In Vitro Evaluation of Loaded Chitosan Membranes for Pain Relief and Infection Prevention

Zoe Lynn Harrison

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IN VITRO EVALUATION OF LOADED CHITOSAN MEMBRANES FOR PAIN RELIEF
AND INFECTION PREVENTION

by

Zoe Lynn Harrison

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

Major: Biomedical Engineering

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PREFACE

The main body of this thesis in Chapter III is a journal article titled “In vitro evaluation of loaded chitosan membranes for pain relief and infection prevention.” This manuscript is to be submitted to Frontiers in Microbiology.
ABSTRACT

Wounds resulting from musculoskeletal trauma cause pain and often infection of damaged tissue. Up to 80% of these infections are due to biofilm formation on wounded tissue. Bacteria within a biofilm can withstand 1000x the minimum inhibitory concentration of antibiotics, demonstrating the need for new therapeutics to prevent and treat these infections. Cis-2-decenolic acid (C2DA) disperses biofilms and can prevent biofilm formation of many microorganisms. Additionally, local anesthetics like bupivacaine have antimicrobial effects against bacteria. This study sought to evaluate modified electrospun chitosan membranes as wound dressings that release C2DA and bupivacaine to prevent infection and alleviate pain associated with wounds. Release profiles of therapeutics were evaluated, with results indicating that membranes release active concentrations of both therapeutics for 72 hours. Though higher concentrations of bupivacaine were not cytocompatible with fibroblasts, membranes were effective in preventing Methicillin-resistant Staphylococcus aureus biofilm growth in vitro.
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CHAPTER I
INTRODUCTION

Statement of Clinical Problem

There is a growing demand for local delivery systems to treat and prevent infection and pain during wound healing. These wounds may result from a surgical procedure or implantation of a medical device, or from other musculoskeletal trauma such as burns. Implantation of medical devices accounts for approximately 2 million healthcare-associated infections annually and can only be effectively treated with removal of the implant and aggressive debridement of surrounding tissue, resulting in significant pain and trauma for patients [1; 2]. Similarly, burn wounds are incredibly susceptible to infection, with approximately 51% of burn victim deaths resulting from wound infection [3]. Formation of antibiotic-resistant biofilm, as well as an alarming rise in multidrug-resistant bacterial strains due to antibiotic overuse, limit the availability of antimicrobials practical for broad-spectrum antibiotic administration to prevent and treat wound infections [4; 5]. Therefore, establishing coverage and reducing pain during early wound management are necessary to effectively reduce both patient costs and trauma.

Bacteria typically colonize wounds by forming a biofilm within 24-72 hours post-injury [6; 7]; therefore, protection of the wound for this window is critical. These biofilm infections, caused by microbial communities adhering to the surfaces of implanted devices, surrounding tissue, and musculoskeletal wounds, are particularly difficult to treat due to their antibiotic resistance. Bacterial attachment is often accompanied by the secretion of a complex, sugary structure termed extracellular polymeric substance (EPS), which functions to protect and encase the community of bacteria in a cohesive polymer matrix [8]. The resulting biofilm is characterized by increased mechanical stability due to the cohesivity of bacteria within the EPS,
and significant interconnection and immobilization of the bacterial cells within it. These immobilized subgroups of bacterial cells within the biofilm, called persister cells, are dormant, non-dividing cells that are resistant to antibiotics [9]. 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 in biofilm compared to planktonic bacteria, and also makes them resistant to immune cell clearance [10].

Developing biofilm maintains equilibrium through growth and dispersal. Diffusible signal factors (DSF) are fatty acid analogs secreted by bacteria to function in a cell-cell communication mechanism, called quorum sensing [11]. It has been observed that some fatty acid signaling factors revert persister cells to a metabolically active state, which in combination with antimicrobials could decrease bacterial viability. These compounds also act to inhibit and disperse biofilms formed by multiple types of microorganisms, meaning they have cross-kingdom efficacy [12]. One well-studied biofilm dispersal signal is cis-2-decenoic acid (C2DA), which has been shown to inhibit biofilm formation and disperse established biofilms of multiple strains [13]. In addition to infection prevention, non-opioid pain management strategies such as delivery of local anesthetics are a top priority for wound treatment, especially as the opioid crisis reaches epidemic proportions [14]. Studies have also confirmed that in addition to analgesic effects, local anesthetics such as lidocaine, ropivacaine, and bupivacaine have inherent antimicrobial effects [15].

Preventative anti-infective strategies in current practice include the local delivery of therapeutics, anti-biofilm coatings on surfaces and precautionary hygienic measures. However, additional strategies are required to improve efficacy of these treatments. Moreover, treatments for existing biofilms are particularly challenging. An established biofilm is often able to survive
aggressive physio-chemical treatments including UV light, heavy metals, acidity, changes in hydration or salinity, and phagocytosis [16; 17; 18; 19; 20]. Currently, antimicrobial therapies have limited efficacy in cases of preformed biofilms such as implant associated osteomyelitis, blood stream infections, chronic pulmonary infections, dental caries, and endocarditis [21]. Long term use of antibiotic therapy can help to manage biofilm infections, but often fails while also increasing the risk of antibiotic resistance and toxic side effects [10]. For instance, Methicillin-resistant *Staphylococcus aureus* (MRSA) is the cause of more than 50% of all invasive infections in burn wounds [1]. The occurrence of biofilm infections can lead to increased hospital time and trauma for patients as well as increased cost of care for hospitalization.

Guided regeneration membranes can provide a covering for healing tissue while also delivering antimicrobials and local anesthetics for use as wound dressings following injury [22; 23]. Chitosan has been investigated as a sustained drug delivery system due to its biocompatibility and physiochemical characteristics and is a promising scaffold to deliver hydrophobic molecules such as C2DA and bupivacaine [24; 25; 26; 27; 28]. Electrospun chitosan membranes loaded with the local anesthetic bupivacaine and C2DA may serve to 1) act as a barrier to microbial contamination, 2) release local anesthetics in a controlled manner that reduce pain and modify the inflammatory response, and 3) release natural, antimicrobial fatty acids that prevent biofilm contamination. These loaded membranes may be used as wound dressings for soft tissue wounds following medical device implantation, musculoskeletal trauma, or burn wounds for prolonged prevention of infection and management of pain.
Hypothesis and Research Objectives

The innovation of the electrospun chitosan membranes utilized in these wound dressings is the chemical process for stabilization to maintain the nanofibrous structure in aqueous environments. In one such modification technique, fatty acid chains are covalently bonded to chitosan to prevent swelling and dissolution of the membrane fibers [29]. The alkyl groups of the fatty acids are hypothesized to form a hydrophobic surface on the core chitosan nanofiber, which may limit aqueous swelling and better retain hydrophobic therapeutics (Figure 1). The hydrophobic surface minimizes adherence of the fibers to wounded tissue, which in turn minimizes damage to fragile tissue during dressing changes. Additionally, electrospun membranes resemble the native fibrous structure of the extracellular matrix to support cell growth and provide increased surface area for drug delivery.

Figure 1. Schematic of acylated membranes loaded with hydrophobic bupivacaine and C2DA.
Due to the hydrophobic nature of C2DA, engineering systems for local delivery have been limited to systems that provide burst release and short duration of release [30]. These results reinforce the idea that biofilm inhibitors are potential infection prophylactic agents that promote wound healing, yet highlight the current gap between benchtop findings and useful drug delivery systems. These modified chitosan wound dressings have significant potential to address this local delivery gap and to extend the antimicrobial activities of C2DA to up to 72 hours between dressing changes. Further, the goal for the local anesthetic component is the extended duration of release through a biomaterial that supports the healing process, allowing for management of pain and initial inflammatory responses. The following objectives are proposed to determine the success of this system:

**Aim 1: Evaluate release profiles when loading therapeutics in a variety of concentrations.**

*Hypothesis 1: Bupivacaine and C2DA release can be tailored by loading concentration.*

*Overall elution goal of 72 hours was selected to match the membrane’s function as a wound dressing.*

**Aim 2: Determine antimicrobial activity of bupivacaine, C2DA, and combinations released from chitosan membranes.**

*Hypothesis 2: Bupivacaine and C2DA released over the course of 72 hours will have antimicrobial effects against MRSA.*

**Aim 3: Determine cellular responses to bupivacaine, C2DA, and combinations released from chitosan membranes.**

*Hypothesis 3: Membranes loaded with C2DA and bupivacaine are non-cytotoxic and promote wound healing responses in fibroblasts.*
CHAPTER II

LITERATURE REVIEW

Biofilm

Biofilms are the cause of 65-80% of all human infections, and thus remain a significant problem in the treatment of wounds [31]. Biofilms can form on biotic or abiotic surfaces, be single or multi-layered, and may contain either homogenous or heterogenous populations of bacteria [32]. While all biofilms are unique on the basis of their attachment surface and microbial components, pathogenic biofilms have a number of similarities in the process of their development and specific phenotypic changes. The analysis of these growth and dispersal mechanisms have been fundamental in discovering novel therapeutics for the treatment and prevention of biofilm infections.

The formation of biofilm begins with the attachment of a single planktonic bacterium to a surface, such as an implanted device or wounded tissue (Figure 2). As microorganisms continue to aggregate on this surface, they secrete an extracellular polymeric substance (EPS) which is a complex network of polysaccharides and proteins that facilitate conglomeration of microbes, assist in bacterial attachment to a surface, and encase bacterial cells to allow the biofilm to continue growing. Further, the EPS protects the biofilm against the immune response while also preventing diffusion of antibiotics and other external forces, deeming the biofilm nearly impenetrable. The forming biofilm reaches full cell density through a unique cell-cell signaling method, termed quorum sensing. Quorum sensing allows for bacteria within the biofilm to determine the population density through recognition of secreted signaling molecules, which they can then use to regulate gene expression as a community [33]. These genetic changes lead to the development of persister cells, which function as yet another mechanism by which
biofilms evade immune cell and antibiotic clearance. Persister cells have a significantly slower growth rate and while they make up only 1% of the total biofilm population, they survive even the greatest external stresses and can therefore repopulate the biofilm even following extensive antimicrobial treatment [9].

