In Vivo Evaluation of Fatty Acid Loaded Chitosan Membranes for Pain Relief and Biofilm Associated Infection Prevention

Yogita Manasa Dintakurthi

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IN VIVO EVALUATION OF FATTY ACID LOADED CHITOSAN MEMBRANES FOR PAIN RELIEF AND BIOFILM ASSOCIATED INFECTION PREVENTION

by

Yogita Manasa Dintakurthi

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

Major: Biomedical Engineering

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Dedication

I would like to dedicate this thesis to my parents whose unwavering love and support have been the driving force behind all of my academic pursuits. My brother who has shared his words of encouragement to finish this thesis.

To my close friends and peers who have always shared their words of advice, driven me to do better and always encouraged me.

And lastly, I would like to dedicate this work to my God, whose unconditional love and grace I could not live without.
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Preface

The purpose of this work is to develop and evaluate the local delivery of molecules that inhibit biofilm formation for the future application of burn wound dressings that can prevent bacterial growth progression and aid in healing.

This thesis submission contains the following chapters: introduction, materials and methods, results, discussion, conclusions and future work. The data presented in this thesis have not been published at the time of writing and are being prepared for submission to Burns Journal.
Abstract

Infections are the leading cause of burn related deaths worldwide. These infections not only complicate micro-environment of a burn wound, but further obscure the wound due to the formation of biofilms at the wound site. Local drug delivery (LDD) has been a highly researched solution due to its advantages in locally treating and preventing infection while avoiding systemic side effects. LDD systems also have advantages for complex wounds, like burns. Chitosan is a natural polymer that is often used in these systems due to its biodegradability, biocompatibility and versatility. Previous research has suggested that short chain unsaturated fatty acids, for example cis-2-decenoic acid (C2DA), can disperse and inhibit formation of biofilms. Furthermore, due to the growing opioid crisis, alternate pain management strategies are crucial. Local anesthetics, like bupivacaine (BUP) have been shown to have inherent antimicrobial effects in addition to their analgesic effects. This project aims to study the in vivo efficacy of electrospun chitosan membranes (ESCMs) that are loaded with C2DA and/or BUP in burn wound applications in comparison to clinically used products.
Table of Contents

LIST OF TABLES ................................................................................................................ XI

LIST OF FIGURES .............................................................................................................. XII

CHAPTER I .......................................................................................................................... 1

INTRODUCTION ................................................................................................................ 1

Statement of Clinical Problem ......................................................................................... 1

Hypothesis and Objectives .............................................................................................. 2

CHAPTER II ......................................................................................................................... 5

LITERATURE REVIEW .................................................................................................... 5

Burn Wounds ...................................................................................................................... 5

Burns in Combat ............................................................................................................... 7

Prevalent Infections ......................................................................................................... 8

Multi-drug Resistance ..................................................................................................... 9

Bacterial Biofilms ........................................................................................................... 10

Fatty Acids as Diffusible Signaling Factors .................................................................. 12

Local Anesthetics ............................................................................................................ 14

Chitosan as a Drug Delivery and Guided Tissue Regeneration Matrix ....................... 15

Current Treatment Methods .......................................................................................... 17

CHAPTER III ...................................................................................................................... 19

MATERIALS AND METHODS ..................................................................................... 19

Membrane Fabrication ................................................................................................. 19

Anhydride Treatment and Loading .............................................................................. 19
LIST OF TABLES

**Table 1**: Treatment groups, time points and number of animals for comb scald wound model. .................................................................................................................................................. 21

**Table 2**: Histological burn depth score ................................................................................................................................................................................................. 23

**Table 3**: Rat Grimace Scale (RGS) ................................................................................................................................................................................................. 24
LIST OF FIGURES

Figure 1: Acylated chitosan nanofiber chemically modified with decanoic acid (yellow) and loaded with C2DA (purple) and bupivacaine (red). (Image created on BioRender.com) ................................................................. 3

Figure 2: Classification of burn wound depth. (Image created on BioRender.com) ........ 5

Figure 3: Image of different zones in a burn wound. (Image found on Google Images) .... 6

Figure 4: Graphic explaining the events leading up to the formation of a mature biofilm (Image created on BioRender.com). ................................................................. 11

Figure 5: Biofilm prevention strategies (Image created on BioRender.com) ................. 12

Figure 6: Structure of cis-2-decenoic acid (Structure drawn using ChemDraw) ........ 13

Figure 7: Structure of a) chitin, b) chitosan and c) functional groups in chitosan where red circles indicate primary hydroxy groups, green circles indicate primary amine groups and blue circles indicate secondary hydroxy groups [66] 16

Figure 8: Placement of burn wounds on rat dorsa. (Image created on BioRender.com) 20

Figure 9: Overview of comb scald model surgical procedure (Image created on BioRender.com) ................................................................................................................. 21

Figure 10: Microbiological load measurement procedure (A) and tissue histological/macroscopic measurement procedure (B). (Image created on BioRender.com) ................................................................................................................. 23

Figure 11: FTIR analysis of electrospun chitosan membranes before (blue) and after (red) the anhydride treatment. Red/blue arrows are for highlighting prior discussed peaks. ................................................................................................................. 26
**Figure 12:** Bar graph showing log reduction in bacterial viability for dressing (dark blue), swab (gray), biopsy (light pink) and overall reduction (green) with different treatments. (N = 6) (*indicates p ≤ 0.05). ...................................................... 28

**Figure 13:** Bar graph showing the histological burn scores for each group tested, including both the treated and contralateral non-treated side of each animal. (N = 6 for all except ECSM + BUP group which is an N = 4). ............................................................... 29

**Figure 14:** Bar graph showing the histological scores in the interspace regions for each group tested, including both the treated and contralateral non-treated side of each animal. (N = 6 for all except ECSM + BUP group which is an N = 4). .... 30

**Figure 15:** Rat grimace scores (RGS) summarized based on treatment. (N = 6 for all except ECSM + BUP group which is an N = 4). ............................................................... 31
CHAPTER I

Introduction

Statement of Clinical Problem

There has been intensive research done over the years to develop new strategies for more effective delivery of drugs. Local drug delivery has recently increased in its popularity due to its advantages in “targeted” treatment and prevention of infection during wound healing. Bacterial biofilms, which are micro-communities of bacteria adhering to implants or tissue, pose a challenge as they create their own micro-environment making traditional antibiotic treatment difficult. Previous research has shown that cis-2-decenoic acid (C2DA), a short chain unsaturated fatty acid, disperses and inhibits formation of biofilms [1, 2]. Furthermore, alternatives to opioid-based pain management are necessary as the US is currently experiencing a severe opioid crisis with more than 80,000 overdose deaths in 2022 alone [3]. Studies have shown that local anesthetics such as bupivacaine, lidocaine, procaine and ropivacaine have inherent antimicrobial effects in addition to their analgesic effects [4, 5].

