7-20-2018

In Vitro Evaluations of the Effects on Chitosan Paste by the Derivatization of Chitosan

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IN VITRO EVALUATIONS OF THE EFFECTS ON CHITOSAN PASTE BY THE DERIVATIZATION OF CHITOSAN

by

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A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

Major: Biomedical Engineering

The University of Memphis

August 2018

DEDICATION

I would like to dedicate this thesis to my family for all of the support that they have provided me throughout the years, especially my loving wife Madison.

ACKNOWLEDGEMENTS

First, I would like to acknowledge my thesis committee for all of the assistance that they have provided me in completing this body of work. It would not have been possible without them. A special thanks to my advisor, Dr. Jennings, that supported me on all of my ideas, helped me chase them, and steered me away from the not so good ones. Also, a special thanks to Dr. Haggard for initially recruiting me to the University of Memphis and getting me interested in this research. I would like to also thank various faculty members across the University of Memphis and University of Tennessee Health Science Center for encouraging me along the way. This work would have also not been possible without assistance from many of my fellow graduate and undergraduate students. I would like to thank Michael Harris, Leslie Pace, and Carlos Wells for all of the assistance they have provided me by teaching me how to perform various studies, assisting with data collection, and analyzing the data. I would also like to thank Nate Webb, Kwei-Yu Liu, Marcin Guzinski, and Bradley Hambly for assistance with the synthesis and characterization of the studied materials. Also, a big shout out to some of the undergraduates in our lab including Brandico Barr, Landon Choi, and Samer Abdulahi for always offering to help with setting up studies and collecting data. Finally, I would like to thank Hope Clippinger and Allison Stocks for all of the support that they have provided.

PREFACE

The main body of this thesis in Chapter III is a journal article entitled “A degradable chitosan derivative based delivery system for local delivery of antimicrobials: Preliminary Evaluations and Assessments” This manuscript was submitted to the Journal of Biomedical Materials Research Part B: Applied Biomaterials.

ABSTRACT

Complex musculoskeletal wounds with high rates of infection can be managed with local delivery systems and systemic antimicrobials. Chitosan has been shown to be biocompatible and biodegradable but exhibits bolus release kinetics. Trimethyl chitosan (TMC) has tailorable degradation properties, and poly(ethylene glycol) diacrylate chitosan (PEGDAc) is cross-linked and exhibits enhanced swelling characteristics. This research investigated a combination of TMC and PEGDAc as an injectable local delivery system. Combination paste eluted active vancomycin and amikacin for 6 and 5 days, respectively, and was degraded after 14 days. Cytocompatibility with NIH3T3 fibroblast and MC3T3 pre-osteoblast cells was above viability standards in ISO 10993-5. Combination paste required 12% of a benchmark force to eject from standard 1 mL syringes. The combination adhered to muscle tissue and was easily removed by irrigation. These preliminary results indicate the combination of TMC and PEGDAc could be further developed for infection prevention.

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CHAPTER I

INTRODUCTION

Statement of Clinical Problem

Bacterial infections are one of the most dreaded complications for any classification of injury or surgical procedure. Injuries sustained from high-energy trauma such as car accidents, sports injuries, or explosive blasts are particularly prone to bacterial colonization.[1,2] These classes of injury typically involve a combination of soft and hard tissue with complex geometries and compromised vasculature that is ideal for bacterial adherence and growth. Administration of systemic antibiotics within 3 hours of sustaining injury has been associated with decreased infection rates.[3,4] If antibiotics are not administered during this time frame or are not effective against the invading pathogen, infection may develop and substantially increase morbidity, mortality, and difficulty of treating the injury. A recent study investigated the incidence of casualties during the wars in Iraq and Afghanistan and found that 77% of all casualties sustained a musculoskeletal wound.[1] Another study showed infection of severe Type III tibial fractures in the U.S. military population led to a significant decrease in the rate of soldiers returning to active duty (24% vs. 44%) compared to uninfected fractures.[5] In the civilian population, musculoskeletal trauma accounts for more than 50% of the total costs on society for nonfatal injuries, and 42% of civilians subjected to high-energy trauma do not return to work 1 year after their injury.[4] Treatment of infected high-energy extremity wounds that affect soft and hard tissues usually requires external fixation and systemic antimicrobials with an adjunctive local antimicrobial delivery system included. Another significant source of bacterial infections is hospital acquired infections, also known as nosocomial infections. The Centers for Disease Control and Prevention published a study that estimated there were nearly 722,000 hospital

acquired infection in 2011.[6] This study reported that surgical site infections are responsible for 21.8% of hospital acquired infections and another 25.6% are due to device-associated infections. Bacteria acquired in hospitals are potentially more resistant to antimicrobials and difficult to treat compared to bacteria found outside of hospitals.[7] Preoperative prophylaxis with systemic antibiotics is the most common method for preventing surgical site infections or device-associated infections.[8] However, if systemic antibiotics fail to prevent the infection, treatment of the resulting infection may include an adjunctive local antimicrobial delivery system.[9]

Currently available treatments for these severe infections depends on the type of injury sustained. For injuries that encompass soft and hard tissues, poly(methyl methacrylate) (PMMA) beads and calcium sulfate pellets are common choices.[10] Superficial injuries that only engage soft tissue can potentially be treated using a range of delivery systems made from materials such as collagen, poly(lactic acid) (PLA), poly(glycolic acid) (PGA), or other polymers. Collagen, PLA, and PGA have been approved by the FDA for a number of biomedical applications, but collagen has been used the most of these materials for soft tissue injures.[11] Each of these systems possesses advantages that make them useful, but they also possess disadvantages that limit their application in musculoskeletal trauma. PMMA has excellent mechanical properties and space-filling capacity, but it does not degrade in the body and necessitates a secondary surgery for removal.[10] Calcium sulfate is a resorbable, osteoconductive material that degrades in a similar time frame as bone formation. However, rapid degradation to calcium ions may cause serous wound drainage in up to 51% of cases.[10,12] Collagen based systems are completely degraded by the body and releases the majority of its therapeutic load, but they are expensive and have weaker mechanical properties compared to synthetic systems.[13] PLA and PGA systems can be tailored to control their release kinetics, degradation properties, and mechanical properties, but

they degrade to acidic waste products that may incite inflammatory responses, and the acidic degradation products accelerate the degradation of the bulk system.[13] Initial management of complex musculoskeletal trauma could be improved by developing a delivery system that is injectable to enhance wound coverage, adhesive to minimize migration from the wound site, degradable in the same time frame as elution kinetics to eliminate removal, and biocompatible to not adversely affect the surrounding tissue.

Chitosan is the second most abundant biopolymer found in nature and has been developed into a multitude of delivery systems.[14,15] Chitosan based delivery systems have been shown to be biocompatible, biodegradable, and compatible with a wide range of therapeutics.[15] However, these systems experience bolus release kinetics, and it would improve infection prevention capabilities if therapeutic release was extended.[10] Chitosan derivatives are chemically modified derivatives of chitosan that impart improved properties to chitosan without sacrificing the inherent biocompatibility and biodegradability.[16] Selected enhanced characteristics that could improve local therapeutic delivery properties include increased swelling ratio, solubility at neutral pH, and range of compatible therapeutics.[17] Trimethyl chitosan has been shown to have enhanced solubility at neutral pH and be able to degrade at the same rate or faster than unmodified chitosan.[18] Poly(ethylene glycol) diacrylate chitosan has been investigated as an adhesive delivery system, and the cross-linked structure provides an enhanced swelling ratio.[19]

Chitosan pastes have previously been developed as an injectable delivery system for complex musculoskeletal trauma. The first generation of this system combined acidic and neutralized chitosan components, and the resulting system was able to elute antimicrobials for 3 days, injectable, adhesive, and enzymatically degradable. However, there were issues with *in vivo* biocompatibility, and future developments were designed to improve biocompatibility. The

following iteration reduced the amount of acidic component and added poly(ethylene glycol) to the neutral component.[20] These modifications ameliorated the biocompatibility issues but resulted in a loss of its advantageous degradation and adhesion characteristics. Attempting to retain the biocompatibility and regain the lost characteristics, the composition of chitosan paste was further modified by reducing the acid content of the acidic component and removing the neutral component.[21] This system experienced a mismatch between elution and degradation by being enzymatically degraded in two days and eluting antimicrobials for 3 days. Combining the mismatch between elution and degradation with a lack of adhesivity, chitosan paste requires further improvement.

Hypothesis and Research Objectives

TMC and PEGDAc were synthesized to assess feasibility as a local delivery system for preventing infection in extremity injury patients with complex musculoskeletal trauma. It was hypothesized that a combination of TMC and PEGDAc would have extended elution and extended degradation compared to non-derivatized paste. Research objectives were to extend elution of antimicrobial agents compared to previously developed systems with a target between 3 and 7 days. Elution for 7 days would provide protection from bacteria during initial wound closure and dressing changes. Another goal is to have complete degradation of the system between 7 and 14 days to allow for initial healing of soft tissue. The final objective is to enhance the adhesive characteristics compared to previously developed systems. Preventing migration of the delivery system from the wound site is crucial for maintaining high concentrations of antimicrobial at the wound site. TMC will allow degradation rate to be tailored by varying the degree of quaternization, and this will provide a mechanism for matching degradation properties and elution kinetics of the local delivery system. Inclusion of PEGDAc will enhance swelling

characteristics and adhesiveness of the material; these additional properties will allow incorporation of more aqueous antimicrobial solution and better adhesion to open wounds. Secondary objectives include maintaining cytocompatibility with representative cell lines, injectability for easy application, and point-of-care loading for increased treatment options. Specific research objectives are the following:

1. Determine if the elution kinetics of active vancomycin and amikacin from a combination of TMC and PEGDAc are extended compared to previously developed systems and maintained between 3 and 7 days.
2. Determine if the enzymatic degradation rate of the combination of TMC and PEGDAc can be tailored to last between 7 and 14 days.
3. Determine if the combination of TMC and PEGDAc is cytocompatible according to ISO 10993-5 with cells lines representative of tissues present in open fractures.
4. Determine if the combination of TMC and PEGDAc is more adhesive compared to previously developed systems.
5. Determine if the combination of TMC and PEGDAc is considered injectable through standard 1 mL syringes according to benchmark force values.

CHAPTER II

LITERATURE REVIEW

Musculoskeletal Trauma

Trauma associated with the musculoskeletal system poses a significant burden on healthcare systems around the globe. In developed countries, this classification of injury is the leading cause of death in patients that are younger than 44 years old.[4] Musculoskeletal trauma caused by high-energy injuries such as car accidents, gun shots, sports injuries, or explosive blasts may result in an open fracture. Court-Brown et al. performed a 15 year retrospective study on open fractures treated at their hospital and reported an incidence rate of 30.7 fractures per 100,000 people per year.[22] Using this figure, approximately 100,000 of these traumatic injuries occur each year in the United States. However, this estimate only encompasses the incidence rate for the civilian population and does not take military personnel into account. Belmont et al. retrospectively reviewed all of the musculoskeletal injuries incurred during the conflicts in Iraq and Afghanistan and found the incidence of open fractures to be 1.27 injuries per 1,000 deployed military personnel per year.[1]

The presence of environmental contaminants, complex geometries, and compromised vasculature make management of open fractures difficult. Initial treatment is to administer systemic antimicrobials as quickly as possible after sustaining the injury to prevent infection. Current clinical guidelines established by both civilian and military trauma surgeons agree that antimicrobials should be administered within 3 hours of injury, and it is an independent risk factor for infection if administered outside of this window.[3,23] To enhance the efficacy of infection prevention, a local delivery system may be placed at the wound site to deliver antimicrobials directly to the site of injury.[10] The next step of management includes irrigation

and debridement of the wound to remove any superficial contaminants or nonviable tissues followed by the placement of a fixation device. Orthopaedic fixation devices facilitate ambulation, promote healing, and prevent further soft tissue injury and may include external fixation, intramedullary nailing, or a combination of plates and screws.[23] Closure of the wound is the final step in managing these traumatic injuries, and delay of this procedure predisposes patients to acquiring nosocomial infections. Despite these established treatment protocols, patients with open fractures remain susceptible to serious complications such as infection, nonunion, malunion, and wound necrosis.[24]

Infection rates for the most severe classification of open fractures can be as high as 50% and result in an elevated risk of complications, duration of hospitalization, number of readmissions, and twice the costs compared to uninfected patients.[25-27] The same factors that make treatment of open fractures difficult also make them prone to infection. Damaged vasculature prevents injured tissues from being perfused by the innate immune system and systemic antimicrobials. Contamination of the wound site places bacteria in close proximity to necrotic soft tissue and devascularized bone. Also, the complex geometries of these injuries hinders the efficacy of irrigation and debridement by providing bacteria with crevices to adhere and provide protection. Necrotic tissues and fixation devices serve as ideal substrates for bacteria to adhere and form a biofilm.[28] The most common pathogens associated with orthopaedic infections belong to the *Staphylococcus* genus, accounting for nearly 67% of infections.[29] *Pseudomonas aeruginosa* (*P. aeruginosa*) has also been found contaminating open fractures, and the combination of these species accounts for nearly 75% of biofilm-based medical device infections.[30] Biofilms are difficult to treat due to several factors including the extracellular polysaccharide matrix, persister cells, horizontal gene transfer, and limited diffusion.[28]

Antimicrobials concentrations required to eradicate these infections can be as high as 1000x the amount required to eliminate planktonic bacteria.[31] These concentrations are impossible to deliver through the systemic vasculature without eliciting adverse effects from the chosen antimicrobials even if the vasculature was not damaged. Therefore, it is imperative to prevent these infections from occurring.

Hospital Acquired Infections

Nosocomial infections, otherwise known as hospital acquired infections, are dreaded complications that affect vulnerable patients while they are receiving treatment within a healthcare facility. The Centers for Disease Control and Prevention published a report that estimated 722,000 of these infections occurred in the United States in 2011.[6] Among these infections, surgical site infections and device associated infections are the most common with incidences of 30% and 25.6%, respectively.[6,8] Device associated infections encompass all medical devices, but the focus here is on devices with an orthopaedic application. Common sites for orthopaedic infections are implanted fracture fixation devices and prosthetic devices used in the management of hip or knee osteoarthritis .While periprosthetic joint infection only occurs in 1% of total hip arthroplasties (THA) and 2% of total knee arthroplasties (TKA), they account for 14.8% and 25.2% of failures, respectively.[32] The volume of THAs and TKAs is projected to grow to 572,000 and 3,480,000 procedures by 2030 in the United States, and the cost of revision surgeries will pose a significant burden on the U.S. healthcare system. Patients that acquire one of these infections will experience an increased length of stay, number of readmissions, risk of complications, and cost of treatment.[27,33]

Patients diagnosed with periprosthetic joint infection can be managed by surgical or nonsurgical treatment methods.[34] Infections caused by low virulence bacteria that are susceptible

to antimicrobials may be treated using systemic antimicrobials and debridement with component retention.[35] However, if the infection is caused by a virulent or antimicrobial resistant bacteria, patients are most often treated using a two-stage revision in the United States.[34] In a two-stage revision, the prosthetic components and bone cement are removed, and a PMMA spacer impregnated with antimicrobials is used to manage the dead space and combined with systemic antimicrobials to eliminate the invading pathogens. The overall cost of this treatment ranges from $60,000 to $100,000 and must be repeated if the infection resurges.[36]

Management of patients with surgical site infections is less invasive and costly compared to patients with periprosthetic joint infection. Superficial infections may only require wound drainage to be effective; whereas, deep infections require managing the site of infection and administering antimicrobials.[8] Treatment of these infections may range from $400 to $30,000 depending on the severity of infection.[11] Infection treatment does not only affect the cost to patients but also substantially increases the costs for healthcare systems.

Prevention of hospital acquired infections is fundamentally different from preventing infections of open fractures. The factors that complicate infection prevention in fractures are not present at surgical sites. These injuries have an adequate blood supply, well defined geometries, and a lack of environmental contaminants.[34] Also, patients at risk for developing an infection are prophylactically administered antimicrobials prior to operation.[11] Theoretically, this measure would reduce the incidence of surgical site infections to 0%, but this is not the case. There are several reasons why this occurs: non-adherence to antimicrobial regimen, inappropriate antimicrobial selection, and antimicrobial-resistant pathogens. The most common pathogens that affect these wounds are *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermis* (*S. epidermis*), and *P. aeruginosa*. There have been many reports of antimicrobial resistance

developing in these microorganisms such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), and multi-drug resistant *P. aeruginosa*.[30,37,38] Resistance can be acquired through many different mechanisms such as inactivation of the antimicrobial, destruction of the antimicrobial, decreased permeability of the bacterial cell membrane, increased efflux of the antimicrobial, and modifications of the target site.[39] Excluding modification of the target site, the other resistance mechanisms are mediated through enzymatic processes which can be saturated at large concentrations of antimicrobials. However, the concentrations required to overcome these mechanisms cannot be achieved through systemic delivery without producing adverse effects.

Local Therapeutic Delivery

Systemic delivery of antimicrobials is an effective treatment for preventing most infections, but it is ineffective for injuries with compromised vasculature, antimicrobial resistant pathogens, or biofilm-based infections. Shortcomings of systemic delivery may be minimized by incorporating local delivery systems as adjunctive therapies. Local delivery systems are capable of achieving concentrations of antimicrobials up to 1000x higher than systemic routes, and these large concentrations are delivered directly to the source of infection.[10,40] Antimicrobials delivered at this concentration may be effective at eliminating bacteria that are normally resistant to lower doses of antimicrobials.[12] Circumventing the systemic vasculature also prevents delivery of high concentrations of antimicrobials to sensitive tissues and reduces their systemic side effects. This is especially important with antimicrobial classes such as aminoglycosides or glycopeptides that have dose-dependent ototoxicity and nephrotoxicity.[41-43] Delivery of antimicrobials from these systems is mediated through diffusion and determined by the initial loading concentration of antimicrobials. This produces an effective release profile for infection

prevention. Initial high concentrations of antimicrobials can eliminate pathogens that are initially colonizing the site, and sustained lower concentrations can prevent additional microbes from settling.

