Characterization of Surface Modified Orthopedic Implants for Their Antimicrobial Properties

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CHARACTERIZATION OF SURFACE MODIFIED ORTHOPEDIC IMPLANTS
FOR THEIR ANTIMICROBIAL PROPERTIES

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Microbiology

by
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December 2017

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ABSTRACT

Contamination of combat trauma wounds with environmental residues can lead to bacterial infection of orthopedic fractures, which causes delay and difficulties in patient treatment. The reported infection rate of the improvised explosive devices (IED) injuries is 91%, and biofilm formation on orthopedic implants can lead to chronic infection with a rate of 40% in fracture wounds. Designing orthopedic implants that can self-regulate local infection and biofilm formation is beneficial for these patients. Polytetrafluoroethylene (PTFE) and biodegradable chitosan with local antibiotic (vancomycin) elution were deposited on the stainless steel and titanium implant samples (coupons) to reduce biofilm formation and bacterial infection. *Staphylococcus aureus* is the most common pathogen associated with orthopedic implant infections. *S. aureus* Seattle 1945 (ATCC 25923) strain was used to evaluate the antimicrobial and anti-biofilm properties of the modified metal coupons using methods such as crystal violet analysis, ultrasound water bath with viable cell counts and confocal laser scanning microscopy. The release rate of vancomycin from the coupons was determined by HPLC analysis of collected leachates from surface modified coupons. *In vitro* studies of antibacterial properties of the coupons showed that PTFE did not provide significant advantages against biofilm formation, but the incorporation of chitosan and vancomycin onto modified surfaces prevented biofilm formation on the coupons. Local drug-release profile of antibiotic doped chitosan showed the concentration of local vancomycin released within the first 48 hours was effective in preventing bacterial attachment onto the coupons.
DEDICATION

To all of those whom I love, and have encouraged me during my study and in my personal life.
ACKNOWLEDGMENT

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>1</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>3</td>
</tr>
<tr>
<td>BACKGROUND</td>
<td>3</td>
</tr>
<tr>
<td>2.1. Combat Trauma</td>
<td>3</td>
</tr>
<tr>
<td>2.2. Implants/DCP</td>
<td>3</td>
</tr>
<tr>
<td>2.3. Biofilm</td>
<td>5</td>
</tr>
<tr>
<td>2.4. Biofilm Formation Stages</td>
<td>5</td>
</tr>
<tr>
<td>2.5. Mechanisms of Biofilm Resistance</td>
<td>7</td>
</tr>
<tr>
<td>2.6. Factors Affecting Biofilm Formation</td>
<td>9</td>
</tr>
<tr>
<td>2.7. Gene Transfer in Biofilm</td>
<td>10</td>
</tr>
<tr>
<td>2.8. <em>Staphylococcus aureus</em></td>
<td>10</td>
</tr>
<tr>
<td>2.9. PTFE</td>
<td>13</td>
</tr>
<tr>
<td>2.10. Chitosan</td>
<td>14</td>
</tr>
<tr>
<td>2.11. Vancomycin</td>
<td>15</td>
</tr>
<tr>
<td>2.12. Combination of PTFE, Chitosan and Vancomycin in Implant Design</td>
<td>17</td>
</tr>
<tr>
<td>2.13. Biofilm Quantification</td>
<td>17</td>
</tr>
<tr>
<td>2.14. MIC Determination</td>
<td>20</td>
</tr>
<tr>
<td>2.15. Vancomycin Release Rate Determination</td>
<td>20</td>
</tr>
<tr>
<td>2.16. Selection of the Implant Type</td>
<td>21</td>
</tr>
<tr>
<td>CHAPTER 3</td>
<td>22</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>22</td>
</tr>
</tbody>
</table>
5.1. Future Work ................................................................. 65

REFERENCES .............................................................................. 67

LIST OF FIGURES

Figure 1. The five stages of biofilm maturation, 1) initial attachment; 2) irreversible attachment; 3) maturation I; 4) maturation II; 5) dispersion (Monroe 2007) ................. 7

Figure 2. Four possible mechanisms of biofilm antibiotic resistance (J. D. Chambless 2006). ............................................................... 8

Figure 3. SEM image of Staphylococcus aureus clusters (Carr. 2012) ......................... 12

Figure 4. Laser confocal image of Staphylococcus aureus clusters ............................ 12

Figure 5. Staphylococcus aureus colonies on TSB plate (left) and S. aureus colonies on an agar plate (right) (HansN. 2012) ..................................................... 13

Figure 6. PTFE structure (Nurioglu 2015) ......................................................... 14

Figure 7. Chitosan structure (Dash 2011) ......................................................... 15

Figure 8. Vancomycin structure (C. B. Liu 2011) .............................................. 17

Figure 9. Metal coupons .......................................................................... 24

Figure 10. Cultivation of bacterial biofilm on metal coupons ............................... 27

Figure 11. A typical crystal violet stained biofilm cultivated on the surface of metal coupons, A. Inoculated; B. Control .................................................. 28

Figure 12. Confocal laser scanning microscopy ............................................... 29

Figure 13. Microscopic views of S. aureus Seattle 1945GFPuvr strain. A. fluorescence microscopy; B. bright field microscopy; C. superimposed images. ......................... 30
Figure 14. AZ100 microscopy and a wide-field fluorescent image of biofilm on metal coupon ........................................... 32

Figure 15. Sonication and viable cell count ................................................................. 34

Figure 16. Glass round coverslips (left) and bacterial growth on glass coverslip (right). 34

Figure 17. Validation of crystal violet experiment ..................................................... 35

Figure 18. Validation of viable cell count experiment ............................................. 35

Figure 19. Crystal violet staining of the biofilm on the metal coupons .................... 41

Figure 20. Crystal violet OD$_{590}$ of tested coupons for three sets of 10 different sample types .................................................................................................................. 41

Figure 21. Crystal violet OD$_{590}$ of tested stainless steel (SS) and titanium (Ti) coupons for three sets of sample types .......................................................................................................................... 42

Figure 22. One series of Z stacks of the biofilm formed on the coupon ................. 43

Figure 23. A 3D structure of biofilm on the metal coupon surface .......................... 43

Figure 24. Cultivation and quantification of biofilm on SS and Ti coupon surfaces by viable cell count method ......................................................................................................................... 47

Figure 25. Viable counts of biofilm recovered from three sets of 22 different metal surface treatments in log scale .................................................................................................................. 47

Figure 26. Viable counts of biofilm recovered from three sets of three different metal surface treatments after 7 days incubation time ................................................................. 48

Figure 27. OD results of crystal violet test for three glass coverslip samples and control without bacteria ................................................................................................................... 49
Figure 28. OD results of crystal violet test for average of three glass coverslip samples and control without bacteria................................................................. 49

Figure 29. One Z stack of LCSM of the biofilm formed on the glass coverslip .......... 50

Figure 30. COMSTAT analysis of biofilm thickness and biomass on glass coverslips ... 51

Figure 31. Viable cell count results of three glass coverslip samples and control ........ 51

Figure 32. Average of viable cell count results of three glass coverslip samples and control ........................................................................................................... 52

Figure 33. A typical microtiter plate result of MIC test .............................................. 53

Figure 34. The antimicrobial property of leachates from 6 vancomycin-surface-modified discs at different 24-hour time points .......................................................... 55

Figure 35. Vancomycin released from SBV coupon in the first and second 24 hours ..... 57

Figure 36. Concentration of vancomycin released from TP5C vancomycin-modified surfaces with different layers of vancomycin on the surfaces during a 4-day study, P ≤ 0.05*................................................................................................................... 60
LIST OF TABLES

Table 1. List of coupon materials and their characteristics ............................................. 23
Table 2. Microscopic Examination of *S. aureus* Seattle 1945<sub>GFPuv</sub> Biofilms on Metal
Coupons. The top-view images were both at 200x. The side-view images were laser
confocal stacked images. ........................................................................................................... 31
Table 3. Wide-field fluorescence and laser confocal images ........................................... 44
Table 4. COMSTAT analysis of biofilm thickness and biomass ........................................ 45
Table 5. COMSTAT analysis of biofilm thickness and biomass on glass coverslips ........ 50
Table 6. Viable cells recovered from vancomycin modified surface at each 24-hour time
points ........................................................................................................................................ 54
Table 7. The antimicrobial property of leachates from 6 vancomycin-surface-modified
discs at different 24-hour time points ..................................................................................... 55
Table 8. Concentration of vancomycin released from six different vancomycin-modified
surfaces during a 7-day study .................................................................................................... 56
Table 9. Concentration of vancomycin released from different vancomycin-modified
TP5C surfaces with different layers of vancomycin during a 4-day study in water matrix
.................................................................................................................................................. 59
CHAPTER 1

