

# Antimicrobial properties of nitric oxide and its application in antimicrobial formulations and medical devices

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**Abstract** This review describes the antimicrobial properties of nitric oxide (NO) and its application as an antimicrobial agent in different formulations and medical devices. We depict the eukaryotic biosynthesis of NO and its physiologic functions as a cell messenger and as an antimicrobial agent of the cell-mediated immune response. We analyze the antimicrobial activity of NO and the eukaryotic protective mechanisms against NO for the purpose of delineating the therapeutic NO dosage range required for an efficacious and safe antimicrobial activity. We also examine the role of NO produced by virulent bacteria in lessening the efficacy of traditional antimicrobials. In addition, we discuss the efficacy

of NO in the healing of infected wounds, describing different NO-producing devices by category, analyzing therapeutic levels, duration of NO production, as well as commercial considerations. Finally, we provide current and future prospects for the design and use of NO-producing devices.

**Keywords** Nitric oxide · Antimicrobial · Bactericidal · Fungicidal · Bacteria · Infection · Wound healing · Patch · Dressing · Device

## Introduction

Since the discovery that endothelium-derived relaxing factor is in fact nitric oxide (NO), it has become evident that this small molecule is a widely distributed and multifunctional cellular messenger (Palmer et al. 1988), that it has a physiologically relevant endogenous metabolism (nitrate–nitrite–nitric oxide pathway; Lundberg et al. 2008), and that it is utilized by the human innate and cellular immune systems as an antimicrobial agent (Anstey et al. 1996; Weller et al. 1996).

Normally, NO is produced enzymatically by nitric oxide synthase (NOS) in the presence of oxygen from the amino acid L-arginine (Palmer et al. 1988). NO is a transitory free radical responsible for the regulation of blood pressure, the control of platelet aggregation (Mollace et al. 1990), and protection against vascular injury caused by tissue deposition of immune complexes (Mulligan et al. 1991) and is used as a broad spectrum antimicrobial agent by both the innate and cell-mediated immune systems (Anstey et al. 1996; Weller et al. 1996). Also, nitrates and nitrites that are consumed in the diet and metabolized by bacteria in the gastrointestinal tract (GIT) are believed to make up bio-inactive pools of NO that

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circulate in blood. These pools help mammals maintain homeostasis of a nitrogen cycle capable of producing NO (Lundberg et al. 2008).

Here, we review the antimicrobial properties of NO and explore its application as an antimicrobial agent in various formulations and medical devices.

### Eukaryotic biosynthesis of nitric oxide

NO is a free radical that is synthesized *in vivo* by a family of NOS that catalyze the oxidation of L-arginine to L-citrulline. There are three nitric oxide synthases produced by eukaryotic cells: (a) endothelial NOS (eNOS), responsible for constitutive NO synthesis in endothelial cells; (b) neuronal NOS (nNOS) responsible for NO synthesis in neural associated cells; and (c) the inducible form, (iNOS), found in epithelial, endothelial, and inflammatory cells, whose expression is upregulated by cytokines, microbes, or bacterial products.

NO is synthesized at different levels based on the pathway and the intended function. At low levels, NO plays a metabolic regulatory role in eukaryotes and as an autocrine, paracrine, and endocrine messenger in a variety of tissues (Lundberg and Weitzberg 2009). In particular, NO initiates a signal transduction pathway in the endothelium, regulating vascular tone and blood pressure, modulating homeostasis and proliferation of vascular smooth muscle cells (Napoli and Ignarro 2009). In leukocytes, NO signaling attenuates inflammatory responses through regulation of cytokine expression. In the GIT, NO functions at more elevated levels as a protective agent against early inflammatory insults and as a protector of a normal, intact mucosal barrier (Alican and Kubes 1996; Lundberg and Weitzberg 2009). Much higher levels are however required to elicit the bactericidal effects on NO. Gaseous (*g*)NO levels are commonly measured in parts per million by volume (ppmV), and 200 ppmV is commonly viewed as the minimal amount required for toxic effects on non-eukaryotic cells (Ghaffari et al. 2007); however, *g*NO at concentrations over 1,000 ppmV can be toxic to eukaryotic cells. In fact, eukaryotic cells have developed protective measures against NO. One of the most notable is metallothionein (MT) production. Metallothionein expression has been shown to be upregulated by eukaryotes in response to oxidative stress among other factors. Metallothionein is a protein thiol induced in cells exposed to cytokines and bacterial products capable of forming iron-dinitrosyl thiolates *in vitro*. Particularly, nucleolus-associated MT may be able to reduce NO-mediated genotoxicity (Schwarz et al. 1995). The overproduction of this protein is one protective mechanism that eukaryotes use against elevated levels of NO.

