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Design and Preliminary Investigation of Crosslinked Chitosan Sponges for Tailorable Drug Delivery and Infection Control

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DESIGN AND PRELIMINARY INVESTIGATION OF CROSSLINKED CHITOSAN SPONGES FOR TAILORABLE DRUG DELIVERY AND INFECTION CONTROL

by

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DEDICATION

I would like to dedicate this thesis to my loving husband, David Parker, and my parents, Tommy and Michelle Cox. Without their love and support throughout my educational pursuits and career, this and many other undertakings would not have been possible.

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ABSTRACT

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Musculoskeletal wound infections can be difficult to treat, often resulting in multiple surgeries and increased costs, and can be complicated by antibiotic resistant bacteria. The aim of this study was to use genipin, alone or with poly(n-isopropylacrylamide) (PNIPAM), to crosslink chitosan sponges for a tailorable, degradable local drug delivery system to treat known musculoskeletal pathogens. Lyophilized uncrosslinked, genipin crosslinked, and PNIPAM/ genipin crosslinked chitosan sponges were evaluated *in vitro* for degradation, antibiotic uptake, elution, biologic activity, and biocompatibility. Crosslinked chitosan sponges exhibited decreased degradation and increased antibiotic uptake and elution. PNIPAM/genipin crosslinked sponges had the highest and prolonged release of antibiotics. Vancomycin and amikacin eluted from all sponges was active against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and did not have significant cytotoxic effects. These results indicate that genipin crosslinked and PNIPAM/genipin crosslinked chitosan sponges have potential as tailorable adjunctive treatments for infection control, suitable for extended degradation and antibiotic release times.

PREFACE

The main body of this thesis is a journal article entitled "Design and Preliminary Investigation of Crosslinked Chitosan Sponges for Tailorable Drug Delivery and Infection Control." This manuscript will be submitted to the Journal of Biomedical Materials Research Part B: Applied Biomaterials.

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CHAPTER 1:

INTRODUCTION

1.1 *Statement of Clinical Problem*

Wound infection and the development of osteomyelitis, a bone infection caused by bacteria or a fungus, can be a challenging problem to treat in orthopaedics, especially with complex musculoskeletal trauma, and can result in multiple surgeries and increased costs.[1, 2] Orthopaedic injuries comprise approximately 65% of total injuries during military combat and osteomyelitis occurs in 2 to 15% of the patients with combat related injuries.[3] Wound infection can also be complicated by wound contamination with antibiotic resistant bacteria, such as Methicillin-resistant *Staphylococcus aureus* (MRSA), which can increase the occurrence of osteomyelitis and nosocomial infections, infections acquired in hospitals.[4] The development of a biodegradable local drug delivery system whose drug release could be tailored depending on intended application, injury, and antibiotic would allow for more controlled and optimized local drug delivery, providing a more efficacious therapy for infection control.

1.2 *Hypothesis*

We hypothesize that *in situ* crosslinking of point of care antibiotic loaded chitosan sponges will allow for tailorable and controlled drug elution, while maintaining activity against resistant organisms, such as *Staphylococcus aureus (S. aureus)*. The sponges will be biocompatible and degradable, in order to eliminate a second surgery for removal of the drug delivery system.

CHAPTER 2:

LITERATURE REVIEW

2.1 *Wound Healing*

A wound can be commonly defined as a body injury that typically results in lacerations to skin and usually contains underlying damage to other tissues. Complex wounds typically involve multiple tissues and often do not heal quickly or even completely.[5] Comorbidities often occur and can contribute to the onset of infection and impair wound healing.[5] Long wound healing times can decrease patients' quality of life, physically, psychologically, and financially.[5] Complex, open orthopedic wounds are often contaminated and infection development is a serious complication.[6] To understand the issues with wound healing and how to correct these issues and improve healing time, it is important to understand the principles of wound healing.

Wound healing involves four stages: haemostasis, inflammation, proliferation, and remodeling. In haemostasis, platelets are activated upon injury and release chemicals such as fibronectin to promote platelet aggregation and platelet derived growth factor (PDGF) and transforming growth factor β (TGF-β) to mediate the wound healing process.[7] Within 24 hours of injury, inflammation begins when neutrophils are recruited to the injury site through chemotaxis and serve as the first line of defense against infection.[7] Neutrophils remove bacteria, foreign materials, and necrotic tissue from the wound.[7] Neutrophils are eventually replaced as the predominant cell type by monocytes, which differentiate into macrophages that also work to remove residual bacteria, foreign material, and non-viable tissue.[7] Both neutrophils and macrophages initiate the proliferative phase by releasing various factors that recruit and activate fibroblasts and

endothelial cells.[7] The proliferation phase of wound healing begins with fibroblast proliferation and collagen deposition in order to form a stable extracellular matrix in the wound. [7] Granulation tissue is formed, epithelial cells construct an epithelial layer over the wound, and endothelial cells initiate angiogenesis.[7] In the remodeling phase, the granulation tissue becomes a scar.[7, 8]

2.2 *Wound Management*

Wound management often provides faster and more comfortable wound healing, which increases patients' quality of life. The common steps utilized in wound management with orthopaedic injuries and surgical sites are debridement, irrigation, dressings, fixation, closure, and antibiotic therapy.[5] All of these steps are utilized by surgeons in order to prevent further damage to the tissues, or even amputation.

Debridement is the surgical removal of foreign debris, necrotic bone and soft tissue from a wound, which creates a sterile healthy wound bed.[5] Patients that undergo debridement within 6 to 24 hours from injury have shown to have a decreased risk for infection.[5, 9] However, there are different opinions about the time dependence of debridement. One study found no correlation between the time to operative debridement to the risk of infection in patients with open high energy lower extremity trauma.[10] Additionally, debridement can sometimes be logistically difficult in patients with complex, multiple trauma.[5]

Irrigation is the use of a solution, usually saline, to cleanse a wound of bacteria and traumatized tissue, and can be delivered either at low or high pressure.[5] While irrigation does decrease the bacterial load, it has been shown that in high pressure

irrigation, the irrigation fluid can spread in a lateral direction, contributing to the development of postoperative edema.[11] Because of the consequential increase of wound infection susceptibility, it is recommended that irrigation not be used as a preventive measure but is reserved for contaminated wounds.[11] While antibiotic solutions can be utilized, saline is often recommended because the antibacterial solutions can create irritation in the wound.[5]

Wounds are also often covered with dressings, to prevent drying of tissue, which can cause cell death, necrosis, and infection.[5, 12] Moist wound healing promotes angiogenesis and epithelialization, thereby improving wound healing for patients.[5] Some of the typical wound dressings include film dressings, foams, alginates, and even chitosan dressings.[5, 13] Some of the dressings contain silver or topical antibiotics, in order to decrease bacterial colonization.[5, 13] However, topical antimicrobials can cause dermatitis and even facilitate *Pseudomonas aeruginosa (P. aeruginosa)* growth.[13] Antimicrobial creams with malic acid or hypochlorite solutions do reduced bacterial colonization; however, they can also increase inflammation of the nearby tissue.[5]

Both fixation and closure prevent further complications with wounds. Fixation stabilizes the bone and allows for weight bearing, rapid bone healing, and the use of the limb before complete healing.[14] Some of the devices utilized in fixation include plates, screws, nails, rods, and pins, depending on which area is being stabilized.[5, 14] While fixation is a critical component of healing, fixation hardware creates another surface on which bacteria can colonize, often resulting in biofilm development.[15] Closure of a wound can decrease the risk of infection and is often conducted with skin allografts or

muscle flaps.[5] Closure with skin grafts promotes vascularization, but needs excellent wound preparation and can have poor appearance and durability.[5]

2.3 *Wound Infection*

Although prevention of infection is the preferred route, treatment of bacterial infections is conducted with antibiotic therapy. *S. aureus* is the most common organism that causes osteomyelitis, but *P. aeruginosa*, *Staphylococcus epidermis*, *Enterococcus* spp., *Streptococcus* spp., *Mycobacterium* spp, and *Enterobacter* spp. are also associated with osteomyelitis, as well as numerous other wound infections.[16, 17] *S. aureus* is a common bacteria and is present in many places; approximately one third of the general population is colonized with the bacteria.[18] Wound healing can also be further complicated by wound contamination with antibiotic resistant bacteria, which can increase the risk of osteomyelitis and nosocomial infections. MRSA is a serious concern in modern medicine and a problem in wound healing.[4] MRSA is a common cause of nosocomial infections, which compose approximately 40-70% of *S. aureus* infections in hospital intensive care units.[19] However, in addition to causing nosocomial infections in patients that are already sick, MRSA also has the capability of causing aggressive infections in children and other young and otherwise healthy people.[20] According to the Center for Disease Control and Prevention (the CDC), MRSA infections are on the rise in hospitals; in 2005, 18,650 patients died in hospitals from MRSA infections.[4]

Another problem associated with wound healing in complex injuries is the possible production of biofilms. According to an estimate by the CDC, approximately 65% of bacterial infections are associated with biofilms.[21] Biofilms are adherent

bacterial populations of single or mixed colonies with an exopolysaccharide matrix.[22] Bacteria can produce biofilms on implants or other surfaces and during the adhesion process, bacteria change their phenotypes.[22] These phenotypic changes enable the bacteria to respond to environmental conditions in varying ways, and communication between bacterial cells can also be established.[22] The communication and reactions to environmental changes of adherent bacteria in biofilms also enables protection against many antibacterial and antimicrobial agents.[23] In biofilms, the metabolic activity of the bacteria is reduced and the adherent bacterial cells ingest less antibiotic than planktonic bacteria with higher metabolic activity, resulting in less susceptibility to antibiotics and an increased minimal inhibitory concentration (MIC).[23] The MIC and minimum bactericidal concentration (MBC) can be defined as the lowest concentrations of antibiotic or antimicrobial that inhibits growth or kills 99.9% of bacteria after an overnight incubation, respectively.[24] Researchers have found the MIC and MBC for nonadherent *P. aeruginosa* to be 1 μg/ml and 50 μg/ml, respectively, but adherent *P. aeruginosa* in biofilms on urinary catheters remained viable after 12 hours of exposure to 1,000 μg/ml of tobramycin.[22, 25] Systemic antibiotics cannot reach such a high concentration at a localized site without toxicity, thereby becoming ineffective against the biofilms.[23]

2.4 *Antibiotic Therapy*

Some of the antibiotics that can be used for treating *S. aureus* include linezolid, minocycline, co-trimoazole, clindamycin, tigeycline, cefazolin, oxacillin, nafcillin, rifampicin, vancomycin, and daptomycin.[26] Clindamycin, co-trimoxazole, linezolid, and

minocycline are typically provided as treatment for skin and soft tissue infections and necrotizing pneumonia.[26] However, there can be resistance issues with clindamycin, co-trimoxazole, linezolid, and minocycline, especially in patients with health care risk factors.[26] Oxacillin, nafcillin, or cefazolin are typically given to patients without health care risk factors while cultures are being taken.[26] Tigecycline is indicated for intra-abdominal infections with no clinical data involving osteomyelitis; resistance to tigecycline has also been reported.[27] Rifampcin is an option for treatment but should not be used without another antibiotic because of the rapid development of resistant bacteria.[26] Daptomycin, a cyclic lipopeptide, has activity against Gram-positive bacteria, including *S. aureus* and MRSA.[27, 26] However, there is little data and experience using daptomycin as a treatment for children under 18 years old.[26] *S. aureus* has remained sensitive to vancomycin overall, until the first vancomycin resistant *S. aureus* strains emerged in the United States in 2002. Vancomycin is a glycopeptide that binds to the D-alanyl-D- alanine cell well precursor used in peptidoglycan crosslinking, which inhibits cell wall synthesis.[28] However, vancomycin remains the antibiotic of choice with clinicians for MRSA, possibly until a new suitable antibiotic is found.[26, 27, 29] For Gram-negative bacteria, such as *Pseudomonas aeruginosa*, aminoglycosides such as gentamycin, kanamycin, tobramycin, and amikacin are often used.[30] Amikacin is commonly used to treat resistant gram-negative bacteria because amikacin has fewer points susceptible to enzymatic attack than the other aminoglycosides.[30]

2.5 *Local Drug Delivery*

Because systemic antibiotics cannot be administered at very high concentrations to fight localized resistant bacteria or biofilms, local drug delivery is emerging as an effective route for treating wounds and minimizing bacterial infections.[31] In addition to issues with antibiotic resistance, toxicity issues can arise with systemic drug delivery because drugs are delivered to the entire body.[31] The organisms that are typically antibiotic resistant with systemic antibiotic delivery concentration levels can be susceptible to the higher levels of antibiotics provided in local delivery.[31] Additionally, the drug can take a longer time to reach the wound when delivered systemically and some avascular areas of the body are unreachable.[31] In severe cases of infection and osteomyelitis, the local blood supply can be compromised and antibiotics cannot be delivered systemically to the infection site.[31] In local drug delivery, ideally the wound area is targeted and the drug therefore has less impact on other parts of the body. The ideal local drug delivery system would be implanted, deliver the antibiotics at appropriate levels, and then degrade so there would be no need for a second surgical procedure.[31]

2.6 *Current Treatment Options*

Several materials are currently being utilized as local drug delivery systems, but many of these materials have disadvantages. Polymethylmethacrylate (PMMA) is one of the most common materials used as a local drug carrier and comes in two forms: antibiotic loaded bone cement for arthroplasties and antibiotic loaded beads for musculoskeletal infections.[2] PMMA beads have shown to reduce the infection rate in severe open fractures from 12% to 3.7% when they were applied in combination with

systemic drug delivery.[6] Antibiotic loaded PMMA bone cement also reduced the revision rate for patients with total hip replacements.[11] The revision rate for patients receiving only systemic antibiotics was 4.3 times greater than for patients receiving systemic and localized drug delivery through the antibiotic loaded bone cement.[32] While PMMA has shown to be successful against bacterial infections, some major disadvantages of the material are the need for surgical removal, possible biofilm formation, and the development of antibiotic resistant bacteria.[23] After the initial high antibiotic release from gentamicin loaded bone cement, the long term, low concentration of antibiotic around implants has shown the potential to develop antibiotic resistant bacteria strains.[23, 33] Antibiotic loaded bone cement has shown bacterial growth in *in vitro* studies, which can easily lead to the development of biofilms on the surface.[34]

Another material used as a local delivery system in orthopaedic applications is calcium sulfate. Calcium sulfate is a biocompatible, biodegradable material with osteoconductive properties that exhibits a bolus release followed by an extended drug release from several hours to weeks, depending on the formulation.[35, 36] Because calcium sulfate degrades over time, the need for surgical removal is eliminated and clinical studies and animal models have shown success with minimizing infection through the use of antibiotic loaded calcium sulfate pellets.[37-39] Though calcium sulfate has been successful at reducing infection, wounds treated with calcium sulfate can develop sterile draining sinuses, mimicking infected draining sinuses, which many surgeons dislike.[40, 41]

2.7 Chitosan

Chitosan, a positively charged linear polysaccharide produced by deacetylation of chitin from crustacean shells, has been studied and utilized extensively in bioadhesion, drug delivery and tissue engineering research and demonstrates promising properties for such biomedical applications.[42] Advantages of chitosan include low cost, biodegradability, high biocompatibility, availability, and functional groups that allow for easy chemical modification.[30] The structure of chitosan is provided in Figure 1.

