THESIS

PHOSPHATIDYLSERINE AND ANTIBIOTIC COATINGS FOR ALLOGRAFT BONE

Submitted by

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ABSTRACT

PHOSPHATIDYLSERINE AND ANTIBIOTIC COATINGS FOR ALLOGRAFT BONE

Osteosarcoma is the most common type of primary bone tumor in humans. Treatment usually involves both surgical resection of the tumor and chemotherapy. Limb sparing often necessitates the use of massive bone allografts, however patients on anticancer drug regimens are at increased risk of infection, non-union and mechanical failure. The purpose of this work was to develop and test antibiotic eluting phospholipid coatings for massive bone allografts which may be useful in revision surgery for patients with osteomyelitis infection. This project was motivated by previous research performed on coatings of the phospholipid 1,2-dioleoyl-sn-glycero-3-phospho-l-serine (DOPS) and the antibiotic Gentamicin Sulfate (GS) applied to metallic implants.

The potential of these coatings to combat infection and enhance osseointegration was evaluated *in vivo* using massive femoral allografts in a murine model with a well established osteomyelitis infection. Phospholipid coatings were applied to decellularized mouse femur segments using an electrospray method. Antibiotic was incorporated between two DOPS layers. The presence of both DOPS and GS was verified by examining the treated allografts with a scanning electron microscope (SEM). Allografts were then prepared and implanted into 50 mice in seven different treatment groups. In four of these treatment groups the mice were deliberately infected with osteomyelitis one week prior to allograft implantation using a genetically modified bioluminescent strain of *Staphylococcus aureus* that enabled tracking of the infection *in vivo*. Mice were sacrificed at 28 days post allograft implantation and allografts were evaluated

histologically. After completing the *in vivo* portion of the study, the antibiotic eluting characteristics of the coatings were analyzed *in vitro* with a total sink elution method and antibiotic in the eluent was quantified using an agar diffusion test.

Results showed that mice receiving antibiotic coated allografts displayed significantly reduced infection up to fifteen days post allograft implantation. Measurable infection remained until the end of the study however and none of the infected mice exhibited any osseointegration with the allograft. These results were most likely due to the severity of the osteomyelitis infection and the rapid elution of the antibiotic from the allografts, as confirmed by the *in vitro* elution study. Osseointegration was observed in the uninfected mice however no statistically significant differences were found between the DOPS coated treatment groups and the uncoated control group. This was attributed to the small sample size of the uninfected groups and the small number of histological sections, and was perhaps exacerbated by inconsistent host-graft apposition. Further research is therefore necessary to validate the potential of DOPS/GS allograft coatings to fight infection and enhance osseointegration.

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Chapter 1 – Introduction

1.1 Project Overview and Motivation

Osteosarcoma is a common bone cancer that is typically treated with a combination of surgery and chemotherapy. Surgical removal of the cancerous tumor often necessitates the removal of large sections of bone and surrounding tissue to ensure a good margin and reduce the incidence of recurrence of cancer. While amputation was once the standard procedure for osteosarcoma, in modern times limb sparing procedures that incorporate massive bone allografts are most often used after surgical resection of the tumor.

Massive bone allografts are typically used for limb sparing procedures, however these allografts are prone to both osteomyelitis, which is infection of bone tissue, as well as mechanical failure due to poor integration between host bone and the allograft. The objective of this work was to develop and test novel osseointegrative, antibiotic eluting allograft coatings for use in limb sparing procedures.

To do this, coatings were studied in an *in vivo* murine model. Mouse femur sections were obtained, decellularized and given coatings of a phospholipid called DOPS (1,2 dioleoyl-sn-glycero-3-phospho-l-serine) which has shown potential to enhance osteogenesis due to its ability to bind calcium and enhance calcium phosphate crystal formation, and Gentamicin Sulfate (GS), an antibiotic effective against *Staphylococcus aureus*, the bacteria responsible for approximately 80% of osteomyelitis cases. These coated allograft femur sections were then implanted into mice in osteomyelitis infected as well as non infected treatment groups. The *S. aureus* used in the study to induce osteomyelitis were genetically modified to express luciferase which enabled

tracking and quantification of the infection over the duration of the study. After 28 days the mice were humanely euthanized and the allografts were removed and analyzed to determine how well they had integrated with the host bone. Later *in vitro* testing was performed on allograft samples to investigate the elution characteristics of the gentamicin.

The following sections provide greater detail about the specific aims of this research as well as additional important background information about bone, fracture healing, osteosarcoma, bone grafts, and various other relevant subjects and investigative methods.

1.2 Background

1.2.1 Bone

Bone is a remarkably dynamic tissue constantly responding to stresses placed on it through a continuous process of resorption of old bone and deposition of new bone. The ongoing process by which bone renews itself is known as remodeling.

Bone is comprised of both a mineralized and non-mineralized phase. The non-mineralized phase is mostly composed of type 1 collagen. The mineralized phase is made up mostly of calcium phosphate in the form of hydroxyapatite.

Microscopically there are two different types of bone tissue, termed woven and lamellar. Woven bone is considered an immature form of bone and has a coarse fiber arrangement with no regular

orientation. Lamellar bone has highly organized stress oriented collagen fibers and makes up most adult bone.

On a macroscopic level, bone is structurally either trabecular or cortical. Cortical bone is stronger and denser than trabecular bone and makes up the outer envelope of cuboid bones and the diaphysis of long bones. The allografts used in this experiment are from the diaphysis of mouse femurs and therefore comprised primarily of cortical bone [1] [2].

The cell types responsible for bone resorption and deposition are osteoclasts and osteoblasts respectively. Other cellular components include osteocytes and osteogenic precursor cells such as mesenchymal stem cells [3]. Osteoblasts deposit new bone by first laying down unmineralized organic matrix called osteoid which subsequently undergoes mineralization. When an osteoblast becomes trapped within surrounding mineralized bone matrix it is termed an osteocyte and these cells then serve in the maintenance of the mature bone

Osteoclasts are the cell type responsible for resorption of bone. They do this by attaching to bone matrix via integrin receptors and releasing several hydrolytic enzymes that dissolve both the organic and inorganic phases of the bone. Multiple osteoclasts working together form a "cutting cone" which moves through the bone forming pits called "Howship's lacunae". This process decreases strain at the front of the cone and increases strain behind. This higher strain along with other chemical signals may be responsible for the recruitment of osteoblasts to deposit new bone behind the cutting cone [4].

1.2.2 Fracture Healing

Bone fracture healing is a process that normally results in complete return of function and unlike soft tissue regeneration does not result in scar formation. Bone regeneration can proceed via two distinct mechanisms; termed primary (direct) and secondary (indirect) bone healing.

Secondary bone healing is the more common mechanism of bone regeneration and occurs in the absence of rigid fixation at the fracture site. This type of bone healing consists of three major phases; inflammation, repair, and remodeling. After a fracture occurs, the site quickly becomes inflamed. This inflammation serves the purpose of hydrostatically holding the fracture in place as well as causing pain and compelling protection of the injury site. Tissue adjacent to the fracture site including osteocytes, damaged periosteum, marrow and other soft tissue becomes necrotic and is reabsorbed. Leukocytes and platelets are drawn to the site and release factors which stimulate angiogenesis. Hematoma forms as well as granulation tissue and the whole process eventually creates a "reparative callus" comprised of fibrous connective tissue, blood vessels, cartilage, and then osteoid and woven bone. This callus serves to mechanically strengthen the injured region during the healing process and will ultimately be replaced by lamellar bone [2].

From approximately 2 days to 2 weeks after fracture, intramembranous ossification occurs. This is where bone formation happens directly without the intermediate formation of cartilage. This process forms part of the callus called the "hard callus" [5]. As this is happening, chondrogenesis is also occurring forming the "soft callus" of cartilage which replaces the fibrous tissue. Between two and three weeks following fracture, endochondral ossification begins.

Calcification of the cartilage results in the formation of a more rigid callus and eventually the cartilage is entirely replaced by woven bone. At this point the remodeling phase begins in which woven bone is replaced with stronger lamellar bone [6].

Primary bone healing is less common and occurs when there is rigid stabilization of the fracture site, resulting in fracture healing without the formation of a reparative callus. This can occur in the presence of direct contact between ends of the fracture or when there is a gap. In gap healing the initial bone laid down is transverse to the original orientation of the lamellar bone and this is then replaced by osteons with the same orientation as the original lamellar bone. In contact healing osteoclasts are able to form cutting cones that cross the fracture, and new haversian systems are laid down directly [1].

This is a simplified description of the process of fracture healing. Much is now known about the complex biochemistry underlying the healing process which involves numerous types of signaling molecules and proteins (SMADs, BMPs, etc.) which serve to recruit and direct the activities various cell types involved in the process of fracture healing.

1.2.3 Osteosarcoma

Osteosarcoma is the most common type of bone cancer [7] and accounts for approximately 15% of all primary bone tumors [8]. It principally affects adolescents however it may occur in patients of all ages [7, 9]. Osteosarcoma most often occurs in the metaphyseal growth regions of the distal femur, proximal tibia and proximal humerus [10] though it can also occur in the pelvis,

shoulder or jaw [11]. The standard treatment for osteosarcoma was once amputation of the affected limb, however advances in technology have enabled limb salvage procedures to become the preferred treatment in industrialized countries [9, 12] and limb salvage is now possible in about 80% of patients [13].

Localized pain and swelling of the affected region are typically presented by patients and accurate diagnosis often involves MRI and CT scans as well as bone scintigraphy [10]. Surgical resection of the neoplasm is usually necessary in conjunction with chemotherapy [14]. The overall expected long term survival rate when both surgery and chemotherapy are employed is approximately 70% in patients with localized disease [10]. About 20% of patients present with distant metastases at the time of diagnosis with the lung being the most frequent site of secondary tumor formation [15]. In patients presenting with metastatic disease long term survival drops to less than 20% [10]. Regional bone metastases or skip metastases are also possible and result in even poorer patient prognoses [16].

Chemotherapy treatment typically involves multiagent regimens of drugs such as cisplatin, doxorubicin and methotrexate, administered for many weeks both before and after surgery [10]. Advancements in multiagent chemotherapy treatments lead to dramatically improved patient outcomes between the 1960s and 1980s however survival rates have not improved significantly over the past several decades [17]. Because most deaths associated with osteosarcoma now result from tumor metastasis much of modern osteosarcoma research has focused on improved understanding of the molecular factors such as the signaling pathways and gene expression

patterns underlying metastatic behavior in order to develop better therapies to target the metastatic disease.

1.2.4 Bone Grafts

Bone grafts are used in numerous types of clinical scenarios. Some examples are massive bone allografts used in limb sparing procedures, spinal fusions, complex fracture repair, repair of bone defects due to traumatic injury, dental implants, and cranial or facial reconstruction. Graft incorporation proceeds via the same principles as fracture healing. Several key terms are used to describe the role of bone grafts and other implantable materials in new bone formation. Osteoconductive refers to the ability of a material to act as a scaffold to conduct bone growth. Osteoinductive refers to the ability to recruit MSCs (mesenchymal stem cells) and stimulate new bone formation [18]. Osteogenic refers to the ability of a material to generate new bone.

There are several types of grafts that can be used in different orthopedic situations. The "gold standard" has long been the autologous bone graft because it offers complete histocompatibility and provides the best osteoconductive, osteoinductive and osteogenic properties, including viable osteogenic cells [19]. The major drawback of autologous bone grafting is that the graft material must be harvested from another site on the individual which can lead to donor site morbidity and is not always an option as in the case of massive bone grafts.

Allografts are obtained from donor bone from a different organism of the same species. Unlike autografts, allografts must be cleaned and processed prior to implantation. Living cells within

the allograft are removed as they would be incompatible with the recipient and thus allografts lack the osteogenic properties of autografts because they no longer possess viable osteogenic cells. One major drawback of allografts is that they may illicit an immune response in the individual receiving the graft [20]. In many cases allografts are the best option although other osteoconductive materials do exist such as demineralized bone matrix, synthetic ceramic or polymer based bone graft substitutes. Because allografts lack living cells they function primarily as an osteoconductive scaffold into which new bone can grow [21]. Although healing and remodeling do take place for several millimeters at the junction between host bone and allograft, full revascularization does not typically occur. Non-union, infection and fracture are common problems associated with allografts [10].

Limb-sparing procedures can improve quality of life both physically and psychologically for surviving cancer patients however implanting allografts for limb-sparing also increases the risk of certain complications. Infection is especially problematic in osteosarcoma patients receiving massive structural allografts because anticancer therapies weaken the immune system thereby increasing the risk of infection.

1.2.5 Osteomyelitis and *Staphylococcus aureus*

Osteomyelitis refers to bacterial infection of bone tissue. Under normal circumstances bone is highly resistant to infection [22], however major trauma may result in contamination of the bone with bacteria that can cause acute or chronic infection and can be difficult to eradicate without antibiotic therapy [23]. *Staphylococcus aureus*, a gram positive facultative anaerobic bacteria is

the pathogen responsible for about 80% of cases of human osteomyelitis [23]. There are several characteristics of *S. aureus* which explain this phenomenon.

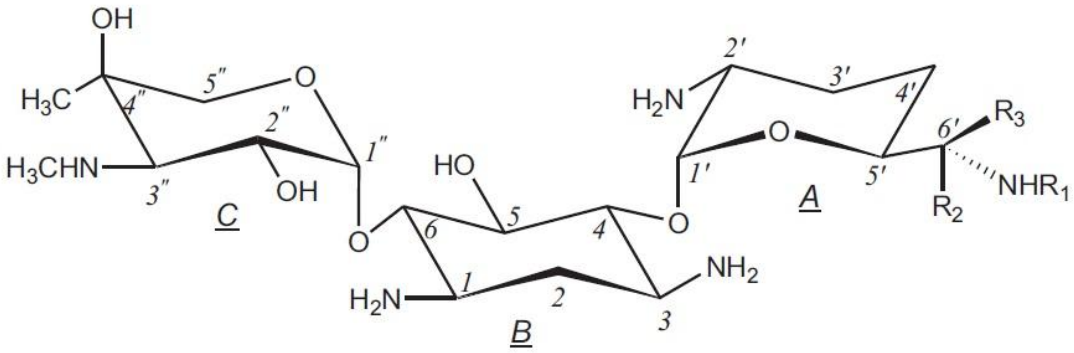
S. aureus expresses cell-surface adhesion proteins that bind to components of bone matrix such as fibronectin, laminin, and collagen. It is also able to be internalized by osteoblasts and survive intracellularly both *in vitro* and *in vivo* [22, 23]. One *in vitro* experiment showed that dead or dying osteoblasts are capable of releasing viable *S. aureus* that may go on to reinfect more osteoblasts in culture. *S. aureus* also secretes toxins that promote local bone resorption and has demonstrated resistance to intravenous antibiotics as well as phagocytosis by host leukocytes [23]. The prevalence of antibiotic resistant strains including ones resistant to potent glycopeptide antibiotics such as vancomycin, is making *S. aureus* increasingly difficult to manage clinically [24].

S. aureus bacteria are also capable of forming biofilms which are composed of extracellular capsular polysaccharides, surface associated adhesins, exoenzymes and exotoxins. These enable the bacteria to resist phagocytosis as well as to adhere to eukaryotic membranes [24]. *S. aureus* is a major cause of wound infections and is capable of causing numerous types of infection from osteomyelitis, bacteremia and endocarditis to infections of the skin and soft tissue [25]. It is very difficult to eradicate once an infection becomes well established and antibiotic coatings which effectively combat bacterial infection could significantly reduce morbidity associated with allograft implants. The experimental model used for the *in vivo* portion of this project was designed to replicate a well established osteomyelitis infection requiring revision surgery (removal of infected allograft and replacement with fresh allograft).

1.2.6 Gentamicin Sulfate

Gentamicin is a member of a class of antibiotics known as aminoglycosides. Aminoglycosides exert their antibiotic effect through several mechanisms most notably by binding 16S rRNA, a component of the 30S ribosomal subunit and preventing it from translating mRNA to protein [26]. These drugs are extremely effective against many types of bacteria and have become the most commonly clinically used broad-spectrum antibiotics; however this widespread use has also led to an increase in antibiotic resistant bacterial strains [27].

Gentamicin is naturally produced by *Micromonospora purpurea* and *M. echinospora*. The structure of gentamicin consists of an animocyclitol ring (B in Figure 1.1) linked by glycosidic bonds to two modified sugar molecules (A and C in Figure 1.1). The term gentamicin does not refer to a single compound but to four major structurally similar molecules and several other minor ones [28]. The major forms of gentamicin are illustrated in figure 1.1.



	R_1	R_2	R_3
gentamicin C_1	CH_3	CH_3	H
gentamicin C_{1a}	H	H	H
gentamicin C_2	H	CH_3	H
gentamicin C_{2a}	H	H	CH_3
gentamicin C_{2b}	CH_3	H	H

Figure 1.1 : Chemical structure of gentamicin, adapted from [29]

Gentamicin is primarily effective against gram negative aerobic bacilli and gram positive cocci [26] such as *S. aureus* and it has been shown to reduce the incidence of osteomyelitis when applied to implants [30]. As noted previously, the primary mechanism of action of gentamicin is to bind to the 30S ribosomal subunit of bacteria and prevent successful translation, however it also exerts other effects such as disrupting the bacterial cell wall, reducing the accuracy of translation, and disturbing DNA and RNA synthesis [26]. Gentamicin sulfate is the salt consisting of gentamicin ionically bonded to sulfate (H_2SO_4) [31].

1.2.7 DOPS

DOPS is the acronym for the phospholipid 1,2 dioleoyl-sn-glycero-3-phospho-l-serine, a type of phosphatidylserine with its phosphate group bound to 1,2 dioleoyl-sn-glycerol as illustrated in the figure below (Figure 1.2.a). Experimental observation as well as molecular dynamics simulations have shown that polar head of the DOPS molecule has a high affinity for binding calcium [32].

In vitro studies on calcium bound phospholipid coatings in the presence of osteoblasts have shown that these coatings increase calcium deposition with phosphatidylserine enhancing calcium deposition more than other phospholipids such as phosphatidylcholine or phosphatidylinositol [33]. Phosphatidylserine has previously been investigated as a coating for metallic implants and has shown promise for enhancing the osseointegrative potential of these implants both *in vitro* [34] and *in vivo* [35]. It has been demonstrated that the presence of phosphatidylserine coatings on titanium creates an effective environment for the formation and

growth of calcium phosphate crystals [36, 37]. The following figure shows the chemical structure of DOPS (Figure 1.2.a) and also how electrosprayed DOPS coatings appear on allografts under SEM at 2000x magnification (Figure 1.2.b).

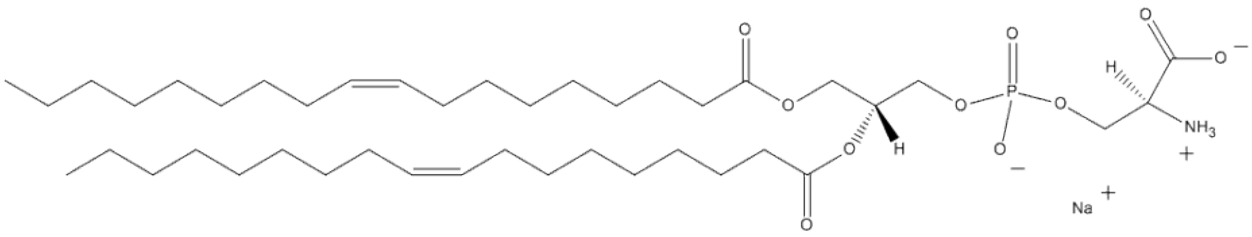


Figure 1.2.a : Chemical Structure of 1,2 dioleoyl-sn-glycero-3-phospho-l-serine adapted from [38]

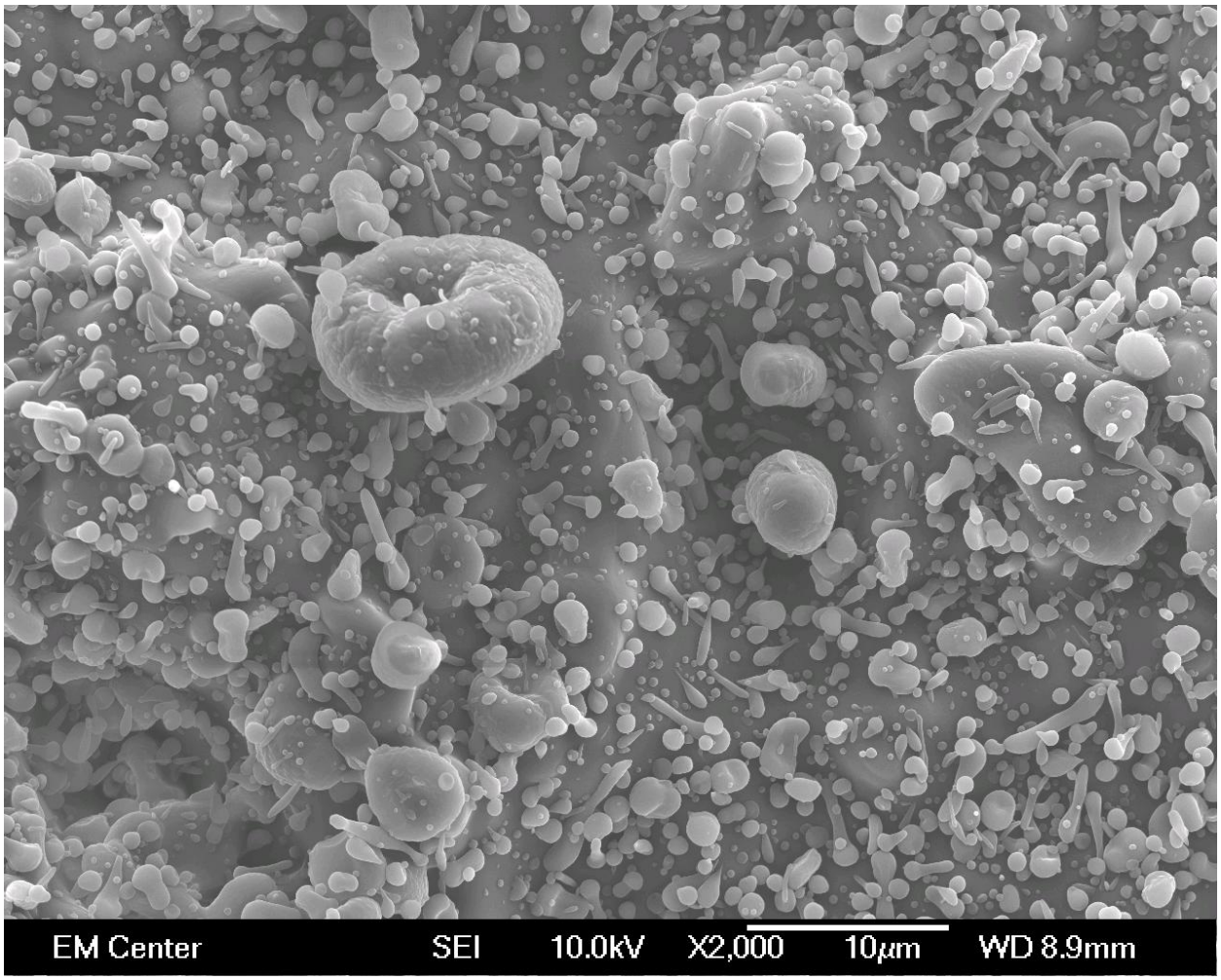


Figure 1.2.b : SEM image of DOPS coating applied to mouse femur allograft

1.2.8 Electrospraying

Electrospraying, also known as e-spraying (or espray) for short, is a method of electrically charging and spraying solutions. Among the numerous uses for this technique is the ability to apply thin uniform coatings to surfaces with non-uniform morphologies [39]. E-spraying works by employing a voltage source to induce an electrical charge in the solution being sprayed and an opposite electrical charge on the surface to receive the coating. By placing the positively charged spray source and negatively charged surface in close proximity the electrical field formed between them enables the positively charged sprayed particles to be attracted towards and deposited onto the negatively charged surface.

The spray will follow the contours of the electrical field and deposit upon the surface much more uniformly than what can be achieved using other application techniques such as dip and drip or aerosol spraying. The physics underlying electrospraying is complex, charging the spray solution may result in any one of several meniscus morphologies at the end of the spray needle and there are several distinct mechanisms by which the jet of material drawn from the meniscus will break into droplets [40, 41].

After the charged droplet of solution has left the spray needle, like charges within the droplet repel one another and the solvent evaporates as the droplet travels with the electric field which decreases the volume in which the charged particles are confined. When the repulsion between like charges overcomes the surface tension of the solvent it causes the droplets to break apart; a phenomenon called “coulomb fission” [40]. This enables the formation of extremely small

droplets and is a major reason why electrospraying is able to produce thinner more even coatings than aerosol spraying.

1.2.9 Previous James Lab Work

Previous work in the James lab has focused on DOPS and Gentamicin coatings and this project is an extension of that research. Dr. David Prawel studied DOPS coatings on titanium, a metal commonly used for orthopedic implants such as hip and knee replacements. Through this research it was found that electrospraying is superior to dip and drip methods for applying thin conformal coatings of DOPS onto thin titanium plates. The ability of DOPS coatings to enhance *in vitro* biomineralization activity of osteoblasts cultured and differentiated from rat mesenchymal stem cells was also investigated. It was found that, in addition to being non-cytotoxic, DOPS coatings are capable of enhancing viability and biomineralization activity of these cells [34] [pg. 230].

Also investigated in this work was the *in vitro* elution profile of gentamicin sulfate loaded between two layers of DOPS coatings. It was found that the majority of elution took place over about three hours using a "perfect sink" elution method and that this profile could be modified by various chemical treatments to the DOPS coatings. The assay method performed was unable to measure absolute quantities of the antibiotic however due to the confounding effects of the DOPS coatings [34] [pg. 128].

Further research by Thomas Triffo [42] involved applying DOPS and gentamicin coatings to stainless steel needles. An *in vivo* study was then performed wherein osteomyelitis was induced (as described in Chapter 2) in treatment mice. Once infection had been established these coated needles were inserted into the femoral medullae of the mice. The infection was monitored over four weeks and the mice were then sacrificed and analyzed histologically. Results from this study indicated that the presence of gentamicin had significantly reduced infection as measured *in vivo* by IVIS (described in the following section) as well as histologically based on inflammation and bacterial colonization [42] [pg. 59].

1.2.10 Xenogen IVIS 100 Imaging System

A special imaging apparatus called the *In Vivo* Imaging System (manufactured by Xenogen), henceforth referred to as IVIS was used to quantify the bacterial infection present in each mouse over the course of the study. The IVIS camera is enclosed in a light blocking chamber and works by detecting bioluminescence. The type of bacteria used in this study to induce osteomyelitis in the treatment mice was a strain of *S. aureus* obtained from Xenogen that is genetically modified to express the bioluminescent protein luciferase.

After placing a subject in the imaging chamber, the IVIS camera detects photons in its imaging field for a specified time (e.g. 1, 3 or 5 minutes) and calculates the average number of photons per second (flux) from each pixel sized region within the imaging field. Where there is relatively high luciferase the IVIS detects a high flux of photons. The relative quantity of bacteria, and hence relative severity of the induced osteomyelitis infection, can therefore be determined

through IVIS imaging. Regions of high flux (high number of detected photons per second per pixel) correspond to high bacterial concentration whereas regions of low flux have few or no bacteria [43]. Infection is quantified by selecting a region of interest (ROI) and summing the total flux of photons through this region. The method for determining ROIs is discussed in detail in Chapter 2.

Although the IVIS chamber blocks out nearly all photons of visible light it is unable to block out high energy photons such as cosmic rays and X-rays which contribute to the background photons detected by the camera. Subjects expressing high luciferase will register levels of photons orders of magnitude higher than background, however subjects with low levels of infection may be difficult to distinguish from subjects with no infection due to the background radiation of high energy photons [44].

1.2.11 MicroCT

Micro Computed Tomography, hereafter referred to as microCT, works on the same principle as a CAT scan. It is a non-destructive technique for imaging bone that functions by taking multiple x-ray images of a sample from all angles [45]. It is capable of reconstructing both 2-dimensional and 3-dimensional models of bone samples and these data can then be used in various types of analysis such as the quantification of bone mineralization [46]. The precision of the machine is as high as 10 micrometer voxel size (a voxel is the three dimensional equivalent of a pixel). These 3 dimensional renderings are a useful complement to histological analysis because they enable visualization of the structure of an entire bone whereas histological analysis is only able

to provide information about 2 dimensional slices. As such, the microCT analysis may serve as corroboration of data obtained through histological analysis.

1.2.12 Gas Chromatography and Electron Capture Detection

Gas Chromatography (GC) is a technique that is useful for precision quantification of chemicals. It works by vaporizing a solution containing the chemical of interest and passing that vapor along with an inert gas such as nitrogen (called the mobile phase) through a long thin column coated on the interior with a stationary phase such as fused silica. The electrostatic interactions between the different components of the vaporized sample and the stationary phase cause each chemical component of the vaporized sample to pass through the column at a unique rate. Thus the purpose of the gas chromatograph is to separate individual chemical components of the mixture so that they each arrive at the end of the column at a different time. Parameters such as mobile phase flow rate, start temperature of the column, and rate of temperature increase can be adjusted to improve results [47].

Several types of detectors can be used at the end of the column to detect the chemical of interest. One commonly used detector is the mass spectrometer [48] and another is the electron capture detector. Electron capture detection (ECD) works by measuring the voltage detected from an electron source such as radioactive nickel-63 [49] which is a beta particle (electron) emitter. These electrons strike molecules in the mobile phase causing an increase in free electrons and thereby increase the voltage at the detector. The voltage is reduced when a molecule capable of capturing electrons passes between the radiation source and the detector. In order to use electron

capture detection to quantify a chemical of interest it must be capable of capturing electrons so the presence of this molecule in the detection chamber will reduce the voltage at the detector [50].

The molecule of interest must be both volatile as well as thermally stable enough to withstand the temperatures it is exposed to in the gas chromatograph. Molecules to be analyzed by GC usually must first undergo a derivatization process in which they are chemically transformed to become more easily volatilized and detectable by the ECD. Frequently this involves adding halogenated moieties to the molecule as the high electronegativity of atoms such as fluorine enables these atoms to capture electrons emitted from the radiation source [51]. By running standards of known concentrations of the molecule of interest through the GC/ECD apparatus it can be determined at what time point the peak corresponding to the molecule of interest passes through the detector. The relationship between area under the peak and quantity of the chemical of interest is then used to quantify samples of unknown concentration.

1.2.13 Agar Diffusion Methods for Antibiotic Quantification

Agar diffusion tests have been used for decades to determine minimum inhibitory concentrations (MICs) of antibiotics against various pathogens as well as to quantify antibiotics in applications ranging from clinical monitoring of serum antibiotic concentrations [52] to ensuring that the antibiotics in animal products are below acceptable limits [53] to measuring concentrations of antibiotics in elution studies [54]. These methods are capable of quantifying antibiotic levels based on an antibiotic sample's ability to inhibit bacterial growth. Agar diffusion tests are

typically performed by first seeding agar plates with a strain of bacteria susceptible to the antibiotic of interest (e.g. *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, etc.). While prepackaged Mueller-Hinton or trypticase soy agar plates are often used for these tests experimenters will sometimes prepare their own agar plates in order to modify the pH or to suspend the test organism at a specified concentration within the agar [55].

Once the plate has been prepared and seeded with bacteria there are two common methods used for introducing antibiotic to the plate. The first is to place antibiotic loaded paper discs onto the agar and the second is to punch wells into the agar and fill these with antibiotic solution. This second "well punch" method has been shown to be accurate at measuring gentamicin using sample quantities as low as 4 microliters [52]. After the antibiotic has been introduced, the plates are incubated, typically at 37 degrees Celsius, to allow the bacteria to grow. After an incubation period which can range from 5 to 24 hours or longer, the plates are observed for bacterial growth. During incubation the antibiotic in the wells (or discs) diffuses through the agar creating a concentration gradient. The bacteria only grow where the concentration of antibiotic is below the MIC. The concentration of antibiotic at any point along the diffusion gradient is dependent upon the quantity and concentration of antibiotic at the source (well or disc) and the diffusion characteristics of the antibiotic in agar. A higher concentration of a given antibiotic leads to a larger region of diffusion above the MIC threshold and therefore larger zones of inhibited bacterial growth.

In order to determine the quantity of antibiotic in a solution of unknown concentration one must run a series of samples of known concentration covering the range of interest (e.g. standards ranging from 1 mg/ml gentamicin to 0.01 mg/ml gentamicin). Based on the areas of the inhibition zones created by these standards a regression line can be generated. Fitting the inhibition zone area formed by a solution of unknown antibiotic concentration to this regression line enables determination of the antibiotic concentration in the unknown solution [56].

1.2.14 *In Vitro* Drug Elution

In the case of antibiotic eluting implants like the ones used in this study, delivering therapeutic levels of antibiotic over a relevant time frame is necessary in order to adequately combat infection [57]. *In vitro* elution studies are commonly used in order to determine the elution characteristics of drugs from implant materials such as bone screws, bone cements, antibiotic eluting beads, etc. While the *in vitro* elution environment does not necessarily mimic the environment experienced by the implant *in vivo*, such experiments can be used to gain a general insight into the elution profile (e.g. whether release occurs as a rapid burst or more steadily over a longer time frame).

