

Reduced bacterial adhesion to fibrinogen-coated substrates via nitric oxide release

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ARTICLE INFO

Article history:

Received 8 May 2008

Accepted 2 July 2008

Available online 26 July 2008

Keywords:

Polymeric nitric oxide release

Bacterial adhesion

Fibrinogen

Antimicrobial

ABSTRACT

The ability of nitric oxide (NO)-releasing xerogels to reduce fibrinogen-mediated adhesion of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli* is described. A negative correlation was observed between NO surface flux and bacterial adhesion for each species tested. For *S. aureus* and *E. coli*, reduced adhesion correlated directly with NO flux from 0 to 30 pmol cm⁻² s⁻¹. A similar dependence for *S. epidermidis* was evident from 18 to 30 pmol cm⁻² s⁻¹. At a NO flux of 30 pmol cm⁻² s⁻¹, surface coverage of *S. aureus*, *S. epidermidis*, and *E. coli* was reduced by 96, 48, and 88%, respectively, compared to non-NO-releasing controls. Polymeric NO release was thus demonstrated to be an effective approach for significantly reducing fibrinogen-mediated adhesion of both gram-positive and gram-negative bacteria in vitro, thereby illustrating the advantage of active NO release as a strategy for inhibiting bacterial adhesion in the presence of pre-adsorbed protein.

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1. Introduction

From orthopedic prostheses to ventricular shunts, modern medical procedures increasingly rely on indwelling devices, many of which reside within the body for extended periods. Unfortunately, infections associated with indwelling medical devices remain a significant clinical challenge [1]. For example, the number of catheter-related bloodstream infections yearly in the U.S. has been estimated at >500,000 [1]. Such infections have a mortality rate of up to 25%; treatment costs alone approach roughly \$28,000 per case [2,3]. Altogether, infections associated with indwelling devices account for nearly half of the two million nosocomial infections that occur in the U.S. each year [4].

Infection of indwelling devices initiated by bacteria occurs in three phases [5]. Immediately after insertion, plasma proteins rapidly coat the surface of the device. The initial interaction between bacterial cells and the adsorbed protein layer is non-specific through a combination of van der Waals, gravitational, and Coulombic forces [6]. During Phase II, bacterial membrane proteins and polysaccharides specifically bind to the proteins on the device surface. Certain bacterial species subsequently secrete a protective exopolysaccharide layer (i.e., form a biofilm) in Phase III that imparts increased antibiotic resistance to the constituent bacterial cells [7]. Biofilm-associated bacterial infections are exceedingly

difficult to treat with conventional antibiotic therapies. Thus, the most promising anti-infective strategies seek to inhibit bacterial adhesion prior to biofilm formation. Reducing bacterial adhesion during the initial 6 h period following implantation is particularly important for avoiding device-associated infection [8]. Indeed, the risk of infection is significantly decreased if the implant is properly integrated into the surrounding tissue prior to colonization of the surface by bacteria.

To combat implant-associated infection, much research has focused on device coatings that reduce initial bacterial adhesion [9]. Passive device coatings have been developed that possess physicochemical properties such that bacteria–substrate interactions become less favorable [9]. While such coatings have shown promise at reducing bacterial adhesion in vitro, their efficacy in vivo has been hindered by the adsorption of plasma proteins that mask the physicochemical properties of the underlying substrate. In contrast, coatings that actively release antimicrobial agents have been shown to reduce bacterial adhesion even in the presence of a surface-adsorbed protein layer [10]. Examples of active release coatings include polymers that release antibodies that block adhesins on the bacterial cell surface [11], silver ions [12], and conventional antibiotics [13]. Unfortunately, the highly selective nature of antibodies and increasing microbial resistance to silver ion [14] and antibiotics [15] warrant research into more effective active release strategies to prevent bacterial adhesion and associated infection.

Polymeric nitric oxide (NO) release has recently been proposed as a strategy to prevent bacterial adhesion and device-associated infection [9]. Relative to traditional antibiotics (e.g., gentamicin,

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cephalothin, etc.), NO has a short half-life in both blood and tissue (on the order of seconds) [16], allowing localized action without systemic effects common to some conventional antibacterial agents [17]. Nitric oxide is an appealing means of inhibiting bacterial adhesion because it is capable of functionally modifying key bacterial cell membrane adhesion proteins that mediate cell–substrate interactions [18]. In addition, NO has been observed to induce the dispersal of bacteria in biofilms, suggesting an antagonistic role in biofilm development [19]. The primary mechanisms by which NO exhibits bactericidal activity include membrane destruction via lipid peroxidation by NO-derived peroxynitrite (ONOO^-) [20], irreparable DNA damage [21], and the inhibition of critical metalloproteins found in bacterial respiratory reactions [22]. Whereas the effectiveness of traditional antibiotics is often undermined by the evolution of resistant bacteria, the more general nature of NO-induced cell death may limit similar development of bacterial resistance to NO [23].