There are many local therapeutic delivery systems available on the market, but they possess drawbacks that limit their efficacy. PMMA is the most commonly used material in the treatment of orthopaedic infections due to its strong mechanical properties and ability to elute therapeutics for extended periods.[44] However, it never degrades in the body, and once the therapeutic load is expended, it serves as an attachment point for bacteria to colonize. $CaSO_4$ is an osteoconductive, resorbable material that eliminates the need for a removal surgery. The most common complaint with this system is the occurrence of serous wound drainage, which has been reported to occur between 15% and 51% of cases.[10,45] Collagen, a natural polymer, is a principal component of the extracellular matrix and has been used extensively in tissue engineering and fabrication of therapeutic delivery systems.[46] The immune system recognizes this polymer and does not elicit an immune response, and the degradation products are easily handled by the body. Drawbacks of this material are weak mechanical properties that prevent fabrication into a supportive construct and relatively high cost. PLA and PGA are common materials used in the fabrication of hydrogels and tissue engineering scaffolds. An advantage of these materials is their mechanical properties can be tailored by varying the stereoregularity of the pendant groups on the polymer.[47] The main disadvantage is the degradation products: lactic acid and glycolic acid. These relatively strong acids elicit an inflammatory response and accelerate the degradation of the local delivery system. Developing a local delivery system that is biocompatible, degrades to easily handled products, and elutes active therapeutic agents is a challenging task, but chitosan shows promise in overcoming the limitations of other therapeutic delivery systems.[16,48,49]

Chitosan

Chitosan is a linear, cationic polysaccharide composed of repeating β-(1-4)-linked glucosamine and N-acetylglucosamine subunits.[15] This versatile material is principally derived from a natural biopolymer, chitin, that composes the exoskeleton of crustaceans and to a lesser extent the cell wall of fungi and some insects.[16] Excluding cellulose, chitin is the most abundant polysaccharide that occurs in nature and serves as an inexpensive, readily available resource.[14] Several million tons of chitin are harvested annually from the exoskeleton of krill, shrimp, lobster, and crab.[15]

Converting chitin to chitosan can be a harsh chemical process that ultimately involves exposing chitin isolated from the exoskeleton shells to concentrated sodium hydroxide or hydrochloric acid, elevated temperatures, and pressure for an extended period of time. The conversion can also be completed using lengthier, less harsh chemical or enzymatic processes. These methods result in deacetylation of the N-acetylglucosamine units or removal of the acetyl groups on the polymer chain. The repeat unit for chitin mainly consists of N-acetylglucosamine units, and may comprise 10% or less of the deacetylated glucosamine units. The consensus for the percentage of glucosamine units required for the polymer to be considered chitosan instead of chitin is 50%.[50]

The degree of deacetylation (DDA) is a defining characteristic of chitosan and has been shown to affect important properties such as biocompatibility, degradation rate, fluid retention, and therapeutic release kinetics.[51-53] Chitosan with 50% DDA has the most rapid degradation rate; whereas, 0% DDA chitin and 100% DDA chitosan exhibit the slowest degradation rate. These differences can be explained by the crystallinity of the polymers. Highly crystalline polymers are more difficult to degrade compared to amorphous polymers. Some methods to

modify DDA are by changing processing conditions such as the concentration of sodium hydroxide, reaction temperature, reaction time, and number of successive reactions.[54-56] Another important parameter that defines the properties of chitosan is the molecular weight of the polymer; this property influences biodegradability, mucoadhesivity, and antimicrobial effects.[53,57,58] Mucoadhesivity increases as the molecular weight of chitosan increases due to increased entanglements of the chitosan chains with mucous proteins. The molecular weight of chitosan can be tailored by using chemical methods, high energy radiation, enzymatic methods, or mechanical treatments.[59-61] Mechanical methods are the most suitable for producing chitosan due to excellent control over the molecular weight and little effect on the DDA.[59] Therefore, tailoring DDA and molecular weight of chitosan is an important consideration for fine-tuning the properties of chitosan for specific applications.

Chitosan possesses two of the most important characteristics of a local therapeutic delivery system: biodegradability and biocompatibility. As such, it has been developed into many different technologies that take advantage of these properties and expand upon them. Some examples include thin films, hydrogels, tissue scaffolds, nanoparticles, sponges, and paste.[48,62-66] These systems have been developed for different therapeutic goals and implemented with some success. However, there are many types of synthetic and natural materials available for fabrication into therapeutic delivery systems. Each material has a distinct set of advantages and disadvantages associated with it that limits its applications, and chitosan is not an exception. A major advantage for chitosan is that it possesses functional groups that allow for its properties to be modulated to the desired application. Once the chemical structure has been modified, it is no longer referred to as chitosan but as a chitosan derivative.[16]

Chitosan Derivatives

Chitosan possesses primary hydroxyl groups, secondary hydroxyl groups, and primary amino groups that are targets for chemical reactions.[16,17] The primary goal of modifying chitosan is to retain the desirable characteristics, biocompatibility and biodegradability, while improving characteristics pertinent to the desired application. In the case of therapeutic delivery systems, modifications aim to tailor the release kinetics, therapeutic compatibility, solubility, and adhesiveness.[19,67-70] Modifications of chitosan can range from cleaving the polymer to grafting long chain polymers to the backbone.[17,71] A major disadvantage of having multiple functional groups available for reaction is the potential for undesirable side reactions to occur.[16,71] Side reactions lead to the production of several minor and major products that have different properties. In most cases functional groups can be conserved by using reversible reactions that conjugate protecting groups to prevent undesirable reactions. Another method to reduce heterogeneity of the final product is to use milder, targeted reaction conditions that show selectivity for functional groups and regioselectivity for specific groups.[72,73] Both methods produce a more homogenous product that is easier to characterize and allows for a more accurate determination of the properties of the final product.

Quaternized Chitosan

Quaternized chitosans are prepared by the addition of alkyl groups to the primary amino group on the backbone of chitosan. By adding three constituent groups to the amino group, chitosan acquires a permanent source of positive charge that supplements its natural cationic properties. The prototypical polymer of this class of derivatives has three methyl groups attached to the primary amino group. Therefore, it is commonly referred to as trimethyl chitosan (TMC). Most synthetic pathways result in undesirable O-methylation or dimethylation of the primary

amino group. The hydrophobic nature of these groups conflict with the hydrophilicity of the trimethylated amino groups and lends amphoteric properties to TMC.[74] The permanent cationic character of trimethyl chitosan has been shown to enhance the antimicrobial properties[75,76], antifungal properties[75,77], mucoadhesion[16,78], and solubility at neutral pH[74,79] compared to chitosan. These benefits are mainly observed when trimethylation only occurs at the primary amino group. If methylation occurs at either of the hydroxyl groups, these beneficial effects are markedly attenuated.[72,78]

Methylation regioselectivity is one of the two principle properties that govern the properties of quaternized chitosan derivatives. The other property is the degree of quaternization (DQ), which is similar to the DDA of chitosan. DQ is a measure of the amount of primary amino groups that have been quaternized and serves as a method for tailoring the properties of this class of derivatives. The antibacterial activity of trimethyl chitosan has been credited to the permanent positive charge resulting from trimethylated amino groups; this is a probable explanation for reports of derivatives with higher DQ possessing enhanced antibacterial effects.[67,80] This makes it seem that maximizing DQ would be desirable, but increasing DQ does not always lead to beneficial effects. There have been reports that high molecular weight trimethyl chitosan with large DQ has cytotoxic effects.[76,78] Also, there are conflicting reports on how DQ affects the mucoadhesivity of this class of derivatives. There have been reports that increasing DQ leads to an increase in mucoadhesivity, but the majority of reports seem to agree that it leads to decreased mucoadhesivity.[16,81] Due to varied effects of DQ on the properties of quaternized chitosans, it is important to consider the final application when selecting the DQ.

Graft Copolymers of Chitosan

Graft copolymers of chitosan are produced by initiating and growing chains of monomer or grafting preformed polymers onto the backbone of chitosan. Methods to produce this class of derivatives include free radical polymerization, polycondensation reactions, copolymerization via grafting of preformed polymers, and other synthetic methods that possess advantages and disadvantages.[16] Derivatives of this class are highly varied and do not typically have similar properties. The most notable graft copolymer of chitosan is produced by addition of poly(ethylene glycol) (PEG) to the backbone. PEG is a useful polymer due to its hydrophilicity, high solubility in water, and biocompatibility. Attaching this polymer to the backbone of chitosan retains its biocompatibility and degradation properties while increasing the solubility of chitosan at neutral pH and swelling ratio.[82,83]

Copolymers of chitosan and PEG have many applications due to the combination of both polymers favorable properties. This class of derivatives has found use in therapeutic delivery,[84] gene product delivery,[85] and as wound dressings.[86] A minor determinant of how adding PEG affects the properties of chitosan is how it was attached. If the PEG was attached via an end group, there might be an additional end group available for crosslinking or interaction with other materials. One study investigated the efficacy of a copolymer of chitosan and PEG for delivery of an anti-cancer drug, methotrexate.[84] This combination delivery system vastly increased the efficacy of the free drug for targeting and treating the studied tumor. Another study investigated the effect of adding PEG to chitosan for the delivery of gene products[85]; the authors found that transfection efficacy was significantly increased and was credited to a reduction in the aggregation of DNA. These studies have investigated the effect of attaching PEG directly to chitosan without end groups. Few studies have investigated attaching PEG with acrylate end

groups to chitosan. The authors investigating the copolymer found that it was able to form thin films that were more adhesive and water soluble compared to chitosan.[19,86]

Current Work

Orthopaedic trauma and total joint replacement patients are at high risks for contracting an infection.[25,26,32] Treating these infections is taxing and expensive for both healthcare systems and patients.[36,87] Due to the characteristics of these infections, the invading pathogens may be refractory to systemic antimicrobials.[4,28] Local delivery of antimicrobials is a popular adjunctive treatment for infection prevention in combination with systemic antimicrobial therapy.[10,88] Local delivery systems currently used in the management of these infections have issues with degradation properties, biocompatibility, and therapeutic options.[10,44,47] Chitosan is a natural biopolymer that has been shown to be able to degrade within the body, biocompatible, and loaded with a breadth of therapeutics.[14,15]

Chitosan paste has been developed as an injectable delivery system for complex musculoskeletal trauma. The first generation paste eluted antimicrobials for 3 days and had limitations with *in vivo* biocompatibility. Future iterations improved on the biocompatibility issue, but modifications to the system resulted in the loss of its advantageous degradation and adhesive characteristics.[20,21] Chitosan possesses reactive functional groups that make it amenable selective chemical modification.[17] Quaternized chitosan derivatives have been shown to enhance the antibacterial properties, solubility, and mucoadhesivity compared to unmodified chitosan.[75,78,79] Copolymers of chitosan and PEG are more soluble at neutral pH, more adhesive, and have an enhanced swelling ratio compared to chitosan alone.[82,83,86] These derivatives have advantages over unmodified chitosan that may be beneficial for local delivery systems. Quaternized chitosan derivatives, a copolymer of chitosan and PEG, or a combination of the two

could allow for tailored elution and degradation characteristics for use as an adjunctive local delivery system of antimicrobials for infection prevention. The aims of this are to (1) produce a local delivery system that elutes active antimicrobials longer than previously developed systems with a target between 3-7 days to provide protection from bacteria during wound closure and initial healing, (2) tailor complete degradation of the system to occur between 7-14 days to maintain antimicrobial elution and facilitate removal before initial healing finishes, (3) retain the biocompatibility of the parent chitosan to allow use as an infection prevention system, (4) regain or exceed the adhesiveness of the first generation chitosan paste to prevent migration from the wound site, and (5) maintain the injectability of the delivery system to allow for enhanced coverage of complex musculoskeletal wounds.

Hypothesis

TMC and PEGDAc were synthesized to assess feasibility as a local delivery system. It was hypothesized that a combination of TMC and PEGDAc could be developed into an injectable delivery system for preventing infection in extremity injury patients with complex musculoskeletal trauma. Research objectives were to extend elution of antimicrobial agents compared to previously developed systems with a target between 3 and 7 days. Elution for 7 days would provide protection from bacteria during initial wound closure and dressing changes. Another goal is to have complete degradation of the system between 7 and 14 days to allow for initial healing of soft tissue. The final objective is to enhance the adhesive characteristics compared to previously developed systems. Preventing migration of the delivery system from the wound site is crucial for maintaining high concentrations of antimicrobial at the wound site. TMC will allow degradation rate to be tailored by varying the degree of quaternization, and this will provide a mechanism for matching degradation properties and elution kinetics of the local

delivery system. Inclusion of PEGDAc will enhance swelling characteristics and adhesiveness of the material; these additional properties will allow incorporation of more aqueous antimicrobial solution and better adhesion to open wounds. Secondary objectives include maintaining cytocompatibility with representative cell lines, injectability for easy application, and point-of-care loading for increased treatment options.

CHAPTER III

A degradable chitosan derivative based delivery system for local delivery of antimicrobials: Preliminary Evaluations and Assessments

Abstract

Refined local delivery systems are needed as adjunctive treatments for severe injuries with high infection rates, such as open fractures. Chitosan systems have been investigated as local delivery systems of antimicrobials for orthopaedic infections but possess mismatches between elution and degradation properties. Derivatives of chitosan were chosen that have enhanced swelling ratios or tailorable degradation properties. A combination of trimethyl chitosan and poly(ethylene glycol) diacrylate chitosan was developed as an injectable local delivery system. Research objectives are eluting antimicrobials for 7 days, degradation as open fracture heals, and remaining cytocompatible. The derivative combination eluted increased active concentrations of vancomycin compared to the non-derivatized chitosan paste, 6 vs. 5 days. The derivative combination degraded slower than non-derivatized in an enzymatic degradation study, 14 vs. 3 days, which increased the antimicrobial delivery profile. Cytocompatibility of the combination with NIH3T3 fibroblast and MC3T3 pre-osteoblast cells are within the cell viability standard set in ISO 10993-5. Combination paste requires an increased ejection force of 9.40N (vs. 0.64N), but this force was within an acceptable injection force threshold of 80N. These preliminary results indicate that the combination paste should be considered for further development into a clinically useful adjunctive local delivery system for infection prevention.

Key Words

Chitosan derivatives, local delivery, infection prevention, trimethyl chitosan, injectable paste

INTRODUCTION

Approximately 100,000 open fractures occur each year in the United States civilian population.[1] Open fractures are traumatic injuries that engage both soft and hard tissues and are more difficult to treat compared to closed fractures due to their complex geometry, compromised vasculature, and possible bacterial contamination. Treatment of these injuries may include irrigation and debridement of the wound site, systemic administration of antimicrobials, and placement of external or internal fixation devices.[2,3] Despite following proper treatment protocols, up to 50% of patients sustaining these injuries will become infected which can result in significant complications such as nonunion, malunion, and wound necrosis.[4-6] The most prevalent pathogens that cause orthopaedic infections are *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). These microorganisms account for nearly 75% of biofilm-based infections in medical devices.[7] Necrotic tissue, devascularized bone, and fracture fixation devices are ideal substrates for bacterial adherence and biofilm formation. Treating infected open fractures is difficult due to compromised vasculature and irregular geometries, and may be further complicated by antimicrobial resistance bacteria and the presence of biofilm.[8-10] Therefore, infection prevention is crucial in the management of patients with open fractures.

Local delivery of antimicrobials has become a popular adjunctive therapy in combination with systemic antimicrobials for infection prevention.[5] Local administration of therapeutic agents reduces systemic toxicity and maximizes concentration at the injury site. Several materials have been fabricated into local delivery systems for the treatment of orthopaedic infections over the past several decades: poly(methyl methacrylate) (PMMA), calcium sulfate ($CaSO_4$), and collagen.[5,11] These materials have been used clinically with some success, but they possess

drawbacks that could be improved. PMMA is incompatible with temperature-sensitive antimicrobials due to heat released during polymerization and requires explantation to facilitate removal from the body.[5] Unless removed in a timely manner, antimicrobials are eluted at subtherapeutic dosages that may promote antimicrobial resistance.[12] $CaSO_4$ is a resorbable biomaterial that eliminates the need for a removal surgery. However, the most common complaint is the production of sterile wound drainage from degradation.[13,14] Collagen is a resorbable biopolymer and an important structural component of the extracellular matrix. Its main drawback is the rapid release of antimicrobial agents does not correspond to its rate of degradation within the body.[5,11]

Chitosan, a natural glycomaterial, is a polysaccharide that is principally derived from the exoskeleton of crustaceans. This abundant biopolymer has been developed into a wide variety of therapeutic delivery systems due its biocompatibility and ability to degrade within the body.[15,16] Other advantages include its mucoadhesivity and intrinsic antimicrobial properties.[17] Release kinetics of antimicrobials from chitosan systems typically exhibit a bolus release and show similar drawbacks as collagen systems.[13] However, the properties of chitosan can be modified due to the presence of reactive functional groups, and derivatives of chitosan can be specifically produced to enhance desirable characteristics while retaining its biocompatibility and degradation properties.[17-19] Quaternized chitosans are water soluble, have enhanced antimicrobial properties, and have been shown to degrade at the same rate or faster than unmodified chitosan.[20,21] Cross-linked chitosans have been shown to extend release kinetics and enhance swelling ratio, but they also extend the degradation profile.[22,23]

In this study, we developed a blend of trimethyl chitosan (TMC) and poly(ethylene glycol) diacrylate chitosan (PEGDAc) to combine the water solubility and degradation properties

of TMC with the enhanced swelling ratio of PEGDAc. When the composite is hydrated with antimicrobial solution, a paste is formed that can be used as an injectable delivery system for preventing infection in complex musculoskeletal trauma. Toward demonstrating feasibility for this combination as an injectable infection prevention biomaterial, elution kinetics, activity of antimicrobials, and degradation of the composite were compared to non-derivatized chitosan controls. Additionally, biocompatibility and injectability were evaluated against clinically relevant standards of 70% viability and 80N of force to assess feasibility of delivery for the complex geometry of open fractures.[24,25]

MATERIALS AND METHODS

Synthesis and fabrication

Two chitosan derivatives were prepared from chitosan (Chitinor AS, Norway) with a molecular weight of 250.6 kDa and degree of deacetylation of 82.46%. The first derivative was *N*-trimethyl chitosan (TMC), a quaternized chitosan derivative, and the other derivative was poly(ethylene glycol diacrylate) chitosan (PEGDAc), a graft copolymer of chitosan. Unmodified chitosan delivery systems previously developed in this lab were prepared using the same batch of chitosan and used as controls.[26,27]

Preparation of trimethyl chitosan

TMC was synthesized according to a method adapted from Verheul et al. with minor modification.[28] This method was chosen to avoid side reactions that are present in other methods for preparing TMC.[29] Chitosan was dissolved at a concentration of 4% (w./v.) in 30.0 mL formic acid, 40.0 mL formaldehyde, and deionized (DI) water and modified using an Eschweiler-Clarke reaction. The solution was heated to 70°C and allowed to stir for 120 hours. The resulting solution containing the intermediate product, dimethyl chitosan (DMC), was evaporated under

vacuum. This solution was gelled using 1.0M NaOH and washed with DI water. DMC was dissolved in DI water adjusted to pH = 5 with 1.0M HCl, and dialyzed for 3 days against DI water. The purified solution was frozen at -80°C and lyophilized for 3 days. To remove particulates, DMC was dissolved in DI water, gelled using 1.0M NaOH, and washed with DI water and acetone. The washed product was suspended in N-methyl-2-pyrrolidone (NMP), and excess iodomethane was added to the solution. This solution was heated to 40°C and allowed to stir for 72 hours. The solution was dropped in a 50:50 mixture of ethanol and diethyl ether to precipitate the final product. TMC was separated from the solution using centrifugation and dissolved in a 5% (w./v.) solution of NaCl. This solution was dialyzed against DI water for 3 days, frozen at -80°C, and lyophilized.

