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Biofunctionalization of silicone polymers using poly(amidoamine) dendrimers and a mannose derivative for prolonged interference against pathogen colonization

Analette I. Lopez, Amit Kumar, Megan R. Planas, Yan Li, Thuy V. Nguyen, and Chengzhi Cai*

Department of Chemistry, University of Houston, Houston, TX 77204, USA

Abstract

Despite numerous preventive strategies on bacterial adhesion, pathogenic biofilm formation remained the major cause of medical device-related infections. Bacterial interference is a promising strategy that uses pre-established biofilms of benign bacteria to serve as live, protective coating against pathogen colonization. However, the application of this strategy to silicone urinary catheters was hampered by low adherence of benign bacteria onto silicone materials. In this work, we present a general method for biofunctionalization of silicone (PDMS) as one of the most widely used materials for biomedical devices. We used mild CO₂ plasma to activate PDMS surface followed by simple attachment of generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers to generate an amino-terminated surface that were maintained even after storage in PBS buffer for 36 days. We then covalently attach a carboxy-terminated mannose derivative to the modified PDMS to promote the adherence of benign *Escherichia coli* 83972 expressing mannose-binding type 1 fimbriae. We demonstrated that dense, stable biofilms of *E. coli* 83972 could be established within 48 h on the mannose-coated PDMS. Significantly, this benign biofilm reduced the adherence of the uropathogenic *Enterococcus faecalis* by 104-fold after 72 hours, while the benign bacteria on the unmodified substrate by only 5.5-fold.

1. Introduction

Catheter-associated urinary tract infection (CAUTI) is the most common type of hospital-acquired infection accounting to ~30% of the infections reported by acute care hospitals in the United States and affecting millions of patients worldwide annually [1-4]. Like other nosocomial infections on medical implants, CAUTI involves the formation of pathogenic biofilms on the surface of urinary catheters. Pathogenic bacteria in the biofilms are embedded in a self-produced polymer matrix, which protect themselves against antimicrobial agents, and facilitate the development of resistance to antibiotics [1, 5-8]. To inhibit biofilm formation, one of the most studied strategies includes incorporation of antimicrobial agents to urinary catheters, which are mostly made of silicone polymers [9-13]. While some of the results encouraged further development, many appeared not promising, especially those relevant to long-term catheter use [2, 3, 14]. A great challenge to overcome is to completely prevent biofouling on the catheters, which blocks the

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* Corresponding author. Tel.: 713 743 2710; Fax: 713 743 2701; cai@uh.edu.

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antimicrobial agents and provides a new platform for growing biofilms [2]. While recent studies on antifouling coatings against bacterial adhesion has made impressive progress [9, 15-17], the rapidly increasing pressure of antibiotic resistance and cost for treatment of CAUTI has called to speed up the development of alternative strategies [7, 18].

One alternative strategy is based on bacterial interference using benign biofilms of probiotic bacteria to protect against the attachment and colonization of pathogens [19-24]. An example of probiotic bacteria, also called “friendly bacteria” or “good bacteria”, is the beneficial bacteria found in the human gut [25]. Despite some of the potential safety concerns, such as gene transfer and pathogenicity, it has been proven that probiotics are vital to proper health maintenance [26, 27]. They have been used as dietary supplements and drug against various bowel disease [25, 26], and investigated for preventing CAUTI and bacterial vaginosis [28-33]. In clinical trials, probiotic *E. coli* 83972 have successfully colonized bladders leading to a significant decrease in the frequency of urinary tract infection without notable side effects [22-24, 34, 35]. As compared to antimicrobial coatings that are prone to leaching and fouling [21, 30, 32, 36], the living biofilms of probiotic bacteria grown on catheters actively produce molecules to outcompete a wide range of aggressive pathogens. This unique feature has rendered bacterial interference one of the most promising approaches for preventing CAUTI.

Despite of its great potentials, the effectiveness of the bacterial interference approach relies greatly on the establishment of a high coverage, stable pre-formed benign biofilms on the surface. Pre-inoculation of urinary catheters with *E. coli* 83972 was used to introduce the benign bacteria to the bladder. However, it has been shown that *E. coli* 83972 and several other benign bacteria adhered less and formed biofilms more slowly on silicone surfaces than common uropathogens [37]. Interestingly, the results were the opposite on solid substrates (glass and polystyrene tissue culture plates) [37]. Thus, the utility of the benign biofilms on silicone catheter to outcompete challenge pathogens had been limited to relatively short bacterial interference time (30 min) [38, 39], while prolonged exposure led to detachment and significant decrease in coverage of the benign *E. coli* on the silicone catheter surface [40].

Biofilm formation is enhanced by the presence of adhesins including type 1 fimbriae present on most *E. coli* strains [41]. However, the wild-type *E. coli* 83972 does not express the common type 1 fimbriae because of its incomplete *fim* operon [42]. A transformed *E. coli* 83972 strain expressing type 1 fimbriae (*fim+* *E. coli* 83972) improved the biofilm formation on urinary catheters [43]. Since type 1 fimbriae binds mannose ligands [44], many *E. coli* strains expressing type 1 fimbriae have a high affinity to mannose-presenting surfaces [45-49]. It has also been demonstrated that the adherence of uropathogens is significantly reduced if a biofilm of *fim+* *E. coli* is pre-coated on the substrates [38, 39], including the mannose-modified single crystal silicon substrates. It has also been demonstrated that biofilms of *fim+* *E. coli* 83972 significantly reduced the adherence of. Therefore, we anticipated that covalent modification of silicone catheters would increase the coverage and stability of the biofilms of *fim+* *E. coli* 83972 and improve the efficiency for bacterial interference.

