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Building an Antifouling Zwitterionic Coating on Urinary Catheters Using an Enzymatically Triggered Bottom-Up Approach

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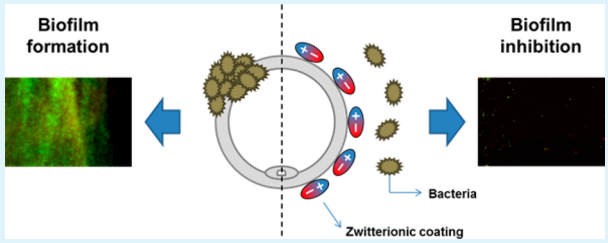
Supporting Information

ABSTRACT: Catheter associated urinary tract infections are common during hospitalization due to the formation of bacterial biofilms on the indwelling device. In this study, we report an innovative biotechnology-based approach for the covalent functionalization of silicone catheters with antifouling zwitterionic moieties to prevent biofilm formation. Our approach combines the potential bioactivity of a natural phenolics layer biocatalytically conjugated to sulfobetaine-acrylic residues in an enzymatically initiated surface radical polymerization with laccase. To ensure sufficient coating stability in urine, the silicone catheter is plasma-activated. In contrast to industrial chemical methods, the methacrylate-containing zwitterionic monomers are polymerized at pH 5 and 50 °C using as an initiator the phenoxy radicals solely generated by laccase on the phenolics-coated catheter surface. The coated catheters are characterized by X-ray photoelectron spectroscopy (XPS), Fourier transformed infrared (FTIR) analysis, atomic force microscopy (AFM), and colorimetrically. Contact angle and protein adsorption measurements, coupled with in vitro tests with the Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* in static and dynamic conditions, mimicking the operational conditions to be faced by the catheters, demonstrate reduced biofilm formation by about 80% when compared to that of unmodified urinary catheters. The zwitterionic coating did not affect the viability of the human fibroblasts (BJ-5ta) over seven days, corresponding to the extended useful life of urinary catheters.



KEYWORDS: *enzymatic polymerization, laccase, antibiofilm coating, urinary catheter, polydimethylsiloxane, artificial bladder*

INTRODUCTION

Biofilms are bacterial communities encased in a self-produced hydrated polymeric matrix. The formation of biofilms include anchoring the microorganisms to a surface followed by a change in gene expression, resulting in a different planktonic state phenotype, and finally secreting adhesive biopolymers to produce the extracellular matrix.[1−3] Such levels of organization and changes at a genetic level provide the bacterial community with a high resistance to both the immune system and antibiotics,[4−6] thus making them a major cause of implant-associated infections. Among the latter, catheter associated urinary tract infections (CAUTIs) related to biofilms are of special relevance as they account for approximately 40% of all hospital-acquired infections and present an incidence of infection of approximately 3−7% of catheterized residents acquiring a new infecting organism every day the catheter remains in situ.[7] Some strategies to face CAUTIs include systematic catheter replacement, which implies patient discomfort and increases treatment costs, or aggressive antibiotic treatments, ultimately inducing drug resistance.[3] The material of choice for catheter production is another key issue as bacteria adhere less to silicone or siliconized catheters than to other catheter materials,[8] especially in short-term catheterization.[9] Despite the general good performance of silicone-based catheters regarding prevention of biofilm formation during short catheterization periods, nonspecific protein adsorption triggers the colonization of the indwelling devices by bacterial communities after prolonged usage. Consequently, different approaches have been developed to minimize the microbial colonization of silicone surfaces, for example, PEGylation[10] coating with modified biopolymers[11] or the use of molecules with antiadhesion properties such as heparin[12] or zwitterionic residues.[13,14] However, polymerization of zwitterionic moieties often entails the use of environmentally hazardous and unstable organic peroxides to initiate the polymerization of their acrylic residues, together with the use of relatively high temperatures, organic solvents, and the need for O_2 removal from the bulk solution.[15−18]

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This study goes beyond the described state-of-the-art by developing an environmentally friendly polymerization process for the building-up of a zwitterionic coating intended to prevent biofilm formation on silicone catheters using laccase as radical initiator. Laccases (E.C. 1.10.3.2) are multicopper oxidases able to oxidize aromatic compounds such as phenols and aminophenols[19,20] with a great potential to be used in white biotechnology owing to the generation of water as the unique byproduct and to their high relative nonspecific oxidation capacity, which may be further increased by the use of mediators.[21] These enzymes use oxygen as electron acceptor to remove protons from the phenolic hydroxyl groups, thus giving rise to radicals that can either rearrange to quinoid structures or spontaneously initiate nonenzymatic coupling to yield dimeric, oligomeric, or polymeric compounds.

In this study, polydimethylsiloxane (PDMS)—the current material of choice for urinary catheters—was plasma-activated and preaminated, allowing subsequent laccase-catalyzed grafting of the natural phenolic compound gallic acid (GA). Subsequently, the tethered GA residues were activated by laccases to phenoxy radicals, triggering an enzymatically initiated radical polymerization of zwitterionic sulfobetaine methacrylate monomers on the silicone catheters in a "grafting from" process. The antibiofilm efficiency of such bioconjugated coating was assessed in vitro in both static and dynamic setups against the Gram-negative (Gram−) *Pseudomonas aeruginosa*, one of the major bacterial species causing nosocomial infections,[22] and against Gram-positive (Gram+) *Staphylococcus aureus*. The stability of the antifouling effect was further evaluated after sterilization and dynamic testing in synthetic urine to mimic the real working conditions of the catheter.