Xerogel polymers modified to release NO have emerged as a class of materials capable of inhibiting bacterial adhesion [24]. While several studies have demonstrated the ability of xerogel-derived NO to reduce bacterial adhesion in vitro [25–28] and limit implant-associated infection in vivo [29], the ability of polymeric NO release to prevent bacterial adhesion in the presence of an adhesion-promoting protein layer has not yet been investigated. Herein, we examine the efficacy of NO release as a strategy to reduce the protein-mediated adhesion of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli*, bacterial species that are collectively responsible for a significant portion of medical device-related infections and whose adhesion to biomaterial substrates is mediated by fibrinogen (Fg) and other plasma proteins [30–33]. By examining the extent of adhesion of *S. aureus*, *S. epidermidis*, and *E. coli* to Fg-coated NO-releasing xerogels, the efficacy of NO release as a strategy to reduce protein-mediated bacterial adhesion is explored.

2. Materials and methods

2.1. Reagents

Water was purified to a resistivity of 18.2 M Ω cm and total organic content of <5 ppb using a Millipore Milli-Q Gradient A-10 water purification system (Bedford, MA). Isobutyltrimethoxysilane (BTMOS) was purchased from Aldrich (St. Louis, MO). *N*-(6-aminohexyl)-aminopropyltrimethoxysilane (AHAP3) was purchased from Gelest (Morrisville, PA). Silanes were stored under nitrogen and used as received. Argon (Ar), nitrogen (N_2), and nitric oxide (NO) gases were purchased from National Welders (Raleigh, NC). Low molecular weight poly(vinyl chloride) (PVC) and tetrahydrofuran (THF) were purchased from Aldrich. *S. aureus* (ATCC #29213), *S. epidermidis* (ATCC #12228), and *E. coli* (ATCC #11775) were purchased from American Type Culture Collection. Human fibrinogen (plasminogen depleted; Lot FIB 12800; 100% clottable) was purchased from Enzyme Research Laboratories (South Bend, IN) at a concentration of 45.76 mg/mL in 20 mM sodium citrate–HCl buffer (pH 7.4), and stored at -80°C . Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Tetrahydrofuran (THF). All other chemicals were purchased from Fisher Scientific (St. Louis, MO) and used as received.

2.2. AHAP3 xerogel synthesis

Xerogels were prepared as described previously [28] by mixing 800 μL of a volume percent mixture of BTMOS and AHAP3 with 800 μL of ethanol (EtOH), 240 μL of H_2O , and 40 μL of 0.5 M hydrochloric acid (HCl). Synthesis of the xerogel polymers was carried out in two steps. First, EtOH, H_2O , HCl, and BTMOS were mixed for 1 h. AHAP3 was then added to this solution, followed by additional mixing for 1 h. Glass microscope slides were cut into 25 mm \times 8 mm sections, sonicated in EtOH for 20 min, dried with N_2 gas, and ozone-cleaned for 30 min in a BioForce UV-cleaner (Ames, IA) before use. A 35- μL aliquot of the sol was cast onto each glass slide. The films were allowed to solidify (i.e., gel) in a fume hood for 15 min, and then dried in an 80 $^\circ\text{C}$ oven for 72 h. The resulting xerogels were stored in a desiccator at ambient temperature prior to further study.

2.3. Nitric oxide donor formation

Diazoniumdiolate-modified xerogels were synthesized by placing the xerogel-coated glass slides into a 500-mL in-house NO reaction vessel [24]. Before exposure

to NO, the reaction chamber was flushed with Ar (5 atm) to remove O_2 . The reactor was then pressurized to 5 atm NO for 72 h. After 72 h, NO was removed from the reaction chamber via copious flushing with Ar. The NO donor-modified xerogels were stored at -20°C under N_2 until use to limit both thermal- and water vapor-initiated diazeniumdiolate decomposition [24]. Control xerogels not modified with diazeniumdiolate NO-donors were stored in a desiccator at ambient temperature.

2.4. Poly(vinyl chloride) coating

Low molecular weight poly(vinyl chloride) (1.0 g) was dissolved in THF (10 mL). Precisely 300 μL of the PVC/THF solution was spin-coated onto control and NO-releasing xerogels using a CHEMAT Technology KW-4A Precision Spin-Coater (Northridge, CA) set to spin at 3.0 krpm. The PVC-coated xerogels were immediately dried with N_2 and returned to -20°C until further study. The approximate thickness of the PVC overlayer was measured using a Zeiss Axiovert 200 Inverted Optical Microscope (Chester, VA).