Cellular responses to loaded electrospun chitosan membranes have previously been shown to promote healing in wound applications such as burns by reducing pain, preventing infection, and modifying inflammatory responses through the release of local anesthetics [6]. The goal of this project is to evaluate the in vivo efficacy of chitosan membranes loaded with C2DA, bupivacaine or both in burn wound applications compared to commercially available alternatives.
Hypothesis and Objectives

Burns are frequently encountered in the modern battlefield and make up to 20% of all combat injuries [7-9]. Additionally, during mass casualty events or combat in austere conditions, proper treatment of burns can be delayed for days to weeks [7-9]. In a global level, after traffic accidents, falls and interpersonal violence, burns are the fourth most frequent type of injury [10]. Before the 20th century, burn wounds had limited care and many patients died due to infections [11]. Recently, regenerative medicine and pharmacotherapy have brought about advancements in burn wound care, however, treatment of burn wounds still remains a challenge. As will be discussed below, the leading cause of death in patients with burn wounds is infection. Therefore, there is a need to identify and develop novel treatment methods to tackle and improve burn wound patient outcomes.

This project is proposing the use of a novel biopolymer membrane, namely electrospun chitosan, with many advantageous features for burn wound care. This semi-permeable membrane can be used as an off-the-shelf product to aid in treatment of second- and third-degree burns. These biopolymer membranes will be able to act as a physical barrier to 1) protect the wound, 2) maintain oxygen permeability, 3) retain moisture at the wound site to enable healing and 4) allow for modification with anti-biofilm agent C2DA and/or analgesics to minimize infection risk and provide pain relief. The main aim of this project is to assess the in vivo efficiency of electrospun chitosan membranes (ESCMs) in a comb scald model against commercially available treatment alternatives for burn wound management.
Figure 1: Acylated chitosan nanofiber chemically modified with decanoic acid (yellow) and loaded with C2DA (purple) and bupivacaine (red). (Image created on BioRender.com)

The novel innovation in these ESCMs is the chemical modification of the membrane with fatty acid molecules (decanoic acid) in order to maintain the nanofibrous structure in aqueous environments. This treatment forms a hydrophobic shell around each nanofiber (occurring due to the long alkyl chains of the fatty acid molecules) and prevents swelling and dissolution of the membrane fibers in aqueous environments (Fig. 1). Additionally, the formed hydrophobic shell allows for retention of hydrophobic therapeutics such as C2DA and Bupivacaine (Fig. 1).

Since the structure of C2DA includes a long alkyl chain, the molecule is highly hydrophobic. As a result, local delivery systems have been confined to burst release profiles during a brief period of time [12]. As will be discussed in the literature review, C2DA has been proven to be useful in the prevention of biofilm related infections, however, there needs to be a prolonged release for better eradication. Our modified ESCM has the potential to address this
gap in drug delivery and has been shown to extend the release profiles of C2DA and bupivacaine for 72 hours [6]. Given these results, we have now established the efficacy of the material *in vitro*, paving the way for in vivo testing.

**Aim 1: Determine the antimicrobial activity of C2DA, bupivacaine, and combinations released from chitosan membranes compared to commercially available products.**

_Hypothesis 1: C2DA and/or bupivacaine released over 72 hours will have better or similar antimicrobial effects against bacteria at the wound site compared to commercially available controls._

**Aim 2: Determine the healing ability of biomaterial compared to commercially available products.**

_Hypothesis 2: C2DA and/or bupivacaine released will help prevent necrosis and spreading of burn wound damage to the interspaces compared to commercially available controls._
CHAPTER II

Literature Review

Burn Wounds

According to the World Health Organization (WHO), an estimated 11 million people suffer from burn wounds each year and 180,000 of them die because of such injuries [11]. Burn wounds are a complex traumatic injury that have various local and systemic effects following its onset. Burn injuries are highly variable in terms of the tissue effected, the severity of the wound and the subsequent side effects [13]. Depending on the depth and severity of the burn – muscle, bone, vascular, dermal, and epidermal tissue can be damaged, followed by pain due to neural damage at the wound site. Majority of burns are a result of flames, i.e. thermal, (55%) and scald burns (40%), with age influencing the cause and extent of trauma occurring [13].

![Classification of burn wound depth](Image created on BioRender.com)

**Figure 2:** Classification of burn wound depth. *(Image created on BioRender.com)*

The classification of burn wounds (Fig. 2) is done according to the degree of involvement of skin and deeper tissues [14]. A first-degree (I°) burn is superficial and affects only the epidermis [11, 13-17]. Second-degree (IIa° and IIb°) burns involve the epidermis and variable thickness of the dermis. IIa° burns are superficial partial burns involving damage of epidermis...
and superficial dermis [11, 13-17]. IIb° burns are deep partial burns which involve damage of the epidermis until the reticular/deep dermis [11, 13-17]. Third-degree burns (III°) are full thickness burns which reach the subcutaneous fat layer [11, 13-17]. Finally, fourth degree burns (IV°), not pictured, is a full thickness including deeper tissues such as muscles, bone, and tendons [11].

A typical burn wound is described as having three zones, i.e. the zone of coagulation, zone of stasis and zone of hyperemia (Fig. 3) [18-20]. The zone of coagulation is the primary site of injury that undergoes necrosis and irreversible tissue damage [19]. The zone of stasis is the surrounding zone that has decreased tissue perfusion and is the outermost region of potentially salvageable tissue [19, 20]. The zone of hyperemia is the last zone with increased perfusion which will recover unless there is another thermal insult [19]. This intermediate zone, i.e. the zone of stasis if remaining hypoperfused can become necrotic resulting in the “spreading” of burn damage.

