**Antimicrobial effects of an acidified nitrite foam on drip flow reactor biofilm**

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**ABSTRACT**

**Background** Nitric oxide (NO) plays critical roles in wound healing, including stimulating vasodilation, angiogenesis and broad antimicrobial activity.

**Aim** To measure the effect of an acidified nitrite foam (ANF) on biofilms created by six different microbes.

**Methods** A novel method of generating, delivering and topically applying NO gas at the point of care was developed using ANF in a mixed bubble foam and was tested in vitro against six common microbial wound pathogens.

**Results** A single 5-minute topical exposure of the NO bubble gas formulation generated a 5.8-log10 reduction of mature biofilm of *Pseudomonas aeruginosa*, a 5.1-log10 reduction of *Staphylococcus aureus* biofilm, a 4.0-log10 reduction of *Staphylococcus epidermidis* biofilm, a 3.2-log10 reduction of *Proteus mirabilis* biofilm, a 2.7-log10 reduction of *Acinetobacter baumannii* biofilm, and a 1.5-log10 reduction of *Candida albicans* biofilm.

**Conclusion** The efficacy of a 5-minute treatment of ANF used on biofilms of *P. aeruginosa*, *A. baumannii*, *S. aureus*, *C. albicans*, *P. mirabilis* and *S. epidermidis* was confirmed. The treatment resulted in a significant reduction in colony-forming units per square centimetre (CFU/cm²) comparable to or surpassing other methods of NO gas application, suggesting NO containing foam’s utility as a point of care solution for chronic wounds with elevated bioburden and biofilms where levels of endogenously produced NO may be insufficient for wound healing completion.

**KEY MESSAGES**

- Nitric oxide (NO) plays a critical role in wound healing, including stimulating vasodilation, angiogenesis and broad antimicrobial activity.
- Deployment of NO as a topical treatment has traditionally been challenging due to NO’s short half-life.
- The micro foam-based means of NO generation, transport and exogenous application takes full advantage of NO’s potential to engage and disrupt biofilms, destroy bacterial pathogens, and serve as a real-time exogenous NO supplementation agent for chronic wounds.
- Acidified nitrite foam (ANF) significantly reduced the colony-forming units per square centimetre (CFU/cm²) of each microbe tested after 5 minutes of treatment.
- *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Proteus mirabilis* seem particularly susceptible to NO antimicrobial actions.
- Could ANF be the antimicrobial solution for managing biofilms?
- How beneficial would a 5-minute treatment of topical NO be for patients suffering with painful and costly chronic wounds?

**INTRODUCTION**

Chronic wounds are complex and inflammatory in nature. High wound bioburden and the development of polymicrobial biofilms result in pathologically prolonged inflammation which plays a significant role in the disruption of the normal healing cascade and wound chronicity1,2. However, these may be overcome by bactericidal treatments, such as that of nitric oxide (NO), which is produced and used by the body to combat biofilm-forming bacteria colonies.

Biofilms are bacteria living in heterogeneous, multicellular communities encapsulated in self-produced extracellular polymeric substances (EPS).1,3 The biofilm’s protective polymer matrix provides an optimal environment for bacteria to evade host immune response and antimicrobial action. Once established, these biofilm colonies surround themselves by free swimming planktonic cells dispersed to colonise new...
When compared to their planktonic counterparts, biofilm colonies generally exhibit greatly increased resistance and up to 10,000 times higher tolerance to immune defences, biocides and antibiotics, thus leading to chronic infections. For this reason, effectual modes of action for eradication of biofilms must include a potentiated broad spectrum antimicrobial mechanism.