Preparation of poly(ethylene glycol diacrylate) chitosan

PEGDAc was synthesized according to the method described in Shitrit et al. with minor modification.[30] Chitosan was dissolved at 1% (w./v.) in a 1% (v./v.) solution of blended lactic and acetic acid (3:1 ratio). This solution was stirred for 24 hours to allow the chitosan to completely dissolve. Then, poly(ethylene glycol) diacrylate (PEGDA) with a molecular weight of 8000 (Alfa Aesar, Massachusetts) was added to the solution at 1% (w./v.) concentration. The solution was stirred for 15 minutes to allow the polymer to dissolve and heated to 60°C for 3 hours. The resulting solution was dialyzed against DI water for 3 days to remove unreacted PEGDA molecules, frozen at -80°C, and lyophilized for 3 days. The freeze-dried product was treated using 0.25M NaOH, and the pH was reduced to neutral using copious DI water. Finally, the neutralized product was frozen at -80°C and lyophilized for 3 days.

Grinding and reconstitution

Final lyophilized products were ground into small flakes and stored in a desiccator. Chitosan derivatives were combined in a 3:1 weight ratio of PEGDAc to TMC. To form the injectable delivery system, the ground products were hydrated using at a ratio of 4.5 mL of liquid per gram of powder. Loss of product was minimized by hydrating the powder directly in syringes using a Luer-Lock coupled system.

Preparation of controls

Two controls were prepared for comparison to the developed system: an injectable paste and lyophilized sponges. Berretta et al. previously developed an injectable system and was prepared according to their methods.[27] Blended chitosan control paste was prepared by dissolving 1% chitosan (w./v.) in 0.85% (v./v.) acetic acid solution and adding 1% (w./v.) PEG (molecular weight = 8000) to the solution. This solution was frozen at -80°C, lyophilized for 3 days, and not neutralized. The PEG blended chitosan control was not mixed with either derivatives. Chitosan sponge controls were prepared according to the methods in Noel et al. with minor modification.[26] Lyophilized sponges were prepared by dissolving 1% chitosan (w./v.) in a 1% (v./v.) blended lactic and acetic acid solution (3:1 ratio). This solution was frozen at -80°C, lyophilized for 3 days, and treated using 1.0M NaOH. The pH of the sponges was brought to neutral using DI water, frozen at -80°C, and lyophilized. Control sponges were hydrated using passive absorption for evaluation.

Fourier transform infrared spectroscopy

A Nicolet iS10 (Thermo Scientific, USA) using attenuated total reflectance (ATR) was used to collect spectra for chitosan, DMC, TMC, PEGDA, and PEGDAc. Samples were prepared by vacuum drying at 40°C for 3 days. Data was collected using a deuterated tryglycine sulfate

(DTGs) potassium bromide detector over the range of 525-4000 cm^{-1} with 64 scans and a resolution of 2 cm^{-1}. Ambient air was used to apply baseline corrections. Spectra were analyzed using Thermo Scientific OMNIC Software.

Nuclear magnetic resonance spectroscopy

The ^{1}H NMR spectra were collected using a JEOL Resonance 400 MHz NMR spectrometer at 25°C. TMC and PEGDAc samples were dispersed at 1% (w./v.) concentration in D_2O with 0.01 mL of DCl added to dissolve the polymers. Solutions were vortexed overnight to ensure complete dissolution.

Antimicrobial elution

Combinations of TMC and PEGDAc and non-derivatized paste were hydrated using a 5 mg/mL combination solution of vancomycin and amikacin (MP Biomedicals, USA). Approximately 0.6 mL of hydrated paste (n = 4) was injected into cell crowns (Scaffdex, Finland) with nylon filters (pore size = 41 µm) attached. Each sample was placed in 5 mL of phosphate buffered saline (PBS), incubated at 37°C, and sampled daily. Upon sampling, each sample was completely refreshed with PBS. Vancomycin was detected and quantified using a high performance liquid chromatography (HPLC) system interfaced with a UV/Vis spectrophotometer. Amikacin was quantified using a previously described method of pre-column derivatization with an o-phthaldialdehyde reagent and subsequent detection with an HPLC system using a fluorescence detector.[31]

Antimicrobial activity

Activity of the eluates against relevant orthopaedic pathogens *S. aureus* (UAMS-1; ATCC 49230) and *P. aeruginosa* (PA; ATCC 27317) was evaluated using zone of inhibition (ZOI) assays. Bacteria were grown separately overnight at 37°C in tryptic soy broth (TSB).

Overnight growth was diluted, 1:10 for *S. aureus* and 1:50 for *P. aeruginosa*, in TSB, and 100.0 µL of these solutions was spread on tryptic soy agar plates. Blank paper discs (diameter = 6.0 mm) were hydrated with 30.0 µL of eluate and placed on the plates. These were incubated for 24 hours at 37°C and photographed. ImageJ software was used to determine the ZOI for each disc.

Enzymatic degradation

Approximately 0.3 mL (n = 3) of each system was hydrated using PBS and placed in petri dishes. Non-derivatized neutral sponges were cut into equal parts (n = 3), hydrated with PBS, and placed in petri dishes. Degradation solution was prepared by dissolving 1 mg/mL lysozyme type VI (MP Biomedicals) and 100 µg/mL Normocin antibiotic/antimycotic in PBS. Then, 5 mL of degradation solution was added to each petri dish, and samples were placed in the incubator at 37°C. At days 1, 3, 5, 7, and 14 samples were taken, aspirated through a nylon filter (pore size = 41 µm), and placed in an oven at 45°C. Fresh degradation solution was replaced every other day by aspirating the old solution through a nylon filter (pore size = 41 µm) and adding 5 mL of fresh solution. After drying, samples were weighed and compared to their initial weight to determine degradation rate (Equation 1). Different samples were used each day due to the destructive nature of the test.

$$Percent\ remaining = \frac{Mass\ after\ degradation}{Original\ dry\ mass} \times 100 \quad (1)$$

Cytocompatibility

NIH3T3 fibroblast and MC3T3 pre-osteoblast cells were seeded at 1 x 10^4 cells/cm^2 in 24-well plates and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 µg/mL Normocin for 24 hours at 37°C and 5% CO_2. Approximately 0.3 mL (n = 3) of each paste was hydrated using sterilized PBS and injected into cell culture inserts (Falcon, pore size = 8 µm) and placed in each well. Cells were exposed to

eluates from the paste for 24 and 72 hours and quantified using a Cell-Titer Glo (Promega) assay. Results were normalized as a percent viability of cells grown on blank tissue culture plastic. Samples were sterilized with ethylene oxide gas (EtO) prior to testing.

Injectability

Injectability of the pastes was evaluated by measuring the maximum ejection force from a standard 1 mL syringe. Approximately 0.9 mL (n = 3) of paste was hydrated using PBS and ejected. Each syringe was placed in an Instron Universal Testing Machine (Instron) with a 500 N load cell and programmed to compress the syringe at a constant rate of 1 mm/second to fully eject the pastes. Force required to fully eject the pastes was measured, and maximum force required was recorded. Syringes filled with air were used for baseline corrections.

Statistical analysis

Statistical analysis of the results was performed using Sigma Plot 14 (Systat Software). Two way analysis of variance (ANOVA) with group and time point as the two factors, and Student-Newman-Keuls post hoc analysis was used to determine statistical differences between groups for degradation rate, antimicrobial elution, and relative cell viability. T-tests were used to assess differences for injectability of the pastes. P values < 0.05 were considered to be statistically significant.

RESULTS

Fourier transform infrared spectroscopy

Characteristic peaks were observed at 1105, 1464, and 2877 cm^{-1} correspond to C-O stretching, C-H bending due to methylene groups, and C-H stretching, respectively (Figure 1). These peaks are present in the spectra for PEGDA and PEGDAc, but they were absent or shifted

in the non-derivatized chitosan sample. Noticeable differences were not observed between the spectrums of DMC, TMC, and chitosan.

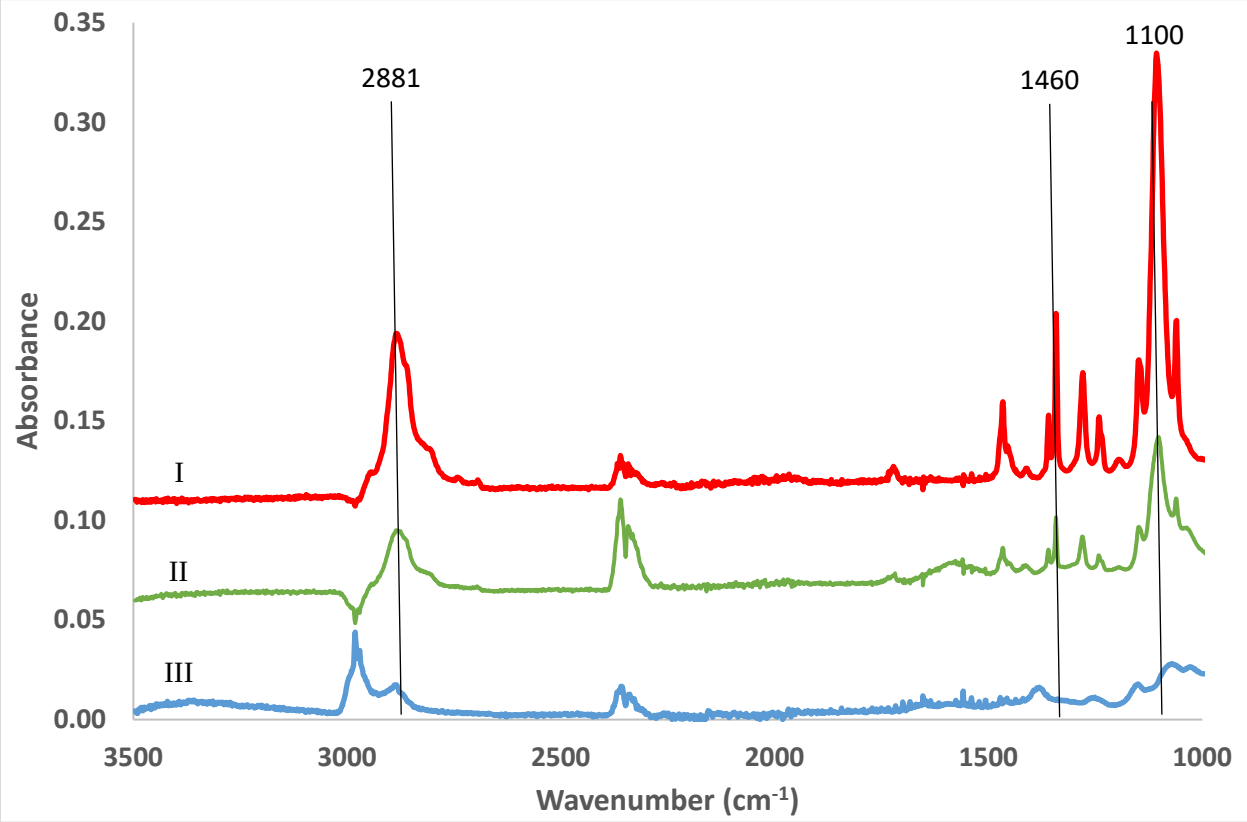

FIGURE 1. ATR-FTIR absorbance spectra for I: PEGDA 8000, II: PEGDAc, and III: unmodified chitosan. Peaks of interest are indicated, and peak at 2350 cm^{-1} is due to CO_2.

Nuclear magnetic resonance spectroscopy

The ^{1}NMR spectra for TMC and PEGDAc disclosed the presence and absence of characteristic peaks. For TMC, the peak at 2.9 ppm corresponds to the dimethyl group, and the peak at 3.2 ppm corresponds to the trimethyl group. The peak at 1.9 ppm is due to the hydrogens of the acetyl moiety. Peaks between 3.5-4.1 ppm are due to the hydrogens present on the chitosan backbone (Figure 2). For PEGDAc, the sharp peak at 3.5 ppm corresponds to the PEG portion of PEGDA, and the peak at 1.9 ppm corresponds to the acetyl moiety. There is an absence of peaks between 5.9-6.5 ppm that would represent the vinyl end group (Figure 3).[30]

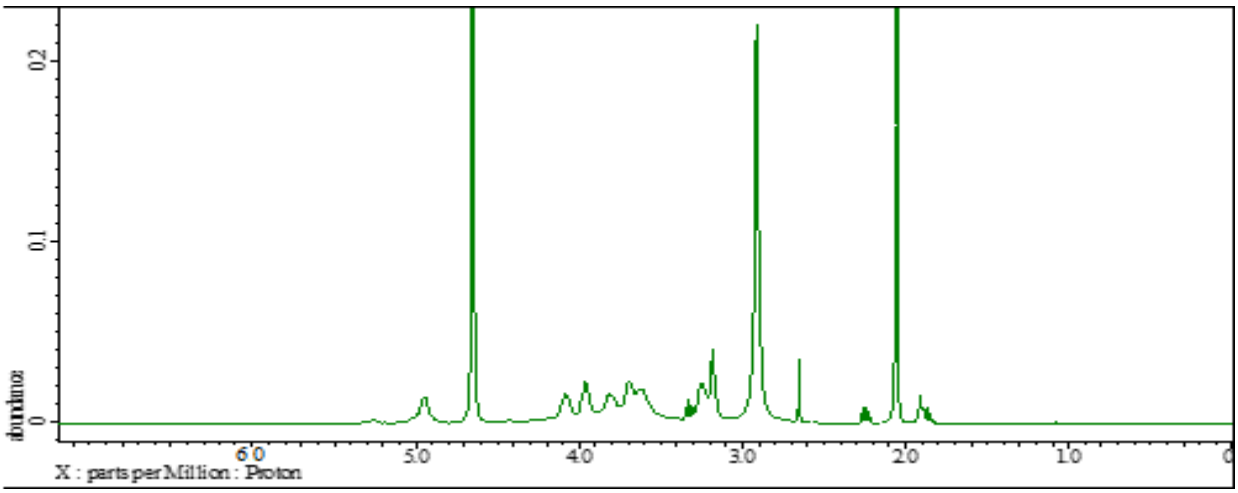

FIGURE 2. NMR spectra of 10 mg/mL TMC dissolved in D_2O/DCl at room temperature.

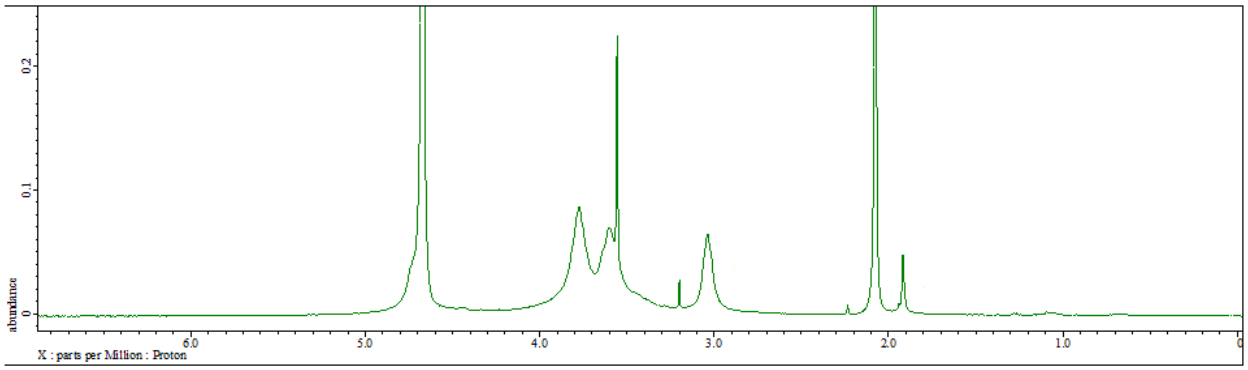

FIGURE 3. NMR spectra of 10 mg/mL PEGDAc dissolved in D_2O/DCl at room temperature.

Antimicrobial elution

Similar release kinetics were observed for both antimicrobials and delivery systems (Figures 4a, 4b, 5a, and 5b). A burst release of antimicrobials was observed on day 1 for both systems that tapered below detectable levels after day 7. Concentrations of vancomycin dipped below the minimum inhibitory concentration (MIC) of *S. aureus* after day 6, and amikacin concentrations dropped below the MIC of *P. aeruginosa* by day 5. Cumulative release of vancomycin from the combination paste was 76.5 ± 13.5% and 76.4 ± 10.6% from the non-derivatized paste. Similar cumulative release profiles were observed for amikacin with the

combination eluting 81.7 ± 13.2%, and the non-derivatized paste eluting 81.6 ± 10.3%. No significant differences were observed between the groups, but the combination paste eluted vancomycin at day 7 while the non-derivatized paste did not.

