Title page

S-nitrosocaptopril Nanoparticles as Nitric Oxide-Liberating and Transnitrosylating Anti-infective Technology

Breanne Mordorski, BA[1]
Robert Pelgrift, BA[2]
Aimee Krausz, BA[1]
Brandon Adler, BA[1]
Alexandre Batista da Costa Neto [2]
Hongying Liang[3]
Leslie Gunther, MSc[4]
Alicea Clendaniel, BS[6]
Stacey Harper, PhD[6,7]
Joel M. Friedman, MD PhD[3]
Joshua D. Nosanchuk, MD[2,5]
Parimala Nacharaju, PhD[3]
Adam J. Friedman, MD[1,3] *

[1]Division of Dermatology, Department of Medicine, Montefiore Medical Center, Bronx, New York, USA
[2]Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, USA
[3]Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York, USA
[4]Analytical Imaging Facility, Albert Einstein College of Medicine, Bronx, New York, USA
[5]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA
[6]Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, Oregon, USA
[7]School of Chemical, Biological and Environmental Engineering, Oregon State University, Corvallis, Oregon, USA

*Corresponding author. Address: Division of Dermatology, Montefiore Medical Center, 111 East 210th Street, Bronx, NY 10467, USA.
Fax: +1 718 944 4219. Phone: +1 718 920 2680
E-mail address: adfriedm@montefiore.org

Short title
Anti-infective S-nitrosocaptopril Nanotechnology

Word count for abstract
146

Complete manuscript word count (including body and figure legends, but excluding abstract/title page/references)
5686

Funding and Conflict of Interest
SLH acknowledges NIH grants ES017552-01A2, ES016896-01, P30 ES03850, T32 ES0007060, and AFRL FA8650-05-1-5041. JDN acknowledges National Institutes of Health/National Institute of Allergy and Infectious Diseases grant 1RC2A1087612-01. AJF acknowledges Dermatology Foundation Career Development Award and Janssen Biotech Research Grant.

JMF, PN, and AJF are co-inventors of the technology described however no financial conflict currently exists.

Abstract

Nitric oxide (NO) is an essential agent of the innate immune system which exhibits multi-mechanistic antimicrobial activity. Previously, increased antimicrobial activity against *E. coli* with nitric oxide releasing nanoparticles (NO-np) was demonstrated when combined with glutathione, versus NO-np alone, due to formation of S-nitrosoglutathione (GSNO), a potent transnitrosylating agent. To capitalize on this finding, the thiol-containing ACE-inhibitor, captopril, was incorporated into the NO-np system to form SNO-CAP-nps, nanoparticles that can both release NO as well as form S-nitrosocaptopril. SNO-CAP-nps demonstrated sustained release of NO and GSNO production in the presence of GSH over time. Both *E. coli* and MRSA were highly susceptible to SNO-CAP-np in a dose-dependent fashion, with *E. coli* being highly sensitive, while demonstrating no toxicity when exposed to zebrafish embryos. Given the significance of *E. coli* in urosepsis, as well as the ability of nanotechnology to overcome biofilm formation, SNO-CAP-np may prove useful against catheter-associated urinary tract infection.

Key words (3-5):

antibacterial, nanotechnology, nitric oxide, nitrosothiols, E. coli

Background

According to conservative estimates by the CDC, 2 million people in the USA are infected by antibiotic-resistant organisms each year, and more than 23,000 of these people die as a consequence of their infection[1]. As antibiotic-resistant infections continue to emerge and the efficacy of our current antibiotics continues to decline, it becomes more apparent that we need to seek other methods of combating bacterial infection. Unfortunately, recent successes in antimicrobial development have been few, and new therapies continue to lag behind the rapid evolution of bacterial resistance genes, which have been found in a wide range of bacterial species[2].

In the ongoing quest for novel antimicrobial therapies, nanotechnology has been quite promising, and nanoparticles can be designed to offer controlled release of a wide variety of antimicrobial agents. Nanoparticles' small size can make them more bioavailability and their increased surface area to volume ratio increases its interaction with the intended target, and thereby its antimicrobial capacity. Furthermore, nanoparticles allow the delivery of therapeutic agents that may not otherwise be soluble *in vivo*, or that may not be stable for long periods of time. For example, nitric oxide (NO) has a very short half-life *in vivo*, but by incorporation into a nanoparticle, it can be spontaneously released at a slow, sustained rate when exposed to an aqueous environment, thus allowing NO to exert its effect over time[3].

In developing new antibacterials, cues have been taken from the innate immune system. For example, naturally secreted antimicrobial peptides and enzymes, which are overcome by resilient bacteria *in vivo*, can be useful therapies if exogenously introduced at higher levels, or with slight modifications[4-6]. As with antimicrobial peptides and enzymes, nitric oxide (NO) is an essential agent of the innate immune system. It is a moderately-reactive radical, and its hydrophobic nature allows it to readily traverse cell membranes[7,8]. NO is generated and released by macrophages, neutrophils, eosinophils, fibroblasts, epithelial cells, endothelial cells, and glial cells as a method of killing or inhibiting the replication of bacteria, fungi and viruses[9-11]. NO has roles in vascular[12] cardiac[13], and neural[14] signaling, exerting its effects through cGMP-mediated as well as non-cGMP-mediated pathways[8,15,16].

NO's specific role in antimicrobial activity has been attributed to several different mechanisms, including reactivity with superoxide anion (forming cytotoxic peroxynitrite), S-nitrosylation of thiol residues in proteins (conformational change), inactivation of enzymes by disruption of iron centers (ribonucleotide reductase, aconitase, ubiquinone reductase), DNA damage, and peroxidation of membrane lipids [9-11]. NO may also exert indirect antimicrobial effects by upregulating IFN-γ[17], as well as superoxide and hydrogen peroxide release by neutrophils[18]. In the context of skin and soft tissue infections (SSTIs), NO's vasodilating properties enable necessary components of the immune system to get to the site of infection, further aiding the overall effort to eradicate the invading organism. Thus, with the application of molecules such as NO, which exert antimicrobial effects by a variety of mechanisms, it is unlikely that microbes will develop resistance, as multiple simultaneous gene mutations would be required to develop in the same microbial cell[19].