In this work, we developed the first method for modifying inert silicone (PDMS) polymers with a mannose derivative. This versatile method can also be applied for surface biofunctionalization of other silicone-based biomedical devices. Silicones are among the most widely used polymeric materials in medical devices, including orthopedic, ophthalmic and aesthetic devices, catheters, drains and shunts [50-52]. However, silicone surfaces are composed of inert alkylsiloxane moieties, thus need to be activated prior to covalent attachment of biomolecules. We describe herein an efficient method to introduce amino

groups on PDMS surfaces, allowing for attachment of a wide variety of biomolecules. We then covalently attached a mannose derivative to this platform, and evaluated the effect of the coating on the coverage and stability of *fim+* *E. coli* 83972, and the ability of these benign biofilms to inhibit the colonization of an *E. faecalis* uropathogen.

2. Materials and Methods

Our method for functionalization of PDMS surfaces is illustrated in Fig. 1. Overall, it is based on a combination of mild carbon dioxide plasma treatment of PDMS followed by attachment of amino-terminated poly(amidoamine) (PAMAM) dendrimers.

2.1. Preparation of PAMAM-modified PDMS surfaces

2.1.1. Preparation of the PDMS substrates—A 10:1 ratio of PDMS base and curing agent (SYLGARD 184 Silicone Elastomer Kit, Dow Corning, Midland, MI) were mixed thoroughly and allowed to stand for at least 30 min or until no bubbles were detected in the mixture. To prepare a thin layer of PDMS, the mixture was gently poured on top of a clean silicon wafer, and then pressed against a film of octadecyltrichlorosilane (OTS) (Sigma Aldrich, St. Louis, MO) pre-formed on another silicon wafer. The OTS monolayer was assembled by immersing a clean silicon wafer in a solution of 0.1 M OTS in toluene for 30 min, followed by rinsing with toluene and drying with argon. The purpose of using an OTS film was to aid in the peeling of the top silicon layer to expose a relatively flat PDMS surface. The mixture was cured at 110 °C for 1 h. After 1 h, the OTS-modified silicon wafer was peeled and the PDMS surface was put in a vial and heated to 110 °C under vacuum to remove contamination from low boiling point components.

2.1.2. Oxidation by CO₂ plasma and attachment of PAMAM dendrimers—CO₂ plasma oxidation was carried out on the PDMS surface using Harrick plasma cleaner (Model PDC-32G, 100 W) with low power setting (6.8 W). The PDMS surfaces were exposed to CO₂ plasma at different exposure times, 15 s, 30 s, 45 s, 60 s, to provide the plasma-treated PDMS substrates, **P₁₅**, **P₃₀**, **P₄₅** and **P₆₀** (Fig. 1), respectively. The resulting oxidized PDMS were immediately immersed in a solution of 1 mg/mL Generation 5 poly(amidoamine) (G5 PAMAM) dendrimer (Dendritech, Inc., Midland, MI) in phosphate buffered saline (PBS) for 1 h to provide the PAMAM-coated surfaces **PP₁₅**, **PP₃₀**, **PP₄₅** and **PP₆₀** (Fig. 1). All the PAMAM-coated surfaces were washed copiously with Millipore water and dried with argon.

2.2. Characterization of PDMS and PAMAM surfaces

2.2.1. Preparation of the standard for PAMAM density calculations—Silicon substrates evaporated with gold films were cut into 1 × 2 cm² area, and treated with oxygen plasma for 1 min. The gold surfaces were directly immersed in a solution of bis(11-azidoundecyl) disulfide (Sigma Aldrich, St. Louis, MO) in absolute ethanol. After 24 h, the films were washed with absolute ethanol, and dried with a flow of argon. An ellipsometer (Rudolph Research, Auto EL III), operated with a 632.8 nm He–Ne laser at an incident angle of 70°, was used for thickness measurement of the azide-terminated self-assembled monolayer (SAM) on gold assuming a refractive index of 1.45 for the organic layers. At least three measurements were taken for each sample. The ellipsometric thickness of the samples was measured to a constant value (13.0 ± 0.5 Å) prior to XPS measurements.

2.2.2. X-ray photoelectron spectroscopy (XPS)—A PHI 5700 X-ray photoelectron spectrometer was equipped with a monochromatic Al Kα X-ray source ($h\nu=1486.7$ eV) incident at 90° relative to the axis of a hemispherical energy analyzer. The spectrometer was operated both at high and low resolutions with pass energies of 23.5 eV and 187.85 eV,

respectively, a photoelectron take off angle of 45° from the surface, and an analyzer spot diameter of 1.1 mm. High resolution spectra were obtained for photoelectrons emitted from C1s, O1s, Si2p and N1s for PDMS and PAMAM surfaces, and Au4f and S2p for the azide-terminated SAM standard. All spectra were collected at room temperature with a base pressure of 1×10^{-8} . Electron binding energies were calibrated with respect to the C1s line at 284.8 eV (C-C). A PHI Multipak software (version 5.0A) was used for all data processing. The high resolution data were analyzed first by background subtraction using the Shirley routine and a subsequent non-linear fitting to mixed Gaussian-Lorentzian functions. Atomic compositions were derived from the high-resolution scans. Peak areas were obtained after subtraction of the integrated baseline and corrected for sensitivity factors.

