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Prevention and Treatment of Virulent Bacterial Biofilms with an Enzymatic Nitric Oxide-Releasing Dressing

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The use of percutaneous medical devices often results in nosocomial infections. Attachment of microorganisms to the surfaces of these medical devices triggers biofilm formation, which presents significant complications to the health of a patient and may lead to septicemia, thromboembolism, or endocarditis if not correctly treated. Although several antimicrobials are commonly used for prevention of biofilm formation, they have limited efficacy against formed biofilms. In this study, we report the use of an enzymatic, gaseous nitric oxide (gNO)-releasing dressing for the prevention and treatment of *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus*, and *Pseudomonas aeruginosa* biofilms. Results show that the bactericidal activity against biofilms of the test strains was dependent on time and rate of gNO release from the dressing. Following 6 h of treatment, gNO-releasing dressings significantly inhibited the growth of test strains relative to vehicle control dressings, demonstrating eradication of bacterial concentrations of up to 10^5 CFU/cm^2. Complete cell death was observed for both prevention of biofilm formation and treatment of 24-h-grown biofilms after 6 h of treatment with the gNO-releasing dressings. Further, gNO-releasing dressings were more efficient against formed biofilms than other antimicrobial agents currently used. These results demonstrate that the gNO-releasing dressing can produce sufficient levels of gNO over a therapeutically relevant duration for maximal bactericidal effects against virulent bacterial strains known to cause nosocomial infections.

In recent years, indwelling catheters, implants, and other transdermal medical devices have become widespread for both short- and long-term uses (16). Despite attempts at sterilization and development of hydrophilic coatings, chronicity of local and systemic infections has been observed due to the formation of bacterial biofilms (23). These complex and dynamic microbial microniches protect the underlying bacteria from the environment through the formation of a slimy matrix known as the extracellular polymeric substance (EPS) (47). Although the composition of the EPS varies among strains, it is primarily composed of polysaccharides, proteins, and nucleic acids (47). These components firmly anchor the biofilm to a given surface and provide protection from antimicrobial agents and host defense mechanisms (37). As the biofilm continues to mature, planktonic bacteria are shed and form biofilms at other sites within the wound bed (12, 21, 43). Moreover, through the continuous release of bacterial toxins and innate proinflammatory molecules, there is increased ancillary tissue damage that results in further biofilm formation in adjacent tissues (13). Consequently, patients are at a greater risk of bloodstream infections (BSI), endocarditis, and even microemboli as biofilm detachment occurs (7, 16, 36). Biofilms may also cause chronic infections due to their multicellular composition in which different species confer protective effects through cooperation (22, 45, 50).

Due to the complexity and variability of biofilms, nonspecific therapies are generally preferred (32). Contemporary therapies, including topically or systemically administered anti-infective agents such as chlorhexidine, povidone-iodine, silver-coated antimicrobial dressings, and antibiotics, are limited in their efficacy and necessitate a new paradigm for treating biofilms (2, 3, 8, 46, 48). *Pseudomonas aeruginosa* and multidrug-resistant organisms (MDROs) such as *Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* (MRSA) represent a serious problem in hospitals due to nosocomial transmission and the difficulty in treating these infections with contemporary therapies (1, 19, 35). Recently, nitric oxide (NO) has been proposed as a novel antimicrobial therapy (24, 25, 42). NO is a biological molecular mediator that is actively produced within the body by a class of nitric oxide synthases (NOS) and serves in cellular messaging in the nervous system (neuronal NOS [nNOS]), regulation of blood pressure (endothelial NOS [eNOS]), and elimination of foreign bodies or pathogens as part of the innate and cell-mediated immune system response (inducible NOS [iNOS]) (38, 49). This reactive free radical has also been shown to be bactericidal against a broad spectrum of both Gram-positive and Gram-negative bacteria (4, 18, 41). It has been postulated that the antimicrobial effect of NO may be due to its efficient diffusion within the biofilm matrix and targeting of cellular components of the underlying bacteria, such as cell membranes, structural proteins, metabolic enzymes, or DNA. Proposed mechanisms include the induction of either nitrosative or oxidative stress (25, 30). Since NO is a lipophilic molecule, it can readily cross into the cell membrane (15, 34). As nitric oxide accumulates and is oxidized to dinitrogen trioxide (N_2O_3), it causes nitrosative stress within the membrane (34). Thiols of cell surface proteins and intracellular proteins may be nitrosated (*S*-nitrosation) or form disulfide bridging between proteins (15, 44). Moreover, lipid peroxidation via the nitryl radical ($NO_2^{\bullet}$) can accelerate membrane destruction; $NO_2^{\bullet}$ can be generated by both the reaction of NO and O_2 and the radical decomposition of per-

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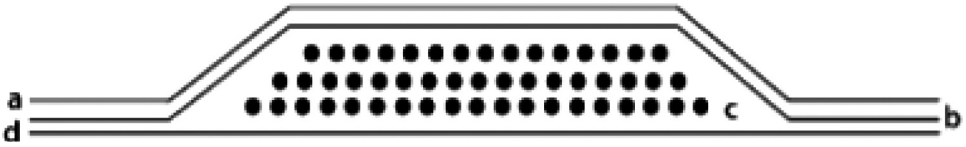

FIG 1 Cross-section of the enzymatic dressing. The dressing components include an outermost aluminized tape layer (a), a polyethylene nylon film (b), the active ingredients (c), and an innermost gas-permeable polyurethane adhesive film (d). Layers b and d were heat sealed to produce a pouch containing the active ingredients.

oxynitrous acid (HOONO) (17, 44). Furthermore, bacterial DNA can be damaged through NO-mediated deamination, via an *N*-nitrosating intermediate (perhaps N_2O_3) or by oxidative cleavage by $OONO^-$ or $NO_2^{\bullet}$ (15, 29).