INTRODUCTION

Biofilm consists of a group of microorganisms attached to a surface within an extracellular polymeric substance (EPS) matrix. Biofilm-associated cells have reduced growth rate and different genes expression than their planktonic cells. Attachment is a complex process and is influenced by different environmental factors and surface characteristics. When the biofilm structure is established, the microorganisms inside it are resistant to various antimicrobial agents. Biofilm formation on orthopedic implants after surgeries is a major problem in the treatment of the patients. It delays the healing process and revision surgeries might be needed to remove the implants with biofilm. Application of surface modified implants with antimicrobial properties can limit bacterial colonization on the implants. The first aim of this study is to characterize the antimicrobial properties of surface modified implants. The combination of polytetrafluoroethylene (PTFE) and chitosan with vancomycin as implants coating should enhance the antimicrobial properties of the implants significantly. We hypothesize that the deposition of PTFE and biodegradable chitosan with local antibiotic elution will enhance the functionality of the implant surface for fixation and bacterial resistance. In order to characterize the biofilm formed on the modified and unmodified surfaces to determine the best surface treatment in terms of preventing biofilm formation, three different quantification methods were utilized. Staphylococcus aureus encoding intracellular GFP was used to evaluate the viability, colonization, and biofilm formation on modified and unmodified metal coupons via crystal violet staining, laser confocal microscopy and viable cell count methods. Protocols for
cultivation and quantification of biofilms on stainless steel (SS) and titanium (Ti), both unmodified and modified, surfaces were developed and validated. In Aim two, the release rates of chitosan and vancomycin were quantified by measuring the concentrations of antimicrobial agent in the leachates of modified surfaces via HPLC. The antimicrobial properties of modified coupons will be further validated in future in vivo rabbit models.
CHAPTER 2
BACKGROUND

2.1. Combat Trauma

Combat trauma is principally distinguished by deep, penetrating injuries that can be contaminated with environmental and ballistic residues. The greatest threat to U.S. soldiers sent into the current U.S. wars is combat-related injuries. These war wounds are mostly open and exposed to environmental dirt and contaminants, so there is a considerable risk of infection for these patients. The infection risk of these injuries is relatively high. Up to 40% of war-trauma patients are affected while in non-war-trauma patients the infection risk is relatively low (Mody, et al. 2009). The infection rate of the improvised explosive devices (IED) injuries is 91%. Infection in the fracture site can cause biofilm formation on the implant surface, which is a severe problem in the treatment of these wounds. Biofilm formation on orthopedic implants may lead to chronic infection, delayed fracture union and revision surgeries (Mody, et al. 2009), which is a serious health problem for the patients and will add to the treatment costs significantly.

2.2. Implants/DCP

Metal internal fixation including dynamic compression plate (DCP) systems are usually used to treat long bone fractures, and are very common in blast injuries (Korzinek 1999), (Giannoudis 2006). Colonization and adhesion of bacteria like Staphylococcus aureus (S. aureus) and Staphylococcus epidermidis (S. epidermidis) on implant surfaces can cause biofilm formation. Designing implants that could prevent local infection and biofilm
formation at the fracture site will decrease the treatment time and healthcare costs for these patients (Belt 2001), (van Loosdrecht 1990). Implants have been developed to improve fracture healing, but it has also been found that they have a different susceptibility to infection (Moriarty 2010). Implant infection has been reported to be one of the reasons for orthopedic implant removal (Le 2014). Limiting the initial microbial adhesion to implant surfaces is critical to defend against early infection (Raphel 2016). Application of non-adhesive surfaces on the implants is one strategy to reduce bacterial attachment. Implant surface coatings such as polyethylene glycol (PEG) has significantly reduced S. aureus adhesion (Yang 2005). Dextran is another coating polymer that has been reported to reduce S. aureus and S. epidermidis adhesion by 50% (Zhang 2008). The effects of PTFE coating on biofilm formation will also be evaluated in this thesis project. Applying anti-adherent coatings alone may allow the bacteria to return, while bactericidal coatings can eliminate the bacteria and prevent future infection (Raphel 2016). Therefore, the combination of anti-adherent surfaces with bactericidal compounds can be more effective. Silver coated implants and chitosan coatings alone or along with other antimicrobial compounds are the most effectively used coatings on implant surfaces (Raphel 2016). Silver has been known to have wide antimicrobial activities (Eckhardt 2013). Chitosan is also a bactericidal polymer that has been combined with other antimicrobial compounds to increase its effects (Ordikhani 2014). Chitosan and vancomycin have been applied on PTFE grafts (Parker 2013), but a combination of PTFE surface coating with chitosan and vancomycin has not been studied before.
2.3. Biofilm

Biofilm is a group of microorganisms in which cells stick together on a surface; these adherents are attached within a self-produced matrix of extracellular polymeric substances (EPS). These EPS usually consist of extracellular DNA, proteins, and polysaccharides. Biofilm was first described by Antonie van Leeuwenhoek as the accumulation of microorganisms in dental plaque in 1686. He is well known for his ability to build microscopes and one of the early scientists to study microscopic life (Slavkin 1997). The special three-dimensional structure of biofilm provides an environment for the bacteria to live as a group of microorganisms together that can form on different living and non-living surfaces in industry, hospitals and natural environments (P. K. Watnick 2000). Presumably this structure serves as protection for microorganisms from the environment and host body to increase their chances of survival. The microorganisms inside this structure will be resistant to antibiotics. They are also protected from the host immune defense (Costerton 2005), (Darouiche 2004). Biofilms can also grow in nutrient conditions that do not permit the growth of planktonic cells and bacterial growth rate is decreased in the biofilm structure (P. R. Watnick 2000).

2.4. Biofilm Formation Stages

Biofilm development has different steps. The first one is the initial attachment. In *Staphylococcus aureus*, the microbial surface components recognizing adhesive matrix molecules (MSCRAMMS) are expressed, that help the bacteria to attach to different
surfaces. The exact attachment mechanism is not known yet, but in the presence of these molecules, the attachment is facilitated (C. T. Heilmann 2003). Polysaccharide intercellular adhesion (PIA) with the chemical composition of poly-N-acetylglucosamine (PNAG) is the main molecule responsible for intercellular adhesion. De-acetylation of N-acetylglucosamine in PIA positively charges the molecule, so PIA attaches by electrostatic interaction to the bacterial surface that is normally negatively charged because of its teichoic acids (Sadovskaya 2005). The ica gene locus is responsible for the production of PIA molecules (Boles 2005). The next step is the maturation phase, which is known by extracellular aggregation of adhesive proteins and polysaccharides with biofilm structural forces that make three-dimensional mushroom-like cell towers and fluid-filled channels between the towers through which nutrients are delivered to deeper biofilm cells. The exact mechanism of this structure forming is not well known yet, but primary findings show the role of the cell to cell signaling by quorum sensing which controls surfactant peptides to build the biofilm structure (Boles 2005). Whenever the cell density is low, the adhesion factors like MSCRAMMs are up-regulated and after colonization of the bacteria, the expression of these molecules are decreased (Boles 2005). The final phase is the dispersal phase, which leads to dissemination of the bacteria to new infection sites (Otto 2008). Single cells or large cell clusters may detach from the biofilm surface area, which is also controlled by the quorum sensing in staphylococci. The five stages of biofilm maturation are shown in Figure 1.
2.5. Mechanisms of Biofilm Resistance

Four possible mechanisms of biofilm antibiotic resistance have been described. The first mechanism is poor antimicrobial penetration in which antimicrobial agents penetrate slowly or incompletely through the biofilm. Consumption of the antimicrobial agents by the biofilm as it consumes other substrates will decrease the concentration of the antimicrobial agents to a level that would be ineffective in the deeper regions of the biofilm (P. S. Stewart 1996). The second mechanism of biofilm protection is stress response defenses that are induced by biofilm-forming bacteria when they encounter an environmental challenge (J. D. Chambless 2006). It has been suggested that slow growth of bacterial cells in the biofilm is due to a general stress response that changes the cells physiologically, so they will be protected from environmental stresses and detrimental agents (Mah 2001). Altered microenvironment and slow growth is the third mechanism by which bacteria in a biofilm are more resistant to antimicrobial agents. Within the biofilm there is a micro-gradient found in the concentration of key metabolic substrates and
products. This leads to slow growing or stationary phase of bacterial cells when they are less susceptible than the bacteria in the growing phase and enables them to survive antibacterial challenges (Wimpenny 1995), (Gilbert 2003). The fourth mechanism of antimicrobial resistance of biofilm is the possibility of a highly protected phenotype of microorganism in a biofilm. Cells in this special state are called persisters (J. D. Chambless 2006). It seems likely that a combination of these factors determines the overall protection of the biofilm. Mechanisms of biofilm tolerance are shown a cross-section of a biofilm (Figure 2). The attachment surface is shown in grey. Yellow phase contains the antibiotic at the top, where antimicrobial penetration is restricted in the presence of EPS. In the green areas, some bacteria change their activity in response to antimicrobial stress. The microenvironment in the deeper area is altered to resist eradication (pink). Persister cells are present in higher concentration in biofilm (violet) (J. D. Chambless 2006).

![Mechanisms of Biofilm Tolerance](image)

Figure 2. Four possible mechanisms of biofilm antibiotic resistance (J. D. Chambless 2006).
2.6. Factors Affecting Biofilm Formation

Biofilms can form on many different surfaces such as living tissues, medical devices, industrial or natural aquatic systems. The ideal environment for the attachment of microorganisms onto a surface is the solid-liquid interface between that surface and a liquid medium like water or blood. The characteristics of the solid surface can also be important in the attachment process. Surface roughness is one of the surface characteristics that affect microorganism’s colonization, the surface area is higher on rough surfaces, and it appears that colonization increases when the surface roughness increases. Surface hydrophobicity can also play a role in the initial attachment; many studies showed that microorganisms attach faster to hydrophobic, nonpolar surfaces such as Teflon and plastics than hydrophilic surfaces like glass or metals (Donlan 2002) (Nurioglu 2015). The results of these studies are contradictory because of the other variables that are always present in biofilm formation and lack of standard methods to evaluate surface hydrophobicity. The liquid medium has characteristics like pH, nutrient level, ionic strength and temperature that affects the attachment of the microorganisms. Seasonal changes affecting biofilm formation in aqueous systems might be ascribed to differences in temperature. A laboratory study also demonstrated more microbial attachments when nutrient concentrations are higher (Otto 2008). Cell surface properties like hydrophobicity, fimbriae and flagella and EPS production can influence the attachment rate. Most bacteria studied are negatively charged and contain hydrophobic surface components (Donlan 2002). Different bacterial strains might vary in hydrophobicity, and some studies did not find a relationship between the
hydrophobicity of the bacterial surface and the extent of initial binding to either a hydrophilic or hydrophobic substrate (N. P. Cerca 2005).

