



Use of nitric oxide nanoparticulate platform for the treatment of skin and soft tissue infections

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The incidence of skin and soft tissue infections (SSTI) due to multi-drug resistant pathogens is increasing. The concomitant increase in antibiotic use along with the ease with which organisms develop mechanisms of resistance have together become a medical crisis, underscoring the importance of developing innovative and effective antimicrobial strategies. Nitric oxide (NO) is an endogenously produced molecule with many physiologic functions, including broad spectrum antimicrobial activity and immunomodulatory properties. The risk of resistance to NO is minimized because NO has multiple mechanisms of antimicrobial action. NO's clinical utility has been limited largely because it is highly reactive and lacks appropriate vehicles for storage and delivery. To harness NO's antimicrobial potential, a variety exogenous NO delivery platforms have been developed and evaluated, yet limitations preclude their use in the clinical setting. Nanotechnology represents a paradigm through which these limitations can be overcome, allowing for the encapsulation, controlled release, and focused delivery of NO for the treatment of SSTI. © 2013 Wiley Periodicals, Inc.

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INTRODUCTION

The incidence of skin and soft tissue infections (SSTI) due to multi-drug resistant (MDR) pathogens is continuing to rise.¹ As a result, antibiotic use has increased in parallel to this trend. For example, a population-based study in Canada demonstrated a 15% increase in physician visits for SSTI and an associated 49% increase in antibiotic prescriptions between 1996 and 2008.² Unfortunately, increasing antibiotic use has become a major driving force in the development of resistant organisms, undermining their very purpose.³

Staphylococcus aureus is the etiologic agent and endemic cause of the majority of SSTI in the United States.^{4,5} The growing rate of methicillin-resistant

S. aureus (MRSA) presents an emergent treatment challenge. Moreover, whereas the majority of MRSA infections between the 1960s and 1990s were hospital-acquired, there has been an exponential increase in community-associated MRSA since the late 1990s,^{2,5} which has led to a greater social and financial burden resulting from hospitalization.⁶ Other pathogens have also demonstrated emerging resistance to many antibiotics. For example, a group of MDR bacteria referred to as the 'ESKAPE' pathogens (*Enterococcus faecalis*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), appropriately named as they 'escape' the effect of a variety of antibacterial drugs, have further complicated the SSTI landscape.¹ This ongoing crisis warrants the development of innovative therapeutic strategies to combat MRSA and resistant microbes implicated in SSTI.

There are several mechanisms through which pathogens overcome antibiotic activity. Resistance to antibiotics can occur via inherent resistance in certain species, such as species that produce penicillinase and are therefore resistant to β -lactam antibiotics.

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Resistance can also occur via *de novo* mutations or by the acquisition of resistance genes via horizontal transfer between microbes.^{1,7} Some resistance mechanisms include direct removal of the drug from the intracellular space, decreased diffusion of the drug via modification or loss of porins, alterations or upregulation of drug target sites, bacterial enzyme drug degradation.⁸ Because excessive antibiotic use is associated with the emergence of and selection for resistance,³ antibiotic overuse and misuse also contributes to the growing problem of bacterial resistance.^{1,4,9}

Nitric oxide (NO) is a diatomic gaseous molecule endogenously produced which, among other properties, exhibits broad spectrum antimicrobial activity. Antibiotic agents that exert multiple mechanisms of antimicrobial action limit pathogens' ability to develop resistance; such drugs are advantageous for this reason. The risk of bacterial resistance to both innate production and exogenous delivery of NO is minimized because NO exhibits multiple mechanisms of antimicrobial action.⁸ However, NO's utility in the clinical setting has been restricted because it is highly reactive and lacks proper vehicles for its delivery and storage,^{10–12} A variety of exogenous NO sources have been developed and studied for antimicrobial efficacy, but limitations preclude their use in the clinical setting. Nanotechnology offers a platform for targeted drug delivery, and extensive research has been conducted to evaluate the efficacy of antibacterial nanoparticles (nps). NO's broad antimicrobial properties and successful incorporation into nps offers a promising solution to the treatment of SSTI.

NITRIC OXIDE

Structure and Chemical Properties

NO is one of the smallest biologically active molecules¹³ and acts on virtually every cell in the body.¹⁴ Because of its lipophilic character and low molecular weight, NO traverses most physiologic barriers with relative ease to reach target cells.^{14,15} Additionally, NO diffuses along its concentration gradient and can therefore cross cell membranes without the need for transport proteins.^{16–18} NO is a natural yet free radical-forming gas and is highly unstable in an oxygen environment: it spontaneously reacts with oxygen or superoxide, forming reactive nitrogen oxide species (RNOS).

NO is endogenously synthesized when one of three distinct nitric oxide synthase (NOS) enzymes induces the oxidation of arginine to citrulline (Figure 1).^{15,17,19–22} Two NOS isoforms, NOS1 and NOS3, are constitutively expressed^{4,15,18,22,23} and are also known by the cell types in which they

