THESIS

IN VIVO EFFICACY OF ANTIBIOTIC-ELUTING PHOSPHOLIPID COATED IMPLANTS

Submitted by

Thomas Triffo

Graduate Degree Program in Bioengineering

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2011

Master's Committee:

Advisor: Susan James

Nicole Ehrhart
Marvin Criswell

ABSTRACT

IN VIVO EFFICACY OF ANTIBIOTIC-ELUTING PHOSPHOLIPID COATED IMPLANTS

Implant-associated infection can be a serious problem for patients that receive orthopedic implants, such as hip and knee replacements. This is a common cause for early implant loosening, which requires revision surgeries and results in an even greater risk of infection. To address this issue, our lab has developed a novel electrospraying technique for applying phospholipid coatings to orthopedic implants. These coatings consist of two layers of 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), with antibiotic loaded in between layers. *In vitro* tests were performed to evaluate how modifications to these coatings affect coating retention, based on a clinically relevant test, and antibiotic elution from these coatings. Coating retention tests were performed by inserting implants through segments of mouse bone and then examining the implants under SEM. Antibiotic elution was performed using a total sink elution combined with OPA assay for detection of antibiotic. These results showed that the coatings that were retained the most and eluted antibiotic slowest were samples that were pre-treated with calcium and were electrosprayed with a mixture of 6:1 DOPS-to-cholesterol. This coating was selected to be used in an *in vivo* study to determine the efficacy of the coatings in treating osteomyelitis. Osteomyelitis was induced

in a murine model using genetically modified bacteria, which allowed tracking of the infection prior to sacrificing the animals via bioluminescent imaging, a technique that makes use of genetically modified bacteria producing luciferin and luciferase which causes emission of photons. It was observed that antibiotic-eluting implants cleared the infection faster than implants without antibiotic during a 4 week study. Also, no kidney damage was observed based on creatinine, blood urea nitrogen, and urine protein tests. Histology confirmed observations from the bioluminescent imaging.

These results show that our antibiotic-eluting implant coatings were able to reduce infection *in vivo* without resulting in adverse effects. Bioluminescent imaging showed significant reduction of emission of photons, $p < 0.05$, in the antibiotic loaded group compared to the control samples. The results also suggest that the implants exhausted their supply of antibiotic at the end of the study, and in future studies a greater amount of antibiotic will be loaded onto the implants.

ACKNOWLEDGEMENTS

We are grateful to the CSU Cancer Supercluster for providing the funding for this work.

I would also like to extend my gratitude to the many people who helped me along the way. Thank you to my advisor Sue James, for guiding me through this project and sharing her enthusiasm with me. Without Sue I never would have had this opportunity, and for that I'm very grateful. Thank you to my other committee members, Nicole Ehrhart and Marvin Criswell, for sharing their time with me and insights on this work. I'm grateful to Debra Kamstock for providing all of our histological analysis for this study, which was certainly no small order. Thank you to Laura Chubb for her invaluable assistance with the mouse study. I'd also like to thank all my friends and colleagues in the James lab who helped me with this work. And finally I'd like to thank my wife Ashley, for always helping me and constantly asking me questions that made me think about this project in different ways.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 Orthopedic Implants

There are more than 4.4 million people with at least one type of orthopedic internal fixation device, such as bone screws, and implant-associated infection is a serious problem in patients that receive orthopedic implants [1]. Osteomyelitis (bone infection) leads to early loosening of implants, which results in the need for a revision surgery. If the infection goes undetected, the bacteria can form a biofilm, which generally requires much greater amounts of antibiotic to clear than osteomyelitis alone. Furthermore, implant surgeries reduce the amount of native bone, with revision surgeries resulting in even more bone loss and a greater risk of infection. Implant failure likelihood is further increased by poor mineralization at the interface between the implant and the bone [2-4]. Thus there is a need for an orthopedic implant capable of treating implant-associated infection while simultaneously encouraging bone growth onto the implant [5].

1.2 Research Objectives

The goal of this research is to create antibiotic-eluting phospholipid coatings for orthopedic implants to treat osteomyelitis. The performance criteria of these implants are divided into two groups, *in vitro* and *in vivo* criteria. The *in vivo* criteria were: decreased emission of photons during bioluminescent imaging (discussed in section 1.6); decreased inflammation and bacterial colonization scores, as well as increased

reactive bone scores based on histology; non-elevated levels during analysis of serum and urine to assess kidney damage. The *in vitro* criteria were: increased amount of coating remaining on samples after insertion through bone segments; elongated elution profile during total sink elution study.

By increasing the coating retention, the coatings will be able to remain on the implant after insertion which should increase the efficacy at treating osteomyelitis. The longer elution profile *in vitro* is desirable because it decreases the rate at which antibiotic elutes out of the coating, which indicates that antibiotic will be eluting over a longer time course *in vivo*. These elution studies don't accurately reflect how long the antibiotic will elute *in vivo*, but shows that the elution profile can be elongated.

For this study, the reduction in emission of photons and the decreased histology scores are the most important performance criteria. The main goal of this work is to create implant coatings that fight osteomyelitis, and the reduction in the amount of bacteria compared to control groups is the greatest indicator of proper performance of these coatings. It has been shown that phospholipids promote bone growth, and as such the reactive bone scores should be higher for mice with DOPS coated implants versus plain stainless steel implants. Also, the kidney and urine results should not be elevated in the antibiotic loaded group compared to the no-antibiotic controls, indicating that no detectable damage was done to the kidneys.

Antibiotic-eluting implant coatings provide local delivery of antibiotic to the infection site, as opposed to systemic dosing which delivers antibiotic throughout the whole body. The advantage of local delivery is that the antibiotic is only delivered to the

target area, which allows for lower levels throughout the body. This decreases the burden on the kidneys, which would have to filter a greater amount of unused antibiotic in the case of systemic dosing. Systemic doses are administered in large doses over short times, whereas locally delivered antibiotics are delivered in comparatively lower doses and are sustained for a longer period of time.

1.3 Previous James Group Work

Dr. David Prawel developed this e-spraying technique for applying 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) to titanium surfaces [6-9]. The chemical structure of DOPS is shown in Figure 1.

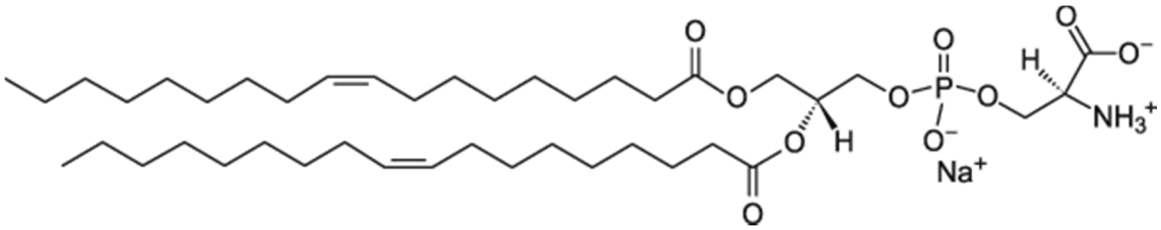

Figure 1: Chemical Structure of DOPS

He discovered that there were many factors that significantly impacted the process, and after many experiments he identified the set of parameters that resulted in what he considered to be ideal coatings. The parameters used were an applied voltage of 12 kV, a syringe pump flow rate of 14 mL/h, a distance from syringe tip to target of 8 cm, and a solution concentration of 20 mM DOPS. It was also observed that the spray time was dependent on the surface area to be sprayed, and for a total surface area of 5

cm^2 the solution was sprayed for 5 minutes. When spraying a different total surface area, the spray time needed to be adjusted accordingly.

In vitro elution studies performed by Dr. Prawel showed that the antibiotic gentamicin dripped onto flat samples eluted very quickly from these coatings. Several experiments showed that the addition of cholesterol to the spray solution resulted in a longer time period for elution, which was desirable to provide therapeutic levels of antibiotic over a clinically relevant time course. It was found by Dr. Prawel that a time course of 10-14 days for antibiotic elution was desired for a clinical application.

Atomic force microscopy (AFM), white light interferometry and scanning electron microscopy (SEM) were used to determine that the e-spray technique can be used to apply coatings that are approximately 8 microns thick per e-spray application layer. AFM and SEM were used by Dr. David Prawel, while a current study is making use of white light interferometry. This thickness could likely be controlled by lengthening or shortening the spray time. The reason for choosing coatings of this particular thickness is the desire to replicate the coatings created by Dr. Prawel. Based on the amount of DOPS sprayed previously to create coatings on titanium, the amount of DOPS was adjusted to provide coatings of the same thickness. Scanning electron micrographs revealed that these coatings are applied uniformly on flat pieces of titanium, and preliminary results suggested that these results are reproducible on 3-D samples. Samples were electro-sprayed twice with phospholipid and the antibiotic gentamicin sulfate (GS) was loaded between the two layers of DOPS. Previous studies of phospholipid coatings prior to Dr. Prawel's work have not attempted to load antibiotic

or other drugs in the coatings, and as such this is an improvement over existing phospholipid coatings. Antibiotic loaded between layers of DOPS has been shown to elute over time, and preliminary evidence has shown the rate of elution can be influenced in a predictable direction by adding cholesterol to the spray mixture [6-9].

1.4 Coating Materials

1.4.1 Phospholipids

It has been shown that phospholipid coatings promote osseointegration *in vivo* into titanium. Studies have shown that phospholipid coatings enhance osteoblast activity and promote mineralization [10-12]. Previous applications of these coatings have been performed using dip and drip coating techniques, which are not conducive to creating uniform coatings on 3-D objects. Also, these techniques result in relatively thick coatings that are not able to resist mechanical stresses without separating from the implant material [13]. Previous studies have led to the conclusion that "thinner coatings need to be applied to ensure the mechanical stability of the implant" [11]. The James Group's electro-spraying (e-spraying) [6] process addresses these issues and improves upon phospholipid implant coatings. DOPS was chosen as the coating material for these implants because it appears to be most effective at enhancing osseointegration [11,14,15].

1.4.2 Calcium and Cholesterol

Dr. Prawel's work focused initially on just e-spraying DOPS onto titanium. Subsequent modifications to this original coating include pre-treating the metal surface

with calcium, and including cholesterol in the coating spray. The purpose of these modifications was to elongate the elution curve and provide better coating retention.

Calcium was included because it has been shown that phosphatidylserine binds to calcium during biomineralization. IR analysis has been used to show that calcium ions ionically bond to the phosphate group of phosphatidylserine [16,17]. The purpose of pre-treating the surface with calcium was to populate the surface with calcium ions, which were believed to be bound to the oxide surface of the metal, and then upon e-spraying samples that the calcium would also bind to the DOPS, creating an ionic bond holding the DOPS on the metal.

Cholesterol was added to the spray blend to create coatings that have a longer elution profile. It was believed that cholesterol would elongate the elution profile because it results in tighter packing of phospholipid films [18]. Cholesterol also has been shown to play a role in stabilizing phospholipids [19]. Thus, it was believed that cholesterol would create a coating that eluted antibiotics more slowly.

1.5 Gentamicin Sulfate

1.5.1 Structure and Function

Gentamicin is an aminoglycoside antibiotic that is shown to be effective at treating infections caused by gram-positive bacteria such as *Staphylococcus* [20]. The structure of this antibiotic is shown in Figure 2.

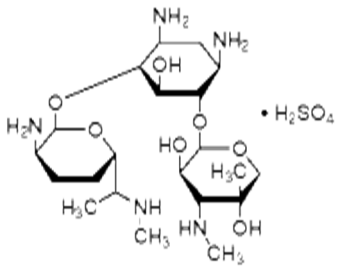

Figure 2: Chemical Structure of Gentamicin Sulfate

This class of antibiotics act on bacteria by being taken up into the bacterial cells and binding to 16S rRNA [21,24]. This binding disrupts the ribosomes, disrupting translation of proteins [21-24]. Gentamicin is removed from the body exclusively by the kidneys, so dosage must be carefully regulated to avoid nephrotoxicity [25]. The minimum inhibitory concentration of gentamicin is 2 μg/mL, and nephrotoxicity occurs above a concentration of 10 μg/mL [26,27]. For local delivery of antibiotic, the desired elution profile is one in which there is an initial burst of antibiotic that creates a concentration at the upper limit of MIC in the tissue, which slowly decreases (but stays above MIC) for 10-14 days.

Staphylococcus Aureus is one of the most common pathogens found in orthopedic infections [28], and for this reason it was used to induce osteomyelitis in mice. As previously stated, gentamicin is an aminoglycoside antibiotic. This class of antibiotics are highly effective against methicillin-resistant pathogens frequently observed in orthopedics, such as *Staphylococcus Aureus*. For these reasons, gentamicin was chosen as the antibiotic for this work.

1.5.2 Detection of Gentamicin

Gentamicin is not a fluorometric molecule, which makes it difficult to detect in solution. There are many techniques to assay for gentamicin, the most common being enzyme-linked assays or high-performance liquid chromatography (HPLC) [29-31]. Enzyme-linked assays are cost prohibitive, which does not make them ideal for performing large numbers of tests. HPLC is a very time-consuming process, making it inconvenient for examining a large number of samples. For detecting gentamicin in a large number of samples, such as would occur during an elution study, a more reasonably priced alternative technique is desirable. Several fluorometric techniques have been developed to address these issues [32,33].

Frutos *et al* reported a fluorometric technique that allows for gentamicin to be detected in solution. Since gentamicin is not a fluorometric molecule, it must first be derivatized with a fluorometric molecule before it can be detected. This technique uses ninhydrin to derivatize gentamicin. This derivitization has been used previously to identify antibiotics that contain primary amines. The ninhydrin complexes with the gentamicin at the primary amine groups, and this complex is able to be detected at wavelengths between 330-400nm. This technique created a reagent containing, among other things, o-phthaldialdehyde. The phthaldialdehyde reagent is mixed with the gentamicin-containing sample and isopropanol in equal parts. This results in formation of a complex with gentamicin that can be detected in the previously described range of wavelengths. For large numbers of samples, this assay is an ideal choice, providing reproducibility with a relatively quick and simple method [33].

One major drawback to this assay is that the complex forms with any molecule that contains a primary amine, not just gentamicin. This is problematic because the phospholipid used to create implant coatings in this study contains a primary amine group, and as the coating comes off during elution it can interfere with the GS assay (i.e., the assays indicates there is more GS than in reality because the reagent is binding to the DOPS). Given that more specific techniques have been shown to be prohibitive for various reasons, it is desirable to find a technique that will separate phospholipid and gentamicin in solution.

Bligh and Dyer developed a lipid extraction technique that makes use of the unique ternary behavior of the mixture of water, methanol, and chloroform as shown in Figure 3.

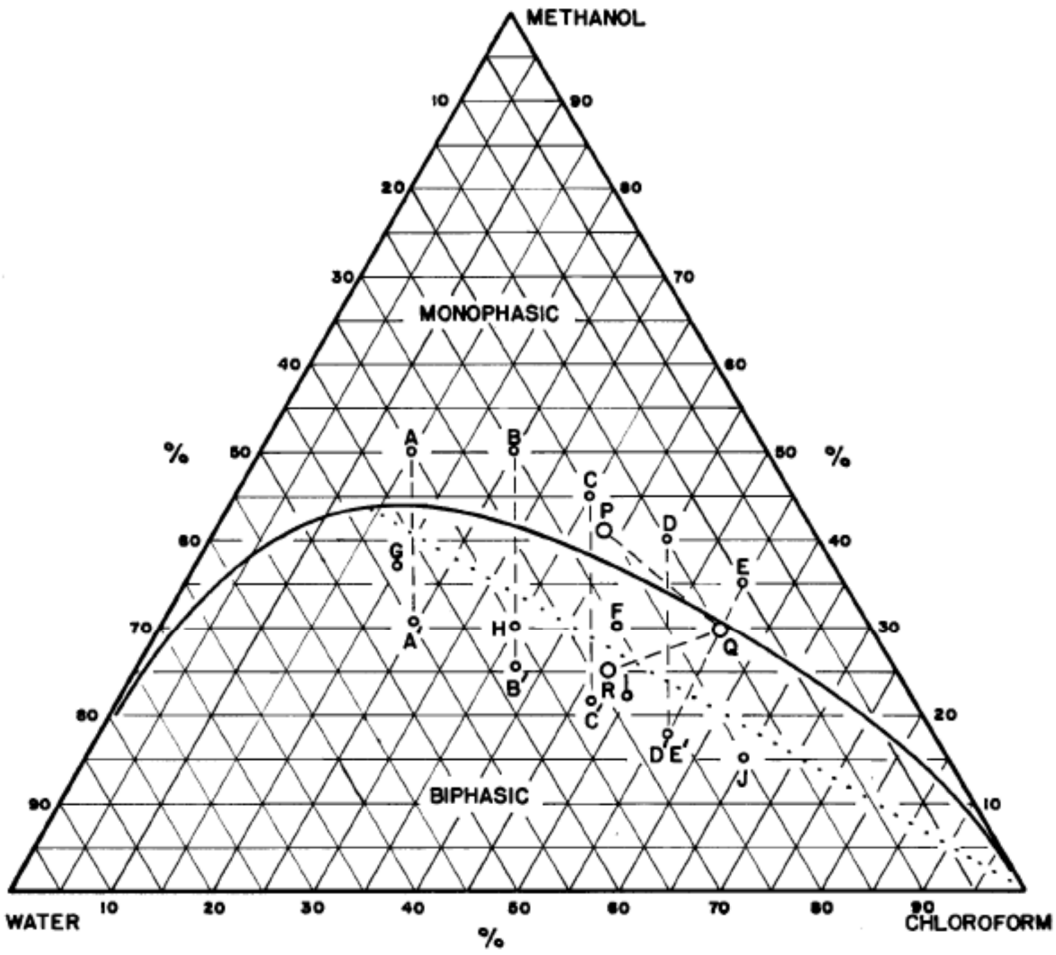

Figure 3: Ternary Phase Diagram for Water, Methanol, and Chloroform[34]

By manipulating the volume fractions of the three chemicals, they were able to create a system that is one liquid phase. This allows for the chloroform and water to mix, and lipids from the water to dissolve into the chloroform where it is far more soluble. Then further manipulation of the volume fractions results in a two liquid phase system, with the chloroform solution containing the lipids on the bottom and the aqueous solution on top. Any molecules that are not soluble in chloroform, such as gentamicin, will

remain in the aqueous phase. Lipid molecules, which are not very soluble in water, will transfer to the chloroform [34].

Gubernator et. al. made use of this extraction to separate amino containing lipids from gentamicin in solution. They examined several different phospholipids that contained primary amine groups, and their results showed that they were able to successfully remove the phospholipids from their gentamicin solution [35]. Including this extraction technique allows the assay developed by Frutos et. al. to accurately quantify the amount of gentamicin eluting from the DOPS implant coatings.

1.6 Bioluminescent Imaging

Bioluminescent imaging is a technique that allows bacteria to be tracked in live animals over time. Traditional animal studies require subjects to be sacrificed at time points during the study. Bioluminescent imaging allows for data collection during the full course of the study before sacrificing any subjects. This technique utilizes genetically modified bacteria that express genes that encode for the enzyme luciferase and its substrate luciferin. The oxidation of luciferin by luciferase results in the emission of photons. These photons are emitted through the tissues of the animals, and are detected by a live imaging camera, in our case a Xenogen IVIS 100 (Caliper Life Sciences, Hopkinton, MA). Then using software from Xenogen the image can be manipulated to isolate the region emitting photons and information can be obtained regarding the number of photons being emitted, which is proportional to the amount of bacteria present. Most bacterial cells do not express these proteins, so when using this technique only the bacteria of interest are detected [36]. Many researchers have used

this technique to track bacteria *in vivo*. Researchers have used luciferase transfected bacteria in a variety of applications [37,38], including investigating the ability of osteomyelitis (bone infection) to suppress osteosarcoma [39,40].