Methods for performing *in vitro* elution studies typically involve first preparing the drug eluting samples (screws, beads, etc.) in the same manner as when they are to be implanted into animals. These samples are then immersed in phosphate buffered saline (PBS) and stored at physiological temperature (37 degrees Celsius). The PBS has a pH similar to that of body fluid and acts as a sink into which the drug can elute. At specified time intervals, the PBS is removed, saved for

analysis, and the sample is immersed in fresh PBS. The time interval of elution is highly variable depending on the material being investigated. Some studies only require hours or days to fully elute the drug of interest while others take weeks or longer.

Other published studies of gentamicin elution have involved a variety of methods to incorporate the antibiotic into numerous types of implant materials. Studies have shown that local sustained antibiotic release lasting several weeks [58, 59] is most effective at remediating infection [60]. Slow sustained release is therefore the most desirable elution profile. In order to achieve this, antibiotic is often incorporated into a three dimensional scaffold, bone cement, or biodegradable polymer. Doing this serves to extend the elution profile as compared to applying the antibiotic as a coating. This is well illustrated by comparing several *in vitro* elution studies performed on different materials. The first study, in which stainless steel screws were coated with the biopolymer chitosan and 2%wt gentamicin, found that the gentamicin was released from the implant in a large burst in the first several hours followed by small but measurable release up to 96 hours elution time [61]. Another study, which involved incorporating the antibiotic into coatings of a biodegradable polymer called PLGA (poly lactic-co-glycolic acid) observed elution for up to two weeks [62]. Finally, a study involving incorporating the aminoglycoside antibiotic tobramycin into bone cement resulted in elution for up to 25 days [54]. All these studies were conducted *in vitro* using the elution process described in the previous paragraph.

1.2.15 Mouse Femur Allograft Model

Mice are often used in experiments involving massive bone allografts and the mouse femoral allograft model used in this project has been well established in the scientific literature. The method usually involves removal of a four [or occasionally five] millimeter segment of mid-diaphyseal femur [63]. Removing a segment of this length ensures that the critical size defect threshold has been surpassed [64]. The segment is then replaced with graft material such as autograft, allograft or isograft and the graft is secured with a [typically 22 gauge] intramedullary pin [65]. When devitalized allografts are used the common procedure is to clean the grafts with 70% ethanol and store at -70 degrees Celsius [66] which reduces antigenic response to the implanted allograft [67]. This method has been used to study numerous types of bone graft treatments ranging from coatings to treatments with stem cells, signaling proteins, gene therapies, etc. [68]. The major advantages of using this model is that it is cost effective and the surgical procedure is relatively simple making it appropriate for experiments involving large numbers of subjects and treatments. Mouse bone does differs from human bone in some ways, for instance it lacks haversian systems and therefore may not be appropriate for some studies, however for most experiments the benefits of low cost and rapid screening enabled by this model outweigh the limitations [68].

1.3 Research Specific Aims

Specific Aim 1: Determine whether the presence of a DOPS coating increases osteogenesis at the interface between allograft and native bone as compared to uncoated allograft in osteomyelitis infected as well as non-infected mouse test subjects.

Hypothesis 1: Previous studies have shown that the presence of DOPS enhances osteoblast activity *in vitro* [33, 34]. The presence of DOPS coatings on the allografts is hypothesized to increase new bone formation and integration between allograft and host bone as determined by histological and microCT analyses.

Specific Aim 2: Demonstrate whether the addition of a gentamicin sulfate coating on the allograft reduces *Staphylococcus aureus* induced osteomyelitis infection in mice.

Hypothesis 2: It is hypothesized that incorporating a GS coating on the allograft will enhance the ability of the mice to combat the induced osteomyelitis infection.

Specific Aim 3: Research and verify an *in vitro* method for gentamicin quantification in eluent of unknown gentamicin concentration that is not confounded by the presence of DOPS in the sample.

Proposed Method: While there are many methods available in the literature which can be used to quantify gentamicin some of these methods are confounded by the presence of DOPS or suffer other shortcomings. Two gentamicin quantification methods which will be explored are gas chromatography electron capture detection (GC/ECD) and agar diffusion.

Specific Aim 4: Determine how quickly the gentamicin sulfate (GS) coating elutes from the allografts using an *in vitro* elution method. Investigate the elution profile of the exterior

(periosteal) gentamicin coating in both the presence and absence of a DOPS coating as well as the elution profile of the interior (endosteal) coating.

Hypothesis 4: *In vivo* results from the IVIS data (Chapter 2) indicated significantly reduced infection and thus the presence of active gentamicin at one day post surgery. Subsequent increase in infection at three days post surgery seems to indicate that gentamicin elution was significantly decreased by this time point. It is therefore hypothesized that the majority of GS eluted from the allograft prior to day three. The presence of a DOPS coating over the GS coating is expected to reduce the elution rate of GS from the exterior of the allograft. Allografts are expected to elute all detectable GS more rapidly using an *in vitro* elution method than during the *in vivo* study however this will not be directly tested.

1.4 Review

This project was motivated by the need for improved outcomes in osteosarcoma patients requiring massive bone allografts. One major problem with allografts in cancer patients is the risk of infection, which is higher than it would otherwise be since these patients usually have compromised immune systems. Another major problem is failure of the allograft to successfully integrate with the host bone.

Results from previous research have indicated that DOPS and gentamicin coatings may be useful for improving osseointegration of such allografts. The purpose of this project was to develop a methodology for applying these coatings to mouse femurs and then investigate the coatings as

discussed in the specific aims section. One aim was to investigate whether allograft coatings of DOPS are capable of enhancing osseointegration as compared to uncoated allografts. Coated allografts were implanted into mice and later analyzed histologically as well as with microCT to accomplish this. The effects of gentamicin coatings were also studied to determine whether such coatings are capable of effectively combating a well established osteomyelitis infection in mice. This was investigated *in vivo* using IVIS to quantify the infection. The elution characteristics of gentamicin from allografts with and without DOPS coatings were also studied using an *in vitro* elution experiment and gentamicin quantification methods adapted from those found in the literature.

Chapter 2 – Implant Coating Method Development and *In Vivo* Study

2.1 Introduction

The purpose of the *in vivo* study was to address specific aims 1 and 2 of this research project.

Specific Aim 1: Determine whether the presence of a DOPS coating increases osteogenesis at the interface between allograft and native bone as compared to uncoated allograft in osteomyelitis infected as well as non-infected mouse test subjects.

Hypothesis 1: Previous studies have shown that the presence of DOPS enhances osteoblast activity *in vitro* [33, 34]. The presence of DOPS coatings on the allografts is hypothesized to increase new bone formation and integration between allograft and host bone as determined by histological and microCT analyses.

Specific Aim 2: Demonstrate whether the addition of a gentamicin sulfate coating on the allograft reduces *Staphylococcus aureus* induced osteomyelitis infection in mice.

Hypothesis 2: It is hypothesized that incorporating a GS coating on the allograft will enhance the ability of the mice to combat the induced osteomyelitis infection.

Significant method development was required prior to beginning the *in vivo* study. The following sections discuss development of the coating application process as well as other methods used during the *in vivo* study.

2.2 Implant Coating Method Development

2.2.1 Cleaning and Decellularization

In order to prepare the DOPS/gentamicin coated allografts it was first necessary to obtain and decellularize donor mouse femurs. During method development these donor femurs were obtained from mice that had been used in other experiments. After the methods for allograft preparation had been developed, 30 "balbC" mice were purchased and used as femur donors for the primary study. This strain of mouse was selected because the femur size was compatible with the allograft recipient strain "Black 6". The balbC mice were euthanized and their femurs immediately removed and prepared according to the described method. All procedures involving live mice were first approved by IACUC (Institutional Animal Care and Use Committee).

The femurs obtained from the donor mice had a great deal of muscle attached at the ends so a hand-held Dremel tool with a 15/16 inch "Dremel 545 Diamond Wheel" head was used to cut off the ends of the femurs where the muscle attachments are located resulting in approximately 1 centimeter long femur segments. These segments had almost no muscle tissue however periosteum remained and the femoral medullae were filled with blood and marrow. Femurs were then stored in a -70 degree Celsius freezer for 72 hours as this has been shown to reduce antigenic response to allografts [20].

All subsequent steps were performed as aseptically as possible in a biosafety cabinet (laminar flow hood). The femurs were cleaned and decellularized by first submerging in 70% ethanol [henceforth referred to as just ethanol] in a petri dish making them easier to clean and less likely to chip. Holding the femurs with Roboz brand model RS-9290 forceps the medullae were

cleaned by sticking a succession of needles through the medullae to remove as much of the marrow as possible. The femurs were then flushed with the ethanol by placing the tip of a 23-gauge needle at the end of the femur and using a syringe to quickly expel about 5 ml of ethanol through the medulla.

Next, the femurs were placed in a 3% hydrogen peroxide solution. The purpose of the hydrogen peroxide is to help in the decellularization process and removal of the periosteum. The periosteum was then removed by placing the femur on a 23-gauge needle, pinching it gently with a kimwipe and pulling the kimwipe away while applying gentle pressure. Subsequently the medulla of each femur was flushed with hydrogen peroxide using the same technique as when flushing with ethanol.

The femurs were again placed in ethanol and flushed several more times until no blood or marrow was visible within the medulla. Sometimes removal of all medullary debris also required scraping with a 23-gauge needle. When the femurs appeared completely clean, they were cut to length with the Dremel diamond saw blade; first the femurs were held at the middle of the diaphysis with the Roboz forceps and cut into approximately 6 millimeter long segments, these were then sanded with the face of the Dremel blade to a length of 4 millimeters. The femurs were then flushed with ethanol once more.

The femurs were then placed in fresh ethanol and sonicated for 30 minutes, rinsed with DI water, placed in hydrogen peroxide and sonicated for 10 minutes, rinsed again with DI water, placed in ethanol and sonicated for 60 minutes. Finally they were placed in a petri dish in the biosafety

cabinet and allowed to dry overnight. Once the femurs were completely dry they were ready to be coated. Figure 2.1 shows femurs at various stages in the preparation process.

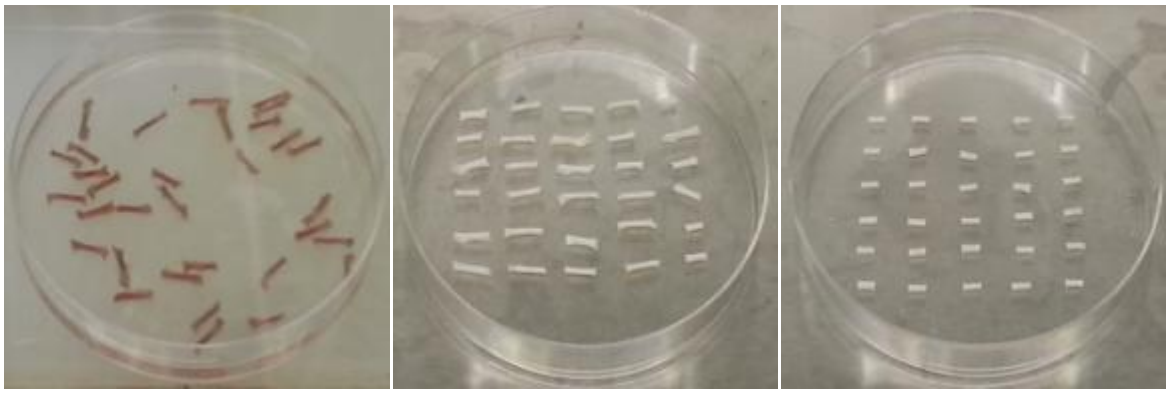


Figure 2.1 : 2.1.a 2.1.b 2.1.c
2.1.a : Femurs in petri dish submerged in ethanol after storage in -70 degree Celsius freezer
2.1.b : After cleaning with ethanol and hydrogen peroxide
2.1.c : After cutting to 4 millimeter length

2.2.2 Determining DOPS Coating Application Parameters

Bone is not as electrically conductive as titanium or steel so it was necessary to test whether bone could be successfully coated using the e-spray process. This was done using a previously constructed apparatus designed to spray thin titanium squares. Cow bones were purchased and cut to an appropriate size (approximately 1 cm x 1 cm x 1 mm) and these were sprayed on the existing e-spray apparatus using the same parameters of voltage, distance, and DOPS solution concentration and extrusion rate as in prior work [34, 42] (12 kilovolts, 8 cm distance from spray tip to sample, 20 millimolar DOPS solution, 14 mL/hr extrusion rate).

An apparatus was then constructed for the purpose of spraying mouse femurs. This initial apparatus was constructed using an electronic breadboard coated with electrical tape with four needles sticking through. These needles were wired together and connected to the negative

output of the voltage source. A syringe pump holding a syringe filled with DOPS/chloroform solution was positioned with the needle above the top of the femur segments. The positive output of the voltage source was connected to the tip of this needle. Initial spray parameters were 12 kV applied voltage, 8 cm distance from needle tip to sample, 20 millimolar DOPS concentration in chloroform, and 14 mL/hr extrusion rate [39]. After some experimentation the extrusion rate was reduced to 7 mL/hr. None of the other parameters was changed.

The electrospray chamber used up to this point consisted of a large acrylic box with a removable front and this presented two problems; first that the ventilation system was inadequate and exposed the user to chloroform inhalation and second that it was too large to fit into a biosafety cabinet. In order to solve these problems, a new electrospray chamber and ventilation system were constructed. Coating application was subsequently performed in a biosafety cabinet in order to ensure that the allografts remained as pathogen free as possible during preparation and chloroform was vented to a fume hood using the ventilation apparatus (Figure 2.3.a on pg. 34).

2.2.3 Determining Gentamicin Coating Application Parameters

In previous research [42] DOPS/gentamicin coated needles were covered with approximately 270 micrograms of gentamicin which was shown to effectively combat, but not fully eliminate an induced *S. aureus* osteomyelitis infection. Based on this result it was decided to increase the amount of applied gentamicin in the hope of fully eliminating the infection. Each allograft received a total of 1 milligram gentamicin, 500 micrograms on the exterior (periosteal) surface and 500 micrograms on the interior (endosteal) surface. While this was a significant increase in

gentamicin it was still well below the single dose LD50 of gentamicin for mice which is approximately 6 milligrams [69]. The probability of 1 milligram of gentamicin causing health problems for the mice was therefore considered unlikely.

It was found that the gentamicin coating could be easily and efficiently applied to the exterior of the allograft by dripping an aqueous gentamicin solution onto it as it rotated on a lathe and then allowing it to dry. In order to apply gentamicin on the interior of the allograft this aqueous gentamicin solution was injected into the medulla of the femur with a micropipette. The solution would stay in place inside the femur due to surface tension. The femur was subsequently suspended with polypropylene forceps and allowed to dry for several hours. Experimental verification of the applied gentamicin is discussed in Chapter 4 (Variation in Applied Gentamicin).

2.2.4 SEM and EDS

The coatings were imaged and verified for uniformity and thickness using a scanning electron microscope. Prepared samples were adhered to aluminum mounts with carbon tape and colloidal graphite and coated with gold using a sputter coater. The thickness of the gold layer applied was initially 15nm however it was later found that 10nm coating thickness was sufficient and this was used for the majority of SEM imaging sessions. Energy dispersive x-ray spectroscopy (EDS) can be performed at the same time as SEM imaging and this was done with several of the samples as well.

2.2.5 Applying the Coatings

Four treatment groups were prepared: No coating, DOPS Only, Gentamicin Only (GS Only), and DOPS with Gentamicin (DOPS/GS). Coatings were prepared the day prior to surgery according to the procedures discussed in the following sections.

2.2.6 No Coating

The no coating allografts had no additional treatment performed after the cleaning and decellularization process and these served as negative controls.

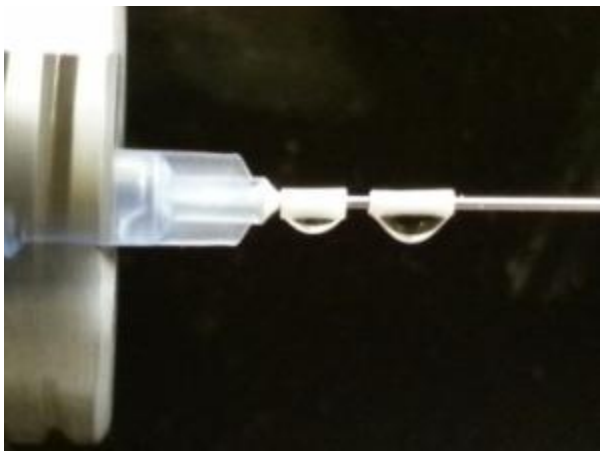
2.2.7 Gentamicin Coating

Gentamicin was applied to both the outer and inner surface of the allografts. Eighty milligrams gentamicin sulfate (GS) salt obtained from Sigma Aldrich was dissolved into de-ionized water to a concentration of 125 mg/mL. This was sterile filtered in a biosafety cabinet using 0.22 micrometer pore size sterile filters and yielding approximately 0.5 milliliters of solution post filtration. This solution was kept refrigerated while not in use.

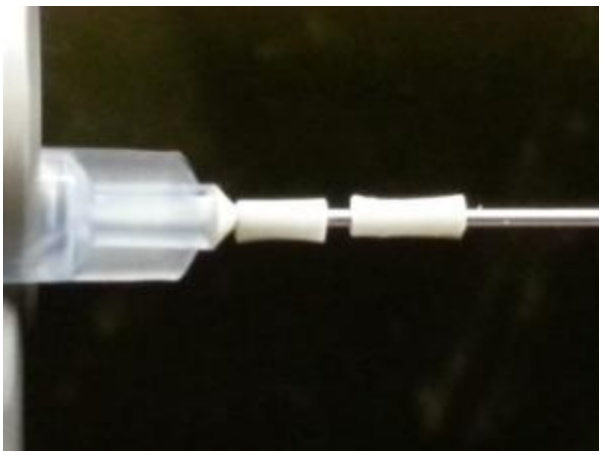
A mini-lathe was sanitized with ethanol and placed in a biosafety cabinet. A 23-gauge needle was placed on the lathe and positioned such that it remained parallel to the ground during rotation. Femur segments were placed on the needle, separated from others by a gap of approximately 1 mm. Four microliters of 125mg/mL GS solution (500 micrograms total GS) was micropipetted onto each femur and the lathe was rotated at a rate of approximately 200 rpm

until the femurs were dry, which took about 30 minutes, as shown in figure 2.2.a and 2.2.b. The purpose of using a lathe was to create an even coating along the entire exterior surface of the allograft.

After the exterior gentamicin coating had been applied the allografts were removed from the lathe, held individually with polypropylene forceps and 4 microliters of gentamicin solution was injected into the medulla of each allograft with a micropipette. These were then placed on a rack as shown in figure 2.2.c and allowed to dry for two hours. Each allograft was thus coated with a total of one milligram of gentamicin; 500 micrograms on the exterior and 500 micrograms on the interior (variation in applied gentamicin is addressed in chapter 4).



2.2.a : Femurs on rotating lathe with GS solution



2.2.b : Femurs 30 minutes later, GS solution has dried

2.2.c : Femurs held by polypropylene forceps to allow interior GS solution to dry

2.2.8 DOPS Coating

The DOPS coatings were applied within the e-spray chamber built for this experiment. Four allografts were sprayed at a time. A 20 millimolar solution of DOPS in chloroform was prepared from DOPS obtained from Avanti. The spray apparatus was set up as shown in figure 2.3.a. Allografts were placed on 23-gauge needles protruding through the bottom of the chamber and these needles were wired to the negative end of the voltage source. Allografts were sprayed for 1 minute at a time and then inverted using the Roboz forceps such that the opposite end of the femur faced up. They were then sprayed for 1 minute again. The purpose of inverting the allografts was to ensure that both ends received a DOPS coating.

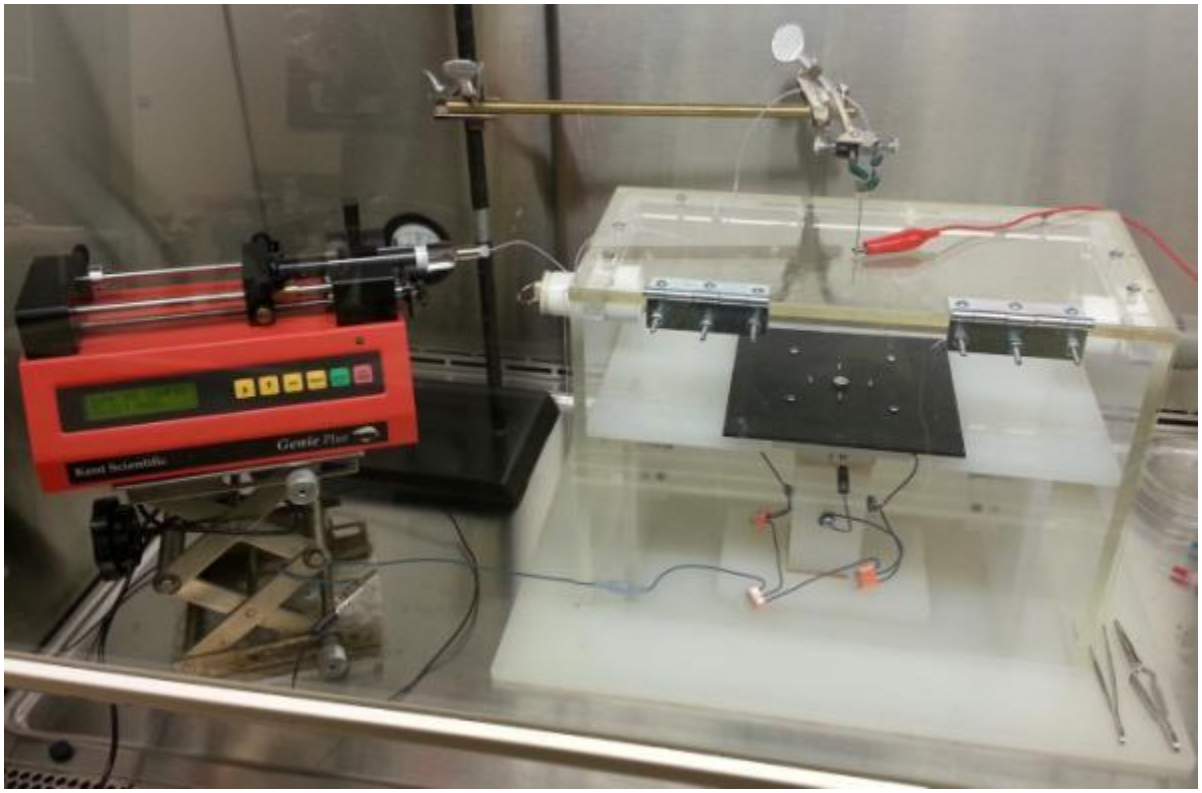


Figure 2.3.a : Electrospray apparatus assembled in biosafety cabinet. Syringe pump is on left, spray chamber on right. Extrusion needle and femur needles are wired to positive and negative voltage source (not shown) respectively.

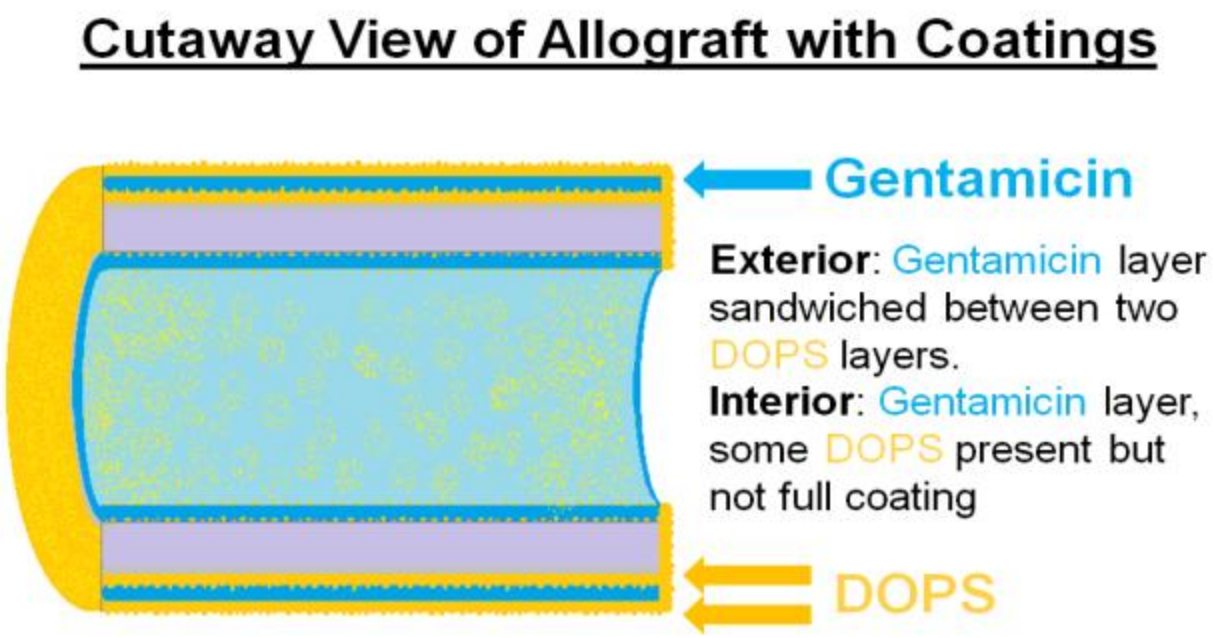


Figure 2.3.b : Diagram of coatings applied on exterior, interior and ends of femur viewed as though femur had been cut longitudinally forming a half-pipe.

Allografts in both the DOPS only and DOPS/GS groups were prepared this way. Those in the DOPS only group were immediately inverted again, sprayed for 1 minute, inverted once more and sprayed for 1 minute. The total spray time for each allograft was therefore 4 minutes. Allografts in the DOPS with gentamicin group were removed after two minutes spray time and gentamicin was applied according to the procedure described above after which they were placed in the e-spray chamber again, sprayed for 1 minute, inverted and sprayed for 1 minute again. Thus the DOPS with gentamicin group had a gentamicin coating sandwiched between two coats of DOPS as illustrated in figure 2.3.b.

After the allografts were prepared they were placed on color coded needles according to coating group. Preparation took place the night before surgery to minimize the time between coating application and surgery. This was done because DOPS coatings have been shown to structurally

rearrange if left for long periods of time [70]. Finally the allografts were UV sanitized in the biosafety cabinet for 20 minutes.

2.3 *In Vivo* Studies

2.3.1 Pilot study

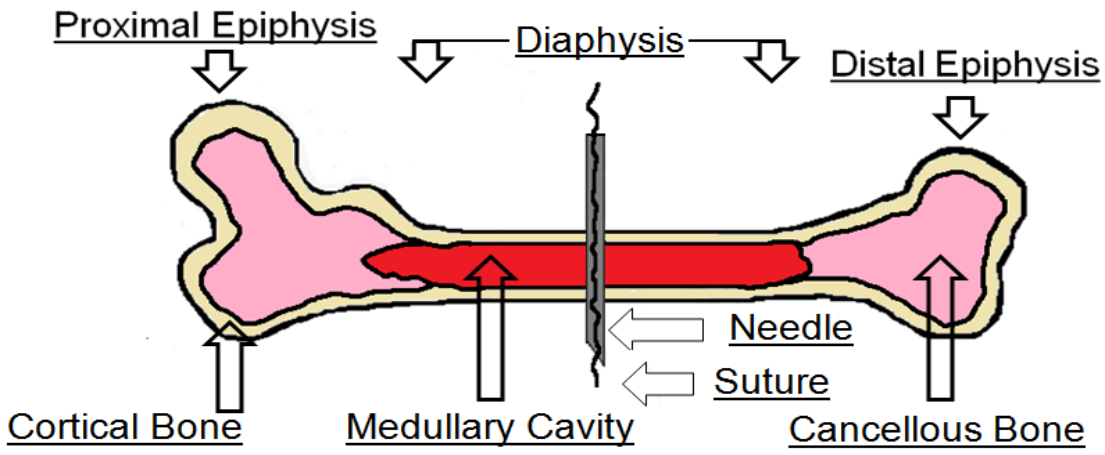
In order to help ensure that the primary study went well, a pilot study was first performed. The pilot study involved 5 mice. DOPS/GS allografts were prepared as they would be for the primary study, the mice were infected with *S. aureus* and a week later they received allografts prepared according to the procedures described in previous and subsequent sections. The pilot study confirmed that the osteomyelitis infection did not cause the infected femurs to become prone to fracture during surgery and also that the dose of gentamicin was not too high.

2.3.2 Induction of Osteomyelitis

Osteomyelitis was induced in 36 mice. *Staphylococcus aureus* was used for this purpose because it is the bacterium responsible for the majority of clinical osteomyelitis cases [23]. The mice were infected according to the following method:

Luciferase transfected *S. aureus* (strain Xen 36 obtained from Xenogen) was grown in 6-well plates. After 48 hours of growth sterile sutures were placed in each of the wells and the bacterial plates were incubated for an additional 24 hours to ensure bacterial adherence to the suture. The mice were anesthetized individually in an isoflurane chamber and placed on a surgery table with

their noses in an isoflurane cone. Using a 25-gauge needle, a hole was drilled by hand laterally through the diaphysis of the left femur. One septic suture was then placed into the end of the needle. The end of the needle was crimped with the suture inside and this was then pulled back through the femur as illustrated in figure 2.4. The ends of the suture protruding from the skin were then cut. Mice were also given numbered ear tags at this point for identification purposes.



2.4 : Diagram of where hole was drilled through femur and bacterial suture was inserted.

This method for osteomyelitis induction was modified from prior work which involved placing a bacterial suture through the epiphysis of the femur and into the medulla. The technique was modified due to concern that using the old technique would result in too great of an infection in the bone which may weaken the bone to the point that it would fracture during allograft implantation surgery. Even with this modified method the possibility of severe weakening of the bone was a concern. One major reason for performing a pilot study was to investigate this possibility and confirm that fracturing of the femurs during surgery would not be a problem.

2.3.3 Allograft Surgery

Mice were first anesthetized with isoflurane and were placed on a surgery table with their noses in an isoflurane cone and secured with several strips of surgical tape (Figure 2.5.a). A sterile cloth was placed over the mouse such that only the left hind leg was exposed. An incision was then made through the skin and muscle to expose the femur (Figure 2.5.b). Forceps were placed under the femur to isolate it from the surrounding muscle tissue (Figure 2.5.c). A Dremel tool with a diamond blade was used to cut a 4-millimeter segment from the middle of the diaphysis of the femur (Figure 2.5.d). A 22-gauge needle was fed through the proximal epiphysis and into the medullary cavity. An allograft was placed on the needle and the end of the needle was stuck into the distal epiphysis (Figure 2.5.e). The excess portion of the needle protruding from the proximal epiphysis was then cut off. The surgical wound was stitched and several surgical staples were used to close the wound (Figure 2.5.f). Mice were then placed in a recovery cage and given a dose of buprenorphine (0.1 mg), an opiate painkiller. A diagram of the locations of the allograft and fixation needle can be seen in figure 2.5.g.

Figure 2.5.a

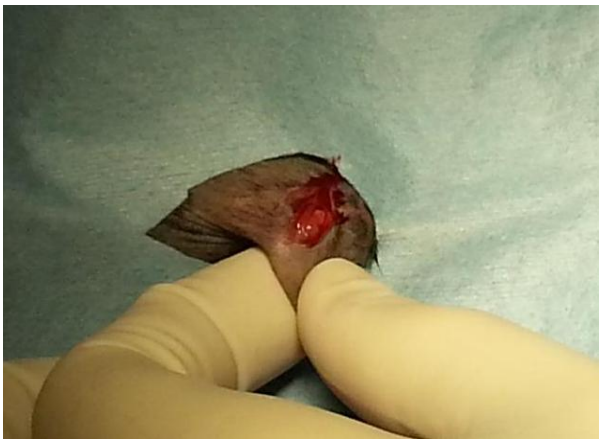


Figure 2.5.b

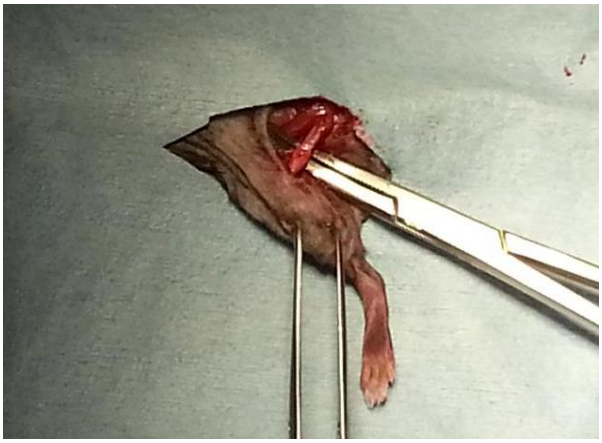


Figure 2.5.c

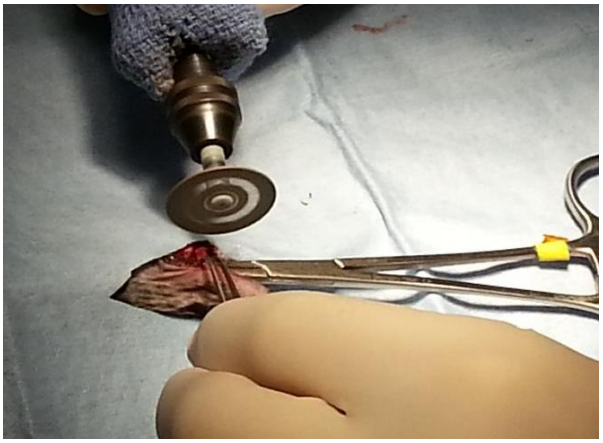


Figure 2.5.d

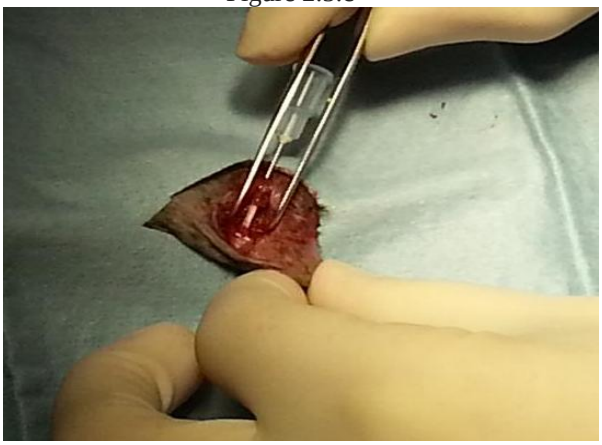


Figure 2.5.e

Figure 2.5.f

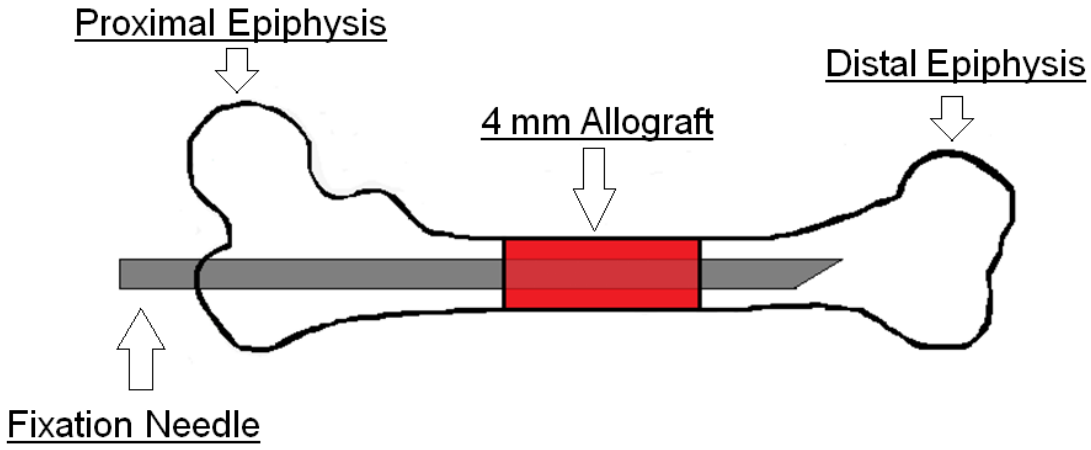


Figure 2.5.g : Diagram of femur with implanted allograft and fixation needle.

