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Antimicrobial Nitric Oxide Releasing Surfaces Based on *S*-Nitroso-*N*-Acetylpenicillamine Impregnated Polymers Combined with Submicron-Textured Surface Topography

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Abstract

A novel dual functioning antimicrobial CarboSil 20 80A polymer material that combines physical topographical surface modification and nitric oxide (NO) release is prepared and evaluated for its efficacy in reducing bacterial adhesion *in vitro*. The new biomaterial is created *via* a soft lithography two-stage replication process to induce submicron textures on its surface, followed by solvent impregnation of the NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), to obtain long-term (up to 38 d) of NO release. The NO release textured polymer surface is evaluated against four bacteria commonly known to cause infections in the hospital settings and results demonstrate that the combined strategy enables a synergistic effect on reducing bacterial adhesion of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* bacteria.

Graphical Abstract

SNAP-impregnated textured polymer films having up to 38 days NO-release were shown to have synergistic effects in inhibiting bacterial adhesion.

Keywords

Nitric oxide; Textured biomaterials; Solvent impregnation; *S*-Nitroso-*N*-acetylpenicillamine; Bacterial adhesion; Antimicrobial surfaces

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Introduction

Bacterial attachment and subsequent biofilm formation play a significant role in health-care related infections within the hospital setting, especially those associated with the use of implanted biomedical devices, such as intravascular (IV) catheters, urinary catheters, prosthetic heart valves, and orthopedic implants.^{1–3} Such infections are extremely difficult to treat by use of antibiotics alone due to biofilm formation on the surface of the devices. Microbial biofilm consists of extracellular polymeric substances (EPS) that are known to be a physical barrier that protects bacteria from traditional antibiotic treatment.^{4,5} It has been suggested that the slow penetration rate of antibiotics through biofilm can enable the expression of genes in the bacteria that mediate antibiotic resistance.⁶ Thus, surgical removal and replacement of an infected implanted devices is often the only resort, causing significant morbidity and mortality, and greatly influencing patient outcome and healthcare costs.^{7,8} Typical bacteria isolated from IV catheters that cause catheter-related bloodstream infections (CRBSIs) include *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*).^{9–12} Table 1 summarizes the characteristics of these four bacteria including their shape, gram staining with or without coagulase, virulence and rate of inducing CRBSIs.

The first step of biofilm formation on the surface of implanted medical devices is bacterial attachment. Indeed, the device should ideally be able to prevent bacterial colonization, especially during the most susceptible first 6 h period after implantation.¹³ Therefore, developing biomaterial surfaces that can resist bacterial attachment represents one of the most straightforward solutions to this problem.⁸ Bacteria can adhere to polymer surfaces via either direct nonspecific interactions or indirect binding to via molecules that first adsorb to the material to which the microbes can then attach. Therefore, one strategy to mediate bacterial adhesion is to modify the material surface properties to inhibit bacterial attachment. One of the most well-known surface texturing techniques that can be applied to polymeric materials creates topographical features that mimic the skin of a shark.^{14,15} This physical modification of a surface can reduce the surface contact area available for bacteria interactions. Xu et al. have demonstrated that submicron-textured surface featuring patterns with pillars having a diameter and spacing of 400/400 nm or 500/500 nm can decrease the adhesion and bacterial colonization of *S. aureus* and *S. epidermidis*.⁸ Helbig et al. have also reported that ordered surface structures (holes, posts or lines) with dimensions similar to bacterial cell size can decrease *S. epidermidis* and *E. coli* bacterial adhesion, regardless of contact time (minutes to hours) or surface hydrophobicity.¹⁶

Another biomimetic approach to develop antibacterial biomaterials is to utilize polymers that can release nitric oxide (NO), mimicking the NO release from nasal epithelial cells, macrophages and endothelial cells, etc.^{3,17–21} Nitric oxide serves as a potent antimicrobial agent in our body's immune response system to combat infectious diseases.^{21–23} NO possesses broad-spectrum antimicrobial/bactericidal activity against both gram-positive and gram-negative bacteria, including methicillin-resistant *S. aureus* (MRSA), without any known resistance.²⁴ NO at low levels (picomolar to nanomolar in solution phase) can also serve as an important signaling molecule in bacterial quorum sensing to minimize bacterial adhesion and disperse mature biofilm.^{25,26} Many NO releasing materials, where NO is

released/generated via decomposition of various NO donors (e.g., *N*-diazoniumdiolates or *S*-nitrothiols, etc.) or reduction of nitrite through electrochemical modulation, have been evaluated for their antibacterial and antibiofilm efficacy *in vitro*.^{27–33} *S*-Nitroso-*N*-acetylpenicillamine (SNAP) is a particularly attractive NO donor species for incorporation into biomedical grade polymers to create NO-releasing materials because of its low cost, safety, and potential for long-term NO release.^{6,30,34,35} Polymers containing SNAP also exhibit exceptional long-term shelf-life stability (8 months) when stored in the dark at 37 °C.³⁰ This is especially true when using CarboSil 20 80A, a low water uptake silicone-polycarbonate-urethane tri-block copolymer.³⁰ Polyurethane block copolymers are widely employed for creating blood-contacting medical devices due to their inherent biocompatibility, which partly results from the microphase separation structure between the thermodynamic immiscibility of hard and soft segments.^{8,36,37} Specifically, SNAP-doped CarboSil IV catheters have been reported to exhibit potent antibacterial activity against *S. aureus*, with 5-log unit reduction of viable bacteria counts on the surface of catheters loaded with SNAP compared to corresponding controls over a 7 d period using a drip-flow biofilm reactor.³⁰

