

# Novel nitric oxide-generating platform using manuka honey as an anti-biofilm strategy in chronic rhinosinusitis

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**Background:** Bacterial biofilms are implicated in the pathogenesis of chronic rhinosinusitis. Nitric oxide (NO) is a key immune effector with potent antimicrobial effects, but a short half-life limits achievement of therapeutic concentrations. We hypothesized that manuka honey (MH) could induce sustained reduction of nitrite to NO causing biofilm disruption and that this effect would be enhanced with the addition of a NO-releasing microparticle.

**Methods:** Porous organosilica microparticles containing nitrosylated thiol groups were formulated (SNO-MP). MH was combined with serial dilutions of nitrite. NO release was evaluated using a NO analyzer. The susceptibility of 2 strains of *Pseudomonas aeruginosa* biofilms to these NO-releasing platforms was evaluated using confocal microscopy. Cell viability and biofilm volume were quantified. Statistical analysis was performed using the Mann-Whitney *U* test with SPSS software.

**Results:** MH with nitrite generated a linear increase in NO formation. SNO-MP induced a bolus release of NO within 5 minutes, followed by a sustained plateau phase. MH with nitrite combined with SNO-MP enhanced NO release

during the plateau phase. MH with nitrite reduced biofilm live cells and volume by 88.5% to 96.9% and 95.1% to 95.6%, respectively, vs control ( $p < 0.0001$ ). SNO-MP reduced live cells and volume by 61.0% to 98.5% and 74.7% to 85.7%, respectively, vs control ( $p < 0.0001$ ). MH with nitrite combined with SNO-MP nearly eradicated biofilm, with a 98.3% to 99.8% (log 1.8-2.6) reduction in viability and a 91.4% to 97.7% decrease in volume ( $p < 0.0001$  vs control).

**Conclusion:** A novel platform that generates NO using MH and nitrite produces a potent anti-biofilm effect, which can be further enhanced with the addition of SNO-MP. © 2019 ARS-AAOA, LLC.

## Key Words:

therapeutics; chronic rhinosinusitis; topical therapy for chronic rhinosinusitis; bacteriology; biofilm

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Chronic rhinosinusitis (CRS) is an inflammatory disease of the paranasal sinuses affecting more than 10% of the US adult population and corresponding to over 26 million people. CRS accounts for 47.9 million ambulatory visits, with 69.3% of these visits resulting in antibiotic prescriptions. Antibiotic overuse can lead to potential adverse effects, such as increased bacterial resistance, with direct and indirect costs of \$20 billion and \$35 billion, respectively.<sup>1</sup> This increasingly worrisome clinical problem and the resultant significant societal cost is a convincing incentive to mitigate antibiotic overuse and develop novel therapeutics that are not susceptible to the development of microbial resistance.

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Bacterial biofilms have been associated with the pathogenesis of CRS, particularly in cases of refractory infection, which may be related to the underlying antibiotic resistance inherent to biofilms.<sup>2–5</sup> Biofilms harbor a 10- to 1000-fold higher resistance to antibiotics than planktonic bacteria secondary to the physical barrier properties of the extracellular matrix, the bacterial stress response, gene transference, and the propagation of antibiotic resistance properties via quorum-sensing.<sup>6–8</sup>

The establishment of biofilms on the sinonasal mucosa likely results in defects in both adaptive and innate immunity, the latter of which represents the first line of defense against infection.<sup>4</sup> Nitric oxide (NO) is a lipophilic, low-molecular-weight, highly reactive, gaseous signaling molecule that is continuously secreted by the upper respiratory tract, especially the nasal mucosa and paranasal sinuses. NO is a key component of the innate immune response as the downstream effector of biofilm-detecting taste receptor activation.<sup>9</sup> In healthy human sinuses, NO is a potent antimicrobial and enhances mucociliary clearance. NO is found at low levels in the sinuses of CRS patients and at significantly higher levels in the sinuses of healthy patients.<sup>10–12</sup>

Manuka honey (MH) is a monofloral honey derived from the manuka tree (*Leptospermum scoparium*), which has garnered attention for its broad-spectrum antimicrobial—including anti-biofilm—and antioxidant effects.<sup>13–15</sup> MH has been integrated into sterile dressings for the treatment of surgical wounds, burns, and ulcers.<sup>16</sup> Interestingly, MH is also mildly acidic and can serve as a proton donor. We hypothesize that MH will reduce nitrite in vitro, thereby generating NO in concentrations that are able to disrupt *Pseudomonas aeruginosa* (PA) bacterial biofilms. We also hypothesize that the combination of MH and nitrite will augment the anti-biofilm capability of a previously described NO-releasing microparticle (SNO-MP).<sup>17</sup>

## Materials and methods

### Isolation of PA bacterial isolates

This study was approved by the institutional review board of the Albert Einstein College of Medicine. PA was obtained from patients diagnosed with CRS as described and was termed PA1.<sup>17</sup> In brief, patients with CRS diagnosed according to the updated adult clinical guidelines for sinusitis were consented.<sup>18</sup> Cultures were obtained from the maxillary/ethmoid sinuses during endoscopic sinus surgery (ESS), plated on tryptic soy agar (TSA), and incubated overnight. Isolates were identified using matrix-assisted laser desorption/ionization time-of-flight (mass spectrometry (Bruker Daltonics, Inc, Billerica, MA). Antibiotic sensitivities were determined using a VITEK 2 system (bioMérieux, Durham, NC). A biofilm-forming reference strain of PA, obtained from the American Type Culture Collection (ATCC 15692), and previously used by other investigators to evaluate anti-biofilm therapeutics, was used in this study.<sup>19</sup>

### Fabrication of NO-releasing particles

SNO-MP was synthesized as described elsewhere.<sup>17</sup> In brief, an organosilica sol-gel monolith was formed containing many covalently attached nitrosated thiol groups. Hydrolyzed trimethyl orthosilicate, hydrolyzed/nitrosated mercaptopropyl-trimethoxysilane, polyethylene glycol, and sodium phosphate were mixed and maintained at room temperature to form a spanning network of siloxane (Si-O-Si) linkages through condensation. The resultant porous sol-gel monolith was lyophilized, dry-milled, and then pestled to a micron-sized powder with particle diameters of 5 to 10  $\mu\text{m}$ . Blank microparticles incapable of NO release (B-MP) were created by performing the aforementioned steps, with the exception of nitrosation.