Figure 3: Image of different zones in a burn wound. (Image found on Google images)
What further complicates this intricate wound environment is the possibility of infection which can become septic and cause further complications. The leading cause of death among patients with burn wounds is an infection; around 75% of all burn related deaths result from infection [15, 17]. Following a burn, many of the intrinsic defense mechanisms against infections, such as defensins secreted by keratinocytes and acidic secretions from sebaceous glands are lost or impaired [16]. As mentioned above, the burn wound itself is a complex microenvironment with biological fluids, i.e., burn wound exudates (BWE). The unique metabolic and cellular profile of BWE creates an environment where pathogens with high versatility can proliferate successfully [17]. This results in the colonization of microorganisms, many of which have been shown to form biofilms at the wound site [16, 17]. The presence of biofilms at the wound site is a major contributing factor to the failure of burn treatment regimens and mortality as a result of burn wound infection [17].

As mentioned above, the depth of burn wounds evolves over time, for example, wounds that start as superficial partial or deep partial burns can progress to deep partial or deep burns respectively over a period of time after the burn injury (zone of stasis conversion) [13]. Histological studies have coined burns to be a dynamic process where necrosis in the zones of stasis is the main contributor for progression [13]. Because of this unique pathophysiology, burn patients need to be recurrently evaluated for the thickness of wounds, which adds to patient discomfort and non-compliance while also increasing medical costs for hospitals.

**Burns in Combat**

Burns are an extensive cause of battlefield injury accounting for up to 20% of combat injury burdens [7, 8, 21]. In combat settings, there is a higher prevalence of thermal burn
injuries. Primarily for combat related burns, 82% of burns are caused due to detonation of an explosive device [21]. Early war casualties list bullet wounds as the main cause for death, however in recent times there have been an increase in explosive wounds, burns and inhalation injuries [22]. In modern warfare, an estimated one of four injuries is caused by burns [23]. Furthermore, definitive treatment for burn injuries for both civilians and soldiers may be delayed for days to weeks, especially in mass casualty situations, combat in austere conditions or remote locations [9]. This delay in receiving definitive care increases the risk of infection and reduces tissue viability due to compromised vasculature at the wound site.

**Prevalent Infections**

As mentioned above, infection is a major cause of morbidity and mortality in burn patients. The loss of the physical barrier provided by skin results in a moist environment, easy access to the wound as well as the patient’s blood stream contributing to infection that may progress to a more severe disease state. Not only does this loss of physical barrier make the skin susceptible endogenous micro-organisms but also exogenous pathogens [24]. Additionally, there is also the activation of an inflammatory cascade that results in reduced cell-mediated and humoral immune functions that are mediated by the macrophages present at the wound [25, 26]. The magnitude of immunosuppression then depends on the severity of the burn injury [27].

At the initial onset of a burn, the wound is sterile because any resident flora is killed by the initial thermal insult [26]. Resident flora within the hair follicles, and sebaceous glands can lead to the initial colonization of the wound. Which paired with the nutrient rich environment and the reduced blood supply can cause a single bacterium to multiply exponentially within 24
hours to colonize the entire wound [26]. Left untreated, this fast colonization can lead to systemic infection.

Consistent with the skin’s natural resident flora, burn wounds are initially infected by gram-positive bacteria, however, over time gram-negative organisms begin to colonize the wound as well [26]. *Pseudomonas aeruginosa* is the most common gram-negative organism that colonizes burn wounds. *P. aeruginosa* which is only present in around 5% of burn wounds at the time of admission into the hospital eventually increases to 70% wound colonization by time of discharge [26]. *Acinetobacter calcoaceticus baumannii* is another gram-negative organism that colonizes burn wounds within the hospital. Gram-positive *Staphylococcus aureus* and gram-negative *Pseudomonas aeruginosa* are the most common pathogenic bacteria found in wound infections along with *Acinetobacter calcoaceticus baumannii* being present in certain locations of military conflict [28].

**Multi-drug Resistance**

One of the biggest challenges of infections to global healthcare is the increase in prevalence of multidrug-resistant bacteria (MDR). The IDSA (the Infectious Diseases Society of America) coins MDR bacteria as “one of the greatest threats to human health worldwide” [29].

Multidrug-resistant bacteria are defined as organisms with immunity to three or more classes of antibiotics [30]. This issue is more prevalent in both gram-negative and gram-positive micro-organisms which include, *A. baumannii*, *E. coli*, *P. aeruginosa*, and *K. pneumonia* (Gram-negative), along with *S. aureus*, *S. pneumoniae*, *E. faecium*, and *E. faecalis* (Gram-positive) [24, 31]. Infections with these types of pathogens are associated with increased mortality and morbidity which are particularly difficult to treat. An increasing rise in MDR bacteria due to
overuse of antimicrobials limits the types and amounts of broad-spectrum antibiotics that can be used in the initial stages of treatment.

Approximately 95% of *P. aeruginosa* organisms exhibit resistance to multiple antibiotics, depending on the burn center [26]. Furthermore, most *A. baumannii* isolates obtained from burn wounds in one study were found to be resistant to broad spectrum antibiotics [32]. Combat casualties are increasingly acquiring nosocomial MDR, gram-negative infections both during treatment and evacuation [33, 34]. Early care is essential to the management and treatment of burn infections as some bacterial populations are able to secrete an extra-polymeric substance (EPS) and form a biofilm at the wound site. Similar to the MDR bacteria, biofilms also exhibit antibiotic resistant traits.

*Bacterial Biofilms*

Biofilms are a structured community of microorganisms that are attached to a surface [35]. Pathogenic bacterial strains like *S. aureus, S. epidermis, P. aeruginosa, and E. coli* form biofilms that account for about 80% of human illnesses [36]. The development of biofilms is a multi-step procedure (Fig. 4). The process begins with the bacteria's initial adhesion to a surface, followed by their colonization, which modifies the expression of certain genes and proteins, and an exponential growth phase. The maturation process involves the secretion of a complex sugary substance, termed extracellular polymeric substance (EPS), that encapsulates and protects the growing microbial community [37]. In the end, surface detachment of the cells occurs in the situations where biofilm production is recycled or restarted onto fresh surfaces.
Figure 4: Graphic explaining the events leading up to the formation of a mature biofilm (*Image created on BioRender.com*).