NO is an endogenous gas transmitter that plays a vital role in wound healing. The reported and previously demonstrated bactericidal capability of NO gas supports myriad reasons for its clinical application. Common exogenous applications of NO rely on some form of acidified nitrite following the pathway shown in the equations below.

\[
2 \text{NO}_2 + 2\text{H}^+ \rightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O}
\]

\[
2 \text{N}_2\text{O}_3 \rightarrow \text{NO}_2 + \text{NO}
\]

Emerging evidence suggests that NO can induce biofilm dispersal, increase bacteria susceptibility to antibiotic treatment, and induce cell damage or cell death via the formation of reactive oxygen or reactive nitrogen species. The antimicrobial activity of NO is due to both nitrosative and oxidative mechanisms which eventually result in the production of dinitrogen trioxide (N$_2$O$_3$) and peroxynitrite (ONOO$^-$). Dinitrogen trioxide induces DNA deamination, while peroxynitrite causes membrane lipid peroxidation.

The full advantage of NO treatment can be accessed on site by applying the acidified nitrite as a foam (ANF). In this context, a foam is ideally comprised of micro bubbles of gas surrounded by thin films of liquid and surfactant. Presenting an ANF requires mixing of one foam derived from a liquid solution of acid, surfactant and water, and a similar foam derived from a solution of nitrite salt, surfactant and water, dispensed by a hand pump that creates a foam when depressed.

These bubbles are comprised of a thin layer of water sandwiched between layers of surfactant molecules. Because the bubble walls are comprised primarily of water and surfactant, the acid and nitrite foams easily coalesce when mixed, exposing the reactants to each other. Importantly, the NO product that is produced is engulfed inside the bubble wall, which forms a cluster of ultra thin filmed bubbles containing NO. These bubbles create an ‘airtight net’ that prevents the entrapped NO from escaping to the ambient air during formation and promotes transport of NO to the tissue site on which the NO bubbles are applied, as illustrated in Figure 1.

**METHODS**

To address the challenges reported herein, a micro foam-based means of NO generation, transport and exogenous application as reported in two US Patents was used. A series of experiments were designed to test whether the effectiveness of ANF is equal to or greater than that reported by other modes of action at disrupting specific biofilms and killing their respective microbial pathogens.

The testing was conducted by the Medical Biofilms Laboratory (MBL) of the Center for Biofilm Engineering (CBE) at Montana State University. Biofilms were created using an in vitro model system, Drip Flow Biofilm Reactor® (DFR 110-6, Biosurface Technologies Corp., Bozeman, MT). The DFR (Figure 2) is designed to model a low shear environment and has been approved by ASTM as a standard method for growing Pseudomonas aeruginosa biofilms (method E-2647-20). Testing was performed using the modified procedure described below because this procedure was found to be more relevant to the treatment of wound biofilms.

Although numerous in vitro wound models have been proposed, none have been clinically validated to predict effectiveness of anti-biofilm treatments. The DFR method for biofilm growth used in this study was based on an ASTM standard method (E-2647-20) that was vetted through the ASTM process, including the evaluation of repeatability and reproducibility based on results from 10 independent laboratories. For this study, changes to the standard method were made to better represent the wound microenvironment, including a collagen-coated surface. The DFR certainly captures one of the most important characteristics of biofilms, tolerance to antibiotics and other antimicrobial agents. The DFR has also recently been used to test other wound care products.

**Pathogens**

Testing was performed on biofilms of *P. aeruginosa* MBL Strain SWR 215 (a clinical chronic wound isolate obtained from a wound biopsy by the Southwest Regional Wound Center in Lubbock, TX and preserved by the MBL), *Acinetobacter baumannii* (ATCC BAA-1797), *Staphylococcus aureus* (ATCC BAA-1556), *Candida albicans* (ATCC 10231), *Staphylococcus epidermidis* (ATCC 35984), and *Proteus mirabilis* (ATCC 7002). These strains are maintained as frozen stock cultures at –80°C in the MBL.

**Treatments**

NOxy Health Products supplied the NO producing foam. The foam was obtained as a mixture of two components (a solution A and a solution B) contained in separate foam pumps. Solution A comprised a solution of citric acid. Solution B comprised a solution of sodium nitrite. Both contained a portion of surfactant. The steps to apply an exogenous NO foam treatment are listed below.
1. Dispense an amount of Solution A foam into an appropriate container by depressing the Solution A pump head consistently and briskly.