EXPERIMENTAL SECTION

Materials. (3-Aminopropyl)-triethoxysilan (APTES), gallic acid (GA), sulfobetaine methacrylate (SBMA; *N*-(3-sulfopropyl)-*N*-(methacryloxyethyl)-*N*,*N*-dimethylammonium betaine), sodium dodecyl sulfate (SDS), ninhydrin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (Spain). Laccase DENILITE II base was provided by Novozymes (Denmark) and was obtained from *Myceliophthora thermophila* expressed in *Aspergillus oryzae*, 8% w/w protein content, activity 0.083 U/mg at 50 °C, and pH 5, where 1 U was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute (ε_{420} = 36 000 L/(mol·cm)). Polydimethyl/vinilmethyl siloxane (PDMS; designated as VMQ polymer by ASTM D 1418) urinary catheters and flat sheets prepared from the same material were supplied by Degania Silicone Ltd. (Israel).

Bacterial Cultures and Growth Conditions. *P. aeruginosa* (ATCC 10145) and *S. aureus* (ATCC 25923) were grown aerobically at 37 °C in a tryptic soy broth (TSB) purchased from Sigma (Spain). This strain was biofilm proficient.

PDMS Functionalization. PDMS stripes prepared from the same material as the urinary catheters were initially used to allow a proper characterization of the coating otherwise hampered by the curved surface of the catheters. Pieces of 3.5 × 1.8 cm were cut and washed in 0.1% (w/v) SDS solution in deionized water for 30 min with constant stirring. After they were washed with deionized water and 96% ethanol, the PDMS stripes were plasma-treated in a radiofrequency reactor operating at 13.56 MHz using O_2 as plasma gas and a constant flow rate of 15 sccm to generate hydroxyl groups on the PDMS surface at an incident power of 100 W for 5 min. Immediately after plasma treatment, samples were transferred to 100 mL of 5% (v/v) APTES in 96% ethanol for 24 h at room temperature. The samples were finally washed with ethanol to remove the unbound APTES molecules. The availability of amino groups on the PDMS surface was confirmed by immersion of the samples into a 2% (w/v) ninhydrin solution leading to a characteristic color change. In a second step, APTES treated PDMS samples were incubated in a 0.04 M GA solution prepared in 0.1 M succinate buffer (pH 5) containing 4.15 U/mL of laccase in a thermostated laboratory shaker at 50 °C for 3 h at 100 rpm. Thereafter, the samples were washed three times with deionized water and dried under nitrogen. The antioxidant activity, as a proof for the presence of free phenolic groups after the enzymatic grafting of GA, was evaluated by incubation of the sample in 60 μM DPPH solution in methanol.[23] Finally, the zwitterionic SBMA was grafted from the phenolated surfaces upon enzymatically initiated radical polymerization of the acrylic residues. Phenolated silicone samples were incubated in 10 mL of 0.75 M SBMA solution in 0.1 M succinate buffer (pH 5) containing 4.15 U/mL of laccase for 24 h at 50 °C and 100 rpm. Contrary to conventional radical initiated polymerizations, degassing of the reaction media was not performed owing to the dependence of laccases on dissolved oxygen to oxidize their substrates. After the treatment, the samples were thoroughly washed with distilled water and rubbed to detach any unreacted monomer. This coating procedure was repeated on the urinary catheters to be used in the biofilm formation tests.

Attenuated Total Reflectance−Fourier Transformed Infrared (ATR−FTIR) Analysis. ATR−FTIR spectra of pristine and modified silicone samples over the 500−4000 cm^{-1} range were collected by a PerkinElmer Spectrum 100 FTIR spectrometer, performing 100 scans for each spectrum at 4 cm^{-1} resolution.

X-ray Photoelectron Spectroscopy (XPS). XPS analysis was performed on a PHI 5500 Multitechnique System (Physical Electronics) using a monochromatic Al Kα X-ray source (hγ 1486.6 eV) placed perpendicular to the analyzer axis and calibrated using the 3d5/2 line of Ag with a full width at half-maximum (fwhm) of 0.8 eV. The analyzed area was a circle of 0.8 mm diameter, and the resolution chosen for the spectra was 187.5 eV of pass energy, 0.8 eV/step for survey spectra, and 0.1 eV/step for each analyzed element. Binding energies (BE) were referenced by setting the C 1s at 284.8 eV. CASA-XPS version 2.3.14 software was used for data processing.

Atomic Force Microscopy (AFM). The morphology of the different surfaces was investigated by AFM using a Dimension 3100 AFM from Veeco operated in tapping mode. The AFM images were analyzed and postprocessed using the Nanotec WSxM software.[24]

Contact Angle. The contact angle of the differently treated samples was measured with a DSA 100 (Krüss, Germany) contact angle analyzer using Krüss DSA3 v1.0.1.3−02 software. Due to the expected high surface potential of the SBMA treated samples, the captive bubble contact angle method was used.[25]

Protein Adsorption Tests. Before and after incubation in artificial urine prepared following the standard UNE-EN 1616,[26] samples were immersed in a 1 mg/mL FITC-BSA urine solution for 30 min to simulate the immediate process of protein attachment preceding the biofilm formation. After it was rinsed with distilled water and dried with nitrogen, the protein attachment on the surface of the silicone samples was evaluated using a NIKON Eclipse Ti−S fluorescence microscope (Nikon Instruments, Inc., The Netherlands) and a fluorescence microplate reader (Tecan, Infinite M200, Austria). In a second approach, samples were immersed in artificial urine for 1 day at 37 °C and analyzed by scanning electron microscopy (SEM) using a JSM 5610 microscope (JEOL Ltd., Japan). The error bars for each data point were the standard deviation of three independent measurements.

