In Vitro Evaluation of Chitosan Membranes Stabilized with Varying Acyl Lengths for Release of Therapeutics for Burn Wound Coverage, Infection Prevention, and Pain Relief

Landon Choi

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IN VITRO EVALUATION OF CHITOSAN MEMBRANES STABILIZED WITH VARYING ACYL LENGTHS FOR RELEASE OF THERAPEUTICS FOR BURN WOUND COVERAGE, INFECTION PREVENTION, AND PAIN RELIEF

by

Landon Reed Choi

A Thesis

Submitted in Partial Fulfillment of the

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Master of Science

Major: Biomedical Engineering

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DEDICATION

I would like to dedicate this thesis to my immediate family who have supported all of my endeavors, my peers who have always given me the drive to do better, and to all the family members and friends that I lost this year who didn’t get to see me get to this point.
ACKNOWLEDGEMENTS

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PREFACE

The main body of this thesis is included as a submission in preparation for International Wound Journal.
ABSTRACT

Adherence of complex bacterial biofilm communities to burned tissue creates a challenge for treatment, with infection causing 51% of burn victim deaths. This study evaluated release of therapeutics from wound care biomaterials and their antimicrobial activity against pathogens *Staphylococcus aureus, Acinetobacter baumannii, and Pseudomonas aeruginosa*. Electrospun chitosan membranes (ESCMs) were fabricated and acylated with chain lengths ranging from 6-10 carbons then loaded with anti-biofilm cis-2-decenoic acid (C2DA) (0.15 mg/membrane with average weight of 4.68 mg) and local anesthetic bupivacaine (0.5 mg/membrane). Combinations of therapeutics released from modified ESCMs at a cumulative amount of 45-70% of bupivacaine and less than 20% of C2DA. Results from bacterial studies suggest that this combination reduced biofilm 10-fold for *S. aureus*, 2-fold for *Acinetobacter baumannii*, and 2-3-fold for *Pseudomonas aeruginosa* by 24 hours. Additionally, dual loaded groups reduced planktonic *Staphylococcus aureus* ~4-fold by 24 hours as well as *Acinetobacter baumannii* ~3-fold by 48 hours.
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CHAPTER I
INTRODUCTION

Statement of Clinical Problem
Burn injury is a common global threat that causes complex wounds leading to complications affecting the whole body. As of 2018, according to the World Health Organization (WHO), it is estimated that 11 million burn injuries occur worldwide, accompanied by 180,000 deaths a year (1). Additionally, non-fatal burns contribute to a leading cause of morbidity, prolonged hospital care, lifelong physical and psychological scarring, resulting in a stigma and an overall decrease in quality of life (2). Thermal burns are the most common type of burn injury; 86% of thermal burn patients require burn center treatment (3). These burns are typically induced from hot liquids (scalds), solids (contact burns), or raw heat (flames). During clinical assessment, the burn will be classified based on severity, depth, location, and pain. Other methods of classifications, used by trauma and emergency medicine providers, entail the Rule of Nines to calculate the total body surface area (TBSA) affected by the burn (4). When a person is moderately to severely burned, their immune system becomes compromised. Skin serves as a protective layer that encases our body and reduces the opportunity for microorganisms, that cause infection, to invade. Once burned, the skin becomes an ineffective barrier, where the chances for life threatening infections to occur are high, especially if exposed to bacteria from debris. Challenges resulting from moderate to severe burn injuries are pain from the site of burn and surrounding areas (depending on severity), infection, inflammatory/immune response, metabolic changes, and distributive shock, all of which can contribute to multiple organ failure (2).

Although a majority of burns typically occur within home or at the workplace, its threat against military personnel during combat has been recognized (5). In combat settings, thermal burns are usually accompanied with explosive injuries (5) such as handheld explosives, bombs, or
vehicular combat. If not properly treated, thermal burns can lead to contamination by adherent bacteria residing from the surrounding environment within viable tissue, which then may colonize the wounded tissue and potentially form Multi-Drug Resistant (MDR) biofilm. Biofilm is a complex community of adherent bacteria that attaches to a surface and is equipped with defense mechanisms against antibiotic therapy. These mechanisms include mechanical strength from the extracellular polymeric substance (EPS) as well as the metabolically dormant persister cells (6), both capable of increasing tolerance to antimicrobials. One of the many challenges of antimicrobial therapy is the resistance to multiple antibiotics obtained by select bacteria strains through the course of antibiotic overuse (7, 8). MDR infections limit options for antibiotics, thus making it more difficult to treat. *S. aureus*, *A. baumannii*, and *P. aeruginosa*, as these are the most prevalent bacterial strains associated with burn injuries from military personnel involved in recent conflicts and all are capable of forming multi-drug resistance and biofilm (9-11).

For military personnel in remote or austere locations the treatment for severe burn injuries can be delayed, which will ultimately decrease chances of salvaging viable tissue, as well as further chances of inducing a life-threatening infection. If not managed properly, post-burn, chances of contamination progressing to infection is likely which can ultimately increase hospital stay and cost for care. It is evident that military medical skills training and available resources must reflect fundamental changes in modern conflicts in preparation for the future (12). Additionally, a proactive infection control approach is essential in burn units (13). Recent reviews and recommendations have identified research gaps and unresolved issues in preventing combat-related infection, including need to improve local antimicrobial delivery, addressing the roles of fungal infections, and biofilm (14, 15). Despite current burn wound (silver based) treatment advantages, the cytocompatibility and infection prevention/eradication associated with some of
these treatments is unfavorable (16-18). With the prevalence of burn injuries and dangerous infections that follow shortly after, there is a need for novel burn treatments that can assist in coverage from further contamination, pain management of moderate burns with minimal damage of the nerves, and increased minimization of difficult contributors to infections, such as biofilm. Cells within biofilms perform cell-to-cell communication behaviors through diffusible signaling factors (DSF) secreted to maintain equilibrium. This communication is termed quorum sensing, and it is responsible for using DSFs to promote and inhibit metabolism and growth depending on biofilm microenvironment (19). Cis-2-decenoic acid (C2DA) is a DSF studied for its property to inhibit and disperse established or emerging biofilm (20-22). Supplementary to infection prevention, pain management was carefully considered. With the use of local anesthetics (LAs) such as bupivacaine, benefits such as inherent antimicrobial effects to modifying the inflammatory response without the use of opioid dependent strategies for pain symptoms can be achieved (23, 24). Extensive research has been done using chitosan, a versatile biopolymer derived from shells of arthropods and used for local drug delivery systems due to its abundance, cytocompatibility, biodegradability (25), and its cationic nature which may contribute to its antimicrobial effect to certain bacteria strains (26). When electrospun, chitosan is dissolved in acid to make a semi-viscous solution that can be fabricated into a membrane of fibers capable of releasing anesthetics, fatty acids, and antimicrobials (27). Research on treating ESCMs with an anhydride solution has been shown to be effective at maintaining fiber stability (28). This is crucial for maintaining coverage from microbial contamination once the ESCM makes contacts with the burn wound injury, as these wounds are usually accompanied by pro-inflammatory fluids as well as blood. In addition to benefits from this treatment, the yielded membrane will be
able to be used as a wrap application, with instant drug delivery once in contact with the affected tissues.

**Hypothesis and Research Objectives**

Electrospun chitosan membranes (ESCM) are made up of layers of fibers spun within the nanometer range, this in return allows for an increase in surface area, ideal for a local drug delivery treatment. In addition to its inherent biocompatible and biodegradable properties, ESCMs have been shown to mimic the extracellular matrix (29) and release hydrophobic therapeutics whilst maintaining stability of fibers once treated with an anhydride solution. Previous investigations of drug release and antimicrobial release has been done with ESCMs, but only when acylated with short chain anhydrides. Specifically in this work we will investigate the following hypothesis and aims.

**Hypothesis:** Electrospun chitosan membranes (ESCM) tailored with acyl lengths ranging from 6 – 10 carbons by reaction of fatty acid anhydride and loaded with both bupivacaine and C2DA may serve to 1) act as a physical barrier from microbial contamination, 2) release antimicrobial local anesthetic to reduce pain and modify the inflammatory response, 3) release natural antimicrobial fatty acids that prevent biofilm contamination, and 4) prolong delivery of these hydrophobic therapeutics for an extended period (Figure 1). ESCMs will provide a sustained release of active therapeutics for up to 3 days, a period relevant to prolonged field care in remote and austere conditions.
Specific Aim 1: Evaluate elution characteristics of bupivacaine and C2DA combination released from ESCM. ESCM modified with hexanoic, octanoic, and decanoic acyl groups will be loaded with therapeutic molecules in combination, followed by evaluation of release over the course of 72 hours using high performance liquid chromatography (HPLC) coupled to charged aerosol or mass spectroscopy detectors.

Specific Aim 2: Evaluate antimicrobial effects of bupivacaine, C2DA, and combinations released from decanoic treated ESCMs. *In vitro* antimicrobial activity of eluates from membranes (alone and combination) will be evaluated against *S. aureus, P. aeruginosa*, and *A. baumannii* strains.
that are commonly pathogenic in burn wounds. Evaluations will include bacterial attachment and bacterial growth assays that our lab has used in previous evaluations of antimicrobial therapies.
CHAPTER II
LITERAURE REVIEW

Burn in the U.S.

In the United States, burn injuries continue to be one of the leading causes of unintentional death and injury, where one civilian fire death occurs every 2 hours 35 minutes (1). Each year in the United States, 1.1 million burn injuries require medical attention (30). According to the Center for Disease Control (CDC), 50,000 of those burn injuries require hospital care with 20,000 having injuries at least 25% total body surface area (TBSA), resulting in approximately 4,500 deaths. Contradictory to the worldwide demographic, in the United States, males are more likely to be burned than females as reported by the American Burn Association (ABA) (1). Additionally, unintentional fire or burn injuries were the fifth leading cause of injury deaths in the United States in 2015 for children aged 1-4, third for those aged 5-9, and eighth leading cause of death for those aged >65 (30). Hospitalizations for burn patients cost the US more than $10.4 billion per year, with treatment costs ranging from 200,000 to 10 million USD, depending on the severity and possible complications (31).

Burn in Combat

Unfortunately, during combat there is a higher frequency of thermal burn related injuries for all parties involved. This is primarily the case for combat related burns, where 63% of burn injuries are caused by explosive device detonation (5) (Figure 2). Despite advancements in protective gear such as helmets and chest armor, these measures are ineffective in preventing death or injury. For example, the armored Kevlar vests from conflicts within Iraq can stop projectiles and bullet rounds efficiently, but those that have survived are left with serious mutilations and handicaps from burns and shrapnel wounds to the unprotected areas (5).
Thermal burns can accompany other injuries such as blast injuries, chemical, flash burns. Atiyeh et al. reports that the potential damage and use of mustard gas is significant, causing large casualties with severe partial thickness burns with slow healing rates. Additional studies note that flash or flame burns are encountered in 45% of the victims of explosions/bombings occurring in confined spaces, 11% in the open, and 14% in bombings associated with structural collapse (5).