2. Dispense an amount of Solution B foam into the same container by depressing the Solution B pump head consistently and briskly.

3. Using a plastic paddle or mixing tool, stir the two foams vigorously for 5 seconds to mix.

4. Apply the mixed NO foam by pouring the foam out of the container onto the site or using a spatula/spoon for a more measured/precise placement of the foam.

### Biofilm growth

Six-channel DFRs, equipped with hydroxyapatite- and collagen-coated (HAC) glass coupons, were operated at 33°C (approximate wound temperature) under aerobic conditions. Hydroxyapatite-coated glass slides, prepared by Clarkson Chromatography, were collagen-coated using a coating matrix kit (Life Technologies Corporation) following the manufacturer’s instructions. Approximately 20 minutes prior to inoculation, sterile media (1%-strength brain-heart infusion broth with 0.5% adult bovine serum) was dripped into each channel and allowed to collect over the coupons. A conditioning layer on the surface of the coupons was observed to form.

Each channel of the reactor was then inoculated with 1mL of an overnight culture of the test organism containing approximately 8x10^10 CFU per mL for the bacteria and approximately 7x10^10 CFU per mL for the yeast. The reactor was then set at a 10° angle and sterile media was dripped through the reactor at a rate of 10mL/hr per channel for 72 hours to mimic the relatively slow fluid flow of wound exudate.

### Biofilm treatment and sampling

For treatment, flow to the DFR was halted and the mixed NO foam or individual solution A and B foams were applied as directed to a coupon. Enough of the foam was applied to completely cover the entire DFR coupon (approximately 20mL of foam). Before applying the foams, the untreated control coupon was removed from the DFR and analysed by plate count, as described below. Following the contact time, the coupon(s) were removed from the DFR and rinsed with phosphate-buffered saline (PBS) to remove residual foam and unattached bacteria.

### Viable plate counts

The number of viable bacteria on each of the coupons was determined by viable plate count. After rinsing, the coupons were placed in 10mL of 2X-strength Dey-Engley (D/E) neutralisation broth. Biofilm on the coupons were scraped and rinsed with the D/E, then sonicated (30 seconds in a 50mL conical centrifuge tube at 60kHz in an ultrasonic cleaner Model CSU3HE, Tuttnauer, Hauppauge, NY), vortexed (2 minutes), and sonicated (30 seconds) to further remove and disaggregate the biofilms. The biofilm suspensions were then serially diluted with PBS and plated on Tryptic Soy Agar (TSA). The plates were then incubated at 37°C for 24–48 hours, following which the number of CFU were counted. Based on the dilution and surface area of the slide, the number of CFU per unit area was calculated and logarithmically (base 10) transformed. Log differences between the treatment and untreated control biofilms were calculated for each experiment set. Each CFU/cm² measurement was reported and is available upon qualified request.

### RESULTS

For each test organism, the viable plate counts in CFU/cm² for an untreated control (no treatment), a Solution A foam treatment, a Solution B foam treatment, and the NO foam mixture treatments were measured. The NO foam treatments were applied to three coupons in the DFR. These six measurements corresponded to the six plates in the DFR. All foam treatments lasted 5 minutes.

To determine if there were differences in the CFU/cm² between the no-treatment or control treatments versus the other treatments, an ANOVA analysis was conducted using the Tukey Honest Statistic Difference (HSD) test on JMP 16.1 (SAS Institute Inc.).

Figure 3 shows the control and each treatment CFU/cm² for each organism tested. Table 1 summarises the average log reduction between the control and the 5-minute NO foam treatments for each organism tested. Table 1 summarises the average log reduction between the control and the 5-minute NO foam treatments.
Figure 3. Log CFU/cm² bar chart of pathogen tests.
which is consistent with the large volume of results of acidified susceptible (2.9 average log reduction), and the C. A. baumannii exposed to ambient conditions, further reduces the potential NO’s short half-life, which measures only seconds when reactivity also imposes limitations on its mode of application.