Cell Culture. The BJ-5ta cells (human foreskin fibroblasts, ATCC-CRL-4001) were maintained in 4 parts Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, 1 mM sodium pyruvate, and 1 part of Medium 199, supplemented with 10% (v/v) of fetal bovine serum (FBS), and 10 g/L Hygromycin B at 37 °C in a humidified atmosphere with 5% CO_2, according to the recommendations of the manufacturer. The culture medium was replaced every 2 days. At preconfluence, cells were harvested using trypsin−EDTA (ATCC-30-2101, 0.25% (w/v) trypsin/0.53 mM EDTA solution in Hank's Balanced Salt Solution (HBSS) without calcium or magnesium) and

seeded at a density of 4.5×10^4 cells/well on a 96-well tissue culture-treated polystyrene plate.

Cytotoxicity by Direct Contact. For biocompatibility evaluation, the BJ-5ta cells (human foreskin fibroblasts, ATCC-CRL-4001) were previously seeded at a density of 4.5×10^4 cells/well on a 24−well tissue culture-treated polystyrene plate. For the cytotoxicity assessment by direct contact, the silicone (control) and SBMA-coated silicone were cut into round pieces (1 cm diameter) and placed in contact with the cells. Then, 0.75 mL of complete growth medium (DMEM) was added, and the tested samples were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 1 and 7 days. At the end of each time period, the cells were examined for signs of toxicity using an AlamarBlue assay kit (Invitrogen). A sample of DMEM subjected to the same conditions was used as a negative control, whereas a hydrogen peroxide solution (30% v/v) prepared in fresh culture medium was used as a toxicity positive control.

Resazurin, the active blue ingredient of the kit, is a nontoxic, cell-permeable compound that, once in a viable cell, is reduced to red colored resorufin. AlamarBlue reagent was diluted in the culture medium (1:10), and the mixture was added to each well after the culture medium containing the samples in contact with cells was aspirated. After the samples were incubated for 4 h at 37 °C, the absorbance at 570 nm was measured in a microplate reader, using 600 nm as a reference wavelength. The quantity of resorufin formed is directly proportional to the number of viable cells. The error bars for each data point were the standard deviation of three independent measurements.

Biofilm Formation Test. Biofilm formation on the modified silicone materials was assessed with *P. aeruginosa* and *S. aureus*. Cell cultures were prepared in TSB and incubated overnight at 37 °C and 130 rpm. After the samples were incubated, the optical density at 600 nm (OD_{600}) was adjusted to 0.3. For the static test, three disks (1 cm diameter) of each sample to be analyzed were fixed to the bottom of a 24 well cell culture plate. Afterward, 500 μL of fresh TSB medium and 500 μL of bacteria solution dilution (10^6 CFU/mL determined by spread plate method) were added to each well. Samples were pushed to the bottom of the well and incubated for 18 h at 37 °C without shaking and washed three times in distilled water.

Dynamic tests were performed in an artificial bladder model.[27] After the catheter was inserted, the bladder model was filled up to the catheter's eye hole level with artificial urine inoculated with *P. aeruginosa* or *S. aureus* to an OD_{600} of 0.01 (10^8 CFU/mL). The system was fed with TSB supplemented artificial urine (1 g/L final TSB concentration) at a constant flow of 0.5 mL/min. After 1 week, the catheter was removed, and total biomass was quantified on three different parts, namely, the tip, the balloon, and the portion below the balloon area, referred to in this paper as urethra.

Quantification of Total Biomass Present in Biofilms. Bacterial biofilms were fixed for 60 min at 60 °C and stained with 500 μL of 0.1% (w/v) crystal violet solution for 30 min.[28] After being washed with water until no dye was released, the silicone pieces were placed in vials containing 1 mL of ethanol for 30 min to redissolve the crystal violet dye fixed on the samples. The absorbance of the resulting solutions was measured at 540 nm. The error bars for each data point were the standard deviation of three independent measurements.

Bacterial Viability in Biofilms. Biofilms were analyzed using the LIVE/DEAD BacLight bacterial viability staining kit L7012 (Invitrogen, Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions. After biofilm development, the samples were incubated with 100 μL of the staining solution containing a 1:1 mixture of propidium iodide and SYTO9 dyes. The green-fluorescing SYTO9 ($\lambda_{exc}/\lambda_{em}$, 480/500 nm) is able to stain nucleic acid in both live and dead bacterial cells. However, red-fluorescing propidium iodide ($\lambda_{exc}/\lambda_{em}$, 480/635 nm) is only able to penetrate damaged membranes of dead bacteria, thus quenching the green fluorescence of SYTO9 dye. Consequently, when analyzed under the fluorescence microscope, the viable bacteria in the biofilm fluoresce green, while the damaged ones appear in red in the images.

RESULTS AND DISCUSSION

PDMS Modification and Characterization. *Plasma Preactivation and Amination.* The enzymatically initiated radical polymerization of SBMA on PDMS aimed at providing the material with antifouling capacity was based on a three step-procedure, of which preamination of the silicone with APTES was the first step. This chemisorption procedure has been described by some authors as likely to take place with a thin water layer on the substrate and not only with hydroxyl groups scarcely present on the surface of the material.[29] However, the use of O_2 plasma to generate Si−OH groups[30] on the surface of the material as anchoring points was necessary to ensure the stability of the coating in the urine media, which will be further discussed below.