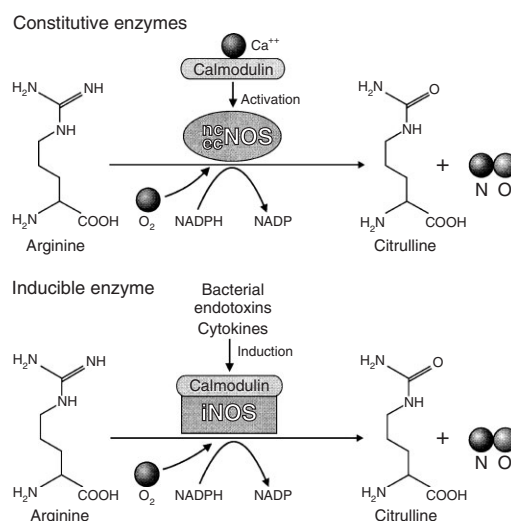


FIGURE 1 | Synthesis of nitric oxide (NO). Endothelial nitric oxide synthase (eNOS) and neuronal NOS (nNOS) calcium-dependent, calmodulin-regulated enzymes. They are constitutively expressed and catalyze the conversion of arginine to citrulline. Inducible NOS (iNOS) converts arginine to citrulline in a calcium-independent fashion, and is activated by bacterial endotoxins and proinflammatory cytokines. (Reprinted with permission from Ref 24. Copyright 1998 Nature Publishing Group)

were enzymatically identified: NOS1 or neuronal NOS (nNOS) from neuronal cells, and NOS3 or endothelial NOS (eNOS), from endothelial cells. Both eNOS and nNOS are calcium-dependent, calmodulin-regulated enzymes,^{23,24} meaning their activity is tightly regulated. When activated, both of these enzymes produce low quantities of NO for short time periods.¹⁵ In these small quantities, NO functions as a signaling molecule.²² NOS2 or inducible NOS (iNOS) was originally discovered in macrophages^{4,24} but is now known to be expressed in many cell types.^{23,24} iNOS generates large quantities of NO in a noncalcium-dependent fashion, and is induced by a wide array of stimulants including proinflammatory cytokines,^{13,20,21,23–25} bacterial polysaccharides and endotoxins,^{13,21,23,25} and neuropeptides²³ (Figure 1), which often act synergistically in iNOS activation.¹⁵

Physiologic Function

NO plays a variety of important physiologic roles including blood pressure regulation, neurotransmission, inhibition of platelet aggregation, immune response, and wound healing.^{12,26,27} Because of its short half-life, measured on the order of seconds, NO's biological impact is determined primarily by its rate of formation.^{14,16} Its site of action is often close to its site of generation, as NO is rapidly scavenged by hemoglobin and myoglobin.^{13,16}

NO initiates multiple cellular signaling cascades, most notably via the soluble guanylyl cyclase (sGC) pathway. In this paradigm, NO binds to sGC, causing increased cyclic GMP levels and activation of protein kinase G. This signaling cascade leads to many downstream effects that facilitate both NO's local biologic activity as a vasodilator and neurotransmitter, as well as its distant impacts as an anti-pyretic.²³ NO can interact with many molecular targets, including protein thiols, heme, nonheme iron, tyrosyl radical proteins, deoxynucleotides, and deoxynucleosides.¹⁴ It can react with glutathione (GSH) and other thiol-containing molecules to form S-nitrosothiols (RSNOs), which function as NO carriers and donors.²⁸ As a free radical, NO can generate potent nitrosylating agents capable of both signaling and cellular damage, such as peroxynitrite (OONO^-) in the presence of superoxide.²³ This effect only occurs at higher concentrations, since as mentioned above, NO is rapidly scavenged in most physiological conditions. RSNOs, S-nitrosylated proteins, nitrosyl-metal complexes, and nitrite may assist in long distance transport of NO. However, the actual NO species, once liberated from these carriers, are short lived.²³

The majority of cutaneous cell types, including adipocytes, endothelial cells, melanocytes, keratinocytes, fibroblasts, Langerhans cells, neutrophils, and macrophages express some isoform of NOS and are therefore able to generate and release NO for a broad array of physiologic processes.^{16,24,26,29} Keratinocytes are the major constituent of the epidermis and express all three NOS isoforms. They produce NO and hydrogen peroxide (H_2O_2) in response to inflammatory stimuli. This likely acts as one of the chief protective mechanisms of the skin, as the epidermis is constantly exposed to foreign matter and organisms. Additionally, NO synthesis on the skin surface may also regulate the growth of cutaneous commensal organisms.²⁴ In the acidic skin environment, reactive nitrogen intermediates are formed, such as nitrous acid (HNO_2), dinitrogen trioxide (H_2NO_3), and peroxynitrite (ONOO^-), which may serve as a nonspecific defense mechanism against cutaneous pathogens.³⁰ In addition, finely regulated responses are also exhibited by NOS species; wound healing is one example. Fibroblasts, found in the dermis, are key regulators of dermal remodeling by synthesizing extracellular matrix, collagen, and fibrin, while orchestrating many of the complex steps of wound healing. Fibroblasts express eNOS, nNOS, and iNOS,²⁴ but this expression is inconsistent across different cells and possibly depends on cell maturation. Due to its widespread distribution, NO can help regulate basic physiological roles such as

establishing and maintaining blood flow, protective responses against invading microorganisms, ultraviolet light-induced melanogenesis, and development of erythema and edema in the setting of a sunburn.²⁴

Antimicrobial Properties and Immune Function

NO has several intrinsic antimicrobial properties and is therefore vital to the body's innate immune response in the defense against invading microbes.^{17,18,24,26,31} One of the main mechanisms is its ability to generate RNOS via spontaneous reactions with oxygen or superoxide. These RNOS include peroxynitrite (OONO^-), RSNOs, nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3^-), and dinitrogen tetroxide (N_2O_4).^{13,19,25} These RNOS are thought to exert NO's antimicrobial effects because they induce nitrosative and oxidative stress that is toxic to microbes.⁸ Peroxynitrite is formed during the oxidative burst in macrophages and is the most highly reactive and potentially cytotoxic of these RNOS.³² RNOS nitrosate protein thiols and modify amino acid residues and thus can inactivate essential enzymes.¹⁵ They can also nitrosylate metal centers (Fe-S), further modifying protein functioning and depleting intracellular iron stores. These events ultimately block essential microbial processes.^{1,21,29,33,34} RNOS also damage microbial DNA, and they do so via a variety of mechanisms, including direct RNOS interaction with DNA, inhibition of DNA repair and replication, and increased synthesis of genotoxic mediators such as alkylating agents and H_2O_2 .^{15,28} OONO^- can also induce DNA strand breaks and abasic sites, among other alterations.^{13,21,28} OONO^- and NO_2 have also been implicated in lipid damage and peroxidation with subsequent disruption of the microbial membrane.^{15,33}