2.3.4 Treatment Groups and Additional Surgery Information

Surgeries were performed on two separate dates spaced three days apart. This enabled IVIS imaging to be performed on concurrent days for all mice for the duration of the study. Twenty-five mice (20 infected, 5 non-infected) were implanted with allografts on the first surgery date and 25 mice (15 infected, 10 non-infected) were implanted on the second surgery date. The type of allograft received by each mouse was randomized by selecting the mice at random from each cage and implanting the allografts in the sequence DOPS/GS, DOPS only, No Coat, GS only. The number of mice in each treatment group is presented in the following table:

Table 2.1

	DOPS+GS	DOPS	No Coat	GS
Infected	10	10	10	5
Uninfected	5	5	5	0

2.3.5 IVIS Imaging

After mice were implanted with the luciferase transfected *S. aureus* suture they were imaged for luciferase expression every 48 hours for six days using a Xenogen IVIS 100 imaging system; allograft implantation surgery took place on day 7. Mice were not imaged on the day of allograft implantation due to concern about overexposure to isoflurane, as each mouse was sedated for about 20 minutes during surgery. The first post-surgery imaging took place the day after surgery; the next imaging took place on day 3 post-surgery. Imaging then took place every 72 hours through day 27. Mice were euthanized on day 28.

The process of imaging involves first opening the IVIS software, initializing the system and setting parameters (Stage on Level A, pixel size medium, imaging time 60 seconds). An individual mouse is then anesthetized with isoflurane and placed into the IVIS with its body centered in the imaging field and its nose in the isoflurane cone within the chamber. The chamber door is then closed and the mouse is imaged for 60 seconds.

The IVIS works by counting the number of photons reaching the camera from each pixel sized region within the imaging field. It then determines the average number of photons per second or flux for each pixel and this data is overlaid upon the preview image to facilitate visualization of the anatomical location of origin of the luminescence. Areas with high flux are colored red or yellow and areas of lower flux are green or blue. If the number of photons detected from a given pixel is below the set threshold, that pixel appears uncolored. Each time a mouse is imaged the data must be reviewed to determine if longer imaging time is required. A good IVIS image should show consistent areas of high and low flux (Figure 2.6.a). If little or no luminescence appears in the 60 second image (Figure 2.6.b) then the mouse is imaged again for 180 seconds (Figure 2.6.c) and then again for 300 seconds if necessary.

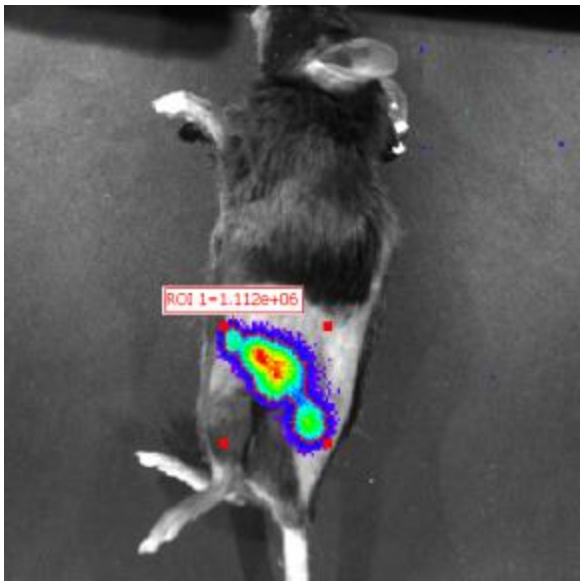


Figure 2.6.a : Good IVIS image

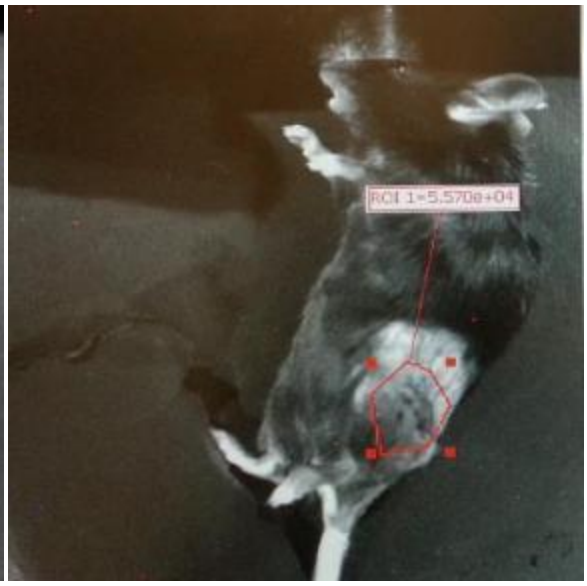


Figure 2.6.b : 1 minute imaging

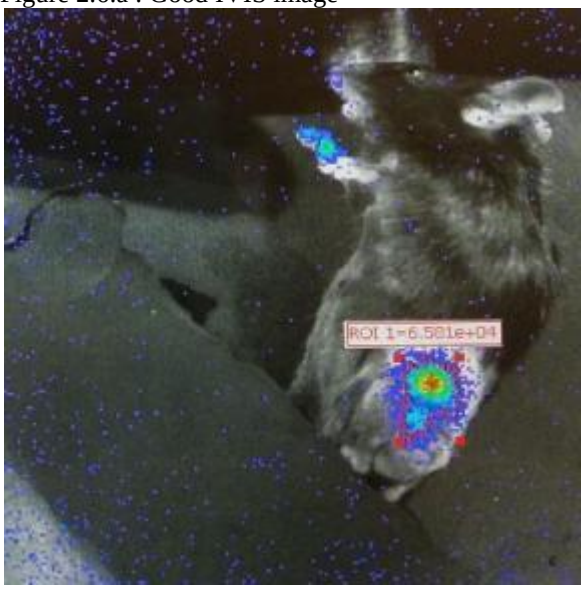


Figure 2.6.c : 3 minute imaging mouse from 2.6.b

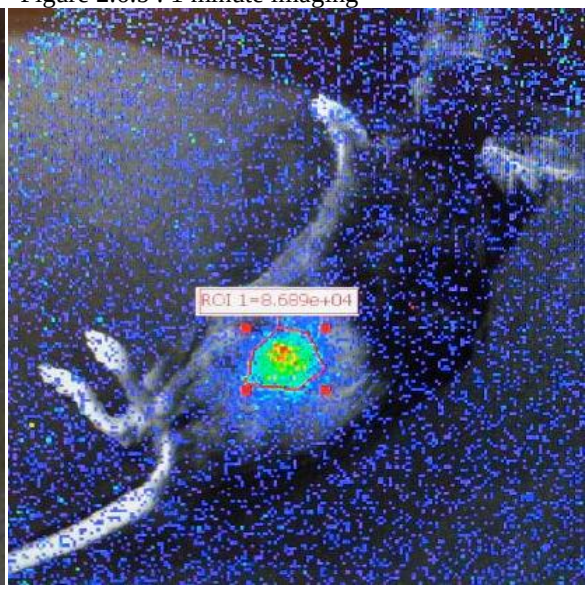


Figure 2.6.d : Minimum set to 10% of maximum

After imaging is complete the mouse is removed from the chamber, its number, treatment day and imaging time (1, 3 or 5 minutes) are recorded with the image file (recall there were two surgery dates so half of the mice are 3 days behind the other half with regard to treatment day) and the mouse is weighed (IACUC rules require euthanasia of mice that lose too much weight). Comparing data from mice imaged for different lengths of time is possible because the flux associated with each pixel is given in units of photons per second. Comparing equivalent regions

from the same mouse for 1 minute and 5 minute imaging times typically yields very similar measures of total flux. The reason for performing longer imaging times is primarily to improve "region of interest" (henceforth referred to as ROI) determination.

After selecting an ROI for a given image, the number generated from that ROI must be saved. Unfortunately, the ROI is not saved automatically and the program has a tendency to randomly crash causing loss of all unsaved data. In order to circumvent this problem screenshots were taken after each ROI was selected so that in the event of a software crash the analysis did not need to be performed again. Once all ROI's were obtained the data was grouped by treatment type (DOPS/GS, DOPS Only, GS Only, and No Coating) and day of study and statistically analyzed using SAS (Statistical Analysis Software). ROI values varied from zero to about 1.1*10^6. In order to help normalize the data these values were transformed to their natural logarithm and it was this logarithmically transformed data on which statistical analysis was performed. Values of zero were reassigned as 1000 to prevent transformation errors.

The SAS code used and an explanation of the analysis procedure (GLM procedure) can be found in Appendix B. Because there are two independent variables (DOPS and GS) the GLM procedure performed a two way ANOVA on the data for each day, the output provided information about the effect of both types of coatings, as well as the interaction between them as a sum of squares. Also included in the output were various diagnostic plots including a residual quantile plot which was used to determine normality, an interaction plot, and a least square means analysis.

2.3.6 Euthanasia

Mice were euthanized on the 28th day after allograft implantation. Mice were anesthetized with isoflurane to ensure minimal suffering. Cervical dislocation was then performed to euthanize each mouse. The dead mice were then placed on the dissection table and the femur, allograft and surrounding muscle tissue was removed. The femurs had been randomly assigned to one of four analysis groups, histopathological analysis, microCT analysis, culturing for *S. aureus*, or cryo-preservation. The following chart illustrates the number of samples per treatment group (some of the femurs in the non-infected groups were analyzed both histologically and by microCT):

Table 2.2

		Histology	MicroCT	Culture	Cryo-Preserve
DOPS/GS	I	3	3	2	2
DOPS	I	3	3	2	2
No Coat	I	3	3	2	2
GS	I	3	3	0	0
DOPS/GS	NI	3	3	0	0
DOPS	NI	3	3	0	0
No Coat	NI	3	3	0	0

"I" indicates infected treatment groups whereas "NI" indicates non-infected treatment groups

The femurs that were to be used for histopathological or microCT analysis were placed in numbered cassettes (the number of the cassette corresponded to the mouse ear tag number). These cassettes were submerged in formalin for tissue fixation. After one week in formalin the pins were removed from the femurs.

The femurs selected for culturing were placed in labeled agar filled culture tubes and these were later sent to the culture lab for analysis.

The femurs selected for cryo-preservation were placed into cryovials, submerged in liquid nitrogen for several minutes then stored at -70 degrees Celsius.

After dissection was complete, the mice were placed into a plastic bag and stored in the lab freezer [as each still had one intact hind limb from which a femur could be taken and used for future experimentation, these femurs were used to complete Specific Aim 4].

2.3.7 MicroCT

MicroCT scanning was performed on three femurs from each treatment group (7 groups, 21 total femurs) using a Scanco brand microCT 80. Femurs were placed into a holder and secured between pieces of foam. A total of four femurs were scanned at a time. After scanning, the femurs were placed back into their cassettes and into formalin for histologic interpretation. These scans were used to digitally create 3 dimensional reconstructions of the femurs (Figure 2.10, pg. 60).

2.3.8 Histology

Three femurs from each of the seven treatment groups were initially prepared for histological analysis. Additional slides were later prepared from the remaining six uninfected femurs (two per uninfected treatment group) for additional osseointegration analysis. After fixation in formalin the bone was decalcified and two slides were made from each femur, one stained with H&E (hematoxylin and eosin) and the other with a gram stain.

The slides were then submitted to a board certified pathologist for analysis and graded on numerous parameters including adequacy of the histological section, presence of allograft, non allograft callus reaction, suture site visibility, inflammation, bacterial colonization and osseointegration. For the latter three categories observational notes were provided where applicable. Representative images were also taken of several slides and these accompanied the pathologist's report. For the initial analysis the histologist was blinded to the treatment group from which each sample was obtained, later an unblinded analysis was also performed.

Along with the qualitative notes, femurs which exhibited osseointegration were given a numerical score indicating the level of osseointegration observed. The scoring scale ranged from 0-3 with 3 indicating high osseointegration and 0 indicating no osseointegration (Figure 2.11, pg. 61). From this data statistical analysis was performed to determine whether there were significant differences in the amount of osseointegration between treatment groups.

Because none of the infected mice exhibited any osseointegration, only the three groups of uninfected mice could be statistically analyzed. Initially only three femurs from each uninfected group were analyzed histologically, however after receiving these osseointegration scores it was decided to have H&E slides prepared and analyzed for the remaining two mice in each uninfected group. This was done to increase the size of the dataset and the statistical power of the analysis. Ultimately osseointegration scores were obtained for 4 mice in the uninfected DOPS treatment group, 4 mice in the uninfected No Coating treatment group, and 3 mice in the uninfected DOPS/Gentamicin treatment group.

2.3.9 Culturing

On the day of euthanasia, two femurs from each of the Infected DOPS/GS, Infected DOPS, and Infected No Coat groups were placed in culture tubes and sent for analysis of bacterial growth. Several tissue samples from the area adjacent to the femur in infected and non-infected mice were randomly selected and also placed in culture tubes and sent to the culture lab. These cultures were incubated for several days and the degree of bacterial growth from each sample was reported as heavy, moderate or light.

2.4 Results

2.4.1 DOPS and Gentamicin Coating, SEM and EDS

Coatings of DOPS and gentamicin were examined under SEM in order to verify the presence of the coatings and to determine coating thickness and verify uniformity. It was found that using the e-spray method was an effective means of applying DOPS coatings to the exterior (periosteal) surface and ends of the allografts (Figure 2.7). The ends of the allograft are where the interface between the host bone and allograft (host-graft apposition) occurs *in vivo* and this is the site of initiation of repair for cortical allograft [21]. The ends of the allograft were therefore considered the most important region to receive the DOPS coating.

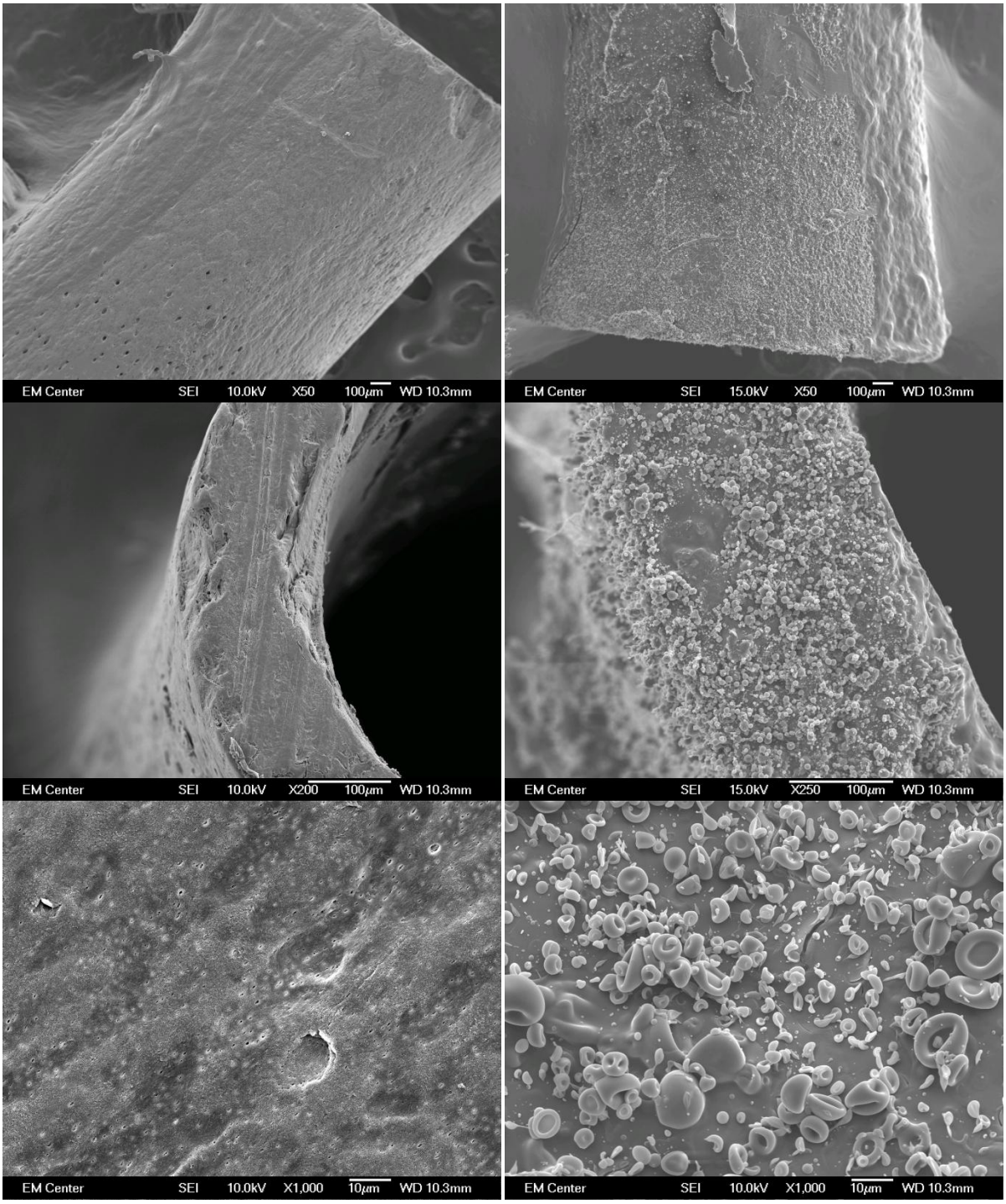


Figure 2.7 : Images on the left are uncoated bone, images on the right are DOPS coated, Top to bottom – low magnification view of femurs, ends of femurs, high magnification of coating.

The morphology of the DOPS coating was distinctly different from that of uncoated bone and the presence of the coating was easily verified with SEM. The thickness of the applied coating of

DOPS was measured by looking at several SEM images of allografts that had been sliced lengthwise. Due to the globular topical morphology of the DOPS coatings the thickness of the coatings are not perfectly uniform and the exterior DOPS Only coating thickness was found to range from approximately 9-12 micrometers using the previously described spray parameters (7ml/hr extrusion rate, 4 minute spray time). This can be seen in figure 2.8.a and 2.8.b.

The gentamicin coating was also observed using SEM and found to adopt a distinct and easily verified morphology. Based on SEM image analysis the thickness of the exterior (periosteal) DOPS/GS/DOPS coating was found to be approximately 15-20 micrometers (Figure 2.8.c). The interior (endosteal) surface of the allograft received negligible DOPS and the gentamicin coating was found to be approximately 15-25 micrometers thick on average (Figure 2.8.d) and exhibited a cracked appearance when imaged with SEM (Figure 2.8.e and 2.8.f).

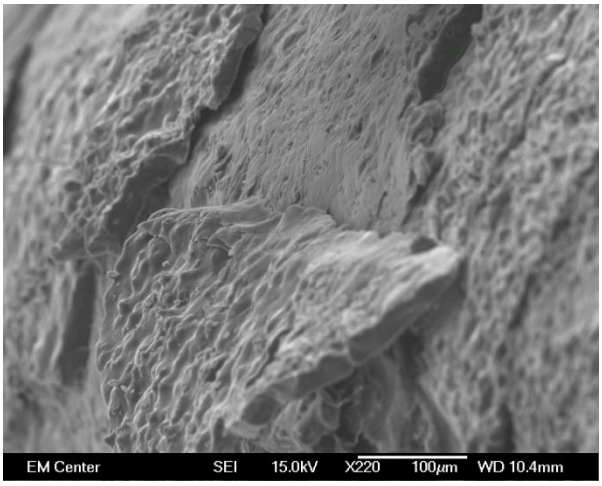


Figure 2.8.a

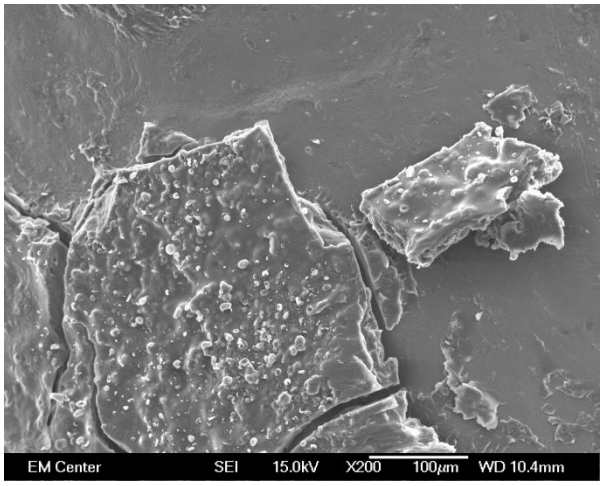


Figure 2.8.b

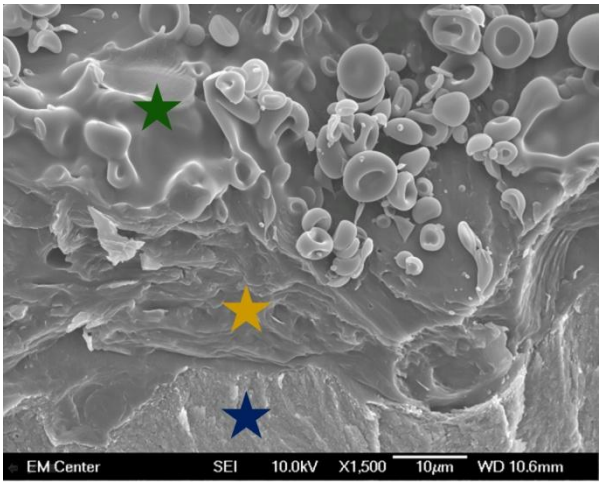


Figure 2.8.c

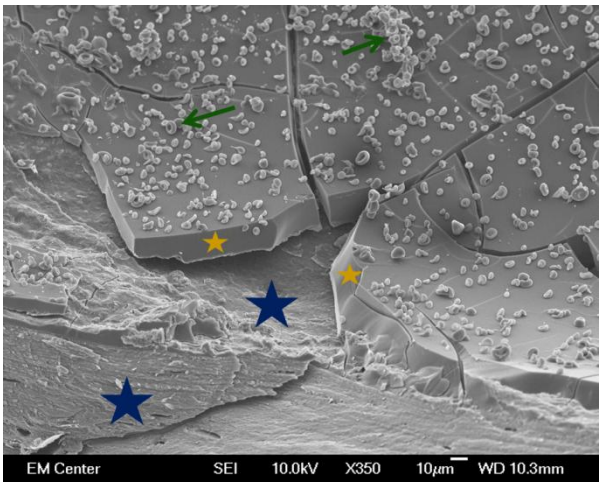


Figure 2.8.d

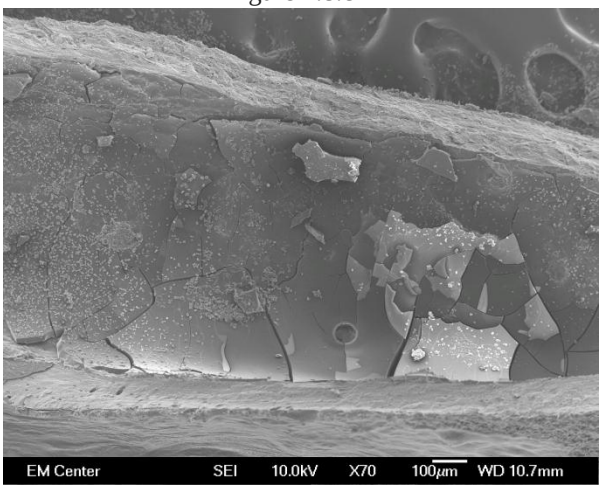


Figure 2.8.e

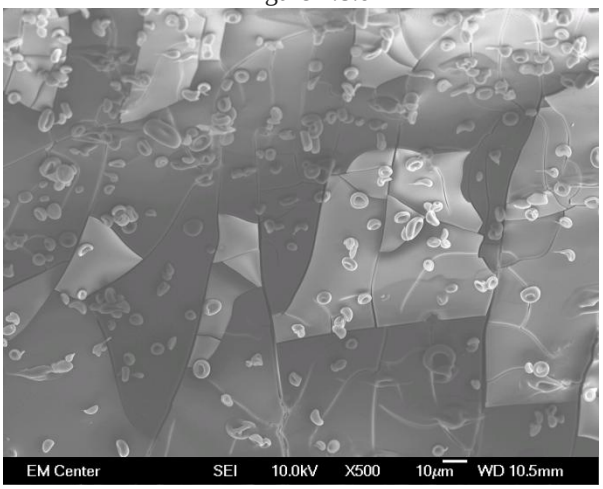


Figure 2.8.f

SEM images like these were used to determine thickness and morphology of the DOPS and gentamicin coatings green indicates DOPS, yellow indicates GS, blue indicates bone
Figure 2.8.a and 2.8.b : Fractured DOPS Only coatings on allograft exterior
Figure 2.8.c : Exterior DOPS/GS/DOPS Coating on allograft sliced lengthwise with razor
Figure 2.8.d : Interior gentamicin coating with some visible DOPS on surface
Figure 2.8.e and 2.8.f : Morphology of interior gentamicin coat at low and high magnifications

2.4.2 Statistical Analysis of Bioluminescence Study (IVIS)

The results of the IVIS data analyses are shown in figure 2.9. The mean luciferase intensity is plotted for each of the four infected treatment groups at each time point. Error bars are one standard deviation.

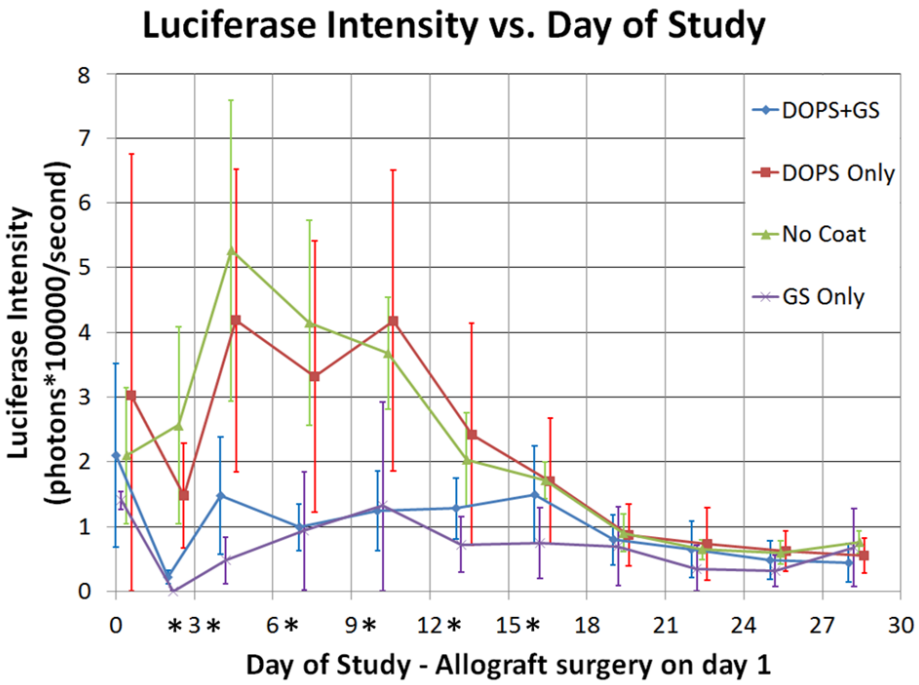


Figure 2.9 : Graph of luciferase intensity on each imaging day including standard deviation error bars. Asterisk next to day indicates a statistically significant difference between GS and non GS treatments. Points on graph are staggered (blue, purple, green, red) to avoid overlap of error bars.

This graph illustrates that mice receiving antibiotic coated allografts exhibited a drastic, statistically significant reduction in infection on the day following allograft implantation. The infection was not completely eliminated however and on the next imaging day (day 3 post-surgery) a significant increase in infection was observed for the gentamicin groups.

The results of the statistical analysis showed with a high degree of confidence (>95%) that mice receiving gentamicin coated allografts displayed reduced luminescence as compared to mice receiving non-gentamicin allografts for the first 15 days of the experiment (indicated by an asterisk next to the day of study).

The statistical analysis also demonstrated that there was a significant interaction between the phospholipid coating and the antibiotic coating on the first day following allograft implantation. The presence of the phospholipid coating on this first day resulted in a higher expression of luminescence as compared to the gentamicin only allografts. Further detail about the statistical analysis performed on the IVIS data can be found in Appendix B.

2.4.3 Histology and MicroCT

The results of the Histological analysis are summarized in the following table:

Table 2.3

Allograft	Infect	Inflammation	Bacterial Col.	H-G App.	Osseointegration
DOPS/GS	I	3,3,3	2,2,2	0,0,0	na,na,na
DOPS	I	3,3,3	2,2,2	0,0,0	na,na,na
GS	I	3,3,1	2,2,0	0,0,0	na,na,na
No Coat	I	3,3,3	2,2,2	0,0,0	na,na,na
DOPS/GS	NI	0,0,1	0,0,0	4,1,0,na,1	1,3,na,na,0
DOPS	NI	0,0,0	0,0,0	2,1,3,na,2	1,1,2,na,1
No Coat	NI	0,0,2	0,0,0	3,2,2,0,1	2,3,2,na,0

The first four rows are infected treatment groups (I = Infected, NI = Not Infected), the next three are uninfected, the numbers correspond to the score received (0,1,2,3 or na = not applicable) by each histologically analyzed sample in a given category (Inflammation, Bacterial Colonization, etc.). Three samples were analyzed initially from each treatment group, two additional uninfected samples were later analyzed for Host-Graft Apposition and Osseointegration in the uninfected groups.

All of the histologically analyzed mice that had been infected with *S. aureus* sutures, with one exception (mouse 731 – from the Infected GS Only group) received a bacterial colonization score of 2 indicating moderate bacterial colonization and an inflammation score of 3 indicating a high degree of inflammation. The one exception (731) showed no bacterial colonization and received an inflammation score of 1.

The notes from the histological analysis indicated [several] common characteristics amongst the infected mice. Gram positive cocci were found as intraosseous colonies and on the endosteal surface of all infected mice (except 731). Although the histological analysis did not specifically identify these gram positive cocci as *S. aureus*, it is unlikely that they would be anything else as none of the mice from the uninfected control groups showed any bacteria on the gram stain analysis.

The analysis of the H&E slides showed necrotic debris admixed with bacterial colonies on the endosteal surface for all of the infected mice. While bacterial colonies were absent in the uninfected mice, there was still occasional necrotic debris. Suppurative (pus forming) inflammation was noted in all of the infected mice and all infected mice (with two exceptions 731 and 713) showed marked host bone resorption with reactive callous formation, which was not observed in any of the uninfected mice. The presence of "rare globular basophilic material" was also noted in the periosteal region which the histologist thought were possibly mineralized myofibers and bone fragments. None of the infected mice showed any osseointegration with the allograft.

The microCT reconstructions supported the histological analysis (Figure 2.10). Mice from the infected treatment groups regardless of allograft type showed significant inflammation and deterioration of the native bone. Union between host bone and allograft appeared absent in all microCT reconstructions from the infected groups. Conversely mice from the three non-infected control groups exhibited visible osseointegration.

