**Antibacterial Action of Nitric Oxide-Releasing Chitosan Oligosaccharides against *Pseudomonas aeruginosa* under Aerobic and Anaerobic Conditions**

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Chitosan oligosaccharides were modified with N-diazeneumdiolates to yield biocompatible nitric oxide (NO) donor scaffolds. The minimum bactericidal concentrations and MICs of the NO donors against *Pseudomonas aeruginosa* were compared under aerobic and anaerobic conditions. Differential antibacterial activities were primarily the result of NO scavenging by oxygen under aerobic environments and not changes in bacterial physiology. Bacterial killing was also tested against nonmucoid and mucoid biofilms and compared to that of tobramycin. Smaller NO payloads were required to eradicate *P. aeruginosa* biofilms under anaerobic versus aerobic conditions. Under oxygen-free environments, the NO treatment was 10-fold more effective at killing biofilms than tobramycin. These results demonstrate the potential utility of NO-releasing chitosan oligosaccharides under both aerobic and anaerobic environments.

*Pseudomonas aeruginosa* is an opportunistic human pathogen that frequently colonizes people with compromised immune systems, such as those with cystic fibrosis (CF) or severe burn wounds (1). The success of *P. aeruginosa* as a pathogen is related to its multitude of virulence factors, which increase adherence to the host cells, induce inflammation, and disrupt the host immune response (1). Furthermore, *P. aeruginosa* is intrinsically resistant to many antibiotics due to low membrane permeability and increased expression of β-lactamases and efflux pumps (2–4). In addition to this native resistance, *P. aeruginosa* readily adapts to antibiotic challenge by acquiring resistance genes (3, 5) and forming protective, cooperative communities known as biofilms (6, 7).

While all antibiotic resistance mechanisms are not fully understood, at least three main factors reduce antibiotic efficacy against bacterial biofilms compared to planktonic cells. First, *P. aeruginosa* in biofilms secretes a protective layer of exopolysaccharides that prevent the diffusion of antibiotics (7). In the context of cystic fibrosis, biofilm-bound *P. aeruginosa* exists predominantly as the mucoid phenotype, characterized by a secreted alginate matrix that provides a physical barrier against the host immune response and antibiotics (8). This exopolysaccharide matrix also prevents the diffusion of oxygen into biofilms, causing *P. aeruginosa* to switch from aerobic to anaerobic respiration (9). The reduced metabolic activity of *P. aeruginosa* undergoing anaerobic respiration protects the bacterium against traditional antibiotics that are most effective against rapidly dividing cells, including aminoglycosides and β-lactams (10, 11). Finally, biofilms produce persister cells (i.e., dormant bacteria that are highly resistant to chemical disinfectants and exhibit multidrug tolerance) much more frequently than planktonic bacterial cultures (3, 7).

The failure of conventional antibiotics to treat *P. aeruginosa* biofilms and infections necessitates the development of new anti-bacterial agents. Nitric oxide (NO), an endogenously produced free radical that can disperse (12, 13) and eradicate (14, 15) biofilms, holds particular promise as an alternative to current antibiotic treatments. Gaseous NO has been repeatedly used to eradicate *P. aeruginosa* infections in small-animal models with no apparent toxicity (16, 17). Under aerobic environments, NO reacts with molecular oxygen, superoxide, and hydrogen peroxide to form highly reactive intermediates (peroxynitrite, nitrogen dioxide, and dinitrogen trioxide). These molecules exert nitrosative and oxidative stresses, such as DNA deamination, nitrosation of membrane and intracellular proteins, and membrane damage via lipid peroxidation, culminating in bacterial death (18–21). Some of these congener molecules, especially peroxynitrite, are more potent antimicrobials than NO alone (19). In anaerobic environments, NO toxicity is less understood. Ren et al. reported that the bacteriostatic mechanisms included modification of iron-sulfur proteins (22). As these proteins are linked to nearly every cellular process, including metabolism, respiration, RNA modification, and DNA repair and replication, their alteration greatly influences bacterial viability (23). The killing activities of NO are expected to be different under aerobic and anaerobic conditions due to the differences in bactericidal mechanisms, but this hypothesis has yet to be studied systematically. As *P. aeruginosa* grows rapidly and forms biofilms under both aerobic and anaerobic conditions, understanding the effects of oxygen on the antibacterial activity of NO is essential for developing NO-based therapeutics.

