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

PRINCIPAL INVESTIGATOR: Jessica Jennings, PhD

CONTRACTING ORGANIZATION: University of Memphis, Memphis, TN

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Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)
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5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Memphis (Herff College of Engineering)
315 Administration Building
Memphis, TN 38152

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
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13. SUPPLEMENTARY NOTES

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. To assess these ESCM, bulk fabrication of ESCM was performed as well as loading treatments (Bupivacaine 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

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
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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 Y2Q7, completed Major Task 1. Completed Major Task 2, subtask 1 complete, subtask 2 complete, subtask 3 complete. Subtask 4 complete.

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

- STATUS: Major Task 3: subtask 1 complete. Subtask 2 completed. Subtask 3 begun, 50% complete, Major Task 4 not started.

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 continues as model observed on separately funded project. Subtasks 3 and 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)

Major Task 1: Investigation of antimicrobial activity of LA therapeutics

Completed. Reported in Q1 and Q7 reports.

Major Task 2: Manufacturing and Characterization

Subtask 1: Fabrication

Completed. Reported in Q1.

Subtask 2: Characterization

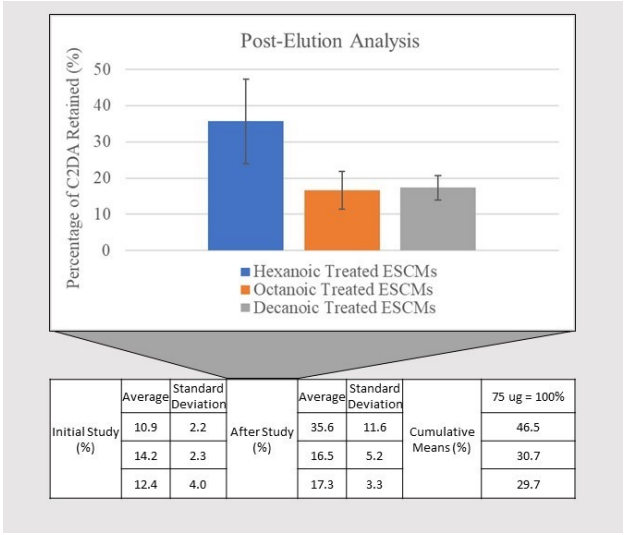
Completed. Reported in Q1, Q2, Q6, and Q7 reports.

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.

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 for the amount of C2DA retained on treated ESCMs, used in initial elution, showed hexanoic ESCMs retaining ~35% of C2DA, octanoic ESCMs retaining ~16% of C2DA, and decanoic ESCMs retaining ~17% of C2DA (Figure 1). Cumulative means summed from initial elution and post elution analysis showed more C2DA loaded on hexanoic treated ESCMs compared to octanoic and decanoic treated ESCMs. Analysis from the samples not used in elution were investigated for loaded therapeutics (Figure 2). Results indicated loading of therapeutics as less than 40% for control group gauze (both therapeutics), additionally mean values of 60% of C2DA and over 100% of theoretic loading bupivacaine were detected for control group chitosan sponge. Experimental groups seemed to have mean values of 50% or more of C2DA loaded on ESCMs and 60% or more of bupivacaine loaded on ESCMs. This analysis of loading may inform future strategies to ensure that loading is consistent for future studies and to interpret the release data. Further, it indicates that some active therapeutics may be retained within the membranes to offer extended protection from contamination.



	Average	Standard Deviation		Average	Standard Deviation		75 ug = 100%
Initial Study (%)	10.9	2.2	After Study (%)	35.6	11.6	Cumulative Means (%)	46.5
	14.2	2.3		16.5	5.2		30.7
	12.4	4.0		17.3	3.3		29.7

Figure 1. Percentage of remaining therapeutic left on samples, post 3-day elution study, and percent cumulative means summed from initial elution (n=5).