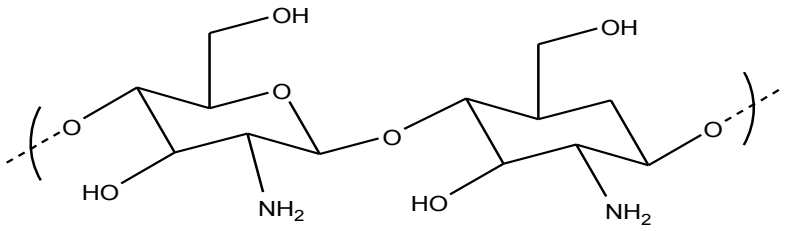

Fig. 1. Chemical structure of chitosan[30]

Chitosan has inherent antibacterial properties[43], has shown to promote wound healing[44, 45] and is presently a Food and Drug Administration approved haemostatic wound dressing utilized by the US military for the control of combat injuries.[46] When used as a drug delivery system, chitosan effectively carries antibiotics and exhibits predictable elution rates and biodegradation.[43] The development of a chitosan local drug delivery system whose drug release could be tailored depending on intended application, injury, and antibiotic has the potential to improve and optimize local drug delivery.

Chitosan has been studied and successfully utilized for local drug delivery in film form,[43, 47, 48] microspheres,[49, 50] and sponges.[51-56] Some of the properties that have shown or have the potential to affect the behavior of chitosan as a local drug delivery system include degree of deacetylation, molecular weight, swelling, crosslinking, antibiotic type, pH, drug concentration of various antibiotics, the time point at which antibiotic is loaded, and type of chitosan.[47, 48, 51, 52, 56] Additionally, combining chitosan with another polymer or chemical modification of chitosan can alter the drug elution rate.[50, 57]

The antibiotic concentration loaded into the sponges can have an impact on the release profile of the drug. In one study, chitosan and ethylcellulose sponges loaded with a low dose of insulin (1 mg) released a higher percentage of drug content than the same sponges loaded with a high dose of insulin (1.75 mg and 2 mg).[52] However, the opposite was true in crosslinked chitosan sponges loaded with platelet derive growth factor BB (PDGF-BB); sponges loaded with 400 ng of PDGF-BB released more of the growth factor than sponges loaded with 100 ng or 200 ng.[53] Because the sponge material and the type of drug typically vary in reported experiments and also influence drug elution, the influence of antibiotic concentration on the drug release rate needs to be investigated on a case by case basis.

The production process of the sponge, especially when the drug is loaded into the sponge, makes a significant difference in the drug elution rate. Multiple studies have shown chitosan sponges to exhibit slower drug release when the antibiotic was loaded during the production of the sponge[52, 56] rather than loaded post production of the sponges. However, one of the goals of this project is to develop a chitosan sponge that is able to be loaded with the drug at the point of care to generate an adaptable drug delivery

system. Drug loading after the sponge is produced would allow the physicians to select and load antibiotics immediately before implantation and would allow for more control of the antibiotics and dosages. Therefore, the best way to create an easily adaptable chitosan drug delivery system is through drug loading at the site of use (point of care loading), allowing the drug to permeate and interact with the sponge, rather than the entrapment of the drug during production of the sponges.

Molecular weight influences numerous properties of a material, including mechanical properties and drug release. Nuthanid and co-workers determined that high molecular weight chitosan lead to films with better mechanical properties than films with low molecular weight chitosan, due to the increased entanglements in the higher molecular weight chitosan.[48] Additionally, in the high molecular weight chitosan, the chitosan with a higher degree of deacetylation also lead to better mechanical properties, most likely due to the denser packing of the additional amino groups.[48]

When studying either films or sponges for use in the body, both swelling and adhesion to tissue are important properties to study. In the previously studied chitosan films, all of the films swelled initially in phosphate buffer solution, and then decreased in volume with time while becoming denser at the same time.[48] Researchers suggest that this decrease in volume is most likely attributed to crosslinking between the amino groups and the phosphate, also explaining the increase in density.[48] Bioadhesion is important in wound healing because the material needs to be able to stay in place in order to deliver the drugs to the target site. In previous research on chitosan sponges, some of the sponges studied in an animal study did not adhere to the local tissues of goats, reducing the effectiveness of the local drug delivery.[58] Increasing the bioadhesion of the

chitosan sponges would allow for the sponge to stay in place and deliver the antibiotics in a controlled manner.

2.8 Crosslinking Chitosan

Crosslinking chitosan can have a significant effect on the drug release profile of a local delivery system. In one study, crosslinking chitosan sponges with 1.33% glutaraldehyde reduced the drug release rate of micronized triamcinolone acetonide at pH 7.4 by over 50%.[51] Crosslinking sponges composed of both chitosan and gelatin with 5% (w/w) glutaraldehyde also reduced the release of the drug, prednisolone, when used in acetic, formic, or lactic acid.[56] Crosslinked chitosan hydrogels can be achieved through small molecule, photo sensitive, enzymatic, and polymer polymer crosslinking.[50] Some small molecule crosslinkers that have been studied with chitosan are glutaraldehyde, diglycidyl ether, diisocyanate, diacrylate, and genipin.[50, 51, 56, 59]

Chitosan hydrogels have also been successfully crosslinked through disulfide bridges using dimethyl 3, 3 dithio bis propionimidate (DTBP) or N-acetyl-L-cysteine (NAC) as the crosslinkers.[60,61] Crosslinked chitosan hydrogel scaffolds prepared with DTBP exhibited similar properties to glutaraldehyde crosslinked chitosan scaffolds, but showed greater strength and less toxicity.[60] The release of insulin and bovine serum albumin could be controlled in chitosan hydrogels crosslinked with NAC by changing the composition, loading, and disulfide bond contents.[61] These NAC crosslinked chitosan hydrogels were porous and biocompatible as well.[61]

Photo-cross linking offers different advantages including safety, low cost, ease of production, and speed. A photo-sensitive chitosan hydrogel was developed by Ono and

researchers through functionalizing chitosan with azide groups, which after ultraviolet (UV) irradiation, are coverted into reactive nitrene groups that bind chitosan's free amino groups.[62] This hydrogel exhibited successful controlled release of growth factors.[62, 63] However, photo-cross linking can require a photosensitizer, which can create additional toxicity issues. Riboflavin phosphate is a photosensitizer that has been researched to crosslink collagen in rabbit and porcine eyes and has not shown immediate toxicity, although long term side effects have not been evaluated.[64] The use of the riboflavin phosphate and UVA at 370 nm created a crosslinked collagen surface, increasing the corneal rigidity.[64] If an additional biocompatible polymer is selected, polymer-polymer cross linking can reduce potential toxicity issues associated with small molecule and photo cross linking. A hydrogel of chitosan and hyaluronic acid has been cross linked through the formation of Schiff bases between the polymers and was stable for at least four weeks and could be loaded with chondroctyes.[65] Polymer polymer crosslinking has also been studied with chitosan and oxidized dextran polysaccharides and alginate.[66, 67]

2.9 *Genipin*

Genipin is a commonly used small linker molecule for crosslinking chitosan and is derived from geniposide by enzymatic hydrolysis with β-glucosidase.[68, 69] The genipin chemical structure is shown in Figure 2.[68, 69] Geniposide is isolated from fruits from the plant, *Gardenial jasminoides* Ellis, and has been used in traditional Chinese medicine.[68] Extracts from the fruits are also used in a food dye known as gardenia blue, which forms a blue pigment through the reaction of genipin with amino acids in the presence of oxygen.[69, 70]

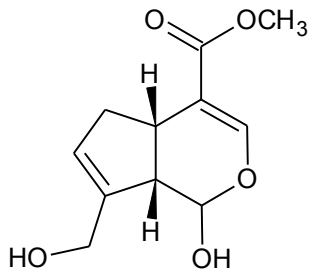

Fig. 2. Genipin chemical structure[69]

Physiological studies have demonstrated that geniposide is converted to genipin in the gastrointestinal tract in rats, with no adverse effects.[69, 71] Genipin crosslinked chitosan microspheres injected intramuscularly into the skeletal muscle of rats exhibited a smaller inflammatory reaction and slower degradation than glutaraldehyde crosslinked chitosan microspheres.[72] In another study, researchers studied the cytotoxicity of genipin and glutaraldehyde to 3T3 fibroblasts and found that genipin was approximately 10,000 times less cytotoxic than glutaraldehyde.[73]

Genipin has been used to crosslink chitosan films and microspheres for heparin removal, protein release, and drug release.[59, 68, 74, 75] Yuan and researchers evaluated the crosslinking of chitosan microspheres with genipin for albumin release and found that as crosslinking time and genipin concentration increased, the overall release of albumin decreased.[68] In addition to drug or protein release, crosslinking chitosan delivery systems with genipin can change other properties. Crosslinking chitosan/poly (ethylene oxide) blended films with genipin resulted in films with greater tensile strength, elongation at

break, increased hydrophobicity, and slower degradation.[59] Chitosan and silk fibroin sponges have also been crosslinked with genipin for cartilage engineering, but sponges formed from chitosan alone have not been studied with genipin.[76]

2.10 *Poly(N-isopropylacrylamide)*

Chitosan has also been studied in combination with other materials, including gelatin,[56] ethylcellulose,[52] collagen and hyaluronan,[54] alginate,[53] and chrondroitin sulfate,[77] to form sponges for local drug delivery systems. Additional polymers are often selected in order to complement the deficiencies of chitosan or to generate a new property for a specific application. One such example is the preparation of "smart" hydrogels by grafting poly(N-isopropylacrylamide) (PNIPAM) onto chitosan.[57] PNIPAM is a thermally responsive polymer, whose chemical structure is shown below in Figure 3, that exhibits a lower critical solution temperature (LCST) and contracts when heated above its LCST.[57] An originally clear PNIPAM solution will also become opaque above its LCST.[78] As a hydrogel, PNIPAM can be a liquid at room temperature and undergo gelation in contact with the body.[57]

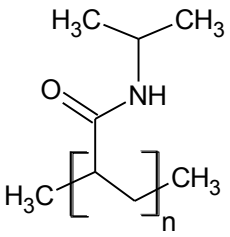

Fig. 3. Poly(N-isopropylacrylamide) chemical structure[79]

Chitosan and PNIPAM have been combined to form semi-interpenetrating networks (semi-IPNs), where one of the polymers is crosslinked, and full interpenetrating networks (full-IPNs) where both polymers are crosslinked.[80, 81] An interpenetrating network is a blend of two polymer networks lacking covalent bonds between the two networks and this type of polymer blend can be utilized to combine properties of different materials.[82] Differences between semi-IPNs and full-IPNS of chitosan and PNIPAM have been found in FTIR spectra, phase transition behavior, and swelling behavior.[81] A semi-IPN of glutaraldehyde crosslinked chitosan and PNIPAM swelled faster than a full-IPN because the PNIPAM was simply embedded in the gel and the swelling behavior was based on swelling of the chitosan.[78, 81] However, in a full-IPN of formaldehyde crosslinked chitosan and methylene bis-acryalmide crosslinked PNIPAM, there was not as much swelling above the LCST because the chitosan swelled while the PNIPAM contracted due to the collapse of the polymer chain.[81] This stress inducing swelling behavior could create other potential problems, such as stress cracking of the hydrogel.[81]

Multiple research studies with PNIPAM based hydrogels have shown minimal cytotoxic effects on various types of cells.[83-85] PNIPAM with iron oxide particles was found to have 85-90% cell viability, as compared to approximately 97% cell viability in cells grown directly on the polystyrene plate.[83] In another study with a PNIPAM based injectable hydrogel, researchers found that rats injected subcutaneously with the polymer solution exhibited an initial acute wound healing phase and the tissue returned to its normal state after 30 days.[84] Polymer solutions of a synthesized, uncrosslinked PNIPAM

and chitosan compound did not significantly inhibit the growth rate of primary cultured corneal epithelial cells.[85]

In the semi-IPNs of chitosan and PNIPAM, PNIPAM is typically the polymer that is crosslinked, either with tetraethylene glycol diacrylate (TEGDA) or methylene bis-acrylamide; however, toxicity issues could arise with these small linker crosslinkers.[80] Chitosan has been crosslinked when combined with PNIPAM in a semi-IPN, but it has been crosslinked with glutaraldehyde, not genipin.[86] Chitosan has also been successfully utilized as a thermo sensitive membrane for a wound dressing in combination with poly(urethane) and PNIPAM.[87] This combination of PNIPAM with chitosan could extend chitosan's properties to injectable biomedical applications and generate a system that would be pH and temperature responsive. It could also be investigated whether the combination of PNIPAM and chitosan could create a sponge that could swell even further when introduced into the body, possibly allowing for better bioadhesion and drug delivery.

2.11 *Conclusions*

Musculoskeletal wound infections continue to be a painful, expensive, and at times deadly medical problem, difficult to treat and further complicated by an increasing emergence of antibiotic resistant bacteria. Debridement, irrigation, fixation, closure, and systemic antibiotic therapy cannot stop all infections, and prevention is a key component to minimizing infection. The delivery of antibiotics, such as vancomycin and amikacin for the inhibition of both gram positive and negative bacteria, through degradable, local delivery systems is ideal because of the possibility of higher antibiotic dosage, as

compared to systemic delivery, and the elimination of an additional surgery to remove the delivery system. The current local delivery systems have their drawbacks, including burst release, possible biofilm formation, need for surgical removal, and the formation of sterile draining sinuses.[23, 40, 41, 88] We hypothesize that crosslinking chitosan sponges with genipin or creating a semi-IPN of genipin crosslinked chitosan and PNIPAM will create a more tailorable adjunctive treatment that allows for antibiotic selection and point of care loading, sustains antibiotic elution and degradation properties, maintains activity against Gram-positive or Gram-negative bacteria, such as *S. aureus* or *P. aeruginosa*, and demonstrates sufficient biocompatibility. The specific aims of this research study are as followed:

1. Create degradable genipin crosslinked and PNIPAM/ genipin crosslinked chitosan sponges that easily absorb antibiotics in one minute of loading
2. Deliver vancomycin and amikacin from the crosslinked sponges through 72 hours and maintain specific biologic activity against *S. aureus* and *P. aeruginosa*, respectively
3. Ensure that neither the crosslinked sponges nor their antibiotic eluates initiate a cytotoxic effect on cells after 24 hours of exposure
4. Evaluate the bioadhesive strength of the uncrosslinked and crosslinked chitosan sponges

CHAPTER 3:

Design and Preliminary Investigation of Crosslinked Chitosan Sponges for Tailorable Drug Delivery and Infection Control

3.1 *Introduction*

Complex musculoskeletal wounds, both in civilian and military populations, can be difficult to treat because they are often contaminated and infection development is a serious complication, which may result in longer healing time, multiple surgeries, and increased costs.[1, 2, 3] Orthopaedic injuries comprise approximately 65% of total injuries during military combat and osteomyelitis, a bone infection caused by bacteria or a fungus, occurs in 2 to 15% of the patients with combat related injuries.[4] Infection is also especially troublesome in complex open extremity fractures from high energy trauma; typical infection rates of civilian open fractures are 17.5-21.2%, while infection rates of closed fractures are only 3.6-8.1%.[5] *Staphylococcus aureus* (*S. aureus*) is the most common bacterial organism that causes osteomyelitis and is very prevalent; approximately one third of the general population is colonized with the bacteria.[6] Wound infections are also often typically polymicrobial and can be further complicated by wound contamination with antibiotic resistant bacteria such as methicillin-resistant *S. aureus* (MRSA).[7] MRSA is a common cause of nosocomial infections, which compose approximately 40-70% of *S. aureus* infections in hospital intensive care units.[8] *S. aureus* has remained sensitive to vancomycin, a glycopeptide that is often the antibiotic of choice with clinicians for MRSA.[9] For Gram-negative bacteria associated with wound infections such as *Pseudomonas aeruginosa* (*P. aeruginosa*), amikacin, an

aminoglycoside, is a commonly selected antibiotic due to fewer points for enzymatic attack than other aminoglycosides.[10]