While the administration of exogenous NO holds promise as a therapeutic, treatment of infections or chronic wounds with gaseous NO is impractical, expensive, and potentially dangerous, as NO mediates other physiological processes (e.g., vasodilation and...
blood clotting) (21, 24–26). Macromolecular scaffolds capable of effectively storing and releasing NO have been developed to enable local delivery (27, 28). The most promising NO release vehicles to date include NO donor-modified N-diazoniumidum silicate nanoparticles (29–31), dendrimers (32–36), and chitosan (15). While silica nanoparticles (14, 37–39) and dendrimers (32–34) are effective as antimicrobials, they do not easily break down and thus have limited potential as inhaled therapeutics. Chitosan-based oligosaccharides represent attractive scaffolds for NO delivery, as they are biodegradable and have low toxicity to mammalian cells (40, 41). We have previously reported that NO-releasing chitosan oligosaccharides are capable of NO storage/release and of eradicating P. aeruginosa biofilms under aerobic environments at concentrations nontoxic to mammalian cells (15). Here, we evaluate the antibacterial efficacy of NO-releasing chitosan oligosaccharides as a function of oxygen availability using nonmucoid, mucoid, and biofilm P. aeruginosa phenotypes.

MATERIALS AND METHODS

Materials. Medium-molecular-weight chitosan, 2-methylaziridine, and tobramycin were purchased from Sigma-Aldrich (St. Louis, MO). Methyltrimeoxisylane (MTMOS) was purchased from Fluka (Buchs, Switzerland). (Heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17 FTMS) was purchased from Gelest (Morrisville, PA). Nitric oxide gas (15 ppm; balance N2), argon (Ar), and nitrogen (N2) gases were purchased from Fisher Scientific (Pittsburgh, PA). All common laboratory salts and reagents were purchased from Acros Organics (Geel, Belgium). Distilled water was purified using a Millipore Milli-Q UV Gradient A-10 system (Bedford, MA). Acetone, collected via centrifugation, and dried in vacuo prior to use.

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Antimicrobial Agents and Chemotherapy

Synthesis of COS-NO. In order to impart NO storage and release, N-diazoniumidum derivatives were formed on the secondary amines of chitosan oligosaccharides (COS-NO) were precipitated in acetone, collected via centrifugation, and dried in vacuo, and stored at –20°C as a yellow powder.

Cheliluminescence detection of NO release. A Sievers (Boulder, CO) 280i chemiluminescence nitric oxide analyzer was used for chemiluminescence detection of NO from COS-NO (1.0 mg) in 30 ml of deoxygenated PBS (pH 6.5) at 37°C. The released NO was carried by N2 gas to the reaction vessel/detector at a flow rate of 80 ml/min. Additional N2 flow was supplied to the sample flask at 200 ml/min to match the collection rate of the instrument. The analysis was terminated when NO concentrations fell below 10 ppb NO/mg COS-NO. Prior to analysis, the instrument was calibrated with air passed through an NO zero filter (0 ppm NO) and 26.8 ppm of NO standard gas (balance N2).

