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TITLE: 3D-Fabricated Nitric Oxide-Releasing Matrix as an Antimicrobial Dressing

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CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX.

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<b>14. ABSTRACT</b> Appropriate wound dressings or the application of bandages at the time of injury is the first line of defense to prevent wound infection caused by microorganisms. These microorganisms are strongly associated with hospital acquired infections contributing to the persistence of debilitating wound infections in both military and non-military patients. Nitric oxide (NO), a highly reactive gas, inherently possesses antimicrobial effect. Due to its volatile nature, storage and delivery of NO has been a great challenge. In this project, we propose to develop nitric oxide releasing polymer matrix, NOmatrix, as an antimicrobial dressing. Briefly, we fabricated nanofibrous NOmatrix via electrospinning system by incorporating biocompatible polymers and NO donor, NONOates. The 3D fabricated NOmatrix was evaluated in vitro for its NO releasing kinetics. The antimicrobial effects of NOmatrix against pathogens including methicillin-resistant <i>S. aureus</i> (MRSA) and <i>P. aeruginosa</i> , were evaluated. The toxicity or safety of NOmatrix was evaluated on fibroblasts derived from human dermal tissues. Lastly, the efficacy of NOmatrix will be evaluated in vivo using a well-established skin infection rodent model. The 3D-fabricated NOmatrix as a dressing, because of its simple and inexpensive fabrication method, will be a major advancement in the rapid and efficient treatment of antimicrobial resistant infections and nosocomial infections.					
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## 1. INTRODUCTION

Wounded military personnel face the high infection rate during the initial hospitalization. Immediate wound care is critical to avoid infections which can lead to prolonged hospitalization, increased morbidity, and slow rehabilitation. Appropriate wound dressings or the application of bandages at the time of injury is the first line of defense to prevent wound infection. Broad-spectrum antibiotics are administered as a prophylactic measure to prevent nosocomial infections during surgical procedures, increasing the risk of developing antimicrobial resistance. These microorganisms are strongly associated with hospital acquired infections contributing to the persistence of debilitating wound infections in both military and non-military patients. Nitric oxide (NO), a highly reactive gas, inherently possesses antimicrobial effect. Due to its volatile nature, storage and delivery of NO have been a great challenge. In this project, we propose to develop nitric oxide releasing polymer matrix, NOmatrix, as an antimicrobial dressing. Briefly, we fabricated NOmatrix as nanofibers via electrospinning system by incorporating biocompatible polymers and NO donor, NONOates. The fabricated NOmatrix was evaluated in vitro for its NO releasing kinetics, which demonstrated the NOmatrix controlled the release of NO. The antimicrobial effects of NOmatrix against pathogens including methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa*, were evaluated to show that NOmatrix was able to inhibit the microbial growth. The minimal toxicity or safety of NOmatrix was demonstrated on fibroblasts derived from human dermal tissues. Lastly, the efficacy of NOmatrix will be evaluated in vivo using a well-established skin infection rodent model. The 3D-fabricated NOmatrix as a dressing, because of its simple and inexpensive fabrication method, will be a major advancement in the rapid and efficient treatment of antimicrobial resistant infections and nosocomial infections.

## **2. Keywords**

Antimicrobial dressing, nitric oxide, NONOates, biopolymers, electrospin, 3D fabrication

### 3. ACCOMPLISHMENTS:

#### What were the major goals of the project?

The major goals of this project focuses on accomplishing the following 3 major tasks as proposed in the SOW:

**Major Task 1.** Fabrication of NOmatrix via electrospinning. Duration: 9 months (months 1-9)

**Major Task 2.** Evaluation of in vitro antimicrobial effect of NOmatrix. Duration: 8 months (months 6-13); 85% completion

**Major Task 3.** *in vivo* evaluation of efficacy in a skin infection mouse model. Duration: 13 months (months 1-4, 10-18); 25% completion

#### What was accomplished under these goals?

In this section, the results accomplished are described for each major task. Specifically, we have accomplished: the following objectives defined under each task.

#### **Major Task 1. Fabrication of NOmatrix via electrospinning**

*Duration: 9 months (months 1-9)*

In this task, the goal was to fabricate NO-releasing NOmatrix and evaluate the release kinetics of NO. Two objectives to follow: **Objective 1.** Electrospinning of NOmatrix as nanofibers; **Objective 2.** Characterization and optimization of NO release kinetics from NOmatrix. Completion of each objective and the outcomes are described below.

#### **Objective 1.** Electrospinning of NOmatrix as nanofibers

Under this objective, NOmatrix was fabricated via the electrospinning process (Figure 1A). Biopolymer solution containing NO donor, NONOates, were prepared then electrospun as nanofibers. The electrospun NOmatrix separated from the collector formed a thin, white sheet as shown in Figure 1B, top and bottom images.

- (a) Selection of biopolymers and nitric oxide donor compounds

For the NOmatrix fabrication, the selection of an appropriate biopolymer is crucial. The ideal candidate of a biopolymer should (1) be highly biocompatible; (2) yield soft yet stable fibers; (3) protect premature NO release. Biopolymers such as polyvinyl pyrrolidone (PVP), poly lactic-glycolic