Due to the great potential of a multi-mechanistic antimicrobial, a considerable effort has been undertaken to harness NO as a therapeutic. Because it is a radical, one may assume NO's effects to be spatially and temporally limited. However, NO's aforementioned reactivity with thiol residues and iron centers can circumvent this issue by allowing its propagation in the form of NO-donating molecules, such as S-nitrosoglutathione (GSNO), S-nitrosoalbumin, S-nitrosylated hemoglobin, and even iron nitrosyl hemoglobin[11,20]. Inspired by S-nitrosylation *in vivo*, a variety of S-nitrosothiol (RSNO) therapeutics have emerged. Their effects are mainly attributed to trans-nitrosylation activity, whereby NO is transferred from one thiol group to another, exhibiting similar activity to NO by acting as long-lasting vasodilators (without drug tolerance), preventing platelet aggregation, and exhibiting antimicrobial effects[21-24].

To protect themselves from GSNO, bacteria employ GSNO reductases and nitroreductases, and may also use the regenerated GSH to protect themselves against oxidative damagefrom oxidants such as from peroxynitrite[25]. Despite these protective mechanisms, it was demonstrated that sustained generation of GSNO from a nitric oxide releasing nanoparticle platform (NO-np) in combination with solubilized GSH was highly effective against bacterial species *in vivo* (*P. aeruginosa*) and *in vitro* (methicillin resistant *S. aureus* (MRSA), *E. coli, P. aeruginosa, K. pneumoniae*)[26,27]. Interestingly, when exposed to an aliquot of GSNO at the same concentration generated from the nanoparticles, no antibacterial activity was observed[26]. Thus, it is likely that *sustained* levels of GSNO are necessary for bactericidal activity.

While the combination of NO-np and GSH was found to be effective both *in vitro* and *in vivo*[26,27], the practical utility of this combination is negated by the instability of GSH in light and ambient temperature, as well as the requirement of this combination to be in suspension, which will ultimately exhaust generated GSNO over time. Therefore, a platform that itself can both release NO and facilitate transnitrosylation would be ideal. To address this need, we designed a NO-generating nanoparticle which incorporates the angiotensin converting enzyme (ACE) inhibitor, captopril. Captopril contains a thiol group that can be nitrosylated to form *S*-nitrosocaptopril (SNO-CAP). There is positive but limited data demonstrating that SNO-CAP itself has potent vasodilating and antiplatelet actions, and that even in this

form it maintains the ability to inhibit ACE[28-30]. As NO is generated and released from the nanoparticle, it is bound up by the captopril sulfhydryl moiety, providing a long lasting NO-donating technology.

Given that nanoparticles have increased reactivity due to their large relative surface area, as well as offer controlled and sustained release, SNO-CAP nanoparticles (SNO-CAP-np) have an increased ability to interact with their intended target and exert their biological impact over an extended period of time. Here, we characterized the SNO-CAP-np's tested, their nitrosylating potential, and evaluated their antimicrobial activity against clinical isolates of *E. coli* and methicillin-resistant *S. aureus* (MRSA). In addition to assessing the efficacy of the SNO-CAP-np's, we also evaluated their toxicity *in vivo* using the model vertebrate, embryonic zebrafish.

Methods

Synthesis of SNO-CAP nanoparticles

A modified tetramethylorthosilicate (TMOS)-based sol-gel method was used to prepare SNO-CAP-np as previously described[3]. Briefly, TMOS (3 mL) was hydrolyzed with 1 mM HCl (0.6 mL) by sonication on an ice-bath. The hydrolyzed TMOS (3 mL) was added to a buffer mixture of 1.5 mL of 0.5% chitosan, 1.5 mL of polyethylene glycol (PEG) 400 and 24 mL of 50 mM phosphate, pH 7.4, containing 0.225 M nitrite and 0.28 M captopril. The mixture was left at room temperature overnight in the dark for polymerization. A pink, opaque sol-gel formed, which was lyophilized and then ball milled in a Pulverisette 6 planetary ball-mill (Fritsch, Idar-Oberstein, Germany) into fine powder. The product was stored at -80^{o} C until use. In addition, nanoparticles synthesized for the *in vivo* toxicity assay also included nanoparticles without nitrite and captopril (control-np), as well as nanoparticles which incorporated Alexa Fluor 568 C5 maleimide dye (Molecular Probes) in lieu of nitrite and captopril (Alexa 568-np). For 1 mL of TMOS, 100 µg of dye was used.

Dynamic light scattering

A suspension of SNO-CAP-np in PBS (2.5 mg/mL) was sonicated, and the size of the particles was measured using dynamic light scattering (DLS). DLS measurement was performed using a DynaPro NanoStar (Wyatt Technology, Santa

Barbara, CA) at 658 nm with an acquisition length of 5 seconds and a total of 40 acquisition attempts. An average particle hydrodynamic diameter (the diameter of the particle along with the shell of closely associated water molecules) and polydispersity were calculated from the results.

Electron microscopy

Nanoparticles were suspended in 100% ethanol and plated on poly-L-lysine coated coverslips. Samples were then critical point dried using liquid carbon dioxide in a Samdri-795 Critical Point Dryer (Tousimis, Rockville, MD), sputter coated with chromium in a Q150T ES Sputter Coater (Quorum Technologies Ltd, United Kingdom), and examined under a Supra Field Emission Scanning Electron Microscope (Carl Zeiss Microscopy, LLC North America) with an accelerating voltage of 3 kV.

In vivo toxicity assay

Zebrafish embryos (*Danio rerio*, wild type, 5D-Tropical strain) were obtained from Sinnhuber Aquatic Research Laboratory, Oregon State University, and exposures and evaluations were conducted according to Truong et al., 2011[31]. Briefly, embryos were dechorionated at 6 hours post-fertilization (hpf) by Protease Type XIV (Sigma Aldrich). Control-np, Alexa 568-np, and SNO-CAP-np were each diluted to 0, 0.016, 0.08, 0.4, 2, 10, 50 and 250 ppm in fish water and vortexed. Each well of a 96-well plate was filled with 150 µL of a given dilution, in addition to one zebrafish embryo at 8 hpf (N = 24 for each dilution). The plates were sealed with Parafilm and incubated at 26.5^{o}C on a 14 h light:10 h dark photoperiod.

Exposures were conducted over 5 days of development which encompasses gastrulation through organogenesis, the periods of development most conserved among vertebrates. All organ systems begin functioning during this time period and all of the molecular signaling pathways are active and necessary for normal development to occur. At 24 hpf, embryos were examined for mortality, developmental progression, notochord development, and spontaneous movement. At 120 hpf, the following larval morphology and behavioral endpoints were examined: body axis, eye, snout, jaw, otic vesicle, heart, brain, somite, pectoral fin, caudal fin, yolk sac, trunk, circulation, pigment, swim bladder,

motility and tactile response. Effects were evaluated in binary notation as either present or not present. Untreated control and exposed groups were compared using Fisher's exact test for each endpoint, and p-value < 0.05 for significance.