While both physical surface modification (topographical surface textures) or chemical modification (nitric oxide release) alone have both proven to be effective approaches to inhibit bacterial adhesion and biofilm formation, the combination of both techniques to create an enhanced dual functioning antimicrobial polymer surface for clinical use may yield an even more effective biomaterial. Since both approaches can reduce bacterial attachment and prevent biofilm formation, single cells either adhered onto catheter surfaces or within the bloodstream, are much more susceptible to antibiotic treatment, which would greatly reduce the possibility of CRBSIs. Toward this goal, we recently reported our efforts to prepare CarboSil films that bear an ordered pillar topography at their outermost top surface and then formed in a “sandwich-like” film configuration with SNAP-doped CarboSil as a middle layer of a tri-layered film configuration.³⁸ Obviously such a process is time-consuming, often requiring 1–2 weeks to reach the desired thickness of each film. Furthermore, the repeated spin-coating of new SNAP-polymer solution on to a dried SNAP-polymer surface can lead to the re-dissolution and re-crystallization of SNAP, causing undesired SNAP degradation during the fabrication process.

Herein, we describe an improved and simpler solvent impregnation method to incorporate NO donor SNAP into the bulk of a textured surface polymer film. Textured CarboSil films without SNAP are first fabricated by a spin-coating⁸ process and then dried. Subsequently, the polymer films bearing the ordered pillar topography on their surfaces are completely impregnated with SNAP by soaking in an optimized SNAP/organic solvent solution. The solvent impregnation of SNAP results in very stable crystals of SNAP within the entire polymer phase and thereby enables long-term NO release (up to 38 d). StreamLine High Resolution (HR) Raman mapping is employed to examine the distribution of SNAP crystals within SNAP-impregnated CarboSil textured films. Further, four common bacteria strains associated with CRBSIs are used to examine the *in vitro* antibacterial activity on these new dual functioning surfaces. While significant reduction in the surface adhesion of all four organisms is observed, it is shown that the combined approach provides a synergistic reduction in the adhesion of *S. epidermidis* and *P. aeruginosa* microorganisms, suggesting

that the new combination surfaces can produce a significantly enhanced antibacterial efficiency than either approach alone.

Experimental Details

Materials

N-Acetyl-D-penicillamine (NAP), sodium nitrite, L-cysteine, sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, copper (II) chloride, ethylenediaminetetraacetic acid (EDTA), tetrahydrofuran (THF) and *N,N*-dimethylacetamide (DMAc) were purchased from Sigma-Aldrich (St. Louis, MO). Methanol (MeOH), methyl ethyl ketone (MEK), hydrochloric acid, and sulfuric acid were products of Fisher Scientific (Hampton, NH). CarboSil 20 80A was obtained from DSM Biomedical Inc. (Berkeley, CA). All aqueous solutions were prepared with 18.2 MΩ-deionized water using a Milli-Q filter from EMD Millipore (Billerica, MA). Phosphate buffered saline (PBS), pH 7.4, containing 138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, and 100 μM EDTA was used for all *in vitro* experiments. Textured silicon wafers were fabricated by RTI International (Research Triangle Park, NC) based on requirements. *S. epidermidis* RP62A (ATCC 35984), *S. aureus* Newman (ATCC 25904), *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 11775) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA).

Preparation of smooth and textured CarboSil PU films

The CarboSil 20 80A PU film surfaces were textured with ordered arrays of pillars using a modified soft lithography two-stage replication molding technique that has been described previously.⁸ Briefly, textured silicon wafers with different ordered array of pillars, either fabricated by RTI International (Research Triangle Park, NC) or Penn State Nanofabrication Lab (State College, PA), were used as the master patterns to prepare the master PDMS molds and the corresponding CarboSil films were then fabricated (see Figure 1A). CarboSil pellets were dissolved in *N,N*-dimethylacetamide (DMAc) at concentration of 15 w/v% (1.5 g PU in 10 mL DMAc) and this solution was spin-coated onto the silicon mold to prepare the textured CarboSil films. To obtain the highest replication efficiency of surface topography, the films were prepared by spin casting CarboSil onto a PDMS mold at 1200 rpm for 1 min in one thin layer first, followed by degassing and curing overnight at room temperature under vacuum. Then, additional layers of CarboSil were added by spin casting at 600 rpm for 1 min, respectively, until reaching the desired thickness of ~700 μm. Each layer was degassed and cured at room temperature under vacuum. The master silicon wafers with pillar geometries having diameter (d), separation (s) and height (h) (d/s/h) values of 400/400/600 nm, 500/500/600 nm and 700/700/300 nm were used as the masters for replicating CarboSil textured films for AFM images. However, only 700/700/300 nm textured films were used in all *in vitro* experiments since the pillars of other two patterns collapsed during SNAP impregnation. Smooth CarboSil films were also prepared by spin-casting against a smooth PDMS mold.⁸

SNAP impregnation of textured CarboSil films

Small disks of films (10 mm in diameter) were punched out from both smooth and textured CarboSil films. As described previously,³⁹ the films were soaked in solutions (70 % MEK and 30 % MeOH) containing different SNAP concentrations (35, 65 and 110 mg/mL) to achieve 5, 10 and 15 wt% SNAP loading in CarboSil PU films, respectively.³⁹ After 2 h of impregnation, the films were removed from the impregnation solution, quickly washed with methanol to remove any residual SNAP solution on the film surface, and allowed to dry up in the ambient environment protected from light exposure.

