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TITLE: Acylated Electrospun Biopolymer Membranes for Burn Wound Coverage, Infection Prevention, and Pain Relief

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5d. PROJECT NUMBER

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14. ABSTRACT
We have developed novel biopolymer membranes with advantageous features (physical coverage, infection prevention, pain relief) for immediate care of burn wounds and during prolonged field care. Electrospun chitosan membranes (ESCM) serve to address burn wound coverage in several ways, including 1) acting as a barrier to microbial contamination, 2) releasing local anesthetics in a controlled manner that reduce pain and modify the inflammatory response, and 3) releasing natural antimicrobial fatty acids that prevent biofilm contamination. In order to assess these ESCM, bulk fabrication of ESCM was performed as well as loading treatments (Bupivicane and/or C2DA) to evaluate elution and antimicrobial properties. Elution results displayed similarities between the single and dual release of C2DA, where controls (sponge & gauze) did not elute therapeutics past 9 hours. Experimental groups (hexanoic, octanoic, and decanoic acylated) eluted therapeutics throughout the study. Antimicrobial results displayed antimicrobial properties of treated membranes against planktonic and biofilm microorganisms.

15. SUBJECT TERMS
biofilm; anesthetic; bupivacaine; electrospinning; chitosan; biomaterial; local drug delivery; wound dressing; infection; Staphylococcus; Pseudomonas; animal model; burn wound; antimicrobial; elution; SEM; FTIR; biopolymer

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1. Table of Contents

1. INTRODUCTION

Burn wounds sustained during combat can become infected and cause significant pain. These traumatic burn injuries can be subject to biofilm infection, which are often antibiotic-resistant and difficult to treat. Dressings may be used as wraps over multiple types of soft tissue wounds, including burns, and have advantages in military wound care in that they are able to remain in place with minimal maintenance during the evacuation process. While proven effective in reducing microbial colonization, current standard of care creams and dressings have drawbacks in that they may delay the wound healing process and they do not address the management of pain.
The purpose of this study is to develop electrospun chitosan membranes (ESCM) loaded with local anesthetics (LA) and the antimicrobial agent cis-2-decenoic acid (C2DA) to serve as burn wound dressings that prevent pain and infection. There are several milestones to demonstrate successful application of loaded ESCM for burn wound treatment in a prehospital setting, including: selecting the most effective LA based on antimicrobial activity, manufacturing and characterizing ESCM, evaluating release of both therapeutics, and determining anti-biofilm properties of loaded ESCM. To ensure these wound dressings do not delay the wound healing process, ESCM will be tested for cytocompatibility with fibroblasts and keratinocytes, collagen and cytokine production will be determined, and monocyte to macrophage differentiation in the presence of loaded ESCM will be investigated. The overall effectiveness of loaded ESCM to prevent burn wound infection in an established contaminated comb scald wound rat model will also be determined. These milestones and the current progress are outlined and described in this report.

2. KEYWORDS

biofilm; anesthetic; bupivacaine; electrospinning; chitosan; biomaterial; local drug delivery; wound dressing; infection; Staphylococcus; Pseudomonas; Acinetobacter; animal model; burn wound; antimicrobial; elution; SEM; FTIR;

3. ACCOMPLISHMENTS

What were the major goals of the project? (Goals to be accomplished and status.)

Specific Aim 1: Evaluate antimicrobial effects of LA, C2DA, and combinations released from ESCM (months 1-12)

- STATUS: completed Y1Q4, completed Major Task 1. Started Major Task 2, subtask 1 complete, subtask 2 in progress and 90% complete, subtask 3 in progress and 80%. Subtask 4 is in progress and 88% complete.

Specific Aim 2: Evaluate dermal and inflammatory cell responses to LA, C2DA, and combinations released from ESCM (months 8-20)

- STATUS: started subtask 1 and 15% complete.

Specific Aim 3: Compare ESCM with and without LAs and C2DA to commercially available casualty care standards in an in vivo contaminated rat comb scald wound model (months 12-36)

- STATUS: Major task 5, subtask 1 is complete. IACUC has been submitted and approved. Subtasks 2-4, yet to start.

What was accomplished under these goals? (Detailed progress and results.)

Specific Aim 1: Evaluate antimicrobial effects of LA, C2DA, and combinations released from ESCM (months 1-12)

5. Major Task 1: Investigation of antimicrobial activity of LA therapeutics

As reported in Q1 report, therapeutics such as bupivacaine, lidocaine, ropivacaine, and C2DA with the final LA concentrations [ranging from 0-10 mg mL-1 and C2DA from 0-500 mg mL1] were evaluated for antimicrobial activity against S. aureus (UAMS-1, a clinical osteomyelitis strain), P. aeruginosa (ATCC # 27317), and multidrug resistant Acinetobacter Baumannii (ATCC # BAA-1710™). For S. aureus, lidocaine and bupivacaine had inhibitory effects, while all three had some efficacy against P. aeruginosa planktonic growth (Fig 1). ropivacaine had the highest efficacy against Acinetobacter planktonic, while both bupivacaine and ropivacaine decreased biofilm formation. These effects were more pronounced when C2DA was combined, with some combinations demonstrating synergism or additive effects. Due to the presence of synergism against S. aureus and effectiveness against both P. aeruginosa and A. baumannii at low concentrations, we have selected bupivacaine for evaluation moving forward in elution and activity studies.

Figure 1. Planktonic and Biofilm antimicrobial activity results from therapeutics with varying concentrations.

6. Major Task 2: Manufacturing and Characterization

Subtask 1: Fabrication

As reported in Q1 report, membranes were electrospun using a high molecular weight chitosan (ChitoLytic) with a degree of deacetylation of 86.5% (Fig 2). After membranes were fabricated, punch-outs of 10 mm diameter discs were treated with pyridine and anhydride solutions with varying acyl lengths (hexanoic, octanoic, & decanoic).

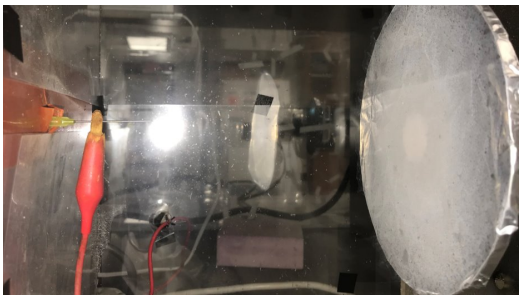


Figure 2. Fabrication of ECSM utilizing an electrospinning set-up.

Subtask 2: Characterization

Treated membranes were imaged with SEM to characterize acylated fibers compared with untreated membranes, with limited fiber swelling suggesting successful modification. FTIR analysis also confirmed the presence of ester bonding and removal of TFA salts, indicating successful acylation reactions for all each of the three anhydride treatments: hexanoic (HA), octanoic (OA), and decanoic (DA) (Fig 3). These quality checks were utilized to ensure consistency before further studies are performed.

NMR has not yet been performed but scheduling with collaborators in the Chemistry department is in progress.