SNO-CAP-np NO release profile

The rate of NO release from SNO-CAP-np was monitored using a chemiluminescent NO analyzer (Sievers NO analyzer, Model 280i, Boulder, CO). SNO-CAP-np were dispersed in 6 mL of PBS at 1 mg/mL concentration. This solution was continuously bubbled with pure nitrogen gas (0.2 L/min). The gas phase was collected into the NO analyzer and the signal was monitored with the software provided by the vendor.

GSNO formation reaction

SNO-CAP-np (20 mg/mL) were suspended in 20 mM GSH/0.5 mM EDTA/PBS solution at room temperature while mixing on a Lab Rotator shielded from light. At 1, 30, 60, 120 and 240 minutes, 10 µL aliquots were taken, diluted to 500 µL in 0.5 EDTA/PBS, and stored at -80° C prior to RPHPLC analysis. Aliquots were also collected in the same fashion from a control suspension of SNO-CAP-np (20 mg/mL) in 0.5 mM EDTA/PBS.

RPHPLC analysis

RPHPLC analysis was performed with a Vydac 218TP C_{18} equipped with a 5 µm analytical column (250 mm x 4.6 mm, W.R. Grace & Co.-Conn., Columbia, MD). Samples were run in an isocratic 10 mM dipotassium phosphate/10 mM tetrabutylammonium hydrogen sulfate, 5% acetonitrile buffer (pH 7.0) at a 0.5 mL/min flow rate, and were detected by UV absorbance at 210 nm.

Peak identities were confirmed by comparing the chromatogram of the GSNO formation reaction to chromatograms of the control reaction, as well as to individual chromatograms of GSH, GSNO, sodium nitrite, captopril, and SNO-CAP (non-np), diluted in 0.5 mM EDTA/PBS. GSNO concentrations were calculated by comparing peak areas during the GSNO formation reaction to the peak area of a GSNO standard of known concentration.

E. coli and MRSA clinical isolates

All clinical isolates were collected from Montefiore Medical Center, Bronx, NY. All samples were obtained with patients' written consent according to the practices and standards of the institutional review boards at the Albert Einstein College of Medicine and Montefiore Medical Center. Eight *E. coli* isolates and 8 MRSA isolates were studied. All strains were stored in TSB with 40% glycerol at -80° C until use, and then grown in TSB overnight at 37° C with rotary shaking at 150 r.p.m.

Susceptibility of E. coli and MRSA to SNO-CAP-np and captopril

For each bacterial strain, one colony of bacteria grown on TSA was suspended in 1 mL TSB. One µL aliquots were transferred to a 100-well honeycomb plate with 199 µL TSB. The TSB contained either 1, 2.5, 5, or 10 mg/mL SNO-CAP-np, or 2.5, 5, or 10 mM captopril, and controls included wells containing bacteria and TSB alone. The background absorbance of each SNO-CAP-np concentration was accounted for by wells containing SNO-CAP-np and TSB alone. No background absorbance was measured for captopril. Prior to plating, all SNO-CAP-np concentrations were sonicated for 1 minute on ice with a Fisher Sonic Dismembrator (model 100, Fisher Scientific, Pittsburg, PA) to disperse the particles. All wells were incubated for 24 h at 37° C and growth was assessed by measuring optical density at 600 nm (OD_{600}) with a microplate reader (Bioscreen C, Growth Curves USA, Piscataway, NJ). P-value < 0.05 by unpaired *t* test was considered significant.

Colony-forming units (CFU) assay

After incubation with either SNO-CAP-np or captopril, 10 µL of bacterial suspension was aspirated from each treatment well of the honeycomb plate. Samples were transferred to an Eppendorf tube with 990 mL PBS and gently vortexed. Controls were collected in the same fashion from wells containing only bacteria and TSB. The suspensions were serially diluted in PBS so that final concentrations were 10^{-6} of the incubated concentration, and 100 µL aliquots were plated on TSA plates for 24 h. CFU's were counted and recorded. Percent survival was determined by comparing

CFU counts of SNO-CAP-np- or captopril-treated bacteria to CFU counts of untreated bacteria. P-value < 0.05 by unpaired *t* test was considered significant.

Results

SNO-CAP-np size characterization

SNO-CAP-np size is congruent with previous data in which our similarly-designed NO-np was measured at 10 nm via transmission electron microscopy. With scanning electron microscopy, on the other hand, results showed nano-aggregates of about 60-80 nm in diameter with individual nanoparticle subunits of about 10 nm (Figure 1B). Dynamic light scattering (DLS) of 2.5 mg/mL SNO-CAP-np revealed an average hydrodynamic diameter of 377.8 nm based on 40 acquisition attempts (Figure 1A). The standard deviation was 16.4 nm (4.3%), proving that SNO-CAP-np are homogenous in size. Since SNO-CAP-np swell with moisture, the average diameter is likely an overestimate.

SNO-CAP-np are non-toxic in vivo

Toxicological impact of control-np, Alexa 568-np, and SNO-CAP-np was assessed using embryonic zebrafish assay (Figure 2, A & B). Embryonic exposures did not elicit any toxic responses in the zebrafish after 5 days of exposure during a sensitive developmental time period. No nanoparticle treatments were significantly difference from untreated controls with respect to mortality, morphology or behavior. Background mortality is maintained below 8.3% in the Harper Laboratory (Oregon State University), which is below the EPA ecological effects test guideline of 10%[32]. Mortality did not differ between groups and was not significantly different than background for any exposure.

SNO-CAP-np NO release profile

The time course of NO formation from SNO-CAP-np in PBS (1 mg/mL) was evaluated over 12 hours via chemiluminescent NO analyzer. Within 2 minutes, the NO concentration peaked at 11.1 μM, and rapidly fell to levels below 4 μM after 4 minutes. NO concentration stabilized at about 2.4 μM after 19 minutes and decayed to a final concentration of about 1.2 μM after 12 h, thus demonstrating sustained NO release over at least 12 hours (Figure 3).