Characterization of surface topography by atomic force microscopy (AFM)

Multimode AFM with a Nanoscope IIIa control system (software version 5.12r3, Veeco, Santa Barbara, CA) was used to examine the surface textures of the CarboSil films by operating in a tapping mode (intermittent contact) using Si probes having aspect ratio of ~ 4:1 (TETRA, K-Tek Nanotechnology, Wilsonville, OR) in air. AFM was also used to image the bacterial adhesion on CarboSil PU surfaces. AFM images were treated and analyzed by off-line AFM software (version 5.12r3, Veeco, Santa Barbara, CA).

Surface wettability

The water wettability of CarboSil PU films was determined as the advancing water contact angle measured by the sessile drop method using a Krüss contact angle goniometer. All measurements were made using water as a probe liquid, with an ~ 8 µL water droplet used for the measurement. Advancing contact angles were obtained by a minimum of eight independent measurements and are presented as mean ± standard deviation.

Cross-Section characterization of SNAP-impregnated CarboSil films by Raman Spectroscopy

StreamLine High Resolution (HR) Raman characterization was utilized to map the two-dimensional SNAP crystal distribution at the cross-section of a 5 wt% SNAP-impregnated CarboSil film and a 5 wt% SNAP-doped CarboSil film. The films were first cut to expose their cross-sections and then embedded into optimal cutting temperature compound (OCT), allowed to harden at -20 °C, and then cut carefully into 30 µm thick slices by a Leica CM3050S research cryostat. StreamLine HR Raman Mapping experiments were conducted by using a Renishaw inVia Raman microscope equipped with a Leica microscope, a RenCam CCD detector and a 785 nm laser employing a 1200 lines/nm grating and a 50 µm slit. Similar to the method previously reported,³⁰ the obtained spectrum of each spot was fitted with pure orthorhombic SNAP spectrum as the reference, and the green areas in the figures presented correspond to regions fitting the characteristic peaks of orthorhombic SNAP.

NO release measurement

Nitric oxide release from the films was measured using a Sievers chemiluminescence Nitric Oxide Analyzer (NOA) 280i (Boulder, CO) using a method described previously.³⁹ Briefly, films with diameter of ~10 mm were placed in the sample vessel immersed in PBS containing 100 µM EDTA at 37°C. Nitric oxide emitted from the film was continuously

purged from the buffer and swept from the headspace using nitrogen gas and a bubbler into the chemiluminescence detection chamber of the NOA. When not being tested with the NOA, the SNAP-doped samples were incubated in PBS under same the conditions while avoiding exposure to light. The NO flux (per unit area of film) was calculated based on assuming a total surface area of the two flat sides of films exposed to solution. All experiments were conducted in triplicate.

Bacterial adhesion on the surface of CarboSil films with different configuration

The methods for culturing and collection of bacteria have been reported previously.⁸ Briefly, bacterial colonies of the four different strains were cultured in tryptic soy broth (TSB) at 37 °C for 24 h and collected by centrifuge at 1360g for 10 min. The collected bacteria were then re-suspended in PBS and diluted to a final concentration of 1×10^7 CFU/mL, as determined by turbidity measurement at 600 nm using a spectrophotometer. All CarboSil films were pre-hydrated in Millipore water for 24 h and conditioned in PBS for 1 h before use. Bacterial adhesion studies were conducted by placing the CarboSil films in a 12-well home-made microwell plate containing 0.5 mL bacteria suspended PBS solution at 37°C for 1 h under near static conditions. After the experiment, the film was washed with PBS 3×, and then fixed in 2.5% glutaraldehyde for 2 h. The films were washed with PBS again before being stained with Gram Stain Stabilized Iodine kit (VWR) and analyzed under an optical microscope (Nikon, Eclipse 80i). Nine optical microscopy images ($91 \mu\text{m} \times 68 \mu\text{m}$) of each film were taken and the number of bacteria adherent on the surface was counted with an average $\pm$ standard error of means used for statistical analysis.

In vitro antimicrobial and antibiofilm test

To test the effect of NO release on bacterial growth, the polymer films were soaked in PBS for 1 h and placed in a 24-well plate. Two ml of corresponding culture medium containing different bacteria with a concentration of 1×10^6 CFU/ml was added and incubated at 37°C with shaking at 180 rpm. At 24 and 48 h time points, 0.20 ml medium was drawn and diluted into 1 ml with sterile corresponding culture medium, and the optical density (OD) was measured at 600 nm, where OD₆₀₀ values indicated the growth of bacteria in the bulk medium. After 48 h incubation, the culture medium was replaced with 2 ml of sterile PBS for 4 times. Then each polymer film was gently transferred into a Corning 15-ml tube with 2 ml of sterile PBS. The tube was vortexed at the highest speed for 1 min to remove adherent bacteria or biofilms from polymer surfaces. The homogenized bacterial solution was serially diluted 10-fold in PBS and 50 μl of dilution was plated onto the corresponding agar plate for viable bacterial counting. All the experiments were conducted in duplicate.

Data analysis

Statistical analysis was performed using SAS software (version 9.4). Means of experimental data were compared by 2-sample t-test and differences were considered statistically significant for $p < 0.05$. Significance is denoted in figures of this paper with one symbol denoting $p < 0.05$, two symbols $p < 0.01$, and three symbols denoting $p < 0.001$. The significance of synergistic or additive effects achieved by combining surface texturing and NO release on the reduction of bacterial adhesion was analyzed using IBM SPSS statistics 22 with a univariate general linear model.