The goal of this work was to test the efficacy of implant coatings created with this novel e-spray technique *in vivo*. Prior to *in vivo* work, it was important to determine how modifications to DOPS coatings impacted coating performance with respect to coating retention and drug elution. Investigation of coating retention provided an *in vitro* evaluation of whether or not the coating would remain on the implant when subjected to clinically relevant forces. It was hypothesized that pre-treating the surface with calcium chloride would result in greater coating retention. An in *vitro* elution study was performed to determine how pre-treating the surface with calcium chloride, as well as the addition of cholesterol to DOPS coating, impacted drug elution. It was hypothesized that calcium chloride pre-treatment would not impact drug elution, while the addition of cholesterol would result in the antibiotic eluting over a longer period of time. The *in vivo* study allowed for evaluation of the efficacy of these antibiotic loaded implants in treating osteomyelitis. It was hypothesized that animals that received an implant loaded with antibiotic would show the greatest reduction of infection compared to DOPS only and stainless steel implants at the end of the study. Also, it was hypothesized that antibiotic loaded implants would release antibiotic over a longer period of time *in vivo* than *in vitro.*

CHAPTER 2: MATERIALS AND METHODS

2.1 Electrospray Technique

2.1.1 Sample Preparation

Before applying DOPS coatings to the metal, stainless steel samples were cleaned and passivated. Briefly, cleaning was performed by sonicating samples in acetone and deionized water. Passivation was performed by bathing samples in 50% nitric acid heated to 71^{o}C for 30 minutes. These samples were then rinsed in deionized water and exposed to lab air for a minimum of 18 hours.

There was evidence from previous work that treating the surface of samples with calcium chloride improved adhesion of the coating. Poor adhesion was a problem noticed during previous *in vitro* cell studies [9]. Stainless steel samples were treated with calcium chloride after they were passivated. Briefly, the samples were bathed in a solution of 2.25 mM calcium chloride in deionized water, which was heated to 50^{o}C, for 1 hour. The samples were then removed from the solution and rinsed 3 times with deionized water, then stored in a desiccator.

There was also evidence from prior work that including cholesterol in the DOPS solution would help increase the time over which the antibiotic would elute from the coating (i.e., would slow down elution). This hypothesis was tested in a 2-week elution study, which is described in detail in a subsequent section. The cholesterol was mixed in with the DOPS in chloroform. The cholesterol was used in either 3:1 or 6:1 ratios of

DOPS-to-cholesterol. Coatings were characterized by scanning electron microscopy (SEM). Before placing samples in the SEM, 10nm of gold was applied to the surface of all samples using an Anatech Hummer VII gold coater (Anatech Ltd., Battle Creek, MI). Samples were placed onto the sample holder using copper tape to secure the ends of the samples to the base of the holder. The SEM used to study samples was a JEOL 6500F scanning electron microscope (JEOL Ltd., Akishima, Tokyo, Japan).

2.1.2 Spraying 2-D Samples

The technique used to apply coatings onto the implants was electrospraying. Briefly, electrospraying is a process that uses a voltage difference to spray a material onto a conductive target. The samples are loaded onto a mounting board, which conducts the voltage into the samples to be sprayed. A syringe containing the spray solution is placed into a syringe pump, which is placed the desired distance from the target. Alligator clips connected to a power supply are hooked up to the syringe and samples as shown in Figure 4.

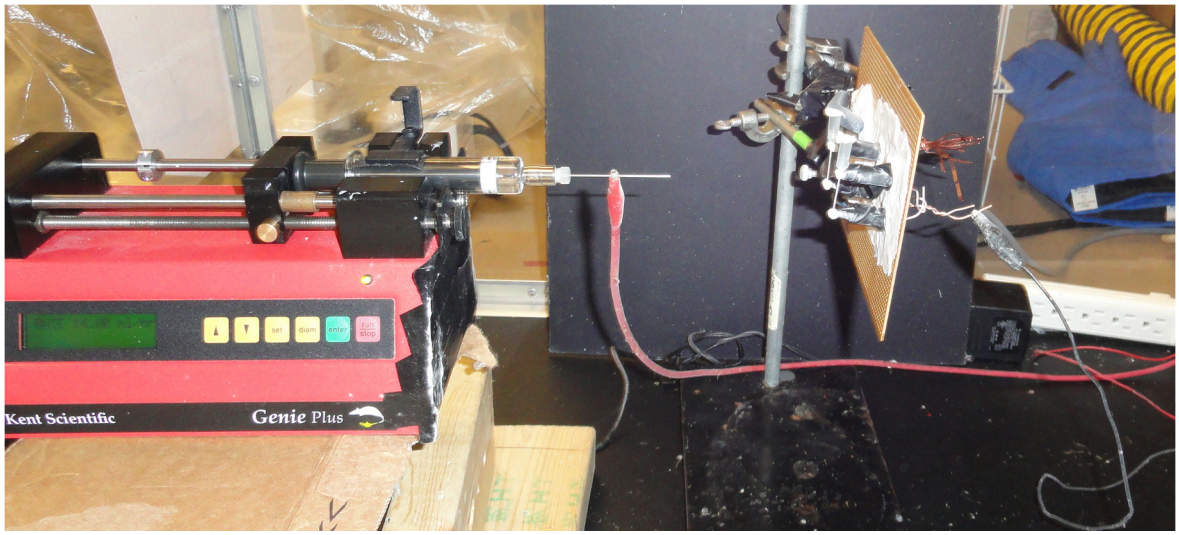

Figure 4: E-spray Apparatus

The syringe pump is set to pump at a desired rate to produce small liquid drops, which become charged by the power supply. The charged liquid droplets, when subjected to an applied electric field above a stability limit known as the Rayleigh threshold, disperse and a fine aerosol spray is carried by the electric field to the target. In this particular application the carrier liquid is chloroform, and as the chloroform is dispersed by the electric field it evaporates, resulting in deposition of only the DOPS on the target samples. This technique is superior to line of sight techniques for spraying 3-D samples because the applied electric field deposits the material on the target along the path of least resistance, which allows the material to wrap around the sample and deposit material uniformly around the sample [9,42]. Previous work has shown that DOPS can be e-sprayed onto titanium. Previous work found the critical parameters for spraying DOPS onto titanium, and these were used as a guideline for spraying DOPS onto stainless steel. For the current work, stainless steel needles were implanted in mice, and so the spray parameters needed to be optimized for stainless steel. Stainless steel squares, type 316/316L rolled stainless steel, 26 gauge, purchased from www.onlinemetals.com, with a 0.5cm side were used for these tests, and 20 samples were sprayed at a time with a total spray time of 5 minutes. Samples were held in contact with the electrodes with UHU tac, a removable adhesive putty manufactured by Saunders. Using the parameters for titanium as a starting point, flat stainless steel samples were sprayed at voltages of 12, 14, and 16kV, while the other parameters were left unchanged. The reason for only changing the voltage was because stainless steel is not as conductive as titanium, so it made sense to try spraying at higher voltages, and

due to the number of possible parameters which could be varied, it was decided that changing only one parameter at a time would be a good strategy to see how changes impacted the coatings. A solution of DOPS in chloroform was prepared in a concentration of 20mM, which was then sprayed at the samples. To optimize the spray parameters, flat stainless steel samples were used. Samples were sprayed two times, allowing sufficient time to dry in between. Some samples were loaded with gentamicin sulfate in between the coatings. These samples were prepared to show that DOPS could be applied on top of GS to form an antibiotic loaded coating. The technique for applying GS depended on the sample used. Flat samples had the antibiotic solution dripped on and then were allowed to dry. Scanning electron microscopy was used to examine coatings, which were compared to coatings on titanium samples. It was found that the voltage that resulted in coatings similar to those observed on titanium was 14kV. Flat samples were only used for comparing coating created using different voltages; all other tests were performed using 3-D samples. Flat samples only received DOPS only coatings.

2.1.3 Spraying 3-D Samples

The implants for the *in vivo* study were stainless steel needles, 22 gauge Kendall Monoject Hypodermic Needles with Polypropylene Hub, which are 3-D objects as opposed to the 2-D squares used to optimize the spray parameters. Stainless steel needles were sprayed two at a time for a total spray time of 45 seconds. These needles were held in place by two alligator clips, one at each end, and sprayed from one

direction without rotating the needle during the process of electrospraying, as shown in Figure 5.

Figure 5: Mounted Samples to be sprayed

To prevent the phospholipid solution from coating the inside of the needle UHU tac (Saunders Mfg. Co., Readfield, ME) was stuck to both open ends of the needle, and this was also placed over the alligator clips holding the needles in place to prevent the phospholipid solution from coating the metal on the alligator clips. This resulted in small sections at the ends of the needle that were not coated in phospholipid. 3-D samples were used for all experiments except for determining the spray parameters. Stainless steel needles had antibiotic applied by the aerosol spray technique, which is described in detail in the next section.

Table 1 provides a list of samples used in different experiments, along with the parameters examined in each study.

Table 1: Samples and Treatments for Different Experiments

Experiment	**Geometry**	**Spray Blend**	**Calcium Pre-Treatment**	**Calcium Post-Treatment**	**Sample Size (per trt)**	**Treatment Groups**
E-Spray Parameters	2-D and 3-D	DOPS	No	No	3	3
Aerosol Spray	3-D	GS in PBS	N/A	N/A	3	14
Coating Retention	3-D	DOPS (1 trt) and DOPS + cholesterol (2 trts)	Yes (2 trts) and no (2 trts)	Yes	3	4
In Vitro Elution, without extraction	3-D	DOPS (4 trts) and DOPS + cholesterol (6 trts)	Yes (6 trts) and no (5 trts)	No	7	11
In Vitro Elution, with extraction (GS Loaded Samples)	3-D	DOPS (2 trts) and DOPS + cholesterol (3 trts)	Yes (3 trts) and no (2 trts)	No	7	5
In Vitro Elution, with extraction (no GS controls)	3-D	DOPS (2 trts) and DOPS + cholesterol (3 trts)	Yes (3 trts) and no (2 trts)	No	1	5
Bioluminescent Imaging	3-D	DOPS + cholesterol	Yes	No	10	3
Histology	3-D	DOPS + cholesterol	Yes	No	5	3
Serum and Urine	3-D	DOPS + cholesterol	Yes	No	5	3

The treatments for these experiments will be discussed in further detail in subsequent sections.

2.2 Aerosol Spray Technique

Application of GS on stainless steel needles was more difficult than on flat samples. Dripping the antibiotic onto the samples would not provide an even distribution on the needle surface, and could potentially wash the coating off the needle. To avoid these potential complications, an aerosol spray technique to apply phospholipid to titanium developed in our lab was adapted to spray GS onto these samples. In short, an artist's airbrush, from the Central Pneumatic 47791 Airbrush Kit purchased from Harbor Freight Tools, was attached to an air compressor with a digital pressure control gauge, which was placed a short distance from the target samples. Samples were sprayed two at a time in short bursts, and the needle was rotated 90^{o} after each burst to provide an even coating. Applying GS to samples 3 at a time was investigated, but the GS was not applied evenly to all samples. The parameters that needed to be optimized in this technique were pressure, amount of fluid sprayed, and distance from aerosol brush to target. The pressures examined over a range of 30-40 psi; the amounts of fluid tested ranged from 50-500µl; the working distances tested were 2 cm and 5 cm. Also tested was the application of small volumes multiple times, to increase the amount of GS on the sample. It was found that the parameters that resulted in the maximum amount of GS on the sample, which was desirable for the *in vivo* study, were 35 psi, 5 cm, and 50µl volume sprayed 8 times for a total volume of 400µl. The parameters used to spray GS on the *in vitro* elution study samples were 35

psi, a distance of 5 cm, and a spray volume of 40µl sprayed 4 times. The concentration of the solution sprayed was 4 w/v % GS in PBS, as this was the maximum solution concentration without resulting in the solution becoming acidic. Amount of GS on these samples was determined by submerging samples in 1 mL of PBS for 15 minutes to wash off the gentamicin. After this time samples were removed and the OPA assay, described in section 2.4, was performed on the PBS. These tests were performed on uncoated stainless steel samples due to the fact that DOPS could cause interference with the OPA assay.

2.3 Coating Retention Study

Retention of coatings was examined by inserting coated implants through sections of mouse bone. This provided information about coating retention in a manner that was clinically relevant. Bone sections were obtained from the lab of Dr. Nicole Ehrhart at the Veterinary Teaching Hospital. The coating retention test initially consisted of sliding sections of bone along the shaft of the needle until the shaft was covered in bone, which required five segments of bone. The segments were gripped between thumb and forefinger of one hand, while the other hand spun the needle 360^{o}. Bone segments were then removed and the samples were then examined under SEM. This technique was later modified by using only one bone segment, which was held in one place while the needle was spun 360^{o}, and then moved to a different position on the needle and spun 360^{o}, continuing this all along the shaft of the needle, as shown in Figure 6.

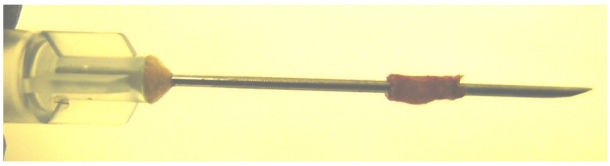

Figure 6: Coating retention test, one segment of bone

Once the bone reached the end of the needle, the needle was cut with tin snips. This way the coating was only exposed to forces due to insertion of the implant into the bone, instead of the additional shear of removing the segment of the bone.

This technique was used to examine the effects of pre-treating sample surfaces with calcium chloride, incorporation of cholesterol in the DOPS solution, and post-treating the coating surface with calcium chloride. Briefly, calcium post-treatment was performed by dipping coated stainless steel needles in 2.25mM calcium chloride solution for 30 seconds. The images obtained from SEM were compared to images of identical samples that were not inserted through bone segments. This allowed identification of a characteristic coating morphology for each treatment, which could then be looked for on the images of samples that had been inserted through bone segments. Energy dispersive x-ray spectroscopy (EDS) was also used to examine surfaces, and confirmed the presence of coating on the samples by detecting the element phosphorus in the DOPS. Micrographs were taken at multiple magnifications ranging from 100x to 5000x, with the majority of micrographs being taken at 100x and 1000x.

Images taken at 100x and 1000x were scored by myself and two other individuals in multiple image sets. The image files were named so that the individuals scoring them

were blinded while assigning scores. A scale of 1-5 was used to score SEM images: 1 indicating less than 25% of sample being coated with DOPS; 2 indicating between 25%-50% of sample being coated with DOPS; 3 indicating between 50%-75% of sample being coated with DOPS; 4 indicating between 75%-100% of sample coated with DOPS; 5 indicating 100% of sample being coated with DOPS. Reference images were provided for each image set to show the evaluators the characteristic microstructure of the various treatment groups prior to insertion through bone segments, and evaluators were informed that it was important to identify the characteristic microstructure of the DOPS coating, and if this could not be observed the image was to be given a score of 1. Statistical analysis for histology results was performed using the X^2 test, since the data obtained is categorical.

2.4 *In Vitro* Elution Study

An *in vitro* elution study was performed to determine the elution characteristics of various coatings on stainless steel needles. The treatments examined are shown in Table 2. Samples containing GS were loaded with approximately 100μg GS, which was applied using the aerosol spray application technique.

Table 2: Elution Study Design

Treatment Group	DOPS conc. (mM)	GS amount (μg)	DOPS:cholesterol mass ratio	Pre-Ca
1	20	100	3:1	Yes
2	20	0	3:1	Yes
3	20	100	6:1	Yes
4	20	0	6:1	Yes
5	20	100	6:1	No
6	20	0	6:1	No
7	20	100	0	Yes
8	20	0	0	Yes
9	20	100	0	No
10	20	0	0	No
11	0	0	0	No

A power analysis was performed using an estimated variance, and an n=10 would have provided a $\beta > 0.8$, however, it was later decided that an n=7 would provide enough statistical significance to determine the coating that resulted in the longest elution time. Subsequent studies would use a higher sample size, if needed, based on the results of this study.

Stainless steel needles had a polypropylene hub, which was removed with tin snips as close to the end as possible to ensure that the full length of coated metal was obtained, and these samples were placed in 12 well plates. 1 mL of PBS was added into the wells, and immediately removed to rinse out GS that wasn't bound within the coating. This wash was performed to calculate the initial amount of GS bound within the coating for a given sample. Then the wells were refilled with 1 mL PBS and placed

into a shaker oven between time points. For the first two hours, the PBS in the wells was removed and replaced every 15 minutes (i.e., total sink condition). Then for the second two hours, time points were taken every 30 minutes. After this, time points were taken every hour for four hours. After the first eight hours, time points were taken every 24 hours for two weeks. Due to the size of the study, it was difficult initially to quickly remove and replace the PBS in the wells. In order to increase the speed of removing and replacing PBS, thus reducing the time samples were exposed to air, two people worked simultaneously on this task. Eluents were placed in 2 mL microcentrifuge tubes, which were stored in a refrigerator.

The o-phthaldialdehyde (OPA) assay is a flourometric assay that has been used to detect the presence of GS in solution. This assay requires that equal amounts of eluent, isopropanol, and OPA reagent be mixed together, and then this mixture can be aliquoted into a well plate and analyzed in a plate reader. For this study, 0.5 mL of eluent was mixed with 0.5 mL isopropanol and 0.5 mL OPA reagent. The volume of mixture placed in each well was 0.2 mL, and each eluent sample was placed in six wells.

The OPA reagent is prepared by mixing the following chemicals in the proportions described in Table 3.

Table 3: OPA Reagent Base Proportions

Chemical Name	**Amount**
O-phthaldialdehyde	2.5g
Methanol	62.5 mL
Mercaptoethanol	3 mL
Sodium borate (1mM)	560 mL

These chemicals are mixed together in a brown glass bottle, as this reagent is photosensitive. After the chemicals are mixed, the reagent needs to be stored in a dark place for 24 hours. After this the reagent can be used, and needs to be used within three days.

Each plate contained 3 sets of GS standards, for the generation of a standard curve for each well plate. The purpose for using standards on each plate is to correct for inter-plate variability. GS standards were prepared in the concentrations reported in Table 4, mixing GS powder in PBS. PBS was used as a zero point on the standard curves. Statistical analysis was performed using a repeated measures ANOVA with Holm-Sidak test.

Table 4: Preparation of GS Standards

Concentration Initial Solution (mg/ml)	Volume Initial Solution (ml)	Concentration Final Solution (μg/ml)	Volume Final Solution (ml)
10	0.25	125	20
10	0.2	100	20
10	0.15	75	20
10	0.1	50	20
10	0.05	25	20

2.5 Lipid Extraction

In order to avoid interference of DOPS while performing the OPA assay a modified Bligh-Dyer extraction was used to separate DOPS and GS into two liquid phases. The Bligh-Dyer extraction makes use of the ability of the water, methanol, and chloroform system to be either one liquid phase or two liquid phases depending on the volume fractions of the components, as shown in the Figure 3. It was shown by Gubernator et. al. that chloroform results in severe interference with the OPA assay, and that by replacing chloroform with dichloromethane the lipids can still be extracted with less interference. Experiments were performed to confirm these results (described below). After the extraction technique was validated it was performed on eluent samples from the *in vitro* elution study, which were stored in a refrigerator after the initial elution. After undergoing extraction the OPA assay was then performed again on

extracted eluent. The extraction technique was performed as described in the Extraction Protocol in Appendix A.

Validation of this technique was performed by mixing known amounts of GS with known amounts of DOPS in solution, then performing the extraction procedure on these samples and examining the results with the OPA assay. Mixed DOPS and GS samples were compared to GS only standards that underwent the extraction procedure. The concentrations of these samples are reported in Table 5. Statistical analysis was performed using a repeated measures ANOVA with the Holm-Sidak test.

Table 5: Extraction Test Sample Concentrations

GS Standards	**Mixed DOPS and GS**	
GS concentration (μg/mL)	GS concentration (μg/mL)	DOPS concentration (μg/mL)
125	125	20
100	100	20
75	75	20
50	50	20
25	25	20
0	0	20

2.6 *In Vivo* Study

2.6.1 Bioluminescent Imaging

To test how effective these implants are at fighting infection, an *in vivo* assessment was necessary. Black-6 mice were used in this study, because they are economical and a well-documented model [36, 37, 43, 44]. The previously mentioned

studies were used to determine the optimal coating parameters, as well as the optimal parameters to create these coatings. The experimental design can be found in Table 6, with each treatment group containing an n=10. CSU ACUC approval was obtained for this *in vivo* study.