According to the Department of Defense (DOD), two large studies from British World War II, involving tank crewmen and Israeli casualties in Lebanon, estimated that about one-third of living wounded casualties sustained burn injuries (32). Another study indicating military personnel, from 2009-2012, in Iraq and Afghanistan having sustained more than 52,000 wounds (33). Explosions account for approximately 78% of these wounds (34), and up to 20% of combat casualties involved burns (35, 36). Causalities in early wars depict bullet wounds as the cause,
but recent wars have been observed indicating an increase in explosive wounds, burns, and inhalation injuries. It is estimated that in modern warfare, one of four injuries is caused by burns (37).

In mass casualty events or combat in austere conditions and remote locations, definitive treatment of burns for service members or civilians could be delayed for days to weeks. This delay from initial wounding to definitive treatment increases the risk of infection and leads to loss of tissue viability because of compromised vasculature (38). Blast injuries alone are at high risk for infection due to cavity formation, tissue destruction, potential foreign bodies, and contaminating pathogens from the skin, clothing, and surrounding soil (39).

**Burn Severity**

Classifications of burn magnitude start with 1st degree (superficial thickness), these burns are mostly associated with sunburns (Figure 3). Second degree burns are divided into two categories, the first being superficial partial thickness burns (affecting the epidermis and parts of the dermis) and second being deep partial thickness burns (affecting deeper parts of the dermis). Both have increased chances of scarring and infection, though it is likely deep partial thickness burns will require surgery if not treated correctly. Third degree burns (full thickness) result in dry leathery skin, requiring surgery to heal, increased infection and scarring, and accompanied with little to no pain (affecting the subcutaneous tissue). Fourth degree burns (subdermal) entail injuries going as far as the muscle or bone, resulting in the loss of the affected region (2).
Skin serves to protect internal tissue by acting as a barrier from contaminating microorganisms that can cause infection. Burned skin, however, loses that barrier function which increases risk for life-threatening infection, especially if bacteria attach to foreign objects such as debris or clothing. Severe burn injuries are followed with a dysregulated immune/inflammatory response, metabolic changes, and distributive shock which can be difficult and lengthy to manage, possibly resulting in multiple organ failure (2, 40).

**Prevalent Infections**

Within the United States, up to 10,000 people die every year from burn-related infections (30). Additionally, infection occurs in approximately one-third of combat casualties, with numbers...
approaching 50% in wounded warfighters requiring admission to an intensive care unit (38, 41). Infection is one of the greatest threats to survival for the burn wound recipient (42). Sepsis or multisystem organ failure resulting from infection is the third most common overall cause of death in combat trauma, and the most common cause of death for those who survive the first 24 hours after injury (42). Current military guidelines recommend that casualties receive treatment within 1 hour (the “golden hour”) (43). If the victim were to survive the initial 72 hours after a burn injury, infections are the most common cause of death (44). This will become increasingly difficult as US conflicts move to remote, austere locations where rapid evacuation to well-equipped medical facilities may not be possible.

Once a victim is burned, it is likely that the wound could be sterile at first depending on the severity (45). Although this may be the case there is still many opportunities for bacteria to invade the injury site postburn. Typically, infection in burns is proportionate to the fraction of body surface injured (44). Although extensive burns mainly involve the skin, as a result, a majority of systems within the body are affected from the immunocompromised status of the burn patient, making it a generalized disorder. More involvement of intensivist and physicians are in burns, unlike any other traumatic wound (45). Burn wound injury involves both nonspecific and specific responses of the immune system. The nonspecific defenses consist of circulating and fixed phagocytic cells and plasma proteins that are integral to the inflammatory response. The specific defense consists of a localized adaptive immune response that contributes to the healing process, clearing of microorganisms from the wound, and has been shown to benefit from the assistance of external aids such as a homograft when burned (45).

In the first days of the postburn hospitalization, more gram-positive organisms predominate, whereas later more resistant gram-negative organisms are found (13). From January 2003 to May
2006, a retrospective cohort study was conducted to evaluate bacteremia in the burn patient population in the US Army Institute of Surgical Research burn center (46). 1,258 patients became bacteremic during their admission to the burn center. Of these, 92 had bacteremia with the highest types of pathogens, such as *Acinetobacter baumannii* complex, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (46). Gram-positive *Staphylococcus aureus* and gram-negative *Pseudomonas aeruginosa* are among the most common pathogenic bacteria found in wound infections, with other gram-negative strains such as *Acinetobacter baumannii* being prevalent in certain locations of military conflict (7). In a recent study between deployed and non-deployed civilians receiving treatment in a military facility, burn injuries were evaluated for incidence and bacteriology of infections. This study revealed the prevalence of *A. baumannii* as the most common organism among military burn victims who were injured during actions in Iraq and Afghanistan (58%), *P. aeruginosa* was most prevalent in patients with burns greater than 60% TBSA, and *S. aureus* in non-combat patients (46%) (35). Additionally, it has been shown that prior to deployment to Iraq or Afghanistan, about 17% of 102 healthy American soldiers on active duty, had acquired an *Acinetobacter* complex colonized on their skin (47). The prevalence of these bacterial strains is common regardless of injury. The moment a victim is burned, the more likely they will be already exposed to bacteria colonizing skin, or from other artifacts such as clothes and debris, to the surrounding environment (48).

**Multi-drug Resistance**

Bacterial infections, particularly the multidrug-resistant (MDR) bacteria, have become a serious challenge to global healthcare (48). The definition for multidrug-resistant bacteria can vary, but in general are organisms classified by the resistance of three or more classes of antimicrobial agents (49). These infections are associated with significant morbidity and mortality, difficult to
An alarming rise in MDR bacterial strains due to antibiotic overuse, limits the availability of antimicrobials practical for broad-spectrum antibiotic administration (7). During the study period of January 2003 to December 2008, Edward et al. performed a 6-year antibiotic susceptibility records review. Results indicated, *Acinetobacter baumannii* (780 isolates [22%]) was the most prevalent organism recovered, followed by *Pseudomonas aeruginosa* (703 isolates [20%]), and *Staphylococcus aureus* (469 isolates [13%]) (9). MDR prevalence rates among these isolates were *A. baumannii* 53%, methicillin-resistant *S. aureus* (MRSA) 34%, and *P. aeruginosa* 15% (9). Another study supporting this, indicated a significant increase in MDR Acinetobacter complex infections among military casualties evacuated from Iraq and Afghanistan, resulting in osteomyelitis, extreme soft tissue contamination of deep wounds and burn infection (50). Not only is *A. baumannii* a prevalent pathogen, but there have also been cases observing the comparisons of *A. baumannii* recovered during hospitalization days 1–5 and 15–30, that revealed higher MDR levels as length of stay increased (48% vs. 75%), however no significant trends were observed for *P. aeruginosa* (9, 13). Increasingly, combat casualties are acquiring nosocomial multi-drug resistant, gram-negative infections during treatment and evacuation (51, 52). Early care for burn infections is vital for managing burn injuries, as some bacteria possess the ability to form an extracellular matrix composed of varying microbials and an array of extracellular polymeric substances (EPS) called biofilm. Similar to the properties of MDR bacteria, bacteria that can form biofilm are also likely to be antibiotic resistant. Bacteria typically colonize burn wounds by forming a biofilm within 48-72 hours post-injury (53, 54); therefore, protection of the burn wound for this duration is critical.
Biofilms and diffusible signal factors

Biofilm is a complex of bacterial communities (Figure 4) that are accumulated in a polymeric matrix (consisting of proteins, polysaccharides, lipids, nucleic acids, and humic substances) (55) produced by bacteria that can be attached to living or inanimate surfaces (56, 57) while being able to colonize in a matter of 2-3 days (53, 54). The EPS not only immobilizes the bacteria but makes its structure mechanically stronger and houses coordination within the biofilm via cell-to-cell communication known as quorum sensing (QS). Quorum sensing involves communication in which accumulation of signaling molecules in extracellular environment leads to regulation of the specific gene expression. Some bacterial species use QS to coordinate the disassembly of the biofilm community (19). This allows bacteria to assess the local cell population density and regulate gene expression by releasing extracellular molecules to facilitate synchronized changes in the bacteria within the biofilm. These transcriptional changes can occur with the exchange of plasmids, which can confer genes for virulence factors, antibiotic resistance, commence the formation, and secretion of the EPS matrix that supports the biofilm (58). In addition, biofilms contain a large subpopulation of persister cells, that remain dormant, which survive antimicrobial treatment (6) and adapt to a slow growth rate through the emergence of small colony variants (59). Because many antibiotics only have activity in metabolically active or dividing cells, the presence of dormant persister cells often increases the minimum inhibitory concentration of antibiotics up to 1000 times higher in biofilms as compared to
planktonic bacteria, and also makes them resistant to immune cell clearance (60). Bacterial biofilm communities differ from the planktonic ones in varying ways such as growth rate, gene expression, transcription and translation because these biofilms communities live in different microenvironments which have higher osmolarity, nutrient scarcity and higher cell density of heterogeneous bacterial communities (60).

Foreign materials embedded in a wound, caused by blast injuries, increases the likelihood of infection by providing surfaces for bacteria to attach and form biofilm. Combat wounds that are contaminated with biofilm-forming bacteria have 30 times greater odds of persistent infection, which increases to 70 times greater odds when polymicrobial biofilm is present (61). Biofilm formation, which increases antibiotic resistance capabilities also causes treatment failure and recurrent staphylococcal infections in burn patients (62). Up to 80% of human bacterial infections are biofilm associated; such infections are most frequently caused by Staphylococcus epidermidis, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli (10). A study in 2019 evaluated the prevalence of biofilm formation for 69 strains of A. baumannii, isolated from burn infections in Iran (Ahvaz), and reported 85.9% (55) of the strains were forming biofilm with variations in magnitude with respect to MDR (11). Based off their results, it was suggested that non-MDR A. baumannii strains tended to form stronger biofilms than MDR strains, while non-MDR strains had greater possibility to produce strong biofilm than MDR strains (11). Biofilm infections are not just seen in combat setting but also seen in domestic injuries, as well as patients who undergo surgery involving implants (11, 58).

Other studies have looked towards the communication method that biofilm utilizes for methods of biofilm eradication. Findings have indicated natural and synthetic drugs that can disrupt formation of exopolysaccharides, virulence factors, or cell wall synthesis (21). In our lab we
have investigated natural and synthetic analogs of fatty acid signaling factors, such as cis-2-decenoic acid (C2DA). C2DA is a short chain fatty acid that acts as a diffusible signal factor (DSF) involved with the regulation of biofilm growth and virulence that can be used to disperse and inhibit biofilm formation (20-22). It has been shown that in addition to its ability to induce biofilm dispersion for multiple pathogenic bacteria (20), C2DA also inhibits bacterial growth and biofilm formation, acting synergistically with antibiotics to inhibit and eradicate infectious biofilm (22). The team at University of Memphis have also demonstrated in preclinical models of biomaterial-associated biofilm infections that, even in the absence of antibiotic, C2DA can prevent infection (63). Although C2DA may seem to have potential to be the key to eradicating biofilm, the efficacy of C2DA is still dose and time dependent. This means that in order to perform successful biofilm prevention or removal, there must be signaling molecules active over extended periods of time. This can prove to be difficult as C2DA has a hydrophobic nature, like other fatty acid DSFs.