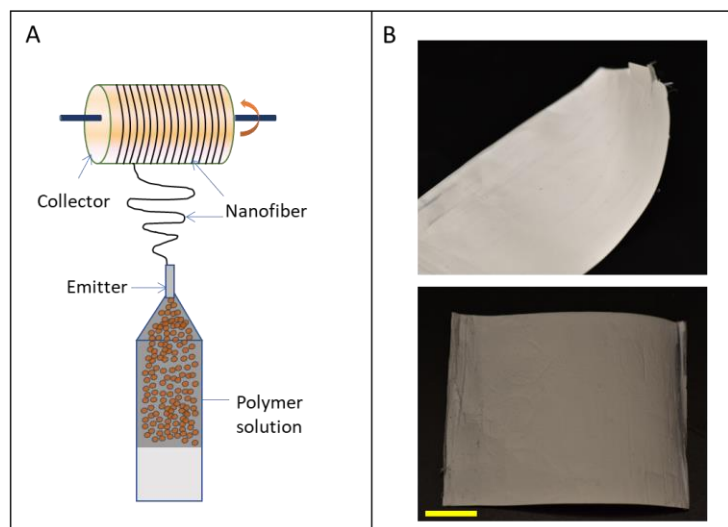


Figure 1. Fabrication of NOmatrix. (A) Schematic of electrospinning system; (B) Images of electrospun NOmatrix sheet, top: side view of the sheet; bottom: a large sheet of NOmatrix. Scale bar = 1cm

acid, polyvinyl alcohol, and cellulose, are widely used in clinical settings, especially in drug delivery systems. Since NO is rapidly release upon contact with water, it is necessary to avoid aqueous solvents in the NOMatrix fabrication. Based on its solubility and compatibility with non-aqueous solvents, two biopolymers were selected, PVP and ethylcellulose (EC), both obtained from Sigma

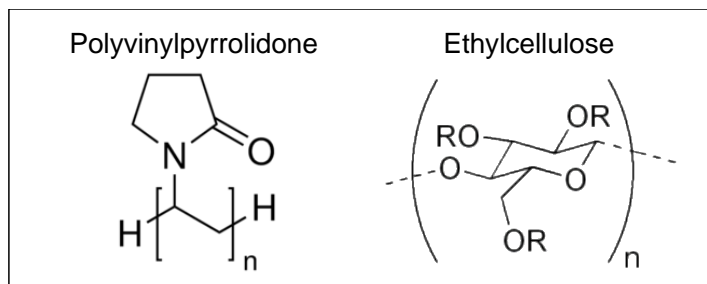


Figure 2. Chemical structures of biopolymers, polyvinylpyrrolidone (PVP) and Ethylcellulose (EC)

Aldrich, St. Louis, MO (Figure 2). PVP has been used as a plasma expander, also can be found in personal hygiene products and pharmaceutical applications. Owing to its polarity, PVP is not only water soluble but also soluble in non-aqueous, organic solvents. EC is cellulose derived synthetic biopolymer which can be found in pharmaceutical formulations as an excipient. EC is not soluble in water but readily soluble in non-aqueous, organic solvents. These selected biopolymers were used to fabricate NOMatrix as a reliable storage and delivery planform of NO.

Diazeniumdiolate, also known as NONOate, is a NO donor compound containing a diolate group that releases two molecules of NO upon contact with water or moisture. NONOates can be chemically modified with functional groups to control the rate of donor decomposition which in turn affects the half-lives of NONOates (Figure 3). In this study, three NO donor compounds with different functional groups, proline (PROLI), dipropylenetriamine (DPTA), and diethylenetriamine (DETA), were selected and incorporated into NOMatrix. PROLI NONOate, DPTA NONOate, and DETA NONOate exhibit NO releasing half-lives of 2s, 3h, and 20h, respectively under physiologically relevant conditions (37 °C and pH 7.4). All NONOates were obtained from Cayman Chemicals, Ann Arbor, MI.

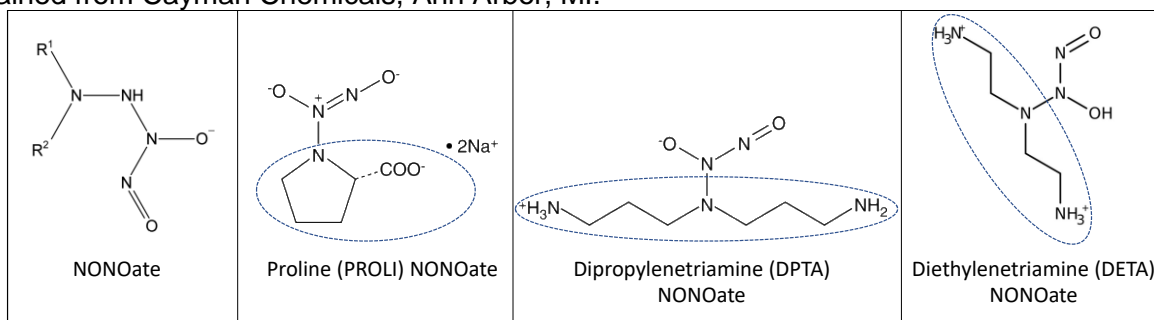


Figure 3. Chemical structures of NO donor compound NONOate and NONOates with various functional groups shown in circles

### (b) Preparation of biopolymer solutions

First, biopolymer solutions either PVP or EC were prepared by dissolving dry polymer powders in anhydrous ethanol (5-10% w/v; Simga Aldrich). Solutions were kept at 50°C on a hot stirring plate (ThermoFisher Scientific, Waltham, MA) for 3-5 hours until the polymers completely dissolved. However, NONOates did not dissolve or disperse well in ethanol. Even though NO is instantly dissociated from NONOates in water, it is quite stable in alkaline solution. NONOates were dissolved in 10mM NaOH and immediately added to the biopolymer solution. Thus prepared NONOate-alkaline solutions were miscible with ethanol forming a homogeneous solution.

(c) Fabrication of NOmatrix as nanofibers via electrospinning system.

The NONOate containing polymer solution was transferred into a syringe for the NOmatrix fabrication. The electrospinning process is a commonly used method to fabricate polymeric fibers for various applications such as textile manufacturing, filter membranes, and scaffolds for tissue engineering. Electrospinning is a straightforward and highly reproducible process to produce polymeric fibers ranging from a few nanometers to micrometers providing a higher surface-to-volume ratio. It also allows easy control of mechanical properties of electrospun fibers such as porosity and mechanical strengths by manipulating processing parameters. The physical properties of the fibers can be adjusted by altering the polymer concentration, the voltage, and the speed of solution injection.