Previously we reported the antibacterial and antifungal activity of a probiotic dressing releasing gaseous NO (gNO) on an array of planktonic cells (28). Though an array of therapies has been tested for the prevention of biofilm formation, evidence of antimicrobial activity of NO for treatment of biofilms does not yet exist (4, 5, 14, 24, 26, 40, 42). Here, we compare the use of an enzymatic gNO-releasing dressing for both prevention of biofilm formation and treatment of 24-h-grown biofilms relative to commercially available therapies including chlorhexidine, a povidone-iodine dressing, silver-coated antimicrobial dressing, gentamicin, and vancomycin. Because of their aforementioned resistance to antiseptics, antibiotics, and proven robustness in a biofilm state, we have selected *P. aeruginosa*, MRSA, and *A. baumannii* as strains against which to test these therapies.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA) was plated on tryptic soy agar and grown in tryptic soy broth (3% Bacto tryptic soy broth, soybean-casein digest medium) (catalog number 211825; BD, Mississauga, Ontario, Canada). *Acinetobacter baumannii* ATCC 19606 and *Pseudomonas aeruginosa* ATCC 10145 were plated on nutrient agar and grown in nutrient broth (0.3% meat extract, 0.5% peptone). Cells were initially inoculated on solid medium (agar) plates and were incubated aerobically at 37°C for 24 h. A single colony was used to inoculate 5 ml of the respective liquid medium and grown overnight (O/N) under aerobic conditions at 37°C.

Measurement of gaseous NO levels. Test dressings designed to release four different gNO levels (dressings A, B, C, and D) and a vehicle control were provided by Micropharma Ltd. (Montreal, Quebec, Canada). The dressings consisted of a 5- by 5-cm pouch containing a solution with the active ingredients including an enzyme, a substrate, and an NO donor (Fig. 1). The dressings were activated when all the ingredients were present in the pouch. After activation, the backings of the dressings were removed, and the dressings were adhered to the top surface of high-density polyethylene (Lee Valley, Canada) assay chambers, consisting of a 3- by 3- by 0.5-cm cavity and a stainless steel sampling port. The dressings were incubated at 37°C, and the assay chambers were sampled every hour, using a 100-μl Hamilton syringe (Reno, NV), for the measurement of gNO production by chemiluminescence with a nitric oxide analyzer (Sievers; GE, Boulder, CO). Duplicate measurements were performed for two dressings containing the same formulation, and gNO concentrations in parts per million by volume (ppmv) were reported relative to a standard calibration curve using known volumes and concentrations of gNO. This method was validated with dressings of different sizes and cavities of different volumes and dressing overlap areas without significant variations in the gNO concentrations.

Measurement of gNO dose release rates. The dressing gNO dose release rates were determined by measures of cumulative nitrite (NO_2^-) concentrations over time resulting from the NO released by water-immersed dressings. Dressings manufactured with four different formulations and vehicle controls were tested in triplicate. Upon activation and after the backings were removed, dressings were immersed in 100 ml of high-performance liquid chromatography (HPLC)-grade water (Caledon Laboratory Chemicals, Ontario, Canada) in capped bottles previously equilibrated to 37°C. A 300-μl sample aliquot was taken from each bottle immediately following dressing immersion, and bottles were incubated at 37°C. Further sample aliquots were taken once every hour for 20 h, with an additional sample taken at 24 h.

To quantify NO_2^- generated by gNO-releasing dressings A, B, C, and D, a Griess assay was used in a 96-well microplate format (20). Briefly, 50 μl of the sample aliquot taken from dressing immersions was added to an equal volume of Griess reagent A (1% sulfanilamide, 5% phosphoric acid) in a microplate well. After a 5-min incubation at room temperature, 50 μl of Griess reagent B [0.1% *N*-(1-naphthyl) ethylene diamine] was added to the same well, and the sample was allowed to incubate a further 5 min at room temperature. Microplates were read in a SpectraMax 250 absorbance microplate reader (Molecular Devices, Sunnyvale, CA) at 540 nm. Absorbance readings were compared to a standard curve prepared using known concentrations of $NaNO_2$. The rate of NO release was expressed as micromoles of NO per unit of dressing area per unit of time as determined from the slope in the nitrite concentration versus time during the linear interval.

Antimicrobial activity of dressing-generated gNO on established biofilms. Overnight liquid cultures of *A. baumannii*, MRSA, or *P. aeruginosa* were diluted in their respective growth media (1/100, vol/vol), and 125 μl of each of the resulting suspensions was transferred into eight wells of a flexible, sterile 96-well polyvinyl plate. Plates were incubated at 37°C and allowed to grow and form biofilms for 24 h. The resulting biofilms were gently washed three times in sterile saline (0.9% [wt/vol] NaCl), and wells containing biofilms were filled with 125 μl of sterile saline. Dressings A, B, C, and D were adhered to the top surfaces of the microplates so that a small headspace was created between the dressings and the saline solution covering the biofilms. The plates were placed in a 37°C incubator so that the treatment proceeded for 6 h. Following treatment, cell viability was assayed by surface plating; dressings were removed, and the wells containing the bacterial biofilms were gently washed three times in sterile saline. The plates were cut into strips of four wells (two strips per strain), which were subsequently submerged in 8 ml of sterile saline within 15-ml sterile conical tubes. The strips were sonicated in a water bath for 30 min and vortexed for 5 s, and three serial 10-fold dilutions were prepared. A volume of 100 μl of each dilution was plated on LB agar plates in duplicate. LB agar plates were incubated at 37°C for 24 h under aerobic conditions, and the resultant colonies were counted. The limit of detection was based on the maximum bacterial cell dilution that resulted in colony formation. Moreover, the dressing with the highest NO release rate, dressing D, was used along with vehicle control dressings to determine ideal efficacious treatment times as described below. The dressings were applied to the microplate for periods of 3 h and 6 h. A 0-h time point corresponds to biofilms that were immediately harvested without exposure to the gNO-generating dressings.