**Figure 2.** Depiction of the biofilm formation and dispersion cycle (reproduced with permission from © Springer Nature Singapore Pte Ltd. 2019) [34].

Despite their clinical burden, biofilms were not first reported until the 1980s, and were not reported in wounds until the 1990s [35; 36]. In 1996, Akiyama et al. published the first article detailing evidence of biofilm in a wound; they found that signs of biofilm formation began just 3 hours after inoculating a mouse dermal wound with *Staphylococcus aureus* (*S. aureus*) [37]. Similarly, in their 2000 study, Rashid et al. recognized horizontal spread and systemic infections resulting from *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilm in rat burn wounds [38]. These early studies of biofilm infections within wounds led to further discoveries throughout the following decades including multi-strain synergy of polymicrobial biofilms and the ability of biofilm to impair wound healing [36].
**Biofilm in burn wounds**

Burns are continuously among the leading causes of injury in the United States; in data published by the Center for Disease Control, 1.1 million burn injuries annually require medical attention, with 50,000 requiring hospitalization. Biofilm infections can be especially devastating during treatment of burn wounds, with approximately 51% of burn victim deaths resulting from complex wound infection [3]. Microorganisms can enter burned dermal tissue through the patient’s own microflora, or through contact with the environment or healthcare workers [39]. After a burn occurs, the protective mechanisms of the skin (i.e. defensins from keratinocytes and acidic secretions from sebaceous glands) are severely impaired or lost entirely, allowing microorganisms to rapidly colonize and form biofilm [40]. While systemic antibiotics are nearly ineffective in treating biofilm, the reduced blood supply to burned tissue further restricts the efficacy of typical antibiotic treatment concentrations [39]. Studies have shown that *P. aeruginosa* isolates from a burn wound developed EPS within 5 hours of inoculation, and had the characteristics of a mature biofilm within 10 hours, demonstrating the necessity of taking rapid preventative measures after a burn occurs [41]. In a clinical study, Moghadam et al. found that approximately 50% of 135 swabbed burn wounds had been colonized by MRSA, indicating the prevalence of drug-resistant biofilm formation in burn wounds [42]. Further clinical studies demonstrated that while debridement of wounds could remove biofilm from wound beds, biofilms recolonized 2 days after this initial debridement [43]. Each of these studies indicates the need for prompt and sustained non-antibiotic methods to treat and prevent biofilm in burn wounds.
Diffusable signal factors and C2DA

As knowledge regarding the development and spread of biofilm has increased, research into potential non-antibiotic therapeutics to treat biofilm has simultaneously expanded. One method in particular is the use of diffusible signal factors (DSF) to both disperse preformed biofilms and prevent biofilm formation entirely [44]. When a biofilm continues to grow and mature, bacteria release these intrinsic DSF fatty acids to trigger release from the growth surface so that bacterial cells can once again become planktonic, travel through the bloodstream, and recolonize another site as biofilm. Studies have shown that DSF also play a role in modulation of bacterial antibiotic susceptibility [11]. DSF can be derived naturally from bacterial strains like Pseudomonas or developed synthetically with slight structural changes that in turn alter their activity. Because DSF are analogs of natural fatty acids molecules, they have the potential to impair microbial growth and subsequent biofilm formation while avoiding the consequence of antibiotic resistance [45].

Considerable investigation into DSF led to the discovery of cis-2-decenoic acid (C2DA) and its anti-biofilm capabilities by Davies et al. (Figure 3) [13]. C2DA is a medium chain fatty acid that belongs to a recently defined group of cis-2-alkenoic acids that are known for triggering communication both within and between different strains of microorganisms [46]. Though its mechanism of action remains unclear, bacterial signaling through C2DA has been shown to modulate at least 666 genes, including those involved with chemotaxis, biofilm attachment, motility, tricarboxylic acid cycle, EPS synthesis, and a number of other pathways essential to biofilm physiology [13; 47]. C2DA was initially investigated for its ability to inhibit biofilm formation of P. aeruginosa and disperse established biofilms, but research has since expanded to determine the efficacy of C2DA against multiple bacterial strains [48]. For example, C2DA has
been shown to inhibit *S. aureus* growth and biofilm formation, using concentrations that are not cytotoxic to fibroblasts [49]. Recent work has begun to indicate that the cis-conformation also increases membrane permeability and could let more small molecule antibiotics into cells, making C2DA a potential adjunctive therapy to work synergistically with common antibiotics [30; 50]. Due to its hydrophobicity and limited solubility, delivery of C2DA has been a challenge, and often requires the use of ethanol as a carrier. A series of *in vitro* studies by Rahmani-Badi et al. confirmed that when combined with antibiotics, C2DA induces dispersal of both *Escherichia coli* and *Klebsiella pneumoniae* biofilms formed on catheters [51]; additionally, this group reported that C2DA combined with chlorhexidine resulted in removal of dental plaque formed by *Streptococcus mutans* and *Candida albicans*, further proving cross-kingdom efficacy of C2DA [52].

![Chemical structure of cis-2-decenoic acid.](image)

**Figure 3.** Chemical structure of cis-2-decenoic acid.

Systems developed to deliver C2DA locally have had variable levels of success, as fatty acids are often difficult to incorporate into conventional materials for sustained release [53]. Chitosan sponges manufactured with a poly(ethylene glycol) carrier successfully release C2DA for up to 5 days, depending on loading concentration, though a substantial burst release was seen during the first day [49]. C2DA and the antibiotic rifampin have been delivered within solid lipid nanoparticles, and results showed that nanoparticles were more successful as a preventative anti-
biofilm agents compared to free forms of the therapeutics; however, this delivery system was not capable of eradicating pre-formed biofilm [54]. Furthermore, C2DA was investigated as an anti-biofilm coating using phosphatidylcholine as a carrier, and completely inhibited S. aureus on titanium pins and bone, both with and without antibiotics, in a murine biofilm-based infection model, though in vitro studies of this coating suggests only a short, burst release of C2DA occurs [55]. Despite the success of C2DA as a biofilm treatment agent in preliminary studies, the challenge to develop a successful delivery system remains.

**Other systems targeting biofilm**

In addition to C2DA, there are a number of other non-antibiotic strategies being studied for their potential to treat and prevent biofilm infections. Aside from the DSF family of signaling molecules, bacteria also release fatty acid biosurfactants that similarly play a role in biofilm formation and dispersion. These includes rhamnolipids, which have anti-adhesive properties that prevent bacteria from attaching to surfaces and forming biofilm [56]. While lower concentrations of rhamnolipids increase bacteria cells’ surface hydrophobicity and thus increase their affinity for other surfaces, higher rhamnolipid concentrations can have the opposite effect and prevent bacterial attachment [57; 58; 59]. Certain peptides and amino acids may also serve to interrupt biofilm development through disruption of bacterial cell walls. Studies have shown that both polyurethane scaffolds and bone grafts loaded with D-amino acids effectively reduce S. aureus biofilm in vitro and in vivo [60; 61]. Another study combined D-amino acids with antibiotics and tested them against clinical isolates of P. aeruginosa and S. aureus from deep tissue wounds, and determined that they are effective in dispersing preformed biofilms and reducing minimum biofilm inhibitory concentration significantly for rifampin, colistin, and ciprofloxacin [62]. Antimicrobial peptides (AMP) function through a similar mechanism and have been studied
clinically in additional to *in vitro* and *in vivo* models [63; 64; 65]. Some clinical applications being tested are a topical gel containing the AMP omiganan to treat skin infections, and the AMP pexiganan to treat infected diabetic foot ulcers [66]. Other applications targeted by AMP systems include urinary, dental, vaginal, oral, orthopedic, and post-surgical infections, through a variety of material delivery systems including poly(methyl methacrylate), polyvinyl alcohol, and calcium phosphate [67; 68; 69; 70; 71; 72; 73; 74; 75; 76].

Enzymes have also been tested due to their potential to target biofilm in early growth stages by preventing EPS production. Glycoside hydrolase enzymes have been tested with *in vivo* wound infection models, with many concluding that while these molecules are functional in dispersing *S. aureus* and *P. aeruginosa* biofilm, they do not eradicate infection unless combined with antibiotics [77; 78; 79]. Protease enzymes have also shown success in treating biofilm because of their ability to cleave peptide bonds, which in turn may remove biofilm from its attached surface [80]. These molecules have proven successful in preventing biofilm formation for MRSA and *Staphylococcus epidermidis* both *in vitro* and *in vivo* [81; 82; 83]. The staphylococcal proteolytic enzyme lysostaphin functions by targeting and cleaving bonds within bacterial cell walls, and is functional in both dispersing and eradicating biofilm of staphylococcal species [84; 85]. Though not yet commercialized for clinical use, lysostaphin has been tested with a number of materials including ceramics, polymers, hydrogels, and wound dressings, and proved capable of reducing infections caused by staphylococcal species [86; 87; 88; 89].