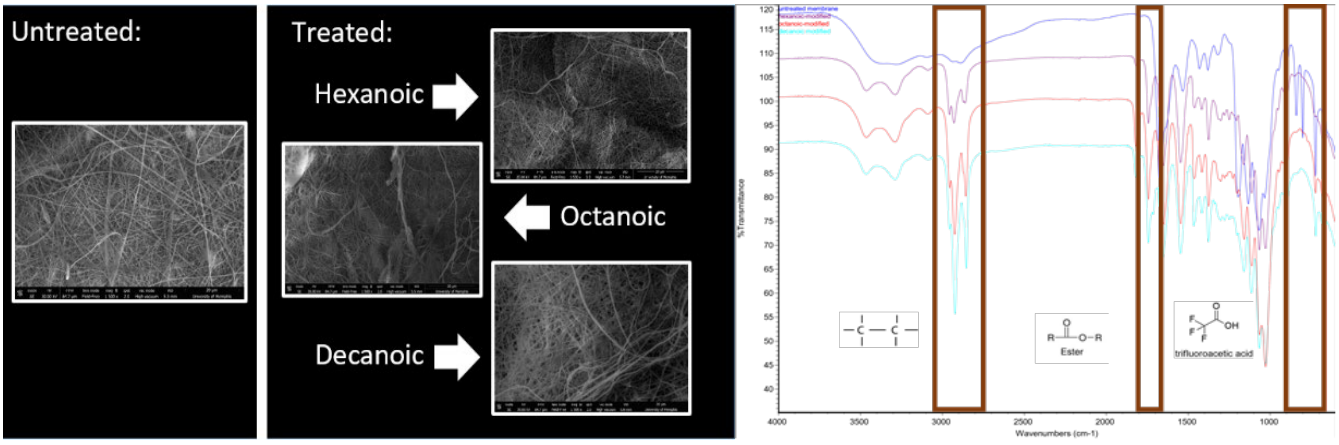


Figure 3. (Left): SEM images taken of experimental membranes. (Right): FTIR analysis of experimental membranes.

Subtask 3: Release

As reported in our Q1 report, preliminary release studies were conducted on HA-ESCM loaded with varying concentrations of C2DA, bupivacaine, or combinations of both therapeutics [loading concentrations of 10,5, 2.5, and 1.25 mg]. Results showed that loading with higher concentrations of bupivacaine produced a larger burst release, which was not observed with lower loading concentrations. C2DA eluted from membranes with no significant burst and showed a sustained release throughout the course of three days, differing by loading concentration. Dual studies showed that the amount of C2DA loaded did not significantly alter the total amount of bupivacaine released and vice versa ($p>0.05$, determined by ANOVA with Holm-Šídák post-hoc tests). 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). See publication appended to this report for further details.

As reported in our Q2 report, due to the high burst release of bupivacaine and high standard deviations seen with C2DA release, new loading concentrations [1.5 mg C2DA/2 mg bupivacaine, 1.5 mg C2DA/1.5 mg bupivacaine, 1 mg C2DA/2 mg bupivacaine, and 1mg C2DA/1.5 mg bupivacaine] was investigated for dual loaded membranes. This assisted in narrowing our loading concentration to 1.5 mg for both treatments moving forward for Q3 report.

As reported in Q3 report, further release studies involved treating membranes with different acyl length fatty acids, loading with both bupivacaine and C2DA, and comparing release from membranes with gauze and chitosan sponge controls. Results showed the octanoic and decanoic modified membranes had a slower release of C2DA, while the other groups, apart from the chitosan sponge, demonstrated a burst release. Octanoic and decanoic modifications prevent high initial release which may be

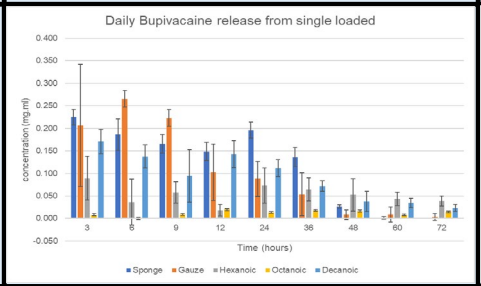
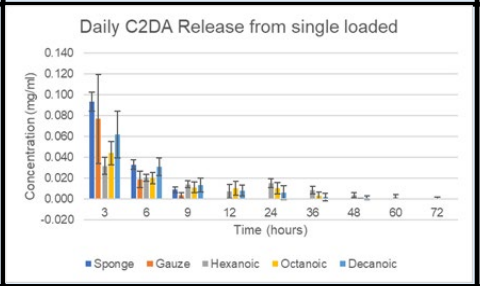
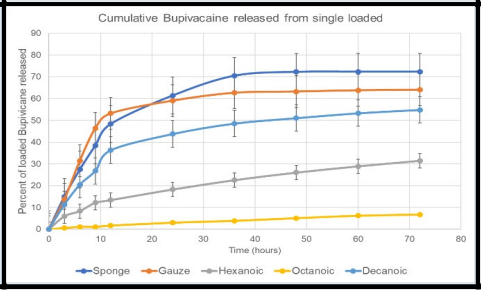
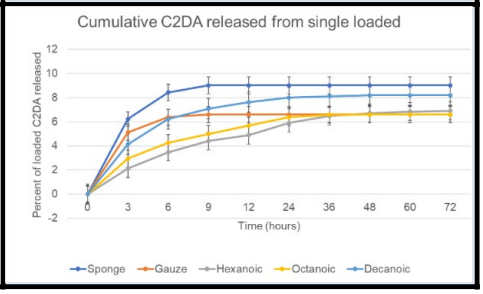
Single Loaded	Bupivacaine	C2DA
Daily Release		
Cumulative Release		

Figure 4. Release of bupivacaine or C2DA from single loaded ESCM and sponge and gauze control materials.

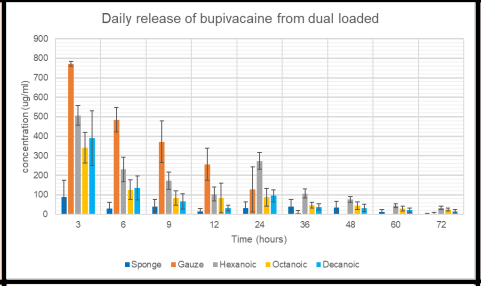
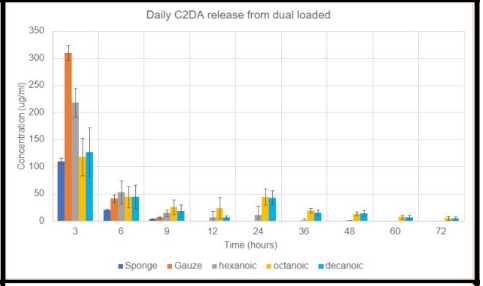
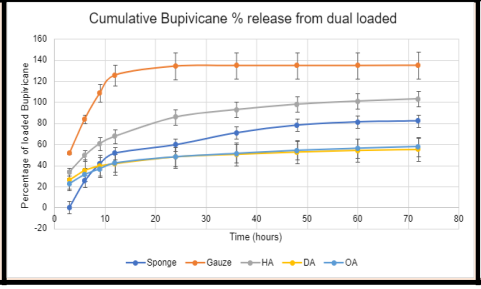
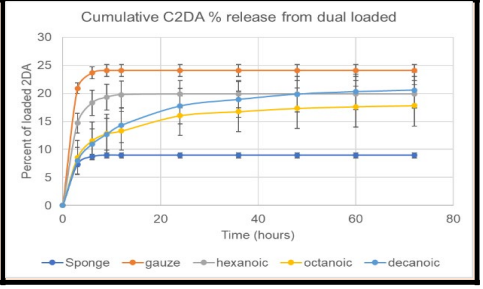
Dual Loaded	Bupivacaine	C2DA
Daily Release		
Cumulative Release		

Figure 5. Release of bupivacaine or C2DA from dual-loaded ESCM and sponge and gauze control materials

beneficial in preventing tissue toxicity during initial release. The chitosan sponge controls showed very minimal release, which may have resulted from a strong association of C2DA to chitosan, or inadequate loading of C2DA. For bupivacaine, membranes of varying acyl lengths released in a similar pattern, with octanoic and decanoic-modified membranes releasing a lower percentage of both therapeutics and at a slower rate. When calculating total amount of bupivacaine released from gauze, erroneous values of 120% total released were calculated, which may be due to a loading error and will be repeated to verify. After Q3, our focus shifted to single-loaded membranes with concentrations of 1.5 mg for each therapeutic (Fig 4). At this concentration, bupivacaine released from decanoic membranes with a high initial burst; a similar release profile was seen for gauze and sponge controls. Other experimental membranes (HA & OA) exhibited a consistent and steady release throughout the duration of the study. For C2DA, results displayed all experimental groups outlasting controls (sponge & gauze) after 9 hours. Additionally, all groups exhibited an initial burst and a diminished concentration after 36 hours. Cumulative data for

C2DA released, depicted all groups releasing less than 10% of the loaded therapeutic. Results from the dual loaded studies, for bupivacaine, showed similarities in release rate for groups OA & DA. For C2DA, one can note that although the controls (sponge & gauze) show a high burst release compared to the experimental membranes, they do not elute therapeutics past 9 hours (Fig 5). This is exhibited as well in the single loaded study. Similar to the single-loaded study, a high initial burst is followed by tapering concentrations, with the exception of the OA and DA membranes releasing at a steady rate throughout the study.