MIC assay. MICs were determined by 2-fold dilution antibiotic titration in a plate assay. Briefly, antibiotic stock solutions of 2 mg/ml chlorhexidine digluconate (Sigma, St. Louis, MO), 1.5 mg/ml vancomycin

(Sigma, St. Louis, MO), and 5 mg/ml gentamicin (Wisent, St. Jean-Baptiste, Quebec, Canada) were prepared in each strain's respective growth medium as described above. A total of 10 2-fold serial dilutions of the antimicrobial stock solutions were subsequently prepared in the same medium, and 50 μl of each dilution was dispensed into the wells of a 96-well plate. A 1:50 dilution of overnight bacterial cultures in the respective growth medium (50 μl) was added to each well containing the antibiotic solutions. One well was filled with 100 μl of a 1:100 dilution of the overnight bacterial culture (positive control), and another well was filled with 100 μl of the growth medium (negative control). The initial inoculum for each strain was the same as the one used for the biofilm experiments and corresponded to exponential growth phase. The plates were then incubated for 24 h at 37°C aerobically. The MIC was determined as the lowest antibiotic concentration that did not present visible bacterial growth. These experiments were performed in triplicate.

Comparison of gNO-releasing dressing to antimicrobial therapies for prevention of biofilm formation. Overnight liquid cultures of the aforementioned bacterial strains were inoculated (1/100, vol/vol) in sterile saline or the appropriate antibiotic solutions as follows. The working concentrations of chlorhexidine, vancomycin, and gentamicin were selected based on the MIC assays described above. Chlorhexidine (Sigma, St. Louis, MO) was prepared to a final concentration of 40 μg/ml for *A. baumannii* and *P. aeruginosa* and at 10 μg/ml for MRSA. Final concentrations of 400 μg/ml and 4 μg/ml vancomycin (Sigma, St. Louis, MO) were used for *A. baumannii* and MRSA, respectively, and 12.5 μg/ml of gentamicin (Wisent, St. Jean-Baptiste, Quebec, Canada) was used for *P. aeruginosa*. A volume of 125 μl of each of the resulting suspensions in the corresponding antibiotics was transferred into eight wells of a flexible, sterile 96-well polyvinyl plate.

Furthermore, 125 μl of the saline-diluted O/N liquid cultures was transferred to four sets of eight wells, where each set was treated with vehicle control dressings, gNO-generating D dressings, silver dressings, or povidone-iodine dressings. For wells treated with silver dressings (Acticoat silver-coated antimicrobial dressing; Smith & Nephew, Hull, England) or povidone-iodine dressings (povidone-iodine prep pads; PDI, Toronto, Ontario, Canada), 4- by 4-mm squares were carefully cut and submerged in each of the wells. The size of the squares was chosen to cover the area of biofilm growth. The gNO-releasing dressings or the vehicle control dressings were adhered to the top surface of the microtiter plate so that a small headspace was created between the dressing and the bacterial suspensions. We demonstrated previously that gNO release levels by a similar dressing are independent of the size and geometry of the cavity. Microplates were then incubated at 37°C for 6 h to allow for any bacterial growth and biofilm formation. Following incubation, gNO-releasing or vehicle control dressings were removed by peeling them off from the plate surface. The povidone-iodine and the silver dressing squares were removed with sterile forceps, and antibiotics were discarded. The microplates were then gently washed in sterile saline. The plates were cut into four-well strips, submerged in sterile saline, sonicated, and serially diluted as described above. A volume of 100 μl of each dilution was plated on LB agar plates in duplicate. LB agar plates were incubated at 37°C for 24 h under aerobic conditions, and the resultant colonies were counted.

Comparison of gNO-releasing dressing to antimicrobial therapies for treatment of 24-h-grown biofilms. O/N liquid cultures of the bacterial strains were diluted in their corresponding growth media and allowed to grow and form biofilms, as described above. The biofilms were gently washed with saline, and the treatments were then applied. Chlorhexidine, vancomycin, and gentamicin were dissolved in saline at the previously determined concentrations, and 125 μl was dispensed into each of the eight wells containing the biofilms. Silver dressings and povidone-iodine dressings were carefully cut into 4- by 4-mm squares and placed on top of the formed biofilms in the wells containing 125 μl of saline (eight wells per strain per condition). The size of the silver dressings and iodine dressings was chosen to cover the area over the biofilm. Vehicle control dressings and gNO-releasing D dressings were adhered to the top surface of the microplates so that a small headspace was created between the dressings and the formed biofilms in 125 μl of saline (eight wells per strain per condition). Microplates were then incubated at 37°C for 6 h. Following incubation, the treatments were removed as described above, the microplates were washed and sonicated, and cells were enumerated as above.

CLSM. Overnight bacterial cultures were diluted (1/100, vol/vol) in corresponding media, and 2 ml of the diluted medium was transferred to six-well plates with each well containing a Rinzl plastic coverslip (Electron Microscopy Sciences, Hatfield, PA) previously treated with 0.1% (wt/vol) gelatin (Mallinckrodt Laboratory Chemicals, Phillipsburg, NJ); biofilms were allowed to form for 24 h at 37°C. The spent medium was gently aspirated, and the biofilms were gently washed with saline. The coverslips containing biofilms of the three reference strains were placed in the assay chambers, covered with 200 μl of saline, and treated with vehicle control dressings or gNO-releasing D dressings for 6 h at 37°C. After treatment removal, biofilms were stained on the coverslips with a Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, OR) according to the manufacturer's specifications. Briefly, saline on the coverslips was aspirated, and the coverslips were washed once with saline and treated with Live/Dead BacLight SYTO 9 and propidium iodide stains for 15 min at room temperature (RT). The concentrations of the stains used for each bacterial strain varied; 3 μl/ml of SYTO 9 stain and 3 μl/ml of propidium iodide were used for MRSA and *P. aeruginosa*, whereas 0.3 μl/ml of SYTO 9 stain and 1.5 μl/ml of propidium iodide were used for *A. baumannii*. Following the incubation, the stain was aspirated, and the coverslips were washed two times with saline and treated with formalin for 30 min. Following fixation, the coverslips were washed an additional two times with saline. Coverslips were mounted on cell slides and immediately observed using confocal laser scanning microscopy (CLSM).

Statistical analysis. All data were presented as the means ± standard deviations (SD). For the time course experiments, data comparisons between treatments with vehicle control dressings and gNO-releasing dressings were analyzed using a Mann-Whitney rank sum test for each time point. For multiple comparisons among various antimicrobial agents, a Kruskal-Wallis analysis of variance (ANOVA) by ranks test was performed. Multiple pairwise comparisons were performed with a posthoc Dunnett test. Significance was set at a P value of < 0.05.