Electrochemical detection of NO release. NO-selective electrochemical sensors were fabricated in house as previously reported (43). Briefly, polished polycrystalline Pt disk electrodes (2 mm) sealed in Kel-F (CH Instruments, Austin, TX) were coated with an NO-selective membrane prepared by mixing MTMOS (60 µl), ethanol (300 µl), 17 FTMS (15 µl), water (80 µl), and 0.5 M hydrochloric acid (5 µl) for 1 h at 25°C. The resulting solution was spread over the Pt electrode and dried overnight at room temperature. Amperometric NO measurements followed, using a three-electrode setup with the NO-selective membrane-modified Pt electrode as the working electrode, a Pt-coiled counter electrode, and an Ag/AgCl reference electrode. The applied potential for NO oxidation was +780 mV versus Ag/AgCl. Immediately prior to use, the NO sensors were calibrated by adding a known amount of PBS saturated with NO gas (1.9 mM) to deoxygenated PBS (pH 6.5). Saturated NO solutions were made on the day of use by degassing PBS (pH 6.5) for 30 min with Ar, followed by 20-min purging with NO gas. The sensors were immersed in 10.0 ml of PBS or LB broth (stirred; 37°C) and polarized at 700 mV versus Ag/AgCl until a stable baseline was achieved prior to the addition of COS-NO. The NO oxidation current was measured every 0.1 s and ceased when the current returned to its background value. Measurement of NO release in an aerobic media was carried out in a Coy anaerobic chamber. Total NO for 1.0-mg COS-NO/ml solutions are reported as the average and standard deviation for 4 or more separate measurements.

Planktonic bacterial assays. Bacteria were grown as overnight cultures, diluted 1:100 in fresh LB broth (with or without nitrate supplementation), and grown to mid-log phase (2 × 108 CFU/ml). These cultures were centrifuged, resuspended in PBS, and diluted to 2 × 106 CFU/ml in PBS. Each suspension was then added to vials containing 2-fold serial dilutions of COS-NO or COS controls and incubated at 37°C for 4 h with gentle shaking. Following treatment, the bacterial solutions were serially diluted, plated on LB agar, and incubated for 24 h at 37°C. Colonies were enumerated using a Flash and Go colony counter (IUL, Farmingdale, NY). The minimum bactericidal concentration after a 4-h exposure (MBC<sub>4h</sub>) was defined as the minimum concentration required to achieve a 3-log-unit reduction in viable bacteria (from 10<sup>9</sup> to 10<sup>6</sup> CFU/ml). The plating-counting method employed has a limit of detection of 2.5 × 10<sup>3</sup> CFU/ml (44). The corresponding NO dose was calculated by multiplying...
the MBC$_{4h}$ of COS-NO (in milligrams per milliliter) with the available NO in aerobic and anaerobic PBS (in micromoles NO per milligram COS-NO).

**Planktonic inhibition assays.** Bacteria were grown as overnight cultures, diluted 1:100 in fresh LB broth, grown to mid-log phase ($2 \times 10^8$ CFU/ml), and diluted to $2 \times 10^6$ CFU/ml in LB broth. The bacterial cultures were then added to vials containing 2-fold serial dilutions of COS-NO or COS controls and incubated at 37°C for 18 h with gentle shaking. The MIC was determined to be the minimum concentration that prevented visible growth, defined as an optical density at 600 nm of $<0.1$. All untreated (control) cultures became visibly turbid during the 18-h growth period. The corresponding NO dose was calculated by multiplying the MIC of COS-NO (in milligrams per milliliter) with the available NO in aerobic and anaerobic broth (in micromoles NO per milligram COS-NO). Nitrate-supplemented LB was used for all stages of bacterial growth and exposure. Anaerobic experiments were performed in a Coy anaerobic chamber.

**Biofilm eradication assays.** Bacteria were grown as overnight cultures, diluted 1:100 in fresh LB broth, and grown to mid-log phase ($2 \times 10^8$ CFU/ml). The bacterial cultures were then diluted to $10^8$ CFU/ml in diluted (25%) LB broth supplemented with 15 mM KNO$_3$ (pH 6.5) and grown for 72 h at 37°C with gentle shaking. The viscous microcolony biofilms formed were easily separated from the growth media via pipetting. The biofilms were harvested by placing a pipette tip near the center of the biofilm and applying suction. The biofilms were then washed by injection into PBS and extracted using the same pipetting procedure to remove planktonic or loosely associated bacteria. The freshly washed biofilms were combined with 750 µl of PBS (pH 6.5); added to vials containing COS, COS-NO, or tobramycin; and incubated with gentle shaking for 18 h at 37°C. After treatment, the biofilms were washed via pipetting in PBS to remove excess antibacterial agent, transferred to 750 µl of PBS (pH 6.5), and gently sonicated to disrupt the biofilm matrix. The dispersed biofilms were vortexed, serially diluted, plated, and enumerated on LB agar. The minimum biofilm eradication concentration (MBEC$_{4h}$) was defined as the concentration that caused a 5-log-unit reduction in viable bacteria (i.e., $10^0$ to $10^5$ CFU/ml) after the 18-h treatment. The corresponding NO dose was calculated by multiplying the MBEC$_{4h}$ of COS-NO (in milligrams per milliliter) with the available NO in aerobic and anaerobic PBS (in micromoles NO per milligram COS-NO).