2.2.3. Contact-angle goniometry—Advancing contact angles were measured at room temperature and ambient relative humidity using a contact angle goniometer (Ramé-Hart model 100) while the pipet tip is still in contact with the water droplet. Millipore water (pH = 7.0) was dispensed and withdrawn using a Matrix Technologies micro-Electrapette 25. At least three measurements were collected for each sample. Initial contact angle measurements were performed immediately after plasma treatment for the oxidized PDMS surfaces (**P**), while the freshly prepared PAMAM surfaces (**PP**) were first washed with Millipore water and dried with argon to remove weakly bound PAMAM from the surface before taking the measurements.

2.3. Long-term stability of PAMAM surfaces

In order to determine the stability of the PAMAM coatings on PDMS, both PAMAM and untreated PDMS surfaces were stored in a 24-well plate containing 0.5 mL of PBS buffer and monitored with XPS and contact angle measurements during time periods of 7, 15, 22 and 36 days. Prior to all measurements, the samples were thoroughly washed with Millipore water and dried with argon to remove any physisorbed contaminants.

2.4. Preparation of mannose-presenting PDMS surfaces

2.4.1. Synthesis of carboxy-terminated mannose with OEG linker (Mann-COOH)—Triphenylphosphine (36.0 mg, 0.077 mmol) was added to a solution of azide-terminated mannose (**Mann-N₃**) [47] (18 mg, 0.038 mmol) in 1 mL of tetrahydrofuran. The mixture was stirred overnight. Subsequent removal of the solvent followed by purification by column chromatography using 20% methanol in dichloromethane gave the amino-terminated mannose (**Mann-NH₂**) (14.3 mg, 85%). ¹H NMR (300 MHz, D₂O) δ 4.01-3.41 (m, 30 H), 3.17 (s, 1 H); ¹³C NMR (75 MHz, D₂O) δ 99.9, 72.7, 70.5, 69.9, 69.5, 69.4, 69.3, 66.7, 66.4, 60.9, 39.1; MS (ESI) *m/z* calculated for C₁₈H₃₇NO₁₁: 443.5; found: 444.5 ([M + H]⁺).

Maleic anhydride (327 mg, 3.26 mmol) was then added to the resulting **Mann-NH₂** (725 mg, 1.63 mmol) dissolved in 1 mL of tetrahydrofuran and the mixture was stirred overnight. The solvent was removed and purification by column chromatography using 20% methanol in dichloromethane gave the carboxy-terminated mannose (502.4 mg, 57%). ¹H NMR (300 MHz, D₂O) δ 3.95-3.77 (m, 5 H), 3.75-3.61 (m, 26 H), 2.5 (m, 4 H); ¹³C NMR (75 MHz, D₂O) δ 179.7, 175.6, 99.9, 72.7, 70.5, 69.9, 69.5, 69.4, 68.8, 66.7, 66.3, 60.9, 38.9, 31.9, 31.7; MS (ESI) *m/z* calcd for C₂₂H₄₁NO₁₄: 543.6; found: 566.4 ([M + Na]⁺).

2.4.2 Coupling of carboxy-terminated mannose to PAMAM-modified PDMS surfaces—PAMAM-coated surfaces (**PP₁₅**, **PP₃₀**, **PP₄₅** and **PP₆₀**, Fig. 1) were placed on separate wells in a 24-well plate. A solution containing 3 mM **Mann-COOH**, 60 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 30 mM *N*-hydroxysuccinimide (NHS) in Millipore water was prepared. A 0.5 mL of this solution was

used to immerse each of the PAMAM surfaces for 2 h. After 2 h, the substrates were washed with Millipore water and dried with argon to provide the mannose-coated surfaces (**MPP₁₅**, **MPP₃₀**, **MPP₄₅** and **MPP₆₀**, Fig. 1).

2.5. Preparation of bacterial strains

Derivative strains of *Escherichia coli* 83972 expressing type 1 fimbriae (*fim+* *E. coli* 83972) [47] and the challenge pathogenic bacteria, a human bloodstream isolate of *Enterococcus faecalis*, which was transformed with pMB158GFP to express GFP [53], were provided by Dr. Barbara W. Trautner at Baylor College of Medicine. For all bacterial assays, a single colony from each bacterial plate was grown in 50 mL of Luria Bertani (LB) (BD, Franklin Lakes, NJ) media containing appropriate antibiotics (20 µg/mL chloramphenicol (Sigma Aldrich, St. Louis, MO) and 4 µg/mL tetracycline (Sigma Aldrich, St. Louis, MO) for benign *fim+* *E. coli* 83972 and *E. faecalis*, respectively). After overnight incubation, the optical density at 600 nm (OD₆₀₀) was adjusted to 0.25 for each bacterial culture, corresponding to a bacterial concentration of 10⁸ CFU/mL.