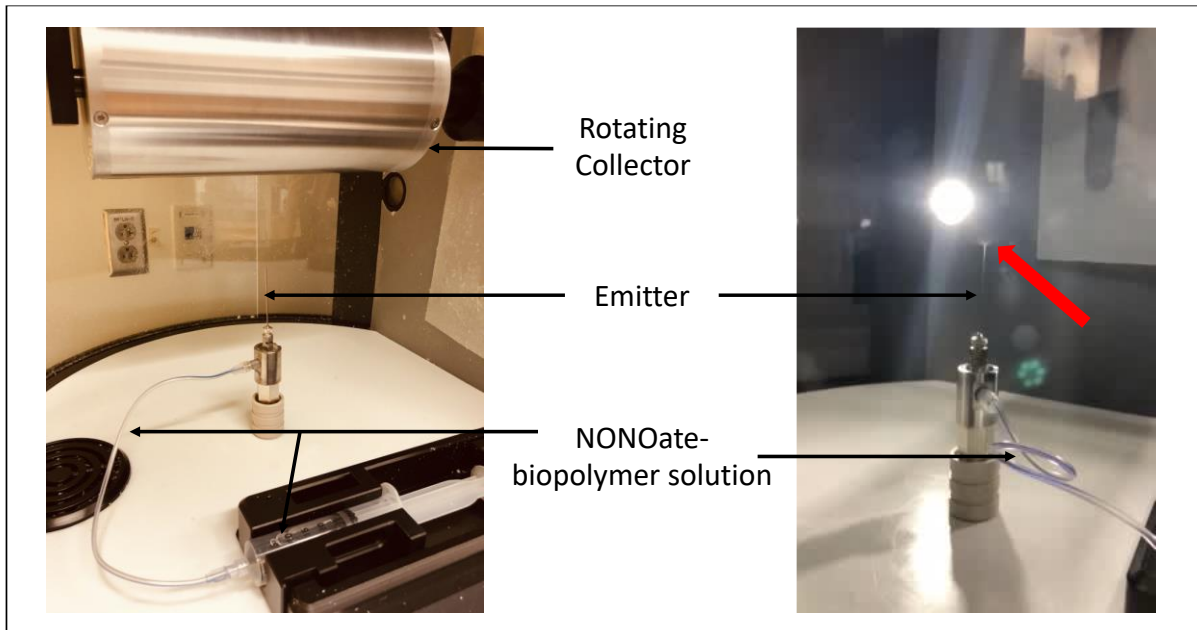


Figure 4. Images of 4SPIN electrospin system. Red arrow points fibers forming from the tip of the emitter when high voltage is applied.

A desk top electrospin system, 4SPIN (Contipro, Czech Republic) is a unique system in which the collector is placed above the emitter (Figure 4). This prevents accidental dripping of polymer solutions on the collected nanofibers in between the spinning process. The tip of the syringe containing a biopolymer solution was connected to a single jet emitter where high electric voltage is applied. Upon applying a high voltage, 10-15kV, the solution was injected at a rate of 30  $\mu\text{L}$  per minute and emitted through the injector tip (19 gauge) then the polymeric fibers were formed as the solvent evaporates. Emitted nanofibers were deposited on a grounded rotating collector at 1000 rpm. The rotating collector aligned nanofibers and maintain the 3D structure of fibers better compared to a static collector (Figure 5D). These collected nanofibers were carefully separated from the collector then were cut into a smaller sheet, NOmatrix. NOmatrices were kept in a sealed container.

**Objective 2.** Characterization and optimization of NO release kinetics from NOmatrix

Under this objective, thus fabricated NOmatrix was characterized and its NO release kinetics in the physiologic conditions were evaluated *in vitro*. The nanofiber formation via

electrospinning was confirmed by scanning electron microscope (SEM). In Figure 5, SEM images revealed that nanofibers formed from electrospinning biopolymer solutions containing PROLI-NONOates, DPTA-NONOates, DETA-NONOates. From Fig 5A-C, it is shown that nanofibers in DETA-NOMatrix had larger diameters ranges from 300nm to 800nm than the nanofibers in PROLI-NOMatrix and DPTA-NOMatrix, diameter ranges from 100nm to 250nm. Figure 5D shows the SEM image of NOMatrix fabricated on a flat, static surface. The nanofibers on a static collector showed randomly arranged fibers with bigger gaps between fibers. This demonstrated the nanofibers in NOMatrix on the rotating collector were aligned better and more packed than the nanofibers on the static collector.

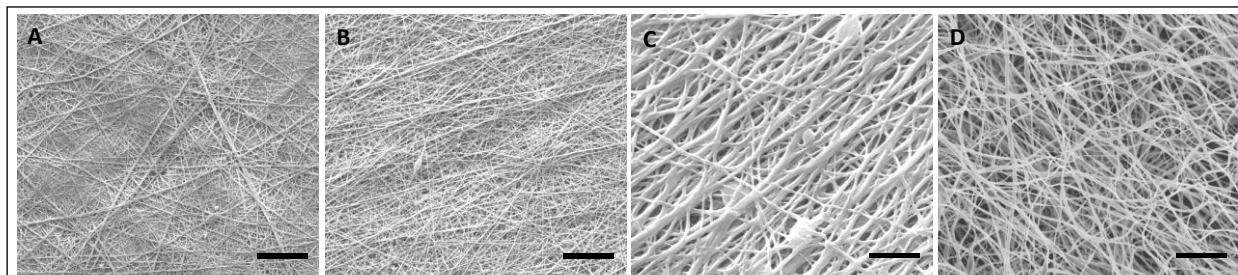


Figure 5. SEM images of electrospun nanofibers of NOMatrix. (A) PROLI-NOMatrix (B) DPTA-NOMatrix (C) DETA-NOMatrix (D) Randomly arranged NOMatrix collected on a static surface. Scale bar = 5  $\mu$ m