Importantly, NO's ability to execute its antimicrobial properties is dictated by its concentration. At low concentrations, NO exerts its antimicrobial properties by acting as a potent immunostimulatory molecule.³³ In this role, NO mediates immune cell differentiation, proliferation and apoptosis, cytokine production, expression of adhesion and co-stimulatory molecules, and synthesis and deposition of extracellular matrix constituents.³⁵ As NO concentration builds secondary to iNOS activation, its inherent antimicrobial properties come into play.¹⁵ The importance of iNOS activation to combat infection was demonstrated by iNOS knockout mice having greater susceptibility to herpes simplex virus infection, higher frequency of viral reactivation, and delayed viral clearance from dorsal root ganglia as compared

to infected heterozygous mice.³⁶ iNOS knockout mice are also more susceptible to Dengue virus infection, and were found to have significantly higher viral loads and greater mortality compared to wildtype mice.³⁷ Similar results were seen in mice treated with the iNOS inhibitor aminoguanidine: treated mice were more susceptible to *Salmonella typhimurium* infection and death.^{38,39} NO provides less feedback inhibition to iNOS compared to eNOS and nNOS, allowing for a bolus production of high NO levels to thwart a microbial threat.¹⁵

Antimicrobial Spectrum

NO has demonstrated activity against a variety of pathogens,¹⁴ including bacteria, viruses, parasites, and fungi.²⁹

Bacteria

A variety of methods of NO delivery have demonstrated its antibacterial effect. Gaseous NO (gNO) was bactericidal against *S. aureus*, MRSA, *Escherichia coli*, Group B *Streptococcus*, and *P. aeruginosa in vitro*.⁴⁰ *In vitro* studies of acidified nitrite, an NO donor, have demonstrated efficacy against *P. aeruginosa*,⁴¹ *Burkholderia cepacia*,⁴¹ *S. aureus*,^{30,41} and *Propionibacterium acnes*.³⁰ The NO-donor β -galactosyl-pyrrolidinyldiazoniumdiolate (β -Gal-NONOate) was bactericidal against *E. coli*.⁴² S-nitrosothiol NO donors demonstrated activity against *P. aeruginosa*,²⁸ coagulase-negative *Staphylococci*,²⁸ *S. aureus*,²⁸ *Serratia marcescens*,²⁸ *Enterobacter aerogenes*,²⁸ *S. typhimurium*¹³ and *E. coli*.¹³ Finally, iNOS-deficient mice failed to inhibit replication of *Listeria monocytogenes*, and succumbed to *Listeria* inocula that were at least 10-fold lower than those lethal to wildtype mice.⁴³

Viruses

NO has demonstrated antiviral activity via a variety of different NO donor molecules. S-nitrosoacetylpenicillamine (SNAP) and 3-morpholino sydnonimine (SIN-1) inhibited Epstein-Barr virus (EBV) protein synthesis and DNA amplification.⁴⁴ SNAP also inhibited the severe acute respiratory syndrome coronavirus replication cycle in a concentration-dependent manner⁴⁵ and reduced porcine parvovirus DNA, protein synthesis, and replication *in vitro*.⁴⁶ Acidified nitrite cream demonstrated a 75% cure rate in patients treated for molluscum contagiosum.⁴⁷

Parasites

There is evidence that microglia inhibit *Toxoplasma gondii* replication by an effector mechanism

that utilizes NO; this is important in cerebral toxoplasmosis.²⁹ In murine macrophages and mice, *Leishmania* proliferation increased when NO synthesis was inhibited.²⁹ Indeed, survival of *Leishmania* within host macrophages depends on the parasite's ability to inhibit host iNOS expression or activity.⁴⁸ Zeina et al.⁴⁹ successfully treated a male patient with cutaneous leishmaniasis with topical glyceryl trinitrate, an exogenous NO donor. The S-nitrosoglutathione (GSNO), S-nitroso-N-acetyl-L-cysteine (SNAC),⁴⁸ and peroxynitrite⁵⁰ have demonstrated leishmanicidal activity *in vitro*. NO-donors can kill other parasites including *Trypanosoma cruzi*⁵¹ and *Plasmodium falciparum*.⁵²

Fungi

NO impedes the growth of *Cryptococcus neoformans*,^{14,53–55} and when N^G -mono-methyl-L-arginine (L-NMMA, a competitive inhibitor of NO synthesis) was added to activated murine macrophages, the *in vitro* production of NO and cryptostatic activity of the macrophages was suppressed.⁵³ NO donor molecules also demonstrate antifungal activity: DETA-NO inhibited the growth of six *Candida* species¹¹ and the NO liberated from acidified sodium nitrite was effective against *Candida albicans*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*.³⁰ Finally, a gNO-producing probiotic patch was fungicidal to *T. mentagrophytes* and *T. rubrum*.²⁵

NO-RELEASING PLATFORMS

The use of exogenous NO for antimicrobial purposes has predominantly been designed to mimic the action of iNOS, i.e., both are designed to synthesize high quantities of NO for an extended period of time.¹⁵ Ideally, NO-generators or donors would be stable at room temperature for easy storage, released predictably at therapeutic doses, delivered effectively to target sites, and cause minimal toxicity.^{15,18} Several classes of natural and synthetic NO donors exist; they include gNO, organic nitrites and nitrates, acidified nitrites, RSNOs, diazeniumdiolates (NONOates), NO-metal complexes, an NO-releasing probiotic patch, and zeolites. Those that have been evaluated for their antimicrobial efficacy are highlighted below.