Results and Discussion

Characterization of SNAP-impregnated and textured CarboSil films

CarboSil films were textured with ordered arrays of pillars using a soft lithography two-stage replication molding technique (Figure 1). Initially, CarboSil films textured with different dimensions, including cylindrical pillars of 400/400/600 nm and 500/500/600 nm dimensions, and square pillars with 700/700/300 nm patterns were prepared, and then each type of film was soaked in impregnation solutions containing 35, 65 and 110 mg/mL SNAP. However, the topography images showed that the cylindrical pillars with dimensions of 400 or 500 nm in diameter and 600 nm in height partially collapsed during the solvent impregnation process, while the shorter and larger square pillars that were 700 nm in size and 300 nm in height remained intact. These results strongly suggest that the mechanical strength of pillars is largely determined by their aspect ratio of height to diameter since the pillars also undergo the swelling/shrinkage process during the SNAP impregnation step. The low aspect ratio of height to diameter (aspect ratio = 0.43 for 700/700/300 nm pattern) can resist the solvent to a greater degree and maintain the pillars intact, while the high aspect ratios (1.2 or 1.5 for 500/500/600 nm, and 400/400/600 nm patterns, respectively) caused the pillars to collapse. As a result, 5 wt%, 10 wt% and 15 wt% SNAP-impregnated smooth and textured (700/700/300 nm) CarboSil films were fabricated for all further characterizations and bacterial adhesion studies.

The surface topography of the pillars after impregnation was further examined by AFM. The topography images show that the pillars are uniformly distributed on the surface with no collapse or surface confined SNAP crystals observed, regardless of the concentration of SNAP used during the impregnation process. Representative AFM 2D topography images of one textured film before and after exposure to 10 wt% SNAP impregnation for 2 h and subsequent drying (for 24 h) are illustrated in Figure 2. The pillar sizes of each type of textured CarboSil PU film was analyzed by AFM offline or Image J software and results are tabulated in Table 2. Interestingly, the size of pillars is somewhat smaller than that before impregnation. The perimeter of pillar top surface decreased ca. 170–320 nm and the area of top surface decreased by about 0.048–0.144 μm^2 ; i.e., the edge length decreased 42–80 nm for square shape pillar. These results suggest that the polymer shrinks during the drying process after impregnation. The shrinkage of polymer is also evidenced from the change of pillar height. The bearing analysis of topography images (Figures 2C and 2D) shows that the height of most of pillars before impregnation is ca. 306 nm, while the height of most of pillars are reduced to about 225–237 nm after impregnation (Table 2). The reduction in height and pillar size decreases the overall roughness of the surface within the $25 \times 25 \mu\text{m}^2$ scanning resolution. The values of RMS (R_q) or arithmetic average of the roughness profile (R_a) decreases about 30%, from 133 nm to 93 nm for RMS and 123 nm to 84 for R_a values, respectively (Table 2). The decrease of pillar height and roughness may affect the bacterial adhesion onto textured surfaces (see below).

The impregnation of SNAP also alters the surface wettability of the CarboSil PU films slightly. The measured water contact angles for 5% SNAP impregnated smooth surfaces are similar to the samples without SNAP impregnation, while the contact angles for the 10% and

15% SNAP samples decreased ca. 6° compared to the samples with 0% and 5% SNAP impregnated (Figure 3). The slight change in water contact angle is probably because SNAP is only slightly hydrophobic⁴⁰, and less hydrophobic than the polyurethane. Surface texturing greatly increased the hydrophobicity of the polymer films due to air trapped in between the pillars. The water contact angle of a regular CarboSil PU textured surface increased from 90.5° to 122.5°. Similar to the smooth films, the impregnation of SNAP into textured surfaces slightly decreases the water contact angle of the films, with the water contact angles of the SNAP-textured surfaces varying in the range of 104–111° (see Figure. 3).

Nitric oxide release from SNAP impregnated CarboSil PU surfaces

Once in contact with moisture or in the presence of light/heat, SNAP decomposes into the disulfide dimer of NAP or (NAP)₂, and releases NO.³⁰ In this study, the NO release flux was measured at 37 °C with the films in PBS until the flux was below 0.5×10^{-10} mol cm⁻² min⁻¹, which is the lower end of the physiological NO flux level exhibited by endothelial cells.⁴¹ NO release varied with the concentration of SNAP incorporated within the PU films. Generally, higher NO fluxes are observed when higher a concentration of SNAP is impregnated into the textured films. The NO release flux gradually decreases with soaking time. The lifetime of NO release from SNAP-impregnated textured films (at or above physiological levels) is maintained for at least 9, 19 and 38 d for 5 wt%, 10 wt% and 15 wt% impregnated films, respectively (see Figure. 4).

