Abstract

The effect of nitric oxide (NO)-release kinetics on the antibacterial activity of NO-releasing silica nanoparticles against the nosocomial pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* is demonstrated. Silica nanoparticles were synthesized and modified to release NO via secondary amine modification with *N*-diazeniumdiolate NO donors. Planktonic *Pseudomonas aeruginosa* and *Staphylococcus aureus* were exposed to a series of NO-releasing silica particles with similar 2 h NO totals but different release kinetics (half-lives). Enhanced antibacterial efficacy was observed against *Pseudomonas aeruginosa* at pH 7.4 for nanoparticles with faster NO-release kinetics. At pH 7.4, minimal bactericidal efficacy was observed against *Staphylococcus aureus*, even for silica nanoparticles storing greater NO payloads. However, antibacterial activity was enhanced by exposing *Staphylococcus aureus* to NO-releasing silica particles at a lower pH (6.4). This was attributed to faster NO-release kinetics at lower pH due to more rapid proton-initiated decomposition of *N*-diazeniumdiolate NO donors. Collectively, these results demonstrate the enhanced NO-mediated bactericidal efficacy of rapid NO-release kinetics against nosocomial pathogens, providing further insight into the design of NO-releasing materials as novel therapeutics to combat hospital-acquired infections.

Introduction

Despite advances in treating bacterial infections, the incidence of nosocomial infections increases annually, resulting in nearly two million cases of infection per year in the United States.[1] In hospitals, biofilms composed of the nosocomial pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* cause systemic infection in burn wounds, surgical sites, and the lungs.[2] In fact, *P. aeruginosa* and *S. aureus* together account for nearly a third of all hospital-acquired lung infections.[2] These infections are currently treated using conventional antibiotics; however, this method is becoming ineffective as the prevalence of antibiotic-resistant bacteria increases.[1] The ramifications of resistance are severe, and it has been estimated that nearly 70% of lethal nosocomial infections are caused by antibiotic-resistant strains of these pathogens.[3] Thus, the lack of suitable current drugs paired with the severity of hospital-acquired infections warrants research into alternative therapeutics.

Nitric oxide (NO), a highly reactive gas endogenously produced during the immune response to invading pathogens, may represent an alternative strategy to treating hospital-acquired infections. The broad-spectrum antibacterial activity of NO stems from its production of peroxynitrite (ONOO⁻) and dinitrogen trioxide (N_2O_3), reactive byproducts which exert oxidative and nitrosative stresses on bacteria.[3] The innate antimicrobial properties of NO, in conjunction with its lack of documented bacterial resistance, make it an ideal candidate for developing novel therapeutics.[4,5] As such, the development of NO-releasing materials that deliver exogenous NO to kill nosocomial pathogens and reduce the incidence of hospital-acquired infections is of interest.

Due to its gaseous and highly reactive nature, NO must be contained in a scaffold to harness its antimicrobial properties. The Schoenfisch lab works on developing NO donor-modified macromolecular scaffolds that store and deliver tunable amounts of NO at physiological conditions for antibacterial applications.[6,7]Silica has attracted attention as an NO delivery vehicle due to its low cytotoxicity and easily tunable physicochemical characteristics.[3] Hetrick et al. first reported the utility of NO-releasing silica nanoparticles as antibacterial agents against the nosocomial pathogens *S. aureus* and *P. aeruginosa*.[3,8] In these studies, Hetrick suggested that certain chemical properties (e.g., NO flux and NO-release kinetics) of the NO-releasing silica nanoparticles influenced NO-mediated antibacterial action.[3,8] Subsequent studies focused on varying these parameters to maximize bactericidal efficacy against pathogenic bacteria. The effect of NO-release kinetics (i.e., half-life) on bactericidal efficacy of NO-releasing silica nanoparticles has been systematically studied against dental pathogens.[10] By synthesizing nanoparticles with similar sizes and NO totals but different half-lives, Backlund et al. reported that the antibacterial activity of NO-releasing silica nanoparticles against oral pathogens depends on NO-release kinetics.[10] Indeed, rapid NO-release kinetics enhanced antibacterial activity against Gram-positive oral pathogens, whereas Gram-negative periodontopathogens were more susceptible to particles with extended NO-release kinetics.[10] While these studies demonstrate the importance of NO-release rates on bactericidal efficacy, the effect of NO-release kinetics on the antibacterial activity of NO-releasing silica particles against nosocomial pathogens has yet to be systematically evaluated.