The incorporation of biopolymers enhanced a long-term storage condition of NO at room temperature for a few weeks. Overall, PVP-NOMatrix showed more controlled fashion of release kinetics than EC-NOMatrix up to 48 hours. This was an unexpected outcome as we initially anticipated ethylcellulose, a water-insoluble biopolymer, to release NO for a longer time in comparison with water-soluble PVP. A burst release of NO from EC-NOMatrix was observed within the first 30 minutes. We believe that the release of NO from EC-NOMatrix within 120 min is caused by NONOates on the surface of NOMatrix (Figure 6A-C). Then the NO release reaches plateau followed by the burst release. It is possible that the hydrophobic EC is repelling water molecules to react with NONOate embedded in the biopolymer. In the case of PVP-NOMatrix, even though PVP disintegrated rapidly NO was released continuously up to 2 days in a controlled fashion. Despite the short half-life of PROLI NONOate, PVP-NOMatrix released NO up to 2 days. For EC-NOMatrices containing DPTA NONOate and DETA NONOate, less than 20% of NO was released within 120 min (Figure 6B&C).

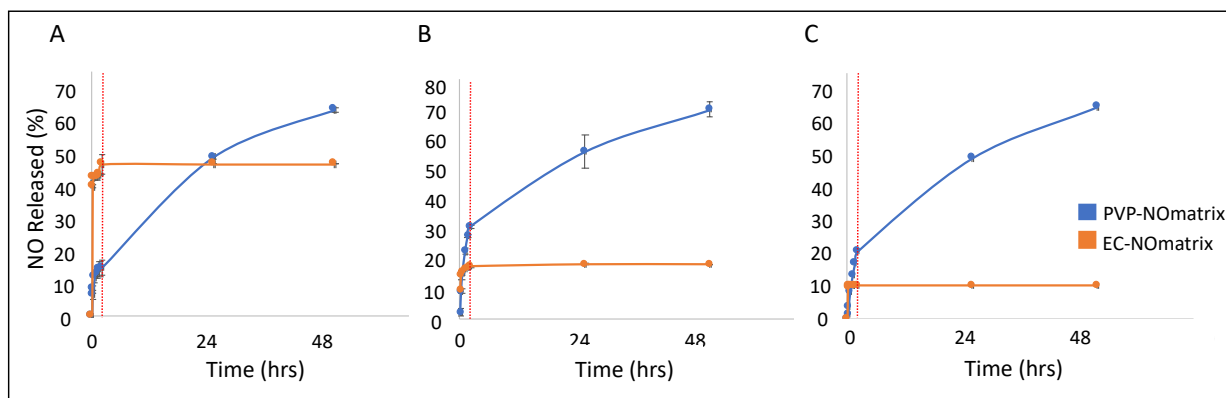


Figure 6. in vitro release of NO from NOMatrix prepared with various NONOates. (A) PROLI-NOMatrix (B) DPTA-NOMatrix (C) DETA-NOMatrix. Red dotted lines show 2 hr marks.

#### (a) Characterization of NOmatrix

In order to confirm the nanofiber formation, NOmatrix was examined by scanning electron microscope (SEM). NOmatrix was cut into 2mm pieces and placed onto a carbon tape mounted on an aluminum stub. Aluminum stubs were then sputter-coated with gold/palladium at 4nm thickness (Leica EM ACE600, Leica Microsystems, Germany). Coated samples were imaged using a Field Emission SEM (SU8230, Hitachi, Japan). Images were obtained using a secondary electron detector, 5-20kV, and at 9mm working distance.

#### (b) Evaluation and optimization of NO release kinetics

Once NOmatrix was prepared, we evaluated the release kinetics of NO from NOmatrix *in vitro*. NOmatrix was cut into a circular disc (6mm diameter) and placed in a dialysis device (Slide-A-Lyzer MINI Dialysis Device, ThermoFisher Scientific, Waltham, MA). The dialysis device containing NOmatrix was placed in a conical tube containing phosphate buffered saline (PBS) and kept in an incubating orbital shaker at 37 °C and 200 rpm (MaxQ 4450, ThermoFisher Scientific, Waltham, MA). All samples were prepared in triplicate. At predetermined time points, small aliquots of the solution in the conical tube were collected and stored at 4 °C until further analyses.

Collected samples were subjected to fluorometric/colorimetric assay using commercially available assay kits (NO Assay, Cayman Chemical, Ann Arbor, MI). A NO fluorometric assay is a sensitive method allowing quantitating NO concentration with the minimum detection limit of 60 nM. In addition, NO colorimetric assay was used to evaluate the higher concentration of NO released from NOmatrix. The released NO undergoes reactions yielding nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) as final products. These assays measure the total NO by combining measured nitrite and nitrate as fluorescence or absorbance intensity. Briefly, the nitrate reductase was added to the collected sample to convert nitrate to nitrite in a 96-well plate. For nitrate measurement, the enzyme cofactors were added prior to the addition of the nitrate reductase. Then 2,3-diaminonaphthalene (DAN) solution was added, followed by NaOH to enhance the signal of the fluorescent products. The fluorescence intensity was measured using a plate reader (CLARIOstar, BMG LABTECH GmbH, Germany) at 360 nm excitation wavelength and 430 nm emission wavelength. For the colorimetric assay, the Griess reagents were used and the absorbance was measured at 540 nm.

### **Task 2. Evaluation of *in vitro* antimicrobial effect of NOmatrix**

*Duration 8 months (months 6-13)*

In this task, fabricated NOmatrix was evaluated *in vitro* for its antimicrobial effect and safety/toxicity in bacterial cultures and cell cultures, respectively. There were two objectives: Objective 1. Evaluation of the antimicrobial effect of NOmatrix and Objective 2. *In vitro* cytotoxicity evaluation of NOmatrix. Detailed outcomes and procedures are described below.

