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Reducing *Staphylococcus aureus* biofilm formation on stainless steel 316L using functionalized self-assembled monolayers

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Abstract

Stainless steel 316L (SS316L) is a common material used in orthopedic implants. Bacterial colonization of the surface and subsequent biofilm development can lead to refractory infection of the implant. Since the greatest risk of infection occurs perioperatively, strategies that reduce bacterial adhesion during this time are important. As a strategy to limit bacterial adhesion and biofilm formation on SS316L, self-assembled monolayers (SAMs) were used to modify the SS316L surface. SAMs with long alkyl chains terminated with hydrophobic (-CH₃) or hydrophilic (oligoethylene glycol) tail groups were used to form coatings and in an orthogonal approach, SAMs were used to immobilize gentamicin or vancomycin on SS316L for the first time to form an “active” antimicrobial coating to inhibit early biofilm development. Modified SS316L surfaces were characterized using surface infrared spectroscopy, contact angles, MALDI-TOF mass

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spectrometry and atomic force microscopy. The ability of SAM-modified SS316L to retard biofilm development by *Staphylococcus aureus* was functionally tested using confocal scanning laser microscopy with COMSTAT image analysis, scanning electron microscopy and colony forming unit analysis. Neither hydrophobic nor hydrophilic SAMs reduced biofilm development. However, gentamicin-linked and vancomycin-linked SAMs significantly reduced *S. aureus* biofilm formation for up to 24 and 48 hours, respectively.

Keywords

stainless steel 316L; self-assembled monolayers; *Staphylococcus aureus*; biofilms; gentamicin; oligoethylene glycol

1. Introduction

Stainless steel 316L (SS316L), is a common material used in biomedical applications such as orthopedic pins, plates and hip replacements.[1] Bacterial biofilms formed on the metal oxide surface of such devices can occur upon or after implantation.[2, 3] Since bacteria local to the surgical site are capable of colonizing the implant surface, limiting initial bacterial attachment perioperatively may reduce device-related infection. The pathogens most commonly associated with orthopedic implant infections are staphylococcal bacteria, such as *Staphylococcus aureus*, which are found on skin and in wounds.[3, 4]

Biofilm development begins with the initial attachment of planktonic, free-floating bacteria to a surface.[3, 5] Adhered bacteria undergo phenotypic changes to become sessile organisms which excrete a protective matrix made up of proteins, DNA, carbohydrates, and polysaccharides that promote surface accumulation of bacteria.[6, 7] Biofilm development facilitates bacterial survival under nutrient-poor, stressful conditions, which occur in the host, and results in structurally-complex, heterogeneous bacterial aggregates associated with a surface. Bacteria within biofilms are more resistant to host immune responses and to antibiotics which target dividing bacteria, making biofilm-associated infections a significant therapeutic challenge.[6, 8, 9] Currently, the most effective treatment for biofilm infection may be removal and replacement of the implant device prolonging the pain and immobility of the patient and imparting enormous costs on both the patient and healthcare facility.[2, 10]

Therefore strategies that limit initial bacterial adhesion and subsequent biofilm formation are needed. Such approaches typically involve either passive or active chemical modification of the device surface and include polymer grafting or self-assembled monolayer (SAM) formation.[8, 11–18] Once the appropriate chemistry for stable SAM formation and immobilization is developed, SAMs may be an effective strategy for altering implant surface properties because of their chemical flexibility and ability to control the interface. SAMs can be used to modify surface properties, such as hydrophobicity, or to immobilize catalysts, polymers or small molecules, which may retain bioactivity using the free end of the molecule (the tail group). Ultimately, multiple chemically and/or biologically active molecules can be delivered locally by SAMs formed on the implant material. Moreover, this approach could allow the simultaneous delivery of antibiotics and osteoblast growth factors to promote both a non-fouling, yet integrative surface.

While SAMs have been used to primarily coat gold, glass and silicon, implant materials used in weight bearing joints must offer strength and durability. Materials such as SS316L are coated by a thin, but strongly adhered oxide layer when exposed to air. Our group has demonstrated that SAM formation on alloy and metal oxide substrates requires conditions

for SAM formation that are specific to each substrate.[19] A major advantage is that the strong bond between the bridging molecules in the monolayer and the oxide surface allow for reliable interfacial control.

Passive strategies have included modifying the interfacial properties of an implant, such as hydrophobicity, by grafting polymer chains or forming SAMs on the surface.[14, 20–22] In this approach, the outermost layer of the implant material is modified to influence the implant-tissue interface while preserving the fundamental bulk properties of the material. Varying the SAM tail groups has been shown to control surface characteristics such as wettability, which may influence bacterial adhesion.[22, 23] Previous studies performed on different substrates comparing hydrophobic and hydrophilic surface modifications on substrates such as gold [24, 25], glass [20, 26], silicone [22], and titanium oxide [15, 27] produced conflicting results. Some studies demonstrated that hydrophobic surfaces retarded bacteria adhesion [28–31], while others demonstrated that hydrophilic modifications were more effective in retarding bacteria adhesion indicating that perhaps each substrate requires unique modifications.[14, 18, 32–34].