**Bupivacaine**

In addition to addressing infection management, another important factor required for burn treatment is pain management. This is especially the case with severe injuries in which nerve damage occurs. When addressing pain management, it is important to consider all aspects of the healing process as certain analgesic drugs could have a worse impact for the patient long-term, like opioids. As the opioid crisis reaches epidemic proportions, non-opioid pain management strategies are a top priority for both military and civilian wound treatment (64). When applied locally to burn wounds through topical sprays or creams, local anesthetics (LA) reduced the need for opioid management of pain symptoms and did not interfere with healing (65). Other studies suggest that LA increase blood perfusion to pre-burn levels (66) and modify the inflammatory
response \((23)\), calming inflammation and reducing edema that contributes to conversion of burn wounds to deeper layers of tissue. Studies have also confirmed that in addition to analgesic effects, local anesthetics such as lidocaine, ropivacaine, and bupivacaine have inherent antimicrobial effects. In this study, a focus on bupivacaine was made for its hydrophobic properties compared to the previously mentioned local anesthetics. In addition to its bacteria response, bupivacaine is known for its slow onset and long-lasting effects \((24)\). Careful loading concentration must be under 2.5-3.5 mg/kg in order to avoid any unwanted cytotoxic effects \((67)\). Similarly to C2DA, local anesthetics such as bupivacaine are incredibly hydrophobic, making these molecules difficult to deliver.

**Chitosan**

Biopolymers have been observed extensively for their potential in drug delivery applications as well as their role in biomaterial science. With the exception of cellulose, chitin is the most abundant polysaccharide found in nature and serves as an inexpensive and readily available resource \((68)\). Chitin is found in the exoskeleton of crustaceans and to a lesser extent the cell walls of fungi and insects \((69)\). Additionally, chitin can be derived into chitosan, a linear cationic polysaccharide copolymer composed of repeating \(\beta\)-(1-4)-linked glucosamine and N-acetylglucosamine units \((68)\). The distinction between chitin and chitosan is the degree of deacetylation (DDA), the percentage of acetyl groups removed; chitin has a DDA less than 10\%, and chitosan has a DDA greater than 50\% \((70, 71)\). Military and civilian healthcare providers currently use commercially available chitosan biomaterials, this is done to promote hemostasis, stimulate wound healing, and prevent infection \((68, 72-74)\). Additionally, chitosan has been studied extensively at the University of Memphis for its applicational use in biomaterials science and drug delivery.
Uses in local drug delivery

The reason chitosan is extensively researched is due to its abundant supply, versatility, biocompatibility, and biodegradability that facilitate its fabrication into local antibiotic delivery systems (25). The medical potential behind chitosan is ever growing as it can be fabricated into hydrogels, chitosan sponge delivery systems (75), magnetic chitosan nanoparticles for targeted drug delivery (76), coatings for musculoskeletal implant fixation hardware, injectable chitosan paste for traumatic injuries (77), and electrospun chitosan membranes (ESCM) (28).

Electrospinning is a cheap and reliable, under specific conditions, technique that entails dissolving a polymer and charging it to extrude the volatile solution on to a grounded plate. This polymeric extrusion produces membranes with fiber diameters in the nano-scale range that mimic the nanofibrous structure of the natural extracellular matrix (ECM) (29). Previous work has shown chitosan nanofibrous membranes as well suited for tissue healing and drug delivery applications due to their increased surface area, high degree of biocompatibility and biodegradability, and ability to mimic the extracellular matrix (29, 78-82). Preliminary evaluations of ESCM have revealed unique properties that make them particularly suited for delivering anesthetics, fatty acids, and antimicrobials (27). The ECSM is ideal for its loading capabilities and fiber mesh work, allowing for a breathable material that fits the need for a burn treatment readily active once administered.

Anhydride Treatment of Chitosan

Although chitosan nanofibrous membranes alone show great promise for their application in drug delivery and tissue engineering, they are still limited due to its strong hydrophilic properties resulting in swelling and loss of nano-fiber structure (28). This swelling is caused by the hydrolysis of chitosan salts (byproduct from spinning) attached to the amine groups of chitosan.
To overcome this issue previous work has demonstrated acylation with short chain fatty acids to the hydroxyl groups of chitosan, in order to protect nanofibers from swelling in aqueous solutions (28). In addition to this protection, findings have shown increased hydrophobic characteristics allowing for extended drug release of hydrophobic molecules (28). An additional study showed fatty acid modified chitosan membranes with good cell attachment and guided bone regeneration potential (83). For the application of burn injury, chitosan nanofibrous membranes can be modified by acylation reactions using fatty acids of different chain lengths. Hexanoic anhydride (HA) is an anhydride treatment that has been used in previous studies, for nanofiber stability, with a number of 6 methylene groups in the fatty acid chain (28). Octanoic anhydride (OA) has a larger fatty acid chain with a number of 8 methylene groups. The last anhydride treatment observed is decanoic anhydride (DA), which has the largest fatty acid chain among the other treatments, with a number of 10 methylene groups.

**Current Treatments**

Because infection has a strong impact on survival of the burn wound victim, initial management strategies have particular focus on antimicrobial therapies. Immediate aid for burns initially includes cleansing with antibacterial soap such as Hibiclens® and the application of topical antimicrobials such as silver sulfadiazine (Silvadene®) or mafenide (Sulfamylon®) cream (84, 85). Other treatments involve the use of nylon dressings such as Silverlon® or Silverseal®, shown to reduce the primary threat of contamination and infection (35). A further advantage of dressings in the military environment is the ability to place the wraps over multiple types of soft tissue wounds, including burns, and leave them in place with minimal maintenance during the evacuation process (86). Although silver sulfadiazine dressings (SSD) are used commonly for second degree burns, they have been shown to potentially impair healing by exerting toxic
effects on keratinocytes and fibroblasts \(^{(16)}\). A systematic review and meta-analysis on children with partial-thickness burns reported that silver treated burn injuries, compared to non-silver treatments, had longer wound healing time, more dressings changed, and longer length of hospital stay \(^{(17)}\). Additionally, it has been reported that the concentration required to eradicate biofilm ranged at 10 to 100 times higher than needed to kill planktonic bacteria \(^{(87)}\). In a recent study, observing the efficacy of SSDs against microbials, it was suggested that alternative antimicrobials with known anti-biofilm properties would be more appropriate for addressing such infections \(^{(18)}\).
CHAPTER III

IN VITRO EVALUATION OF VARYING ACYLATED LENGTH CHITOSAN MEMBRANES RELEASING THERAPEUTICS FOR BURN WOUND COVERAGE, INFECTION PREVENTION, AND PAIN RELIEF

ABSTRACT

Adherence of complex bacterial biofilm communities to burned tissue creates a challenge for treatment, with infection causing 51% of burn victim deaths. This study evaluated release of therapeutics from wound care biomaterials and their antimicrobial activity against pathogens Staphylococcus aureus, Acinetobacter baumannii, and Pseudomonas aeruginosa. Electrospun chitosan membranes (ESCMs) were fabricated and acylated with chain lengths ranging from 6-10 carbons then loaded with anti-biofilm cis-2-decenoic acid (C2DA) (0.15 mg/membrane with average weight of 4.68 mg) and local anesthetic bupivacaine (0.5 mg/membrane). Combinations of therapeutics released from modified ESCMs at a cumulative amount of 45-70% of bupivacaine and less than 20% of C2DA. Results from bacterial studies suggest that this combination reduced biofilm 10-fold for S. aureus, 2-fold for Acinetobacter baumannii, and 2-3-fold for Pseudomonas aeruginosa by 24 hours. Additionally dual loaded groups reduced planktonic Staphylococcus aureus ~4-fold by 24 hours as well as Acinetobacter baumannii ~3-fold by 48 hours.

KEYWORDS

biofilm; infection; anesthetic; bupivacaine; C2DA; electrospun; chitosan; biomaterial; anhydride; local drug delivery; burn wound dressing; S. aureus; A. baumannii; P. aeruginosa; combat
INTRODUCTION

Burn injury is a common global threat that causes complex wounds that may affect the whole body. As of 2018, according to the World Health Organization (WHO), it is estimated that 11 million burn injuries occur worldwide, accompanied by 180,000 deaths a year (1). Additionally, non-fatal burns contribute to a leading cause of morbidity, prolonged hospital care, lifelong physical and psychological scarring, resulting in a stigma and a negative impact on quality of life (2). For individuals with moderate to severe burns, challenges such as infection, inflammatory/immune response, metabolic changes, and distributive shock are all factors that can lead to multiple organ failure (2). This is especially the case for individuals in severe conditions and/or combat settings where delayed care could result in a loss of viable tissue and increase chances of obtaining a life-threatening infection.

Infections with multi-drug resistant biofilms are threats for burn wound injuries as they present challenges for post burn therapeutic care. Biofilm refers to a complex community of adherent bacteria that consist of a single or multiple bacterial strains, that attaches to a surface while colonizing within a matter of 2-3 days (53, 54). Up to 80% of human bacterial infections are biofilm associated; such infections are most frequently caused by Staphylococcus epidermidis, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli (10). Combat wounds that are contaminated with biofilm-forming bacteria have 30 times greater odds of persistent infection, which increases to 70 times greater odds when polymicrobial biofilm is present (61). Biofilms’ resistance mechanisms entail mechanical strength from the extracellular polymeric substance (EPS) as well as protection from antibiotic clearance via metabolically dormant persister cells (6). Current treatment for burns include topical antimicrobial therapies such as silver sulfadiazine (Silvadene®) or mafenide (Sulfamylon®) cream (84, 85) followed by
application of wound dressings. Although silver sulfadiazine dressings (SSD) are used commonly for second degree burns, they have been shown to potentially impair healing by exerting toxic effects on keratinocytes and fibroblasts (16). A systematic review and meta-analysis on children with partial-thickness burns reported that silver treated burn injuries, compared to non-silver treatments, had longer wound healing time, more dressings changed, and longer length of hospital stay (17). Additionally, it is suggested that alternative antimicrobials with known anti-biofilm properties would be more appropriate (18).

Bacteria within biofilm release intrinsic signaling molecules, termed diffusible signal factors (DSF), to detach from the surface and allow for biofilm colonization throughout other areas of the body (88). This cell-to-cell communication is known as quorum sensing, and it is performed by biofilm to assess and address the microenvironment it resides in (19). In our labs we investigated cis-2-decenoic acid (C2DA), a short chain fatty acid that acts as a DSF involved with the regulation of biofilm growth and virulence that can be used to disperse and inhibit biofilm formation (20-22). Former research has been done towards evaluating C2DA’s antimicrobial effects, results showed C2DA can prevent infection when acting alone and synergistically with other antibiotics (63, 89). In addition, the cis conformation contributes to membrane permeability, allowing passage of small molecule antibiotics into the cell (22).