#### **Objective 1. Evaluation of the antimicrobial effect of NOmatrix**

Under this objective, the *in vitro* antimicrobial effect of NOmatrix was evaluated using the bacterial cultures of Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA), and Gram-negative *Pseudomonas aeruginosa* (Figures 7&8). The growth curve of MRSA in Figure 7A showed moderate inhibition of NOmatrixes on MRSA growth over 24 hours. Interestingly, PROLI-NOmatrix prepared with EC was the most effective with 50% reduction in microbial concentration after 24 hours of treatment (Figure 7B). Considering the *in vitro* release kinetics,

approximately 50% of NO from PROLI-NOmatrix prepared with EC was released within the first two hours. Although further investigation is needed to confirm, it seems that in order to inhibit the growth of MRSA, it requires a large quantity of NO at an early stage of growth.

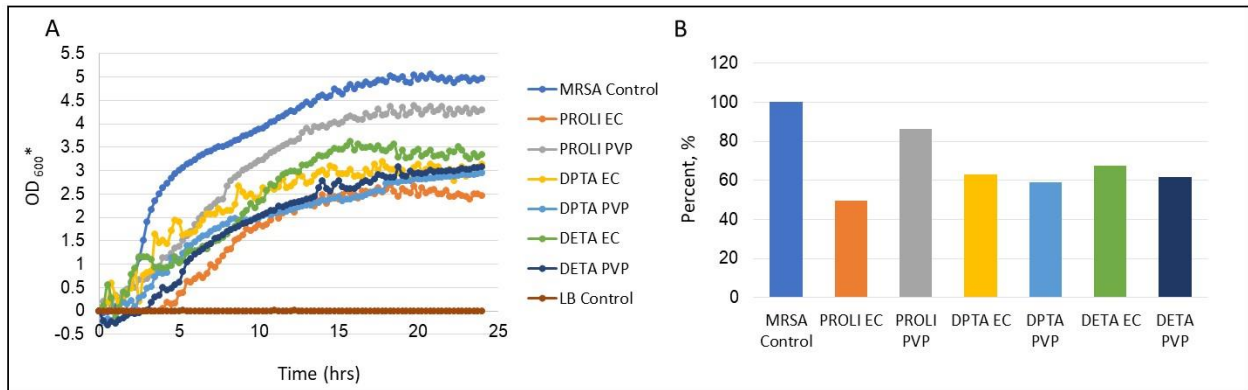


Figure 7. Antimicrobial effect of NOmatrix on MRSA over 24 hours  
 (A) Growth curve of MRSA treated with NOmatrixes measured as microbial concentration at OD 600nm  
 (B) Comparison of MRSA concentration after 24 hours

Compared to MRSA, *P. aeruginosa* was more susceptible to NOmatrix. In Figure 8A, the growth curve showed inhibition of *P. aeruginosa* over 24 hours. In cases of DPTA-NOmatrix and DETA-NOmatrix, *P. aeruginosa* concentrations were less than 20% of the untreated control (Figure 8B). Referring to the in vitro release kinetics, NO release from DPTA-NOmatrix and DETA-NOmatrix prepared with PVP showed more controlled and continuous release than those prepared with EC. This suggests the continuous release of NO is necessary to inhibit *P. aeruginosa* while a large dose of NO is needed to inhibit MRSA. The difference may be due to the structure of cell walls in Gram-positive and Gram-negative microorganisms. This study demonstrated the need to consider different antimicrobial requirements are needed to inhibit microbial growth of MRSA and *P. aeruginosa*.

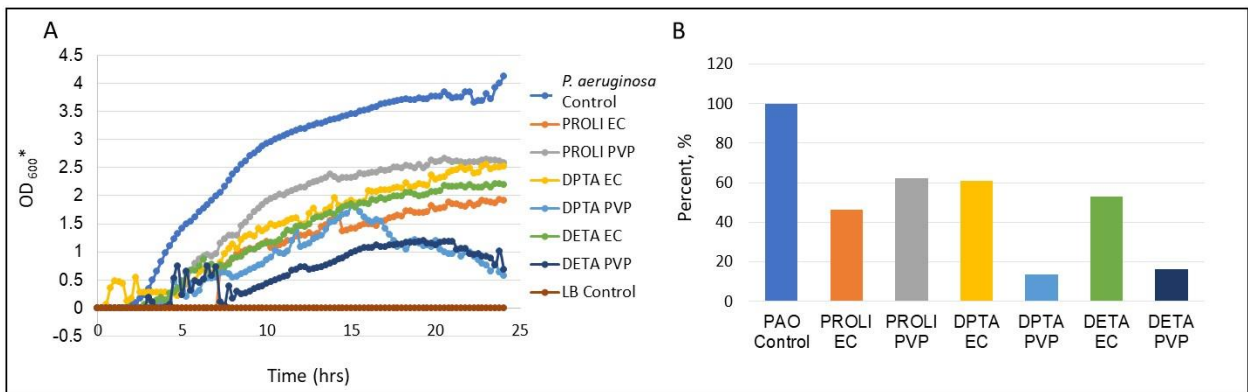


Figure 8. Antimicrobial effect of NOmatrix on *P. aeruginosa* over 24 hours  
 (A) Growth curve of *P. aeruginosa* treated with NOmatrixes measured as microbial concentration at OD 600nm  
 (B) Comparison of *P. aeruginosa* concentration after 24 hours

(a) Bacterial culture

MRSA and *P. aeruginosa* stocks in 50% glycerol were kept frozen at -80 °C until the experiments. Generally two days before the experiment, each strain was streaked on an LB

agar plate and incubated at 37 °C overnight. A single colony was picked and inoculated in 3mL of LB broth and incubated at 37 °C overnight in a shaker (250 rpm).