2.7. Gene Transfer in Biofilm

Biofilms provide a good environment for transferring of the extrachromosomal DNA (plasmids) through conjugation as the cells in the biofilm are closer and cell-to-cell contact is more possible. These plasmids may encode for resistance to antimicrobial compounds (Donlan 2002).

2.8. *Staphylococcus aureus*

The majority of biofilm-associated infections are caused by Staphylococci, which is the most common bacteria on the human surfaces like skin and mucous, and therefore can easily enter the body through surgical cuts or infect the medical devices that enter these surfaces (Otto 2008). *Staphylococcus* is a gram-positive, round shape, non-motile facultative anaerobe bacterium that can grow without the need for oxygen. It is a member of Firmicutes and normal body flora (Masalha 2001). It has grape-like clusters under the microscope and produces large, round, golden-yellow colonies and does not form spores (Ryan, Medical microbiology 2004), (Figure 3 - Figure 5). *Staphylococcus aureus* is a ubiquitous bacterial species and it often exhibits hemolysis when grown on blood agar plates (Ryan, Medical microbiology 2004). Alexander Ogston identified *Staphylococcus* for the first time in 1880 in surgical pus in Scotland. Binary fission is the way that *S. aureus*
reproduces asexually; the daughter cells remain attached to one another and appear as clusters (Varrone JJ 2014). Staphylococcus can cause many diseases from mild skin infections to serious, life-threatening infections. \textit{S. aureus} is one of the most common causes of hospital-acquired infections and the most common pathogen associated with orthopedic implant infections (Ribeiro 2012). \textit{Staphylococcus aureus} can produce a multilayered biofilm embedded within a glycocalyx or slime layer. It is an important clinical pathogen due to its resistance to antibiotics. The mortality rate of 25\% infection associated with \textit{S. aureus} has been reported in the US (Belt 2001). It is the main cause of osteomyelitis or infection of the bone. \textit{S. aureus} Bacteria can enter through the blood or direct inoculation during surgery, trauma or an old or chronic infection (Archer 2011). Application of antimicrobial compounds to resolve the \textit{S. aureus} infection is not effective in most cases (Archer 2011). Preventing biofilm formation or removing formed biofilm by surgery are the practical options to solve this issue. \textit{S. aureus} strain Seattle 1945GFP is used for evaluation of colonization, and biofilm formation on modified and unmodified implant surfaces. \textit{Staphylococcus aureus} subsp. \textit{aureus} ATCC 25923 with the designation Seattle 1945 is a clinical isolate that is used as a standard laboratory testing control strain. It is sensitive to a variety of antibiotics, including methicillin (Treangen 2014). This bacterium contains green fluorescent protein (GFP), which makes it possible to use fluorescent microscopy to exam the presence and growth of bacteria and evaluate the biofilm formation.
Figure 3. SEM image of *Staphylococcus aureus* clusters (Carr. 2012)

Figure 4. Laser confocal image of *Staphylococcus aureus* clusters
2.9. PTFE

PTFE is a synthetic fluoropolymer of tetrafluoro ethylene, which has many industrial and clinical applications. It is a high molecular weight hydrophobic compound made of carbon and fluorine. It has been frequently used as a coating for surgical interventions like catheters as it is non-toxic and biocompatible, while also possessing the ability to interfere with the bacterial adhesion (Pavithra 2008). It has been shown that biofilm formation is reduced when PTFE was applied as an anti-adherent coating. PTFE provides an anti-adherence, microbe repelling surface and has been used in medical implants (Berry 2000), (A. E. Demling 2010). PTFE structure is shown in Figure 6.
2.10. Chitosan

Chitosan is a biopolymer (linear polysaccharide) composed of N-acetyl-2-amino-2-deoxy-d-glucopyranose and 2-amino-2-deoxy-d-glucopyranose. Chitosan can be produced commercially by deacetylation of chitin, the exoskeleton of crustaceans (such as crabs and shrimp) and fungi cell walls. It is a non-toxic, biocompatible biomaterial that has been shown to have antimicrobial properties against planktonic microbes (Rabea 2003), (Şenel 2004). The exact anti-microbial mechanism of chitosan is not clear yet (Carlson 2008), but some possible mechanisms have been studied. There are different theories to explain chitosan’s antimicrobial mechanism. More likely, chitosan’s anti-microbial mode of action is not limited to a single target molecule. It has been shown that the initial contact between the polycationic chitosan and negatively charged cell wall polymers leads to disruption of the equilibrium of cell wall dynamics. In addition, binding of chitosan to cell wall polymers could trigger secondary cellular impacts that destabilize and disrupt bacterial membrane function leading to cellular components leakage and dysfunction of the whole cellular system (Raafat 2008). As the resistance of chitosan-coated surfaces to biofilm formation by S. aureus has been reported, using chitosan would provide a flexible platform for designing coatings to protect implant surfaces from infection (Carlson 2008).
active primary amino groups on the molecule are reactive, sites for a variety of side group attachments are provided. Chitosan can be degraded by enzymes that can hydrolyze glucosamine–glucosamine, glucosamine–N-acetyl-glucosamine and N-acetyl-glucosamine–N-acetylglucosamine linkages. The amino group in chitosan has a charge density dependent on pH which makes it a bioadhesive that binds to negatively charged surfaces. Chitosan biodegradation plays a role when it is applied to drug delivery systems. This potential can be utilized as a carrier for controlled release of various therapeutic compounds such as antibiotics (Dash 2011). Chitosan structure is shown in Figure 7.

![Chitosan structure](image)

Figure 7. Chitosan structure (Dash 2011)

2.11. Vancomycin

Vancomycin is an antibiotic used to treat several bacterial infections especially for treatment of serious, life-threatening infections by gram-positive bacteria unresponsive to other antibiotics (González 1999). Vancomycin is produced by soil bacterium *Amycolatopsis orientalis*. It is a type of glycopeptide antibiotic and works by blocking the proper biosynthesis of the cell wall in Gram-positive bacteria and a few of gram-negatives.
Peptidoglycan layer of the cell wall is rigid because of its cross-linked structure consists of N-acetyl muramic acid (NAM) and N-acetyl glucosamine (NAG) monomers. Vancomycin binds to the NAM and NAG building blocks and inhibits cross-linking of peptidoglycan layers. Because of that, the peptidoglycan layers will be less rigid and more permeable leading to leakage of cellular contents and death of the affected bacteria (Watanakunakorn 1984). Vancomycin has been shown to be effective against Staphylococcus aureus (C. B. Liu 2011). The in vivo half-life of vancomycin is 4-6 hours, and it is removed via renal extraction (Bratzler 2005). The approximate half-life of vancomycin in vitro at 37°C is 9 days (White 1988), and the saturation limit of vancomycin is 17 mg/ml in solutions with pH of 7.5 (Faustino 2008). These are some of the vancomycin parameters that need to be considered when designing the experiments. Vancomycin structure is shown in Figure 8.
2.12. Combination of PTFE, Chitosan and Vancomycin in Implant Design

The combination of PTFE and chitosan with vancomycin as implants coating would increase antimicrobial properties of the implants significantly. We propose that the deposition of polytetrafluoroethylene (PTFE) and biodegradable chitosan with local antibiotic elution will enhance the functionality of the implant surface for fixation and bacterial resistance.

