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Targeted Release of Tobramycin from a pH-Responsive Grafted Bilayer Challenged with *S. aureus*

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Abstract

A stimuli-responsive, controlled release bilayer for the prevention of bacterial infection on biomaterials is presented. Drug release is locally controlled by the pH-responsiveness of the bilayer, comprised of an inner poly(acrylic acid) (PAA) monolayer grafted to a biomaterial and cross-linked with an outer chitosan (CH) brush. Tobramycin (TOB) is loaded in the inner PAA in part to minimize bacteria resistance. Because biofilm formation causes a decrease in local pH, TOB is released from PAA and permeates through the CH which is in contact with the biofilm. Antibiotic capacity is controlled by the PAA thickness which depends on PAA brush length and the extent of cross-linking between CH and PAA at the bilayer interface. This TOB-loaded, pH responsive bilayer exhibits significantly enhanced antibacterial activity relative to controls.

Keywords

Bilayer; Stimuli-Responsive Functional Coating; Drug delivery; Tissue Engineering; Antibiotics

INTRODUCTION

Intravenous catheters, endotracheal tubes, prosthetic heart valves, and joint prostheses are medical devices that save millions of patients' lives.^{1–5} However, all implanted devices increase the patients' risk for device-associated bacterial infections with, at a minimum, the associated inflammation, tissue destruction and prolonged antibiotic treatments.^{1–5} These infections arise when microorganisms adhere to the implant and initiate quorum sensing to

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Supporting Information.

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ultimately form a biofilm, a cluster of bacteria embedded within a polysaccharide-rich matrix (Figure 1A). As the biofilm matures, bacteria are disseminated so that the biofilm serves as a nidus of infection (Figure 1A). Formation of this biofilm radically alters antimicrobial therapies as: (1) The adherent bacteria encased within the biofilm are able to evade host defenses.^{1–5} (2) Once adherent, bacteria alter their metabolic status and become recalcitrant to antibiotics.^{1–12} (3) Once encased in a biofilm, this antibiotic recalcitrance is exacerbated by biofilm-based mechanisms where biofilm associated proteins and polysaccharides limit bacterial exposure to antibiotics.^{6–12}

To prevent bacterial adherence (Figure 1AI), antibacterial coatings for contact killing, adhesion resistance, or both, have been developed.^{13–27} To enhance the bactericidal activity of antimicrobial therapies against biofilms, electrical fields and biofilm dispersal agents have been used.^{27–35} In addition, controlled-release systems have been developed that are in clinical use, such as the antibiotic eluting cements used for prosthetic joint infections^{36–38} and silver-impregnated wound dressings for chronic ulcers.^{39–41} Controlled-release polymer coatings have been prepared from polyelectrolyte multilayers (PEMS) using a layer-by-layer processing method.^{42–47} By incorporating a degradable polymer into the PEMS, the rate of erosion has been controlled, allowing prolonged drug delivery.⁴⁶ For example, to treat *S. aureus* bone infection, a PEMS coating was developed that had extended release of gentamicin (70% after 3 days).⁴⁶ Recently layer-by-layer deposition of tannic acid combined directly with cationic antibiotics was examined using dipping and spin-assisted methods on silicon wafers coated with an adhesive priming layer.⁴⁷ This novel application of PEMS uses multiple dip-deposited and spin-deposited steps to build up the film thickness which transitions from smooth to rough at about 35 to 45nm. We emphasize that this multi-deposition approach is distinct from the bilayer explored in the present study. Nevertheless this is a promising approach to prepare antibacterial coatings where cationic antibiotics are mixed and directly coordinated with a complementary anionic small molecule.

Stimuli responsive polymers respond to specific biological stimuli by changes in molecular conformation, structure, and chemistry.^{48–55} For example, responsive polymers have been used for macro and nanoscale drug delivery systems,^{42–47,56,57} and antibacterial and biocompatible implant coatings.^{58–61} By grafting responsive polymers to biomaterials, novel coatings that respond to environmental cues can be designed. For example, a poly(acrylic acid) (PAA) brush collapses upon lowering pH resulting in a thinner layer.^{49–55,62–64} Similarly, we have reported that brushes of chitosan (CH) or quaternary modified chitosan (CH-Q) swell as pH decreases resulting in a thicker layer.^{58,59} The key to this pH response is that PAA is anionic, whereas CH and CH-Q are cationic. Also polymer brushes have been shown to respond to changes in temperature and ionic strength.^{48,62–64} In addition, mixed polymer brushes, i.e., at least two chemically different polymers grafted to the same substrate, show changes of the surface composition and wetting behavior after treatment in different solvents.⁵⁵

Formation of biofilm can create local conditions in microenvironments, substantially different from those in the surrounding solution; the microenvironments in biofilms become more acidic (Figure 1AII), due to the production of acids resulting from bacterial metabolism.^{65–67} The present study introduces a novel bilayer brush (Figure 1B), which is