GSNO formation reaction

SNO-CAP-np (20 mg/mL) were incubated with GSH (20 mM) during which aliquots were taken at 1, 30, 60, 120 and 240 minutes and characterized by RPHPLC as described in the methods. GSH and GSNO peaks (labeled 1 and 2) were identified in the chromatogram of the reaction mixture by comparing the individual components separately (Figure 4A). Small unidentified peaks in the reaction mixture chromatogram are likely oxidized products of GSH and GSNO, such as glutathione disulfide (GSSG). Unreacted nitrite peaks were not found in the reaction mixture, as confirmed by RPHPLC analysis of sodium nitrite. Pure captopril and SNO-CAP (non-np) samples analyzed by RPHPLC did not yield any useful peaks, as we discovered that neither captopril nor SNO-CAP bind to the Vydac C_{18} column.

The time course of GSNO formation from the SNO-CAP-np + GSH reaction mixture was determined by comparing the peak area of a GSNO standard to reaction mixture chromatograms at successive time points. The production of GSNO levels greater than 6.5 mM was instantaneous, and reached peak levels of approximately 7.5 mM GSNO within half an hour. Relatively constant levels of GSNO were maintained by the reaction mixture for at least 4 h (Figure 4B).

SNO-CAP-np inhibits E. coli and MRSA growth

E. coli and MRSA strains were incubated with various concentrations of SNO-CAP-np (1, 2.5, 5 or 10 mg/mL) or captopril (2.5, 5, or 10 mM) for 24 h (Figure 5). For both species, all concentrations of SNO-CAP-np significantly inhibited bacterial growth compared to controls in a dose-dependent manner for up to 24 h. Overall, *E. coli* was visibly more sensitive than MRSA, and 10 mg/mL SNO-CAP-np lead to 100% growth reduction for both species. Interestingly, captopril showed an effect on *E. coli* growth in a dose dependent fashion, which was significant for all concentrations of captopril after 12 h. Captopril did not have an effect on the MRSA isolates tested.

SNO-CAP-np are bactericidal against E. coli

After incubation with either SNO-CAP-np or captopril, *E. coli* suspensions were diluted and plated on TSA, and CFU's were quantified after 24 h (Figure 6, A & C). Percent survival compared to control for 1, 2.5, 5 and 10 mg/mL SNO-CAP-np was 79.6, 30.2, 5.5 and 0.3%, respectively. Reduction in *E. coli* survival by 2.5, 5 and 10 mg/mL SNO-CAP-np was

statistically significant (P = 0.0007, <0.0001 and <0.0001). Furthermore, 2.5 mg/mL SNO-CAP-np exhibited a significant effect compared to 1 mg/mL (P = 0.03); 5 mg/mL exhibited significant effect compared to 2.5 mg/mL (P = 0.009); and 10 mg/mL exhibited a significant effect compared to 5 mg/mL (P = 0.035). Percent *E. coli* survival compared to control for 2.5, 5 and 10 mM captopril alone was 85.0, 83.6 and 59.1%, respectively. Only the 10 mM concentration of captopril significantly inhibited *E. coli* growth (P = 0.026).

SNO-CAP-np are bactericidal against MRSA

After incubation with either SNO-CAP-np or captopril, MRSA suspensions were diluted and plated on TSA, and CFU's were quantified after 24 h (Figure 6, B & D). Percent survival compared to control for 1, 2.5, 5 and 10 mg/mL SNO-CAP-np was 90.7, 67.1, 40.6 and 0.4%, respectively. Reduction in MRSA survival by 5 and 10 mg/mL SNO-CAP-np was statistically significant (P = 0.02 and 0.0003). In addition, 10 mg/mL SNO-CAP-np significantly inhibited MRSA growth compared to 5 mg/mL (P = 0.003). Captopril alone did not show a significant effect on MRSA (99.3, 95.6 and 69.5% MRSA survival for 2.5, 5 and 10 mM captopril).

Discussion

Due to the continued threat of antibiotic resistance, it is necessary to pursue technologies that employ innovative, multi-mechanistic methods to kill bacteria and limit the chance for adverse events associated with their use. In the current study, SNO-CAP-np demonstrated sustained nitrosylation activity via production of GSNO in the presence of GSH, which was maintained at a steady concentration over an extended period of time. In addition, SNO-CAP-np released and maintained significant levels of NO over at least 12 hours. SNO-CAP-np exhibited significant dose-dependent antimicrobial activity against *E. coli* and MRSA over 24 h, with full inhibition of both species for at least 24 h at a concentration of 10 mg/mL of nanoparticles. Unlike past studies evaluating the impact of nitroso-species on gram positive and negative bacteria, here *E. coli* was more sensitive to SNO-CAP-np than MRSA, suggesting a unique interaction and potential impact of this NO donating compound on a recalcitrant, clinically relevant organism. Importantly, SNO-CAP-np were found to be non-toxic *in vivo,* further supporting their potential use in the clinical setting.

In considering this technology's activity, SNO-CAP-np's antimicrobial properties are most likely due to transnitrosylation activity as well as NO release. However, it must be considered that SNO-CAP-np may also release free captopril. A previous study noted modest antimicrobial activity of captopril against *E. coli* and *S. enterica*[33]. Here, a modest but significant effect against *E. coli* was demonstrated, but not witnessed with MRSA. CFU killing assays revealed that captopril significantly killed *E. coli* only at the highest concentration (10 mM), yet the highest concentration of SNO-CAP-np studied (10 mg/mL) could contain a maximum of only 2.76 mM free captopril. Therefore, it seems unlikely that captopril's antimicrobial properties significantly contributed to the antimicrobial activity of SNO-CAP-np. However, it is important to consider the limitation of our captopril control treatments, as they contained only free captopril rather than captopril in nanoparticle form (CAP-np). As previously mentioned, the increased bioavailability offered by nanoparticle encapsulation can increase the antimicrobial capacity of an agent, and therefore it is not implausible that low levels of captopril contributed SNO-CAP-np antimicrobial activity. In addition to its capacity as a NO-donor, the utilization of captopril as an antimicrobial adjunct should continue to be explored in future studies.

As mentioned above, while *E. coli* was more sensitive than MRSA to SNO-CAP-np, *E. coli* was actually the least sensitive of five bacterial species tested in a previous study with NO releasing nanoparticles (NO-np) alone, and overall, gram negative species were less sensitive (*S. pyogenes* > *E. faecalis* ≈ *K. pneumoniae* > *P. aeruginosa* > *E. coli*)[34]. In a similar study, NO-np were combined in solution with glutathione (GSH) to form nitrosoglutathione (GSNO) and demonstrated significantly improved antimicrobial activity against *E. coli,* indicating the important role of nitroso-intermediates in the eradication of gram negative organisms[26]. Interestingly here, *E. coli* was inhibited to an even greater extent by SNO-CAP-np. The increased antimicrobial efficacy of SNO-CAP-np against *E. coli* may be attributable to improved SNO-CAP-mediated transnitrosylation activity, as opposed to free formed GSNO by NO-np.