2.6. Adherence assay of *E. coli* 83972 and *E. faecalis*

The ability of *fim+* *E. coli* 83972 and *E. faecalis* to adhere to mannose-coated (**MPP**) and PDMS surfaces was assessed by the following adherence assay. Each surface was placed in a 15 mL centrifuge tube containing 10 mL of LB media with appropriate antibiotics, and inoculated with either *fim+* *E. coli* 83972 or *E. faecalis* to obtain a bacterial concentration of 10⁵ CFU/mL (10 µL of the bacterial suspension with an OD₆₀₀ of 0.25 were added to 10 mL of LB in each tube). The surfaces were incubated with the respective bacteria for 18 or 48 h with shaking at 37 °C. After incubation, the surfaces were rinsed three times in PBS prior to mounting on a glass slide for microscope imaging. In addition to the microscopy-based assay, a standard plate-based counting was performed to obtain the viable bacterial counts of *fim+* *E. coli* 83972 that adhered on the surfaces. Thus, the surfaces following the final rinse with PBS were transferred to a 1 mL solution of 0.01% sodium dodecyl sulfate (SDS), and sonicated for 10 min. Serial dilutions from the sonicated bacterial suspension (10⁻¹, 10⁻² and 10⁻³) were prepared and 10 µL of each dilution was plated in duplicates on LB agar with chloramphenicol antibiotic. The plates were incubated at 37 °C, and the bacterial colonies formed were counted after 24 h.

2.7. Bacterial interference assay

Bacterial interference assays were performed with some modifications to an established protocol for *in vitro* bacterial interference [38, 39]. The surfaces were first placed in 10 mL of LB broth containing 20 µg/mL of chloramphenicol antibiotic and 10⁵ CFU/mL bacterial suspension of *fim+* *E. coli* 83972. The benign bacteria were allowed to adhere to the surfaces for a period of 18 or 48 h with shaking at 37 °C. The surfaces with pre-formed *fim+* *E. coli* biofilms were transferred to 10 mL sterile LB (no antibiotics) in separate 15-mL tubes containing 10⁵ CFU/mL bacterial suspension of the challenge *E. faecalis* pathogen. The tubes were continuously shaken at 37 °C for 24 h. After the first 24 h, the surfaces were transferred to new 15 mL centrifuge tubes containing 10 mL of freshly prepared challenge pathogen suspension (10⁵ CFU/mL), and then re-incubated with shaking at 37 °C. This procedure was done three times such that the total time for bacterial interference is 72 h. At the end of the bacterial interference, the surfaces were rinsed three times in PBS, and then imaged using a fluorescence microscope.

2.8. Microscopy imaging of the surfaces

The images from the bacterial adherence and interference assays were obtained using the 40× objective and the reflected brightfield and FITC filters of a Nikon 80i Microscope

(Nikon Instruments, Melville, NY). NIS Elements software (Version 3.0, Nikon Instruments, Melville, NY) and a CoolSnap HQ2 camera (Photometrics, Tuscon, AZ) were used for image acquisition and analysis. Differentiation of the two strains used in the bacterial interference experiment was possible since the *E. faecalis* expresses GFP ($\lambda_{em} = 535$ nm) while the *fim+* *E. coli* 83972 is non-fluorescent. Live/dead imaging was performed by adding 1.5 μ L of 15 μ M propidium iodide, a dye which show red fluorescence for membrane-compromised cell death, on the surfaces having the pre-formed *fim+* *E. coli* 83972 prior to viewing under the microscope.

3. Results and Discussion

3.1. Preparation and characterization of PAMAM surfaces

Oxygen plasma has been commonly used on inert silicone to generate hydrophilic functional groups, primarily silanols on the surface [54-57]. However, O₂ plasma is often accompanied by a fast recovery to full hydrophobic surface. The loss of hydrophilicity over time, known as hydrophobic recovery, is a common phenomenon after plasma treatment of silicone [58, 59]. It is a major problem for surface functionalization of silicone polymers [60-63]. Several mechanisms to explain hydrophobic recovery had been proposed [58, 64], including the migration of the native polymer from the bulk to the surface to replace the fragmented, oxidized polymers on the surface [54, 61]. This phenomenon significantly diminishes the stability of the surface functionalization of silicone. Indeed we initially used O₂ plasma for activation of silicone, but the results were not satisfactory, that is, no detectable presence of oxidized carbon species in the C1s narrow scan spectrum (data not shown). In search for a milder method, we found that the seldom used carbon dioxide plasma might be a potential alternative to activate organic/polymer surfaces [65-69]. Not only that it greatly reduces the degradation of silicone polymer [70], it also tends to generate more carboxylic acids on both alkylsiloxane monolayers [65] and polyethylene surfaces [68, 69]. Moreover, successive CO₂ plasma treatments afforded fully wettable surfaces [68, 70], which is contrary to O₂ plasma. As a plausible explanation for these results, O₂ plasma generates more reactive radical species compared to CO₂ plasma [70], leading to more degradation of the polymer.

We systematically optimized the CO₂ plasma exposure times (Fig. 1) prior to attaching the PAMAM dendrimers. The resulting plasma-treated surfaces were characterized by XPS and water contact angle measurements. The C1s and Si2p narrow scan spectra of the oxidized PDMS were shown in Fig. 2a and 2b, respectively. Evidence of oxidation was observed with the appearance of small peaks at binding energies higher than 284.8 eV in the C1s narrow scan spectra compared to untreated PDMS (Fig. 2a), corresponding to C-O (286.2 eV), C=O (288.0 eV) and O-C=O (289.2eV). On the other hand, silicone was also oxidized as shown by the increase in the peak area (Fig. 2b) towards a higher binding energy (~ 104 eV). Partial degradation of carbon can also be observed as given by the decrease in atomic concentration of carbon. This can be explained by possible removal of the methyl groups during the oxidation of the silicone and replacement by hydroxyl groups. Similar to the report in the literature [58], a fully wettable surface was obtained after 45 s of CO₂ plasma treatment (**P₄₅**) as given by the measured advancing water contact angle ($< 10^\circ$) (Table 1). However, relatively shorter CO₂ plasma exposure times (**P₁₅** and **P₃₀**) led only to partially wettable surfaces (Table 1).