2.5. Characterization of nitric oxide release

A chemiluminescent nitric oxide analyzer (NOA; Sievers Model 280; Boulder, CO) was used to quantify NO release from the PVC-coated diazeniumdiolate-modified xerogels. The xerogel-coated glass substrates were placed in a vessel containing deoxygenated phosphate-buffered saline (PBS, pH 7.4) maintained at 37 $^\circ\text{C}$ using a heated water bath. The PBS vessel was purged continuously with N_2 gas. The NOA was calibrated with an atmospheric sample passed through a NO zero filter (0 ppm) and a NO gas standard (24.1 ppm; balance N_2).

2.6. Bacterial adhesion studies

Bacteria were cultured in tryptic soy broth (TSB) at 37 $^\circ\text{C}$, pelleted by centrifugation, rinsed with H_2O , resuspended in a solution of PBS with 15% (v:v) glycerol, and stored at -80°C . From the -80°C stock, secondary cultures were grown in TSB for ~ 12 h. A 1 mL portion of the mature colony was inoculated into 200 mL of TSB and grown to approximately 10^8 colony-forming-units (CFU) per mL. Bacterial cell concentrations were measured by optical density ($\text{OD}_{\lambda=600\text{nm}} \approx 0.2$ for *S. aureus* and *S. epidermidis*; $\text{OD}_{\lambda=600\text{nm}} \approx 0.5$ for *E. coli*). The resulting culture was collected by centrifugation and resuspended in PBS. Serial dilutions and plating of the resuspended bacteria on tryptic soy agar verified that the concentration of viable cells was consistent between experiments.

The PVC-coated NO-releasing and control xerogels were soaked in PBS for 90 min at 37 $^\circ\text{C}$ to initiate a steady level of NO release. The xerogels were immediately incubated with gentle shaking in 5 mL of a 20 $\mu\text{g}/\text{mL}$ solution of either human Fg or BSA in PBS (pH 7.4) for 90 min at 37 $^\circ\text{C}$. After removal from the protein solution, the xerogels were immersed in 5 mL of a bacterial suspension at 37 $^\circ\text{C}$ for 90 min with gentle shaking.

2.7. Phase contrast microscopy

Following incubation in the bacterial suspension, the xerogels were removed, rinsed by gently immersing the slides in H_2O to remove loosely adhered bacteria, and then dried with a stream of N_2 . Adhered bacteria were imaged via phase contrast optical microscopy using a Zeiss Axiovert 200 Inverted Microscope equipped with a Zeiss AxioCam. For each xerogel composition (i.e., NO flux), three different xerogel-coated slides with PVC overlayers were imaged. Five randomly selected regions ($215 \times 270 \mu\text{m}^2$) on each slide were used to calculate an average bacterial surface coverage. The images were digitally processed by thresholding to make white cells contrast sharply with the dark background. Percent surface coverage was translated from the number of white versus black pixels in the thresholded image and normalized relative to controls (scored as 1.0).

2.8. Water contact angle measurements

Static water contact angle measurements of both untreated and protein-treated (20 $\mu\text{g}/\text{mL}$ Fg or BSA in PBS, pH 7.4 for 90 min) PVC-coated control xerogels were acquired with a KSV Instruments Cam 200 Optical Contact Angle Meter (Helsinki, Finland).

2.9. Fibrinogen surface coverage measurements

To determine the relative amount of Fg adhered to both control and NO-releasing substrates, Fg was fluorescently labeled [34] using an Alexa Fluor 488 Protein Labeling Kit (Invitrogen; Eugene, OR) as per the manufacturer's instructions [35]. A volume of 500 μL of a protein solution prepared by mixing 10% fluorescently tagged Fg and 90% unlabeled Fg (20 $\mu\text{g}/\text{mL}$ total Fg concentration) was deposited on both control and NO-releasing PVC-coated 40% AHAP3/BTMOS xerogels ($n = 3$ of each) that had been previously immersed in PBS for 90 min at 37 $^\circ\text{C}$. After 90 min at 37 $^\circ\text{C}$, the substrates were removed from the Fg solution and gently rinsed with PBS to remove loosely adhered protein. Adsorbed Fg was then eluted by immersing the slides in a 5% solution of sodium dodecyl sulfate (SDS) [36] in PBS at 37 $^\circ\text{C}$ for 90 min. The fluorescence intensity of the SDS/PBS solution was monitored with

a Varian/Cary Eclipse Spectrofluorometer (Palo Alto, CA). An excitation wavelength of 495 nm was employed, and fluorescence emission was monitored from 515 to 525 nm [35].

2.10. Statistical analysis

All data are expressed as mean values \pm one standard deviation and were analyzed for significance ($p < 0.05$) with a two-tailed Student's *t*-test.

3. Results and discussion

3.1. Poly(vinyl chloride)-coated xerogel characteristics

A spin-coated PVC overlayer was used to ensure surface uniformity of xerogel samples composed of different proportions of aminosilane (i.e., AHAP3) and alkoxy silane backbone (i.e., BTMOS). As such, differences in bacterial adhesion may be attributed solely to NO release and not other potentially significant changes in substrate surface properties. Our lab has previously reported the benefits of a polymer overcoat for enhancing the stability of amino-siliconalkoxide-modified xerogels upon immersion in aqueous solution [28]. For the xerogel substrates described herein, neither delamination nor degradation of either the PVC or xerogel film was observed. As measured by optical microscopy, the average thickness of the PVC layer was $15 \pm 4 \mu\text{m}$.