Subtask 4: Antimicrobial studies

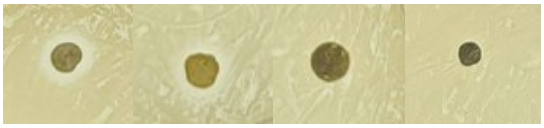


Figure 6. ZOI analysis on disks loaded with therapeutics.

Initial Zone of Inhibition assays determined the preliminary bacterial inhibition from C2DA loaded membranes (Fig 6), and also confirmed that bupivacaine is poorly soluble. After further evaluation of the experimental groups, an observation on the decanoic modified membrane showed a slower and more sustained release, thus we performed antimicrobial studies of DA modified membrane loaded with 1.5 mg of BUP or C2DA or both and compared with both loaded and unloaded sponge, and gauze. This study was performed including 24h and 48h S. aureus, P. aeruginosa, and A. baumannii. The A. baumannii/S. aureus (48 hr)/P. aeruginosa SEM images and P. aeruginosa data are currently being analyzed (Fig 7). Progress of completed work is estimated at 88%. We are also ready to quickly repeat studies, should cell viability studies indicate the need for adjusted loading.

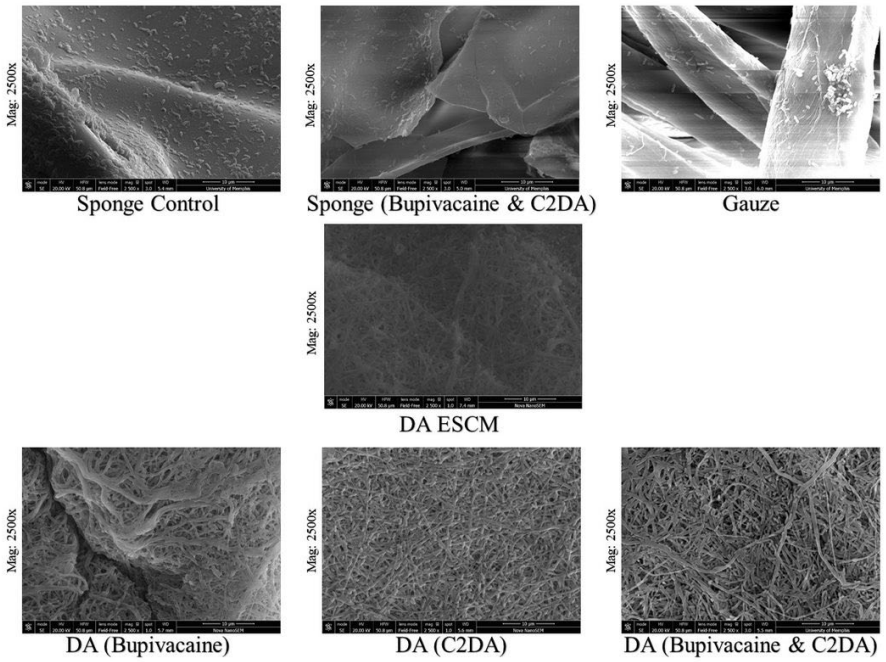


Figure 7. SEM images of control and DA membranes after exposed to P. aeruginosa after 24 hours.

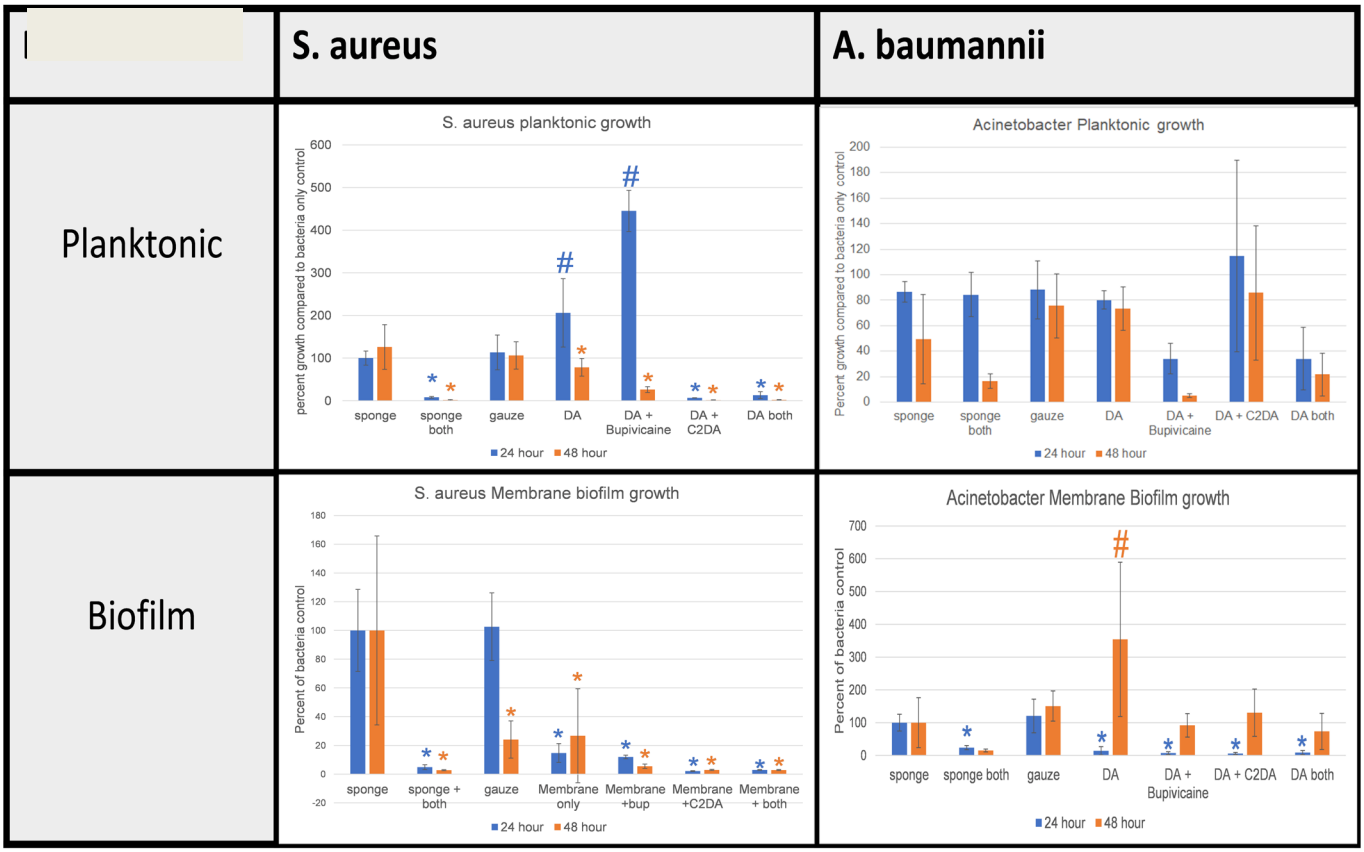


Figure 8. Planktonic and Biofilm results from antimicrobial exposure for 24 and 48 hours (*: significant difference less than control (Sponge), #: significant difference greater than control (Sponge)).