Over the years, numerous inherent biofilm characteristics have been found to have an impact on antibiotic resistance. First, the biofilm matrix acts as a diffusion barrier, preventing antibiotics from reaching their targets [38]. Second, altered microenvironments within the biofilm prevent rapid proliferation of microbes [38]. Third, a small subpopulation of bacteria within the biofilm seem to differentiate into persister cells which are dormant, non-dividing cells with reduced sensitivity to antibiotics [38]. Microbial cells within in a biofilm, especially persister cells, have shown to have 10-1000 times more antibiotic resistance than their planktonic counterparts [37].

The enhanced extent of virulence and pathogenicity in biofilms require novel methods of controlling the formation and development of biofilms. Current strategies for prevention of biofilms in practice include three different methodologies (*Fig. 5*). First method is prevention of adhesion through anti-biofilm coatings on the surface [39]. Second method is disruption of survival of the attached biofilm through the use of antibiotics and antimicrobials [39]. And
finally, third, disruption of formed biofilm through the use of known biofilm dispersing compounds [39].

Figure 5: Biofilm prevention strategies (Image created on BioRender.com)

Treatments of existing biofilms are particularly challenging because an established biofilm is able to survive physiochemical aggressions including UV light, pH changes, changes in hydration or salinity and phagocytosis by immune cells [40-43]. Although long term antibiotic suppression can help control biofilm infections, it frequently fails and raises the risk of harmful side effects and antibiotic resistance. As a result, the presence of biofilm infections most times leads to extended hospitalization and trauma for the patients along with increased cost of care for hospitals.

Fatty Acids as Diffusible Signaling Factors

A class of medium chain fatty acids, also known as diffusible signaling factors (DSFs), have been found to have biofilm-dispersing as well as inhibiting properties. DSFs are naturally
secreted by bacteria to function in cell-cell communication within the biofilm. The presence of these molecules, and similar molecules, naturally in trace amounts can reduce the chances for resistance when used as non-antibiotic alternatives for infection treatment. DSF’s may even be used synergistically with antibiotic therapy to ensure complete treatment of infections [2, 44, 45]. Cis-2-decenoic acid (C2DA) (Fig. 6) is a well-researched biofilm dispersion agent that has demonstrated abilities to prevent *P. aeruginosa* from forming biofilms and dispersing already present biofilms of various other strains [46, 47]. The ability of these fatty acids to disperse biofilms of multiple different bacterial strains and fungi suggests they have a cross-strain and cross-kingdom efficacy [1, 46, 48-50]. This cross-kingdom efficacy is thought to stem from the fact that many fatty acid DSFs contain a *cis*-unsaturated double bond at the 2-position (Fig. 6) which allows for inter-species signaling, while the branching and chain length differences effect the specificity [47].

![Structure of cis-2-decenoic acid](https://example.com/structure-cis-2-decenoic-acid.png)

**Figure 6:** Structure of cis-2-decenoic acid (*Structure drawn using ChemDraw*).

Persister cells, as described above are responsible for the antibiotic tolerant and resistant nature of biofilms. C2DA has also been found to “awaken” persister cells within biofilms of *P. aeruginosa* and *E. coli* [51]. This cell awakening is said to be supported by an increase in metabolic activity of persister cells, however, C2DA allows for this transition without increasing cell number. Furthermore, given that most antibiotics target actively dividing cells, combination therapy with antimicrobials and C2DA shows a significantly greater decrease in persister cell viability when compared to antibiotic treatment alone [51].
Bacterial growth inhibition increases when free carboxyl moieties are present along with lower degrees of saturation [52]. It has also been determined that cis isomers are more inhibitory than the trans isomers [52]. Furthermore, for aerobic, cocci, and gram-positive bacteria in particular, the action of cis-unsaturated fatty acids to enhance bacterial inhibition is noteworthy [52-54]. This inhibitive action of cis-isomer and C2DA in particular can be attributed to the fact that these fatty acids can incorporate into the membranes of growing bacteria thereby affecting the membrane’s permeability [12, 55]. A cis double bond does not allow free rotation of the carbons involved; this characteristic physiologically induces a kink within the hydrocarbon tail of the fatty acid affecting the permeability of bacterial membranes. As a result, using C2DA with antimicrobials allows for increased drug penetration into the bacterial cells, this in turn enables the drug to perform its function more effectively. Overall, the use of C2DA could be a solution or a supplement in the treatment of biofilms.

Local Anesthetics

Local anesthetics (LAs) reversibly block the generation and propagation of neural impulses by blocking voltage-gated sodium channels [4, 56]. Besides pain management, the antimicrobial and anti-inflammatory action of LAs has been well documented in several studies [4, 5, 55, 57]. Due to their analgesic efficacy, opioids are central in the management of wound and procedure pain in burn patients [58-60]. Given that the opioid crisis has been attaining epidemic proportions currently, it is essential to determine non-opioid pain management strategies [3]. LAs when applied locally as topical sprays or creams reduced the requirement for opioids without hindering the healing process [60]. Additionally, some studies suggest that LAs may raise blood perfusion levels at the wound site and alter the inflammatory response,
decreasing edema, that may cause burn wound damage to penetrate to surrounding and deep tissue layers in both animal and human trials [55]. Research has confirmed that in addition to the analgesic and anti-inflammatory action, LAs can also function to have anti-microbial effects. For example, studies with lidocaine and procaine have shown to cause loss of viability of E. coli [5]. Similarly, the use of lidocaine or bupivacaine significantly reduced edema compared to controls along with having antimicrobial and antifungal effects [4, 55, 61]. Some studies indicated that LAs combined with antibiotics did not interfere with their activity [62].

