolecules. The final attachment method, KLVA, was designed as a comparison method to further evaluate the effectiveness of adsorption versus covalent attachment for the antibiotic as well as a comparison for the success of the first two schemes in attaching both biomolecules in comparable amounts. DRIFT analysis after each step of immobilization confirmed that both biomolecules were successfully attached utilizing all three attachment methods.

Bacterial turbidity tests with *S. aureus* indicate that VLKL is successful at inhibiting bacterial growth in culture (p < 0.05), however it is not as effective as the standard dosage, VS, (p < 0.001). Both KLVL and KLVA were more effective than the standard dosage and VLKL at inhibiting bacterial growth in culture. Similar results were seen in the zone inhibition assay in which VLKL had a statistically significantly smaller zone of inhibition than VS, KLVL and KLVA, and no statistical significance was seen between the other 3 samples. This indicates that VLKL is the least effective in inhibiting bacterial growth in culture. The decrease in activity of VLKL over the other samples is most likely due to the interaction of the peptide with the antibiotic during deposition. Vancomycin interacts with D-ala-D-ala termini of the growing bacterial peptidoglycan cell wall by hydrogen bonding to oxygen and hydrogens mainly from the backbone of the peptide. Results from the single modification of CA with vancomycin indicate that the antibiotic is linked to the surface with its active site that interacts with the D-ala-D-ala substrate oriented away from the surface, open at the interface. Therefore, when the
KRSR(C) peptide is immobilized after the vancomycin has been immobilized to the surface, the peptide may be non-specifically interacting and hydrogen bonding to the active site of vancomycin inhibiting its ability to interact with the natural substrate thus decreasing its antibacterial activity relative to the other two sample types.

Osteoblast adhesion studies showed that on Day 1, all three modification schemes were successful in increasing live cell adhesion compared to the unmodified CA surface. However, KLVL significantly increased live and total cell adhesion compared to all other sample types. On Day 4, KLVL increased live cell adhesion compared to all other samples types and increased total cell adhesion compared to unmodified CA and KLVA. On Day 7, KLVL again increased both live and total cell adhesion compared to all sample types. The osteoblast adhesion results indicate that the KLVL attachment method is the most successful at increasing osteoblast adhesion on the CA surface. The VLKL and KLVA methods are also able to increase cell adhesion at some time points; however they are not as successful as the KLVL method. The interaction between vancomycin and KRSR(C) that decreased the antibacterial activity of the vancomycin in the bacterial tests is also the cause of the decreased activity of KRSR(C) to increase cell adhesion in the VLKL and KLVA samples. The hydrogen bonding interaction between vancomycin and KRSR(C) would inhibit the ability of the peptide to properly interact with the osteoblast proteoglycans.

The results from the bacterial and osteoblast tests indicate that all three sample types could be viable options for decreasing bacterial adhesion. However, the optimal attachment method for both increasing osteoblast adhesion and decreasing bacterial adhesion is the KLVL method in which KRSR(C) is immobilized in the 4th step and the
vancomycin is immobilized in the 5th step. This attachment method resulted in modified calcium aluminate samples that optimally increased osteoblast attachment and decreased bacterial growth.

5.5 Conclusions

Porous calcium aluminate scaffolds were cast at room temperature and three different schemes were successfully used to attach both vancomycin and KRSR to the calcium aluminate surface using multi-step solution deposition methods. DRIFT was used to confirm all steps were successful in each of the 3 attachment methods. KLVA and KLVL were more effective than the standard dosage of the antibiotic reducing S. aureus growth in culture. In the zone inhibition tests, KLVA and KLVL produced zones of inhibition comparable to the standard dosage; however VLKL produce a significantly smaller zone. Osteoblast cell adhesion tests indicated that although all three sample types were able to increase live and total cell adhesion on CA at least one of the time points, KLVL was able to increase live and total cell adhesion compared to all sample types on Days 1 and 7 and compared to most sample types on Day 4. It was determined that the optimal attachment method to increase cell adhesion and decrease bacterial growth was KLVL.
5.6 References


15. Miljkovic, N. D.; Cooper, G. M.; Hott, S. L.; Disalle, B. F.; Gawalt, E. S.; Smith, D. M.; McGowan, K.; Marra, K. G., Calcium aluminate, RGD-modified calcium aluminate,


Chapter 6: Conclusions

6.1 Physical Optimization of Calcium Aluminate

The physical properties of the calcium aluminate material were modified and optimized for use as a bone scaffold material through the casting procedure. Five different mixtures (A-E) were created by varying the ratio of the starting material aggregates and then subjecting the mixtures to one of four different heat treatments. The average pore sizes of the material were determined by optical microscopy and ranged from approximately 100 to 290 microns. The heat treatments did not affect the average pore size of the material. The two mixtures with the largest pore sizes were eliminated for further analysis due to their lack of mechanical strength.

