Sol−Gel Derived Nitric-Oxide Releasing Materials that Reduce Bacterial Adhesion

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Despite sterilization and aseptic procedures, bacterial infection remains a major impediment to the utility of medical implants including catheters, artificial limbs, and subcutaneous sensors.[1] In fact, over half of all nosocomial infections are coupled to implanted medical devices.[1,2] Implant-associated infections are commonly the result of microbial biofilms that form at the site of incision and insertion. These biofilms cause persistent, chronic illnesses with many acute, universal symptoms, making diagnosis difficult. Since mature biofilms are resilient against the immune system and conventional antibiotic treatments, numerous materials have been developed to passively resist the initial cellular adhesion stage of biofilm formation.[3−7] Diffusible antimicrobial agents, such as antibiotics[8−10] and polyclonal antibodies,[11] integrated into porous materials have recently been shown to actively prevent microbial adhesion at the implant site. The effectiveness of such local-release therapies is limited, however, due to the increasing resistance of bacteria to antibiotic therapy and the specificity associated with antibodies.[2] Since nitric oxide (NO) is directly involved in the destruction of bacteria during phagocytosis,[12] polymeric NO-release may represent an innovative strategy for fighting implant-related infections by preventing bacterial adhesion. Furthermore, as a result of NO's short half-life in blood (<1 s),[13] the effects of NO release would be localized at the implant interface, thus avoiding systemic toxicity issues. Herein, we report the ability of NO-releasing materials to actively reduce *Pseudomonas aeruginosa* adhesion.

Previous strategies for preparing NO-releasing materials are based on incorporating NO donors known as diazeniumdiolates as uniform dispersions into hydrophobic polymers.[14,15] These NO-donors are stable as solids in the absence of hydrogen ion sources, but react spontaneously with water to produce NO and residual amines.[16,17] The NO-release characteristics of these materials are controllable and have proven effective in reducing platelet adhesion by mimicking the inner surface (endothelium) of healthy blood vessels.[18] Recent work indicates that the in vivo performance of intravascular sensors is improved by coating the sensors with thin polymeric films that release NO.[19,20] Unfortunately, noncovalently entrapped NO donors and decomposition byproducts can leach from the polymer matrix into solution.[19] To address this potential toxicity concern, we demonstrate the synthesis of diazeniumdiolates that are covalently linked to a siloxane polymer using sol−gel chemistry. Due to mild synthesis conditions and tremendous chemical flexibility,[21,22] sol−gel methods represent a unique strategy for preparing materials that release NO.

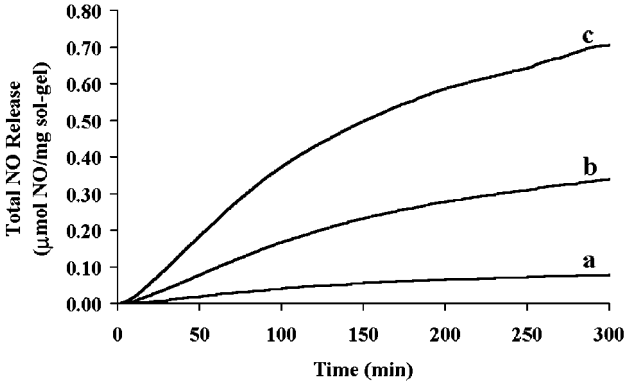

Figure 1. Total nitric oxide release (real time) for (a) 25% DET3/75% BTMOS, (b) 35% DET3/65% BTMOS, and (c) 45% DET3/55% BTMOS (v/v) NO-releasing sol−gel films.

Aminosilane-based sol−gels were prepared by combining variable amounts of (3-trimethoxysilylpropyl) diethylene- triamine (DET3) and isobutyltrimethoxysilane (BTMOS), reported as % DET3 (v/v) ranging from 15 to 45%. Sol−gels were synthesized with the addition of 3:1 molar ratio of water:silane followed by mixing for 5 min. A 30 μl aliquot of the silane mixture was cast onto an ozone-cleaned glass slide (ca. 0.45 cm^2) and allowed to gel, dry, and age under atmospheric conditions for 5−7 days, resulting in 0.8 ± 0.2 mm thick films. The amine groups in these sol−gel cast films were then converted to diazeniumdiolates by exposing the sol−gel coated glass slides to 5 atm NO for 72 h in a high pressure reaction chamber as described previously by Hrabie et al.[17] Diazeniumdiolate formation was confirmed by the appearance of a characteristic band at 250 nm in the solid-state UV−vis absorbance spectra of analogous films cast on quartz slides.[23] Controls were prepared similarly without exposure to NO and therefore did not exhibit an UV−vis band at 250 nm. The rate of NO release was measured using a NO chemiluminescence analyzer and a solution cell aspirated with N_2. DET3-diazeniumdiolate sol−gels release NO continuously up to 24 h. As shown in Figure 1, the NO release is tunable over a wide range by varying the amount of aminosilane (DET3) in the sol−