### Manuka honey formulation preparation

MH containing a minimum of 0.083 mg/mL methylglyoxal (MGO; Comvita, New Zealand) was stored at room temperature in the dark prior to use. Stock MH solution was made by dissolving MH in phosphate-buffered saline (PBS) at 37°C on a rotator to make a 14% wt/vol MH solution for a final minimum MGO concentration of 0.012 mg/mL.

### Measurement of NO release rate

NO release rates were measured using a NO analyzer (Sievers 280i; GE Instruments, Boulder, CO). Samples were dispersed in 5 mL of tryptic soy broth (TSB) or MH 14% wt/vol in TSB at room temperature. Release kinetics were analyzed using Sievers NOAnalysis software (GE Instruments) with 12 measurements per minute recorded for a 280 total minutes.

### Titration of nitrite dose

To determine the optimal concentration of nitrite for use in subsequent biofilm experiments, we first evaluated the efficacy of serial dilutions of nitrite, in combination with a fixed concentration of MH, using a colony-forming unit (CFU) assay. An overnight-grown culture of the PA1 strain in TSB was diluted to 10<sup>8</sup> cells/mL based on McFarland equivalence turbidity standard No. 0.5 (Thermo Fisher Scientific, Waltham, MA) and then adjusted to 10<sup>5</sup> cells/mL in TSB. Suspensions were treated with MH, nitrite, and serial dilutions of nitrite from 10 mmol/L to 1  $\mu\text{mol/L}$  combined with MH. Untreated bacteria were used as negative controls. Bacterial suspensions were mixed using a tube rotator to ensure uniform distribution of bacteria and then incubated at 37°C for 6 hours. Suspensions were then diluted in PBS and plated on TSA. Bacterial viability was determined by counting the number of colonies established on an agar plate after overnight incubation at 37°C.

### Biofilm preparation

A culture of PA, grown overnight in TSB, was diluted to 10<sup>8</sup> cells/mL. The bacterial suspension was then adjusted to 10<sup>5</sup> cells/mL in TSB and inoculated into an 8-well coverglass system (LabTek II Chambered #15; Thermo Fisher

Scientific). The chamber system was incubated at 37°C for 48 hours. The media was replenished after 24 hours to maintain bacterial viability.

### Evaluation of MH and nitrite, alone and combined on biofilm viability

Established biofilms were divided into 4 treatment groups: (1) negative control with untreated bacterial suspension; (2) MH; (3) nitrite (10 mmol/L); and (4) MH administered with nitrite (10 mmol/L). Bacteria were treated with the appropriate treatment solution, as noted earlier, for 6 hours in complete darkness at 37°C, followed by washing with PBS. Staining was performed using the BacLight Live/Dead Bacterial Viability kit (Invitrogen, Carlsbad, CA). The Live/Dead kit solution constituting of Syto9 and propidium iodide was then applied to each well of the chamber slide system for 1 hour at room temperature in darkness. After incubation, each sample was rinsed with PBS and incubated in 10% buffered formalin for 20 minutes at room temperature in darkness. Afterward, samples were washed with PBS for evaluation with a confocal laser scanning microscope (CLSM). This experiment was repeated in triplicate for both the PA1 and ATCC 15692 strains.

### Evaluation of MH with nitrite platform combined with NO-releasing microparticles on biofilm viability

Established biofilms were divided into 4 treatment groups: (1) negative control with untreated bacterial suspension; (2) B-MP (10 mg/mL); (3) SNO-MP (10 mg/mL); (4) MH with SNO-MP (10 mg/mL); (5) MH with nitrite (10 mmol/L); and (6) MH with nitrite (10 mmol/L) combined with SNO-MP (10 mg/mL). SNO-MP, whether blank or active, was applied at a concentration of 10 mg/mL, based on previously published data demonstrating efficacy against planktonic bacteria at this concentration.<sup>17</sup> Biofilms were treated as described earlier before evaluation with CLSM. This experiment was repeated at least twice with the PA1 and ATCC 15692 strains.

### Biofilm quantification

Images of biofilms were taken using CLSM (TCS SP5; Leica Microsystems, Wetzlar, Germany) configured to a 488-nm argon laser with a 63× objective. Five to 10 image stacks (Z stacks) of each individual sample were taken corresponding to the areas of highest biofilm growth. Each Z stack consisted of 0.5-μm individual sliced images of the same area of tissue. Volocity software (PerkinElmer, Waltham, MA) was used to quantify biofilm viability and biomass in each Z stack.

### Statistical analysis

Statistical analysis was performed using SPSS version 21.0 (IBM Corp, Armonk, NY). Differences between treatment

groups were analyzed using the nonparametric Mann-Whitney *U* test. Statistical significance was set at  $p \leq 0.05$ .

## Results

### NO-release kinetics

A NO analyzer was used to quantify NO release from each formulation in real time over the course of 300 minutes (Fig. 1). As anticipated, nitrite alone (5 mmol/L) did not release NO with <0.1 part per million (ppm) NO detected (mean,  $0.004 \pm 0.001$  [range, 0.003-0.009] ppm). MH can reduce nitrite to generate NO at low levels as evidenced by the observed linear increase in NO release over the course of 280 minutes (mean,  $0.020 \pm 0.007$  [range, 0.005-0.034] ppm). The addition of blank microparticles to the combination of MH and nitrite produced a similar linear increase in NO levels (mean,  $0.016 \pm 0.004$  [range, 0.005-0.022] ppm). SNO-MP was used at a concentration of 5 mg/mL as higher concentrations, when combined with MH, produced gas bubbles resulting in a noisy baseline, which precluded accurate NO-release measurements. SNO-MP (5 mg/mL) demonstrated a bolus release of NO over the first 5 minutes, peaking at 21.0 ppm at 2.2 minutes, followed by a logarithmic decay to a sustained plateau release phase (mean,  $3.114 \pm 1.064$  [range, 0.004-21.000] ppm). The addition of MH to SNO-MP (5 mg/mL) appeared to retard the initial bolus release with a peak NO concentration of 3.9 ppm achieved at 15.3 minutes (mean,  $2.917 \pm 0.497$  [range, 0.003-3.900] ppm), followed by a logarithmic decay and sustained plateau phase akin to that seen with SNO-MP alone. Combining SNO-MP (5 mg/mL) with MH and nitrite (5 mmol/L) resulted in a maximum NO concentration of 12.5 ppm at 1.5 minutes followed by a gradual decline to a sustained plateau phase. Of note, this final combination was able to sustain higher levels of NO release relative to SNO-MP for approximately 180 minutes.