3.2. Nitric oxide release

Xerogels enable careful study of the effects of NO release on bacterial adhesion because the NO flux from such materials is readily tunable by altering the identity and/or concentration of the aminosilane precursor [24]. The AHAP3/BTMOS xerogel composition was selected based on the superior solution stability of the ensuing xerogel film compared to other combinations of amino and alkoxy silanes [24]. By tuning the amount of AHAP3 relative to BTMOS during xerogel synthesis, this system also provides a range of constant NO fluxes ($10\text{--}30 \text{ pmol cm}^{-2} \text{ s}^{-1}$) over which to study bacterial adhesion.

To prepare substrates with a range of NO surface fluxes, xerogels composed of 10–40% AHAP3 were synthesized, modified to diazeniumdiolate NO donor form, and coated with a PVC overlayer. As shown in Fig. 1, increasing the proportion of AHAP3 resulted in a greater NO surface flux, thus enabling the investigation of the relationship between NO flux and bacterial adhesion. The NO release profiles during the 90 min period from 3 to 4.5 h following xerogel immersion in PBS buffer are depicted in the inset of Fig. 1. This period represents the incubation time during which the bacterial adhesion experiments were performed. The average NO flux of each of the four xerogel samples fell within $10\text{--}30 \text{ pmol cm}^{-2} \text{ s}^{-1}$, levels of NO previously reported to significantly reduce *Pseudomonas aeruginosa* adhesion [28]. Of note, the NO flux for each of the four xerogels remained relatively constant throughout the 90-min bacterial adhesion assay, with 10, 20, 30, and 40% AHAP3 xerogels generating an average NO flux of approximately 11, 18, 23, and $30 \text{ pmol cm}^{-2} \text{ s}^{-1}$, respectively, during the 3–4.5 h period after PBS immersion.

3.3. Fibrinogen-mediated bacterial adhesion

The pervasiveness of Fg as a mediator of bacterial adhesion [31–33] makes it appealing to include in studies aimed at elucidating the influence of polymeric NO release on protein-mediated bacterial adhesion. Fibrinogen promotes bacterial adhesion by bridging the biomaterial surface with bacterial cell membrane receptors specific to Fg [37]. Such interactions are responsible for bacterial adhesion to medical devices in vivo, and bacteria possessing the ability to specifically bind surface-adsorbed Fg have been found

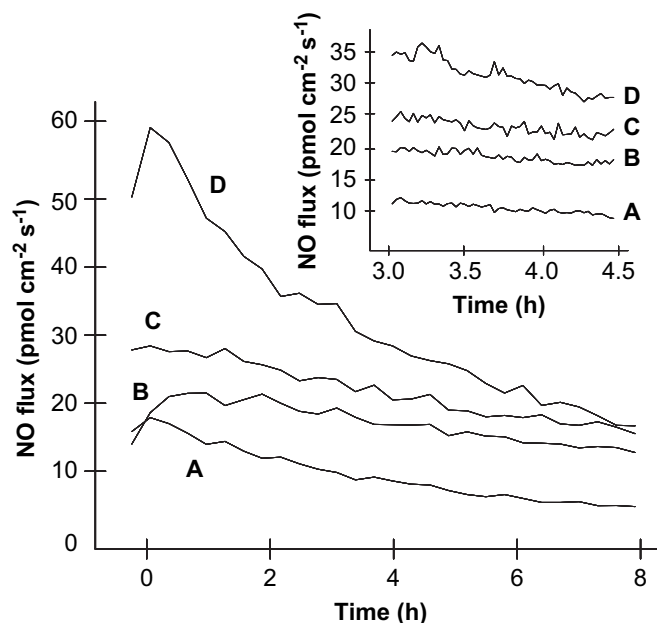


Fig. 1. Nitric oxide release profiles for 10 (A), 20 (B), 30 (C), and 40% (D) AHAP3 xerogels (v/v, balance BTMOS) from $t = 0$ to $t = 8$ h. Inset: NO release from $t = 3$ to $t = 4.5$ h, corresponding to the period during which xerogels were incubated in bacterial suspensions.

to be responsible for significantly more clinical orthopedic and cardiovascular device-associated infections than those without Fg-binding proteins [38]. Fibrinogen activity (i.e., function) has been shown to be susceptible to oxidative species [39], providing further motivation for its use in studies elucidating NO's effect on bacterial adhesion.