### Microbial biosynthesis of nitric oxide

Bacterial and eukaryotic NOS are structurally and mechanistically related and catalyze the oxidation of L-arginine to L-citrulline to produce NO. Although bacterial NOS (bNOS) lack an essential reductase domain, bacteria use eukaryotic cellular reductases to generate NO *in vivo* (Gusarov et al. 2009). Gusarov et al. recently found that low levels of NO, produced by bNOS with the help of eukaryotic reductases, may protect bacteria against oxidative stress applied by systemic antibiotics and increase the resistance of bacteria to broad spectrum antibiotics.

While synthesis of bacterial NO is an important factor on the antimicrobial landscape, other approaches to NO production may prove more relevant protecting humans against infection. The reduction of nitrate to nitrite and its subsequent dismutation to NO appears to be an important protective process on skin and within the GIT (Benjamin et al. 1997; Doel et al. 2004; Sobko et al. 2006; Xu and Verstraete 2001). Weller et al. showed that NO is generated on the surface of human skin and proposed that nitrate in sweat is reduced to nitrite by bacterial nitrate reductases in anaerobic niches, with the resulting nitrite converted to NO (Weller et al. 1996). These authors showed that NO synthesis is not inhibited by arginine analogs and NO production is increased by topical acidification of the skin surface. In addition, patients on long-term antibiotics show significantly reduced NO production on skin. These studies concluded that NO generation on skin is dependent on nitrate reduction to nitrite by bacteria and subsequent nitrite reduction by acidification. The same studies proposed a physiologic role of NO in the inhibition of infection by improving cutaneous T-cell function, keratinocyte differentiation, and skin blood flow (Weller et al. 1996).

NO can also be generated in the GIT of mammals from inorganic nitrate and nitrite by the action of commensal bacteria (Sobko et al. 2006). Under anaerobic conditions, bacteria presenting nitrate reductase activity can generate nitrite anions from nitrates present either in saliva or ingested. The produced nitrite results in NO once it reaches the stomach due to the acidic environment. Consequently, NO enters the blood stream through the mesenteric circulation and gets reconverted to nitrate, which can then be eliminated in urine, or gets recycled to the saliva where it is concentrated and becomes available for further NO generation (Lundberg et al. 2008). Lactic acid bacteria can generate high amounts of NO from nitrites due to the acidification of the environment as a result of fermentation (Xu and Verstraete 2001). Production of NO in the GIT from nitrites and nitrates was observed in the presence of probiotics such as *Lactobacilli* and *Bifidobacteria* (Sobko et al. 2005). Finally, ammonia-oxidizing bacteria such as *Nitrosomonas* produce NO from ammonia and hydroxylamine (Kampschreur et al. 2006).

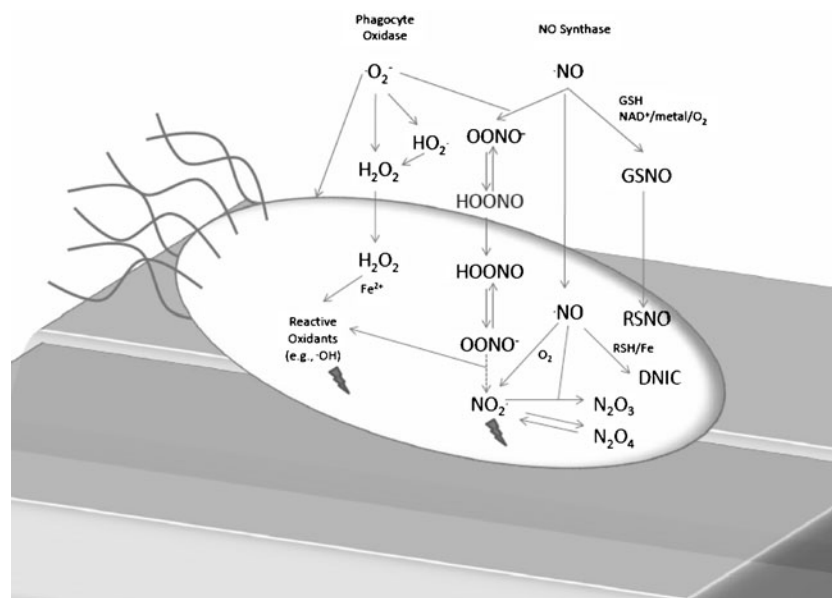
## Antimicrobial applications of nitric oxide—prospects and limitations