RESULTS

Design and evaluation of gNO-generating dressings. To investigate the gNO release profile of the supplied dressings, gNO production was monitored over 22 h. One formulation (dressing D) generated above 200 ppmv of gNO following 3 h of incubation at 37°C and maintained the gNO release for over 22 h (Fig. 2A), whereas the other formulations did not surpass the 200 ppmv of gNO production. A target concentration of 200 ppmv of gNO was selected based on previous observations that showed bactericidal activity against planktonic bacteria at these release levels (18). As bacterial cells embedded in biofilms, they would likely be more resistant to minimally effective concentrations. However, rates of gNO release expressed in nmoles/cm^2/h may be more meaningful for a prospective topical dressing. The dressing's rate of gNO release was determined from measurements of accumulated NO_2^- over time, using an assay in which the dressings were submerged in water immediately after activation. The initial rate of gNO release varied between 0 and 73 nmol of gNO released per cm^2 per hour, and gNO release increased for up to 24 h (Fig. 2B).

Dressing-generated gNO levels and bacterial biofilm susceptibility. To determine biofilm susceptibility to gNO levels, bacterial viability was investigated in 24-h-grown biofilms of the reference bacterial strains. Upon treatment, cell viability was inversely correlated to the dressing's gNO-release levels. Following 6 h of treatment, a gradual gNO dose-response effect was observed for

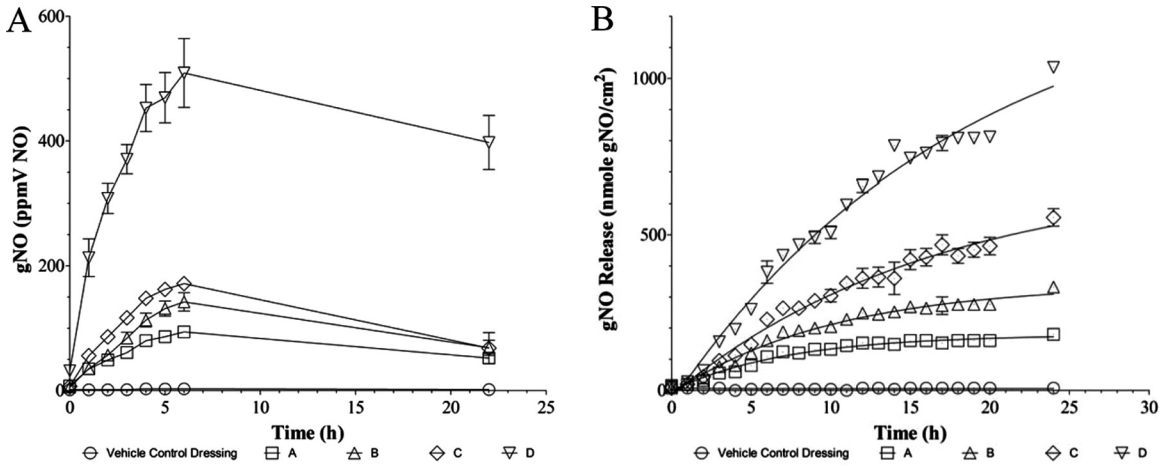

FIG 2 gNO release profiles of four different dressing formulations (A, B, C, and D, corresponding to squares, triangles, diamonds, and inverted triangles, respectively) and vehicle control dressings (circle). Generated gNO levels were determined by chemiluminescence (Sievers instrument; GE) and are reported as parts per million by volume (ppmv) of gNO produced. The data are means ± SD ($n = 2$) of independent preparations of each dressing with duplicate measurements. (B) gNO release rates of four different dressing formulations (A, B, C, and D corresponding to inverted triangles, diamonds, triangles, and squares, respectively) and vehicle control dressings (circle). gNO release rates of 24, 34, 42, and 73 nmol/cm^2 · h corresponded to dressings A, B, C, and D, respectively. The gNO release rates were determined from cumulative measures of NO_2^- produced in aqueous solution in contact with submerged dressings at indicated time points using a Griess assay, as described in Materials and Methods. The values indicate the means ± SD of independent dressing preparations ($n = 3$).

all three microorganisms. No reduction or only minimal reduction in the viabilities was seen for biofilms treated with vehicle control dressings, whereas a significant bactericidal effect (no detected viabilities at dilution 0) was observed for biofilms of all reference strains treated with dressings with the highest initial release rate of gNO (73 nmol/cm^2 · h; dressing D). The gNO release rates correspond to releasing concentrations of above 200 ppmv, which was previously reported to have bactericidal activity against planktonic bacteria (18) (Fig. 3). Untreated controls (biofilms unexposed to any treatment) (data not shown) were analyzed, and the results were comparable with the results obtained with the vehicle control dressings, with no decrease in biofilm viabilities under the same experimental conditions. Based on these results, we selected the dressings with the highest gNO release rate (dressings "D") and the vehicle controls for the following sets of experiments.

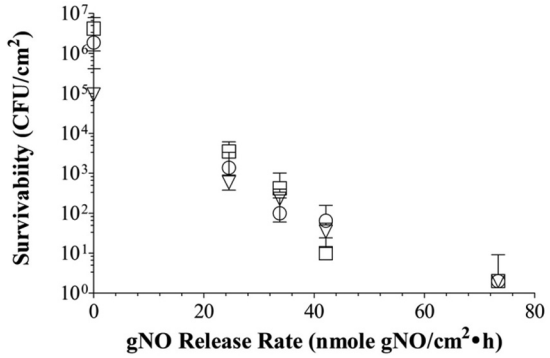

FIG 3 The effect of the gNO dose release rates on the bactericidal activity of gNO-releasing dressings against 24-h-grown biofilms of *A. baumannii* ATCC 19606 (squares), methicillin-resistant *S. aureus* ATCC 43300 (inverted triangles), and *P. aeruginosa* ATCC 10145 (circles). gNO release rates of 24, 34, 42, and 73 nmol/cm^2 · h corresponded to dressings A, B, C, and D, respectively. Bacterial viabilities (CFU/cm^2) were determined by spread-plating on LB agar plates after an overnight incubation at 37°C. The values indicate means ± SD of independent trials ($n = 2$).