Gaseous NO

Ghaffari et al.²⁶ designed a gNO exposure chamber to test the antimicrobial efficacy of gNO on common clinical pathogens. Constant exposure to 80 ppm of gNO inhibited *P. aeruginosa* and *S. aureus* growth, and gNO was bactericidal at 160 ppm.²⁶ gNO

delivered at 200 ppm for 24 h was bactericidal against a variety of clinically relevant pathogens, including *S. aureus*, MRSA, *E. coli*, Group B *Streptococcus*, *P. aeruginosa*, and *C. albicans*.⁴⁰ When gNO was administered intermittently to *S. aureus*, *P. aeruginosa*, and *E. coli* in short durations and at high doses (160 ppm), the same bactericidal effect was demonstrated compared to continuous gNO delivery. However, it took 10 h longer to achieve this effect.⁷ Furthermore, the utility of intermittent gNO treatment may be limited *in vivo* because the time between treatments may permit bacterial replication; this possibility warrants further investigation. The efficacy of continuous gNO treatment was also evaluated *in vivo*: *S. aureus* was inoculated into full-thickness wounds in the New Zealand white rabbit; wounds were then treated with 200 ppm of gNO for 8 h a day for three consecutive days. Treatment caused significant reduction in wound bacterial burden.⁵⁶ Although effective as an antimicrobial, gNO is limited because of its expense, required delivery from a gas tank, length of time required for treatment, requirement for nonambulation during therapy, and potential toxicity to host cells from the production of NO₂ and development of methemoglobinemia.^{7,23,25,27,40} Furthermore, gNO is not the best candidate for topical antimicrobial therapy because its short half-life prevents delivery to deep wounds.⁸

Organic NO Donors: Nitrates and Nitrites

Organic NO donors include nitroglycerin, isosorbide dinitrate, isosorbide 5-mononitrate, and sodium nitroprusside and have long been used to treat cardiovascular disease. There is a paucity of research examining the potential of these NO donors as antimicrobials, although two reports indicate that they have limited antibacterial and biofilm disrupting capabilities.¹⁵ Organic nitrates are limited because of the well-known side effect of tachyphylaxis after continuous and prolonged use, and sodium nitroprusside has the feared side effect of cyanidosis.²³ The availability of alternative NO donors that are more easily administered and cause fewer side effects decreases the likelihood that organic NO donors will be further investigated for antimicrobial efficacy.

Acidified Nitrite

Acidified nitrite creams generate NO via the reaction between an acid and nitrite.¹⁵ In an *in vitro* investigation, the addition of nitrite increased the microbicidal activity of acid solutions containing common cutaneous pathogens, including *S. aureus*,

P. acnes, *C. albicans*, *T. rubrum*, and *T. mentagrophytes*.³⁰ This NO donor is also effective in killing *P. aeruginosa* and *B. cepacia*.⁴¹ Acidified nitrite cream has demonstrated efficacy in human studies of tinea pedis,⁵⁷ tinea versicolor,⁵⁸ molluscum contagiosum⁴⁷ and MRSA.⁵⁹ These creams are advantageous because they are easily applied and because they are effective against several pathogens. However, they are limited because the ingredients must be mixed together immediately prior to use¹⁵ and because they have been associated with skin irritation after application.^{15,18,21}

S-nitrosothiols

RSNO include a variety of NO donors that all possess an NO moiety bound to a thiol (sulfhydryl group)²³; NO is released when this bond is cleaved. Although NO release does not occur spontaneously, it can transpire in physiologic conditions. NO release can be induced by light with a wavelength of 550–600 nm, direct reaction with ascorbate, or copper ion-mediated decomposition.^{15,23} In addition to releasing NO, RSNO can participate in transnitrosylation, the process of transferring NO to another thiol group.¹⁵ This has important implications in the skin, as thiol groups are abundant in the cysteine-rich stratum corneum.¹⁸ Two RSNOs (see Figure 2), S-nitrosoglutathione (GSNO), and S-nitroso-N-acetylcysteine (SNAC) were evaluated for antimicrobial efficacy, and demonstrated effective inhibitory and bactericidal effects against *P. aeruginosa*, coagulase-negative *Staphylococci*, *S. aureus*, *Serratia marcescens*, and *E. aerogenes*. SNAC had greater antimicrobial activity compared to GSNO in all clinical isolates tested.²⁸ GSNO and SNAC are also active against *Leishmania major* and *Leishmania amazonensis*.⁴⁸ Despite demonstrated antimicrobial efficacy, RSNO are limited in their utility to treat SSTI because thiols spontaneously form disulfide bonds in the presence of heat and water, requiring their refrigeration as powder until they are ready for use.¹⁵ Additionally, light, heat and enzymes such as superoxide dismutase and a variety of dehydrogenases can induce premature NO release from the NO-thiol bond.⁴

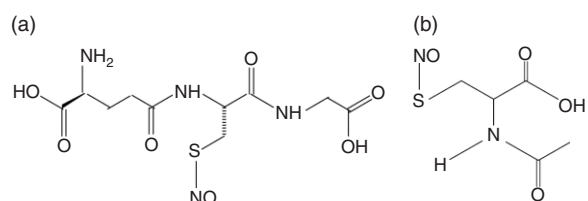


FIGURE 2 | (a) S-nitrosoglutathione (GSNO) structure and (b) S-nitroso-N-acetylcysteine (SNAC) structure. (Reprinted with permission from Ref 60. Copyright 2004 Wiley-Blackwell)