Topical creams and sprays in their typical formulations offer immediate relief but their effects wear off rapidly and need to be reapplied [60]. Furthermore, issues with neurotoxicity and short half-lives have significantly limited the applications of LAs clinically [63]. To overcome these barriers several Drug Delivery Systems (DDSs) have been developed to encapsulate LAs and administer large doses released slowly to provide their effects over a prolonged amount of time. Therefore, depending on the application, the choice of appropriate drug delivery matrix is important for success of this type of system.

Chitosan as a Drug Delivery and Guided Tissue Regeneration Matrix

Chitosan, particularly N-deacetylated chitosan, is a derivative of chitin which is extracted from the shells of shrimp and other crustaceans [64-66]. The structure of chitosan (Fig. 7) consists of repeated units of β-1-4-linked D-glucosamine and N-acetyl-D-glucosamine units [65, 66]. The number of N-acetyl-D-glucosamine units depends on the degree of deacetylation performed during the chitosan derivation process. The availability of amenable functional groups, namely primary amine as well as both primary and secondary hydroxyls allow for chemical modifications without changing the polymerization [64]. Recently, chitosan-based
biomaterials have piqued the interest of researchers due to its high biocompatibility, biodegradability, antibacterial and anti-inflammatory properties [64, 67].

![Structure of a) chitin, b) chitosan and c) functional groups in chitosan](image)

**Figure 7:** Structure of a) chitin, b) chitosan and c) functional groups in chitosan where red circles indicate primary hydroxyl groups, green circles indicate primary amine groups and blue circles indicate secondary hydroxyl groups [66]

Chitosan has been shown to have antimicrobial properties against *E. coli*, *P. aeruginosa*, and *S. aureus* [65]. The underlying mechanism of action behind its antimicrobial nature is not entirely understood. Several theories have been proposed, however, most studies seem to agree that the antibacterial properties may be mostly attributed to the cationic nature of the molecule [65, 68, 69]. The number of amino groups in the molecule can cause changes in membrane permeability leading to cell death via leakage of intracellular components [65].

Despite the preliminary effectiveness of C2DA as a biofilm treatment agent, a hurdle remains, signaling molecules and need to be active for prolonged amounts of time to be effective biofilm preventive agents. The hydrophobic nature of C2DA has posed a challenge in extended-release applications. Similarly, LAs (ex. bupivacaine) are also highly hydrophobic, complicating
extended-release applications. Chitosan could offer a natural way to incorporate a biodegradable, anti-biofilm, and analgesic material directly onto the wound serving as a local drug delivery matrix and a source for guided tissue regeneration.

Current Treatment Methods

Pharmacological treatment regimens are specific to a patient’s status, including current medical condition, pulmonary status, burn severity, medical history of the patient, and current medications taken by the patient [11]. The first line of pain treatment is the administration of opioid analgesics, particularly morphine [11]. However, due to the high prevalence of opioid-induced hyperalgesia as a side effect, opioid administration should be limited [11]. For minor burns, post treatment pain therapeutics include acetaminophen or non-steroidal anti-inflammatory drugs (NSAIDs). If the burn wound remains open, infection is a large concern. To combat this issue, a broad-spectrum antibiotic is also administered along with analgesics.

Early excision and split skin grafting are a primary treatment for deep dermal and full thickness burns [11]. The most common sources for wound coverage are human cadavers and pig skin, however, these sources carry a risk of viral and bacterial disease transmission. Acellular fish skin grafts have recently become an alternative source for xenograft in deep dermal and full thickness burns [11]. Specifically, North-Atlantic (NA) cod grafts are useful because there are no known prion, bacterial, or viral diseases that can be transmitted to humans from NA cod fish [70]. The collagen present in the decellularized NA cod graft plays a key role in the wound healing process. Furthermore, the antibacterial, antiviral properties and acceleration of 3D cell ingrowth by fish skin grafts make them very relevant to severe burn injuries [71].

There are multiple different types of dressings being studied by researchers currently to help alleviate infection risk involved in burn wound treatment, without hindering the healing
process. One example of dressings are hydrogel dressings. These dressings have a multimodal impact in that they may be applied to practically all sections of the body, have cooling and wound covering capabilities, come in a variety of sizes, and can remove heat from the wound by convection and evaporation [72]. It is well known that a moist environment is needed at the burn wound site for proper healing, which is offered by a hydrogel. However, this increased moisture content can increase the number of microorganisms able to infect the wound and hinder the healing process.

Recently, silver products have been used for their beneficial effects against bacteria [73]. Silver-containing creams, such as silver sulfadiazine, have been favored as topical ointments for large burn wounds. Due to its widespread usage in managing bacterial infections, silver sulfadiazine swiftly became the choice of treatment in burn wounds. Now, the cream is sold commercially as a water-soluble ointment. Similarly, a dressing that is available in the market, Silverlon®, is marketed to be a nylon dressing plated with slow-releasing Ag ions [73, 74]. When used during evacuation of military burn casualties, Silverlon® dressings trended toward a decreasing rate of wound infection in full thickness burns and did not contribute to burn related complications [75].

Chitin based dressings have been on the rise recently in burn wound treatment due to its biocompatible, biodegradable, and antibiotic nature. Products such as the Sentrex BioSponge® and electrospun chitosan membranes have been used as a semi-permeable dressing. Chitosan is able to not only maintain a sterile environment but also optimize healing conditions and prevent wound contamination [11]. Chitosan and chitin are known to stimulate the wound healing process and have resulted in a significant reduction in treatment time with minimal scarring in various animals [11].
CHAPTER III

Materials and Methods

Membrane Fabrication

Membranes were electrospun using 86% degree of deacetylation (DDA) chitosan flakes purchased from ChitoLytic. The flakes were solubilized in 70% (v/v) trifluoroacetic acid (TFA) and 30% (v/v) dichloromethane (DCM) solution for 16 hours. The solution was then vortexed and then centrifuged to remove undissolved chitosan to yield a clear viscous solution. The solution transferred to a 10 ml syringe and electrospun to 16 cm diameter and ~0.7mm thickness (~12mL of spinning solution) as previously described [76]. The chitosan/TFA solution was ejected at a steady flow rate of 0.028 mL/min and at 18-26 kV with constant monitoring of the Taylor cone to ensure quality membranes. Chitosan nanofibers were collected on a non-stick aluminum foil attached to a grounded metal wheel rotating at 8.4 rpm.