Prior to probing the influence of NO release, the fundamental effect of pre-adsorbed Fg and BSA on bacterial adhesion was evaluated. Bacterial adhesion to control (i.e., incapable of NO release) surfaces pre-treated with Fg was compared to controls pre-treated with BSA under the same conditions to differentiate between specific Fg-mediated interactions and general protein–protein interactions. As shown in Fig. 2, pre-treatment of the PVC surface

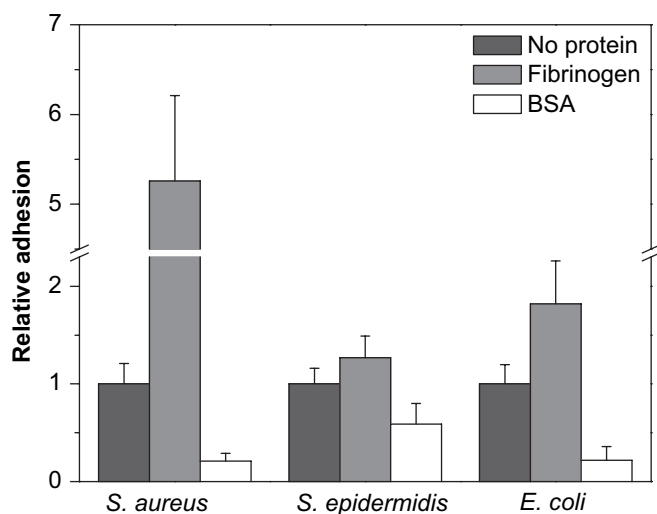


Fig. 2. Effect of pre-adsorbed fibrinogen and BSA on *S. aureus*, *S. epidermidis*, and *E. coli* adhesion to PVC-coated control 40% AHAP3 xerogels (v/v, balance BTMOS) at 37°C . For each bacterial species, the difference in adhesion between substrates with no pre-adsorbed protein and both fibrinogen and BSA-coated substrates was significant ($p < 0.05$).

with BSA resulted in significantly reduced bacterial adhesion for each of the three species tested. Such reduction is consistent with the observations of others [40] and may be the result of decreased surface hydrophobicity in the presence of the BSA layer [41]. Indeed, the water contact angle for bare PVC and BSA-coated surfaces was $78.6 \pm 4.0^\circ$ to $35.4 \pm 3.1^\circ$, respectively, indicating a decrease in surface hydrophobicity upon BSA modification.

In contrast, the extent of bacterial adhesion was greater to substrates with pre-adsorbed Fg compared to substrates without protein or those modified with BSA (Fig. 2). The most significant increase was observed in the case of *S. aureus*, where adhesion to Fg-coated substrates was more than 5 times that of uncoated controls. Baumgartner et al. reported similar results for *S. aureus* adhesion to polyurethane surfaces with pre-adsorbed Fg [42]. In a similar study, *S. epidermidis* adhesion to control catheters without pre-adsorbed functional Fg was approximately half that observed at Fg-coated catheters [43]. Our data also indicate that the presence of adsorbed Fg increases *S. epidermidis* adhesion by $\sim 26\%$, substantially less than the >5 -fold increase observed with *S. aureus*. Nevertheless, the Fg-mediated increase in *S. epidermidis* adhesion proved significant ($p < 0.05$). The adhesion of *E. coli* to Fg-treated PVC-coated xerogels was $\sim 83\%$ greater than control PVC-coated xerogels. Collectively, the data indicate that the increase in bacterial adhesion is the result of specific Fg-mediated interactions between the bacterial cells and the substrate [44] and not non-specific interactions between bacterial cells and the protein layer as adhesion was reduced in the presence of BSA and enhanced in the presence of Fg. The increased bacterial adhesion at Fg pre-treated substrates was also not the result of greater hydrophobic interactions, since the water contact angle was reduced from $78.6 \pm 4.0^\circ$ to $50.0 \pm 4.5^\circ$ for bare PVC and Fg-coated substrates, respectively, after Fg treatment. Increased bacterial adhesion due to specific bacteria–Fg interactions is consistent with the observations of others [31–33,40].

3.4. Inhibition of bacterial adhesion via NO release

While previous studies have established that NO release reduces bacterial adhesion in buffer [25–28,45], none have directly examined the antibacterial efficacy of NO-releasing surfaces in the presence of an adhesion-promoting protein conditioning layer. A primary advantage of active release antibacterial coatings is the ability to reduce bacterial adhesion in the presence of adsorbed proteins [9,10]. The studies described below were thus conducted to assess the ability of NO release to prevent bacterial adhesion in the presence of an adhesion-promoting protein layer.