able to respond to this localized acidification which results from bacterial metabolism.^{65–67} This polymer bilayer brush is comprised of an inner PAA monolayer cross-linked with an outer CH brush that is grafted to planar and tubular oxide and polymer surfaces, respectively. The inner anionic polymer and outer cationic polymer exhibit orthogonal swelling behavior that allows this bilayer brush to serve as a pH-responsive depot for antibiotics. Figure 1B shows the bilayer construction with the end-grafted PAA brush (red) cross-linked to the outer CH (blue), respectively. CH is a natural polymer that inhibits blood coagulation and inflammatory response upon blood contact (i.e., biocompatible)^{60,61} and resists the attachment of bacteria.^{68–70} Chitosan is insoluble at pH 7 and therefore forms a glassy outer layer under physiological conditions.⁵⁸ The inner PAA layer (anionic polymer) is loaded with cationic antibiotics via electrostatic attraction and forms crosslinks with the outer CH layer resulting in a stable bilayer. Tobramycin (TOB), a multi-cationic aminoglycoside antibiotic^{71,72} is chosen as a model drug for loading in PAA. We propose that bacterial colonization and biofilm formation on these polymer brushes initiate localized acidification of the bilayer surface. This acidification causes the initially glassy CH layer to hydrate and swell so that the TOB stored in the PAA layer is able to diffuse into the biofilm and kill bacteria as represented in Figure 1B. Because bacterial adhesion may depend on modulus,⁷³ the viscous CH layer can further impair bacterial adherence and biofilm formation. In this manuscript, we characterize, the pH-responsive bilayer swelling and collapse, TOB uptake, storage, and release, and antibacterial efficacy of the CH/PAA bilayer.

MATERIALS AND METHODS

Materials and Bacterial Strains

N-Type, (100) oriented silicon wafers (CZ silicon, dopant; Ph, 20–30 Ω resistivity) were purchased from Silicon Quest International. QCM sensor crystals, an AT-cut piezoelectric quartz crystal (14 mm in diameter and 0.3 mm thickness) coated with a 50 nm thick layer of silicon dioxide, were purchased from Biolin Scientific, Inc. Chitosan Chitoclear Cg-10 (M_w = 60 kDa and degree of deacetylation = 87%) was received from Primex ehf., Iceland. 3-Aminopropyltriethoxysilane (APTES, 98%), dichlorodimethylsilane (99.5%), chloroplatinic acid (99.9%), tobramycin (TOB), copper (I) bromide (CuBr, 99.9%), copper (II) bromide (CuBr₂, 99.9%), *tert*-butyl acrylate (*t*-BA, 98%), allyl 2-bromo-2-methylpropionate (98%), *N,N,N',N''*-pentamethyldiethylenetriamine (PMDETA, 99%), trifluoroacetic acid (TFA, 99%), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS, 98%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 99%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 99.5%), and anhydrous toluene (99.8%) were purchased from the Aldrich Chemical Co. Ultrapure water (Millipore Direct-Q, 18 ML cm resistivity) was used for the preparation of all solutions. Acetone (Extra Dry, 99.8%, AcroSeal™, ACROS Organics™), Polyurethane (Nalgene 280 PUR Tubing), Trypticase Soy broth (TSB), Bacto™ Agar, Phosphate buffered saline (PBS), chloramphenicol, penicillin, and Kirby Bauer discs were purchased from Fisher Scientific. *S. aureus* strains ATCC® 25923™ (ATCC, Manassas, VA.) and AH1710 (kind gift from A. Horswill) were used in bacterial experiments.

Preparation of the Bilayer, Comprised of an Inner Polyacrylic Acid (PAA) Monolayer Cross-linked with an Outer Chitosan (CH) Brush Grafted to Biomaterials

Silicon oxide surfaces (silicon wafers, SiO₂-coated QCM sensor crystals) were cleaned by immersion in piranha solution, rinsed with ultrapure water, dried with N₂, and exposed to UV-Ozone. An alkyl halide initiator, 3-(chlorodimethylsilyl) propyl-2-bromoisobutyrate, was prepared by chloroplatinic acid catalyzed hydrosilylation of allyl 2-bromo-2-methylpropionate with chlorodimethylsilane according to a procedure described in the literature.^{62–64} Initiator immobilization on silicon oxide surfaces was prepared by immersion of the wafers into a initiator solution in anhydrous toluene at 70 °C for 12 hours under N₂, followed by rinsing in toluene to remove impurities on the surface. To use well-defined PAA polymer brushes with two different high molecular weights, surface-initiated ATRP of *tert*-butyl acrylate (*t*-BA) with added free initiator described in ref 60 and 61 was performed for ~6 hours and ~12 hours, respectively. *Tert*-Butyl acrylate (*t*-BA) was purified by passing through a basic alumina column. Copper(I) bromide was purified by washing sequentially with acetic acid and diethyl ether, filtering and drying, and was stored under vacuum before use. Freshly distilled and deoxygenated *t*BA (350 mL, 2.38 mol), extra dry acetone (250 mL), PMDETA (612 μL, 3.0 × 10⁻³ mol), and the free initiator (moisture-quenched 1-(chlorodimethylsilyl)propyl-2-bromoisobutyrate) were added to the solids (CuBr (322 mg, 2.24 × 10⁻³ mol), CuBr₂ (15 mg, 6.60 × 10⁻⁵ mol)) and the homogeneous green mixture was transferred into a Schlenk flask containing the initiator-functionalized silicon oxide surfaces, that was subsequently heated to 60 °C under nitrogen with stirring. Molecular weight and molecular weight distribution of surface-grafted chains were determined by size exclusion chromatography (SEC) using free *Pt*BA from SI-ATRP of *t*BA with added free initiator. The number-average molecular weight (*M_n*) and the polydispersity index (PDI) of polymer brushes are summarized in Table S1 (SI). For PAA brushes, hydrolysis of *Pt*BA polymer brush was performed by using trifluoroacetic acid (TFA) solution for 12 hours. CH was grafted onto PAA brush via Sulfo-N-hydroxysuccinimide (Sulfo-NHS) ester functionalization of acrylic acid of PAA brush,⁶⁰ followed by quenching the reaction with basic solution (pH ~8, adjusted by using 1N NaOH solution). In addition, PAA coated PU (PAA/PU) and CH grafted PAA/PU (CH/PAA/PU) tubes were prepared using previously published methods.⁶⁰

Surface Characterization

The thicknesses and refractive index (*n*) of dry substrate on the surface were measured by using an alpha-SE ellipsometer (J.A. Woollam Co., Inc., Lincoln, NE) equipped with a wavelength range from 380 to 900 nm (70° angle of incidence). Contact angles were measured by using a 1 μL sessile drop method. Fourier transform infrared (FTIR) spectra of the surface-grafted polymers before and after the hydrolysis of *Pt*BA on silicon wafers were recorded using an attenuated total reflection accessory as a sampling system on a Perkin Elmer infrared spectrophotometer (Spectrum RX I FTIR system) at a resolution of 8 cm⁻¹ averaging 256 scans. Data were analyzed using Omnic E.S.P v5.2 software.