Local antibiotic delivery is emerging as an effective route for treating wounds and minimizing bacterial infections because systemically delivered antibiotics cannot be administered at very high concentrations to fight localized resistant bacteria or biofilms.[11] The higher levels of antibiotics provided in local delivery, as compared to systemic delivery, may kill bacteria before antibiotic resistance develops.[11] In addition to issues with antibiotic resistance, toxicity issues can arise with systemic drug delivery because drugs are delivered to the entire body.[12] In severe cases of infection and osteomyelitis, the local blood supply is compromised to the extent that antibiotics cannot be delivered systemically.[13] In local drug delivery, the local wound area is targeted and thus drugs are more effective and have less impact on other parts of the body. Polymethylmethacrylate (PMMA) and calcium sulfate are two common local drug delivery systems, but both materials have disadvantages. PMMA sometimes needs to be surgically removed and there is also potential for biofilm formation and antibiotic resistant bacteria development.[1, 12] Wound treated with calcium sulfate may develop sterile draining sinuses, mimicking infected draining sinuses, which many surgeons disfavor.[14-17]

Chitosan, a positively charged linear polysaccharide produced by deacetylation of chitin from crustacean shells, is a low cost, biodegradable, and biocompatible material that has shown success in local drug delivery systems. [18] When used as a drug delivery system, chitosan effectively carries antibiotics and exhibits predictable elution rates and biodegradation.[19] In a lyophilized sponge delivery system, chitosan has shown to

predictably release antibiotics and effectively inhibit the growth of *S. aureus* and *P. aeruginosa*.[20]

Crosslinking chitosan can have a significant effect on the drug release profile of a local delivery system. In one study, crosslinking chitosan sponges with 1.33% glutaraldehyde reduced the drug release rate of micronized triamcinolone acetonide at pH 7.4 by over 50%.[21] Genipin is another commonly used small linker molecule for crosslinking chitosan and has been shown to be 10,000 times less cytotoxic on 3T3 fibroblasts than glutaraldehyde. [22] Genipin has been used to crosslink chitosan films and microspheres for heparin removal, protein release, and drug release.[23-27] Chitosan and silk fibroin sponges have also been crosslinked with genipin for cartilage engineering, but sponges formed from chitosan alone have not been studied with genipin.[28]

Chitosan and poly(N-isopropylacrylamide) (PNIPAM), a thermally responsive polymer, have also been combined to form semi-interpenetrating networks (semi-IPNs), where one of the polymers is crosslinked, and full interpenetrating networks (full-IPNs) where both polymers are crosslinked.[29, 30] In the semi-IPNs of chitosan and PNIPAM, PNIPAM is typically the polymer that is crosslinked, either with tetraethylene glycol diacrylate (TEGDA) or methylene bis-acrylamide; however, toxicity issues could arise with these small linker crosslinkers.[29] Chitosan has been crosslinked when combined with PNIPAM in a semi-IPN, but it has been crosslinked with the more toxic glutaraldehyde, not genipin.[31]

In this *in vitro* investigation, we evaluated the properties of genipin crosslinked chitosan sponges and genipin crosslinked chitosan sponges with PNIPAM for a local antibiotic delivery system. The sponges' degradation properties were ascertained to

determine *in vitro* degradation timing and Fourier Transform Infrared Spectroscopy (FTIR) analysis was conducted to examine the crosslinking reaction of the chitosan sponges. To determine if the sponges could effectively release the antibiotics, vancomycin and amikacin, and inhibit the growth of infectious bacteria, the antibiotic uptake, elution, and activity against specific strains of *S.aureus* and *P. aeruginosa* were studied. In order to assess short term cytotoxicity, the impact of the sponges and antibiotic eluates on the cell viability of 3T3 fibroblasts was evaluated.

3.2 *Materials and Methods*

Materials

Chitosan powder at 71% degree of deacetylation (DDA) with 1480 cP intrinsic viscosity and approximately 426,800 g/mol molecular weight was obtained from Primex (Iceland) and genipin was obtained from Wako (Richmond, VA). Poly (n-isopropylacrylamide) (PNIPAM) with a molecular weight of approximately 10,000-15,000 g/mol was purchased from Sigma Aldrich (St. Louis, MO). All other reagents were purchased from Fisher Scientific (Pittsburg, PA) and were of analytical grade. Vancomycin, amikacin, and 2 x crystallized chicken white egg lysozyme were obtained from MP Biomedicals (USA) and trypticase soy broth (TSB) was purchased from Beckton, Dickinson, and Company (Franklin Lakes, NJ). NIH 3T3 mouse fibroblasts were purchased from ATCC (Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and 100x penicillin (10,000 units/mL), streptomycin (10 mg/mL), and amphotericin B (25 μg/mL) solution were purchased from Fisher Scientific. The Live/Dead® stain kit for mammalian cells and the Cell Titer-Glo®

Luminescent Cell Viability assay were obtained from Invitrogen (Carlsbad, CA) and Promega (Madison, WI), respectively.

Sponge Preparation

Three different chitosan sponge test groups were created: an uncrosslinked chitosan sponge (control), a genipin crosslinked chitosan sponge (genipin crosslinked), and a sponge with genipin crosslinked chitosan mixed with PNIPAM (PNIPAM/genipin crosslinked) To create the sponges, a 1% (w/v) chitosan solution was prepared by dissolving the chitosan powder in 1% (v/v) blended lactic/ acetic acid (75:25), under constant stirring for 24 hours. In order to remove any insoluble chitosan from the solution, the solution was filtered through a 180 μm nylon screen and allowed to degas at ambient temperature for approximately one hour. For the uncrosslinked chitosan sponges, 20 mL of the filtered 1% (w/v) chitosan solution was cast into separate fluted aluminum weighing dishes (Fisher Scientific, 42 mL) and placed into a -80°C freezer (C90-14A31; Kendro Lab Products, Asheville, NC) for one hour.

In order to crosslink the chitosan, genipin was added to the 1 % (w/v) filtered chitosan solution in order to reach a genipin concentration of 5 mM, determined from previous formulation research. The genipin and chitosan solution was stirred for 3.5 hours at 35°C before 20 mL of the solution was cast into aluminum weighing dishes and placed in the -80°C freezer for one hour. As the crosslinking time increased, a blue color developed in the genipin and chitosan solution. To make sponges from genipin crosslinked chitosan and PNIPAM, PNIPAM was added to deionized water to reach 0.4% (w/v), a concentration determined from previous formulation research. Lactic acid, acetic acid, and chitosan were then added to the PNIPAM and deionized (DI) water solution in

order to reach a solution of 1 % (w/v) chitosan, 1% (v/v) blended acid (75:25 lactic to acetic acid), and 0.4% (w/v) PNIPAM. After mixing for 24 hours, the chitosan and PNIPAM solution was filtered through a 180 μm nylon mesh. Next, genipin was added to the solutions to reach a concentration of 5 mM, solutions constantly mixed for 1.5 hours and then degased at ambient temperature for approximately one hour. The PNIPAM/ genipin crosslinked chitosan solution was cast into separate aluminum weighing dishes in 20 mL amounts, covered with parafilm, and allowed to continue crosslinking at ambient temperature for a cumulative 24 hours of crosslinking. The same blue color development seen in the genipin crosslinked chitosan sponges appeared in the PNIPAM/genipin and chitosan solution and the solutions were placed into a -80°C freezer for one hour.

After all three types of sponges were frozen at -80°C for one hour, they were removed from the freezer and placed into a freeze-dryer (FreeZone 2.5; Labconco, Kansas City, MO) for 48 hours. The sponges were removed after lyophilization and soaked in 0.2 sodium hydroxide (NaOH) solution and rinsed in distilled water until neutral pH was achieved. However, the sponges used in the direct contact cytotoxicity testing were soaked in 1 M NaOH because of residual acidity in the uncrosslinked chitosan sponges. The hydrated sponges were refrozen at -80°C for one hour and then relyophilized for 48 hours. The sponges were removed and sterilized by low dose gamma irradiation (25.9-29.6 kGy).

Sponge Degradation

To measure the effect of crosslinking on the degradation, unloaded uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked chitosan sponges, were subjected to degradation testing. Samples with an approximate initial dry weight of 30.30 mg were obtained from three replicate sponges of each type. One sample from each replicate sponge type was measured for change in mass at each time point. Pieces of dehydrated uncrosslinked and crosslinked chitosan sponges were weighed on a Mettler Toledo XS205 Dual Range scale (Columbus, OH) and then submerged in 10 mL of phosphate buffered saline (PBS) supplemented with 100 µg/mL lysozyme, 100 units/mL penicillin, 0.1 mg/ml streptomycin, 0.25 µg/mL amphotericin B. Lysozyme is an enzyme present in the human body and has shown to quickly degrade chitosan *in vitro*.[32] The samples were incubated at 37°C for six weeks and samples (n=3/group) were taken at 1, 2, 3, and 6 weeks. In order to maintain sufficient lysozyme activity, the lysozyme solution was completely refreshed each week. At each time point, the lysozyme solution was removed and the sponges were washed with 10 mL deionized water, in order remove any residual salts from the PBS. To remove the deionized water wash solution, the sponges were placed in fluted aluminum weighing dishes, with known weights, in a convection oven at 30°C for one hour. After one hour, the sponges were placed in a dessicator for 24 hours and the new sponge weights were measured. The percent of the sponge remaining at each time point was determined using the following equation:

$$\text{Percent remaining (\%)} = \left(\frac{\text{Sponge weight at x weeks}}{\text{Initial dry sponge weight}}\right) \times 100$$

Evaluation of Crosslinking

The presence of crosslinking in the chitosan sponges was determined by Fourier Transform Infrared Spectroscopy (FTIR). FTIR spectra of the sponges were recorded using diffuse reflectance (DRIFT) FTIR on a Nicolet Magna IR Spectrometer 550 (ThermoScientific) with a helium neon class II laser. The sponges were ground into powder, dried in a convection oven at 30°C, and combined with potassium bromide. The spectra were acquired with 64 scans and a resolution of 4 cm^{-1} and OMNIC™ software was used to analyze the results.

Antibiotic Uptake and Elution

Because wound infections are often polymicrobial with both Gram-positive and Gram-negative bacteria present, both single and dual antibiotic loaded sponges were tested. To determine the sponges' antibiotic uptake and elution characteristics, 5 mg/ml solutions or either vancomycin, amikacin, or both 5 mg/ml vancomycin and 5 mg/ml amikacin were loaded into sponges. Using three replications of each sponge type for each of the three antibiotic solutions, sponges of known weights were submerged in 10 mL of the antibiotic solution for one minute. After one minute, the sponge was removed and the volume of unabsorbed antibiotic solution was measured. The volume of antibiotic uptake was normalized by the sponge weight.

Immediately after the 1 minute *in situ* antibiotic loading, the sponges were completely submerged in 20 mL of sterile PBS and incubated at 37°C. At 1, 3, 6, 24, 48, 72, and 96 hours the PBS was completely refreshed, saving 1 mL aliquots at -20°C for

analysis of released antibiotics. Fluorescence polarization immunoassay (TDxFLx; Abbott Labs; Abbott Park, IL) was utilized to quantify the concentration of antibiotics eluted from the sponges with amikacin and vancomycin specific reagent kits.

Antibiotic Activity

The specific antibiotic activity of the eluates against *S. aureus* (Cowan I strain) and *P. aeruginosa* (ATCC 27317) was tested using turbidity assays. Dilutions (1:10) of vancomycin eluates were tested against *S. aureus* and 1:10 dilutions of amikacin eluates were tested against *P. aeruginosa*. Vancomycin eluates were added in two hundred microliter amounts to 1.75 mL of trypticase soy broth (TSB) along with 50 µL inoculum of *S. aureus* containing approximately $2x10^6$ colony forming units (CFU). Amikacin eluates (200 µL) were added to 1.75 mL of TSB and 50 µL inoculum of *P. aeruginosa* containing approximately $2x10^6$ CFU. Positive controls with bacteria but no antibiotic eluates and negative controls with only sterile TSB were also prepared. All samples were mixed and incubated at 37°C for 24 hours. After incubation, the samples were mixed again and the absorbance at 530 nm was recorded.

In vitro cytotoxicity

For cytotoxicity testing of the antibiotic eluates from the sponges, NIH 3T3 fibroblasts were grown in DMEM, supplemented with 100 units/mL penicillin, 0.1 mg/ml streptomycin, 0.25 µg/mL amphotericin B, and 20% FBS in an incubator at 37°C and 5% CO_2. Twenty percent FBS was used instead of 10% FBS because of eventual dilution of the serum with the antibiotic eluates. The cells were then seeded at 3×10^4 cells/cm^2 in 96 well plates made of tissue culture polystyrene (TCP) and allowed to attach overnight . Each antibiotic eluate from each elution time point (n = 3) from the sponges and a

negative control of sterile 1 x PBS solution (n = 9) were added in 100 µl volumes to the wells containing cells and growth media, for a total of 200 µl of media and eluate or PBS in each well, and allowed to incubate for 24 hours. After incubation, the culture media was removed and replaced with serum free DMEM and an equal volume of Cell Titer-Glo® Reagent Solution. The cell solutions were transferred into opaque 96 well plates and the luminescence was read at 590 nm in a 96 well plate reader (Bio-Tek Instruments Inc; Ontario, Canada). The results were reported as cells/cm^2, which was determined from a standard curve generated by a known dilution of cells.

Direct contact assay using NIH 3T3 fibroblasts was used to evaluate any potential cytotoxicity of the experimental crosslinked sponges as compared to control sponges. Cells were grown in DMEM, supplemented with 10% FBS, and 100 units/mL penicillin, 0.1 mg/ml streptomycin, 0.25 µg/mL amphotericin B. The cells were seeded at 3 x 10^4 cells/cm^2 in 48 well TCP cell culture plates and allowed to attach for 24 hours to establish a monolayer. A standard size hole punch (2" Reach Punchline; McGill, Inc.; Marengo, IL) was used to cut 6 mm in diameter samples of each sponge with an average weight of approximately 6 mg. The samples (n = 3 for each sponge type) were soaked in approximately 2 ml of 70% ethanol for over one hour and then rinsed with sterile PBS, warmed to 37°C, four times. The third PBS rinse was extended to 30 minutes so that any remaining ethanol could be removed through diffusion. The sponges were placed in direct contact with the cell monolayer. Polytetrafluoroethylene (PTFE) disks, with a 6 mm diameter and 4 mm thickness were utilized as controls. After 24 hours of incubation at 37°C and 5% CO_2, the culture media was removed and replaced with serum free DMEM and an equal volume of Cell Titer-Glo® Reagent solution. The cell solutions

were transferred to an opaque 96 well plate and the luminescence was read at 590 nm on a plate reader. The results were reported as cells/cm^2 determined from a standard curve generated by a known number of cells and percent cell viability, with the cells exposed to the PTFE disks as 100% viable cells. Live/Dead® staining was also utilized to qualitatively assess the cells' viability (n = 2) and was conducted at 100 x magnification with a fluorescent light microscope (Nikon Eclipse ® TE300; Tokyo, Japan), a digital camera (Q Imaging Retiga Fast Color ® 1394; British Columbia, Canada) and imaging computer software (BioQuant OSTEO II ®; Nashville, TN). Green and red fluorescence of cells, from the calcein AM and Eth D-1 reagent staining, indicated viable and non viable cells, respectively.