2.13. Biofilm Quantification

In order to compare different surface treatments of orthopedic implants, standard quantification methods are needed. There are different methods to quantify biofilms on
solid surfaces in vitro, They can be divided into three main groups; microscopic methods, cultural based procedures and biochemical investigations (K. K. Doll 2016). Each different method has its advantages and disadvantages and therefore can be used for different purposes. Six most popular quantifying methods to analyze biofilm of S. aureus bacteria are listed as follows: CLSM, sonication and CFU, enzymes and CFU, resazurin, BacTiter-Glo™ and crystal violet. (K. K. Doll 2016). In BacTiter-Glo™ method equal volumes of PBS and the BacTiter-Glo™ reagent are added to the sample with biofilm. Followed by 5 min incubation at ambient temperature, and luminescence is measured using a multi-mode reader. This method has been reported to be costly, but not time consuming (K. K. Doll 2016). Adding resazurin solution (a fluorescent dye) in PBS to the samples and incubating at 37 °C is another biochemical method to analyze the biofilm. Fluorescence is then measured using a multi-mode reader. This method is not costly and not time consuming (K. J. Doll 2016). For a rapid high-throughput screening of antibacterial approaches, the viability assays resazurin and BacTiter-Glo™ are very suitable. Application of enzyme to detach bacteria followed by CFU counting is another quantifying method of biofilm. This method is time consuming and moderately costly (K. K. Doll 2016). Crystal violet (CV) staining, a low cost, not complicated and fast method for analysis of biofilm, was first described by Christensen et al. (G. Christensen 1985). CV is a basic protein dye that binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (Li 2003). CV stains both live and dead cells as well as the matrix so it can not be used to evaluate functional biofilm (Pitts 2003). The method is basically based on staining the biofilm formed on a surface with CV, then rinse a few times to remove non-attached
bacterial cells, wash with 95% ethanol to release the CV and then recording the optical density (OD) with a spectrometer (Z. L. Xu 2016). Confocal laser scanning microscopy (CLSM) is a technique for taking high-resolution optical images. As it can provide z-sectioning of thick specimens, it is a suitable method for analyzing thickness, biomass and structure through the 3D images or cross-sectional views of the biofilm. It has been used extensively to increase the understanding of biofilm characteristics (Mueller LN 2006). Development of the new analytical methods and software increased the quantitative output of these images (Mueller LN 2006). COMSTAT is a program that enables the quantification of the biofilm based on CLSM images. COMSTAT source code is used to read the “tif” images based on the number of pixels in the image columns and rows and the z-step size. An automatic threshold is used in MATLAB to separate the bacteria and surface sequences (Ross 2014, Heydorn A1 2000). Sonication and viable cell count is another method used to analyze biofilm formed on the implants. It is more complicated and time-consuming than crystal violet method, but less costly and faster than laser confocal microscopy (N. P. Cerca 2004), (C. G.-R. Heilmann 1996). The method is based on recovering the live bacteria from the surface biofilm by detaching them through a sonication technique followed by plate counting(CFU) method (K. J. Doll 2016). The low-moderate sonication power (20-40 kHz) for a brief time (2-10 minutes) of bacterial suspension in the water bath, has been shown to have the optimum effect on bacterial detachment. Longer exposure time or more ultrasound power leads to disruption of the bacterial cell wall (E. Joyce 2011), (Geir Bjerkan 2009). Based on the accessibility, cost and required time each method, crystal violet, LCSM and Sonication with viable cell count
were the three methods used in this study to quantify biofilm formed on the different sample treatments.

2.14. MIC Determination

MIC is the lowest concentration of an antimicrobial, antibiotic or bacteriostatic drug that inhibits the visible growth of a microorganism after overnight incubation. It is different than the minimum bactericidal concentration (MBC), which is the concentration resulting in microbial death (Tripathi 2013). The MIC of a compound is determined by preparing solutions of the compound at constantly increasing concentrations in a series of tubes and incubating the solutions with the cultured bacteria separately for 24 hrs. The turbidity of the solutions indicates the amount of microbial growth, and no bacterial growth is when the solution is clear. When the concentration of the antimicrobial compound increases the turbidity of the solution decreases until the concentration reaches the MIC, that is, when the microorganism cannot grow at or above that concentration. Minimum inhibitory concentration of chitosan and vancomycin need to be determined in this study as we need to apply the concentration more than the MIC in order to prevent bacterial growth and subsequent biofilm formation on the implants.

2.15. Vancomycin Release Rate Determination

Chitosan is a biodegradable compound that is used as a carrier molecule for controlled-release of vancomycin from the surface of the implants. Monitoring the release rate of
vancomycin helps us to have a proper understanding of the effects of surface modifications and track the antibacterial activities of the implants through time. High-performance liquid chromatography (HPLC) is the method used in this study to determine the release rate of vancomycin. An \textit{in vitro} elution method by placing the disks in water and collecting the leachates at different time periods and analyzing with HPLC to determine the concentration of released vancomycin is a common method that has been used previously in different studies (Hsu 2014), (Oyaert 2015), (Abu Tariq 2010).

2.16. Selection of the Implant Type

Based on the different biofilm quantification methods and the results of vancomycin release rate, the best implant surface treatment is determined and will be evaluated \textit{in vivo} in a rabbit fracture model.
CHAPTER 3
MATERIALS AND METHODS

3.1. Titanium and Stainless-Steel Round Shape Coupons

Stainless steel, titanium, and titanium alloys (titanium with 6% aluminum and 7% niobium, TAN) are the most common materials used for fracture fixation implants (Hayes 2010). In this study biofilm was grown on different titanium and stainless-steel coupons with aluminum in different particles sizes (90 µm and 50µm) coated with PTFE chitosan and vancomycin that have been tested for their antimicrobial properties. The surface modifications evaluated were alumina blasting (50 and 90 µm particle-size) with CoBlasted PTFE and chitosan. The coupons dimensions are 1 cm diameter and 1mm height. Stainless steel and titanium surfaces for bacteriostatic and biofilm regulation in vitro was quantified. Different surface treatments including: stainless-steel (S), titanium (T), Alumina in different particle sizes (5 for 50 and 9 for 90 µm particle-size), PTFE (P), chitosan (X) and Vancomycin (V) have been prepared. Sample types and their characteristics are listed in Table 1. The metal coupons’ shape and design is illustrated in Figure 9.
Table 1. List of coupon materials and their characteristics

<table>
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<tr>
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</table>
3.2. Bacterial Strain

For quantification the growth of biofilm on the stainless-steel and titanium sample coupons, a methicillin-susceptible *Staphylococcus aureus* strain Seattle 1945 (ATCC 25923) was used. A clinical isolate, that is used as a standard laboratory testing control. This bacterium contains green fluorescent protein (GFP), thus presence, growth and colonization of bacteria can be evaluated using fluorescent microscopy.

3.3. Bacterial Growth on the Coupons

*Staphylococcus aureus* ATCC 2593 stock cultures were streaked on Tryptic
Soy Agar (TSA) +1% glucose plates and incubated overnight at 37°C to obtain isolated colonies. A single colony was transferred to Tryptic Soy Broth (TSB) media and grown overnight in a shaker incubator at 37°C and 125 rpm to the concentration around 1x10⁹ CFU/ml inoculum. The overnight cultures were used to inoculate the experimental coupons. Stock cultures were frozen in 1:1 of 20% sterile glycerol and media. Bacteria were recovered in 10 ml of media and incubated for 24 h at 37°C.

3.4. Coupon Cleaning Procedure

Prior to use, coupons were cleaned and sterilized. Individual coupons were soaked in a 70% Ethanol solution for 10 minutes and then washed in 1x Phosphate buffered saline (PBS) solution, then soaked in ethanol solution and rinsed with PBS again and left in sterile petri dishes to dry.

3.5. Coating the Underside (Unmodified) Surface of the Coupons with VALAP

Since only one side of each metal coupon was modified, i.e., co-blasted, biofilm growth on the unmodified side of the coupons needed to be minimized. The coating of the unmodified surfaces of each coupon with VALAP, a biologically inert material consists of vaseline, lanolin and paraffin wax in equal ratio, that has been shown to prevent bacterial attachment (Jung 2014), was further optimized to ensure that only the biofilm form on the modified surfaces were quantified.
3.6. Cultivation and Quantification of Biofilm

Cultivation and quantification of biofilm growth on metal coupon surfaces were conducted as follows; briefly, 24-well flat-bottom non-tissue-culture treated microtiter plates were used to minimize binding of bacteria onto the wells and to facilitate the cultivation of biofilm on metal coupon surfaces. Coupons were dipped gently into the melted VALAP until the bottom side was covered. Each coupon was then placed into a separate well of the 24-well plate. 600 µl of TSB+1% glucose was added to each well to cover the entire surface of the coupon. The concentration of the overnight *S. aureus* was determined using a Bio-Rad SmartSpec 3000 spectrophotometer and $10^3$ bacterial cells were added to each well containing coupon and the plate was kept in an incubator at 37°C for 24 hours. Washing of metal coupons after growth of biofilms was by dipping the coupons in wells containing 500 µl of D.I. water. This process was repeated 3 times to wash away planktonic bacteria. The biofilms on metal coupons were then observed using an epi-fluorescence microscope or a laser confocal microscope. For quantification of biofilm, the crystal violet staining method or viable cell count experiment was used. A positive control, i.e., a well-containing TSB and bacterial inoculum, and a negative control, i.e., a well-containing TSB without bacteria were included for each individual experiment. Cultivation of bacterial biofilm on metal coupons is shown in Figure 10.
3.7. Quantification of Biofilm Via Crystal Violet Staining

For quantification of biofilm growth on metal coupons using crystal violet method, they were processed as follows: After the last wash to remove unbound planktonic cells, the coupons were placed into empty wells and allowed to air-dry at 37°C for 30 minutes. Metal coupons were placed into wells containing 600 µl of 0.1% (w/v) aqueous crystal violet solution and stained for 5 minutes at room temperature. The metal coupons were then washed 3 times in wells containing 600 µl of D.I. to remove excess stains and air-dried at 37°C for 30 minutes. 600 µl of 100% ethanol was added to the wells containing coupons to dissolve the bound crystal violet and the plate was placed on a shaker (50 rpm) at room temperature for 10 minutes to detach the adherent biofilm growing on the coupons. 100 µl
of each solution was transferred into a 96-well microtiter plate and the biofilm quantified using a Synergy H1 Hybrid Microplate Reader reading at 590 nm. A typical result of crystal violet stained biofilm on the metal coupons is illustrated in Figure 11.