In-Situ SE Measurement

According to the literature,^{59, 74–76} a homemade liquid cell was used to measure the thickness and refractive index (n) of the PAA layer or CH/PAA bilayer on silicon wafer in different pH solutions (the same pH solutions used for QCM-D). In situ SE measurements were carried out using an Alpha-SE ellipsometer. The liquid cell accuracy was verified by measurements of spin-coated polystyrene films according to the literature.^{74–76} The error in the thickness measurement was determined to be within $\pm 3\%$. To understand the pH-dependent swelling behavior of PAA monolayer, the percent swelling was calculated from the dry thickness and the pH-dependent swollen thicknesses determined by in situ SE according to the previously published methods.⁵⁹ The thickness, refractive index (n), and % swelling of the PAA brush are summarized in Table S2 (SI). The pH-dependent thicknesses and refractive index (n) of CH/PAA bilayer were also determined by in situ SE. The percent water content of the CH/PAA bilayer at each pH was calculated from the refractive index (n) according to the previously published methods.⁵⁹ The thickness, refractive index (n), and % water content of the CH/PAA bilayer are summarized in Table S3 (SI).

QCM-D Measurement

The QCM-D measurement is based on the resonance frequency change of a vibrating quartz crystal, a piezoelectric material, when mass is deposited on it. The deposited mass, m , has a relationship with the frequency change, f , according to the Sauerbrey equation,⁷⁷

$$\Delta m = -C \frac{\Delta f_n}{n}$$

where C is the mass sensitivity constant ($C = 17.7 \text{ ng}\cdot\text{cm}^{-2}\cdot\text{Hz}^{-1}$ for an AT-cut, 5 MHz crystal) and n is the vibrational mode number ($n = 1, 3, 5, \dots$). In addition, the dissipation change, D_n , the loss of energy stored in a vibration cycle, indicates the physical characteristics of the deposited layer such as viscosity, elasticity, and so on. If D_n is more than 2.0×10^{-6} , the layer is viscoelastic. The physical properties (thickness, shear modulus, and viscosity) of the viscoelastic layer can be estimated fitting between the QCM-D experimental data (f_n/n and D_n) and a Voigt-based viscoelastic model incorporated in Q-Sense software Q-Tools.^{77–81} On the contrary, if D_n is less than 2.0×10^{-6} , the layer is an elastic film. The physical properties (mass) of the elastic layer can be calculated using the Sauerbrey equation.⁷⁷ First, an E4 QCM instrument (Q-Sense Inc., Gothenburg, Sweden) was used to measure dry areal mass before and after TFA hydrolysis of the PtBA brush. The frequency and dissipation were measured after drying samples I, II, and III as shown in Figure S2 (SI). The data from regions I, II, and III were combined using Q-soft (Q-Sense) in Figure S2 (SI). The grafting densities (ρ) of polymer brushes which were estimated using dry areal masses of the layers are summarized in Table S1. Second, QCM was used for the *in-situ* monitoring of TOB loading at low pH, TOB during storage at pH 7, and TOB release upon returning to low pH in a PAA monolayer and CH/PAA bilayer on SiO_2 -coated QCM sensor crystals in Figure 4. Our prior studies demonstrated that the PAA monolayer and CH/PAA bilayer exhibit reversible characteristics upon cycling the pH from 7 to 4 and back to 7, consistent with stable films.⁸² Solution pH was measured with a dual pH/conductivity

meter (Dever Instrument Co. USA). Solution pH was adjusted by titration with 1 M NaOH and 1 M HCl. pH 4.0 solution containing TOB was prepared by adding 10 μ L of 1 N HCl to 0.3 mM TOB solution (40 mL). All different pH solutions were degassed by sonication. The liquid medium was pumped by peristaltic pump at a rate of 100 μ L/min through a flow cell with the sensor crystal. The temperature of the system was controlled to 21 ± 0.02 °C. Last, to quantify uptake and release of TOB by the PAA monolayers and CH/PAA bilayers, the frequency and dissipation were measured after drying samples at time points denoted I, II, and III in Figure 4, and the areal masses of TOB stored and TOB retained in layers are summarized in Table 1 (Figures S4 and S5 (SI)).

Bacterial Culture

Staphylococcus aureus ATCC® 25923™ or GFP-expressing *Staphylococcus aureus* AH1710 was grown at 37°C with agitation in trypticase soy broth (TSB) (supplemented with 8 μ g/mL of chloramphenicol for maintenance of the GFP-expressing plasmid in AH1710) and 1% glucose. Using a 0.5 McFarland standard (a turbidity standard where a 0.5 McFarland $\sim 1 \times 10^8$ colony forming units (CFU)/mL), *S. aureus* were diluted to 10^8 CFU/mL and further diluted in PBS as indicated.

