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Superhydrophobic nitric oxide-releasing xerogels

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ABSTRACT

Superhydrophobic nitric oxide (NO)-releasing xerogels were prepared by spray-coating a fluorinated silane/silica composite onto *N*-diazeniumdiolate NO donor-modified xerogels. The thickness of the superhydrophobic layer was used to extend NO release durations from 59 to 105 h. The resulting xerogels were stable, maintaining superhydrophobicity for up to 1 month (the longest duration tested) when immersed in solution, with no leaching of silica or undesirable fragmentation detected. The combination of superhydrophobicity and NO release reduced viable *Pseudomonas aeruginosa* adhesion by >2-logs. The killing effect of NO was demonstrated at longer bacterial contact times, with superhydrophobic NO-releasing xerogels resulting in 3.8-log reductions in adhered viable bacteria vs. controls. With no observed toxicity to L929 murine fibroblasts, NO-releasing superhydrophobic membranes may be valuable antibacterial coatings for implants as they both reduce adhesion and kill bacteria that do adhere.
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1. Introduction

A combination of surface roughness and low surface energy yields "superhydrophobic" materials that are characterized by high water contact angles (⩾150°) [1]. Due to their non-wetting properties, such surfaces have proven useful for a wide range of applications, including droplet direction in microfluidics [2], antifouling coatings [3] and drug release [4,5]. The characteristics that govern a water droplet's behavior on a superhydrophobic interface are described by the Cassie-Baxter model [6]. Water droplets rest over a pocket of air trapped within the micro- and/or nanoscopic valleys of the surface. This property tends to make superhydrophobic materials resistant to fouling from debris, cells and biomolecules [7–9].

The ability for such interfaces to resist bacterial adhesion holds great promise for biomedical applications [10]. For example, microbial proliferation on an implant is responsible for many of the two million hospital-acquired infections that occur annually [11]. Researchers have sought to address this problem by designing interfaces that reduce bacterial adhesion passively or release antimicrobial agents [12]. While superhydrophobic interfaces certainly reduce bacterial adhesion [7], they are not able to kill the bacteria that do adhere, so such bacteria are still able to form deadly biofilms [13]. In contrast, antimicrobial agents released from a surface are able to actively kill bacteria, but generally only over finite periods (e.g. the duration of the drug release). By combining passive and active approaches simultaneously, we hypothesize that the resulting interface will exhibit improved antimicrobial efficacy.

Nitric oxide (NO) is a broad-spectrum antimicrobial agent that inhibits bacterial adhesion [14], kills bacteria [15] and reduces the incidence of implant infections in vivo [16]. To contend with NO's high reactivity and short biological half life [17], we and others have developed NO-releasing macromolecules and coatings to facilitate controlled NO release [18]. For example, silica xerogels formed from aminosilane precursors represent a template for storing and releasing NO. When exposed to high pressures of NO, the secondary amine sites within this polymer are converted to *N*-diazeniumdiolate NO donors [19]. In water, the NO donors decompose to yield the parent amine along with two equivalents of NO. Herein, we prepared superhydrophobic surfaces that actively release NO and evaluated their ability to decrease bacterial adhesion. We also examined how a superhydrophobic coating on top of an NO-storage reservoir can be employed to control and extend NO release kinetics.

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2. Materials and methods

2.1. Materials

Isobutyltrimethoxysilane (BTMOS), methyltrimethoxysilane (MTMOS) and low molecular weight poly(vinyl chloride) (PVC; average m.w. ~48,000) were purchased from Sigma Aldrich (St. Louis, MO). *N*-(6-Aminohexyl)aminopropyltrimethoxysilane (AHAP), tetraethylorthosilicate (TEOS) and (heptadecafluoro-1,1,2,2-tetrahydrodecyl) trimethoxysilane (17-FTMS) were acquired from Gelest (Tullytown, PA). Milli-Q water was purified from distilled water to a resistivity of 18.2 MΩ cm and a total organic content of <5 ppb using a Millipore Milli-Q UV Gradient A-10 system (Bedford, MA). Nitric oxide gas was purchased from Praxair (Bethlehem, PA). Standardized NO (26.85 ppm, balance N_2), argon (Ar) and nitrogen (N_2) gases were acquired from Airgas National Welders (Durham, NC). *Pseudomonas aeruginosa* (ATCC #19413) was purchased from American Type Culture Collection (Manassas, VA). Fibroblast cells (L929) were acquired from the UNC tissue culture facility (Chapel Hill, NC). Dulbecco's modified essential medium (DMEM), (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS), phenazine methosulfate (PMS), tryptic soy broth and tryptic soy agar were obtained from Becton, Dickinson and Company (Sparks, MD). All other reagents were analytical grade and used as received.

2.2. Synthesis and characterization of superhydrophobic NO-releasing xerogels

Glass slides served as the underlying substrate for all coatings. Slides were cut to dimensions of 9×25 mm^2 and cleaned via successive sonication in water, ethanol and acetone. The substrates were then dried with N_2 and cleaned with UV/ozone for 20 min using a Bioforce TipCleaner (Ames, IA).