Another novelty of this work is the use of the generation 5 amino-terminated poly(amidoamine) (G5 PAMAM) dendrimers to cross-link the activated silicone polymers on the surface, thereby reducing their diffusion away from the surface. In addition, G5 PAMAM dendrimers possess ~ 128 amino groups on its periphery [71-73], which could be utilized for both immobilization onto the oxidized surface and bioconjugation (Fig. 1). Immobilization of the dendrimers was achieved by simply immersing the activated silicone

substrate in a PBS solution of PAMAM for 1 h. Alternatively, we also attached PAMAM *via* amidation in the presence of activating agents (EDC/NHS). Although the nature of the bonding of the dendrimers on the samples prepared without EDC/NHS remains unclear, it possibly involves a combination of multiple electrostatic interactions and covalent bonding through imine formation as illustrated in Fig. 1.

The surfaces before and after modification were characterized by XPS and water contact angle measurements. The results from XPS confirmed the presence of PAMAM by the appearance of N1s peak at around 400 eV in the wide scan XPS spectra corresponding to both amine and amide nitrogen in PAMAM as well as increase in the amide C=O peak at 288 eV in the deconvoluted C1s narrow scan spectra. The peak areas corresponding to the oxidized carbon species (286.5 and 288 eV, corresponding to C-O and C=O, respectively) in the C1s spectra of the PAMAM surfaces were also observed to increase with increasing plasma treatment time, signifying a greater density of attached dendrimers for longer exposure to CO₂ plasma. The PAMAM surface was determined by XPS using a reference monolayer of bis(11-azidoundecyl) disulfide, which is an azide-terminated alkyl monolayer on gold, with a known density of nitrogen on the film surface (Supplementary information). The chemisorbed PAMAM molecules on the surface was presumed to be a compressed ellipsoidal shape similar to those in a monolayer [74]. Considering the nominal diameter of G5 PAMAM, which is 54 Å, the cross-sectional area occupied by each G5 PAMAM dendrimer is 2290 Å² [75]. Thus, the density of PAMAM to completely cover a 1 cm² area is estimated to be 4.4×10^{12} molecules/cm², and the coverage of the PAMAM on the oxidized PDMS surfaces ranges from 34% to 77%. The advancing water contact angles were obtained right after PAMAM attachment and the results were summarized in Table 1. The values were comparable to a reported initial water contact angle ($40^\circ \pm 2^\circ$) of a generation 4 PAMAM dendrimer with a relatively high coverage (82.2%) [76]. The hydrophilicity of the PAMAM surfaces also increased with the plasma treatment time, which supported a higher PAMAM density for the surface with longer CO₂ plasma exposure (PP₆₀).

3.2. Stability of adsorbed PAMAM on PDMS surfaces

The long-term stability of PAMAM films over 5 weeks in PBS solution was also monitored by XPS and contact angle measurements. Fig. 3a shows a huge drop in PAMAM density within 7 days in PBS, especially for PP₄₅ and PP₆₀, indicating desorption of some PAMAM moieties that were weakly adsorbed on the surfaces. Afterwards, the PAMAM densities were almost constant for over 5 weeks. Supporting these results were the contact angles measured for almost the same period of time. In Fig. 3b, it can be seen that the highest increase in advancing water contact angles was after the first 7 days. These results are consistent with the loss of most of the PAMAM moieties during the first week as observed from the calculated PAMAM densities using XPS. The incremental increase in the water contact angle for the PAMAM surface in the following days may be attributed to the hydrophobic recovery of PDMS. The diffusion of the untreated polymer to the surface from the bulk may have caused a slight covering of the PAMAM adsorbed on the surface rendering the hydrophobicity.

Table 1 shows that even after 36 days, greater than 50% of the PAMAM molecules remained on the surfaces, corresponding to a ~45% surface coverage. It should be noted, however, that the PAMAM density values did not vary with the CO₂ plasma exposure time except for PP₁₅, which had the least initial PAMAM density. These results imply that while a greater amount of carboxylic may presumably form with longer CO₂ plasma treatment time, it was not necessary to attach more PAMAM on the surface. Due to the multiple amino groups in the PAMAM, it could be potentially adsorbed even with a lower concentration of the carboxylic acid on the surface. Surprisingly, the final PAMAM densities were also

comparable to the ones covalently-linked with EDC/NHS (Table S1). Thus, substantial amount of PAMAM coated on oxidized PDMS surface remained stable over a long period of time (36 days) when stored in PBS.