The antimicrobial effect of NO has been suggested by diverse observations. First, NO production by inducible NOS has been stimulated by proinflammatory cytokines such as IFN $\gamma$ , TNF- $\alpha$ , IL-1, and IL-2 as well as by a number of microbial products like lipopolysaccharide or lipoteichoic acid (Fang 1997). Second, elevated expression of NO in animal models improved the abilities of host to fight infectious agents and inhibited microbial proliferation, overall improving the host response (Anstey et al. 1996; Evans et al. 1993a, b). Third, in vitro studies demonstrated that inhibition of NO synthases resulted in impaired cytokine-mediated activation of phagocytic cells and reduction of bactericidal and bacteriostatic activity (Adams et al. 1990). Finally, direct administration of NO donor compounds in vitro induced microbial stasis and death. Importantly, NO-dependent antimicrobial activity has been demonstrated in viruses, bacteria, fungi, and parasites (De Groote and Fang 1995; Fang 1997).

One of the plausible mechanisms of antimicrobial activity of NO involves the interaction of this free radical (and a reactive nitrogen intermediate) with reactive oxygen intermediates, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide (O<sub>2</sub><sup>-</sup>) to form a variety of antimicrobial molecular species (Fig. 1). In addition to NO itself, these reactive antimicrobial derivatives include peroxyntirite (OONO<sup>-</sup>), *S*-nitrosothiols

(RSNO), nitrogen dioxide (NO<sub>2</sub>), dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>), and dinitrogen tetroxide (N<sub>2</sub>O<sub>4</sub>). It has been shown that these reactive intermediates target DNA, causing deamination, oxidative damage including abasic sites, strand breaks, and other DNA alterations (Juedes and Wogan 1996). Reactive nitrogen intermediates can also react with proteins through reactive thiols, heme groups, iron–sulfur clusters, phenol or aromatic amino acid residues, or amines (Ischiropoulos and al-Mehdi 1995). Peroxynitrite and NO<sub>2</sub> can oxidize proteins at different sites. Additionally, NO can release iron from metalloenzymes and produce iron depletion. NO-mediated inhibition of metabolic enzymes may constitute an important mechanism of NO-induced cytostasis. Moreover, nitrosylation of free thiol groups may result in inactivation of metabolic enzymes (Fang 1997). Also, the antibacterial effect of NO was shown through a variety of mechanisms such as *S*-nitrosothiol-mediated inhibition of spore outgrowth in *Bacillus cereus* (Morris and Hansen 1981), and several protein targets of nitrogen reactive species have been found in *Salmonella typhimurium* (De Groote and Fang 1995).

In addition to the antibacterial activity of NO, several examples of the antiviral, antifungal, and anti-parasitic effects of NO have been described in the literature. Kawanishi et al. demonstrated the antiviral activity of NO when they showed that NO donors inhibited Epstein–Barr virus late protein synthesis and amplification of DNA preventing viral replication as a result of peroxyntirite



**Fig. 1** Mechanisms of interactions between phagocyte-derived reactive oxygen species and NO-derived species in antimicrobial activity. The schematic shows possible reactions between products originating from phagocyte oxidase and NO synthase. *Fe* iron, *GSH* glutathione, *GSNO* *S*-nitrosoglutathione, *H2O2* hydrogen peroxide, *HOONO* peroxyntirous acid, *NAD* nicotinamide adenine dinucleotide, *NO* nitric oxide, *NO2* nitrogen dioxide, *N2O3* dinitrogen trioxide, *N2O4*