Effective time of treatment. To evaluate the effect of the treatment time of gNO-releasing dressings on biofilms, viabilities of the three studied strains were evaluated without application of treatment (0 h), as well as at 3 h and 6 h after the administration of the dressings. Whereas *A. baumannii* and *P. aeruginosa* biofilms treated with vehicle control dressings maintained cell viabilities of approximately 2×10^5 and 1×10^6 CFU/cm^2, respectively, for the 6-h duration of the experiment, MRSA biofilms had a reduction of 1 log unit from 1×10^6 CFU/cm^2 to 1×10^5 CFU/cm^2 when treated with vehicle control dressings for 6 h.

Biofilm viability of all reference strains treated with gNO-releasing dressings presented a 3-log decrease following a 3-h treatment ($P = 0.029$, Mann-Whitney rank sum test for all three strains). Following a 6-h treatment with gNO-releasing dressings, biofilm viability of *A. baumannii* and MRSA decreased by 4 log units, whereas *P. aeruginosa* biofilms decreased by approximately 5 log units in cell viability (Fig. 4) ($P = 0.029$, Mann-Whitney rank sum test for all three strains).

Comparison of gNO-releasing dressings to antimicrobial therapies for prevention of biofilm formation. The gNO-releasing dressings were compared to therapeutically used antimicrobial treatments, including povidone-iodine dressings, silver dressings, and antibiotics such as chlorhexidine, gentamicin, and vancomycin, for the prevention of biofilm formation. The antibiotic concentrations were chosen based on MICs for planktonic bacteria in exponential growth phase. First, MICs were determined as described previously. For *A. baumannii*, determined MICs of chlorhexidine, gentamicin, and vancomycin were 15.6, 9.8, and 190 μg/ml, respectively. MICs of the same antibiotics for MRSA were 3.9, 4.9, and 1.5 μg/ml, respectively. Finally, the MICs of chlorhexidine and gentamicin for *P. aeruginosa* were 15.6 and 4.9 μg/ml, respectively. *P. aeruginosa* is resistant to vancomycin

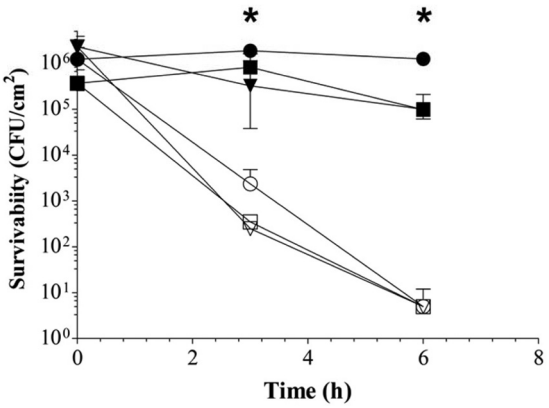

FIG 4 Treatment of a 24-h-grown biofilms of *A. baumannii* ATCC 19606 (squares), methicillin-resistant *S. aureus* ATCC 43300 (inverted triangles), and *P. aeruginosa* ATCC 10145 (circles) using vehicle control dressings (filled symbols) or gNO-releasing dressings (open symbols) for 0, 3, and 6 h at 37°C. Dressing D corresponding to 73 nmol/cm^2 · h of gNO release was used. The antimicrobial effect of the treatment was determined by spread plating on LB agar plates after an overnight incubation at 37°C. The values indicate the means ± SD of independent trials ($n = 2$). *, $P = 0.029$.

up to 1,320 μg/ml. The working chlorhexidine concentrations in this set of experiments were 40, 10, and 40 μg/ml for *A. baumannii*, MRSA, and *P. aeruginosa*, respectively. For vancomycin, the working concentrations were 400 and 4 μg/ml for *A. baumannii* and MRSA, respectively. Since *P. aeruginosa* is resistant to vancomycin, gentamicin was used instead at a working concentration of 12.5 μg/ml. Application of vehicle control dressings, silver dressings, and iodine dressings for prevention of *A. baumannii* biofilm formation demonstrated final viabilities of 7.5×10^6 CFU/cm^2, 1×10^2 CFU/cm^2, and 1.5×10^1 CFU/cm^2, respectively. Conversely, other treatments completely prevented biofilm formation (Fig. 5A) ($P = 0.002$, Kruskal-Wallis ANOVA by ranks; $P < 0.05$ for chlorhexidine, vancomycin, and NO-releasing dressing versus vehicle control dressing, Dunnett's test). MRSA biofilm formation was prevented using the gNO-producing dressing and significantly decreased using vancomycin and chlorhexidine ($P < 0.001$, Kruskal-Wallis; $P < 0.05$ for NO-releasing dressing, chlorhexidine and vancomycin versus vehicle control dressing, Dunnett's test); other treatments did not inhibit MRSA biofilm formation. Viabilities of 1.1×10^7 CFU/cm^2, 2.4×10^4 CFU/cm^2, 1.4×10^4 CFU/cm^2, 6×10^1 CFU/cm^2, 5 CFU/cm^2, and below the detection limit at dilution 0 were determined for MRSA biofilms after application of vehicle control dressings, silver dressing, iodine dressing, chlorhexidine, vancomycin, and gNO-releasing