Diazeniumdiolates

These synthetic NO donors are easily produced via a reaction between NO and a variety of different amines. NONOates are stable under ambient conditions but release two molar equivalents of NO spontaneously when exposed to aqueous solution.^{19,23,61,62} Rates of NO release can be controlled by modulating various parameters including pH, temperature, and the structure of the nucleophile to which the NO is complexed.^{19,27} β -Gal-NONOate demonstrated higher bactericidal activity against *E. coli* compared to conventional NONOate.⁴² (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) is a NONOate that inhibited growth of six *Candida* species, and is synergistic when used in concert with azole antifungal drugs.¹¹ NONOates are advantageous because they spontaneously release NO in biological milieus at predictable and dependable rates,^{11,19,63} they are easy to prepare, have an excellent shelf life⁶² and structural diversity.⁶³ Yet the formation of methemoglobin potentially limits their use, as well as the risk of pulmonary and systemic toxicity secondary to the production of NONOate metabolites.²⁷ For example, the N-nitroso byproduct of O(2)-vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO) is a hepatocarcinogen.^{15,63} The availability of other, less toxic NO donors with antimicrobial efficacy minimizes NONOate use for this purpose.¹⁵

NO Probiotic Patch

The probiotic patch is a simple and cost effective method for generating gNO at effective doses. It exploits the metabolic activity of *Lactobacillus fermentum*, a lactic acid-producing bacterium. The lactic acid reacts with nitrite salts present in the gas-permeable patch to produce gNO. The patch was bactericidal against *E. coli*, *S. aureus*, *P. aeruginosa*, and MRSA, and resulted in almost complete death of *A. baumannii*. It was also fungicidal toward *T. mentagrophytes* and *T. rubrum*.²⁵ Patch application to *S. aureus*-infected full-thickness wounds in the New Zealand white rabbit caused significant decrease in wound area but a nonsignificant decrease in wound bacterial burden compared to controls.⁶⁴ A major limitation to this system is the fact that the rate of gNO production depends on the activity of *L. fermentum* in each patch; this introduces variability in peak NO synthesis between patches.¹⁵

Zeolites

These are a new class of NO donors and consist of a framework of metal ions that can bind gNO

and store it until exposure to water.²³ The rate and extent of NO release can be altered by modifying pore size and the metal ions within the lattice.¹⁸ They are advantageous because of their stability, large storage capacity for NO and modifiable rate of NO release.^{4,15,23} Zeolites are effective against MSSA, MRSA, *P. aeruginosa*, and *C. difficile*,⁶⁵ among others.¹⁵ More investigations are necessary to further elucidate zeolites' antimicrobial properties and potential for utility in the treatment of SSTI.⁴

Despite the efficacious antimicrobial activity of these NO donors, many have limitations, including instability on the skin surface,⁶⁰ release of NO in low or inconsistent concentrations,¹⁵ short duration of action,^{8,60} expense,²³ and toxicity.⁴⁰ Nanoparticulate platforms represent a unique way of circumventing some of these limitations.

NANOTECHNOLOGY AND NITRIC OXIDE

Nanotechnology represents a platform from which to deliver drugs to promote wound healing and treat infections, including SSTI. Because of their small size and high surface-to-volume ratio, nps allow for targeted delivery of antimicrobial products.⁶⁶ As previously mentioned, nps can be exploited for antibacterial use in two main ways: some nps have inherent antimicrobial properties, whereas others can serve as vehicles to deliver traditional antibiotics. The efficacy of antimicrobial nps is promising and suggests that the encapsulation of nontraditional antimicrobial agents may be similarly efficacious. The incorporation of NO into nps presents an innovative avenue for the treatment of SSTI.

The nps that either generate or donate NO are advantageous over previously developed NO donor molecules for several important reasons. Firstly, the rate and duration of NO release can be modified by alterations in np size, composition and surface hydrophobicity.¹⁰ Secondly, toxicity can be minimized by varying the ingredients used for np synthesis. Thirdly, np synthesis can incorporate specific functional groups to maximize targeted delivery as well as to enable medical imaging.¹⁰ Finally, nps are advantageous because their small size enables them to surpass biological barriers that impede targeted delivery of drugs in other forms.

Nitric Oxide-releasing Nanoparticles Hybrid NO-releasing Nanoparticles

Friedman et al.¹⁶ developed hybrid hydrogel/glass composite NO-releasing nanoparticles (NO-nps)

through which encapsulated sodium nitrite is thermally reduced to NO within the polymeric nps.¹⁶ This platform is based on established silane-based sol–gels made from either tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS). Sol–gel refers to the transition of a system from a liquid ‘sol’ into a solid gel phase.^{23,67} Sol–gels are capable of trapping proteins and other large molecules, yet they remain porous to smaller molecules like NO, which can limit their drug delivery capabilities. To overcome this limitation and minimize porosity, glass-forming sugars and polysaccharides like chitosan can be added during sol–gel synthesis to essentially plug up these pores.^{23,67} The resulting glassy properties are also of benefit because the matrix promotes the thermal reduction of nitrite to NO, as well as NO retention and sustained release.^{16,23} The final NO-np formulation is stored in a powder form. The NO remains trapped in the matrix when dry, permitting easy storage. Upon exposure to an aqueous environment, NO release is initiated as the nps swell from taking on water.^{4,16,31} The rate and total quantity of NO release can be modified by altering the synthesis steps, such as changing the concentration of nitrite or polyethylene glycol’s (PEG) molecular weight and/or concentration.^{4,15} For example, the utilization of larger PEGs increase pore size, allowing for a rapid bolus-type NO release pattern, whereas NO-nps made with smaller PEGs demonstrated a slower, sustained NO release over time. NO-nps were minimally toxic to treated human lung fibroblasts and reconstituted human epidermis *in vitro*.^{16,68} Human lung fibroblasts treated with NO-nps *in vitro* demonstrated minimal toxicity compared to those cultured with media and control particles. This suggests that these NO-nps are therapeutic agents safe for topical application.¹⁶ Furthermore, no clinical adverse events were reported in murine models of infection treated with NO-nps. The ease of synthesis, storage, administration and control over NO release makes NO-nps attractive for a broad range of clinical scenarios, including the treatment of SSTIs.