dinitrogen tetroxide, *O2* molecular oxygen, *•OH* hydroxyl, *OONO<sup>-</sup>* peroxyntirite, *RSNO* *S*-nitrosothiol. These oxygen and nitrogen reactive species are the principle cause of bacterial cell death produced by modifying DNA, inactivating metabolic enzymes, or damaging structural proteins among other mechanisms. The figure was modified from Fang (1997)

formation (Kawanishi 1995). Activity against parasites was established with a murine model of leishmaniasis; NO and superoxide produced by macrophages lead to a peroxynitrite-related anti-parasitic effect (Augusto et al. 1996), and the use of a topical NO donor glyceryl trinitrate was successfully used to treat cutaneous leishmaniasis (Zeina et al. 1997). Finally, the antifungal effects are evidenced by recent observations that indicate that murine macrophages exert antifungal activity against *Candida* through peroxynitrite synthesis (Vazquez-Torres et al. 1996).

### Effect of NO on infected wounds

Common sites of infections are wounds. Wound healing is a complicated process relying heavily on the integration of a multitude of control mechanisms, events, and factors. Inflammatory cells, keratinocytes, fibroblasts, and endothelial cells, as well as many enzymes and growth factors, must interact seamlessly for the normal healing process to occur (Blackytny and Jude 2006). Several pathological conditions, including diabetes, venous stasis, and infection are associated with cellular and molecular changes that ultimately disrupt normal wound healing (Blackytny and Jude 2006). One of these changes is the pathological change in the regulation of NO during the wound healing process (Blackytny and Jude 2006). During normal healing, the production of NO radical shows a very distinct time course with initially high concentrations which aid in inhibiting and clearing bacterial infection followed by lower levels of the free radical allowing for the normal wound healing processes to take place. If a wound fails to heal or becomes infected, however, the body maintains high levels of circulating NO and the wound enters a vicious cycle preventing it from healing (Stenzler and Miller 2006).

Infected wounds pose a specific and significant problem as systemic and topical antibiotics have become increasingly less effective against common pathogens. A worldwide increase in drug resistant strains of bacteria since the introduction of antimicrobial agents has documented this well-accepted trend (Anstead et al. 2007; Gorwitz 2008; Nordmann et al. 2007). Several topical antimicrobial agents such as colloidal silver polymyxins or dye compounds have also been used and incorporated into wound dressings; however, these antimicrobial agents often have difficulty reaching the wound bed and thus are not able to break up bacterial biofilms that plague chronic wounds. Due to these deficiencies in common antimicrobial agents, alternative treatments are needed.

It has recently been shown that topical exposure of NO to infected wounds can be beneficial in promoting healing and preparing the wound bed for treatment and recovery (Stenzler and Miller 2006). The application of exogenous gas has been shown to reduce microbial infection, manage

**Table 1** Main NO-producing devices and their capability to maintain NO levels within the therapeutic window and information on therapeutic duration and commercial considerations

Type of device	Therapeutic window	Therapeutic duration	Commercial considerations	Reference
Endogenous production	Production limited by endogenous nitric oxide synthase (NOS) cellular machinery and availability of arginine	Production of endogenous levels limited to synthesis by eukaryotic NOS	Cost: dependent on cost of endogenous NO production stimulating molecule Ease of use: cream application	(Gosselink et al. 2005)
Free gas	Complete control of gNO levels within desired therapeutic window	Maintains levels in therapeutic window for desired therapeutic duration; however, limited by time patient can remain non-ambulatory	Cost: expensive device: tank, regulator, computer control system Ease of use: complicated set up and use of device, patient non-ambulatory during therapy, tethered to device	(Ghaffari et al. 2006, 2007)
Adsorption/release	Production of therapeutic levels dependent on conditions (exudates, temperature, etc.)	High levels of gNO early with relatively short half life	Cost: moderate Ease of use: patch/dressing application	(Bhide 2006; Friedman et al. 2008; Morris et al. 2005; Shabani et al. 1996; Wheatley et al. 2006)
Chemical	Poor control of therapeutic levels with short production dictated by chemical reaction	High levels of gNO early with relatively short half life	Cost: inexpensive materials Ease of use: patch/dressing application	(Gribbe et al. 2008; Hardwick et al. 2001; Li and Lee 2010; Weller et al. 2001)
Cell and enzyme	Excellent control of gNO levels within a therapeutic window dictated by enzymatic reaction	Maintains therapeutic levels of gNO for 24–72 h	Cost: inexpensive materials Ease of use: patch/dressing application	(Jones et al. 2010)