Detecting Tobramycin (TOB) Elution at Physiologic pH

2 inch sections of tubes were filled with PBS for ~ 24 hours at 37 °C. 20 μ L volumes of PBS containing any eluted antibiotic were then loaded onto blank Kirby Bauer discs, which were dried under sterile conditions at room temperature for 24 hours. Kirby Bauer discs, including a control disc containing TOB or penicillin, were then placed on TSB agar streaked with *S. aureus* ATCC® 25923™ at a concentration of 10^6 CFU/mL. Zones of Inhibition were recorded after 24 hours of growth at 37 °C.

Bacterial Challenge of Tubes

~ 2 in. sections of tube were loaded with 1 mL of *S. aureus* AH1710 and incubated for 12 hr at 37 °C in order to allow biofilm formation on the inner lumen of the tubes. Tubes were then removed and washed lightly, with sterile PBS (3 times) in order to remove non-adherent bacteria. Next, the tubes were sliced horizontally into 2mm-thick, washer-like rounds, and fixed with 4% PFA prior to microscopic examination.

Confocal Laser Scanning Microscopy (CLSM) of Biofilms

Z-stack microscopy of the lumen of the tubes was performed on samples using an Olympus Fluoview 300 (Olympus America Inc., Center Valley, PA) confocal laser scanning microscope. Three dimensional images were reconstructed using a 4 $\times$ lens, with 500 μ m sections at 25 μ m intervals.

Antimicrobial Activity of PAA, TOB Loaded PAA (PAA_{TOB}), CH/PAA, and TOB Loaded CH/PAA (CH/PAA_{TOB}) Polymer Brushes

APTES (as a control), PAA, PAA_{TOB}, CH/PAA, or CH/PAA_{TOB} polymer brush surfaces were challenged with a surface layer of 100 μ L of either 10^4 or 10^5 CFU/mL of *S. aureus* ATCC® 25923™ in TSB for 2 hours, at 37 °C. Next, excess fluid was aspirated from the

surfaces, and the surfaces were swiftly inverted such that the challenged surface made direct contact with fresh TSB agar plate. Plates were then incubated at 37 °C to permit colony growth overnight. After 24 hours, the TSB plates were imaged using an ImageQuant LAS4000 for macroscopic imaging of bacterial colonies present at the polymer/agar interface. For these images, light areas represent bacterial growth, dark areas where no growth was visible. Quantification was performed using ImageJ analysis by image thresholding and determination of percentage white vs black.

RESULTS AND DISCUSSION

Characterization of CH/PAA Bilayer

The CH/PAA bilayer is prepared by first growing a polymer brush from the substrate. As shown in Figure 2, two different molecular weight PAA brushes (PAA^I: $M_n = 134$ kg/mol; PAA^{II}: $M_n = 59$ kg/mol in Supplementary Table S1) were end-grafted to silicon oxide using surface-initiated atomic transfer radical polymerization (SI-ATRP) of *tert*-butyl acrylate (*t*-BA) which was then hydrolyzed using trifluoroacetic acid (TFA) to produce the anionic PAA brushes using published procedures.^{62–64} Attenuated Total Reflectance-FTIR (ATR-FTIR) was used to characterize the surface-grafted polymers before and after the hydrolysis of Poly(*tert*-butyl acrylate) (PtBA). ATR-FTIR results (Figure S1, Supporting information (SI)) are consistent with complete conversion of PtBA to PAA in good agreement with the literature.^{62–64} The grafting densities of two PAA brushes as well as the percent hydrolysis of PtBA brush were estimated by Quartz Crystal Microbalance with Dissipation (QCM-D) (Table S1, Figures S2 (SI)). QCM-D results showed that the dry areal masses of PtBA^I and PAA^I brushes were 31.1 ± 1.1 $\mu\text{g}/\text{cm}^2$ and 17.6 ± 0.5 $\mu\text{g}/\text{cm}^2$, respectively (Table S1 (SI)), which corresponds to 98.8% hydrolysis of PtBA to yield PAA. The grafting densities of PtBA^I and PAA^I were indistinguishable, namely 0.78 ± 0.03 chains/nm² and 0.79 ± 0.02 chains/nm², respectively, confirming that hydrolysis does not detach grafted chains (Table S1 (SI)). The PAA^{II} brush exhibited higher grafting density (grafting density: 0.85 ± 0.03 chains/nm²) than PAA^I.

Spectroscopic ellipsometry (SE) and contact angle measurements of PtBA and PAA brushes yielded the expected values of thickness, reflective index (*n*), and contact angle (Tables S1 and S2 (SI)). Namely, the PAA brush has a lower dry thickness and water-contact angle than the PtBA brush. Using *in-situ* SE, the pH-dependent swelling of the PAA^I brush was then investigated. The thickness was 388 nm at pH 3.8, 369 nm at pH 5.7, 396 nm at pH 7.2, and 475 nm at pH 10.2 (Table S2 (SI)). As pH increased, the estimated percent swelling and water content of the PAA^I brush increased from 281% to 367% and 78% to 93%, respectively (Figure S3 (SI)). Over a pH range of 3.8 to 10.2, the PAA^I brush was thicker and underwent greater swelling relative to cationic brushes of CH and CH-Q.^{58,59} These pH-responsive swelling properties of PAA and CH monolayers were then used to design and synthesize CH/PAA bilayers that served as a biocompatible, pH-controlled antibiotic release system.