**Statistical analysis.** All data are expressed as the mean ± 1 standard deviation and were analyzed for significance ($P < 0.05$) with a two-tailed Student $t$ test.

**RESULTS**

**Nitric oxide release from COS-NO in media.** Nitric oxide release from COS-NO was measured via chemiluminescence in deoxygenated PBS (pH 6.5) at 37°C to yield total NO release payloads of $0.86 \pm 0.05 \mu$mol NO/mg, with an overall release duration of 10.2 ± 2.7 h (see Fig. S1 in the supplemental material). While NO release from macromolecular scaffolds is generally measured in deoxygenated medium, measuring the amount of bioavailable (i.e., nonscavenged) NO is critical for elucidating the biocidal dose-response relationship of NO under the intended conditions, as NO is rapidly scavenged by oxygen and proteins in biological media (45). Unfortunately, foaming associated with nutrient-rich medium makes chemiluminescence detection difficult and irreproducible (43). Thus, we turned to amperometric NO detection to carry out NO measurements in broth (LB).

In the absence of NO scavenging (i.e., in deoxygenated PBS), amperometric measurements revealed a total NO payload of $0.34 \pm 0.17 \mu$mol/ml from the 1.0-mg/ml solution of COS-NO over 4 h (Fig. 1). As might be expected, both the total NO payload and the release duration of COS-NO measured via amperometry were reduced compared to chemiluminescence detection. These decreases are common for electrochemical sensors that are based on the diffusion of NO to the working electrode and the inherent loss of NO to the ambient atmosphere (43).

Under aerobic conditions, oxygen scavenging reduced the amount of free NO available in PBS by approximately 35% ($0.22 \pm 0.08 \mu$mol NO/ml). Nutrient broth (LB) further diminished the available NO payload via scavenging of the NO by proteins in the broth. The amount of NO available in anaerobic LB broth was reduced to $0.14 \pm 0.08 \mu$mol NO/ml, a 66% reduction relative to aerobic PBS. Further reductions ($0.027 \pm 0.017 \mu$mol/ml) were observed in aerobic LB broth due to reaction of NO with oxygen.

**Bactericidal action of the COS scaffold.** To confirm that NO and not the scaffold was responsible for the observed bacterial killing, all bacterial assays were performed using NO-releasing and control (i.e., non-NO-releasing) chitosan oligosaccharides. In MBC$_{4h}$ assays of planktonic cells, COS did not influence bacterial viability at $1 \times$ or $10 \times$ the MBC$_{4h}$ of COS-NO, indicating that the
chitosan oligosaccharide alone was not bactericidal (see Fig. S2 in the supplemental material). Similarly, bacterial viability was not reduced upon treatment of the biofilms with 4.0 mg COS/ml (1 × the MBE_{18}h) for 18 h under both aerobic and anaerobic conditions (see Fig. S3 in the supplemental material). Based on these data, the bactericidal activity of COS-NO was attributed solely to the effects of NO and not to toxicity of the COS scaffold.