Table 6: *In Vivo* Study Experimental Design

Infection Status	**Implant Received**
Osteomyelitis	Uncoated Stainless Steel
Osteomyelitis	6:1 DOPS-to-chol, pre-Ca, w/o GS coated Stainless Steel
Osteomyelitis	6:1 DOPS-to-chol, pre-Ca, w/GS coated Stainless Steel
No Osteomyelitis	Uncoated Stainless Steel
No Osteomyelitis	6:1 DOPS-to-chol, pre-Ca w/o GS coated Stainless Steel
No Osteomyelitis	6:1 DOPS-to-chol, pre-Ca w/GS coated Stainless Steel

Osteomyelitis was induced in mice by creating a whole perpendicular to the femur and placing a section of suture that had been incubating in genetically modified *Staphylococcus Aureus* inside the femur. An incision was made over the bone, and then a hole was made in the femur. The hole in the femur was created by using two needles of increasing size, first an 18 gauge Kendall Monoject Hypodermic Needle followed by a 20 gauge Kendall Monoject Hypodermic Needle, then once the hole was large enough the suture was placed in the tip of the smaller needle, which was used to position the suture in the bone. The skin around the suture site was stapled shut. Animals that were

not infected had a section of sterile suture inserted inside their femurs. After sutures were inserted, the mice were allowed 7 days for the infection to become established. The group of mice that received a sterile suture and stainless steel implants were performed several months after the other treatment groups.

The genetically modified bacteria were modified with genes that encoded for expression of the enzyme luciferase and its substrate luciferin. The purpose of using genetically modified bacteria was that it allowed for the use of bioluminescent imaging to track the infection over time without sacrificing any mice prior to the end of the study. The oxidation of luciferin by luciferase results in the production of light. For this experiment, a Xenogen IVIS 100 camera (Caliper Life Sciences, Hopkinton, MA) was used to detect the light emitted by this reaction, which was used to quantify the infection over time.

Prior to implantation all implants were exposed to UV light for 17.5 hours to sanitize the implants. Phospholipids can not be sterilized with typical methods such as autoclaving or ethylene oxide, as it results in degradation of the phospholipid. It has been shown that phospholipids could be exposed to UV for up to 48 hours without any degradation [41], and so UV exposure was chosen to destroy any biological contaminants on the implants. UV exposure does not provide the same degree of sterilization as terminal sterilization techniques such as autoclaving. To verify the efficacy of UV exposure to sterilize samples, coated implants were exposed to UV and then incubated in cell culture media to observe whether or not bacteria were present. Implants were exposed to UV for 17.5 hours, and then incubated in culture media for 24

hours. In order to test whether or not UV irradiation truly sterilizes samples bacteria would first need to be placed on the samples which would then be subjected to this procedure. However, it was not possible to perform this test, and the procedure described previously was performed to observe after applying a coating to a sample and exposing it to UV if cells would grow in culture.

Once the infection was established, implants were placed inside the appropriate mice. This procedure involved creating a pilot hole in the head of the femur using a 20 gauge needle, and then inserting the coated implant through the length of the femur. Excess implant was cut off, and the skin around the implant site was stapled shut. After implantation, animals were imaged every 48 hours for 28 days. After 28 days, mice were sacrificed and femurs containing the implant and kidneys were harvested. Femurs were also imaged after they were harvested.

Prior to sacrifice, a cardiac punch was performed to draw blood from all the mice. Approximately 0.5 mL blood was obtained from each animal, and placed in a centrifuge tube containing a gel. Upon spinning the tube down, the gel rose to the top of the tube, removing any cells from the blood. Also prior to sacrifice urine was collected from each mouse. Upon sacrifice, it was noted that some of the bladders were still rather full, and so a syringe was used to collect the remaining urine. Serum and urine from five mice randomly chosen from each treatment group were sent to the CSU Diagnostic Lab. Tests run on these samples were for blood urea nitrogen, creatinine, and urine protein. Of the harvested femurs, half were chosen to undergo histology while the other half were cut up and placed in culture tubes. The purpose of culturing

these samples was to see if any bacteria remained in the samples that were not being detected by the camera. Statistical analysis was performed using ANOVA repeated measures with the Holm-Sidak test.

2.6.2 Histology

Histological analysis was performed on femurs removed from the mice at the end of the study. Kidneys were also removed from the mice for histological analysis, however, after serum and urine were analyzed it was determined that it was not necessary to perform histology on the kidneys. Decalcified histology was performed on the femurs, and as such the bones were decalcified in formalin prior to preparation. The stains used to evaluate the bones were H&E and a gram stain. The H&E stain was used to observe cells of the mice and inflammation, while the gram stain provided information about bacterial colonization.

Histological evaluation was performed using a scale developed specifically for this project by the evaluator to evaluate inflammation and bacterial colonization. The scale ranged from 0-3: 0 indicating no inflammation or bacterial colonization on the sample; 1 indicating mild inflammation or bacterial colonization on the sample; 2 indicating moderate inflammation or bacterial colonization on the sample; 3 indicating marked inflammation or bacterial colonization on the sample. All histology slides were evaluated for inflammation and bacterial colonization, and the uninfected controls were also evaluated for bone growth onto the implant. This scale allows the histological images to be converted to semiquantitative information, upon which statistics can be performed. Statistical analysis for histology results was performed using the X^2 test,

since the data is categorical. All statistical analyses were performed using SigmaPlot software.

CHAPTER 3: RESULTS

3.1 Electrospray Technique

Figure 7A shows a DOPS coating applied to a flat stainless steel sample at a voltage of 12 kV. Figure 7B shows a DOPS coated surface created using 14 kV. Figure 7C shows a DOPS coated surface created using 16 kV. Figure 7D shows a piece of flat stainless steel that was not coated.

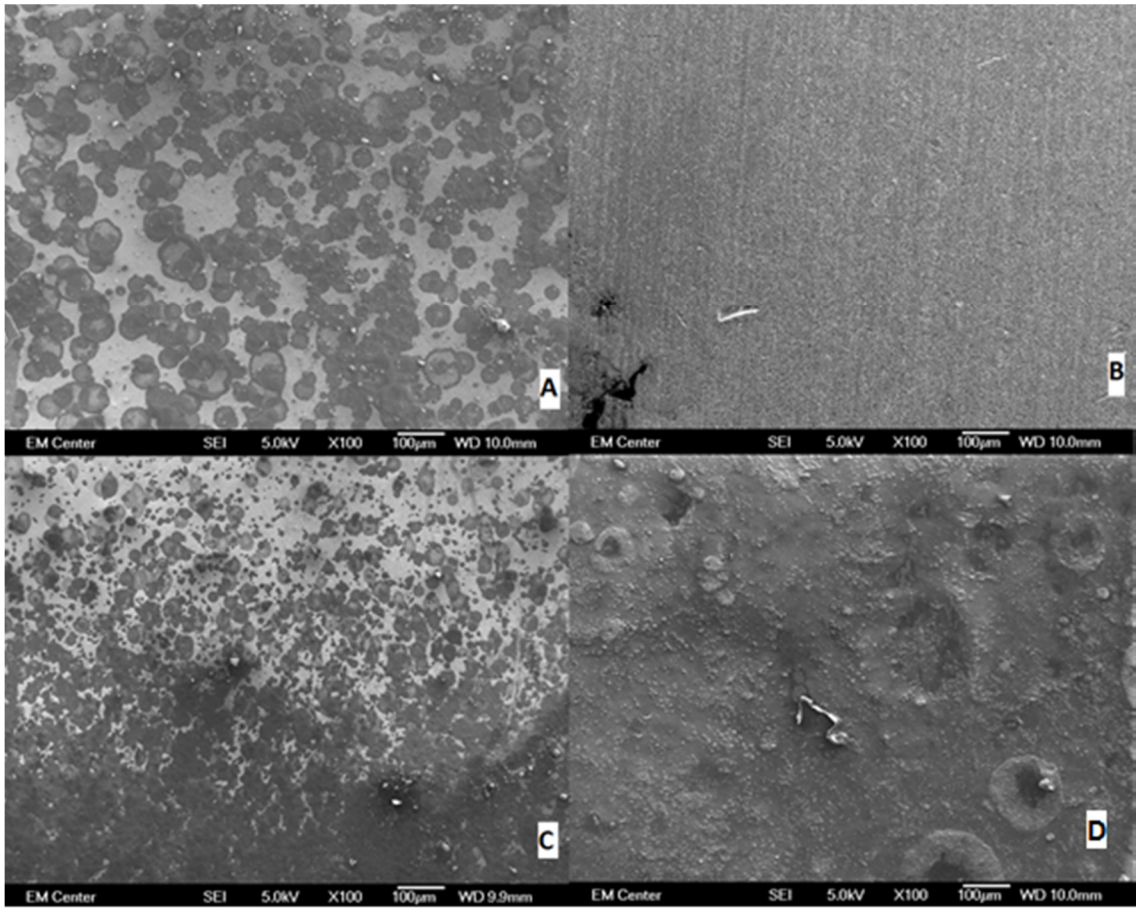

Figure 7: SEM images of flat stainless steel samples: A) DOPS coating sprayed at 12kV at 100x, B) DOPS sprayed at 14kV at 100x, C) DOPS sprayed at 16kV at 100x, and D) a plain stainless steel sample at 100x.

Samples were successfully sprayed at all voltages, as can be observed by comparing Figure 7 images A-C to Figure 7-D. However, the different voltages resulted in differences between the samples. All voltages tested resulted in coatings being applied to the surface, with coatings that appear similar. It was observed that coatings sprayed at 12kV and 16kV samples had uneven coatings, as indicated by areas of the sample that had more coating than others, which was not observed on the 14 kV sample. The 14 kV sample appeared to have an even coating, and the coating morphology was similar to those created by Dr. Prawel on titanium samples. Based on these results, 14 kV was chosen as the voltage at which subsequent samples were sprayed.

Figure 8 shows a needle electrosprayed with a 6:1 DOPS-to-cholesterol blend. Cholesterol was included in the spray mixture to modify the applied coatings in such a way that would increase the time over which GS would elute from the coating. Another modification made to the sample in Figure 8 was a calcium chloride pre-treatment. The purpose of this pre-treatment was to bind calcium to the metal surface, which would then bind to the phospholipid as it was sprayed onto the metal.

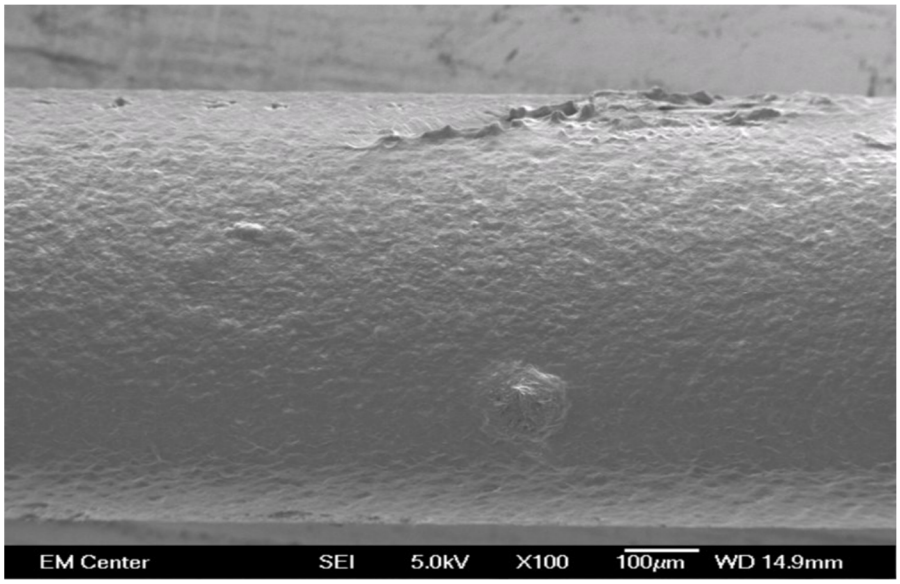

Figure 8: Needle Coated with 6:1 DOPS-to-cholesterol w/Ca pre-treatment, 100x

When spraying stainless steel needles, samples were not rotated while the coating was applied. SEM images revealed that an even coating was applied all around the needle, as can be partially observed in Figure 8. Figure 9 provides a higher magnification of the 6:1 DOPS-to-cholesterol coating, showing details of the coating microstructure. The formation of this spindle-like microstructure was not observed on plain DOPS coatings, but rather appeared only with the addition of cholesterol and calcium pre-treatment.

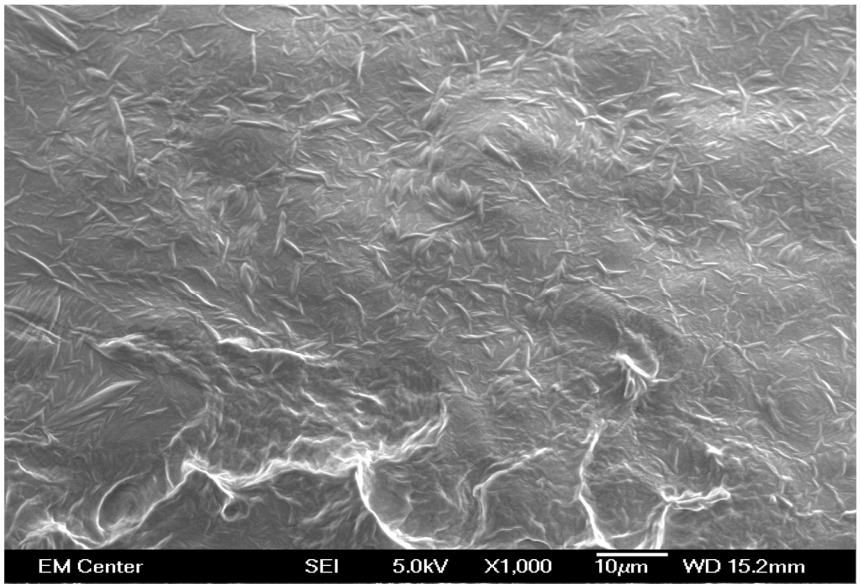

Figure 9: Needle Coated with 6:1 DOPS-to-cholesterol w/Ca pre-treatment, 1000x

Figure 10 is an image of a plain stainless steel needle.

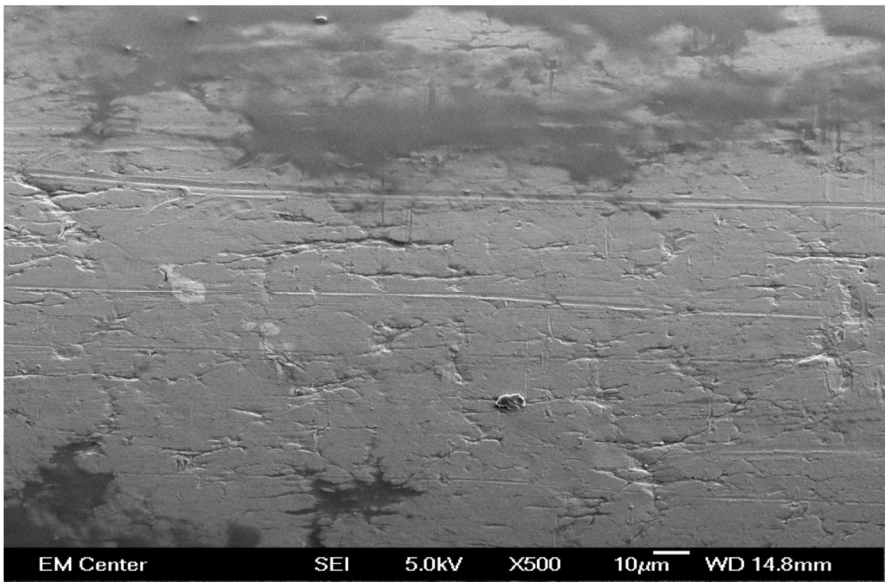

Figure 10: Stainless Steel Needle, 500x

Figures 8-10 show that by adding cholesterol to the electrospray solution the morphology of the coating is drastically altered.

3.2 Aerosol Spray Technique

The parameters that were varied in these experiments were the air pressure, the distance the air brush was held from the samples being sprayed, and the volume of GS solution sprayed at the samples. Initially the whole volume of fluid was sprayed on the samples. The samples in Table 7 were all sprayed two samples at a time. Table 8 shows data from samples that were sprayed multiple times with a smaller amount of fluid, allowing time to dry in between applications. It was observed that the maximum amount of GS was applied when the samples were sprayed 8 times with a volume per spray of 50µl. The air pressure was increased to 35 psi to avoid fluid from getting stuck in the spray tube connecting the fluid reservoir and the air brush tip.

Table 7: Aerosol Spray, Single Application

Air Pressure (psi)	Distance from Target (cm)	Volume of Solution (ml)	Amount GS on Sample (µg)
30	2	0.5	74
30	5	0.5	164
30	2	0.25	32
30	5	0.25	219
40	2	0.25	41
40	5	0.25	104
30	5	0.2	240
30	5	0.1	172
38	5	0.1	77

One issue that was noticed with spraying large volumes all at one time was that there was little consistency between the two samples being sprayed. It was found that one needle would have much more GS than the other, which was likely due to different amounts of fluid run off. Tables 7 and 8 present the average amount of GS on the two samples that were sprayed. It was observed that differences between the two simultaneously sprayed samples were as large as 140μg of GS. Spraying small volumes multiple times provided more consistency between the samples, and thus was the preferred method.

Table 8: Aerosol Spray, Multiple Applications

Air Pressure (psi)	Distance from Target (cm)	Volume of Solution per spray (ml)	Number of Times Sprayed	Amount of GS on Sample (μg)
35	5	0.05	4	141
35	5	0.05	8	270
35	5	0.03	5	84
35	5	0.1	4	188
35	5	0.04	4	97

3.3 Coating Retention Study

Images of the needles pushed through bone segments were used to identify whether or not the coating adhered to the metal surface. Figure 11 shows a coated needle after being inserted through a segment of mouse bone. From the image it can be seen that part of the coating was removed after the insertion process, as can be

observed in the areas of stainless steel, as indicated by the arrows. Figure 12 provides a higher magnification image of this sample. Beneath the residual marrow that stuck to the sample, the characteristic microstructure of the DOPS-cholesterol coating can be observed. While the coating may have been disturbed in some areas by the insertion process, Figure 12 suggests that large parts of the coating remain intact.

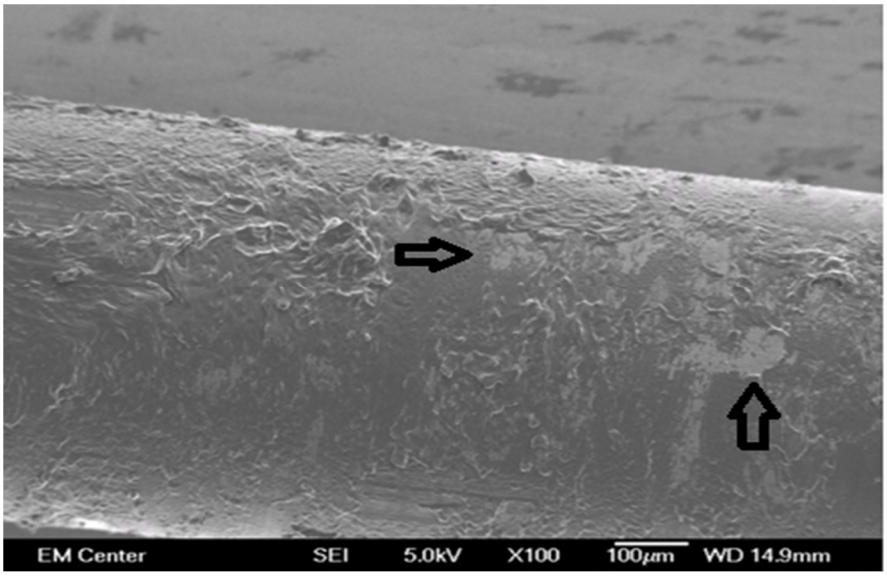

Figure 11: 6:1 DOPS-to-cholesterol w/Ca pre-treatment, inserted through bone, 100x. Arrows indicate areas where coating was removed by insertion process.

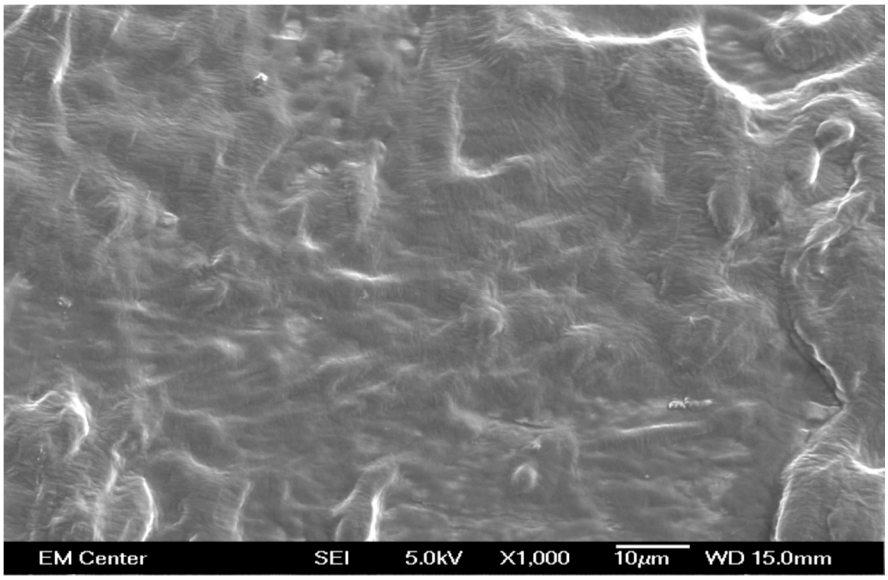

Figure 12: 6:1 DOPS-to-cholesterol w/Ca pre-treatment, inserted through bone, 1000x

The image in Figure 13 shows an uncoated needle that was inserted through a bone segment.