Statistical Analysis

All quantitative data are expressed as mean ± standard deviation ($n \geq 3$ for all groups). Two way analysis of variance (ANOVA) was used on data from degradation, antibiotic uptake, elution, and eluate cytoxicity testing to determine the differences between antibiotic or time and sponge independent variables and one way ANOVA was utilized on sponge cytotoxicity data. If statistically significant differences were detected, then the Holm-Sidak post hoc analysis was used for multiple comparisons. Analysis was performed using 2004 SigmaStat (San Jose, CA) and Microsoft Excel software (Microsoft, Inc, Redmond, WA) and $p < 0.05$ was considered statistically significant.

3.3 *Results*

The results of the degradation study are shown in Figure 1, demonstrating the percent remaining of the mass of the original uncrosslinked and crosslinked chitosan

sponges. Both types of crosslinked sponges exhibited statistically significant differences from the uncrosslinked chitosan control sponges at each time point ($p < 0.0001$), and from each other at week 3 ($p = 0.014$). Statistically significant differences in degradation over time appeared at week 6 in the genipin crosslinked and PNIPAM/ genipin crosslinked sponges ($p < 0.0001$). The uncrosslinked sponges demonstrated a fast initial degradation, with only an average of 4.49 ± 2.58 weight (wt) % remaining at week one and an average of 2.62 ± 1.17 wt% remaining at week six. Both types of crosslinked sponges showed a slower degradation, with 78.8 ± 1.15 and 73.9 ± 1.27 wt% remaining at week one and 64.74 ± 6.50 and 60.96 ± 0.80 wt% remaining at week six in the genipin crosslinked and PNIPAM/ genipin crosslinked sponges, respectively.

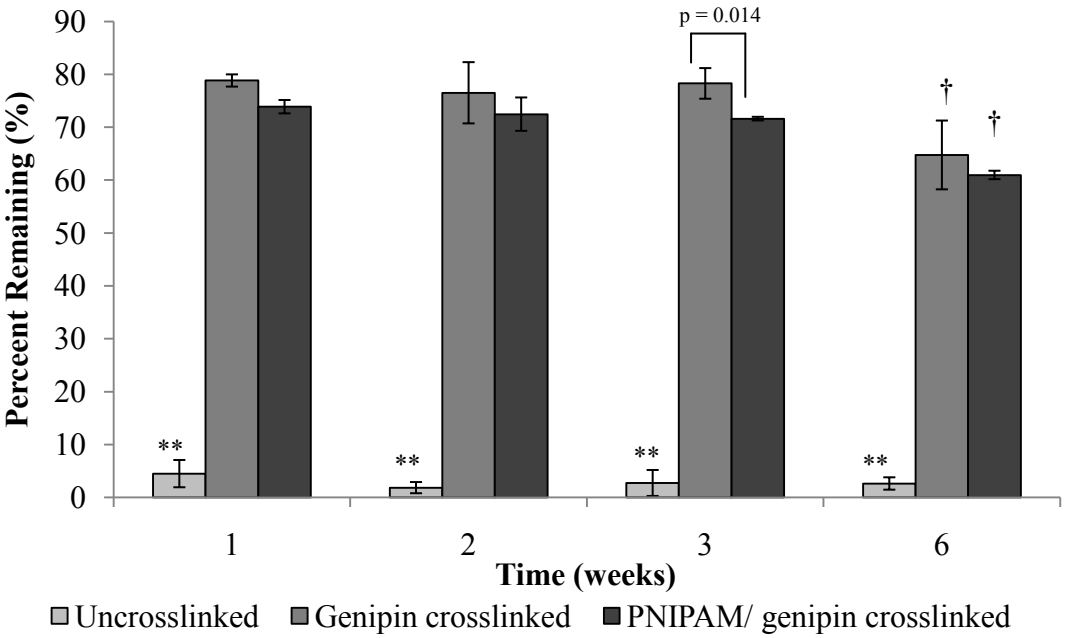

Figure 1. The degradation of uncrosslinked and crosslinked chitosan sponges without antibiotics in 100 µg/ml lysozyme solution represented as mean ± standard deviation. n = 3 for all groups (** vs. all others, $p < 0.0001$; † vs. other timepoints in same sample, $p < 0.0001$)

Infrared spectra of uncrosslinked and crosslinked chitosan sponges are shown in Figure 2. Significant absorbance was observed at 1739, 1576, 1472, 1210, 1052, 921, and 669 cm^{-1}. The peaks at 1576 and 1210 cm^{-1} were assigned to primary amine scissoring and C-N stretching in aliphatic amines, respectively. The peak at 1052 cm^{-1} corresponded to C-O stretching and the 669 cm^{-1} peak was assigned to N-H wag in primary and secondary amines. The absorbance peaks at 1472, and 921 cm^{-1} did not correspond to peaks previously found to be associated with genipin crosslinking of chitosan.[27]

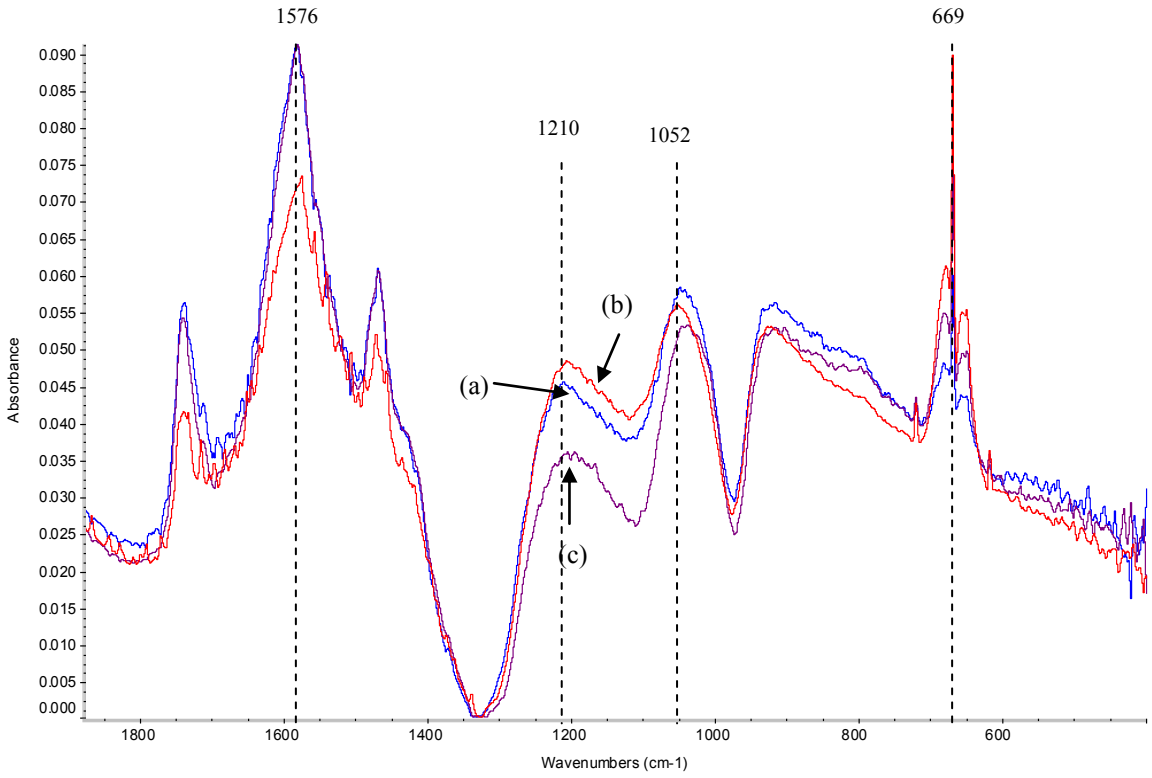

Figure 2. FTIR spectra of (a) uncrosslinked chitosan sponge (blue), (b) genipin crosslinked chitosan sponge (purple), and (c) PNIPAM/genipin crosslinked chitosan sponge (red) (n = 2 for all groups)

The absorbance peaks at 1739 and 1576 cm^{-1} decreased slightly upon the addition of genipin to the chitosan sponges and decreased significantly from the addition of PNIPAM and genipin to the chitosan sponges. Decreases in absorbance were also measured for the peak at 1052 cm^{-1} from the incorporation of PNIPAM and genipin, as well as genipin alone, into the uncrosslinked sponges, accompanied by an increase in absorbance at 669 cm^{-1} from crosslinking the chitosan sponges. The absorbance peak at 1210 cm^{-1} exhibited an increase in absorbance from the addition of PNIPAM and genipin into the chitosan sponges, and a decrease in absorbance from the addition of only genipin to the uncrosslinked chitosan sponges.

The uptake of vancomycin, amikacin, and dual loaded vancomycin and amikacin solutions by the uncrosslinked and crosslinked chitosan sponges is reported as milliliters of antibiotic solution absorbed in one minute per gram of chitosan sponge (Figure 3). The uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked chitosan sponges absorbed an overall average of 2.78 ± 1.19, 5.44 ± 1.35, 5.74 ± 0.61 mL/min of antibiotic solution (non-normalized to weight), respectively. Both types of crosslinked chitosan sponges exhibited significantly higher antibiotic uptake (mL/min/g) than the uncrosslinked chitosan sponges ($p < 0.0001$), but both the genipin crosslinked sponges and the PNIPAM / genipin crosslinked sponges were statistically similar. There were no statistically significant differences between antibiotics absorbed in each sponge type

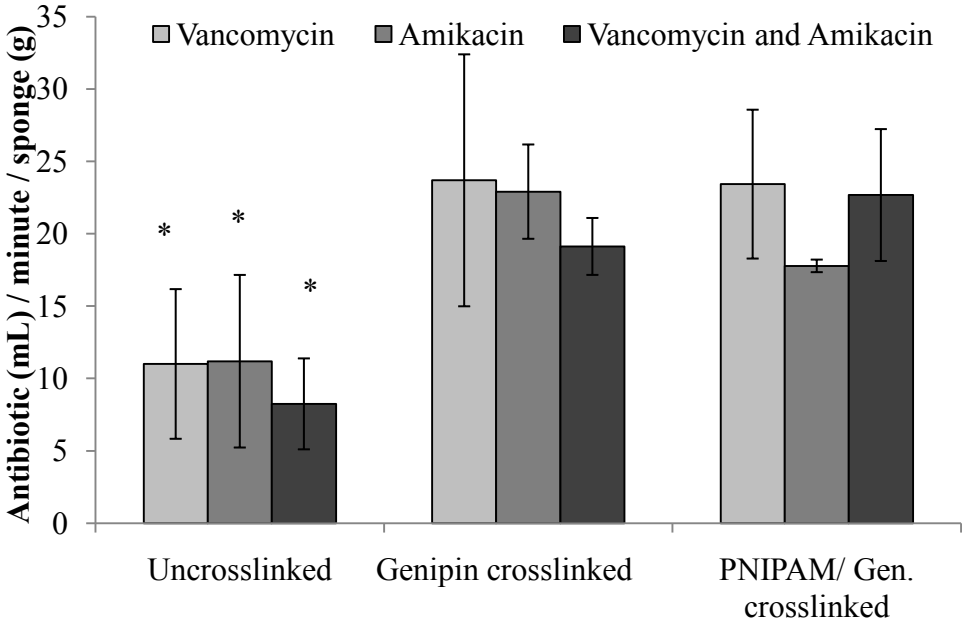

Figure 3. The antibiotic uptake of vancomycin, amikacin, and dual loaded vancomycin and amikacin solutions by uncrosslinked and crosslinked chitosan sponges, normalized to sponge weight, represented as mean ± standard deviations (n = 4 for all groups). The uncrosslinked chitosan sponges absorbed significantly less antibiotic solution than both types of crosslinked sponges (*vs all other crosslinked sponges, $p < 0.001$).

The elution studies indicated that the all three types of sponges eluted the highest levels of vancomycin and amikacin at the 1 hour time point, after which the antibiotic release levels were reduced (Figures 4 and 5). The PNIPAM/genipin crosslinked chitosan sponges, either single loaded, dual loaded, or both, eluted significantly higher quantities of vancomycin than the uncrosslinked chitosan sponges through 96 hours (Figure 4). The PNIPAM/ genipin crosslinked chitosan sponges also released significantly more vancomycin than the genipin crosslinked sponges from 24 hours to 96 hours of antibiotic release. The dual loaded PNIPAM/ genipin crosslinked chitosan sponges also eluted significantly more amikacin than the uncrosslinked chitosan sponges from 24 hours through 96 hours (Figure 5).

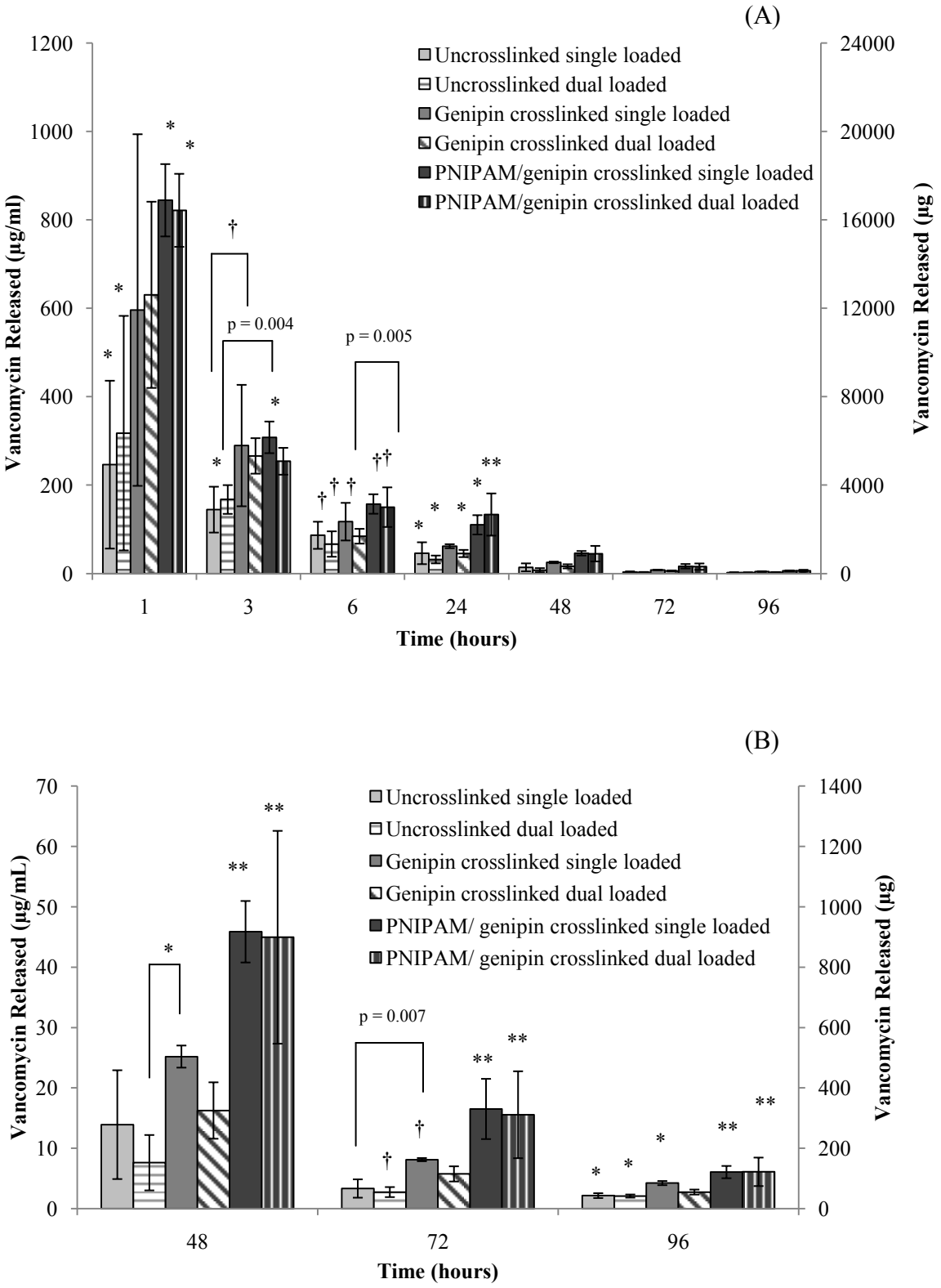

Figure 4. The vancomycin elution from (A) 1 to 96 hours and (B) an expanded view of 48 to 96 hours from uncrosslinked and crosslinked chitosan sponges, both single and dual loaded, represented as mean ± standard deviation (n = 3 for all groups). (* vs. each other, $p \leq 0.001$; † vs. each other, $p \leq 0.003$; ** vs. all others, $p \leq 0.001$)