3.3. Bacterial adherence of benign *fim+* *E. coli* 83972 and *E. faecalis*

In order to demonstrate the utility of the PAMAM-coated surfaces as a universal platform for attachment of biomolecules, we have prepared a mannose derivative (**Mann-COOH**) consisting of an oligo(ethylene) glycol (OEG) linker and a carboxylic acid terminal group, to serve as the ligand for the type 1 fimbriae that mediates the attachment of the benign *fim+* *E. coli* 83972 to the surface. OEG was used as a linker to minimize nonspecific adsorption of bacteria to the surfaces while the carboxyl group was for covalent attachment to the amino groups of the PAMAM *via* amidation. The resulting mannose-coated surfaces (**MPP**) were subjected to bacterial adherence assay using both the benign bacteria and the challenge pathogen (*E. faecalis*). After a preliminary screening (data not shown) of the mannose-coated (**MPP**) surfaces derived from different PAMAM surfaces (**PP**), we selected **MPP₄₅** as the mannose-presenting model surface to perform the succeeding bacterial adherence and interference assays. In the first set of bacterial adherence experiments, we observed the attachment of the *fim+* *E. coli* 83972 to **MPP₄₅** surfaces derived from **PP₄₅** surfaces (Fig. 1), which have been freshly prepared or stored in PBS for 40 days and compare the results to the corresponding PDMS control. All the sample surfaces were incubated with a 10^5 CFU/mL concentration of the benign bacteria for 18 h at 37°C. The results were evaluated using a microscopy-based method and the selected brightfield images are shown in Fig. 4a-4d. Using the Nikon Elements software, the results of bacterial counting showed that the adherence of *fim+* *E. coli* 83972 to **MPP₄₅** surfaces (3.7×10^3 *E. coli*/field) was increased by 2.2 fold compared to adherence to unmodified PDMS substrates (1.3×10^3 *E. coli*/field, $*P < 0.001$, T-test). Surprisingly, the results for the **MPP₄₅** surfaces derived from the **PP₄₅** surfaces stored in PBS for 40 days showed no substantial difference from the freshly prepared surfaces for both PDMS and mannose surfaces ($*P > 0.1$, $*P = 0.1$, respectively, T-test). Similarly, the **MPP₄₅** surfaces (3.3×10^3 *E. coli*/field) resulted in better adherence than the PDMS surfaces (1.1×10^3 *E. coli*/field, $*P < 0.001$, T-test). From the earlier results, we have shown that PAMAM densities decrease after storage in PBS for 36 days. However, since the results showed almost comparable attachment of the benign bacteria on the **MPP₄₅** surfaces derived from the freshly prepared and PBS-stored **PP₄₅**, it can be presumed that either a threshold mannose density was reached for bacterial attachment or comparable mannose densities were achieved regardless of the difference in PAMAM coverage. We had also compared the freshly **MPP** surfaces derived from **PP** surfaces stored in PBS for 40 days to their corresponding **PP** surfaces derived from amidation. Enhanced adherence was observed for the **MPP** surfaces than the **PP** surfaces while comparable attachment were seen among the **MPP** surfaces (Fig. S2.).

The relatively short duration of the bacterial adherence (18 h) did not allow the benign bacteria to completely cover the mannose surface. Thus, we extend the incubation time to 48 h to permit further growth of the benign bacteria and to see whether it is capable to form biofilms on the mannose surface during this time. We hypothesize that in order for these benign bacteria to serve as a durable protective coating against the pathogens; they must form a dense (high coverage), stable biofilm on the surface. The results for the 48 h adherence assay are summarized in Fig. 5. Since counting individual bacterium as done in the previous set of experiments was impossible due to crowding and formation of more bacterial aggregates that overlap each other in this case, the percent area covered by the bacterial aggregates per field was taken as the quantitative measure to compare the surfaces. The results showed enhanced adherence of the benign bacteria on the **MPP₄₅** surface (Fig. 5b) compared with the PDMS surface (Fig. 5a) (1.5-fold increase in area, $*P < 0.001$, T-test).

However, compared to the results from the 18 h incubation with the benign bacteria, the enhancement of adherence was lower (1.5-fold *versus* 2.2-fold) during the 48 h adherence assay. We attribute this discrepancy to the limitation of the quantitative measure of the adherence in this case. During the 48 h incubation, while we have seen that the benign bacteria had increased adherence on the PDMS surface, a dense biofilm of benign bacteria was also found in the mannose surface. Most bacteria in the crowded biofilm on the mannose surface was observed to have changed their orientation to the vertical standing up position (circular) from the horizontal lying position (elongated) as mostly seen in the PDMS to maintain contact with mannose. This observation of the vertical orientation of the bacteria is remarkable. It was reported that the vertical orientation enhances motility of the bacteria which facilitates biofilm formation [77]. The difference on bacterial orientation caused an underestimation for bacterial adherence on the mannose surface by a factor of 2-3 as compared to the PDMS surface. In our attempt to have a more accurate quantification of the bacteria on the surface, we performed a plate-based adherence assay on both PDMS and **MPP₄₅** surfaces. A detergent solution (0.01% SDS) and sonication (10 min) were used to detach the benign bacteria from the surfaces. The results are plotted in Fig. 5D. The benign bacteria showed better adherence to **MPP₄₅** surfaces (2.3×10^5 CFU/mL) by 2.3-fold compared with PDMS (1.0×10^5 CFU/mL). This data still represent an underestimate of the adherence on the mannose surface due to strong binding of the bacteria to this surface resulting in incomplete detachment of the bacteria as revealed by microscopic images (data not shown).