When addressing pain management, it is important to consider all aspects of the healing process as certain analgesic drugs such as systemic opioids could have a detrimental impact on the patient long-term. Local anesthetics (LA) have been investigated as an alternative to opioids due to their efficacy in post-burn care in the form of topical sprays or creams, and their lack of interference with the healing process (65). Additional benefits of using LAs include increasing blood perfusion to pre-burn levels (66), its ability to modify the inflammatory response (23) and
calm inflammation, to reducing edemas that contribute to the conversion of burn wounds to deeper layers of tissue. Studies have also confirmed that in addition to analgesic effects, local anesthetics such as lidocaine, ropivacaine, and bupivacaine have inherent antimicrobial effects (23). An additional benefit from LAs, such as the case for bupivacaine, is its slow onset and long-lasting effects (24). Similar to C2DA, local anesthetics such as bupivacaine are incredibly hydrophobic, making these molecules difficult to deliver.

Chitosan is extensively researched due to its abundant supply, versatility, biocompatibility, and biodegradability that facilitate its fabrication into local antibiotic delivery systems (25). Military and civilian healthcare providers currently use commercially available chitosan biomaterials, this is done to promote hemostasis, stimulate wound healing, and prevent infection (68, 72, 73, 90). The medical potential behind chitosan is ever growing as it can be fabricated into hydrogels, chitosan sponge delivery systems (75), magnetic chitosan nanoparticles for targeted drug delivery (91), coatings for musculoskeletal implant fixation hardware, injectable chitosan paste for traumatic injuries (77), and electrospun chitosan membranes (ESCM) (92, 93).

Electrospinning is a cheap and reliable technique that entails dissolving a polymer and charging it to extrude the volatile solution on to a grounded plate where a multi-layered membrane is fabricated via nano-scale fiber collection. Previous work has shown chitosan nanofibrous membranes as well suited for tissue healing and drug delivery applications due to their increased surface area, high degree of biocompatibility and biodegradability, and ability to mimic the extracellular matrix (29, 78-81, 94). Preliminary evaluations of ESCM have revealed unique properties that make them particularly suited for delivering anesthetics, fatty acids, and antimicrobials (27). As-spun membranes are modified via acylation using fatty acids of different chain lengths (via attachment to chitosan’s hydroxyl groups) to protect from swelling and
dissolution of membrane fibers in aqueous solutions at low pH (caused by hydrolysis of chitosan salt formed at the amine group of chitosan during solution prep) (27). Modification via acylation surrounds the core chitosan nanofiber with a hydrophobic surface and minimizes adherence of the fibers to wounded tissue, which is ideal for patient care during dressing changes. Prior research has gone into evaluating modified ESCM with short chain fatty acids, with success of extended release of hydrophobic therapeutics (28, 93). Based on preliminary studies for cytocompatibility of ESCMs loaded with different levels as well as published research on release of these molecules in single- and dual-loaded contexts (93) we selected the following concentrations: 0.15 mg of C2DA and 0.5 mg of bupivacaine per 10 mm diameter membrane with an average weight of 4.68 mg cut in half.

Electrospun chitosan membranes tailored with acyl lengths ranging from 6 – 10 carbons by reaction of fatty acid anhydride and loaded with both bupivacaine and C2DA may serve to 1) act as a physical barrier from microbial contamination, 2) release antimicrobial local anesthetic to reduce pain and modify the inflammatory response, 3) release natural antimicrobial fatty acids that prevent biofilm contamination, and 4) prolong delivery of these hydrophobic therapeutics for an extended period. These loaded membranes may be used as wound dressings for soft tissue wounds following 2nd and 3rd degree burns (with 10% and 1% TBSA respectively) for prolonged prevention of infection and management of pain. In this study we sought to determine release profiles of therapeutics from chitosan membranes and their ability to prevent S. aureus (UAMS-1; ATCC 21121), A. baumannii (BAA;1710) and P. aeruginosa (PA; ATCC 27317) growth and biofilm formation. Experimental groups within this study consist of the varying acylated ESCMs. Control groups consist of a threaded gauze like material one might find within a first-aid kit, a
commercially available chitosan sponge to compare differing chitosan delivery products, and bacterial growth with no material present for the bacterial analysis.

**MATERIALS AND METHODS**

**Synthesis and Fabrication**

**Preparation of Electrospun Chitosan Membranes**

Membranes were electrospun using a 311.5 kDa chitosan (ChitoLytic, Ajax, Ontario, Canada) with a 86.5%-degree deacetylation (Figure 5). Chitosan was dissolved overnight at 5.5 (w/v) %, of 70% (v/v) trifluoroacetic acid & 30% (v/v) dichloromethane. Solution was vortexed then centrifuged to remove any undissolved chitosan particulates. Afterward the solution was transferred to a 10 mL syringe and electrospun into 15 mm diameters and ~ 0.7 mm (>10 ml spinning solution) thick membranes as previously described (27). Briefly, chitosan/TFA solution was ejected with a flow rate of 0.015-0.03 ml/min and at 14-26 kV with constant monitoring of the Taylor Cone to ensure good quality membranes. Fibers were collected on a non-stick aluminum foil attached to a grounded metal wheel rotating at 8.4 RPM by an AC motor to ensure even and random distribution.

**Anhydride Treatment**

Discs, 10 mm in diameter were punched out and subjected to post-spinning treatment as previously described using hexanoic (HA), octanoic (OA), or decanoic (DA) fatty acid anhydrides (27, 92). After membranes have undergone the anhydride treatment, membranes are
lyophilized (FreeZone 2.5) for one day or more to be later stored in Ziplock bags or in desiccators. After treatment, samples are loaded with 5.071 µl/ml of C2DA and/or 16.67 mg/ml of bupivacaine via ethanol evaporation (30µL, intervals of 10µL every 5 minutes). These concentrations result in 0.15 mg of C2DA and/or 0.5 mg of bupivacaine. One half of each specimen is used for characterization via elution/bacterial analysis and the other half of each specimen was used for quantifying loading.

**Scanning Electron Microscopy**

Treated and untreated membranes were imaged with Scanning Electron Microscopy (SEM) (Nova NanoSEM650, FEI) to characterize fibers on the surface of the ESCMs. To prepare the SEM samples, membranes were placed on flat metal stubs to later be sputter coated (EMS Quorum/Q 150T ES plus) with argon and nitrogen. After images were taken via SEM, fiber width was recorded and compared to scale bars from the same SEM image to accurately measure the width. Three SEM images were analyzed for each group and 20 fibers from each SEM image were measured. Fiber measurements were done via ImageJ software (NIH).

**Fourier Transform Infrared Spectroscopy**

Treated membranes were observed via a PerkinElmer Frontier FT-IR spectrometer, with a diamond crystal, in Attenuated Total Reflectance (ATR) mode to analyze the bonds on the surface of ESCMs, and to verify success of fabrication and treatment (Waltham, MA) via ester bonds, decreased presence of chitosan salts, and an increase in carbon chain.

**Water Contact Angle**

Water contact angle was performed to determine changes in ESCM hydrophobicity following each anhydride treatment (HA, OA, and DA). Treated membranes were placed on a VCA
Optima (AST Products, Billerica, MA, USA) stage where a water droplet (5µL) was slowly dropped on top of the sample and left for 5 minutes (n=3 per treatment group). Contact angle for each sample was determined using VCA OptimaXE software.

**X-ray Photoelectron Spectroscopy**

X-ray Photoelectron Spectroscopy (XPS) was utilized to look at the surface of the samples for the percentage of elements associated with treated groups. In addition to the elemental analysis, XPS results can be used to calculate the Degree of Substitution (DOS) (27). Analysis of the samples was performed using a ThermoScientific K-Alpha XPS system equipped with a monochromatic X-ray source at 1486.6 eV, corresponding to the Al Ka line. The X-ray power of 75 W at 12 kV was used for all experiments with a spot size of 400 mm². The base pressure of the K-Alpha instrument was at 7.5 x 10^{-10} mBar. The instrument was calibrated to give a binding energy of 84.0 eV for Au 4f7/2 and 284.8 for the C1s line of adventitious (aliphatic) carbon present on the nonsputtered samples. Photoelectrons were collected from a takeoff angle of 900 relative to the sample surface. A series of XPS spectra were acquired in the Constant Analyzer Energy mode. The survey spectra were collected at a pass energy of 200 eV and an energy step size of 1.0 eV, while the high resolution (HR) core level spectra of C 1s, O 1s, etc. were taken at a 40 eV pass energy, an energy step size of 0.1 eV, and using an average of 40 scans (n=2). The XPS data acquisition was performed using the Avantage v5.995 software provided with the instrument. Degree of substitution was calculated according to Eq. (1):

\[
A\%C: A\%N = \left( \frac{6 + px}{1} \right) \times 0.865 + \left( \frac{8 + px}{1} \right) \times 0.135
\]
where $A\%C$ is atomic percentage of carbon, $A\%N$ is atomic percentage of nitrogen, $p$ is the number of carbons in the chain, and $x$ is the degree of substitution.

**Elution Profile**

Loaded half samples (samples with a diameter of 10 mm, cut in half) ($n=5$ per group) were weighed and placed in sterile phosphate buffered saline (PBS) with 10% bovine growth serum (HyClone, Logan, Utah), eluates were collected by complete solution change at time points of 3, 6, 9, 12, 24, 36, 48, 60, and 72 h. The other half of each sample was saved for a single time point analysis following a 100% ethanol wash. The concentration of C2DA and bupivacaine in the eluates was measured with high performance liquid chromatography (HPLC) using a ThermoScientific Dionex Ultimate 3000 Series HPLC system and a BDS Hypersil reversed-phase C18 column (particle size of 5µ) (250 x 4.6 mm) (run time = 7 min, injection volume = 10 µL, wavelength = 197 nm, dilution factor = 1.0). All eluate concentrations were normalized to standard curves with known concentrations of C2DA and bupivacaine.

**Antimicrobial Activity**

Loaded and unloaded samples ($n=4$) were placed in 24 well plates and inoculated with 0.5 ml tryptic soy broth (TSB) containing $10^6$ colony forming units (CFU) of *S. aureus* (UAMS-1; ATCC 21121), *A. baumannii* (BAA;1710) or *P. aeruginosa* (PA; ATCC 27317). After incubating at 37°C for 24 or 48 hours, membranes were removed from wells, rinsed twice with sterile PBS, and sonicated for 5 min at 40 kHz (Fisher Scientific Ultrasonic Bath, 9.5 L) to remove biofilm-associated bacteria. Quantification of biofilm was determined using BacTiter-Glo® Microbial Cell Viability Assay (Promega). To quantify the presence of viable planktonic bacteria, supernatants from wells containing membranes and bacteria were removed after 24- or
48-hours exposure to membranes and added to a new 96 well plate, then combined with BacTiter-Glo®. Biofilm growth on tissue culture plastic for wells containing membranes was further analyzed to determine biofilm formation at sites distant from membranes. After membranes and supernatants were removed, wells were rinsed with PBS (2 times) and attached biofilm was quantified using BacTiter-Glo®. Results were normalized as a percent viability versus bacterial cells grown in untreated wells.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software Incorporation, La Jolla, CA, USA) and Xrealstats (Add-in) through Excel. Data was assessed first by performing Shapiro-Wilk normality test, followed by Brown-Forsythe equal variance test. If both passed, data was further analyzed with a one-way analysis of variance (ANOVA) followed by Holm-Sidak’s post-hoc analysis to detect significant between experimental groups ($\alpha = 0.05$). If normality and equal variance were not passed, data was analyzed using Kruskal-Wallis ANOVA on ranks ($\alpha = 0.05$), followed by Tukey post-hoc test or Dunn. Comparisons against control groups were performed with one-way ANOVA followed up with Dunnett C.