Ninhydrin tests confirmed the presence of amino groups in the APTES treated samples by the formation of Ruhemann's purple, which was also in the intraluminal section of the catheter (Figure S1, Supporting Information). The amination was further supported by the presence of two dominating vibrational modes found around 1575 and 1485 cm^{-1} and the shoulder at 1330 cm^{-1} in the ATR−FTIR spectra of the samples (Figure 1), assigned to surface amino groups that form

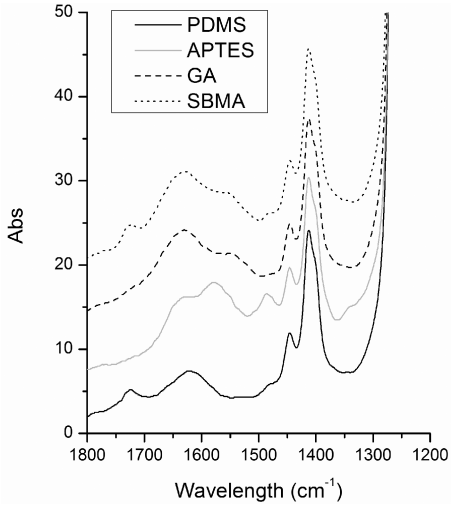

Figure 1. ATR−FTIR spectra of untreated silicone (PDMS), APTES, GA, and SBMA coated samples.

bicarbonate salts with atmospheric CO_2.[31] The oxidation upon air exposure of such salts gives rise to the formation of imine groups responsible for the absorption peak at around 1650 cm^{-1}. The XPS spectrum of the APTES treated samples (Figure 2a) also revealed the presence of free surface amino groups (BE = 399.1 eV, Table 1).[32,33]

Table 1. N 1s Energy Binding of the Deconvoluted High-Resolution XPS Spectra and Functional Group Assignment

	APTES	GA	SBMA
BE (eV)/%	$-NH_2$ (399.1/100)	ar−C═N (399.4/45.5)	ar−C═N (399.4/22.4)
		ar−C−N (400.1/17.4)	ar−C−N (400.1/7.9)
		$C-NH_3^+$ (401.5/37.1)	$C-NH_3^+$ (401.5/21.1)
			$-N^+(CH_n)_3$(402.6/48.6)

Phenolation. The available amino groups were used to immobilize GA on the surface of PDMS. In a previous work, we

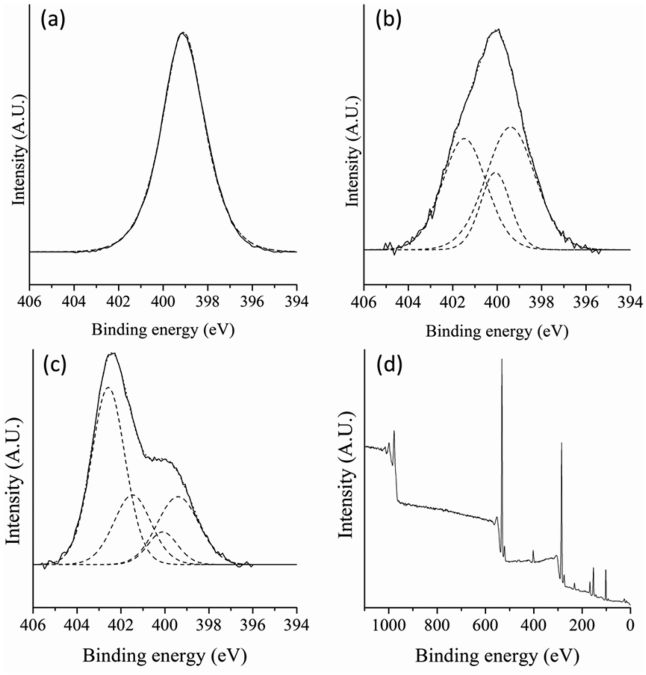

Figure 2. High resolution N 1s XPS spectra of (a) APTES, (b) GA, (c) and SBMA coated PDMS samples and (d) survey spectra of SBMA treated PDMS.

reported the grafting of the phenolic compound catechol on preaminated cellulose fibers using laccase.[34] The rationale behind the process was the enzymatic oxidation of phenols into reactive *o*-quinones, which are prone to undergo nonenzymatic reactions with nucleophiles such as amino groups resulting in Michael addition and/or Schiff-base coupling.[35−38] In the present work, GA—a natural phenolic compound that can be found as part of hydrolyzable tannin family, a group of phenolics with known antibacterial activity[39]—was grafted onto the catheter material. The lack of reactivity of the GA-modified samples toward ninhydrin indirectly confirmed the covalent bonding of GA to the free amino groups present in APTES-modified samples, while the antioxidant capacity of GA-coated PDMS determined in the stable free radical DPPH assay (Figure S2, Supporting Information) further proved the presence of phenolic moieties on the surface of the material. After the GA-coated sample was placed in a 60 μM DPPH solution prepared in methanol, the original violet color (λ_{max} = 517 nm) diminished, accounting for antioxidant properties of the material, as the extent of the reaction depends on the hydrogen-donating ability of the sample.[23] Thus, the DPPH assay indicated (i) the presence of GA on the surface of the samples and (ii) the availability of nonoxidized phenolic groups prone to form phenoxy radicals serving as initiators in the "grafting from" polymerization of acrylic monomers upon oxidation with laccase.

The disappearance of the bands accounting for free amino groups in the ATR−FTIR spectra of the samples (i.e., at 1575, 1485, and 1330 cm^{-1}) coupled with the presence of two main bands at 1650 and 1550 cm^{-1}, which are attributed to the C═N stretching mode of imines[40,41] and the aromatic ring C═C stretching, further supported the presence of GA (Figure 1). The existence of imine groups accounts for the prevalence of Schiff-base reactions between the amino groups of APTES-modified samples and the oxidized GA molecules, whereas a Michael addition reaction is less likely to take place because the only two free carbons in the aromatic ring susceptible to undergo this kind of coupling are sterically hindered.