Analysis for antimicrobial results were evaluated comparing materials to the control group (Sponge) (Fig 8). Evaluation of S. aureus antimicrobial activity exhibited significant differences in bacterial viability for all ESCM materials (24 & 48 hours), besides gauze, within the planktonic study. Additionally, out of all the significant differences, only DA and DA + Bupivacaine were the only groups to have a significant difference above the control. As for the biofilm growth, all groups besides the gauze, at 24 hours, were significantly decreased compared to the control.

Evaluation of A. baumannii antimicrobial activity exhibited no significant differences for both 24/48-hour planktonic growth. Unlike the planktonic growth, biofilm growth for A. baumannii showed sponge + both and all DA membranes as significantly different below the control at 24 hours. Additionally, only DA (not treated) at 48 hours remained significantly different but was reported significantly different above control. A common trend between the data collected, indicated the unloaded therapeutics may have minimal antimicrobial activity on their own, as they were similar to sponge and gauze groups. The presence of C2DA facilitates the inhibition of biofilm for S. aureus. In contrast, planktonic Acinetobacter is not as sensitive to C2DA as S. aureus. Significant differences were caluclated with ($p>0.05$, determined by ANOVA with Holm-Šídák post-hoc tests).

Specific Aim 2: Evaluate dermal and inflammatory cell responses to LA, C2DA, and combinations released from ESCM (months 8-20)
Studies to investigate dermal and inflammatory responses to ESCM are underway but incomplete. We have cultured both NHDFs and NHEKs and tested with membranes but are in the process of tailoring loading concentrations to enhance cytocompatibility with both cell types. At 1.5 mg loading, bupivacaine is almost at the 70% cell viability for both cell types, but C2DA alone and combined at 1.5 mg loading appears toxic to both cell types (Fig 9). Studies to determine dose response for each therapeutic are being repeated to determine final loading thresholds. After this is determined, we will reload membranes to repeat cytocompatibility, then proceed with collagen and cytokine assays.

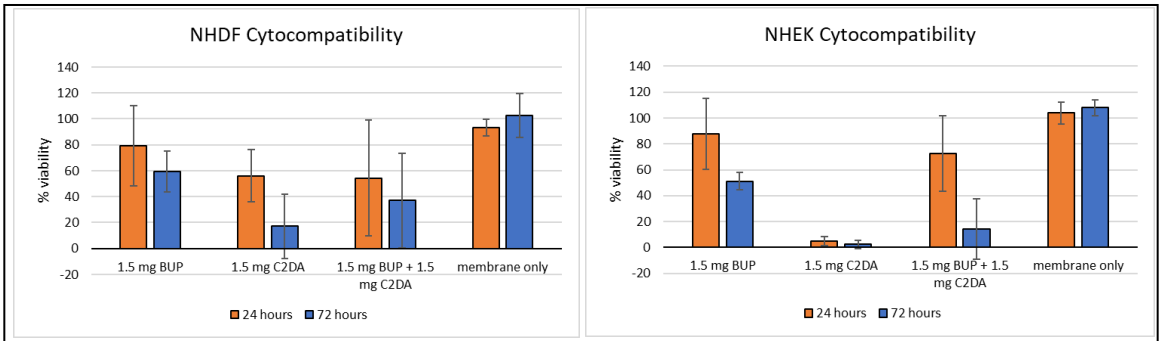


Figure 9. Percent viability of primary dermal fibroblasts (NHDF) and keratinocytes (NHEK) after exposure to decanoic-modified membranes loaded with 1.5 mg bupivacaine, 1.5 mg C2DA, or a combination of both.

Specific Aim 3: Compare ESCM with and without LAs and C2DA to commercially available casualty care standards in an in vivo contaminated rat comb scald wound model (months 12-36)
Not completed. We have obtained approvals from UM IACUC and ACURO. Postdoctoral position will begin in August.

What opportunities for training and professional development has the project provided?

This project has a variety of synthesis and analysis methods needed to evaluate the appropriate samples. Due to this, our staff/students, even if not funded through this grant, have been given the opportunity to learn and operate many of the equipment and studies. Our undergraduate students have been a key example of this, as they have been trained and tasked to assist with studies to reduce analysis time and to learn for future analysis. The following are a list of undergraduates and interns that had assisted in the elution and antimicrobial studies:
Kelli Faust-Bastidas, Mackenzie Edwards, Emily Coleman, Brittany Spencer, Lydia Ross, John David Ross, Bharat Jothlingham, Omar Mohamed
This also applies to graduate students, as they have been tasked with training undergrads by producing SOPs and leading a majority of the studies. The following list of graduate assistants and their professional development activities stemming from this project:
Zoe Harrison — Cell culture experience aiding in her dissertation and for future applications
Landon Choi — Fabrication of a new electrospinning box
Rabeta Yeasmin — Project Class learning about antimicrobial assays to assist in her doctoral research
Ezzuddin Abuhussein — Project class learning about elution and HPLC analysis
Carlos Wells — Synergy studies have allowed for additional data included in his dissertation
Additionally, some of our undergraduate students have been able to present research related to this project on campus at the Student Research forum, held virtually this year, and in return gained experience with presenting scientific research at conferences. The following are students that have presented with their presentation titles and conference:
Student Research Forum:
Emily Coleman—Assessment of cis-2-decenoic acid and local anesthetics for synergistic antimicrobial and biofilm reduction effects [won first place in undergraduate category]
John-David Ross —Acylation treatment of cis-2-decenoic loaded chitosan membranes for the inhibition of biofilm growth
Lydia Ross—Loading chitosan membranes with local anesthetic and antibiofilm therapeutics
Omar Mohamed—Evaluation of the antimicrobial properties of Acyl-modified chitosan membranes

How were the results disseminated to communities of interest?

The following are the ways the PI and collaborators have shared information regarding this project with the scientific community and the general community:

The PI has submitted an abstract, which was accepted for poster presentation at MHSRS in August. However, we were recently informed that this conference has been cancelled.
Recent progress and potential translational activities have been included in the University of Memphis Research Foundation Board meetings, as well as a brief YouTube video along with other researchers in the Herff School of Engineering during Herff Advisory Council meetings. The project goals were also presented as part of a presentation by the PI on Biomaterials at Girls Experiencing Engineering and the UrbanSTEM Collaborative, which were held virtually this year.
Additionally, see publications and/or presentations.

Plans for the next reporting period to accomplish the goals

We plan to finish subtask 2 & 4 of Specific Aim 1, Major Task 2. Subtask 2 will conclude after completion of NMR, which has been delayed due to the scheduling with the Chemistry department. Subtask 4 will conclude after SEM of the rest of the samples.
We plan to continue efforts towards subtask 1, 2, and 3 of Specific Aim 2, Major Task 3. Subtask 1 will be analyzing human dermal cell response to treated ECMs. Subtask 2 focuses on collagen production, which will be performed after obtaining supernatants from subtask 1. Subtask 3 will consist of cytokine production, which will also be pending on subtask 1 supernatants.
We will begin subtask 1 of Specific Aim 2, Major Task 4. Subtask 1 will be performed to determine the ECM's effects towards cell activation and pro-healing with inflammatory cells. We also will be working with co-investigator Bumgardner on planning for the animal study, since for a separate funded project we will start evaluating materials in the rat comb scald model.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The development of a biopolymer modification strategy to tailor drug release over time impacts the field of biomedical engineering and drug delivery. Our understanding of material properties and how they may be applied could lead to the development of other functional materials for antimicrobial or other therapeutic delivery. Chitosan is a biodegradable, antimicrobial, and versatile biopolymer. In this project we electrospin chitosan solution into a fibrous membrane, one that has an increased surface area, woven to be breathable, and capable of loading therapeutics and other agents. Biomaterial fabrication strategies such as electrospinning may be useful in burn wound dressings, orthopaedic wraps, or tissue engineering scaffolds. Our evaluation of the effect of materials and released therapeutics on dermal and inflammatory cells shapes our understanding of the potential benefits and risks of these novel dressings as we move toward translating them into the clinic and battlefield use.
A key impact of this project is the development of clinical tools to protect the patient from infection and promote healing. Our understanding of antimicrobial material characteristics has been expanded to evaluate both the planktonic and biofilm formation characteristics they inhibit or promote. As we further investigate the interactions between biofilm inhibitors and anesthetic molecules, we can design materials and loading strategies to heal and protect burn wounds, as well as other combat or non-combat-related injuries. Our investigation of multiple different strains of pathogenic bacteria impacts the field of microbiology in adding knowledge of the effects of locally delivered therapeutics on bacterial viability and biofilm formation. Similarities or differences in response can guide development of therapeutics and may impact clinical guidelines for infection prevention and treatment. Our observation of altered exopolymeric substance production in response to these materials could lead to new understanding of the biofilm response for these microorganisms.