### PA isolate

A clinical strain of PA derived from a CRS patient and used in earlier work evaluating SNO-MP (PA1) and a reference PA strain, ATCC 15692, were used in this study.<sup>17</sup> PA1 demonstrated an antimicrobial resistance typical of PA species without an acquired resistance to fluoroquinolones.

### Dose optimization of nitrite with MH

As anticipated, nitrite (10 mmol/L) administered alone did not have a significant effect on PA1 CFU (Fig. 2). Monotherapy with MH resulted in a nonsignificant 2.3-log reduction in CFU vs controls ( $p = 0.083$ ). The addition of nitrite to MH, resulting in the generation of NO, produced a dose-dependent reduction in CFU relative to controls at all concentrations of nitrite (reduction of 4.1-6.8 log, from 1 μmol to 10 mmol/L nitrite, respectively;  $p < 0.05$ ). Given these findings, nitrite (10 mmol/L), alone or in combination with MH, was used for all biofilm experiments.

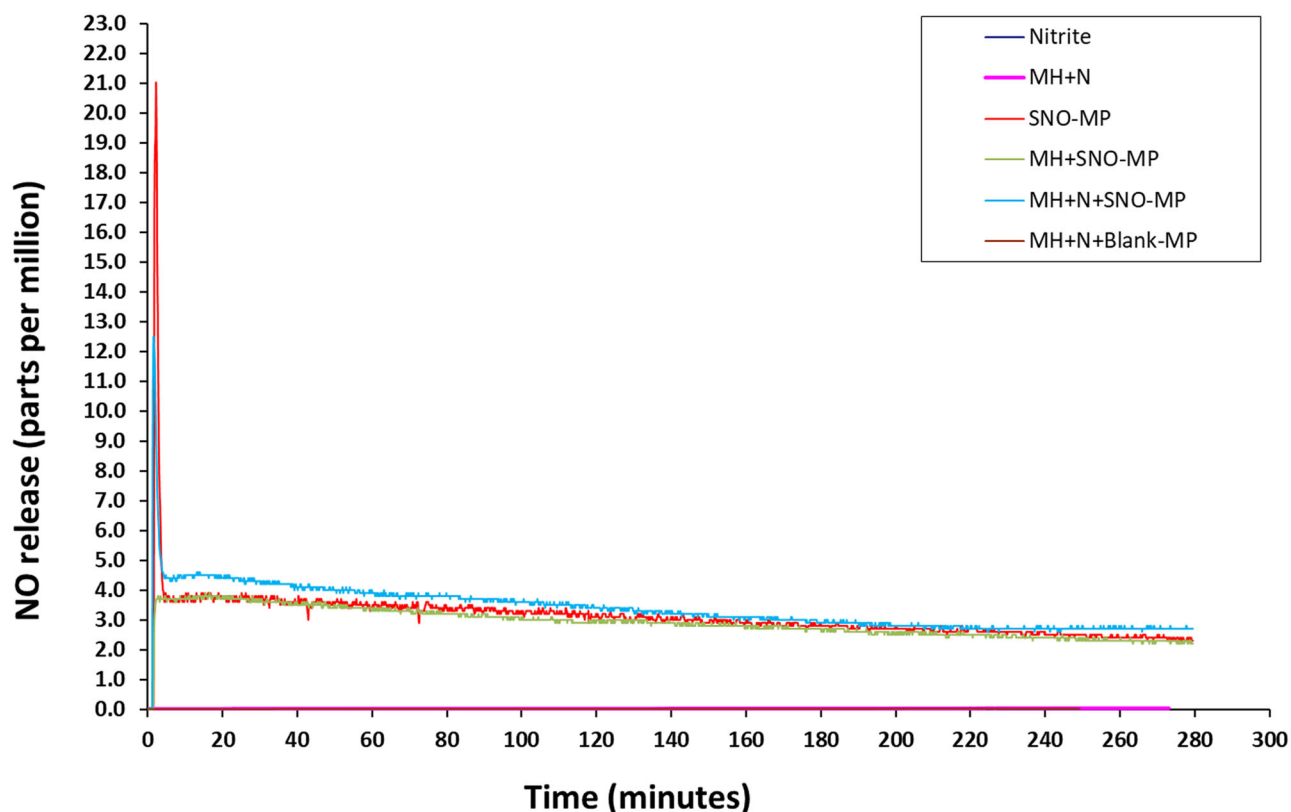


**FIGURE 1.** NO-release curves measured using a NO analyzer over the course of 280 minutes at neutral pH in vitro. Treatment groups included: (i) nitrite alone in tryptic soy broth; (ii) MH with nitrite; (iii) SNO-MP in tryptic soy broth; (iv) NO-releasing SNO-MP with MH; (v) SNO-MP with nitrite and MH; and (vi) blank microparticle (blank-MP) incapable of NO release with nitrite and MH. Nitrite was used at 5 mmol/L concentration, MH at 14% wt/vol, and SNO-MP and blank-MP at 5 mg/mL. MH = manuka honey; NO = nitric oxide. SNO-MP = porous organosilica microparticles containing nitrosylated thiol groups.