To create the CH/PAA bilayer, CH was cross-linked to the previously grafted PAA brush. Figure 2 (bottom) shows that the PAA brushes are first activated by sulfo-NHS ester-functionalization, exposed to CH, and cross-linking then quenched by basic solution (pH ~8,

adjusted by using 1N NaOH solution). Figure 2 shows the molecular structure of PAA after esterification (I) and the cross-link (II) between PAA (red) and CH (blue). For this CH/PAA swelling study, PAA^I brush was used to make CH/PAA^I bilayer. The dry thickness and reflective index (n) values of the CH and PAA^I layers were 13 nm, 1.478 and 52 nm, 1.533, respectively (Table S3 (SI)). Figure 3 shows the *in-situ* thickness and water content as a function of pH (pH range: from 2.6, 5.8, 7.1, to 10.2) measured by *in-situ* SE (Specific values are also given in Table S3 (SI)). Upon increasing pH from 5.8 to 7.1, the CH thickness and water content decreases strongly from 158 nm to 37 nm, and 97% to 48%, as shown in Figures 3a and 3b, respectively. Upon further increasing the pH from 7.1 to 10.2, the CH thickness increases slightly and the water content decreases slightly, suggesting a discrepancy. We attribute this small inconsistency to the interfacial region (Figure 2) which is ignored in the modeling. Similarly, upon lowering the pH from 5.8 to 2.5 the thickness slightly increases whereas the water content remains similar in CH. Thus, the dominant pH response for the outer CH layer is between pH 5.8 and 7.1. In contrast to CH, the thickness and water content of the inner PAA^I layer increases from 113 nm to 290 nm and 45% to 83%, respectively, upon increasing pH from 5.8 to 7.1. Similar to CH, the thickness and water content of PAA^I shows the greatest response to pH in this region. For both layers, swelling and contraction were completely reversible upon cycling the pH between 2.6 and 10.2.

The pH response of CH and PAA^I in the bilayer can be understood by protonation and deprotonation of NH₂ functional groups of CH and COOH functional groups of PAA, respectively. For CH, as pH decreases (i.e., approaches the pK_a of CH, ~6.5), the amines become protonated (NH₃⁺) resulting in electrostatic repulsion and swelling. This pH-dependent swelling of the CH in CH/PAA^I is similar to that previously observed for a CH monolayer grafted directly to silicon oxide.⁵⁸ For the PAA layer in CH/PAA^I, as pH decreases (i.e., approaches the pK_a of PAA, 4.2), the equilibrium between COO⁻ and COOH shifts towards COOH, and therefore the overall charge of PAA^I chains decrease and excluded volume is reduced. This behavior for the PAA^I in the bilayer is similar to that observed for a PAA monolayer (Figure S3 (SI)). Thus, the pH-dependent swelling behavior of the PAA^I brush is orthogonal to the CH layer, i.e., the CH and PAA^I layers in the bilayer exhibit complementary pH-dependent swelling.

Evaluation of TOB Uptake and Release

To evaluate antimicrobial uptake and release (Figure 1B), *in-situ* QCM-D was used to measure TOB loading at low pH, during storage at pH 7, and TOB release upon returning to low pH in a PAA^I monolayer and CH/PAA^I bilayer as shown in Figure 4. After establishing a baseline of 0 at pH 3.8, TOB was introduced into the QCM flow cell at pH 4.0 (arrow 1 in Figure 4a). Correspondingly, the frequency, $f_3/3$, and dissipation, D_3 , of the PAA^I brush decreased (Figure 4a), indicating an increase in mass and rigidity, respectively. The mass increase is due to the uptake of TOB and reaches saturation after 15 min. The increase in the PAA^I brush stiffness is consistent with the multi-cationic TOB (five amine functional groups having pK_a values of 6.2, 7.4, 7.4, 7.6, and 8.6)⁷¹ forming electrostatic crosslinks between the carboxylate anions of PAA^I. This electrostatic cross-linking is extremely fast, occurring within several minutes and is similar to the behavior of a cationic CH brush

exposed to a multi-anion small molecule, such as citrate.⁵⁸ As the pH increases from 4.01 to 7.0 (arrow 2 in Figure 4a), $f_3/3$ decreases slightly, whereas D_3 remains nearly constant, indicating that the TOB initially loaded into PAA^I is retained near physiological pH 7.0. This result suggests that at pH 7, the carboxyl acid groups of the PAA ($pK_a = 4.2$) are predominantly deprotonated. Because these carboxylate anions have a strong electrostatic interaction with the multi-cationic TOB, the film remains thin and rigid. Upon decreasing pH from 7.0 to 3.8 (arrow 3 in Figure 4a), $f_3/3$ increases rapidly during the initial 20 min., and then more slowly for times up to 40 min (arrow III in Figure 4a). This release profile shows diffusion of TOB out of PAA^I at low pH. At pH 4, this result suggests that the carboxylate anions of PAA are partially protonated and that electrostatic interactions between PAA and multi-cationic TOB decrease. The weakening of PAA-drug interaction strength promotes TOB release via diffusion, during which D_3 is constant. This result suggests that the residual multi-cationic TOB retains strong electrostatic crosslinks with anionic carboxylic groups and, correspondingly, the layer remains thin and rigid.

The QCM-D result for the CH/PAA^I bilayer is shown in Figure 4b. On exposing the bilayer to TOB (arrow 1 Figure 4b), the frequency, $f_3/3$, decreases whereas the dissipation value,

D_3 , slightly increases, indicating an increase in mass and only a slight increase in viscoelasticity, respectively. The mass increase is attributed to the facilitated uptake of TOB due to electrostatic cross-linking between PAA^I and TOB. The small increase in viscosity indicates that the outer CH layer is only slightly more swollen and does not adsorb TOB. When pH increases from 4.0 to 7.0 (arrow 2 in Figure 4b), $f_3/3$ increases and D_3 decreases. Similar to that observed for a CH monolayer,⁵⁸ this behavior is attributed to collapse of the CH layer at pH 7. This collapse is due to the insolubility of CH at high pH (above ~6.5) which results in the expulsion of water and the formation of a rigid outer layer.