**RESULTS**

**Scanning Electron Microscopy**

SEM imaging confirmed consistent fiber diameter with minimal swelling between the different treatments and control (ESCM), resulting in no significant differences between fiber diameters. (Figure 6).
Fourier Transform Infrared Spectroscopy

For the untreated membranes, characteristic peaks were observed at 720-840, 1790, 1670, 2300-3600, 1100 and 1200 cm\(^{-1}\) correspond to C-F stretching, C=O stretching, C=O making chitosan salt, broad O-H stretching, and large peaks caused from TFA. For the treated membranes, characteristic peaks were observed at 1742, ~3460 and ~3290, 1650, 1545, and 2900 cm\(^{-1}\) corresponding to C=O of ester, N-H stretching of amine (NH\(_2\)), C=O of amide, and N-H bend of chitosan amide, and acyl carbon chains at the surface of the membrane. All TFA peaks were absent or drastically decreased (Figure 7).
Each water drop stayed fixed on treated membranes for 5 minutes and all treated membranes exhibited hydrophobic properties (Figure 8).

Figure 8.) Contact angle images of anhydride treated ESCMs and their corresponding averaged left/right angles ± the standard deviation (N=3).
X-ray Photoelectron Spectroscopy

Results from XPS showed an increase in carbon detected starting with untreated through decanoic treated groups at binding energy 277-292 eV (Figure 9). The various anhydride treatments lead to an increased C:N ratio which was then used to calculate the degree of substitution (DOS) (Table 1) using equation 1. This increase in C:N ratio resulted in a decrease in DOS, with all values resulting less than 2.

![Figure 9: XPS results for the anhydride treated groups, untreated, and chitosan powder.](image)

Table 1.) Anhydride treated ESCMs with their averaged corresponding degree of substitution ± the standard deviation (N=2).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hexanoic Anhydride</th>
<th>Octanoic Anhydride</th>
<th>Decanoic Anhydride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of Substitution (%)</td>
<td>1.51 ± 0.14</td>
<td>1.25 ± 0.39</td>
<td>1.13 ± 0.09</td>
</tr>
</tbody>
</table>
**Bupivacaine Elution Profile**

Results showed a high burst release of bupivacaine from the controls with chitosan sponge eluting no more by 36 hours and gauze eluting out to the end of the 3-days (Figure 10). Experimental groups show a burst release within the first 3 hours followed with tapering concentrations throughout the 3-day elution. Octanoic anhydride treated membranes exhibited the highest burst release of bupivacaine from the experimental groups, followed by release of lower concentrations over the course of 3 days. Cumulative release showed significant differences between control groups vs. hexanoic and decanoic treated groups, while no significant differences were observed for octanoic group. Control groups released loading concentration within the first day, while all experimental groups released approximately 45% of the payload or more by the end of the 3 days.
Figure 10.) Bupivacaine elution profile. A.) Bupivacaine release over the course of 3 days, B.) bupivacaine release over the course of 3 days (upper limit of 50 ug/mL), C.) cumulative release of bupivacaine over the course of 3 days. Values plotted are the means ± the standard deviation. Significant differences are represented by stars as shown above (p=0.05).

Significant differences were determined by Kruskal-Wallis ANOVA on ranks, followed by Dunn test.

**C2DA Release**

Results from the 3-day release (**Figure 11**) showed all samples start with a burst release within the first 3 hours then tapering concentrations at 6 hours. By 24 hours, only the experimental groups released C2DA. C2DA release was not detected for any groups within the 12-hour
timepoint or after 24 hours. Cumulative release of C2DA, showed a significant difference between octanoic and chitosan sponge vs. hexanoic group. Additionally, all samples only released less than 20% of the C2DA payload.

Figure 11.) C2DA A.) release over the course of 3 days and B.) cumulative release of C2DA over the course of 3 days. Values plotted are the means ± the standard deviation. Significant differences are represented by stars as shown above (p=0.05). Significant differences were determined by Kruskal-Wallis ANOVA on ranks, followed by Dunn test.
Antimicrobial Activity

*S. aureus*

Results from the *S. aureus* planktonic analysis showed all groups having significantly lower growth compared to bacteria control within the first 24 hours (Figure 12). By the second day, only groups loaded with both C2DA and bupivacaine had significantly lower planktonic growth compared to control. As for the biofilm associated on the wells of the plates, control groups were similar to untreated bacteria, while experimental groups show high variability within the first 24 hours. Significant differences over the control were detected for decanoic + C2DA by 24 hours, by 48 hours all groups showed similar growth rates to that of the bacteria control outside from gauze. Biofilm associated with membranes showed both chitosan sponge at 24 hours and 48 hours significantly higher than the rest of the groups. Additionally, all of the ESCM resulted in the least amount of *S. aureus* biofilm viability at both 24 and 48 hours. Images of the sample’s surface was taken via SEM showing more colonies present on the surface of the samples within the first 24 hours as opposed to the 48 hours, this is also seen with the biofilm within the wells (Figure 13 & Figure 14).
Figure 12.) *S. aureus* viability at 24 and 48 hours. A.) Planktonic viability, B.) biofilm found in the wells, and C.) biofilm found on samples. Values plotted are the means ± the standard deviation. Significant differences against bacteria control are represented by stars as shown above (p=0.05). Significant differences were determined by one-way ANOVA, followed by Dunnett C for figures with controls and Kruskal-Wallis ANOVA on ranks, followed by Tukey post-hoc test for figures with no controls. (BUP=Bupivacaine).
Figure 13.) SEM images of *S. aureus* (yellow) bacteria on samples at 24 hours. A.) Denotes the groups not loaded with therapeutics, B.) denotes the groups loaded with a single therapeutic, C.) denotes the groups loaded with both therapeutics. Images were taken at random with a magnification of 2500x and a spot size of 1.
Figure 14.) SEM images of *S. aureus* (yellow) bacteria on samples at 48 hours. A.) Denotes the groups not loaded with therapeutics, B.) denotes the groups loaded with a single therapeutic, C.) denotes the groups loaded with both therapeutics. Images were taken at random with a magnification of 2500x and a spot size of 1.
A. baumannii

Results from the A. baumannii planktonic analysis showed all groups to be similar to that of the bacteria control at 24 hours (Figure 15). At 48 hours, the groups loaded with the combination of C2DA and bupivacaine were the only groups significantly lower to the bacteria control. This was also the case for the biofilm plate analysis at 24 hours, where the combination of therapeutics inhibits the most growth compared to the other loaded or unloaded groups. By the end of 48 hours, all groups were similar to that of the bacteria control. The biofilm associated with the membranes showed that the chitosan sponge and gauze was significantly higher than all of the other groups within the first 24 hours. Within 48 hours, Decanoic + bupivacaine showed a significant decrease in viability vs. gauze and Decanoic ESCM, while the chitosan sponge (combo) showed a significant decrease in viability vs. groups gauze, Decanoic ESCM, and Decanoic + C2DA. Images of the sample’s surface was taken via SEM showing continual growth from 24 hours to 48 hours, as seen with the biofilm growth on the membranes (Figure 16 & Figure 17).
Figure 15.) *A. baumannii* viability at 24 and 48 hours. A.) Planktonic viability, B.) biofilm found in the wells, and C.) biofilm found on samples. Values plotted are the means ± the standard deviation. Significant differences against bacteria control are represented by stars as shown above (p=0.05). Significant differences were determined by one-way ANOVA, followed by Dunnett C for figures with controls and Kruskal-Wallis ANOVA on ranks, followed by Tukey post-hoc test for figures with no controls. (BUP=Bupivacaine).
Figure 16. SEM images of A. baumannii (purple) bacteria on samples at 24 hours. A.) Denotes the groups not loaded with therapeutics, B.) denotes the groups loaded with a single therapeutic, C.) denotes the groups loaded with both therapeutics. Images were taken at random with a magnification of 2500x and a spot size of 1.
*P. aeruginosa*

*P. aeruginosa* planktonic analysis for 24 hours showed all groups having growth similar to that of control, by 48 hours the chitosan sponge had significantly higher growth than the control (Figure 18). Biofilm plates were later analyzed and results for 24 and 48 hours showed similar
results to the bacteria control. Biofilm growth on the plates showed signs of decreased growth by the end of the 48 hours. Lastly the samples were analyzed for potential biofilm, results from the 24-hour analysis showed significant differences for chitosan sponge and chitosan sponge (combo) showing higher growth vs. Decanoic + C2DA and Decanoic (combo). In addition to the 24-hour analysis, significantly higher growth was seen for gauze vs. Decanoic + C2DA. The 48-hour analysis showed significantly higher growth for Decanoic + C2DA against all other groups besides Decanoic ESCM and Decanoic (combo). Images of the sample’s surface taken via SEM showed high growth within the first 24 hours as well as biofilm formation to a reduced but still present growth by 48 hours, as seen with the data above. (Figure 19 & Figure 20).
Figure 18.) *P. aeruginosa* viability at 24 hours. A.) Planktonic viability, B.) biofilm found in the wells, and C.) biofilm found on samples. Values plotted are the means ± the standard deviation. Significant differences against bacteria control are represented by stars as shown above (p=0.05). Significant differences were determined by one-way ANOVA, followed by Dunnett C for figures with controls and Kruskal-Wallis ANOVA on ranks, followed by Tukey post-hoc test for figures with no controls. (BUP=Bupivacaine).
Figure 19.) SEM images of *P. aeruginosa* (green) bacteria on samples at 24 hours. A.) Denotes the groups not loaded with therapeutics, B.) denotes the groups loaded with a single therapeutic, C.) denotes the groups loaded with both therapeutics. Images were taken at random with a magnification of 2500x and a spot size of 1.
Figure 2. SEM images of *P. aeruginosa* (green) bacteria on samples at 48 hours. A.) Denotes the groups not loaded with therapeutics, B.) denotes the groups loaded with a single therapeutic, C.) denotes the groups loaded with both therapeutics. Images were taken at random with a magnification of 2500x and a spot size of 1.
DISCUSSION

Overall results of these studies indicate that anhydride modified ESCMs show promise as a wound dressing for burn injury applications. Loading ESCMs with therapeutics via ethanol evaporation makes them readily useable in the context of combat-related wounds. Furthermore, local delivery of anesthetics from ESCMs would have potential to alleviate the unfavorable dependency that follows the use of systemic opioid use and management (23, 24) for pain symptoms, while C2DA delivery may prevent infection post-burn. With minimal swelling of anhydride-treated fibers between the experimental groups, we can further tailor ESCMs to be more stable and maintain biological active loading capabilities (27). Washing ESCMs after treatment successfully removed TFA salts, allowing for a decrease in cytotoxic effects while maintaining hydrophobic properties (92, 95). The DOS calculation showed all of the experimental groups resulting in values less than 2 indicating more viable functional groups available on chitosan, possibly due to steric hinderance making it harder for anhydrides with longer carbon chains to react and bind to chitosan. In return, this allows for potential loading of more or other therapeutics to further develop this novel burn wound dressing. Additionally, the intrinsic biofilm inhibitory properties and release of antimicrobial and anti-inflammatory agents may act as a potential burn wound dressing that cuts down on healing time and physiological scarring.