![Figure 11](image_url)

**Figure 11.** A typical crystal violet stained biofilm cultivated on the surface of metal coupons, A. Inoculated; B. Control

### 3.8. Quantification of Biofilm Using Confocal Laser Scanning Microscopy (CLSM)

The fluorescence property of *S. aureus* Seattle 1945GFPuvr strain was verified using a Zeiss laser confocal microscope (Figure 12). Confocal laser scanning microscopy (CLSM) is a technique for obtaining high-resolution optical images with depth selectivity (Z stacks). The main feature of confocal microscopy is its ability to acquire in-focus images from selected depths. Washing metal coupons after the growth of biofilms is completed by dipping the coupons in wells containing 600 µl of D.I. water. This process is repeated 3 times to wash away planktonic bacteria. The biofilms on metal coupons then observed using an epi-fluorescence microscope or a laser confocal microscope. Images acquired point-by-point and reconstructed with the computer, allowing three-dimensional
reconstructions of topologically complex objects. Three-dimensional images gave us a (an idea) view of biofilm structure on different surface treatments. Interpreting the images through measuring some features of bacterial biofilm using COMSTAT software gave us the ability to determine the best surface material and treatment of the disks that can prevent or reduce biofilm formation on the metal implants.

Figure 12. Confocal laser scanning microscopy

The fluorescence property of *S. aureus* Seattle 1945<sup>GFP<sup>uvr</sup></sup> strain was verified using a Zeiss laser confocal microscope and the result illustrated in Figure 13 and Table 2.
Figure 13. Microscopic views of *S. aureus* Seattle 1945$^{\text{GFPuvr}}$ strain. A. fluorescence microscopy; B. bright field microscopy; C. superimposed images.
Table 2. Microscopic Examination of *S. aureus* Seattle 1945<sup>GFPuvr</sup> Biofilms on Metal Coupons. The top-view images were both at 200x. The side-view images were laser confocal stacked images.

<table>
<thead>
<tr>
<th>Metal Coupon</th>
<th>Epi-fluorescence Microscopy (Top Views)</th>
<th>Laser Confocal Microscopy (Side Views)</th>
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</thead>
<tbody>
<tr>
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<td><img src="image" alt="Titanium Top View" /></td>
<td><img src="image" alt="Titanium Side View" /></td>
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<tr>
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<td><img src="image" alt="Stainless Steel Top View" /></td>
<td><img src="image" alt="Stainless Steel Side View" /></td>
</tr>
</tbody>
</table>

To analyze the biofilm distribution on coupon surfaces, wide-field fluorescence images were taken using Nikon AZ100 epi-fluorescence microscope and the images were analyzed using the ImageJ software (Figure 14). The signal thresholds, e.g., optimized brightness and saturation settings were standardized in all image acquisition and analysis. Thresholding was applied with manual adjustment. Threshold limits were maintained at a constant value for all the images (Carson 2010), (Rasband 1997-2007).
3.9. Biofilm Thickness and Biomass Analysis

To analyze the average thickness and biomass of biofilm formed on coupon surfaces, multiple laser confocal image stacks were analyzed using the COMSTAT software. The image acquisition and data analysis were standardized and optimized, e.g., size of inoculum, incubation time, and the number of stacks. There are some features that are calculated by COMSTAT; Bio-volume, which is defined as the number of biomass pixels in all images of a stack multiplied by the voxel size \[(\text{pixel size})_x \times (\text{pixel size})_y \times (\text{pixel size})_z\] and divided by the substratum area of the image stack. The resulting value is biomass volume divided by substratum area \((\mu \text{m}^3/\mu \text{m}^2)\). Bio-volume represents the overall volume of the biofilm, and provides an estimate of the biomass in the biofilm. Area occupied by bacteria in each layer: This is the fraction of the area occupied by biomass in each image of a stack and the substratum coverage is the area coverage in the first image.
of the stack. Substratum coverage reflects how efficient is the colonization of bacteria on the substratum. Thickness distribution and mean thickness: This function locates the highest point (μm) above each (x,y) pixel in the bottom layer containing biomass. Hence, the thickness is defined as the maximum thickness over a given location, ignoring pores and voids inside the biofilm. Mean biofilm thickness provides a measure of the spatial size of the biofilm and is the most common variable used in biofilm literature (Russ 2016).

3.10. Quantification of Biofilm Using Viable Cell Count Method

A viable count test was performed to quantify biofilm formation on the coupons since this method yields more consistent and sensitive results than the crystal violet method. We followed the Bjerkan method with modification; Briefly, after cultivation of biofilm on the coupon surface, each disk was placed into a 15ml sterile conical tube containing 5ml of PBS and gently vortexed for 15 seconds to rinse off planktonic cells. After addition of 5ml of PBS, each tube was then subjected to sonication for 5 minutes. This wash 1 solution (W1) was serially diluted 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ folds. 100 μl from each undiluted and diluted sample was plated on TSA and incubated at 37°C for 48hr. Each disk was washed a second time in 5ml PBS in ultrasound water bath for another 5 minutes yielding wash solution W2. The washing process was repeated for the third time yielding W3. Viable counts of W2 and W3 was conducted the same way described for W1. Numbers of colonies were counted and the total cell number of each coupon was calculated (Bjerkan 2009). By calculating the recovered bacterial cells from different treatments, we could compare the surface treatments. Sonication and viable cell count experiments are shown in Figure 15.
3.11. Validation of the Methods

To validate the biofilm quantifying methods, round shape glass coverslips with 10 mm diameter and 0.16-0.19 mm thickness were used with three different quantifying methods including crystal violet, laser confocal and viable cell count as described for the metal coupons. The results were obtained to determine if the methods utilized are reproducible and consistent. The typical setups for biofilm quantification methods are shown in Figure 16 - Figure 18.
Figure 17. Validation of crystal violet experiment

Figure 18. Validation of viable cell count experiment
3.12. Determination of the Minimum Inhibitory Concentration (MIC) of Chitosan

The MIC of chitosan was measured to determine whether the chitosan released from the samples is sufficient to affect the bacterial biofilm formation. The experiment is mainly preparing a series of two-fold dilutions of chitosan from high concentration to low in a 96-well plate and then cultivate the bacteria in the wells, incubate them and observe the growth in each well. The turbidity of each well determines whether the bacteria has grown or not. Since the solubility of chitosan in water is low, chitosan was first dissolved in acetic acid added to subsequent growth media for analysis. The MIC of acetic acid was determined first in order to select an appropriate concentration of acetic acid that is not inhibitory when used as a solvent for chitosan. Two-fold serial dilutions of acetic acid starting from 4M to 1mM solutions were prepared in 96-well plates using fresh liquid media as the diluents. Each well was then seeded with 1x10⁵ CFU of test bacteria. The plates were incubated overnight at 37°C and inspected the following morning. Acetic acid at sub-MIC (1/4 MIC) concentration was used as the solvent for chitosan in subsequent experiments. Chitosan did not easily dissolve in lower concentrations of acetic acid, so a more concentrated stock was prepared first. 0.01g of chitosan powder was added to 200 μl of 0.5M acetic acid in a tube. This tube was then put on a heat block for 30-60 minutes until the chitosan was fully dissolved. However, once the chitosan was dissolved in the acid, the solution would be too viscous to pipette into different holders, so 2 ml of purified water was added to the solution, and then the tube was placed back on the heat block until the solution became less viscous. Two-fold serial dilutions of chitosan was prepared after that, and added to a 96-well plate in total volume of 200 μl for each well (100 μl of chitosan solution plus 100 μl of 2x media.
and 2x10⁵ CFU/ml bacterial inoculum). There were two controls including the following: one well filled with solution of the Cation-adjusted Mueller Hinton broth (CAMHB) media with fresh bacteria, and one well filled with solution of just the CAMHB media without bacteria. Once all the solutions were placed in the 96-well plate, it was placed in an incubator for 24 hours to allow the bacteria to grow. The well plate was inspected after for bacterial growth, and the data was recorded.

3.13. Determination of the MIC of Vancomycin

The MIC of vancomycin was measured to determine whether the vancomycin released from the samples is sufficient to affect the bacterial biofilm formation. The experiment entails mainly preparing a series of two-fold dilutions of vancomycin from high to low concentration in a 96 well plate and then cultivate the bacteria in the wells, incubate them and observe the growth in each well. The turbidity of each well determines if the bacteria has grown or not. 0.01 gram of vancomycin powder was dissolved in 1 ml DI water, two-fold serial dilutions of vancomycin solution were prepared after that, and added to a 96-well plate in total volume of 200 μl for each well (100 μl of vancomycin solution plus 100 μl of 2x media and 2x10⁵ CFU/ml bacterial inoculum). There were two controls including the following: one well filled with a solution of the CAMHB media with fresh bacteria, and one well filled with a solution of just the CAMHB media without bacteria. Once all the solutions were placed in the 96-well plate, it was placed in an incubator for 24 hours to allow the bacteria to grow. The well plate was inspected after for bacterial growth, and the data was recorded.
3.14. Vancomycin Release Rate Study

The release rate of vancomycin from the modified surfaces was evaluated to assess whether the amount of vancomycin deposited on modified implant surfaces would be sufficient to maintain the anti-biofilm property for 7 days. In one experiment, vancomycin discs were tested for an entire week to determine if the amount of vancomycin remained on the discs at different time periods was sufficient to prevent biofilm formation on the coupons. The experimental design is as follows: seven (one for each day of a continuous week) individual coupons (TBV type, TBV1-TBV7) were placed (one each) into individual microtiter plate wells containing 600 µl of TSB medium plus 1% glucose. The well containing disc TBV1 was immediately inoculated with 1000 Staph. aureus cells and sealed to prevent evaporation of media. The amount of biofilm formed on disk one after 24 hrs. of incubation at 37° C was determined. Leachates from individual wells containing coupons number 2-7 were removed after 24 hrs. and replaced with 600 µl of fresh TSB medium and TBV2 was inoculated with 1000 Staph. aureus cells; the amount of biofilm formed after the second 24 hrs. incubation at 37° C was determined for disk number 2, Discs 3, 4, 5, 6, and 7 were processed the same way after 48, 72, 96, 120, and 144 hrs., respectively.