In contrast to passive coatings, active strategies use molecules such as antibiotics or nitric oxide donors bound to the surface or localized within a polymer coating [35, 36] to limit bacterial adhesion during and after device implantation.[12, 37, 38] Previous studies concerning the immobilization of vancomycin on titanium (Ti) and Ti alloys are promising, but the chemistry for surface immobilization used previously requires the antibiotic to be stable at high temperatures.[39]

In this study, both passive and active approaches were used to test abrogation strategies for biofilm formation on the SS316L surface using SAMs. First, the interfacial properties of the metal oxide surface of SS316L were manipulated using SAMs with different functional tail groups and alkyl chain lengths to determine which passive, nonspecific approach might be appropriate for SS316L. This was accomplished using molecules with hydrophobic methyl tail groups of 18 or 28 carbons and molecules with hydrophilic tri- or penta-(ethylene glycol) pendant groups (Figure 1a). Second, the oxide surface was modified to deliver antibiotics commonly used in orthopedic applications, to specifically retard biofilm formation. Gentamicin or vancomycin was immobilized on the surface of SS316L using strongly-bound carboxylic acid terminated SAMs through a procedure leading to amide bond formation without the need for high temperatures (Figure 1b). In this way, antibiotics were targeted locally, to the site of potential bacterial adhesion and implant infection.[40–42] These strategies may therefore be clinically relevant.

2. Experimental section

2.1. Materials and methods

Gentamicin sulfate (Sigma Aldrich), Vancomycin Hydrochloride (Sigma Aldrich), octadecylphosphonic acid (ODPA, Alfa Aesar), octacosanoic acid (OCA, Sigma Aldrich), 16-phosphonohexadecanoic acid (COOH-PA, Sigma Aldrich) and α -Cyano-4-hydroxycinnamic acid (CHCA, Fluka) were used as received. EG3PA and EG5PA were synthesized as described in Appendix A. 200-proof ethanol (Pharmco), ACS reagent grade methanol (VWR) and acetone (Fisher Scientific) were used as received and optima grade tetrahydrofuran (THF; Fisher Scientific) was distilled over sodium and benzophenone before use. Stainless steel 316L (SS316L) foil of 0.5 mm thickness was purchased from Goodfellow, Inc. (Oakdale, PA US) and prepared as described below.

2.2. SS316L coupon preparation and cleaning

SS316L foil (Fe 66.01%, Cr 19.19 %, Ni 9.17 %, Mn 3.22% and Mo 2.42%) was sanded and cut to 1 cm² coupons as described.[19, 43] The SS316L coupons were cleaned by rinsing in acetone and sonication in methanol for 30 minutes followed by immersion in boiling methanol for 10 minutes to remove organic residue and metal dust from the surface. The clean SS316L coupons were placed in a 100 °C oven to dry for 8–12 hours before further use.

2.3. Self-assembled monolayer formation procedures

2.3.1. Octadecylphosphonic acid (ODPA; Figure 1a)—A 1 mM solution of ODPA was prepared in distilled tetrahydrofuran (THF). SS316L coupons were heated to 100 °C for 24 hours before placement in the ODPA solution for 5 minutes. Coupons were then removed from solution and returned to the oven (100°C) for 24 hours to remove excess solvent before surface characterization.

2.3.2. Octacosanoic acid (OCA; Figure 1a)—A 1 mM solution of OCA was prepared in distilled THF and heated to 50°C on a hot plate. SS316L coupons were cooled on ice (0 °C) for 1 hour and transferred to the heated solution of OCA, which was maintained at 50 °C for 2 hours. The coupons were removed from solution and placed in an oven at 100 °C for 24 hours to remove excess solvent

2.3.3. Triethylene glycol terminated phosphonic acid (EG3PA; Figure 1a)—A 1 mM solution of EG3PA was prepared in methanol. SS316L coupons were heated to 100 °C for 24 hours before placing in the EG3PA solution for 15 minutes. Excess solvent was removed from the coupons as above (2.3.2).

2.3.4. Pentaethylene glycol terminated phosphonic acid (EG5PA; Figure 1a)—A 1mM solution of EG5PA was prepared in methanol. SAMs of EG5PA were prepared on SS316L using the method described above for EG3PA coatings.

2.3.5. Gentamicin and vancomycin-linked SAMs on SS316L—SS316L coupons were placed in a 1mM solution of 16-phosphonohexadecanoic acid (COOH-PA) that was prepared in distilled THF at room temperature for 1 hour. Coupons were removed and placed in an oven at 100 °C for 12 hours to remove excess solvent before characterization. Coupons with stable COOH-PA SAMs were immersed in 2-propanol containing 0.2 M pentafluorophenol (PFP) and 0.1 M 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC). The reaction was stirred on ice while with nitrogen bubbling through for 1 hour. Coupons were removed and dried under vacuum (0.1 Torr) for 30 minutes. Gentamicin or vancomycin was then linked to the COOH-PA SAM by placing the coupons in an absolute ethanol solution containing 2 mg/mL of gentamicin or 2 mg/mL of vancomycin. The reaction was stirred with nitrogen bubbling through for 1.5 hours and coupons were rinsed in ethanol to remove unbound antibiotic. Steps in the antibiotic attachment sequence were confirmed using diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) (below).

2.4. Surface characterization techniques

2.4.1. Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy—A Nexus 470 Fourier Transform Infrared Spectrometer with the diffuse reflectance (DRIFT) attachment was used to confirm the modifications of the surface by analyzing the methylene

and functional group stretching regions of molecules attached to the surface. Each sample was analyzed using 256 scans with 4 cm^{-1} resolution.