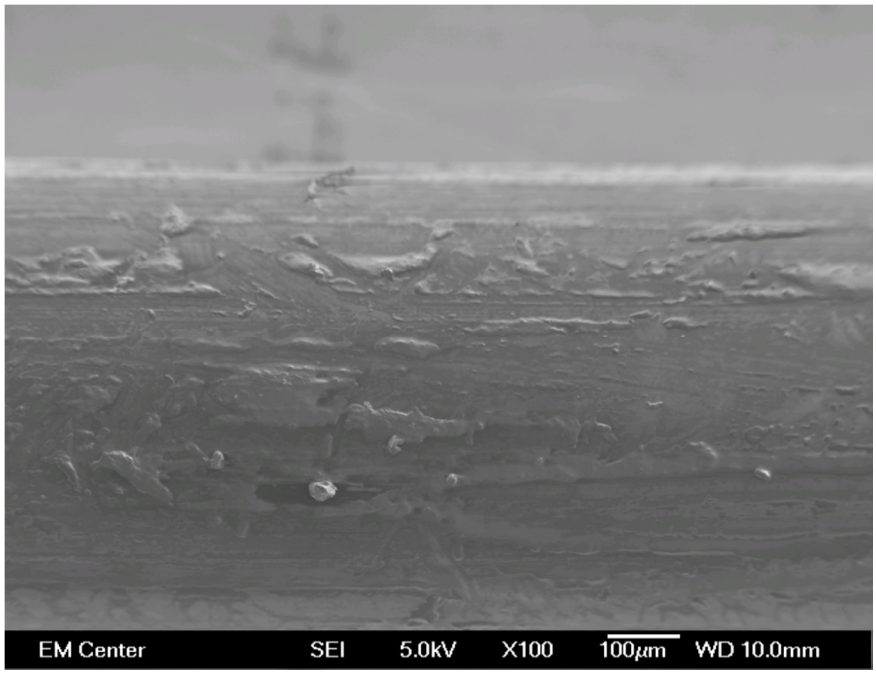

Figure 13: Stainless steel needle, inserted through bone, 100x

Comparing this image to the plain stainless steel needle in Figure 10, it can be seen that the process of inserting a needle through a section of bone results in the deposition of residual marrow from the bone segment onto the needle. This observation supports the conclusion that the coating remained on the sample in Figure 12, as the characteristic microstructure can be clearly observed through the residual marrow that remained on the sample.

Statistical analyses of the image scores from all evaluators showed that at images taken at 1000x there was no statistical difference between the various DOPS coated samples, but all the DOPS coated samples were significantly different than the stainless steel group. Scores from images taken at 100x from all three evaluators showed that all the coated samples were scored significantly higher than stainless steel, $p < 0.05$. Results from image scoring are presented in Table 9.

Table 9: Coating Retention Image Scores

Treatment	Magnification	Tom	Mike	Justin	Combined
DOPS	100x	2.5 ± 0.65	3.75 ± 0.25	4.0	3.42 ± 0.29
6:1 DOPS-to-chol, pre-Ca	100x	3.29 ± 0.29	3.71 ± 0.13	4.21 ± 0.21	3.74 ± 0.14
6:1 DOPS-to-chol, pre-Ca, post-Ca	100x	3.55 ± 0.28	3.55 ± 0.16	3.82 ± 0.18	3.64 ± 0.12
Stainless steel	100x	1.0	2.4 ± 0.51	1.8 ± 0.58	1.73 ± 0.28
DOPS	1000x	3.33 ± 0.76	3.83 ± 0.48	4.17 ± 0.48	3.78 ± 0.33
6:1 DOPS-to-chol, pre-Ca	1000x	3.5 ± 0.52	3.8 ± 0.29	4.2 ± 0.25	3.83 ± 0.22
6:1 DOPS-to-chol, pre-Ca, post-Ca	1000x	3.0 ± 0.55	3.33 ± 0.44	3.44 ± 0.44	3.26 ± 0.27
Stainless steel	1000x	1.0	2.5 ± 0.22	3.0 ± 0.52	2.17 ± 0.27

It was observed that there were no significant differences, $p > 0.05$, in the image scores between evaluators at 1000x magnification. It was also observed that the other two evaluators gave scores greater than 1 to the stainless steel control images. While scoring the images the author was also blinded; however, due to familiarity with the images, and the process of renaming image files so the other evaluators would be blinded, the author was not truly blinded.

3.4 *In Vitro* Elution Study, Without Extraction

The *in vitro* elution study was performed to determine the effects of cholesterol and calcium chloride pre-treatment, on elution time. Because the absorbance values are very sensitive to the amounts of chemicals used to prepare the reagent, it is difficult

to compare absorbance values from groups that were analyzed with different batches of OPA reagent. For this reason statistical analysis was not performed on these results. However, the shapes of the curves were examined, and used to determine the effects of calcium pre-treatment and cholesterol. The results of the 3:1 DOPS-to-cholesterol samples are presented in Figure 14 and Figure 15. Figure 14 shows that by time point 1.5 the samples have reached a plateau value, suggesting that there is no longer any GS being released from the coatings. Figure 15, which represents samples not loaded with GS, shows signal detection over a greater time period than Figure 14.

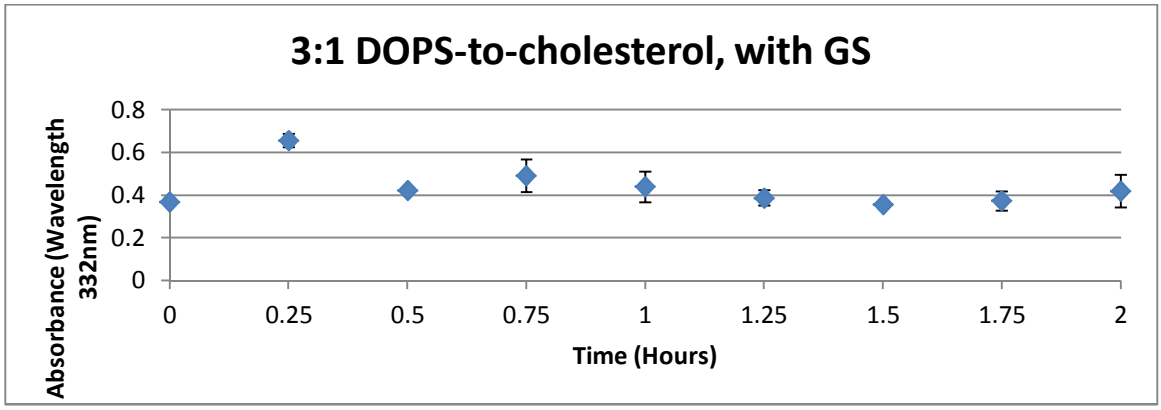

Figure 14: Absorbance values of 3:1 DOPS-to-cholesterol GS loaded samples with a calcium chloride pre-treatment.

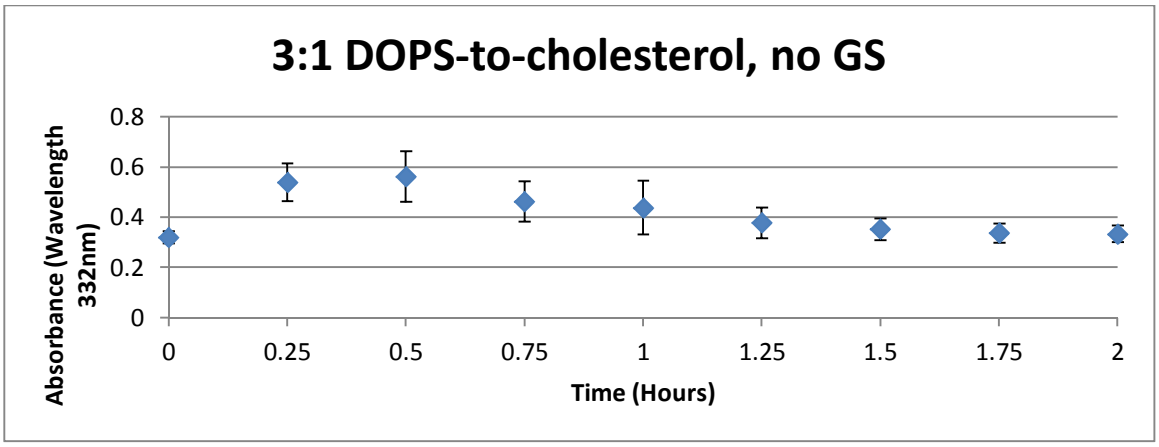

Figure 15: 3:1 Absorbance values of DOPS-to-cholesterol no GS control samples with a calcium chloride pre-treatment.

Figure 16 has continually decreasing absorbance values over the full 2 hours, indicating that GS is still being released from the coating. Figure 17 appears very similar to Figure 15, indicating that coatings made up of 6:1 DOPS-to-cholesterol have approximately the same amount of coating coming off in solution as the 3:1 coatings. The information in these figures suggests that the 6:1 coatings retain GS over a longer period of time than the 3:1 coatings. The 3:1 coatings have eluted almost all of their GS at hour 32, whereas the 6:1 coatings appear to have more GS.

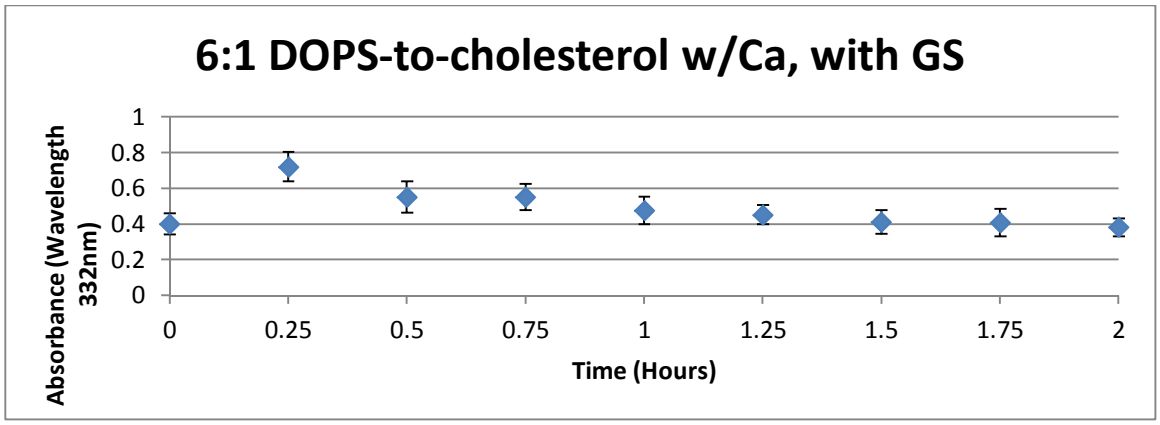

Figure 16: Absorbance values of 6:1 DOPS-to-cholesterol GS loaded samples with a calcium chloride pre-treatment.

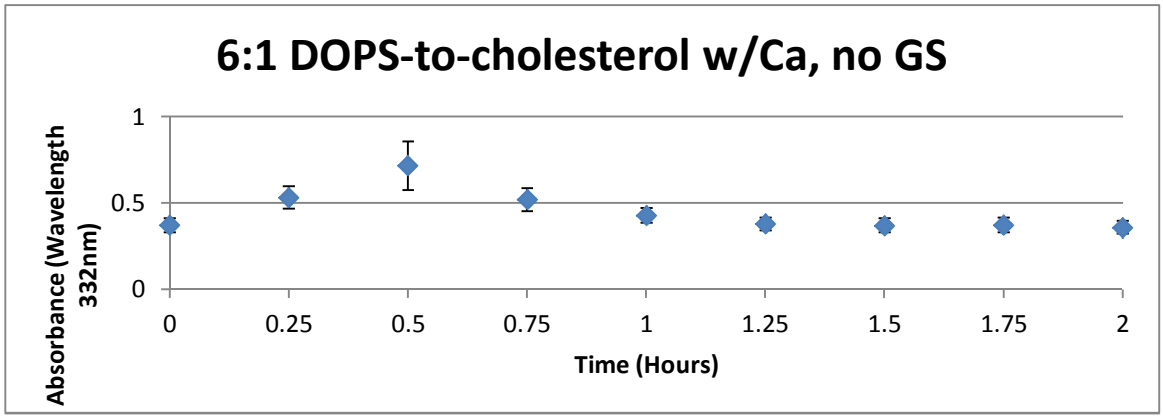

Figure 17: 6:1 Absorbance values of DOPS-to-cholesterol no GS control samples with a calcium chloride pre-treatment.

Data from samples that were coated in a 6:1 DOPS-to-cholesterol mixture but did not receive a calcium chloride pre-treatment are shown in Figures 18 and 19. Figures 17 and 19 do not appear to be different from each other. However, Figure 16 appears to still be decreasing in absorbance at the end of 2 hours, whereas a plateau absorbance appears to have been reached in Figure 18.

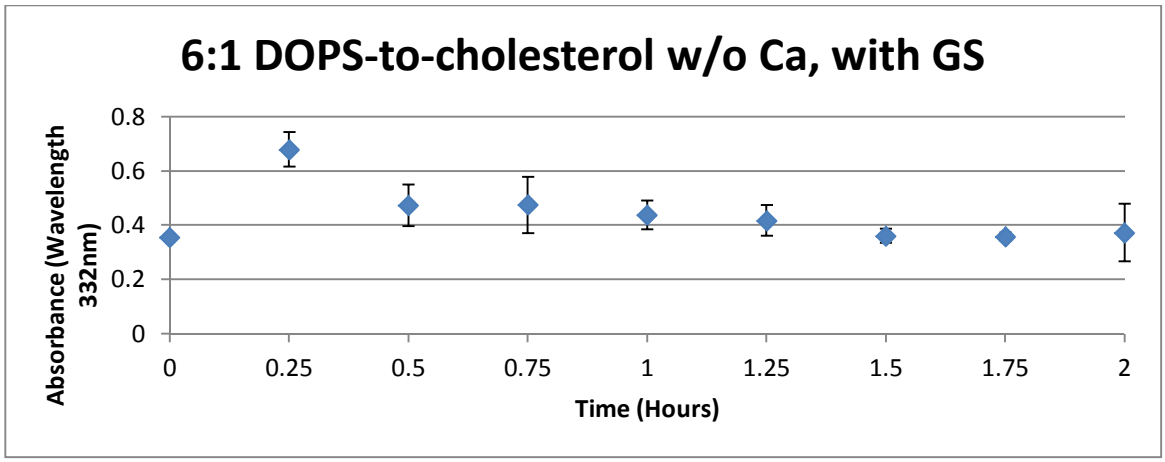

Figure 18: Absorbance values of 6:1 DOPS-to-cholesterol GS loaded samples without a calcium chloride pre-treatment.

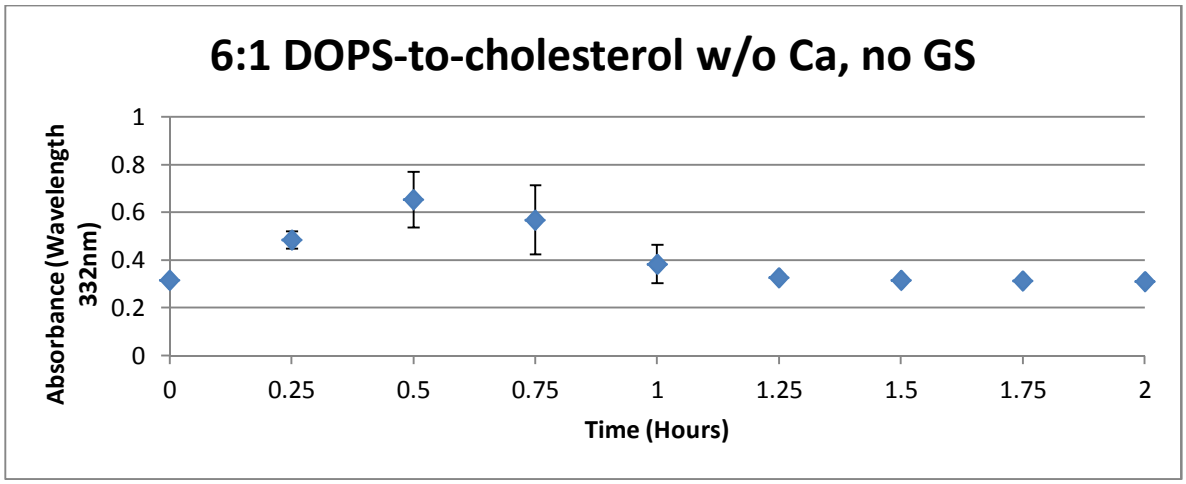

Figure 19: Absorbance values of 6:1 DOPS-to-cholesterol no GS control samples without a calcium chloride pre-treatment.

Figures 20 and 21 are absorbance values over time from samples that were coated with DOPS only without a calcium pre-treatment.

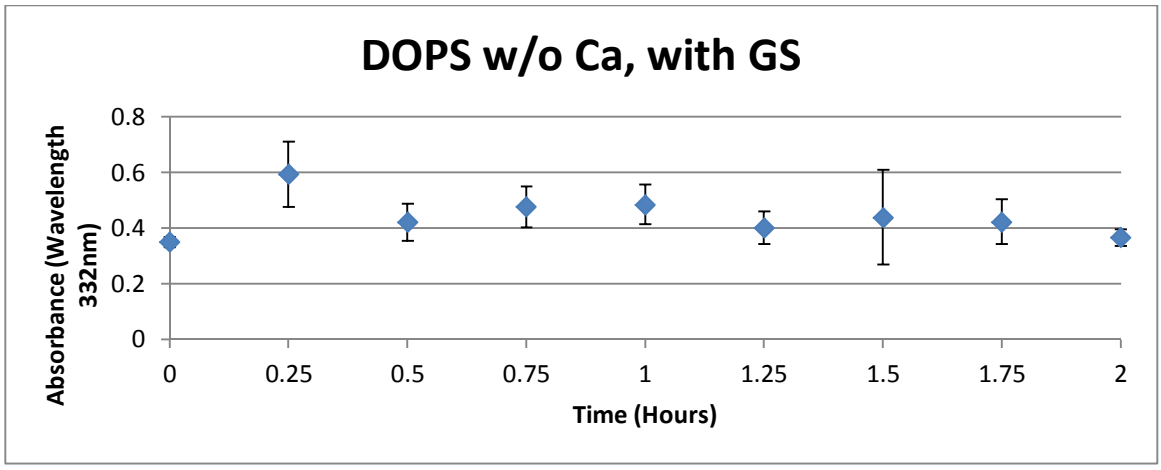

Figure 20: Absorbance values of DOPS without a calcium chloride pre-treatment, GS loaded samples.