The analysis of the N 1s core spectra after the grafting of GA (Figure 2b) reveals the disappearance of the peak corresponding to the free nonprotonated amino groups (BE = 399.1 eV) and the formation of three new peaks at 399.4, 400.1, and 401.5 eV attributed to the formation of aromatic imine bonds after Schiff base formation, aromatic C−N bonds due to Michael type addition,[42] and protonation of the remaining free amino groups, respectively. This protonation is a consequence of the high pK_a of APTES (9.6 after incubation in GA solution pH 5).

Although GA does not possess a remarkable antibacterial activity, the present approach serves not only as development of a per se functional material but also as proof-of-concept for the use of other phenolics. This multilayer coating approach would allow combining natural antibacterial activity of plant-derived polyphenols with antifouling activity of zwitterions.

Zwitterionic Functionalization. The zwitterionic coating of the PDMS catheters was based on the radical polymerization of SBMA monomers initiated from phenoxy radicals present on the phenolated silicone surface. The generation of such radicals has already been reported for the coupling of phenolic-based compounds (e.g., lignin or tannic acid to acrylate compounds)[43−46] in a laccase/organic peroxide combined system, but to the best of our knowledge, this is the first time that a methyl methacrylate monomer has been polymerized from a phenolic using solely the radical generation capacity of laccase. The presence of SBMA on the catheters was indirectly indicated by the partial loss of antioxidant activity in the DPPH assay when compared to that of GA modified samples (Figure S2, Supporting Information) due to the loss of a hydrogen atom during the phenoxy radical generation and subsequent polymerization of SBMA monomers from the phenolated surface (Scheme S1, Supporting Information). This was further confirmed by the peak at 1725 cm^{-1} in the FTIR spectrum featuring the C═O stretching of the carbonyl group (Figure 1). The evolution of this peak over time was used to monitor the extent of the polymerization reaction (Table S1, Supporting Information). The presence of sulfur in the XPS survey spectra (BE = 168.0 eV, Figure 2d) along with the presence of a new peak at 402.6 eV in the deconvoluted XPS spectrum (Figure 2c), assigned to the quaternary ammonium group in the SBMA structure,[47] further supported the successful polymerization reaction.

The morphology of the obtained coating was further studied using AFM. The 3D topographic images of the surface (Figure 3) clearly showed that the roughness of PDMS sample (Figure 3a) is decreased upon incubation with GA (Figure 3b) and even further after polymerization of SBMA from the phenolic moieties present on the silicone (Figure 3c). Topographic profiles taken from these images are shown in Figure 3d. Once again, the effect of the modification of the surface with GA and SBMA is clearly visible. The roughness of the different surfaces varies from 3 nm in the case of PDMS to 1.8 nm for GA and finally to 0.3 nm for SBMA. The AFM analysis revealed that the polymerization of SBMA zwitterionic moieties from the plasma-activated and phenolated PDMS surface resulted in a highly homogeneous and continuous coating. It is worth noting that when the coating is built on PDMS without plasma preactivation, the effect is exactly the opposite, that is, the different treatment levels increase the roughness of the surface (Figure S3, Supporting Information). This is explained by the inherent low density of anchoring points naturally present on pristine PDMS surfaces, leading to the formation of isolated polymeric islets. As a result, a homogeneous although not

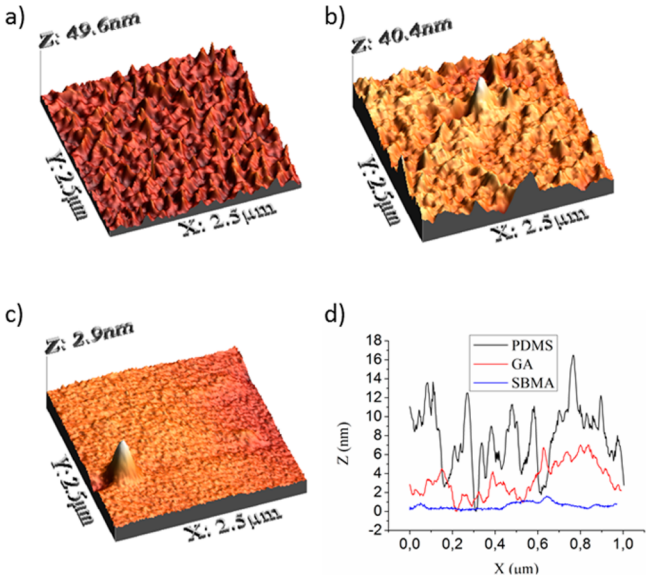

Figure 3. Three-dimensional AFM images of (a) untreated silicone (PDMS), (b) GA-, and (c) SBMA-coated catheter samples. (d) Topographic profiles of these samples.

continuous coating is obtained in the absence of plasma pretreatment.

Initial Protein Attachment. Proteinuria, or the presence of plasma proteins in urine, is a common condition in patients presenting UTIs. Such proteins are capable of adsorbing onto hydrophobic surfaces, such as silicone, serving as a conditioning layer favoring the attachment of bacteria and subsequent biofilm growth. The hydrophilicity (a property required for materials intended to present low organic matter adsorption)[12] of the resulting surfaces after the different modification steps was evaluated with the captive bubble method (Figure 4).