NO’s concentration gradient and impedes the delivery of NO to the wound. The NO micro bubble foam formulation overcomes the serious diffusion barrier that is caused by a viscous mass of gel or cream topical formulations of NO. Gels and creams impede diffusion of NO out of those formulations to the wound bed. In contrast, the NO foam micro bubbles cover the entire wound surface, ensuring intimate contact with and sealing off the complicated surface topology of the wound from ambient air, while simultaneously releasing a predetermined dosage in direct proximity to the targeted bioburden. This mode of action purports to offer the advantages and none of the disadvantages previously encountered in exogenous supplementations.

CONCLUSIONS
The data reported above confirms that ANF is an effective antimicrobial agent against the tested biofilms. To date, P. aeruginosa, A. baumannii, S. aureus, C. albicans, P. mirabilis, and S. epidermidis biofilms were tested against a 5-minute treatment of ANF and all the microbes experienced a significant log reduction in CFU/cm² at least equivalent to or greater than that of other means of NO gas application. These results demonstrate that a single, 5-minute treatment of topical NO gas foam was effective in significantly reducing CFUs of in vitro biofilms.

This study demonstrated that exogenous foam-based NO supplementation took full advantage of NO’s potential to engage and disrupt biofilms and destroy bacterial pathogens, and suggests that foam-based NO may be a point of care solution for rescue of chronic wounds stuck in the inflammation stage of wound healing due to high bioburden and biofilms. Exogenous foam-based NO supplementation may also serve as a resource in those instances where endogenous production of NO is sufficient to start but becomes insufficient to complete the wound healing sequence. Given the advantages of the ANF system and its effectiveness detailed herein, it is hoped that ANF will become a preferred treatment in the clinician’s toolbox to fight planktonic and biofilm infections.

Limitations
Biofilms were grown using a DFR model based on the ASTM-approved standard method E-2647-20 to test anti-biofilm effectiveness. Although modifications to ASTM E-2647-20 were made to better mimic the wound environment, no in vitro method has been developed that can completely capture the in vivo complexities of a wound. There is no in vitro wound model that has been validated to predict the effectiveness of antibiofilm agents for human chronic wounds.

In the method described herein, D/E neutralising broth is used for biofilm recovery because it contains a surfactant (Tween 80) which helps remove and disperse the microorganisms as well as inactivating a variety of antimicrobial agents. Inadequate neutralisation can be indicated in plate count results, with no counts at low dilutions but counts at medium to high dilutions. No evidence of this was seen, but the neutralising effect of the D/E broth was not validated for this specific formulation. NO concentration gradient and impedes the delivery of NO to the wound.
Historically, some antimicrobial treatments have performed well in a laboratory setting but perform less well against biofilms in vivo. It is thought that the biofilm penetrates the substrate it is growing on, thereby yielding extra protection from an antimicrobial agent. In other cases, the in vivo environment may neutralise the antimicrobial agent, reducing its effectiveness against the biofilm pathogen.

Further animal model and/or human in vivo data is required to ascertain the effectiveness of topical NO gas therapies as potential benefits in chronic wound care. Testing has been completed to affirm the ANF’s full capabilities against an ex-plant porcine dermal model. A First-In-Human study has been initiated in Israel to confirm the safety and efficacy of ANF in humans.

CONFLICT OF INTEREST STATEMENT

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mr Miller, Dr Bell and Prof Schultz report that financial support was provided by NOxy Health Products. Mr Miller, Dr Bell and Prof Schultz report a relationship with NOxy Health Products that includes consulting or advisory and equity or stocks. Mr Miller and Dr Bell have patents US10052348 and US10751364 issued to NOxy Health Products.

CONSENT IN FULL

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