The main advantage of SNAP impregnation into CarboSil PU films is the longevity of NO release and high storage stability. In our previous study, we prepared SNAP-textured CarboSil films via a layer-by-layer method and spin-coating, where the SNAP was doped within the middle layer of a “sandwich like” CarboSil film bearing a textured pattern on the top surface.³⁸ This process was found to be extremely time-consuming and shortened the NO release lifetime (in the range of 2 to 10 d), which is probably due to the repeated dissolution and recrystallization of SNAP during fabrication, leading to SNAP degradation. The solvent impregnation technique described herein allows SNAP incorporation into CarboSil polymer via a one-step method, and the impregnated SNAP molecules are embedded in the entire bulk of the material as the solvent evaporates.³⁹ Since SNAP can partially dissolve in the CarboSil polymer with a solubility limit of ~3.4–4 wt%,³⁰ the SNAP is present in the materials in the form of both dissolved SNAP as well as crystalline SNAP, the latter which forms a stable polymer-crystal composite that can enable long-term NO release.

The SNAP crystal distribution in the textured CarboSil film was further analyzed by Raman mapping. Figure 5 illustrates the two-dimensional Raman map of the cross-section of a 5 wt % SNAP-doped textured CarboSil film (Fig. 5A) and 5 wt% SNAP-impregnated film (Fig. 5B). The former SNAP-doped textured film was prepared by the layer-by-layer spin-coating method previously reported³⁸. As shown in Figure 5, after the solvent impregnation process, the SNAP molecules/crystals are “inserted” into the interspace between CarboSil 20 80A polymer chains,³⁹ with a lamellae structure of the PDMS phase and the polycarbonate/polyurethane hard segment mixed phase.⁴² This process may result in a slightly less uniform

crystal distribution in the 5 % SNAP-impregnated films when compared to the 5 wt% SNAP-doped films prepared by the previously reported spin coating technique.³⁹

Antibacterial adhesion of dual functional surfaces

Bacterial adhesion to the SNAP-textured films was assessed with four strains of bacteria under near static conditions in PBS and evaluated by determining the bacteria counts per unit area. On the regular smooth polymer film surfaces without NO release, *P. aeruginosa* and *E. coli* exhibit the highest and lowest bacterial adhesion among the four strains examined, respectively. Bacterial adhesion of *P. aeruginosa* is almost one order of magnitude higher than that of *E. coli*, suggesting that the specific strain of bacteria significantly influences the adhesion of the microbes on the polymer surface, although both of these strains have a similar rod shape (Figures 6a and 6d) and both are gram negative. The adhesion of *Staphylococcus* strains appears to be similar, although adhesion of *S. aureus* is slightly greater than that of *S. epidermidis*. Overall, the presence of a textured surface significantly reduces the bacterial adhesion. The 700/700/300 nm surface texture alone reduced the surface bound bacteria counts by 49 %, 28 %, 52 % and 27 % for *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *E. coli*, respectively, after only 1 h of incubation (see Figures 6a–d). This decrease in bacterial adhesion is likely due to the reduction in available surface contact area since the dimensions of textured pillars are smaller than the size of the bacterial cells. It should be noted that rod shape bacterial cells may have access into the gap between pillars, leading to an orientation of any bacterial cells attached on the textured surfaces (see below).

Nitric oxide release inhibits bacterial growth and reduces bacterial adhesion with increasing of SNAP concentration within the polymer films and the concomitant increase of NO release flux. The degree of reduction of bacterial adhesion on the combined NO release (15 wt% SNAP) and textured surfaces increased to 88 %, 61 %, 85 %, and 85 %, for the same four bacteria over the 1 h test period, suggesting that the combination of physical modification and NO release substantially enhances the inhibition of bacterial adhesion. Further, the highest inhibition rates occur when the concentration of impregnated SNAP is increased to 15%.

To study the effect of the combination of physical surface modification and NO release on bacterial adhesion, we used a univariate general linear model to analyze the significance of interaction of both factors, and the results are summarized in Table 3. The statistical analysis indicates that either the surface texturing or the NO release alone has a significant effect on the inhibition of bacterial adhesion, regardless of bacteria strain. There was a significant interaction between these two factors (i.e., the combination of texturing and NO release) for *S. epidermidis* ($p=0.037$) and *P. aeruginosa* ($p<0.001$), but the effect was non-significant for the interaction of both factors for *S. aureus* ($p=0.199$) and *E. coli* ($p=0.089$). These results indicate that surface texturing and NO release produces an additive effect for reducing the adhesion of *S. aureus* and *E. coli*. It should be noted that the mechanism of achieving synergy between texturing and NO release for preventing bacterial adhesion over a short time period (1 h) is not entirely clear and the different effects of these two factors on different bacteria strains still needs further investigation.

Antimicrobial and antibiofilm properties of dual functional surfaces

An *in vitro* antimicrobial test was conducted to compare the influence of the NO releasing polymer films and a series of control films on growth of planktonic bacterial cells through the measurements of bacterial turbidity of media. Figure 7 illustrates the values of OD₆₀₀ of media incubated with different strains and SNAP-impregnated or control polymer films after 24 and 48 h. Results showed that the inhibition of microbial growth by NO releasing films is dependent on the bacterial strain and SNAP content in the polymer films. NO releasing films impregnated with 10 and 15% SNAP significantly inhibit the growth of *S. epidermidis* in medium, as the values of OD₆₀₀ of media from these cultures were as low as 1/60 of the value of control (smooth 0%), suggesting the bacteria in bulk media solution were inhibited by the NO release (Fig. 7a). However, there is no significant inhibition of *S. epidermidis* by the films with 5% SNAP impregnated, probably due to the low NO releasing flux rates. Significant inhibition of bacterial growth was seen from the media incubated with *E. coli* (Fig. 7d). The values of OD₆₀₀ of culture media incubated with 10 and 15% SNAP impregnated films were significantly smaller than the values of media with control. It is interesting to see that the NO release had no significant inhibition of growth of *S. aureus* and *P. aeruginosa* in the bulk media. Both strains grow fast even in the incubation of 10 and 15% SNAP impregnated films (Fig. 7b and 7c). This is likely due to the low concentration of NO dissolved in the solution being insufficient to inhibit the growth of strains, suggesting NO effects may be strain dependent.