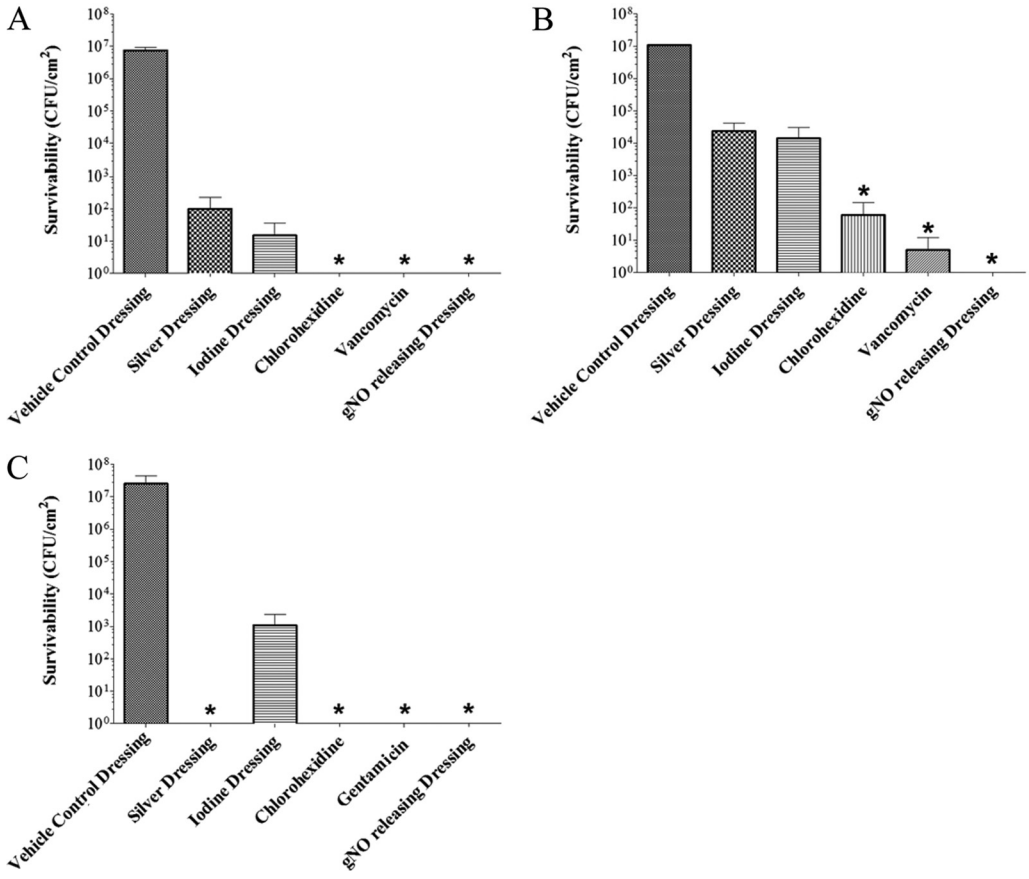

FIG 5 Comparison of the activity of different antimicrobial therapies for prevention of biofilm formation by virulent bacterial reference strains. An array of antimicrobials was applied to suspensions of *A. baumannii* ATCC 19606 (A), methicillin-resistant *S. aureus* ATCC 43300 (B), and *P. aeruginosa* ATCC 10145 (C) for 6 h at 37°C. Dressing D corresponding to 73 nmol/cm^2 · h of gNO release was used as the gNO-releasing dressing. The values indicate the means ± SD of independent trials ($n = 2$) ($P < 0.002$ Kruskal-Wallis ANOVA by ranks test; *, $P < 0.05$ Dunnett's test).

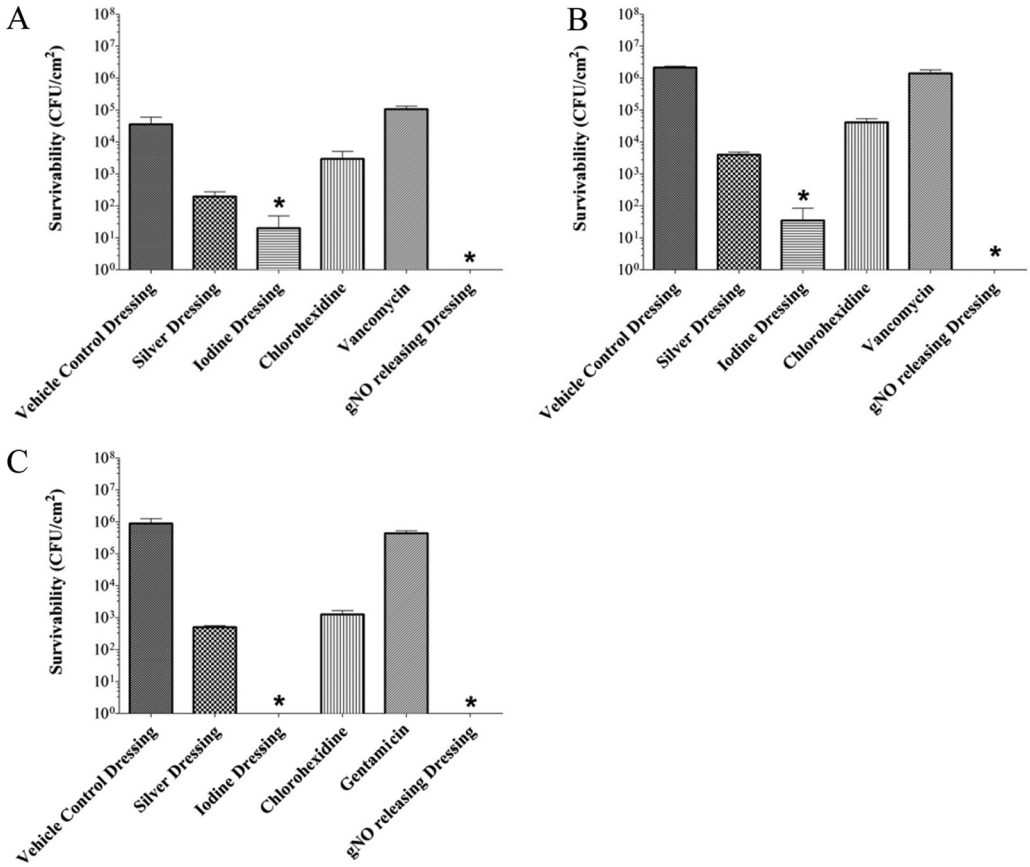

FIG 6 Comparison of the antibiofilm activity of different antimicrobial therapies for treatment of 24-h-grown biofilms of virulent bacterial reference strains. An array of antimicrobials was applied to biofilms of *A. baumannii* ATCC 19606 (A), methicillin-resistant *S. aureus* ATCC 43300 (B), and *P. aeruginosa* ATCC 10145 (C) for 6 h at 37°C following 24-h biofilm formation. Dressing D corresponding to 73 nmol/cm^2 · h of gNO release was used as the gNO-releasing dressing. The values indicate the means ± SD of independent trials ($n = 2$) ($P < 0.001$, Kruskal-Wallis ANOVA by ranks test; *, $P < 0.05$ Dunnett's test).