NO-releasing Silica Nanoparticles

Shin et al.¹⁹ prepared synthetic NO-releasing silica nps via a sol–gel process. The drug delivery potential of silica is attractive because of its chemical and structural versatility, as well as the fact that it is nontoxic.¹⁹ TEOS or TMOS was combined with aminoalkoxysilane, ethanol or methanol, water and ammonia; the amine functional groups were then converted to NONOates. This technology is advantageous for two reasons: first, because it is capable of storing large quantities of NO. Second, because np size (20–500 nm), half-life (0.1–12 h) and release

kinetics (15–30 h) can be altered by modifications in the synthetic process such as temperature, pH and the type and concentration of ingredients.^{10,19}

NITRIC OXIDE-RELEASING NANOPARTICLES IN THE TREATMENT OF SOFT TISSUE INFECTIONS

In Vitro Data

The hybrid NO-nps developed by Friedman et al. exhibited *in vitro* efficacy against a variety of gram-positive and gram-negative bacteria, including MSSA,⁶⁹ MRSA,⁶⁹ *Streptococcus pyogenes*,¹ *E. faecalis*,¹ *A. baumannii*,⁷⁰ *K. pneumoniae*,¹ *E. coli*¹ and *P. aeruginosa*.¹ This nanoparticle platform was also effective against *C. albicans in vitro*.⁷¹

NO-releasing silica nps demonstrated greater bactericidal efficacy against *P. aeruginosa* when compared to a nonencapsulated small molecule NO donor 1-[2-carboxylato]pyrrolidin-1-yl]diazene-1-ium-1,2-diolate (PROLI/NO).¹⁰ Cytotoxicity studies with mouse fibroblasts confirmed that NO-releasing silica nps are nontoxic to these mammalian cells at concentrations capable of killing *P. aeruginosa*, while PROLI/NO was toxic to host cells at bactericidal concentrations.¹⁰ Smaller NO-releasing silica nps (50 nm) were more effective in killing *P. aeruginosa* compared to larger nps with identical NO release profiles.¹² Importantly, several bacterial species tested (MSSA, MRSA, *S. epidermidis*, *E. coli*, and *P. aeruginosa*) were unable to develop resistance to NO from silica nps after multiple exposures and colony passages.⁸

The silica-based nps effectively killed biofilm-forming pathogens, demonstrating greater than a 99% kill rate of biofilm cells of *P. aeruginosa*, *E. coli*, *S. aureus*, *S. epidermidis*, and *C. albicans*, with the greatest efficacy against *P. aeruginosa* and *E. coli*. Biofilms represent a serious therapeutic impediment given their ability to block drug penetration as well as permit transfer of resistance genes between communal cells.⁷² It is hypothesized that the ease with which NO diffuses across biological membranes may allow for its enhanced penetration into biofilms compared to traditional antibiotics.³² Therefore, an NO-delivering platform may be one avenue to address this challenge.⁷²

In Vivo Models

Excisional Wound Infections

Friedman et al.’s hybrid NO-nps have been investigated in multiple murine infection models. When

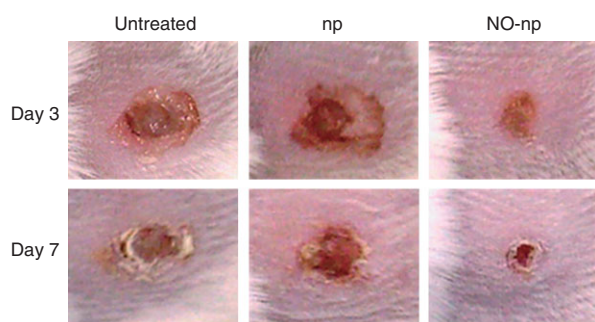


FIGURE 3 | Nitric oxide-releasing nanoparticles (NO-nps) accelerated healing in methicillin-resistant *Staphylococcus aureus* (MRSA)-infected excisional wounds. Wounds were untreated, treated with nanoparticles without NO (np), or treated with NO-np. (Reprinted with permission from Ref 69. Copyright 2009 Nature Publishing Group)

applied to a murine model of MRSA-infected full-thickness wounds, NO-np-treated wounds clinically demonstrated accelerated wound closure (Figure 3) and significantly lower bacterial burden as compared to controls. Histological examination of wounded tissue showed that those infected wounds treated with NO-np had less inflammation, more organized granulation tissue, and less destructive changes to dermal architecture than in controls.⁶⁹

In an analogous study, these NO-nps were applied to a murine model of MDR *A. baumannii*-infected full-thickness excisional wounds. Similar to their effect on MRSA-infected wounds, NO-nps significantly increased the rate of wound healing (Figure 4), even more so than in the MRSA-infected wounds, decreased wound bacterial loads, and inhibited collagen degradation.⁷⁰ *A. baumannii* is an increasingly common etiologic agent of nosocomial infections, and is also implicated wound infections in soldiers deployed in Iraq and Afghanistan. Its resistance to many antibiotics complicates treatment of such infections;⁷⁰ therefore, the success of topical NO-nps in the treatment of *A. baumannii* wound infections is promising.

