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Morphological analysis of the antimicrobial action of nitric oxide on Gram-negative pathogens using atomic force microscopy

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Abstract

Atomic force microscopy (AFM) was used to study the morphological changes of two Gram-negative pathogens, *Pseudomonas aeruginosa* and *Escherichia coli*, after exposure to nitric oxide (NO). The time-dependent effects of NO released from a xerogel coating and the concentration-dependent effects rendered by a small molecule that releases NO in a bolus were examined and compared. Bacteria exhibited irregular and degraded exteriors. With NO-releasing surfaces, an increase in surface debris and disorganized adhesion patterns were observed compared to controls. Analysis of cell surface topography revealed that increasing membrane roughness correlated with higher doses of NO. At a lower total dose, NO delivered via a bolus resulted in greater membrane roughness than NO released from a surface via a sustained flux. At sub-inhibitory levels, treatment with amoxicillin, an antibiotic known to compromise the integrity of the cell wall, led to morphologies resembling those resulting from NO treatment. Our observations indicate that cell envelope deterioration is a visible consequence of NO-exposure for both Gram-negative species studied.
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Keywords: Antimicrobial; Atomic force microscopy; Gram-negative; Morphology; Nitric oxide

1. Introduction

Nitric oxide (NO) is a highly reactive diatomic radical endogenously produced by the enzyme-catalyzed oxidation of L-arginine to L-citrulline. It has been implicated as a mediator in multiple physiological processes, ranging from regulatory roles in the cardiovascular and nervous system to the inducible host response to infection [1,2]. Various therapeutic properties attributed to NO, including tumor cytotoxicity [3,4], antimicrobial activity, and improved wound healing and tissue integration at implant sites [5], may prove beneficial in a number of pharmacological applications [6,7]. Due to its reactivity, diverse regulatory roles and short half-life in blood (<1 s) [8], the ability to target therapeutic NO delivery locally is critical. Nitric oxide donating compounds, such as *N*-diazeniumdiolates [6] and *S*-nitrosothiols [9,10], decompose to release NO and hence serve as vehicles for its storage and transport. A number of materials, including nanoparticles [11,12] films and coatings [13,14] and small molecules [15,16], have employed NO-donor chemistry with varied physicochemical and NO-release properties.

The role of NO in the innate immune response is a conserved feature through a wide range of species, from *Drosophila* to human [17], In mammals, macrophages and other immune cells produce NO in response to invading pathogens [18]. The antimicrobial properties of NO may be elicited by direct modification of biomacromolecules or by formation of reactive nitrogen species (RNS) via reaction with oxygen (O_2) or superoxide (O_2^-) [19]. These RNS may render nitrosative stress by the formation of compounds such as dinitrogen trioxide (N_2O_3) and oxidative stress via the formation of peroxynitrite ($ONOO^-$) [19–22]. The spectrum of potential bactericidal mechanisms is thus broad, encompassing DNA damage resulting from deamination of deoxyribonucleotides, protein damage via numerous potential reactive sites (e.g. heme groups, thiols,

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amines) that disrupts normal cellular transport and metabolism, and membrane damage propagated by radical lipid peroxidation. The local physiological environment plays a key role in determining the metabolic pathways available to NO, and it would thus be expected that the bactericidal mechanism(s) of NO produced endogenously in phagosomal compartments would differ from NO released extracellularly (e.g. from an implanted biomaterial) as a result of differences in local conditions and substrates available in the biological milieu.

In vitro, NO has proven a potent antimicrobial agent effective against a range of microorganisms, including both Gram-negative and Gram-positive bacteria. Gaseous NO was found to be toxic against a number of pathogenic species, including *Candida albicans* and methacillin-resistant *Staphylococcus aureus* [23]. *N*-Diazeniumdiolate-modified NO-releasing surfaces have been shown to reduce initial *Pseudomonas aeruginosa* adhesion relative to controls [24–26] and kill those that do adhere [27]. Nitric oxide release from silica nanoparticles has been characterized by significant toxicity to bacterial cells with reduced toxicity to L929 mouse fibroblasts [28]. While the bactericidal effects of NO and NO-releasing biomaterials have been demonstrated repeatedly, details on the primary targets resulting in bacterial cytotoxicity and the corresponding cellular effects of NO on microbial species remain speculative.