In the aforementioned study with NO-np + GSH[26], it was demonstrated that the combination formed sustained levels of GSNO which, in contrast to bolus GSNO of the same concentration, were capable of significant antimicrobial activity[26]. However, a therapeutic suspension of NO-np + GSH would not be practical, as NO donation by the nanoparticles would form free GSNO, and in solution, both GSH and GSNO can decay to GSSG and become depleted. On

the other hand, the SNO-CAP-np platform can release NO as well as facilitate transnitrosylation *within* the nanoparticle, thus providing a stable, long lasting reservoir of both NO and RSNO (SNO-CAP).

Although some studies have discussed the antimicrobial activity of NO-releasing therapeutics (NONOates, nitroprusside) interchangeably with that of RSNO therapeutics (GSNO, SNO-Cys), it has become increasingly clear that their actions are distinctly different. While the former acts primarily via free NO release, the later acts primarily via transnitrosylation[35]. As such, different bacterial stress responses have been observed in the presence of NO alone versus RSNO, and are marked by distinct changes in bacterial gene expression[36,37].

In *E. coli* for example, free NO perturbs regulatory proteins including NsrR, NorR, Fnr, Fur and SoxR/S, while RSNO primarily perturbs methionine biosynthesis. Small but detectable perturbations of NsrR and NorR by RSNO are presumptively due to relatively small amounts of free NO released, but do not contribute to RSNO defense in a significant manner.[35,37]

Free NO inhibits NsrR, an iron-sulfur protein that blocks expression of Hmp, a bacterial flavohemoglobin with dioxygenase activity. When Hmp is expressed in the presence of NO, it can convert NO to nitrate under aerobic conditions, undermining its role in the innate immune response. NO also activates iron-containing NorR, a protein that upregulates expression of flavorubredoxin and an associated flavoprotein, which reduce NO to N_2O under microaerobic and anaerobic conditions[38,39]. NO-mediated inhibition of Fnr, an iron-sulfur protein, also upregulates Hmp[37,38], and NO-mediated inhibition of the iron-containing Fur protein upregulates genes involved in iron acquisition and reconstruction of iron-containing proteins that may have been damaged by NO[37]. SoxR, on the other hand, is an iron-sulfur transcription factor activated by either oxidation or nitrosylation to stimulate SoxS transcription, which then activates transcription of the Sox regulon[38,40]. The regulon includes super oxide dismutase, which detoxifies superoxide and peroxynitrite, as well as DNA repair enzymes that repair damage specific to oxidative (but not nitrosative) stress. Although it induces oxidative stress-specific defense mechanisms, activation of the Sox regulon confers NO defense, thus preventing free NO from exerting its full antimicrobial effect[41].

In contrast to free NO, RSNO acts primarily via transnitrosylation activity, leading to an accumulation of *S*-nitrosylated cysteine and homocysteine (SNO-Cys and SNO-Hcy). This disrupts *E. coli's* methionine biosynthesis pathway and upregulates genes involved in methionine biosynthesis. In fact, supplementation with exogenous methionine was shown to strongly attenuate GSNO's effect on *E. coli* growth. In contrast, genes for methionine biosynthesis were not upregulated in response to platforms that release free NO, nor did exogenous methionine offer protection from such treatments[35,37].

These differences in NO- versus RSNO-induced stress responses offer insight into mechanisms of NO- versus RSNO-mediated bacterial killing. For example, RSNO-induced genes indicate a depletion of cellular methionine reserves via transnitrosylation, which may affect the synthesis of new proteins. NO-induced genes, on the other hand, indicate a role for disruption of iron-containing proteins as well as formation of peroxynitrite, as previously discussed. Furthermore, lack of methionine biosynthesis perturbation in response to free NO confirms that NO alone does not result in *S*-nitrosylation. Rather, NO must be transferred from a NO-donor, such as SNO-CAP, in order for intracellular *S*-nitrosylation to occur.

In addition to unique nitrosative stress responses in *E. coli*, cellular entry of NO versus RSNO may also affect their antimicrobial efficacy. While NO freely diffuses across bacterial membranes, GSNO utilizes the Dpp transporter (dipeptide permease) for internalization. Dpp deficient *E. coli* mutants treated with bactericidal concentrations of GSNO did not experience significant growth inhibition, enzymes involved in methionine biosynthesis were not upregulated, and NO sensitivity remained unchanged[35]. The ease of cellular entry offered by the Dpp transporter may contribute to GSNO's potency against *E. coli*. In addition, these results confirm that transnitrosylation of intracellular targets, rather than NO release, is the main mode of RSNO-mediated growth inhibition. Furthermore, it raises the question as to how other RSNO's, such as SNO-CAP, enter bacterial cells.

Together, these findings demonstrate that the antimicrobial mechanisms of NO and RSNO are distinctly different, and that compensatory bacterial responses for one versus the other do not overlap to a significant extent. Since *E. coli* growth inhibition following NO-np treatment was less pronounced[34], *E. coli* is likely better equipped to neutralize free NO and its effects, such as disruption of iron centers and peroxynitrite formation, than RSNO-mediated transnitrosylation effects which lead to methionine depletion. SNO-CAP-np not only have the ability to produce sustained levels of free NO, but also offer sustained transnitrosylation activity via SNO-CAP production. This combination, in addition to increased bioavailability due to nanoparticle encapsulation, can overwhelm *E. coli's* innate defense mechanisms and produce substantial bactericidal effects.

In contrast to *E. coli*, there is significantly less literature regarding MRSA's nitrosative stress response when treated with free NO versus RSNO. It is known that S*. aureus* is one of the few bacterial species with a nitric oxide synthase (bNOS), and bNOS-deficient mutants have increased susceptibility to host neutrophils, antimicrobial peptides, as well as antibiotics that act on the cell envelope (vancomycin and daptomycin). Furthermore, b-NOS mutants demonstrate reduced virulence and smaller abscess generation in mice, in addition to *decreased* susceptibility to aminoglycosides[42], indicating a complex relationship between intracellular NO and MRSA pathology. MRSA's defense against the antimicrobial effects of NO includes Hmp[43], as well as NO-inducible lactate dehydrogenase which facilitates maintenance of redox homeostasis in the presence of nitrosative stress[44]. While previous experiments demonstrated a significant difference in MRSA treated with NO-releasing therapy (NO-np) versus RSNO-generating therapy (NO-np + GSH)[26], the difference was not exceedingly pronounced, suggesting that both NO and RSNOs share equally important roles in the innate immune response.