2.4.2. Contact angle analysis—Contact angle analysis was used to evaluate the wetting properties of SAMs formed on SS316L. A Video Contact Angle (VCA) 2000 instrument was used to determine the advancing contact angle of deionized water (dH_2O) with the surface. A syringe was used to deposit a $1\text{ }\mu\text{L}$ droplet of dH_2O on the surface and the advancing contact angle (θ) the liquid made with the surface was measured.[44, 45] Contact angles were acquired in three different spots on each of three samples. The mean and standard deviation for each modification were calculated and compared to the control SS316L surface.

2.4.3. Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis—MALDI-TOF MS was used to differentiate between monolayer and multilayer films of COOH-PA, ODPA, EG3PA, and EG5PA.[46] An Agilent Technologies (G3250AA) LC/MSD-TOF mass spectrometry system with model G1969A AP MALDI source was used in positive mode. Spectra were collected using a capillary voltage of 3300 V, fragmentor voltage of 330 V, and skimmer voltage of 60 V. Nitrogen drying gas (325°C) purged the instrument at a rate of 10 L/min. The SAM-modified SS316L coupons were attached to the MALDI target with double sided tape. A matrix solution of 10 mg/mL of CHCA was prepared in distilled tetrahydrofuran and spotted in $1\text{ }\mu\text{L}$ droplets on the modified coupons using a dried droplet method. Three areas on each of three samples were examined.

2.4.3. Atomic force microscopy (AFM)—To determine the surface coverage of antibiotic in the antibiotic-linked SAMs, a Molecular Imaging PicoSPM system was used to image coupons before and after modification. For AFM imaging, SS316L coupons were sanded as described above and additionally polished using a 1.0, 0.3, and $0.05\text{ }\mu\text{m}$ Al_2O_3 suspensions on a micro cloth. The coupons were then cleaned as described above and imaged in noncontact mode with silicon cantilevers (Force constant: 21–98 N/m, Nanosensors Inc.). Three $500 \times 500\text{ nm}$ areas on each of three coupons were examined. Images were thresholded using Gwyddion software in order to calculate the surface area covered by the linked gentamicin. Values obtained for linked gentamicin were compared to the unthresholded image area (0.25 nm^2). Additionally, based on the proposed attachment scheme of gentamicin, molecules in each viewing area consistent with the size of gentamicin (135 nm) were counted to evaluate the extent of reacted surface groups.

2.5. Bacteria adhesion studies

2.5.1. Substrate sterilization—Control SS316L and passive modified coupons (ODPA, OCA, EG3PA, EG5PA) were sterilized by immersion in 70% ethanol for 15 minutes. Coupons linked with gentamicin or vancomycin were removed from the final reaction step described above (200-proof ethanol) under sterile conditions and rinsed in 70% ethanol to remove unbound antibiotic. Coupons were left under sterile conditions until use. DRIFT spectroscopy confirmed that this sterilization procedure did not remove the antibiotic or affect the stability of the SAMs on the surface.

2.5.2. Bacterial strain and growth conditions—Frozen stocks of the osteomyelitis isolate *S. aureus* UAMS-1 OSSA ATCC 49230 (Gillaspay et al. 1995) were plated on tryptic soy agar (TSA) plates and incubated overnight at 37°C and 5% CO_2 . Individual colonies were picked and grown overnight in TSB with gentle shaking. Growth curves (OD and CFUs mL^{-1}) were correlated using regression analysis as described [47] and calibration curves were used to estimate the inoculated number of *S. aureus* cells and validated by

plating. To simulate physiological conditions in the host, the culture inoculum for biofilm challenge of the coupons was diluted with supplemented TSB containing 0.1 M NaCl, 0.08% glucose to the desired optical density (OD_{600}) using a Beckman DU 650 UV-Vis spectrophotometer (Beckman Coulter Inc., Indianapolis, IN US) for all experiments. All experiments were carried out at 37°C and 5% CO_2 .

2.5.3. Assessment of biofilm development

2.5.3.1. Confocal laser scanning microscopy (CLSM): To initially screen surface modifications, *S. aureus* biofilms were visualized directly on the SS surface using the BacLight bacterial viability kit (Invitrogen) and CLSM. Triplicate coupons were incubated in polystyrene Petri dishes (Falcon) containing (2.5×10^8) *S. aureus* mL^{-1} and incubated at 37°C and 5% CO_2 with gentle shaking. After the challenge period, coupons were rinsed with Hank's buffered salt solution (HBSS) with Ca^{+2} and Mg^{+2} to remove planktonic cells and adherent bacteria were stained with BacLight prepared according to the manufacturer's instruction. Coupons were rinsed, covered with fresh HBSS and immediately imaged with a Leica DM RXE microscope attached to a TCS SP2 AOBS confocal system (Leica Microsystems, Exton, PA) using 20× and 63× water immersion lenses and the sequential scanning mode as described.[48, 49] Five images were collected per coupon and experiments were performed in triplicate (N=45) for each time point and analyzed using the Leica LCS software and COMSTAT.[50] COMSTAT thresholding was used to eliminate background noise in the image stacks and thresholded image stacks were individually compared with the original image files to confirm the elimination of noise but not relevant data.[50] The biofilm parameters maximum thickness and biomass were determined for each image stack and averaged over the image area ($2500 \mu m^2$) for each image and further averaged (5 images per coupons and 3 coupons per experiment (N=15) for triplicate experiments to yield mean average thickness and biomass for each different surface modification and compared with the unmodified SS control.