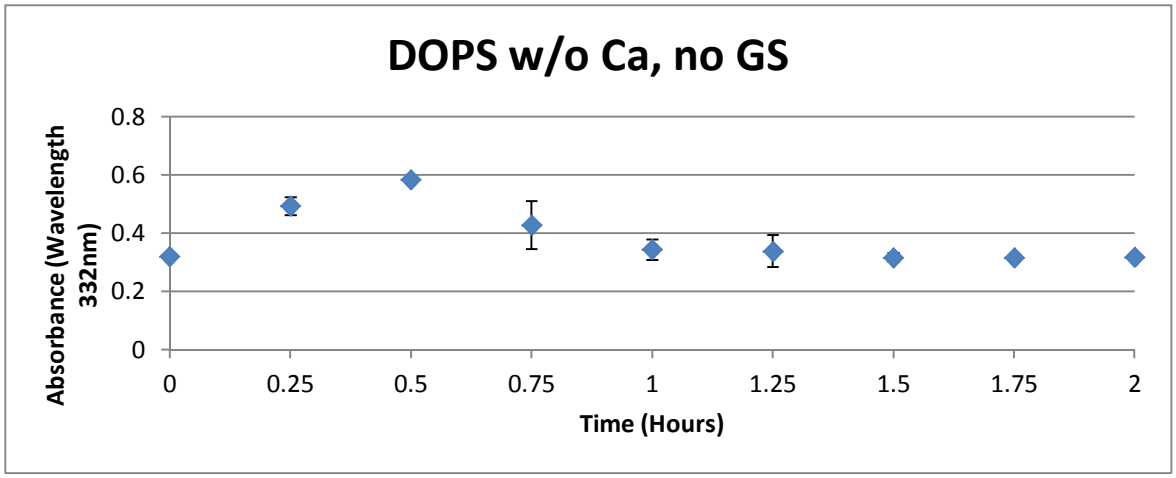

Figure 21: Absorbance values of DOPS without a calcium chloride pre-treatment, no GS control samples.

Figures 22 and 23 show similar coatings as the samples in Figures 20 and 21, but these samples did receive a calcium pre-treatment. These data show that the samples without GS caused larger signal for a longer time than the samples with GS. Figure 22 shows a very sharp spike in the absorbance value at time 0.25, and then plateaus.

Without the ability to discriminate between signal detection caused by DOPS and signal detection caused by GS the results in Figures 20-23 are not useful.

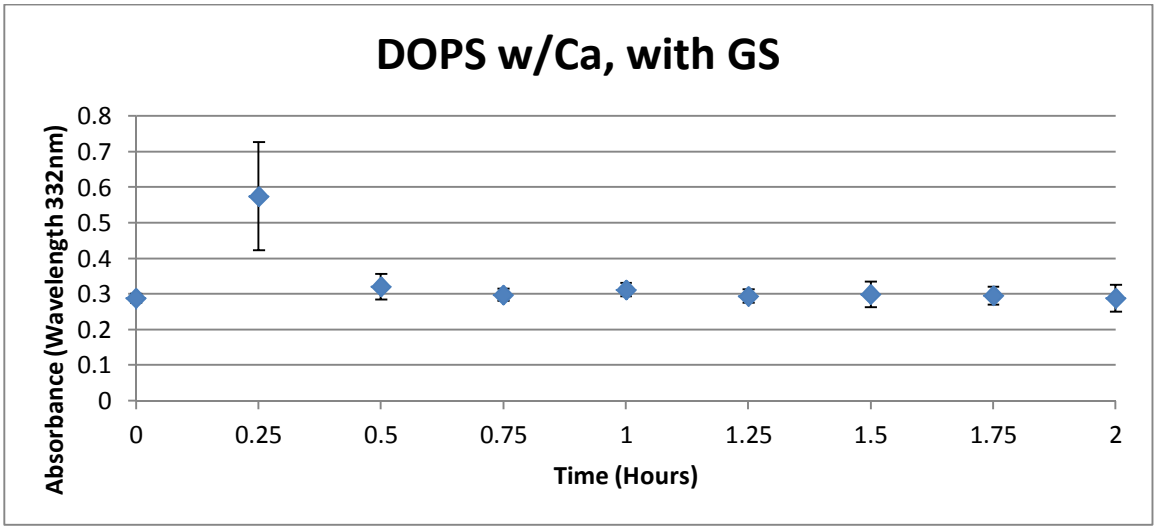

Figure 22: Absorbance values of DOPS with a calcium chloride pre-treatment, GS loaded samples.

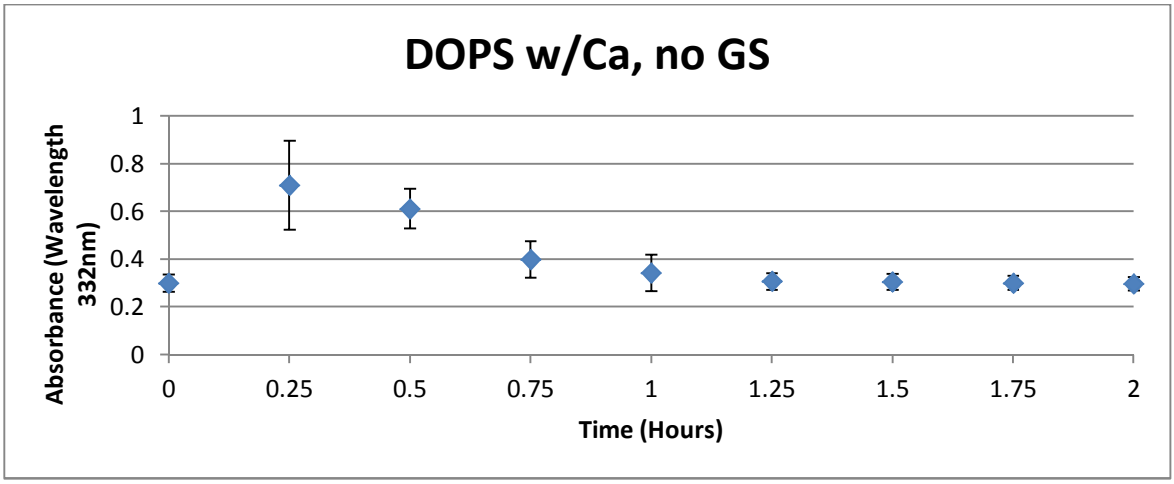

Figure 23: Absorbance values of DOPS with a calcium chloride pre-treatment, no GS control samples.

Figure 24 shows that the amount of signal that comes from plain stainless steel samples is negligible and does not impact the results presented in previous figures.

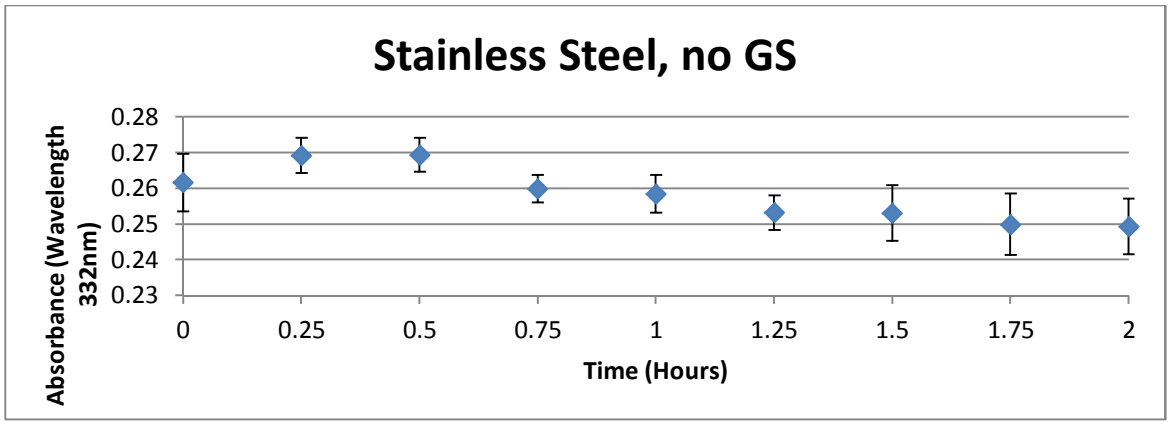

Figure 24: Absorbance values of stainless steel samples.

3.5 *In Vitro* Elution Study, With Extraction

The results of the validation of the extraction show that this technique is effective in separating DOPS and GS in solution. Values over the range of 0-75 µg/mL were the most accurate when comparing the mixed samples to the standards, as is shown in Figure 25. The curve fits through the data points overlap, and the values of the slopes for both fits are the same.

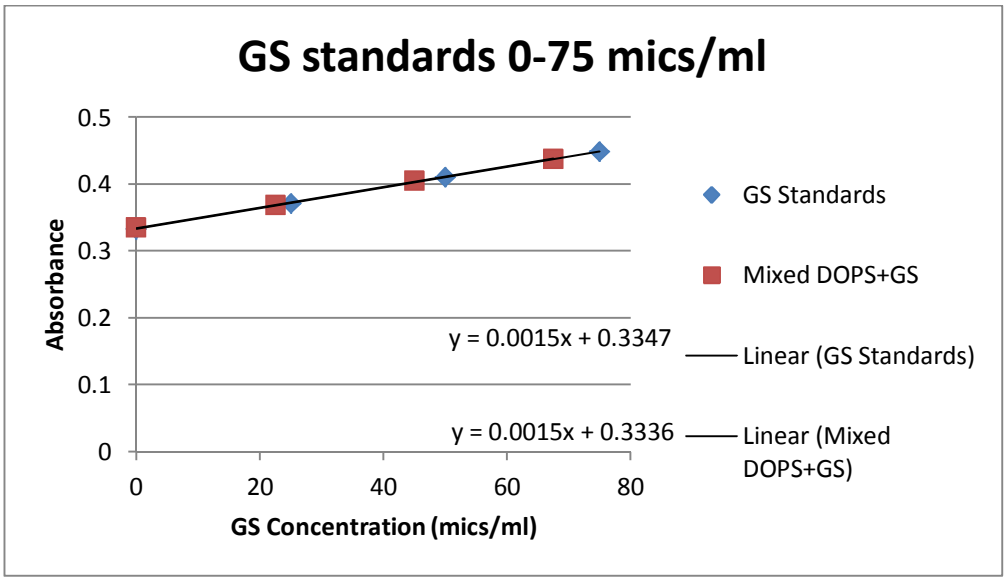

Figure 25: Lipid extraction test, GS standards and mixed DOPS+GS

After the extraction was shown to effectively separate DOPS from GS in solution, it was applied to all eluent samples that were obtained during the elution study. It was observed that most samples continued to show GS eluting from the sample over the full duration of the two week study, resulting in a cumulative GS much greater than the 100µg applied. For this reason, the results of the *in vitro* elution with extraction are reported in terms of absorbance, instead of amount of GS. Figure 26 shows the results of performing the OPA assay on samples that had been extracted.

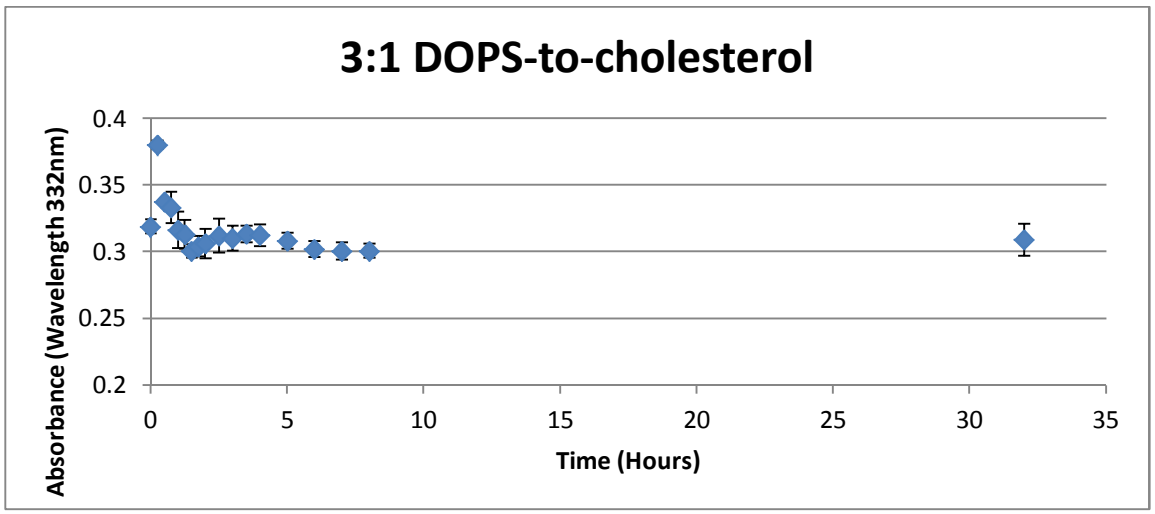

Figure 26: Absorbance of extracted eluent, 3:1 DOPS-to-cholesterol with a Ca pre-treatment

Because of the wavy behavior of the curve at later time points, which was observed in all treatment groups, it was decided that only the first two hours would be looked at. Figure 27 shows the same information as Figure 26, focusing on the first 2 hours of the elution study.

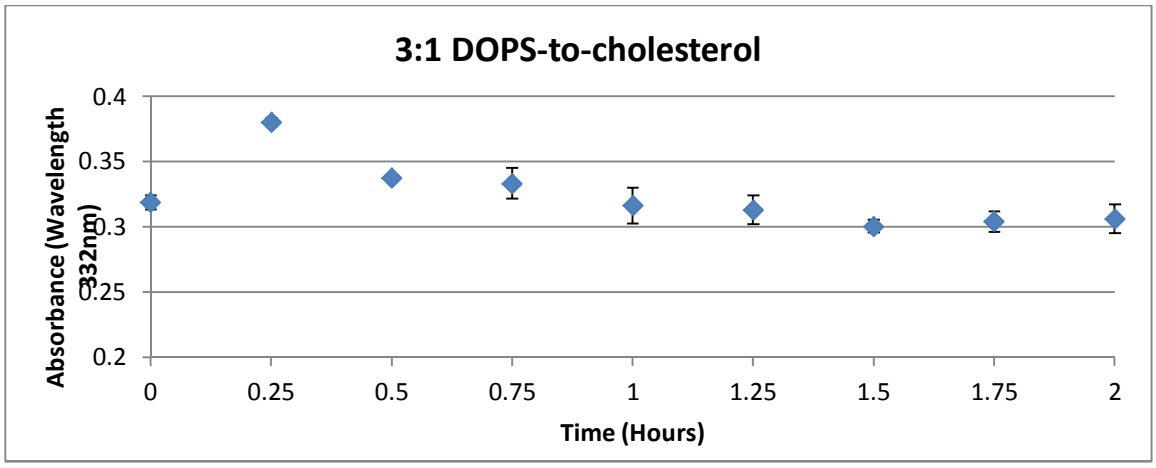

Figure 27: Absorbance of extracted eluent, 3:1 DOPS-to-cholesterol with a Ca pre-treatment, first 2 hours.

Figures 28 and 29 show 6:1 DOPS-to-cholesterol coated samples, with and without a calcium pre-treatment, respectively.

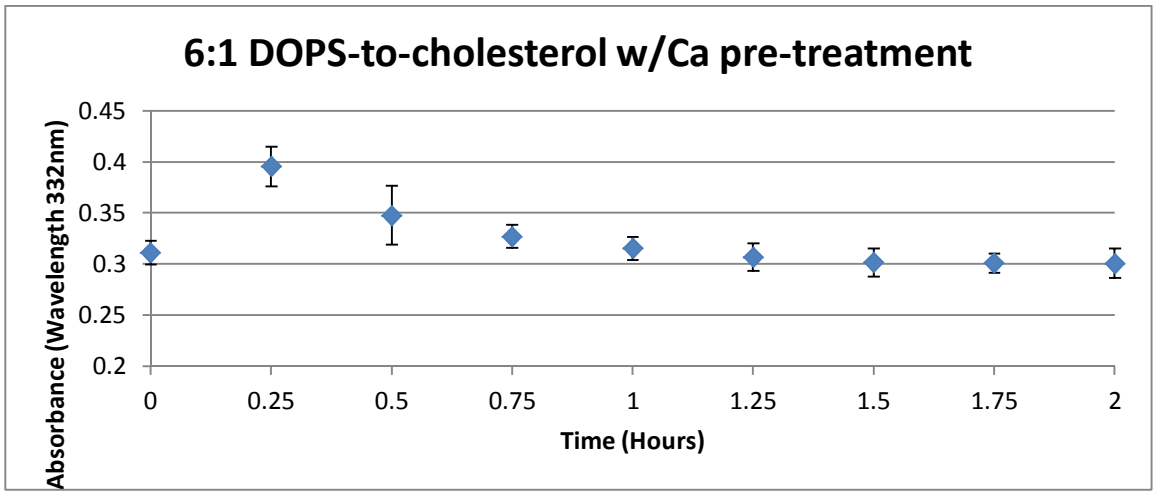

Figure 28: Absorbance of extracted eluent, 6:1 DOPS-to-cholesterol with a Ca pre-treatment.

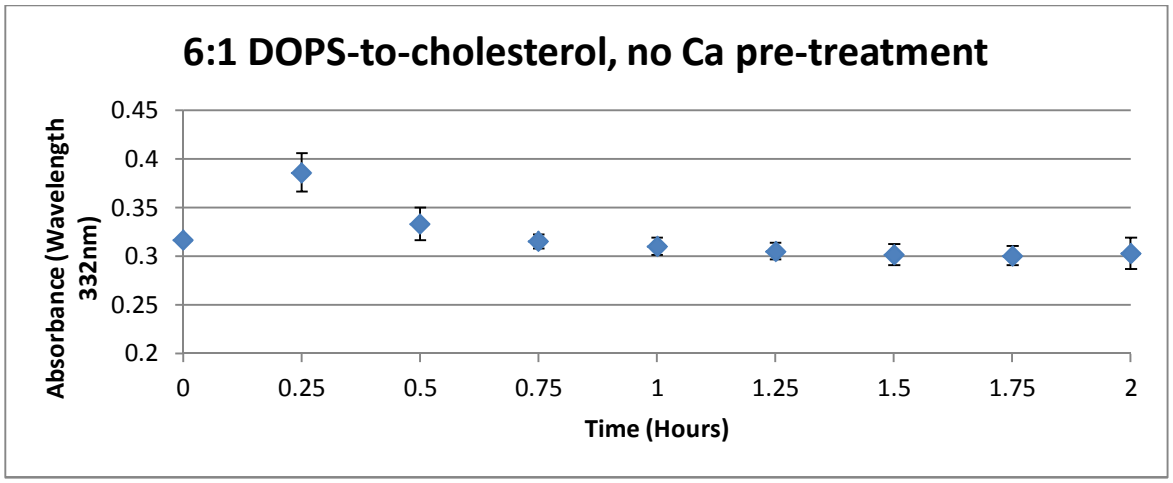

Figure 29: Absorbance of extracted eluent, 6:1 DOPS-to-cholesterol, without a calcium pre-treatment

Figures 30 and 31 show DOPS only coated samples with and without a calcium pre-treatment, respectively.