Anhydride Treatment and Loading

Membranes were cut into 7.5cm × 4cm rectangles and treated with a 50/50 solution of pyridine to decanoic anhydride as previously described [76]. After the anhydride treatment, membranes were lyophilized (FreeZone 4.5 Plus) for 24 hours and stored in Ziploc bags.

Stored membranes were UV sterilized prior to loading with therapeutics. Ethanol (200 proof) was used to dissolve and load therapeutics onto cut, sterilized membranes. Membranes were loaded with either 40mg/mL C2DA (Biosynth), 40mg/mL of BUP, or a combination of both treatments and placed to dry aseptically in a laminar flow hood.
Fourier Transform Infrared Spectroscopy

In order to analyze the bonds on the surface of ESCMs and confirm the success of fabrication and treatment, treated membranes were observed using a PerkinElmer Frontier FT-IR spectrometer equipped with a diamond crystal in Attenuated Total Reflectance (ATR) mode. This allowed for the observation of ester bonds, a decrease in the presence of chitosan salts, and an increase in the carbon chain.

In vivo Comb Scald Model

Adult male and female Wistar rats weighing 250-350g (total 30) were used for the entire experimental protocol (Table 1). The rats were anesthetized via isoflurane inhalation and the dorsal area was shaved and cleaned with isopropanol and betadine. A brass comb with 4 prongs (1cm × 2cm separated by 5mm notches) was used to create symmetric burn wounds (Fig. 8).

We used a brass comb wound model previously established by Regas et. al [77]. The comb was placed in boiling water and placed on the dorsa for 40 seconds, this procedure was repeated for each side of dorsa (Fig. 9). Burns were inoculated with \( P. \) aeruginosa \( (10^3 \) CFUs) delivered

Figure 8: Placement of burn wounds on rat dorsa. \( (Image \ created \ on \ BioRender.com) \)
through 20μL of bacteria suspended in saline to each burn wound. The scalded skin on one side served as the no-treatment control which was determined randomly during the surgeries.

**Figure 9**: Overview of comb scald model surgical procedure (*Image created on BioRender.com*)

The treatment side was dressed in either the ESCM or the commercially available alternative treatment. Dressings were sized to cover the four burn wounds and the interspaces in-between (zones of stasis). Silver sulfadiazine groups had applications of the cream twice daily.

**Table 1**: Treatment groups, time points and number of animals for comb scald wound model.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Time points</th>
<th>Number total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>Control sides of dorsa for each animal</td>
<td></td>
</tr>
<tr>
<td>Silverlon dressing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silver sulfadiazine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESCM alone</td>
<td>1 (3 days post-burn)</td>
<td>6/group (3 male &amp; 3 female)</td>
</tr>
<tr>
<td>ESCM with C2DA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESCM with combination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESCM with Bupivacaine</td>
<td></td>
<td>4 (3 male &amp; 1 female)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>34</td>
</tr>
</tbody>
</table>
**Burn Wound CFU Counting**

Animals were euthanized by CO$_2$ inhalation 3-days post-surgery. Three types of samples were collected for CFU (colony forming unit) counting. Burn wounds were first swabbed across all 4 burns across the treatment and no-treatment sides and placed in separate tubes containing sterile saline. Next, the wound dressings were collected in separate tubes containing sterile saline for sonication and CFU counts. Finally, prior to homogenization and CFU measurement, an 8mm biopsy punch was utilized to extract tissue from each burn region (Fig. 10A).

Obtained samples from each animal were diluted via serial dilution procedure and 100µL of each dilution was plated on a Tryptic soy agar plate for 24 hours at 37ºC prior to analysis. The number of colonies on each plate were counted and recorded in an excel spreadsheet for comparison and statistical analysis.

**Burn Wound Histology**

Animals were euthanized by CO$_2$ inhalation 3-days post-surgery. Wounds were photographed with a ruler set to measure of area and width of wound as well as zones of stasis using ImageJ software (Fig. 10B). Macroscopic assessments were documented, for example redness around wound, necrosis etc., prior to fixation in 10% formalin. Excised samples were embedded in paraffin and sectioned prior to staining with hematoxylin and eosin. BioQuant image analysis software was used for imaging histological slides. Length of epithelial layer, presence of blood vessels, necrosis and inflammatory cell infiltration were assessed, and the sections were given a score from 1-5 in analogy to clinical grading (Table 2).
Figure 10: Microbiological load measurement procedure (A) and tissue histological/macroscopic measurement procedure (B). (Image created on BioRender.com)

Table 2: Histological Burn Depth Score

<table>
<thead>
<tr>
<th>Score</th>
<th>Burn depth</th>
<th>Clinical grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Epidermis</td>
<td>First degree</td>
</tr>
<tr>
<td>2</td>
<td>Superficial dermis</td>
<td>Second degree – 2a</td>
</tr>
<tr>
<td>3</td>
<td>Intermediate dermis</td>
<td>Second degree – 2b</td>
</tr>
<tr>
<td>4</td>
<td>Deep dermis</td>
<td>Third degree</td>
</tr>
<tr>
<td>5</td>
<td>Muscle</td>
<td>Fourth degree</td>
</tr>
<tr>
<td>6</td>
<td>Subcutaneous</td>
<td></td>
</tr>
</tbody>
</table>

Rat Pain Assessment

The Rat Grimace Scale (RGS) is widely used to best quantify pain behaviors of rats. RGS utilizes four facial action units including orbital tightening, nose flattening, ear changes, and
whisker changes and quantifies them on a scale from 0-2 to numerically evaluate a rat's well-being. Along with these four factors, the lack of grooming (rough hair/coat, porphyrin staining) was also assessed and scored for these rats using the same scoring system. A summary of the RGS that was used is outlined in Table 3 below.