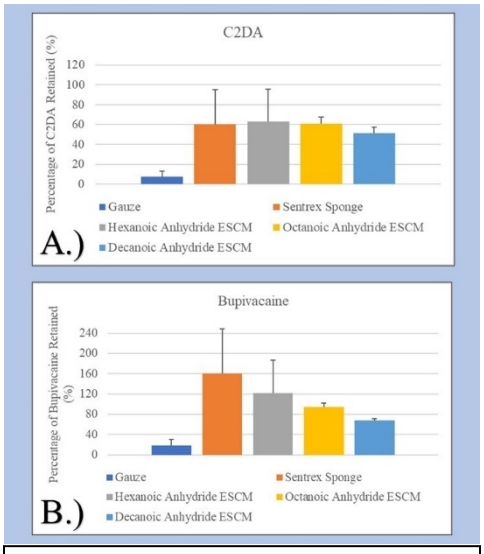


Figure 2. Percentage of loaded therapeutics on half samples not used in the initial elution. A.) Percentage of C2DA loaded on samples and B.) percentage of bupivacaine loaded on samples (N=2).

In a repeat of the elution studies with lower concentrations due to our initial cell culture viability studies showing toxicity of higher loading levels, patterns of elution were similar. Therapeutics elute out of gauze up to 3 days with low concentrations by 24 hours, while chitosan sponge ceases to elute by 36 hours (Figure 3). There was elution for gauze group to 40 µg within the first 3 hours followed by a 4x decrease while chitosan sponge elution was below 25 µg within the first 3 hours followed by a 3-4x decrease. Results also indicate the experimental groups releasing detectable concentrations of bupivacaine out to 3 days, with octanoic membranes releasing up to 70 µg within the first 3 hours followed by a 7x decrease as well as the highest amount eluted between the other groups until hour 60. As for the release/mass for C2DA, unlike the bupivacaine data, all experimental groups seemed to show a higher burst release than the control groups (Figure 4). All groups eluted less than 5 µg of C2DA within the first 3 hours, but traces of bupivacaine were only detected for the experimental groups by 24 hours.

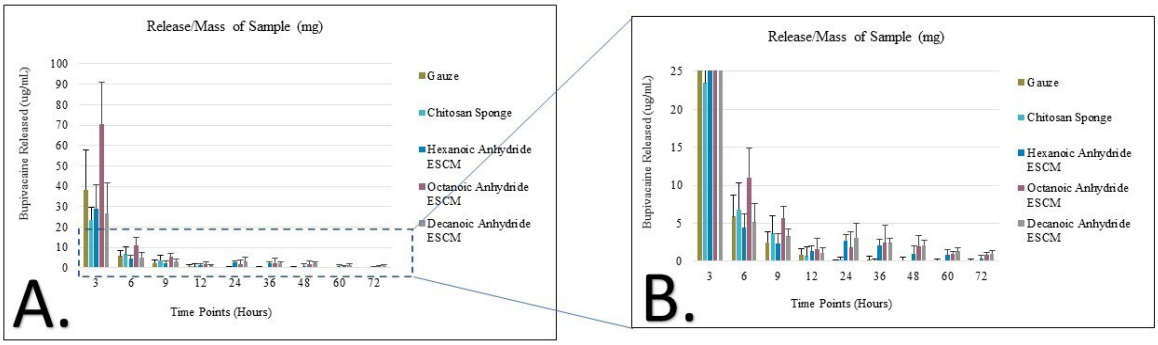


Figure 3. Three days of bupivacaine release A.) divided by weight per mass of sample, B.) bupivacaine release over the course of 3 days divided by weight per mass of sample (upper limit of 25 µg/mL). Values plotted are means ± standard deviation.

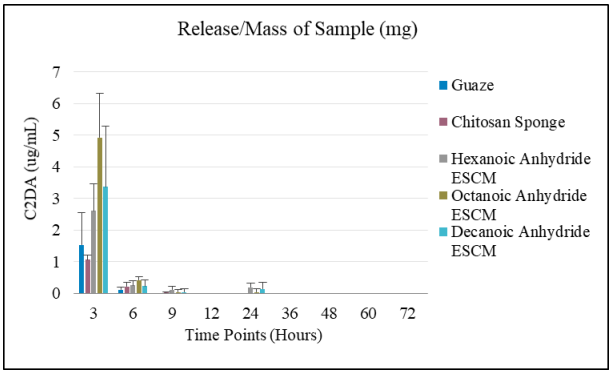


Figure 4. Three days of C2DA release divided by weight per mass of sample. Values plotted are means ± standard deviation.