Herein, we report the bactericidal efficacy of NO-releasing silica nanoparticles as a function of NO-release kinetics against planktonic cultures of *P. aeruginosa* and *S. aureus*. Nitric oxide-releasing silica nanoparticles were synthesized with similar sizes (~150 nm) and modified

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to yield similar 2h NO totals, but different release kinetics (half-lives). The 2 h minimum

bactericidal concentrations (MBC_{2h}) were determined against *P. aeruginosa* and *S. aureus* to

evaluate the role of NO-release kinetics on antibacterial activity independent of total NO release.

This study provides insight into the design of future NO-releasing materials to combat

nosocomial infections.

Methods

    I.   *Synthesis of nitric oxide-releasing silica particles*

The synthesis of NO-releasing silica particles has been reported previously.[10,11] Briefly,

hybrid silica particles were synthesized using a Stöber method, involving the co-condensation of

the aminosilanes *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAP3), *N*-

methylaminopropyltrimethoxysilane (MAP3), and *N*-(6-aminohexyl)aminopropyl-

trimethoxysilane (AHAP3) with a tetramethylorthosilicate (TMOS) alkoxysilane backbone.[12]

Silanes (TMOS with either AEAP3, MAP3, or AHAP3) were combined and injected in a bolus

into a reaction mixture containing water (27.84 mL), ammonium hydroxide (9.8 mL) and EtOH

to a total volume of 100 mL. After 2 h, the reaction was poured off and collected via

centrifugation. The particles were centrifuged and the supernatant decanted twice more to

remove unreacted silanes and residual solvent. After the third centrifugation, the supernatant was

decanted once more and the particles were dried under vacuum overnight. The next day, the

resultant secondary amine-modified particles (50 mol% MAP3/TMOS, 60 mol%

AHAP3/TMOS, 80 mol% AEAP3/TMOS) were collected, massed, and stored in a parafilmed

microcentrifuge tube under ambient conditions.

The hybrid silica nanoparticle secondary amines were then modified with *N*-diazeniumdiolate NO donors, which spontaneously release NO under physiological conditions.[11] A portion (30 mg) of the particles was suspended in a solution of 9:1 *N,N*-dimethylformamide (DMF) to methanol (MeOH) with 25 μL of sodium methoxide. The solution was then placed in a Parr reaction container connected to an in-house NO reactor and flushed with argon six times to remove oxygen in solution. The solution was then held under 10 atm of NO for three days with constant stirring. After three days, the unreacted NO was removed from the solution by purging the chamber with argon six times once more. The particles were collected via centrifugation and subsequent decanting of the supernatant. The pellet was centrifuged and the supernatant decanted twice more to remove residual solvent and NaOMe. The resulting *N*-diazeniumdiolate-modified silica nanoparticles were dried for 2 h and stored in a -20°C freezer in a vacuum-sealed bag until use.

II. Hybrid particle characterization

Dynamic light scattering (DLS) was utilized to measure particle size (i.e., hydrated diameter) and monodispersity.[10] Samples were suspended in water at either 0.1 or 0.2 mg/mL, and sonicated for 20 min prior to analysis at room temperature. Particle morphologies and sizes were also evaluated using Scanning Electron Microscopy.

2.4 Characterization of nitric oxide release

The release kinetics (half-lives) of the particles were characterized using a Sievers 280i chemiluminescence nitric oxide analyzer, which measures NO release in real time.[10] Briefly, ~1 mg of NO-releasing material was added to a flask containing 30 mL of deoxygenated phosphate buffered saline (PBS; pH = 7.4 or 6.4). The flask was purged continuously with N_2 at 80

mL/min to carry liberated NO to the analyzer. Analysis was terminated when NO levels decreased to <10 ppb/mg.