**Table 2** Mechanisms of gNO production by various devices

Type of device	Mechanism	Description of NO production
Endogenous production	$\text{L-Arg} + \text{NADPH} + \text{H}^+ + \text{O}_2 \xrightarrow{\text{NOS}} \text{NOHLA} + \text{NADP}^+ + \text{H}_2\text{O}$ $\text{NOHLA} + \frac{1}{2}\text{NADPH} + \frac{1}{2}\text{H}^+ + \text{O}_2 \rightarrow$ $\text{L-citrulline} + \frac{1}{2}\text{NADP}^+ + \text{NO}(g) + \text{H}_2\text{O}$	NO is synthesized in vivo by a family of NO synthases (NOS) that catalyze the oxidation of L-arginine to L-citrulline
Free gas	$\text{NO}(p) \rightarrow \text{NO}(g)(\text{atm})$	NO is stored under pressure and released over time at controlled levels
Adsorption/release	$\text{Zeolite } x\text{NO} \rightarrow x\text{NO}(g)$	Transition metal-exchanged zeolites adsorb and store NO (up to 1 mmol of NO/g of zeolite); stored NO is released on contact with an aq. environment; the release of the NO can be tuned by altering the chemical composition, amount of water, temperature, and pH
Chemical production	$3\text{NO}_2^- + 3\text{H}^+ \xrightleftharpoons{k_{\text{eq}}} 2\text{NO}(g) + \text{H}_2\text{O} + \text{NO}_3^-$	The acidification of nitrite salts produces nitric oxide gas at a rate dependent on availability of nitrite, protons, water, temperature, and pH
Chemical release	Chitosan – based NONOate + H <sub>2</sub> O → 2NO (g)	Chitosan-based diazenium diolates (NONOates) are synthesized by reacting NO with modified chitosan polymers and release NO in aq. media; the rate at which NO is released is dependent on amount of water, temperature, and pH
Chemical release	$2\text{RSNO} \rightarrow 2\text{NO}(g) + \text{RSSR}$	Interpolymer complexes with nitrosothiols derived from glutathione are decomposed in the presence of light, metal, or heat. The rate of NO release is dependent on the wound temperature
Cell and enzyme	$\text{Glucose} \xrightarrow{\text{L.fermentum}} \text{lactic acid}$ $3\text{NO}_2^- + 3\text{H}^+ \xrightleftharpoons{k_{\text{eq}}} 2\text{NO}(g) + \text{H}_2\text{O} + \text{NO}_3^-$	Pobiotic bacteria and enzymatic systems can be used to produce organic acids which will dismutate nitrite salts releasing NO over time depending on temperature and pH

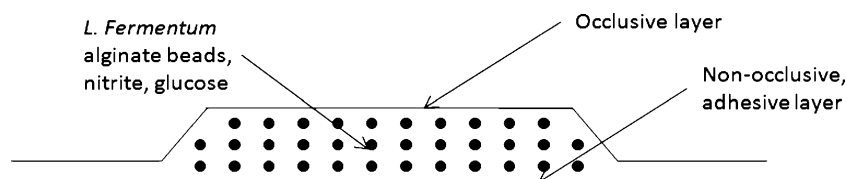
exudates and secretions by reducing inflammation, and regulate the formation of collagen (Stenzler and Miller 2006). Furthermore, regimens have been proposed for the treatment of chronic wounds with gNO which specify high and low treatment periods to first reduce the microbial burden and inflammation and increase collagenase expression to debride necrotic tissue and then restore the balance of NO and induce collagen expression aiding in the wound closure respectively (Stenzler and Miller 2006).

### Design of NO-producing devices

Devices for production and delivery of topical NO have been constructed in several arrangements. A considerable

challenge in the delivery of NO is that NO rapidly oxidizes in the presence of oxygen (O<sub>2</sub>) to form NO<sub>2</sub>, which is highly toxic, even at low levels. A device for the delivery of NO must be anoxic, preventing NO from oxidizing to toxic NO<sub>2</sub> and preventing the reduction of NO which is required for the desired therapeutic effect (Stenzler and Miller 2006). Thus, since NO will react with O<sub>2</sub> to convert to NO<sub>2</sub>, it is desirable to have minimal contact between the gNO and the outside environment.