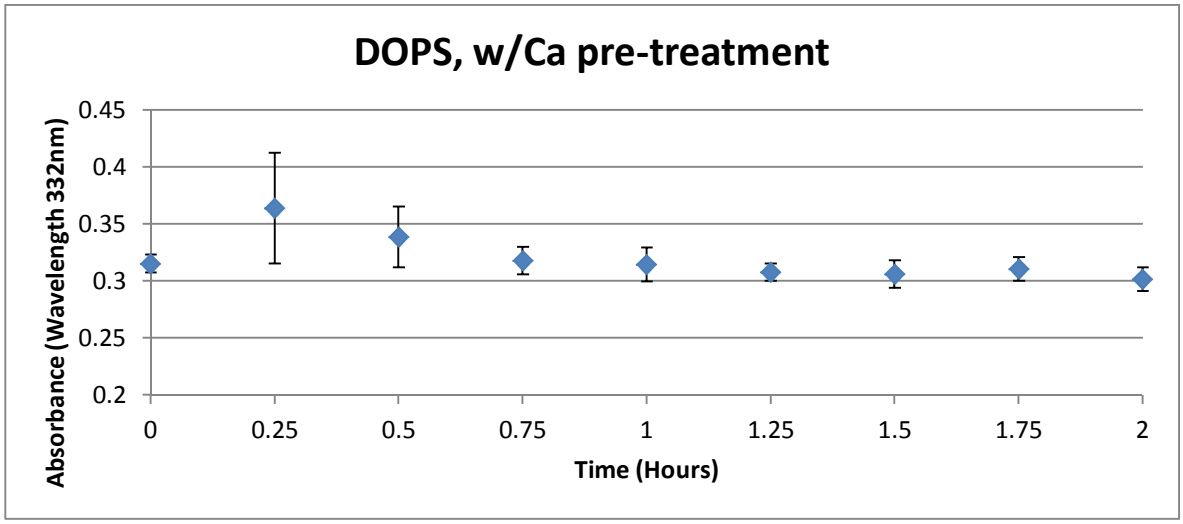

Figure 30: Absorbance of extracted eluent, DOPS only coatings with a calcium pre-treatment

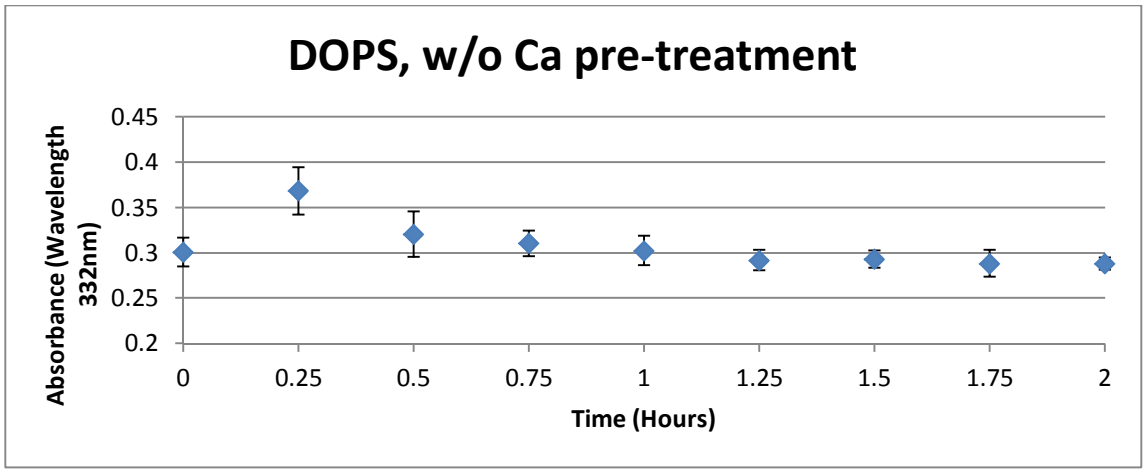

Figure 31: Absorbance of extracted eluent, DOPS only coatings without a calcium pre-treatment

Figures 27-31 all show an initial spike in absorbance, indicating that GS is coming out of the coating. All samples also show a decline to a plateau value, which appears to be an absorbance of approximately 0.3. Because of this similar plateau absorbance value, statistical analysis was performed. There does not appear to be a difference in the absorbance values between treatments over time based on these figures. Statistical analysis shows that there are significant differences, $p < 0.05$, between the 3:1 DOPS-to-cholesterol w/Ca treatment group and the 6:1 DOPS-to-cholesterol w/o Ca and DOPS only w/o Ca treatment groups at 0.75 hours. No differences were observed between the Ca pre-treatment groups. It was also observed that the DOPS w/Ca group had a significantly higher absorbance than the DOPS w/o Ca group, $p < 0.05$, at time 1.75.

Figure 32 shows the absorbances measured from no GS controls, one sample from each of the treatment groups reported above.

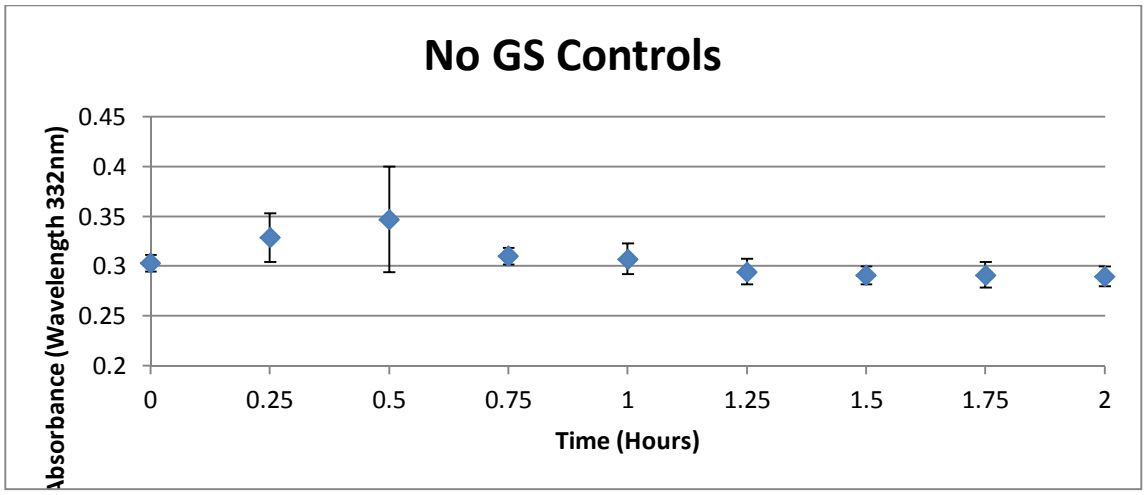

Figure 32: Absorbance of extracted eluent, no GS controls

This figure shows that there are measured absorbance values in a pattern similar to those observed in the GS containing treatments. Statistical analysis showed some significant differences, $p < 0.05$, between the no GS controls and all the cholesterol containing treatments at time 0.25, as well at time 0.75 between the no GS controls and the cholesterol and calcium pre-treatment groups.

3.6 *In Vivo* Study

3.6.1 Bioluminescent Imaging

Assessment of sterility of implants exposed to UV irradiation was done by observation of samples immersed in media in a well plate. The media contained a dye that changed color after being metabolized by cells. The color of the media did not change after incubating the implants for 48 hours, indicating that no cells were growing in these wells. Also, the wells were observed macroscopically for any visible growth. Nothing was observed, another indicator that there were no cells on the implants.

Based on these results, it was determined that after UV irradiation the implants were sufficiently free of bacteria.

It was observed that the infection remained localized over the femur of the mice for the duration of this study. Figure 33A shows a mouse 1 day prior to receiving an antibiotic loaded implant. The colored pixels in the image indicate luminescence, a result of the oxidation of luciferin by luciferase, which indicates the presence of bacteria within the mouse. Figure 33B shows the same mouse at the end of the study. This particular mouse received an implant that was loaded with GS, and the image shows that there are no pixels over the femur of the mouse.

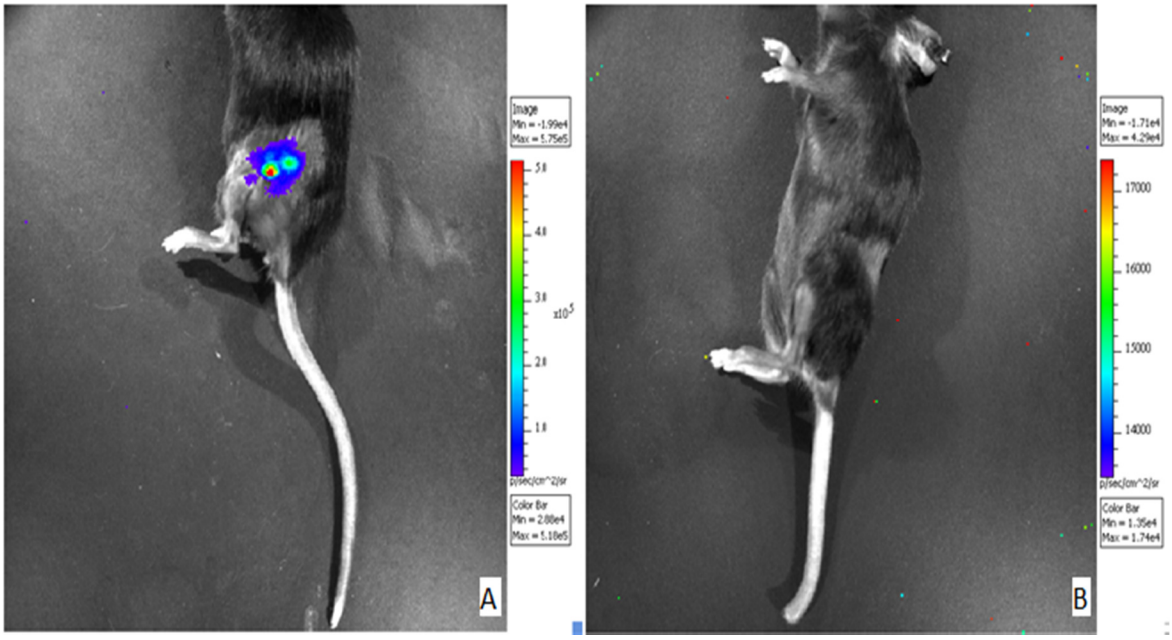

Figure 33: Mouse from DOPS+GS treatment group at A) 1 day pre-implant and B) 28 days post-implant. Image A shows a scale of flux that is one order of magnitude greater than the flux scale of image B.

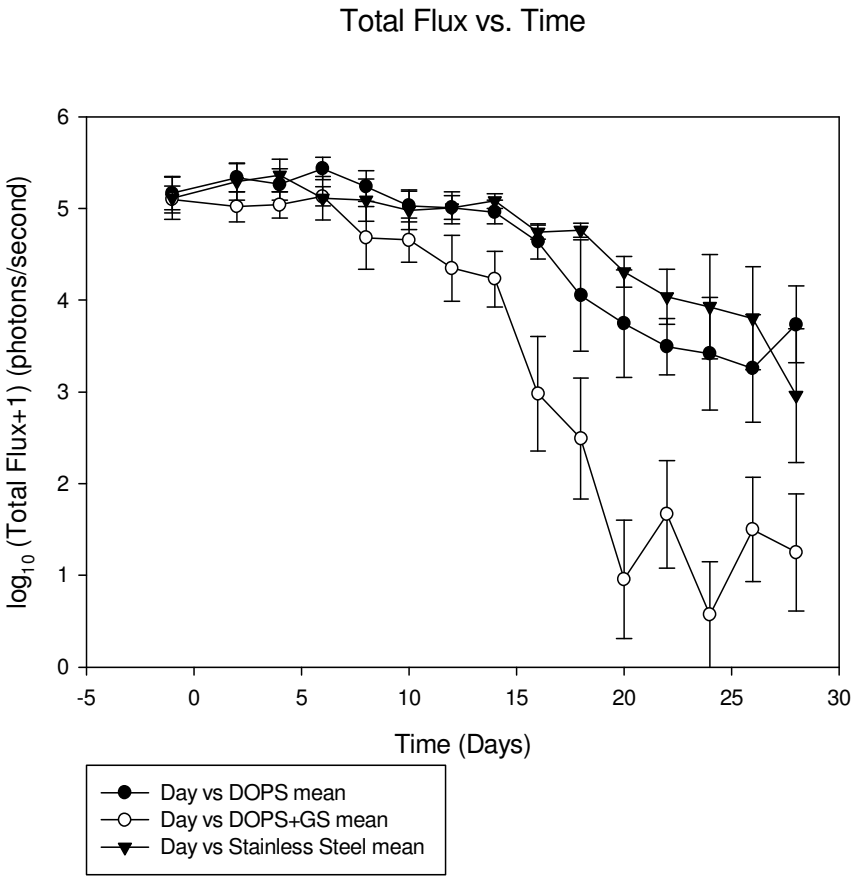

Figure 34: Total Flux vs. Time

Figure 34 provides a more quantitative representation of the data for all the mice during the *in vivo* study. The total flux is a value of total photons being detected by the camera, and is recorded by the Xenogen software. Total flux is a measure of how much bacteria is present at a given time point. The data at day -1 indicate the total flux at 1 day prior to inserting the implants. This shows that 1 day before implantation the mice were emitting a large number of photons, showing that the infection had been established. At day 14 the difference between the DOPS+GS treatment group and the other two treatment groups is statistically significant, $p < 0.05$, and continues to be

significant up until day 28. At day 28, the difference between DOPS+GS and stainless steel is significant, however, the difference between DOPS+GS and the DOPS only group is not statistically significant, nor is the difference between the DOPS only group and the stainless steel implant group. These differences indicate that the reduction in bacteria observed at day 14 is a result of the antibiotic in the implants, not the DOPS coating. If this were due to the DOPS, both the antibiotic loaded and nonantibiotic-loaded groups would show significant differences when compared to the stainless steel group. The difference observed on day 28 shows that the immune system of the mice does play a role in clearing the infection, and over time can successfully reduce the amount of bacteria present.

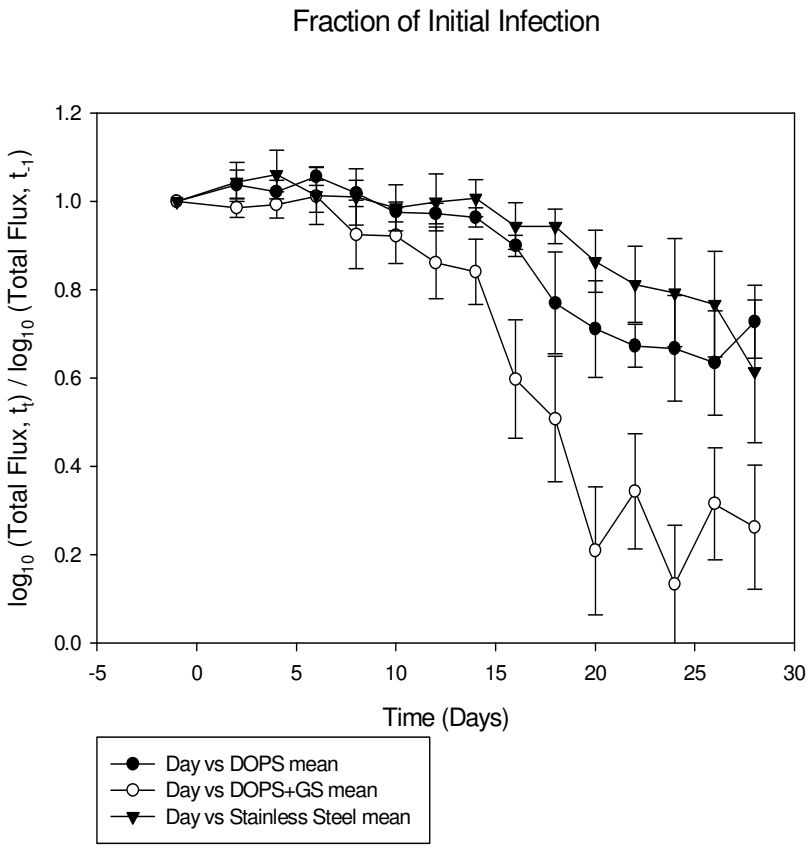

Figure 35: Fraction of Initial Flux vs. Time.

Figure 35 shows results that are similar to those presented in Figure 34. Again, the difference between the DOPS+GS treatment and the other two treatments are statistically significant starting at day 14 and continue to be significant up to day 28. On day 28 the difference between the DOPS+GS treatment and the DOPS only treatment are not significant, but the difference between the DOPS+GS treatment and the stainless steel treatment are significantly different. This figure supports the results from Figure 34, indicating that reduction in bacteria over time in the DOPS+GS group was due to the presence of the antibiotic. Also, both of these figures show at day 28 that there is no significant difference between the DOPS+GS and the DOPS only group. This suggests that the DOPS plays some role in reducing the amount of bacteria present over time.

Table 10: Urine and Serum Data

Treatment Group	Urine Protein (mg/dL)	Creatinine (mg/dL)	Serum BUN (mg/dL)
Uninfected, DOPS implant	278 ± 109	Approx. 0.1	26.8 ± 1.6
Uninfected, DOPS+GS implant	292 ± 92	<0.1	21.4 ±1.5
Infected, DOPS implant	183 ± 31	<0.1	21.4 ± 1.8
Infected, DOPS+GS implant	228 ± 66	<0.1	28.8 ± 1.3
Infected, Stainless Steel implant	212 ± 71	<0.1	21.2 ± 2.8

The results presented in Table 10 are from the serum and urine samples taken from the various treatment groups after sacrifice. These data show that there is no significant difference in the urine protein values between the treatment groups. There are some significant differences between the serum BUN values, but all values reported are within acceptable ranges. Serum BUN and urine protein levels indicate that the animals did not suffer kidney damage during the course of this study. Creatinine values were below the limit of detection of the tests, but upon consulting with a clinician it was observed that these values were not indicative of kidney damage.

These results show the clinical efficacy of these antibiotic loaded implants in treating established osteomyelitis. Implants loaded with antibiotic resulted in significantly lower values of flux than the other treatment groups, indicating that these implants were more effective at fighting the infection than the immune system of the animals alone. Also these implants did not result in any kidney damage, which suggests that more antibiotic could be loaded onto the implants to more effectively clear the established osteomyelitis.

3.6.2 Histology

Histology results for animals in which osteomyelitis was induced are reported in Table 11.

Table 11: Histology Results for Inflammation and Bacterial Colonization

Treatment Group	Infection Status	Inflammation Score (Average ± SEM)	Bacterial Colonization Score (Average ± SEM)
DOPS+GS	Osteomyelitis	1.66 ± 0.33	1
DOPS	Osteomyelitis	2.25 ± 0.48	1.75 ±0.25
Stainless Steel	Osteomyelitis	2 ± 0.408	1.5 ±0.29
DOPS+GS	Uninfected	0	0
DOPS	Uninfected	0	0
Stainless Steel	Uninfected	0	0

Statistical analysis was performed on these results, and it was found that there were no statistical differences for either inflammation scores, $p = 0.990$, or bacterial colonization scores, $p = 0.584$. It can be observed from the data that the DOPS+GS treatment group tended to have a lower average inflammation score and a lower bacterial colonization score than the treatments without antibiotic. Power analysis was performed after histology scores were obtained and it was found that for inflammation scores the power was 0.246, and for bacterial colonization scores the power was 0.622. Results for the uninfected control groups show that there is no inflammation or bacterial colonization.

The histology results for bone growth on the uninfected controls samples are presented in Table 12.

Table 12: Histology Results for Reactive Cortical and Trabecular Bone

Treatment Group	Reactive Cortical Bone Score	Reactive Trabecular Bone Score
DOPS+GS	1.2 ± 0.2	0.2 ± 0.2
DOPS	1.4 ± 0.25	0
Stainless Steel	1.25 ±0.25	0.25 ± 0.25

Statistical analysis showed that there were no significant differences between treatments in either reactive cortical bone scores, $p = 0.856$, or reactive trabecular bone, $p = 0.321$. *Post hoc* power analysis revealed that for reactive cortical bone scores the power was 0.289, and for reactive trabecular bone the power was 0.608.

CHAPTER 4: DISCUSSION

4.1 Electrospray Technique

SEM images show that phospholipid coatings were successfully applied to flat stainless steel samples via electrospraying. Results from coatings applied to flat samples showed that the only parameter that needed to be changed for e-spraying on stainless steel was the voltage. Due to stainless steel being less conductive than titanium the voltage needed to be raised to 14kV to apply a uniform coating. No other parameters needed to be changed. Samples sprayed with voltages of 12kV and 16kV resulted in uneven coatings, with more DOPS on one side of the sample. Potential reasons for these uneven coatings are poor contact between the samples and the electrode, or poor conductivity through the sample, particularly in the case of the 12kV samples. Without sufficient voltage through the sample, the potential difference would not be sufficient to pull the phospholipid evenly across the coating surface. For 16kV samples, the voltage could be so high that the phospholipid was pulled around the target samples towards the copper electrodes in the back of the mounting board, applying phospholipid to the outside of the coatings. Since orientation of samples during electrospraying was not noted, it is impossible to know if this is what caused these observations. Once the parameters for electrospraying were obtained, these parameters were used to apply coatings to stainless steel

needles. It was found that the same parameters were successfully used to apply phospholipid coatings to these samples, and the coatings were found to be uniform on all sides of the sample. The samples were electrosprayed only from one direction, and the samples were not turned to expose a different side of the sample. This shows that the electrospray process can successfully be used to evenly coat 3-dimensional objects, with the charged molecule being sprayed seeking out the path of least resistance. This showed that electrospraying could be used to apply coatings to stainless steel needles, which were used as intramedullary rods in the *in vivo* experiment.

The surface of the target and the coating mixture were both modified, with the intent of improving retention of the coating on the surface as well as increasing the time over which GS elutes from the coating. The uneven surface of the stainless steel sample in Figure 7D suggested that a passivation process was needed for subsequent samples, to remove any surface scale and grease as well as provide a uniform oxide layer. Passivation was performed on the stainless steel to remove grease and scale from the metal, as well as to form a uniform oxide layer on the metal surface. After passivation the surfaces were treated with calcium chloride. The calcium, which is a divalent cation, is believed to bind to the oxygen molecules of the surface oxide, and when surfaces are sprayed with phospholipid one bond is formed between the calcium and the DOPS while one bond remains between the calcium and the stainless steel. Pre-treating the samples with calcium and including cholesterol in the phospholipid solution altered the coating morphology significantly. The inclusion of cholesterol in the coating resulted in a spindle-like microstructure, which was not observed when DOPS alone was sprayed.