2.5.3.2. Viable bacterial counts: Three stainless steel coupons of each modification were placed in polystyrene Petri dishes as above containing 2.5×10^4 *S. aureus* mL^{-1} and incubated at 37°C and 5% CO_2 with gentle shaking for 2, 6, 24, 48 and 72 hours. Coupons were removed at each time point, rinsed and immediately transferred to a sterile tube containing TSB. Adherent bacteria were detached by 3 cycles of sonication for 5 minutes, followed by vortexing for 15 seconds to break up aggregated bacteria. The bacterial suspension was serially diluted in TSB. Dispensed onto duplicate TSA plates using the drop plate method (Herigstad *et al.* 2001) and incubated overnight. Experiments were repeated in triplicate for each time point.

2.5.3.3 SEM: Coupons were immersed in 2% glutaraldehyde/phosphate buffered saline (PBS) at 4 °C, rinsed, placed in sterile well plates, covered with 1% osmium tetroxide/PBS for 1 hour, rinsed again and dehydrated in an ethanol series.[51] Coupons were transferred to 1mL of HMDS for 10 minutes, removed to a new sterile well plate and stored in a desiccator until imaging using a Hitachi S-3400N-II SEM under vacuum. Images were collected under high vacuum at $5000 \times$ magnification with a 7.00 kV accelerating voltage and a working distance between 4.6–4.8 mm.

2.5.3.4. Statistics: A one way analysis of variance (ANOVA) with a Bonferroni post-hoc test was used to determine statistical difference at the $p < 0.05$ level of significance for each experiment.

3. Results

3.1. SAM characterization

3.1.1. DRIFT spectroscopy—SAM formation and stability was initially characterized using DRIFT spectroscopy. Peaks in the methylene (CH_2) region of the DRIFT spectra were used to indicate if an ordered SAM with aligned alkyl chains was present on the SS316L surface (Figure 2a). The SAMs are ordered if peaks assigned to ν_{CH_2} asymmetric and ν_{CH_2} symmetric are present at $< 2918 \text{ cm}^{-1}$ and $< 2850 \text{ cm}^{-1}$, respectively.[52, 53] Modified coupons were examined using DRIFT after initial deposition and after the solvent rinse (10 minutes) and sonication (15 minutes) to determine if the SAMs were strongly bound at the surface. Table 1 shows that the modification conditions developed herein generated ordered SAMs that were stable through both the rinse and sonication in solvent steps with peaks corresponding to ν_{CH_2} asymmetric $< 2918 \text{ cm}^{-1}$ and ν_{CH_2} symmetric $< 2950 \text{ cm}^{-1}$. Additionally, the SAMs were stable to rinsing in protic solvents such as ethanol and in 0.1M HCl, indicating that they were stable to corrosive environments.

Additionally, the binding mode of the molecules with the surface was assessed using DRIFT spectroscopy by comparing the DRIFT spectra of the SAMs with that of the solid, unbound molecules.[19, 43] For example, solid, unbound molecules with phosphonic acid head groups exhibit peaks in the DRIFT spectra corresponding to bond stretching of P=O at 1260 cm^{-1} , P-O at $1077, 1007 \text{ cm}^{-1}$, and P-OH at 952 cm^{-1} . [19, 54] The presence or absence of these peaks in the DRIFT spectra of the SAMs was used to determine the binding mode of the SAMs with the surface (Table 2). The DRIFT spectra of EG3PA, EG5PA, and COOH-PA SAMs exhibited one broad peak present at approximately 1050 or 1079 cm^{-1} , which is characteristic of group P-O stretches.[54, 55] These data, coupled with the lack of peaks attributed to the P=O and P-OH stretches at 1260 cm^{-1} and 952 cm^{-1} , respectively, indicated that there was only one P-O species present on the surface. Therefore, the phosphonic acid head group of the EG3PA, EG5PA, and COOH-PA SAMs are strongly bound to the surface in a tridentate fashion (Figure 2b). Further, it was confirmed that the carboxylic acid tail groups of COOH-PA SAMs were not binding to the surface, since peaks in the DRIFT spectra at 1690 and 1460 cm^{-1} indicated unbound carboxylic acid present at the interface and importantly, available for further chemical reaction.[16, 56]

The stable COOH-PA SAMs that were formed were used to link gentamicin or vancomycin to the surface. Briefly, EDC was used to immobilize PFP to the carboxylic acid tail groups of the COOH-PA SAM (Figure 1b).[57] The DRIFT spectra of the PFP solid contained peaks corresponding to the bond stretching of O-H at 3550 cm^{-1} , C=C-F at 2975 cm^{-1} , and C=C at 2609 and 2470 cm^{-1} . When PFP was immobilized, the C=C-F and C=C peaks were present, but the spectra lacked an O-H peak at 3350 cm^{-1} indicating that PFP was attached to the SAM (Figure 3). PFP was then displaced by an amine group of gentamicin or vancomycin, forming an amide bond between the COOH-PA SAM and the antibiotic. The coupons were rinsed to remove any physisorbed antibiotic and DRIFT spectra were collected for the solid antibiotics and coupons after gentamicin or vancomycin attachment and solvent rinse in ethanol (10 minutes) to demonstrate that the antibiotic was stably bound to the surface.