2.5 Bactericidal efficacy of nitric oxide-releasing silica nanoparticles

P. aeruginosa and *S. aureus* were cultured aerobically. Bacterial cultures were grown overnight in tryptic soy broth (TSB) at 37°C. A 500 µL aliquot of this solution was reinoculated into 50 mL of fresh TSB, incubated at 37°C, and grown to 10^{8} colony-forming units per milliliter (CFU/mL) as determined by optical density (OD) at 600 nm. *P. aeruginosa* cultures were pelleted via centrifugation, resuspended in distilled water, and diluted to 10^{6} CFU/mL in Tris-PBS (pH = 7.4) supplemented with 250 µL TSB. For *S. aureus,* 10^{8} CFU/mL bacteria cultures were pelleted via centrifugation, resuspended in distilled water, and diluted to 10^{6} CFU/mL in PBS (pH = 7.4). Nitric oxide's antibacterial activity against *S. aureus* was also tested in PBS (pH = 6.4) supplemented with 125 µL TSB. For both *P. aeruginosa* and *S. aureus,* Bacteria solutions were then added to vials containing either NO-releasing or control (non-NO-releasing) silica nanoparticles, sonicated to disperse particles, and incubated aerobically at 37°C with moderate shaking under static (non-growth) conditions. After 2 h, these solutions were diluted and 100 µL were plated on tryptic soy agar plates and incubated at 37°C. Viable colonies were enumerated the following day on agar plates using a Flash and Go colony counter. The concentration resulting in a 3-log reduction in bacterial viability to below 2.5 x 10^{3} CFU/mL was determined to be the MBC_{2h}.

Results and Discussion

I.   Nitric oxide-releasing silica nanoparticle characterization

The Stöber method was utilized to synthesize hybrid alkoxysilane/aminosilane silica

nanoparticles (50 mol% MAP3/TMOS, 60 mol% AHAP3/TMOS, and 80 mol%

AEAP3/TMOS).[12] For clarity, the particles will hereafter be referred to as MAP3, AHAP3, and

AEAP3. Synthetic parameters were tuned to produce particles of similar sizes (~150 nm) and a

high degree of monodispersity, as indicated by low polydispersity index (PDI) values (≤0.06;

Table 1). As previous reports have demonstrated that particle size influences bactericidal

efficacy, maintaining a constant particle size was crucial to this study.[13,14] Thus, by tuning the

identity of the aminosilane precursors while maintaining constant nanoparticle diameters, we

were able to evaluate NO-mediated bactericidal efficacy independent of particle size.

Aminosilane	Diameter[a] (nm)	Diameter[b] (nm)	PDI[b]
MAP3	166 ± 14	197 ± 7	0.04 ± 0.02
AHAP3	174 ± 15	239 ± 14	0.05 ± 0.02
AEAP3	125 ± 13	148 ± 9	0.06 ± 0.02

Table 1. Silica nanoparticle characterization. Results presented as mean ± standard deviation for
n = 3 or more pooled experiments. [a]Geometric diameter estimated using scanning electron
microscopy. [b]Hydrodynamic diameter and particle PDI measured in water using DLS.

Due to the proton-initiated NO release from *N*-diazeniumdiolate NO donors, NO-release kinetics depend on aminosilane structure.[11] As such, NO release from MAP3, AHAP3, and AEAP3 silica nanoparticles were quantified using an NOA to obtain NO-release totals, 2 h NO-release totals, and NO-release kinetics (half-lives).[10] The total NO release for the MAP3, AHAP3, and AEAP3 particles was 0.23, 0.26, and 0.41 µmol/mg, respectively (Table 2). More importantly, 2 h NO-release totals were similar (~0.20 µmol/mg) across all three particle systems, corresponding to the NO dose delivered during the 2 h MBC assays. The NO-release half-lives were significantly longer for AEAP3 (91.8 min) compared to MAP3 (31.6 min) and AHAP3 (42.2 min). The extended NO-release kinetics characteristic of AEAP3 were attributed to intramolecular hydrogen bonding between neighboring cationic amines on the aminosilane scaffold stabilizing the *N*-diazeniumdiolate NO donor and inhibiting rapid NO donor decomposition.[10,15] The bactericidal efficacies of MAP3, AHAP3, and AEAP3 were thereby compared as a function of NO-release kinetics, independent of particle size and 2 h NO-release totals.