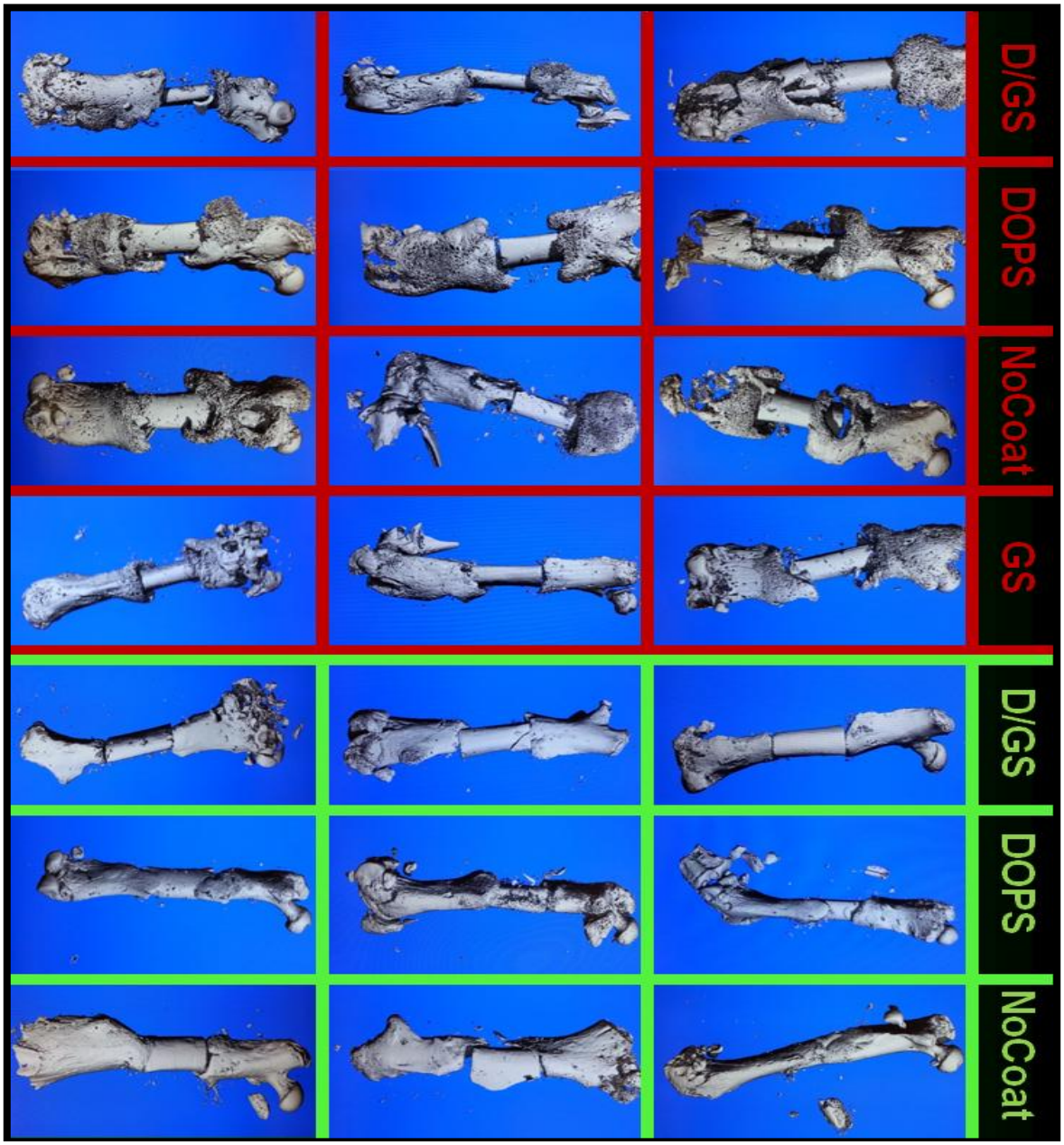


Figure 2.10 : Three dimensional MicroCT reconstructions of femurs from each treatment group. Red headings indicate infected treatment groups. Green headings indicate non-infected treatment groups.

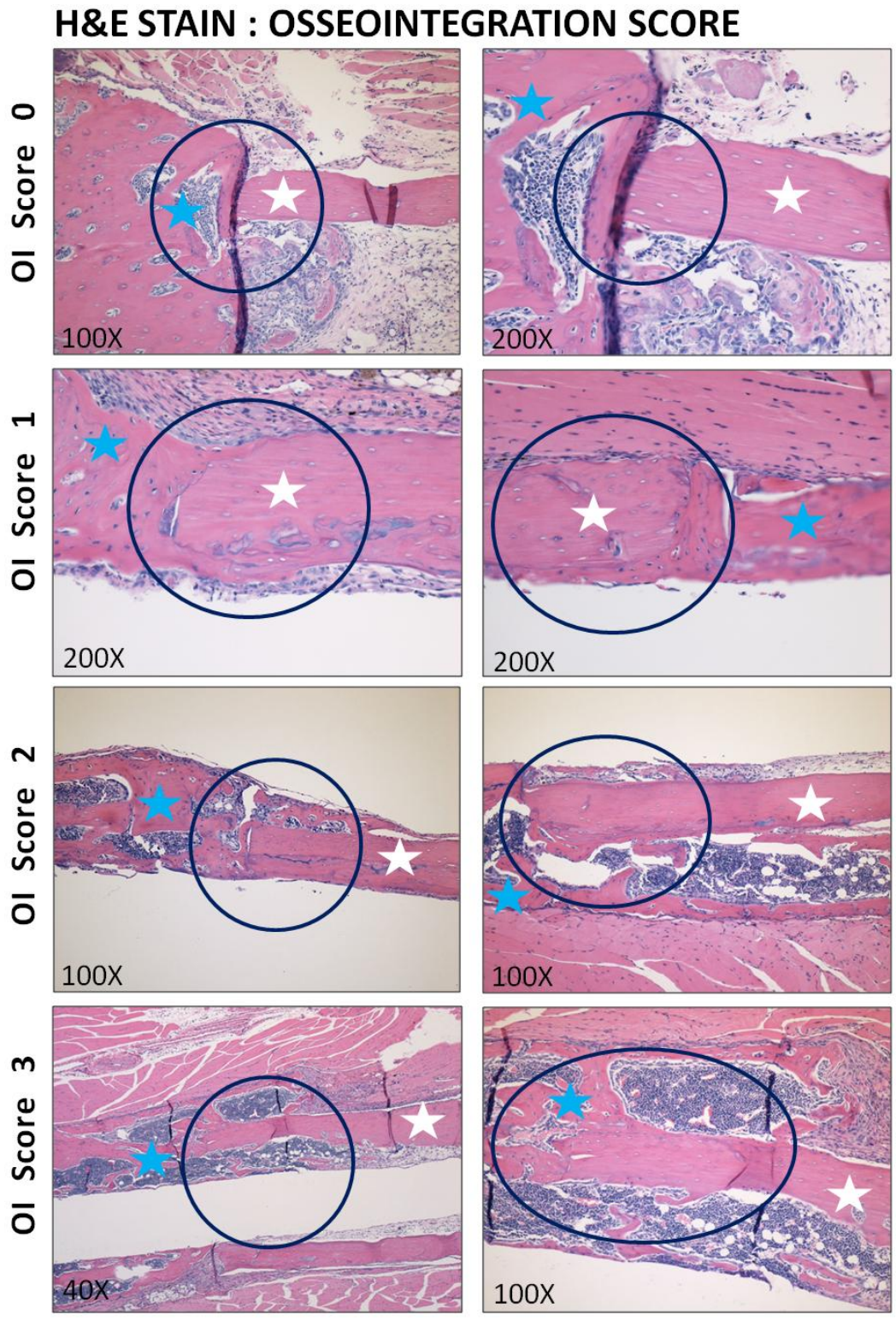


Figure 2.11 : H&E stain with representative osseointegration scores of 0-3. Blue star indicates native bone, white star indicates allograft, blue circle indicates location of osseointegration.

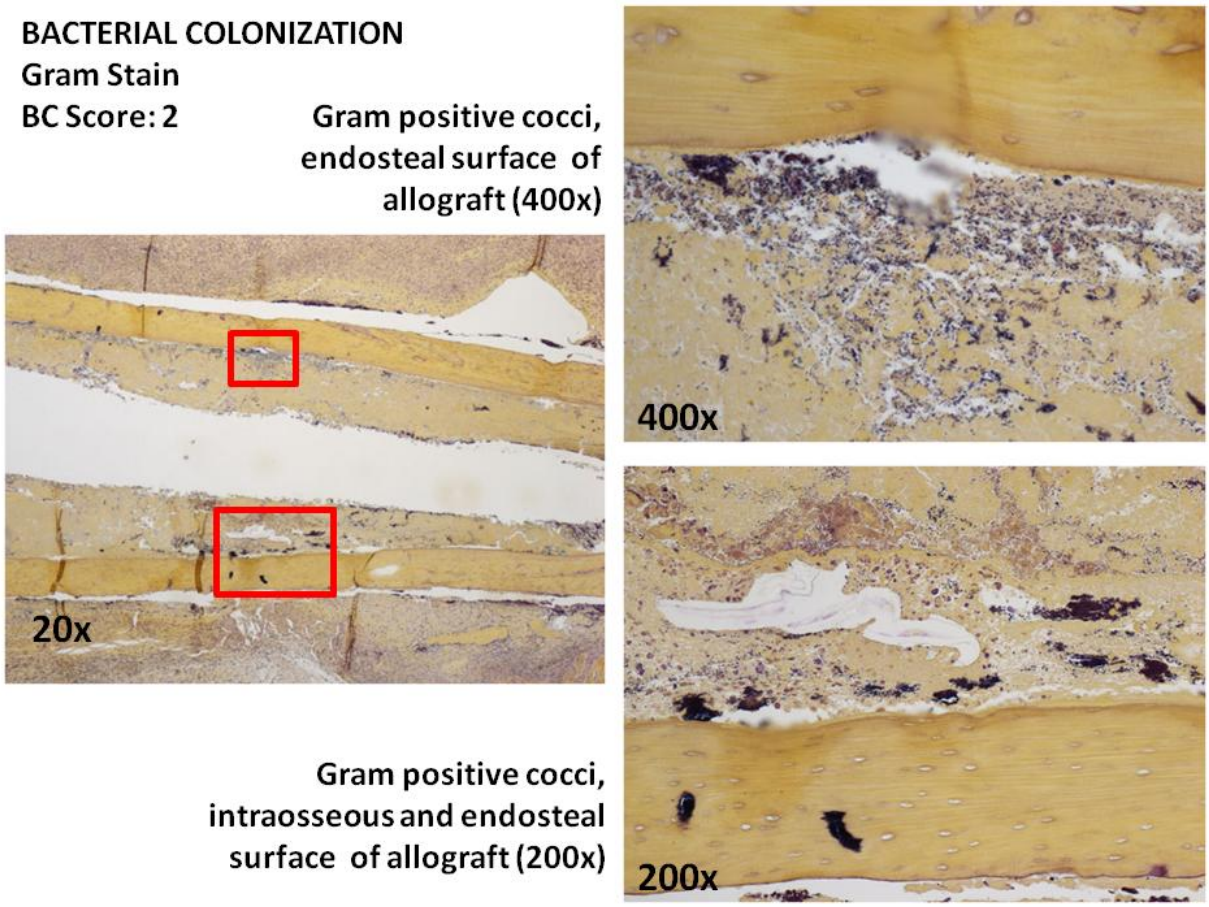


Figure 2.12 : Gram stain with visible bacterial colonies (which appear black), the two images on the right are blown up from the red boxed regions in the left image.

Two outliers were noted in the unblinded analysis: mouse 731, an infected mouse that showed no identifiable bacteria and minimal inflammation (score of 1), and mouse 909, an uninfected mouse that showed moderate inflammation (score of 2). Aside from these two outliers histological results were consistent (i.e. all scores were identical) within the infected and uninfected groups with regard to inflammation, bacterial colonization and callus formation. The location of the bacteria in the infected treatment groups was noted as always being associated with the allograft either on the endosteal surface or intraosseously, but bacteria were not observed in the surrounding soft tissue (Figure 2.12). Also endosteal acellular debris were noted as present versus variably present and viable endosteal stroma absent versus present in infected

and uninfected groups respectively. The lack of viable endosteal stroma in infected mice was thought to have been caused by extensive tissue necrosis as well as bacteria along the endosteal surface.

Variability in the stability of the allograft due to the fixation method may have contributed to the inconsistent osseointegration scores in the uninfected mice. For all three uninfected treatment groups, osseointegration was present to varying degrees, however no significant differences in osseointegration were observed between groups. It was noted that viable endosteal stroma was most prolific in mice with the highest osseointegration scores.

2.4.4 Statistical Analysis of Histological Osseointegration Scores

The uninfected groups (all of which contained samples exhibiting osseointegration) were compared using a Wilcoxon rank sum test, a non-parametric test for comparing group means that does not assume normality of the sample distribution. The analysis was performed using SAS. The SAS code can be found in Appendix B.

The Wilcoxon test works by first ranking each input value and then performing analysis on those ranked valued. Since there were 11 input values in three groups this means that the highest scoring value would receive a rank of 11 and the lowest would receive a value of 1. Due to the discrete scoring system for osseointegration however there were inevitably numerous ties (e.g. two scores of 3 resulting in a tie between ranks 11 and 10 so each received a rank of 10.5). The

qualitative nature of the histological osseointegration analysis necessitates such a scoring system however this scoring system also makes it more difficult to obtain statistical significance.

The test was run multiple times comparing all three groups as well as each two group combination however no significant differences in osseointegration between means could be concluded based on the p-values obtained from the Wilcoxon rank sum test, meaning the test failed to reject the null hypothesis that mean osseointegration scores were the same between groups.

2.4.5 Femur Culturing

Two femurs were cultured, one on each euthanasia day from the Infected DOPS/GS, Infected DOPS, and Infected No Coat groups. Also cultured were several muscle tissue samples from the area adjacent to the femurs of several randomly chosen mice as well as tissue samples from 731 (the mouse that seemed to have cured the infection) and an uninfected control mouse.

The results were at first perplexing. All the cultured femurs and tissue samples from the first euthanasia day showed heavy growth while the femurs from the second euthanasia day showed moderate growth and the tissue samples from the control mouse and 731 showed light growth. The cause of these results was discovered to be that the cultures from the first euthanasia day had not been submitted to the culture lab until the following day which had allowed extra time for growth. While this confounds the results somewhat there is still some useful information to be gleaned.

Assuming that heavy growth from the samples on the first euthanasia day corresponds approximately with moderate growth from the second euthanasia day it can be said that all of the samples showing heavy or moderate growth still had significant quantities of *S. aureus* infection present at the time of euthanasia. The control tissue sample and tissue sample from 731 showed only light growth. Because the control tissue sample had never been exposed to *S. aureus*, the fact that it exhibited any growth can most likely be attributed to unintentional contamination and this is also likely the cause of the light growth from tissue sample 731 as this mouse seemed to have completely cleared the infection based on both IVIS data and histological analysis.

2.5 Discussion

2.5.1 E-spray

The e-spray method worked quite well for applying even, consistent coatings of DOPS to the allografts in this study, but the scalability of this process is questionable. It was noted in the very first attempts at e-spraying using thinly cut slices of cow bone that the thickness of the bone affects the efficiency of the e-spray process (thicker sections of bone receive less coating). Although the method works remarkably well on thin mouse femurs, it may not be appropriate for anything much larger than that. It is possible that modifying some of the spray parameters could change this, for instance by increasing the applied voltage; however given that the e-spray process was hindered by bone of just a couple millimeters in thickness this method may not be appropriate for spraying large allografts such as a human femur or humerus.

This is not necessarily a major problem because if DOPS coatings are found to be useful for enhancing osteogenesis other methods of applying the coatings such as aerosol spraying could be employed for larger bones. Because the present study involved coating a large number of very small allografts it would have been significantly more difficult to attempt aerosol spraying due to the size and shape of the mouse femurs. Also DOPS is relatively expensive and it would have been wasteful to aerosol spray because much of the spray solution would have been lost as overspray. For larger bones however this would not be as much of a problem because the larger size would enable greater ease of aerosol spray application with less overspray.

It may be possible to make large bones more amenable to e-spraying by increasing electrical conductivity, for instance if titanium pins were inserted into large allografts then these could be attached to a voltage source and perhaps make the e-spraying perform better. While e-spraying does tend to produce a more even coating and finer spray particles than aerosol spray it is also possible that aerosol spraying would be sufficient to create effective DOPS coatings. Further analysis would be necessary to determine whether coating application method impacts the efficacy of the coatings and this should be investigated if future experiments are performed involving larger allografts that prove to be incompatible with the e-spray process. Additionally other coating application methods such as ultrasonic vaporization or supercritical fluid deposition could be investigated.

2.5.2 Induction of Osteomyelitis

Two methods of osteomyelitis induction were initially considered. The method ultimately selected involved implanting bacterial sutures laterally through the femurs of the study mice. The other method involved placing bacterial sutures through the proximal epiphysis into the femoral medullae of the mice as in Thomas Triffo's study [42]. The choice to use the lateral suture method was based on a concern with using that using the proximal epiphysis method for this study could result in too severe of an infection inside the femur potentially weakening the bone to the point that it would become prone to fracture during the allograft implantation surgery.

A pilot study was performed using the lateral suture method of infection and it was found that fracture during allograft implantation was not a problem. In retrospect it would have been a good idea to perform a larger pilot study to test both osteomyelitis induction methods and perhaps others as well. Doing so would have established whether the medullary suture method did indeed cause excessive bone deterioration and proneness to fracture and would have demonstrated whether severity and distribution of infection differed significantly between the two methods.

There are other methods for osteomyelitis induction in the literature which were not considered during discussions on the topic but which may suit the needs of the experiment better than the one used. One such method is to inject a small amount of bacterial broth directly into the femoral medulla. This method has been found to work well in rats and rabbits and has also been adapted to mice [71, 72]. This method may be a better way to induce an osteomyelitis infection

for studies such as this because it does not involve placing a foreign body (suture) into the animal. The presence of such a foreign body increases the ability of infection to establish itself because it provides a surface for bacterial attachment and biofilm formation [71].

2.5.3 IVIS and ROI Discussion

When luciferase expression is high the IVIS system works quite well, imaging for 60 seconds is sufficient, and it is easy to see where the ROI boundary should be however when luciferase expression is low ROI determination becomes problematic.

This problem is illustrated in figure 2.13 (pg. 70). ROI3 illustrates background radiation, ROI2 is a large region with flux marginally higher than background however because the region is so large and diffuse the amount of background contributing to the flux is significant so the region was ignored. The smaller region in the image (ROI1) with a flux of 3.265*10^3 photons per second was included although it may have been more appropriate to not include any region from this image and to call the flux zero.

The difficulty arises in determining when to label a region as having zero flux. The method of ROI determination used seems to occasionally ignore the fact that there is some small quantity of luciferase present however including such regions where background contributes significantly to total flux would yield skewed results. While the IVIS works great for detecting high luciferase expression levels it becomes increasingly less reliable as the lower detection limit is approached and background noise becomes significant.

Luciferase levels in many of the infected mice were approaching this lower limit towards the end of the study and histological analysis showed that bacterial infection was still “moderate” in all infected mice at the time of euthanasia. If an infection level considered “moderate” approaches the lower detection limits of the instrument this calls into question whether IVIS imaging is appropriate for this type of experiment. Since the infection is located deep underneath muscle and inside bone it seems likely that much of the luminescence from the luciferase could be absorbed by these tissues and this would explain why a “moderate” level of infection registered such low luciferase intensity when imaged with IVIS.

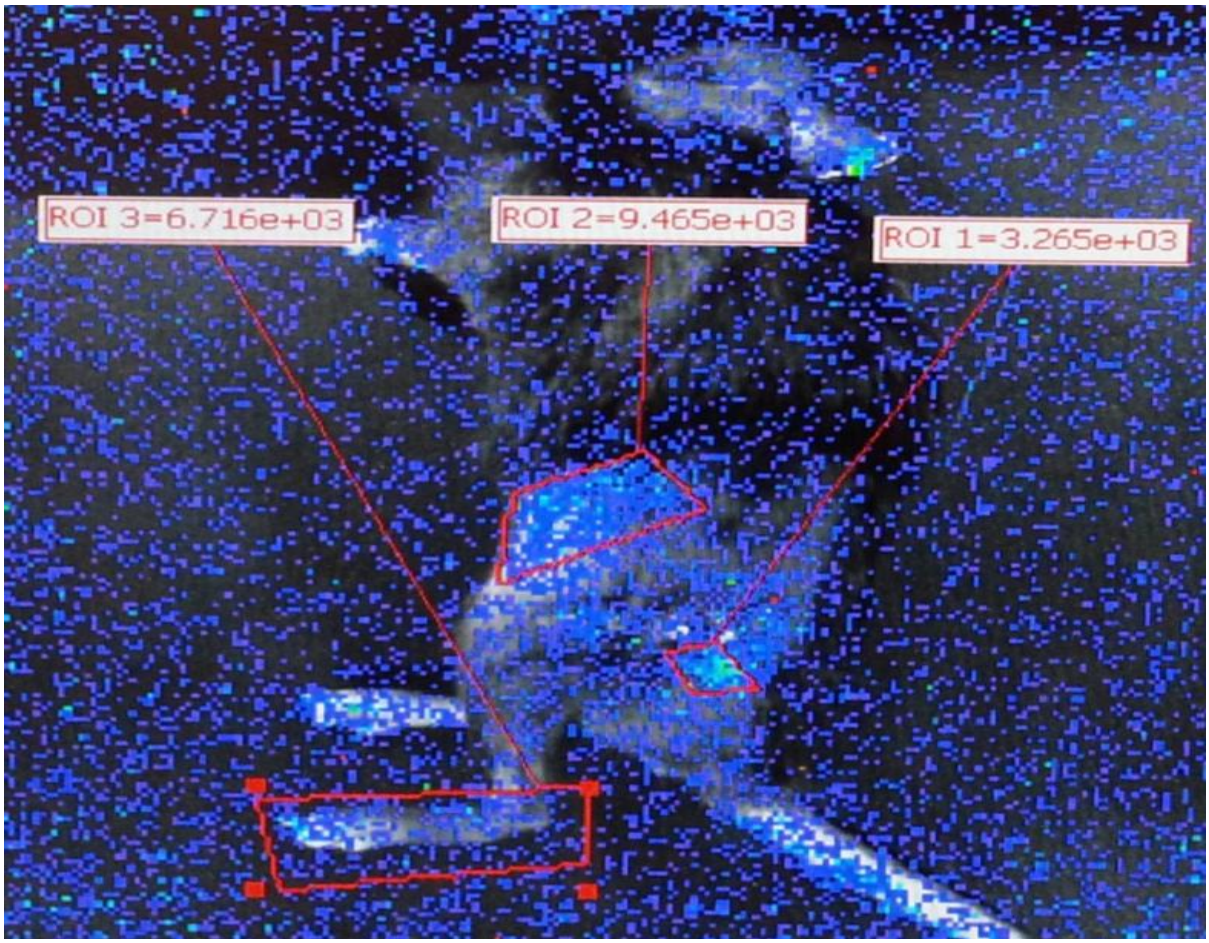


Figure 2.13 : Mouse IVIS image with low luciferase expression

2.5.4 Allograft Fixation

The 4 millimeter femoral graft model has been used for many years in mice and has been shown to be an effective model for experimenting with numerous types of graft treatments. The combination of severe osteomyelitis infection and brief duration of antibiotic presence were likely the major factors which prevented successful osseointegration in the infected treatment groups in this experiment. That being said, it seems possible that the intramedullary fixation method used may have promoted the persistence of endosteal bacteria as foreign bodies often serve as locations for bacterial adhesion and biofilm formation. Many of the infected mice had abscesses where the needle protruded from the proximal epiphysis as in figure 2.14 (taken after euthanasia) and this area was often where the highest luciferase expression was observed during IVIS imaging. Since the end of the needle was prone to abscess and bacterial growth it could have acted as a conduit for bacteria to make their way back into the medulla of the allograft in the gentamicin treated mice that had initially seen reduced infection rates. While this was not conclusively determined it is a reasonable explanation for the ubiquitous presence of bacterial colonies on the endosteal surface of the allografts in the infected mice and absence of bacterial colonies on the periosteal surface.

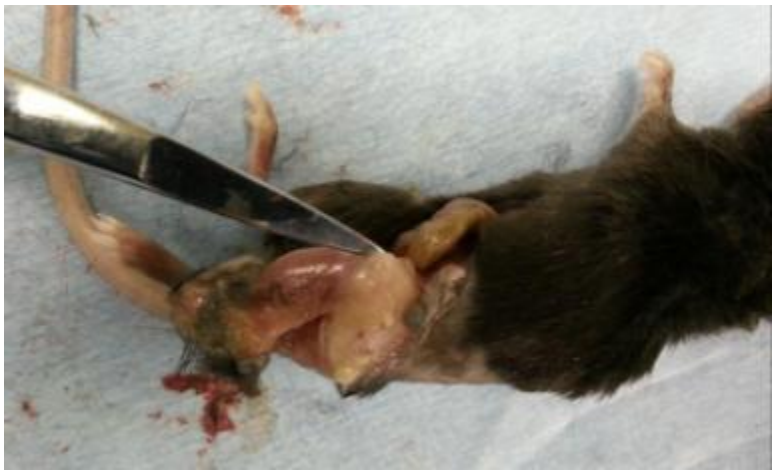


Figure 2.14 : Large abscess at insertion point of fixation needle

In the non-infected treatment groups the major complication was inconsistent host-graft apposition. On each histological slide there were four locations where apposition between host bone and allograft could be present (Figure 2.15) however it was not uncommon for only one or even none of these locations to exhibit proper apposition (see Table 2.3 on page 52). MicroCT reconstructions indicated that sometimes host-graft apposition was present only partially (see Figure 2.10 on page 54) so it is possible that the inconsistent host-graft apposition was an artifact of where the H&E slide happened to be taken from on a given femur. Because of this it is possible that this inconsistency would not have been observed if multiple H&E slides (taken from different levels on the femur) were to be analyzed from each mouse. This would have provided a more complete understanding of the host-graft apposition and osseointegration present throughout the entire femur but also would have added significantly to the cost of the analysis.

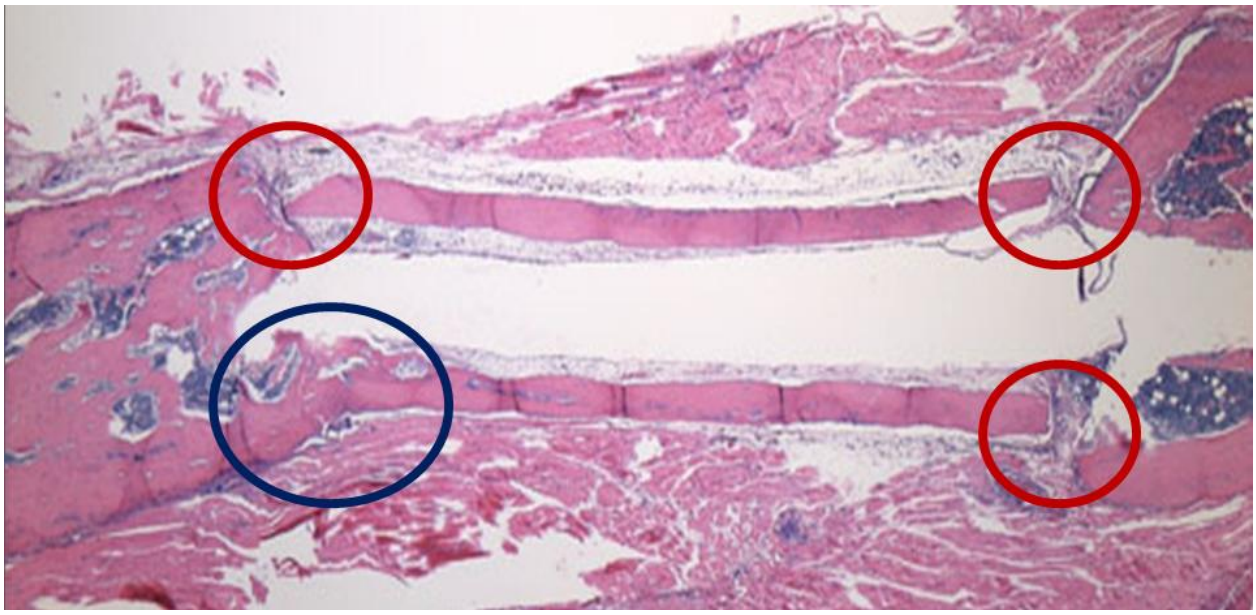


Figure 2.15 : Of the four potential locations (circled) in this image where host-graft apposition could be present, it was only present at one (blue) and absent in the other three (red)

Since the intramedullary pin fixation method has been used successfully in many other experiments involving allografts it seems unlikely that the fixation method would be solely responsible for the inconsistent host-graft apposition and osseointegration results. It is possible however that these inconsistent results were due largely to the short four week duration of the experiment. Allografts heal much more slowly than autografts; another study involving this same fixation method showed that at five weeks only 85% of allografts achieved healing at the host-graft junction [63] and most studies found in the literature evaluated allograft incorporation at significantly longer time points between nine and eighteen weeks [65] [67] [73] [74].

In future experiments it may be worthwhile to look at other allograft fixation models. Using plate and screw fixation rather than securing the allograft with an intramedullary pin may yield more consistent host graft apposition, albeit at the cost of obtaining expensive hardware and using a larger test animal. Since the DOPS coating used in this experiment is hypothesized to work on the principle of improving calcium phosphate crystal formation it may perform better under such rigid fixation conditions. If primary bone healing could be achieved the coating would be able to more immediately provide calcium to bone depositing osteoblasts rather than having to first weather the progression of inflammation, callus formation, osteoid deposition and other processes associated with secondary bone healing.

Since multiple factors may have contributed to the osseointegration outcomes observed in this experiment it is not possible to definitively say what role any individual factor played. Fixation method should be one factor taken under consideration in the design of future studies as should duration of the study, number of animals per treatment group and scope of the histological

analysis. It is possible that increasing the sample size and experimental duration may yield better results in testing DOPS allograft coatings using this fixation method. It is also possible that this fixation method is not ideally suited for some experimental conditions such as when attempting to eliminate an established osteomyelitis infection.

2.6 Conclusions

The purpose of the study was to investigate whether DOPS coatings on allografts enhance bone regrowth and also whether incorporating gentamicin sulfate coatings can successfully reduce an established osteomyelitis infection. While the GS coatings did demonstrate efficacy in reducing osteomyelitis they were unable to fully eliminate the infection. This may have been due to the dosage, infection method, fixation method, elution kinetics or other factors. The study was unable to demonstrate whether DOPS coatings are capable of enhancing allograft osseointegration. Only the uninfected control mice showed any osseointegration and there were only 5 mice in each uninfected treatment group. This small sample size and low number of histological sections made statistical significance difficult to achieve from the histological data. Further research with larger sample sizes and perhaps longer *in vivo* experimental duration will be necessary to determine whether DOPS coatings are potentially useful for enhancing allograft osseointegration.

Chapter 3 – Gentamicin Quantification Method Development

3.1 Introduction

Data obtained from the *in vivo* study (both from IVIS and histological analysis) showed that the infection was not successfully eliminated in the majority of test subjects receiving antibiotic coated allografts. This fact motivated further investigation into the elution profile of the antibiotic from the allografts and lead to specific aims 3 and 4 of the project:

Specific Aim 3: Research and verify a method for gentamicin quantification in samples of unknown gentamicin concentration that is not confounded by the presence of DOPS in the sample.

Specific Aim 4: Determine how quickly the Gentamicin Sulfate coating elutes from the allografts using an *in vitro* elution method. Investigate the elution profile of the exterior (periosteal) gentamicin coating in both the presence and absence of a DOPS coating as well as the elution profile of the interior (endosteal) coating.

It was hypothesized that the GS elution profile from the allograft interior may be different from the exterior elution profile. Incorporating both exterior and interior coatings of gentamicin would therefore have resulted in data where two distinct elution profiles were combined and this would confound the data analysis. In order to obtain better data three treatment groups were analyzed. These groups were Exterior DOPS/GS, Exterior GS Only, and Interior GS Only. Recall that the amount of DOPS applied to the interior of the allografts was negligible and

therefore the interior GS elution profile would presumably be the same regardless of whether DOPS had been applied.

3.2 Method Development: Gentamicin Quantification

3.2.1 Introduction

Previous research in the James lab has employed a fluorometric method involving reaction of the gentamicin molecule with ortho-phthaldialdehyde [75] however this method was found to be unreliable in the presence of DOPS [42] and it was determined that other methods of gentamicin quantification should be investigated.

A review of the literature yielded many available methods for this purpose. These methods ranged from radiometric assays to HPLC with mass spectrometry [29, 76] to gas chromatography with electron capture detection [49, 77] to microbial assays [56] as well as numerous others. The gas chromatography electron capture detection method (GC/ECD) was initially selected.

3.2.2 GC/ECD Method, Results and Discussion

Gas chromatography electron capture detection methods were initially chosen for their high sensitivity and seemingly simple derivatization reaction sequence. The purpose of the derivatization reactions is to replace hydroxyl and amine functional groups on the gentamicin molecule with different functional moieties. The derivatized gentamicin is then extracted into an

organic solvent (hexane) and is amenable to vaporization and detection with the electron capture detector.

After the organic phase with the derivatized gentamicin is injected into the gas chromatograph it is heated to vaporize the solution which travels with a carrier gas (nitrogen) through a long (~30 meter length) thin (~0.25 millimeter inner diameter) column lined with fused silica before reaching the ECD detector. The results are obtained as voltage at the detector as a function of time. This voltage is reduced when molecules capable of capturing the radiated electrons pass through the detector. Ideally there should be a distinct region of the curve for which the area under the curve increases with increasing concentration of the molecule of interest. Figure 3.1.a is an example of an output obtained from the GC/ECD.