Solid gentamicin yielded a peak in the spectrum corresponding to the 1° amine bend at 1631 cm^{-1} with a shoulder peak at 1550 cm^{-1} and attachment was confirmed by the shift of the shoulder to a well-defined peak at 1536 cm^{-1} indicative of amide bond formation (Figure 4a). Further, the peak for the 1° amine bend at 1631 cm^{-1} was reduced in intensity in relation to the peak at 1536 cm^{-1} , suggesting that gentamicin was attached by the primary amines present in the molecule. Vancomycin attachment was similarly evaluated. Briefly, peaks attributed to the phenol rings were present for the aromatic ring (C=C at 1500 cm^{-1})

and the C-O stretch at 1235 cm^{-1} (Figure 4b) in the spectrum. The peak at 1585 cm^{-1} in the spectrum corresponds to the C-OO⁻ stretch in vancomycin. Finally, distinct and enhanced amide peaks corresponding to the C-N stretch and N-H bend were present in the spectrum at 1420 and 1651 cm^{-1} , respectively, indicating that the molecules is bound to the SAMs through amide bond formation.

3.1.2 Contact angle analysis—Contact angle analysis was used to indicate the interfacial wetting behavior of stable SAMs formed on SS316L. The average water contact angles and standard deviations are reported in Table 1. SAMs with methyl terminated alkyl chains (ODPA, OCA) were hydrophobic, exhibiting increased contact angles ($97 \pm 4^\circ$ and $113 \pm 9^\circ$, respectively) compared with unmodified SS316L, which possessed a hydrophilic surface oxide layer ($68 \pm 9^\circ$). SAMs with terminal carboxylic acid (COOH-PA), and tri- and penta-(ethylene glycol) moieties (EG3PA, EG5PA) exhibited contact angles similar to the hydrophilic control SS316L surface, further suggesting that COOH groups on the COOH-PA modified coupons, which had an average contact angle of $73 \pm 7^\circ$, had not reacted with the surface, but were presented as carboxylic acid tail groups for reaction at the interface.

3.1.3 MALDI-TOF MS—In order to further assess uniformity of the surface modifications, MALDI-TOF MS was used to differentiate between mono- and multilayer passive films.[46] Surface-bound molecules were desorbed and ionized from the coupon surface with α -cyano-4-hydroxycinnaminic acid (CHCA) matrix. Molecules aggregated in multilayers on the substrate surface result in m/z peaks of the dimer molecular weight of the parent ion ($2M^+$) or the dimer molecule plus one cation (H^+ , Na^+ , or K^+). Conversely, if a monolayer is present on the coupon surface, only m/z peaks at the monomer molecular weight of the parent ion (M^+) or the monomer molecule plus one cation (H^+ , Na^+ , or K^+) should be present. The presence of monomer peaks and the lack of any dimer peaks indicated that COOH-PA, ODPA, EG3PA, and EG5PA (Figure 5) formed monolayer films (Table 4).

3.1.4 AFM—AFM imaging was used to further confirm the presence of and evaluate the amount of gentamicin and vancomycin that was linked to the surface compared to the unmodified SS316L (Figure 6a–c). Thresholding the images collected on the antibiotic-linked films was used to provide information on the extent of reacted COOH-PA tail groups. Based on the proposed attachment scheme (Figure 1b) the total surface area occupied by gentamicin or vancomycin was evaluated and compared to the unthresholded image. It was determined that gentamicin covered an average surface area of $0.094 \pm 0.001\text{ }\mu\text{m}^2$ representing 37.6% compared to unthresholded image area ($0.25\text{ }\mu\text{m}^2$). Vancomycin covered an average surface area of $0.10 \pm 0.01\text{ }\mu\text{m}^2$, representing 40% of the image area of $0.25\text{ }\mu\text{m}^2$. For both gentamicin and vancomycin-linked films, the antibiotic molecules in each viewing area were shown to be closely packed.

3.2. Bacteria adhesion studies

3.2.1 Confocal laser scanning microscopy (CLSM)—CLSM was used to initially assess biofilm growth on the ODPA, OCA, EG3PA, EG5PA, and antibiotic-linked coupons. The images displayed live (SYTO-9 - green) and dead (propidium iodide (PI) - red) bacteria and were used to evaluate trends in biofilm viability and composition up to 72 hours. At 24 hours, the hydrophobic ODPA SAMs (18 carbons) appeared to have similar biofilm density as the control, while the longer chain OCA SAMs (28 carbons) displayed localized areas of concentrated biofilm density, where bacteria cells aggregated in large clusters (Figure 7). SAMs that presented hydrophilic interfaces (EG3PA, EG5PA) appeared to have an increased density of viable *S. aureus* biofilm growth compared with control SS316L and the

length of the pendant glycol did not appear to be influential in retarding biofilm development (Figure 7). These trends were consistent through 72 hours (data not shown).

In contrast, CLSM images indicated antibiotic-linked coupons either exhibited an increase in nonviable bacteria, as shown by cells stained red with PI, or reduced viable bacteria adhesion compared with unmodified SS up to 48 h (Figure 7).