The release of bupivacaine from the dual-loaded experimental ESCMs was similar to another study in which ESCMs treated with hexanoic anhydride (different loading concentrations) resulted in a burst release (93). Later the study inferred that this burst release is possibly due to excess bupivacaine drying on top of the membranes rather than being loaded in between the acylated fibers (93). As for the cumulative release of bupivacaine, control groups eluted the total
amount loaded just within 24 hours, while the experimental groups eluted out no more than 45-70% by the end of the study. This extended release may provide the patient with pain relief for a longer amount of time and allow for an increase in time between dressing change in dire situations. Although the dual-loaded experimental ESCMs do not release their full payload, for a patient with a compromised immune system, this release may provide a more guided growth not hindered by high concentrations affecting immune/skin cells during the healing process than the release kinetics observed by the control groups.

C2DA release from dual-loaded experimental ESCMs exhibited a burst release within the first 3 hours, followed by a ~3x-4x decrease in concentration by the next timepoint. Additionally, only the experimental groups eluted through the 24-hour timepoint. Furthermore, no C2DA was detected at the 12-hour timepoint which was likely due to most of the soluble therapeutic releasing before that timepoint. These results may be confirmed in future studies using another detection method, such as LC/MS. Our work, though somewhat contrary to similar studies of acylated chitosan membranes, are similar to another local drug delivery system that loaded C2DA onto phosphatidylcholine coatings, resulting in a burst release at first then tapering concentrations afterwards (63). Unlike the cumulative bupivacaine release, C2DA’s cumulative release displayed all groups eluting less than 20% of the loaded payload. This suggests that the samples are holding onto C2DA more tightly, possibly due to the chain length and configuration of the anhydride treatment on the hydroxyl group of chitosan allowing for attachment of long carbon chains dictating its time-dependent release. Future studies will be performed to measure the C2DA within the samples more accurately via HPLC detection post chemically/physically destroying the samples for detecting therapeutics that may reside within the samples.
Additionally, although not fully understood, the synergistic effect (22) from loading C2DA with bupivacaine may play a role in release kinetics.

Activity against strains prevalent in burn injuries suggest these biomaterials would be useful in burn injury infection prevention. Similar antimicrobial activity against S. aureus was reported in a study performed by Harrison et al., where the same therapeutics and method of delivery were used, but at much higher concentrations. In this study, nearly all groups had decreased growth within 24 hours, even groups not loaded with any therapeutic (93), indicating that materials themselves were inhibitory to bacteria, but not bactericidal at these concentrations. In contrast our study looked at the response by 48 hours, where the only biomaterials with significant antibacterial properties were those loaded with both therapeutics. This suggests that the combination of therapeutics may have additive or synergistic effects for inhibition of biofilm. Unlike S. aureus, A baumannii’s planktonic results showed no groups with significant differences against the control by 24 hours, which may be due to lower susceptibility of this strain to both bupivacaine and C2DA. Similar to S. aureus, only the groups loaded with both therapeutics had significant less growth than the control by 48 hours. This however was not seen for the P. aeruginosa planktonic data suggesting the combination and concentration of both therapeutics seems to be more effective at inhibiting S. aureus and A. baumannii planktonic bacteria over planktonic P. aeruginosa. This is likely the case due to P. aeruginosa being one of the gram-negative strains that become harder to treat as time increases versus other skin related infections from burn wounds (13). Although reports of high concentrations of C2DA have found that this agent increases membrane permeability (96), P. aeruginosa among other strains produce and utilize these DSFs (97). This would explain the lack of inhibition of P. aeruginosa at such concentrations compared to that of other strains. It is likely that results from gauze are due to the
processing method of the gauze, as they are bleached for color and sterility purposes. When dealing with gram negative strains such as *A. baumannii*, it seems that the combination of therapeutics perform the best at reducing bacterial viability. The lack of inhibitory effect against the gram-negative strains may also be explained by the burst release seen in the elution studies, where a burst release of C2DA is followed by tapering concentration within the first day. The reduction of *S. aureus* and *P. aeruginosa*’s biofilm in the wells, below the samples, at 48 hours may be due to the increase in time resulting in more available therapeutic in the wells. In addition to more therapeutics being released, it is also important to note that most of these samples are hydrophobic and float within the TSB media, resulting in an increase of therapeutics reaching the bottoms of the wells via diffusion over time. The higher growth of *S. aureus* and *A. baumannii* biofilm on the chitosan sponge suggests that the material alone was not able to reduce the viability of biofilm for both timepoints as opposed to the experimental groups such as the decanoic acid modified ESCM, indicating it may have an inhibitory effect on its own. The reduction of *P. aeruginosa* biofilm growth by 48 hours, caused by the groups loaded with both therapeutics, suggests that for some strains an additive effect of both therapeutics may benefit reducing biofilm than when these therapeutics act alone.

These *in vitro* evaluations investigated combination and individual loading of therapeutics for control and experimental groups to aid in pain relief and infection prevention as a burn wound dressing application. Results displayed combo loaded ESCMs, of varying acylation modification, exhibiting a burst release of therapeutics followed by a steady release of bupivacaine and C2DA. In austere locations or combat settings, where medical attention may be delayed, this may be advantageous for minimizing infections from further contamination as well as potential biofilm growth within the first 24 hours of administration, with local anesthetics remaining active over
the course of 72 hours, until further care is available. Additionally, since this material is light-weight and does not elute out all active therapeutics at once, a loading concentration can be tailored around the percentage of release to avoid any cytotoxic effects. For bacterial studies, ESCMs were modified with decanoic anhydride to extend release, while minimizing a burst release of therapeutics. Based on findings from the bacterial studies it is suggested that the combination of therapeutics play a major role in biofilm prevention for select strains such as *S. aureus, A. baumannii, and P. aeruginosa*, whether that is from direct contact or diffusion in aqueous solutions. Future studies will focus on effects on cells and tissues *in vitro* and *in vivo*. To conclude, this *in vitro* study denotes the potential of varying acylated ESCMs, especially decanoic-modified, to be used as a burn wound dressing by releasing hydrophobic therapeutics for prevention of bacterial/biofilm growth and pain relief.
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**Conflicts of Interest**
We have no conflicts of interest to declare.
CHAPTER IV
DISCUSSION

Fatty acid anhydride treated ECSMs ranging from 6-10 carbons in length were capable of being loaded with C2DA and bupivacaine in combination or individually, to prevent biofilm formation. Additionally, with the loading of the therapeutics performed via ethanol evaporation, ESCMs can be readily available for immediate use. Local anesthetic and C2DA loaded wound dressings may alleviate the unhealthy dependency that follows the use of opioid use and management (23, 24) for pain symptoms, as well as provide antimicrobial properties to further address infection post-burn. With minimum swelling of fibers between the experimental groups, we can further tailor ESCMs to be more stable and maintain loading capabilities (27). In addition to treating the ESCMs with an anhydride solution, the washes after treatment remove TFA salts allowing for a decrease in cytotoxic effects whilst maintaining its hydrophobic property post treatment (92, 95).

Although the DOS does not reach 2 for all of the experimental groups, possibly due to steric hinderance, this allows for potential loading of more or other therapeutics to further develop this novel burn wound dressing. This combination of therapeutics plus the material housing it, provides physical coverage from bacterial contamination and may act as a potential burn wound dressing that cuts down on healing time and physiological scarring via its intrinsic infection resistance as well as the resistance and anti-inflammatory benefits gained from the therapeutic agents, against certain pathogens growing biofilm and planktonic bacteria.

Prior to this study, preliminary results showed that varying acylated ESCMs (hexanoic, octanoic, and decanoic) are capable of loading and releasing single and dual loaded therapeutics (with concentrations of 1.5 mg) such as bupivacaine or C2DA alone or in combination (Appendix A) (Appendix B). Within the first 3 hours, dual-loaded experimental ESCMs exhibited a slight burst release of bupivacaine similar to that of preliminary single and dual release of bupivacaine.
loaded ESCMs. This burst release at the first timepoint was followed by a tapering yet steady release over the course of the 72 hours. As for the cumulative release of bupivacaine, control groups were able to elute the total theoretical amount loaded just within 24 hours, while the experimental groups eluted out no more than 45-70% within 72 hours. A common trend in the release data exhibits control groups with high burst releases lasting to 24 hours, while experimental groups exhibit burst release followed by lower and more consistent concentrations lasting to the 72 hours. Although the dual-loaded experimental ESCMs do not release their full payload, this release can likely exhibit less toxicity for a patient suffering from an impaired immune system caused by a moderate to severe burn than the high burst release performed by the control groups.

C2DA release from dual-loaded experimental ESCMs exhibited a burst release similar to that of the control groups within the first timepoint, followed by a ~3x-4x decrease in concentration by the next timepoint. As seen in preliminary data for both single and dual release, only the experimental groups elute past the 9-hour timepoint. These results, although loaded with different concentrations, are similar to another local drug delivery system that loaded C2DA with phosphatidylcholine coatings, resulting in a burst release at first then tapering concentrations afterwards (76). Unlike the cumulative bupivacaine release, C2DA’s cumulative release exhibited none of the groups eluting more than 20% of the theoretical loading concentration. This suggests that the samples are holding onto C2DA more tightly than originally expected, possibly due to the chain configuration from the decanoic anhydride treatment and the C2DA. Future studies will be performed to more accurately measure the C2DA within the samples.

Antimicrobial efficacy studies performed under similar conditions with concentrations of 1.5 mg of C2DA, 1.5 mg of bupivacaine, or a combination of the two (Appendix C) against pathogens
S. aureus and A. baumannii showed that higher concentrations of these therapeutics may be necessary to effectively decrease planktonic viability at both timepoints for S. aureus. The inhibition of planktonic S. aureus for all groups loaded with combinations of therapeutics in this study confirms that these two therapeutics may have additive effects. A baumannii’s planktonic growth inhibition at 48 hours may indicate that it is less susceptible to these therapeutics at low concentrations. Lower susceptibility was also supported in preliminary results for planktonic A. baumannii. The absence of inhibition of planktonic P. aeruginosa data indicates that these therapeutics do not inhibit P. aeruginosa at these concentrations, which is in line with other studies of P. aeruginosa inhibition (89, 97-99).