Effect of oxygen on bactericidal action of NO against planktonic 

*P. aeruginosa.* The biocidal action of NO was evaluated with respect to the oxygen concentration in the treatment medium by exposing planktonic cultures to COS-NO in both aerobic and anaerobic PBS (pH 6.5) (Table 1). When grown under aerobic conditions, the concentration of COS-NO required to inhibit growth was reduced in anaerobic environments, but the efficacy of NO was unchanged for most of the isolates tested. However, there was no statistical difference in the NO dose required to inhibit growth for most of the strains tested (Fig. 2B). However, two isolates (Fig. 2B, asterisks) showed a statistically significant increase in the NO dose required to inhibit growth under anaerobic conditions less than or equal to those under aerobic conditions for all 10 isolates (Fig. 2A). There was no statistical difference in the NO dose required to inhibit growth for most of the strains tested (Fig. 2B). However, two isolates (Fig. 2B, asterisks) showed a statistically significant increase in the NO dose required to inhibit growth under anaerobic conditions relative to aerobic conditions. Overall, COS-NO was more effective at inhibiting growth under anaerobic environments, but the efficacy of NO was unchanged for most of the isolates tested.

Inhibition of planktonic *P. aeruginosa* growth by COS-NO. 

MIC assays were performed to evaluate the efficacy of COS-NO during bacterial growth under aerobic and anaerobic environments (Table 2). Under aerobic conditions, the nonmucoid phenotype was more tolerant of COS-NO than the mucoid strain, with inhibitory doses of 800 μg COS-NO/ml (0.022 ± 0.014 μmol NO/ml) versus 400 μg COS-NO/ml (0.011 ± 0.007 μmol NO/ml), respectively. Anaerobic conditions decreased the MIC to 100 μg COS-NO/ml (0.014 μmol NO/ml) for both phenotypes. While the COS-NO concentration required to inhibit growth was reduced in anaerobic environments, the NO dose delivered was not statistically lower, indicating that NO lost to reaction with oxygen accounts for the increased MICs against COS-NO under aerobic conditions.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth medium</th>
<th>Aerobic MBC_{4h} (μg COS-NO/ml)</th>
<th>NO dose* (μmol NO/ml)</th>
<th>Anaerobic MBC_{4h} (μg COS-NO/ml)</th>
<th>NO dose* (μmol NO/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonmucoid</td>
<td>Aerobic</td>
<td>100</td>
<td>0.022 ± 0.008</td>
<td>100</td>
<td>0.034 ± 0.017</td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
<td>100</td>
<td>0.022 ± 0.008</td>
<td>100</td>
<td>0.034 ± 0.017</td>
</tr>
<tr>
<td>Mucoid</td>
<td>Aerobic</td>
<td>100</td>
<td>0.022 ± 0.008</td>
<td>100</td>
<td>0.034 ± 0.017</td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
<td>200</td>
<td>0.044 ± 0.016</td>
<td>200</td>
<td>0.068 ± 0.033</td>
</tr>
</tbody>
</table>

*P. aeruginosa* cultures were grown in LB broth (plus 15 mM KNO_{3}) under aerobic or anaerobic conditions and then exposed to COS-NO in PBS (pH 6.5) for 4 h under aerobic or anaerobic conditions.

TABLE 2 Influence of oxygen on the inhibitory efficacy of COS-NO*

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Nonmucoid strain</th>
<th>Mucoid strain</th>
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<tbody>
<tr>
<td></td>
<td>MIC (μg COS-NO/ml)</td>
<td>NO dose* (μmol NO/ml)</td>
</tr>
<tr>
<td>Aerobic</td>
<td>800</td>
<td>0.022 ± 0.014</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>100</td>
<td>0.014 ± 0.008</td>
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</table>

*Bacterial cultures in mid-log-phase growth were diluted to 2 × 10^9 CFU/ml in LB broth (plus 15 mM KNO_{3}) with COS-NO and grown for 18 h under aerobic or anaerobic conditions. The MIC was determined as the concentration of COS-NO that visibly inhibited growth.