(b) Evaluation of antimicrobial effect of NOmatrix:

MRSA and *P. aeruginosa* were cultured as planktonic cells in 3mL of LB broth at 37 °C until the optical density (OD) at 600nm reached 0.3-0.6 (exponential growth phase). The bacterial culture was appropriately diluted and 200 µL of culture was transferred to each well in a 96-well plate. To each well, a 6 mm NOmatrix was added. The plate was placed in a temperature-controlled plate reader (CLARIOstar, BMG LABTECH GmbH, Germany) and its OD at 600nm was measured for 24 hours at a 15-minute interval. Fresh LB with NOmatrix was added to empty wells to determine the interference of NOmatrix, however, the presence of NOmatrix did not affect the optical density.

**Objective 2.** In vitro cytotoxicity evaluation of NOmatrix

The safety/toxicity of the NOmatrix was evaluated in and human dermal fibroblast cells (HDFs). Since NOmatrix will be applied on the skin as a wound dressing and dermal fibroblasts will be exposed to NOmatrix, fibroblast cells are selected to evaluate the safety of NOmatrix. Figure 9 shows the percent viability of HDFs after 24 hours of incubation with NOmatrices measured as fluorescence intensity. Overall, NOmatrices did not have a serious toxic effect on HDFs when compared to the untreated control, with a minimum of 85% viability of HDFs treated with DEPA-NOmatrix.

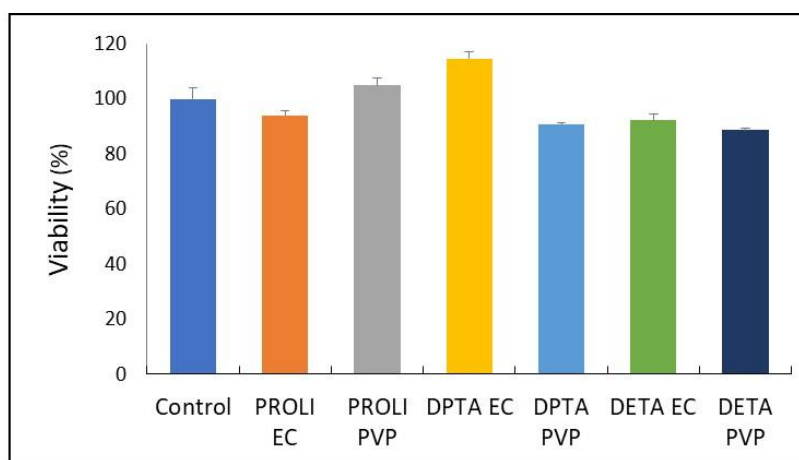


Figure 9. Safety/toxicity of various NOmatrices on HDFs

(a) Cell culture

Toxicity/safety of NOmatrix will be evaluated in human and mouse cell models. Human dermal fibroblast cells (HDFs) were cultured as monolayers in tissue-culture treated 75 cm<sup>2</sup> flask and maintained at 37°C with 5% CO<sub>2</sub>. All culture media and cell culture reagents were obtained from Invitrogen, ThermoFisher Scientific. Once cells reached 85% confluency, cells were trypsinized and passaged. For further evaluations, trypsinized cells were appropriately diluted and depending on the size of well, 10<sup>4</sup>-10<sup>5</sup> cells were added to each well. Once cells were added, cells were incubated overnight so that cells could adhere to the surface. HDFs used in the evaluation were between passages 3-6.

(b) Evaluation of cytotoxic effect of NOmatrix in human fibroblasts

To each well, a 6 mm circular disc of NOmatrix was carefully added in a biosafety cabinet. The well plate was covered and incubated for 24 hours in the incubator. For Live/Dead assay, cells media and remnants of NOmatrix were removed and cells were incubated with Live/Dead cell indicating fluorescence probes for 30 minutes (Live/Dead Cell Viability Assay kit, Life

Technologies). The fluorescence intensity for live cells was measured using a plate reader (excitation 494nm and emission 517nm).

(c) Evaluation of cytotoxic effect of NOmatrix in murine fibroblasts

This experiment is not completed. L929 mouse fibroblast cells will be cultured as monolayers in a flask. Cells will be trypsinized and appropriately diluted and depending on the size of well,  $10^4$ - $10^5$  cells will be added to each well in a well plate. After the overnight incubation, NOmatrix will be placed in each well and incubated for 24 hours. Toxicity of NOmatrix will be measured by labeling live and dead cells with fluorescence probes using a plate reader.

**Task 3. In vivo evaluation of efficacy in a skin infection mouse model (25% completion)**

*Duration 13 months (months 1-4, 10-18)*

In this task, the in vivo efficacy of NOmatrix will be evaluated in a skin infection mouse model. In order to begin the experiment involving animals, we first obtained approvals from Institutional Animal Care and Use Committee of Baylor College of Medicine on February 12, 2019 and the USAMRMC Animal Care and Use Review Office (ACURO) on March 27, 2019.

Objective 1. Submit documents for IACUC/ACURO approval

Animal protocol, AN-7916, titled Nitrix Oxide Releasing Matrix as an Antimicrobial Wound Dressing was submitted to IACUC and ACURO and received approvals.

Following objectives will be completed during the next funding period:

Objective 2. Development of a skin infection mouse model

Objective 3. Treat of NOmatrix

Objective 4. Analysis of efficacy and histology of treated mice

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

"Nothing to Report"

**How were the results disseminated to communities of interest?**

"Nothing to Report"

**What do you plan to do during the next reporting period to accomplish the goals?**

During the next reporting period, following objectives will be accomplished.

Major Task 2. Evaluation of in vitro antimicrobial effect of NOmatrix

Objective 2. In vitro cytotoxicity evaluation of NOmatrix

(C) Evaluation of cytotoxic effect of NOmatrix in murine fibroblasts

This experiment is not completed. L929 mouse fibroblast cells will be cultured as monolayers in a flask. Cells will be trypsinized and appropriately diluted and depending on the size of well,  $10^4$ - $10^5$  cells will be added to each well in a well plate. After the overnight incubation, NOmatrix will be placed in each well and incubated for 24 hours. Toxicity of NOmatrix will be measured by labeling live and dead cells with fluorescence probes using a plate reader.