**Table 3: Rat Grimace Scale (RGS)**

<table>
<thead>
<tr>
<th></th>
<th>Not Present (0)</th>
<th>Present (1)</th>
<th>Pronounced (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orbital tightening</td>
<td><img src="orbital.jpg" alt="Image" /></td>
<td><img src="orbital1.jpg" alt="Image" /></td>
<td><img src="orbital2.jpg" alt="Image" /></td>
</tr>
<tr>
<td>- Display a narrowing of orbital area, manifesting as partial or complete eye closure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nose/Cheek flattening</td>
<td><img src="nose.jpg" alt="Image" /></td>
<td><img src="nose1.jpg" alt="Image" /></td>
<td><img src="nose2.jpg" alt="Image" /></td>
</tr>
<tr>
<td>- Less bulging of nose and cheek</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ear changes</td>
<td><img src="ear.jpg" alt="Image" /></td>
<td><img src="ear1.jpg" alt="Image" /></td>
<td><img src="ear2.jpg" alt="Image" /></td>
</tr>
<tr>
<td>- Ears curl and angle forwards or outwards.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Space between ears may appear wider</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whisker change</td>
<td><img src="whisker.jpg" alt="Image" /></td>
<td><img src="whisker1.jpg" alt="Image" /></td>
<td><img src="whisker2.jpg" alt="Image" /></td>
</tr>
<tr>
<td>- Whiskers move forward from baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Whiskers may clump together</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- May loose natural “downward” curve</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 3 (continued):** Rat Grimace Scale (RGS)

<table>
<thead>
<tr>
<th>Lack of grooming</th>
<th>Not Present (0)</th>
<th>Present (1)</th>
<th>Pronounced (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of porphyrin staining around eyes and nose</td>
<td><img src="image.jpg" alt="Image" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Statistical Analysis**

All statistical analyses were 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 with non-parametric analysis using the 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.
CHAPTER IV

Results

Confirmation of Anhydride Treatment

FTIR was utilized to confirm the success of reaction performed. For the untreated membrane (blue, Fig. 11), characteristic peaks were observed at 1652, 2800-3360 cm⁻¹ corresponding to C=O stretching and broad O-H stretching respectively. The treated membrane (red, Fig. 11) characteristic peaks were observed 1740, 3297, 1650, 1547, and 2800-2900 cm⁻¹ which correspond to an ester C=O bond, N-H stretching of NH₂, an amide C=O bond, the N-H bond of a chitosan amide and acyl carbon chains on the surface of the membrane.

Figure 11: FTIR analysis of electrospun chitosan membranes before (blue) and after (red) the anhydride treatment. Red/blue arrows are for highlighting prior discussed peaks.
Microbiological Data: Colony Forming Units (CFUs)

Viable bacterial cell concentrations were estimated by counting CFUs on both the treated and non-treated sides of each rat. The log reduction of bacteria of the treatment compared to the contralateral non-treated control was calculated using Equation 1 below.

Equation 1: Formula to calculate log reduction

\[
\text{Log reduction} = \log\left(\frac{\text{control}}{\text{treatment}}\right)
\]

The percent reduction of bacteria on the treated side compared to the contralateral non-treated control was calculated using Equation 2 below.

Equation 2: Formula to calculate percent reduction (% reduction)

\[
\%\text{reduction} = \left(\frac{\text{control} - \text{treatment}}{\text{control}}\right) \times 100
\]

The log reduction values for each group (Fig. 12) indicate the efficacy of each treatment type with respect to an untreated control. Similarly, a higher percent reduction means more bacterial cells were eliminated by the treatment. Percent reduction and log reduction are commonly used interchangeably to describe the efficacy of a certain drug or treatment. An overall log reduction was calculated as an average of the mean log reductions in each of the three samples, i.e. biopsy, dressing and swab, to determine the overall antimicrobial activity of the dressing. Silver sulfadiazine cream produced an overall log reduction of 2, which translates to approximately 99% decrease in bacterial viability compared to the non-treatment control. Similarly, Silverlon produces a log reduction of 1 which is around 90% decrease in bacterial viability compared to the control. ESCM alone group produced an overall log reduction of -0.6 meaning that the treatment may be having an opposite effect and causing increased proliferation of bacteria compared to the control. The chitosan groups loaded with C2DA (ESCM + C2DA), bupivacaine
(ESCM + BUP), and a combination (ESCM + Combo) produced a log reduction of 0, 0.6, and 0.5. These values translate to an overall approximate percent reduction value of 0%, 75% and 70% respectively. There were no statistically significant differences between any groups in the Biopsy and Swab samples. There was a statistically significant difference found between Silver Sulfadiazine cream and ESCM group in the Dressing samples.

**Figure 12**: Bar graph showing log reduction in bacterial viability for dressing (dark blue), swab (gray), biopsy (light pink) and overall reduction (green) with different treatments. (n = 6)

(*indicates p ≤ 0.05)

**Histological Data: Burn Depth Scores**

Histological evaluation of burn depth (Fig. 13) at day 3 showed infiltration of immune cells, i.e., neutrophils, extending until the deep dermis layers for all animals at 3 days post burn.
Figure 13: Bar graph showing the histological burn scores for each group tested, including both the treated and contralateral non-treated side of each animal. (n = 6 for all except ECSM + BUP group which is an n = 4).

The interspaces between each burn were also scored to determine whether there was any spread of damage to the zones of stasis between the burns. There were no statistically significant differences between the groups however, the ESCM + BUP and ESCM + Combo groups seemed to have prevented the spread of damage beyond the intermediate and deep dermal layers respectively.
Figure 14: Bar graph showing the histological scores in the interspace regions for each group tested, including both the treated and contralateral non-treated side of each animal. (n = 6 for all except ECSM + BUP group which is an n = 4).

Pain Assessment Data: Rat Grimace Scores

A total of 34 animals were scored using the RGS scoring method. All 5 facial action unit scores were added to have an overall pain score from 0-10, with 10 meaning the animal is in intense pain and 0 being no pain. A summary of the findings is presented below (Fig. 15). When making comparisons between treatments, ESCM alone and ESCM + Combo groups seemed to have produced similar analgesic affects when compared to the commercially used alternatives.
Figure 15: Rat grimace scores (RGS) summarized based on treatment. (n = 6 for all except ECSM + BUP group which is an n = 4).
CHAPTER V

Discussion

Confirmation of Anhydride Treatment

The FTIR analysis presented in this thesis demonstrated the successful anhydride treatment of the electrospun chitosan fibers. The appearance of acyl carbon chain peaks at ~2800 cm\(^{-1}\) (Fig. 9) in the treated membranes along with the appearance of a clear ester C=O peak at ~1740 cm\(^{-1}\) indicate the reaction, i.e. an O-acylation, was able to successfully take place. This finding is in agreement with the literature review which state the appearance of a ester C=O peak at around 1735-1755 cm\(^{-1}\) is indicative of a successful O-acylation of chitosan as opposed to an N-acylation [78-80].