COMSTAT image analysis was used to quantitatively evaluate the average thickness (μm) and biomass ($\mu\text{m}^3/\mu\text{m}^2$) of *S. aureus* biofilms formed on the modified coupons.[50] Data were evaluated separately for live and dead bacteria. At 24 hours, hydrophobic or hydrophilic passive SAMs did not significantly reduce biofilm growth on SS316L reflected by the averaged thickness and biomass of live and dead bacteria within the biofilm on the modified coupons ($p < 0.05$). In contrast, antibiotic-linked coupons significantly reduced both live and dead biofilm bacteria (Figure 8a,b). Gentamicin-linked coupons decreased average thickness and biomass by over 70% in comparison with the unmodified coupons which had an averaged thickness of $0.37 \mu\text{m}$ and biomass of $0.11 \mu\text{m}^3/\mu\text{m}^2$. Meanwhile, vancomycin-linked were the most effective at reducing biofilm growth compared to the unmodified SS316L, which exhibited a biomass that was 10 times greater at 24 hours.

At 48 hours, no significant changes were seen in the biomass or average thickness of the hydrophobic (ODPA, OCA) and hydrophilic (EG3PA, EG5PA) SAMs compared to the unmodified SS316L. Gentamicin-linked coupons exhibited a 27% reduction in average live biomass. Vancomycin-linked coupons however, continued to demonstrate significantly reduced biomass and average thickness ($0.06 \mu\text{m}^3/\mu\text{m}^2$ and $0.21 \mu\text{m}$) compared with unmodified SS316L and other SAM-modified coupons indicating that vancomycin-linked SAMs remain effective at inhibiting *S. aureus* biofilm formation for longer periods of time.

Past 72 hours, the biofilms that developed on passive hydrophobic and hydrophilic SAMs remained comparable to the unmodified control SS316L and did not significantly change from previous time points. Furthermore, gentamicin-linked SAMs were no longer effective toward inhibiting biofilm growth, displaying statistically similar biomass ($0.16 \pm 0.01 \mu\text{m}^3/\mu\text{m}^2$) and average thickness ($0.621 \pm 0.052 \mu\text{m}$) to the unmodified SS316L which had values of $0.15 \pm 0.01 \mu\text{m}^3/\mu\text{m}^2$ and $0.49 \pm 0.03 \mu\text{m}$, respectively. Only vancomycin-linked SAMs continued to display significantly less biofilm growth, evidenced by lower biomass and average thickness ($0.057 \pm 0.006 \mu\text{m}^3/\mu\text{m}^2$, $0.21 \pm 0.02 \mu\text{m}$ at the $p < 0.05$ level of significance).

3.2.2 SEM—SEM was used to further evaluate the physical properties of biofilms formed on the control and modified SS316L coupons. Biofilm growth was assessed at short (2, 6 hour) and longer time points (24, 48, 72 hour) in order to more finely resolve the adhesion and development of biofilms on the modified surfaces. Hydrophobic (ODPA, OCA) and hydrophilic surface modifications (EG3PA, EG5PA) had similar biofilm coverage to the unmodified control SS316L at both short and long time points. Gentamicin-linked SAMs in contrast exhibited fewer adhered cells and aggregate biofilm colonies through 24 hours compared to unmodified SS316L. Vancomycin-linked SS316L likewise displayed reduced numbers of adhered cells at early time points which extended through 48 hours (Figure 9).

3.2.3 Colony forming units—After screening the functional effectiveness of the different surface modifications to reduce biofilm development by *S. aureus* using image analysis, viable bacteria within the biofilms on control and modified SS316L surfaces were enumerated up to 72 hours. The passive modifications were not statistically different compared with unmodified SS316L at any time point using CFU analysis, which was consistent with previous data. (Table 5; Figure 8c).

In contrast, antibiotic-linked surfaces (gentamicin and vancomycin) exhibited a significant (99%) reduction in *S. aureus* bacterial adhesion and biofilm development up to 6 hours (average of 1.0×10^1 CFU/cm² compared with the control average of 1.3×10^6 CFU/cm²) (Figure 8c, Table 5). At 24 hours, the difference in biofilm development on gentamicin-linked coupons was more modest (9.4×10^5 CFU/cm²) compared to unmodified SS316L (4.3×10^6 CFU/cm²) and was no longer significantly different at 48 hours. Meanwhile, vancomycin-linked coupons continued to significantly reduce biofilm formation by 99% up to 48 hours and 51% at 72 hours. These data corroborated the microscopic data with COMSTAT analysis and indicate that gentamicin-linked coupons demonstrated a statistically significant reduction in viable adherent *S. aureus* within the biofilm up to 24 hours and that vancomycin-linked coupons remained effective for longer periods.

4. Discussion

SS316L is a common orthopedic biomaterial whose interaction with surrounding biological tissues and fluids may be improved through the functionalization of the surface. Here we investigated chemical modification approaches using SAMs to reduce bacterial adhesion and subsequent biofilm formation on the oxide surface of SS316L present on implant materials. [19, 43, 58] These approaches took the form of both passive surface modifications, which present molecules at the interface which control surface properties, but interact nonspecifically in a biological environment, and active surface modifications, which present biologically active molecules such as antibiotics, peptides or proteins to mediate biologically-specific interactions. These strategies have not been used previously to modify metal oxide surfaces on SS316L.