The coatings were observed to be applied evenly across the coating of the sample surface, however higher magnification images revealed that the coatings are bumpy. This resulting change in morphology could be beneficial for osseointegration. Osteoblasts have been shown to prefer rougher surfaces [38-40], such as nanotubes, so perhaps this spindle-like microstructure provides a more favorable surface than DOPS alone.

4.2 Aerosol Spray Technique

The application of GS in between layers of phospholipid coating is very important, considering that these implant coatings are designed to treat infection at the surgical site. For flat samples GS was dripped on. However, for the stainless steel needles to be used as implants in the *in vivo* study dripping would not provide an even application of GS. Electrospraying GS in water and PBS was briefly investigated, but initial efforts did not result in consistent coatings and due to time constraints this technique was abandoned for the aerosol spray technique. This technique was successfully used to apply GS in varying amounts on stainless steel needles, about 100µg on samples used for the *in vitro* elution study and about 270µg on samples used for the *in vivo* mouse study.

The parameters that were varied to apply the desired amount of GS were air pressure, distance from target, and amount of fluid sprayed. It was observed that a pressure of about 35 psi was ideal for application of GS solution. A higher pressure resulted in too much air pressure on the sample, which resulted in GS solution being blown off the needle. This is also the reason for the greater distance resulting in more

GS being applied to the sample. Holding the brush too close resulted in too much air pressure on the sample and thus loss of GS solution. It was found that spraying too much fluid on the sample resulted in saturation of the samples. In fact it was observed during spraying of the samples that drops of solution would run off the tip of the needle. To avoid sample run off, the least amount of fluid possible was sprayed at samples. 200µl of fluid was sprayed on samples, but one set of samples was sprayed all at once while another set was sprayed four times with 50µl, allowing sufficient time to dry in a vacuum desiccator in between applications. Even though needles sprayed multiple times resulted in less GS on the surface than those with a comparable volume of fluid sprayed all at once, this technique resulted in less fluid run off and as such was chosen as a superior technique. For the *in vivo* study, 300µg of GS was desired to be loaded on the samples. However, 270µg GS was the maximum amount of GS that could be applied to samples with the aerosol procedure developed to date, and so this was deemed sufficient.

4.3 Coating Retention Study

Retention of the coating on the metal surface is a major concern given that these coatings will be applied to samples that will be used as intramedullary rods in mice, and ultimately to be applied to bone implants to be used in humans. If the coating does not adhere well to the implant surface, it will likely come off during the implantation process. Pre-treating the metal surface with calcium chloride to bind calcium to the surface of the metal was shown to improve coating retention. Stainless steel needles that were not calcium treated resulted in SEM images that did not show any coating on

the surface after being inserted through segments of bone, whereas stainless steel needles that were calcium treated showed the characteristic microstructure of DOPS-cholesterol coatings after being pushed through segments of bone. This shows that calcium pre-treatment does in fact improve retention of the coating on the surface.

All images of samples inserted through bone show that excess marrow from the bone samples was left on the stainless steel samples. Only 6 samples of bone were obtained for this preliminary coating retention study, and the samples were used more than once. In order to provide the most accurate assessment of coating retention using this allograft insertion technique, one bone sample needs to be obtained for each stainless steel needle to ensure no marrow is removed from a sample prior to inserting a coated needle. Repeating this test as described above would provide the most accurate information regarding coating retention. Another way to determine coating retention would be to examine the stainless steel needles removed from the mice used in the *in vivo* study under SEM to see whether or not the characteristic microstructure could be observed. One potential difficulty with this is that these needles will have experienced double the shear force as samples in the preliminary coating retention study, due to the fact that these samples will have been inserted and removed from the mice femurs.

Evaluating these images on a 1-5 scale with three evaluators, two of whom were completely blinded, showed that there were indeed differences between the various types of DOPS coatings. Blind evaluators were given images to score with no information regarding whether the sample in the image was coated or uncoated, and if

the sample was coated no information about the coating was provided. Conversely, nonblinded evaluators have some knowledge about the sample in the image, and this knowledge could introduce some bias when scoring. Because nonblinded evaluators could have bias while scoring images, it was important to have blind evaluators to provide complete objectivity when scoring images.

Based on the initial evaluation of images, without any scoring, it appeared as though the 6:1 DOPS-to-cholesterol coating with a calcium pre-treatment resulted in the greatest coating retention, and was selected for the *in vivo* study because of this. When the scores from all three evaluators were combined it was observed that a DOPS only coating was just as effective as the modified coating; however, it was observed that stainless steel only samples inserted through bone segments were scored as having coating by the two blinded evaluators. This is likely due to a poor explanation to the two blind evaluators of how to identify the coating microstructure beneath the residual biological material that results from inserting a coated implant through bone segments.

4.4 *In vitro* Elution Study

The first set of assays from the *in vitro* elution study resulted in questionable data due to the fact that the OPA assay detects both DOPS and GS. This assay makes use of a reaction that targets a primary amine group, which is present in both DOPS and GS. The assay does not have any discriminatory power, so it cannot be known whether the signal detected by the assay is due to DOPS in solution or GS in solution. However, the data still provide some insights into the behavior of these coatings and the effects of modifications to the coatings.

Some samples in the elution study received a calcium pre-treatment whereas some did not. When samples of 6:1 DOPS-to-cholesterol coatings with and without a calcium pre-treatment are compared there does not appear to be a difference in how the coatings elute antibiotic. It can also be observed that the samples that receive a calcium pre-treatment appear to lose more coating than samples without a calcium pre-treatment. This suggests that the process of pre-treating the surface with calcium chloride results in some disruption of the coating at some point away from the metal-phospholipid interface. Some sort of depth profiling or adhesion testing technique should be performed to compare samples with and without a calcium pre-treatment to better understand this result. The samples that are coated with DOPS only show some very strange results.

This study also yielded information about how the inclusion of cholesterol affects the elution of GS from the coatings. Comparing the 3:1 DOPS-to-cholesterol coatings to the 6:1 DOPS-to-cholesterol coatings it can be observed that the 6:1 curve appears to still be releasing GS, while the 3:1 curve appears to have eluted all of the GS loaded onto those samples. Even though this test did not have the power to discriminate between DOPS or GS, the conclusions stated above were the rationale used to support the use of a 6:1 DOPS-to-cholesterol coating for the *in vivo* study.

One problem with this study is that stainless steel needles were placed in a flat well plate, which was then placed in a shaker oven to agitate the fluid in the wells. This resulted in the needles rolling in the well plates, which created additional mechanical forces on the coating that would not be present in a biological system. This study

should be repeated with flat samples, so as to avoid these excess forces on the coating and provide more accurate information about how the antibiotic elutes from these coatings. The other major problem with this study is the assay that is used to detect GS. Since this assay interacts with any compound containing a primary amine it detects not only GS but DOPS as well. GS contains five primary amines while DOPS only has one, but tests with known amounts of DOPS and GS suggested that the assay does not interact with each primary amine in GS (data not shown). For this reason the initial elution data are not very reliable, and thus the lipid extraction technique was developed.

Despite some of these results being flawed, this study showed that modifications to the implant coatings can be used to control the time over which GS elutes from the coatings. The inclusion of cholesterol resulted in an increase in the elution time, but a 6:1 ratio of DOPS-to-cholesterol appeared to result in a longer elution time than coatings with a 3:1 ratio. It was shown that calcium pre-treatment does not affect the elution of GS from these coatings and might disrupt coating adhesion so it was not used to prepare the *in vivo* samples.

Adding the lipid extraction did not result in better data, suggesting that there are other factors that are confounding this experiment. This is best observed in Figure 32, which should have a constant absorbance value since there is no GS on the samples and the extraction removes DOPS from the eluent. Yet there is still some signal being detected by the assay, which indicates that there is likely a problem with the OPA reagent. While statistical differences were observed at a few time points between several treatment groups, these results do not suggest that any one coating is

preferable. Also, given that the control groups showed no significant difference from most treatments at all time points, and all treatments at time point 0.5, these results are unreliable. Figure 25 shows evidence that the lipid extraction procedure is effective, which suggests that there is a problem elsewhere. One potential source of error is with the OPA assay. Another source of error could be the fact that the same eluent was used for both studies, with a 6 month period of time after the no extraction study before the study that included lipid extraction. Eluent was stored in a refrigerator during this time period.

Dr. Prawel used the OPA assay during in his work, and he modified the original OPA reagent by reducing the sodium borate (boric acid) concentration by half. The purpose of doing this was to allow the assay to be used to detect smaller amounts of GS, however, no work was done to see how this impacted the life of the reagent. Since the OPA reagent has a limited shelf life, perhaps altering the borate concentration decreased the shelf life. This is one potential source of error that could contribute to the results observed in the elution study. Another potential source of error has to do with the pH. It has been shown that the OPA reagent has maximum sensitivity under alkaline conditions, which could be very important given that boric acid is a component of the reagent. Furthermore, the eluent is GS in PBS, which is buffered to keep the pH at 7.4. This could be yet another reason for the results observed from the elution study. These issues need to be addressed before the OPA assay can be used to accurately quantify the elution of GS from these coatings [48].

4.5 *In vivo* Study

The *in vivo* study showed very promising results that suggest the antibiotic loaded implants helped reduce the amount of bacteria present in the mice. Differences between the DOPS+GS treatment and the other two treatments are significant at around day 14 and continue up until the very last day of the study. This shows that the reduction in infection is due to the presence of antibiotic in the coating, not just the immune system of the mice. Figure 34 shows that there is a plateau in the values of total flux from day 20 to the end of the study for the antibiotic loaded samples. This plateau suggests that the supply of antibiotic in the coating is running out. It is important to note that in order to detect later generations of bacterial cells using bioluminescent imaging, the plasmid must be passed from the parent cell to the daughter cells. Over time, the plasmid will eventually lost and cells will no longer be able to be detected via bioluminescent imaging. However, given that this is only a 28 day study loss of plasmid is likely not a problem in this work. Since the total flux values plateau instead of beginning to increase, the infection is not becoming reestablished. Thus, the amount of antibiotic eluting from the implant is either very low or completely exhausted. Because these animals are immunocompetent, it is possible that the supply of antibiotic is completely exhausted and the immune system of the animals is keeping the remaining bacteria from becoming reestablished infection. The trends of the non-antibiotic treatment groups showing the immune systems of the mice actively fighting infection makes it more likely that the reason for the plateau observed in the DOPS+GS

group is exhaustion of GS in the coating, and action of the immune system prevents the total flux values from increasing.

Additionally, the serum and urine results show that the mice did not suffer any kidney damage from too much antibiotic, suggesting that more GS could be loaded onto these samples in a future study in an effort to totally clear the infection, rather than just reduce it. However, there are limitations to using creatinine, BUN, and urine protein tests to assess kidney damage. The values of these tests could be reported in the normal range, but the only way to truly know whether or not kidney damage has occurred is to perform histology on the kidneys. For the purpose of this study, however, it was determined that the BUN, creatinine, and urine protein values were sufficient to suggest no damage was done to the kidneys. These data suggest that this coating technique produces coated implants that are able to effectively treat an established infection without resulting in damage to the mice, and that these coatings will be effective at preventing infection after an implant surgery. Furthermore, these results are evidence that these coatings can be studied in a larger animal model.

One problem that was encountered during the *in vivo* study is that many mice did not have implants at the end of the study. This is likely due to the fact that the mice can scratch at the surgical site, and potentially pulled the implant out. Without knowing when the implant was removed data from these mice may not be accurate. Mice that removed their implants during the study may have a high infection rate, but that could be due to the implant being removed, not poor implant performance. Another issue encountered was the scale not being equal on each image, which caused some

confusion over whether or not there was infection present in a mouse at a given time point. The images obtained do not tell the whole story, and the numerical values reported by the Xenogen software are a more accurate representation of whether or not bacteria are present. Finally, the tissue between the femur and the camera could be masking some of the photons being emitted by the oxidation of luciferin by luciferase.

Another limitation of this study is that it does not compare the efficacy of local delivery of antibiotics in treating osteomyelitis to the efficacy of using systemic antibiotics. There was no treatment group in this study that received systemic antibiotics to treat osteomyelitis, and as such it is not known whether these antibiotic-eluting implants are more or less effective than systemic antibiotics. In the future, it would be recommended that a treatment group with mice that were being treated with systemic antibiotics were included to compare the two methods.

The histology results show that while there are no statistical differences between the infected groups, the DOPS+GS group has lower average values for both inflammation score and bacterial colonization score. *Post hoc* power analysis showed that the power was low for these tests, below the desired power of 0.8. This explains why there are trends in the data that can be observed, but no statistical significance. This suggests that this group has less bacteria present than the others, which confirms the observations from the bioluminescent imaging data. Histological scoring shows that bacteria are still present in the femurs of these animals, and that the DOPS only group has higher inflammation and bacterial colonization scores than both the DOPS+GS and stainless steel treatment groups. Bioluminescent imaging is useful for reducing the

number of animals in a given study, but needs to be supplemented with other techniques such as histology to fully understand the infection progression. Furthermore, the histology results showed that the DOPS coating did not provide any increased osseointegration. The *post hoc* power analyses revealed that, like the inflammation and bacterial colonization scores, the power for these tests was below the desired value of 0.8. One way to increase the power would be to increase the number of samples that undergo histology. Aside from the observation that there is more reactive cortical bone than reactive trabecular bone, there are no observable trends in the data that would suggest one particular treatment providing enhanced osseointegration. This is likely due to the fact that these femurs had the pins manually removed before being analyzed, which could have resulted in damage to the bone growing onto the implants. It was anecdotally reported by the experimenters that removal of the implants was difficult. In removing the implants, because removal was difficult perhaps the bone that had grown onto the implants was damaged and possibly pulled out with the implant. To confidently say that there is no enhanced osseointegration on implants with DOPS coatings, another testing method would be required to verify these results, such as micro CT. However, these results suggest that DOPS coated implants do not improve osseointegration compared to stainless steel implants.

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

Implant-associated infection is a serious problem for patients receiving orthopedic implants. This work sought to address this issue by creating an antibiotic-eluting phospholipid coating for implants. These coatings were applied to stainless steel needles that were used as intramedullary implants in a murine model. These coatings were applied via electrospray, which was initially used to apply phospholipid coatings to titanium samples. The electrospray parameters were tuned to apply coatings on stainless steel that are morphologically similar to those previously applied to titanium. Also, it was demonstrated that this technique can be used to apply phospholipid coatings to two-dimensional and three-dimensional samples, and the coating was observed to be applied uniformly on all samples sprayed. The major conclusions of this work are as follows:

- Antibiotic-eluting successfully treated osteomyelitis *in vivo*
- No kidney damage was observed
- Implants ran out of antibiotic prior to the end of the study
- Calcium pre-treatment and inclusion of cholesterol in spray solution improved coating retention and elongated *in vitro* elution profile

Bone insertion tests were used to examine the effects of various modifications to implant coatings on clinically relevant coating retention. Both qualitative observation

and semiquantitative analysis were used to study coating retention. Initially qualitative observations were used to determine that a calcium pre-treatment improved the amount of coating that remained on the implant after insertion through bone, and this was later confirmed statistically with blinded image scoring. The semiquantitative analysis revealed that the combination that provided the greatest amount of coating retention on implants was a 6:1 DOPS-to-cholesterol spray mixture onto samples that received calcium pre-treatments.

A total sink elution study was performed on samples to determine how modifications to the coating impacted antibiotic elution. Despite the lack of discriminatory power between DOPS and GS when using the OPA assay, the results showed that a spray mixture of 6:1 DOPS-to-cholesterol yielded the longest time of antibiotic elution while still having some antibiotic within the coating, suggesting that more will come out at longer times. Also, while it did not appear that pre-treating surfaces with calcium had any impact on antibiotic elution the results showed that samples that were pre-treated with calcium had more DOPS coming off of the surface than those that were not pre-treated with calcium. This suggests that calcium pre-treatment disrupts the bonding of phospholipid molecules in the coatings, thus making it easier for the coating to come off. However, the results of the coating retention study show that calcium pre-treatment is needed for the coating to withstand insertion through bone. These contradictory results could indicate that the calcium pre-treatment successfully holds the coating onto the metal more effectively at the metal-coating interface, but further from the interface the bonding within the coating is

disrupted. However, due to the fact that the elution without extraction results are confounded by the presence of DOPS, they are not as reliable as the coating retention results. As such, the coating retention results are more trustworthy, and the calcium pre-treatment was necessary for *in vivo* performance. One potential solution to this problem is to co-spray calcium and phospholipid when applying coatings.

It was shown that these coatings were successfully able to treat osteomyelitis *in vivo*. Bioluminescent imaging showed that osteomyelitis was successfully induced using genetically modified *Staphylococcus Aureus*, and over 28 days it was shown that the mice with antibiotic loaded implants reduced the luminescent signal faster than those without. The infection was never completely cleared in any treatment group, suggesting that the antibiotic loaded onto the implants was exhausted prior to the end of the study. Histological analysis confirms what was observed in the bioluminescent images, in that there were still bacteria present in all infected mice and that the antibiotic-loaded treatment group had levels of inflammation and bacterial colonization that were lower than the other treatments. Tests run on serum and urine showed values that were within normal ranges. This suggests that antibiotic was not accumulating and causing significant damage to the kidneys as measured by serum and urine tests. Histological analysis of the kidneys themselves would confirm there is no kidney damage which is not showing up in urine and serum. All of these results from the *in vivo* study show that antibiotic-eluting phospholipid coated implants were able to effectively treat osteomyelitis while not exhibiting any adverse effects.

5.2 Future Work

Due to the promising results of this work I would recommend repeating this work in a larger animal model, which is the next step in getting these implant coatings into a clinical setting. When performing these experiments I would recommend again using an animal model with a functional immune system. The fact that all the implant groups showed a reduction in bacteria over time might suggest that an immunocompromised model would be more favorable. However, an immunocompromised animal would not be desired as the results obtained from this animal would not be clinically relevant. The results from an immunocompromised animal in this case would be analogous to those obtained from an *in vitro* cell study. In order to observe how effective these implant coatings are versus uncoated implants, the immune system of the animal would be required. This would provide a clinically relevant evaluation of the efficacy of these antibiotic-eluting coatings.

If this work is to be repeated in a murine model, it is recommended that more antibiotic be loaded onto the samples. Due to the bioluminescent imaging and histology results showing that infection was still present, and conclusion that the antibiotic supply was exhausted prior to the end of the study, future experiments would require a greater antibiotic load in order to completely clear the infection. This would show whether or not the implant coatings are capable of completely clearing an established case of osteomyelitis. Also, it is recommended that a treatment group be included in which the animals with osteomyelitis are treated with systemic antibiotics so the two methods can be compared. Additional future work would be to repeat the *in vivo* study to determine

how effective these implant coatings are at clearing a biofilm. A biofilm is a network of bacteria in a polysaccharide matrix, and can result when an infection is left untreated. Biofilms are much more difficult to clear than a typical infection, requiring higher doses of antibiotics. It would be worthwhile to see if these implant coatings can be used to deliver antibiotic locally to clear a biofilm.

Furthermore, an additional experiment worth performing would be a minimum inhibitory zone study *in vitro*. This test would be used to supplement the *in vitro* elution studies, which had several problems with the assay used. By performing the minimum inhibitory zone study qualitative data could be obtained on whether or not the amount of GS in solution at a given time point during an elution study was able to kill bacteria. This experiment would not provide quantitative data on how much GS was present, but would provide an accurate time frame over which the implants were eluting GS at or above MIC.

REFERENCES

1. Kurtz, S., et. al., *The future burden of hip and knee revisions.* American Academy of Orthopaedic Surgeons, 2006. 73(SE53).

2. Vasudev, D.V., et al., *In vivo evaluation of a biomimetic apatite coating grown on titanium surfaces.* Journal of Biomedical Materials Research Part A, 2004. 69A(4): p. 629-636.

3. Goyenvalle, E., et al., *Osteointegration of femoral stem prostheses with a bilayered calcium phosphate coating.* Biomaterials, 2006. 27(7): p. 1119-1128.

4. Frenkel, S.R., et al., *Osseointegration on metallic implant surfaces: Effects of microgeometry and growth factor treatment.* Journal of Biomedical Materials Research, 2002. 63(6): p. 706-713.

5. Richards, R.G., *Introduction: Implants and infection in fracture fixation "ten years on".* Injury, Int. J. Care Injured, 2006. 37(S1—S2).

6. Chen, C.H., Kelder, E.M., and Schoonman, J., *Electrostatic sol-spray deposition (ESSD) and characterisation of nanostructured TiO2 thin films.* Thin Solid Films, 1999. 342(1-2): p. 35-41.