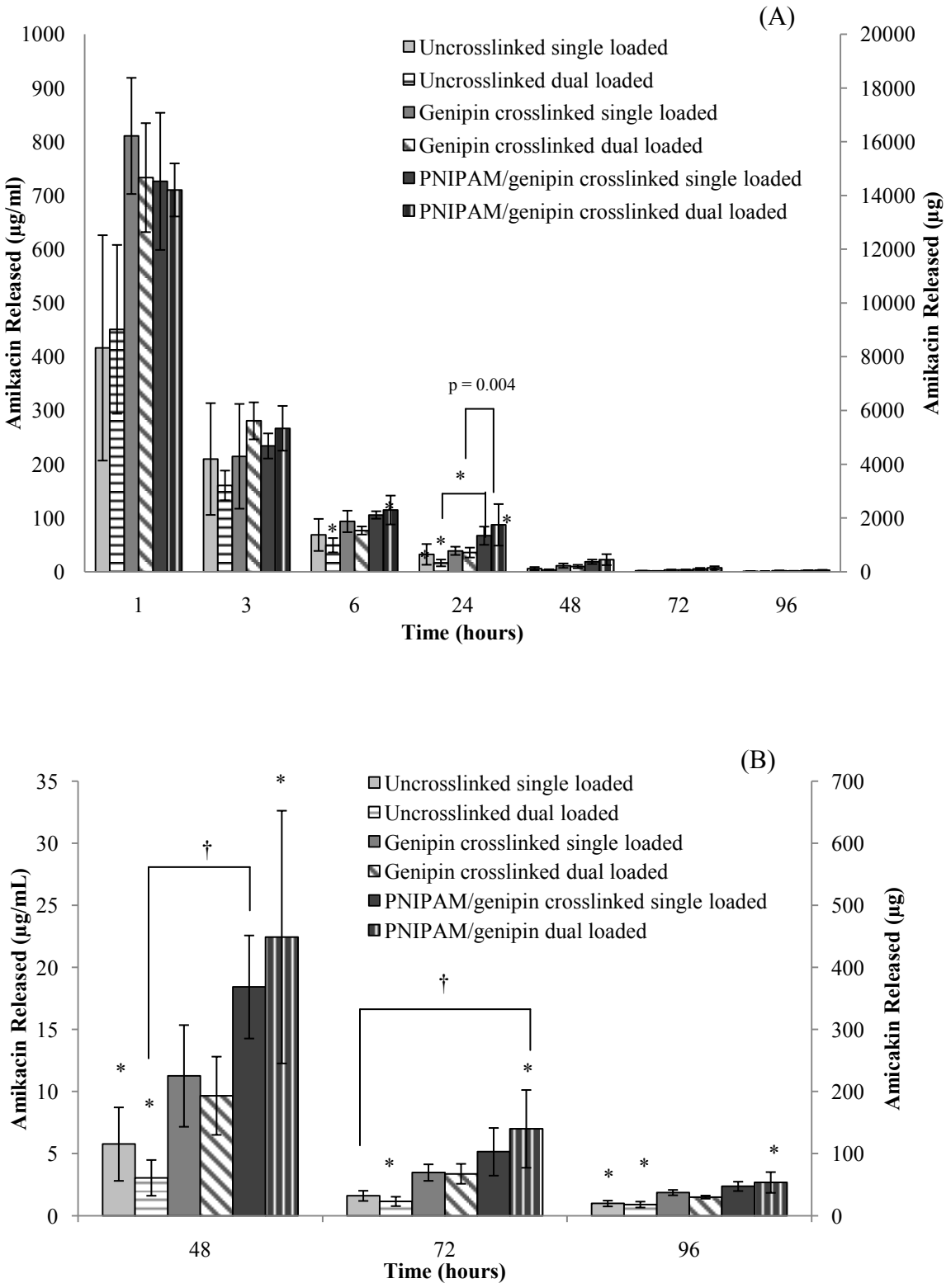

Figure 5. The amikacin elution from (A) 1 to 96 hours and (B) an expanded view of 48 to 96 hours from uncrosslinked and crosslinked chitosan sponges, both single and dual loaded, represented as mean ± standard deviation (n = 3 for all groups). (* vs. each other, $p \leq 0.001$; † vs. each other, $p \leq 0.003$; ** vs. all others, $p \leq 0.001$)

The PNIPAM/genipin crosslinked sponges exhibited a higher percent total release of single loaded vancomycin and amikacin that was loaded into the sponges than the uncrosslinked and genipin crosslinked sponges, but did not elute more dual loaded vancomycin and amikacin than the other two sponge types (Table 1).

Table 1. Total % release of antibiotics from uncrosslinked and crosslinked sponges (n = 3 for all groups)

	Uncrosslinked	Genipin crosslinked	PNIPAM/genipin crosslinked
Vancomycin Single Loaded	74.64 ± 29.99	71.87 ± 10.87	100 ± 4.68
Amikacin Single Loaded	87.24 ± 12.22	83.23 ± 4.93	89.78 ± 10.03
Vancomycin and Amikacin	90.45 ± 33.69	94.05 ± 7.54	93.53 ± 1.60

From activity testing, the vancomycin eluates from the uncrosslinked chitosan sponges, both single and dual loaded, demonstrated sufficient activity against *S. aureus* with no bacterial growth through 24 hours (Table 2). Vancomycin eluates from genipin crosslinked sponges and PNIPAM/genipin crosslinked sponges were active against *S. aureus* through 48 and 72 hours, respectively. Amikacin eluates from single loaded uncrosslinked sponges, dual loaded genipin crosslinked sponges, and both single and dual loaded PNIPAM/ genipin crosslinked sponges were active against *P. aeruginosa* through 24 hours (Table 3). Eluates from the dual loaded uncrosslinked sponges and single loaded genipin crosslinked chitosan sponges only inhibited bacterial growth through 6 hours.

Table 2. Average activity of 1:10 dilutions of vancomycin eluates from uncrosslinked and crosslinked chitosan sponges against *S. aureus* (n = 3 for all groups)

Staphylococcus aureus		Time (hours)						
Sponge type	**Loading**	**1**	**3**	**6**	**24**	**48**	**72**	**96**
Uncrosslinked chitosan	Single	-	-	-	-	+	+	+
Uncrosslinked chitosan	Dual	-	-	-	-	+	+	+
Genipin crosslinked chitosan	Single	-	-	-	-	-	+	+
Genipin crosslinked chitosan	Dual	-	-	-	-	-	+	+
PNIPAM/genipin crosslinked chitosan	Single	-	-	-	-	-	-	+
PNIPAM/genipin crosslinked chitosan	Dual	-	-	-	-	-	-	+

(-) represents no bacterial growth detected (+) bacterial growth detected

Table 3. Average activity of 1:10 dilutions of amikacin eluates from uncrosslinked and crosslinked chitosan sponges against *P. aeruginosa* (n = 3 for all groups)

Pseudomonas aeruginosa		Time (hours)						
Sponge type	**Loading**	**1**	**3**	**6**	**24**	**48**	**72**	**96**
Uncrosslinked chitosan	Single	-	-	-	-	+	+	+
Uncrosslinked chitosan	Dual	-	-	-	+	+	+	+
Genipin crosslinked chitosan	Single	-	-	-	+	+	+	+
Genipin crosslinked chitosan	Dual	-	-	-	-	+	+	+
PNIPAM/ genipin crosslinked chitosan	Single	-	-	-	-	+	+	+
PNIPAM/ genipin crosslinked chitosan	Dual	-	-	-	-	+	+	+

(-) represents no bacterial growth detected (+) bacterial growth detected

The effects of the vancomycin and amikacin eluates, both single and dual loaded, from the uncrosslinked and crosslinked chitosan sponges on 3T3 fibroblasts after 24 hours of exposure are shown in Figure 6. All three types of antibiotic eluates, vancomycin single loaded, amikacin single loaded, and vancomycin and amikacin dual loaded, exhibited similar effects on the number of cells as compared to the PBS control from 3 to 96 hours. In the single loaded vancomycin and amikacin eluates from the PNIPAM/ genipin crosslinked sponges, there was even a consistent growth of cells beyond the PBS control and the eluates from the uncrosslinked and genipin crosslinked chitosan sponges. At 1 hour, the highest antibiotic elution concentration, there were significantly fewer cells, as compared to the PBS control, from all three types of antibiotic eluates released from all three sponges. This lower cell number corresponds to higher levels of antibiotics present in the first time point, which have been reported to be cytotoxic at high levels.[33]

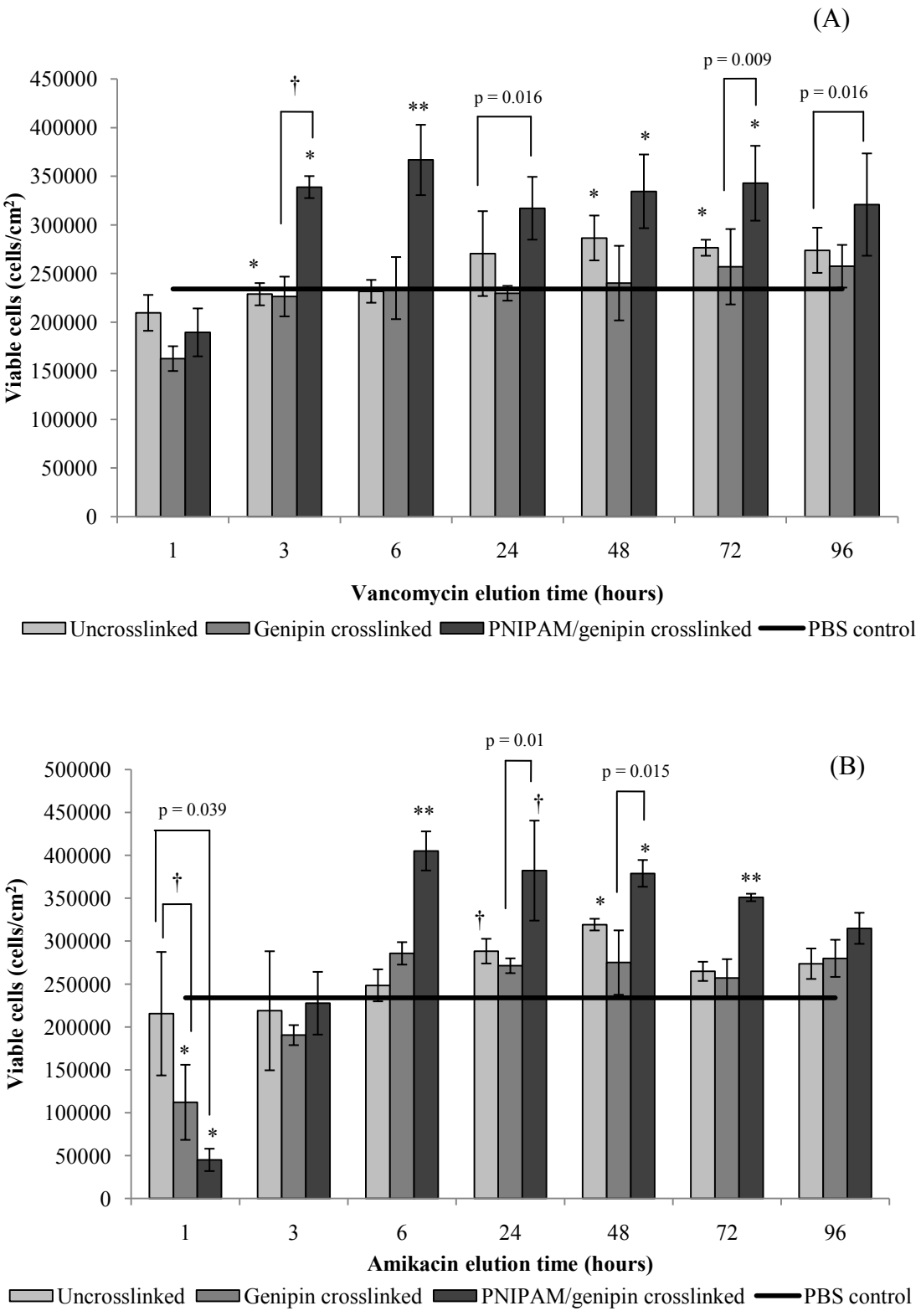

Figure 6. Number of viable cells determined with Cell-Titer Glo® assay after 24 hours of exposure to (A) vancomyin eluates, (B) amikacin eluates and (C) dual loaded vancomycin and amikacin eluates from uncrosslinked and crosslinked chitosan sponges represented as mean ± standard deviation. n = 3 for all groups (** vs. all others, $p \leq 0.001$; *, $p \leq 0.001$; †, $p \leq 0.003$)

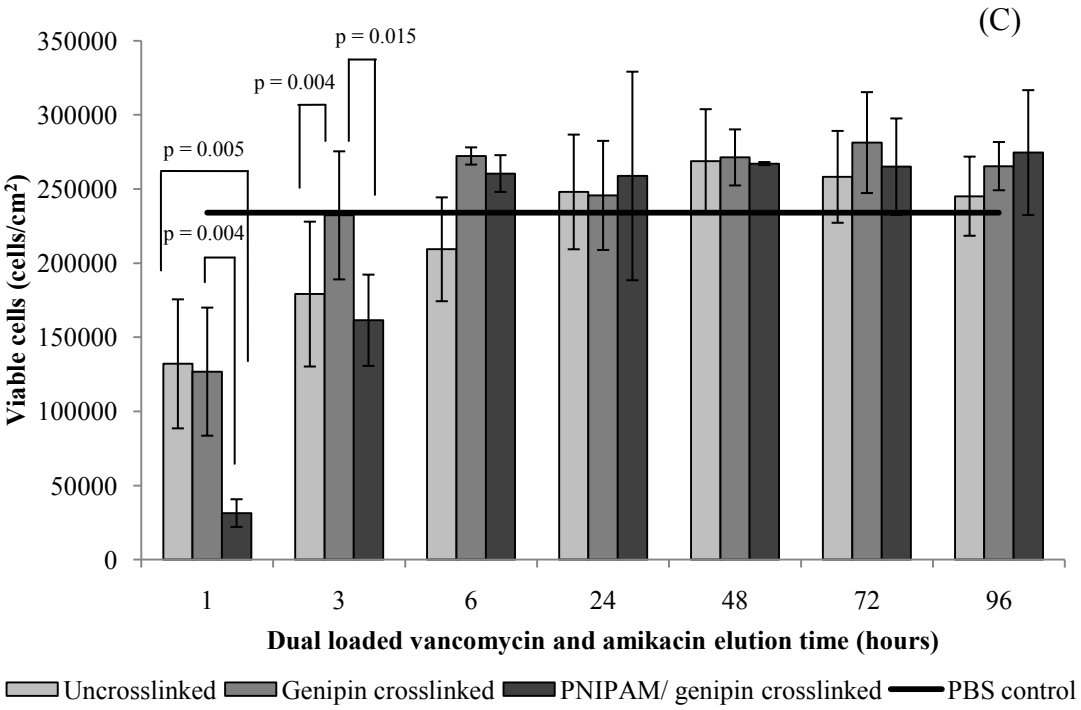

Figure 6. Number of viable cells determined with Cell-Titer Glo® assay after 24 hours of exposure to (A) vancomyin eluates, (B) amikacin eluates, and (C) dual loaded vancomycin and amikacin eluates from uncrosslinked and crosslinked chitosan sponges represented as mean ± standard deviation. n = 3 for all groups (** vs. all others, $p \leq 0.001$; *, $p \leq 0.001$; †, $p \leq 0.003$)

The effect on cell viability after 24 hours of direct contact to uncrosslinked and crosslinked chitosan sponges, with no antibiotics loaded, is provided in Table 4. All three types of sponges exhibited higher number of cells than the control biomaterial, PTFE, with significant differences between the uncrosslinked and PNIPAM/genipin crosslinked sponges and PTFE ($p < 0.0001$). Significant differences were also seen between the uncrosslinked and genipin crosslinked chitosan sponge ($p = 0.00053$).