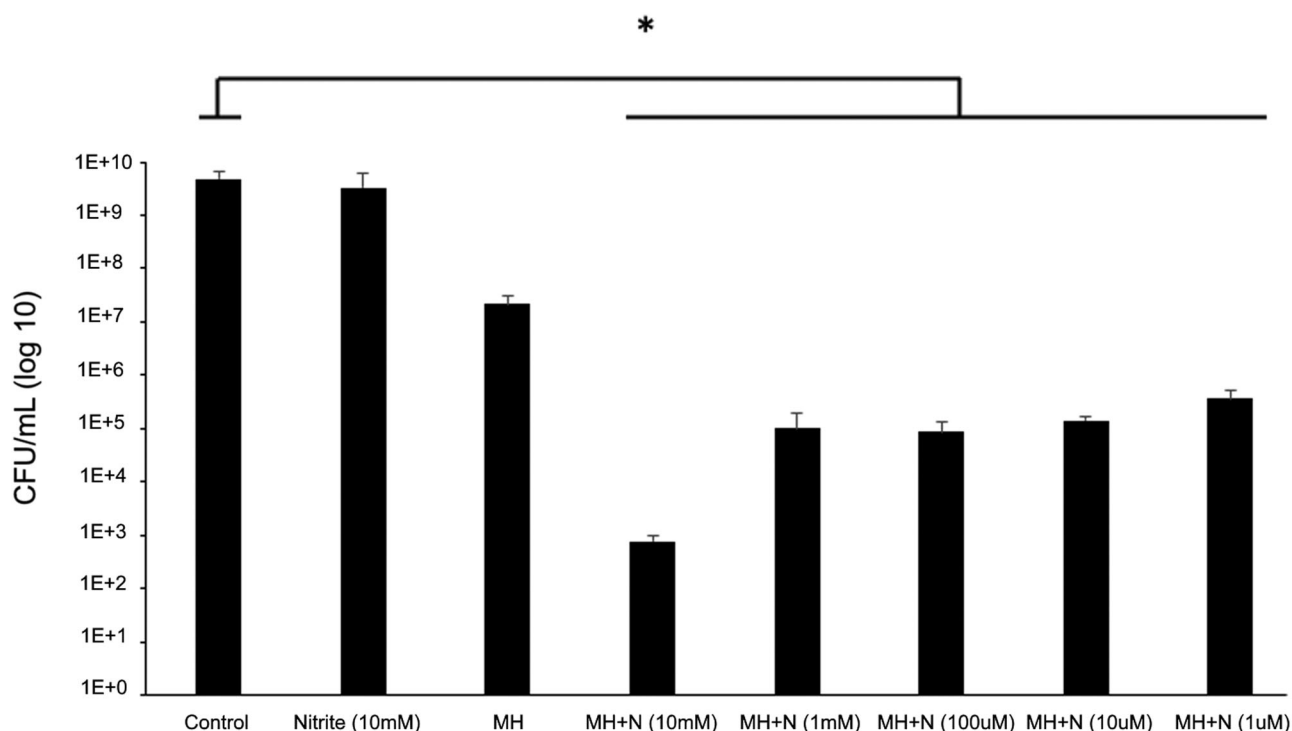


**FIGURE 2.** CFU assay demonstrating antimicrobial efficacy of nitrite, MH, and MH with serial dilutions of nitrite against *Pseudomonas aeruginosa* planktonic isolates. CFU = colony forming units; control = untreated bacteria in tryptic soy broth; MH = manuka honey; N = nitrite. \* $p \leq 0.05$ .

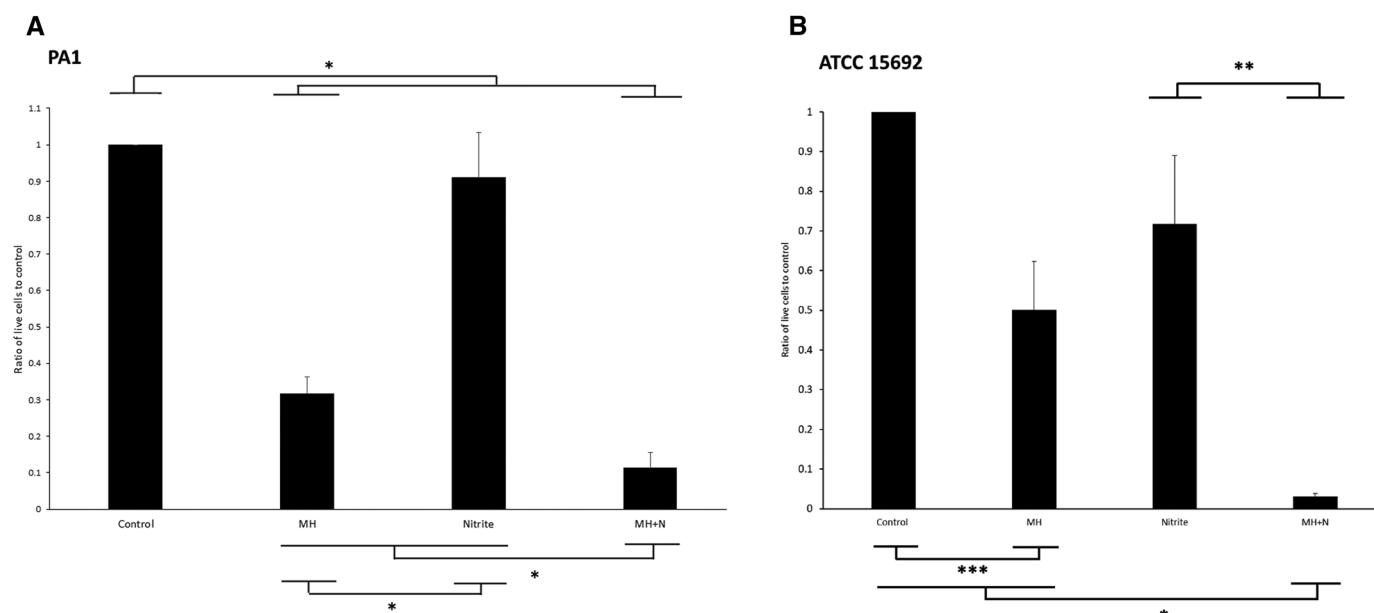


**FIGURE 3.** Ratio of live cells in each treatment group to live cells in the control group after treatment of PA biofilm determined by live/dead staining and CLSM in the (A) PA1 strain and (B) ATCC 15692 strain. ATCC = American Type Culture Collection; CLSM = confocal laser scanning microscopy; control = untreated bacteria in tryptic soy broth; MH = manuka honey (14% wt/vol); N = nitrite (10 mmol/L); PA = *Pseudomonas aeruginosa*. \* $p \leq 0.0001$ ; \*\* $p \leq 0.001$ ; \*\*\* $p \leq 0.002$ .