Nanomaterials have also been investigated extensively as a means to deliver therapeutics for applications as diverse as cancer therapy, neurodegenerative disorders, and ophthalmology [90; 91; 92; 93]. Because these particles can be reactive, have a large surface area to volume ratio, and have alterable chemical and physical properties, they are also ideal carriers to deliver
therapeutics to disrupt biofilm; further, their nanoscopic size allows them to penetrate the EPS and accumulate within the biofilm to effect growth directly [8; 94; 95]. Polymer-based nanoparticles have been used to deliver antibiotics though they do not have intrinsic antibacterial properties; in contrast, metal nanoparticles alone are well-known to be inherently antimicrobial [95; 96; 97]. Despite cytotoxicity concerns, silver nanoparticles have shown promise as factors to reduce biofilm formation for both gram-positive and gram-negative bacteria strains, and in skin wounds [98; 99]. Similarly, zinc oxide particles have been used successfully in wound treatments in both in vitro and in vivo assessments [100]. Magnetic nanoparticles have been fabricated from iron oxide and heat up when exposed to magnetic fields to induce local hyperthermia that, when combined with antibiotics, can successfully eradicate biofilm. These particles have been modified with other therapeutics including D-amino acids or polymers such as chitosan, chitin, and polyethylene glycol, which increased biofilm eradication but could result in adverse effects associated with elevated temperatures [95; 101; 102].

Metabolites have been used as a means to stimulate persister cells within biofilm, so that they regain susceptibility to aminoglycoside antibiotics. The sugar alcohols mannitol, erythritol, and fructose combined with antibiotics have shown promise in the treatment of lung, dental, and wound infections caused by variable strains of bacteria [103; 104; 105]. Most recently, Pace et al. investigated mannitol delivery from chitosan pastes, and saw reduction in S. aureus biofilm both in vivo and in vitro [106]. Other anti-biofilm metabolites include Manuka and Ulmo honey, which have additional antimicrobial properties because they contain hydrogen peroxide, and are effective against wound pathogens like S. aureus, P. aeruginosa, Enterobacter cloacae, and Proteus mirabilis, though they were ineffective against Enterococcus faecalis [107; 108; 109].
Honey has been investigated for delivery through numerous biomaterial systems including hydrogels, cryogels, and electrospun membranes [110].

**Local anesthetics for pain relief**

In addition to infection prevention, non-opioid methods to treat pain are currently of great clinical relevance, especially as the opioid crisis reaches epidemic proportions. Approximately 4% of the adult US population misuses prescription opioids; by 2015 more than 33,000 deaths were attributed to opioid overdose, with that number increasing to 64,000 in 2017 [111; 112; 113]. Because of the opioid crisis, a number of alternative therapies have been investigated for the treatment of acute pain associated with post-surgical wounds and musculoskeletal trauma. A 2018 clinical study concluded that low doses of the dissociative anesthetic ketamine provided comparable effects in pain relief to morphine [114]. Topical, pain-relieving naltrexone cream has also proven successful in accelerating wound healing in *in vivo* studies [115]. Recently, delivery of local anesthetics has shown success as a non-opioid pain management approach. Local anesthetics function by blocking voltage-gated sodium channels and thus temporarily blocking nerve conduction through nociceptive afferent nerves [116]. These therapeutics are divided into two main groups based on their chemical structures: amides and esters. Amides are typically safer and more widely used as they lead to less severe side effects and have low rates of patient allergic reactions [117].

Bupivacaine, which is categorized as an amide anesthetic, is known for its slow onset and lengthy duration of action [15]. Bupivacaine, as well as other local anesthetics, is hydrophobic which increases both its potency and duration of action, but also increases its toxicity and makes it difficult to deliver in sustained quantities [118]. Clinical studies have shown that laparotomy patients treated with liposomal bupivacaine had a 30% reduction in
opioid use during the first 72 hours following surgery [119]. Furthermore, bupivacaine has been shown to have both prophylactic and bactericidal effects against varying strains of microorganisms [120]. Some studies also suggest that local anesthetics may increase blood perfusion levels [121] and modify the inflammatory response [122], calming inflammation and reducing edema that could lead to conversion of burn wounds to deeper layers of tissue.

Figure 4. Chemical structure of bupivacaine.

There are several formulations of creams and transdermal patches that contain local anesthetics, with some available for over-the-counter use [123; 124]. Typical formulations of topical creams and sprays provide rapid relief, but effects are limited in duration and require reapplication. A number of drug delivery systems have been developed in an attempt to provide a sustained release of local anesthetics due to the neurotoxicity and short half-lives often associated with these molecules [125; 126]. However, few of these systems are projected to enter clinical trials due to issues commonly associated with the use of local anesthetics such as plasma stability, blood brain barrier permeability, and cardiotoxicity [116; 127]. One system showing initial in vitro success loaded poly(ethylene glycol) and gelatin matrices with bupivacaine and silver sulfadiazine to manage pain and prevent infection. Results of this study indicated that the
A combination of therapeutics did not alter the antimicrobial effects of silver sulfadiazine, and enhanced the release of bupivacaine [128]. Pek et al. tested poly(D,L-lactic-co-glycolic acid) (PLGA) and poly(L-lactic acid) (PLLA) microspheres loaded with bupivacaine in vivo for treating postoperative pain relief after knee surgery, and saw a sustained release of therapeutic concentrations of bupivacaine for two weeks post-surgery, with no adverse side effects [129]. Bupivacaine has also been incorporated into ultra-high molecular weight polyethylene in joint prosthetics to provide pain relief following joint replacement surgery, but this material has limited efficacy in other applications like wound dressings [130]. Bupivacaine has also been encapsulated into microspheres, [131; 132] polyanhydride disks, [133], and liposomes [134], which all resulted in a significant burst release, an issue often seen with hydrophobic therapeutics. As demonstrated by the varying results of these studies, it is clear that the selection of the appropriate drug delivery carrier is essential to the success of bupivacaine delivery systems.

**Chitosan and electrospun chitosan membranes**

Chitosan, a linear polysaccharide biopolymer, could offer a way to incorporate biodegradable, anti-biofilm, pain management therapeutics directly into wounds (Figure 5). Chitosan is derived from chitin, a linear polysaccharide of N-acetylglucosamine units, that is located within the exoskeletons of crustaceans and arthropods as well as the cell walls of fungi and yeast. This high availability means chitin and chitosan are both extremely inexpensive to obtain [135]. Chitosan can be manufactured from chitin via partial or full deacetylation, with a required minimum of 50% degree of deacetylation (DDA) before the polymer is considered chitosan rather than chitin. In its final form, chitosan is comprised of randomly distributed β-(1-4)-2-amino-2-D-glucosamine (deacetylated) and β-(1-4)-2-acetamido-2-D-glucoseamine.
(acetylated) units, and is soluble in dilute acids [136]. Chitosan’s solubility in aqueous solutions has led to its successful fabrication into several forms including sponges, pastes, thin films, nanoparticles, and hydrogels. Some of the medical applications currently being investigated at the University of Memphis include the following: chitosan sponge delivery systems [137], magnetic chitosan nanoparticles for targeted drug delivery [138], coatings for musculoskeletal implant fixation hardware [139], guided bone regeneration mats [140], and injectable chitosan paste [106].

Chitosan has been shown to display antimicrobial properties against *Escherichia coli*, *P. aeruginosa*, *Staphylococcus epidermidis*, and *S. aureus*, which may be due in part to its cationic nature [141]. It has also been demonstrated that low-molecular-weight chitosan can penetrate bacterial cell walls, bind with DNA, and inhibit DNA transcription and mRNA synthesis [142]. High molecular weight chitosan has been shown to bind to the negatively charged components on the bacterial cell wall, forming an impermeable layer around the cell, changing cell permeability and blocking transport into the bacterial cell [135]. This broad-spectrum antimicrobial activity and low toxicity to human cells makes chitosan a promising antimicrobial agent; however, varying results based on the molecular weight and degree of deacetylation of the chitosan used, as well as variation based on type and age of bacteria tested, make conclusions indefinite [135]. Though the exact mechanism of chitosan’s antimicrobial properties is still being researched and debated, these properties make chitosan a favorable material choice when fabricating wound healing membranes.

Electrospinning is a membrane fabrication method that is used frequently because it creates micro- and nano-scale fibers, which are similar to the nanoscale structures within native tissue and thus often lead to efficient tissue regeneration and compatibility [143]. During the
process of electrospinning, an electrical field is applied between a metallic needle and a collector surface [144]. Due to the charge between the needle and collection surface, when a polymeric liquid droplet is ejected from the needle, the charged fluid jet is ejected in the shape of a conical protrusion called a Taylor cone [145]. The high surface charge allows the fluid stream to be whipped around so that the stretched nanofiber is deposited on the collection surface with a random pattern. The resulting fibers can differ greatly based on temperature and humidity of the reaction site, as well as the electrical voltage, distance between the tip and collector, solution feeding rate, and a variety of other factors [146]. Chitosan solutions can be electrospun to produce nanofibrous membranes of varying thickness [140; 143]. These nanofibrous membranes resemble the native fibrous structure of the extracellular matrix to support cell growth and provide increased surface area for drug delivery.