The antibiofilm properties of NO releasing polymers were assessed by the viability of bacteria adherent on the film surfaces after static incubation in culture medium for 48 h, and by the bacterial counts per unit area (mm²) as summarized in Table 4. As expected from the antimicrobial test, good inhibition rates of bacterial attachment and biofilm formation were seen for strain *S. epidermidis*. Without NO release, the surface texturing alone inhibits 60.4% of bacterial adhesion/growth in biofilms. The 5% SNAP impregnation slightly increased the inhibition rates, while 10 and 15% SNAP impregnations dramatically inhibited the growth of bacteria on polymer surfaces, up to 97.0%. Compared to the smooth surfaces with same load of SNAP, the texturing has small increase in inhibition (2–3%), but this is not surprised for static condition. In this experiment, the polymer films sat at the bottom of plate well and the planktonic cells can easily settle on the textured surfaces. This phenomenon was also seen in other bacterial strains. Interestingly, *S. aureus*, another grape-shape bacterium, shows lower inhibition rates compared to *S. epidermidis*, with the highest inhibition rate being only ~60% (Table 4). This is consistent with the results of the antimicrobial test (Fig. 7b).

The antibiofilm property of dual functional surfaces was also seen in the rod-shape bacterial strains, *P. aeruginosa* and *E. coli* (Table 4). Without NO release, the surface texturing contributed about 29.8% and 31.1% inhibitions of biofilms, respectively, but NO release dramatically inhibited the bacterial adhesion and growth on film surfaces. We found the dual functional surfaces inhibited about 94% of *P. aeruginosa* and 80% of *E. coli* for 2 d incubation. It should be mentioned that NO release has no significant influence on growth of cells for *P. aeruginosa* in the bulk medium (Fig. 7c), but largely inhibited the growth/adhesion of bacteria on the surface. Although the mechanism of NO release on antibiofilm is

not clearly understood, the surface texturing and NO release near the surface synergistically disrupt the bacterial adhesion and biofilm formation, which has been confirmed by the following AFM characterization results.

AFM characterization of adhered bacteria on dual functional surfaces

The above optical microscopic approach demonstrates the spatial distribution and local quantity of adherent bacteria, and results showed that surface texturing and NO release, either alone or combination, significantly reduce the bacterial adhesion. To further examine the bacterial adhesion behavior, bacterial cells on CarboSil films was examined by AFM. Figures 8 and 9 show representative AFM images of grape-shaped *S. epidermidis* and rod-shaped *P. aeruginosa* bacteria adhered to CarboSil films with different configurations. Consistent with the optical microscopic examinations (above), the bacteria counts on polymer surfaces visibly decrease with the increasing SNAP concentration (0, 5, 10, 15 wt %), for both smooth and textured films. For CarboSil films with the same SNAP concentration, fewer bacteria attach to the surfaces with a textured pattern than onto the smooth surfaces.

The shape and size of given bacterial cells influence their adhesion to various surfaces. The grape-shaped *Staphylococcus* strains are mostly present in small clusters (2 or more cells) on the textured film surfaces, and none of the cells or cell-clusters appears to be trapped in the space between pillars, regardless of the concentration of SNAP impregnation (Figure 8). On smooth surfaces, more cell clusters are observed on the films without NO release, and fewer clusters are adhered on the surfaces with increased rates of NO release. In contrast to grape-shaped bacterial cells, a large quantity of rod-shaped *P. aeruginosa* bacteria are clustered and adhered on the smooth surface without NO release (SNAP 0%) (Figure 9a). However, the surface texturing and NO release seem to disrupt the clustering of the bacteria, and generally only single cell or small clusters with two or three cells are observed on these surfaces (Figure 9b–h). Of note, it is interesting to observe that the bacterial cells are trapped in between the grooves of the pillars for the textured films without NO release (SNAP 0%) (Figure 9i). This suggests that the bacterial cells tend to maximize their contact area for adhesion on the surface and the resulting orientation of the cells is aligned with the surface pattern features. However, when the films exhibit NO release (SNAP 5–15%), it was surprising to see that the *P. aeruginosa* cells only adhere on the very top surface of the pillars, and no cells are observed trapped within the spaces between pillars. This observation strongly suggests that the release of NO may serve as a signaling molecule to inhibit bacterial interaction with the surface, leading to the significant reduction in the number of adhered bacteria.

The AFM images further demonstrate the reduction of bacterial adhesion on textured and NO releasing films. Taken together, the data show that both approaches can interrupt the interactions between cells and inhibit cluster formation on surfaces. Nitric oxide release further inhibits the interaction of cells and the surface, and reduces the adhesion, although the surface texturing may increase the contact area for rod-shaped bacterial cells. It should be noted that only one textured pattern was tested in this study and hence it will be important in the future to examine and compare the influence of different geometries of the pillar

topographical design, including pillar diameter (d), separation distance (s) and pillar height (h), on the bacterial adhesion properties in the future. This will require further optimization of the SNAP impregnation process to reduce significant changes in surface pillar dimensions as a result of the polymer swelling and shrinking processes associated with this method.