Similar to previous work with *P. aeruginosa* [28], an inverse relationship between NO release and bacterial adhesion was observed for the three bacterial species examined herein (Figs. 3 and 4). Fluxes of NO between 10 and $30 \text{ pmol cm}^{-2} \text{ s}^{-1}$ resulted in statistically significant decreases in bacterial adhesion (Table 1). At a NO flux of $30 \text{ pmol cm}^{-2} \text{ s}^{-1}$ (the greatest flux examined), adhesion of *S. aureus* and *E. coli* was decreased by 96 and 88%, respectively, relative to bacterial adhesion to Fg-treated xerogel controls. Plots of relative bacterial adhesion versus NO flux for *S. aureus* and *E. coli* demonstrated a negative correlation between NO release and bacterial adhesion (Fig. 3). Overall, the NO release reduced *S. aureus* adhesion to a greater extent than *E. coli* adhesion. *S. aureus* was also more susceptible to the effects of NO release at lower fluxes. For example, the adhesion of *S. aureus* was reduced by $\sim 40\%$ at a NO flux of $10.6 \text{ pmol cm}^{-2} \text{ s}^{-1}$ (relative to controls) while *E. coli* adhesion was reduced by only $\sim 20\%$ (versus controls) at an identical NO flux. Comparison of the effectiveness of NO release at reducing adhesion by the different bacterial species was accomplished by relating the slopes from the linear regression equations derived from the plots of relative adhesion versus NO flux. For *S. aureus* and *E. coli* adhesion, the slope of the linear regression

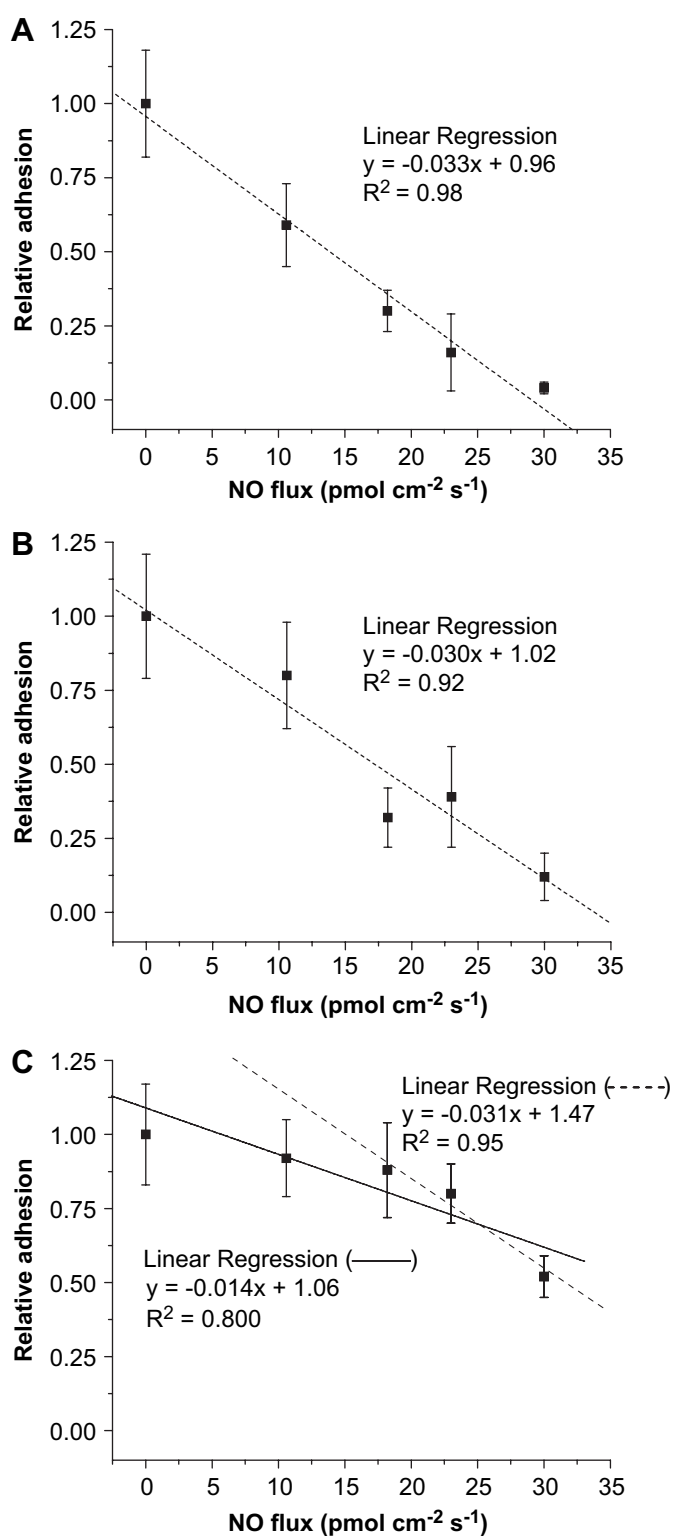


Fig. 3. Influence of NO surface flux on *S. aureus* (A), *E. coli* (B), and *S. epidermidis* (C) adhesion to PVC-coated NO-releasing xerogels.

was -3.3 ± 0.3 and $-3.0 \pm 0.5\%$ bacterial surface coverage per $\text{pmol cm}^{-2} \text{ s}^{-1}$ of NO flux, respectively. Despite greater overall surface coverage for *S. aureus*, the decrease in relative adhesion with increasing NO flux was statistically equivalent for both *S. aureus* and *E. coli*, indicating that the marginal effect of increasingly higher NO fluxes is similar for both species.