3.15. High-Performance Liquid Chromatography (HPLC) Analysis

To determine the release-rate of vancomycin and whether vancomycin contained in the leachates from individual modified surfaces was sufficient to inhibit the growth of test bacteria Staph. aureus, leachates from individual discs collected at different time points
were analyzed for their vancomycin contents as follows: different vancomycin-surface-modified discs were placed (one each) into microtiter plate wells containing 600 µl of TSB medium with 1% glucose and sealed to prevent evaporation of media, at each 24-hour time points, leachates were removed from each well for analyzing vancomycin content via HPLC; 600 µl of fresh TSB was added back to each well and the same steps were repeated a total of 7 days. For HPLC analysis a LC System; Shimadzu LCMS-8040, Prominence Series with ESI interface and autosampler was used. The Kinetix XB C18 (100 x 3mm, 2.6um) column was used, and the solvents were A:0.1% Formic Acid and B: Methanol, as aqueous and organic solvents respectively. Certified standards were run to optimize the method.

3.16. Statistical Analysis

IBM SPSS statistic software version 24 was used for statistical analysis. To analyze the results of biofilm quantifying methods and the results of validating of the methods, a one-way ANOVA test is run with a Tukey post-hoc analysis. The one-way ANOVA test is used to determine whether there are significant differences between the means of data.
CHAPTER 4

RESULTS

4.1. Biofilm Quantification Results

The effect of PTFE+chitosan+vancomycin on stainless steel and titanium surfaces for bacteriostatic and biofilm regulation in vitro was quantified. We validated that these materials can be used to limit bacterial colonization of implant surfaces in vitro in a S. aureus bacterial model. The viability of S. aureus cultivated in growth media on these surfaces over time was quantified. The S. aureus biofilm formation on individual modified/unmodified metal disk surfaces over time was quantified via crystal violet staining, laser confocal microscopy, and viable cell count methods. Since only one side of each metal coupon was modified, i.e., co-blasted, biofilm growth on the unmodified side of the coupons needs to be minimized. The coating of the unmodified surfaces of each coupon with VALAP was used in biofilm analysis, that has been shown to prevent bacterial biofilm formed. VALAP was applied to ensure that only the biofilm formed on the modified surfaces were quantified.

4.2. Crystal Violet Data

S. aureus Seattle 1945 formed biofilms on the surface of metal coupons. Crystal violet dye has been utilized to stain bacterial biofilms. Stained biofilm was dissolved in ethanol to recover the crystal violet for quantification using a microplate reader. The concentration of crystal violet recovered is proportional to the amount of biofilm on metal surfaces. The results of crystal violet staining are shown in Figure 19 - Figure 21. The experiment was
performed in three replications for ten different surface treatments including, SB: stainless steel disk; SA5C: 50µm CoBlast stainless steel; SA9C: 90µm CoBlast stainless steel; SP5C: 50µm CoBlast-PTFE stainless steel; SP9C: 90µm CoBlast-PTFE stainless steel, TB: titanium disk; TA5C: 50µm CoBlast titanium; TA9C: 90µm CoBlast titanium; TP5C: 50µm CoBlast-PTFE titanium; TP9C: 90µm CoBlast-PTFE titanium.

Figure 19. Crystal violet staining of the biofilm on the metal coupons

Figure 20. Crystal violet OD$_{590}$ of tested coupons for three sets of 10 different sample types
Figure 21. Crystal violet OD$_{590}$ of tested stainless steel (SS) and titanium (TI) coupons for three sets of sample types

Based on the collected data, no significant difference was observed among 10 different treatments. It appears that titanium base materials exhibited better biofilm suppression than stainless steel (Error! Reference source not found.).

4.3. Confocal Laser Scanning Microscopy Data

After bacterial growth on ten different modified surface coupons for 24 hours, the coupons were washed three times with DI water to eliminate the unattached bacteria from the surfaces. The coupons were then placed into glass bottom chambers upside down while using glass coverslips as spacers to elevate the coupons thus preventing direct contact of biofilms to the bottom of the chamber to avoid changing the biofilm structure. Images were taken using CSLM with a 63x objective and oil immersion. For each coupon, 3 image
stacks were taken. One typical image of Z stack series of the biofilm formed on the coupon is shown in Figure 22, and Figure 23 shows a 3D structure of biofilm on the metal coupon surface.

Figure 22. One series of Z stacks of the biofilm formed on the coupon

Figure 23. A 3D structure of biofilm on the metal coupon surface
4.4. Biofilm Thickness and Biomass Analysis

To analyze the average thickness and biomass of biofilm formed on coupon surfaces, multiple laser confocal image stacks were taken and analyzed using COMSTAT software. The data is shown in Table 3.

Table 3. Wide-field fluorescence and laser confocal images

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<tr>
<td>TP5C</td>
<td><img src="TP5C.png" alt="Image" /></td>
<td>TP9C</td>
<td><img src="TP9C.png" alt="Image" /></td>
</tr>
<tr>
<td>SA9C</td>
<td><img src="SA9C.png" alt="Image" /></td>
<td>TA5C</td>
<td><img src="TA5C.png" alt="Image" /></td>
</tr>
<tr>
<td>SB</td>
<td><img src="SB.png" alt="Image" /></td>
<td>TB</td>
<td><img src="TB.png" alt="Image" /></td>
</tr>
</tbody>
</table>
To enumerate the average biofilm thickness and biomass on different coupon surfaces, the wide-field fluorescence images taken using Nikon AZ100 epi-fluorescence microscope were analyzed using the ImageJ software. The results are shown in Table 4.

Table 4. COMSTAT analysis of biofilm thickness and biomass

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Average Thickness (µm)</th>
<th>Biomass (µm³/µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB</td>
<td>3.67705</td>
<td>1.88372</td>
</tr>
<tr>
<td>SA5C</td>
<td>9.00548</td>
<td>2.43798</td>
</tr>
<tr>
<td>SA9C</td>
<td>5.92908</td>
<td>3.64167</td>
</tr>
<tr>
<td>SP5C</td>
<td>1.437513</td>
<td>0.28256</td>
</tr>
<tr>
<td>SP9C</td>
<td>6.98783</td>
<td>1.75675</td>
</tr>
<tr>
<td>TB</td>
<td>5.73802</td>
<td>1.92333</td>
</tr>
<tr>
<td>TA5C</td>
<td>5.09406</td>
<td>1.22100</td>
</tr>
<tr>
<td>TA9C</td>
<td>5.18069</td>
<td>0.74439</td>
</tr>
<tr>
<td>TP5C</td>
<td>10.86349</td>
<td>2.35303</td>
</tr>
<tr>
<td>TP9C</td>
<td>8.39567</td>
<td>1.28102</td>
</tr>
</tbody>
</table>

From the collected data, different surface treatments showed different amounts of biofilm formed on them. These results are based on one set of 10 different surface treatments. No specific trend between the treatments was observed. In terms of preventing biofilm formation, it seems that SP5C showed better results than the other sample types.
There were less biofilm thickness and biomass observed on this sample type. The sample type with the highest biofilm thickness observed was TP5C and the sample type with the most biofilm biomass was SA9C.

4.5. Viable Cell Count Data

Evaluation of modified implant surfaces for their anti-biofilm properties was performed by viable cell count method; Three sets of 22 different modified implant surfaces including stainless steel and titanium with and without vancomycin deposition were tested. No significant advantages against biofilm formation on any specific modified surfaces have been observed EXCEPT those with vancomycin modified surfaces where no viable bacteria were recovered. Viable counts of biofilm recovered from various surface-modified stainless steel and titanium coupons for three complete sets are shown in Figure 24 and Figure 25. Biofilms recovered on unmodified, 50µm CoBlast, 90µm CoBlast, 50µm CoBlast-PTFE, 90µm CoBlast-PTFE, chitosan and vancomycin modified stainless steel or titanium surfaces are consistent with previous reported results. In addition, no viable cells were recovered from vancomycin modified stainless steel or titanium surfaces. Results from low inoculum (10^3 cells per coupon) and short duration (24 hours) indicate that chitosan modified surfaces do not provide any advantages in preventing biofilm formation by S. aureus strain Seattle 1945. In order to assess whether chitosan modified surfaces provide advantage in preventing biofilm formation during long-term-exposure, we conducted a 7-day exposure study where titanium, chitosan-modified titanium and vancomycin titanium coupons were incubated in growth media inoculated with 10^3 cells
for 7 days. No significant difference in the number of cells recovered from the two coupon
types were observed. (Figure 26). For both experiments ANOVA analysis with SPSS
software of the collected data did not show significant difference between different surface
treatments (α=0.05).