Subtask 4: Antimicrobial studies
Zone of Inhibition, biofilm formation, and planktonic cell viability studies were reported in Q3-7 reports.

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. Cytocompatibility with NHDF and NHEK cells has previously been reported, with viability improved when C2DA and BUP are delivered together instead of alone. RAW264.7 rat monocytes were studied in this quarter and had similar results, with simultaneous delivery of both molecules increasing viability over delivering either molecule alone.

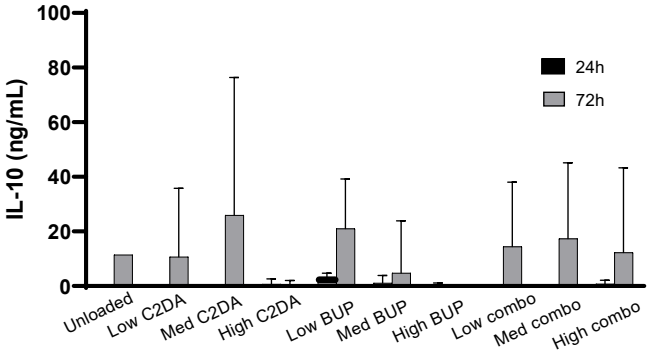
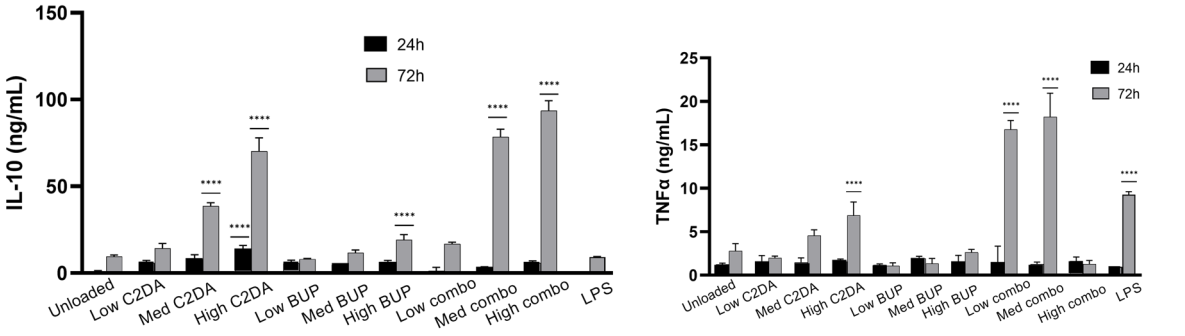


Figure 5. IL-10 production by NHEK in contact with DA-ESCM for 24 or 72 hours (n = 4). Individual data points are shown as bars representing mean and error bars representing standard deviation. No significant differences were determined between groups..

Collagen production of fibroblasts cultured with the loaded membranes was reported in Q7. Further studies have been performed to evaluate cytokine production of NHEK and RAW264.7 cells using ELISAs. The production of cytokines IL-10 and IL-12 were evaluated at 24 and 72 hours in NHEK cell culture. No cytokine production was detectable at 24 hours. After 72 hours, IL-10 was seen in all groups that delivered both C2DA and BUP (Figure 5), however, it was not statistically different than controls with only membranes in culture. No IL-12 response was seen in any of the NHEK groups. RAW264.7 cells showed increased IL-10 production after 72 hours in all groups that had high concentrations of C2DA, with minimal production at 24 hours. The RAW264.7 cells also showed increases in TNF-alpha after 72 hours in simultaneous delivery groups delivering low to medium concentrations of the therapeutic molecules (Figure 6).