NO Release Scaffold	$t[NO]^a$ (µmol/mg)	$[NO]_m^b$ (ppb/mg)	$t_{1/2}^c$ (min)	$t[NO]_{2h}^d$ (µmol/mg)
MAP3	0.23 ± 0.04	900 ± 300	32 ± 5	0.21 ± 0.01
AHAP3	0.26 ± 0.05	1700 ± 1000	42 ± 5	0.20 ± 0.05
AEAP3	0.41 ± 0.05	1400 ± 200	92 ± 14	0.21 ± 0.02

Table 2. Characterization of NO-releasing silica particles in PBS (pH = 7.4 at 37 °C) by means of a chemiluminescent nitric oxide analyzer. Results shown for 50 mol% MAP3, 60 mol% AHAP3, and 80 mol% AEAP3 NO-releasing silica particles are presented as mean ± standard deviation for n = 3 or more pooled experiments. [a]Total amount of NO released. [b]Maximum NO flux achieved. [c]Time to release half of total NO payload. [d]Total amount of NO released after 2 h.

II.   *Kinetic-dependent killing of planktonic Pseudomonas aeruginosa*

To study the role of NO-release kinetics on bactericidal efficacy, 2 h bacteria killing assays were conducted against nosocomial pathogens in Tris-PBS (pH = 7.4; 37 ^{o}C). *P. aeruginosa* was readily susceptible to NO, with bactericidal NO doses < 2 µmol/mL (Figure 1). Furthermore, bactericidal efficacy was found to be dependent on NO-release kinetics (Figure 1). The most effective killing of *P. aeruginosa* was achieved using NO-releasing MAP3 particles with the most rapid NO-release kinetics. The minimum bactericidal concentration (MBC_{2h}) for NO-releasing MAP3 was 4 mg/mL, corresponding to a bactericidal NO dose of 0.8 µmol/mL. This bactericidal NO dose is lower that of both NO-releasing AHAP3 and AEAP3, which required NO doses of 1.6 and 3.2 µmol/mL, respectively, to kill *P. aeruginosa.* We hypothesize that the rapid release kinetics characteristic of MAP3 (31.6 minutes) compared to AHAP3 (42.2 min) and AEAP3 (91.8) facilitates enhanced killing. More rapid NO-release kinetics also account for the enhanced bactericidal efficacy of AHAP3 compared to AEAP3.

While rapid NO-release kinetics improved bactericidal efficacy against *P. aeruginosa*, other Gram-negative pathogens have been shown to be more susceptible to extended NO-release kinetics. Backlund et al. previously reported that the Gram-negative periodontopathogens *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* are more susceptible to silica particles with extended NO-release kinetics.[10] However, *P. aeruginosa* does not follow the same trend as these Gram-negative species. This difference could be due to the natural NO detoxification mechanisms of *P. aeruginosa*. As a denitrifying bacterium, *P. aeruginosa* possesses the enzyme nitric oxide reductase (NOR) as a component of its electron transport chain.[16] Since NOR functions to protect *P. aeruginosa* from low levels of intracellular NO, larger instantaneous concentrations of NO (fast release kinetics) are required to overload NOR