Figure 24. Cultivation and quantification of biofilm on SS and Ti coupon surfaces by
viable cell count method

Figure 25. Viable counts of biofilm recovered from three sets of 22 different metal
surface treatments in log scale
Figure 26. Viable counts of biofilm recovered from three sets of three different metal surface treatments after 7 days incubation time

4.6. Validation of the Methods

To validate the biofilm quantifying methods, round shape glass coverslips were used with three different quantifying methods, i.e., crystal violet staining, laser confocal microscopy and viable cell count as previously described for the metal coupons. These experiments were conducted to determine whether the used methods are reproducible and consistent in terms of analyzing the amount of biofilm formed on the coupons. Results are showed in Figure 27 - Figure 32 and Table 5.
Figure 27. OD results of crystal violet test for three glass coverslip samples and control without bacteria

Figure 28. OD results of crystal violet test for average of three glass coverslip samples and control without bacteria
Figure 29. One Z stack of LCSM of the biofilm formed on the glass coverslip

Table 5. COMSTAT analysis of biofilm thickness and biomass on glass coverslips

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Average Thickness (µm)</th>
<th>Biomass (µm³/µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>3.87302</td>
<td>1.58352</td>
</tr>
<tr>
<td>Sample 2</td>
<td>3.28549</td>
<td>1.37056</td>
</tr>
<tr>
<td>Sample 3</td>
<td>4.90568</td>
<td>2.23520</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 30. COMSTAT analysis of biofilm thickness and biomass on glass coverslips

Figure 31. Viable cell count results of three glass coverslip samples and control
Figure 32. Average of viable cell count results of three glass coverslip samples and control

To validate the consistency and reproducibility of the methods biofilm was grown on the glass coverslips and analyzed using three quantification methods to evaluate biofilm formed on the metal coupons. The results showed that the biofilm formed successfully on the glass coverslips Figure 29. Quantification of biofilm formed on the glass coverslips showed the consistency and reproducibility of the used methods. All the data falls within the range of quantified biofilm of the metal coupons. In the crystal violet test the OD results of three glass coverslips were in the range of (0.398333 - 0.525) with standard deviation of 0.065509. The viable cell count for total cells recovered from the glass coverslips result fell within (7.59E+05 - 1.21E+06 cells) with standard deviation of 231902.7. CLSM data showed the biofilm thickness range of (3.28549 - 4.90568 µm) and standard deviation of 0.820223, biomass evaluation of biofilm formed on the glass coverslips were within (1.37056 - 2.2352 µm3/µm2) with standard deviation of 0.450489. All in all, the obtained
data for each different method are close (based on small standard deviation that indicates the data points are close to the mean, and 90% of the obtained data fall within the standard deviation) and this demonstrates the consistency and reproducibility of the methods.

4.7. MIC Results

The MIC of chitosan against *S. aureus* Seattle 1945 has been determined to be 790 µg/ml, and the MIC of vancomycin against *S. aureus* Seattle 1945 has been determined to be 1.22 µg/ml. A typical result of MIC test is shown in Figure 33.

![Figure 33. A typical microtiter plate result of MIC test](image)

4.8. Vancomycin Growth Inhibition Test and Release Rate

Since vancomycin modified surfaces exhibited significant anti-biofilm properties, we evaluated the release rate of vancomycin from metal coupons to assess whether the amount of vancomycin deposited on modified implant surfaces would be sufficient to maintain the anti-biofilm property for 7 days. In one experiment, seven vancomycin discs of the same type (TBV) were tested for 7 consecutive days to determine whether the amount of
vancomycin remained on the discs at different time periods was sufficient at preventing biofilm formation on the coupons.

Table 6. Viable cells recovered from vancomycin modified surface at each 24-hour time points

<table>
<thead>
<tr>
<th>Disc Type</th>
<th>Time (hour)</th>
<th>Viable Count (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBV-1</td>
<td>0-24</td>
<td>Not detected</td>
</tr>
<tr>
<td>TBV-2</td>
<td>24-48</td>
<td>5.18E+05</td>
</tr>
<tr>
<td>TBV-3</td>
<td>48-72</td>
<td>2.59E+06</td>
</tr>
<tr>
<td>TBV-4</td>
<td>72-96</td>
<td>4.59E+05</td>
</tr>
<tr>
<td>TBV-5</td>
<td>96-120</td>
<td>4.26E+05</td>
</tr>
<tr>
<td>TBV-6</td>
<td>120-144</td>
<td>1.83E+06</td>
</tr>
<tr>
<td>TBV-7</td>
<td>144-168</td>
<td>5.01E+05</td>
</tr>
</tbody>
</table>

As shown in Table 6, the amount of vancomycin remained on TBV discs in the period of 0-24 hours in 600 µl TSB media was sufficient in preventing biofilm formation on the disc surface while the amount of vancomycin remained on TBV surfaces in the period of 24-48, 48-72, 72-96, 96-120, 120-144 and 144-168 hours was not sufficient in preventing biofilm formation on the disc surface. To determine the release-rate of vancomycin and whether vancomycin contained in the leachates from individual modified surfaces was sufficient to inhibit the growth of test bacteria *Staph. aureus*, leachates from individual
discs collected at different time points were analyzed for their antimicrobial properties and vancomycin contents. Results are shown in Figure 34 and Table 7.

Figure 34. The antimicrobial property of leachates from 6 vancomycin-surface-modified discs at different 24-hour time points

Table 7. The antimicrobial property of leachates from 6 vancomycin-surface-modified discs at different 24-hour time points

<table>
<thead>
<tr>
<th>Time/Disc Type</th>
<th>SBV</th>
<th>SP5CV</th>
<th>SP9CV</th>
<th>TBV</th>
<th>TP5CV</th>
<th>TP9CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>No Growth</td>
<td>No Growth</td>
<td>No Growth</td>
<td>No Growth</td>
<td>No Growth</td>
<td>No Growth</td>
</tr>
<tr>
<td>Day 2</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>Day 3</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>Day 4</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>Day 5</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>Day 6</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>Day 7</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Growth</td>
</tr>
</tbody>
</table>
As shown in Table 7, the amount of vancomycin released from 6 different vancomycin-modified discs in the period of 0-24 hours into 600 µl TSB medium was sufficient in preventing the growth of *Staph. aureus* inoculated while the amount of vancomycin released during 24-48, 48-72, 72-96, 96-120, 120-144, 144-168 hours was not sufficient in preventing the growth of *Staph. aureus* inoculated.

Table 8. Concentration of vancomycin released from six different vancomycin-modified surfaces during a 7-day study

<table>
<thead>
<tr>
<th>Time/Coupon Type</th>
<th>SBV</th>
<th>SP5CV</th>
<th>SP9CV</th>
<th>TBV</th>
<th>TP5CV</th>
<th>TP9CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>10.997 (ppm)</td>
<td>4.904</td>
<td>7.886</td>
<td>5.180</td>
<td>7.921</td>
<td>4.841</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.245</td>
<td>0.0595</td>
<td>0.098</td>
<td>0.087</td>
<td>0.102</td>
<td>0.123</td>
</tr>
<tr>
<td>Day 3</td>
<td>BDL(^1)</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>Day 4</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>Day 5</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>Day 6</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>Day 7</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
</tr>
</tbody>
</table>

\(^1\) Below Detection Limit
As shown in Table 7, Table 8 and Figure 35, SBV appeared to release the highest amount of vancomycin among the coupon types tested and the majority of vancomycin was released during the first 24 hours.

![Graph showing vancomycin release from SBV coupon in the first and second 24 hours.](image)

**Figure 35.** Vancomycin released from SBV coupon in the first and second 24 hours

As it is shown in Figure 35, most of vancomycin is released in the first 24 hours of coupon exposure to the medium. So, coupons with thicker coatings of the chitosan and vancomycin (five new variations) were fabricated in an effort to extend the release of vancomycin. The amount of vancomycin deposited on the coupons was increased, but the density of vancomycin within the co-deposited chitosan remained the same. The same leachate experiments were conducted to see whether the release rate of the new
vancomycin samples is different from the old samples. Based on the results of experiments, we can conclude that vancomycin-modified surfaces exhibited the best anti-biofilm and antimicrobial properties. However, the majority of vancomycin was released from the modified surface during the first 24 hours. We have modified the vancomycin deposition process to produce vancomycin-modified surfaces with higher concentration of vancomycin and repeated the evaluation process to select for the final candidates for testing in the animal models. Since vancomycin modified surfaces exhibited significant anti-biofilm properties, we evaluated the release rate of vancomycin from to assess whether the amount of vancomycin deposited on modified implant surfaces would be sufficient to maintain the anti-biofilm property for 7 days. In our previous experiments, we observed that most of the vancomycin in different sample types was released within the first 24 hours. In order to prolong the release of vancomycin and to enhance the antimicrobial activity, implants modified with 1, 3, 6, 9, or 12 layers, and another one deposited under high-power (HP) setting have been prepared and evaluated. We have examined the vancomycin release rate of different TP5C samples coated with various layers of vancomycin. During HPLC analysis of vancomycin TP5C samples with different layers of vancomycin, we have learned that the TSB medium containing the vancomycin leachate interact with the HPLC column matrix and interfere with the detection. The release rate of vancomycin was repeated using water as the sample matrix. The results are shown in Table 9 and Figure 36.
Table 9. Concentration of vancomycin released from different vancomycin-modified TP5C surfaces with different layers of vancomycin during a 4-day study in water matrix