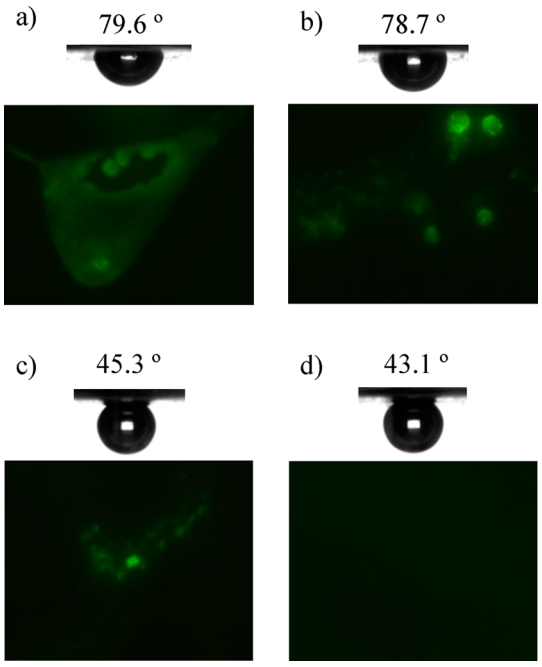

Figure 4. Air contact angle measurement and fluorescence micrographs (magnification 40×) of (a) untreated silicone catheter sample, (b) APTES-, (c) GA-, and (d) SBMA-treated samples. Fluorescence micrographs were taken after incubation in 1 mg/mL of FITC-labeled BSA urine solution.

This technique is advantageous relative to the sessile drop method because the coated silicone samples remain in their wet state, simulating the environment found in the real application of the catheters. Low contact angles found for GA- and SBMA-coated samples are in agreement with the high content of hydrophilic groups (i.e., surface exposed hydroxyl groups and zwitterionic moieties, respectively). Contact angle measurements of samples stored for 5 months gave similar results to freshly prepared samples (43.7°). The polymerization of SBMA residues on the surface, instead of using solely conventional plasma treatments to increase PDMS hydrophilicity, prevented the hydrophobic recovery upon aging samples, a well-known drawback of PDMS plasma-treated surfaces.[48]

Fluorescence microscopy was used to evaluate the protein adsorption on the untreated and treated silicone samples using FITC labeled BSA protein. Pictures a, b, and c (Figure 4) show the fluorescent-labeled protein attached to the blank, APTES-, and GA-treated samples, respectively. On the contrary, protein adhesion on the surface of the zwitterion (SBMA) modified specimen was not observed. These results demonstrate that hydrophilization of the catheter surface is not sufficient to prevent the protein and other organic matter deposition as $\pi-\pi$ interactions between aromatic residues of proteins, and phenolics present in the GA-treated samples led to BSA adsorption, thus forming a conditioning film that has been shown to alter the material surface properties and affect the microbial attachment.[49] The rationale of the nonfouling behavior of zwitterionic materials lies in their capacity to form a hydration layer via electrostatic interaction capable of disrupting hydrophobic interactions between proteins and the surface of the material, thus preventing the formation of a conditioning film and ultimately preventing the colonization of pathogenic bacteria on the catheter. The key factors that determine this electrostatic interaction between zwitterions and water molecules include balanced charge and minimized dipole.[50]

The adhesion of proteins capable of forming a conditioning layer on catheters was also evaluated by SEM (Figure S4, Supporting Information). After the samples were incubated for 1 day at 37 °C in simulated urine (ISO UNE EN 1616)[26] supplemented with peptone, only the pristine silicone showed deposition of protein-salt aggregates, further supporting the antifouling properties of the enzymatically generated zwitterionic coating.

To test the stability and durability of the coating, we incubated samples of SBMA-modified silicone with and without plasma preactivation in artificial urine at 37 °C for up to 7 days. Subsequently, the samples were immersed for 30 min in 1 mg/mL of FITC-BSA urine solution, and the amount of protein adsorbed onto their surfaces was monitored by fluorescence measurements at λ_{exc} = 495 nm and λ_{em} = 525 nm. Figure 5 shows a clear decrease of protein adsorption on SBMA-coated samples compared to the untreated silicone samples before incubation in artificial urine. However, when the samples were immersed in the protein solution after 1, 2, and 7 days of incubation at 37 °C in urine, the antifouling properties of SBMA-coated samples without plasma preactivation diminished progressively. Such loss of the antifouling performance was not observed on the samples coated after the plasma preactivation step. Those samples showed a steady antifouling behavior after 1 week incubation in urine, thus pointing out the positive effect of PDMS plasma preactivation on the stability of the final coating.

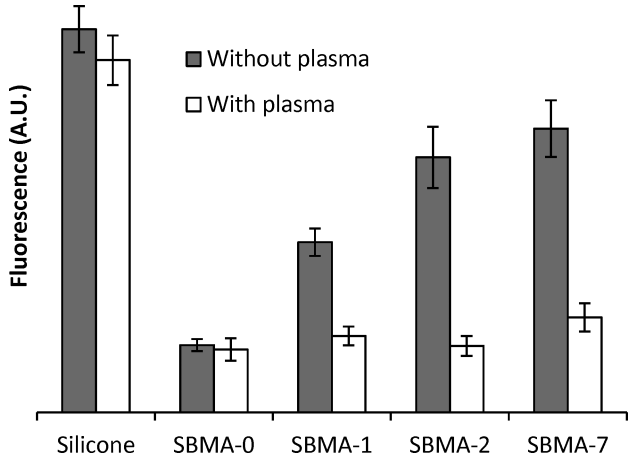

Figure 5. Fluorescence intensity (λ_{exc} = 495 nm; λ_{em} = 525 nm) of the FITC-BSA protein adsorbed on untreated silicone catheter and on SBMA-coated samples before incubation in artificial urine (SBMA−0) and after one (SBMA−1), two (SBMA−2), and seven (SBMA−7) days of incubation in artificial urine at 37 °C.