### Efficacy of MH and nitrite against PA biofilms

Nitrite, when administered alone, did not significantly reduce biofilm cell viability in either PA strain. In PA1, MH alone demonstrated a significant antibiofilm effect, reducing the number of live cells by 68.2% relative to control ( $p < 0.0001$ ) (Fig. 3A). The combination of MH and nitrite produced an additional anti-biofilm effect with a reduction in cell viability of 88.5%, 87.4%, and 63.9% relative to control, nitrite alone, and MH alone, respectively ( $p < 0.0001$ ). Treatment of ATCC 15692 with MG reduced live cells by 49.9% relative to control ( $p < 0.002$ ) (Fig. 3B). MH combined with nitrite produced a reduction in cell viability of 96.9%, 95.7%, and 93.9% relative to control ( $p < 0.0001$ ), nitrite alone ( $p < 0.001$ ), and MH alone ( $p < 0.0001$ ), respectively.

The anti-biofilm efficacy of MH combined with nitrite was demonstrated by a reduction in biofilm volume in PA1 (Fig. 4A and B). Relative to control, MH combined with nitrite produced a 95.1% reduction in biofilm volume ( $p < 0.0001$ ). This represented an improvement over the 85% reduction, relative to control, induced by MH alone ( $p < 0.0001$ ) or the nonsignificant 43.9% reduction associated with nitrite treatment alone. MH with nitrite produced a 66.4% and 91.3% reduction in volume compared with MH alone ( $p < 0.001$ ) and nitrite alone ( $p < 0.0001$ ), respectively. In ATCC 15692, MH alone produced a nonsignificant 13.0% reduction in biofilm volume relative to control (Fig. 4C and D). The combination of MH with nitrite induced 95.6%, 95.7%, and 95.0% reductions in biofilm volume relative to that in the control ( $p < 0.0001$ ), nitrite-alone ( $p < 0.002$ ), and MH-alone ( $p < 0.0001$ ) groups, respectively.

### Combination of NO-generating and NO-releasing platforms

In a previous study, we described a NO-releasing microparticle with proven efficacy against multiple strains of planktonic bacteria.<sup>17</sup> In the current series of experiments we have demonstrated that SNO-MP has efficacy against PA1 biofilms (Fig. 5A). SNO-MP produced a 61% reduction in cell viability relative to control ( $p < 0.0001$ ), but, interestingly, this was not significantly better than the 55.2% reduction in viable cells on treatment with blank microparticles incapable of NO release. The addition of MH to SNO-MP resulted in an additive effect, producing a 90.0% reduction in live cells compared with controls ( $p < 0.0001$ ). The most potent anti-biofilm treatment in PA1 was the combination of MH and nitrite with SNO-MP. This combination nearly eradicated the biofilm with a 99.76% (log 2.61) reduction in cell viability relative to controls ( $p < 0.0001$ ), a 99.4% (log 2.2) reduction relative to SNO-MP alone ( $p < 0.0001$ ), and a 97.6% (log 1.6) reduction relative to MH combined with SNO-MP in the absence of nitrite ( $p < 0.0001$ ).

Comparatively, studies on ATCC 15692 showed that SNO-MP was even more effective against this strain than against PA1, producing a 98.4% (log 1.8) reduction in cell viability vs control ( $p < 0.0001$ ) (Fig. 5B). This compares favorably with the 71.3% reduction in live cells produced by blank microparticles relative to control ( $p < 0.0001$ ). The addition of MH to SNO-MP or MH and nitrite to SNO-MP produced similar 99.2% (log 2.1) and 98.3% (log 1.8) reductions in cell viability, respectively, vs control ( $p < 0.0001$ ), and these were not significant improvements over SNO-MP monotherapy.