Initial designs utilized observations regarding the acidification of nitrite in sweat on skin and reproduced the gNO generating mechanism by acidifying nitrite salts with agents such as ascorbic acid (Gribbe et al. 2008; Hardwick et al. 2001; Li and Lee 2010; Weller et al. 2001). Other attempts have been made to increase endogenous production of NO



**Fig. 2** Antimicrobial gNO-producing probiotic patch containing lyophilized *L. fermentum* alginate microbeads and a solution containing glucose, NaCl, and sodium nitrite, in a heat-sealed pocket made

between a non-occlusive layer, in contact with the skin, and an occlusive layer of polyethylene (Jones et al. 2010)



by stimulating eukaryotic synthesis of NO from arginine (Gosselink et al. 2005). Also, a number of groups have tried adsorbing the free gas under pressure to various polymeric compounds or trapping NO under pressure in silica metal chambers until the time of delivery (Bhide 2006; Hetrick et al. 2008; Morris et al. 2005; Shabani et al. 1996; Wheatley et al. 2006). Another group used a combination of chemical generation of gNO from nitrite using electrons from glucose with an adsorption method so that in situ produced NO is incorporated to a chitosan-tetramethylorthosilicate matrix which is lyophilized to form nanoparticles. In this case, the NO is released when the matrix becomes in contact with an aqueous phase (Friedman et al. 2008). Still other groups have tried to control the delivery of free gas using a tank of compressed NO, controller, regulator, delivering the gas through a tube to an adhesive patch (Ghaffari et al. 2006; Ghaffari et al. 2007). Recently a novel gNO-producing device, using enzymatic and cellular systems (Jones et al. 2010), has shown promise due to design simplicity and therapeutic efficacy; however, its clinical potential in humans is yet to be investigated. A review of these devices is provided in Table 1 which outlines the categories of NO-producing devices, presents information regarding the capability of a device to maintain NO levels within the therapeutic window and therapeutic duration, and discusses commercial considerations. Table 2 presents the mechanisms for categories of NO-producing devices and presents examples of NO generating mechanisms for each. Finally, antimicrobial gNO-producing probiotic patches have been developed which produce >200 ppmV gNO for more than 24 h at RT (Jones et al. 2010). These patches contain lyophilized *Lactobacillus fermentum* alginate microbeads and a solution containing glucose, NaCl, and sodium nitrite, in a heat-sealed pocket made between a non-occlusive layer, in contact with the skin, and an occlusive layer of polyethylene (Fig. 2).

### Current status, future trends, and prospects

It is becoming increasingly clear that NO plays an important role in human-specific and nonspecific immunity and that it is a particularly good broad spectrum antimicrobial agent. Evidence for endogenous NO production on skin, within the gut, and by the cellular immune system to protect and fight disease is growing. Furthermore, the importance of systemic nitrate, nitrite, and nitrosylated compounds in blood is becoming better understood and the implications in fighting infectious disease more apparent. Stimulation of endogenous NO or application of exogenous NO to infected human tissue appears to be an effective method for treating microbial infections; however, considerable hurdles with design of a commercially viable device

exist. Current design trends aim to achieve therapeutic antimicrobial levels below what is considered toxic to eukaryotic cells. One of the largest hurdles in bringing antimicrobial NO-producing products to the clinic is the consistency of gNO release at therapeutic levels. The majority of adsorption release or chemical release technologies require activation by moisture, heat, light, or other factors that are difficult to control in a clinical setting. These challenges are compounded by the fact that most devices release high levels initially and weaning levels over time making it difficult to ensure bactericidal levels of gNO are sustained for sufficient duration. To date, the only consistent delivery of gNO is through direct application of gNO from a tank delivered via tube and topical applicator. Unfortunately, this renders patients non-ambulatory and bears significant cost. These hurdles can be overcome with novel gNO activation and delivery systems. Emerging technologies based on controlled release or reaction systems, such as enzymatic systems, may provide sustained consistent therapeutic release over time, while utilizing inexpensive mass producible constituents. In addition, systems should be carefully designed with stable and safe components to ensure extended shelf life and stability. Such devices may prove commercially viable in treating a wide variety of bacterial, fungal, parasitic, and viral infections and in healing infected wounds.

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