What was the impact on other disciplines?

With the advancement in evaluating therapeutics and biomaterials, related to the anti-bacterial/anti-biofilm affects they possess, knowledge gained in this project may apply to other engineering materials that require antimicrobial properties. For instance, electrospun chitosan modified with antimicrobials may be used in water filtration media in civil engineering.

What was the impact on technology transfer?

Discussions and negotiations have been in progress with Chitolytic to acquire a license to further develop this into a commercially available bandage. Additional progress entails discussions with Tripler Air Force Base Researchers regarding potential collaborations for moving forward.

What was the impact on society beyond science and technology?

By introducing a biomaterial to address wound healing we are supporting improved patient care through the use of biomaterials in medical treatments. As we move closer to our goal, these therapeutics could help a wide range of people that have been burned and in need of antimicrobial/pain relief. This could impact society by improving overall patient outcomes and reducing the costs of burn therapies to patient and society.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

The primary cells ordered to confirm the macrophage immune response are reported to have variable nitric oxide response, contrasting with other macrophage cell lines, which was proposed as a measure of inflammation in aim 2. Therefore, in addition or as an alternative to measuring nitric oxide through the Griess assay, we will image cells and use Cell Profiler free software to classify macrophage phenotype by shape.

Actual or anticipated problems or delays and actions or plans to resolve them

Issues with characterizing antimicrobials through the HPLC have been resolved resulting in a SOP for the other assistants in the lab. Restrictions in ESCM fabrication have been addressed and a new electrospinning box was constructed with the intent to reduce the membrane synthesis by half the time. The postdoctoral associate who has accepted the offer will not be able to move and begin until August. There have been some delays in synthesis/characterization within the chemistry department due to non-functional air conditioning unit (making the work environment unsafe to operate equipment), and collaboration for the solid-state NMR analysis.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

TOTAL PROTOCOL(S): 1
PROTOCOL (X of Y total):
IACUC Protocol Number: **0865**

ACURO Protocol Number: MB190046.e001
Protocol PI: Jessica Jennings, PhD
Protocol Site: University of Memphis
Protocol Title: Acylated Electrospun Biopolymer Membranes for Burn Wound Coverage, Infection Prevention, and Pain Relief
Number of Animals Approved for Use: 120
IACUC Initial APPROVAL DATE: 1/21/2021 (expires 01/21/2024)
ACURO initial APPROVAL DATE: 4/21/2021
RENEWAL APPROVAL DATES: Due 4/21/2024
AMENDMENTS:
- **One to update protocol to both male and female rats**

adverse events or unanticipated problems:
- **None.**

Significant changes in use of biohazards and/or select agents

Not applicable.

6. PRODUCTS

Journal publications

1. Harrison, ZL, Bumgardner, JD, Fujiwara, T, Baker, DL, and Jennings, JA, *In vitro evaluation of loaded chitosan membranes for pain relief and infection prevention.* J. Biomed. Mater. Res. B Appl. Biomater., 2021.
    a. Original manuscript
    b. Published
    c. Directly related to SOW, specific aim 1
    d. DoD funding acknowledged
2. Wells, CM, Harrison, ZL, Coleman EC, Jennings, JA, Antimicrobial and anti-biofilm efficacy of local anesthetics combined with cis 2 decenoic acid against Staphylococcus aureus, Pseudomonas aeruginosa, and Acinetobacter baumannii. Frontiers in Cellular and Infection Microbiology.
    a. Original manuscript
    b. Under Review
    c. Directly related to SOW, specific aim 1
    d. DoD funding acknowledged

Books or other non-periodical, one-time publications

Nothing to report

Other publications, conference papers, and presentations

1. Choi L, Wells C, Harrison Z, Bumgardner J, Fujiwara T, and Jennings J, Choi L*. April. 2021. *Acylation of electrospun chitosan membranes with medium chain fatty acids*. Society for Biomaterials Annual Meeting, Virtual-Chicago
    a. Talk
    b. Presented
    c. Directly related to SOW, specific aim 1
    d. DoD funding acknowledged
2. Harrison Z, Bumgardner J, Fujiwara T, Baker D, and Jennings J, Harrison Z*. April. 2021. *In vitro evaluation of loaded chitosan membranes for pain relief and infection prevention*. Society for Biomaterials Annual Meeting, Virtual-Chicago
    a. Talk
    b. Presented
    c. Directly related to SOW, specific aim 1
    d. DoD funding acknowledged
3. Harrison Z, Bumgardner J, Fujiwara T, Baker D, and Jennings J, Harrison Z*. February. 2021. *In vitro evaluation of loaded chitosan membranes for infection prevention*. Orthopaedic Research Society Annual Meeting, Virtual
    a. Talk
    b. Presented
    c. Directly related to SOW, specific aim 1
    d. DoD funding acknowledged

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Jessica Amber Jennings
Project Role: PI
Researcher Identifier: https://orcid.org/0000-0002-2760-6948
Nearest person month worked: 0.75
Contribution to Project: Project management and supervision of graduate assistants

Name: Daniel Baker
Project Role: co-I
Researcher Identifier:
Nearest person month worked: 0.3
Contribution to Project: Synthesis of C2DA and input on detection methods

Name: Tomoko Fujiwara
Project Role: co-I
Researcher Identifier: https://orcid.org/0000-0002-3329-0361
Nearest person month worked: 0.3
Contribution to Project: Analysis of FTIR and input on fabrication methods

Name: Joel Bumgardner
Project Role: co-I
Researcher Identifier:
Nearest person month worked: 0.25
Contribution to Project: Input on electrospinning and modification of chitosan, analysis of release results

Name: Landon Choi
Project Role: MS graduate assistant (funded from this grant)
Researcher Identifier:
Nearest person month worked: 1.5
Contribution to Project: Release Studies, electrospinning of chitosan, performing acylation treatments, and SEM

Name: Zoe Harrison
Project Role: PhD graduate assistant (funded by departmental funds)
Researcher Identifier: https://orcid.org/0000-0002-5276-450X
Nearest person month worked: 1.5
Contribution to Project: Release studies and analysis, SEM imaging

Name: Rabeta Yeasmin
Project Role: PhD graduate assistant (funded on another grant, Project course work)
Researcher Identifier:
Nearest person month worked: 1
Contribution to Project: Antimicrobial studies of modified membranes

Name: Ezzuddin Abuhussein
Project Role: PhD graduate assistant (funded on another grant, Project course work)
Researcher Identifier:
Nearest person month worked: 0.5
Contribution to Project: HPLC and analysis

Name: Brian C. Hoffman
Project Role: PhD graduate assistant (Chemistry)
Researcher Identifier:
Nearest person month worked: 0.5
Contribution to Project: synthesis of C2DA

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Each of the investigators are also now part of an active award that started in March. Specifics are listed below:

Jessica Amber Jennings

Project Title: Development of acylated nanofibrous chitosan membranes for pre-hospital wound coverage, infection inhibition, and pain mitigation	
New award	Total Award Period Covered: 3/15/21 –3/14/23
Project Objective(s): 1. Determine and characterize the stability of membranes modified by direct acylation with C2DA (ECX) and determine the release kinetics of C2DA and bupivacaine from ECX. 2. Determine antimicrobial efficacy of ECX against gram-negative and gram-positive pathogens, potential synergism with bupivacaine versus biofilm, and protection of ECX surface from biofilm. 3. Determine efficacy of ECX with and without bupivacaine in preventing infection in an *in vivo* biofilm-associated composite tissue infection model.	
Award Amount:	Time Committed to the Project Per Year: 3.5%
Name of Awarding Agency/Funder: Peer Reviewed Medical Research Program	
Address of Awarding Agency/Funder: 1077 Patchel StreetFort Detrick, MD 21702-5024	
Contracting/Grants Officer: Annmarie Gersch	
Overlap: NA	

Daniel Baker

Project Title: Development of acylated nanofibrous chitosan membranes for pre-hospital wound coverage, infection inhibition, and pain mitigation	
New award	Total Award Period Covered: 3/15/21 –3/14/23
Award Amount:	Time Committed to the Project Per Year: 2%
Name of Awarding Agency/Funder: Peer Reviewed Medical Research Program	
Address of Awarding Agency/Funder: 1077 Patchel StreetFort Detrick, MD 21702-5024	
Contracting/Grants Officer: Annmarie Gersch	
Overlap: NA	

Tomoko Fujiwara

Project Title: Development of acylated nanofibrous chitosan membranes for pre-hospital wound coverage, infection inhibition, and pain mitigation	
New award	Total Award Period Covered: 3/15/21 –3/14/23
Award Amount:	Time Committed to the Project Per Year: 2%
Name of Awarding Agency/Funder: Peer Reviewed Medical Research Program	
Address of Awarding Agency/Funder: 1077 Patchel StreetFort Detrick, MD 21702-5024	
Contracting/Grants Officer: Annmarie Gersch	
Overlap: NA	

Joel Bumgardner

Project Title: Development of acylated nanofibrous chitosan membranes for pre-hospital wound coverage, infection inhibition, and pain mitigation	
New award	Total Award Period Covered: 3/15/21 –3/14/23
Award Amount:	Time Committed to the Project Per Year: 1%

Name of Awarding Agency/Funder: Peer Reviewed Medical Research Program
Address of Awarding Agency/Funder: 1077 Patchel StreetFort Detrick, MD 21702-5024
Contracting/Grants Officer: Annmarie Gersch
Overlap: NA

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHART

9. APPENDICES

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RESEARCH ARTICLE

Society For Biomaterials WILEY

In vitro evaluation of loaded chitosan membranes for pain relief and infection prevention

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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 1,000 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 hr, 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
anesthetic, biofilm, biomaterial, bupivacaine, chitosan, electrospun, infection, local drug delivery, staphylococcus, wound dressing

1 | INTRODUCTION

There is a growing demand for local delivery systems to treat and prevent infection and alleviate pain during wound healing.[1] 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.[2,3] 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 1,000× the typical minimum inhibitory concentration of antibiotics.[4] Once infection is present, the removal of implanted materials and/or aggressive debridement of wounded tissue are often the only successful strategies for treating the infection.[5,6] Long term antibiotic use can help to manage biofilm infections, but often fails while also increasing risk of antibiotic resistance and toxic side effects.[7] Furthermore, overuse of aminoglycoside antibiotics can lead to nephrotoxic and ototoxic side effects.[8] 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.[9] 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.[10,11] Previous studies have investigated delivery of C2DA in other local delivery systems, including chitosan/polyethylene glycol (PEG) sponges and phosphatidylcholine coatings.[4,12,13] 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 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.[16–18] 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.[1,16] 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.[19] There are several formulations of creams and transdermal patches that contain local anesthetics, with some available for over-the-counter use.[20,21] 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.[22] 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.[23] 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,[24] 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 (a) act as a barrier to microbial contamination, (b) release local anesthetics in a controlled manner that reduce pain and modify the inflammatory response, and (c) 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.

2 | MATERIALS AND METHODS

2.1 | 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 26 kV as previously described.[23,25] 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.[23] 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.[24] Membranes were loaded with either C2DA (31.25, 62.5, 125, or 250 μg), bupivacaine (1.25, 2.5, 5, or 10 mg), or a combination of both treatments. Therapeutic concentrations were dissolved in ethanol (200 proof) and applied to membranes; membranes were then dried aseptically in laminar flow hood to allow ethanol evaporation, leaving therapeutics incorporated within the membrane fibers.[24]

Our target loading was to be within a therapeutic window for daily release that does not exceed the concentration that could be cytotoxic but still maintains antimicrobial activity. In previous studies, C2DA concentrations of 500 μg/ml completely inhibited *S. aureus* growth, with biofilm inhibition observed at lower concentrations in the nanomolar range.[11] Since concentrations of 1 mg/ml or above were toxic to mammalian cells, we chose a total dose that would be 4-fold lower than this dose. The maximum single dose of bupivacaine is 175 mg, but can have therapeutic activity at lower doses.[26] Higher concentrations, up to 20 mg/ml, are only used for instances like spinal anesthesia; therefore amounts released are adequate for wound treatment applications. 0.5% bupivacaine is a standard clinical concentration and is not detrimental to wound healing, which correlates with a

concentration of 5 mg/ml.[27] Our concentration selected for the high concentration of bupivacaine for combination loading is 35-fold lower than the maximum dose.

2.2 | 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 hrs. The 72 hrs elution period was selected as the maximum time that may elapse between wound dressing changes, which are typically changed daily or every other day.[28] The concentration of C2DA and bupivacaine in the eluates was measured with high performance liquid chromatography (HPLC) using a ThermoScientific Dionex Ultimate 3,000 Series HPLC system (Figure S1 and Table S1). All eluate concentrations were normalized to standard curves with known concentrations of C2DA and bupivacaine.

2.3 | Zone of inhibition studies

To determine the baseline antimicrobial characteristics of the HA-ESCM, membrane groups were evaluated in modified Kirby-Bauer zone of inhibition (ZOI) 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 10^5 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 hrs. Resulting zones were measured using ImageJ software and compared to vancomycin controls as well as paper disk controls.

2.4 | Biofilm assays

Biofilm inhibitory properties were tested by direct inoculation of freshly loaded HA-ESCM and membranes after elution in PBS for 72 hrs. 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°C for 24 hrs, membranes were removed from wells, rinsed twice with sterile PBS, and sonicated for 5 min at 40 kHz (Fisher Scientific Ultrasonic Bath, 9.5 L) to remove biofilm-associated bacteria. Quantification of biofilm was determined using BacTiter-Glo® Microbial Cell Viability Assay (Promega).

The presence of viable planktonic bacteria was determined for wells with membranes. Supernatant from wells containing membranes and bacteria was removed and added to a new 96 well plate, then combined with BacTiter-Glo® to quantify the amount of planktonic bacterial growth after 24 hrs 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.

2.5 | 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 1×10^4 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 hrs at 37°C and 5% CO2. After overnight incubation, membranes were placed within the upper chamber of transwells. After 24 hrs, 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.

2.6 | Statistical analysis

Statistical analysis was performed using SigmaPlot and GraphPad Prism 7.2 software (GraphPad Software Incorporation, La Jolla, CA). 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.

3 | RESULTS

3.1 | Elution

3.1.1 | 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 1a). 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.