and elicit NO-mediated killing.[10,16] Conversely, Gram-negative bacteria that do not possess this intracellular mechanism (e.g., *A. actinomycetemcomitans* and *P. gingivalis*) benefit from a slower build-up of NO.[10] The differential sensitivity to NO-release kinetics between *P. aeruginosa* and other Gram-negative pathogens may enhance the selective targeting of future NO-based therapeutics against different types of bacterial infections.
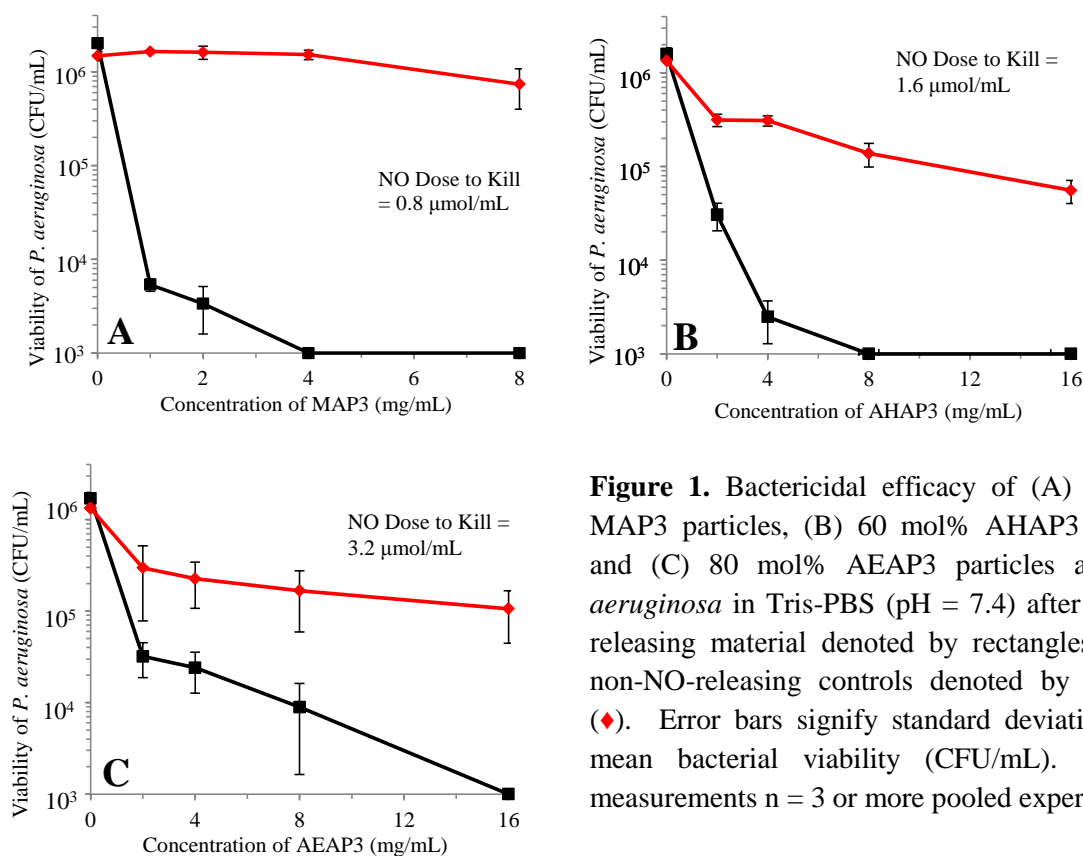

Figure 1. Bactericidal efficacy of (A) 50 mol% MAP3 particles, (B) 60 mol% AHAP3 particles, and (C) 80 mol% AEAP3 particles against *P. aeruginosa* in Tris-PBS (pH = 7.4) after 2 h. NO-releasing material denoted by rectangles (■) and non-NO-releasing controls denoted by diamonds (♦). Error bars signify standard deviation of the mean bacterial viability (CFU/mL). For all measurements n = 3 or more pooled experiments.