Results from the biofilm growth, within the wells, for S. aureus exhibited a decrease in growth by the 48-hour timepoint for gauze and experimental groups. It is likely that the reason for gauzes difference to the bacteria control is due to the processing method of the gauze, as they are bleached for color and sterility. The initial inhibition of A. baumannii biofilm may be due to burst release seen in the elution studies, where a burst release is followed by tapering concentration within the first day. The lack of inhibition of A. baumannii by 48 hours is consistent with the low susceptibility observed for planktonic growth. As for P. aeruginosa’s results, all groups at both timepoints were classified as similar to the bacteria control, followed by a decrease in growth for all groups by 48 hours. It is also important to note that most of these samples float at the top of the well, meaning biomaterials do not make contact with the bottoms of the wells except via diffusion of released therapeutics, which may take time to come into effect.

The final bacterial analysis consisted of quantifying the amount of biofilm on the membranes followed by SEM and pseudo coloring the bacteria attached to the samples. The
enhanced growth of *S. aureus* biofilm on chitosan sponge alone indicates that the chitosan material itself is not strongly antimicrobial. The lower growth of biofilm on ESCM and especially those loaded with both therapeutics is consistent with planktonic inhibition of *S. aureus*. Preliminary results also confirm similar reduction of biofilm viability for *S. aureus* in the presence of loaded ESCM. The decrease in *A. baumannii* biofilm viability for the experimental groups within the first 24 hours, but not the 48 hours, is also confirmed in the preliminary data and indicates that the effects of these therapeutics may be short-lived for *A. baumannii*. The initial biofilm inhibition of *P. aeruginosa* biofilm growth at 24 but not 48 hours may also indicate that the effects of these therapeutics are short-lived for this gram-negative strain. The combo loaded chitosan sponge and ESCM had consistent biofilm preventive effects for *S. aureus* and *A. baumannii* and *P. aeruginosa*, further indicating potential additive or synergistic effects and that the loaded therapeutics have more effect than the chitosan sponge/ESCM biomaterial antimicrobial properties.

These *in vitro* evaluations investigated combo and individual loading of therapeutics for control and experimental groups to aid in pain relief and infection prevention as a burn wound dressing application. Results displayed combo loaded ESCMs, of varying acylation modification, exhibiting a burst release of therapeutics followed by a steady release of bupivacaine and C2DA for up to 3-days and 1-day respectfully, whereas controls usually taper and cease to elute after ~48 hours and 9 hours respectfully. Additionally, the samples used in the elution study, as well as the halves that were not, were analyzed post ethanol rinse to more accurately determine what payload remained in the samples. Results indicated samples were holding on to more C2DA than originally released in elution study (*Appendix D*). Despite the weight difference between the control and experimental groups, the experimental groups eluted the same amount if not more
per mg (Appendix E). Results from bacterial studies suggest experimental groups and control groups exhibit short-lived antibiofilm effects, outside of the groups loaded with the combination of therapeutics. Based on these findings it is suggested that the combination of therapeutics play a major role in biofilm prevention for select strains such as S. aureus, A. baumannii, and P. aeruginosa, whether that is from direct contact or diffusion in aqueous solutions. To conclude, this in vitro study denotes the potential of varying acylated ESCMs to be used as a burn wound dressing, by releasing hydrophobic therapeutics for prevention of bacterial/biofilm growth and pain relief.
CHAPTER V
CONCLUSIONS

These in vitro evaluations investigated combination and individual loading of bupivacaine and C2DA for electrospun chitosan membranes treated with fatty acid anhydrides (ranging from 6-10 carbons) and control groups (chitosan sponge and gauze) to aid in pain relief and infection prevention as a burn wound dressing application. Results demonstrated that combo loaded ESCMs, of varying acylation modification, exhibiting a burst release of therapeutics followed by a steady release of bupivacaine and C2DA for up to 3-days and 1-day respectfully, whereas controls taper and cease to elute after ~48 hours and 9 hours respectfully. As for the cumulative release of bupivacaine, control groups eluted the total theoretical amount loaded just within 24 hours, while the experimental groups eluted out no more than 45-70% by the end of the study. C2DA’s cumulative release exhibited none of the groups eluting more than 20% of the theoretical loading concentration, suggesting that the samples are holding onto C2DA more tightly than expected. Additionally, experimental groups and control groups exhibited minimum antibiofilm effects, unless loaded with the combination of therapeutics. Results from bacterial studies suggest that this combination reduced bacterial attachment 2-fold for Acinetobacter baumannii, 2-3-fold for Pseudomonas aeruginosa, S. aureus 10-fold by 24 hours, and S. aureus 4-fold by 48 hours. Additionally dual loaded groups reduced planktonic growth for Staphylococcus aureus ~4-fold by 24 hours and ~2 fold by 48 hours as well as Acinetobacter baumannii ~3-fold by 48 hours. Based on these findings it is suggested that the combination of therapeutics play a major role in biofilm prevention for select strains such as S. aureus, A. baumannii, and P. aeruginosa, whether that is from direct contact or diffusion in aqueous solutions. To conclude, this in vitro study denotes the potential of varying acylated ESCMs to be
used as a burn wound dressing, by releasing hydrophobic therapeutics for the prevention of bacterial/biofilm growth and pain relief.
CHAPTER VI
FUTURE WORK

Future studies for this project entail reconsideration of loading concentrations, *in vivo* studies, and perfecting the chitosan solution for ideal membrane fabrication. Additional future studies include cytocompatibility studies using human dermal fibroblasts and keratinocytes. Additionally, effects of membranes, bupivacaine, and C2DA on immune cells will be explored through assays to measure effects on the polarization of human macrophages toward inflammatory phenotype (M1) or anti-inflammatory, pro-healing phenotype (M2) to determine whether membranes induce healing and/or direct inflammatory responses that might lead to burn wound conversion. *In vivo* characterization of dual loaded ESCMs entail a comb scald wound model inoculated with bacteria and compared to commercially available wound care materials. In collaboration with the University of Memphis Chemistry department, other synthetics analogs of C2DA have been developed as well as new procedures for measuring therapeutics, so future studies may include incorporation of these analogs into chitosan membranes and evaluation of their potential to prevent and eradicate biofilm more effectively than C2DA. Although combo loaded ESCMs seem promising there are still limitations, such as no *in vivo* results, and many unanswered questions like how would octanoic treated ESCMs fare against said bacteria? How would the release and bacterial results have looked like if treated with two different anhydrides? With a DOS of 2, what else could be loaded that would promote healing? Lastly, instead of ethanol drying process, what other methods for loading therapeutics should be considered? Future studies will focus on these questions while fabrication of ESCMs, possible reconsideration to load any other therapeutics or change the current concentrations or anhydride, cell studies, as well as *in vivo* work are underway.
References


APPENDIX A: Preliminary Elution Profile of Single Loaded Membrane

Rationale and Methods

Previous elution investigations of C2DA and Bupivacaine were conducted to ascertain the release profile of hexanoic anhydride treated membranes with concentrations under cytotoxicity levels (up to 20 mg for bupivacaine and 500 µg for C2DA) (93). As a result of the high burst release of bupivacaine and high standard deviations seen with C2DA release from previous studies, new loading concentrations [1.5 mg C2DA, 1.5 mg bupivacaine] was investigated for single loaded membranes. Loaded membranes were treated with varying anhydrides (hexanoic, octanoic, and decanoic). Loaded membranes (n=5 per group) were placed in sterile phosphate buffered saline (PBS) and eluates were collected by complete solution change at time points of 3, 6, 9, 12, 24, 36, 48, 60, and 72 h. The concentration of C2DA and bupivacaine in the eluates was measured with high performance liquid chromatography (HPLC) using a ThermoScientific Dionex Ultimate 3000 Series HPLC system and a BDS Hypersil reversed-phase C18 column (particle size of 5µ) (250 x 4.6 mm) (run time = 7 min, injection volume = 10µL, wavelength = 197 nm, dilution factor = 1.0). All eluate concentrations were normalized to standard curves with known concentrations of C2DA and bupivacaine.

Results

Initial release studies involved treating membranes with different acyl length fatty acids, single-loading with bupivacaine/C2DA separately (Figure 21 & 22), and comparing release from membranes with gauze and chitosan sponge controls (chitosan sponge). Results showed bupivacaine released from decanoic membranes with a high initial burst; a similar release profile was seen for gauze and sponge controls. Other experimental membranes (HA & OA) exhibited a
consistent and steady release throughout the duration of the study. For C2DA, results displayed all experimental groups outlasting controls (sponge & gauze) after 9 hours. Additionally, all groups exhibited an initial burst and a diminished concentration after 36 hours. Cumulative data for C2DA released, depicted all groups releasing less than 10% of the loaded therapeutic.

**Figure 21.** Release of bupivacaine from single loaded ESCMs, sponge, and gauze control materials.

**Figure 22.** Release of C2DA from single loaded ESCMs, sponge, and gauze control materials.
Conclusions

Bupivacaine eluted from treated membranes over the course of 72 hours, while C2DA is eluted over the course of 36 hours. Decanoic treated membranes look promising as they are able to maintain release of hydrophobic therapeutics after initial release. Results from the C2DA profile indicates that perhaps C2DA is residing in the loaded samples as only less than 10% comes out over the course of 72 hours. For future studies, dual-loaded concentrations will be utilized and assessed.
APPENDIX B: Preliminary Elution profile of Dual Loaded Membranes

Rationale and Methods

After the single-loaded release studies, membranes with different acyl length fatty acids were dual-loaded with bupivacaine and C2DA together (Figure 35 & 36) to compare release profiles from membranes with gauze and chitosan sponge controls. [1.5 mg C2DA/1.5 mg bupivacaine] was investigated for dual loaded membranes. Loaded membranes (n=5 per group) were placed in sterile phosphate buffered saline (PBS) and eluates were collected by complete solution change at time points of 3, 6, 9, 12, 24, 36, 48, 60, and 72 h. The concentration of C2DA and bupivacaine in the eluates was measured with high performance liquid chromatography (HPLC) using a ThermoScientific Dionex Ultimate 3000 Series HPLC system and a BDS Hypersil reversed-phase C18 column (particle size of 5µ) (250 x 4.6 mm) (run time = 7 min, injection volume = 10 µL, wavelength = 197 nm, dilution factor = 1.0). All eluate concentrations were normalized to standard curves with known concentrations of C2DA and bupivacaine.

Results

Results showed the octanoic and decanoic modified membranes had a slower release of C2DA, while the other groups, apart from the chitosan sponge, demonstrated a burst release. The chitosan sponge controls showed very minimal release, which may have resulted from a strong association of C2DA to chitosan, or inadequate loading of C2DA. For C2DA, one can note that although the gauze shows a high burst release compared to the experimental membranes, it does not elute therapeutics at past 9 hours. This is exhibited as well in the single loaded study. For bupivacaine, membranes of varying acyl lengths released in a similar pattern, outside of
hexanoic, with octanoic and decanoic-modified membranes releasing a lower percentage of both therapeutics and at a slower rate (Figure 23 & 24).

**Dual loaded – Bupivacaine**

**Daily Release**

Figure 23.) Release of bupivacaine from dual loaded ESCMs, sponge, and gauze control materials.