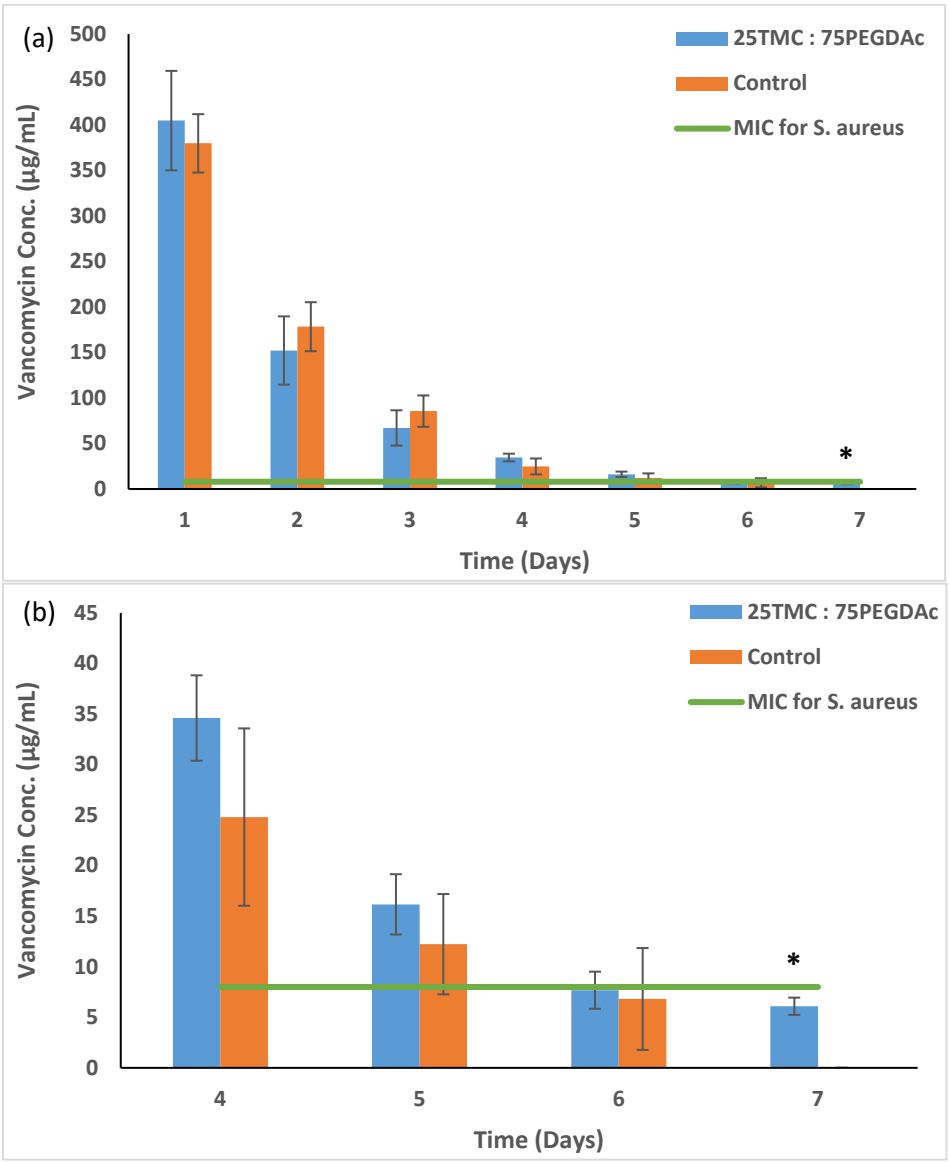

FIGURE 4. *In vitro* elution of vancomycin. (a) full elution profile for days 1-7. (b) shows zoomed in elution profile for days 3-7. Data are presented as mean ± standard deviation (n = 4). * denotes significant differences from control ($p < 0.05$).

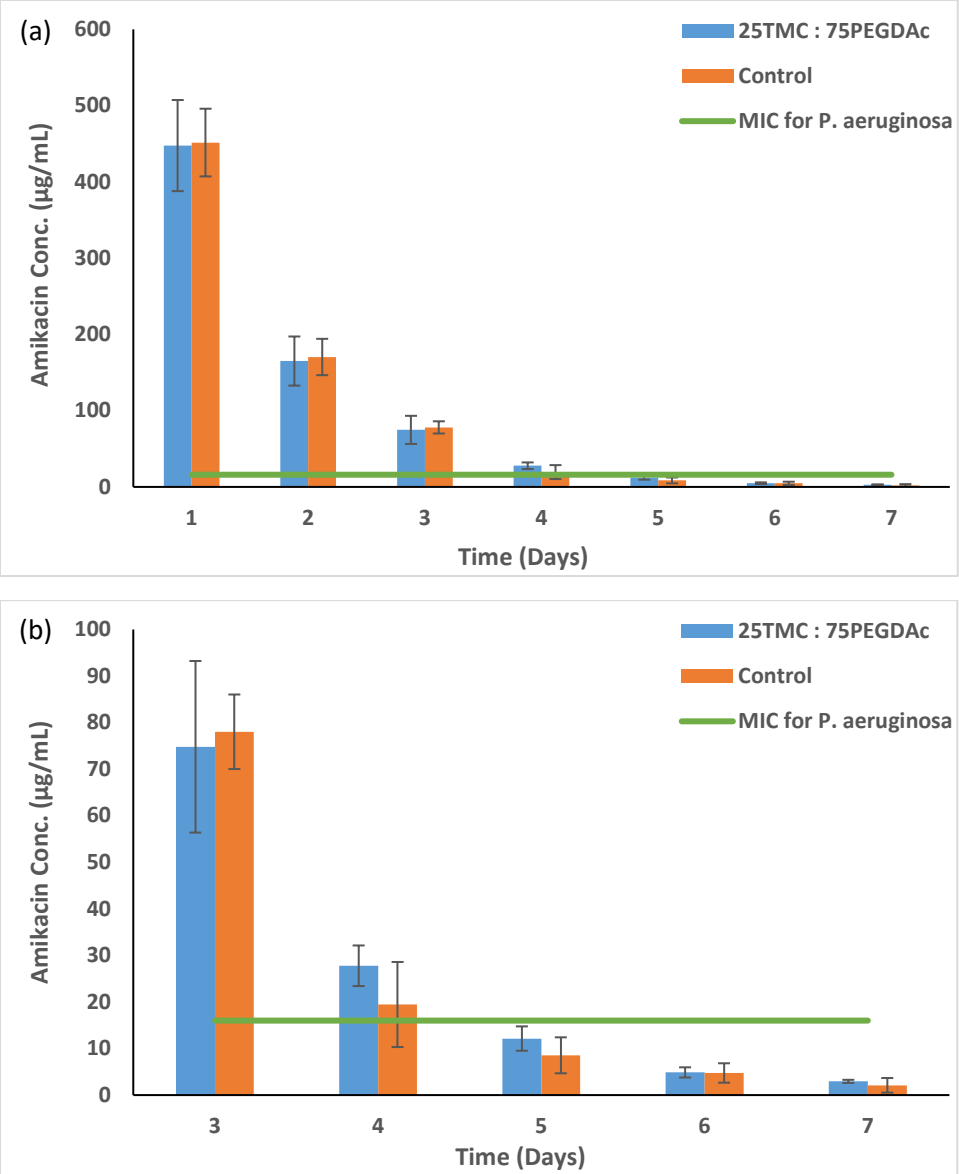

FIGURE 5. *In vitro* elution of amikacin. (a) full elution profile for days 1-7. (b) shows zoomed in elution profile for days 3-7. Data are presented as mean ± standard deviation (n = 4).

Antimicrobial activity

Large zones (> 10mm) were observed for both systems on day 1 that tapered off to clinically irrelevant zones (< 1mm). Combination paste inhibited growth of *S. aureus* for 6 days while the non-derivatized paste only prevented growth for 5 days (Table 1). Both combination and non-derivatized paste produced zones for 5 days against *P. aeruginosa* (Table 2).

TABLE 1. Zone of inhibition results for *S. aureus*. Zones greater than 10mm are indicated with +++, larger than 6mm are indicated with ++, larger than 1mm are indicated with +, and smaller than 1mm are indicated with -.

Formulation	Eluate Sample Time (Day)						
	1	2	3	4	5	6	7
25TMC : 75 PEGDAC	10.1 ± 0.8 +++	8.3 ± 0.5 ++	6.7 ± 0.5 ++	4.4 ± 0.4 +	2.3 ± 0.5 +	1.4 ± 0.4 +	0.0 ± 0.0 -
Control	10.0 ± 0.6 +++	8.1 ± 0.7 ++	5.1 ± 0.5 +	3.6 ± 0.8 +	2.3 ± 1.1 +	0.5 ± 0.9 -	0.0 ± 0.0 -

TABLE 2. Zone of inhibition results for *P. aeruginosa*. Zones greater than 10mm are indicated with +++, larger than 6mm are indicated with ++, larger than 1mm are indicated with +, and smaller than 1mm are indicated with -.

Formulation	Eluate Sample Time (Day)						
	1	2	3	4	5	6	7
25TMC : 75 PEGDAC	13.1 ± 0.5 +++	9.9 ± 0.8 ++	7.4 ± 0.8 ++	3.5 ± 0.7 +	1.5 ± 0.4 +	0.0 ± 0.0 -	0.0 ± 0.0 -
Control	14.0 ± 0.4 +++	10.5 ± 0.9 +++	6.0 ± 1.9 +	2.9 ± 1.1 +	1.2 ± 0.7 +	0.0 ± 0.0 -	0.0 ± 0.0 -

Enzymatic degradation

Combination paste was almost completely dissolved/degraded by day 14 with 5.55 ± 1.93% remaining of the original mass (Figure 6). A decrease in mass was observed for each day which is in contrast to the non-derivatized paste. Non-derivatized paste was degraded to 10% of its original mass by day 5 and remained there for the duration. The non-derivatized sponge did not experience any observable degradation. By day 14, it weighed 138.26 ± 2.07% of its original mass, and this is possibly due to the salts in PBS being retained and crystallizing in the sponge.

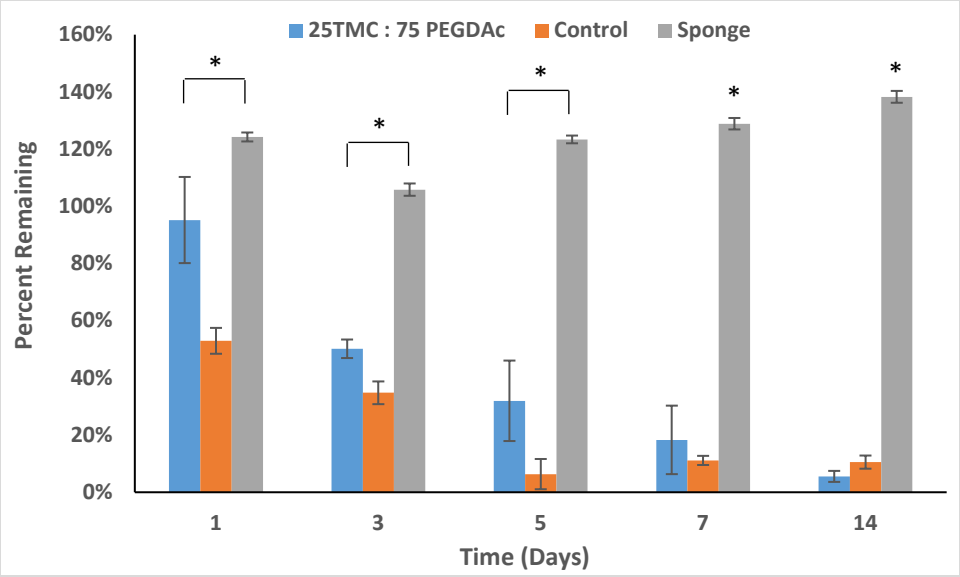

FIGURE 6. *In vitro* enzymatic degradation of chitosan-based delivery systems. Results are presented as mean ± standard deviation (n = 3). * denotes significant differences from control ($p < 0.05$).

Cytocompatibility

Each of the delivery systems evaluated were cytocompatible according the criteria established in ISO 10993-5.[24] Cellular viability was based on cells not exposed to chitosan-based biomaterials. Viability of NIH3T3 fibroblasts cells was reduced by approximately 12 and 13% after being exposed to combination paste for 24 and 72 hours, respectively (Figure 7). There was no reduction in viability for the non-derivatized sponge and paste after 24 hours, but there was approximately a 25 and 30% reduction after 72 hours. Similar results were observed with MC3T3 pre-osteoblast cells with combination paste producing a 4 and 17% reduction in viability on days 1 and 3, respectively (Figure 8). Again, there was not a reduction in viability for the non-derivatized sponge and paste after 24 hours, but there was a reduction of 6 and 16% after 72 hours.

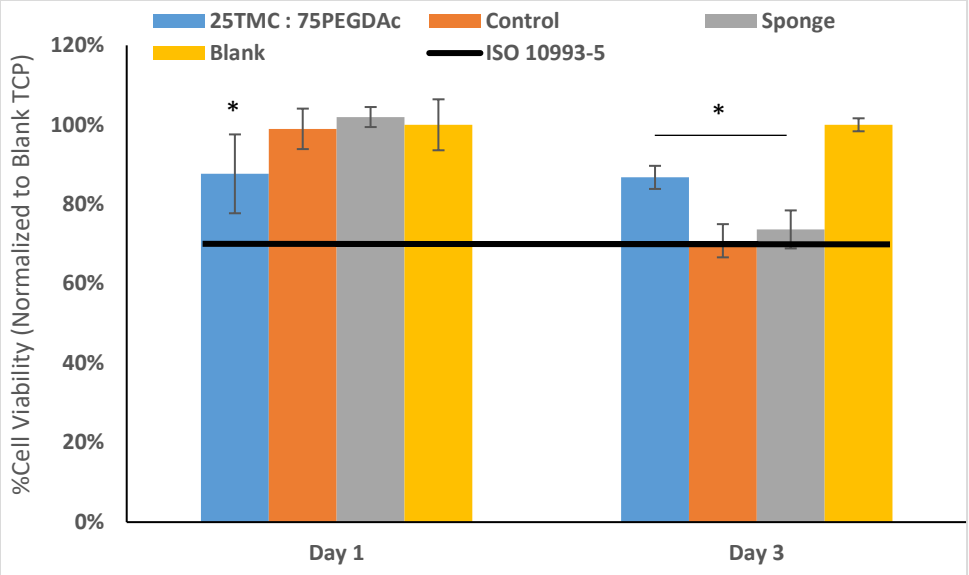

FIGURE 7. *In vitro* cytocompatibility evaluation with NIH3T3 fibroblast cells. Results are presented as mean ± standard deviation normalized to cells grown on blank tissue culture plastic (n = 3). * denotes significant differences from blank tissue culture plastic ($p < 0.05$). The black bar shows the threshold value outlined in ISO 10993-5 for cytocompatibility.

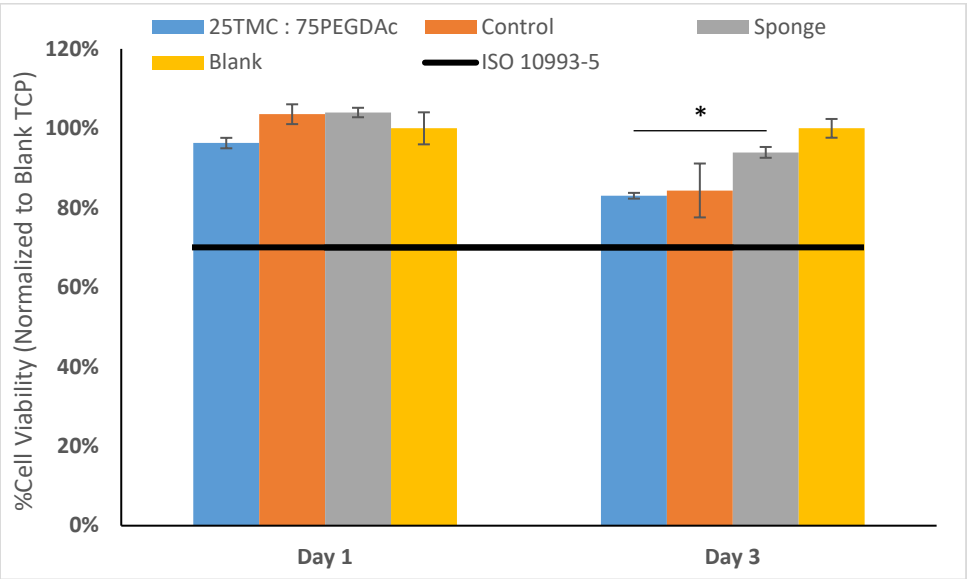

FIGURE 8. *In vitro* cytocompatibility evaluation with MC3T3 pre-osteoblast cells. Results are presented as mean ± standard deviation normalized to cells grown on blank tissue culture plastic

(n = 3). * denotes significant differences from blank tissue culture plastic ($p < 0.05$). The black bar shows the threshold value outlined in ISO 10993-5 for cytocompatibility.

Injectability

After applying baseline corrections, non-derivatized paste required 0.64 ± 0.22N to eject, and the combination paste required 9.40 ± 0.83N (Figure 9). Both of the evaluated injectable delivery systems are below clinically relevant values for ejection force.

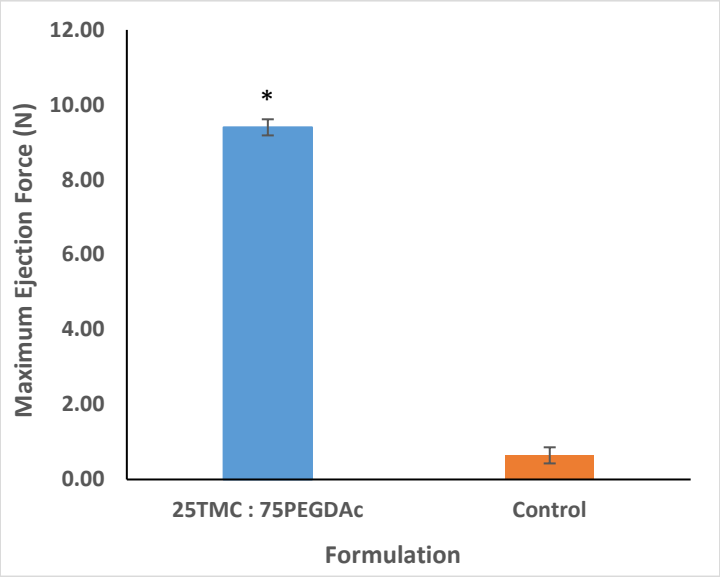

FIGURE 9. Injectability assessment of combination paste. Results are presented as mean ± standard deviation (n = 3). * denotes significant differences from control ($p < 0.05$).

DISCUSSION

This study sought to determine elution, degradation, and injectability of a derivatized chitosan paste composite containing TMC and PEGDAc to assess feasibility as an injectable local delivery system for antimicrobials. The combination exhibited an extended elution profile of vancomycin and increased degradation time compared to the non-derivatized paste without sacrificing the injectability or cytocompatibility of the delivery system. These enhanced properties may be due to the characteristics of the selected chitosan derivatives.[29,32] These

preliminary results indicate that the combination of TMC and PEGDAc should be considered for further investigation as a local delivery system for use in infection prevention in severe injuries with high infection rates.