Given the demonstrated utility of SNO-CAP-np against *E. coli in vitro*, its role at the bedside is rather apparent given that *E. coli* is the major cause of urinary tract infections (UTIs) and gram-negative bacteremia[45]. UTI's are a persistent issue in the setting of indwelling urinary catheters, and bacteria can become especially resistant as many catheterized patients require multiple courses of antibiotics[46]. Primary antibiotics used to treat UTI currently include ciprofloxacin (Cipro) and trimethoprim-sulfamethoxazole (TMP/SMX, or Bactrim), each of which have very specific targets by which

they exert antimicrobial activity. Ciprofloxacin inhibits DNA gyrase and DNA topoisomerase IV, both of which are necessary for DNA replication, by trapping them on DNA[47]. On the other hand, TMP/SMX inhibits two successive enzymes in the folate biosynthesis pathway, thereby depriving bacteria that are incapable of scavenging folate from their host. However, such specifically-targeted antimicrobial mechanisms are especially prone to resistance development. In addition, both ciprofloxacin and TMP/SMX resistance can arise by acquisition of an efflux pump[48,49]. As such, significant levels of microbial resistance have been demonstrated to both fluoroquinolones, including ciprofloxacin, and TMP/SMX[50,51]. In contrast, SNO-CAP-np exert antimicrobial activity by a variety of non-specific mechanisms, which make resistance unlikely. Furthermore, previous studies with NO-np[52] demonstrate that the nanoparticles are capable of bacterial cell wall damage and lysis, thus negating the function of potential efflux pumps.

Biofilms are another antimicrobial resistance mechanism employed by bacteria, and catheter associated UTI typically includes the formation of biofilms on both the inner and outer surfaces of urinary catheters. Biofilms allow bacteria to withstand even high concentrations of antibiotic, especially bacteria in deeper layers of the biofilm, by secreting a protective matrix of extracellular polymeric substance (EPS). The relatively anaerobic conditions in the deeper layers also lower growth rates, making the cells less susceptible to typical antibiotics. Furthermore, by lowering the antibiotic agent below minimum inhibitory concentration (MIC), but not quite to zero, EPS may also facilitate the procurement of new resistance mechanisms[53,54].

Current antibiotics for UTI can neither penetrate these biofilms, nor do they have potential to incorporate into catheters themselves or as a catheter lock. However, studies with nanotechnology have had promising results. For example, NO-np made of silica eradicated *E. coli* biofilms that had already been formed[55], and silver nanoparticles (Ag-np) inhibited the formation of *E. coli* biofilms on plastic catheters[56]. Unlike current antibiotics, nanoparticles may be able to permeate EPS and reach the lower levels of biofilms in sufficient quantities. In addition, the increased bioavailability of the nanoparticle-encapsulated agent may enable it to kill bacteria even in the deepest biofilm layers. Given SNO-CAP-np effectiveness against *E. coli*, and the previous success of nanotechnology in biofilm prevention and eradication, it may be beneficial to investigate SNO-CAP-np utility in combating E. coli infections in urosepsis.

As with any new nanotechnology application intended for close contact with humans, it is prudent to assess the potential to cause harm. Thus, the toxicity of the SNO-CAP-np were investigated as a precaution for this potentially useful nanotechnology application. The embryonic zebrafish model was selected as the ideal vertebrate model because it has proven sensitive for measuring toxicity of many nanomaterials including metals such as gold and silver, and metal oxides such as titanium and tin[57]. A database of over 100 nanomaterials has been compiled using this model organism for the study of nanomaterial-biological interactions (nbi.oregonstate.edu) which illustrates that this model is amenable to detect toxicity from nanoparticle exposures. It is very promising that no such toxicity was observed from the SNO-CAP-np.

Conclusion

SNO-CAP-np were found to be non-toxic in a preclinical toxicity assay. SNO-CAP-np synthesized for these studies generated GSNO over time at sustained concentrations in the presence of GSH. SNO-CAP-np killed both *E. coli* and MRSA in a dose-dependent fashion likely through the transnitrosylation activity of SNO-CAP. The SNO-CAP-np platform was not toxic to a model vertebrate which further enhances its usefulness in a clinical setting as a broad-spectrum antimicrobial. In addition to potential utility in the setting of SSTI's, SNO-CAP-np may be useful in combating catheter-associated UTI's, given the particular sensitivity of *E. coli* and the ability of nanoparticles to prevent and overcome biofilm formation.

References

1. Antibiotic resistance: a final warning. *Lancet.* Sep 28 2013;382(9898):1072.

2. Gootz TD. The global problem of antibiotic resistance. *Critical reviews in immunology.* 2010;30(1):79-93.

3. Friedman AJ, Han G, Navati MS, et al. Sustained release nitric oxide releasing nanoparticles: characterization of a novel delivery platform based on nitrite containing hydrogel/glass composites. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society.* Aug 2008;19(1):12-20.

4. Movert E, Wu Y, Lambeau G, Touqui L, Areschoug T. A novel bacterial resistance mechanism against human group IIA-secreted phospholipase A2: role of Streptococcus pyogenes sortase A. *Journal of immunology.* Dec 15 2011;187(12):6437-6446.

5. Hunter P. Where next for antibiotics? The immune system and the nature of pathogenicity are providing vital clues in the fight against antibiotic-resistant bacteria. *EMBO reports.* Aug 2012;13(8):680-683.

6. Wanniarachchi YA, Kaczmarek P, Wan A, Nolan EM. Human defensin 5 disulfide array mutants: disulfide bond deletion attenuates antibacterial activity against Staphylococcus aureus. *Biochemistry.* Sep 20 2011;50(37):8005-8017.

7. Fang FC. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *The Journal of clinical investigation.* Jun 15 1997;99(12):2818-2825.

8. McDonald LJ, Murad F. Nitric oxide and cyclic GMP signaling. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine.* Jan 1996;211(1):1-6.

9. De Groote MA, Fang FC. NO inhibitions: antimicrobial properties of nitric oxide. *Clin Infect Dis.* 1995;21 Suppl 2:S162-165.

10. Liew FY, Cox FE. Nonspecific defence mechanism: the role of nitric oxide. *Immunology today.* Mar 1991;12(3):A17-21.

11. Bogdan C. Nitric oxide and the immune response. *Nature immunology.* Oct 2001;2(10):907-916.

12. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature.* Jun 11-17 1987;327(6122):524-526.