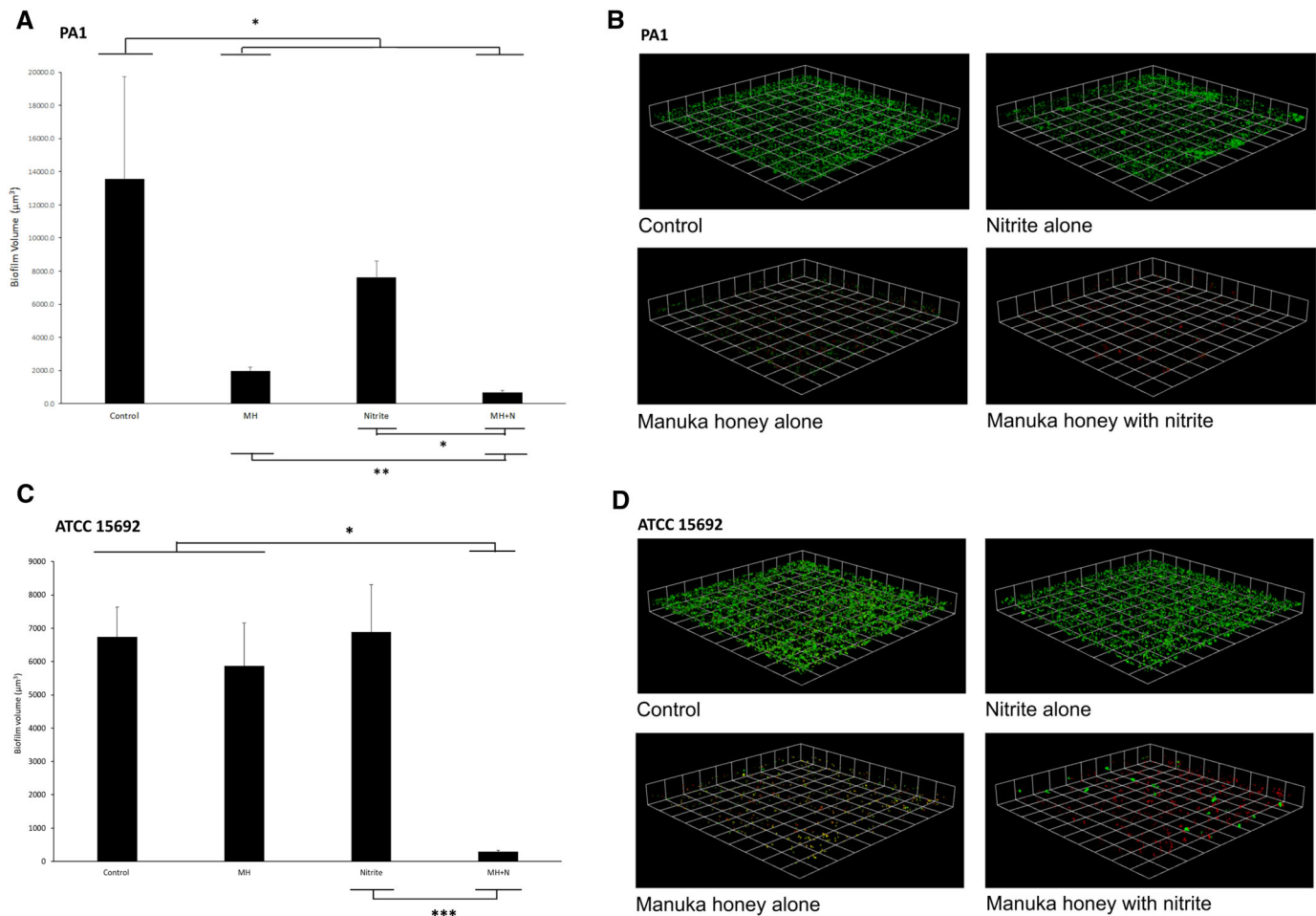


**FIGURE 4.** Biofilm volume ( $\mu\text{m}^3$ ) in the control group and after treatment of *Pseudomonas aeruginosa* biofilms determined by live/dead staining and CLSM in the (A) PA1 strain and (C) ATCC 15692 strain. Representative CLSM images of PA biofilms with 3-dimensional structures reconstructed from Z stacks in the (B) PA1 strain and (D) ATCC 15692 strain. Red indicates dead cells. ATCC = American Type Culture Collection; CLSM = confocal laser scanning microscopy; control = untreated bacteria in tryptic soy broth; green = live cells; MH = manuka honey (14% wt/vol); N = nitrite (10 mmol/L); PA = *Pseudomonas aeruginosa*. \* $p \leq 0.0001$ ; \*\* $p \leq 0.001$ ; \*\*\* $p \leq 0.002$ .

The combination of MH with nitrite and SNO-MP was also the most effective intervention in terms of biofilm volume reduction in PA1, producing a 97.7% decrease in volume relative to controls ( $p < 0.0001$ ) (Fig. 6A and B). This result compared favorably to the 95.1% reduction produced by MH with nitrite in the absence of the microparticle ( $p = 0.012$ ) and was superior to the combination of MH and SNO-MP without the presence of nitrite (85.4% reduction in biofilm volume vs control,  $p < 0.0001$ ). Importantly, the addition of MH with nitrite to the SNO-MP platform improved on the efficacy of the latter, which, when administered alone, produced a 74.7% reduction in biofilm volume. ATCC 15692 biofilm volume was significantly reduced with SNO-MP monotherapy (85.7% vs control,  $p < 0.0001$ ) (Fig. 6C and D). This effect was bolstered by the addition of MH to SNO-MP (97.6% vs control,  $p < 0.0001$ ), although augmentation of NO release by using MH with nitrite and SNO-MP failed to further reduce biofilm volume (91.4% vs control,  $p < 0.0001$ ). As with cell viability, SNO-MP, MH

with SNO-MP, and MH and nitrite with SNO-MP were statistically comparable in their volume-reduction effects.

## Discussion

Biofilms have long been implicated in CRS. Earlier studies have demonstrated that PA and *Staphylococcus aureus* biofilms correlate with poor clinical improvement after ESS, and those patients with recalcitrant CRS are more likely to harbor biofilm-forming species.<sup>2,20</sup> Biofilm-positive patients have statistically worse sinus symptoms, require additional postoperative visits, and necessitate treatment with multiple antibiotics.<sup>21</sup> Morphologically, the mucosa of CRS patients with biofilm-positive disease is markedly damaged compared to those without biofilm growth on their mucosa with the resultant impairment in ciliary function promoting bacterial persistence.<sup>22,23</sup> Consequently, there has been considerable focus on the development of novel anti-biofilm therapeutics.

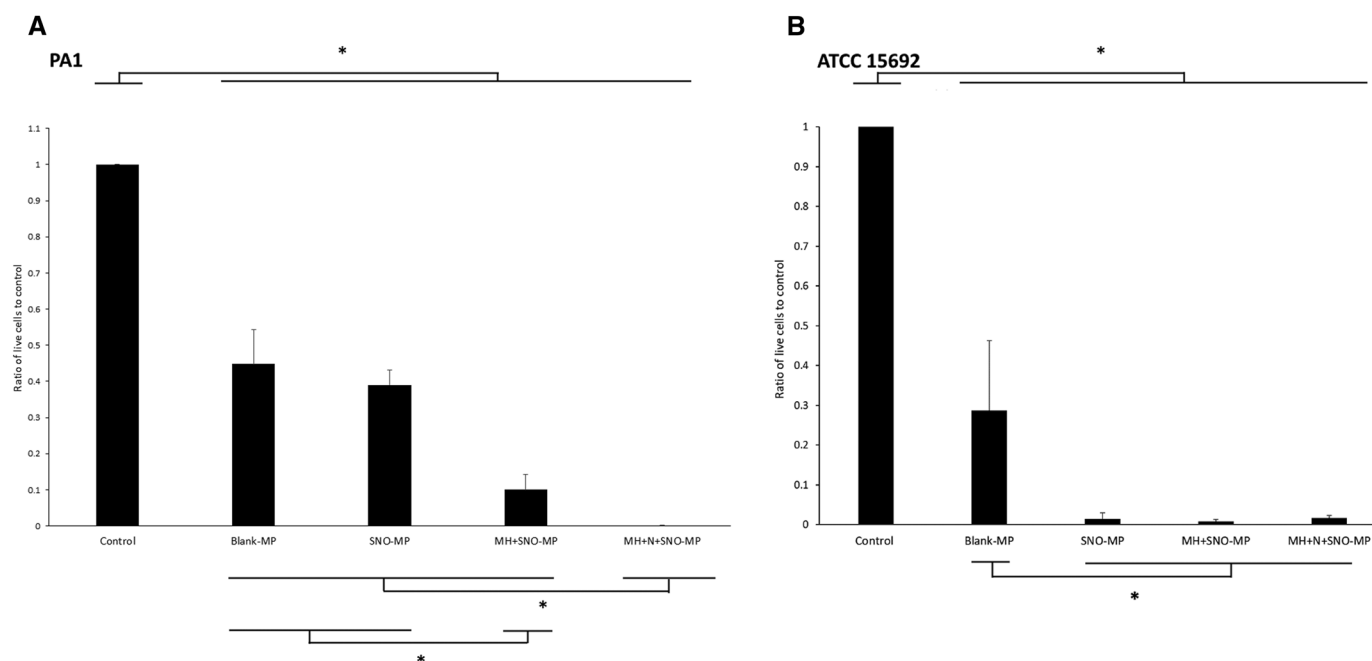


**FIGURE 5.** Ratio of live cells in each treatment group to live cells in the control group after treatment of PA biofilm determined by live/dead staining and CLSM in the (A) PA1 strain and (B) ATCC 15692 strain. ATCC = American Type Culture Collection; Blank-MP = blank microparticles incapable of nitric oxide release; CLSM = confocal laser scanning microscopy; control = untreated bacteria in tryptic soy broth; MH = manuka honey (14% wt/vol); N = nitrite (10 mmol/L); PA = *Pseudomonas aeruginosa*; SNO-MP = nitric oxide-releasing microparticles. \* $p \leq 0.0001$ .