Results from the FTIR and NMR analyses provided evidence that the modifications were successfully performed. PEGDAc required both FTIR and NMR results to provide confirmation that the synthesis was performed successfully. FTIR spectra showed peaks that were representative of ether bonds in PEGDA and PEGDAc, and these were absent in unmodified chitosan. NMR demonstrated that PEG was present in the polymer by the peak at 3.5 ppm. The absence of a group of peaks between 5.9 – 6.5 ppm is indicative that there were no free acrylate groups present.[30] This may indicate that all of the PEGDA molecules present are cross-linking the chitosan backbone. NMR results alone provided evidence that TMC was successfully synthesized. Peaks at 2.9 ppm and 3.2 ppm were assigned to the dimethyl and trimethyl groups, respectively. Sharp peaks were absent at 3.4 and 3.5 ppm which would indicate that side reactions at the hydroxyl groups did not occur.[28] However, there was evidence that residual NMP was still present in TMC.

A primary goal of this research was to reduce the mismatch between elution kinetics and degradation rates that affect chitosan-based delivery systems.[5] Elution kinetics for these systems typically exhibit an initial bolus release that is followed by low levels of release until the system is degraded.[27,33] PEGDA has previously been investigated as a cross-linked hydrogel and a cross-linking agent with a variety of systems to sustain drug delivery.[23,32,34] In preliminary work not contained in this manuscript, our group showed that PEGDAc had twice the swelling ratio of unmodified chitosan, and other groups have produced similar results.[23,32] This could help explain the extended release profile observed compared to unmodified chitosan and other injectable

chitosan paste studies.[27,35] Chen et al. investigated a complex of thiolated chitosan, PEGDA, and β-glycerophosphate as an injectable hydrogel.[23] Their study showed an extended release profile of a model protein that exhibited a tapered bolus release. The tapered release may be due to the size of the protein which would have a lower diffusion coefficient.[32]

MIC values reported in this study were lab generated values produced by exposing the bacteria to serial dilutions of the antimicrobials used. Activity observed for eluate samples indicate that incorporation of antimicrobials into the system did not inactivate them. Chitosan has been reported to possess antimicrobial properties, and quaternized chitosan derivatives, such as TMC, have been shown to have enhanced antimicrobial effects compared to chitosan.[15,21,36,37] The boost in activity observed against *P. aeruginosa* could possibly be due to an additive antimicrobial effect between amikacin and TMC, but the antimicrobial activity of the delivery system was not evaluated in this study.

Previous studies evaluated the release of vancomycin and amikacin from unmodified chitosan pastes for 3 days, but degradation properties for these systems did not match their elution kinetics. Berretta et al. showed almost complete degradation after day 2, and Rhodes et al. showed continued degradation out to day 10 which was their final time point.[27,35] Preliminary evaluations demonstrated that increasing the reaction time from 24 to 72 hours slowed the degradation rate of TMC. Previously developed systems degraded too rapidly, and it was determined that the degradation rate needed to be slowed.[27] Extended degradation of the system under investigation could be due to a combination of the increased molecular weight of TMC and the cross-linking of PEGDAc.[38] The 14 day degradation was within the time frame of initial healing, but this may mean that the material would remain after antimicrobials were expended. This could be ameliorated by using TMC that had been reacted for less time, Concentrations of

lysozyme used in this study were higher than physiological levels, and this might indicate that it would take longer than 14 days to degrade.[39] However, Stinner et al. demonstrated that chitosan sponges were almost completely degraded in an *in vivo* model of infection in 42 hours. Whereas, in this study, there was no measureable degradation after two weeks. *In vitro* evaluations lack the complexity to accurately mimic clinical scenarios, and this makes translation of *in vitro* degradation results difficult to translate to *in vivo* degradation time.

Another goal was to maintain the biocompatibility of chitosan after derivatization. Early studies of TMC reported that this material was cytotoxic, and that increasing DQ enhanced the cytotoxic effects.[21,40,41] Synthetic methods used to prepare TMC for the studies that showed cytotoxicity produced methylation at the amino and hydroxyl groups. More recent evaluations use synthetic procedures that specifically target the amino group and report minimal to no cytotoxic effects.[42-44] Mazzoccoli et al. reported that concentrations of PEGDA between 20-40% weight percent reduce cellular viability by 20-64% as the concentration increased.[45] PEGDAc produced in this study was manufactured using PEGDA at a 1% weight percentage and showed no cytotoxicity in preliminary studies. The apparent reduction in viability for each group and between days was higher than expected. It was noted during the experiment that cell culture media turned pinker for the non-derivatized sponge and yellower for non-derivatized paste, which would indicate increased alkalinity and acidity, respectively. Combination paste absorbed some of the media and could have sequestered growth factors from the cell culture media, or residual solvent could have been leached out of the device. Also, these evaluations used a static fluid flow model that would result in extended exposure of the cells to the materials.

Preparation of an injectable delivery system offers distinct advantages over other systems currently used for the treatment of open fractures. Hydration immediately prior to application

allows for a clinician to choose which antimicrobials are incorporated. Also, the hydration ratio can be tailored to modify the injectability properties. By hydrating paste with more aqueous antimicrobial solution, the injection force may be lowered and more antimicrobials can be delivered. MacDonald et al. determined the maximum ejection force of female healthcare workers using a standard chuck grip to be 79.5N.[25] Force values reported in this study are almost 10 times lower than this threshold value. Previous studies that have investigated injectable chitosan paste reported values for ejection force ranging from 30-150N.[27,35] Injectability of the system allows for complete coverage of open fractures that may possess complex geometries. This is an advantage over other delivery systems that are not able to conform to these wounds.

CONCLUSIONS

TMC and PEGDAc were synthesized, characterized, and developed into an injectable paste. *In vitro* evaluations indicate that the combination of TMC and PEGDAc may be developed into an improved local delivery system for infection prevention. The derivative combination's ability to elute active concentrations of vancomycin and amikacin, cytocompatibility with representative cell lines, and injectability through standard 1 mL syringes demonstrates its feasibility as an injectable antimicrobial delivery system. Capability to elute antimicrobials beyond 3 days make the system applicable for infection prevention in open fractures, and degradability within 14 days facilitates removal in a similar time frame to initial healing of these injuries. Injectability and degradability of the system provides a distinct advantage over other systems such as PMMA or $CaSO_4$ currently used for open fractures. Future studies will investigate the clinical potential of this system by using *in vivo* assessments for biocompatibility and infection prevention efficacy.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Marcin Guzinski and Bradley Hambly for assistance with TMC synthesis and characterization, Landon Choi for assistance with data collection, and Leslie Pace and Michael Harris for general research assistance. Research reported in this publication was supported by the National Institutes of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number R01AR066050. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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CHAPTER IV

DISCUSSION

The purpose of this study was to evaluate two chitosan derivatives, TMC and PEGDAc, as potential local antimicrobial delivery systems for infection prevention. Initial evaluations demonstrated that a combination of the two derivatives was superior to either one alone (Appendix B), and that a weight ratio of 75% PEGDAc 25% TMC was superior to both 50% PEGDAc 50% TMC and 25% PEGDAc 75% TMC (Appendix C). Efficacy of the combination was determined by evaluating elution kinetics of vancomycin and amikacin, activity of eluted antimicrobials, enzymatic degradation characteristics, cytocompatibility with representative cell lines, injectability from 1 mL syringes, and adhesivity to muscle tissue (Appendix E). Combining TMC and PEGDAc resulted in elution of active vancomycin and amikacin for 6 and 5 days and successfully met the first objective of extending elution. Increasing the reaction time for fabrication of TMC and cross-linking chitosan with PEGDA resulted in complete degradation by day 14; this is at the later end of the criteria established in the second research objective. Derivatization of chitosan did not result in any cytotoxicity according to ISO 10993-5. Cytocompatibility evaluations were performed with cell lines representative of tissues present in open fractures according to the third research objective. Incorporation of cross-linking and a quaternized moiety increased adhesivity compared to previous iterations of chitosan paste in line with the fourth research objective. Injectability characteristics of the combination of TMC and PEGDAc were conserved and approximately 10% of a benchmark force in accordance with research objective five. Enhanced properties observed for the combination paste are likely due to the properties of the derivatives.[16,19] These preliminary evaluations indicate that the combination

of PEGDAc and TMC has much potential to be developed for local delivery of antimicrobials to prevent infection.

Results from the FTIR and NMR analyses provided evidence that the synthesis of TMC and PEGDAc were successfully performed. NMR spectroscopy was used to characterize TMC, obtain a quantitative value for DQ, and assess if O-methylation had occurred. The synthetic procedure used to produce TMC was adapted from previous methods outlined in Verheul et al.[72] The authors reported a linear relationship between 40-60% DQ and reaction time of 24-72 hours. While TMC produced in this study was allowed to react for 72 hours under similar conditions to Verheul et al, the low DQ of 12% in the present study conflicts with the linear relationship reported. DQ was calculated using previous methods to be 12% and was lower than expected, and there was no evidence of O-methylation.[89] . This could possibly be explained by the differences in molecular weight and DDA of the starting materials. Verheul et al. used chitosan with a molecular weight of 42 kDa and DDA of 93%, and the chitosan used in this study had a molecular weight of 250.6 kDa and DDA of 82.46%. The conversion of dimethyl chitosan to trimethyl chitosan calls for iodomethane to be added in excess, and the amount called for in Verheul et al. was used. This may not have provided iodomethane in excess to produce the desired DQ. FTIR and NMR analysis were used to provide evidence that PEGDAc was successfully synthesized. In the FTIR spectra, characteristic peaks were observed at 1105, 1464, and 2877 cm^{-1} and correspond to C-O stretching, methylene C-H bending, and C-H stretching, respectively. PEGDA and PEGDAc possessed these peaks that are characteristic of ethers, and they were not present in chitosan. NMR analysis demonstrated that PEG was present in the polymer by the peak at 3.5 ppm. The absence of a peak group between 5.9-6.5 ppm indicates the

absence of free acrylate groups.[19] This may indicate that all of the PEGDA residues are cross-linking the chitosan backbone, and that none of the acrylated PEG chains are pendant groups.

Combining TMC and PEGDAc was designed to extend the elution kinetics compared to previously developed chitosan pastes. This goal was established to ameliorate the mismatch between elution and degradation that affect local delivery systems.[10,88] Chitosan-based systems typically exhibit an initial bolus release that is followed by low levels of release.[21,90] Berretta et al. and Rhodes et al. previously worked on different formulations of unmodified chitosan paste and showed elution of active vancomycin and amikacin for 3 days.[20,21] Huang et al. and Hoque et al. investigated chitosan derivative based hydrogels that eluted vancomycin for 7 and 14 days, respectively.[91,92] Huang et al. and Hoque et al. used Schiff base reactions to covalently attach vancomycin to chitosan. This could possibly explain the extended release profile observed in these studies, and the reduction observed for cumulative release profiles, 50% and 60%, respectively. Elution kinetics for the combination paste lasted for 7 days, and there was a cumulative release of approximately 76% of vancomycin and 82% of amikacin. This could be due to the PEGDAc component of the paste. PEGDA has been investigated as a cross-linked hydrogel and a cross-linking agent with different local delivery systems to sustain therapeutic delivery.[83,93,94] Preliminary studies demonstrated that PEGDAc had twice the swelling ratio compared to unmodified chitosan (Appendix A), and other groups have produced similar results.[83,93] Chen et al. investigated a complex of thiolated chitosan, PEGDA, and β-glycerophosphate as an injectable hydrogel.[83] Their study showed an extended release profile of a model protein that exhibited an attenuated burst release. The evaluated systems were only loaded with two classes of antimicrobials, and this limits the ability to generalize these results to

other classes of antimicrobials. Vancomycin and amikacin were chosen because they are used in the management of patients with complex musculoskeletal injuries or MRSA infections.[4,38]

MIC values used for reference in the elution study were lab generated values that were produced using serial dilutions of the antimicrobials. The observed activity indicates that incorporation of aqueous antimicrobial solution did not diminish their antimicrobial effects. Chitosan has been reported to possess antimicrobial properties, and quaternized chitosan derivatives, such as TMC, have been shown to have enhanced antimicrobial effects.[15,80,95] Additional activity observed against *P. aeruginosa* beyond where the MIC was reached could be due to an additive effect between TMC and amikacin. Zhang et al. and Wahid et al. investigated chitosan derivative based hydrogels that displayed antimicrobial effects.[96,97] Wahid et al. provided evidence that the antimicrobial effect was due to increased concentrations of zinc ions, but Zhang et al. showed that the antimicrobial activity could be attributed to quaternized chitosan.

Degradation of chitosan-based delivery systems is a major advantage over PMMA and $CaSO_4$, but matching elution and degradation properties is difficult. Previous studies of chitosan paste demonstrated elution of antimicrobials for 3 days, but degradation properties did not match their elution kinetics. Berretta et al. showed almost complete degradation after day 2, and Rhodes et al. showed continue degradation out to their final time point of 10 days.[20,21] Preliminary evaluations demonstrated that degradation of TMC could be slowed by increasing the reaction time for 24 to 72 hours. Degradation of previously developed systems was too rapid, and this led to incorporation of TMC that had been reacted for longer.[21] Enhanced molecular weight of TMC and the cross-linked structure of PEGDAc could be contributing to the enhanced degradation time. The 14 day degradation observed for this system allows for antimicrobials to be delivered

and complete degradation to occur as initial healing ceased. Degradation time could be shortened by exchanging the TMC for one that had been reacted for less time. Degradation solution used in this study had a lysozyme concentration of 1 mg/mL which is much higher than physiological levels.[98] Other factors could affect the *in vivo* degradation rate, and this makes translating *in vitro* results difficult to physiological degradation. Another limitation was the exclusive use of *in vitro* assessments for the efficacy and biocompatibility of this system. *In vitro* evaluations lack the complexity to accurately mimic clinical scenarios, but they are invaluable models for collecting preliminary data during initial evaluations.

Biomaterials used in infection prevention need to demonstrate cytocompatibility before they can be implemented. Combination paste showed a reduction in relative cell viability compared to cells grown on blank tissue culture plastic, but the relative viability remained above the standard of 70% viability.[99] Preliminary study results demonstrated that the DQ of TMC could be modified to control the degradation rate without decreasing cytocompatibility (Appendix A). This contradicts early studies with TMC reporting that the material was cytotoxic, with increasing cytotoxicity as DQ increased.[80,100,101] However, the synthetic methods used to prepare the TMC used in studies that reported cytotoxicity produced non-specific methylation of both the amine and hydroxyl groups. More recent evaluations use synthetic procedures that target the amine group to eliminate the cytotoxicity of TMC.[78,102,103] Preliminary evaluations appeared to show that increasing the content of TMC decreased cytocompatibility, but the apparent differences between the groups were not significant (Appendix C). Preliminary results also demonstrated that PEGDAc did not lead to an increase in cytotoxicity and may have exerted a pro-proliferative effect on NIH3T3 fibroblasts compared to controls (Appendix A). Mazzoccoli et al. reported that concentrations of PEGDA between 20-40% weight percent reduced cellular

viability by 20-64% as concentration increased.[104] PEGDAc evaluated in this study was manufactured using PEGDA at 1% weight percentage, and there was no evidence of cytotoxicity in preliminary studies. Observations noted during this study were that the non-derivatized sponge and non-derivatized paste produced a change in color of the cell culture media with pH indicator. Non-derivatized sponge turned the media pink which would indicate increased alkalinity due to the sponges not being completely neutralized. Non-derivatized paste turned the cell culture media yellow, which indicates increased acidity, likely due to the acetic acid content. Reduced cell viability for the combination paste could be due to residual solvents remaining in the system. Combination paste also absorbed some of the media, which could have resulted in sequestering of growth factors from the media. A limitation of this evaluation is that static fluid flow conditions result in extended exposure of cells to materials and solvents, and thus may not accurately predict cell response in an *in vivo* wound.

Injectable delivery systems provide complete wound coverage that other systems currently used in the management of complex musculoskeletal trauma such as PMMA and $CaSO_4$ do not offer. Hydration immediately prior to application allows for a clinician to incorporate their choice of antimicrobials depending on the suspected bacteria, and the hydration ratio can be adjusted to modify the injectability. By providing more aqueous antimicrobial solution, the force required to eject the paste may be decreased and increases the amount of antimicrobials available. MacDonald et al. determined the maximum ejection force of female healthcare workers using several different grip methods to range from 79.5-104.7N. Force values reported in this study are lower than this threshold value. Previous studies that have investigated injectable chitosan paste report values for ejection force ranging from 30-150N. [20,21] Injectability of the system allows for complete coverage of open fractures that may have complex geometries.

However, coverage may not be maintained if the delivery system does not adhere to the wound site and migrates. TMC and PEGDAc have been reported to have enhanced mucoadhesive properties compared to chitosan.[19,105] Pardeshi et al. reported a 3.4-folds increase in adhesivity for TMC compared to chitosan and was attributed to ionic interactions between TMC and mucin proteins.[105] Shitrit et al. demonstrated a 3-fold increase in adhesivity for PEGDAc, and the authors attributed this to entanglement with PEGDA chains and a Michael addition reaction between free acrylate groups and mucin proteins.[19] Combination paste incorporates both of these moieties, and this could explain the enhanced adhesion observed compared to the non-derivatized chitosan paste (Appendix E). Combination paste can easily be removed by conditions similar to irrigation performed in the management of complex musculoskeletal trauma. Whereas, the non-derivatized paste was resistant to removal by PBS irrigation, and principally removed by gravity.

CHAPTER V

CONCLUSIONS

TMC and PEGDAc were successfully synthesized, characterized, and developed into an injectable paste. Initial studies were performed to determine the optimal combination of TMC and PEGDAc according to preliminary elution and cytocompatibility results (Appendices A, B, and C). *In vitro* evaluations indicate that weight percentages of PEGDAc and TMC can be adjusted to achieve target goals for elution, degradation, and cytocompatibility. In addition to the capability to elute antimicrobials, degradability, and cytocompatibility, combination paste is injectable through standard 1 mL syringes and adheres to muscle tissue, demonstrating its feasibility as an injectable delivery system. Elution kinetics lasting longer than 3 days makes the system applicable for protecting open fractures from infection during wound closure and dressing changes. Capability to degrade within 14 days facilitates removal from these wounds in a similar time frame to initial healing, and cytocompatibility allows the combination to be used as an infection prevention biomaterial without eliciting deleterious adverse effects. Injectability provides greater wound coverage which is an advantage over other delivery systems currently used in the management of open fractures such as PMMA or $CaSO_4$. Enhanced mucoadhesion is an improvement over previously developed chitosan pastes and reduces migration of the delivery system from the wound site. These *in vitro* evaluations indicate that the combination of TMC and PEGDAc has potential to be developed into a local delivery systems for infection prevention in complex musculoskeletal trauma.