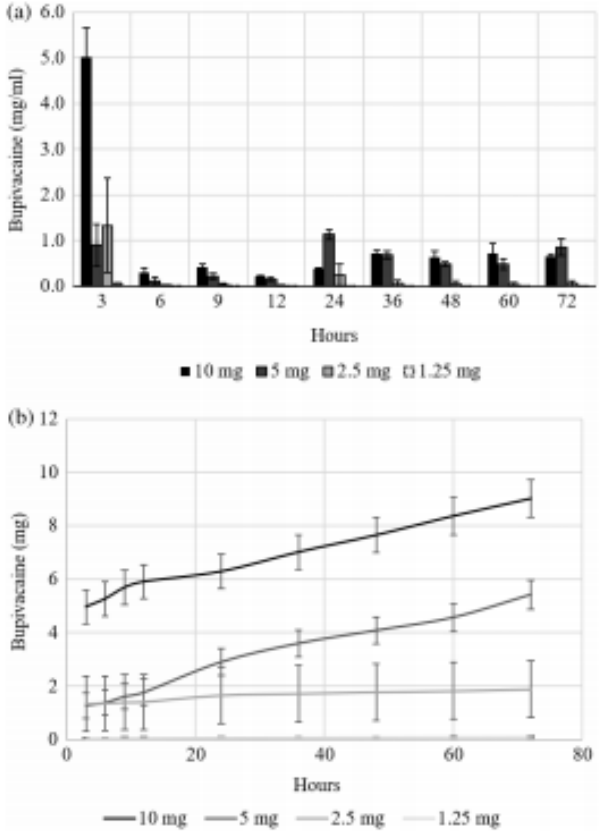

FIGURE 1 Graphs detailing (a) bupivacaine release and (b) cumulative bupivacaine release by HA-ESCM loaded with 10, 5, 2.5 or 1.25 mg of bupivacaine throughout 72-hour elution study. Release was determined by high performance liquid chromatography (HPLC). Data is represented as mean ± standard deviation (n = 5). Lines connecting points are intended to guide the eye

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 1b).

3.2 | C2DA elution

C2DA eluted from membranes with no significant burst, showing a sustained release throughout the course of 3 days, differing by loading concentration (Figure 2a)). Percentage of total therapeutic released was determined for each group following 72-hour elution (Table 1). Following results of this elution study, 250 μg and 125 μg loading concentrations were selected for future tests as these produced the highest cumulative release of C2DA(Figure 2b).

TABLE 1 Release percentages for each membrane group

Loading amount		Percent released	
Bupivacaine (mg)	C2DA (μg)	Bupivacaine (mg)	C2DA (μg)
10	0	90.14	N/A
5	0	~100	N/A
2.5	0	75.36	N/A
1.25	0	7.47	N/A
0	250	N/A	46.25
0	125	N/A	70.08
0	62.5	N/A	~100
0	31.25	N/A	22.09
5	250	72.22	~100
5	125	66.68	~100
2.5	250	93.56	~100
2.5	125	75.29	~100

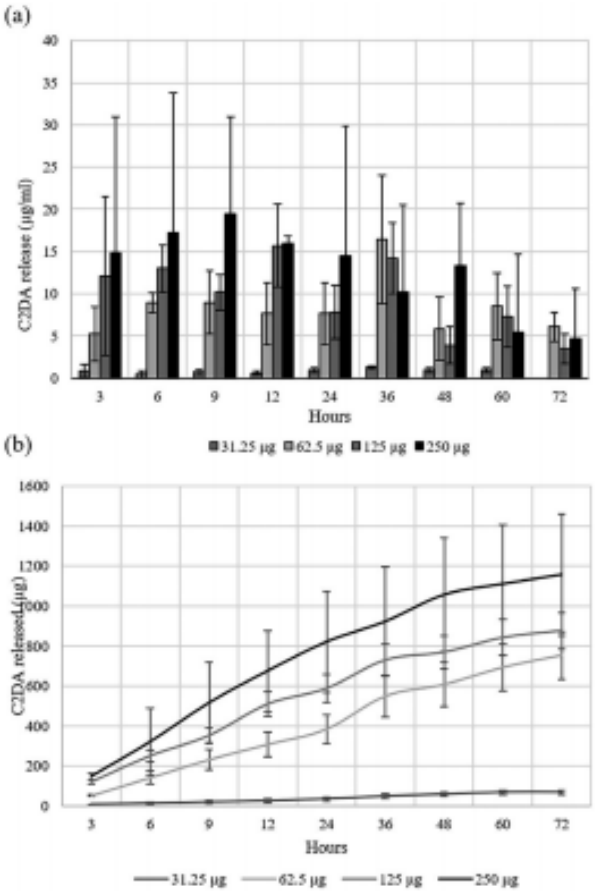

FIGURE 2 Graphs detailing (a) C2DA release and (b) cumulative C2DA release by HA-ESCM loaded with 250, 125, 62.5, or 31.25 μg of C2DA throughout 72-hour elution study. Release was determined by high performance liquid chromatography (HPLC). Data is represented as mean ± standard deviation (n = 5). Lines connecting points are intended to guide the eye

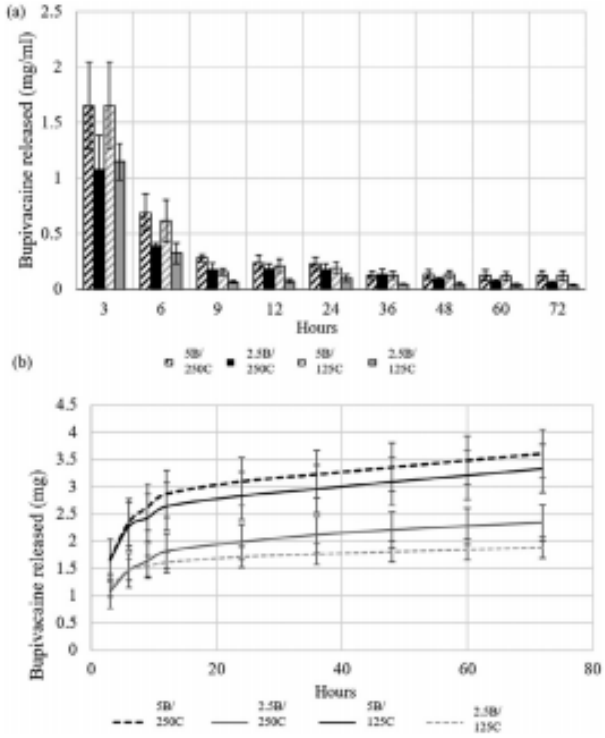

FIGURE 3 Graphs of (a) bupivacaine release and (b) cumulative bupivacaine release from dual-loaded HA-ESCM. Data is represented as mean ± standard deviation (n = 5). Lines connecting points are intended to guide the eye

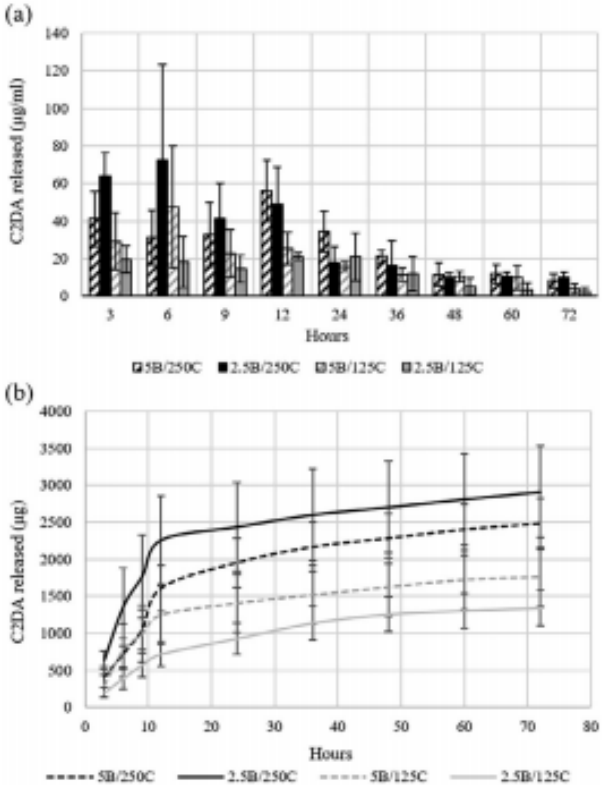

FIGURE 4 C2DA release from dual-loaded membranes. Graphs of (a) C2DA release and (b) cumulative C2DA release from dual loaded membranes modified by hexanoyl acylation. Data is represented as mean ± standard deviation (n = 5). Lines connecting points are intended to guide the eye. Significance was determined by ANOVA with Holm-Šídák post-hoc tests

3.3 | 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-Šídák post-hoc tests) (Figure 3). Percentage of total therapeutic released was determined for each group following 72-hour elution (Table 1). 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.