dressing, respectively (Fig. 5B). Complete prevention of *P. aeruginosa* biofilm formation (undetected viabilities at dilution 0) was achieved with the silver dressing, chlorhexidine, gentamicin, and gNO-releasing dressing ($P < 0.001$, Kruskal-Wallis; $P < 0.05$ for silver dressing, chlorhexidine, gentamicin, and gNO-releasing dressing versus vehicle control dressing, by Dunnett's test), while after application of the vehicle control dressing and iodine dressing, biofilm viabilities of 2.5×10^7 CFU/cm^2 and 1.1×10^3 CFU/cm^2 were observed, respectively (Fig. 5C). These results show that gNO-releasing dressings perform better than commercially available dressings on similar areas in the prevention of biofilm formation under the experimental conditions. In addition, antibiotic treatments prevent biofilm formation by killing planktonic bacteria. Although the treatments were targeting planktonic cells forming biofilms for the selected antibiotic concentrations (2- to 2.5-fold above MIC), incomplete inhibition may not have been seen due to the length of the treatment (6 h compared to the 24-h duration of the MIC assay).

Comparison of gNO-releasing dressing to antimicrobial therapies for treatment of biofilm formation. In contrast to prevention of biofilm formation, treatment of formed biofilms displayed distinct results. Notably, treatment of 24-h-formed *A. baumannii* and MRSA biofilms demonstrated that the gNO-releasing dressing achieved undetectable viabilities at dilution 0, while the iodine dressing resulted in significant cell death ($P < 0.001$, Kruskal-Wallis ANOVA by ranks test; $P < 0.05$ versus vehicle control dressings, Dunnett's test) (Fig. 6A and B). Similarly, the gNO-releasing dressing and the iodine dressing were the only treatments capable of achieving significant bactericidal activity when applied to 24-h-formed MRSA biofilms ($P < 0.001$, Kruskal-Wallis ANOVA by ranks test; $P < 0.05$ versus vehicle control dressings, Dunnett's test) (Fig. 6B). Finally, the 6-h treatment of 24-h-grown *P. aeruginosa* biofilms with gNO-releasing dressings and iodine dressings resulted in undetectable viabilities at dilution 0, whereas the other therapies yielded biofilms with viable cells ($P < 0.001$, Kruskal-Wallis ANOVA by ranks test; $P < 0.05$ versus vehicle control dressings, Dunnett's test) (Fig. 6C). Overall, these results show that gNO-releasing dressings and povidone-iodine dressings of similar areas present significant antibiofilm activities against the tested strains as opposed to silver dressings of similar areas that did not reduce biofilm viabilities significantly. Antibiotic solutions at higher concentrations than the determined MICs for planktonic bacteria did not present antimicrobial activity against the 24-h-grown biofilms.

Confocal laser scanning microscopy. Following 24-h bacterial growth and biofilm formation, biofilm thickness for *A. baumannii*, MRSA, and *P. aeruginosa* varied between 20 to 40 μm, as estimated by confocal microscopy. Microbial viabilities were visu-

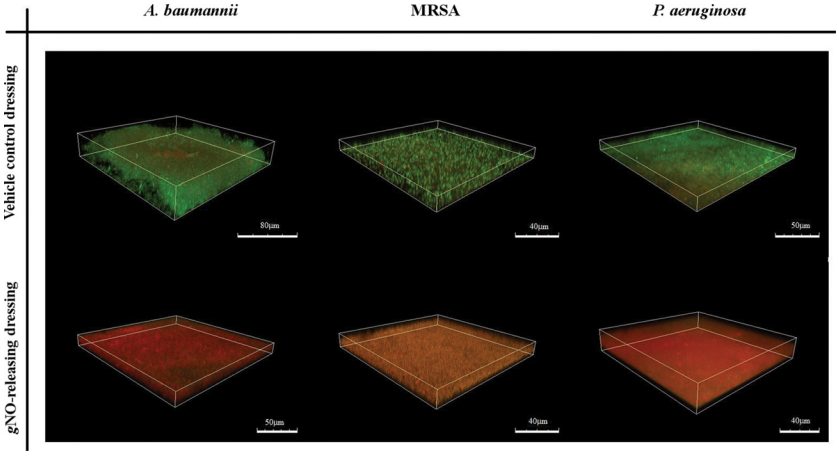

FIG 7 Three-dimensional view of confocal laser scanning micrographs of 24-h biofilms of *A. baumannii*, MRSA, and *P. aeruginosa*, treated with either vehicle control dressings or gNO-releasing dressings for 6 h at 37°C and stained with a Live/Dead BacLight fluorescent kit for bacterial viability. Live cells are stained in green, and dead cells are stained in red.

alized by confocal laser scanning microscopy after treatment of biofilms with gNO-releasing dressings or vehicle control dressings. Exposure of the biofilms to the gNO-releasing dressings resulted in substantial cell death, as indicated by red fluorescence, compared to treatment with vehicle control dressings, where most cells survived, as indicated by green fluorescence (Fig. 7). These results, as observed for the three studied strains, confirm the viability measurements as determined by surface plating.