Membranes may be modified via acylation, during which fatty acid chains are covalently bonded to chitosan to prevent swelling and dissolution of the membrane fibers [29; 147]. Acylation forms a hydrophobic surface on the core chitosan nanofiber that minimizes adherence of the fibers to wounded tissue, which in turn minimizes damage to fragile tissue during dressing changes [147]. Additionally, the modification of membranes via acylation provides the potential to retain and provide a controlled release of hydrophobic therapeutics. For instance, Murali et al. investigated simvastatin release from electrospun chitosan membranes acylated with different short chain fatty acids, and found each to release a sustained amount of the therapeutic for greater than 30 days, though release amount differed by the type of short chain fatty acid modification performed [148]. Due to previous success of modified electrospun chitosan membranes in delivering hydrophobic therapeutics, these membranes may be applicable in improving delivery of the hydrophobic agents C2DA and bupivacaine.
Despite chitosan’s beneficial attributes as a wound dressing, there are also limitations to consider when developing such applications. For example, chitosan is soluble only in diluted organic or inorganic solvents that are below its pKa of 6.3 [149]. Furthermore, chitosan materials vary greatly in terms of molecular weight, polydispersity index, degree of deacetylation, and moisture content depending on the manufacturer, so products and studies involving chitosan can be inconsistent [150]. Similarly, manufacturing conditions such as humidity and temperature can greatly alter the final chitosan product. This final product is also often post-processed with sterilization, thermal processing, or chemical modifications, all of which can also lead to differing material properties [150].

**Figure 5.** Chemical structure of chitosan composed of β(1→4) linked units of (A) N-acetyl-D-glucosamine and (B) D-glucosamine [151].

Hexanoic acid-treated electrospun chitosan membranes (HA-ESCM) loaded with both bupivacaine and C2DA may serve to 1) act as a barrier to microbial contamination, 2) release local anesthetics in a controlled manner that reduce pain and modify the inflammatory response, and 3) release the natural antimicrobial fatty acid C2DA to prevent biofilm formation. These loaded membranes may be used as wound dressings for soft tissue wounds following medical device implantation, musculoskeletal trauma, or burn wounds for prolonged prevention of infection and management of pain.
CHAPTER III

IN VITRO EVALUATION OF LOADED CHITOSAN MEMBRANES FOR PAIN RELIEF AND INFECTION PREVENTION

ABSTRACT

Wounds resulting from surgeries, implantation of medical devices, and musculoskeletal trauma result in pain and can also result in infection of damaged tissue. Up to 80% of these infections are due to biofilm formation either on the surface of implanted devices or on surrounding wounded tissue. Bacteria within a biofilm have intrinsic growth and development characteristics that allow them to withstand up to 1000 times the minimum inhibitory concentration of antibiotics, demonstrating the need for new therapeutics to prevent and treat these infections. Cis-2-decenoic acid (C2DA) is known to disperse preformed biofilms and can prevent biofilm formation entirely for some strains of bacteria. Additionally, local anesthetics like bupivacaine have been shown to have antimicrobial effects against multiple bacterial strains. This study sought to evaluate hexanoic acid-treated electrospun chitosan membranes (HA-ESCM) as wound dressings that release C2DA and bupivacaine to simultaneously prevent infection and alleviate pain associated with musculoskeletal trauma. Release profiles of both therapeutics were evaluated, and membranes were tested in vitro against Methicillin-resistant Staphylococcus aureus (MRSA) to determine efficacy in preventing biofilm infection and bacterial growth. Results indicate that membranes release both therapeutics for 72 hours, and release profile can be tailored by loading concentration. Membranes were effective in preventing biofilm growth but were toxic to fibroblasts when loaded with 2.5 or 5 mg of bupivacaine.
KEYWORDS
biofilm; anesthetic; bupivacaine; electrospun; chitosan; biomaterial; local drug delivery; wound dressing; infection; Staphylococcus

INTRODUCTION

There is a growing demand for local delivery systems to treat and prevent infection and alleviate pain during wound healing [116]. Biofilm infections, caused by microbial communities adhering to the surfaces of implanted devices, surrounding tissue, and musculoskeletal wounds, are particularly difficult to treat due to their antibiotic tolerance [32; 152]. Up to 80% of all human infections are due to the formation of biofilm; these infections remain particularly difficult to treat due to phenotypic changes that make bacteria within a biofilm resistant to 1000x the typical minimum inhibitory concentration of antibiotics [8]. Once infection is present, the removal of implanted materials and/or aggressive debridement of wounded tissue is often the only successful strategy for treating the infection [153; 154]. Long term antibiotic use can help to manage biofilm infections, but often fails while also increasing risk of antibiotic resistance and toxic side effects [10]. Furthermore, overuse of aminoglycoside antibiotics can lead to nephrotoxic and ototoxic side effects [155]. Thus, the occurrence of biofilm infections can lead to increased hospital time and trauma for patients, as well as increased cost of care.

Bacteria within a biofilm release intrinsic signaling molecules, termed diffusible signal factors (DSF), to trigger dispersal from the attachment surface and allow for biofilm colonization throughout other areas of the body [11]. Previous research has shown that the DSF cis-2-decenoic acid (C2DA), a short chain fatty acid, disperses mature biofilm and inhibits biofilm formation, making this molecule a promising therapeutic to prevent wound infections [13; 156].
Previous studies have investigated delivery of C2DA in other local delivery systems, including chitosan/polyethylene glycol (PEG) sponges and phosphatidylcholine coatings [8; 139; 157]. However, these systems are limited due to the substantial burst release of C2DA, resulting in a quick depletion of the therapeutic. First-order kinetic (i.e. burst) release is associated with diffusion driven release; because phosphatidylcholine is very transient on the surface and can form micelles to dissociate and diffuse away from the surface, C2DA diffuses with it, leading to a substantial burst. Loading C2DA within chitosan/PEG sponges also appeared to encourage diffusion of C2DA into surrounding media, indicating the engineering challenge of developing a delivery system that avoids a burst release of C2DA.

In addition to infection prevention, non-opioid pain management strategies such as local delivery of anesthetics are a top priority for wound treatment, especially as the opioid crisis reaches epidemic proportions [14]. Studies have also confirmed that in addition to analgesic effects, local anesthetics like bupivacaine have inherent antimicrobial effects [15]. A number of drug delivery systems have been developed in an attempt to provide a sustained release of local anesthetics to combat the neurotoxicity and short half-lives often associated with these molecules when delivered systemically [117; 125; 127]. However, few of these systems are projected to enter clinical trials due to issues commonly associated with the use of local anesthetics such as plasma stability, blood brain barrier permeability, and cardiotoxicity [116; 127]. For instance, Pek et al. tested poly(D,L-lactic-co-glycolic acid) (PLGA) and poly(L-lactic acid) (PLLA) microspheres loaded with bupivacaine in vivo for treating postoperative pain relief after knee surgery, and saw a sustained release of therapeutic concentrations of bupivacaine for two weeks post-surgery, with no adverse side effects [129]. Bupivacaine has been incorporated into ultra-high molecular weight polyethylene in joint prosthetics to provide pain relief following joint
replacement surgery, but this material has limited efficacy in other applications like wound dressings [130]. There are several formulations of creams and transdermal patches that contain local anesthetics, with some available for over-the-counter use [123; 124]. Though formulations of topical creams and sprays provide rapid relief, effects are limited in duration and require reapplication.

Because of the limited therapeutic release duration provided by previous systems, a delivery system is needed to reduce the initial burst release and provide a sustained release of these infection and pain preventing molecules. Chitosan is a biocompatible polymer that has been investigated in several applications, including wound and bone healing [135]. Electrospun chitosan membranes can provide a template for healing tissue, coverage of wounds, and drug delivery following injury. These nanofibrous membranes resemble the native fibrous structure of the extracellular matrix to support cell growth and provide increased surface area for drug delivery. Membranes are modified via acylation, during which fatty acid chains are covalently bonded to chitosan to prevent swelling and dissolution of the membrane fibers [29]. Acylation forms a hydrophobic surface on the core chitosan nanofiber that minimizes adherence of the fibers to wounded tissue, which in turn minimizes damage to fragile tissue during dressing changes. Additionally, the modification of membranes via acylation provides the potential to retain and provide controlled release of hydrophobic therapeutics. Due to previous success of modified electrospun chitosan membranes in delivering hydrophobic therapeutics [148], these membranes may be applicable in delivering the hydrophobic compounds (or agents) C2DA and bupivacaine.

Chitosan membranes loaded with both bupivacaine and C2DA may serve to 1) act as a barrier to microbial contamination, 2) release local anesthetics in a controlled manner that reduce
pain and modify the inflammatory response, and 3) release the antimicrobial fatty acid C2DA to prevent biofilm formation. These loaded membranes may be used as wound dressings for soft tissue wounds following medical device implantation, musculoskeletal trauma, or burn wounds for prolonged prevention of infection and management of pain. In this study we sought to determine release profiles of therapeutics from chitosan membranes, their ability to prevent Methicillin-resistant *Staphylococcus aureus* (MRSA) growth and biofilm formation, and their compatibility with fibroblast cells.

**RESULTS**

**Elution**

*Bupivacaine elution*

When bupivacaine was loaded into HA-ESCM without C2DA, it eluted with an initial burst release for the highest concentration, but a reduced burst for lower concentrations (Figure 4). This was followed by a sustained release for the 10 mg and 5 mg loading groups at levels averaging approximately 0.5 mg per day per membrane disc (Figure 6). Percentage of total bupivacaine released was markedly lower for the 1.25 mg loaded group compared to the three higher concentrations (Table 1). Following results of this elution study, 5 mg and 2.5 mg bupivacaine loading concentrations were selected for dual-loaded membranes as these produced a sustained release with a lower burst than the 10 mg loading concentration, and a higher percentage release than the 1.25 mg loading group.
Figure 6. Graphs detailing (A) bupivacaine release and (B) cumulative bupivacaine release by HA-ESCM loaded with 10, 5, 2.5 or 1.25 mg of bupivacaine throughout 72-hour elution study. Release was determined by high performance liquid chromatography (HPLC). Data is represented as mean ± standard deviation (n=5). Lines connecting points are intended to guide the eye.
**Table 1.** Percentage of total bupivacaine released for each loading concentration throughout 72-hour elution study.