The three remaining Mixtures A, B, and C that had been subjected to the four heat treatments were then analyzed using a four point bend (modulus of rupture) and a cold crushing test for their mechanical strength. It was found that the smaller the average pore size of the mixture was, the greater the modulus of rupture and cold crushing strength of the material were. It was also determined that dry heat treatments at 650 °C decrease the strength of the material whereas autoclaving (a wet heat treatment) increased the modulus of rupture of the material.

Mixtures A-C were evaluated for their biological properties. The degradation in phosphate buffered saline was evaluated and all mixtures were found to be stable for up to 6 months. They were also evaluated for cell adhesion on Days 1, 4, and 7 and it was found that on Day 4, Mixture A with an average pore size of approximately 100 µm increased cell adhesion compared to Mixtures B and C.
After reviewing the results of the various physical and biological tests, Mixture A was chosen over Mixtures B and C for the remaining studies due to the increased strength of the material as well as the increased cell adhesion on Day 4. This project determined that the optimal pore size of the material was approximately 100 µm and that autoclaving was an acceptable sterilization method for the calcium aluminate material due to its impact on increasing the strength of the material.

### 6.2 Immobilization and Effect of Cell Adhesion Peptides

The surface of Mixture A was successfully covalently modified with cell adhesion peptides through a three-step solution deposition method. Diffuse Reflectance Infrared Fourier Transform Spectroscopy confirmed cell adhesion peptides RGD and KRSR with added terminal cysteine residues were attached to the CA surface. The peptide modified surfaces were then evaluated for their biological activity through cell adhesion tests, cell proliferation tests, and Scanning Electron Microscopy.

Fibroblast test results indicated that both RGD and KRSR increased live fibroblast adhesion on CA when compared to the control unmodified CA. Osteoblast tests showed that KRSR increased *live* osteoblast adhesion compared to all other samples and also increased *live* osteoblast adhesion compared to fibroblast adhesion on Days 1 and 4. KRSR modified CA increases *total* osteoblast adhesion compared to unmodified CA and compared to RGD modified CA on Days 1 and 4. These results show that KRSR would be more beneficial in bone regenerating applications than RGD because it is cell-specific to osteoblasts whereas RGD in a non-cell specific peptide.
Finally, the effect of KRSR on osteoblast morphology was evaluated using SEM. RGD does not allow for cell spreading unless combined with another cell adhesion peptide, PHSRN, so it was important to determine if the immobilization of the KRSR cell adhesion peptide alone could increase cell spreading on the CA surface. SEM images showed that the osteoblasts present on the unmodified CA surface were rounded whereas the osteoblasts that were present on the KRSR modified surface were spread. Cell spreading is necessary for cell proliferation and the addition of the KRSR peptide to the CA surface increased cell spreading which would increase cell proliferation that is necessary for tissue regeneration.

After reviewing the results of all of the cell adhesion peptide tests, it was concluded that immobilizing the KRSR cell adhesion peptide onto the CA surface preferentially increased osteoblast adhesion over fibroblast adhesion and increased osteoblast spreading. Therefore, KRSR was the most beneficial cell adhesion peptide for optimizing CA as a bone scaffold material.

6.3 Immobilization and Effect of Antibiotics

The surface of Mixture A was successfully covalently modified with antibiotics using a three-step solution deposition method. DRIFT spectroscopy confirmed that vancomycin and ampicillin were attached to the CA surface. Both antibiotics were also successfully adsorbed onto the CA surface in a one-step solution deposition method which was also confirmed by DRIFT spectroscopy. The activity of vancomycin which is effective against gram-positive bacteria was determined using *S. aureus*, a common gram-positive bacteria found in implant related infections. The activity of ampicillin,
which is effective against gram-positive and some gram-negative bacteria was
determined using *S. aureus* and *E.coli*, a gram-negative bacteria associated with some
implant infections and also other common infections. *S. aureus* bacterial turbidity results
indicated that unmodified CA and CA with adsorbed or covalently linked vancomycin
inhibited bacterial growth in culture. These results show that CA has some inherent
antibacterial activity that would be beneficial in an implant material and help reduce
infection without any type of added antibiotic therapy. The bacterial turbidity results
along with the zone inhibition results also showed that the vancomycin modified samples
were more effective than a standard dose of vancomycin at inhibiting bacterial growth.

Based on the antibacterial activity data, it was determined that CA modified with
vancomycin, either covalently or adsorbed onto the surface, was the most effective at
inhibiting bacterial growth. Linking or adsorbing the antibiotics on the surface of an
implant would decrease the need for prescribed systemic antibiotics after surgery. This is
extremely beneficial as it would decrease bacterial resistance to antibiotic because the
required dosages would be smaller.

Based on the mass spectrometry data, vancomycin that was covalently linked to
the CA surface released slower than the vancomycin that was adsorbed to the CA surface.
This is because adsorption is a weaker physical interaction that is susceptible to shear
flow and diffusion whereas the covalent linkage is much stronger and less susceptible to
these release methods. These results combined with the comparable antibacterial activity
of both samples in the *S. aureus* tests indicated that covalently linking the antibiotic
would be more beneficial in a physiological environment by releasing the antibiotic more
slowly and allowing it to be at the site of implantation for longer periods of time.
Covalently linking the antibiotic also prevents the vancomycin from diffusing to other tissues thus reducing bacterial resistance to antibiotics by reducing systemic delivery.