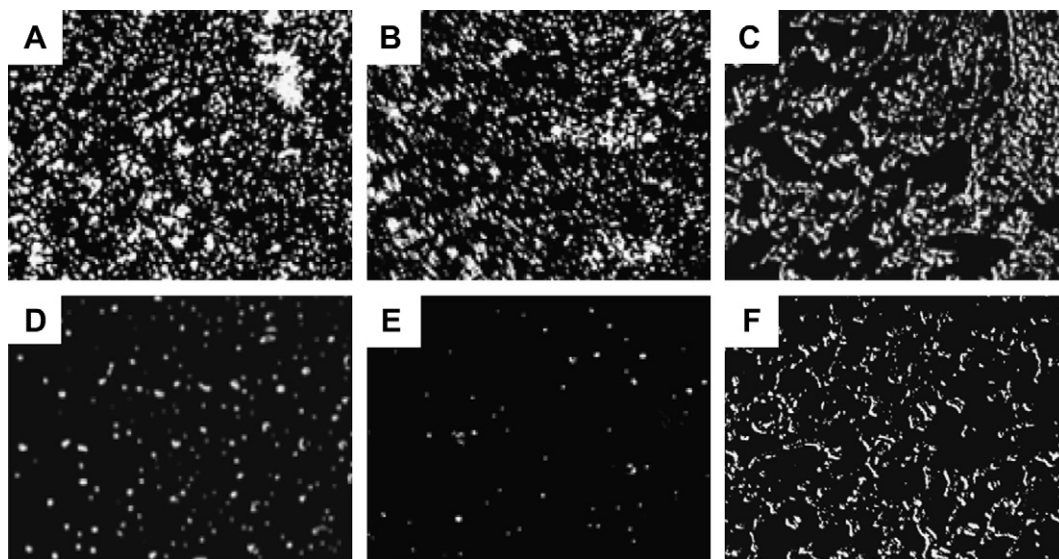


Fig. 4. Representative phase contrast images of *E. coli* (A, D); *S. aureus* (B, E); and *S. epidermidis* (C, F) adhesion to fibrinogen-coated control (A, B, C) and NO-releasing (D, E, F) 40% AHAP3 xerogels (v/v, balance BTMOS) at 37 °C. Cells are white. Images are 215 × 270 μm².

Table 1

Fibrinogen-mediated *S. aureus*, *S. epidermidis*, and *E. coli* adhesion to PVC-coated NO-releasing and control AHAP3 xerogels (balance BTMOS)

Average NO flux (pmol cm ⁻² s ⁻¹)	<i>S. aureus</i> surface coverage			<i>E. coli</i> surface coverage			<i>S. epidermidis</i> surface coverage		
	Absolute (%)	Relative to control	<i>p</i> -value ^a	Absolute (%)	Relative to control	<i>p</i> -value ^a	Absolute (%)	Relative to control	<i>p</i> -value ^a
0 (control)	14.1 ± 2.5	1.00 ± 0.18	–	21.2 ± 5.1	1.00 ± 0.21	–	15.3 ± 2.6	1.00 ± 0.17	–
10.6 ± 0.7	8.3 ± 2.0	0.59 ± 0.14	8 × 10 ⁻⁵	17.0 ± 3.8	0.80 ± 0.18	8 × 10 ⁻³	14.1 ± 2.0	0.92 ± 0.13	8 × 10 ⁻²
18.2 ± 0.8	4.2 ± 1.0	0.30 ± 0.07	2 × 10 ⁻⁷	6.8 ± 2.1	0.32 ± 0.10	3 × 10 ⁻⁹	13.5 ± 2.4	0.88 ± 0.16	3 × 10 ⁻²
23 ± 1	2.3 ± 1.8	0.16 ± 0.13	6 × 10 ⁻⁹	8.3 ± 3.6	0.39 ± 0.17	1 × 10 ⁻⁸	12.2 ± 1.5	0.80 ± 0.10	3 × 10 ⁻⁴
30 ± 2	0.56 ± 0.28	0.04 ± 0.02	5 × 10 ⁻⁹	2.5 ± 1.7	0.12 ± 0.08	8 × 10 ⁻¹¹	8.0 ± 1.1	0.52 ± 0.07	3 × 10 ⁻⁹

^a Two-tailed Student's *t*-test for statistical significance.