* Determined via amperometry. The values are presented as means ± standard deviations for 3 or more pooled experiments.
Biofilm eradication by COS-NO and tobramycin. As bacterial biofilms exist in both aerobic and anaerobic environments (46), it was important to determine how oxygen concentrations affected the antibiofilm activity of COS-NO. Under aerobic conditions, highly viscous microcolony biofilms were formed (\( \sim 250 \mu l \) in volume) with bacterial viability of \( 4.0 \pm 0.6 \times 10^8 \) and \( 2.5 \pm 0.5 \times 10^8 \) CFU/ml for nonmucoid and mucoid phenotypes, respectively. Of note, nitrate supplementation was required to prevent phenotypic switching from the mucoid to the nonmucoid phenotype (47). Under anaerobic growth conditions, we were unable to cause the bacteria to form robust biofilms even after 7 days of growth.

Bacterial biofilms were exposed to COS-NO for 18 h in PBS (pH 6.5) under aerobic or anaerobic conditions. The MBEC\(_{18 \, h}\) values for the two phenotypes were \( 4,000 \mu g \) COS-NO/ml and \( 1,000 \mu g \) COS-NO/ml (\( 0.88 \pm 0.33 \) and \( 0.34 \pm 0.17 \) \( \mu mol \) NO/ml) under aerobic and anaerobic conditions, respectively (Table 3). These results indicate that NO released from a scaffold is equally effective at eliminating biofilms derived from nonmucoid and mucoid strains. Moreover, NO is significantly more effective at eliminating biofilms in the absence of oxygen.

The MBEC\(_{18 \, h}\) of tobramycin against biofilms was determined under the same conditions (i.e., 18-h exposure in PBS (pH 6.5) under aerobic or anaerobic conditions). The MBEC\(_{18 \, h}\) values for the two phenotypes were \( 200 \) and \( 800 \mu g/ml \) for nonmucoid and mucoid strains, respectively (Table 4). Both strains required greater tobramycin levels (1,600 \( \mu g/ml \)) to eradicate bacterial biofilms under anaerobic conditions.

DISCUSSION

We have previously reported on the antibacterial activity of NO against planktonic and biofilm-based \( P. \) aeruginosa (14, 15, 39). However, little is understood regarding how oxygen and the bacterial phenotype impact NO’s efficacy. Such knowledge is critical in the development of NO-based therapeutics. Water-soluble NO-releasing chitosan oligosaccharides were used as the NO release scaffold in the studies described here due to their biocompatibility and ability to be degraded \( in \) \( vivo \) (40, 41, 48). Although chitosan is a known bactericidal agent, the reduced molecular mass (to ensure water solubility) and 2-methylaziridine modification (for NO donor addition) resulted in a material with no bactericidal activity (see Fig. S2 and S3 in the supplemental material).

It is well known that NO reacts with oxygen and superoxide to form highly reactive intermediates that facilitate bacterial killing through oxidative and nitrosative stresses (19, 49). As oxygen plays an integral role in the antibacterial action of NO, anaerobic environments may reduce the biocidal efficacy of NO (20, 50). However, NO also reacts with oxygen to form nitrate and nitrite. These seemingly paradoxical roles of oxygen in NO-mediated killing are not fully understood. Therefore, we carried out electrochemical measurements of NO under aerobic and anaerobic conditions to quantify the amounts of bioavailable NO. Under aerobic conditions, NO is significantly more effective at eliminating biofilms in the absence of oxygen.
aerobic conditions, the measured NO decreased by 35% compared to anaerobic conditions (Fig. 1). To elucidate the effects of oxygen availability in treatment media, bacteria were first grown aerobically and then exposed to COS-NO in aerobic or anaerobic PBS. Identical concentrations of COS-NO were required to kill P. aeruginosa regardless of the treatment conditions. Due to the reaction of NO with oxygen, the bioavailable concentration of NO (i.e., the NO dose) was slightly, but not significantly, higher under anaerobic conditions (Table 1). As such, oxygen availability in the treatment medium has no statistically significant effect on the bactericidal activity of NO released from COS-NO.