**Cumulative Release**

**Dual loaded – C2DA**

**Daily Release**

Figure 24.) Release of C2DA from dual loaded ESCMs, sponge, and gauze control materials.
Conclusions

Results from the dual loaded studies, for bupivacaine, suggest that octanoic and decanoic modifications prevent high initial release which may be beneficial in preventing tissue toxicity during initial release. Similar to the single-loaded study, a high initial burst is followed by tapering concentrations, with the exception of the OA and DA membranes releasing at a steady rate throughout the study. Additionally, when single loaded membranes release C2DA there is a noticeable decrease in the amount of C2DA released compared to when it is released in combination with bupivacaine. When calculating total amount of bupivacaine released from gauze, erroneous values of 120% total released were calculated, which may be due to a loading error and will be repeated to verify.
APPENDIX C: Preliminary Bacterial Evaluations of Loaded Membranes

Rationale and Methods

C2DA/Bupivacaine alone and combination was evaluated for its effects against varying strains such as *S. aureus*, *A. baumannii*, and *P. aeruginosa*. Decanoic anhydride was the anhydride treatment of choice due to its performance in the prior elution studies. The groups were evaluated for their frequency of planktonic bacteria, biofilm on the sample, and biofilm on the wells. Samples were loaded with either 1.5 mg bupivacaine, 1.5 mg of C2DA, or a combination of the two. Membranes (n=3) were placed in 24 well plates and inoculated with 0.5 ml tryptic soy broth (TSB) containing $10^6$ colony forming units (CFU) of *S. aureus* (UAMS-1; ATCC 49230), *A. baumannii* (BAA;1710) and *P. aeruginosa* (PA; ATCC 27317) respectively. After incubating at 37°C for 24 hours, membranes were removed from wells, rinsed twice with sterile PBS, and sonicated for 5 min at 40 kHz (Fisher Scientific Ultrasonic Bath, 9.5 L) to remove biofilm-associated bacteria. Quantification of biofilm was determined using BacTiter-Glo® Microbial Cell Viability Assay (Promega). The presence of viable planktonic bacteria was determined for wells with membranes. Supernatant from wells containing membranes and bacteria was removed and added to a new 96 well plate, then combined with BacTiter-Glo® to quantify the amount of planktonic bacterial growth after 24- & 48-hours of exposure to membranes. Biofilm growth on tissue culture plastic for wells containing membranes was further analyzed to further determine effects on biofilm formation at sites off of the membrane itself. After membranes and supernatant were removed, wells were rinsed with PBS and attached biofilm was quantified using BacTiter-Glo®. Results were normalized as a percent viability versus bacterial cells grown in untreated wells and also compared to a control group of chitosan sponges and gauze.
Preliminary antibacterial studies had been done on *P. aeruginosa*, but due to human error the values for the pathogen were not available for this study.

**Results**

Analysis for antimicrobial results were evaluated comparing materials to the control group (Sponge) (Figures 25 & 26). Evaluation of *S. aureus* antimicrobial activity exhibited significant differences in bacterial viability for all ESCM materials (24 & 48 hours), besides gauze, within the planktonic study. DA and DA + Bupivacaine were the only groups to have a significant difference above the control. As for the biofilm growth, all groups besides the gauze, at 24 hours, were significantly decreased compared to the control. Evaluation of *A. baumannii* antimicrobial activity exhibited no significant differences for both 24- and 48-hour planktonic growth. Unlike the planktonic growth, biofilm growth for *A. baumannii* showed sponge + both and all DA membranes as significantly different below the control at 24 hours. DA (not treated) at 48 hours remained significantly different but was reported significantly different above control. Significant differences were calculated with (p>0.05, determined by ANOVA with Holm-Šídák post-hoc tests). With the addition of the quantitative data for the antimicrobial study, one sample was kept from each group to analyze the surface for possible biofilm via SEM (Figures 39-43). All pathogens were imaged besides *S. aureus* at 24 hours due to human error. From what was imaged we can see clear housing of bacteria for all groups, as well as clumps of bacteria that adhere to the surfaces of these groups indicating formations of biofilm (Figures 27-31).
Figure 25.) Planktonic and Biofilm results from antimicrobial *S. aureus* exposure for 24 and 48 hours. (\*: significant difference less than control (Sponge), \#: significant difference greater than control (Sponge))

Figure 26.) Planktonic and Biofilm results from antimicrobial *A. baumannii* exposure for 24 and 48 hours. (\*: significant difference less than control (Sponge), \#: significant difference greater than control (Sponge))
Figure 27.) SEM membranes exposed to *P. aeruginosa* for 24 hours.

Figure 28.) SEM membranes exposed to *P. aeruginosa* for 48 hours.
Figure 29.) SEM membranes exposed to *A. baumannii* for 24 hours.

Figure 30.) SEM membranes exposed to *A. baumannii* for 48 hours.
Conclusions

A common trend between the data collected, indicated the unloaded therapeutics may have minimal antimicrobial activity on their own, as they were similar to sponge and gauze groups. The presence of C2DA facilitates the inhibition of biofilm for S. aureus. As expected, the membrane displays and addresses biofilm growth with antibacterial effects, but for A. baumannii perhaps the concentration needs to be increased or these therapeutics are not as efficient for inhibition of A. baumannii. Although the presence of C2DA facilitates the inhibition of biofilm for other common burn pathogens, planktonic A. baumannii did not appear to be as sensitive to C2DA as pathogens such as S. aureus and P. aeruginosa.
APPENDIX D: Loading and Retention after Elution

Rationale and Methods

As a result of the high standard deviations seen with the most recent elution study, a single time point elution study was conducted to determine the difference between the theoretical amount loaded and how much was released/retained from the other half membranes not used in the study as well as those used after elution. This was performed by adding 500µL of 100% EtOH to tubes containing the combo loaded samples, to be later vortexed, and plated. The concentration of C2DA and bupivacaine in the eluates was measured with high performance liquid chromatography (HPLC) using a ThermoScientific Dionex Ultimate 3000 Series HPLC system and a BDS Hypersil reversed-phase C18 column (particle size of 5µ) (250 x 4.6 mm) (run time = 7 min, injection volume = 10 µL, wavelength = 197 nm, dilution factor = 1.0). All eluate concentrations were normalized to standard curves with known concentrations of C2DA and bupivacaine.

Results

Results from post-elution indicated all control groups indicating no peaks for both therapeutics, additionally all experimental groups indicated a split peak at a later retention time, possibly due to a difference in buffer solution. Based off of the standards of C2DA also indicating split peaks and a prior study recognizing split peaks via HPLC for DSFs (100), peaks were analyzed as C2DA. Results for the amount of C2DA retained on treated ESCMs, used in initial elution, showed hexanoic ESCMs retaining ~35%, octanoic ESCMs retaining ~16%, and decanoic ESCMs retaining ~17% (Figure 32). Cumulative means summed from initial elution and post elution analysis showed more C2DA loaded on hexanoic treated ESCMs compared to octanoic and decanoic treated ESCMs. Analysis from the samples not used in elution (other halves) were
investigated for loaded therapeutics (Figure 33). Results indicated loading of therapeutics as less than 40% for control group gauze (both therapeutics), additionally mean values of 60% of C2DA and over 100% of bupivacaine was detected for control group chitosan sponge. Experimental groups seemed to have mean values of 50% or more of C2DA loaded on ESCMs and 60% or more of bupivacaine loaded on ESCMs.

Figure 32.) Percentage of remaining therapeautical left on samples, post 3-day elution study, and percent cumulative means summed from initial elution (n=5).
Results from the post-elution analysis showed signs of hexanoic treated ESCMs giving off more C2DA than the longer chain treatments such as octanoic and decanoic groups. This correlates with the cumulative release of C2DA where hexanoic group releases the smallest percentage out of the experimental groups. Results from the half samples, not used in the elution, indicated a very low percentage of both therapeutics for control group gauze. This is likely due to human error, as the inert loose fibers of the gauze material proves to be difficult to cut, let alone load with ethanol-based techniques. As for the chitosan sponge material, one can see similar results to experimental groups in terms of loading C2DA; conversely this does not seem to be the case for

Figure 33.) Percentage of loaded therapeutics on half samples not used in the initial elution. A.) Percentage of C2DA loaded on samples and B.) percentage of bupivacaine loaded on samples (N=2).

Conclusions
bupivacaine as it remains to have the highest mean and standard deviation compared to the other samples. Lastly, the sum of mean percentages from the elution study show differences compared to percentages from samples not used in the study. This difference is likely due to the therapeutics not releasing, therapeutics are releasing yet are attracted to the plastic of the sample containers, or therapeutics are not staying on the material when loaded. Additionally, FTIR results from previous work show chitosan derived delivery systems not fully ridding of C2DA after the ethanol procedure, although this would only account for a small percentage. Future studies for the ethanol washing protocol could introduce an immediate and delayed rinse as well as a method pertaining biotin or some fluorescent molecule to help visualize payload.
APPENDIX E: Elution per weight of sample

Rationale and Methods

As per protocol membrane halves were cut in half and weighed before loading. From there we divided the groups elution release by their corresponding weights to analyze the amount of release for each groups weight over the course of 3 days for both therapeutics. The concentration of C2DA and bupivacaine in the eluates was measured with high performance liquid chromatography (HPLC) using a ThermoScientific Dionex Ultimate 3000 Series HPLC system and a BDS Hypersil reversed-phase C18 column (particle size of 5µ) (250 x 4.6 mm) (run time = 7 min, injection volume = 10 µL, wavelength = 197 nm, dilution factor = 1.0). All eluate concentrations were normalized to standard curves with known concentrations of C2DA and bupivacaine.

Results

Results show gauze eluting out to 3 days with low concentrations by 24 hours, while chitosan sponge ceases to elute by 36 hours (Figure 34). Gauze group eluted out to 40 µg within the first 3 hours followed by a 4x decrease while chitosan sponge eluted below 25 µg within the first 3 hours followed by a 3-4x decrease. Results also indicate the experimental groups releasing detectable concentrations of bupivacaine out to 3 days, with octanoic membranes releasing up to 70 µg within the first 3 hours followed by a 7x decrease as well as the highest amount eluted between the other groups until hour 60. As for the release/mass for C2DA, unlike the bupivacaine data, all of the experimental groups seemed to show a higher burst release than the control groups (Figure 35). All groups eluted less than 5 µg within the first 3 hours, but traces of bupivacaine were only detected for the experimental groups by 24 hours.
Figure 34.) Bupivacaine release over the course of 3 days A.) divided by weight per mass of sample, B.) bupivacaine release over the course of 3 days divided by weight per mass of sample (upper limit of 25 µg/mL). Values plotted are the means ± the standard deviation.

Figure 35.) C2DA release over the course of 3 days divided by weight per mass of sample. Values plotted are the means ± the standard deviation.
Conclusions

Despite weighing 2-3 times less than control groups, experimental ESCMs indicated the same if not higher release/mass of sample (mg). This study helps give an idea of how the bulk application and release from these products may act. Of course, when going up in scale there are many other factors involved such as making sure these concentrations fall under cytocompatibility levels with skin and immune cells to ensuring equal distribution of loading said therapeutics, and further testing in vivo. Additionally, this light weight will help in a variety of ways such as lighter packaging/equip weight to more manipulation for a burn wrap dressing application.
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