III.   Efficacy of nitric oxide-releasing silica particles against nosocomial pathogens

Previous reports have shown that Gram-positive species demonstrate reduced susceptibility to NO treatment compared to Gram-negative pathogens.[9,17] For example, Backlund et al. previously reported the enhanced bactericidal efficacy of NO-releasing silica particles against the Gram-negative periodontopathogen *Aggregatibacter actinomycetemcomitans* compared to the Gram-positive dental pathogen *Streptococcus mutans*.[17] Indeed, a similar trend was observed for nosocomial pathogens, as planktonic cultures of Gram-positive *S. aureus* were not susceptible to any of the aforementioned NO-releasing silica particles (MAP3, AHAP3, and AEAP3) tested against Gram-negative *P. aeruginosa*. To enhance antibacterial activity against *S. aureus,* the NO payload of the most rapid-releasing particle system (MAP3) was increased by increasing the mol% of the aminosilane (Table 3). Thus, by using NO-releasing 70 mol% MAP3 particles with higher 2 h NO totals, we compared the susceptibility of *P. aeruginosa* and *S. aureus* to NO. While the bactericidal concentration of NO-releasing 70 mol% MAP3 was 2 mg/mL against *P. aeruginosa*, an increased concentration of 48 mg/mL was required to achieve 3-log killing of *S. aureus* (Figure 2). The difference between the bactericidal NO doses for *P. aeruginosa* (1.6 µmol/mL) compared to *S. aureus* (42.2 µmol/mL) highlights the enhanced antimicrobial activity of NO against Gram-negative bacterial species.

NO Release Scaffold	t[NO]^a (μmol/mg)	[NO]$_m$^b (ppb/mg)	t ½^c (min)	t[NO]$_{2h}$^d (μmol/mg)
50 mol% MAP3	0.22 ± 0.04	900 ± 300	31.6 ± 4.8	0.21 ± 0.01
70 mol% MAP3	0.84 ± 0.14	9200 ± 4300	19.8 ± 3.3	0.81 ± 0.15

Table 3. Characterization of NO-releasing silica particles in PBS (pH = 7.4 at 37 °C) by means of a chemiluminescent nitric oxide analyzer. Results shown for 50 mol% and 70 mol% MAP3 NO-releasing silica particles and presented as mean ± standard deviation for n = 3 or more pooled experiments. [a]Total amount of NO released. [b]Maximum NO flux achieved. [c]Time to release half of total NO payload. [d]Total amount of NO released after 2 h.
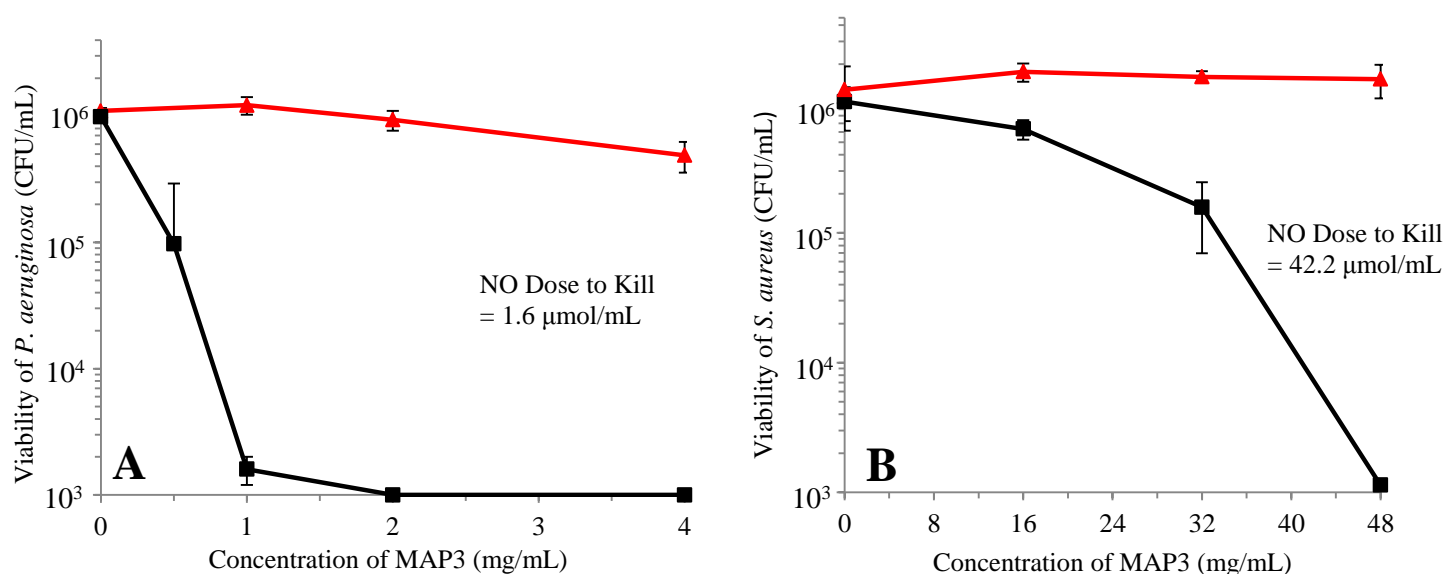