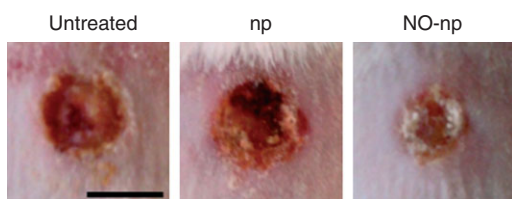


FIGURE 4 | Nitric oxide-releasing nanoparticles (NO-nps) accelerated healing in *Acinetobacter baumannii*-infected excisional wounds. Wounds were untreated, treated with nanoparticles without NO (np), or treated with NO-np, 3 days post-infection. (Reprinted with permission from Ref 70. Copyright 2010 Landes Bioscience)

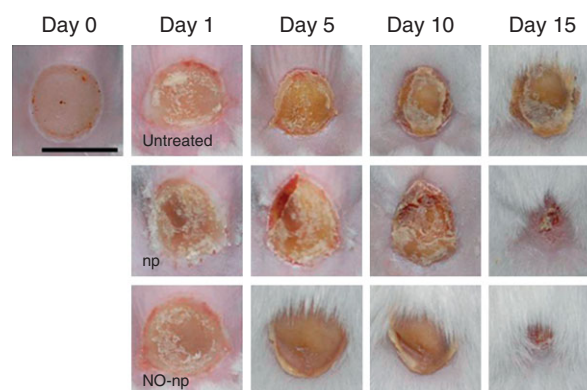


FIGURE 5 | Nitric oxide-releasing nanoparticles (NO-nps) accelerated healing in *Candida albicans*-infected burn wounds. Wounds were untreated, treated with nanoparticles without NO (np), or treated with NO-np. Bar = 5 mm. (Reprinted with permission from Ref 71. Copyright 2012 Frontiers)

Burn Wound Infections

The hybrid NO-nps were found to be effective in treating burn wounds infected with *C. albicans*. Treated wounds healed significantly faster than control wounds (Figure 5) and had significantly lower fungal burden. Histological analysis demonstrated less suppurative inflammation and more fibrin deposition in NO-np-treated groups, with an associated increase in collagen content. Interestingly, mice in the control groups clinically demonstrated fungal transmission from the burn site (on their backs) to their paws as indicated by erythema and white maceration. This finding highlights the importance of quickly and effectively treating these infections to eliminate potential dissemination.⁷¹

Abscesses

MRSA is a common pathogen also associated with deeper bacterial infections, such as intradermal, and intramuscular abscesses. Because of their biofilm-like character and poor perfusion, abscesses are often difficult to treat with conventional antibiotics. In light of this, Friedman et al.'s hybrid NO-nps were evaluated for the treatment of both of these clinical entities in murine model. Both topical and intradermal NO-np application significantly reduced intradermal abscess area (Figure 6) and bacterial burden. Treatment resulted in improved preservation of dermal and subcutaneous architecture, with less inflammation, and bacterial presence on histologic exam.⁶⁸

In a mouse model of MRSA-infected intramuscular abscesses, both topical and intralesional administration of the hybrid NO-nps also significantly decreased MRSA burden within the muscle compared

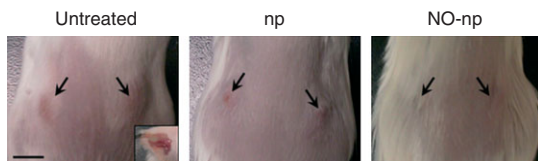


FIGURE 6 | Nitric oxide-releasing nanoparticles (NO-nps) decrease methicillin-resistant *Staphylococcus aureus* (MRSA)-infected intradermal abscess area. Abscesses were untreated, treated with nanoparticles without NO (np), or treated with NO-np, day 4. Arrows denote abscesses; inset demonstrates a representative purulent abscess 4 days after MRSA infection. Bar = 5 mm. (Reprinted with permission from Ref 68. Copyright 2009 Public Library of Science)

to control mice and, in animals treated with systemic vancomycin, a commonly used systemic antibiotic for MRSA SSTIs. NO-np-treated mice demonstrated clinically accelerated abscess clearance based on visual decrease in abscess size and purulence compared to other treatment groups (Figure 7). Histologically, intralesional NO-np administration resulted in less muscle necrosis, granulomatous inflammation, and decreased bacterial load compared to control mice. While vancomycin did have a significant impact on the intramuscular abscesses as compared to untreated, the outcome was not to the extent as those animals treated with the NO-nps.³³

RSNO NANOPARTICLES

RSNOs are NO-donating compounds that are generated from the reaction of NO with a thiol.

S-nitrosoglutathione (GSNO) is an S-nitrosothiol, and functions as an NO donor that can transfer the nitrosonium ion to thiol moieties on proteins in a process called *trans* S-nitrosylation.¹⁵ GSNO's main activity is nitrosation of sulfhydryl-containing cellular proteins. In doing so, GSNO can reversibly block enzyme and protein functioning and disable key pathogen machinery. To counteract the threat to cell viability that results from nitrosation of critical cellular elements, bacteria employ GSNO reductases⁴⁰ and nitroreductases, and also regenerate GSH.³⁴