CHAPTER VI

FUTURE WORK

Future evaluations should further investigate the nature of how PEGDA interacts with chitosan. The initial idea was that PEGDA chains would freely hang from the backbone of chitosan and allow free acrylate groups to react with mucin proteins. Some of the chains may be freely hanging, but it is likely that the majority of the PEG chains are acting as cross-linking agents, which is evidenced by the enhanced swelling ratio of PEGDAc and the absence of acrylate groups in the NMR spectra. Another study should investigate the effects of adding additional iodomethane during the synthesis of TMC. This may address the lower than expected DQ observed. Also, additional purification steps should be incorporated to remove residual NMP from TMC.

Chitosan used to synthesize TMC and PEGDAc was chosen because it had been previously used to fabricate other local delivery systems. DDA and molecular weight are important parameters of chitosan that helps to determine its properties. Modification of these two parameters allows for tailoring of the properties chitosan before derivatization and may produce a more efficacious delivery system. Furthermore, this study systematically investigated a limited number of formulations. Combinations were chosen by mixing the desired components in a 50:50 weight ratios and adjusted by further 50:50 splitting of the two components. Following this scheme, a 62.5% PEGDAc 37.5% TMC and an 87.5% PEGDAc 12.5% TMC should be evaluated to determine their characteristics. All evaluations were performed using a hydration ratio of 4.5 mL of solution per gram of dried paste based on preliminary studies. Increasing the hydration ratio of this system may provide a method to reduce ejection force and increase antimicrobial loading, thus enhancing injectability and extending elution.

Evaluation performed for this study could be further expanded to provide a more robust characterization of the local delivery system. Vancomycin and amikacin were chosen as antimicrobials since they are commonly used in the prophylaxis of complex musculoskeletal trauma and in combination have activity against Gram positive and Gram negative bacteria. Future studies may determine elution kinetics of other antimicrobials, such as tetracyclines, macrolides, and first and second generation cephalosporins. In a similar vein, future studies could evaluate efficacy of the delivery system in treating other pathogenic bacteria, such as methicillin resistant strains of *S. aureus*, the enterococci genus, and *Acinetobacter baumannii*. While we have investigated chitosan derivatives as antimicrobial delivery systems in this work, the possible additive antimicrobial effect between TMC and amikacin could be explored in future microbiological investigations. Degradation characteristics of the evaluated system did not meet the target of 14 days, and this could be ameliorated by using TMC that had been reacted for less time than TMC used in this study. Cytocompatibility evaluations in this study were limited to cells representative of connective tissue and bone tissue, but future studies could explore more in depth inflammatory responses that could be triggered by the combination of chitosan derivatives. Additional cytocompatibility studies will investigate the effects on cellular viability by incorporating antimicrobials into the system. Injectability was assessed from standard 1 mL syringes for application to external injuries, but future studies will investigate delivery through different gauge needles for subcutaneous applications.

Future studies for the evaluated combination of PEGDAc and TMC will incorporate *in vivo* evaluations of biocompatibility and infection prevention efficacy. A rodent model will be used to evaluate the safety of the device when incorporated into a living system without infection present. If the delivery system is proven to be biocompatible, its efficacy can be evaluated in an

in vivo model of infection prevention. Early studies will focus on preventing infection from occurring by placing the delivery system alongside bacteria before they establish an infection. Later studies will focus on eliminating an already established infection. These studies would have bacteria inoculated prior to placement of the local delivery system with infection already established.

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APPENDIX A: Preliminary Refinement of TMC and PEGDAc

Rationale

The characteristics of TMC and PEGDAc can be adjusted by modifying the reaction conditions. TMC can be tailored by varying the DQ to affect properties such as mucoadhesivity, solubility, and antibacterial activity.[70,76,79] The synthetic procedure used to manufacture TMC in this study is able to control DQ by changing the duration of reaction.[72] PEGDAc can be modified by adjusting the molar ratio of PEGDA molecules to amine groups on chitosan. This ratio has been demonstrated to affect swelling ratio, mucoadhesivity, and cytocompatibility.[19,93,104] Previous studies by Verheul et al. and Shitrit et al. were used to determine initial reaction conditions, and further modifications were performed after initial evaluations demonstrated success. Different groups of TMC were produced by changing the reaction time for each group to 24, 48, or 72 hours. PEGDAc groups were synthesized by modifying the molar ratio to 23.5, 47, or 94 free amine groups to PEGDA molecules. Three varieties of each derivative were synthesized to determine if DQ or the molar ratio of PEGDA to amine groups had an effect on antimicrobial elution, degradation rate, swelling ratio, or cytocompatibility.

Antimicrobial elution

Lyophilized constructs (n = 4) of TMC and PEGDAc were quartered and hydrated for 2 minutes using a 1 mg/mL solution of vancomycin. Each quarter was added to a petri dish with 5 mL of PBS and placed in an incubator at 37°C. Sampling was performed daily for 7 days with complete refreshment of PBS at each time point. Vancomycin was detected and quantified using an HPLC system interfaced with a UV/Vis spectrophotometer. Statistical analysis was performed using one way ANOVA with a SNK post-hoc test to perform comparisons between groups. Differences were considered significant if p-values were less than 0.05.

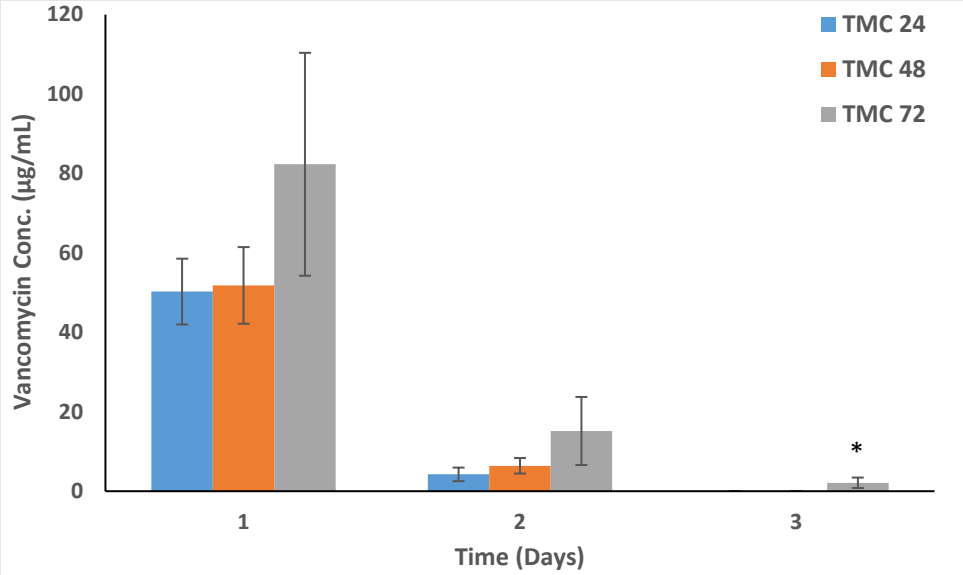

FIGURE 10. Preliminary *in vitro* elution results of vancomycin from TMC groups. Data are presented as mean ± standard deviation. * denotes significant differences from other groups.

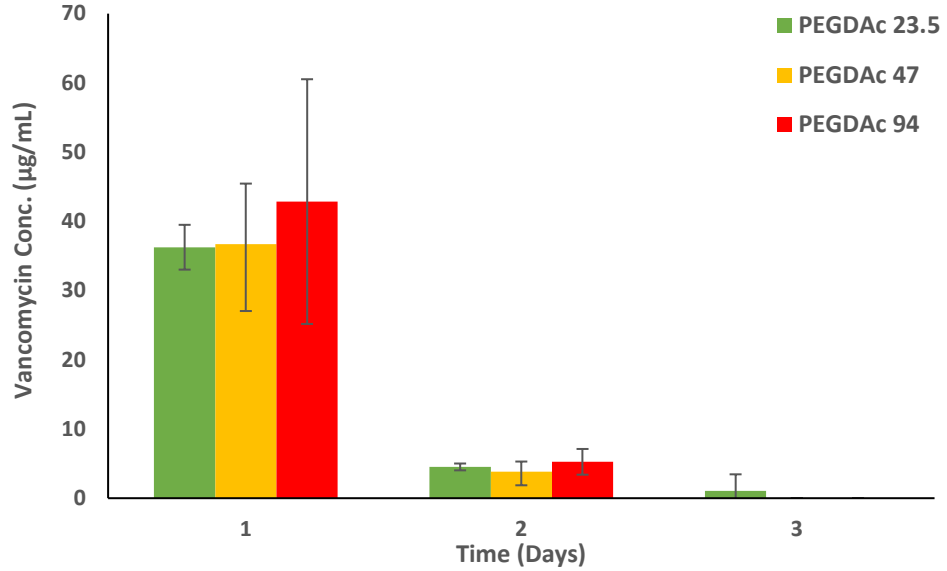

FIGURE 11. Preliminary *in vitro* elution results of vancomycin from PEGDAc groups. Data are presented as mean ± standard deviation. * denotes significant differences from other groups.

Oxidative degradation

Five lyophilized sponges from each group were quartered (n = 4), weighed, and hydrated for 2 minutes in PBS. Samples were immersed in an oxidative degradation solution consisting of 0.1M $CoCl_2 \cdot 6H_2O$ and 3% H_2O_2.[106] TMC groups were sampled at 1, 2, 3, 4, and 6 hours by removal from the solution, rinsing with PBS, and drying in a vacuum oven at 45°C. PEGDAc groups were sampled in a similar manner at 0.5, 1, 1.5, 2, and 3 hours. After samples dried for 3 days, they were weighed to calculate percent remaining. Statistical analysis was performed using one way ANOVA with a SNK post-hoc test to perform comparisons between groups. Differences were considered significant if p-values were less than 0.05.

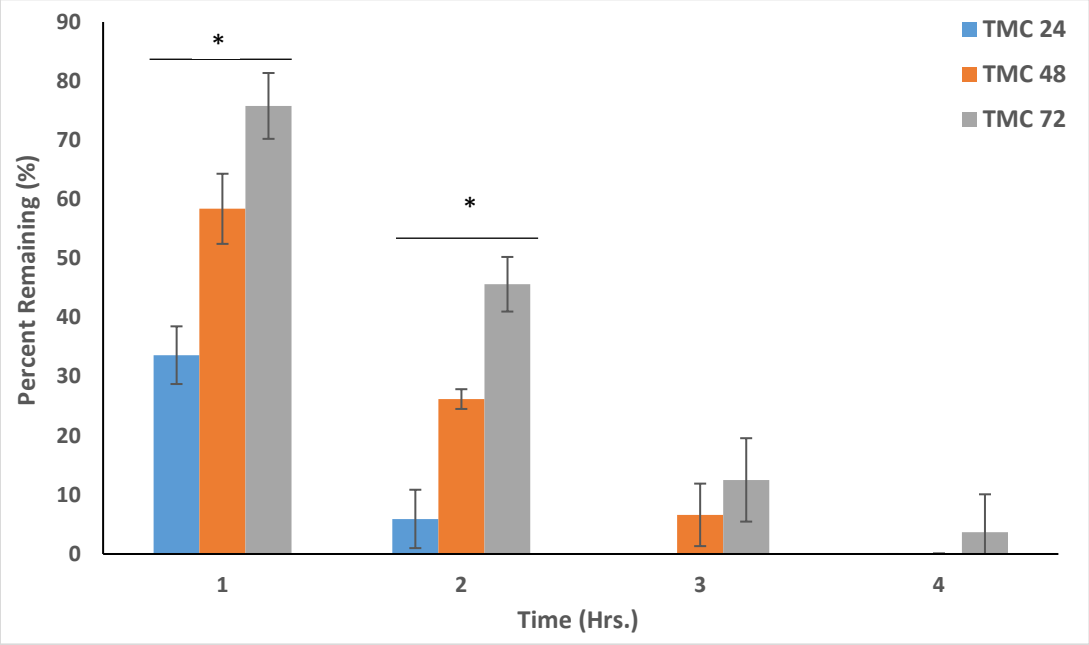

FIGURE 12. Preliminary *in vitro* accelerated degradation study of TMC groups. Data are presented as mean ± standard deviation. * denotes significant difference between each group.

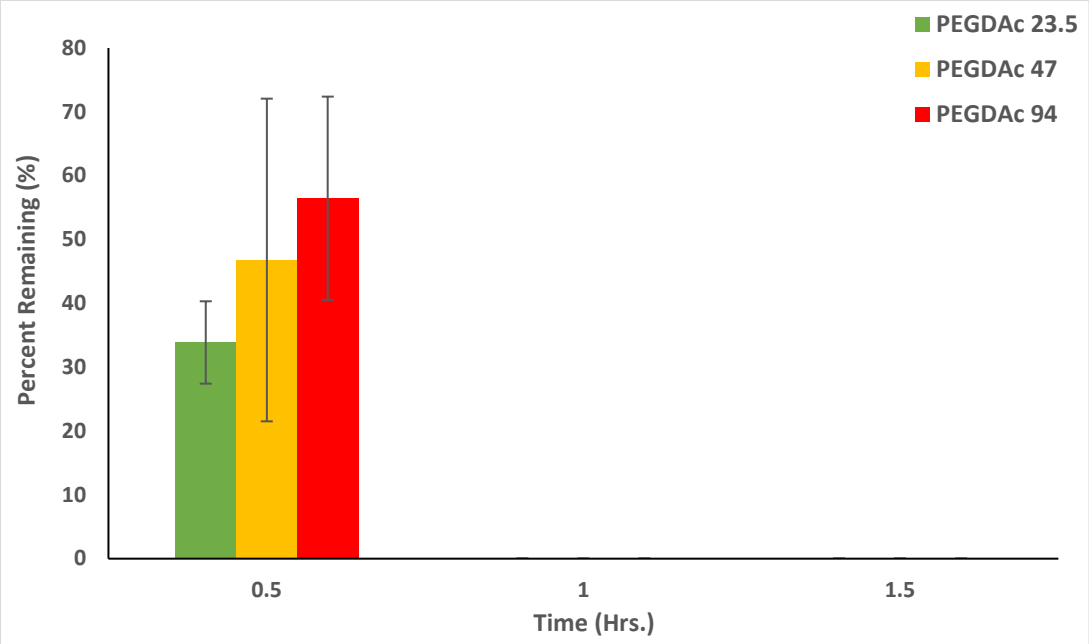

FIGURE 13. Preliminary *in vitro* accelerated degradation evaluation of PEGDA groups. Data are presented as mean ± standard deviation. * denotes significant difference from each group.

Swelling ratio

Sponges from each group were quartered (n = 4), weighed, and hydrated in PBS for 2 minutes. Excess fluid was allowed to drip off before obtaining the wet mass. Fluid uptake was calculated as the difference between the wet and dry masses. Swelling ratio was calculated by normalizing the fluid uptake to the initial mass. Statistical analysis was performed using one way ANOVA with a SNK post-hoc test to perform comparisons between groups. Differences were considered significant if p-values were less than 0.05.

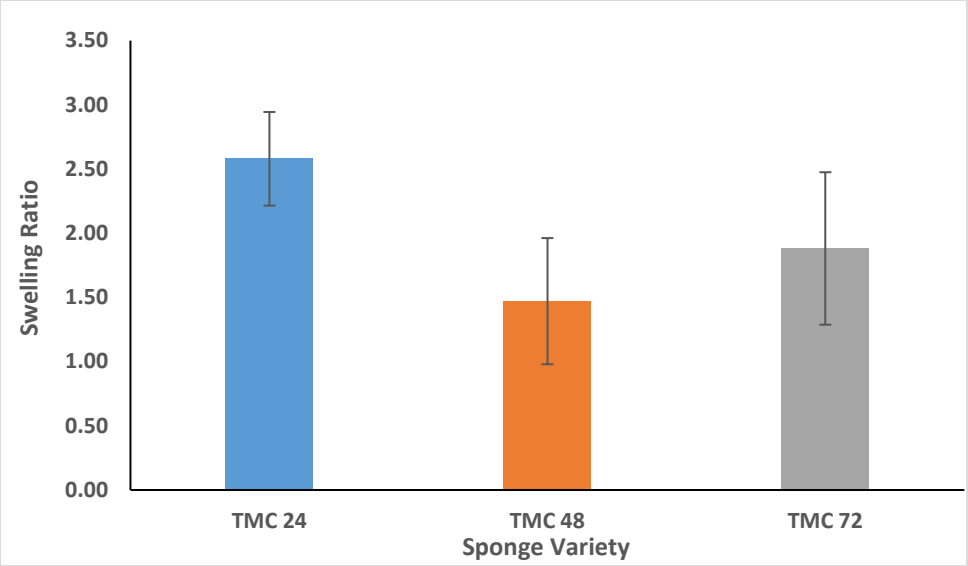

FIGURE 14. Preliminary *in vitro* swelling ratio results of TMC groups. Data are presented as mean ± standard deviation. * denotes significant differences from each group.

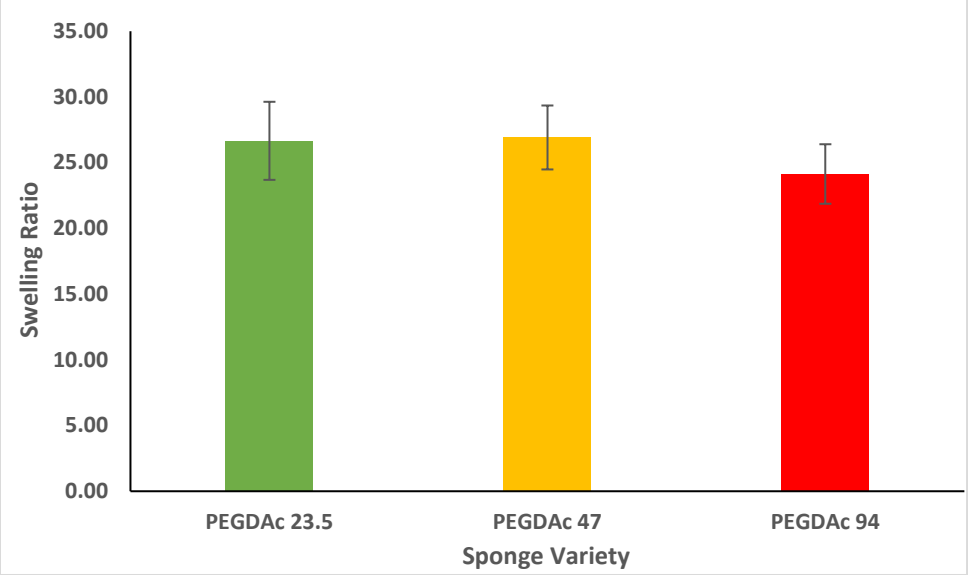

FIGURE 15. Preliminary *in vitro* swelling ratio results of PEGDAc groups. Data are presented as mean ± standard deviation. * denotes significant differences from each group.