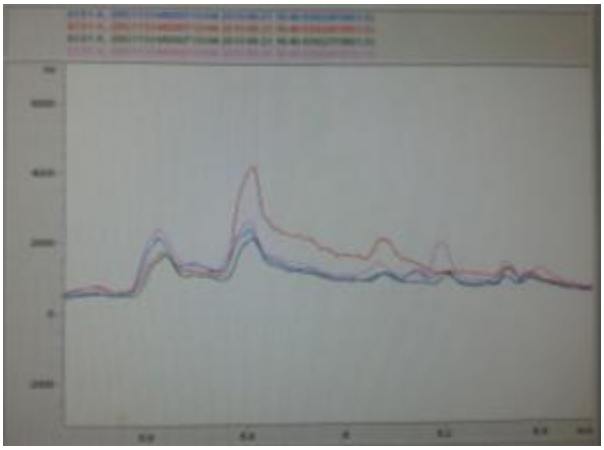


Figure 3.1.a – Data output from GC/ECD

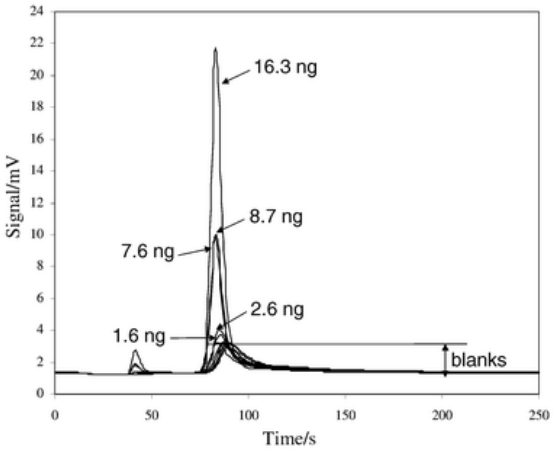


Figure 3.1.b – Sample of good GC/ECD data

The data in figure 3.1.a shows the curve generated between 5.4 and 6.5 minutes after injection into the GC for four samples of different quantities of derivatized gentamicin solution ranging from 20ng to 160ng derivatized gentamicin (blue=20ng, red=40ng, green=80ng, pink=160ng). For comparison figure 3.1.b (adapted from [78]) shows what data should look like in order to be able to accurately create a calibration curve to measure unknown concentrations of an analyte.

The data obtained from the GC/ECD experiments was all similar to that depicted in figure 3.1.a and the data could not be used to generate a calibration curve due to the fact that the peaks were not well defined and there was no single peak that consistently increased in area with increasing analyte concentration. It was therefore not possible to proceed with this method.

The GC/ECD method was attempted numerous times however none of the results obtained were usable. This may have been due to problems with the derivatization such as unsuccessful quenching of unreacted HFBI or TFAA, the temperature ramp not being optimally set to separate components within the organic phase injected into the column, issues with the specific GC column used in the experiment, or some other confounding effect. The GC/ECD method was ultimately abandoned after obtaining good results from another method (agar diffusion) discussed next.

3.2.3 Agar Diffusion Microbial Inhibition Method Introduction

Microbial growth inhibition has long been used to quantify numerous types of antibiotics, including gentamicin, and many sources were found on this topic. The method discussed in the following sections was adapted from a paper about determination of MICs (minimum inhibitory concentrations) of antibiotics [56].

The method involves seeding Mueller-Hinton agar plates with *Staphylococcus epidermidis*, punching wells in the agar and injecting gentamicin containing solution into the wells. The plates are then incubated for 24 hours at 37 degrees Celsius allowing the bacteria to colonize the

plate except where antibiotic concentration is too high. This forms "inhibition zones" around the gentamicin containing wells. The sizes of the inhibition zones are used to determine antibiotic concentration.

The following sections will discuss method development (which involved multiple small scale pilot studies), results from pilot studies, and a discussion of how method parameters were ultimately selected for use in the final gentamicin elution study (Chapter 4).

3.2.4 Development of Agar Diffusion Method

A total of five pilot experiments were performed and numerous adjustments made to the agar diffusion gentamicin quantification method prior to using it for the full scale elution study (Chapter 4). This section details how each of these pilot experiments was performed. The key changes that were made between each experiment are summarized in table 3.1. The major changes from the initial pilot were the bacterial strain used, the diameter of agar plate, inhibition zone measurement method, measurement of bacterial broth light absorbance with a plate reader, and method of bacterial broth preparation.

Table 3.1

Pilot #	Bacteria	Plates	Measurement	Absorbance	Broth Prep
1	*E. coli, S. epidermidis*	100 mm	Ruler	No	TSA Slant
2	*S. epidermidis*	100 mm	Ruler	No	TSA Slant
3	*S. epidermidis*	150 mm	Ruler	No	TSA Slant
4	*S. epidermidis*	150 mm	ImageJ	Yes	TSA Slant
5	*S. epidermidis*	150 mm	ImageJ	Yes	LB Broth
Full Study	*S. epidermidis*	150 mm	ImageJ	Yes	LB Broth

Pilot 1: The method was first attempted using 100 millimeter diameter Mueller-Hinton agar plates which were seeded with bacteria obtained from the CSU Microbiology Immunology and Pathology department. Both *E. coli* and *S. epidermidis* were examined.

Bacteria were obtained growing on TSA (trypticase soy agar) slants. Bacteria from each TSA slant were prepared in a broth of sterile saline and visually matched to a 0.5 McFarland Standard. Then 200 microliters of this solution was pipetted onto the agar plate and spread over the entire surface.

Six two-fold serial dilutions of gentamicin in Milli-Q water were prepared ranging in concentration from 1 mg/mL to approximately 0.03 mg/mL. Twenty microliters of these solutions was pipetted into 3mm diameter wells punched in the agar plates such that each plate had one well with 1mg/mL solution, one with 0.5mg/mL solution, etc. Two additional control spread plates (one for *E. coli* and one for *S. epidermidis*) were prepared, however gentamicin solution was not added to these plates. The agar plates were incubated at 37 degrees Celsius for 24 hours. They were then removed from the incubator, observed, photographed and the diameters of the inhibition zones were measured with a ruler to the nearest 0.5 millimeter.

Pilot 2: *S. epidermidis* was seeded onto fourteen 100 mm diameter agar plates. This experiment tested several variables. The lower gentamicin concentration was decreased to 0.0075 mg/mL. DOPS was added to several of the wells to determine whether it affects inhibition zone size. Also the volume of eluent added to the wells was varied from 5 microliters to 40 microliters.

Pilot 3: The experiment was performed again with four 150 mm plates. This time 400 microliters of bacterial saline solution was spread over the plate to accommodate the larger area. A new well punching device was used and 10 microliters of antibiotic solution was injected into each well. A lower concentration of 0.0032 mg/mL gentamicin was also tested. The diameters of the inhibition zones were measured using a ruler to a precision of 0.5 millimeters.

Pilot 4: Allograft samples were prepared according to the procedure described in Chapter 2 with exterior coatings of DOPS/GS or GS Only (500 micrograms total GS on the allograft exterior only). Two DOPS/GS and two GS Only allografts were placed in PBS at 37 degrees Celsius and eluent was collected and replaced with fresh PBS at time points of 1, 4 and 24 hours. Bacterial broth was prepared in sterile PBS and the absorbance of 0.3 mL of this broth was measured with a BMG Labtech FLUOstar Omega plate reader to be 0.12 at 600 nm. Three 150 mm diameter agar plates were swabbed with 400 microliters of this bacterial broth and 12 wells were punched in each plate. An "antibiotic ladder" (serial dilution of antibiotic concentrations used to create a calibration curve) was run on each plate along with the eluent samples. The plates were then incubated for 24 hours. Inhibition zones were photographed and the photographs were analyzed using image analysis software called "ImageJ" ([79] version 1.47 from http://imagej.nih.gov/ij/). Gentamicin concentration of the eluent samples was calculated by fitting the inhibition zone size from these samples to the calibration curve generated from the antibiotic ladder.

Pilot 5: Three allografts were prepared as described in chapter 2 with interior coatings of gentamicin (500 micrograms per allograft). These were placed in PBS at 37 degrees Celsius and eluent was obtained at time points of 0.5, 1, 3, 24 and 42 hours. *S. epidermidis* was grown for 24

hours in lysogeny broth (LB) to ensure that the bacteria would grow well when plated onto agar. The LB was diluted with sterile PBS and absorbance of the mixture at 600 nm was measured to be 0.35. Seven-hundred and fifty microliters of this diluted PBS/LB mixture was spread onto agar plates, 12 wells punched per plate, and eluent run along with an antibiotic ladder for all eluent samples from this pilot as well as for eluent samples obtained in pilot 4 (Pilot 4 yielded poor bacterial growth so these eluent samples were run again in order to obtain better data). Plates were incubated for 24 hours at 37 degrees Celsius, photographed and the photographs were analyzed with ImageJ. Gentamicin content of the eluent samples was then calculated from this data.

3.3 Results

Table 3.2 summarizes the results obtained from each pilot as well as conclusions about these results and methodological changes made between pilot studies:

Table 3.2

Pilot #	**Notes and Conclusions**
1	Method seems promising, *S. epidermidis* works better than *E. coli*
2	Method not confounded by DOPS, 100 mm diameter plates too small to accommodate the necessary number of wells
3	150 mm diameter plates work much better, Method is accurate from 1 mg/ml to about 0.01 mg/ml gentamicin, Using ruler to measure inhibition zones does not yield sufficient precision, Need better method of bacterial broth preparation
4	First experiment with eluent of unknown concentration, Used freshly obtained *S. epidermidis* from ATCC, ImageJ analysis improves accuracy substantially, tried standardizing bacterial concentration with plate reader absorbance at 600 nm, bacterial growth was unexpectedly poor
5	Grew bacteria in lysogeny broth for 1 day and plated dilution of this broth, which solved the bacterial growth problem
Full Study (Chapter 4)	3 treatment groups (24 samples), 6 time points (20 mins, 40 mins, 1 hr, 4 hrs, 24 hrs, 48 hrs), bacteria prepared in LB broth diluted with PBS to absorbance of 0.36

Pilot 1: Proof of concept was successful. The results showed a relationship between gentamicin concentration and inhibition zone size, but the high degree of inhibition zone overlap prevented accurate measurements of inhibition zone size and results were not graphed. It was also found that *S. epidermidis* provides better results than *E. coli* because it forms larger inhibition zones with more distinct boundaries.

Pilot 2: DOPS was added to several of the plates in this experiment and the results indicated that DOPS does not inhibit the growth of bacteria or affect the size of the inhibition zone formed by the gentamicin. At the lowest gentamicin concentration of 0.0075 mg/mL it was observed that a small inhibition zone was present indicating that this is above the lower detection threshold. The volume of eluent added to the wells ranged from 5 microliters to 40 microliters. Volumes larger than 15 microliters required larger diameter wells and these were more difficult to punch in the agar. Therefore 3mm diameter wells were selected to be used for future experiments. Ten microliters of gentamicin solution was found to be the ideal amount to add to these wells because it fills them almost fully but does not overflow them. Several of the plates from this experiment using 3 mm wells and 10 microliters GS solution are shown in the figure 3.2.

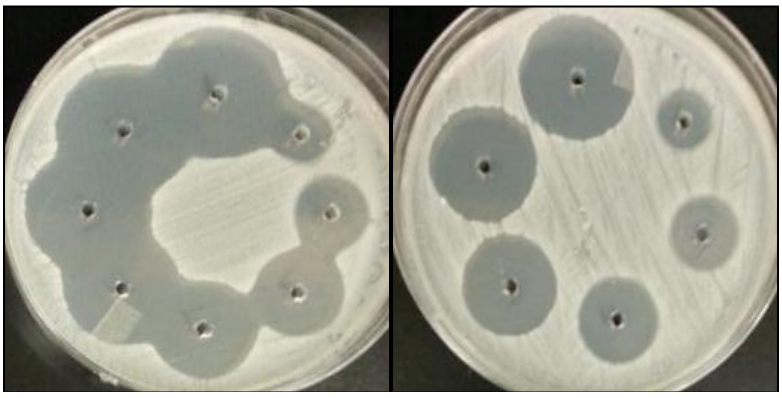


Figure 3.2 : The plate on the left had eight 2-fold dilutions of GS solution ranging from 1.00 mg/ml to 0.0075 mg/ml, the plate on the right had six 2-fold dilutions ranging from 0.25 mg/ml to 0.0075 mg/ml. All wells in these two plates were filled with 10 microliters of antibiotic solution.

Four of the plates run in this experiment tested a series of eight two-fold dilutions of gentamicin ranging in concentration from 1 mg/mL to 0.0075 mg/ml. Ten microliters of antibiotic solution was added to each well. The diameters of the inhibition zones were measured with a ruler to a precision of 0.5 millimeters and the data graphed as the log of gentamicin concentration versus inhibition zone diameter to yield figure 3.3. The high R squared value of the regression line served as evidence that this method could be used to accurately quantify gentamicin. In order to run more samples per plate it was decided that larger 150 millimeter diameter agar plates should be used for future experiments.

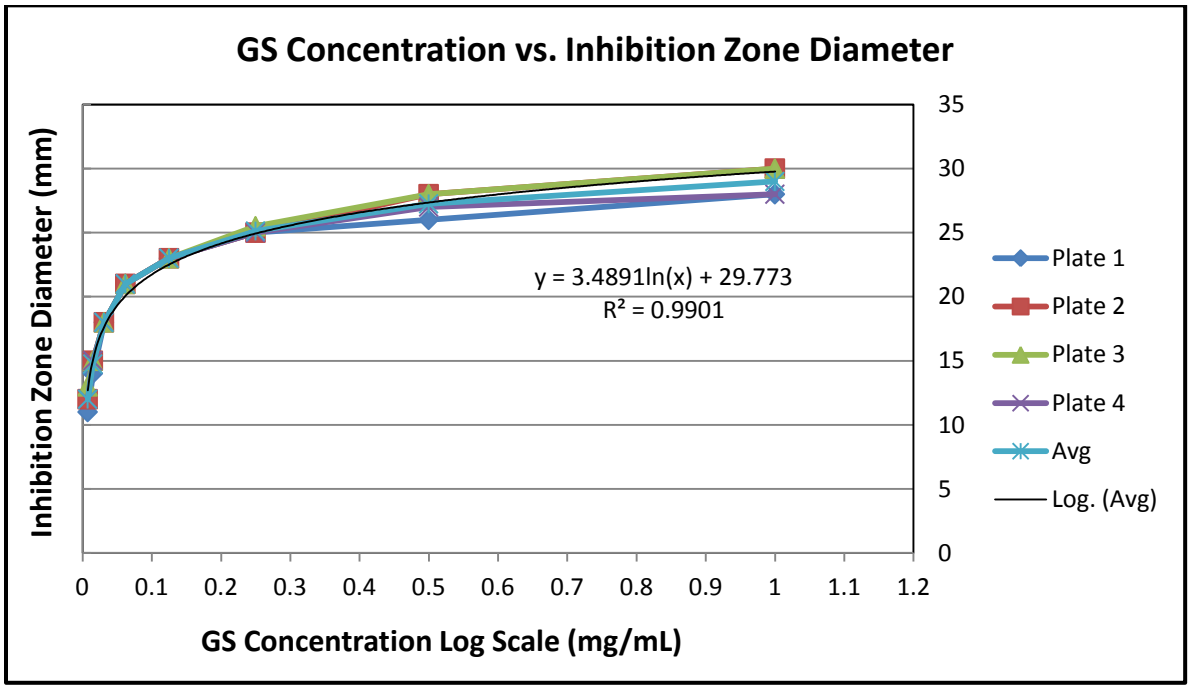


Figure 3.3 : Each plate had 1 set of standards run at concentrations of 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.062 mg/ml, 0.031 mg/ml, 0.015 mg/ml and 0.0075 mg/ml

Pilot 3: A well punching device consisting of a 20 milliliter volume syringe connected to PVC tubing with a 3mm diameter aluminum pipe at the end of the tube was made and found to work better than pipette tips for punching wells into the agar. One-hundred and fifty (150) millimeter

diameter plates enabled two sets of standards to be run on each of 4 plates and the resulting inhibition zone diameters were measured with a ruler to a precision of 0.5 mm and the results are plotted in the figure 3.4. The method of measuring inhibition zone diameter with a ruler was determined to be too imprecise and it was decided to photograph plates after incubation and measure inhibition zone size with image analysis software in future experiments.

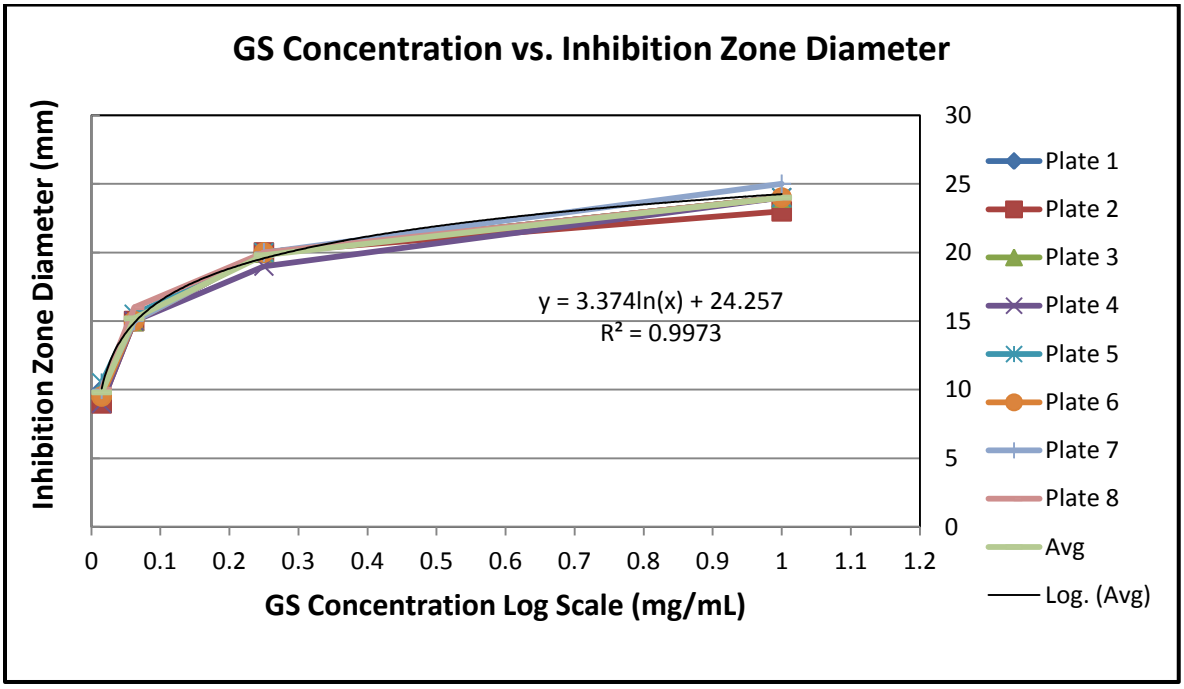


Figure 3.4 : Each of 4 plates had 2 sets of standards run at concentrations of 1 mg/ml, 0.25 mg/ml, 0.062 mg/ml and 0.015 mg/ml

Pilot 4: This was the first experiment in which gentamicin was eluted from the allografts and the gentamicin concentration of the unknown eluent was determined by fitting the inhibition zone size to the regression curve of an antibiotic ladder run on the same plate. The first time this eluent was analyzed bacterial growth on the plates was poor and usable data was not obtained. The same eluent was tested again while performing pilot 5 and this time yielded good bacterial growth and usable results. These results are shown in the figure 3.5.

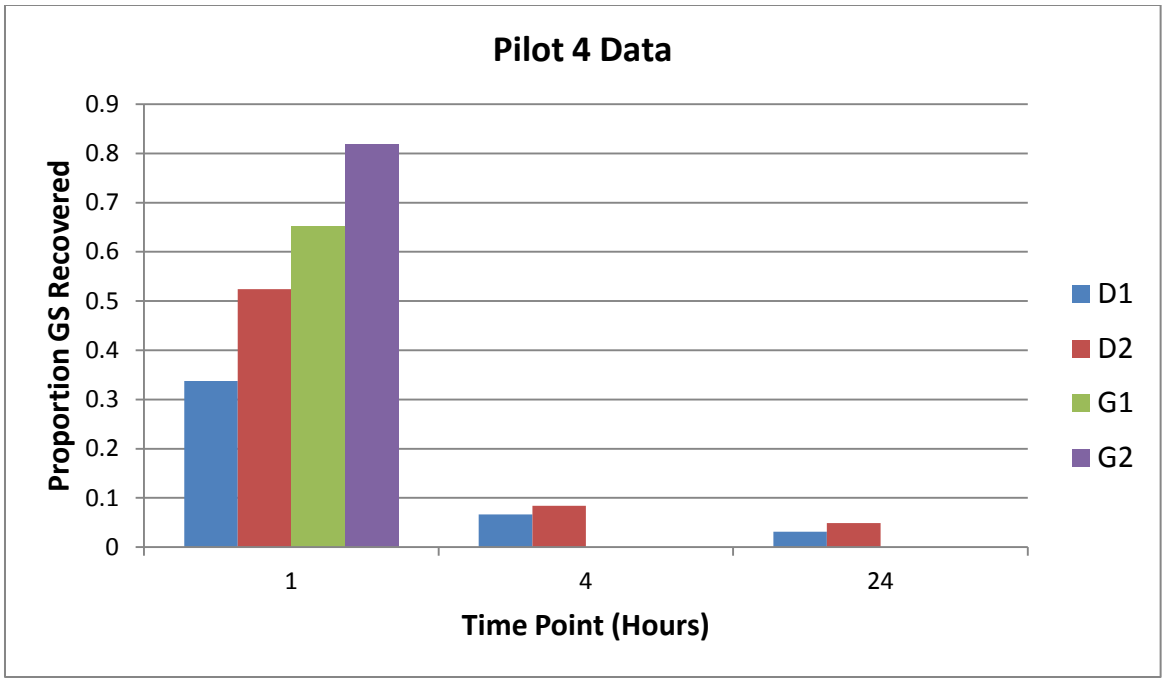


Figure 3.5 : Results of pilot elution test 4, D1 and D2 are DOPS/GS allografts 1 and 2, G1 and G2 are Gentamicin Only allografts 1 and 2

Pilot 5: The additional step of growing the bacteria in LB broth prior to seeding it on the agar plates was found to solve the problem of low bacterial growth and poorly defined inhibition zones. Measurable amounts of gentamicin were eluted from two of the three interior gentamicin coated allografts for the first half hour, and gentamicin continued to elute from the third allograft until the 24 hour time point. These data are illustrated in figure 3.6. Cumulative recovery of gentamicin was found to be 80%, 86% and 87% for samples 1,2 and 3 respectively. Due to the small sample size statistical outlier analysis could not be performed.

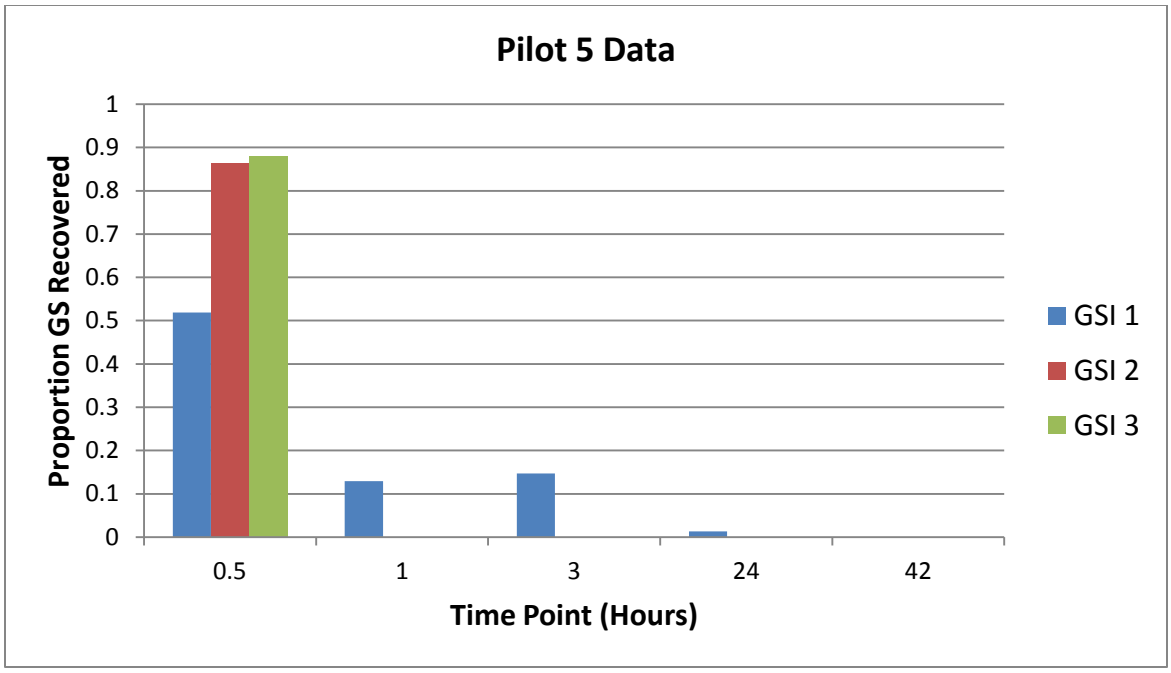


Figure 3.6 : Results of pilot elution test 5, eluent samples at 5 time points from three allografts with interior gentamicin only coatings were tested

3.4 Discussion

Perhaps the most important factor determining the success of the agar diffusion method for antibiotic quantification of unknown solutions is obtaining an accurate calibration curve with a high coefficient of determination (R squared). R squared values above 0.99 are typical when everything is done right. The condition of the bacteria was a problem during the method development phase. While making bacterial broth directly from a TSA slant works well when the slant is fresh, the bacteria become less viable the longer they are stored resulting in poor quality data. The best way to overcome this issue is to transfer bacteria from the TSA slant to LB broth, incubate for a day, add several milliliters of the inoculated LB broth to sterile PBS solution and use this mixture to plate the bacteria. The ratio of LB broth to PBS used in the final elution experiments was approximately 1:5. Matching the turbidity of the broth by measuring

the absorbance at 600 nm with a plate reader enabled consistent results to be obtained in experiments performed on different days.

The method of spreading the bacteria was also found to be important. Nearly perfectly circular inhibition zones with clear boundaries can usually be obtained but in order to do so it is important to not disrupt the agar at all while streaking the plate as this can lead to asymmetries in the inhibition zones which makes later measurements more difficult. Disruption of the agar sometimes occurs when streaking a plate on which the bacterial inoculum has substantially evaporated. Using 750 microliters of bacterial broth and a wet swab was found to be ideal for covering the surface of the 150 millimeter diameter plates without the broth evaporating too quickly.

Due to the logarithmic equation used for the calibration curve the gentamicin quantity corresponding to measurements of the inhibition zones at high concentrations could vary considerably (5%-10%) with only a small increase in inhibition zone size. Therefore accurate measurement of the inhibition zones is crucial to obtaining good results, particularly at high gentamicin concentrations. Taking photographs (Figure 3.7) of the agar plates after incubation and analyzing the inhibition zones with ImageJ greatly improved the accuracy of the method. Averaging multiple measurements of the same eluent on different plates was also done in the final elution experiments (Chapter 4) to improve results for eluent samples containing high gentamicin concentration.

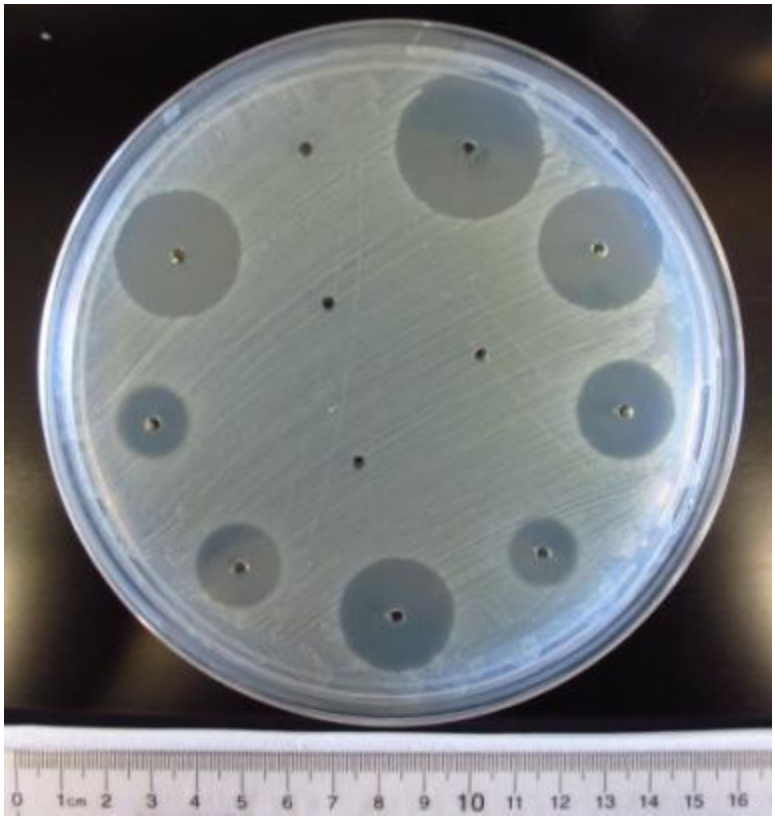


Figure 3.7 : Image of 150mm Mueller Hinton Agar plate with antibiotic ladder (Four inhibition zones on right side of image) and eluent after 24 hours incubation time. Scale is determined by photographing each plate next to a ruler. Images were analyzed using ImageJ.

If all these issues are taken into account and good lab technique is used throughout every step of the process, this method can be used to successfully measure unknown quantities of gentamicin. While the precision of the method is not as high as some other methods reported in literature such as GC/ECD, it was sufficient for the purposes of this experiment because extremely high precision was not essential to testing the hypotheses under investigation. The agar diffusion method is much more cost effective than other methods such as ELISA, radiometric assays, HPLC, etc. and can easily be scaled up to measure many samples at once. Additionally it does not require performing sensitive chemical reactions or involve toxic chemicals. For these reasons this method was ideally suited for the needs of this experiment.

3.5 Conclusions

Several methods of gentamicin quantification were considered and two were attempted. The GC/ECD method was problematic from the outset and never yielded usable results. Conversely the agar diffusion method showed great promise as a means of quantifying gentamicin from the first pilot test. Numerous issues required consideration in developing the method to be as accurate, precise and efficient as possible. The strain of bacteria, type of agar plate, well punching procedure, bacterial inoculum preparation, and data analysis methods were all selected based on running the experiment numerous times and identifying where flaws existed and methodological improvements could be made. Ultimately a method was developed that is capable of quantifying the amount of gentamicin in samples of unknown concentration. The agar diffusion method discussed in this chapter was then used to address the fourth and final specific aim of this project.

Chapter 4 – Measuring *In Vitro* Gentamicin Elution

4.1 Introduction

The elution study addresses the fourth and final specific aim of this project:

Specific Aim 4: Determine how quickly the Gentamicin Sulfate coating elutes from the allografts using an *in vitro* elution method. Investigate the elution profile of the exterior (periosteal) gentamicin coating in both the presence and absence of a DOPS coating as well as the elution profile of the interior (endosteal) coating.

Hypothesis 4: *In vivo* results from the IVIS data (Chapter 2) indicated significantly reduced infection and thus the presence of active gentamicin at one day post surgery. Subsequent increase in infection at three days post surgery seems to indicate that gentamicin elution was significantly decreased by this time point. It is therefore hypothesized that the majority of GS eluted from the allograft prior to day three. The presence of a DOPS coating over the GS coating is expected to reduce the elution rate of GS from the exterior of the allograft. Allografts are expected to elute all detectable GS more rapidly using an *in vitro* elution method than during the *in vivo* study however this will not be directly tested.

4.2 Methods

4.2.1 Variation in applied Gentamicin

Although allografts for the elution experiment were intended to be loaded with 500 micrograms gentamicin (either on the exterior or the interior) the actual quantity applied to each allograft

presumably varied over some range due to limitations of precision with the equipment used as well as human error. In order to quantify this variability in applied gentamicin an experiment was performed with the goal of obtaining "complete elution" from the allografts; meaning to elute all gentamicin that could possibly come off the allograft into a single aliquot of PBS. Additionally this experiment was intended to demonstrate whether a significant difference in total elution would be observed between DOPS coated and non DOPS coated samples as it was thought that DOPS could potentially bind some of the gentamicin in such a way that it would be unable to diffuse into the eluent, an idea supported by the data from pilot study 4.

Four samples were prepared with DOPS/GS and GS Only coatings and these were placed in 1 ml PBS and incubated at 37 degrees Celsius for 28 hours, the eluent was also agitated during the pipetting to ensure as much elution as possible. This eluent was tested to determine gentamicin concentration. In order to improve statistical analysis additional samples were later prepared and tested for "complete elution" as well for a total of 8 samples in each of the three treatment groups DOPS/GS, GS Only exterior and GS Only interior. After correcting for evaporative losses of PBS from the initial 1 milliliter (approximately 0.1 milliliter evaporated over the course of 28 hours incubation) the data in figure 4.1 were obtained.