DISCUSSION

In the present study, we report for the first time the use of an enzymatic gNO-releasing dressing as an antimicrobial device against biofilms of three clinically relevant antibiotic-resistant microorganisms: *A. baumannii*, MRSA, and *P. aeruginosa*. The gNO production profiles of the dressings were characterized, and formulations produced different levels of gNO and allowed for gNO release in a sustainable manner for up to 18 h. In order to attain bactericidal gNO concentrations in a timely manner, as well as a prolonged gNO release above a target level, dressings that demonstrated gNO production higher than 200 ppmv for 22 h were selected; an amount over 160 ppmv of gNO maintained for at least 6 h has been observed to lead to effective antimicrobial activity (18, 33). These dressings presented a peak of approximately 325 ppmv of gNO and an initial release rate of 73 nmol/cm^2/h during the linear interval of 18 h. Other NO-releasing technologies, such as silica nanoparticles (24) and NO-impregnated 6-mm diameter catheters (42), have been tested for antimicrobial activity against biofilms. The silica nanoparticles released up to 8 μmol per mg within 20 min, with a peak of maximum NO production at approximately 6 min. The impregnated catheters released NO at a much lower rate of approximately 0.1 μmol/cm during the first hour with an exponential decay over 3 days. By 30 h, these catheters released approximately a total of 0.25 μmol/cm, which represents an average of 8.3 nmol/cm/h.

In the present work, we analyzed the bactericidal effects of the enzymatic gNO-releasing dressings at various gNO release levels after a 6-h exposure to biofilms of the aforementioned bacteria that had been previously grown for 24 h. Upon bacterial viability quantification through surface plating, substantial bacterial death was observed for both planktonic bacteria and bacteria in biofilms using the dressings with the highest gNO release rate. These observations were consistent for all strains tested. In addition, a time course was run and confirmed that 6 h was ample treatment time for highly significant bacterial cell death when biofilms previously grown for 24 h were treated with gNO-releasing dressings compared to vehicle control dressings, where a minimal bactericidal effect was noticeable. Antimicrobial activity within biofilms was observed as early as after 3 h of treatment with active dressings as opposed to results with vehicle controls. Other NO-releasing technologies tested the survivability of biofilms or prevention of biofilm formation only at 24 h after application of the corresponding therapies (24, 42). Moreover, cell viabilities in biofilms of *Burkholderia cepacia*, *P. aeruginosa*, and *S. aureus* were decreased approximately 5 logs, 2 to 4 logs, and 2 logs, respectively, only after 24 h treatment with 60 mM sodium nitrite (31).

A growing body of data has shown that virulent bacterial strains can attach to the wound site within minutes and form strongly attached microcolonies within 2 to 4 h of initial injury (6, 11). Contemporary therapies (chlorhexidine digluconate dressings, povidone-iodine dressings, silver-coated antimicrobial dressings, and antibiotics) have been limited in their efficacy, and the use of NO has been shown *in vitro* as an efficient broad-spectrum agent primarily in the prevention of biofilms (4, 24, 42). The gNO-releasing dressing reported in this study has demonstrated a potential as a more efficient antimicrobial agent than other widely available therapies against *A. baumannii*, MRSA, and *P. aeruginosa* biofilms and, upon further validation, may potentially serve as a novel antimicrobial therapy for wound infections. This dressing can be easily adhered to skin and placed on top of catheter lines, and because of the high diffusivity of NO (3×10^{-5} cm^2 s^{-1}) (51), this agent may likely permeate the thin catheter barrier and exert its antimicrobial activity.

In addition to antimicrobial properties, NO plays a role as a signaling molecule in a variety of processes, including cell proliferation, cell death, vasorelaxation, inflammation, and angiogenesis, as well as in other pathways involved in wound healing (9, 10). It is generally accepted that NO stimulates cell migration, proliferation, and vasorelaxation at low levels and promotes cell death

at high levels (10). Exposure to NO at high concentrations for long periods of time may therefore cause tissue damage to the host cells. We have recently tested a probiotic NO-releasing dressing of similar construction and gNO release (200 to 500 ppmv) for the healing of infected ischemic wounds in New Zealand rabbits and demonstrated both efficacy and safety of the devices (27). In addition to accelerated wound healing in the presence of the gNO-releasing device after a 3-week treatment, there was a reduction in exudate and microbial load compared to vehicle controls. Further, no indications of skin or systemic toxicity were seen, as analyzed by histopathology and assessment of blood samples for safety parameters. In the same study, methemoglobin levels after treatment were below detection limits. Other studies analyzed the toxicity of NO-releasing silica nanoparticles with antibiofilm activities in fibroblast cultures, with no observable detrimental results (24). These results indicate that NO could be used safely to treat infected wounds over extended periods of time.

The enzymatic gNO-releasing dressings were efficacious at eliminating biofilm-based microbes. Comparison of the gNO-releasing dressing to commercially available chlorhexidine digluconate, povidone-iodine dressings, silver-coated antimicrobial dressings, vancomycin, gentamicin, and vehicle control dressings was performed for both prevention of biofilm formation and treatment of 24-h-grown biofilms. For prevention experiments, where appreciable biofilm formation had not occurred and bacteria were primarily planktonic, all tested antibiotics and the NO-releasing dressing were efficient at achieving significant or complete bacterial death; however, only gNO-releasing dressings and iodine dressings presented efficacious antibacterial activity when biofilms of all tested strains that had been grown for 24 h were treated. A previous study using a novel *in vitro* model of infected skin wounds shows that cadexomer iodine dressings were the only therapeutics capable of reducing formed biofilms, while other tested agents, such as silver dressings, polyhexamethylene biguanide (PHMB) dressings, or doxycycline monohydrate-releasing dressings, were ineffective (39). Although the mechanism through which NO prevents bacterial adhesion and treats formed biofilms is unknown, several hypotheses have been postulated. It is presumed that through reactive intermediate species such as dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4), NO disrupts bacterial adhesion proteins to prevent surface adhesion (15). Again, due to its elevated diffusion coefficient and solubility (51), NO can permeate biofilms and target cellular components such as cell membrane, structural proteins, metabolic enzymes, or DNA by nitrosative or oxidative stress and therefore kill biofilm bacteria (25).

These results demonstrate a novel approach to eradicate three virulent bacteria of clinical relevance when they are present within a biofilm matrix *in vitro*. The use of this enzymatic gNO-releasing dressing demonstrates antibiofilm activity that is superior to commonly available therapies on the market. Although this testing model may present limitations, given the duration of the dressing's gNO release and *in vitro* efficacy, this device may potentially have a broad application in hospital, long-term care, and home settings. Nonetheless, further *ex vivo* or *in vivo* biofilm studies are desired to corroborate the current *in vitro* results.

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