<table>
<thead>
<tr>
<th>Loading amount</th>
<th>Percent released</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg</td>
<td>90.14%</td>
</tr>
<tr>
<td>5 mg</td>
<td>~100%</td>
</tr>
<tr>
<td>2.5 mg</td>
<td>75.36%</td>
</tr>
<tr>
<td>1.25 mg</td>
<td>7.47%</td>
</tr>
</tbody>
</table>

**C2DA elution**

C2DA eluted from membranes with no significant burst, showing a sustained release throughout the course of three days, differing by loading concentration (Figure 7). Percentage of total therapeutic released was determined for each group following 72-hour elution (Table 2).

Following results of this elution study, 25 μg and 12.5 μg loading concentrations were selected for future tests as these produced the highest cumulative release of C2DA.
Figure 7. Graphs detailing (A) C2DA release and (B) cumulative C2DA release by HA-ESCM loaded with 25, 12.5, 6.25, or 3.125 μg of C2DA throughout 72-hour elution study. Release was determined by high performance liquid chromatography (HPLC). Data is represented as mean ± standard deviation (n=5). Lines connecting points are intended to guide the eye.

Table 2. Percentage of total C2DA released for each loading concentration throughout 72-hour elution study.

<table>
<thead>
<tr>
<th>Loading amount</th>
<th>Percent released</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 μg</td>
<td>46.25%</td>
</tr>
<tr>
<td>12.5 μg</td>
<td>70.08%</td>
</tr>
<tr>
<td>6.25 μg</td>
<td>~100%</td>
</tr>
<tr>
<td>3.125 μg</td>
<td>22.09%</td>
</tr>
</tbody>
</table>

Bupivacaine/C2DA combination elution

When bupivacaine and C2DA were dually loaded into membranes, bupivacaine released with an initial burst of about 1.6 mg for the 5 mg loading concentration and 1 mg for the 2.5 mg
loading concentration, followed by a much lower sustained release. Though there were slight differences of bupivacaine release depending on C2DA concentration, the amount of C2DA loaded did not significantly alter the total amount of bupivacaine released (p>0.05, determined by ANOVA with Holm-Šidák post-hoc tests) (Figure 8). Percentage of total therapeutic released was determined for each group following 72-hour elution (Table 3). For groups loaded with 5 mg of bupivacaine, release was significantly higher for single-loaded membranes starting at the 24-hour timepoint. However, the opposite was true for membranes loaded with 2.5 mg bupivacaine.
Figure 8. Graphs of (A) bupivacaine release and (B) cumulative bupivacaine release from dual-loaded HA-ESCM. Data is represented as mean ± standard deviation (n=5). Lines connecting points are intended to guide the eye.

Table 3. Percentage of total bupivacaine released for each dual-loaded concentration throughout 72-hour elution study.

<table>
<thead>
<tr>
<th>Loading amount</th>
<th>Percent released</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg B/25 μg C</td>
<td>72.22%</td>
</tr>
<tr>
<td>5 mg B/12.5 μg C</td>
<td>66.68%</td>
</tr>
<tr>
<td>2.5 mg B/25 μg C</td>
<td>93.56%</td>
</tr>
<tr>
<td>2.5 mg B/12.5 μg C</td>
<td>75.29%</td>
</tr>
</tbody>
</table>

C2DA released from dual loaded membranes with a slight burst during the first 12 hours, followed by a lower sustained release for the last 60 hours. As seen with bupivacaine release from dual loaded membranes, there were slight differences of C2DA release depending on
bupivacaine loading concentration, but the amount of bupivacaine loaded did not significantly alter the total amount of C2DA released. Further, there was no significant difference between C2DA release from single or dual-loaded membranes (p>0.05, determined by ANOVA with Holm-Šídák post-hoc tests) (Figure 9). Percentage of total therapeutic released was determined for each group following 72-hour elution (Table 4).
**Figure 9.** C2DA release from dual-loaded membranes. Graphs of (A) C2DA release and (B) cumulative C2DA release from dual loaded membranes modified by hexanoyl acylation. Data is represented as mean ± standard deviation (n=5). Lines connecting points are intended to guide the eye. Significance was determined by ANOVA with Holm-Šídák post-hoc tests.

**Table 4.** Percentage of total C2DA released for each dual-loaded concentration throughout 72-hour elution study.

<table>
<thead>
<tr>
<th>Loading amount</th>
<th>Percent released</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg B/25 μg C</td>
<td>~100%</td>
</tr>
<tr>
<td>5 mg B/12.5 μg C</td>
<td>~100%</td>
</tr>
<tr>
<td>2.5 mg B/25 μg C</td>
<td>~100%</td>
</tr>
<tr>
<td>2.5 mg B/12.5 μg C</td>
<td>~100%</td>
</tr>
</tbody>
</table>

**Zone of inhibition**

Results of Kirby-Bauer Zone of Inhibition studies (Table 5) show highest therapeutic release by groups containing 25 μg of C2DA, while unloaded membranes, membranes loaded with just bupivacaine, and membranes loaded with the lower C2DA concentration did not produce significant zones. Representative images are included to demonstrate zones produced by different membranes groups (Figure 5).
Table 5. Table indicating 24-hour Zone of Inhibition (mm) against Methicillin-resistant S. aureus, measured using ImageJ software (NIH), for each membrane type (n=4). ± represents standard deviation. * indicates significantly higher zone than paper disk control, as determined by one-way ANOVA with Holm-Šídák post-hoc tests (p<0.05).

<table>
<thead>
<tr>
<th>Loading concentration</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg BUP</td>
<td>0.84± 0.48</td>
</tr>
<tr>
<td>2.5 mg BUP</td>
<td>0.62 ± 0.42</td>
</tr>
<tr>
<td>25 μg C2DA</td>
<td>3.09 ± 0.29*</td>
</tr>
<tr>
<td>12.5 μg C2DA</td>
<td>1.23 ± 0.25*</td>
</tr>
<tr>
<td>5 mg BUP + 25 μg C2DA</td>
<td>3.48 ± 0.98*</td>
</tr>
<tr>
<td>5 mg BUP + 12.5 μg C2DA</td>
<td>0.16 ± 0.07</td>
</tr>
<tr>
<td>2.5 mg BUP + 25 μg C2DA</td>
<td>2.99 ± 0.09*</td>
</tr>
<tr>
<td>2.5 mg BUP + 12.5 μg C2DA</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Unloaded membrane</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Paper disk control</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Vancomycin control*</td>
<td>4.19 ± 0.49</td>
</tr>
<tr>
<td>Controls</td>
<td>Negative (blank)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td><strong>Bup 0</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Bup 2.5</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Bup 5</strong></td>
<td></td>
</tr>
<tr>
<td><strong>C2DA 0</strong></td>
<td></td>
</tr>
<tr>
<td><strong>C2DA 12.5</strong></td>
<td></td>
</tr>
<tr>
<td><strong>C2DA 25</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 10.** Representative images of ZOI for each membrane group and control groups.

**Biofilm growth assays**

For the first study measuring viability of planktonic *S. aureus*, all membranes freshly loaded with therapeutics resulted in significantly less viable bacteria (*p*<0.05) compared to both gauze and chitosan sponge controls. Unloaded membranes prior to 72-hour elution, as well as membranes with 25 μg of C2DA after 72-hour elution, resulted in less viable planktonic bacteria growth than was seen in the gauze controls (**Figure 11**).

The following study quantified biofilm growth on membranes after 24-hour incubation with *S. aureus*. Almost all membrane groups allowed significantly less biofilm growth (*p*<0.05)
compared to gauze and chitosan sponge controls. The only exception was the unloaded membranes after 72-hour elution, which was only significantly lower than the gauze controls (Figure 12).

The final biofilm study quantified growth of biofilm in wells beneath membranes. Most membrane groups allowed for significantly less biofilm growth (p<0.05) in wells compared to both chitosan sponge and gauze controls, with the exception of the post-elution unloaded group and the post-elution group loaded with 2.5 mg of bupivacaine (Figure 13).

![Graph showing normalized bacterial viability](image)

**Figure 11. Planktonic bacteria growth.** Graphical representation of planktonic *S. aureus* growth in direct contact with membranes or controls (n=4). Amount of viable bacteria was quantified based on metabolic activity by measuring ATP production. Results were then normalized as a percentage compared to untreated control wells. ** indicates significant difference (p<0.05) between groups and both gauze and chitosan sponge controls. * indicates significant difference (p<0.05) between groups and gauze controls. Significance was determined by ANOVA with Holm-Šidák post-hoc tests.
**Figure 12. Biofilm growth on membranes.** Graphical representation of *S. aureus* biofilm growth on membranes or controls (n=4). Amount of viable bacteria was quantified based on metabolic activity by measuring ATP production. Results were then normalized as a percentage compared to untreated control wells. ** indicates significant difference (p<0.05) between groups and both gauze and chitosan sponge controls. * indicates significant difference (p<0.05) between groups and gauze controls. Significance was determined by ANOVA with Holm-Šidák post-hoc tests.
Figure 13. Biofilm growth in wells. Graphical representation of *S. aureus* biofilm growth in wells beneath membranes or controls (n=4). Amount of viable bacteria was quantified based on metabolic activity by measuring ATP production. Results were then normalized as a percentage compared to untreated control wells. *a* indicates groups that have significantly less biofilm growth (p<0.05) than both *b* and *c* groups. *b* indicates groups that have significantly less biofilm growth (p<0.05) than *c* groups. Significance was determined by ANOVA with Holm-Šídák post-hoc tests.