While the oxygen concentration in the exposure medium did not alter the bactericidal efficacy of NO, the presence of oxygen during bacterial growth did influence P. aeruginosa susceptibility to NO. Anaerobic growth conditions reduce the efficacy of current antibiotics by altering certain properties of the bacteria, such as alginate production (46) and metabolic rates (10). To separate these factors, MBG assays were performed under nonnutritive conditions to minimize the effects of bacterial metabolism on the bactericidal activity of NO. When bacteria were grown under anaerobic conditions, the efficacy of NO released from the chitosan oligosaccharide scaffold was decreased against the mucoid, but not the nonmucoid, phenotype, indicating that growing mucoid bacteria without oxygen significantly alters their defense against NO (Table 1). Worlitzsch et al. previously reported that P. aeruginosa produces a protective alginate exopolysaccharide that is 50% thicker when grown anaerobically (46). As alginate restricts the diffusion of oxygen, the increased thickness of this protective layer could potentially prevent NO diffusion into the bacteria, therefore requiring a larger NO dose for killing.

To study the role of anaerobic growth on the efficacy of NO and COS-NO, we evaluated the inhibition of P. aeruginosa growth by COS-NO in nutrient-rich medium under both aerobic and anaerobic conditions. In contrast to the static conditions of MBG assays, bacteria are actively growing during inhibition assays. Comparison of MIC values under oxygen and oxygen-free environments showed that the efficacy of COS-NO was enhanced under anaerobic conditions while there was no statistical difference in the corresponding NO dose. This behavior was observed in the laboratory strains (Table 2) and most of the clinical isolates tested (Fig. 2). As the efficacy of NO is not reduced under anaerobic environments, NO-based treatments represent a potential alternative to current antibiotic treatments, including amino glycop sides and β-lactams, which are less effective under anaerobic conditions because their mechanism of action requires actively dividing cells (51, 52).

It is important to characterize the antibiofilm activities of antibacterial agents, as P. aeruginosa exists as biofilms on medical implants (e.g., catheters) (53, 54), on burn wounds (55, 56), and in the airways of patients with cystic fibrosis (57, 58). As shown in Table 4, mucoid biofilms are significantly more resilient against tobramycin than nonmucoid biofilms. Hentzer et al. attributed decreased antibiotic efficacy against mucoid strains to the overproduction of alginate (59). As has been previously reported (60, 61), low-oxygen conditions further decrease the effectiveness of tobramycin (the MBEC18h is increased to 1,600 μg/ml under anaerobic conditions). While tobramycin is a highly effective anti-pseudomonal agent, these factors compromise its ability to kill P. aeruginosa biofilms in oxygen-free environments. In contrast, NO released from the chitosan scaffold was equally effective at eradicating mucoid and nonmucoid biofilms. Similarly, the antibiofilm activity was not reduced under anaerobic environments (Table 3). Furthermore, the NO dose required for biofilm eradication under anaerobic conditions is 0.34 ± 0.17 μmol NO/ml, 10-fold lower than that of tobramycin (3.42 μmol/ml) (Tables 3 and 4).

In conclusion, these studies examined the susceptibilities of nonmucoid, mucoid, and biofilm P. aeruginosa phenotypes to NO-releasing chitosan oligosaccharides as a function of oxygen availability. The antibacterial activity of NO-releasing chitosan oligosaccharides was enhanced in oxygen-free environments, despite a concomitant decrease in the number of possible mechanisms available to kill bacteria (i.e., fewer toxic by-products from the reactions of NO and oxygen). Furthermore, the antibiofilm action of NO was more effective than that of tobramycin and was not influenced by the bacterial phenotype. When combined with NO’s significant biocidal action against P. aeruginosa, these results suggest that NO-releasing chitosan oligosaccharides may represent a potential alternative to traditional antibiotics, particularly when treating biofilms or in low-oxygen environments. We are currently seeking to enhance the NO payloads and evaluate the effects of NO release kinetics on the antibacterial efficacy of chitosan oligosaccharides.

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Mark H. Schoenfisch declares a competing financial interest: he is a co-founder and member of the board of directors and maintains a financial interest in Novan Therapeutics, Inc. Novan Therapeutics, Inc. provided partial funding for this research and is commercializing macromolecular nitric oxide storage and release vehicles for dermatological applications.

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