Figure 2. Bactericidal efficacy of 70 mol% MAP3 particles in Tris-PBS (pH = 7.4) against (A) *Pseudomonas aeruginosa* and (B) *Staphylococcus aureus* after 2 h exposure. NO-releasing particles denoted by rectangles (■) and non-NO-releasing controls denoted by triangles (▲). Error bars signify standard deviation of the mean bacterial viability (CFU/mL). For all measurements n = 3 or more pooled experiments.

The greater susceptibility of Gram-negative pathogens to treatment by NO lends further support toward the future use of NO as an antibacterial therapeutic. Conventional antibiotics are more effective at eradicating Gram-positive species, causing Gram-negative infections to spread and commonly remain untreated.[1] Furthermore, Gram-negative species are more likely to develop antibiotic resistance compared to Gram-positive pathogens, reducing the availability of effective treatment strategies for Gram-negative infections.[1] Thus, the greater antibacterial activity of NO-releasing therapeutics against Gram-negative species demonstrates the promise NO holds as an alternative strategy for treating hospital-acquired Gram-negative bacterial infections.

IV.   *Kinetic-dependent killing of planktonic Staphylococcus aureus*

Backlund et al. previously observed that more rapid NO-release kinetics at lower pH enhanced bactericidal efficacy against Gram-positive *Streptococcus mutans*.[10] Due to the similar decreased susceptibility to NO, we hypothesized that Gram-positive *S. aureus* would also be susceptible to larger NO doses with faster NO-release kinetics. As increased bactericidal efficacy was observed against *S. aureus* using 70 mol% MAP3 particles with greater 2 h NO totals (~0.80 μmol/mg), NO-release kinetics were accelerated by decreasing solution pH (6.4). Since *N*-diazeniumdiolate NO donors undergo proton-initiated decomposition, the increased proton concentration at pH 6.4 causes decomposition to occur more rapidly.[10,15] Accordingly, MAP3 NO-release kinetics were enhanced at pH 6.4, including a greater initial NO flux (9200 to 25961 ppb/mg) and a shorter half-life (19.8 to 4.2 min) (Table 4). 2 h NO totals remained similar at both pHs, allowing bactericidal efficacy to be evaluated independent of total NO payload.

pH	$t[NO]^a$ (µmol/mg)	$[NO]_m{}^b$ (ppb/mg)	$t_{1/2}{}^c$ (min)	$t[NO]_{2h}{}^d$ (µmol/mg)
7.4	0.84 ± 0.14	9200 ± 4300	19.8 ± 3.3	0.81 ± 0.15
6.4	0.85 ± 0.17	25961 ± 7500	4.2 ± 0.2	0.85 ± 0.17

Table 4. Characterization of MAP3 NO-releasing silica particles in PBS (37 °C) at different pH values (7.4 and 6.4) using a chemiluminescent nitric oxide analyzer. Results shown for 70 mol% MAP3 NO-releasing particles and presented as mean ± standard deviation for n = 3 or more pooled experiments. [a]Total amount of NO released. [b]Maximum NO flux achieved. [c]Time to release half of total NO payload. [d]Total amount of NO released after 2 h.