Antibacterial coatings are rapidly emerging as an important component of the global mitigation strategy for bacterial pathogens. Recent advances in material science and biotechnology have led to the great progress in designing new surfaces with antibacterial properties.⁴³ Many coatings developed so far have employed releasing bactericidal agents such as silver ions and antibiotics. However, the release-based antibacterial coatings often face the challenges in controlled release, multi-functionality, and long-term stability, although the silver coatings have been reported with long term stability.^{44,45} Furthermore, a problem with many similar bactericidal surfaces is the attachment of dead microorganisms remaining on the antibacterial coating, which can trigger immune response and inflammation, as well as block a coating's active functional groups.³ A unique attribute of NO releasing materials is that they can significantly reduce bacterial adhesion and prevent mature biofilm formation when released from the biomaterial surface, even at very low concentration. This may allow the bacteria to be susceptible to conventional antibiotics treatment and improve patient outcomes. The impregnation of SNAP into polyurethane films has shown long-term controlled release of NO up to 38 d in buffer (for 15% SNAP-PU, see Figure 4), indicating the potential to control bacterial adhesion after long term exposure in an aqueous environment. However, this study focused on the initial bacterial responses to NO releasing polyurethane biomaterial surfaces, and successfully demonstrated reductions in bacterial adhesion on surfaces within 1 h and decreases in viability of cells on surfaces with 2 d of exposure. It will be interesting to extend these studies to further time points, but such long term device studies are beyond the scope of this set of experiments.

Conclusion

A novel dual functioning CarboSil polymer surface with combined texturing and NO release was prepared by fabricating polymeric films bearing ordered submicron (700/700/300 nm) pillars on the surface, and then subsequently solvent impregnating the films with the NO donor SNAP for long-term NO release. This approach is much more time-efficient and produces more stable and longer-term NO release at physiological conditions than an earlier approach.³⁸ Both surface texturing and NO release alone can significantly reduce bacterial adhesion of *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli*, respectively. However, a synergistic effect is observed between texturing and NO release with respect to the adhesion of *S. epidermidis* and *P. aeruginosa* cells. Such a dual functioning surface should provide a promising new approach to develop novel biomaterial that can reduce bacterial adhesion, and thus can potentially significantly decrease the risk of device-related infections.

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Figure 1. Schematic diagrams of a soft lithography two-stage (A & B) replication molding technique employed to prepare textured CarboSil films, followed by SNAP impregnation of the CarboSil films.

Figure 2. 2D AFM images of textured PU surface before (A) and after (B) impregnation of SNAP, and bearing analysis of sample before (C) and after (D) impregnation of SNAP (scale bar = 5 μm). Bearing analysis generates a histogram of feature height based upon the occurrence of pixels at various heights over a sample and reveals how much of a surface lies above or below a given height. The red and green cursors represent the height levels of surface features with maximum bearing area histograms near the base and the top, respectively. The distance between two cursors represents an average height of the pillars.

Figure 3. Water contact angle of smooth and textured (700/700/300 nm pattern) CarboSil films with different concentrations (0, 5, 10 and 15 wt%) of SNAP impregnation.

Figure 4.
NO release profiles at physiological conditions for 5 wt% (A), 10 wt% (B) and 15 wt% (C) SNAP-impregnated CarboSil films with 700/700/300 nm textures.

Figure 5. StreamLine HR Raman mapping results for fitting of the cross section of A) 5 wt% SNAP-doped CarboSil films, and B) 5 wt% SNAP-impregnated CarboSil films, under 50× objective. Green areas represent the distribution of crystalline SNAP, which is less uniform in the 5 wt% SNAP-impregnated CarboSil films.

Figure 6. Summary of bacterial adhesion on CarboSil PU films; (a) *P. aeruginosa*; (b) *S. aureus*; (c) *S. epidermidis*; and (d) *E. Coli* under near static condition at 37 °C for 1 h, represented as the number of adhered bacteria per 10⁴ μm² of polymer film.

Figure 7. Optical density of bacterial culture medium measured at 600 nm wavelength after being incubated with various Carbosil films for 24 and 48 hrs. (a) *S. epidermidis*, (b) *S. aureus*, (c) *P. aeruginosa*, and (d) *E. coli*. (*: p<0.01)

Figure 8. Representative AFM images of *S. epidermidis* adhesion on CarboSil films with different configurations after 1 h incubation (scale bar is 10 μ m). To better observe the bacterial orientation on the textured pattern surface, magnified images are provided in the rightmost column (15 μ m $\times$ 15 μ m scan size).

Figure 9. Representative AFM images of *P. aeruginosa* adhesion on CarboSil films with different configurations after 1 h incubation (scale bar is 10 μm). To better observe the bacterial orientation on the textured pattern surface, magnified images for *P. aeruginosa* (side-view) are provided in rightmost column (15 μm $\times$ 15 μm scan size).

Table 1.

Summary of characteristics of four test bacteria.