Another important concern for an effective bacterial interference is the viability of the benign bacteria on the surfaces. In order to address this concern, we have conducted a simple viability test *via* live/dead imaging using propidium iodide to detect the presence of bacteria with compromised cell membranes. While we have already shown earlier through the plate-based counting method the viable counts of the benign *fim+* *E. coli* on both **MPP₄₅** and PDMS surfaces, this live/dead imaging procedure was performed due to the ease of screening all the **PP** and **MPP** surfaces rather than doing the more tedious plating. Using this simple test, the bacteria having compromised cell membranes (dead bacteria) show red fluorescence at 562-588 nm. The overlay of the red fluorescence with the brightfield images reflects the ratio of the dead to the live bacteria on the surfaces. Results showed that in both PDMS and **MPP₄₅** surfaces, only few bacteria (< 10%) were found to have compromised cell membranes (Fig. 5a-5c), signifying low toxicity to the benign bacteria. PAMAM dendrimers, on the other hand, have been reported to inhibit the growth of Gram negative bacteria [78, 79]. A modified minimum inhibitory concentration (MIC) assay [78] was used to test various concentrations (3.1 - 400 µg/mL) of the PAMAM dendrimer for antibacterial activity against the benign *fim+* *E. coli* 83972. The MIC value obtained is 200 µg/mL, suggesting a very low toxicity for the unmodified PAMAM dendrimer to the benign bacteria. Even these PAMAM dendrimers might increase their local concentration on the surface, similar to **MPP₄₅** and PDMS surfaces, the **PP₄₅** surfaces gave similar results wherein ~10% of the adherent bacteria were found to have compromised cell membranes (data not shown).

Bacterial adherence assay was also performed on uropathogenic *E. faecalis*. These bacteria were found to be commonly associated with implantable device-related infections and thus used in our bacterial interference assay as a challenge organism [38]. The results of the fluorescence-based microscopy assay showed greater adherence of *E. faecalis* to **MPP₄₅** surface compared to PDMS (15.7-fold increase in area, $*P < 0.001$, T-test) (Fig. 6). However, the probable mechanism for the observed preferential attachment of *E. faecalis* to mannose surfaces has yet to be explored.

3.4. Bacterial interference by the benign *fim+* *E. coli* 83972

Bacterial interference were initially performed on surfaces that were pre-coated with the benign *fim+* *E. coli* for a period of one week and two weeks. However, the interference assays were performed at relatively short exposure time with the challenge pathogen (30 min or 24 h incubation). Successful results showed almost no *E. faecalis* attachment on both PDMS and **MPP₄₅** surfaces (Fig. S3). In an attempt to stretch the capabilities of the benign biofilms to prevent the attachment and subsequent colonization of *E. faecalis*, we have subjected the surfaces to a longer 72 h incubation with the challenge pathogen. To ensure that there were no depletion of the nutrients in the media and that enough viable challenge pathogen is present during the length of the assay, the surfaces were transferred to new LB media inoculated with freshly prepared suspension of *E. faecalis* (10^5 CFU/mL) every 24 h. Prior to the 72 h challenge with *E. faecalis*, both **MPP₄₅** and PDMS surfaces were incubated with the benign *fim+* *E. coli* for 18 or 48 h. This comparison (18 *versus* 48 h) was made to establish the importance of creating a high coverage, stable biofilms of benign bacteria prior to bacterial interference. Representative images from the microscopy-based assay and quantification of results are shown in Fig. 7. As a control, the *E. faecalis* was also grown for 72 h on both surfaces but without a pre-established biofilm of the benign bacteria. Upon treatment of the unmodified PDMS and the mannose-modified PDMS surface **MPP₄₅** with *E. coli* 83972 for 18 h, *E. faecalis* adherence was reduced by 2.4-fold (in area of *E. faecalis*, $*P < 0.001$, T-test) and 16.9-fold (in area of *E. faecalis*, $*P < 0.001$, T-test), respectively. Hence, even the coverage was low, the existence of *E. coli* 83972 successfully impeded the growth of *E. faecalis* on both PDMS and mannose surfaces. On the other hand, the bacterial interference was greatly improved when higher coverages of benign *fim+* *E. coli* (~ 50% to almost full area coverage for PDMS and **MPP₄₅**, respectively) were pre-formed on the surfaces after 48 h of adherence. Significantly, such coating of *E. coli* 83972 biofilms resulted in a 5.4-fold (in area, $*P < 0.001$, T-test) decrease of *E. faecalis* adherence on PDMS surface while a 104-fold (in area, $*P < 0.001$, T-test) decrease on the mannose-modified surface **MPP₄₅**. The durability of the benign biofilms during bacterial interference for 72 h reflect their remarkable stability on the mannose-modified surfaces. As a general observation, the surface area coverages of the benign bacteria decreased in the presence of the challenge pathogen. The decrease, however, was much more evident for the PDMS surfaces which did not form stable benign *E. coli* biofilms. For instance, the coverage of the adherent benign bacteria on PDMS was greatly decreased from $(55.1 \pm 3.7)\%$ to $(7.2 \pm 2.8)\%$ during bacterial interference for 72 h. On the other hand, there is only a slight decrease from $(82.5 \pm 1.3)\%$ to $(77.1 \pm 5.9)\%$ coverage of the benign biofilms even after continuously challenging with a high concentration (10^5 CFU/mL) of *E. faecalis* for 72 h. These remarkable results were attributed to the greatly enhanced stability of the benign biofilms on the **MPP₄₅** surfaces compared to PDMS.