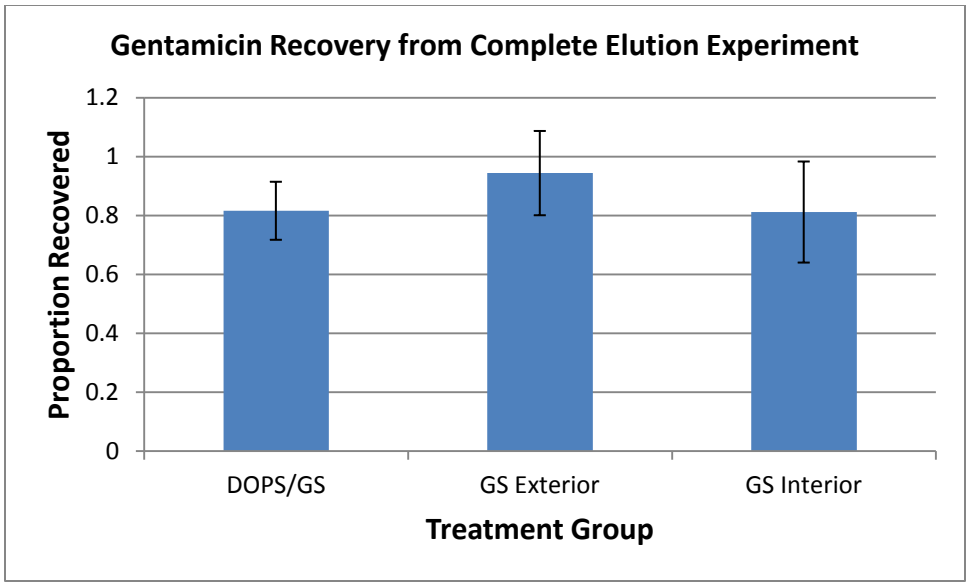


Figure 4.1 : Recovery of gentamicin from "full elution" test, 1 indicates 100% recovery, standard deviation error bars are included for each treatment group

While mean recovery varied somewhat between treatment groups statistically significant differences in recovery were not present at alpha=0.05. ANOVA was performed on this data and yielded a p-value of 0.097.

4.2.2 Full Scale Elution Experiment

The pilot data (Pilot 4, previous chapter) was statistically analyzed using a java applet for computing required sample size ([80] http://www.stat.uiowa.edu/~rlenth/Power). The data used from the pilot study to perform this calculation was sample size (n=2 per group), sample standard deviations ($\sigma 1 = 0.36$, $\sigma 2 = 0.15$), estimated difference between sample means (0.8), and two tailed alpha=0.05. Running a two-sample t-test on these numbers yielded a required sample size of 6 per treatment group to obtain statistical power > 0.95. Because this estimate was based on such a small pilot sample size (n=2 per group) it was decided to perform the full scale elution

experiment on 8 samples from each of the 3 treatment groups (DOPS/GS Exterior, GS Exterior, GS Interior) rather than the minimum required sample size of 6 per group.

Samples were prepared according to the methods described in Chapter 2 (though only outer or inner GS coatings were applied but not both). Each sample received 500 micrograms gentamicin as either an outer GS coating with DOPS, an outer GS coating without DOPS or an inner GS coating without DOPS ("GS Only treatment groups" henceforth refers to both the GS Exterior and GS Interior groups). Samples were placed in 1 mL PBS at 37 degrees Celsius and the eluent was saved and replaced with fresh PBS at time points of 20 minutes, 40 minutes, 1 hour, 4 hours, 24 hours and 48 hours.

Eluent was tested for gentamicin content using the agar diffusion method described in Chapter 3. *Staphylococcus epidermidis* was prepared in LB broth and incubated for 24 hours. This broth was diluted with PBS and absorbance at 600 nm of 300 microliters broth in a 96 well plate was measured to be 0.36. Agar plates were prepared by pipetting 750 microliters of this broth onto the agar and spreading the broth using a sterile cotton swab to cover the entire surface. Twelve wells were punched in each agar plate. The first 6 wells were filled with an antibiotic ladder of 10 microliters gentamicin solution at concentrations of 1, 0.5, 0.375, 0.25, 0.125, and 0.0625 milligrams per milliliter, the inhibition zones around these wells were later used to create the calibration curve. The rest of the wells were each filled with 10 microliters of eluent of unknown gentamicin concentration obtained from the 24 treatment samples (8 each of DOPS/GS, GS Exterior, GS Interior) at each time point. The plates were incubated for 24 hours and photographed. The images were analyzed to create a calibration curve for each plate and

determine the concentration of gentamicin in the wells containing eluent of unknown gentamicin concentration. This was done for each eluent sample. Statistical analysis was then performed on the data. Allografts were also analyzed with XPS (X-ray Photoelectron Spectroscopy) and SEM in order to confirm complete gentamicin elution.

4.3 Results

The quantity of gentamicin recovered at each time point was calculated and also summed for each treatment group to determine total recovery. The amount recovered for each treatment group at each time point is shown in figure 4.2, cumulative recovery for each treatment group is shown in figure 4.3.

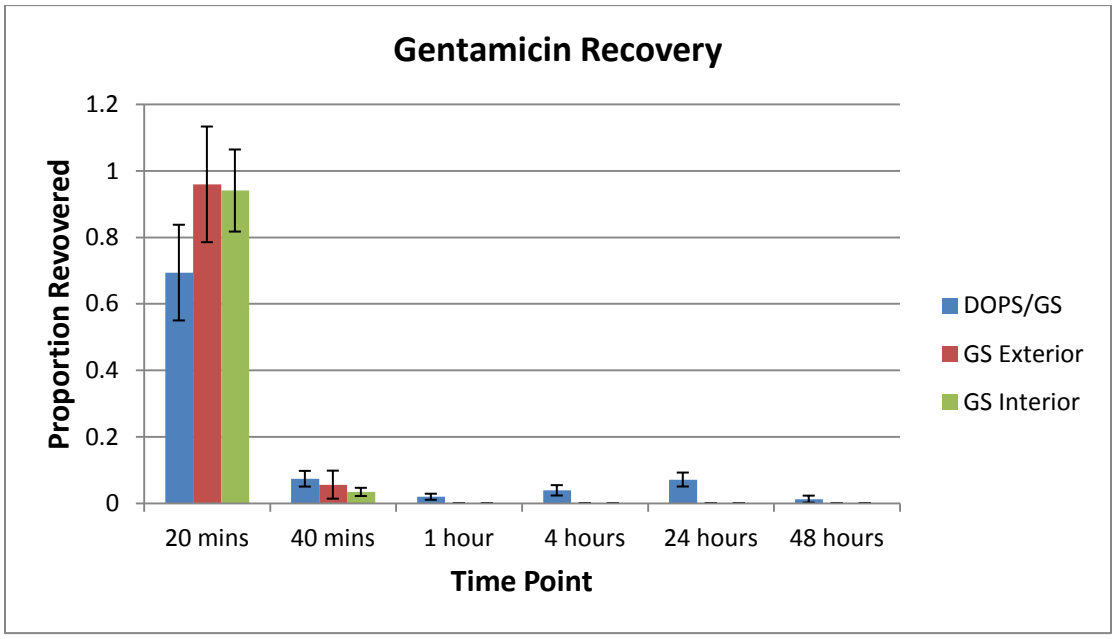


Figure 4.2 : Gentamicin recovery at each time point, standard deviation error bars are included

The data clearly show a difference between the DOPS/GS treatment group, which exhibited measurable elution at all time points, and the two GS Only treatment groups which eluted all measurable gentamicin in the first two time points (20 minutes and 40 minutes).

Performing ANOVA on this data yielded a p-value of 0.003 at the 20 minute time point and 0.04 at the 40 minute time point indicating a significant difference in gentamicin elution between the DOPS/GS group and the GS Only groups at these two time points (the only time points for which the GS Only samples eluted detectable gentamicin).

When total recovery was calculated no significant differences were observed between groups. This result was expected based on the results discussed in the "Variation in Applied Gentamicin" section. The mean total gentamicin recovery and standard deviation (σ) was calculated for the DOPS/GS, GS Exterior and GS Interior groups as 91% (σ = 16%), 102% (σ = 18%) and 97% (σ = 13%) respectively as illustrated figure 4.3.

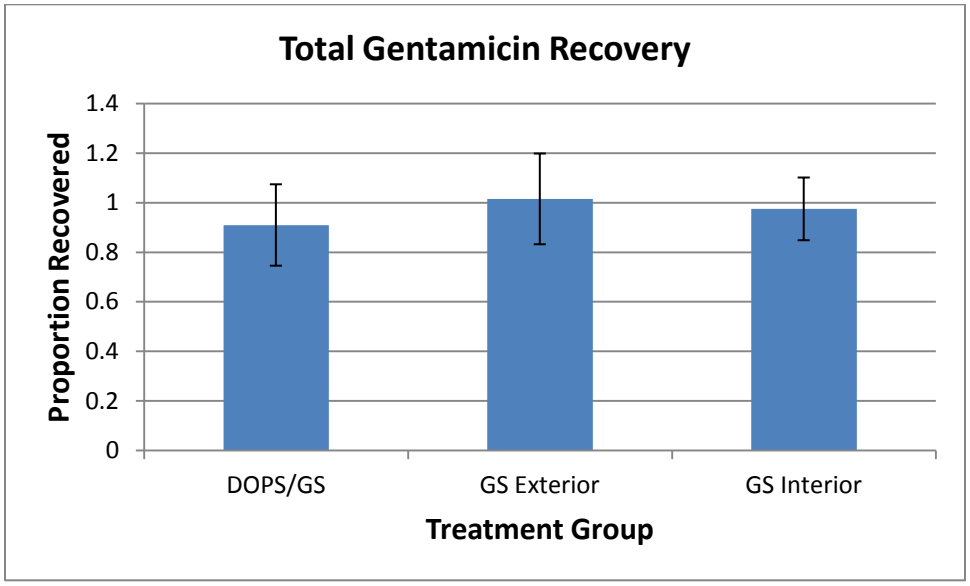


Figure 4.3 : Total recovery for each of the 3 treatment groups for the final elution experiment, standard deviation error bars are included

XPS was performed comparing three control samples (one uncoated allograft, one with a DOPS coating and one with a GS coating) to samples that had undergone the elution process (two DOPS/GS, two GS exterior and one GS interior). Sulfur was notably present on the newly prepared GS coated sample which had not undergone elution. This result was to be expected due to the sulfate ion present in the gentamicin sulfate salt. The sulfur peak was not present on any of the samples that had undergone the elution process however. The Nitrogen, Oxygen and Carbon peaks were observed at high resolution in order to determine whether any unique signals could be attributed to the gentamicin molecule (as some of these atoms are in unique electronic environments in the gentamicin molecule as compared to the DOPS molecule) however none were found. XPS data can be found in Appendix C. SEM was also performed on several samples post-elution. The post-elution GS Only (interior and exterior) samples exhibited no differences in appearance compared to uncoated bone. The DOPS/GS samples retained much of the DOPS coating although the coating morphology had been drastically altered by the elution process.

4.4 Discussion and Conclusions

The results of this experiment demonstrate that the DOPS coating does alter the elution characteristics of the gentamicin from the allografts. Gentamicin is highly soluble in water and the coating eluted quite rapidly from the allografts in both GS Only groups (exterior and interior coating). The DOPS coating did serve to reduce the elution rate of the gentamicin however the majority still came off the allograft by the 20 minute time point. The DOPS coated allografts continued to release measurable gentamicin at a slow but measurable rate for the 1 hour, 4 hour

and 24 hour time points. At the last time point gentamicin detection was zero for three of the samples and only about 0.01 mg/mL for the other five.

Six of the twelve wells on each plate were ultimately used to generate the calibration curve (concentrations of 1, 0.5, 0.375, 0.25, 0.125, and 0.0625 mg/mL gentamicin were used for the standards). While this curve worked well it may have been better to use 0.75 mg/mL rather than 1 mg/mL for the highest concentration standard. It was found that the 1 mg/mL concentration standard sometimes deviated from the calibration curve more than the other points and 0.75 mg/mL would have been closer to the detection range of interest in the experiment.

It was noted that the DOPS sometimes flakes off into the PBS during the elution experiment. Since the DOPS comes off so easily in PBS solution it could potentially do so *in vivo* as well which calls into question the viability of DOPS coatings and indicates that they may need to be mechanically strengthened in order to better withstand the environment encountered *in vivo* as well as during *in vitro* testing. This issue was minimized in the final elution experiment by gently removing the allografts from the wells prior to adding or removing the PBS solution, thus reducing the coatings exposure to turbulence during the eluent replacement process.

While the difference in elution rate between the DOPS/GS and GS Only treatments was statistically significant it seems unlikely that the presence of DOPS coatings like those used in this study would be capable of extending the release profile of the gentamicin enough to eliminate a well established osteomyelitis infection as this requires slow steady release over much longer timescales than those observed [59]. Overall the data from the elution experiment

supports the idea that antibiotic elution from the allografts *in vivo* was rapid for all gentamicin coated treatment groups. This rapid elution profile was one of the factors discussed at the end of Chapter 2 as a potential reason for why the induced osteomyelitis infection was not eliminated during the *in vivo* study and investigating this elution profile in greater depth was the reason for conducting the elution experiment.

Cumulative gentamicin recovery from the treatment groups varied slightly however the differences in total recovery were not statistically significant. In order to test whether any gentamicin remained on the post-elution samples XPS and SEM were performed however neither of these methods indicated gentamicin remaining on the allografts. This was of greatest interest in the DOPS/GS samples due to the fact that the DOPS coating remained on the post-elution allografts and could potentially have contained bound gentamicin. XPS was only able to detect the presence of sulfur (part of the sulfate ion of the gentamicin sulfate salt from which the coating solution was prepared) and not the actual gentamicin molecule so this method was unable to preclude the possibility of some gentamicin remaining in the DOPS coating. When the XPS and SEM evidence along with the cumulative recovery totals are taken together it seems unlikely that any clinically relevant quantity of gentamicin remained in the DOPS/GS samples or the GS Only samples post-elution.

The best explanation for the differences observed in mean recovery (Figure 4.3) between treatment groups is that two of the samples in the DOPS/GS group had received too little gentamicin during preparation and one of the GS Exterior samples had received too much. When these three samples are removed from the statistical analysis all three treatment groups

yield mean recoveries between 96% and 99% and standard deviations for DOPS/GS and GS Exterior are much smaller as shown in figure 4.4.

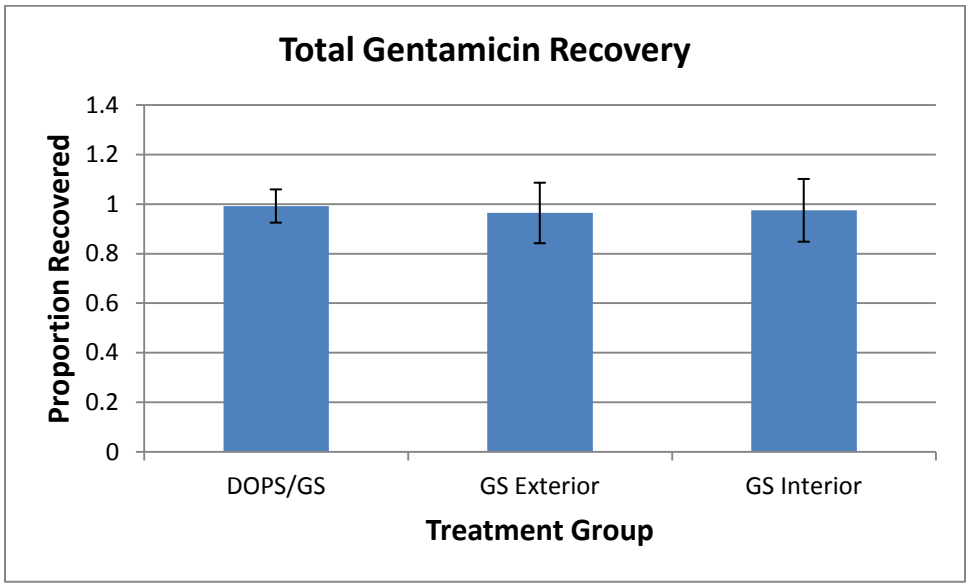


Figure 4.4 : Total recovery for each of the 3 treatment groups for the final elution experiment with standard deviation error bars and extreme values omitted. Excluding the two most extreme data points from the DOPS/GS group increases mean recovery from 91% to 99% and decreases standard deviation from 16% to 7%. Excluding the single most extreme data point from the GS Exterior group reduces mean recovery from 102% to 96% and decreases standard deviation from 18% to 12%. GS Interior had no extreme data points and is unchanged from Figure 4.3.

Chapter 5 – Conclusions and future work

5.1 Conclusions

This research was motivated by the clinical need to improve outcomes for patients receiving massive bone allografts. Complications such as osteomyelitis infection and non-union are common in osteosarcoma patients receiving such allografts. Patients receiving massive allografts who develop osteomyelitis may require additional surgery to remove and replace infected tissue with fresh allograft. It was hypothesized that loading coatings of the antibiotic gentamicin sulfate (GS) onto allografts would enable elimination of a well established osteomyelitis infection. It was also hypothesized, based on previous research in the James lab, that coating allografts with the phospholipid DOPS would improve allograft osseointegration with the host bone.

Ultimately the GS coatings did demonstrate efficacy in reducing detectable infection, however they were unable to fully eliminate the infection. In order to further investigate the release profile of gentamicin from the allografts an *in vitro* elution experiment was performed and the quantity of antibiotic in the eluent was measured using an agar diffusion method. The principles for quantifying antibiotics using agar diffusion were found in literature however significant methodological development was necessary in order to ensure that the method would perform adequately for the needs of this experiment.

The *in vitro* elution test demonstrated that gentamicin elution from the allograft was quite rapid. Experiments such as this have been used to examine antibiotic elution from many kinds of orthopedic materials and while the *in vitro* elution conditions are different from *in vivo*

conditions the results from this test serve as compelling evidence that gentamicin also eluted rapidly from the allografts *in vivo*. Since local sustained antibiotic release is most effective at remediating infection [60] and the serum half-life of gentamicin is on the order of hours [81] this means that a rapid elution profile would result in high local levels of antibiotic that would quickly decline. Such an elution profile would most likely reduce infection in a single burst but not be sufficient to fully eliminate infection. Because this is exactly what was seen with IVIS, rapid elution of the antibiotic from the allograft *in vivo* is presumably largely responsible for the inability of the antibiotic coating to successfully eliminate the severe infection. While this release profile may be inappropriate for eliminating established osteomyelitis it may be effective as a prophylactic coating to reduce the likelihood of developing infection in newly implanted allografts.

The intramedullary pin allograft fixation method may have contributed to the resilience of the infection by providing a surface conducive to bacterial attachment and biofilm formation. The presence of abscesses at the needle insertion point as well as the histological findings of bacteria located intraosseously and on the endosteal surface of the allograft but absent from the periosteal surface and surrounding muscle tissue all support this idea that the fixation method played a role in enabling the infection to persist.

The study was unable to demonstrate whether DOPS coatings are capable of enhancing allograft osseointegration. The infected mice exhibited severe bone deterioration and there was no osseointegration between host bone and allograft in any of the infected mice. Only the uninfected control mice showed osseointegration however statistically significant differences

between treatment groups were not obtained. This was probably partially due to the small sample size of 5 mice per uninfected treatment group, analyzing only one histological section (slice) per group, and the discrete 0-3 scoring system used to assess osseointegration, all of which decreased the likelihood of obtaining statistical significance. Inconsistent host-graft apposition was also a factor that may have contributed to the highly variable osseointegration scores within treatment groups and the lack of statistical significance. Because of these inconclusive results further research will be necessary to determine whether DOPS coatings are potentially useful for enhancing allograft osseointegration.

5.2 Future Work

The analysis of the results obtained throughout this project has identified where methodological improvements are possible and provided clarity on how future studies should be conducted.

While the current study attempted to test novel allograft coatings for both osseointegrative enhancement potential and osteomyelitis reduction capability, testing these variables individually in two separate studies may yield more conclusive results. The next study should forego inducing osteomyelitis in the test animals and examine only whether DOPS enhances allograft osseointegration *in vivo*, a second study could then look at eliminating osteomyelitis. There were two major factors that were identified as contributing to the inconclusive osseointegration results of the uninfected control groups in this study. These were poor host-graft apposition and small sample size. The four week experimental duration may have been responsible for the inconsistent host-graft apposition by not allowing sufficient time for osseointegration to take

place. The fixation method used may have contributed to this result as well. Modifying experimental duration, fixation method, or both should be considered in the design of future experiments. Additionally there should be more test subjects in each treatment group and multiple histology sections per subject so as to ensure high confidence in the statistical analysis. In determining the number of test subjects to use it will be necessary to consider the critical effect size as well as the limitations imposed by the discrete 0-3 osseointegration scoring system.

If DOPS does prove to have a significant effect on osseointegration then the incorporation of antibiotics into DOPS coatings should be examined. The agar diffusion gentamicin quantification method developed for this project should be used in conjunction with elution experiments to characterize the antibiotic release profiles of treated allografts prior to further *in vivo* experiments. Based on elution results from this study it is clear that gentamicin sandwiched between two DOPS layers is not an effective delivery system for eliminating established infection because elution is far too rapid. While such rapidly eluting coatings may be useful as prophylactic measures the elution profile will need to be altered to release antibiotic over longer timescales in order to be effective at eliminating established osteomyelitis infection. Perhaps this can be accomplished by incorporating antibiotic into a polymer layer that is applied to the allograft, by incorporating other materials with the DOPS to improve coating durability, or by some other means.

Only after both the osseointegrative ability of DOPS coatings has been established and the proper antibiotic elution profile has been obtained should the coatings be tested together *in vivo* in subjects with induced osteomyelitis. The duration of the experiment, test animal, method of

allograft fixation, method of osteomyelitis induction and desired severity of infection prior to allograft implantation should all be carefully considered to increase the conclusiveness of the experiment.

While this is a lot of preliminary work to undertake it is necessary to be incremental and rigorous in the development of these coatings if they are to ultimately be successful. Eliminating the potentially confounding factors identified throughout this research project, testing the coatings individually and optimizing the elution profile of gentamicin is the most promising path towards realizing the full potential of these coatings for improving allograft osseointegration.

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Appendix A: Standard Operating Procedures

Section Contents:

Note: Most of the procedures described are best performed in a biosafety cabinet to avoid contamination, UV or ethanol sanitize everything prior to using in biosafety cabinet, 70% ethanol, kimwipes and gloves are necessary for all of these protocols except A.6.

A.1: Mouse Femur Dissection and Preparation

Scope and Purpose

This SOP describes how to dissect, clean, decellularize and cut mouse femurs to prepare them for use in allograft experiments. Coatings of DOPS and gentamicin may later be applied to these prepared femurs.

Note: Femur cleaning is time consuming, it is most efficient to clean and prepare all experimental femurs at once to avoid having to repeat the process.

Materials

- Femur Donor Mice
- Kimwipes
- 70% Ethanol
- 3% Hydrogen Peroxide
- Several Plastic Petri Dishes
- Beakers
- Parafilm
- 23 Gauge Needles
- 22 Gauge Needles
- Plastic Syringe (5 or 10 mL)

Equipment

- Roboz RS-9290 Stainless Steel Forceps
- Surgical Scissors
- Dremel Rotary Tool
- 15/16 inch Dremel 545 Diamond Wheel
- -70 Degree Celsius Freezer
- Biosafety Cabinet
- Sonicator

Procedure

Mouse Dissection and Femur Cryostorage

1 - Obtain mice to use as femur donors.

2 - If mice are frozen allow them to thaw, place frozen mice in warm water to thaw faster.

3 - Remove femur, the fastest way to do this is by cutting tissue from around femur with surgical scissors and breaking the hip and knee joints by bending backwards (the opposite direction they usually bend).

4 - Remove muscle from femur by pinching gently with a Kimwipe and pulling femur through pinched fingers, it is only necessary to remove muscle from the diaphysis. The epiphyses will be cut off later.

5 - Repeat this process for all donor mice.

6 - Place femurs at -70 Celsius for 72 hours (this reduces immune response to allografts).

Cleaning, Decellularizing and Cutting Femurs

1 - Retrieve femurs from freezer.

2 - Perform subsequent work in a sanitized biosafety cabinet if allografts are to be used *in vivo* or are to remain pathogen free for other reasons.

3 - Place femurs in petri dish submerged in 70% ethanol for several minutes, this reduces likelihood of chipping (Figure A1- Left).

4 - Hold femur with forceps, using the diamond Dremel blade cut away femoral epiphyses leaving diaphysis approximately 1 cm in length.

5 - Place femur back in ethanol, repeat for all femurs.

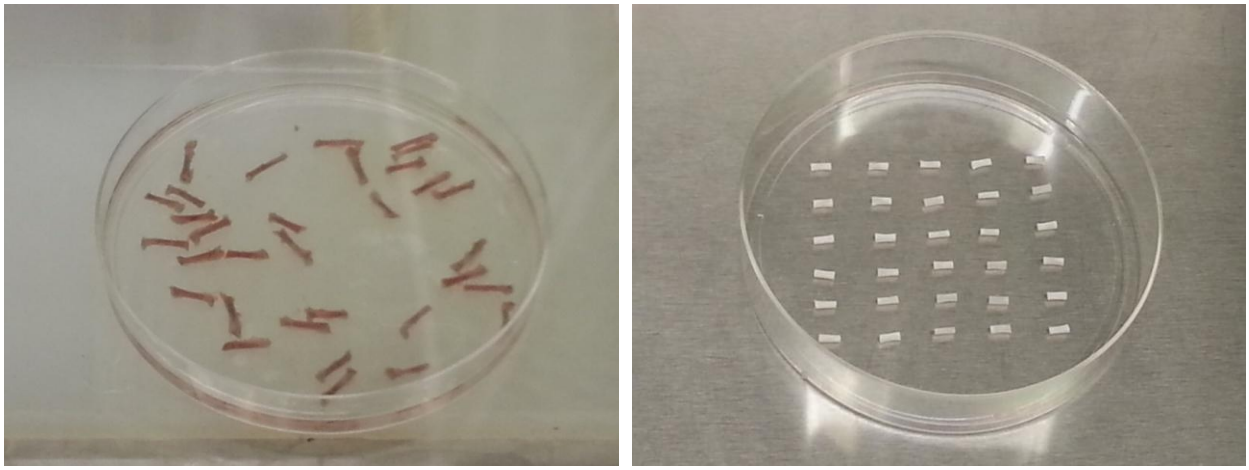


Figure A1: Left: Femurs after removal from donor mice Right: Femurs after cleaning procedure

6 - Use a syringe fitted with a 23 gauge needle to mechanically remove marrow from medulla, first hollow out a channel using a drilling motion with needle, remove needle from syringe, fill syringe with ethanol, reattach needle (removing the needle makes filling the syringe much faster) and flush medulla with ethanol from both ends until most of the debris has been removed.

7 - When all femurs have been cleaned with ethanol place them into 3% hydrogen peroxide for several minutes this will loosen the periosteum.

8 - Place femur on 22 gauge needle, pinch with Kimwipe and pull through to remove periosteum, repeat for all femurs.

9 - Flush medullae several times with 3% hydrogen peroxide.

10 - Place back into ethanol and flush several more times using a 22 gauge needle, by this point the femur should be entirely clean.

11 - Inspect femur and make sure there is no blood, marrow or other debris on interior or exterior, continue flushing with ethanol and hydrogen peroxide until femur is clean, sometimes it is necessary to again use the needle to mechanically dig away debris stuck in the medulla.

12 - When femur is clean hold with Roboz forceps (Figure A2) and use Dremel to cut away excess leaving femur approximately 6 mm long.

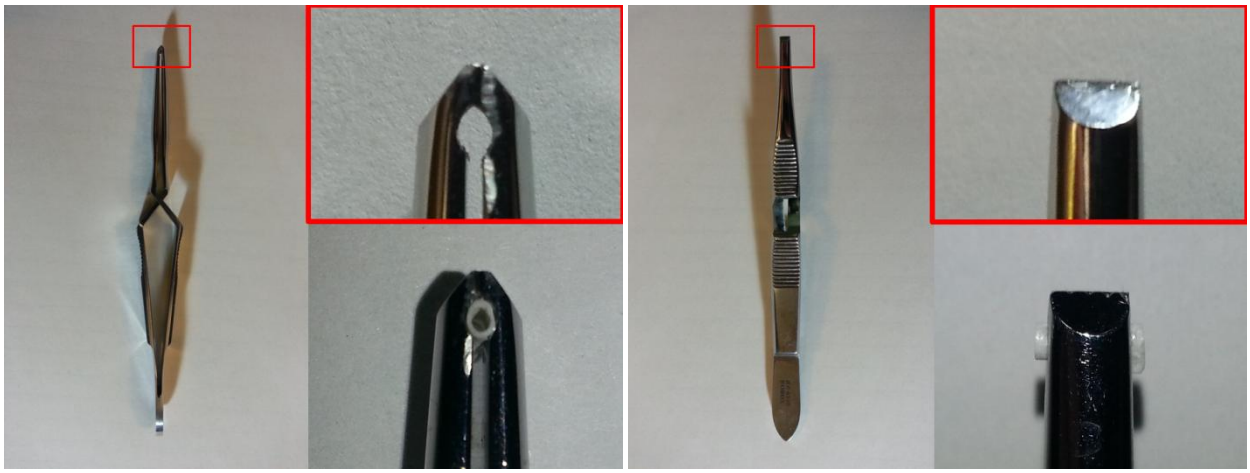


Figure A2: These forceps work better than any other tool to handle mouse femurs because they have a groove at the tip which is the perfect size to hold the femurs and because they grip in the absence of applied pressure

13 - Continuing to hold with Roboz forceps and stabilize using index finger so that the femur cannot slide through the forceps, use Dremel to sand down femur first from one end, then from the other until it is 4 mm in length.

14 - Flush once more with ethanol to remove any debris from the sanding process.

15 - Place femurs in ethanol and sonicate for 30 mins, rinse with sterile water.

16 - Place femurs in hydrogen peroxide and sonicate for 10 mins, rinse with sterile water.

17 - Place femurs in ethanol and sonicate for 60 mins.

18 - Femurs are now clean and decellularized.

19 - Allow to dry completely (Figure A1 - Right).

20 - Apply coatings or store for later use on capped 22 gauge needles to maintain sterility.

A.2: Applying DOPS Coating to Allografts

Scope and Purpose

This SOP describes how to electrospray DOPS coatings onto mouse femur segments which have been prepared as described in the SOP "Mouse Femur Dissection and Preparation"

Note about E-spray Chamber: Outer clear portion of spray chamber was made from Acrylic, interior was made from Polypropylene, black spray plate is Acrylonitrile Butadiene Styrene (ABS), these materials were purchased from Fort Collins Plastics Inc. Spray chamber was designed to be as modular as possible so it would be easy to modify if used for other electrospray projects.

Materials

- DOPS Solution in Chloroform (20 millimolar)
- Chloroform
- Cleaned femur segments

Equipment

- Roboz RS-9290 Stainless Steel Forceps
- E-spray Apparatus (Voltage Source, Spray Chamber, Ventilation System)
- Syringe Pump and Stand
- Glass Spray Syringe and Additional Syringe Components (plunger, needle, tubing)
- Biosafety Cabinet (optional)
- Fume Hood

Procedure

1 - Set up e-spray apparatus as shown in Figure A3 with spray chamber, syringe pump and voltage source (if sterility is a concern clean and sanitize everything and set up in biosafety cabinet to ensure that femurs do not become contaminated, UV biosafety cabinet).

2 - Attach ventilation apparatus, vent to fume hood.