Cytocompatibility

NIH3T3 fibroblasts were seeded at 10^4 cells/cm^2 in 24-well plates and grown in DMEM supplemented with 10% FBS and 100 µg/mL of Normocin for 24 hours. Coupons (diameter = 6mm) were punched from each derivative, placed in cell culture inserts (pore size = 8um), and placed in each well. Cells were exposed to the coupons for 24 hours and quantified using Cell-Titer Glo assay. Results were expressed as a percentage of cells grown on blank tissue culture plastic. Statistical analysis was performed using one way ANOVA with a SNK post-hoc test to perform comparisons between groups. Differences were considered significant if p-values were less than 0.05.

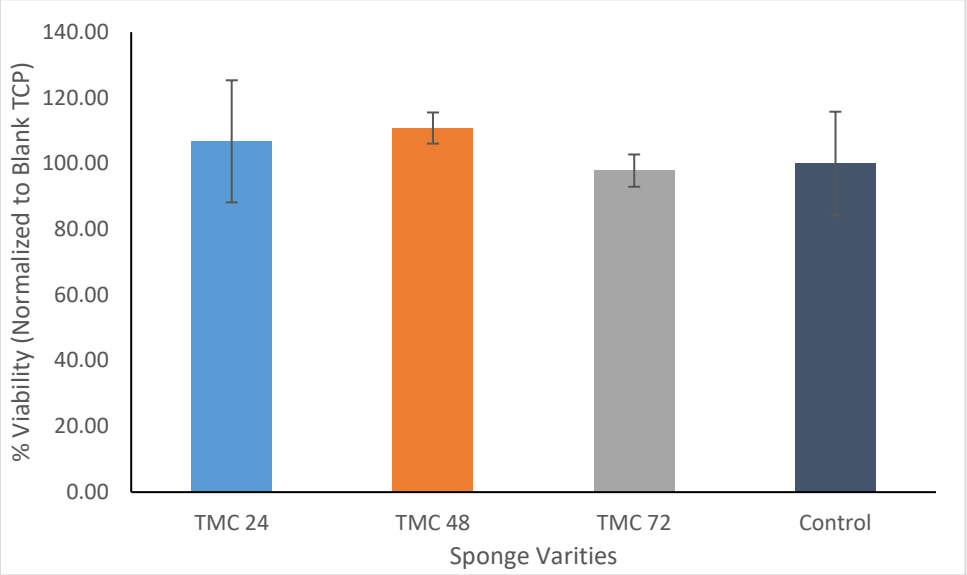

FIGURE 16. Preliminary *in vitro* cytocompatibility results with NIH3T3 fibroblasts for TMC groups. Data are normalized to cells grown on blank tissue culture plastic and represented as a mean ± standard deviation. * denotes statistical significance from all groups.

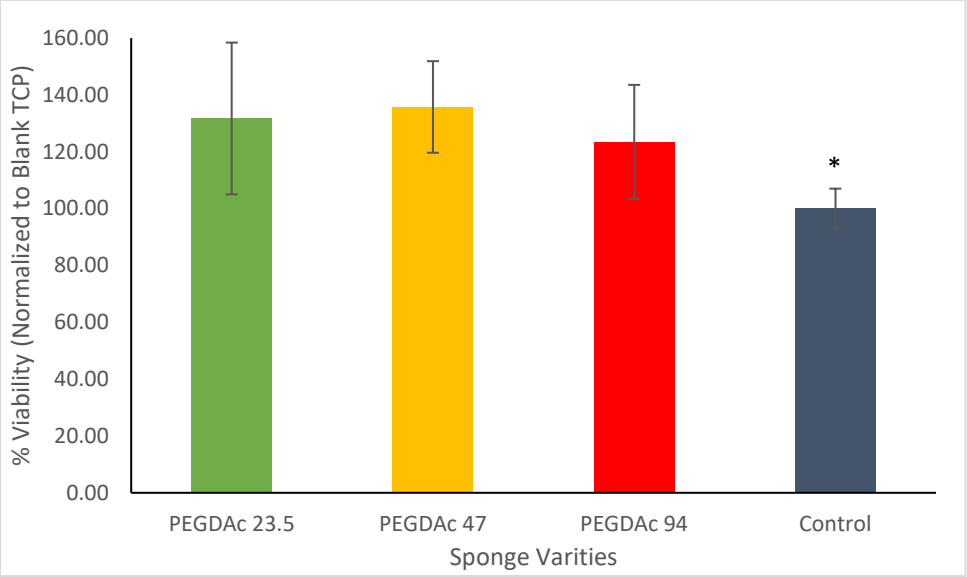

FIGURE 17. Preliminary *in vitro* cytocompatibility results with NIH3T3 fibroblasts for PEGDAc groups. Data are normalized to cells grown on blank tissue culture plastic and represented as a mean ± standard deviation. * denotes statistical significance from all groups.

Conclusion

All TMC formulations were able to elute antimicrobials (Figure 8), degradable in an oxidative environment (Figure 10), uptake fluid (Figure 12), and cytocompatible with cells representative of connective tissue (Figure 14). The only significant differences observed between the three formulations were in the degradation study. TMC 48 and TMC 72 lasted significantly longer than TMC 24. TMC 72 appeared to last longer than TMC 48, but this difference was not significant. Each PEGDAc formulation was found to be able to elute antimicrobials (Figure 9), degrade in an oxidative environment (Figure 11), uptake fluid (Figure 13), and not be cytotoxic to NIH3T3 fibroblasts (Figure 15). No significant differences were observed between the PEGDAc formulations in any of the evaluations. Previous paste formulations degraded before they could deliver their antimicrobial payload, and it would be

advantageous to extend the degradation profile to match the elution properties. Therefore, TMC 72 and PEGDAc 47 were chosen for continued evaluation.

APPENDIX B: Preliminary Paste Combination Evaluations

Rationale

TMC and PEGDAc show promise as lyophilized sponges for local antimicrobial delivery, but their efficacy as paste delivery systems still needed to be evaluated. Using an injectable paste over a lyophilized sponge offers several advantages: enhanced wound coverage for complex musculoskeletal injuries, decreased diffusion distance of antimicrobials, and multiple starting products can be mixed to obtain desired properties. Wound coverage and decreased diffusion distance have already been discussed, but the concept of mixing different starting products has not been evaluated. Previous evaluations showed that TMC had an extended degradation profile compared to unmodified chitosan, and that PEGDAc had twice the swelling ratio compared to unmodified chitosan. Each derivative alone, a combination of the derivatives, a previously developed paste[21], and combinations of the previous paste and derivatives were evaluated for their ability to elute antimicrobials and cytocompatibility with NIH3T3 fibroblasts.

Antimicrobial elution

Approximately 600 mg of each paste variety was hydrated using a 5 mg/mL solution of vancomycin. Combinations were prepared by mixing 300 mg of the respective dried materials. Dried powder was hydrated using a ratio of 4.5 mL solution per gram of dried powder except for PEGDAc alone, which required 7.0 mL solution per gram. Approximately 0.6 mL of hydrated paste (n = 4) was injected into cell crowns (Scaffdex) with nylon filters (pore size = 41 μm) attached. Each sample was placed in 5 mL of PBS, incubated at 37°C, and sampled daily. Upon sampling, each sample was completely refreshed with PBS. Vancomycin was detected and quantified using an HPLC system interfaced with a UV/Vis spectrophotometer. Statistical

analysis was performed using one way ANOVA with a SNK post-hoc test to perform between group comparisons. Differences were considered significant if p-values were less than 0.05.

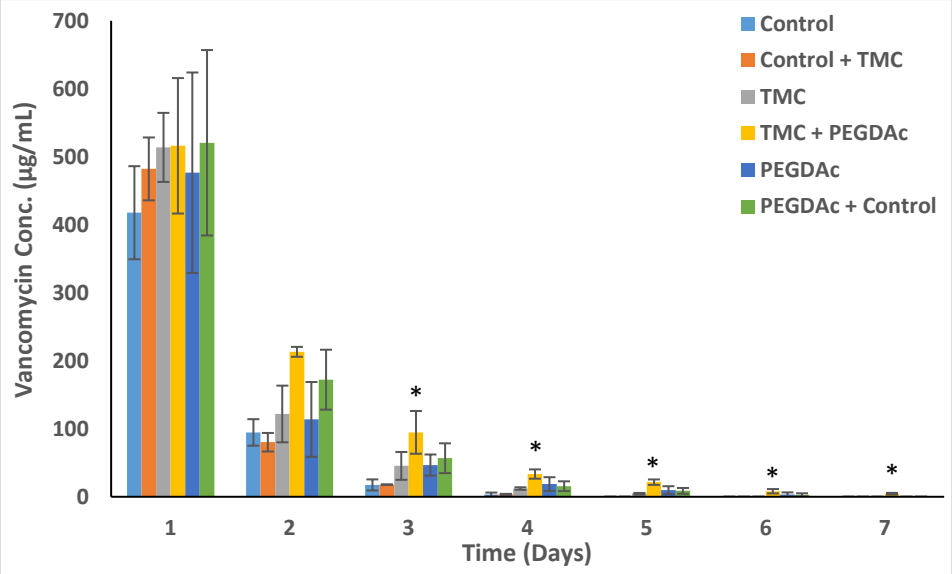

FIGURE 18. Full scale (Days 1-7) preliminary *in vitro* elution results of vancomycin from chitosan derivative combinations pastes. Data are presented as mean ± standard deviation. * denotes significantly higher concentrations compared to all other groups.

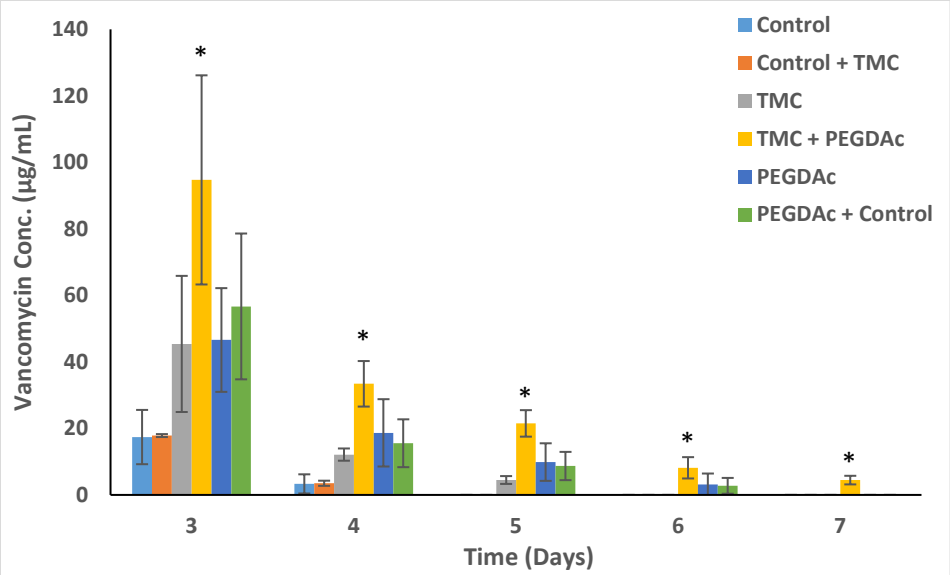

FIGURE 19. Zoomed in (Days 3-7) preliminary *in vitro* elution results of vancomycin from chitosan derivative combinations pastes. Data are presented as mean ± standard deviation. * denotes significant differences from all other groups.

Cytocompatibility

NIH3T3 fibroblast cells were seeded at 10^4 cells/cm^2 in 24-well plates and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 100 µg/mL of an antibiotic/antimycotic solution for 24 hours. Approximately 300 mg of dried material was hydrated using a PBS solution. Hydrated volumes were split into three equal volumes (~0.5 mL) and injected into cell culture inserts (Falcon, pore size = 8 µm) and placed in each well. Cells were exposed to pastes eluates for 24 hours and quantified using Cell-Titer Glo (Promega) assay. Results were expressed as percentage of cells grown on blank tissue culture plastic. Controls in this study was unmodified chitosan paste (control) and an unmodified chitosan sponge (sponge).

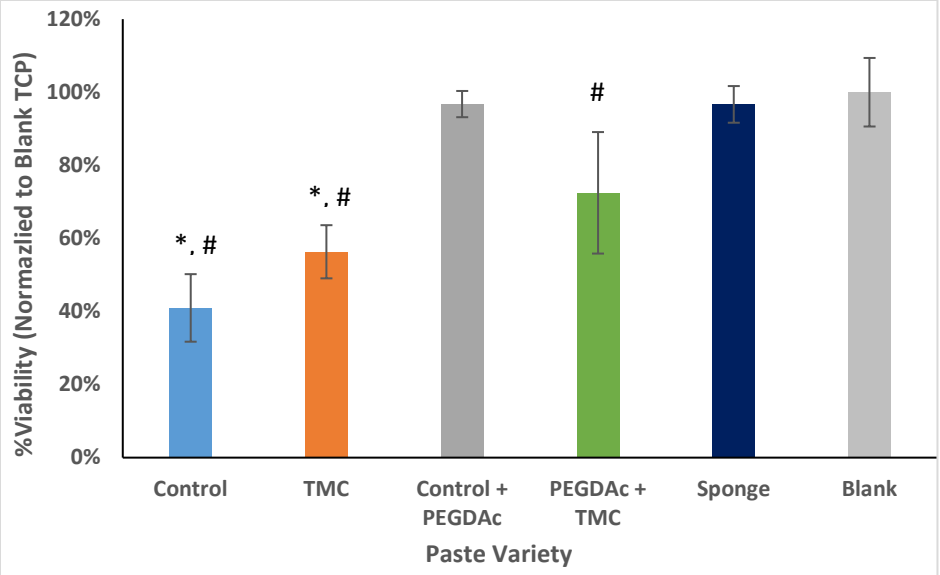

FIGURE 20. Preliminary *in vitro* cytocompatibility results with NIH3T3 fibroblasts for pastes that elute vancomycin. Data are normalized to cells grown on blank tissue culture plastic and represented as a mean ± standard deviation. * denotes statistical significance vs. sponge, and # denotes statistical significance vs. blank.

Conclusion

Combination of control and TMC paste was eliminated by the elution study due to only eluting for 4 days (Figure 16 and 17). Control would also be eliminated by this criteria, but it remained as a historical control. PEGDAc was not carried forward to the cytocompatibility study due to difficulty with handling properties. Both the control and TMC were eliminated in the cytocompatibility evaluation. Both pastes performed under the benchmark set out by ISO 10993-5. The combinations of PEGDAc and TMC and PEGDAc and control both eluted for six days and were cytocompatible according to ISO 10993-5. The combination of PEGDAc and TMC eluted longer than PEGDAc and control (7 vs. 6 days), but it displays a lower relative viability (72 vs. 97%) (Figures 17 and 18). However, there was one replicate that performed poorly compared to the other two, and if this point was excluded, it would be (82% vs. 97%). Further

research efforts are focused on the combination of PEGDAc and TMC with the possibility of PEGDAc and control being evaluated in the future.

APPENDIX C: Refinement of PEGDAc and TMC Combination

Rationale

A combination of PEGDAc and TMC paste was superior to other evaluated pastes, but other combinations of these two derivatives have not been tested. The 50:50 combination was above the cytocompatibility benchmark established in ISO 10993-5, but it could be possibly be improved by adjusting the ratio of PEGDAc to TMC. This is evidenced by the improved cytocompatibility observed when PEGDAc was mixed with either the control paste or TMC. Three combinations of PEGDAc and TMC were produced by modifying the weight ratios: 25% PEGDAc 75% TMC, 50% PEGDAc 50% TMC, 75% PEGDAc 25% TMC. These were evaluated to see if there was an effect on antimicrobial elution or cytocompatibility.

Antimicrobial elution

Approximately 600 mg of each paste variety was hydrated using a 5 mg/mL combination solution of vancomycin and amikacin. Combinations were prepared by mixing the appropriate weight percentages of dried material. Powder was hydrated using a ratio of 4.5 mL solution per gram of dried powder. Approximately 0.6 mL of hydrated paste (n = 4) was injected into cell crowns (Scaffdex) with nylon filters (pore size = 41 μm) attached. Each sample was placed in 5 mL of PBS, incubated at 37°C, and sampled daily. Upon sampling, each sample was completely refreshed with PBS. Vancomycin was detected and quantified using an HPLC system interfaced with a UV/Vis spectrophotometer. Amikacin was quantified using a previously described method of pre-column derivatization with an o-phthaldialdehyde reagent and subsequent detection with an HPLC system using a fluorescence detector.[107] Statistical analysis was performed using one way ANOVA with a SNK post-hoc test to perform comparisons between groups. Differences were considered significant if p-values were less than 0.05.

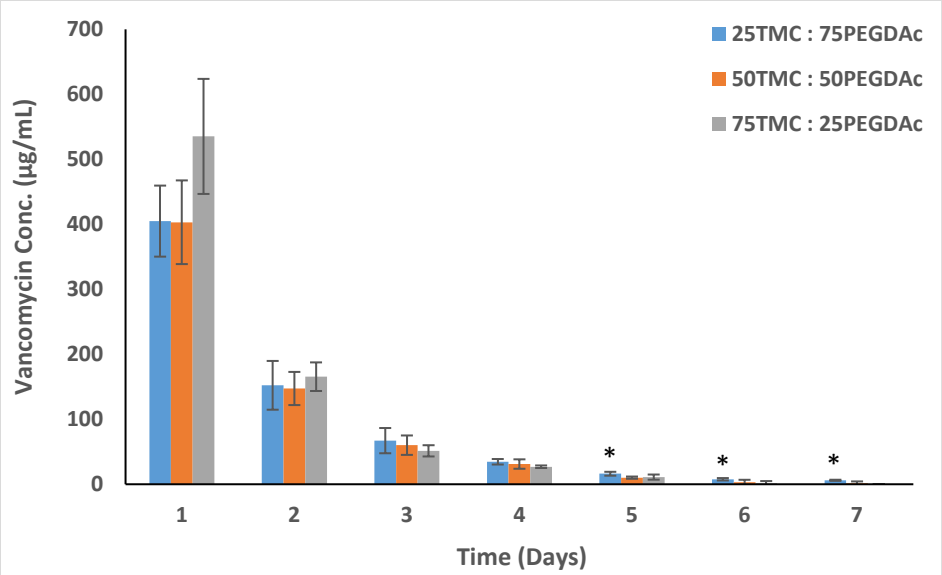

FIGURE 21. Full scale (Days 1-7) preliminary *in vitro* elution results of vancomycin from chitosan derivative combinations pastes. Data are presented as mean ± standard deviation. * denotes significantly higher concentrations compared to all other groups.