Morphological analyses of bacteria aid in understanding mechanisms of antibiotic action by allowing visualization of changes in the appearance of the microbe undergone subsequent to treatment. While electron microscopy has been employed toward this end for decades [29–31], atomic force microscopy (AFM) has been used with increasing frequency [32–37]. As a surface characterization tool, AFM is ideal for morphological studies of surface-adhered bacteria as it allows cells to be imaged in situ with high resolution without requiring chemical drying, metal coating or exposure to ultrahigh vacuum. An added benefit of AFM is the flexible and adaptable nature of cantilevers as transducers that allow detection of other physical (e.g. elasticity) or chemical (e.g. charge distribution) surface parameters simultaneously with the acquisition of height information. Atomic force microscopy has been applied to visualizing the antimicrobial action of peptides [32–34], chitosan [35], quantum dots [36] and the β-lactam antibiotics penicillin and amoxicillin [37].

Herein, we report a morphological analysis of *P. aeruginosa* and *Escherichia coli* after exposure to NO released from two *N*-diazeniumdiolate-modified materials: a small molecule NO-donor derived from proline (PROLI/NO) and an NO donor-modified xerogel surface coating. The diazeniumdiolate moiety stores two molecules of the antimicrobial agent NO on each functionalized amine. Exposure to proton sources such as buffer and blood catalyzes the release of NO. Using topographical surface mapping and nanometer-scale height resolution, changes in bacteria shape and surface roughness were studied as a function of exposure time, material, and quantity of NO released.

2. Materials and methods

2.1. Materials

Ethanol and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Argon, NO, nitrogen (N_2) and a 25.7 ppm gaseous NO standard in N_2 were purchased from National Welders (Raleigh, NC). *N*-(6-Aminohexyl) aminopropyltrimethoxysilane (AHAP3) was obtained from Gelest (Tullytown, PA). Amoxicillin was obtained from Fluka (Buchs, Switzerland). Isobutyltrimethoxysilane (BTMOS), L-proline, sodium methoxide and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). The amino- and alkoxysilanes were stored over desiccant. The above chemicals were used without further purification. Distilled water was purified with a Millipore Milli-Q UV Gradient A-10 system (Bedford, MA) to a resistivity of 18.2 MΩ cm.

2.2. Cell culture

Pseudomonas aeruginosa (ATCC #19143) and *E. coli* (ATCC #53323) were obtained from American Type Culture Collection (Manassas, VA) and cultured in tryptic soy broth (TSB). Stock cultures were prepared and stored at −80 °C for subsequent experiments. A 1 ml aliquot from an overnight culture was inoculated in ~100 ml of TSB and incubated at 37 °C for 3–5 h until the culture reached mid-exponential log phase as determined from optical density at 600 nm ($OD_{600} = 0.2 \pm 0.1$), corresponding to $\sim 10^8$ colony forming units (cfu) ml^{-1}.

2.3. Synthesis of xerogel films

Glass slides were coated with a 40 vol.% (of total silane content) AHAP3/BTMOS xerogel film via a two-step process as described by Marxer et al. [13]. Briefly, 120 μl BTMOS was mixed with 60 μl of water, 200 μl of ethanol and 10 μl of 0.5 M HCl for 1 h. Then 80 μl of AHAP3 was added, and the solution was mixed for an additional hour. Glass slides were cut into sections (13 × 17.5 mm), rinsed with ultrapure water and ethanol, dried under a stream of nitrogen and cleaned for 30 min in a ultraviolet (UV)–ozone cleaner (BioForce, Ames, IA). To cast a film, 40 μl of the sol was pipetted onto clean glass slides, dried for 30 min at ambient temperature and cured at 85 °C for 3 days. Control xerogel films were stored in desiccators at 22 °C.

2.4. NO-donor synthesis and characterization

Xerogels were modified to release NO by exposing the films to 5 atm of NO for 72 h as previously described [13]. The NO chamber was flushed twice with 5 atm of

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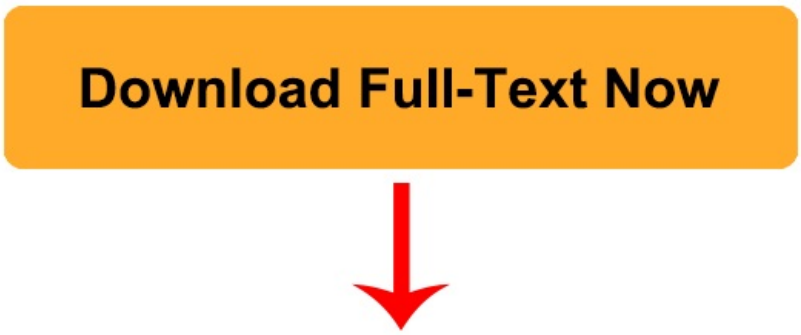

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