Upon decreasing pH from 7.0 to 3.8 (arrow 3 in Figure 4b), D_3 increases for the bilayer, indicating that CH re-swells upon returning to pH 3.8. In addition, $f_3/3$ decreases rapidly suggesting a “net” increase in mass due to the swelling of CH. Because TOB release may occur simultaneously with CH swelling, a method to decouple these confounding contributions has been developed as described in the next section. In addition, after releasing drug (arrow III), on re-exposing the TOB solution, $f_3/3$ and D_3 return to the original values of the first exposure of the TOB solution, which means that the drug load, store, and release are reversible.

To quantify uptake and release of TOB by the PAA^I monolayer and CH/PAA^I bilayer, the frequency and dissipation were measured after drying samples at time points denoted I, II, and III in Figure 4 (Figures S4 and S5 (SI)). In addition to PAA^I ($M_n = 134$ kg/mol in Table S1), a lower molecular weight monolayer denoted PAA^{II} ($M_n = 59$ kg/mol in Table S1) was prepared to investigate the effect of PAA chain length (molecular weight) on TOB uptake and release. The areal mass and carboxylic acid areal densities for PAA^I are 17.6 ± 0.5 $\mu\text{g}/\text{cm}^2$ and 1475 ± 44 [COOH]/ nm^2 , whereas for PAA^{II}, they are 8.3 ± 0.2 $\mu\text{g}/\text{cm}^2$ and 695 ± 21 [COOH]/ nm^2 , respectively (Table 1). For PAA^I and CH/PAA^I brushes, the areal TOB mass and areal TOB density after the TOB uptake are $17,231 \pm 236$ ng/cm^2 (222 molecules/ nm^2), and 1865 ± 38 ng/cm^2 (24 molecules/ nm^2), respectively (Table 1 and Figure S4 (SI)). Using carboxylic acid areal density of PAA^I (~1475/ nm^2), a TOB loading

of 222 molecules/nm² is plausible. Namely, if five carboxylic acid groups coordinated with one TOB, which has five amino groups, the maximum loading of TOB into PAA^I would be 295 molecules/nm². Using the same approach, the loading of TOB in CH/PAA^I is 24 molecules/nm², which is more than ten times less than in the PAA^I monolayer. This result suggests that carboxylic acid groups required for coordination with TOB are greatly reduced because of the crosslinking between CH and PAA^I (Figure 2II). By comparison, the areal TOB masses and areal TOB densities at pH 7.0 (point II in Figure 4) after TOB uptake by PAA^{II} and CH/PAA^{II} layers are $5,577 \pm 150$ ng/cm² (72 molecules/nm²), and 1558 ± 26 ng/cm² (20 molecules/nm²), respectively, as shown in Table 1 and Figure S5. Relative to the carboxylic acid groups in PAA^{II} (695 ± 21 [COOH]/nm²), the amount of TOB loaded into PAA^{II} (72 molecules/nm²) is reasonable. As previously mentioned, if five carboxylic acid groups from PAA coordinate with TOB, the maximum loading of TOB into PAA^{II} is 139 molecules/nm².

The amount of TOB released from PAA^I and CH/PAA^I after 30 min exposure to pH 3.8 (point III in Figure 4) was determined. The areal mass and density of residual TOB are $11,560 \pm 122$ ng/cm² (149 molecules/nm²) and 1195 ± 60 ng/cm² (15 molecules/nm²), respectively. Thus, the TOB concentration after 30min decreases by 33% and 36% in PAA^I and CH/PAA^I, respectively. The residual TOB concentrations in PAA^{II} and CH/PAA^{II} after 30 min (point III in Figure 4) are $4,174 \pm 164$ ng/cm² (54 molecules/nm²), and 807 ± 31 ng/cm² (10 molecules/nm²), respectively. Compared with PAA^I and CH/PAA^I, PAA^{II} and CH/PAA^{II} layers contain less TOB after loading and less TOB after drug release. This difference can be attributed to the lower carboxylic acid density of PAA^{II}, suggesting that antibiotic loading can be tuned by controlling the molecular weight of the PAA brush chain via SI-ATRP.

Evaluation of Antibacterial Activity

The ability of TOB loaded PAA and CH/PAA brushes to be stimulated to release drug and kill adherent bacteria was evaluated using two distinct surface samples; polyurethane (PU) tubes and silicon oxide surfaces (silicon wafers and SiO₂-coated QCM sensors). First, to confirm that TOB was not released at physiological pH in the bacterial growth incubator (temperature = 37 °C), disk diffusion bioassays^{83–85} were performed (see Figure S6 (SI)). PU, PAA coated PU (PAA/PU), CH grafted PAA/PU (CH/PAA/PU), TOB loaded PAA (PAA_{TOB}/PU), TOB loaded CH/PAA/PU (CH/PAA_{TOB}/PU) tubes were prepared using previously published methods⁶⁰ and TOB loaded as described above for *in-situ* QCM-D (Figure 4). Tubes were filled with sterilized PBS (pH 7) at 37 °C for 1 hr and bathing solution tested for the presence of TOB as detected by inhibition of bacterial growth. Specifically, Kirby Bauer filter discs^{83–85} were impregnated with undiluted samples, dried and placed on *S. aureus* (ATCC®25923™) inoculated agar plates to determine if TOB was present. Antibiotics would elute out of the disc to inhibit bacterial growth, resulting in a zone of inhibition (ZOI). As shown in Figure S6, the absence of a ZOI from these discs was used to confirm that the TOB release from these tubes remained negligible at pH 7 for PAA_{TOB}/PU and CH/PAA_{TOB}/PU, consistent with QCM-D results showing that TOB loaded PAA and TOB loaded CH/PAA layers did not release the drug at pH 7 (Figure S6 (SI)) (Figure 4).