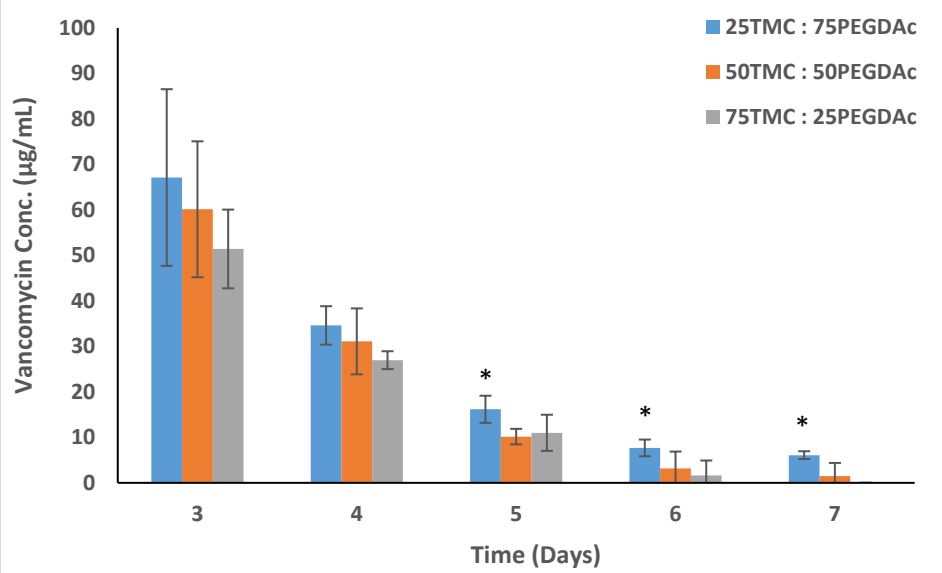

FIGURE 22. Zoomed in (Days 3-7) preliminary *in vitro* elution results of vancomycin from chitosan derivative combinations pastes. Data are presented as mean ± standard deviation. * denotes significant differences from all other groups.

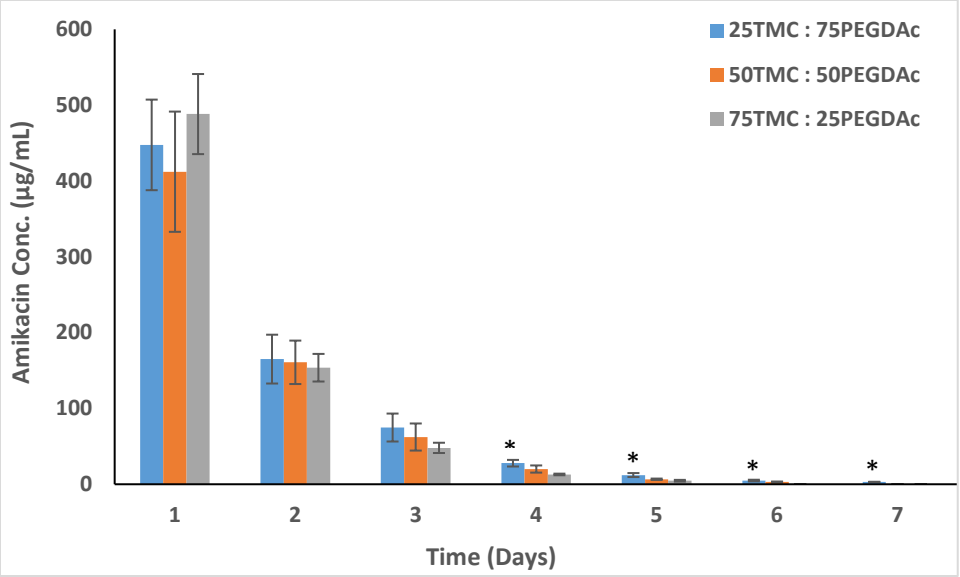

FIGURE 23. Full scale (Days 1-7) preliminary *in vitro* elution results of amikacin from chitosan derivative combinations pastes. Data are presented as mean ± standard deviation. * denotes significantly higher concentrations compared to all other groups.

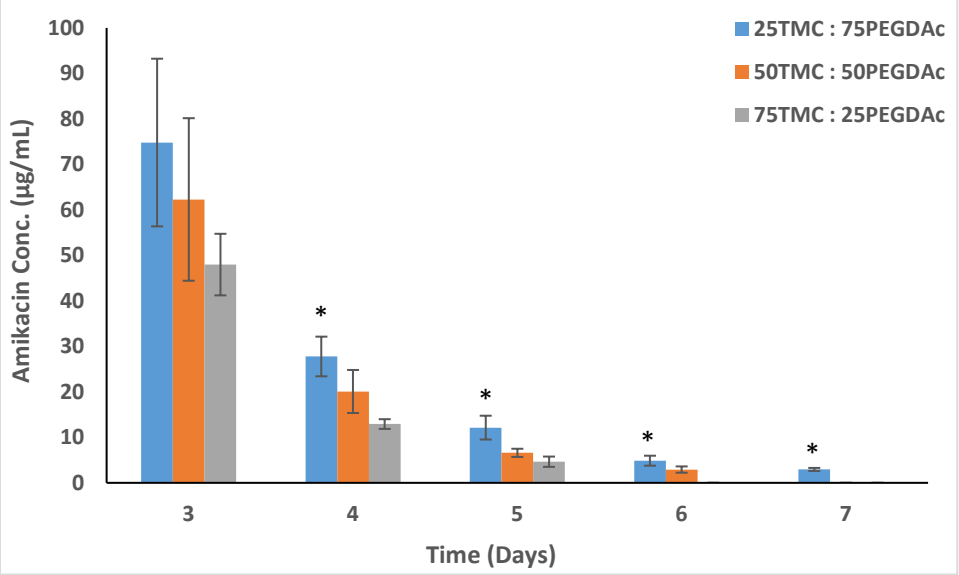

FIGURE 24. Zoomed in (Days 3-7) preliminary *in vitro* elution results of amikacin from chitosan derivative combinations pastes. Data are presented as mean ± standard deviation. * denotes significant differences from all other groups.

Cytocompatibility

NIH3T3 fibroblast cells were seeded at 10^4 cells/cm^2 in 24-well plates and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 100 µg/mL of an antibiotic/antimycotic solution for 24 hours. Approximately 150 mg of dried material was hydrated using a PBS solution. Hydrated volumes were split into three equal volumes (~0.3 mL) and injected into cell culture inserts (Falcon, pore size = 8 µm) and placed in each well. Cells were exposed to pastes eluates for 24 hours and quantified using Cell-Titer Glo (Promega) assay. Results were expressed as percentage of cells grown on blank tissue culture plastic. Statistical analysis was performed using one way ANOVA with a SNK post-hoc test to perform comparisons between groups. Differences were considered significant if p-values were less than 0.05.

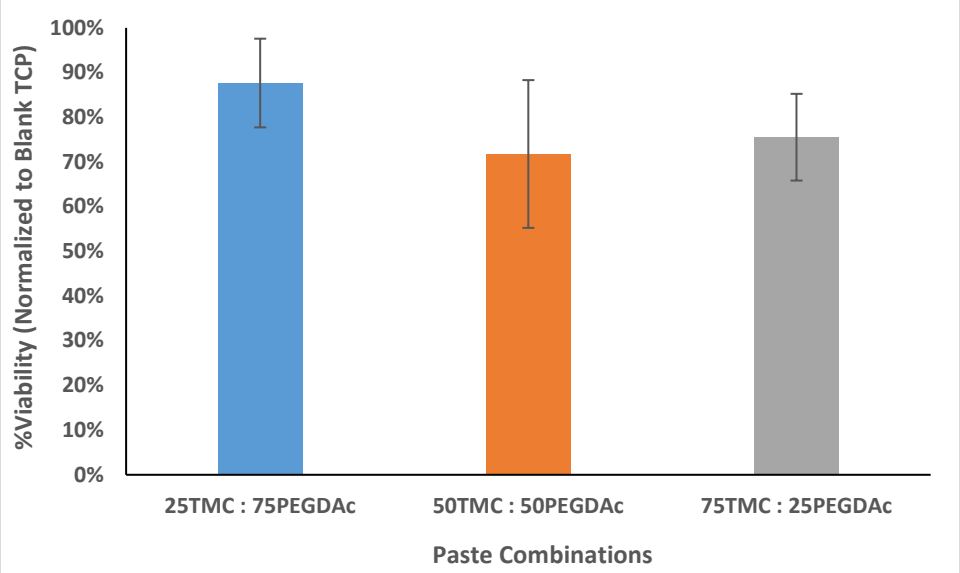

FIGURE 25. Preliminary *in vitro* cytocompatibility results of chitosan derivative combinations with NIH3T3 fibroblasts. Data are normalized to cells grown on blank tissue culture plastic and represented as a mean ± standard deviation. * denotes statistical significance from other groups.

Conclusions

The combination consisting of 75% PEGDAc and 25% TMC was superior to the other two combinations in eluting vancomycin and amikacin (Figures 19, 20, 21, and 22). This combination eluted significantly higher concentrations at days 5, 6, and 7 for both antimicrobials. No significant differences were observed for cytocompatibility with NIH3T3 fibroblasts, but it appeared that 75% PEGDAc and 25% TMC had a higher relative viability compared to the other combinations (87% vs. 72% and 76%) (Figure 23). Due to the enhanced elution properties and cytocompatibility of 75% PEGDAc and 25% TMC, further research efforts are focused on comparing this combination to previously developed systems.

APPENDIX D: Ninhydrin Assay

Rationale

TMC and PEGDAc are both produced by derivatizing chitosan at the primary amino group present on the glucosamine subunit.[19,72] The ninhydrin assay is used for the detection of primary amine groups, and it was determined that this assay could be used as a useful indicator for reaction success and efficiency.[108,109] When the ninhydrin reagent is heated in the presence of a primary amine groups, a purple reaction product is formed that can be detected using a UV/Vis spectrophotometer. A ninhydrin test was used to determine the amount of free amino groups remaining after reaction, which is proportional to the degree of reaction.

Methods and results

Ninhydrin solution was prepared by dissolving 2% (w./v.) ninhydrin reagent in dimethyl sulfoxide and combined with an acetate buffer (pH = 5.4) to adjust the concentration to 1.5% (w./v.). Samples (1% w./v.) were dissolved in deionized water and combined with ninhydrin solution in a 1:1 ratio and heated in a hot water bath at 100°C for 30 minutes. After cooling to room temperature, reacted solutions were diluted 1:15 using isopropyl alcohol and absorbance at 570 nm was determined using a Biotek Synergy H1 microplate reader. The amount of free amino groups was determined using a standard curve produced from known chitosan weight percentages vs. absorbance.

A straight line was obtained when the absorbance of the standard chitosan solutions was plotted against their concentrations. Absorbance of the PEGDAc solution was not different compared to the highest concentration of chitosan (3.46 vs. 3.53). Absorbance for DMC and TMC was similar to the absorbance value for the blanks (0.05 vs. 0.03), and there was no color change.

Conclusions

PEGDAc absorbance not being significantly different from the highest concentration of chitosan is not surprising due to the molar ratio of PEGDA to amino groups present on chitosan, 1:47. If the reaction was performed with 100% efficiency, there would have only been a 2.1% change in the number of free amino groups. However, the results for TMC were positive for indication of reaction. The first step in producing TMC is a quantitative dimethylation of the amino groups. The absorbance of TMC was not significantly different from the blank; this indicates that no free amino groups were present. Results from this study provide extra support to the FTIR and NMR analyses that the derivatizations were successfully performed.

APPENDIX E: Adhesion Testing

Rationale

Complex musculoskeletal trauma wounds have complex geometries that make clinically used orthopaedic biomaterials less effective.[4] An injectable delivery system offers the distinct advantage of enhanced wound coverage because it can be ejected directly into the wound site. If the delivery system is not adhesive, it may migrate from the wound site and not deliver antimicrobials to the affected area. It would be advantageous if an injectable delivery system could be developed that would adhere to the wound site until a clinician was able to irrigate and debride the wound site. TMC has been shown to have enhanced mucoadhesivity compared to unmodified chitosan, but the effects of DQ on mucoadhesivity is still debated.[16,78] PEGDAc has also been shown to have enhanced mucoadhesivity compared to unmodified chitosan and thiolated chitosan, the most adhesive class of chitosan derivatives.[16,19] Therefore, a combination of 75% PEGDAc and 25% TMC was evaluated to determine if mucoadhesivity was enhanced compared to an unmodified chitosan delivery system.

Methods and results

Fresh muscle tissue was harvested from the hind legs of New Zealand White rabbits and used as a model tissue for muscle tissue in a complex musculoskeletal injury. Tissues were coated in approximately 4 mL of fetal bovine serum (FBS) to simulate surface proteins. Dried powder was hydrated using 4.5 mL of PBS per gram of dried powder, and approximately ~0.9 mL of paste (n = 3) was ejected on the tissue. Paste was allowed to sit for 1 minute to visually assess adhesion to the muscle tissue. After assessing initial adhesion, 10 mL of PBS was applied to the paste to simulate irrigation used in the management of complex musculoskeletal injuries. This step was repeated three times, or until the paste was removed from the tissue.

Unmodified control pastes were unable to maintain their initial position, and gravity immediately began to dislodge them (Figure 25a). Addition of PBS facilitated the removal of paste from the tissue. The combination of PEGDAc and TMC immediately adhered to the tissue and showed no evidence of movement after 1 minute (Figure 25b). Addition of PBS directly to the paste completely removed it from the tissue, and adding PBS to the tissue above the paste produced swelling and gradual removal (Figure 25c).

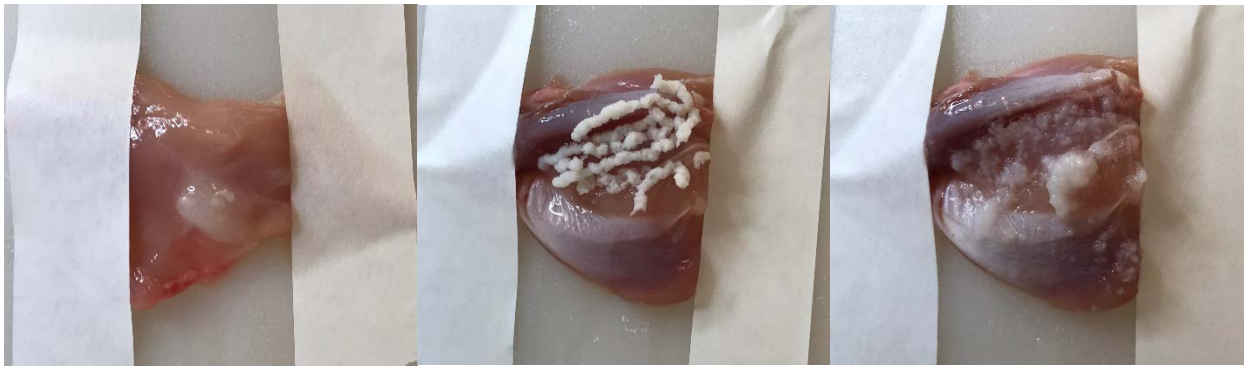

FIGURE 26. Pastes ejected onto representative tissue. (a) Image of control paste. (b) Image of combination paste prior to PBS wash. (c) Previous image immediately after PBS wash

Conclusions

Combination paste successfully adhered to representative muscle tissue prior to simulated irrigation with PBS. This is advantageous over the control paste that had difficulty adhering to the simulated wound site and was unable to be washed away. Combination paste could be applied immediately after injury occurs, and it would remain in place until a clinician was ready to irrigate and debride the wound site. Then, more combination paste could be applied to the clean wound site for further infection prevention, and it could be removed by additional irrigation or degradation within the body depending if the wound was left open or closed. An interesting observation was made when the hydration ratio was increased to 7.0 mL from 4.5 mL

per gram of dry paste. The resulting paste was easier to inject, held more antimicrobial solution, and was more resilient to irrigation with PBS (Figures 26a and 26b).

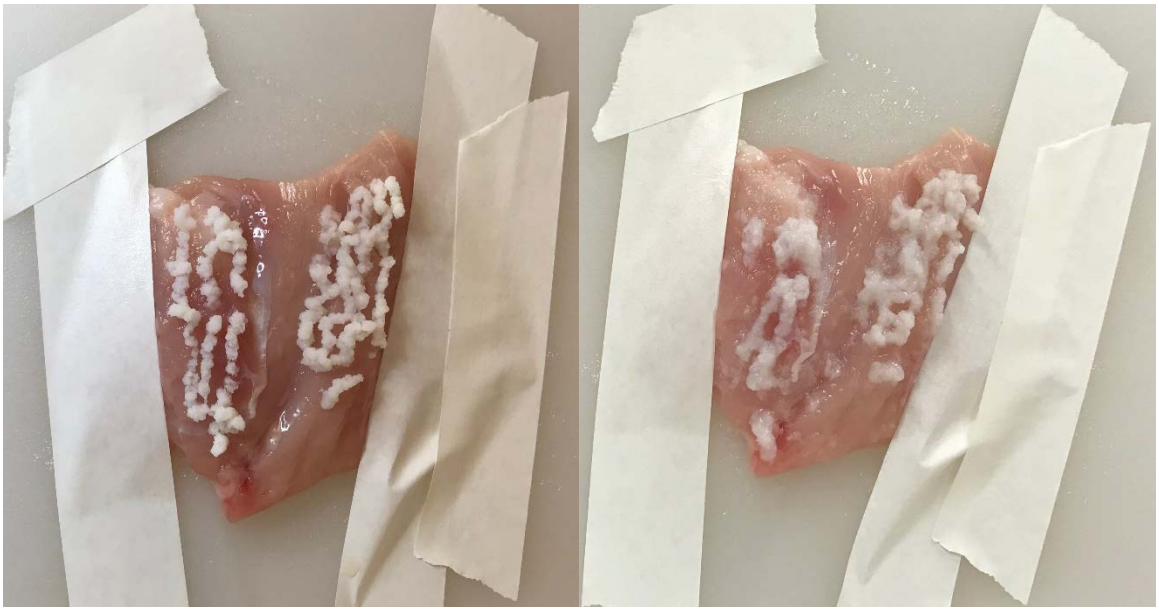

FIGURE 27. Pastes with different hydration ratios ejected onto representative tissue. (a) 4.5 mL on left and 7.0 mL on right (b) Pastes after irrigation with PBS