The antibacterial efficacy of NO against *S. epidermidis* adhesion to Fg-treated xerogels was less than that observed for *S. aureus* and *E. coli*. Although the absolute decrease in *S. epidermidis* adhesion over the range of NO fluxes studied was not as large, greater NO fluxes (23 and 30 pmol cm⁻² s⁻¹) maintained a stronger inhibitory effect on adhesion (Table 1). Using the data points at all five NO fluxes to generate a linear regression equation for *S. epidermidis*, a slope of $-1.4 \pm 0.4\%$ bacterial surface coverage per pmol cm⁻² s⁻¹ of NO flux was determined. A second linear regression equation determined using only the adhesion data for the three highest fluxes, the region where adhesion appears to be more strongly influenced by increasing NO flux, revealed a slope of $-3.1 \pm 0.6\%$ bacterial surface coverage per pmol NO cm⁻² s⁻¹. The slope from the abbreviated data set was statistically similar to the slopes from the *E. coli* and *S. aureus* adhesion plots, supporting the hypothesis that a strong, negative correlation exists between the relative adhesion of *S. epidermidis* and NO flux, but only at elevated NO fluxes. It is thus evident that fluxes of NO greater than a threshold value of ~ 18 pmol cm⁻² s⁻¹ are necessary to inhibit *S. epidermidis* adhesion to the same extent as *S. aureus* and *E. coli*. The uniformity of the linear regression slopes and the ability to inhibit the adhesion of both gram-positive and gram-negative bacteria suggests a general mode of NO's anti-adhesion activity, albeit requiring different NO fluxes for different species. Studies of additional bacterial species may help to further elucidate this hypothesis. Importantly, our data suggest that polymeric NO release is an effective strategy for reducing the Fg-mediated adhesion of both gram-positive (e.g., *S. aureus*, *S. epidermidis*) and gram-negative (e.g., *E. coli*) bacteria despite the differences in membrane properties and adhesive proteins that distinguish these classifications of bacteria [46].

To exclude the possibility that variations in bacterial adhesion are the result of differences in the amount of adsorbed Fg at control and NO-releasing surfaces, a fluorescently labeled protein adsorption experiment was used to quantify Fg adhesion. Congruent with the procedure for testing bacterial adhesion, PVC-coated control and NO-releasing xerogels were immersed in PBS at 37 °C for 90 min, and then transferred to a 20 μg/mL solution of Fg. The Fg solution consisted of 90% (v/v) unlabeled human Fg and 10% Alexa Fluor 488-modified human Fg. Following 90 min immersion in the Fg solution, the substrates were transferred to a 5 wt% SDS solution [36] and incubated for 90 min at 37 °C to remove the protein from the substrate. Fluorescence intensity measurements revealed no significant difference between samples in which control and NO-releasing substrates had been incubated (measured fluorescence intensities of 1.6 ± 0.5 and 1.4 ± 0.1 AU for control and NO-releasing samples, respectively; $p > 0.05$). The data indicate that the observed reduction in bacterial adhesion must be attributed solely to the surface flux of NO. Whether the xerogel-derived NO alters the structure or function of adsorbed Fg requires more study. Recent work in our laboratory has focused on elucidating NO's influence on fibrinogen function in solution, particularly its polymerization to fibrin [47]. In both nitrosoglutathione (a NO donor) and control (i.e., glutathione) solutions, fibrin polymerization was impaired to the same extent, suggesting that NO itself does not directly interfere with Fg function.

4. Conclusions

Nitric oxide release from xerogel polymer surfaces reduced *S. aureus*, *S. epidermidis*, and *E. coli* adhesion in the presence of pre-adsorbed Fg, a plasma protein known to mediate implant-

associated infection. Reduction of *S. aureus* and *E. coli* adhesion correlated strongly with NO surface flux in the range of 0–30 pmol cm⁻² s⁻¹. A NO surface flux of 30 pmol cm⁻² s⁻¹ reduced the relative surface coverage of *S. aureus* and *E. coli* by 96 and 88%, respectively. The relationship between bacterial adhesion and NO flux for *E. coli* and *S. aureus* fits a linear model with a common rate of change (approximately -3% relative surface coverage per pmol cm⁻² s⁻¹ of NO flux). While *S. epidermidis* adhesion did not correlate strongly with NO flux throughout the range of 0–30 pmol cm⁻² s⁻¹, a strong correlation for *S. epidermidis* was observed in the range of 18–30 pmol cm⁻² s⁻¹. These data suggest that NO release is more effective at inhibiting the adhesion of *S. aureus* and *E. coli* than *S. epidermidis* in the presence of pre-adsorbed fibrinogen.

Acknowledgements

This research was supported by the National Institutes of Health (NIH EB000708). The authors gratefully acknowledge the support of the Frances C. and William P. Smallwood Foundation (G.W.C.) and Pfizer (E.M.H.). We thank Prof. Alisa Wolberg and Robert Campbell for the generous gift of fluorescently-labeled fibrinogen, and acknowledge Prof. Kevin Weeks for use of his spectrofluorometer.

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