C2DA released from dual loaded membranes with a slight burst during the first 12 hrs, followed by a lower sustained release for the last 60 hrs. 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 4). Percentage of total therapeutic released was determined for each group following 72-hour elution (Table 1).

3.4 | Zone of inhibition

Results of Kirby-Bauer ZOI studies (Table 2) show highest therapeutic release by groups containing 250 µg of C2DA, while unloaded membranes, membranes loaded with just bupivacaine (BUP), 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 S1 and Table S1).

3.5 | 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 250 μg of C2DA after 72-hour elution, resulted in less viable planktonic bacteria growth than was seen in the gauze controls (Figure 5).

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 6).

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 7).

TABLE 2 Table indicating 24-hour Zone of Inhibition (mm) against Methicillin-resistant *S. aureus*, measured using ImageJ software (NIH), for each membrane type ($n = 4$)

Loading concentration	Zone of inhibition (mm)
5 mg BUP	0.84± 0.48
2.5 mg BUP	0.62 ± 0.42
250 μg C2DA	3.09 ± 0.29[a]
125 μg C2DA	1.23 ± 0.25[a]
5 mg BUP + 250 μg C2DA	3.48 ± 0.98[a]
5 mg BUP + 125 μg C2DA	0.16 ± 0.07
2.5 mg BUP + 250 μg C2DA	2.99 ± 0.09[a]
2.5 mg BUP + 125 μg C2DA	0.16 ± 0.03
Unloaded membrane	0.15 ± 0.01
Paper disk control	0.00 ± 0.00
Vancomycin control[a]	4.19 ± 0.49

[a]Indicates significantly higher zone than paper disk control, as determined by one-way ANOVA with Holm-Šídák post-hoc tests ($p < 0.05$).
Note: ± represents standard deviation.

3.6 | 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 8).

4 | 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

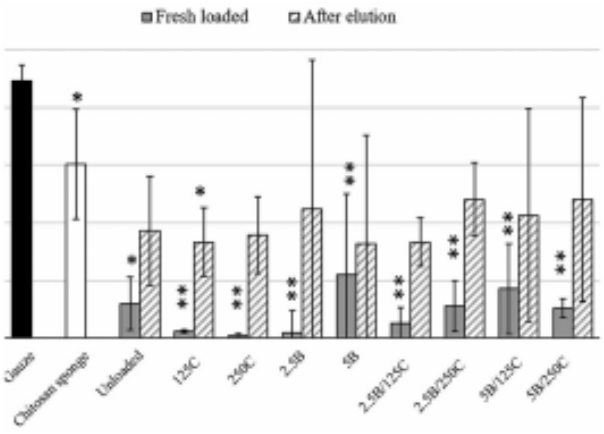

FIGURE 5 Planktonic bacteria growth. Graphical representation of planktonic *S. aureus* growth in direct contact with membranes or controls ($n = 4$). Amount of viable bacteria was quantified based on metabolic activity by measuring ATP production. Results were then normalized as a percentage compared 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-Šídák post-hoc tests

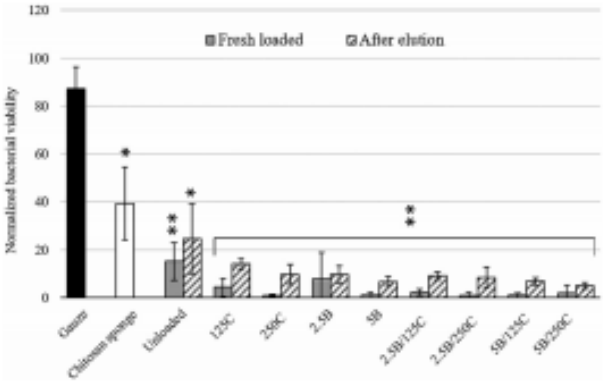

FIGURE 6 Biofilm growth on membranes. Graphical representation of *S. aureus* biofilm growth on membranes or controls ($n = 4$). Amount of viable bacteria was quantified based on metabolic activity by measuring ATP production. Results were then normalized as a percentage compared to untreated control 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-Šídák post-hoc tests

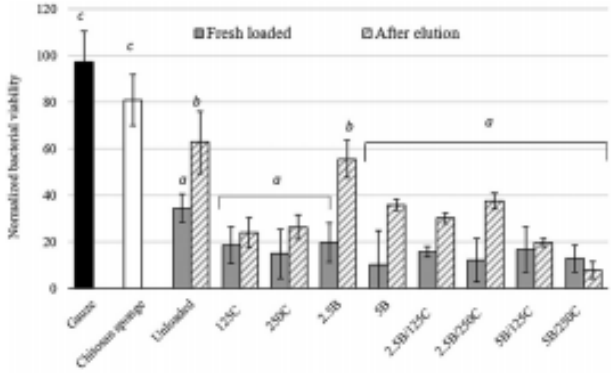

FIGURE 7 Biofilm growth in wells. Graphical representation of *S. aureus* biofilm growth in wells beneath membranes or controls ($n = 4$). Amount of viable bacteria was quantified based on metabolic activity by measuring ATP production. Results were then normalized as a percentage compared to untreated 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

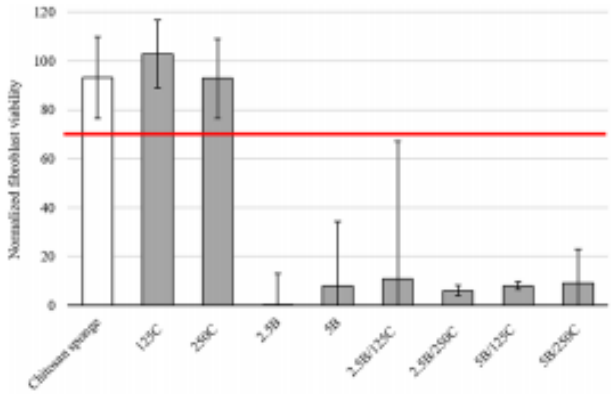

FIGURE 8 Hexanoic acid-treated electrospun chitosan membranes (HA-ESCM) cytocompatibility with fibroblasts. Graphical representation of cytocompatibility results for each membrane type when evaluated with L929 fibroblasts cells ($n = 5$). Amount of viable fibroblasts was quantified based on metabolic 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

opioids, which often leads to unwanted consequences like opioid misuse, dependence, and consequential addiction.[29] 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.[30] 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 hrs 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.[31] 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.[32] 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,[11] 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 hrs.[12] 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, though limit of detection studies indicate that the methods used can reliably detect C2DA at concentrations as low as 5 μg/ml. 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.[24] 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.[33]

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 hrs 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.[34] These results are consistent with previous studies that found nanomolar amounts of C2DA to be active against bacteria.[10,35,36] 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.[32] Due to the higher burst release seen during the first 6 hrs 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.[37] 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.[32,37] 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.[17] Bupivacaine specifically is known to be more toxic, especially in tissues with high aerobic demand and low tolerance for hypoxia.[38]

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.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [repository name e.g "figshare"] at http://doi.org/[doi], reference number [reference number].

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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