In this study, we have demonstrated that MH, in combination with nitrite, generates a potent anti-biofilm effect against 2 strains of PA: ATCC reference 15692 and clinical strain PA1 isolated from a CRS patient. Furthermore, we found that SNO-MP, previously shown to eradicate planktonic bacterial isolates, is also capable of disrupting PA biofilms, producing a 61% and 75% reduction in the number of viable cells and biofilm volume, respectively, in PA1 and an even more robust 98.4% and 85.7% reduction in viable cells and biofilm volume, respectively, in ATCC 15692.<sup>17</sup> Although a standard has yet to be established in the literature beyond which biofilm killing is considered clinically efficacious, a reduction of  $\geq 99.9\%$  (3 log) of viable bacteria is considered bactericidal.<sup>24</sup> The combination of SNO-MP with MH and nitrite approaches this threshold in PA1, inducing a log 2.6 reduction in viable cells and a 98% decrease in biofilm volume. ATCC 15692 appeared highly susceptible to the levels of NO produced by SNO-MP, with no significant increase in anti-biofilm activity achieved through augmentation of NO flux by adding MH or MH and nitrite to SNO-MP. Indeed, SNO-MP-containing treatments produced a log 1.8 to 2.1 reduction in biofilm cell viability and an 85.7% to 97.6% reduction in biofilm volume. These findings are particularly compelling in the context of PA biofilms, which exhibit a 100- to 1000-fold higher minimum bactericidal threshold than planktonic forms.<sup>25</sup>

MH has been shown to induce a 91% reduction in PA biofilms in vitro and has demonstrated efficacy against biofilms in a sheep model of CRS.<sup>13,26</sup> MH concentrations

between 15% and 30% wt/vol exhibit antibacterial effects.<sup>27</sup> Our results confirm that MH is capable of significantly reducing biofilm viability and volume relative to controls. However, PA biofilms treated with MH developed subpopulations of bacteria exhibiting resistance to MH that could be sustained through subsequent generations.<sup>28</sup> In contrast, there has yet to be compelling evidence demonstrating biofilm resistance to NO. The importance of NO in protecting against biofilms is suggested by its role as the end-effector of taste receptor activation. Sweet and bitter taste receptors are present on discrete solitary chemosensory cells throughout the upper airway and detect quorum-sensing molecules produced by biofilms leading to the downstream production of NO.<sup>29–31</sup> More recently, genetic variation in upper airway taste receptor function has been shown to correlate with the severity of CRS in patients.<sup>9,32</sup> The mechanism of effect of NO is multifaceted and includes the inactivation of enzymes responsible for bacterial replication, reaction with oxygen to produce toxic species, and binding with superoxide  $O_2^-$  radicals to form the strong oxidant peroxynitrite.<sup>33</sup>

To our knowledge, this is the first study to demonstrate water-initiated reduction of nitrite to NO using MH as a proton donor. Although the mean volume of NO produced was approximately 150-fold lower than that released by SNO-MP, the release profile increased in a linear manner over the duration of the measurement period of 280 minutes. This was anticipated as the loss of NO gas into the atmosphere further drives the equilibrium of the reduction reaction toward the right, producing more NO. A similar