Cytocompatibility

All membrane types except those loaded with only C2DA were below the accepted 70% viability value when normalized to the blank standard, in accordance with the ISO 109935 Biological Evaluations of Medical Devices standard when evaluating biomaterials against fibroblasts; the C2DA loaded membranes and the chitosan sponge control all had significantly higher viability than any groups containing bupivacaine (Figure 14).
Figure 14. HA-ESCM cytocompatibility with fibroblasts. Graphical representation of cytocompatibility results for each membrane type when evaluated with L929 fibroblasts cells (n=5). Amount of viable fibroblasts was quantified based on metabolic activity by measuring ATP production. Results were then normalized as a percentage compared to untreated control wells. Error bars indicate standard deviation and the red bar line represents the accepted value of 70% according to ISO 10993-5.

DISCUSSION

HA-ESCM were capable of being loaded with C2DA and bupivacaine, individually or in combination, to prevent biofilm formation. This material shows prospective use as a wound dressing following surgery, implantation of a medical device, or musculoskeletal trauma. Loading therapeutics via ethanol evaporation allows HA-ESCM to be usable almost immediately, indicating their potential for patient-specific loading at time of care. Local anesthetic-loaded wound dressings may alleviate the need for patients to manage pain with
systemic opioids, which often leads to unwanted consequences like opioid misuse, dependence, and consequential addiction [158]. In addition to providing physical coverage from bacterial contamination, the use of the antimicrobial biopolymer chitosan as wound dressing material provides intrinsic infection resistance. This resistance only increases with the addition of bupivacaine, which kills bacteria at higher concentrations, as well as C2DA, which disperses bacteria to prevent biofilm formation [120]. Together, these three antimicrobial components function to prevent pain and infection associated with wounds, which in turn may decrease the need for revision surgeries or tissue debridement required after development of complex biofilm infections.

The slight burst seen during the first 6 hours of membranes loaded with bupivacaine only may indicate that bupivacaine is being loaded in excess of the amount soluble within the acyl layer of chitosan membranes. This may imply that rather than loading within spaces between acylated fibers, the excess bupivacaine could dry on top of membranes and thus release instantly from the surface. Median values of 5 mg and 2.5 mg of bupivacaine were chosen because the 10 mg loading concentration saw the highest proportional burst release of 5 mg, which based on other studies was likely a toxic amount [159]. When combining bupivacaine with C2DA, release followed a similar pattern, with a slight burst during the first timepoint followed by a sustained average release of about 0.25 mg per time point, which is below previously reported toxic levels of 0.6 mg/ml [160]. C2DA release from membranes loaded with only C2DA followed a zero-order release profile, contrasting with previous attempts to deliver C2DA. For instance, C2DA release from chitosan sponges was sustained throughout 5 days, but also saw a burst release during the first day [156], and phosphatidylcholine coatings loaded with C2DA saw a similar variable release profile, with the majority of C2DA releasing as a burst during the first 24 hours.
Furthermore, combining both therapeutics may cause interactions that alter release profiles, as demonstrated by the slightly lower bupivacaine release when dually loaded with C2DA. When C2DA was loaded with bupivacaine, release followed a first-order release pattern, differing from the zero-order release seen when C2DA was loaded alone. High variability in C2DA elution may be decreased by modifying HPLC protocols to improve detection of C2DA at low quantities. Furthermore, extraction can be used to resuspend C2DA in more accurate, detectable concentrations. Similar studies have shown that hexanoic anhydride-treated membranes retain more therapeutics compared to membranes treated with other short chain fatty acids such as acetic anhydride and butyric anhydride; thus, modifying with another fatty acid may allow for higher cumulative release of hydrophobic therapeutics [148]. Future studies will include repeated tests with other concentrations of each therapeutic to better elucidate release mechanisms, which in turn may explain inconsistencies. Tests will also be repeated using other elution media, including fetal bovine serum (FBS)-containing media, since the presence of protein is known to affect the release of hydrophobic substances [161].

The minimal zones (< 1 mm) around bupivacaine-loaded membranes suggest that bupivacaine does not diffuse from membranes to the same extent as C2DA, though small zones may indicate potential effectiveness in preventing bacterial attachment. While this test is useful in recognizing initial interactions between materials and bacteria, zones are highly dependent on diffusion so results may not give the full scope of antimicrobial characteristics. For further confirmation of initial antimicrobial results, three separate but related biofilm assays were conducted. First, there was significantly more viable planktonic bacteria for all groups after elution compared to the freshly loaded membranes, which may be due in part to the slight initial burst release of therapeutics seen by all groups; higher concentrations released during the first 24
hours may be sufficient in killing bacteria that contacts the membranes, rather than just preventing growth on the membranes. Biofilm assays determining biofilm growth on the membranes demonstrated that the membrane materials and therapeutics were all capable of inhibiting biofilm growth to a significant extent. Inhibition after the full course of elution for loaded membranes suggests that membranes are still retaining a small but active amount of these antimicrobials. Viability quantification of biofilm growth on wells beneath membranes showed that growth was minimal for all membrane groups compared to gauze and control chitosan sponge, suggesting that biofilm inhibitors are released from membranes at amounts that would keep biofilm from forming on sites distant to the material, which is beneficial in wound healing. Other studies have strongly suggested that electrospun chitosan nanofibers can interact with bacterial cell walls to rupture and cause leakage of intracellular components, which may explain why even unloaded chitosan membrane groups were successful in preventing biofilm growth [162]. It is also worth noting that the total amount of C2DA loaded within membranes is lower than that loaded into previous delivery systems [139; 156; 157], but antimicrobial activity is maintained. These results are consistent with previous studies that found nanomolar amounts of C2DA to be active against bacteria [13; 52; 163]. Future studies can expand on these to evaluate different types of strains of pathogenic microorganisms and image biofilm formed on materials using SEM or fluorescence assays.

Results of cytocompatibility studies showed that the concentrations of C2DA were compatible with fibroblasts, whereas both concentrations of bupivacaine were toxic to fibroblasts. While unfavorable, the results demonstrating bupivacaine’s toxicity to fibroblasts were consistent with other studies. At concentrations of 0.3 mg/ml, bupivacaine is compatible with fibroblasts but reduces cell viability below 25% at a 0.6 mg/ml concentration [160]. Due to
the higher burst release seen during the first 6 hours of elution, it is feasible that bupivacaine loading may affect fibroblast growth during the first few hours. However, other dermal cell types such as keratinocytes may be more tolerant to higher concentrations of bupivacaine [164]. The toxic effect of bupivacaine could be addressed by either determining a lower functional loading concentration, or trying other local anesthetics with less toxic effects on fibroblasts, such as lidocaine or ropivacaine [160; 164]. High serum concentrations of all local anesthetics can cause mitochondrial dysfunction and disturbed oxidative phosphorylation, both of which can lead to seizures, cardiac arrhythmias, and hypotension [117]. Bupivacaine specifically is known to be more toxic, especially in tissues with high aerobic demand and low tolerance for hypoxia [165].

This pilot study investigating loaded HA-ESCM for pain relief and infection prevention suggests that membrane materials and loaded membranes are capable of preventing MRSA growth on their surface. Loaded HA-ESCM were also sufficient in releasing active amounts to inhibit biofilm on surfaces beneath membranes, indicating their potential use as dressings to prevent biofilm colonization of open tissue. Despite the initial signs of success of this delivery system, some limitations remain, including generalization of results in vitro to in vivo effects, assessment of just one bacterial strain, and evaluation of a limited number of loading scenarios. Future studies will determine loading capacity and loading efficiency for both to design loading for a sustained release profile and minimal cytotoxicity. Lower concentrations of bupivacaine will be tested with human fibroblasts and keratinocytes as well as immune cells to ensure this system’s cytocompatibility. Bacterial studies will be repeated with S. aureus validate results, in addition to new assays with other bacterial strains such as Pseudomonas aeruginosa and Acinetobacter baumannii. Overall, this in vitro study indicates the potential success of loaded
HA-ESCM in releasing therapeutics and preventing microbial growth, making it a promising wound dressing material to provide pain relief and infection prevention.

MATERIALS AND METHODS

Fabrication

Membranes were electrospun using a 71% degree of deacetylation, 311.5 kDa chitosan (Primex) at 5.5 (w/v) % in 70% (v/v) trifluoroacetic acid - 30% (v/v) dichloromethane solution at 26kV as previously described [140; 147]. Membranes were spun to 15 cm diameters and ~ 0.7 mm (30 ml spinning solution) thickness and treated using a 50-50 solution of pyridine and hexanoic anhydride [147]. Membranes were punched into 1 cm diameter discs and UV sterilized prior to contact with fibroblasts or bacterial cells. Ethanol (200 proof) was used for loading therapeutics [148]. Membranes were loaded with either C2DA (3.125, 6.25, 12.5, or 25 μg), bupivacaine (1.25, 2.5, 5, or 10 mg), or a combination of both treatments, then dried aseptically in a laminar flow hood.

Elution

Elution studies were conducted on HA-ESCM loaded with varying concentrations of C2DA, bupivacaine, or combinations of both therapeutics. Loaded HA-ESCM (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 (Table 6). All eluate concentrations were normalized to standard curves with known concentrations of C2DA and bupivacaine.
Table 6. HPLC settings used for detection of bupivacaine and C2DA.