<table>
<thead>
<tr>
<th>Day/Vancomycin layers</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>HP-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repetition</td>
<td>1  2  3</td>
<td>1  2  3</td>
<td>1  2  3</td>
<td>1  2  3</td>
<td>1  2  3</td>
<td>1  2  3</td>
</tr>
<tr>
<td>Day 1</td>
<td>9.55 11.33 7.56</td>
<td>19.91 16.58 17.87</td>
<td>16.43 19.42 28.62</td>
<td>74.645 70.01 77.57</td>
<td>96.21 79.395 119.415</td>
<td>41.155 74.58 37.94</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.24 0.314 0.311</td>
<td>0.361 0.731 0.575</td>
<td>0.505 0.549 1.169</td>
<td>1.789 1.697 2.143</td>
<td>1.506 3.135 4.101</td>
<td>0.738 1.675 0.937</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.116 0.141 0.146</td>
<td>0.148 0.142 0.135</td>
<td>0.137 0.146 0.172</td>
<td>0.179 0.172 0.171</td>
<td>0.191 0.238 0.209</td>
<td>0.156 0.218 0.156</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.111 0.116 0.113</td>
<td>0.126 0.119 0.12</td>
<td>0.117 0.124 0.132</td>
<td>0.124 0.12 0.124</td>
<td>0.124 0.139 0.129</td>
<td>0.129 0.146 0.126</td>
</tr>
<tr>
<td>Sum</td>
<td>10.017 11.901 8.13</td>
<td>20.545 17.572 18.7</td>
<td>17.189 20.239 30.093</td>
<td>76.737 71.999 80.008</td>
<td>98.031 82.907 123.854</td>
<td>42.178 76.619 39.159</td>
</tr>
</tbody>
</table>

Based on the above analysis we can conclude that majority of the vancomycin on different implant surface was released within the first 24 hours, 1, 3 and 6 layers are significantly different from 9 and 12, but they are not significantly different from each other and HP. 9 and 12 are not significantly different from each other and HP was just significantly different from 12, but not from 1, 3, 6 and 9 layers. The amount of vancomycin released from 9 and 12 layers within the second 24 hours was also considerable. ANOVA analysis was done with SPSS software for different surface treatments (α=0.05).
Figure 36. Concentration of vancomycin released from TP5C vancomycin-modified surfaces with different layers of vancomycin on the surfaces during a 4-day study, $P \leq 0.05^*$
CHAPTER 5
DISCUSSION

Biofilm formation on the surface of orthopedic implants can cause serious problems for patients, especially for patients with war injuries as their wounds are open and biofilm formation bacteria such as *S. aureus*, the most common pathogen associated with orthopedic implant infections, can enter the body and establish biofilm structure on the implant surface. Chronic infection, delay in the treatment, and revision surgeries to remove the implant are problems that can be prevented if the biofilm formation is inhibited on the implant surfaces. Once the biofilm is formed it becomes resistant to antibiotics. The protective mechanisms in biofilms against antibiotics are not completely understood, but are hypothesized to be influenced by reduced penetration ability due to biofilm structure, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells (P. S. Stewart 2002), (Høiby 2010). In this thesis work, application of PTFE for its anti-adherent properties, chitosan for its antibacterial effects and its ability to act as a carrier molecule for vancomycin was studied. PTFE coatings have been shown to reduce the bacterial adhesion on medical devices in clinical studies (Berry 2000), (A. E. Demling 2010). In this study, we did not observe significant difference between PTFE coated and non-PTFE coated surfaces. There are other studies with the same finding (Fuchslocher Hellemann 2013). Although PTFE is a biocompatible, nontoxic compound that provides a hydrophobic surface for clinical devices, some studies have shown that proteins adhere quickly to PTFE surfaces (Müller 2009). There are proteins in the surface of the bacteria and therefore they can attach to the PTFE surfaces. These results contradict with some
clinical findings indicating that biofilm formation is reduced on anti-adherent PTFE coated surfaces (A. E. Demling 2010). Our findings did not show any significant advantage of PTFE coated surfaces in terms of biofilm prevention. This could be due to hydrophobic characteristic of *S. aureus* bacteria (Reifsteck 1987), and since hydrophobic bacteria prefer hydrophobic materials, application of PTFE in this case has a negative impact. The other compound used in this study as the surface coating was chitosan, a biocompatible, biodegradable natural polysaccharide with antibacterial properties. The MIC of chitosan in this study was determined to be 790 µg/ml. Thus, the chitosan used should prevent bacterial growth at this and higher concentrations. Results obtained from using different biofilm quantifying methods in this study did not demonstrate any significant advantage of using chitosan as a biofilm inhibitor. There are various factors that could influence the results. The fact that chitosan is not water soluble and it dissolves in low concentration of acetic acid (1.0M) while the *in vitro* studies were conducted in water-based TSB media thus, the absence of significant biofilm inhibitory contributed by chitosan could be due to low its solubility in test media. The other possibility is that the concentrations of chitosan on the coupons were not enough to prevent bacterial growth in 600 µl of TSB media (our experiments’ condition). It should be considered that chitosan was used as a carrier molecule for the antibiotic as well. Vancomycin was the antibiotic used in this study, which has been shown to be effective against *Staphylococcus aureus* (C. B. Liu 2011). Vancomycin modified surfaces successfully prevented biofilm formation on the implant surfaces. The results from three biofilm quantifying methods showed the complete prevention of bacterial growth on the implant surfaces. LCSM scanning of the modified
surfaces with vancomycin did not detect any GFP signal from these coupons, while for the other surface treatments the biofilm formed all over the coupon surfaces. No bacterial cell was recovered from the vancomycin treated surfaces. Crystal violet is a quick and simple method best for initial analysis of the modified surfaces. It is sensitive to pipetting and evaporation of the liquids and thus, not suitable when evaluation of higher number of samples are needed, e.g., more than 10 samples. For 22 treatments and three replications of each, keeping the experiment condition the same for all the samples became challenging and not feasible. For these reasons, we switched to using viable cell count method in subsequent experiments, which is a more accurate method for quantification of biofilms. LCSM was the other method used to evaluate biofilm formation. This method enabled us to exam the biofilm structures and biofilm characteristics such as biofilm thickness and biomass. Although the accuracy of the LCSM method has been shown to be considerably better, the problems lie in that it is both time-consuming and costly in comparison with the other two methods. Minimum area of at least 100000 µm² should be imaged to have representative data of the biofilm formed on one surface (Heydorn 2000). Each stack area is about 10000 µm² and it takes 30 minutes to one hour to take the entire Z stacks for one area. With at least 10 different stacks of each sample type to be imaged and 22 different sample types to be evaluated, the required time to complete such task is considerable. Therefore, the viable cell count method was the method of choice for completion of all biofilm quantification analysis and the data was used in combination with the physical property data for selection of the final surface-modified implants for in vivo studies in a rabbit model. However, the number of sample types to be evaluated in animal study will
be limited and LCSM would be a more suitable method of choice for analyzing the recovered implants (explants). The MIC for chitosan for *S. aureus* has been reported in range of 20 µg/ml to 1250 µg/ml (Shanmugam 2016). In this study, it is determined to be 790 µg/ml. We determined the vancomycin MIC to be 1.22 µg/ml, which is within the range of previously reported vancomycin MIC for *S. aureus* (0.5µg/ml- 2 µg/ml) (Moise 2007).

The results of antimicrobial property test of the leachates from 6 vancomycin-surface-modified discs at different 24-hour time points showed that the amount of vancomycin released from 6 different vancomycin-modified discs in the period of 0-24 hours into 600 µl TSB medium was sufficient in preventing the growth of *S. aureus* inoculated while the amount of vancomycin released during 24-48, 48-72, 72-96, 96-120, 120-144, 144-168 hours was not sufficient in preventing the growth of *Staph. aureus* inoculated. It is worth noting that the localized release of vancomycin from implant surfaces into adjacent tissues in vivo could potentially be higher. The release rate of vancomycin samples was tested in vitro by collecting the leachates from different vancomycin surface treatments for one week. HPLC analysis of the collected leachates showed that the majority of vancomycin was released in the first 24 hrs. of coupon exposure to the medium. Therefore, coupons with thicker vancomycin coatings were fabricated and evaluated (3, 5, 6, 9 and 12 layers). The HPLC results of vancomycin samples with more vancomycin layers showed that the majority of the vancomycin on different implant surface was released within the first 24 hours. 1, 3 and 6 layers are significantly different from 9 and 12, but they are not significantly different from each other and HP (5 layers). 9 and 12 are not significantly
different from each other and HP was just significantly different from 12, but not from 1, 3, 6 and 9 layers. The amount of vancomycin released from 9 and 12 layers within the second 24 hours was also considerable. One of the aims of this study is to identify implant core material with specific surface modification that could prevent biofilm formation. *In vitro* analysis showed the combination of biodegradable chitosan and vancomycin is successful in preventing biofilm formation in the first 24 hours. We also know that bacterial attachment is an important stage of biofilm formation (Otto 2008), and the first 6 hours after surgery are critical period for preventing early infection, as the introduced pathogens have not yet begun rapid proliferation (Raphel 2016). The amount of vancomycin released within the first and second 24 hours of the coupon exposure was enough to eliminate 1000 cells (bacterial inoculum used in the experiments) *S. aureus* bacteria in 600 µl TSB. Culturing 100 µl of the leachates after 24 hours of inoculation with bacteria on TSA plates did not show any growth of bacteria, indicating the bacterial cells could not survive in the leachate after 24 hours. Based on data obtained from these *in vitro* studies, TB (as the control), TBV and TA5CV each with 9 layers of vancomycin depositions were selected to further evaluate their performance *in vivo*. The results obtained from *in vivo* studies in rabbits will reveal the effectiveness of the chitosan plus vancomycin implant surface coated.

5.1. Future Work

Based on different *in vitro* anti-bacterial analysis of the modified surfaces and other factors and characteristics that affects biofilm formation on the implants, such as surface
roughness, area, charge and hydrophobicity or hydrophilicity of the surfaces that will be accessed by the bioengineering group, the best treatments will be selected for the animal study. Overall, the goals are to determine the pre-clinical viability of optimized DCP surfaces using short- and long-term *in vivo* bacterially-challenged rabbit fracture models.
REFERENCES