Major Task 3. in vivo evaluation of efficacy in a skin infection mouse model

## Objective 2. Development of a skin infection mouse model

A full thickness excision wound (1.5 cm x 1.5 cm) will be created on the back of the Balb/c mouse. For microbial infections, 10<sup>4</sup> CFU of MRSA or *P. aeruginosa* will be directly inoculated onto the wound.

## Objective 3. Treatment of NOmatrix

The in vivo efficacy of NOmatrix will be evaluated in a skin infection mouse model. Onto the infection sites in mice, NOmatrix will be placed and will be covered with a surgical tape to secure the NOmatrix in place and mice will be monitored daily. Untreated mice will be used as a control.

## Objective 4. Analysis of efficacy and histology of treated mice

Wound closure will be monitored daily by taking photographs. After four days of infection, bioburden will be examined by plating wound swab on an agar plate. After seven days of infection, animals will be euthanized and skin tissue sections will be harvested and will be subject to hematoxylin and eosin staining. Also, the CFU from the wounded skin will be determined by homogenizing the tissue and plating on an agar plate.

#### **4. IMPACT**

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

The findings and results from this study thus far made an impact on a theory that the antimicrobial effect of nitric oxide may have different influences on microorganisms based on the cell wall structures of Gram-positive and Gram-negative bacteria. This finding can lead to further investigations to study the effect of nitric oxide on bacterial cell walls.

##### **What was the impact on other disciplines?**

"Nothing to Report"

##### **What was the impact on technology transfer?**

"Nothing to Report"

##### **What was the impact on society beyond science and technology?**

"Nothing to Report"

#### **5. CHANGES/PROBLEMS:**

The progress of the project was delayed due to unexpected delays in animal surgical procedure training and surgical suite arrangement/assignment as the institution's vivarium was undergoing constructions for expansion. This was resolved as of March 13, 2020. However, the institution halted all new animal experiments on March 18, 2020 and eventually halted all laboratory experiments starting on March 23, 2020 due to the global impact of COVID-19.

If possible, a no cost extension of the project will be requested.

#### **6. PRODUCTS:**

##### **Journal publications.**

"Nothing to Report"

##### **Conference abstract**

CS Shin, D Wang, R Lee, G Acharya. Development of Nitric Oxide Releasing Polymer Matrix (NOmatrix) as an Antimicrobial Dressing. Controlled Release Society Annual Meeting 2020, June 27-July 1, Las Vegas, NV. Accepted.

##### **Presentations**

Crystal Shin: Invited seminar in the Department of Biochemistry and Molecular Biology: "Biomaterials: Fabrication Strategies for Biomedical Applications" at

University of Texas UTHealth – McGovern Medical School, Houston, TX. Date:  
 March 9, 2020.

**Books or other non-periodical, one-time publications.**

"Nothing to Report"

**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	<b>Crystal S. Shin, Ph.D.</b>
Project Role	Principal Investigator
Researcher Identifier	
Nearest person month worked	3
Contribution to the Project	Directed and oversaw the project performance of all experiments defined under Tasks 1, 2. Performed the fabrication and characterization of NOmatrix, evaluation of antimicrobial effect. Reviewed and analyzed the experimental results. Prepared the animal protocol for IACUC/ACURO submission and reviewed for the approval.
Funding Support	
Name	<b>Ghanashyam Acharya, Ph.D.</b>
Project Role	Co-Principal Investigator
Researcher Identifier	
Nearest person month worked	2
Contribution to the Project	Directed and oversaw experiments in Tasks 1 & 2, including electrospinning process, NO release studies, evaluation of NOmatrix in bacterial and cell cultures. Also designed the experiments, reviewed and analyzed the experimental results.
Funding Support	

Name	<b>Barbara Trautner, M.D., Ph.D.</b>
Project Role	Co-Principal Investigator
Researcher Identifier	
Nearest person month worked	1
Contribution to the Project	Directed and oversaw experiments in Tasks 1 & 2, focusing on microbial culture selections, and method developments on evaluation of antimicrobial effect and provided guidance on a skin wound infection animal model development.
Funding Support	
Name	<b>Daniel Wang</b>
Project Role	Undergraduate Researcher/Student Volunteer
Researcher Identifier	
Nearest person month worked	4
Contribution to the Project	Daniel is an undergraduate researcher from Rice University in Houston, TX. He was sponsored by BCM's SMART Program in the Summer of 2019 and continued during the fall semester as a student volunteer researcher. Daniel fabricated NOmatrix and performed the in vitro release studies. Also maintained cell culture and performed the viability test.
Funding Support	BCM SMART Program/Research Course

**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**

**COLLABORATIVE AWARDS:**

"Not Applicable"

## 9. APPENDICES

### **Appendix 1:** Conference Abstract

CS Shin, D Wang, R Lee, G Acharya. Development of Nitric Oxide Releasing Polymer Matrix (NOmatrix) as an Antimicrobial Dressing. Controlled Release Society Annual Meeting 2020, June 27-July 1, Las Vegas, NV. Accepted.

## Development of Nitric Oxide Releasing Polymer Matrix (NOMatrix) as an Antimicrobial Dressing

Controlled Release Society Annual Meeting 2020, June 27-July 1, Las Vegas, NV.

**Introduction:** Misuse and overuse of conventional antimicrobial agents has accelerated the progression of nosocomial infections due to the hospital acquired antimicrobial resistance. In an effort to overcome antimicrobial resistance, a highly reactive gas, nitric oxide (NO), has been explored as an alternative antimicrobial agent (1). NO, produced endogenously by immune cells, possesses multiple antimicrobial mechanisms which decrease the likelihood of developing antimicrobial resistance. Although NO is the ideal candidate as an antimicrobial agent, its volatile nature limits the delivery of NO (2). The main objective of this study was to develop NO releasing polymer matrix (NOMatrix) as an antimicrobial dressing which can efficiently store and deliver NO in a controlled fashion.