While the bactericidal concentration of NO-releasing 70 mol% MAP3 was 48 mg/mL against *S. aureus* at pH 7.4, faster NO-release resulted in enhanced killing (3-log) of *S. aureus* at pH 6.4 at a reduced concentration of 32 mg/mL MAP3 particles (Figure 3). Thus, killing was enhanced at pH 6.4 (bactericidal NO dose 27.2 µmol/mL) compared to pH 7.4 (bactericidal NO dose 42.2 µmol/mL) due to the enhanced *N*-diazeniumdiolate NO donor decomposition and faster NO-release kinetics. The benefit of fast NO-release kinetics for more effective killing of both *P. aeruginosa* and *S. aureus* may influence the design of future NO-releasing therapeutics for treatment of hospital-acquired infections implicating both of these nosocomial pathogens.
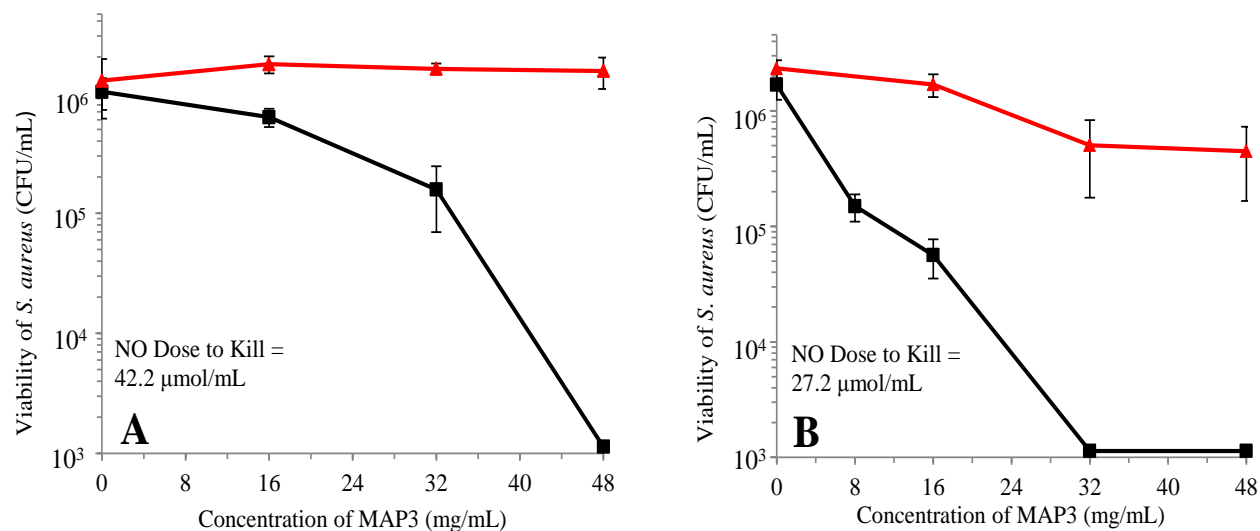
Figure 3. Bactericidal efficacy of 70 mol% MAP3 particles against *S. aureus* in (A) Tris-PBS (pH = 7.4) and (B) PBS (pH = 6.4) after 2 h exposure. NO-releasing material denoted by squares (■) and non-NO-releasing controls denoted by triangles (▲). Error bars signify standard deviation of the mean bacterial viability (CFU/mL). For all measurements n = 3 or more pooled experiments.

Conclusions

Our study details the effect of NO-release kinetics (half-lives) of NO-releasing silica particles on bactericidal efficacy against nosocomial pathogens. By synthesizing ~150 nm silica particles with similar 2 h NO totals but different half-lives, we evaluated the effect of release kinetics independent of size or total NO storage. Nitric oxide delivered from silica particles was significantly more effective at killing *Pseudomonas aeruginosa* compared to *Staphylococcus aureus.* However, faster NO-release kinetics enhanced bactericidal efficacy against both nosocomial pathogens, albeit at lower NO doses for *P. aeruginosa.* The greater antibacterial activity of NO-releasing materials with rapid release kinetics against nosocomial pathogens and the selectivity of NO against Gram-negative species warrant attention with respect to the future design of NO-releasing therapeutics to treat hospital-acquired infections.

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