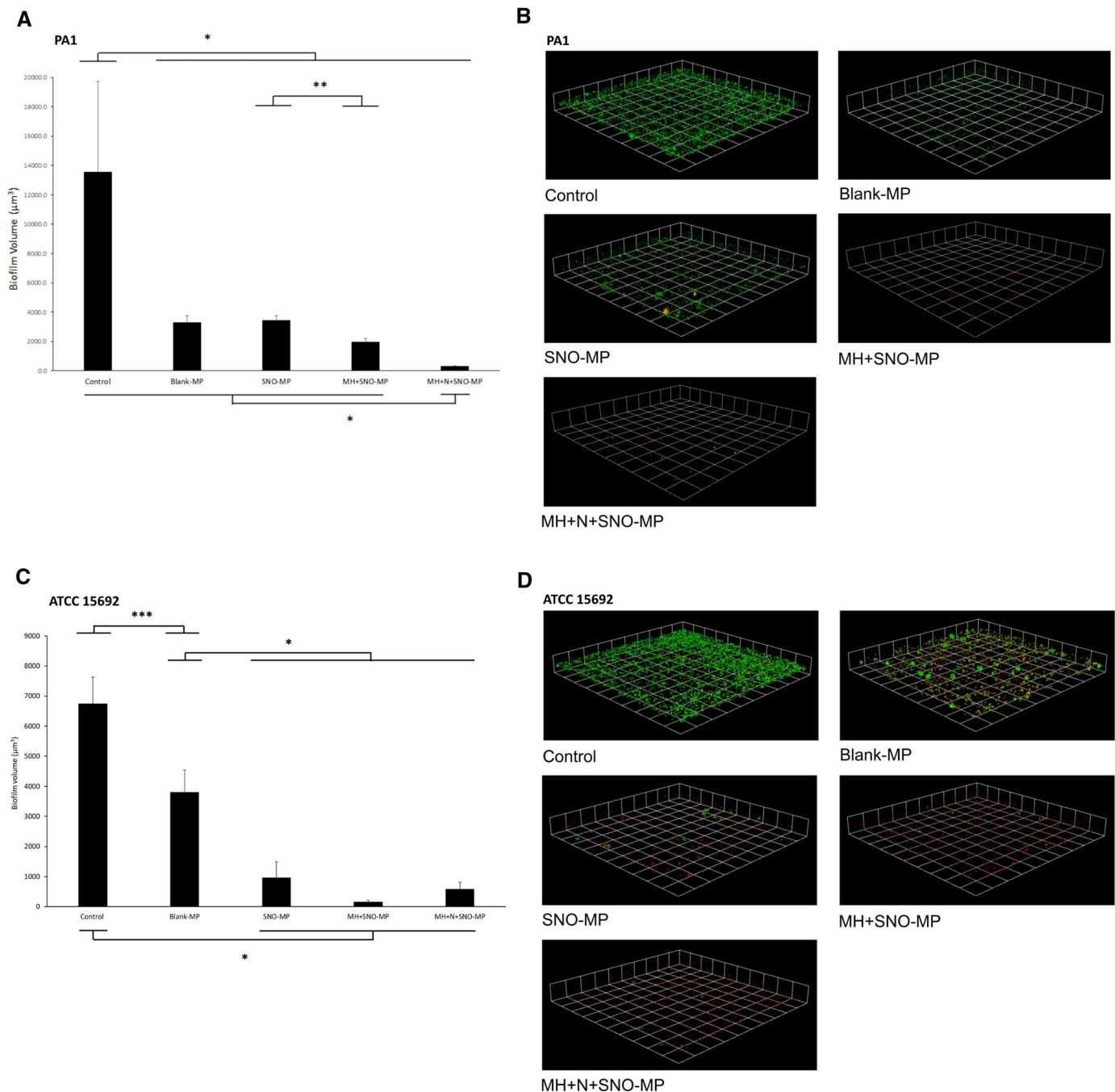


**FIGURE 6.** Biofilm volume ( $\mu\text{m}^3$ ) in the control group and after treatment of PA biofilms determined by live/dead staining and CLSM in the (A) PA1 strain and (C) ATCC 15692 strain. Representative CLSM images of PA biofilms with 3-dimensional structures reconstructed from Z stacks in the (B) PA1 strain and (D) ATCC 15692 strain. Green indicates live cells and red indicates dead cells. ATCC = American Type Culture Collection; Blank-MP = blank microparticles incapable of nitric oxide release; CLSM = confocal laser scanning microscopy; control = untreated bacteria in tryptic soy broth; MH = manuka honey (14% wt/vol); N = nitrite (10 mmol/L); PA = *Pseudomonas aeruginosa*; SNO-MP = nitric oxide-releasing microparticles. \* $p \leq 0.0001$ ; \*\* $p \leq 0.002$ ; \*\*\* $p \leq 0.02$ .

profile may be seen in vivo where the rapid scavenging of NO may, paradoxically, enhance its production through chemical reduction.<sup>34</sup>

SNO-MP monotherapy demonstrated a potent antibiofilm effect, likely related to the large bolus release of NO within minutes of administration, followed by a sustained plateau phase. Interestingly, this effect did not appear to be entirely related to NO release, as evidenced by

the nonsignificant difference in cell viability and biofilm volume reduction in PA1 relative to blank microparticles incapable of NO release. Interestingly, SNO-MP was significantly more effective than the blank microparticle against the ATCC 15692 strain, suggesting that, in some bacterial strains, there may be an added benefit—beyond any intrinsic effect of the microparticles—with NO release.

We have previously seen anti-biofilm effects of blank particles designed by our group and postulate a potential deleterious effect of the particle itself on the biofilm lifecycle.<sup>33,35</sup> This effect may be mediated by thiol groups in the blank microparticle, which harbor intrinsic antioxidant and antimicrobial properties. This has been demonstrated in *N*-acetylcysteine, a well-characterized molecule that, like our blank microparticle, contains thiol groups.<sup>36,37</sup> Thiols can irreversibly react with the disulfide bonds of key bacterial proteins involved in growth. The thiol groups can also react with proteins governing the production of extracellular polysaccharide, thus preventing the production of the biofilm extracellular matrix and impairing the adhesion of biofilms to surfaces and thereby interrupting the biofilm lifecycle.

Interestingly, the combination of MH with SNO-MP, and without nitrite, impeded NO release relative to the SNO-MP particle alone. This reduction in NO release may be related to the viscosity of MH, which not only affects viscosity of the media in which the microparticles dissolve, but, by penetrating the matrix of the microparticle, affects the internal viscosity of the particle as well. As internal viscosity increases, the NO dissociating from the microparticle has a higher probability of recombining with the thiyl radical to reform nitrosothiol rather than escaping into the surrounding media in a process termed geminate recombination. Nevertheless, the reduction in NO release did not appear to negatively impact the anti-biofilm effect.

Combining MH with nitrite and SNO-MP induced the most potent anti-biofilm effect in PA1. NO release from SNO-MP was, again, compromised by a likely viscosity-related mechanism. However, the addition of MH with nitrite had the benefit of sustaining NO release at a higher level during the plateau phase than was observed with SNO-MP alone. This additional NO during the plateau phase cannot be attributed solely to the addition of NO production by MH-mediated nitrite reduction to that released by SNO-MP as the magnitude of the increase was greater

than expected. We hypothesize that the NO generated by the reduction of nitrite re-nitrosated the thiyl radicals in the microparticle, refreshing the nitrosothiol groups, and allowing for a boost in NO release from SNO-MP.

Weaknesses of this study are the absence of antibiofilm efficacy testing of NO-releasing platforms on bacterial species beyond PA. The evaluation of efficacy against bacteria and even fungi associated with refractory CRS would enhance the clinical translatability of our in-vitro results. These experiments, due to time and cost constraints, did not evaluate whether the anti-biofilm effect of the described platforms was sustained over protracted periods.

## Conclusion

In this study we have described the design of a novel platform that disrupts biofilm by using MH to reduce nitrite to NO. We demonstrated the synergistic anti-biofilm effect of this platform when combined with a NO-releasing microparticle producing near-total eradication of PA biofilms. Our future experiments will involve the use of a refined lipophilic NO-releasing particle which is 200 to 400 nm in size, conferring an increased ability to penetrate bacterial cell membranes. Furthermore, we continue to develop particle-based platforms that combine in-situ NO production—akin to that produced with MH and nitrite in the current study—with the delivery advantages inherent to nanoparticles with the goal of prolonged and increased NO release. 🌐

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