Table 4. Cell viability, determined by Cell-Titer Glo® assay, after 24 hours of exposure to uncrosslinked and crosslinked chitosan sponges, and PTFE disks

Sample	Viable cells (cells/cm^2)	Cell Viability (%)
Uncrosslinked†	107585 ± 3474	140.05 ±4.52
Genipin crosslinked†	88273 ± 7438	114.91 ±9.68
PNIPAM/genipin crosslinked	99335 ± 15022	129.31 ± 19.56
PTFE control*	76816 ± 7964	100 ± 10.37

Data represented as mean ± standard deviation (n = 3 for all groups), [a] Cells exposed to PTFE corresponded to 100% cell viability (†, $p = 0.00053$; * vs. all others except genipin crosslinked, $p < 0.0001$)

Live/Dead® staining also revealed that the cells exposed to the uncrosslinked and crosslinked chitosan sponges exhibited similar cell shape and viability as the cells exposed to the PTFE disks (Figure 7). The uncrosslinked and crosslinked chitosan sponges exhibited low cytotoxicity, as indicated by the few number of dead cells present in all samples.

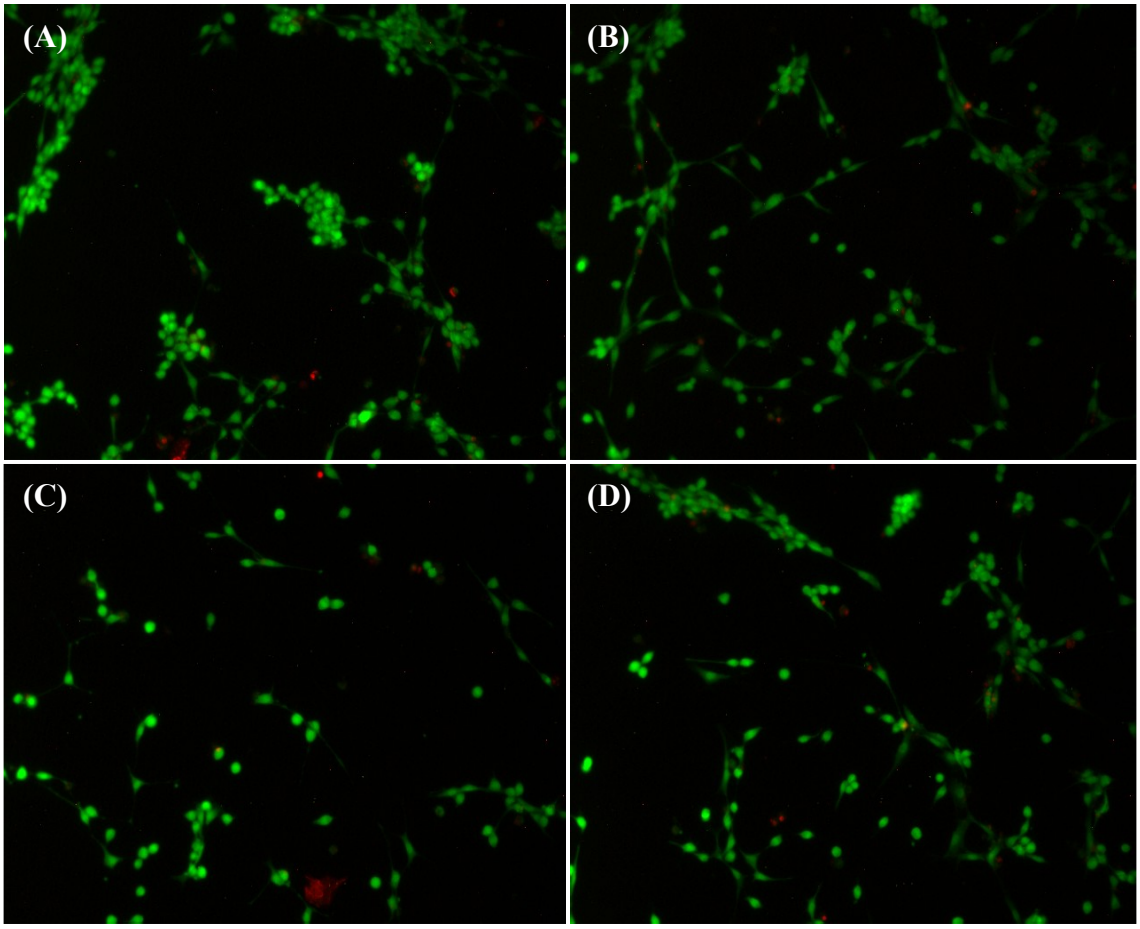

Figure 7. Representative fluorescence overlay images of 3T3 fibroblasts adjacent to test samples with Live/Dead® stain after 24 hours of exposure to (A) uncrosslinked chitosan sponge, (B) genipin crosslinked chitosan sponge, (C) PNIPAM/ genipin crosslinked chitosan sponge, and (D) PTFE disk (known biomaterial control); green and red represent viable and non viable cells, respectively. (100x magnification)

3.4 *Discussion*

The treatment of musculoskeletal infections is becoming complicated by the increased emergence of antibiotic-resistant bacteria and increases the need for local drug delivery over systemic delivery. The current local delivery systems have their drawbacks, including burst release, possible biofilm formation, need for surgical removal,

and the formation of sterile draining sinuses.[12, 14, 15, 20] This *in vitro* study proposes the creation of a local drug delivery system with tailorable elution and degradation properties, by crosslinking chitosan sponges either with genipin alone, or as a semi- IPN with genipin crosslinking and PNIPAM incorporation. These crosslinked sponge delivery systems need to be degradable, able to absorb and elute biologically active antibiotics , and exhibit low cytotoxicity.

In order to determine sponge degradation, the *in vitro* degradation properties were investigated. The finding of this study was that both genipin crosslinked and PNIPAM/genipin crosslinked chitosan sponges degrade slower with 64.74 ± 6.50 and 60.96 ± 0.80 wt% remaining respectively, in comparison to 2.62 ± 1.17 wt% of the uncrosslinked chitosan sponges remaining at week six. No previous reported studies have evaluated the *in vitro* degradation properties of genipin crosslinked chitosan sponges. However, these results follow the same trend reported in an *in vivo* study of uncrosslinked and glutaraldehyde or genipin crosslinked chitosan microspheres.[34] Mi and researchers found that, after intramuscular injection of chitosan microspheres into the skeletal muscle of Wistar rats, the uncrosslinked and glutaraldehyde crosslinked chitosan microspheres were severely degraded at 20 weeks post-operation, while the genipin crosslinked microspheres were significantly less degraded.[34] While the slower degradation in the crosslinked sponges was expected, the degradation of the uncrosslinked chitosan sponges is much higher than values reported for chitosan films and sponges found in previous reports.[32, 35, 36] Slow or rapid sponge degradation can impact active agent elution and the response of pathogenic bacteria within a wound.[37] Tomihata and researchers reported films of 1 wt% chitosan (73% DDA) in 1% acetic acid

to have approximately 90 wt% remaining after 50 hours in 4 mg/ml lysozyme solution.[32] Another study found films made from 1.5 wt% chitosan in 1% lactic acid and 1% acetic acid to have approximately 50 wt% and 25 wt% remaining after 100 hours in 100 µg/ml lysozyme solution. [35] Smaller lyophilized 1 wt% chitosan sponges (5 x 10 mm) had 18.08 ± 4.28 % weight loss after 24 hours in 8 mg/ml lysozyme solution.[36]

In order to determine that crosslinking of the chitosan sponges occurred, FTIR spectra were obtained from all three sponge types. The increase in absorbance of the N-H wag peak at 669 cm^{-1} from primary and secondary amines in the spectra of the crosslinked sponges, as compared to the uncrosslinked, could illustrate an increase in secondary amine groups in the amide linkages during crosslinking. As seen in previously reported research, the crosslinking reaction is demonstrated by the decrease in absorbance of the primary amine peak at 1576 cm^{-1} and the C-O stretching peak at 1052 cm^{-1}.[27] When crosslinking occurs, the chitosan loses primary amines and the ester group in genipin is converted to amide linkages.[27, 38] Oxygen radical-induced polymerization of genipin could explain the decrease in absorbance of the C-N stretching peak at 1210 cm^{-1} with the addition of genipin to the chitosan sponge. The green/blue color development as crosslinking occurred could also be an indicator of the genipin polymerization, or simply the reaction of genipin with primary amines.[27, 38]

Antibiotic uptake and elution are two of the most important properties for a local drug delivery device and have been investigated in many chitosan delivery systems, including sponges[20], microspheres[23, 39], hydrogels[31], and films[35]. It has been previously reported that point of care antibiotic loading, loading of antibiotics during surgery or treatment, of local drug delivery systems provides the advantage of the physician's

ability to select an antibiotic based on the patient's needs.[40] The similar values in antibiotic uptake between both crosslinked sponges suggests that the increase in antibiotic uptake, compared to the uptake in the uncrosslinked sponges, is due more to the genipin crosslinking than to the presence of PNIPAM. Noel et al previously reported vancomycin and amikacin uptake to be approximately 7 mL/min in uncrosslinked chitosan sponges constructed with 25 mL of 1% (w/v) chitosan solution; however, after the normalization of uptake to approximate sponge weight, the uptake values of both antibiotics in the uncrosslinked sponges are similar to those reported in this research.[20]

The increase in antibiotic uptake in both of the crosslinked chitosan sponges corresponds to an overall increase in antibiotic elution as well. The results of this elution study differ from previous research with antibiotic or albumin pre-loaded genipin crosslinked chitosan microspheres; as crosslinking increased the overall antibiotic or protein release decreased.[23, 39] However, this difference is expected because the antibiotic and protein were both pre-loaded during construction of the microspheres, as opposed to point of care loading after the microspheres were created. In another reported study, the overall drug release increased as glutaraldehyde crosslinking increased in full-IPN hydrogels of bis(acrylamide) crosslinked PNIPAM and glutaraldehyde crosslinked chitosan with point of care loading.[31] The concentration of vancomycin released after 1 hour from the uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked chitosan sponges in this study is approximately 75, 40.8, and 16.2% lower than vancomycin released after 1 hour, 1007.4 ± 162.8 μg/ml, from uncrosslinked sponges previously reported in the Noel study.[20] The same study reported the uncrosslinked chitosan sponges released 881.5 ± 15.4 μg/ml of amikacin after 1 hour, which is

approximately 52, 8, and 18% higher than the amikacin released from the uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked sponges in this study, respectively. However, if the previously reported elution values from the Noel study are normalized to approximate sponge weight, there should be no significant differences from this research.[20]

The biologic activity of the eluted vancomycin and amikacin was verified by measuring the effective activity against specific strains of *S. aureus* and *P. aeruginosa*, with MICs of 0.5 – 1 μg/ml and 4 μg/ml, respectively.[20] The single loaded vancomycin eluates from the uncrosslinked, genipin crosslinked, and PNIPAM/ genipin crosslinked chitosan sponges inhibited growth of *S. aureus* through 24, 48, and 72 hours, respectively. Single loaded amikacin eluates from the uncrosslinked, genipin crosslinked, and PNIPAM/ genipin crosslinked chitosan sponges inhibited growth of *P. aeruginosa* through 24, 6, and 24 hours, respectively. Noel et al previously reported that vancomycin and amikacin eluates from uncrosslinked chitosan sponges inhibited *S. aureus* and *P. aeruginosa* through 72 and 48 hours, respectively.[20] However, the longer inhibition times correspond to higher levels of antibiotics released from larger sponges, and after normalization to sponge weight, the inhibition times would be similar to the inhibition found in this study, based on the MIC levels. [20] As previously mentioned, the activity assay was conducted with a 1:10 dilution of the antibiotic eluates; this dilution factor accounted for bacterial growth when original eluates were above MIC levels.

The cytotoxicity testing of the sponges and the antibiotic eluates was investigated in order to assess the biocompatibility of the sponges, the antibiotics, and any potential leachable substances from the sponges. The direct contact cytotoxicity test with the

sponges resulted in viable cells and low cytotoxicity when exposed to all three sponge types for 24 hours. Cell viability percentages for L929 fibroblasts exposed to genipin crosslinked chitosan and silk fibroin sponges for 24 hours were previously reported to be 79.1 ± 13.3, 53.5 ± 21.1, and 69.5 ± 29.7% cell viability for sponges with 80/20, 50/50, and 20/80 chitosan to silk ratios, respectively.[28] However, the reported cell viability percentages were calculated with cells grown directly on tissue culture plastic as the control 100% cell viability.[28] When cells exposed to PTFE controls represent 100% cell viability, the previously reported cell viability percentages are similar to the cell viability percentages for the uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked sponges in this study.[28] The increased number of viable cells exposed to the uncrosslinked and crosslinked sponges compared to the PTFE control could be caused by the increased structural porosity in the sponges versus the solid PTFE disks. The cytotoxicity test with the antibiotic eluted from the sponges also resulted in viable cells and low cytoxicity. The PNIPAM/genipin crosslinked chitosan sponge eluates resulted in a large number of viable cells, indicating that the PNIPAM/genipin crosslinked chitosan sponges harbor a favorable environment for cell proliferation with these study conditions.