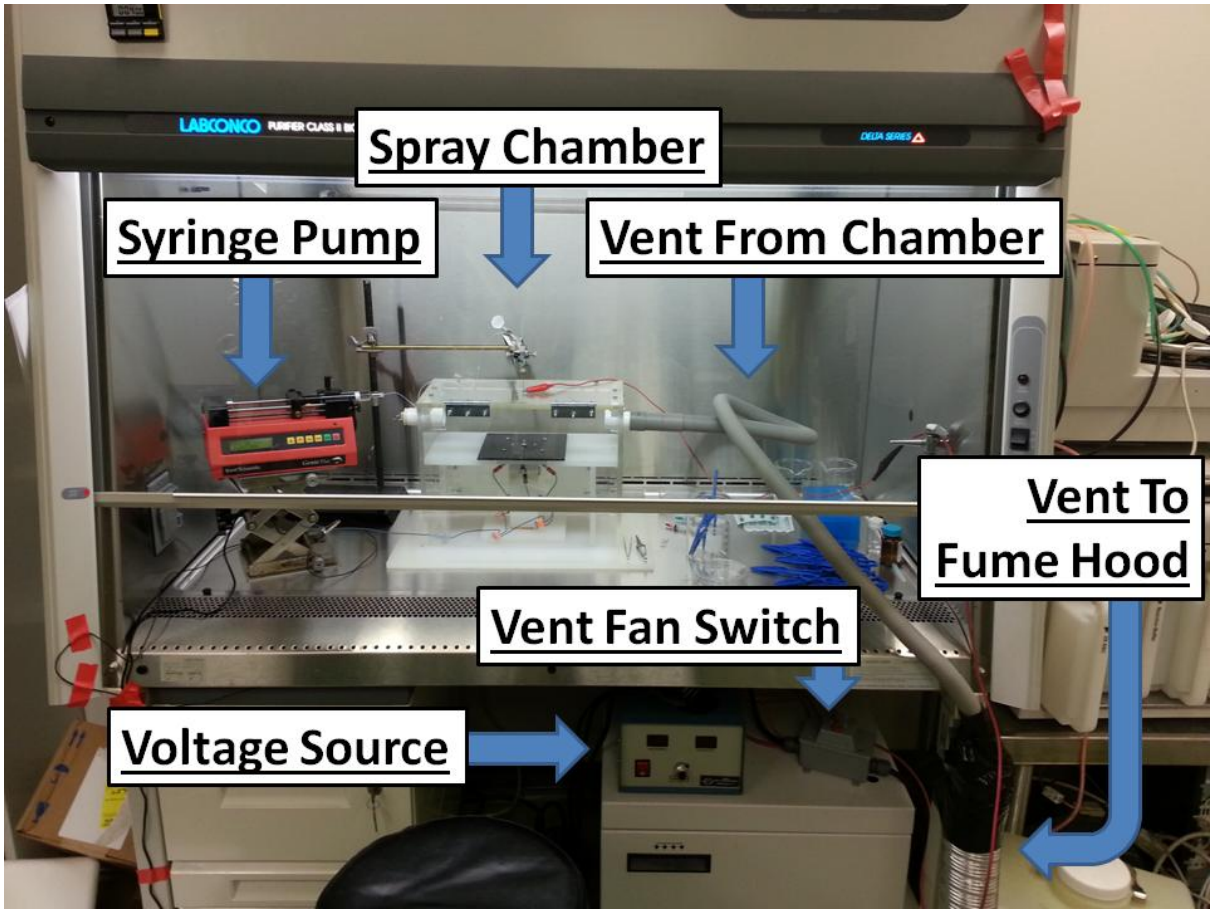


Figure A3: Electrospray apparatus and ventilation apparatus set up in biosafety cabinet

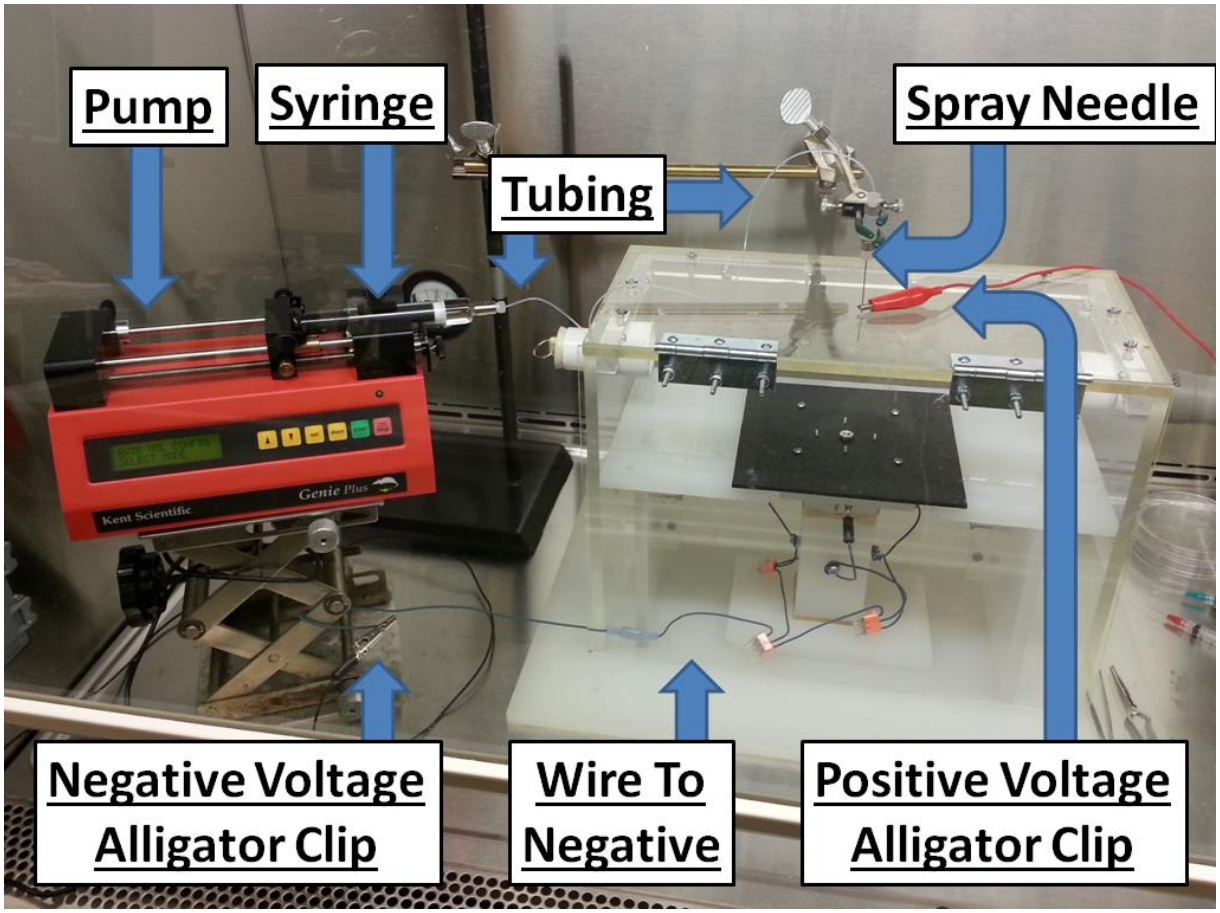
3 - Fill syringe with appropriate volume of 20mmol DOPS solution in chloroform (femurs are sprayed 4 at a time with the currently constructed apparatus, total spray time is 4 minutes at 7 ml/hr extrusion rate to coat 4 femurs with DOPS).

4 - Set syringe pump to 7 mL/hr extrusion rate if necessary.

5 - Attach tubing between syringe and spray needle, purge tubing of air bubbles, secure syringe to pump, insert spray needle into chamber, attach positive voltage to spray needle (Figure A4)

6 - Attach alligator clips to four 23 gauge needles sticking through Spray Plate.

7 - Place four cleaned 4 millimeter femur segments on 23 gauge needle tips on spray plate in espray chamber, adjust spray needle so vertical distance from femur segments is 8 centimeters.



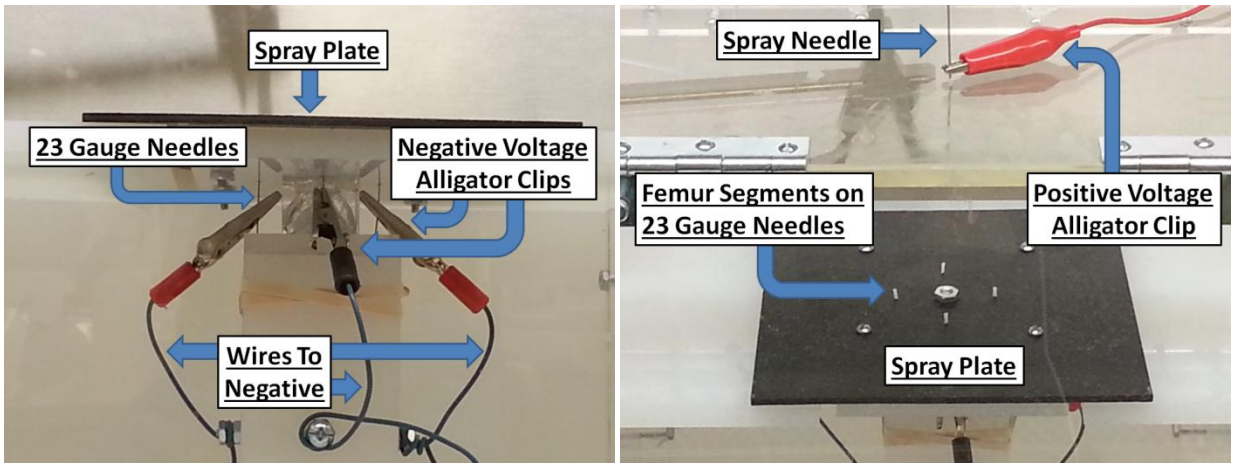


Figure A4: Top: Fully assembled spray chamber and syringe pump wired to voltage and ready to e-spray DOPS
Bottom Left: Close up of wiring below spray plate Bottom Right: Top of spray plate with allografts on needles

8 - Turn on voltage source, set voltage to 12 kilovolts, **BEWARE HIGH VOLTAGE.**

9 - Start syringe pump, spray femurs for 1 minute, turn off pump, turn off voltage (spray is difficult to see but can be visualized as a cone exiting the spray needle, spray can also be verified by looking for any small bubbles traveling through the syringe tubing or turning off the voltage and making sure that fluid drips from the spray needle).

10 - Open ventilation cap on e-spray chamber and turn on ventilation fan for 15 seconds to evacuate chloroform from chamber, turn off ventilation fan.

11 - Make sure voltage is turned **OFF**, open front of chamber invert femurs on needles with forceps so opposite end faces up.

12 - Repeat steps 8-10 again.

13 - **IF FEMURS ARE TO RECEIVE GENTAMICIN COATING** follow instructions for "Applying Gentamicin Coatings to Allografts" then place femurs back on spray needles

14 - Repeat steps 8-11 two more times.

15 - Allografts have now been DOPS sprayed for a total of 4 minutes, place onto surgical needles, UV sanitize in biosafety cabinet (Figure A6 - Right), finally cap needles.

A.3: Applying Gentamicin Coating to Allografts

Scope and Purpose

This SOP describes how to apply exterior (periosteal) and interior (endosteal) coatings of gentamicin to mouse femur allografts.

Note: The purpose of using the lathe for the exterior coating is to obtain even gentamicin distribution on the entire exterior of the allograft, in order to accomplish this it is necessary for the gentamicin droplet applied to each allograft to hang below the allograft as it rotates on the lathe thus evenly distributing the solution as it dries (see Figure A5).

The gentamicin solution droplets applied to the allograft may stick to and spin with the allograft if the droplet volume applied is too small or the bone is too dry. Therefore it is easiest to use a gentamicin solution droplet volume of about 10 microliters. Also it helps to wet (with sterile water) the bare allografts onto which the coating is to be applied so as to prevent the droplet sticking to the allograft (remove excess water after wetting, only do this with bare allografts, not DOPS coated ones, the DOPS coated allografts are not likely to exhibit the droplet sticking problem as water will not stick on the lipid layer as it does on dry bone).

Materials

- Cleaned femur segments (or 2 minutes electrosprayed DOPS coated allografts)
- Gentamicin Sulfate Powder
- Milli-Q or Distilled Water

- Several Small Vials
- Millipore 0.22 micrometer pore size sterile filter

Equipment

- Roboz RS-9290 Stainless Steel Forceps
- Polypropylene Forceps (one pair per allograft)
- Mini-Lathe
- Micropipettes (10 microliter, 100 microliter, 1 milliliter) and tips
- Biosafety Cabinet

Procedure

Gentamicin Solution

1 - Prepare gentamicin sulfate solution to desired concentration by mixing Gentamicin Sulfate Powder with Milli-Q Water in a vial (for the interior coating this solution will be micropipetted into the femoral medullae which can only hold about 4 microliters of fluid, the *in vivo* experiment described in Chapter 2 used a concentration of 1.25 mg/mL, therefore 4 microliters contained 500 micrograms gentamicin).

2 - Filter the solution if necessary through a Millipore 0.22 micrometer pore size sterile filter (if using a sterile filter prepare an additional 0.5 milliliters of gentamicin solution because approximately this volume will be lost in the filter).

3 - Dilute the portion of gentamicin solution that is to be used for the exterior coatings such that 8-10 microliters of solution contains the desired coating quantity of gentamicin.

4 - Store solutions in capped vials, use immediately or keep refrigerated.

Exterior Coating

1 - Set up mini lathe (if sterility is a concern clean and sanitize first and set up in biosafety cabinet, due to space limitations this will require a separate biosafety cabinet from the electrospray apparatus).

2 - Attach a 22 gauge needle to the lathe and adjust such that it remains parallel to ground and doesn't wobble when lathe rotates (to prepare more than 4 samples at once use a rubber flask stopper and stick several needles through it, attach this to the needle on the lathe).

3 - Place 4 femur segments (cleaned or with DOPS coating) on the needle on the lathe, separate femurs by about 1 mm, for allografts without a DOPS coating dip in sterile water to wet surface, then remove excess prior to placing on lathe (wetting the dry allografts makes coating application easier).

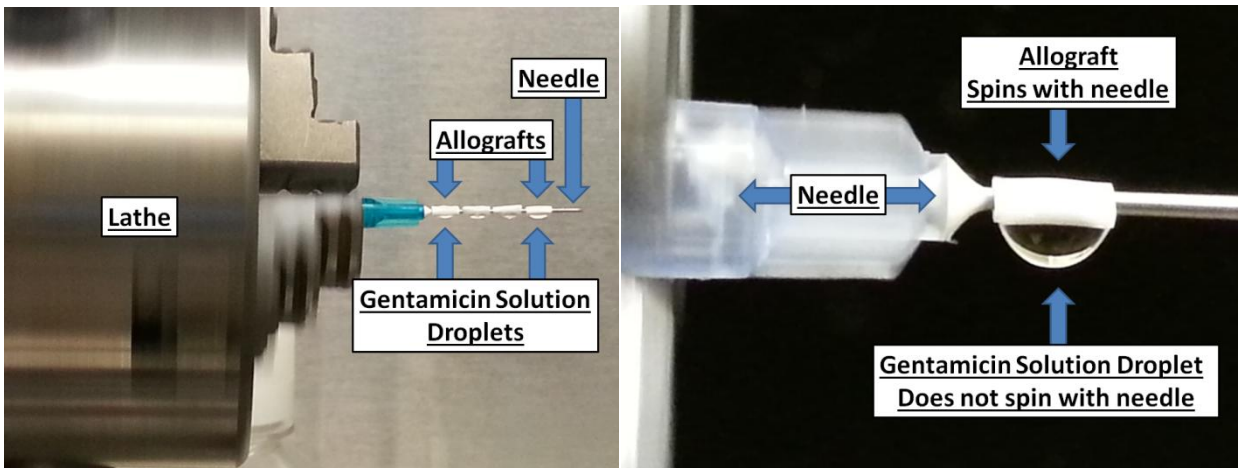


Figure A5: Left: Four allografts with applied gentamicin spinning on lathe Right: Zoomed in on allograft with large gentamicin solution droplet to illustrate how the droplet should hang below as the allograft spins

4 - Using a micropipette place a droplet of gentamicin solution with the desired amount of gentamicin on the exterior of each allograft, this is most easily accomplished using volumes of 8 microliters or more.

5 - When all allografts have received gentamicin solution spin lathe at approximately 150 RPM until the droplets dry (make sure that the femur rotates on the needle and that the droplet remains below the femur throughout rotation).

6 - Allow the droplet to completely dry.

Interior Coating

1 -Remove allografts from lathe, hold with polypropylene forceps using the tip of the forceps where the grips are the thinnest (lock forceps around something such as capped needles or a pencil for several minutes prior to using for this step to loosen grip and reduce incidence of breaking allografts).

2 - Micropipette 4 microliters of gentamicin solution into the medullae of the allografts (the surface tension of the solution allows formation of a small meniscus that may protrude from both ends of the allograft, if this meniscus touches the forceps it will break and the solution will bleed onto the forceps).

3 - Suspend forceps on rack for 2 hours until the gentamicin solution has evaporated leaving a coating on the inside of the femur (as in Figure A6 - Left).

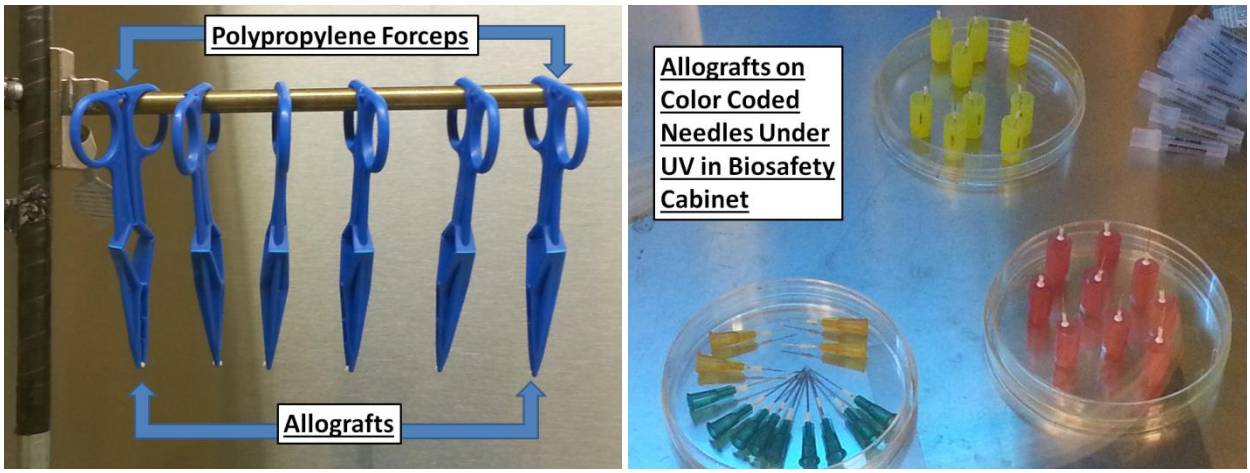


Figure A6: Left: Allografts hanging as interior gentamicin coating dries Right: UV Sanitizing coated allografts

4 - Be delicate with the allograft as both the exterior and interior gentamicin coatings are easily disrupted, place allografts onto sterile needles (23 gauge or smaller after application of interior coating).

5 - For DOPS coated allografts electrospray the second DOPS coating (step 14 from SOP "Applying DOPS Coating to Allografts").

6 - Allografts are now ready for *in vivo* implantation or *in vitro* experimentation, place on individual labeled needles (sanitize allografts with biosafety cabinet UV light if desired as in Figure A6 - Right), cap the needles.

A.4: Gentamicin Elution from Allografts

Scope and Purpose

This SOP describes how to perform *in vitro* elution of gentamicin from coated allografts.

Materials

- Allografts with gentamicin coating
- 22 or 23 Gauge needles
- Beaker for waste needles
- 48 well plate
- Sterile Phosphate Buffered Saline (PBS)
- 1.5 mL Eppi Tubes (autoclaved)
- Eppi Tube Rack

Equipment

- Biosafety Cabinet
- Roboz RS-9290 Stainless Steel Forceps
- Small needle nose pliers
- Diagonal cutters
- Micropipette (1 mL) and tips (sterile)
- Incubator

Procedure

1 - Select treatment groups for elution experiment (the treatment groups discussed in Chapter 4 were DOPS/GS Exterior Coating, GS Exterior Coating, and GS Interior Coating).

2 - Prepare test allografts as described in SOP's A.1, A.2 and A.3.

3 - Perform subsequent steps in biosafety cabinet.

4 - Clean tools with ethanol prior to use.

5 - Prepare holders for each allograft, start with 22 or 23 gauge needle, cut off sharp end and then cut to approximately 2 cm length with diagonal cutters, using needle nose pliers bend about 0.5 cm of needle to form "L" shape.

6 - Dispose of waste portions of needle in beaker (later dispose in sharps container).

7 - Once all bent needle allograft holders are prepared place them individually into wells of a 48 well plate.

8 - Place one test allograft on each needle holder.

9 - Add 1 mL PBS to each well with an allograft as shown in Figure A7 (in order to minimize the effect of turbulence it is best to remove the allograft/holder, fill the empty well with PBS and then gently place the allograft/holder back into the well).

10 - Make sure allograft is fully submerged in PBS.

11 - Add PBS in this manner to all wells with allografts.

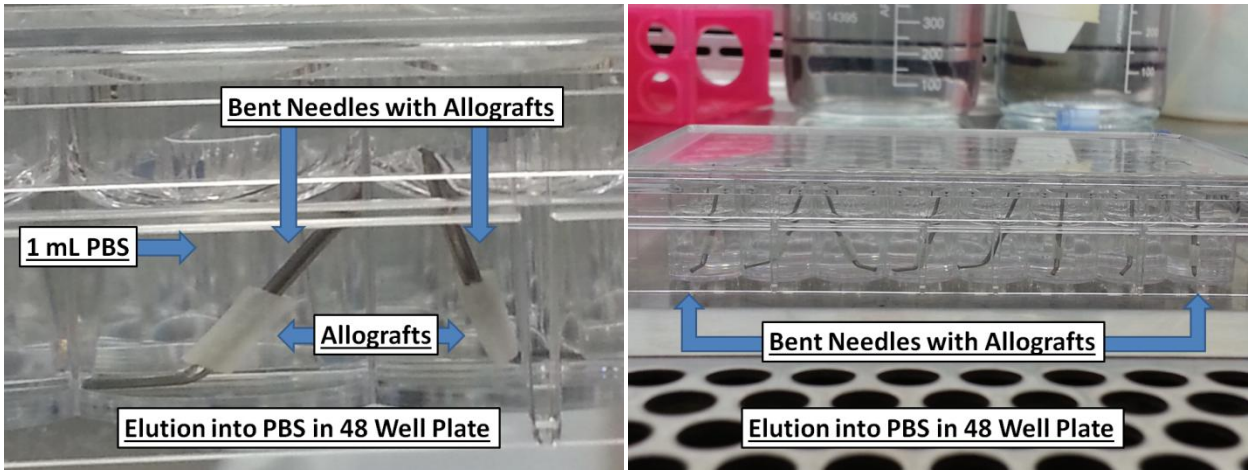


Figure A7: Left: Close up image of allografts on bent needle holders submerged in PBS
Right: Row of submerged allografts on holders in 48 well plate

12 - Cover 48 Well plate, incubate at 37 degrees Celsius.

13 - At selected time points (e.g. 20 minutes, 40 minutes, 1 hour, 4 hours, 24 hours and 48 hours) remove 48 well plate from incubator and place in biosafety cabinet, save PBS eluent from each well in labeled 1.5 mL Eppi tube and replace with fresh PBS according to the aforementioned technique.

14 - After eluent has been collected for all time points allografts may be stored for further analysis (SEM, EDS, XPS, etc.).

15 - Eluent samples should be stored refrigerated and analyzed for gentamicin content (via the agar diffusion method, SOP A.5) as soon as reasonably possible, long term storage may reduce potency of gentamicin.

A.5: Agar Diffusion Bacterial Inhibition Zone Method for Gentamicin Quantification

Scope and Purpose

This SOP describes how to quantify gentamicin content in solutions of unknown gentamicin concentration.

Note: This SOP is broken into three parts. It is necessary to begin Bacterial Preparation (part 1) one day prior to performing the Agar Plate (part 3) portion of the experiment. Gentamicin Standards Preparation (part 2) should also be completed prior to Agar Plate Preparation.

Note: The method of gentamicin quantification described in this SOP only works if the bacteria grow well, it is a good idea to make sure that the bacteria being used is viable by performing a test run prior to any large scale experiment (i.e. prepare LB bacterial broth, seed agar plates, incubate 24 hrs and observe good bacterial growth).

Part 1: Bacterial Preparation

Materials

- *Staphylococcus epidermidis*
- Lysogeny Broth (LB)

- Sterile Phosphate Buffered Saline (PBS)
- Sterile Cotton Swabs
- Bacterial Culture Tube
- Culture Tube Rack

Equipment

- Biosafety Cabinet
- Incubator with shaker

Procedure

1 - Obtain bacterial strain for study, *Staphylococcus epidermidis* may be obtained from the CSU Microbiology department or from ATCC (http://www.atcc.org/Products/All/12228.aspx).

2 - Prepare bacteria according to instructions in ATCC protocol, culture bacteria on Trypticase Soy Agar (TSA) slant, refrigerate when not in use.

3 - One day prior to performing inhibition zone experiment swab TSA slant and place swab in growth tube with several milliliters Lysogeny Broth (LB), agitate to disperse bacteria, remove swab, cap tube and place in 37 degree Celsius incubator/shaker for about 24 hours (prepare fresh bacterial broth [steps 3 and 4] immediately before each use and **DO NOT** reuse at a later date).

4 - The LB should now appear turbid, mix with sterile Phosphate Buffered Saline (PBS) at a ratio of about 5:1 PBS:LB, measure absorbance at 600nm with plate reader (an absorbance of 0.36 for 300 microliters of PBS/LB bacterial broth in a 96-well plate was used for the Chapter 4 experiment).

Part 2: Gentamicin Standards Preparation

Materials

- Gentamicin Sulfate Powder
- Sterile Phosphate Buffered Saline (PBS)
- 1.5 mL Eppi Tubes (autoclaved)
- Eppi Tube Rack

Equipment

- Biosafety Cabinet
- Micropipette (1 milliliter) and tips (sterile)
- Refrigerator

Procedure

Note: The gentamicin standards are used to create a calibration curve against which solutions of unknown gentamicin concentration are compared, six standards were used in the elution experiment (Chapter 4) with concentrations of 1.0, 0.5, 0.375, 0.25, 0.125 and 0.0625 mg/mL gentamicin, the antibiotic concentrations of the standards should be modified according to experimental needs, using a high concentration of 0.625 mg/mL or 0.75 mg/mL may yield a better calibration curve than using 1.0 mg/mL.

1 - Prepare 3 milliliters "stock" gentamicin solution in sterile PBS at a concentration of 1mg/mL.

2 - Label six sterile Eppi tubes 1.0, 0.5, 0.375, 0.25, 0.125 and 0.0625 mg/mL and place in rack.

3 - In the first tube add 1 mL from the stock solution.

4 - In the second tube add 500 microliters stock solution and 500 microliters PBS.

5 - In the third tube add 375 microliters stock solution and 625 microliters PBS.

6 - Continue in this fashion to make 0.25, 0.125 and 0.0625 mg/mL concentration solutions.

7 - Refrigerate the gentamicin standards when not in use.

Part 3: Agar Plate Preparation, Incubation and Analysis

Materials

- Bacterial broth in culture tube from "Bacterial Preparation" steps
- Culture Tube Rack
- 150 Millimeter Diameter Mueller Hinton Agar Plates
- Two Beakers for waste agar and used pipette tips
- Sterile Cotton Swabs
- Eppi Tube Rack
- Gentamicin Standards (in 1.5 mL Eppi Tubes)
- Gentamicin Unknowns (from elution experiment in 1.5 mL Eppi Tubes)
- Kimwipes
- Sharpie
- Parafilm

Equipment

- Micropipettes (10 microliter, 1 milliliter) and tips (sterile)
- BMG Labtech FLUOstar Omega Plate Reader and 96-well plate for use with plate reader
- Agar Plate Well Puncher

- Well Punch Pattern Guide
- Incubator
- Digital Camera

Procedure

1 - Wet a sterile cotton swab with PBS:LB Broth (from "Part 1: Bacterial Preparation"), pipette 750 microliters of this broth onto a 150 mm diameter Mueller Hinton agar plate and streak evenly across plate, first use the wet cotton swab to crudely spread the PBS:LB, then streak the entire plate using a raster pattern, rotate plate 45 degrees, streak again, rotate again, streak again.

2 - Lay agar plate on "Well Punch Pattern Guide" (Figure A8 - Bottom Right) and use "Agar Plate Well Puncher" (Figure A8 - Bottom Left) to punch 12 wells, to punch a well first cover the open hole on the 3-way connector with index finger, punch to bottom of agar with aluminum pipe, retract syringe plunger, remove aluminum pipe from agar to create well (expel agar into waste beaker when necessary, the purpose of the 3-way connector is to make purging the waste agar easier).

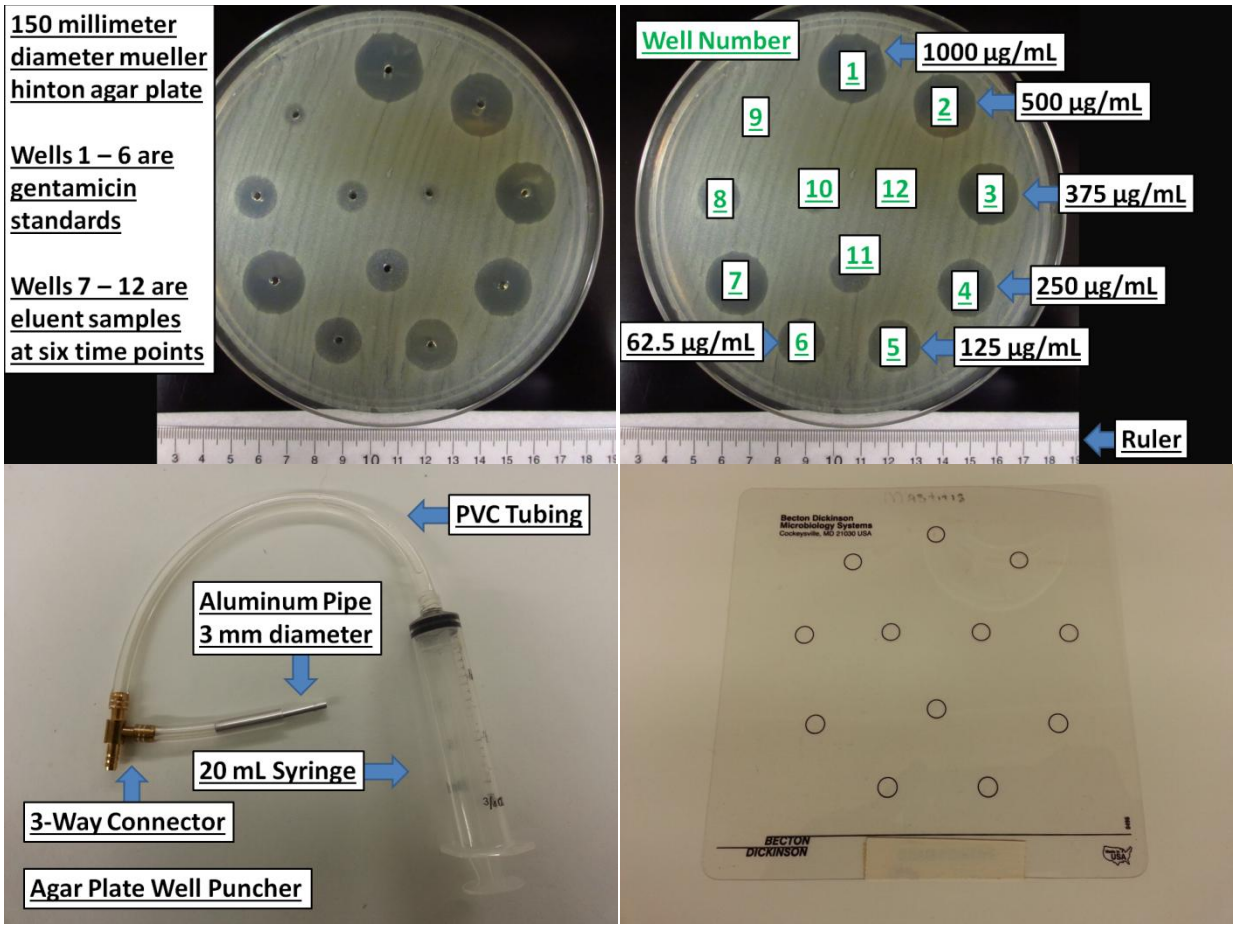


Figure A8: Top Left: Plate with inhibition zones after incubation for 24 hours, note the position of the wells
Top Right: Same plate with wells numbered and wells with gentamicin standards indicated
Bottom Left: Well Puncher used in agar diffusion experiment Bottom Right: Well Punch Pattern Guide

3 - Mark the first well with a sharpie on the bottom of the plate.

4 - Micropipette 10 microliters of the six gentamicin standards into the first six wells, into the next six wells micropipette 10 microliters from eluent samples of unknown gentamicin concentration, be sure to place the micropipette tip into the well when expelling gentamicin solution (otherwise the droplet may contact the top edge of the well and bleed onto the top of the agar), **USE PIPETTE TIPS ONLY ONCE** (to prevent any cross contamination).

5 - Label plate with relevant treatment information and bacterial strain used.

6 - Repeat this process of seeding bacteria, spreading bacteria, punching wells, and micropipetting gentamicin standards into the first 6 wells and unknowns into the next six wells

for all unknown samples (six unknowns fit on each plate, it is advisable to test suspected high concentration unknowns in duplicate or triplicate and average the results to improve precision).

7 - When finished preparing agar plates, place all plates into incubator at 37 degrees Celsius for 24 hours.

8 - Dispose of waste agar and pipette tips, clean beakers, well puncher, pattern guide, biosafety cabinet etc. with ethanol, UV biosafety cabinet.

9 - After 24 hours remove plates from incubator, take digital photographs of each plate.

10 - First photograph plate with cover (cover should be labeled with relevant info about plate).

11 - Next photograph plate without cover, when photographing plate make sure image is not blurry, take several images if necessary, include a ruler in the photo for setting scale when analyzing images, take images from directly above plate.

12 – Cover plates, wrap shut with Parafilm and save in refrigerator or dispose of plates in biohazard waste (red waste bags which are autoclaved).

13 - Analyze digital images with ImageJ according to SOP A.6 (ImageJ and Inhibition Zone Data Analysis).

A.6: ImageJ and Inhibition Zone Data Analysis

Scope and Purpose

This SOP describes how to analyze images obtained according to protocol A.5 using ImageJ and then use this data to create a calibration curve and quantify gentamicin content in solutions of unknown gentamicin concentration.

Materials/Equipment

- Digital images of agar plates with inhibition zones
- Computer with ImageJ program and
- Microsoft Excel or other spreadsheet application

Procedure

1 - Open ImageJ, load an image of a plate, use the straight line tool, zoom in on the ruler in the image and draw a line 5 centimeters in length, select analyze from the menu, then select set scale. The number of pixels of the line that was just selected should be entered in the "distance in pixels" box, set known distance to 5.

2 - Select the oval tool and draw an oval over the first zone of inhibition, select analyze from the menu and then measure, this will pull up a new window that will show the area of the selected inhibition zone.

3 - Continue to do this for all zones on the plate, keep track of which measurement corresponds to which zone, save these results and input into an excel worksheet.

4 - Using the data obtained from the inhibition zones around the gentamicin standard wells construct a calibration curve, plot the concentration of antibiotic versus the inhibition zone area

5 - Obtain the equation for the logarithmic trendline (in the form $y=a*\ln(x)+b$ where y = inhibition zone area and x = gentamicin concentration).

6 - Fit the inhibition zone areas of the unknowns to this trendline to determine antibiotic concentration of the unknowns.