Secondary amine-modified xerogels were prepared via a two-step, one-pot reaction. First, 378 μl of BTMOS was prehydrolyzed in 633 μl of ethanol, 190 μl of water and 31.7 μl of 0.5 M hydrochloric acid for 1 h. Following prehydrolysis of the backbone silane, 255 μl of AHAP was added and mixed for an additional 1 h. Afterwards, 40 μl of the resulting sol was cast onto a glass substrate, cured on the bench for 1 h, and further dried and cured in an oven for 3 days at 70 °C. After drying, films were modified with *N*-diazeniumdiolate NO donors via reaction with high-pressure NO gas. Amine-modified xerogels were placed in a Parr hydrogenation bomb and purged copiously with argon gas. Xerogels were then exposed to 10 atm NO for 3 days to form *N*-diazeniumdiolate NO donors at 2° amine sites. The *N*-diazeniumdiolate NO donor-modified xerogels were purged again with argon to remove unreacted NO. For bacteria experiments, non-superhydrophobic control and NO-releasing xerogels were coated with low-molecular-weight PVC to ensure identical surface attributes between the two groups [15,20]. Briefly, 400 mg of PVC was dissolved in 4 ml of tetrahydrofuran, then 300 μl of the resulting solution was spin coated on the xerogels at 3000 rpm for 10 s, and dried in vacuo for 24 h. The xerogels were stored under nitrogen at −20 °C until further use.

Fluorinated silica particles were synthesized via the Stöber method by co-condensing TEOS and 17-FTMS. In a 50 ml round-bottom flask, ethanol (30 ml) was combined with 12 ml of ammonium hydroxide (28 wt.% in water). To this solution, a mixture of 17-FTMS (690 μl) and TEOS (973 μl) was added via syringe pump (0.056 ml min^{-1} over 30 min). Following dropwise addition of the silane, the reaction was allowed to proceed for an additional 90 min to yield 30 mol.% 17-FTMS (balance TEOS) particles. Particles were collected via centrifugation at 2355*g* for 5 min, washed three times in ethanol via the same centrifugation regimen and dried under a vacuum overnight.

Control and NO-releasing xerogels were made superhydrophobic by spray-coating the xerogels with a mixture of fluorinated silica particles and silane precursors. First, 0–1000 mg of 30 mol.% 17-FTMS (balance TEOS) particles was suspended in ethanol (9.4 ml) via 30 min of ultrasonication. Next, 17-FTMS (221.4 μl), MTMOS (199.7 μl), water (2.00 ml) and 0.1 M hydrochloric acid (200 μl) were added to the suspension and allowed to react for 90 min. After reaction, the suspension was spray-coated using an Iwata HP-BC PLUS airgun with a nitrogen feed pressure of 6 bar at a distance of 30 cm. The nozzle pass rate over each substrate was approximately 2.5 cm s^{-1} (i.e. the entire vertical distance of the xerogels was covered in 1 s). Six, 12, 18 or 24 layers were made with the spraygun over each xerogel. Following coating, the resulting superhydrophobic xerogels were dried on the bench for ~5 min and placed in vacuo for 48 h.

2.3. Xerogel characterization

Static water contact angles were determined from images obtained with a KSV Instruments Cam 200 Optical Contact Angle Meter (Helsinki, Finland). For each film, measurements were taken in at least $n = 3$ locations. To assess long-term contact angle stability, superhydrophobic NO-releasing xerogels were immersed in phosphate-buffered saline (PBS) at 37 °C for 7, 14, 21 or 28 days. For each time point, the xerogels were removed from the soak solutions and the static water contact angles measured again.

Top-down images of the surfaces were acquired using a Hitachi S-4700 cold cathode field emission scanning electron microscope at an accelerating voltage of 2 kV. Cross-sectional images of the superhydrophobic coatings were collected using an FEI Quanta 200 field emission gun environmental scanning electron microscope at an accelerating voltage of 10 kV in high vacuum mode. To estimate the thickness of the superhydrophobic layers, a portion of the coating was cleanly removed from the substrate using a razor blade, leaving a sharp interface between the underlying substrate and superhydrophobic material. The samples were then coated with a 3 nm conductive Au/Pd film, mounted onto EM specimen stubs and placed onto a sample stage such that the coated side was nearly perpendicular to the detector. The distance from the base of the substrate to the top of the coating was measured for two coatings at $n \geqslant 30$ randomly selected locations using ImageJ software.

The surface roughness of the non-superhydrophobic and superhydrophobic xerogels was measured using atomic force microscopy (AFM). Root-mean-square roughness (R_{RMS}) was calculated from 20 μm^2 image fields acquired in air on an Asylum MFP-3D atomic force microscope operating in AC mode. The microscope was equipped with an Olympus AC240TS silicon beam cantilever with a spring constant of 2 N m^{-1}.

A silicon leaching assay was used to assess the chemical stability of the xerogels [19] Glass substrates, NO-releasing xerogels and superhydrophobic NO-releasing xerogels were submerged in 10 ml of PBS at 37 °C for 7, 14, 21 or 28 days. At set periods, the Si content in each soak solution was analyzed using an inductively coupled plasma optical emission spectrometer (Teledyne Leeman Prodigy ICP-OES; Hudson, NH), with calibration standards ranging from 0 to 10 ppm Si (as sodium silicate) at a wavelength of 251.611 nm.

The release of NO from the xerogels was measured using a Sievers 280i nitric oxide analyzer (NOA; Boulder, CO). Approximately 30 ml of PBS (pH 7.4, 37 °C) was placed in a round-bottom flask and deoxygenated by supplying nitrogen through a porous glass frit at a rate of 80 ml min^{-1}. The xerogels were submerged in the buffer and the NO liberated/released was carried to a pre-calibrated NOA by an additional stream of nitrogen gas supplied

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