Bacteria Strain	Shape	Gram Staining	With or without coagulase	Virulence and % responsible for CRBSIs
<i>S. aureus</i>	grape	positive	coagulase positive	40 % ^{9,10}
				25 % in children ^{46,47}
				22 % ⁴⁸
				20 % is responsible for CLABSIs ⁴⁹ **
				MRSA accounts for >50 % of all <i>S.aureus</i> infection ⁵⁰
<i>S. epidermidis</i>	grape	positive	coagulase negative	1 in 4 <i>S.aureus</i> colonization on IV catheters develop to bacteremia if not treated w/antibiotics ^{46,51}
				< 34 % in children ^{46,47} *
				< 37 % ⁴⁸ *
				< 8 % ¹⁰ *
				< 31 % is responsible for CLABSIs ⁴⁹ **
<i>P. aeruginosa</i>	rod	negative	coagulase negative	22 %, responsible for BSIs in ICU in US, causing 2 billion annually in US ¹²
				16 % ¹⁰
				5.5 % ⁴⁸
<i>E. coli</i>	rod	negative	n/a	8 % ¹⁰
				6 % is responsible for CLABSIs ⁴⁹ **

* The data reported in the papers are for coagulase negative *Staphylococci* (CoNS) in general, in which one of the most important strain is *S. epidermidis*, therefore the % responsible for CRBSIs by *S. epidermidis* is reported as “< % for CoNS”.

** CLABSIs is central line associated blood stream infections

Table 2.

Surface features of Carbosil textured PU films before and after SNAP impregnation as observed by Atomic Force Microscopy. (Scan size for analysis: 25 μ m $\times$ 25 μ m)

SNAP (%)	Size of pillar top surface		Pillar height [*] (nm)	Overall surface roughness	
	Perimeter (nm)	Area (μ m ²)		RMS (R_q) (nm)	R_a (nm)
0	3193 $\pm$ 363	0.722 $\pm$ 0.153	305.8 $\pm$ 12.7	132.7 $\pm$ 10.7	123.4 $\pm$ 9.6
5	3023 $\pm$ 302	0.674 $\pm$ 0.128	231.0 $\pm$ 1.4	92.6 $\pm$ 2.2	84.2 $\pm$ 2.2
10	2861 $\pm$ 315	0.578 $\pm$ 0.122	224.9 $\pm$ 6.4	94.8 $\pm$ 3.3	85.8 $\pm$ 2.8
15	2991 $\pm$ 354	0.654 $\pm$ 0.141	236.5 $\pm$ 5.2	103.8 $\pm$ 0.2	94.6 $\pm$ 0.2

^{*} Measured from bearing analysis of AFM images.

Table 3.

Statistical analysis of the bacterial adhesion results. X1 represents texture and X2 (0, 1, 2, 3) represents 0, 5, 10 and 15 wt% of SNAP in films. The result shows that surface texturing and NO release produced a synergistic effect on bacterial adhesion reduction for *S. epidermidis* and *P. aeruginosa*, while an additive effect was observed on the reduction for *S. aureus* and *E. coli*.

Parameter	Significance			
	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
X1*X2	0.199	0.037	<0.001	0.089
X1	<0.001	0.031	<0.001	<0.001
[X2=0]	<0.001	<0.001	<0.001	<0.001
[X2=1]	<0.001	0.041	<0.001	0.001
[X2=2]	0.013	0.101	0.108	0.492
[X2=3]	0 ^a	0 ^a	0 ^a	0 ^a
[X2=0] * X1		0.049	<0.001	
[X2=1] * X1		0.577	0.004	
[X2=2] * X1	n/a	0.527	0.136	n/a
[X2=3] * X1		0 ^a	0 ^a	

^a. This parameter is set to zero because it is used as reference.

Table 4.

Bacterial viability on various Carbosil films which were incubated in strain media for 48 h. *The inhibition rates were compared to the corresponding control smooth sample without SNAP impregnations.

Strain	Carbosil Samples		Bacteria counts (CFU/mm ²)		Inhibition rates (%)*
	SNAP content	Pattern	Mean	STDEV	
<i>S. epidermidis</i> RP62A	0 %	Smooth	6.61E+07	2.22E+06	N/A
		700/700/300	2.61E+07	3.39E+06	60.4
	5 %	Smooth	1.05E+07	5.61E+05	84.0
		700/700/300	1.79E+07	5.37E+06	72.9
	10 %	Smooth	3.50E+06	9.33E+05	94.7
		700/700/300	2.00E+06	5.93E+05	97.0
	15 %	Smooth	3.69E+06	9.37E+05	94.4
		700/700/300	2.45E+06	7.54E+05	96.3
<i>S. aureus</i> Newman	0%	Smooth	3.38E+08	1.54E+08	N/A
		700/700/300	2.47E+08	1.62E+08	26.9
	10%	Smooth	1.36E+08	7.04E+06	59.9
		700/700/300	1.87E+08	1.84E+07	44.8
	15%	Smooth	1.57E+08	6.23E+07	53.5
		700/700/300	2.25E+08	3.82E+06	33.4
	0 %	Smooth	4.59E+08	1.36E+07	N/A
		700/700/300	3.22E+08	1.21E+07	29.8
<i>P. aeruginosa</i>	5 %	Smooth	2.28E+08	2.99E+07	50.3
		700/700/300	2.78E+08	1.94E+07	39.5
	10 %	Smooth	3.16E+07	4.35E+06	93.1
		700/700/300	2.86E+07	2.11E+06	93.8
	15 %	Smooth	2.71E+07	1.82E+06	94.1
	0%	Smooth	2.11E+07	2.99E+06	N/A
		700/700/300	1.45E+07	2.66E+06	31.1
<i>E. coli</i>	10 %	Smooth	3.48E+06	1.62E+06	83.5
		700/700/300	5.47E+06	1.63E+06	74.0
	15 %	Smooth	5.82E+06	1.15E+06	72.4
		700/700/300	4.10E+06	9.82E+05	80.5