For passive surfaces, SAM formation protocols were developed for OCA, and the ethylene glycol terminated monolayers. The protocol for ODPA was developed previously.[43] Uniform SAMs of COOH-PA, ODPA, OCA, EG3PA, and EG5PA that were stable through solvent rinse and sonication were formed on the metal oxide surface of SS316L. The data for the SAMs formed here are consistent with the conclusion that the alkyl chains are ordered (Table 1). If the CH₂ asymmetric stretch is 2918 cm^{-1} ν CH₂ asymmetric stretch is 2850 cm^{-1} the alkyl chains are considered ordered. [52,53] For example, the CH₂ asymmetric peak for OCA and EG3PA are at 2914 cm^{-1} and the symmetric at 2947 cm^{-1} indicating that the alkyl chains are ordered. Ordered alkyl chains led to the presentation of ordered interfaces that consistently and effectively altered the surface properties of the outermost layer of the SS316L alloy as measured by contact angle.

For active, specific anti-bacterial surfaces, the COOH-PA monolayers presented reactive COOH at the interface where a series of reactions (Figure 1) resulted in the covalent immobilization of the antibiotic to the oxide surface via an amide bond to the monolayer. This was confirmed by infrared spectroscopy. AFM imaging of the surface clearly shows the bound vancomycin and gentamicin molecules on the surface.

The two different functional approaches were then tested for their ability to retard bacterial adhesion and biofilm development by *S. aureus* using a biologically-relevant challenge regime and were assessed by CLSM, SEM and CFU. CLSM imaging and COMSTAT analysis suggest that passive hydrophobic or hydrophilic SAMs were not effective at limiting biofilm formation regardless of tail group functionality or chain length. CFU analysis performed at 2, 6, 24, 48 and 72 hours further indicated that passive hydrophobic (ODPA, OCA) and hydrophilic (EG3PA, EG5PA monolayers were not statistically different from the control SS316L at any time point. SEM images are consistent with these data that there is no difference in bacteria adhesion and colony formation over 72 hours. These data are consistent with the microscopic and COMSTAT analysis confirming that, passive SAM

monolayers did not abrogate biofilm formation on SS316L and suggest that a passive chemical modification strategy using SAMs may not be sufficient to control biofilm development on SS316L and that an active antimicrobial approach such as the one developed in this study may be more useful. This result may be due to the roughness of the underlying SS316L coupon compared to other substrates such as gold, glass and silicon that have previously indicated that hydrophobic or hydrophilic modification alone retarded bacterial adhesion.[22, 33, 59, 60] Additionally, SAM coating of the SS316L oxide layer is only one molecule thick which may not be enough to compensate for the microscale surface roughness of SS316L.

The biologically-active strategy with the antibiotic-linked SAMs on SS316L was more promising, resulting in significantly decreased *S. aureus* biofilm formation. Based on COMSTAT results, gentamicin-linked SAMs reduced the average thickness and biomass of biofilm by 70% at 24 hours when compared to control and vancomycin-linked SAMs reduced average thickness by 99.9% at 48 hours. Figures 5 and 6, clearly demonstrate that there were significantly fewer bacteria present on the surface and, of those present, more were dead on antibiotic-modified surfaces. This is consistent with the antibiotic retaining its activity while bound to the surface in the presence of constant challenge with bacteria. CFU data further indicate that gentamicin- and vancomycin-linked SS316L coupons exhibited significantly reduced biofilm at 24 hours reducing viable *S. aureus* by 99% up to 6 hours. The viability of adherent bacteria remained approximately 50% of the control up to 24 hours with gentamicin compared with the unmodified SS316L control. At 48 hours, only vancomycin-linked coupons remained significantly effective while gentamicin-linked SS316L was not significantly different than control SS316L. Thus gentamicin appears to be effective at inhibiting biofilm formation at early time points (2, 6 hours), while vancomycin-linked coupons were effective for longer time points (6–48 hours).

Finally, our assay system used an experimental physiologically relevant model designed to be a worse-case scenario that constantly challenged the surface with bacteria in a protein rich medium. Although bacteria encounter protein rich environments during orthopedic surgery, they would be unlikely to encounter a constant challenge of bacteria during this time point. Our results therefore are promising since they indicate that SAMs on the oxide layer of SS316L may dramatically reduce bacterial adhesion.

Summary

Alloys used as orthopedic implant devices, such as SS316L, are covered by an oxide layer that is susceptible bacterial adhesion and biofilm formation by common pathogens such as *S. aureus*. In a chemical approach the surface modifications developed herein using SAMs effectively altered the interfacial properties of the alloy in attempt to limit bacterial attachment to the surface. It was demonstrated that SAMs presenting either a hydrophobic or hydrophilic interface did not affect biofilm formation on SS316L when compared with the unmodified control surface. Active films, in which the antibiotics gentamicin or vancomycin were bound at the surface, however, significantly reduced bacterial adhesion and biofilm formation up to 48–72 hours. Since infection of the implant device occurs around the time of implant surgery, limiting initial bacterial attachment during early time points may represent an important strategy in retarding biofilm infection. However this is just the first step in creating an effective multi-functional surface for SS316L resulting in a more effective biomaterial. Using orthogonal surface chemistry, other active groups can be added to the surface to provide an enhanced local environment for reducing bacterial adhesion which promoting specific host cell adhesion and eventual bone growth and intercalation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A: Tri- and pentaethylene glycol terminated phosphonic acid (EG3PA and EG5PA) synthesis