3.5 *Summary and Conclusions*

This *in vitro* study demonstrates that crosslinking chitosan sponges with genipin or genipin and PNIPAM creates novel, point of care antibiotic loaded, crosslinked sponges which exhibit potential for use as a degradable adjunctive therapy for musculoskeletal infection control. Crosslinking chitosan sponges with genipin and genipin crosslinking in the presence of PNIPAM resulted in an increase in vancomycin

and amikacin uptake, release of antibiotics for improved bactericidal efficacy against known pathogens, and a decrease in degradation, as compared to uncrosslinked chitosan sponges. The crosslinking of the chitosan sponge appears beneficial when a slower degradation or an increased release of vancomycin or amikacin is desired, and the addition of PNIPAM to genipin crosslinked chitosan sponges even further increases, and sustains, the release of vancomycin or amikacin. The novel crosslinked chitosan sponges and their antibiotic eluates elicited minimal cytotoxic response from cells, similar to other biomaterials. Limitations of this study are, 1) the short duration of the cytotoxicity study, 2) the biologic activity assays were only conducted against planktonic bacteria, not adherent bacteria typically associated with biofilms, and 3) the translation of *in vitro* research results to *in vivo* efficacy. For an accurate evaluation of clinical performance, *in vivo* studies with the crosslinked chitosan sponges should be pursued in the future. In order to fully characterize the properties of the crosslinked sponge delivery systems, further investigations into the swelling and more extended degradation and elution studies of the sponges are needed.

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CHAPTER 4:

CONCLUSIONS

In this preliminary *in vitro* study, novel genipin crosslinked sponges and sponges made from a semi-interpenetrating network of genipin crosslinked chitosan and poly(n-isopropylacrylamide) were developed for potential use as nontoxic, degradable, local drug delivery systems to minimize or treat musculoskeletal infections. Both crosslinked sponges demonstrated decreased degradation, when compared to the uncrosslinked chitosan sponges. The genipin crosslinked and PNIPAM/genipin crosslinked sponges absorbed and released more vancomycin and amikacin than the uncrosslinked sponges. The incorporation of PNIPAM into a genipin crosslinked sponge also prolonged the release of both antibiotics and the antibiotics' corresponding specific biologic activity. The antibiotic eluates from all of the sponges exhibited effective biologic activity against the known musculoskeletal pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and, along with the sponges, did not induce any short term cytotoxic effects.

The preliminary *in vitro* data from this study demonstrates that the incorporation of the nontoxic crosslinker genipin, either alone or with the thermo-responsive polymer PNIPAM, into chitosan sponges creates tailorable drug delivery systems that may show future promise for *in vivo* studies and translation to clinical evaluations. The ability of a physician to select a type of sponge, antibiotic, and antibiotic concentration based on a patient's needs could potentially result in faster and more efficacious treatment of infections, and even a possible reduction of antibiotic resistance development in the patient. While additional *in vivo* and *in vitro* studies are needed in order to assess the true effectiveness and long term biocompatibility of these crosslinked sponges, this

preliminary study has demonstrated that crosslinking chitosan sponges, with genipin and PNIPAM and genipin, is beneficial when a more sustained degradation and antibiotic elution time is desired.

CHAPTER 5:

FUTURE WORK

In order to fully evaluate the properties of the crosslinked sponges, additional *in vitro* materials characterization tests are needed, including swelling measurements, more extended degradation studies with non loaded and antibiotic loaded sponges, and more extended elution studies, with additional antibiotics. The bioadhesive strength of the sponges when loaded with PBS and antibiotics also needs to be assessed, with a greater number of samples. Long term cytotoxicity and cell proliferation tests would provide beneficial information about the impact of the sponges on cells before additional *in vivo* tests are conducted. Specifically, an *in vivo* degradation study in rats would provide a more accurate degradation profile of the sponges and local tissue response. In addition to an *in vivo* degradation study, a proof of principle infected mouse catheter animal model would demonstrate the true efficacy of sponges as a local antibiotic delivery system preventing and treating bacterial infections.

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APPENDIX A:

Crosslinking Chitosan Sponges with Dimethyl 3, 3 dithio bis propionimidate (DTBP)

Introduction

Chitosan hydrogels have also been successfully crosslinked through disulfide bridges with dimethyl 3, 3 dithio bis propionimidate (DTBP), but the hydrogels were not in a lyophilized sponge form.[62] Crosslinked chitosan hydrogel scaffolds prepared with DTBP exhibited similar properties to glutaraldehyde crosslinked chitosan scaffolds, but showed greater strength and less toxicity.[62] Therefore, DTBP crosslinked chitosan sponges were created and evaluated through a vancomycin elution screening study.

Materials and Methods

DTBP and Tris were purchased from Sigma Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). A 5 mM DTBP solution was prepared by adding 0.03865 grams of DTBP to 25 mL of 0.2 M Tris solution, at a pH of 9, and mixing. A lyophilized and neutralized 1 % (w/v) chitosan sponge, with 71% DDA, was submerged into the DTBP solution and allowed to crosslink at ambient temperature for approximately 25 hours. The sponge was then rinsed multiple times with deionized water, until a neutral pH was reached. The sponges were then placed in the -80°C freezer for one hour and then lyophilized for 24 hours.

The DTBP crosslinked sponge, with a weight of 0.1739 grams, was submerged into ten milliliters of a 5 mg/ml vancomycin solution for one minute. Immediately after vancomycin loading, the sponge was submerged in 20 mL of sterile 1 x PBS and was incubated at 37°C for the duration of the study. One milliliter aliquots were taken at 1, 2, 25, and 72 hours, with complete PBS refreshment at each time point. The elution

samples were immediately frozen after acquisition. Fluorescence polarization immunoassay (TDxFLx) with a vancomycin specific reagent kit was used to quantify the vancomycin released from the sponge.

Results and Discussion

The 5 mM DTBP crosslinked chitosan sponge was smaller in size, with a 50% smaller diameter, than the uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked chitosan sponges and appeared less porous. A photograph of the crosslinked sponge was taken with a digital camera, as seen in Figure 1. The DTBP crosslinked sponge absorbed only 0.5 mL of the vancomycin solution, which was 82.0, 90.8, and 91.3% less than average volume of antibiotic solution absorbed by the uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked chitosan, respectively.

Fig. 1. Photograph of 5 mM DTBP crosslinked chitosan sponge

The low amount of vancomycin released from the DTBP crosslinked chitosan sponge can be seen in Figure 2. However, this low elution was expected due to the low antibiotic uptake. Because of the reduced apparent porosity, vancomycin uptake and elution, the DTBP crosslinked chitosan sponges did not appear to be effective for a local drug delivery system and were not studied more extensively. While the DTBP crosslinked sponge was not absorbent enough to effectively release antibiotics, it might demonstrate greater potential as a tissue engineering scaffold because of better strength and handling properties, as compared to the uncrosslinked and genipin crosslinked chitosan sponges.

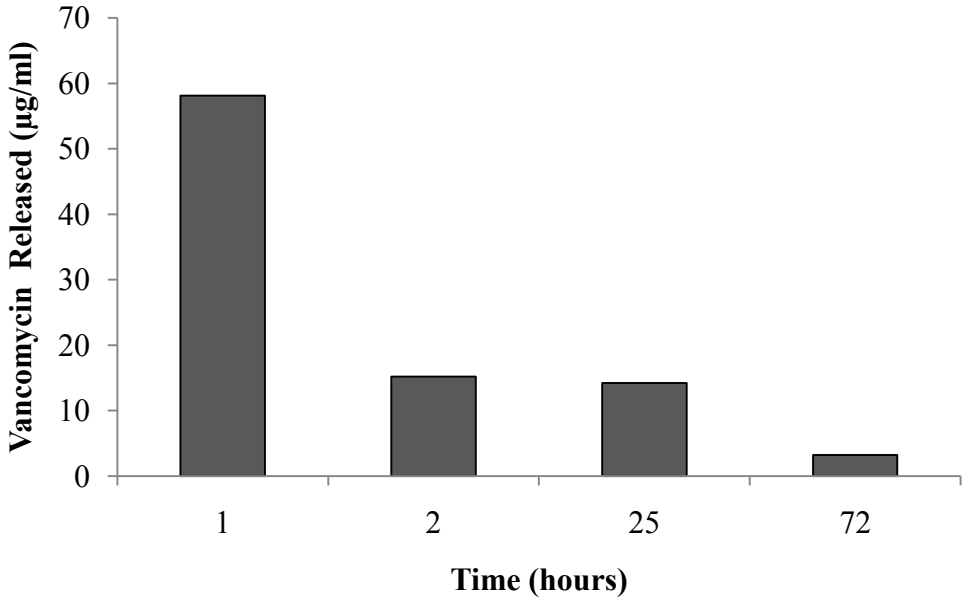

Fig. 2. The vancomycin elution from a DTBP crosslinked chitosan sponge (n = 1)

APPENIDIX B:

Bioadhesion Testing

Introduction

Bioadhesion is critical in musculoskeletal infection control because the material needs to be able to stay in place in order to deliver the drugs to the target site. In previous research on chitosan sponges, some of the sponges studied in an animal study did not adhere to the local tissues of goats, reducing the effectiveness of the local drug delivery.[60] Measuring the bioadhesive strength of the sponges *in vitro* could provide a method of comparison between the uncrosslinked and crosslinked chitosan sponges before *in vivo* testing.

Experimental methods of analyzing and measuring bioadhesion vary greatly and no standard test methods have been developed.[92] Thus, it can be difficult to compare data from different experiments. Various types of mechanical tests have been developed for testing bioadhesion, both *in vivo* and *in vitro*. One research group developed a customized piece of equipment for bioadhesion tensile testing, where mouse skin was placed on either side of the hydrogel and bonding strength of the hydrogels was reported as the maximum detachment force.[93] While studying a fucoidan-chitosan hydrogel for burn healing applications, researchers used a Texture Analyzer and chicken back skin and calculated the work of adhesion as the area under the curve of a force distance plot.[94] Tensile and shear measurements are the most common mechanical tests utilized for bioadhesion testing.[92] In this preliminary study, a standard tensile testing method was modified for bioadhesive strength measurements of the uncrosslinked and crosslinked chitosan sponges.

Materials and Methods

USDA grade boneless beef chuck steak was purchased from a local grocery store, Kroger, and was allowed to equilibrate to ambient laboratory temperature. The steak was cut into pieces to fit on a stage fixture, with a 7.62 cm diameter, attached to a Instron 33R, model 4465, Universal Testing Machine (Norwood, MA). The steak was attached to the stage with Scotch® double sided tape. A washer style disk made of ultra high molecular weight polyethylene (UHMWPE), with a full diameter, a cut out inner diameter, and thickness of 7.62, 3.06, 0.635 cm, respectively, was centered and fixed onto the top of the steak, held in place temporarily with standard office binder clips (Figure 3). The UHMWPE washer contained one rough side for attachment to the meat, and one smooth side so as not to interfere with the adhesive strength of the sponges. The binder clips were removed from the meat and UHMWPE washer approximately one minute before testing and were replaced between each sponge bioadhesion test. The UHMWPE washer was clamped onto the steak until the meat was raised just slightly higher than the disk itself, as seen in Figure 3, so that the sponges would only attach to the steak. In order to keep the meat from drying, the meat was sprayed with deionized water, which was allowed to absorb for approximately 5-10 minutes, between bioadhesion testing of each sponge. A similar fresh piece of chuck steak was cut and fixed to the testing machine every time a sponge group was changed.

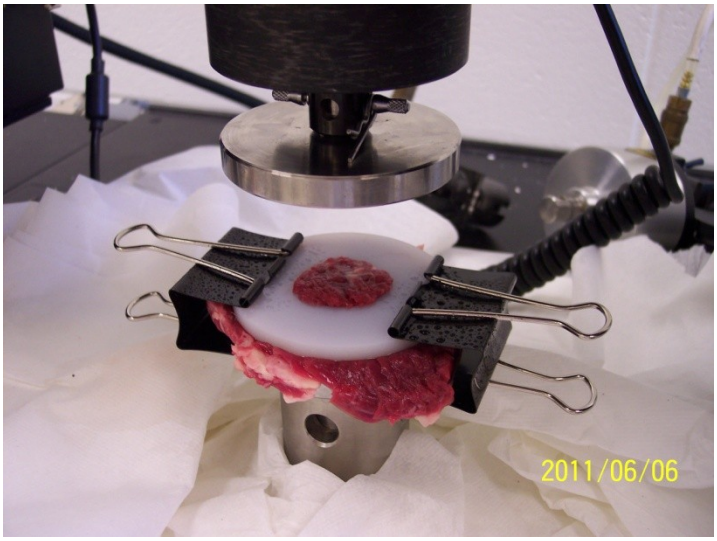

Fig. 3. Bioadhesion testing preparation by temporarily clamping boneless chuck steak and a UHMWPE washer with binder clips, in order to keep the two materials in place and to raise the meat above the washer.

Three replications of uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked chitosan sponges were submerged in 10 mL of deionized water for 1 minute and centered and attached to the top stage of the universal testing machine with Scotch® double sided tape (Figure 4). Using a 500 N load cell automated by Instron Bluehill software, a compression preload of approximately 15 N was applied to each sponge. Immediately after applying the preload force, the sponge and the meat were pulled apart in tension at a rate of 50 mm/min. Data was recorded every 30 milliseconds and was provided in maximum force (N) values, which was divided by the surface area of the exposed steak to determine adhesive strength (kPa). All quantitative data are expressed as mean ± standard deviation ($n \geq 3$ for all groups). Statistical analysis of the

bioadhesion data was conducted using one way ANOVA and Holm-Sidak post hoc analysis. Analysis was performed using 2007 Microsoft Excel software (Microsoft, Inc, Redmond, WA) and a p value of < 0.05 was considered statistically significant.