7. Prawel, D.A., James, S.P., and Popat, K.C., *Novel electro-spray technique for applying phospholipid coatings to titanium*. *Society for Biomaterials*, P. Phillip B. Messersmith, Editor. 2010: Seattle, WA. p. 65.

8. Prawel, D.A., James, S.P., and Popat, K.C., Patent 13041960, Filed March 7, 2011: USA.

9. Prawel, D.A., *A drug eluting osseointegrative phospholipid coating for orthopedic implants*, Doctoral Dissertation, Bioengineering, Colorado State University, Spring, 2011.

10. Camolezi, F.L., et al., *Construction of an alkaline phosphatase-liposome system: a tool for biomineralization study.* International Journal of Biochemistry & Cell Biology, 2002. 34(9): p. 1091-1101.

11. Santin, M.e.a., *Calcium-binding phospholipids as a coating material for implant osteointegration.* J. R. Soc. Interface, 2005. 3: p. 277-281.

12. Satsangi, A., et al., *Osteoblast response to phospholipid modified titanium surface.* Biomaterials, 2003. 24(25): p. 4585-4589.

13. Merolli, A., et al., *In vivo assessment of the osteointegrative potential of phosphatidylserine-based coatings.* Journal of Materials Science: Materials in Medicine, 2006. 17(9): p. 789-794.

14. Eanes, E.D., *Mixed phospholipid liposome calcification*, Bone and Mineral, 1992. 17: p. 269-272.

15. Hung, C.T., et. al., *Intracellular Ca2+ stores and extracellular Ca2+ are required in the real-time Ca2+ response of bone cells experiencing fluid flow*. Journal of Biomechanics. 29(11): p. 1411-1417.

16. Poste, G., Allison, A.C., *Membrane-fusion,* Biochimica Et Biophysica Acta, 1973. 300(4): p. 421-465.

17. Dluhy, R.A., et. al., *Fourier-transform infrared spectroscopic studies of the effect of calcium-ions on phosphatidylserine*, Biochemistry, 1983. 22(26): p. 6318-6325.

18. Vemuri, S., Rhodes, C.T., *Preparation and characterization of liposomes as therapeutic delivery systems: a review,* Pharmaceutica Acta Helvetiae, 1995. 70: p. 95-111.

19. Grit, M., Crommelin, D.J.A., *Chemical stability of liposomes: implications for their physical stability,* Chemistry and Physics of Lipids, 1993. 64(1-3): p. 3-18.

20. Hancock, R.E.W., *Aminoglycoside uptake and mode of action – with special reference to streptomycin and gentamicin I. Antagonists and mutants*, Journal of Antimicrobial Chemotherapy, 1981. 8(4): p.249-276.

21. Shakil, S., Khan, R., et. al., *Aminoglycosides versus bacteria – a description of the action, resistance, mechanism, and nosocomical background*, Journal of Biomedical Science, 2008. 15: 5-14.

22. Hahn, F.E., Sarre, S.G., *Mechanism of action of gentamicin*, Jounral of Infectious Diseases, 1969. 119(4-5): p. 364-369.

23. Tangy, F., Moukkadem, M., et. al., *Mechanism of action of gentamicin components. Characteristics of their binding to Escherichia coli ribosomes*, European Journal of Biochemistry, 1985. 147: p.381-386.

24. Jana, S., Deb, J.K., *Molecular understanding of aminoglycoside action and resistance*, Applied Microbial Biotechnology, 2006. 70: p. 140-150

25. Obatomi, D.K., Plummer, D.T., *Renal damage caused by gentamicin: a study of the effect in vitro using isolated rat proximal tubular fragments*, Toxicology Letters, 1995. 75: p. 75-83.

26. Chang, H.I., Lau, Y.C., et. al. *Controlled release of an antibiotic, gentamicin sulphate, from gravity spun polycaprolactone fibers*, Journal of Biomedical Materials Research Part A, 2008. 84(1): p. 230-237.

27. Ruszczak, Z., and Friess, W., *Collagen as a carrier for on-site delivery of antibacterial drugs*, Advanced Drug Delivery Reviews, 2003. 55: p. 1679-1698.

28. Soundrapandian, C., Datta, S., Sa, B., *Drug-eluting implants for osteomyelitis*, Critical Reviews in Therapeutic Drug Carrier Systems, 2007. 24(6): p. 493-545.

29. Jin, Y., Jang, J., et. al., *Development of ELISA and immunochromatographic assay for the detection of gentamicin*, Journal of Agricultural and Food Chemistry, 2005. 53: p. 7639-7643.

30. Ratcliff, R.M., Mirelli, C., et. al., *Comparison of five methods for the assay of serum gentamicin*, Antimicrobial Agents and Chemotherapy, 1981. 19(4): p. 508-512.

31. Kaale, E., Long, Y., et. al., *Gentamicin assay in human serum by solid-phase extraction and capillary electrophoresis*, Electrophoresis, 2005. 26: p. 640-647.

32. Wang, H.Y., Liu, L.D., et. al., *Determination of gentamycin by synchronous derivative fluorimetry*, Talanta, 2000. 52: p. 201-209.

33. Frutos, P., Torrado, S., et. al., *A validated quantitative colorimetric assay for gentamicin*, Journal of Pharmaceutical and Biomedical Analysis, 2000. 51: p. 1149-1159.

34. Bligh, E.G., Dyer, W.J., *A rapid method of total lipid extraction and purification*, Canadian Journal of Biochemistry and Physiology, 1959. 37(8): p. 911-917.

35. Gubernator, J., Drulis-Kawa, Z., Kozubek, A., *A simply and sensitive fluorometric method for determination of gentamicin in liposomal suspensions*, International Journal of Pharmaceutics, 2006. 327: p. 104-109.

36. Doyle, T.C., Burns, S.M., Contag, C.H., *In vivo bioluminescence imaging for integrated studies of infection*, Cellular Microbiology, 2004. 6(4): p. 303-317.

37. Jang, K., Lee, K., *et al*, *In vivo tracking of transplanted bone marrow-derived mesenchymal stem cells in a murine model of stroke by nioluminescence imaging*, Journal of Korean Neurosurgical Society, 2010. 48: p. 391-398.

38. Cook, S.H., Griffin, D.E., *Luciferase imaging of a neurotropic viral infection in intact animals*, Journal of Virology, 2003. 77(9): p. 5533-5538.

39. Sottnik, J.L., U'Ren, L.W., *et al*, *Chronic bacterial osteomyelitis suppression of tumor growth requires innate immune responses*, Cancer Immunology, Immunotherapy, 2010. 59: p. 637-378.

40. Sottnik, J.L, Duval, D.L., *et al*, *An orthotopic, postsurgical model of luciferase transfected murine osteosarcoma with spontaneous metastasis*, Clinical and Experimental Metastasis, 2010. 27: p. 151-160.

41. Satsangi, N., Satsangi, A., *et al, Osteoblast response and calcium deposition on phospholipid modified surfaces*, Journal of Materials Science: Materials in Medicine, 2004. 15: p. 693-697.

42. Feng, Q.J., *Electrohydrodynamic behaviour of a drop subjected to a steady uniform electric field at finite electric reynolds number*, Proceedings: Royal Society of London A, 1999. 455(**1986**): p. 2245-2269.

43. Ponnaiya, B., Cornforth, M.N., Ullrich, R.L., *Radiation-induced chromosomal instability in BALB/c and C57BL/6 mice: the difference is as clear as black and white*, Radiation Research, 1997. 147(**2**): p. 121-125.

44. Black, B.L., Croom, J., *et al*, *Differential effects of fat and sucrose on body composition in A/J and C57BL/6 mice*, Metabolism, 1998. 47(**11**): p.1354-1359.

45. Lincks, J., Boyan, B.D., *et al*, *Response of MG63 osteoblast-like cells to titanium and titanium alloy is dependent on surface roughness and composition*, Biomaterials, 1998. 19(**23**): p. 2219-2232.

46. Oh, S., Daraio, C., *et al*, *Significantly accelerated osteoblast cell growth on aligned TiO_2 nanotubes*, Journal of Biomedical Research Part A, 2006. 78A(**1**): p. 97-103.

47. Oh, S. and Jin, S., *Titanium oxide nanotubes with controlled morphology for enhanced bone growth*, Materials Science and Engineering: C, 2006. 26(**8**): p. 1301-1306.

48. Kaale, E., et. al., *Gentamicin assay in human serum by solid-phase extraction and capillary electrophoresis*, Electrophoresis, 2005. 26: p. 640-647.

APPENDIX A: STANDARD PROTOCOLS

A.1 : Electrospray Protocol

Materials and Supplies

- 1010 Gastight Glass Syringe and plunger
- Pipettor Gun and 5mL graduated pipette
- 50mL beaker , 100mL beaker, small vials with Teflon tabbed lids
- Samples to be sprayed
- Teflon sticky tack
- Cotton swabs and kim wipes
- Chloroform (Mallinckrodt Incorporated), phospholipid (Avanti Polar Lipids, Inc) and cholesterol (Alfa Aesar)
- Mounting board
- Electrospraying apparatus and needle

Methods

**Always wear gloves*

Chemical Set Up

1. Set up clean work space in hood with Kimwipes
2. Clean all glass wear (syringe, beakers, pipette, vials) thoroughly with chloroform before beginning to avoid any possible contamination
3. Rinse needle with chloroform to make sure to remove any remaining solution from previous use, and ensure needle works properly
4. Weigh and label all titanium samples.
5. Clean the non-labeled side of all titanium samples with chloroform.

6. Clean sample mounting board. Make sure the area where the samples will be attached is free of any old phospholipid and all tape is in place covering any leads you will not be using.
7. Attach clean samples to mounting board. For 2-D samples do so using Teflon tack stuck around the metal contact, ensuring that they are touching the contact. For 3-D samples load 2 needles into the alligator clips, making sure to connect clips to both the top and bottom of the needles. Place Teflon tack around the contact points, and the hub of the needle. If there is no connection, no phospholipid will get on the samples. Clean samples again with chloroform on a cotton swab after attaching.
8. Connect needle tube to syringe.
9. Prepare the DOPS/Chloroform solution as required. Keep container closed as much as possible to avoid (rapid) evaporation of the mixture.
10. Transfer the solution to the syringe by sucking the material from the container into the syringe. Do not push the plunger into the tube; carefully turn the syringe so the plunger is down and syringe tube is up, so the free air in the tube is upward; push the plunger into the syringe tube, pushing the air out the needle, until the chloroform mixture in the syringe is nearly all the way up the tube to the needle.

<u>Electro-Spray Apparatus Set up</u>

1. Check the pump rate on pump (*every third or fourth use you will want to check the pump rate and ensure calibration)
2. Put syringe in place on pump and clamp needle into holder in apparatus

3. Clamp sample mounting board in place

a. Align needle such that it points at the center of the ring of samples on the sample mounting board

b. Adjust distance from needle tip to sample mounting board

c. Recheck alignment, and then distance again.

4. Attach ground wires to syringe and sample mounting board

5. Attach power wire to needle tip

6. Check that Voltage on power supply is set as required

7. Double check samples (make sure they are touching) and clean one last time with chloroform.

8. Double check all connections: that solder board and syringe are grounded and needle is powered

9. Push pump activator manually until you see fluid start to move again in tube, this is to make sure the pump activator is in contact with the syringe plunger

10. Turn on power supply

11. Turn on pump

12. Watch for a spray cone, once you see the spray cone start the timer and close the door to the apparatus

13. When the prescribed time has run out, turn off the pump and turn off the power supply

14. Remove ground and power wires, and remove sample mounting board

15. Remove samples from the sample mounting board

16. Reweigh samples

17. Clean the sample mounting board

Clean Up

1. Push a few mls of chloroform through the syringe, into a waste beaker
2. Clean all glass wear (beakers, pipette, vials) and needle with chloroform
3. Very carefully clean syringe
4. Store needle with E-spraying apparatus
5. Rinse glass wear with lab soap and then rinse in DI water
6. Dry carefully and store
7. Double check that E-spray apparatus is clean and clean up area in hood
8. Do one last check of lab and make sure you have stored everything properly

A.2 : Stainless Steel Cleaning and Passivation Protocol

Materials and Supplies

- 70% nitric acid (EMD Chemicals)
- Deionized water
- Acetone (Mallinckrodt Incorporated)
- Ethanol (Pharmaco-Aaper)
- Liquinox
- Hot plate with digital temperature control
- Sonicator
- 3 250mL beakers
- Large petri dish

Methods

Note: Passivate 20 samples or less in one beaker (more than this results in significant degradation of plastic piece connecting needle to hub)

And remember to use glassware, not plastic.

1. Sonicate 30 minutes in 100 ml Acetone (stirring vigorously every 5 minutes)
2. Rinse in tap water
3. Sonicate 15 minutes in 100 ml ~5% Liquinox (stirring vigorously every 5 minutes)
4. Rinse in Di water until no evidence of soap
5. Sonicate 15 minutes in 100 ml DI water (stirring vigorously every 5 minutes)
6. Rinse twice in ethanol
7. Rinse once in acetone

8. Blow dry with lab air
9. Prepare a 100 mL solution of 50% nitric acid in a 250mL beaker
   i. Note: It is important to use a 250mL beaker to allow needles to almost float in the solution.
   ii. Note: Always acid to water, **never add water to acid**
10. Heat 50% nitric acid solution on a hot plate to 71^{o}C.
   i. Note: This usually takes about 15 to 20 minutes to heat up, so to save time start heating this solution before sonicating samples in DI water.
11. Place no more than 20 needles in 50% nitric acid solution, set stirring to 200 rpm (no stir bar) and let sit for 30 minutes.
12. Remove samples from solution and quench in three sequential DI water baths, stirring vigorously in each bath.
13. Remove samples from final water bath and arrange on an uncovered petri dish. Leave exposed to air for a minimum of 18 hours.
14. After 18 hours in air, store samples in a desiccator.

A.3 : Calcium Pre-Treatment Protocol

Materials and Supplies

- Calcium chloride (Fisher Scientific)
- Deionized water
- Samples to be calcified
- 3 250mL beakers
- Hot plate with digital temperature control

Methods

1. Samples must be passivated before calcification (see passivation protocol)
2. Prepare a solution of 2.25mM $CaCl_2$ in DI water.

a. Note: I found that making 100mL of solution and placing 20 samples in this solution works well.

3. Heat solution on a hot plate to 50^{o}C
4. Place samples in heated solution, set stirring to 200rpm (no stir bar needed, needles are magnetic) and let sit for 1 hour.
5. Remove samples from solution and rinse 3 times with DI water to remove unbound ions.
6. Store samples in a desiccator or vaccum oven to dry (ideally store in a vacuum desiccator)
7. Once samples are dry, store covered in a desiccator.

A.4 : Coating Retention Protocol

A.4: Coating Retention Protocol

Materials and Supplies

- Proper number of bone segments
- Needles to be tested
- Gloves
- Tin snips
- Adequate 12 well plates

Methods

1. Slide bone segment over the end of the needle, making sure that the tip is just coming out the other end of the medullar cavity.
2. Grip bone segment between thumb and forefinger of one hand, using the other hand to rotate the needle 360 degrees.
3. Slide bone segment towards the hub of the needle. Make sure to slide bone to a position that is just next to the position that was previously covered.
4. Again, grip bone segment between thumb and forefinger of one hand, using the other hand to rotate the needle 360 degrees.
5. Repeat this procedure until the bone segment reaches the hub of the needle.
6. Using the tin snips, carefully cut the needle at the bone and place this into the well plate.

A.5: Lipid Extraction Protocol

Materials and Supplies

- Dichloromethane (Mallinckrodt Incorporated)
- PBS (Sigma Life Sciences)
- Methanol (Fisher Scientific)
- 3 100mL beakers
- Sufficient tube racks for samples
- Sufficient 2mL centrifuge tubes
- Centrifuge
- 1mL adjustable-volume pipettor
- Sufficient tips, standard and aerosol-filter
- Chloroprene gloves, lab coat, and lab glasses

Methods

1. Pipette 200 mL of sample into a 2 mL centrifuge tube.
2. Add to each tube 500 mL of methanol and 250 mL dichloromethane.

i. Note: Dichloromethane is a volatile compound, and aerosol filter tips should be used when pipetting this chemical. Also, the first time dichloromethane is aspirated into the pipette tip it rapidly drips out of the tip. After the first aspiration the chemical does not drip out of the tip, and as such the tip should be "primed" with dichloromethane.

3. Vortex tubes for 1 minute.

i. Note: Cover tubes on rack with foil while vortexing to prevent tops from opening.

4. Add to each tube 250 mL PBS and 250 mL dichloromethane.

5. Vortex tubes for 1 minute.

6. Centrifuge tubes for 5 minutes at 10,000rpm.

a. Top layer is aqueous phase (GS containing) and bottom layer is dichloromethane (DOPS containing)

7. Transfer 0.5 mL aqueous phase into a new 2 mL centrifuge tube for OPA assay.

i. Note: Perform this step carefully, so as not to remove any of the dichloromethane layer. Sample should be pulled from the middle of the aqueous phase.

ii. Note: For one set of 24 samples this procedure takes approximately 30 minutes.

iii. Note: Dichloromethane is a volatile chemical, and aerosol filter tips must be used when pipetting this solution. Also, tips must be “primed” by aspirating the amount of dichloromethane to be pipetted prior to transferring dichloromethane to the sample tubes.

A.6 : OPA Assay Protocol

Materials and Supplies

- Sufficient OPA Reagent (see below)
- Sufficient 96-well plates
- Sufficient 2 mL centrifuge tubes
- Sufficient Gentamicin Sulfate standards (see below)
- Plate reader that can read at 332 nm
- O-phthaldialdehyde (Sigma Life Sciences)
- Methanol (Fisher Scientific)
- Mercaptoethanol (Sigma Life Sciences)
- Sodium tetraborate decahydrate (Sigma-Aldrich)
- Gentamicin Sulfate (Sigma Life Sciences)
- Isopropanol (Mallinckrodt Incorporated)

Methods

OPA Assay

1. Mix desired quantity of OPA Reagent at least 24 hours prior to planned OPA assay. (see Mixing OPA Reagent below)
2. Combine equal amounts of eluent or GS standard being tested with OPA Reagent and isopropanol.

a. If vial containing eluent being tested is large enough, add OPA Reagent and isopropanol in amounts equal to amount of eluent being tested, into vial containing

eluent being tested. For example, in DOPS-GS elution studies, we typically have 0.5 ml of GS eluent in a 1.5 ml vial. In this case, we would add 0.5 ml of OPA Reagent and 0.5 ml of isopropanol into the vial containing the 0.5 ml of GS eluent.

3. Carefully (to avoid dripping or splashing into any adjacent wells) plate 0.2 ml of each GS standard concentration into the first three wells of each row of a 96-well plate. There are five [GS], so the first three wells of five rows should be occupied. Leave the 7^{th} and 8^{th} rows blank.
4. Carefully (to avoid dripping or splashing into any adjacent wells) plate 0.2 ml of each eluent time point into six wells of each column of the same 96-well plate, starting with column 4 and continuing to the end of the plate.
5. If eluent time point samples remain, start a new plate, repeating step 4 above until all eluent time points are plated. When starting a new plate, repeat step 3 above for each plate.
6. Set plate reader to read at 332 nm. Read all plates.
7. Discard samples and clean up.

<u>Mixing OPA Reagent</u>

NOTE: OPA Reagent has shelf life of only three days. Base Proportions

2.5 g o-pthaldialdehyde
62.5 ml methanol
3 ml mercapoethanol
560 ml borate (1 mM)

(for Borate mixing, see Mixing Borate below)

Typical volume mixed for elution studies

4% of base proportions above
2.5*.04=0.1 g o-pthaldialdehyde
62.5*.04=2.5 ml methanol
3*.04=0.12 ml mercapoethanol
560*.04=22.4 ml borate (1 mM)
Yields 2.5+.12+22.4=25.02 ml OPA Reagent

Mixing Borate

1 mM borate consists of 381.37 g borate in 1 L diH_2O

For 200 ml, use 0.0763 g borate

Notes:

Absorbances from different plates cannot be compared. Standards curves from one plate must be used to convert absorbances to GS concentration, which can then be compared from plate-to-plate.

OPA reagent preparation is very sensitive to amounts of o-phthaldialdehyde. Different amounts of OPA in the reagent could cause the absorbance values of blank PBS to vary. This is why plate-to-plate absorbances can't be compared, but rather must be converted to GS concentration.