<table>
<thead>
<tr>
<th></th>
<th><strong>Bupivacaine</strong></th>
<th><strong>C2DA</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td>C18 100x2.1mm</td>
<td>C18 100x2.1mm</td>
</tr>
<tr>
<td><strong>Mobile phase</strong></td>
<td>Buffer : Acetonitrile (50 : 50)</td>
<td>Buffer : Acetonitrile : 10% Methanol (5 : 75 : 20)</td>
</tr>
<tr>
<td><strong>Buffer</strong></td>
<td>KH₂PO₄ + N(CH₂CH₃)₃ + H₂O + H₃PO₄</td>
<td>MeOH+ H₂O + H₃PO₄</td>
</tr>
<tr>
<td><strong>Wavelength</strong></td>
<td>220 nm</td>
<td>210 nm</td>
</tr>
<tr>
<td><strong>Flow rate</strong></td>
<td>1 mL/min</td>
<td>0.3 mL/min</td>
</tr>
<tr>
<td><strong>Column oven temperature</strong></td>
<td>45°C</td>
<td>45°C</td>
</tr>
</tbody>
</table>

**Zone of inhibition (ZOI) studies**

To determine the baseline antimicrobial characteristics of the HA-ESCM, membrane groups were evaluated in modified Kirby-Bauer zone of inhibition assays: freshly loaded membranes, blank paper disc controls, and vancomycin-loaded paper disk controls. Overnight growths of bacteria (Methicillin-resistant *S. aureus* (MRSA) (ATCC 33591)) at concentrations of 10⁵ colony forming units (CFU) were combined in trypticase soy broth (TSB) and added to tryptic soy agar plates to form bacterial lawns. HA-ESCM loaded with bupivacaine, C2DA, or a combination of both were placed on bacterial lawns and incubated at 37°C for 24 hours. Resulting zones were measured using ImageJ software and compared to vancomycin controls as well as paper disk controls.
Biofilm assays

Biofilm inhibitory properties were tested by direct inoculation of freshly loaded HA-ESCM and membranes after elution in PBS for 72 hours. Membranes were placed in 48 well plates and inoculated with 0.5 mL tryptic soy broth (TSB) containing $10^6$ colony forming units (CFU) of *S. aureus*. After incubating at $37^\circ$ C for 24 hours, membranes were removed from wells, rinsed twice with sterile PBS, and sonicated for 5 minutes 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 hours 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.

Cytocompatibility

An adaptation of ISO 10993-5 (“Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity”) was used to evaluate membrane cytocompatibility with fibroblasts. HA-ESCM were evaluated alone, as well as loaded with bupivacaine, C2DA, or combinations of both. Fibroblasts (L929, Lonza) were seeded at $1 \times 10^4$ cells/ cm$^2$ in 12-well
plates and grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and 100 µg/mL of Normocin antibiotic/antimitotic solution for 24 hours at 37 °C and 5% CO2. After overnight incubation, membranes were placed within the upper chamber of transwells. After 24 hours, wells were imaged microscopically, and cell viability was quantified using the CellTiter-Glo® viability assay (Promega). Results were normalized as a percent viability versus cells grown on blank tissue culture plastic and also compared to a control group of chitosan sponges.

**Statistical analysis**

Statistical analysis was performed using SigmaPlot and GraphPad Prism 7.2 software (GraphPad Software Incorporation, La Jolla, CA, USA). 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 (α = 0.05). If normality and equal variance were not passed, data was analyzed using Kruskal-Wallis ANOVA on ranks, followed by Tukey post-hoc test.
Funding

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Acknowledgments

The authors would like to thank Joel D. Bumgardner PhD, Daniel Baker, PhD, and Tomoko Fujiwara, PhD for the guidance in study design. Additionally, they would like to thank Alexis Johnson, Emily Coleman, Carlos Wells, and Landon Choi for the assistance with data collection and analysis.

Conflicts of Interest

We have no conflicts of interest to declare.
CHAPTER IV

DISCUSSION

Hexanoyl-acylated electrospun chitosan membranes were capable of being loaded with C2DA and bupivacaine, individually or in combination, to prevent biofilm formation. This material shows prospective use as a wound dressing following surgery, implantation of a medical device, or musculoskeletal trauma. Loading therapeutics via ethanol evaporation allows membranes to be usable almost immediately, indicating their potential for patient-specific loading at time of care. Local anesthetic-loaded wound dressings may alleviate the need for patients to manage pain with systemic opioids, which often leads to unwanted consequences like opioid misuse, dependence, and consequential addiction [158]. In addition to providing physical coverage from bacterial contamination, the use of the antimicrobial biopolymer chitosan as wound dressing material provides intrinsic infection resistance. This resistance only increases with the addition of bupivacaine, which kills bacteria at higher concentrations, as well as C2DA, which disperses bacteria to prevent biofilm formation [120]. Together, these three antimicrobial components function to prevent pain and infection associated with wounds, which in turn may decrease the need for revision surgeries or tissue debridement required after development of complex biofilm infections.

Prior to this study, preliminary results showed that HA-ESCM are capable of being loaded with and subsequently releasing maximal concentrations of both C2DA and bupivacaine (Appendix 1). The slight burst seen during the first 6 hours of membranes loaded with bupivacaine only may indicate that bupivacaine is being loaded in excess of the amount soluble within the acyl layer of chitosan membranes. Median values of 5 mg and 2.5 mg of bupivacaine were chosen because the 10 mg loading concentration saw the highest proportional burst release
of 5 mg, which based on other studies was likely a toxic amount [159]. When combining bupivacaine with C2DA, release followed a similar pattern, with a slight burst during the first timepoint followed by a sustained average release of about 0.25 mg per time point, which is below previously reported toxic levels of 0.6 mg/ml [160]. C2DA release from membranes loaded with only C2DA followed a zero-order release profile, contrasting with previous attempts to deliver C2DA. For instance, C2DA release from chitosan sponges was sustained throughout 5 days, but also saw a burst release during the first day [156], and phosphatidylcholine coatings loaded with C2DA saw a similar variable release profile, with the majority of C2DA releasing as a burst during the first 24 hours [139]. Furthermore, combining both therapeutics may cause interactions that alter release profiles, as demonstrated by the slightly lower bupivacaine release when dually loaded with C2DA. When C2DA was loaded with bupivacaine, release followed a first-order release pattern, differing from the zero-order release when C2DA was loaded alone. Similar studies have shown that hexanoic anhydride-treated membranes retain more therapeutics compared to membranes treated with other short chain fatty acids such as acetic anhydride and butyric anhydride; thus, modifying with another fatty acid may allow for higher cumulative release of hydrophobics [148]. Future studies will include repeated tests with other concentrations of each therapeutic to better elucidate release mechanisms, which in turn may explain inconsistencies. Tests will also be repeated using other elution media, including fetal bovine serum (FBS)-containing media, since the presence of protein is known to affect the release of hydrophobic substances [161].

The minimal zones around bupivacaine-loaded membranes suggest that bupivacaine does not diffuse from membranes to the same extent as C2DA, though they appear effective in preventing bacterial attachment. While this test is useful in recognizing initial interactions
between materials and bacteria, zones are highly dependent on diffusion so results may not give the full scope of antimicrobial characteristics. For further confirmation of initial antimicrobial results, three separate but related biofilm assays were conducted. Preliminary studies indicated the success of these membranes in preventing *P. aeruginosa* biofilm formation and suggested that bupivacaine and C2DA may have a synergistic relationship in preventing microbial growth (Appendix 2-3). After adapting these early studies for *S. aureus*, there was significantly more viable planktonic bacteria for all groups after elution compared to the freshly loaded membranes, which may be due in part to the slight initial burst release of therapeutics seen by all groups; higher concentrations released during the first 24 hours may be sufficient in killing bacteria that contacts the membranes, rather than just preventing growth on the membranes. Biofilm assays determining biofilm growth on the membranes demonstrated that the membrane materials and therapeutics were all capable of inhibiting biofilm growth to a significant extent. Inhibition after the full course of elution for loaded membranes suggests that membranes are still retaining a small but active amount of these antimicrobials. Viability quantification of biofilm growth on wells beneath membranes showed that growth was minimal for all membrane groups compared to gauze and control chitosan sponge, suggesting that biofilm inhibitors are released from membranes at amounts that would keep biofilm from forming on sites distant to the material, which is beneficial in wound healing. Other studies have strongly suggested that electrospun chitosan nanofibers can interact with bacterial cell walls to rupture and cause leakage of intracellular components, which may explain why even unloaded chitosan membrane groups were successful in preventing biofilm growth [162]. It is also worth noting that the total amount of C2DA loaded within membranes is lower than that loaded into previous delivery systems [139; 156; 157], but antimicrobial activity is maintained. These results are consistent with
previous studies that found nanomolar amounts of C2DA to be active against bacteria [13; 52; 163]. Future studies can expand on these to evaluate different types of strains of pathogenic microorganisms and image biofilm formed on materials using SEM or fluorescence assays.