We then asked if *S. aureus* were able to colonize our materials. PU, PAA/PU, CH/PAA/PU, PAA_{TOB}/PU, and CH/PAA_{TOB}/PU tubes were incubated with $\sim 10^5$ colony forming units/mL (CFU/mL) of *S. aureus* AH1710 (a strain of *S. aureus* containing a plasmid coding for green fluorescent protein)⁸⁶ and incubated at 37 °C overnight. Figure 5 shows representative images of fluorescent bacteria (top row) images merged with bright field images (bottom row) of the adherent bacteria on the surfaces, as imaged by confocal laser scanning microscopy. PU, PAA, CH/PAA tubes all showed green fluorescence, indicating bacterial adherence and growth on these materials. In contrast, no fluorescence was observed from the PAA_{TOB}/PU tube, indicating that PAA_{TOB}/PU killed all bacteria. For the CH/PAA_{TOB}/PU tube, weak fluorescence was present suggesting that even though bilayer loading was almost 10× less than the PAA_{TOB} monolayer, bacteria attachment and growth is greatly limited.

We further evaluated the effects of TOB loading on bacterial colonization of surfaces previously characterized by QCM-D. Amino-propyl-triethoxy-silane (APTES control), PAA^I, PAA^I_{TOB}, CH/PAA^I and CH/PAA^I_{TOB} surfaces were exposed to *S. aureus* (ATCC®25923™) for 2 hr at 37 °C. Figure 6 shows optical microscopy images for surfaces exposed to $\sim 10^4$ (I) and $\sim 10^5$ CFU/mL (II). Although slightly fewer at $\sim 10^4$ CFU/mL, bacteria attached to APTES, PAA^I, and CH/PAA^I, but not to PAA^I_{TOB} at either $\sim 10^4$ or $\sim 10^5$ CFU/mL; CH/PAA^I_{TOB} is only able to prevent colonization at $\sim 10^4$ CFU/mL. Bacterial coverage of the surfaces was measured after growth under a thin film of TSB agar overnight. Percentage of area covered was determined using NIH imageJ software. As shown by the bar graph in Figure 6, after exposure to $\sim 10^4$ CFU/mL (yellow bars), the surface coverages are 57%, 48% and 67% on APTES, PAA^I and CH/PAA^I, respectively. At $\sim 10^5$ CFU/mL, the surface coverages are 88%, 77% and 92% on APTES, PAA^I and CH/PAA^I, respectively. Although chitosan (CH) and chitosan derivatives have been reported to show antibacterial activity,^{58,59,68–70} adhesion of bacteria to CH/PAA^I is greater than to APTES and PAA^I at both low and high concentrations. The outer layer (CH) appears to maintain an environment favorable for biofilm formation and strong bacterial adhesion in neutral medium (pH 7) at an initial stage of bacterial exposure. CH can act as a polycationic biocide, but it shows antibacterial activity only in an acidic medium because of its poor solubility and high rigidity above pH 6.5.^{58,69,70} Water-soluble quaternized chitosan (CH-Q; soluble in both acidic, basic, and physiological circumstances), which has been shown to have antibacterial properties due to its polycationic structure as well as its high swelling property,^{58,59} may serve as a good candidate for the outer layer. Importantly, *S. aureus* colonies did not grow on PAA^I_{TOB} at either concentration of bacteria, indicating that TOB release from PAA^I_{TOB} was sufficient to kill adherent bacteria. For CH/PAA^I_{TOB}, *S. aureus* colonies did not grow at 10^4 CFU/mL, but 46% of the surface is covered at 10^5 CFU/mL. Thus, TOB release from CH/PAA^I_{TOB} was sufficient to eradicate adherent bacteria only at the lower bacterial concentration; percent coverage, however, was decreased below the coverage measured with controls. Importantly, these results demonstrate that antibiotic release from thin monolayer and bilayer grafted layers is sufficient to kill adherent bacteria. In future studies, the activity of the bilayer system will be tuned by varying the loading of TOB.

CONCLUSIONS

We have developed a simple, efficient approach to generate multifunctional grafted polymer bilayers on biomaterials by chemically combining a biopolymer as an outer layer and a synthetic polymer as an inner layer. We have imparted pH-responsive, biocompatible properties to the outer layer, which, in a clinical application is typically in direct contact with biological tissue. We have also designed pH-responsive, drug-tunable uptake and release properties into the inner layer, which, in a clinical application, is typically in direct contact with the biomaterial and could only be accessed through the outer layer.

Antibacterial tests demonstrated that model drug (e.g., TOB) loaded into the bilayer did not release at physiological pH (pH 7), yet with bacterial growth and its accompanying local acidification, the TOB-loaded constructs could be stimulated to release sufficient drug to eliminate bacteria from the biomaterial surface. The amount of drug loaded, which is a tunable property of the brush layers described, was sufficient to eradicate and/or decrease bacterial adhesion, depending on the size of the challenge. Although beyond the scope of the present study, the antibiotic loading capacity can be increased by increasing the thickness of the PAA layer, by increasing the brush length, and by decreasing the cross-linking of CH and PAA at the bilayer interface. The approach to generate multifunctional grafted polymer bilayers on biomaterials is versatile and applicable to stimuli-responsive macroscale drug delivery systems and stimuli-responsive nano-carriers for drug delivery as well as implantable medical devices having direct contact with living tissue for biomedical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Bacterial infection on biomaterials involves (I) bacterial attachment, (II) bacterial colonization and biofilm formation, and (III) biofilm detachment for bacterial proliferation. (B) pH-responsive, drug release polymer bilayer system has an outer layer of chitosan (CH, blue), which provides biocompatibility and hemocompatibility. This layer minimizes blood coagulation and inflammation when a biomaterial comes in direct contact with biological tissue and provides initial resistance against bacterial infection. An inner layer of poly (acrylic acid) (PAA, red) is grown from the biomaterial using surface initiated atomic transfer radical polymerization (SIATRP). The molecular weight can be varied to tune the loading amount of tobramycin (TOB, green) which is electrostatically attracted to the PAA at pH 7. The depicted drug release mechanism is that bacterial colonization and formation of a biofilm on the TOB loaded CH/PAA bilayer causes a local decrease in pH near the infected area (Figure 1B. II). The reduced local pH triggers the outer CH layer to swell and reduces the electrostatic attraction between PAA and TOB (Figure 1B. III). TOB loaded in PAA releases and diffuses into biofilm to kill the bacteria (Figure 1B. IV). In summary, TOB is loaded at pH ~4.5, retained at pH 7 and released at low pH.