GSNO serves as a stable reservoir for NO donation and is advantageous compared to NO because S-nitrosothiol half lives are measured in minutes to hours, compared to the seconds-long half-life of free NO.²⁷ Additionally, as described above, GSNO is a potent nitrosating agent, conferring it with antimicrobial activity that threatens microorganism viability. In fact, the antimicrobial efficacy of GSNO in solution against *E. coli* has been previously reported.³⁴ To elucidate GSNO's impact on bacterial growth and survival, Friedman et al. evaluated the ability of the hybrid NO-nps to generate GSNO in the presence of GSH.³⁴ When combined with GSH, NO-np not only formed GSNO, but also produced significant concentrations of GSNO over an extended time period (greater than 24 h). This is likely secondary to the controlled and sustained release of NO from the NO-np, which corresponds to steady GSNO formation. The mixture of NO-np with GSH significantly inhibited the growth and/or survival of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* compared

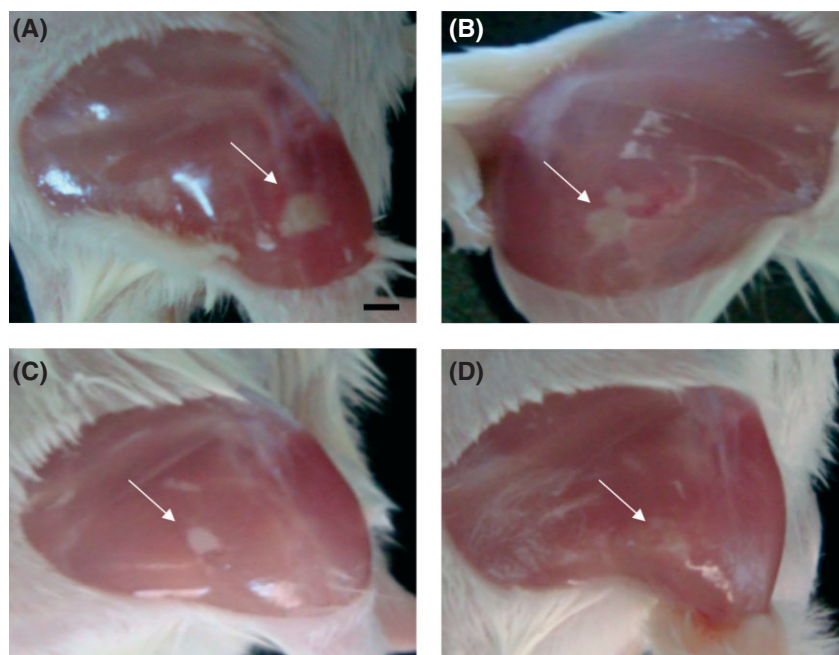


FIGURE 7 | Nitric oxide-releasing nanoparticles (NO-nps) decrease methicillin-resistant *Staphylococcus aureus* (MRSA)-infected intramuscular abscess area. Induced MRSA-infected intramuscular abscesses were clinically evaluated on day 4 after infection. These images, untreated (a), treated with vancomycin (b), treated topically with NO-nps (c), or treated intralesionally with NO-nps (d) are representative of the clinical appearance of these lesions. Arrows denote abscesses. (Reprinted with permission from Ref 33. Copyright 2012 Landes Bioscience)

to controls and NO-np alone. *K. pneumoniae* was the most resistant to this formulation, whereas *P. aeruginosa* was the most susceptible and exhibited no growth over 24 h.³⁴

Given the static and cidal activity of NO-np-generated GSNO *in vitro*, the efficacy of this platform was evaluated in the previously described excisional wound model infected with an MDR clinical isolate of *P. aeruginosa*. Wounds treated with NO-np + GSH exhibited significantly accelerated wound closure clinically and histologically, as well as lower bacterial burden based on tissue cultures when compared to NO-np-treated and control wounds. The finding that NO-np + GSH was more effective than NO-np correlates to the *in vitro* data in that *P. aeruginosa* may be more sensitive to nitrosothiols as opposed to NO. In both the *in vitro* and *in vivo* setting, NO-nps + GSH had greater antimicrobial activity compared to NO-nps alone. This may be because GSNO is a more stable reservoir for NO and because it is a potent nitrosating agent, capable of rendering microbial proteins inactive. Additionally, GSNO can be actively taken up by microbial systems that usually function to import GSH. This enables GSNO to reach intracellular bacterial targets that NO cannot access. These results are promising and further highlight the versatility and applicability of NO-nps to a wide array of clinical scenarios.⁷³

CONCLUSION

The rise of pathogen resistance to our antimicrobial armamentarium and the economic burden of infections due to MDR organisms underscore the need for the development of innovative therapeutics

to circumvent this problem.^{10,68} Nitric oxide is an attractive approach to combating this medical epidemic due to its multiple mechanisms of both static and cidal activity against a broad range of organisms. NO's small size and hydrophobic nature enable it to rapidly traverse bacterial membranes, where it can significantly impact and interfere with cell function. Importantly, it has been shown that multiple bacterial species do not develop resistance to exogenous NO even after multiple exposures and cell passages—it is therefore unlikely that resistance would develop, as it would require multiple mutations to occur simultaneously.⁸ Despite the proven antimicrobial efficacy of a variety of NO donors, many have limitations that preclude their use in clinical settings. Recent advances in NO delivery, particularly the use of nanotechnology, are promising. The ease of nanoparticle production, storage, administration, and modulation render it an attractive therapeutic modality for SSTI. Its design for local application minimizes the risk for systemic toxicity associated with traditional, systemically administered antibiotics. The proven *in vitro* and *in vivo* antimicrobial efficacy provide further evidence that NO-based nanotechnologies have the potential to treat SSTI caused by a variety of pathogens, including those with resistance to traditional antibiotics. Their therapeutic use in combat and/or disaster situations in which specialized medical care or technology is not readily available would be ideal given the breadth of physiologic, and importantly, antimicrobial, activities.¹⁷ NO-nps are an innovative approach and promising solution to the treatment of SSTI in the setting of escalating bacterial resistance.

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