4. Conclusions

We have developed a versatile and practical method for biofunctionalization of PDMS based on mild CO₂ plasma treatment followed by simple attachment of PAMAM dendrimer to create an amino-terminated platform with remarkable long-term stability. We then attached a carboxy-terminated mannose derivative to the platform. The resulting mannose-presenting PDMS surfaces enhanced the adherence and biofilm formation of the benign *fim+* *E. coli* 83972. These pre-established biofilms of the benign bacteria were remarkably stable even under challenging by a high initial concentration (10^5 CFU/mL) of the uropathogenic *E. faecalis*. Note that this pathogenic concentration warrants the diagnosis of CAUTI. Furthermore, the benign biofilms reduced the adherence of *E. faecalis* on the mannose surface by 104-fold after 72 h, while the benign bacteria on the unmodified substrate by only 5.5-fold. Our results clearly demonstrated the great importance of the establishment of a

dense, stable biofilm of the benign bacteria prior to bacterial interference. The strategy presented in this work may be extended to modify silicone-based medical devices for preventing pathogenic biofilm formation leading to infection. The bacterial interference approach offers great potential in solving bacterial infection problems, particularly those associated with prolonged use of medical devices where conventional attachment of antimicrobials has been restricted by the development of microbial resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Outline of this work. **(A)** Preparation of mannose-presenting silicone (PDMS) surfaces by CO₂ plasma treatment for 15, 30, 45, 60 s to provide the activated surfaces **P_n**, respectively (n = plasma treatment time), followed by immersion in a solution of G5 PAMAM dendrimers containing ~128 NH₂ groups, leading to the corresponding amino-terminated PAMAM surfaces **PP_n**, and then attachment of **Mann-COOH** to afford the corresponding mannose-modified silicone surfaces **MPP_n**. **(B)** Preparation of benign biofilms of *E. coli* 83972 and demonstration of bacterial interference, *i.e.*, the exclusion of pathogens from the benign biofilm, using *E. faecalis* as a model.

A.

B.

Fig. 2.
(A) C1s and (B) Si2p narrow scan XPS spectra of PDMS and oxidized PDMS (P₁₅-P₆₀) surfaces.

A.

B.

Fig. 3. Monitoring of (A) PAMAM density by XPS and (B) advancing water contact angles of the PDMS and PAMAM (PP) surfaces measured over a period of 36 or 38 days storage in PBS.

Fig. 4. Representative reflected brightfield images of *fim+* *E. coli* 83972 obtained after 18 h incubation on (A, B) PDMS and (C, D) MPP₄₅ surfaces. PDMS were either (A) freshly prepared or (B) stored in PBS for 40 days. MPP₄₅ surfaces were derived from (C) freshly prepared or (D) stored in PBS for 40 days PP₄₅ surfaces. (E) Results represent the mean of at least 2 experiments where 10 fields were imaged for each sample surface.

Fig. 5.

Representative reflected brightfield images of *fim*⁺ *E. coli* 83972 obtained after 48 h incubation on (A) PDMS and (B) MPP₄₅ surfaces. The overlay of the red fluorescence images on brightfield represents membrane-compromised bacteria. (C) Results represent the mean of at least 3 experiments where 10 fields were imaged for each sample surface. (D) Results represent the mean of 3 experiments done in duplicates.

Fig. 6. Representative green fluorescence images of *E. faecalis* obtained after 18 h incubation on (A) PDMS and (B) MPP₄₅ surfaces. (C) Results represent the mean of at least 3 experiments where 10 fields were imaged for each sample surface.

Fig. 7.

Representative overlay of green fluorescence images on brightfield obtained after 72 h incubation of *E. faecalis* (B, C, E, F) with or (A, D) without pre-formed *E. coli* 83972 on (A-C) PDMS and (D-F) MPP₄₅ surfaces. The benign bacteria were grown for (B, E) 18 h or (C, F) 48 h on the PDMS and MPP₄₅ surfaces. (G) Results represent the mean of at least 3 experiments where 10 fields were imaged for each sample surface.

Scheme 1.
Preparation of **Mann-COOH** from **Mann-N₃**.

Table 1

Characterization of PDMS, CO₂ plasma-treated PDMS (**P**) and PAMAM-modified surfaces (**PP**) using XPS and water contact angle measurements.

Surface	^a PAMAM density (E+12 molecules/cm ²)		Advancing water contact angles, °	
	0 days	36 days	0 days	38 days
PDMS			104 ± 1	107 ± 1
P₁₅			< 10	
P₃₀			< 10	
P₄₅			11 ± 1	
P₆₀			28 ± 4	
PP₁₅	1.5 ± 0.1	1.0 ± 0.3	93 ± 3	98 ± 1
PP₃₀	2.9 ± 0.1	1.8 ± 0.2	68 ± 1	90 ± 4
PP₄₅	3.4 ± 0.1	1.9 ± 0.1	54 ± 3	88 ± 3
PP₆₀	3.3 ± 0.1	2.0 ± 0.5	43 ± 1	78 ± 1

^a See Supplementary information for the equation used to estimate the density, assuming that the adsorbed PAMAM molecules are flat with a uniform thickness and that the nitrogen atoms are evenly distributed along the surface.