7 - Repeat this process for all images, generating a new calibration curve for each plate to solve for the unknowns on that plate.

8 - Gentamicin has now been quantified in the unknown solutions, further analysis may be performed with this data (such as statistical analysis of elution treatment groups, determining cumulative elution for samples, etc.).

APPENDIX B: SAS Code and Additional Statistics Information

Statistical Analysis Software (SAS) programs were used twice over the course of this project. IVIS Data was analyzed using the GLM Procedure to perform a multivariate analysis of variance on the data, more information about the GLM procedure may be found in the User's Guide on the SAS website (http://support.sas.com/index.html). The program code used is shown below:

```
data mouse;
 input day phos $ anti $ lnlum; /* variables: day of study, phospholipid (yes or no), antibiotic (yes or no),
natural log of luminescence */

datalines; /* sample data not included */
-1 yes yes 12.1216
/* Data input, 396 lines included luminescence data from all 36 infected mice at 11 time points */
27 no yes 10.8893
;
proc print data=mouse; /* print data to screen */
run;
proc sort data=mouse; by day; /* sort data by day */
run;
ods graphics on; /* enable graphics output */
proc glm data=mouse plots=diagnostics;
by day;
class phos anti; /* define classes for model statement */
model lnlum = phos anti phos*anti; /* output diagnostic plots for each day */
lsmeans anti/ pdiff cl; /* output least square means, p-values for differences between least square means
and confidence limits */
output out=data1 student=stu; /* create new data set (data1) and saves diagnostic measures calculated
after fitting model, defines studentized residuals */
run;
proc univariate data=data1 normal; /* output descriptive statistics for specified dataset (data1) */
by day;
var stu;
run;
ods graphics off; /* disable graphics output */
```

Below is a sample of the program output from the above code, some parts have been omitted for brevity:

The GLM Procedure

day=1

Class Level Information		
Class	**Levels**	**Values**
phos	2	no yes
anti	2	no yes

Number of Observations Read	36
Number of Observations Used	36

The GLM Procedure

Dependent Variable: lnlum

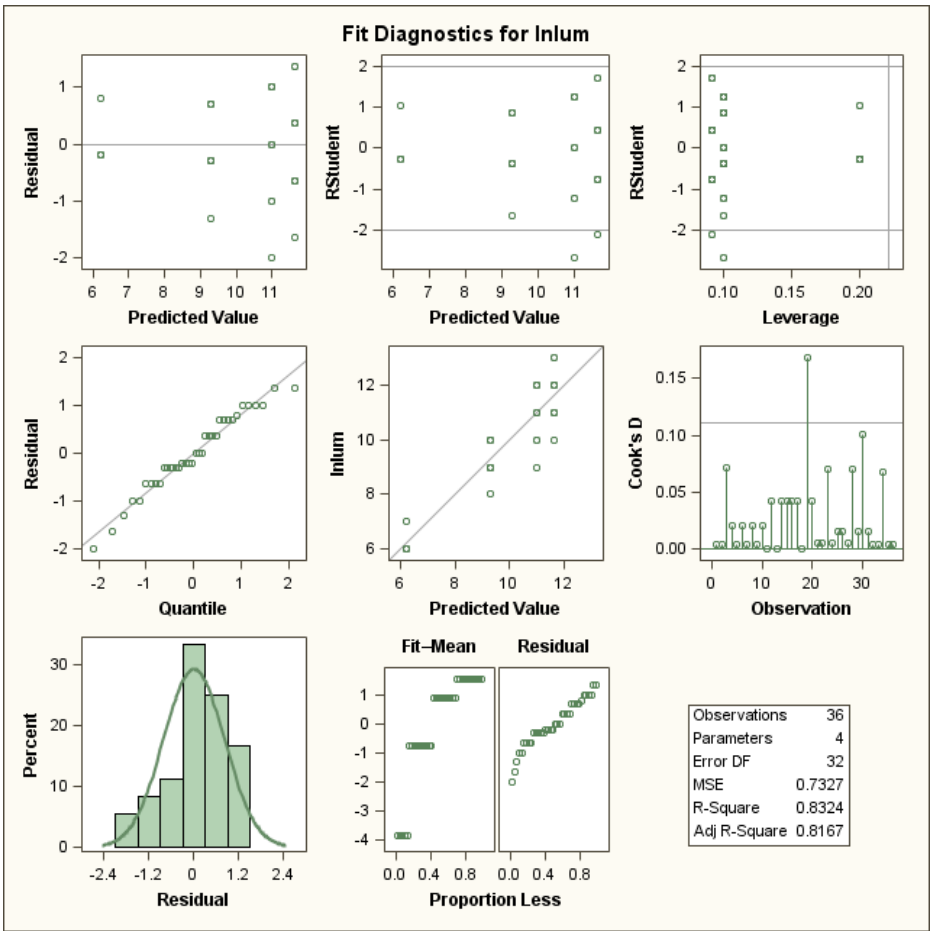
day=1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	116.4434343	38.8144781	52.98	<.0001
Error	32	23.4454545	0.7326705		
Corrected Total	35	139.8888889			

R-Square	Coeff Var	Root MSE	lnlum Mean
0.832399	8.512326	0.855962	10.05556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
phos	1	0.40138889	0.40138889	0.55	0.4646
anti		187.60416667	87.60416667	119.57	<.0001
phos*anti		128.43787879	28.43787879	38.81	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
phos	1	12.3638047	12.3638047	16.87	0.0003
anti		1103.7415825	103.7415825	141.59	<.0001
phos*anti	1	28.4378788	28.4378788	38.81	<.0001



The GLM Procedure

Least Squares Means

day=1

anti	lnlum LSMEAN	H0:LSMean1=LSMean2 Pr > |t|
no	11.3181818	<.0001
yes	7.7500000	

anti	lnlum LSMEAN	95% Confidence Limits	
no	11.318182	10.937279	11.699085
yes	7.750000	7.272513	8.227487

Least Squares Means for Effect anti				
i	j	Difference Between Means	95% Confidence Limits for LSMean(i)-LSMean(j)	
12		3.568182	2.957378	4.178986

SAS was also used to perform a Wilcoxon Rank Sum Test on the osseointegration data from the histological analysis of uninfected mice. The program code used is shown below:

```
data OIscore;
input group $ score; /* treatment group name and score */
datalines;
nocoat 3 nocoat 2 nocoat 2 nocoat 0 nocoat 1
DOPS 2 DOPS 1 DOPS 3 DOPS 2 DGS 4
DGS 1 DGS 0 DGS 1
;
/* input data for each group, each input (e.g. nocoat 3) should be on its own line for the code to run
properly */

proc print data = OIscore; /* output data */
run;
proc npar1way wilcoxon data=OIscore;
class group;
var score;
exact wilcoxon;
run; /* run wilcoxon rank sum test for all three groups */

proc npar1way wilcoxon data=OIscore;
where group ne 'DOPS'; /* exclude DOPS group */
class group;
var score;
exact wilcoxon;
run; /* run wilcoxon rank sum test for other two groups */
proc npar1way wilcoxon data=OIscore;
where group ne 'DGS'; /* exclude DGS group */
class group;
var score;
exact wilcoxon;
run; /* run wilcoxon rank sum test for other two groups */
proc npar1way wilcoxon data=OIscore;
where group ne 'nocoat'; /* exclude nocoat group */
class group;
var score;
exact wilcoxon;
run; /* run wilcoxon rank sum test for other two groups */
```

This code performed the Wilcoxon Rank Sum Test on all three groups as well as each two group combination. Below is a sample of the program output from the above code, some parts have been omitted for brevity:

Obs	group	score
1	nocoat	3
2	nocoat	2
3	nocoat	2
4	nocoat	0
5	nocoat	1
6	DOPS	2
7	DOPS	1
8	DOPS	3
9	DOPS	2
10	DGS	4
11	DGS	1
12	DGS	0
13	DGS	1

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable score Classified by Variable group					
group	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
nocoat	5	34.50	35.0	6.621643	6.9000
DOPS	4	33.00	28.0	6.281842	8.2500
DGS	4	23.50	28.0	6.281842	5.8750
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	0.7974
DF	2
Asymptotic Pr > Chi-Square	0.6712
Exact Pr >= Chi-Square	0.7006

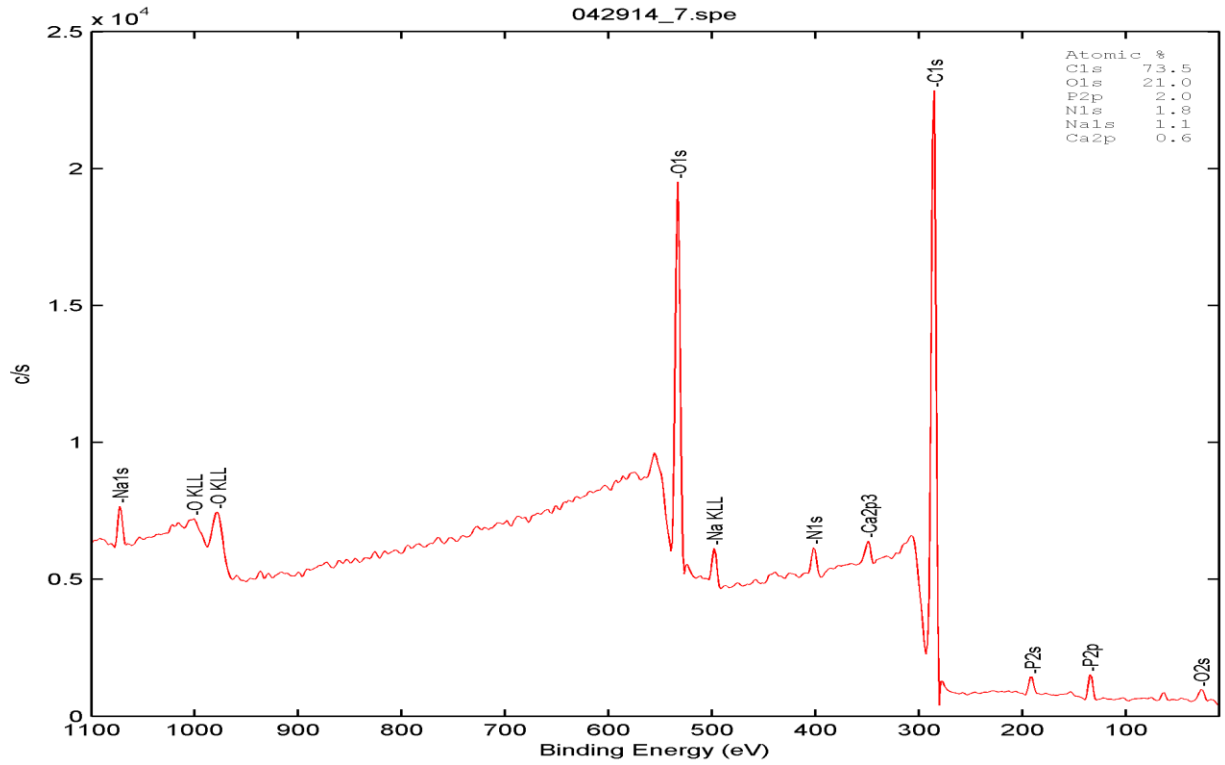
APPENDIX C: XPS Data

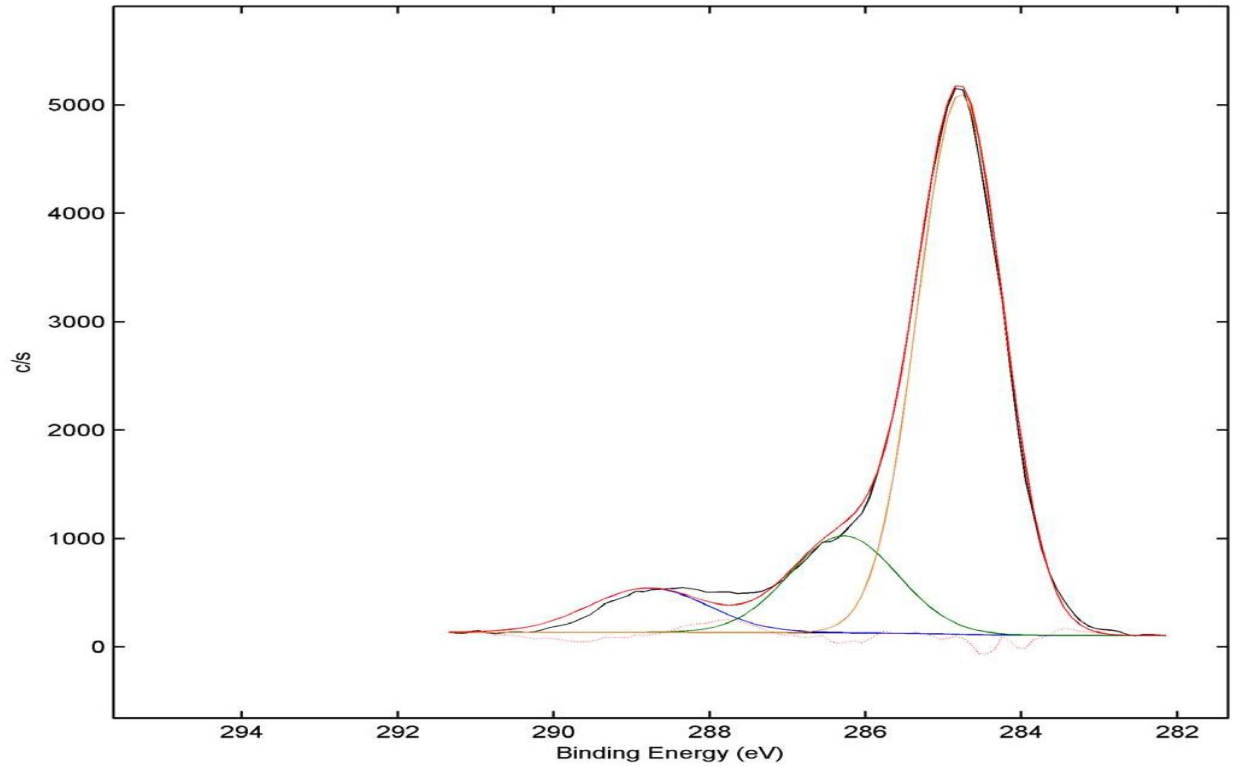
X-ray Photoelectron Spectroscopy (XPS) was performed on several allografts that had undergone the *in vitro* elution process (DOPS/GS and GS Exterior) as well as three controls (DOPS, GS, and uncoated bone). Both survey spectra and Carbon 1S high resolution spectra were obtained for each sample, these are presented on the following pages. The top spectrum on each page is the survey, the bottom is the high resolution C1S.

The important features to note in the survey spectra are the presence of a calcium peak on all samples except the one with a gentamicin coating. The gentamicin coated sample is the only one for which a sulfur peak is present as would be expected because the sulfate salt of gentamicin was used to prepare the coating.

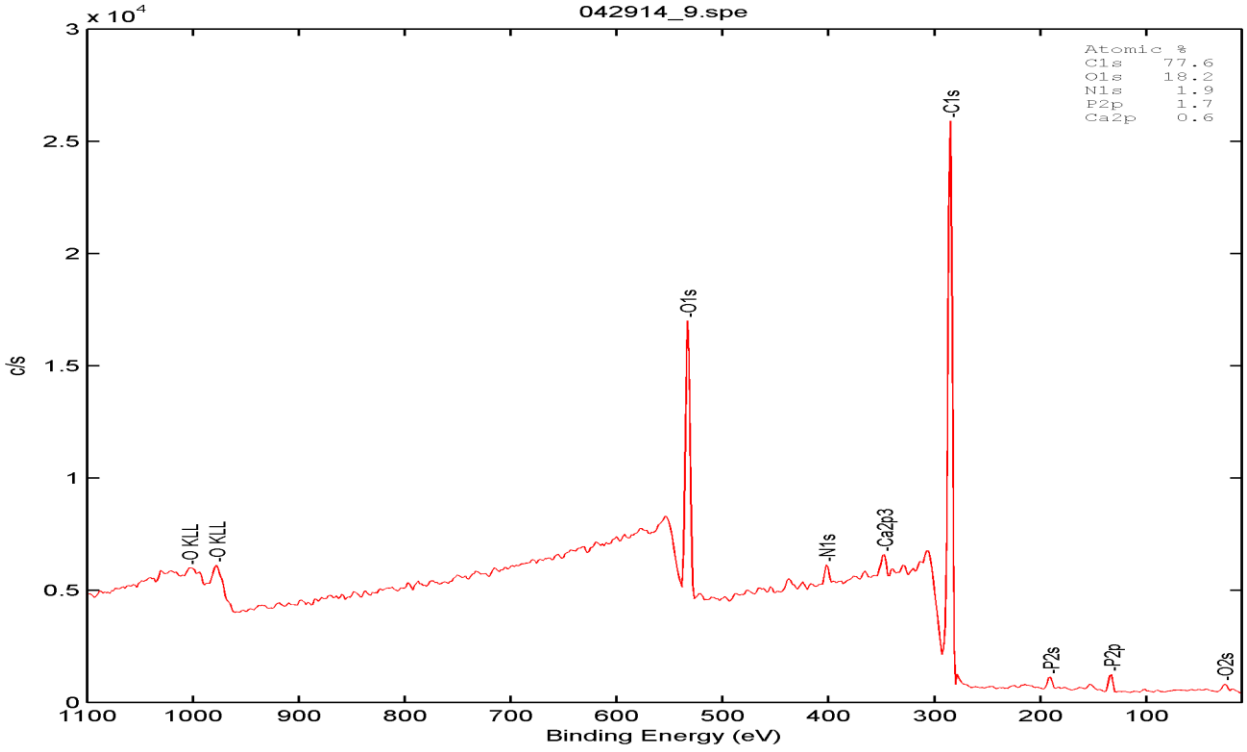
On the C1S spectra the important features are that the DOPS coated sample (1) and the DOPS after elution sample (2) are practically identical, indicating that the DOPS remained on the sample after the elution process, an observation confirmed with SEM imaging. Also the uncoated bone (3) and gentamicin after elution (4) samples look very similar indicating that after elution the surface of the gentamicin coated sample was very similar to that of uncoated bone. The gentamicin coated sample (5) that had not undergone elution exhibits a much larger peak at 286 eV than any of the other samples as would be expected due to the numerous amine groups present on the gentamicin molecule.

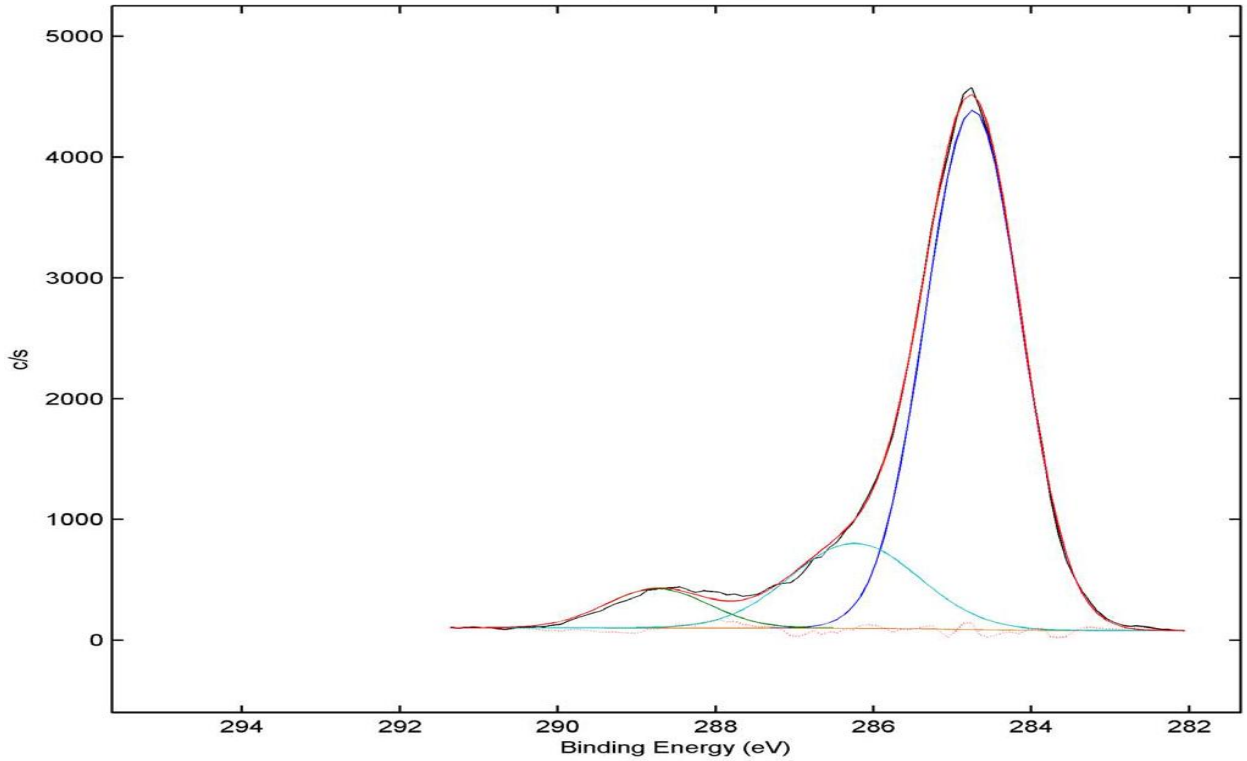
Sample 1 – DOPS Coated



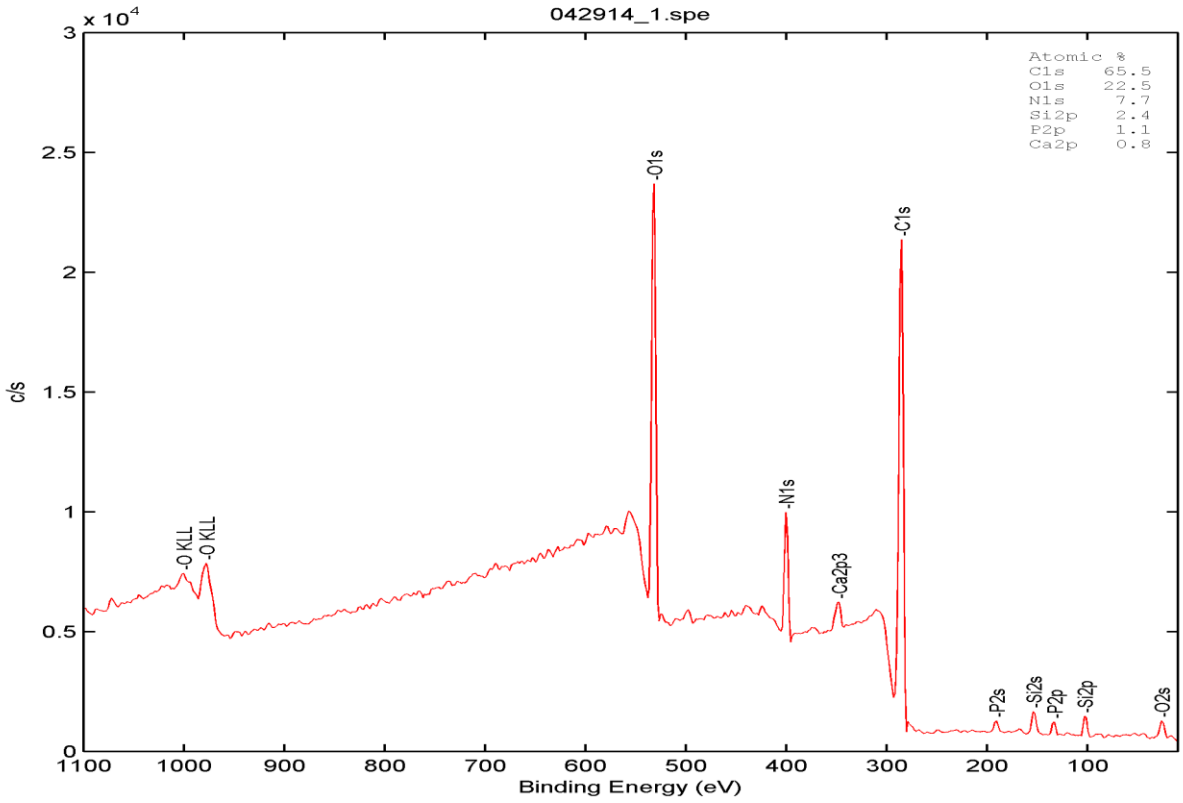


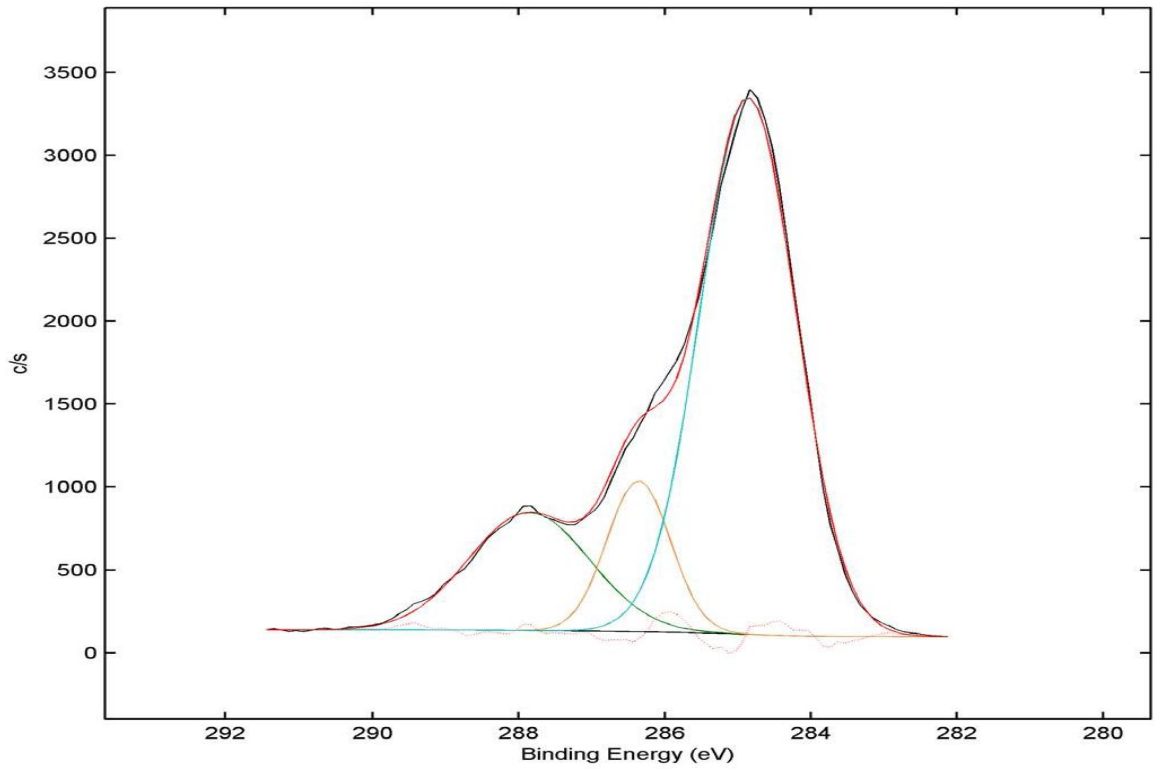
Sample 2 – DOPS Coated After Elution



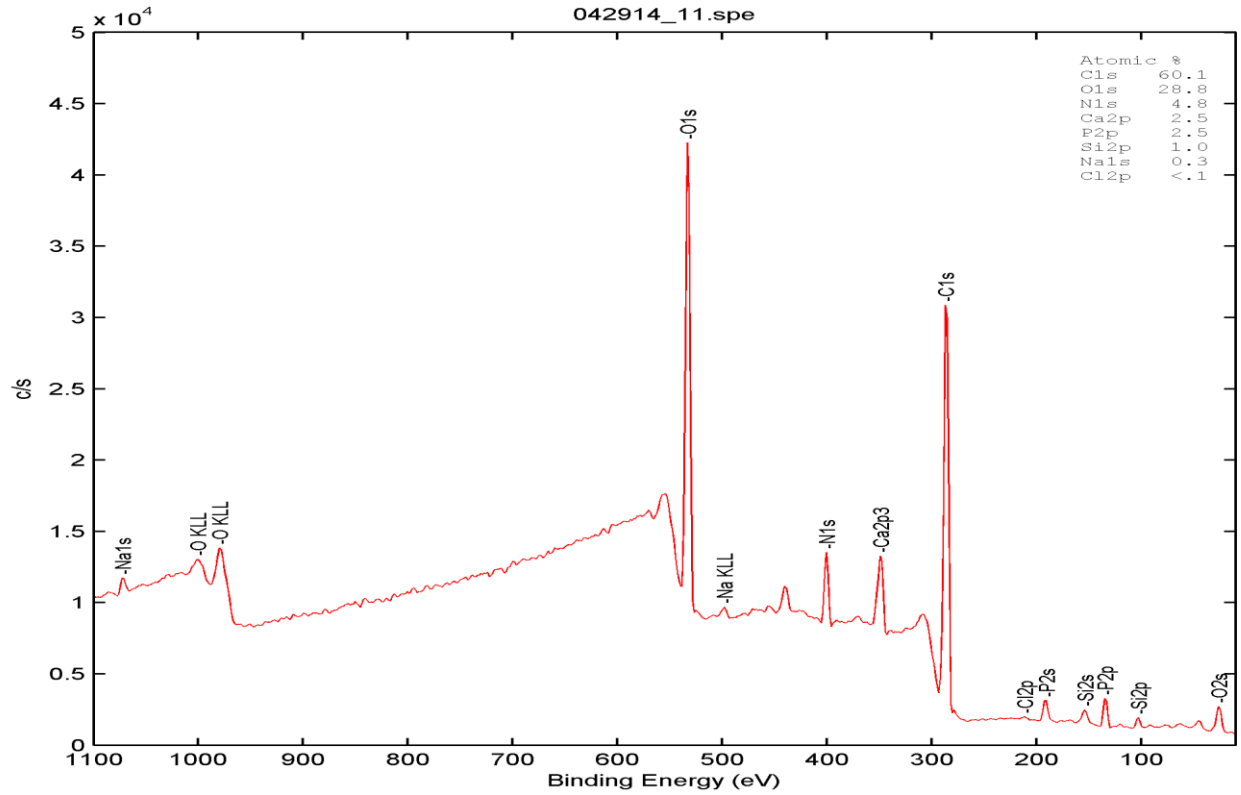


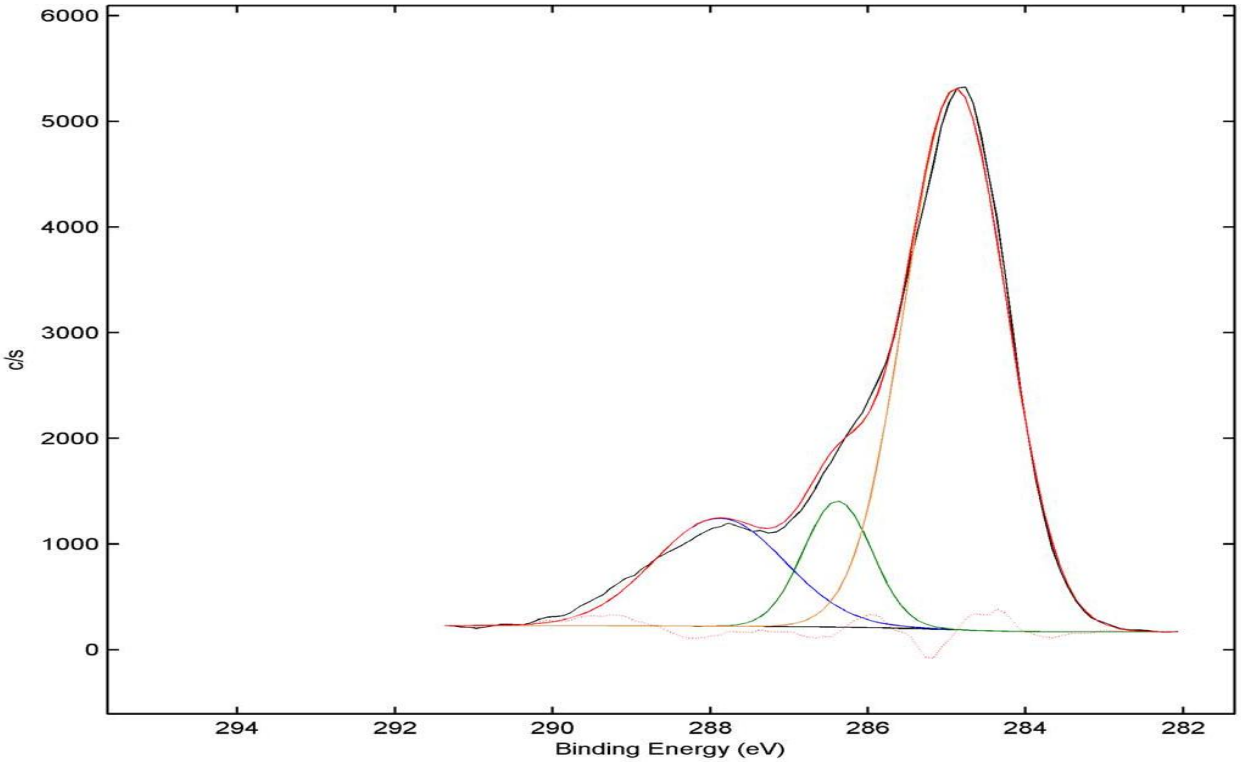
Sample 3 – Uncoated Bone





Sample 4 – Gentamicin Coated After Elution





Sample 5 – Gentamicin Coated