13. Barouch LA, Harrison RW, Skaf MW, et al. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature.* Mar 21 2002;416(6878):337-339.

14. Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature.* Oct 25 1990;347(6295):768-770.

15. Gordge MP, Hothersall JS, Noronha-Dutra AA. Evidence for a cyclic GMP-independent mechanism in the anti-platelet action of S-nitrosoglutathione. *British journal of pharmacology.* May 1998;124(1):141-148.

16. Wanstall JC, Homer KL, Doggrell SA. Evidence for, and importance of, cGMP-independent mechanisms with NO and NO donors on blood vessels and platelets. *Current vascular pharmacology.* Jan 2005;3(1):41-53.

17. Diefenbach A, Schindler H, Rollinghoff M, Yokoyama WM, Bogdan C. Requirement for type 2 NO synthase for IL-12 signaling in innate immunity. *Science.* May 7 1999;284(5416):951-955.

18. Lee C, Miura K, Liu X, Zweier JL. Biphasic regulation of leukocyte superoxide generation by nitric oxide and peroxynitrite. *The Journal of biological chemistry.* Dec 15 2000;275(50):38965-38972.

19. Pelgrift RY, Friedman AJ. Nanotechnology as a therapeutic tool to combat microbial resistance. *Advanced drug delivery reviews.* Nov 30 2013;65(13-14):1803-1815.

20. Pawloski JR, Hess DT, Stamler JS. Export by red blood cells of nitric oxide bioactivity. *Nature.* Feb 1 2001;409(6820):622-626.

21. Henry PJ, Drummer OH, Horowitz JD. S-nitrosothiols as vasodilators: implications regarding tolerance to nitric oxide-containing vasodilators. *British journal of pharmacology.* Nov 1989;98(3):757-766.

22. Kowaluk EA, Poliszczuk R, Fung HL. Tolerance to relaxation in rat aorta: comparison of an S-nitrosothiol with nitroglycerin. *European journal of pharmacology.* Dec 15 1987;144(3):379-383.

23. Richardson G, Benjamin N. Potential therapeutic uses for S-nitrosothiols. *Clinical science.* Jan 2002;102(1):99-105.

24. Gaston B. Nitric oxide and thiol groups. *Biochimica et biophysica acta.* May 5 1999;1411(2-3):323-333.

25. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature.* Mar 22 2001;410(6827):490-494.

26. Friedman AJ, Blecher K, Schairer D, et al. Improved antimicrobial efficacy with nitric oxide releasing nanoparticle generated S-nitrosoglutathione. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society.* Nov 30 2011;25(4):381-386.

27. Chouake J, Schairer D, Kutner A, et al. Nitrosoglutathione generating nitric oxide nanoparticles as an improved strategy for combating Pseudomonas aeruginosa-infected wounds. *Journal of drugs in dermatology : JDD.* Dec 2012;11(12):1471-1477.

28. Cooke JP, Andon N, Loscalzo J. S-nitrosocaptopril. II. Effects on vascular reactivity. *The Journal of pharmacology and experimental therapeutics.* Jun 1989;249(3):730-734.

29. Loscalzo J, Smick D, Andon N, Cooke J. S-nitrosocaptopril. I. Molecular characterization and effects on the vasculature and on platelets. *The Journal of pharmacology and experimental therapeutics.* Jun 1989;249(3):726-729.

30. Shaffer JE, Lee F, Thomson S, Han BJ, Cooke JP, Loscalzo J. The hemodynamic effects of S-nitrosocaptopril in anesthetized dogs. *The Journal of pharmacology and experimental therapeutics.* Feb 1991;256(2):704-709.

31. Truong L, Harper SL, Tanguay RL. Evaluation of Embryotoxicity Using the Zebrafish Model. In: Gautier J-C, ed. *Drug Safety Evaluation: Methods and Protocols.* Vol 691. New York, NY: Humana Press; 2011:271-279.

32. Ecological Effects Test Guidelines: OPPTS 850.1075 Fish Acute Toxicity Test, Freshwater and Marine: United States Environmental Protection Agency; 1996.

33. Uda NR, Creus M. Selectivity of Inhibition of N-Succinyl-l,l-Diaminopimelic Acid Desuccinylase in Bacteria: The product of dapE-gene Is Not the Target of l-Captopril Antimicrobial Activity. *Bioinorganic chemistry and applications.* 2011;2011:306465.

34. Friedman A, Blecher K, Sanchez D, et al. Susceptibility of Gram-positive and -negative bacteria to novel nitric oxide-releasing nanoparticle technology. *Virulence.* May-Jun 2011;2(3):217-221.

35. Jarboe LR, Hyduke DR, Tran LM, Chou KJ, Liao JC. Determination of the Escherichia coli S-nitrosoglutathione response network using integrated biochemical and systems analysis. *The Journal of biological chemistry.* Feb 22 2008;283(8):5148-5157.

36. Flatley J, Barrett J, Pullan ST, Hughes MN, Green J, Poole RK. Transcriptional responses of Escherichia coli to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. *The Journal of biological chemistry.* Mar 18 2005;280(11):10065-10072.

37. Pullan ST, Gidley MD, Jones RA, et al. Nitric oxide in chemostat-cultured Escherichia coli is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. *Journal of bacteriology.* Mar 2007;189(5):1845-1855.

38. Spiro S. Nitric oxide-sensing mechanisms in Escherichia coli. *Biochemical Society transactions.* Feb 2006;34(Pt 1):200-202.

39. Helmick RA, Fletcher AE, Gardner AM, et al. Imidazole antibiotics inhibit the nitric oxide dioxygenase function of microbial flavohemoglobin. *Antimicrobial agents and chemotherapy.* May 2005;49(5):1837-1843.

40. Hausladen A, Privalle CT, Keng T, DeAngelo J, Stamler JS. Nitrosative stress: activation of the transcription factor OxyR. *Cell.* Sep 6 1996;86(5):719-729.

41. Nunoshiba T, deRojas-Walker T, Wishnok JS, Tannenbaum SR, Demple B. Activation by nitric oxide of an oxidative-stress response that defends Escherichia coli against activated macrophages. *Proceedings of the National Academy of Sciences of the United States of America.* Nov 1 1993;90(21):9993-9997.

42. van Sorge NM, Beasley FC, Gusarov I, et al. Methicillin-resistant Staphylococcus aureus bacterial nitric-oxide synthase affects antibiotic sensitivity and skin abscess development. *The Journal of biological chemistry.* Mar 1 2013;288(9):6417-6426.