A **B**

Figure 6. Production of (A) IL-10 and (B) TNF-α by monocytes in contact with DA-ESCM for 24 or 72 hours (n = 4). Individual data points are shown as bars representing mean and error bars representing standard deviation. Concentrations were normalized based on viability for each sample. **** indicates significantly higher cytokine production compared to unloaded control, detected using one-way ANOVA with Tukey's post hoc tests ($p < 0.0001$).

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. Cytocompatible concentrations of C2DA and bupivacaine have now been obtained for use in the animal model.

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:
Emily Coleman, Brittany Spencer, Brenna Ballard, Peter Engstrom, Isabella Bianca-Reano, Payton Freeman, Gustavo Jimenez Cienfuegos
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 — Conducting elution and defending thesis centered around this work.
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:
Gustavo Jimenez Cienfuegos – Evaluation of the Effectiveness in a Novel Anti-microbial Against Bacterial Biofilm

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, September 12-15, 2022.

Recent progress and potential translational activities have been included in the University of Memphis Research Foundation Board meetings, Additionally, see publications and/or presentations.

Plans for the next reporting period to accomplish the goals

We plan to finish subtask 3 of Major Task 3, and begin subtask 2 of Major Task 5.
Subtask 3 of Major Task 3 consists of cytokine production studies using a MAGPIX panel measuring multiple targets. Major Task 4, subtask 1 will follow with a similar method looking at cytokine production of monocytes.

Major Task 5, subtask 2 is performing the animal scald model. We have been working with co-investigator Bumgardner on planning for the animal study, observing a separately funded project to evaluate materials for the scald model. The animal model will continue.

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. A meeting with the CEO of this small business will occur in August next quarter. We are also connecting with investigators at Tripler Air Force Base regarding potential collaborations 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

NMR was proposed to study the degree of substitution of carboxylic acids on the surface of the chitosan membranes. This equipment is no longer available, and XPS was used to determine elemental ratios on the surface so that a degree of substitution could be calculated as reported in Q7.

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

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; additional amendment in June 2022 to add investigators and new students**

adverse events or unanticipated problems:

- **None.**

Significant changes in use of biohazards and/or select agents

Not applicable.

6. PRODUCTS

Journal publications

1. 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 Revision
    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, Bumgardner J, Fujiwara T, and Jennings J, Evaluation of acyl-modified chitosan membranes loaded with cis-2-decenoic acid and bupivacaine for infection prevention. in Joint Symposium of the Society for Biomaterials and Japanese Society for Biomaterials. 2022. Honolulu, HI.
    a. Poster
    b. Presented
    c. Directly related to SOW, specific aim 1
    d. DoD funding acknowledged

2. Choi L*. December 2021. *Evaluation of acyl-modified chitosan membranes loaded with cis-2-decenoic acid and bupivacaine for infection prevention*. St. Jude Children's Research Hospital Advances in Antibacterial Discovery Virtual Symposium, Memphis, TN
    a. Talk
    b. Presented
    c. Directly related to SOW, specific aim 1
    d. DoD funding acknowledged

3. Abuhussein E, C, Coleman E, and Jennings J, Abuhussein E*. *Antimicrobial and anti-biofilm efficacy of local anesthetics combined with cis-2-decenoic acid against Staphylococcus aureus*. Orthopaedic Research Society Annual Meeting. February 2022. Tampa, FL.
    a. Poster
    b. Presented
    c. Directly related to SOW, specific aim 1
    d. DoD funding acknowledged

4. Yeasmin R, Choi L, Harrison Z, Coleman E, and Jennings J. *Antimicrobial study of acylated chitosan nanofibers loaded with local anesthetics and cis-2-decenoic acid against Staphylococcus Aureus*. Orthoapaedic Research Society Annual Meeting. February 2022. Tampa, FL.
    a. Poster
    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

Name: Emily C. Montgomery
Project Role: MS graduate assistant
Research Identifier: https://orcid.org/0000-0003-1389-0586
Nearest person month worked: 1.2
Contribution to Project: Release and antimicrobial studies

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

Nothing to report.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHART

9. APPENDICES