**Methods:** In this study, we developed NOMatrix using biopolymers via 3D-bioprinting technology. Diazeniumdiolate (NONOate) was used as a NO donor compound. The incorporation of biopolymers was to control the release of NO and to protect NONOate from any water or moist present in the environment. Briefly, the biopolymer solutions (polyvinylpyrrolidone and ethylcellulose) containing NONOates with various functional groups were prepared in organic solvents. Thus prepared solutions were used to bioprint NOMatrix as an antimicrobial dressing. NOMatrix was evaluated for the in vitro NO release kinetics using a fluorescent based NO assay kit. The in vitro antimicrobial effect of NOMatrix was evaluated in bacterial cultures of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* and the toxicity of NOMatrix was evaluated in human fibroblasts.

**Results:** The 3D-bioprinting technology allowed precise fabrication of NOMatrix without the use of water. Once NOMatrix was printed, we evaluated the release kinetics of NO from NOMatrix. NONOates were conjugated with different functional groups; proline (PROLI), diethylenetriamine (DETA), and dipropylenetriamine (DPTA). Overall, PVP-NOMatrix showed more controlled fashion of release kinetics than EC-NOMatrix. Despite the short half-life of PROLI NONOate, PVP-NOMatrix released NO up to 2 days. For EC-NOMatrices containing DPTA NONOate and DETA NONOate, less than 20% of NO was released within 120 min (Figure 1). This study showed that NOMatrices inhibited the growth of MRSA and *P. aeruginosa* (Figure 2). PVP-NOMatrix was more efficient in preventing the growth of *P. aeruginosa* than EC-NOMatrix while PROLI-NOMatrix showed 50% inhibition of MRSA. Both PVP-NOMatrix and EC-NOMatrix showed minimal toxicity in human fibroblast cells.

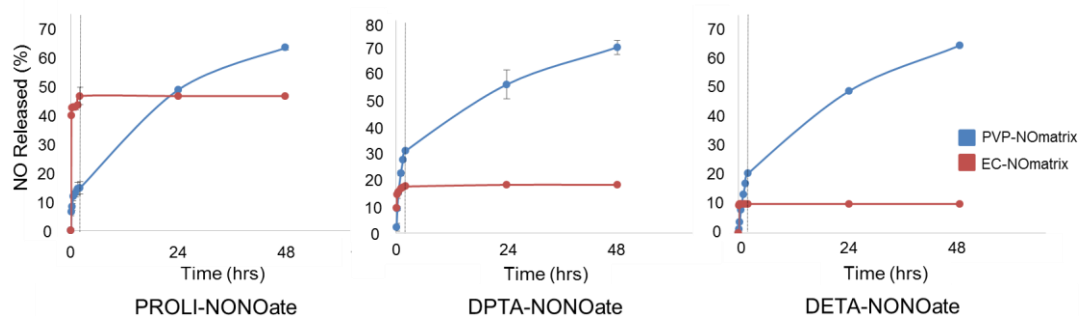


Figure 1. In vitro release of NO from NOMatrix prepared with three different NONOates.

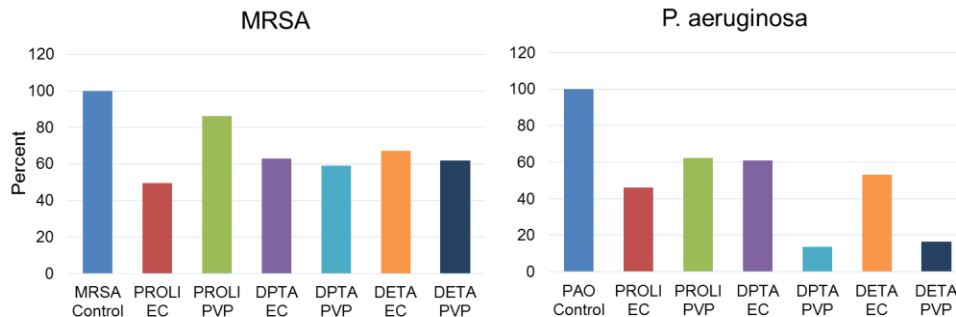


Figure 2. Microbial Concentration Comparison (OD<sub>600</sub>) after 24 hours

Conclusion: We demonstrated that the development of NO releasing biopolymers as an antimicrobial bandage. This strategy can control the initial burst release and extended NO release from NOmatrix and inhibit the growth of MRSA and *P. aeruginosa*.

Acknowledgements: This work was supported by Department of Defense (E01 W81XWH1910091) and the Michael E. DeBaakey Department of Surgery at Baylor College of Medicine.

#### References

1. Martinez, L. R., Han, G., Chacko, M., Mihu, M. R., Jacobson, M., Gialanella, P., Friedman, A. J., Nosanchuk, J. D. & Friedman, J. M. Antimicrobial and healing efficacy of sustained release nitric oxide nanoparticles against *Staphylococcus aureus* skin infection. *J. Invest. Dermatol.* 129, 2463–2469 (2009).
2. Schairer, D. O., Chouake, J. S., Nosanchuk, J. D. & Friedman, A. J. The potential of nitric oxide releasing therapies as antimicrobial agents. *Virulence* 3, 271–9 (2012).

Presenter Biography: Crystal Shin is an Assistant Professor in the Michael E. DeBaakey Department of Surgery, Baylor College of Medicine in Houston, TX. Her research focuses on developing biomaterial based delivery systems with enhanced therapeutic efficacy.

#### Learning Objectives:

Recognize the potential use of nitric oxide-releasing matrix

Demonstrate the use of biopolymer for the nitric oxide delivery

Assess the advantage of 3D fabrication technology