Finally to determine the biocompatibility of all modifications, all antibiotic modified samples and unmodified CA were incubated with osteoblasts for 1, 4 and 7 days. It was found no modifications decreased live cell attachment and therefore are non-toxic and acceptable for use in bone scaffold applications.

6.4 Immobilization of Two Bioactive Molecules

KRSR and vancomycin were the most effective surface modifications to the physically optimized CA to increase osteoblast adhesion and inhibit S. aureus bacterial growth. Three different schemes were develop to simultaneously immobilize both biomolecules onto the CA surface. Two of the schemes covalently attached both biomolecules and utilized the same first three steps for the immobilization process. Following the third step, the VLKL attachment method, then attached vancomycin in the fourth step, followed by KRSR in the fifth step. The KLVL attachment method attached KRSR in the fourth step followed by vancomycin in the fifth. The third and final double immobilization scheme, KLVA, linked KRSR to the surface following the protocol for immobilization of a single cell adhesion peptide, followed by adsorption of vancomycin onto the peptide modified surface. The three different schemes were utilized to investigate any loss in activity of either biomolecule that could be due to the order in which the two biomolecules were attached. DRIFT confirmed that the attachment of both biomolecules was successful under all three attachment schemes. After confirming that the three attachment methods were successful, the bioactivity of both biomolecules was
evaluated utilizing the experiments that had been completed to determine the activity of the single biomolecule modification.

Bacterial turbidity and zone inhibition tests showed that KLVL and KLVA were more effective than VLKL and the standard vancomycin dosage at inhibiting bacteria growth. Osteoblast tests results showed that KLVL increased live and total osteoblast adhesion more effectively than the other two double immobilization schemes. Based on these results, the activity of both biomolecules is affected by the order in which they are immobilized. In this case, if KRSR if immobilized after the vancomycin, it interacts with the active site of the antibiotic and inhibits its ability to reduce bacteria growth. A similar effect is seen when KRSR is individually linked to the surface followed by adsorption of vancomycin. The KLVL attachment scheme allows both biomolecules to be present at the CA surface in an effective and active form to increase osteoblast adhesion and decrease bacteria growth and is the optimal double immobilization scheme for the CA surface.

6.5 Impact and Future Directions

During the course of this project, the CA material was physically optimized for use as a bone scaffold material. The single immobilization of the antibiotic, vancomycin, and the cell adhesion peptide, KRSR, onto the CA surface are evidence that the material can be further improved by the easy covalent addition of useful and relevant biomolecules.

It is well known that cell adhesion peptides that can preferentially bind certain types of cells would be the most useful peptides in biomaterials applications to recruit specific cells and decrease unwanted cell adhesion. The increase over a seven day period
in osteoblast adhesion to KRSR modified CA compared to unmodified and RGD modified CA is the longest time period reported thus far. This is also true for the increase in osteoblast adhesion to KRSR modified CA compared to fibroblast adhesion over four days. These results are further evidence that KRSR combined with CA produces an osteoblast cell-specific surface that would be useful in bone regeneration.

The covalent immobilization of vancomycin to the CA surface that remains active after autoclave sterilization is significant progress in the search to reduce implant associated infections due to biofilm formation. The covalent linkage allows for smaller effective doses of antibiotic and for a local delivery, both of which are important factors when fighting bacterial infections.

The co-immobilization of vancomycin and KRSR shows that two biomolecules can be simultaneously attached to a surface using two different linker systems in a minimal amount of steps. The results also show that the order of attachment can make a significant difference in the activity of both biomolecules. The ability to immobilize multiple biomolecules with independent functions on a biomaterial surface has never been shown before and is extremely useful in creating biomaterials for specific tissue regenerating applications. In this case, the addition of the two biomolecules created a CA surface that can increase osteoblast adhesion and inhibit bacteria growth. Both of which are necessary for a successful bone regeneration scaffold.

All of the modification schemes used to modify the CA surface are also applicable to any surface that contains reactive hydroxyl and μ-oxo groups. Many existing metallic biomaterials contain these types of functional groups on their surfaces and these
modification schemes could easily be applied to improve upon the currently approved materials.

In the future, an *in vivo* critical size bone defect model is needed to evaluate the ability of the doubly modified CA surface to increase bone growth in a physiological environment. Similarly, an implant-associated infection *in vivo* model is necessary to determine the antibacterial activity of the modified scaffolds in a physiological environment. If possible, a combined critical size bone defect and implant associated infection model would be the most advantageous to fully evaluate the potential of the doubly modified CA surface.

Future studies to evaluate and improve the osteoinductive, osteointegrative, and osteogenic properties of the unmodified and modified CA should also be investigated to further optimize the material for use as a bone scaffold.