Figure 2.

Scheme for surface-initiated atom transfer radical polymerization (SI-ATRP) of *tert*-butyl acrylate (*t*BA) from silicon oxide and hydrolysis of PtBA, followed by grafting CH (blue) onto PAA (red) via (sulfo-NHS) ester functionalization of acrylic acid. Bottom row, far right: The bilayer structure of the end-grafted PAA brush (red), a middle cross-linked region (yellow circles) and CH brush (blue). Cartoons I and II show the PAA functionalization (red) and crosslinking of PAA and CH (yellow), respectively.

Figure 3.

In situ SE brush thickness (a) of CH (blue) and PAA^I (red) in the CH/PAA^I bilayer as a function of solution pH. (b) Percent water content of CH (blue) and PAA^I (red) determined from the reflective indexes of each layer as a function of solution pH. The inner PAA (red) swells at high pH and collapses at low pH. In contrast, the outer CH layer (blue) collapses at high pH and swells at low pH. In the lower images, the double arrows represent the reversible pH-dependent swelling responses of CH and PAA in the bilayer below (left) and above (right) pH 7.

Figure 4.

Traces of $f_3/3$ (blue) and D_3 (red) for PAA^I (a) and CH/PAA^I (b) surfaces versus time upon sequentially changing the solution pH. Arrows 1, 2, and 3 represent the change of pH from 3.8 to pH 4.0 (with TOB), from pH 4.0 (with TOB) to pH 7.0 and from pH 7.0 to pH 3.8, respectively. pH 3.8 and pH 7.0 of the aqueous medium were adjusted by addition of 0.1 N HCl and 0.1 N NaOH under salt free condition; pH 4.0 was prepared by adding 10 μ L of 1 N HCl in 0.3 mM TOB solution (40 mL). Arrows I, II, and III represent the time points

at which the films were dried and areal masses were measured as given in Table 1 and Figure S4 and S5.

Figure 5.

Representative confocal fluorescence images (a), and confocal images merged with bright field images (b) of fluorescent bacteria (*S. aureus* AH1710) on PU, PAA/PU, PAA_{TOB}/PU, CH/PAA/PU and CH/PAA_{TOB}/PU tubes (left to right) after overnight culturing. Each tube was cut open for viewing. Both images (a and b) have the same scale.

Figure 6.

Optical micrograph images of bacterial colonies on APTES, PAA^I, PAA^I_{TOB} (222 TOB molecules/nm²), CH/PAA^I, and CH/PAA^I_{TOB} (24 TOB molecules/nm²) after exposure to ~10⁴ (top row, I) and 10⁵ (bottom row, II) CFU/mL bacterial solutions (*S. aureus* ATCC[®] 25923[™]). The size is 7.255 × 6.765 mm². B) The bar graph shows the surface coverage of bacteria colonies from optical images analyzed using imageJ. Both PAA^I_{TOB} and CH/PAA^I_{TOB} prevent bacterial attachment and growth at ~10⁴ CFU/mL. At 10⁵ CFU/mL, PAA^I_{TOB} prevents bacterial attachment/growth whereas CH/PAA^I_{TOB} shows a lower coverage than all other surfaces.

Table 1

TOB uptake and release by PAA monolayers and CH/PAA bilayers

Mono or Bilayer	TOB Stored		TOB Retained		% TOB Released
	Areal mass ^{a)} (ng/cm ²)	Areal density (molecules/nm ²)	Areal mass ^{a)} (ng/cm ²)	Areal density (molecules/nm ²)	
PAA ^I	17231 ± 237	222 ± 3	11560 ± 122	149 ± 2	33 ± 0
CH/PAA ^I	1865 ± 38	24 ± 0	1195 ± 60	15 ± 1	36 ± 4
PAA ^{II}	5577 ± 150	72 ± 2	4174 ± 164	54 ± 2	25 ± 1
CH/PAA ^{II}	1558 ± 26	20 ± 0	807 ± 31	10 ± 0	48 ± 1

^{a)} Areal mass measured by QCM-D based on dried layers. Mean values and standard deviations calculated from three modes, f_H/n ($n = 3, 5, 7$); PAA^I brush: areal mass = $17.6 \pm 0.5 \mu\text{g}/\text{cm}^2$, dry thickness = $145 \pm 4 \text{ nm}$, $[\text{COOH}]/\text{nm}^2 = 1475 \pm 44$; PAA^{II} brush: areal mass = $8.3 \pm 0.2 \mu\text{g}/\text{cm}^2$, dry thickness = $68 \pm 2 \text{ nm}$, $[\text{COOH}]/\text{nm}^2 = 695 \pm 21$. Methods are described as given in Table S1 and Figure S2, S4, and S5 (SI).