43. Richardson AR, Dunman PM, Fang FC. The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. *Molecular microbiology.* Aug 2006;61(4):927-939.
44. Richardson AR, Libby SJ, Fang FC. A nitric oxide-inducible lactate dehydrogenase enables Staphylococcus aureus to resist innate immunity. *Science.* Mar 21 2008;319(5870):1672-1676.
45. Johnson JR, Stell AL. Extended virulence genotypes of Escherichia coli strains from patients with urosepsis in relation to phylogeny and host compromise. *The Journal of infectious diseases.* Jan 2000;181(1):261-272.
46. Nicolle LE. Catheter-related urinary tract infection. *Drugs & aging.* 2005;22(8):627-639.
47. Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiology and molecular biology reviews : MMBR.* Sep 1997;61(3):377-392.
48. Pakzad I, Zayyen Karin M, Taherikalani M, Boustanshenas M, Lari AR. Contribution of AcrAB efflux pump to ciprofloxacin resistance in Klebsiella pneumoniae isolated from burn patients. *GMS hygiene and infection control.* 2013;8(2):Doc15.
49. Kohler T, Kok M, Michea-Hamzehpour M, et al. Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy.* Oct 1996;40(10):2288-2290.
50. Edelsberg J, Weycker D, Barron R, et al. Prevalence of antibiotic resistance in US hospitals. *Diagnostic microbiology and infectious disease.* Nov 15 2013.
51. Perfetto EM, Gondek K. Escherichia coli resistance in uncomplicated urinary tract infection: a model for determining when to change first-line empirical antibiotic choice. *Managed care interface.* Jun 2002;15(6):35-42.
52. Martinez LR, Han G, Chacko M, et al. Antimicrobial and healing efficacy of sustained release nitric oxide nanoparticles against Staphylococcus aureus skin infection. *The Journal of investigative dermatology.* Oct 2009;129(10):2463-2469.
53. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *International journal of antimicrobial agents.* Apr 2010;35(4):322-332.
54. Donlan RM. Biofilms: microbial life on surfaces. *Emerging infectious diseases.* Sep 2002;8(9):881-890.
55. Hetrick EM, Shin JH, Paul HS, Schoenfisch MH. Anti-biofilm efficacy of nitric oxide-releasing silica nanoparticles. *Biomaterials.* May 2009;30(14):2782-2789.
56. Roe D, Karandikar B, Bonn-Savage N, Gibbins B, Roullet JB. Antimicrobial surface functionalization of plastic catheters by silver nanoparticles. *The Journal of antimicrobial chemotherapy.* Apr 2008;61(4):869-876.
57. Nanomaterial-Biological Interactions Knowledgebase. ONAMI & Oregon State University; 2010. http://nbi.oregonstate.edu/.

Figure Legends

Figure 1. Size characterization of SNO-CAP-np. **(A)** SNO-CAP-np diameter was measured via dynamic light scattering (DLS). The average diameter weighted by intensity was 377.8 ± 16.4 nm, and the curve represents 40 acquisition attempts. Since SNO-CAP-np swell with moisture, the diameter is likely an overestimate. **(B)** SNO-CAP-np were visualized via scanning electron microscopy (accelerating voltage 3 kV). Results show nano-aggregates about 60-80 nm in diameter with individual nanoparticle subunits of about 10 nm. The white scale bar represents 100 nm.

Figure 2. SNO-CAP-np are non-toxic *in vivo***. (A)** Percent mortality as a function of exposure concentration and treatment material (N = 24). Mortality was not significant for any nanomaterial across the concentrations tested. **(B)** Zebrafish embryos (120 hpf) exposed to 250 ppm of nanomaterial. **(i)** Untreated **(ii)** Control-np **(iii)** Alexa 568-np **(iv)** SNO-CAP-np.

Figure 3. NO release from SNO-CAP-np in PBS (1 mg/mL) was evaluated over 12 hours via chemiluminescent NO analyzer (Sievers NO analyzer, Model 280i).

Figure 4. GSNO formation reaction. **(A)** RPHPLC analysis of the SNO-CAP-np + GSH reaction. Twenty mg/mL SNO-CAP-np with 20 mM GSH was incubated at room temperature, as was a control suspension of SNO-CAP-np. Their respective chromatograms represent aliquots taken after one minute and diluted 50x. GSH and GSNO standards were analyzed by RPHPLC at 0.1 mM. Peaks 1 and 2 in the SNO-CAP-np + GSH reaction were identified as GSH and GSNO, respectively. Small peaks in between peaks 1 and 2 are likely oxidation products of GSH and GSNO. **(B)** Time course of GSNO formation. GSNO peak area was evaluated for SNO-CAP-np + GSH reaction mixture at various time points and compared to the GSNO standard to determine real quantities of GSNO release. GSNO concentrations were plotted at 1, 30, 60 and 240 minutes (120 minute time point was omitted).

Figure 5. *E. coli* and MRSA are susceptible to SNO-CAP-np. **(A)** *E. coli* with SNO-CAP-np **(B)** MRSA with SNO-CAP-np **(C)** *E. coli* with captopril **(D)** MRSA with captopril. *E. coli* and MRSA suspensions were incubated at 37° C for 24 h with and without various concentrations of SNO-CAP-np (1, 2.5, 5 and 10 mg/mL) or captopril (2.5, 5 and 10 mM). OD_{600} was plotted every 4 h, and background OD_{600} for SNO-CAP-np in TSB was subtracted. Eight strains each of *E. coli* and MRSA were evaluated, and each condition was measured in triplicate. Error bars represent SEM.

Figure 6. CFU assay. **(A)** *E. coli* with SNO-CAP-np **(B)** MRSA with SNO-CAP-np **(C)** *E. coli* with captopril **(D)** MRSA with captopril. After *E. coli* and MRSA were incubated at 37° C for 24 h with either SNO-CAP-np or captopril in TSB (one colony/mL diluted 200-fold), 10 µL was aspirated and further diluted 10^6-fold in PBS. The dilutions were plated in 100 µL aliquots on TSA, and colony forming units (CFU's) were quantified following 24 h incubation at 37° C. CFU counts are expressed as percent survival compared to control. Eight strains each of *E. coli* and MRSA were evaluated, and each condition was plated in triplicate. Error bars represent SEM. Symbols denote p-value significance (*P = 0.0007, **P < 0.0001, †P = 0.02, ††P = 0.0003, #P = 0.026) as calculated by unpaired *t* test analysis.