Results of cytocompatibility studies showed that the concentrations of C2DA were compatible with fibroblasts, whereas both concentrations of bupivacaine were toxic to fibroblasts. While unfavorable, the results demonstrating bupivacaine’s toxicity to fibroblasts were consistent with other studies. At concentrations of 0.3 mg/ml, bupivacaine is compatible with fibroblasts but reduces cell viability below 25% at a 0.6 mg/ml concentration [160]. Due to the higher burst release seen during the first 6 hours of elution, it is feasible that bupivacaine loading may affect fibroblast growth during the first few hours. However, other dermal cell types such as keratinocytes may be more tolerant to higher concentrations of bupivacaine [164]. The toxic effect of bupivacaine could be addressed by either determining a lower functional loading concentration, or trying other local anesthetics with less toxic effects on fibroblasts, such as lidocaine or ropivacaine [160; 164]. High serum concentrations of all local anesthetics can cause mitochondrial dysfunction and disturbed oxidative phosphorylation, both of which can lead to seizures, cardiac arrythmias, and hypotension [117]. Bupivacaine specifically is known to be more toxic, especially in tissues with high aerobic demand and low tolerance for hypoxia [165].
CHAPTER V

CONCLUSIONS

This pilot study investigating loaded chitosan membranes for pain relief and infection prevention suggests that membrane materials and loaded membranes are capable of preventing MRSA growth on their surface. These membranes were also sufficient in releasing active amounts to inhibit biofilm on surfaces beneath membranes, indicating their potential use as dressings to prevent biofilm colonization of open tissue. Despite the initial signs of success of this delivery system, some limitations remain, including generalization of results in vitro to in vivo effects, assessment of just one bacterial strain, and evaluation of a limited number of loading scenarios. Future studies will determine loading capacity and loading efficiency for both to design loading for a sustained release profile and minimal cytotoxicity. Lower concentrations of bupivacaine will be tested with human fibroblasts and keratinocytes as well as immune cells to ensure this system’s cytocompatibility. Bacterial studies will be repeated with S. aureus validate results, in addition to new assays with other bacterial strains such as Pseudomonas aeruginosa and Acinetobacter baumannii. Overall, this in vitro study indicates the success of loaded chitosan membranes in releasing therapeutics and preventing microbial growth, making it a promising wound dressing material to provide pain relief and infection prevention.
CHAPTER VI

FUTURE WORK

There are numerous studies planned to continue the evaluation of electrospun chitosan membranes and related anti-biofilm systems. As previously mentioned, elution studies and biofilm assays will be performed using other therapeutic concentrations as well as other bacterial strains. Cytocompatibility studies will be repeated 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 promote healing and/or direct inflammatory responses that might lead to burn wound conversion. For in vivo characterization of this delivery system as a wound dressing, a comb scald wound model will be performed and compared to commercially available wound care materials. The scald wounds will be inoculated with bacteria to determine the antimicrobial characteristics of loaded membranes as well as their functionality in healing burn wounds. In collaboration with the University of Memphis Chemistry department, other synthetics analogs of C2DA have been developed, 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. Finally, other methods of attachment of fatty acids and membranes will be investigated, such as direct acylation of C2DA or analogs to membrane or click chemistry reactions to directly attach the reactive azide group of chitosan with the reactive alkyne tail groups of synthetic DSF analogs.
APPENDIX A

Initial Release Profile of Bupivacaine from Membranes

Rationale and Methods

Prior to investigations into release profiles of different bupivacaine and C2DA concentrations from membranes, preliminary data was required to determine whether either would release at all, and if so to what extent. While it is ideal for therapeutic concentrations of bupivacaine to release daily to ensure pain relief, bupivacaine is known to have a series of unwanted side effects including neurotoxicity and cardiotoxicity, so it is essential to ensure daily and cumulative release fall below toxic ranges. The loading amount for bupivacaine was chosen to be 20 mg, scaled down from the maximum clinical dose used in spinal anesthesia and thus larger than would be necessary for wound care, but would provide a preliminary indication of the maximum possible release from membranes. The maximum non-toxic C2DA concentration has been reported to as 500 μg/mL, so this concentration was selected for initial loading. Elution studies were conducted on membranes loaded with either 20 mg bupivacaine or 500 μg of C2DA. 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 (Table 6). All eluate concentrations were normalized to standard curves with known concentrations of C2DA and bupivacaine. Percent release was calculated to determine how much of both therapeutics was being retained by membranes.
Results

Bupivacaine eluted from membranes with an initial burst release followed by a sustained release, indicating that the local anesthetic will continue to release from the membrane for at least three days (Figure 15). C2DA eluted from membranes somewhat similarly to bupivacaine, with an initial burst during the first 12 hours followed by a gradual decrease in release throughout the course of three days (Figure 16).

Figure 15. Graphs of elution of (A) hourly bupivacaine release and (B) cumulative bupivacaine released by membranes loaded with 20 mg bupivacaine and modified by hexanoyl acylation.
Figure 16. Graphs of elution of (A) hourly C2DA release and (B) cumulative C2DA released by membranes loaded with 500 μg C2DA and modified by hexanoyl acylation.

Conclusions

C2DA and bupivacaine both eluted from membranes over the course of 72 hours using initial maximum loading concentrations of each. These results indicate the success of this system in releasing hydrophobic therapeutics, though high variability in C2DA release at most time point indicates the need for repeated tests to confirm accuracy of results. For future studies, lower concentrations of each therapeutic will be utilized and assessed.
APPENDIX B

Synergy between Bupivacaine and C2DA analog

Rationale & Methods

When delivering combinations of multiple therapeutics together, it is first helpful to determine whether their relationship is additive, synergistic, or antagonistic. Knowledge of potential synergy invites the possibility of trying different and possible lower loading concentrations of each. Prior to loading both therapeutics into membranes, synergy between bupivacaine and a diffusible signal factor analog of C2DA (2-heptylcyclopropane-1-carboxylic acid) was investigated against *P. aeruginosa*. In this study, bupivacaine was added to wells in a series of concentrations and used either alone or in combination of 250 μg of the C2DA analog (n=4 per group) (*Table 7*). Wells were inoculated with $10^5$ colony forming units from an overnight growth of *P. aeruginosa* (ATCC strain 27317) combined in trypticase soy broth (TSB). Turbidity readings were performed using a Biotek Synergy™ H1 microplate reader, with increased turbidity readings indicating a higher number of viable cells. Percent viability was determined using positive (TSB alone) and negative (ethanol) controls.
Table 7. Loading concentrations of membranes used to test synergy of bupivacaine and C2DA analog against *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Positive control</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bupivacaine concentration (μg/ml)</td>
<td>500</td>
<td>500</td>
<td>1000</td>
<td>1000</td>
<td>2000</td>
<td>2000</td>
<td>TSB with no added bacteria</td>
<td><em>P. aeruginosa</em> + ethanol</td>
</tr>
<tr>
<td>C2DA analog concentration (μg/ml)</td>
<td>0</td>
<td>250</td>
<td>0</td>
<td>250</td>
<td>0</td>
<td>250</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results

Results of synergy studies indicate that combining the C2DA analog with bupivacaine increased antimicrobial efficacy over either treatment alone against *P. aeruginosa* (**Figure 17**). There was significantly less bacterial growth in wells containing bupivacaine combined with the C2DA analog compared to bupivacaine only at the 0.5 mg/ml concentration.

**Figure 17.** Growth of *P. aeruginosa* after 24 hours in trypticase soy broth with added bupivacaine with and without C2DA analog at 250 μg/ml. Asterisk indicates significant difference (p<0.05) between groups determined by Kruskal-Wallis ANOVA with Tukey
Conclusions

While these studies were informative as initial indicators of anti-biofilm potential and synergy, they were not directly applicable to the direct results of this study as they were not used within the context of the membranes. However, understanding the baseline antimicrobial characteristics of these therapeutics was beneficial in determining whether local anesthetics and diffusible signal factors are functional in combination, and ensuring that they do not act antagonistically. Further, while *P. aeruginosa* behaves differently than *S. aureus*, these preliminary studies informed later decisions in combining therapeutics to test against other strains. Future studies related to this test may investigate a broader range of therapeutic concentrations against other bacterial strains.
APPENDIX C

Biofilm Studies with *P. aeruginosa*

**Rationale & Methods**

Before conducting experiments with *S. aureus*, effectiveness of eluted membranes against *P. aeruginosa* was investigated. C2DA is known to be effective in dispersing *P. aeruginosa*, so tests were performed to ensure that enough therapeutics were released to prevent biofilm growth of this species. First, membranes were loaded with either 25 μg of C2DA, 10 mg of bupivacaine, or a combination of both, and were placed in phosphate buffered saline to release therapeutics for 72 hours. After the elution study, membranes were placed in 48 well plates and inoculated with a 0.5 mL solution of 10^6 colony forming units (CFU) of *P. aeruginosa* in tryptic soy broth (TSB). After incubating at 37 °C for 24 hours, membranes were removed from wells, rinsed twice with sterile PBS, and sonicated for 5 minutes at 40 kHz (Fisher Scientific Ultrasonic Bath, 9.5 L) to remove biofilm-associated bacteria. PrestoBlue™ viability reagent was used to compare viable bacteria attached to each membrane type. Briefly, the PrestoBlue™ reagent interacts with the reducing environment associated with viable bacterial cells and produces fluorescence directly proportional to the number of viable cells present in each well. Fluorescence was read with a Biotek Synergy™ H1 microplate reader.

**Results**

Even after the majority of bupivacaine and C2DA were released from the membranes during the 72-hour elution study, biofilm formation was significantly lower for each membrane group (C2DA only, bupivacaine only, and C2DA combined with bupivacaine) as compared to non-loaded (Figure 18).
Figure 18. Biofilm on membranes after 72-hour elution. *P. aeruginosa* biofilm on membranes loaded with bupivacaine, C2DA, or a combination of both, after 72 hours of elution. Bars represent average fluorescence of PrestoBlue™ Viability reagent of bacteria removed by sonication from membranes 24 hours after bacterial challenge. Asterisks indicate significant difference (p<0.05) between groups and non-loaded membrane control determined by ANOVA with Holm-Šidák post-hoc tests.

Conclusion

These results provided a preliminary understanding of interactions between C2DA and bupivacaine. Because these membranes were tested with gram-negative *P. aeruginosa*, they were not included with proceeding tests using gram-positive *S. aureus*. This data informs future studies that will include other gram-negative bacterial strains, and supports the hypothesis that membranes loaded with C2DA and bupivacaine will be effective in preventing gram-positive, gram-negative, and potentially polymicrobial biofilm.
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