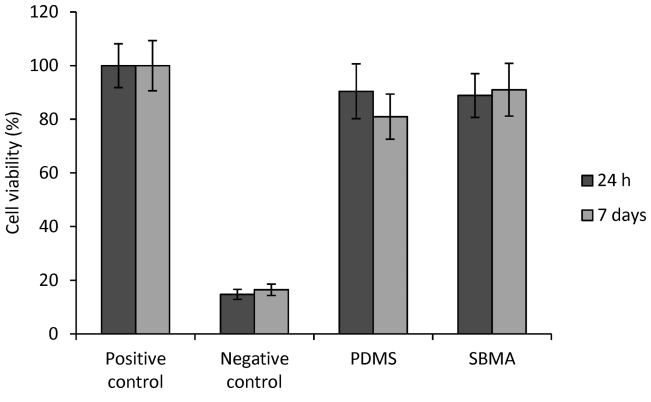

Figure 6. Viability of fibroblasts (BJ-5ta) after being in contact with pristine PDMS and SBMA-coated silicone for 24 h and 7 days.

Biocompatibility Evaluation. The biocompatibility of the SBMA coating is an essential parameter for biomedical applications of the developed material. It is important to ensure that the coatings do not cause adverse effects upon interaction with human cells. The results of the direct contact study following fibroblast incubation with the silicone samples showed no cytotoxicity, regardless of the contact time (Figure 6). Neither 24 h nor 7 days of contact induced cell toxicity, suggesting that the prolonged usage of modified catheters in vivo would not imply any biocompatibility concern.

Biofilm Inhibition. To assess the ability of the coating to inhibit biofilm formation, we carried out two static biofilm tests with *P. aeruginosa* and *S. aureus* by incubating the corresponding bacteria with differently treated catheter samples for 18 h. Though longer incubation time could be considered, in practice, the biofilm formation starts only few hours after catheterization. Thus, avoiding the deposition of microorganisms from the very beginning of the process is of primary importance. Following the incubation, a bacterial viability test was performed, and direct fluorescence microscopy observation of the biofilms enabled the qualitative evaluation of biofilm formation (Figure 7).

After incubation in the presence of *P. aeruginosa*, which shows a higher adherence to silicone catheters than other Gram− bacteria,[7,51] the untreated silicone sample was covered by a dense and continuous bacterial film. Upon incubation with *S. aureus*, another pathogen frequently isolated in patients undergoing urinary tract infections,[52] a similar biofilm structure was observed. The different nuances in the brightness of the images (Figure 7a,d) indicate the organization of the bacteria in multiple layers already forming a three-dimensional biofilm structure with several focal planes at different heights within 18 h (Figure S5, Supporting Information). The GA samples still showed bacteria colonization on the surface; however, according to the microscopy observations, its organization was in only one level, showing a two-dimensional growth typical for early stages of biofilm formation. Nevertheless, this initial

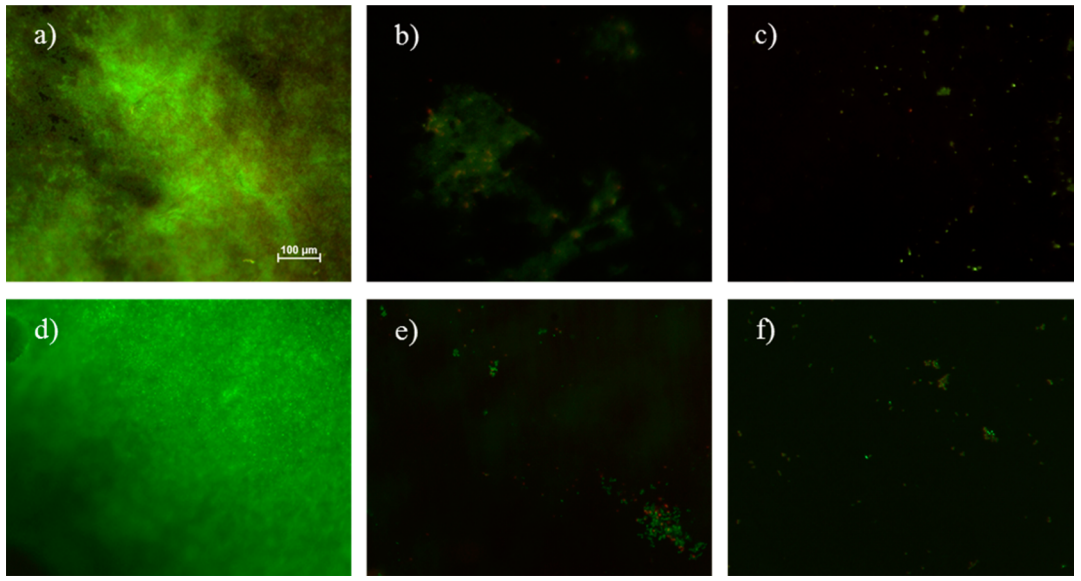

Figure 7. Fluorescence microscopy imaging of (green) live and (red) dead bacteria (a−c, *P. aeruginosa*; d−f, *S. aureus*) after 18 h of incubation of the differently treated silicone catheter samples (magnification, 40×). (a and d) Untreated silicone, (b and e) phenolic-treated silicone, and (c and f) zwitterion-treated silicone.

attachment of the bacteria was in good agreement with the adsorption of proteins on GA-treated surfaces (discussed in the previous section), which is the first event in bacteria attachment and initiation of biofilm formation.[53] In contrast, only a reduced population of bacteria could be observed on the surface of the SBMA-coated material, when compared to pristine PDMS material, due to its nonfouling, antibiofilm properties. Quantitatively, only 35 and 42% of the biofilm attached on the silicone controls was observed on SBMA-treated samples incubated in the presence of *P. aeruginosa* and *S. aureus*, respectively (Figure 8a). The antibiofilm activity of SBMA-coated