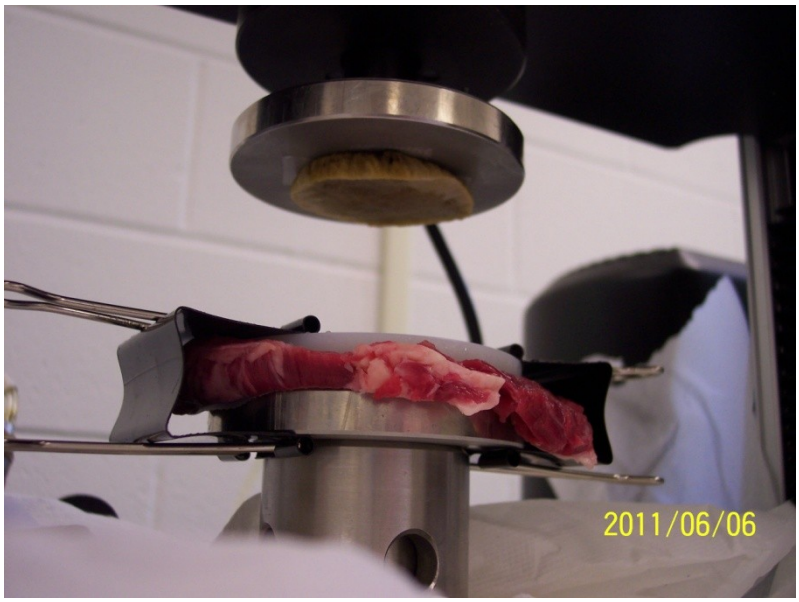

Fig. 4. Bioadhesion testing fixtures with a genipin crosslinked chitosan sponge attached to the top cylindrical fixture of the universal testing machine

Results and Discussion

The bioadhesive strength in kPa of each type of chitosan sponge is provided in Figure 5. The maximum force required for the uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked chitosan sponges to detach was 11.17 ± 3.18, 7.33 ± 1.46, and 8.44 ± 0.99 N, respectively. There was a significant difference between the uncrosslinked and genipin crosslinked chitosan sponges ($p = 0.001$). Crosslinking the chitosan sponge appears to slightly reduce the bioadhesive strength, not increase the

bioadhesion as originally hypothesized. The maximum detachment force of the all three sponge types are higher than peak detachment force reported for 2 % (w/v) chitosan films made with either 2 % (w/v) acetic or 1% (w/v) lactic acid, 0.47 ± 0.03 and 0.71 ± 0.02 N, respectively.[95] However, the maximum detachment forces of the uncrosslinked and crosslinked chitosan sponges found in this study were lower than the average peak detachment force value, 46.93 N, found for 1% chitosan sponges (85% DDA) made with 2% acetic acid.[96] The bioadhesion testing methods used to obtain the maximum detachment force values in the two previous studies are similar to the modified tensile test presented here, but the two previous studies utilized a texture analyzer and chicken pouch tissue or rabbit thigh muscle.[95, 96]

While *in vitro* bioadhesion tests provide useful material characterization properties, the wide variety of testing methods and types of data seen in previous research makes direct comparisons between studies difficult. However, modified *in vitro* bioadhesion tests do provide a faster and easier method of comparison between different materials, as compared to *in vivo* studies. Limitations of this modified tensile test for bioadhesion include 1) the test was conducted with a large load cell and needs to be ran with a smaller load cell for more accurate results, 2) to better simulate physiological conditions, the sponges need to be loaded with PBS, instead of deionized water, and 3) the bioadhesion of the sponges should be tested on different types of tissues. Additionally, while *in vitro* bioadhesion tests provide useful material characterization properties, the most accurate bioadhesion test is an *in vivo* animal model. *In vitro* results can be difficult to translate to *in vivo* studies and bioadhesion characteristics *in vivo*, especially in a musculoskeletal wound, are different from *in vitro* bioadhesion properties.

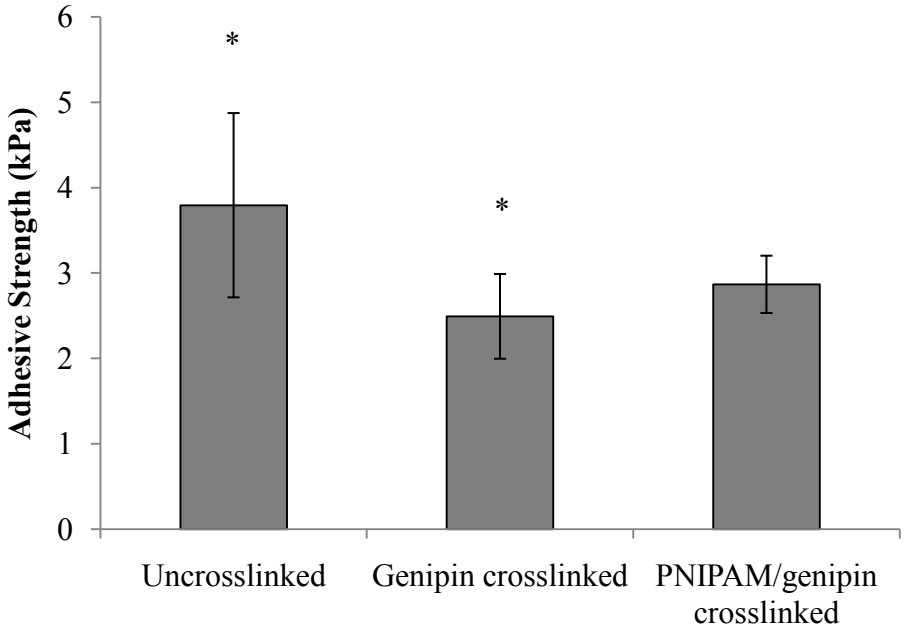

Fig. 5. Adhesive strength (kPa) of uncrosslinked, genipin crosslinked, and PNIPAM/ genipin crosslinked chitosan sponges to USDA grade boneless chuck steak, represented as mean ± standard deviation with n = 3 for all groups (*, p = 0.001)

APPENDIX C:

Photographs of Uncrosslinked and Crosslinked Chitosan Sponges

Photographs of the uncrosslinked, genipin crosslinked, and PNIPAM/genipin crosslinked were acquired with a Kodak Easy Share DX4530 digital camera. Neutralized uncrosslinked, genipin crosslinked, and PNIPAM/ genipin crosslinked chitosan sponges can be seen in Figure 6. The genipin crosslinked chitosan sponge exhibits a green color, due to the crosslinking reaction. The PNIPAM/ genipin crosslinked chitosan sponge exhibits a slightly bluer color than the genipin crosslinked chitosan sponge.

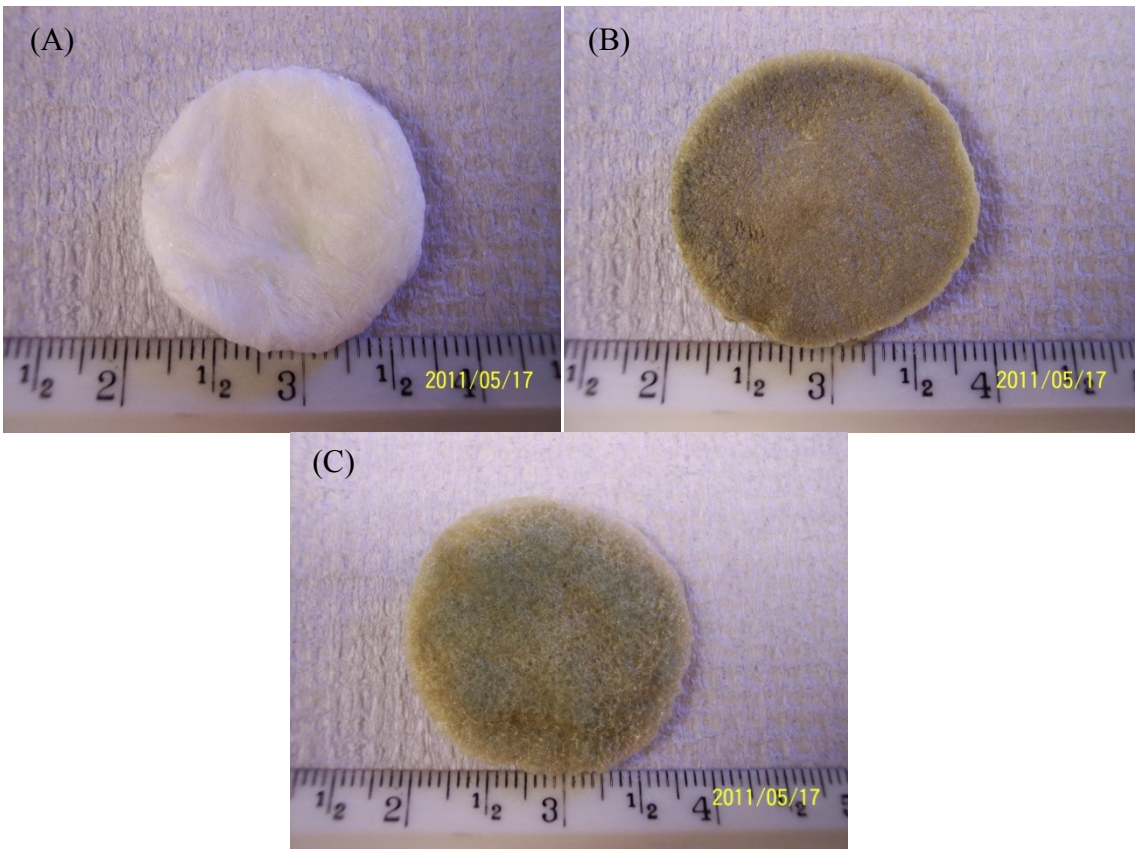

Fig. 6. Photographs of neutralized (A) uncrosslinked chitosan sponge (B) genipin crosslinked chitosan sponge and (C) PNIPAM/ genipin crosslinked chitosan sponge

APPENDIX D:

Water Uptake in the Uncrosslinked and Crosslinked Chitosan Sponges

In addition to antibiotic solution uptake studies, the uptake of deionized water into the uncrosslinked and crosslinked sponges was investigated to determine if antibiotics are absorbed differently than water. The same procedure for uptake was followed as outlined in the materials and methods section of Chapter 3, except 10 mL of deionized water was used in place of 10 mL of 5 mg/ml antibiotic solution. The volume of water absorbed into the sponges and the volume of water absorbed, normalized to the weights of the sponges, is provided in Table 1. Significant differences were seen between the uncrosslinked and genipin crosslinked sponges ($p = 0.0015$) and between the uncrosslinked and PNIPAM/genipin crosslinked sponges ($p = 0.0017$).

Table 1. Deionized (DI) water uptake in uncrosslinked and crosslinked chitosan sponges (n = 3 for each group)

Sponge	DI Water Uptake (mL)	DI Water Uptake (mL/ g of sponge)
Uncrosslinked *, †	1.63 ± 0.39	7.86 ± 2.51
Genipin crosslinked*	4.83 ± 1.27	20.52 ± 5.80
PNIPAM/genipin crosslinked†	5.35 ± 0.07	29.41 ± 1.00

Data represented as mean ± standard deviation (*, $p = 0.0015$; †, $p = 0.0017$)

The volume of water absorbed by the sponge normalized to sponge weight was lower in the uncrosslinked and genipin crosslinked chitosan sponges than the volume of antibiotic solution absorbed (see Figure 4 in Chapter 3). However, the PNIPAM/genipin

crosslinked sponges absorbed more water (mL/g) than antibiotic solution (mL/g). The introduction of PNIPAM into the genipin crosslinked sponges might make the sponge more absorbent overall because of the increase of interpenetrating polymer chains, but could also be causing steric hindrance issues with the antibiotics.

APPENDIX E:

Degradation of Uncrosslinked Chitosan Sponges Neutralized in 1 M NaOH

The high degradation of the uncrosslinked chitosan sponges reported in Chapter 3, significantly higher than values previously reported, raised concerns about residual acidity in the sponges. In order to evaluate the neutralization process and the resulting degradation, uncrosslinked chitosan sponges were constructed by the same procedure as noted in Chapter 3 and neutralized in 1 M, instead of 0.2 M, NaOH. The sponges were submerged in the NaOH solution for approximately two to three minutes, until bubbles were no longer emerging from the sponges. The sponges were then submerged in approximately 750 mL of distilled water and stirred constantly on a stir plate. After ten minutes, the pH of the water was tested using an Accumet basic AB15 pH meter and electrode and the distilled water was refreshed. The sponges were allowed to stir in the distilled water for approximately 30 more minutes, the water was refreshed again and the same procedure was followed two more times. Both the pH of the water and the pH of the sponges were tested using a pH electrode and pH strips. The sponges were returned to the -80°C freezer for one hour, and then re-lyophilized for 48 hours.

The sponges were cut into pieces, with three replicates for each time point, and tested for degradation following the same protocol outlined in Chapter 3. Because the originally tested uncrosslinked sponges degraded so quickly, degradation samples were analyzed after 1, 3, 5, 7, and 14 days, instead of after 1, 2, 3, and 6 weeks.

The results from degradation study on the neutral uncrosslinked chitosan sponges are provided in Figure 7. The percent remaining of the sponges neutralized in 1 M NaOH is significantly higher than the percent remaining of the sponges neutralized in 0.2 M

NaOH seen in Chapter 2. After one week in the lysozyme solution, 92.24 ± 0.66% of the original 1 M NaOH neutralized sponges remained, while only 4.49 ± 2.28% of the 0.2 M NaOH chitosan sponges remained. However, there were no significant differences between the degradation times of the 1 M NaOH neutralized sponges ($p = 0.99$).

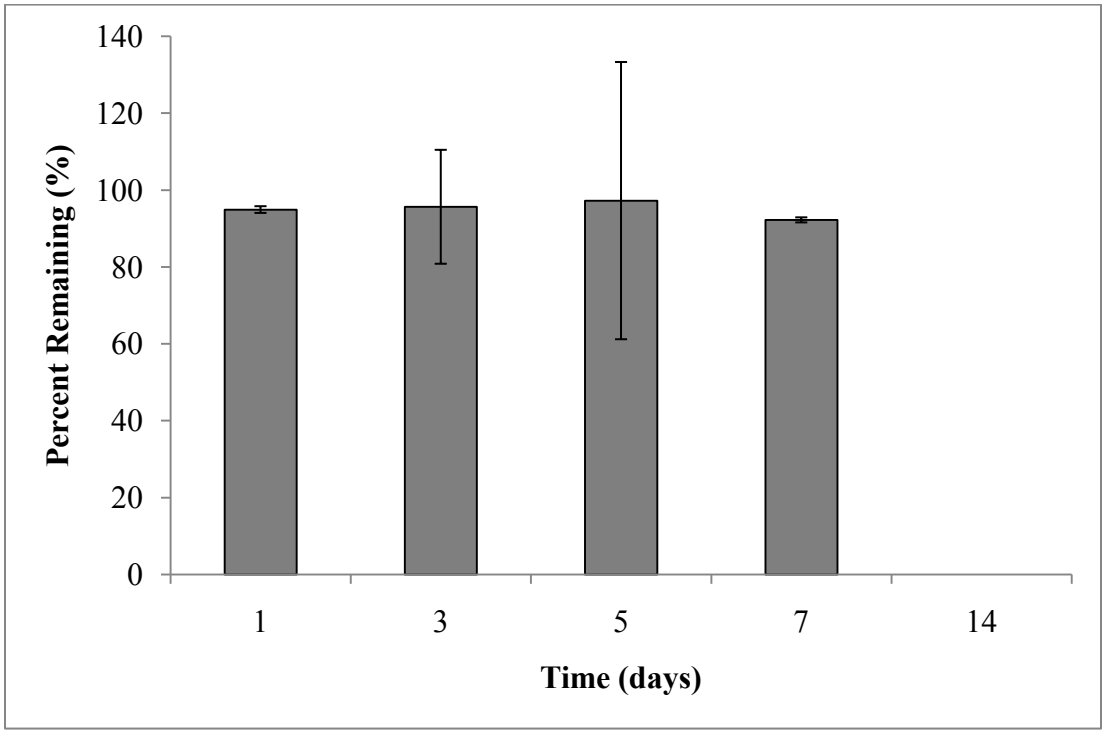

Fig. 7. The degradation of unsterilized and uncrosslinked chitosan sponges, neutralized 1 M NaOH, without antibiotics in 100 µg/ml lysozyme solution represented as mean ± standard deviation. n = 3 for all groups ($p = 0.99$)

These degradation results indicate there is a difference in the acidity of the sponges, when neutralized in different concentrations of NaOH. While sterilization might also be a factor in the differences in degradation, sterilization should theoretically result in a decrease in degradation, not an increase, because of the small amount of

crosslinking induced through the gamma irradiation process. Although direct comparisons with the crosslinked chitosan sponges cannot be made because of sterilization differences and a shorter study time, the degradation trend of completely neutralized uncrosslinked chitosan sponges can be seen through this preliminary study. For comparison with the crosslinked sponges, another *in vitro* degradation study should be conducted with sterilized, 1 M NaOH neutralized, uncrosslinked chitosan sponges for six weeks.