Release Profiles of Loaded Molecules

Previous studies done have shown that the membranes release both therapeutics, C2DA and Bupivacaine, over the span of three days (i.e. 72 hours) [6]. Loaded drugs displayed an initial burst release of therapeutic for about 3-6 hours followed by a slower release of therapeutics for around 72 hours [6]. A 1cm ESCM disc was used for characterization of the release profiles as well as determination of the ideal concentration of drugs loaded. The 40mg/ml concentration of drugs loaded onto the rectangle membranes in this thesis was a value scaled up from these initial studies. Furthermore, the release profiles established in previous assays were done in aqueous environments, however, the difference in application, i.e., topical/dermal, release may be different.
Microbiological Data: Colony Forming Units (CFUs)

When looking at the Biopsy sample results alone, the electrospun chitosan membranes loaded with Bupivacaine or a combination of bupivacaine and C2DA, seemed to have reduced bacterial viability in the wound better than commercially available controls. This finding agrees with literature as silver sulfadiazine has been found in some studies to have limited wound penetration [81, 82]. Furthermore, there was a significant difference in log reduction between the silver sulfadiazine cream and the ESCM alone group in the dressing samples, indicating that the electrospun chitosan membranes alone without any drugs loaded produced an opposite effect, leading to an increase in bacterial viability on the dressing. These results support literature review as these silver containing dressings and creams are known to prevent the colonization of bacterial colonies [83-85]. Since silver sulfadiazine is applied daily on the wound, the renewed release of silver, approximately 3,176 ppm, prevents the dressing and burn from bacterial infection and proliferation [85]. However, the mesh-like properties of ESCMs act as a substrate for attachment of cells and subsequent proliferation, thereby potentially aiding biofilm formation in this particular setting [86]. The Swab sample data indicate that the commercially used controls are able to prevent colonization of bacteria on the apical surface of the wound. However, due to the vast spread of the data in the ESCM + BUP and ESCM + Combo groups, concrete conclusions about treatment efficacy cannot be made.

The vast spread of the data points may be attributed to the fact that there may have been cross-contamination between the treated and non-treated sided on the animal as well as cross contamination from the rat’s surroundings in the cages. Future work will need to explore methods to properly secure the wound dressing and prevent cross contamination between the two sides and the surroundings.
**Histological Data: Burn Depth Scores**

Histological burn depth was primarily evaluated on the basis of inflammatory cell infiltration into the skin and scored based on the depth of tissue that was infiltrated [87]. The burn scores indicated that there was consistent infiltration of inflammatory cells, i.e., neutrophils, 3 days after the infliction of the burn wound. This infiltration extended till the deep dermis layers across all the treatment groups tested. According to **Fig. 12**, the dressings were not able to prevent the damage from the initial burn. However, data in **Fig. 13** suggests that the dressings were able to prevent the damage from spreading to the healthy tissue, i.e., zones of stasis/interspaces. This infiltration of immune cells in the zone of coagulation may be indicative of the amount of damage occurred [88]. However, given the timeline of burn wound healing, 3 days post injury is still within the timeframe of the acute phase response (APR) of wound healing [89]. A characteristic trait of the acute phase response includes rapid and abundant recruitment of inflammatory cells to aid in the repair and removal of necrotic tissue. The presence of inflammatory cells to this extent 3-days post wound infliction is supported by literature. Excision of tissue samples 7 days and 14 days post wound infliction would be beneficial to understanding the long-term efficacy of the electrospun chitosan treatments.

**Pain Assessment Data: Rat Grimace Scores**

Compared to the commercially available treatments, no ESCM groups seemed to produce statistically significant increases in analgesic effects. However, the ESCM alone and ESCM + Combo groups seem to have produced similar analgesic effect compared to the two commercially available alternatives. This difference in analgesic effects may be attributed to the burn model used. The animals were burnt on both sides, however since only one side received
any treatment, the overall analgesic effect cannot be properly quantified or determined. Other burn models which utilized the rat grimace scale to score pain behaviors of rats had control (i.e. untreated) and treated rats separately [90, 91]. Future changes to the burn model used may help properly address these differences and appropriately quantify the analgesic effects of each treatment.

Conclusion

The comparable qualities of each molecule, chitosan, bupivacaine and cis-2-decanoic acid may still be enough to replace these “gold standard” commercially used products as the risk of long-term cytotoxic effects of silver are still present. The materials tested in this thesis produced similar bactericidal, healing and pain relief abilities when compared to the clinically used products. Additionally, the release of therapeutic molecules over 3 days is beneficial especially when immediate care of wounds is difficult. Overall, the application of electrospun chitosan membranes in burn wound management is an emerging practice that requires more testing but seems to offer a non-toxic alternative to current standards, while delivering similar analgesic, healing and anti-biofilm effects.
CHAPTER VI

Future Work

The desired effect for these electrospun materials would be to significantly reduce the viability of bacteria at the wound site while enabling the healing process. Recovery of a burn wound can take anywhere from a few weeks to a month depending on the severity of the injury [89]. Furthermore, an overactive systemic APR after a burn injury can be detrimental to the healing process as it may cause damage to the surrounding tissues, delay the wound healing process and contribute to the severity of scarring [88, 89]. The reduction, transition and control of immune cells are crucial for dampening the initial surge of inflammation and the establishment of a healthy healing process [88]. Further experiments should involve study of the analgesic, healing and anti-biofilm effects of electrospun chitosan membranes over the course of 7-14 days to further understand their long-term benefits in the healing of burn wounds. Additionally, future studies should establish a protocol to prevent the cross contamination of wounds between the non-treated and treated sides to obtain an accurate representation of the antibacterial abilities compared to commercially used alternatives.

Acknowledgements

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References