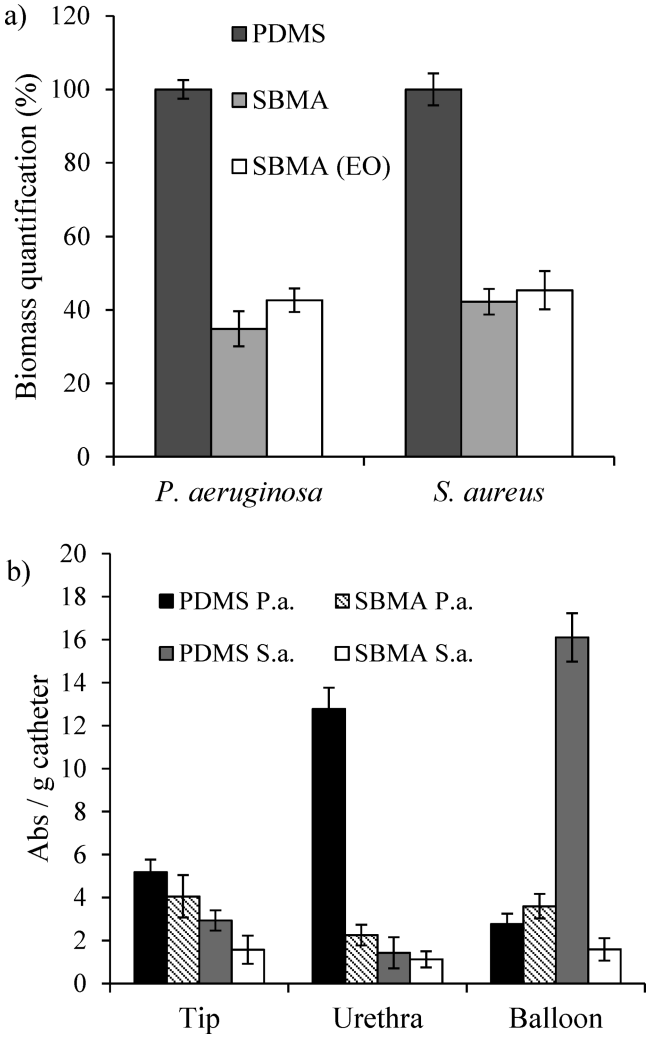

Figure 8. (a) Relative biofilm attachment measured in static conditions on PDMS control and SBMA-modified silicone material before and after EO sterilization after incubation with *P. aeruginosa* and *S. aureus*. Biofilm attached on PDMS control was set as 100%. (b) Biofilm measurement on PDMS control and SBMA-modified catheters after incubation with *P. aeruginosa* (P.a.) and *S. aureus* (S.a.) in dynamic conditions expressed as crystal violet absorbance per gram of catheter.

samples was also assessed after ethylene oxide sterilization (i.e., the usual method industrially employed for sterilization of medical devices), obtaining similar results to those observed for unsterilized samples (Figure 8b).

After static evaluation of biofilm growth, pristine and SBMA-modified silicone catheters were placed in the artificial bladder model for 1 week. The inhibition of biofilm growth on SBMA-coated catheters in a dynamic system mimicking the real usage conditions of the product was comparable to that in static conditions (Figure 8b). Although biofilm prevention in the tip of the catheter after SBMA coating was limited when compared to pristine PDMS catheters currently used in medical practice, this prevention was increased by about 80% in the urethra part in the case of *P. aeruginosa*. This fact can be explained by the preference of this bacteria to form biofilms at the liquid−air interface, like many other aerobic microorganisms.[54] Thus, the overall amount of biofilm present in the PDMS catheter tip was much lower than that present in the urethra part, which constantly presents a liquid−air interface due to the low urine flow. In the case of the facultative anaerobic *S. aureus*, however, the most significant biofilm reduction (about 90%) was found in the balloon of the catheter, which is inflated when the catheter is inside the bladder to hold the whole catheter. This part of the catheter is constantly immersed in urine without being in contact with air, and contrary to *P. aeruginosa*, *S. aureus* is prone to build biofilms under these conditions.

CONCLUSIONS

PDMS urinary catheters were surface functionalized with zwitterionic moieties in a novel enzymatic approach, and their efficiency to prevent biofilm formation was demonstrated. In the first step, PDMS was O_2 plasma preactivated and aminated with APTES, allowing for the subsequent grafting of in situ generated *o*-quinones by oxidation of GA with laccases. The final functionalization step comprised the enzymatic generation of radicals on the GA modified samples, capable of initiating the radical polymerization/grafting of SBMA on the catheter surface at a pH of 5 and a temperature of 50 °C, without the need for any organic peroxide initiator or harsh process conditions. In each step of the process, the modifications obtained on the catheters were evaluated by spectroscopic and colorimetric methods. The resulting biocompatible catheter material showed increased hydrophilicity and reduced protein adsorption, two important parameters governing the formation of bacterial biofilms on medical indwelling devices, along with coating stability upon urine incubation and ethylene oxide sterilization. The biofilm formation on the SBMA-coated samples was reduced by about 80% compared to the biofilm produced on the urethra of uncoated PDMS catheters by *P. aeruginosa* and by about 90% in the case of biofilm produced on the catheter balloon by *S. aureus* in a dynamic setup simulating the real usage conditions of a urinary catheter.

ASSOCIATED CONTENT

Supporting Information

Scheme of the coating on PDMS catheters; evolution of C═O peak intensity at 1725 cm^{-1} over polymerization reaction time; APTES-treated catheter pictures after ninhydrin test; DPPH concentration after incubation with pristine PDMS and APTES-, GA-, and SBMA-coated catheter samples; AFM roughness profiles of PDMS and GA- and SBMA-coated samples; SEM micrographs of untreated and SBMA-coated silicone after incubation in artificial urine; *S. aureus* biofilm fluorescence micrographs showing different focal planes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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