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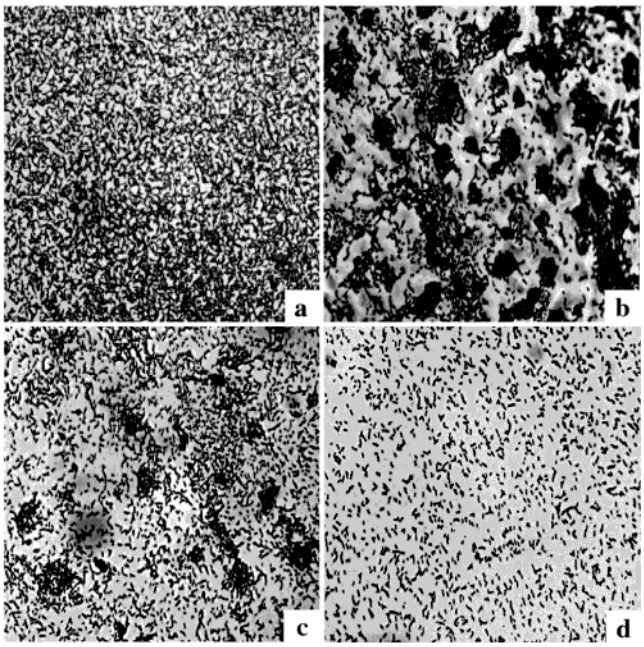

Figure 2. Optical microscopy images of *P. aeruginosa* adhesion (dark) to (a) 15%, (b) 30% DET3 control sol−gels, (c) 15%, (d) 30% DET3 NO-releasing sol−gels. Images are 167 × 167 μm^2.

gel. Within error (not shown), the amount of NO release is proportional to the % DET3.

To assess material stability, the Si content from phosphate-buffered saline (PBS) soak solutions was measured as a function of sol−gel immersion time using direct current plasma (DCP) emission spectroscopy. Since the diazeniumdiolate precursors (aminosilanes) are covalently linked to the polymer backbone, the detection of Si would indicate material instability. Notably, the solution concentration of Si was undetectable (<1 ppm) over the first 3 h of immersion. At longer immersion times, however, the Si concentration was measured to be 26.2 ± 0.1 ppm, indicating slight material fragmentation. Of note, we have recently determined that by first initiating the hydrolysis of BTMOS (by combining appropriate amounts of BTMOS, water, and ethanol) prior to adding DET3, the formation of sol−gel films with superior stability is readily achievable. Indeed, the concentration of Si in the soak solutions of such-formed sol−gels after 24 h immersion is not detectable.

P. aeruginosa was selected for bacterial adhesion studies because it is a well-characterized, medically relevant bacterium known to actively form biofilms.[24] Both control and NO-releasing sol−gels were immersed into PBS solutions for 1 h prior to being exposed to 10^9 colony forming units (cfu)/ml *P. aeruginosa* PBS solutions for 30 min. Following immersion, the adhered bacteria were fixed in 2% glutaraldehyde and then stained with safranin. Representative optical microscopy images for control and NO-releasing sol−gels are shown in Figure 2. Ubiquitous cell distribution (Figure 2a) and numerous dense cell clusters (Figure 2b) were common on the controls, regardless of amine content. Cell association is specifically known to be an essential attribute associated with biofilm maturation.[24] In contrast, a substantial decrease in both the bacterial adhesion and the amount of cell association was observed on surfaces that release NO (Figure 2c and d), suggesting that NO release inhibits adhesion and cell−cell interaction. As shown in Table 1, significant cell adhesion (ca. 60% coverage) was observed on the controls regardless of the amine content, indicating the amount of DET3 does not influence *P. aeruginosa* adhesion. The bacterial coverage on NO-releasing sol−gels was significantly less than controls for each concentration of DET3 studied ($p \ll 0.001$). The antimicrobial action of NO is further supported by reduced adhesion with increased DET3 concentration (and thus NO release). Of note, the antimicrobial efficiency of NO with respect to reduced *P. aeruginosa* adhesion appears to plateau between 25 and 30% DET3, although additional studies are necessary to establish optimal NO flux. To ensure that the NO oxidation product, nitrite (NO_2^-), is not responsible for the reduced bacterial adhesion, analogous experiments were performed by immersion of DET3 sol−gel films (controls) into 10^9 cfu/ml *P. aeruginosa* PBS solutions containing 0.3 mM $NaNO_2$ for 30 min. This concentration of nitrite corresponds to the total concentration of NO released from the diazeniumdiolate-modified sol−gels and readily oxidized to NO_2^- during the time of the above experiments as measured using the Griess spectrophotometric assay.[25] The bacterial coverage for controls immersed into *P. aeruginosa* PBS solutions containing NO_2^- was not significantly different ($p > 0.9$), suggesting NO is acting as the antimicrobial agent.

Table 1. Analysis of *P. aeruginosa* Adhesion to DET3 Nitric Oxide-Releasing Sol−Gels

	bacterial coverage[a]		ANOVA
% DET3	control	NO-releasing	*p*-value
15	67 ± 17	35 ± 15	2.5×10^{-8}
20	60 ± 11	23 ± 11	1.7×10^{-18}
25	55 ± 10	14 ± 7	2.0×10^{-25}
30	61 ± 14	17 ± 10	3.6×10^{-20}

[a] Percent bacterial coverage as measured by the percent opaqueness in a given image area (167 × 167 μm^2). The averages and standard deviations were determined from 10 images from 3 different surfaces ($n = 30$).

The astounding pace of analogous discoveries on the physiological roles of NO[26] demands new methods for generating controlled NO release. Sol−gel chemistry may provide a versatile method for preparing stable and tunable NO-releasing materials. Most significantly, polymeric NO release reduces *P. aeruginosa* adhesion. Although complete inhibition of bacterial adhesion was not demonstrated, these experiments were performed at high cell concentrations and low NO-release rates. We are currently exploring the antimicrobial diversity of NO by investigating the adhesion of other bacterial species as a function of NO release. Localized NO release may prove to be an effective strategy for lessening the frequency of biofilm formation and implant-related infections.

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Supporting Information Available: Details on the synthesis of DET3 sol−gels films, contact angle measurements and the procedure for *P. aeruginosa* adhesion studies (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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