Pentadecanolide (98%), hydroiodic acid (47%), triethylphosphite (98%), p-tosyl chloride (99%), triphenylphosphine (PPh₃, 99%), and triethylene glycol (99%) were obtained from Sigma Aldrich. Pyridine (99.8%) and trimethylsilyl bromide (TMS-Br, 97%) were obtained from Fluka. Pentaethylene glycol (98%), anhydrous dichloromethane (DCM, 99.9%) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 98%) were obtained from Acros Organics. Sodium azide (NaN₃, 99%) was obtained from Alfa Aesar. Acetic acid (HOAc, 99.9%), sulfuric acid (H₂SO₄, 99.9%), and sodium hydroxide (NaOH, 99%) were obtained from Fisher. EG3PA (Figure 1) and EG5PA (Figure 2) were synthesized as described below. ¹H NMR were collected on a Bruker AVANCE™ II 500 MHz NMR spectrometer with multinuclear broad band observe (BBO) and inverse triple resonance (TXI) probes (5 mm diameter) with variable temperature control, pulse field gradients and automatic tuning and matching. All spectra were collected using deuterated chloroform (CDCl₃; Acros Organics, 99.8%) as the solvent unless otherwise indicated. Mass spectra of the final products were collected using MALDI-TOF MS as described in *Materials and Methods* by dissolving the product (EG3PA or EG5PA) in methanol with a 10 mg/mL of CHCA matrix.

2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (1)

Triethylene glycol (42.46 g, 0.283 mol) was added to an oven dried round bottom flask and the flask was purged with argon. Tetrahydrofuran (THF) (20 mL), pyridine (20 mL, 0.296 mol) and tosyl chloride (36.18 g, 0.190 mol) were added to the flask and the reaction was allowed to proceed for 2.5 hours at room temperature. The solution was rinsed with 1 M sodium hydroxide (NaOH) (50 mL) and THF was removed under reduced pressure. The sample was then extracted into chloroform (CHCl₃). The organic layers were then combined, dried over sodium sulfate, and concentrated to give a yellow oil. The resulting oil was purified via flash column chromatography (90:10 ethyl acetate:hexanes) to produce 9.4 g (22.1%) of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (**1**) as a clear oil. ¹H NMR δ 2.45 (s, 3H), 3.5–3.7 (m, 14H), 4.18 (t, 2H), 7.34 (d, 2H), 7.79 (d, 2H).

2-(2-(2-azidoethoxy)ethoxy)ethanol (2)

2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (**1**) (8.4 g, 0.0120 mol) was added to a round bottom flask, diluted with 95% ethanol (150 mL), and sodium azide (1.66 g, 0.0255 mol) was added to the flask and the reaction was allowed to reflux for 24 hours. The solvent was reduced by ~120 mL and 1 M NaOH (25 mL) was added to the flask. The reaction was then extracted with chloroform. The organic layer was dried over sodium sulfate and concentrated, which produced 4.3 g (51.2%) of 2-(2-(2-azidoethoxy)ethoxy)ethanol (**2**) as a clear oil. ¹H NMR δ 3.40 (t, 2H), 3.36–3.7 (m, 14H).

2-(2-(2-aminoethoxy)ethoxy)ethanol (3)

2-(2-(2-azidoethoxy)ethoxy)ethanol (**2**) (4.3 g, 0.0248 mol) was added to an oven dried round bottom flask and the flask was purged with argon. Anhydrous dichloromethane (DCM, 10 mL) and triphenylphosphine (1.97 g, 0.0751 mol) were added to the flask and the reaction was allowed to proceed at room temperature for 12 hours. Water (5 mL) was then added to the flask until a white precipitate formed. The organic layer was removed via rotary evaporation. The aqueous layer was rinsed twice with toluene (10 mL) and concentrated to give 2-(2-(2-aminoethoxy)ethoxy)ethanol (**3**) as a clear oil (yield < 96.8%). ^1H NMR δ 2.89 (t, 2H), 3.56 (t, 2H), 3.6–3.7 (m, 12H).

Methyl 15-iodopentadecanoate (4)

Pentadecanolide (3.04 g, 0.0126 mol) was added to a round bottom flask and diluted with hydroiodic acid (7.5 mL, 0.0997 mol), acetic acid (20 mL), and the reaction was allowed to reflux for 3 hours. Acetic acid was removed under reduced pressure and the sample was diluted with DCM (50 mL), and rinsed with 10% sodium sulfate. The organic layer was dried over sodium sulfate and concentrated via rotary evaporation to produce a white solid. The solid was diluted with methanol : hexanes (10:1), sulfuric acid (3 drops), and set to reflux for 48 hours. Solvent was then removed via rotary evaporation to produce a white solid that was then washed with water and extracted into CHCl_3 . The organic layers were then combined, dried over sodium sulfate, and concentrated to give a white solid. The resulting solid was purified via flash column chromatography (5:95 ethyl acetate : hexanes) to produce 3.43 g (68.4%) of ethyl 15-iodopentadecanoate (**4**) as a white solid. ^1H NMR δ 1.2–1.4 (m, 25H), 1.62 (m, 2H), 1.82 (m, 2H), 2.30 (t, 2H), 3.19 (t, 2H), 3.67 (s, 3H).

Methyl 15-(diethoxyphosphoryl)pentadecanoate (5)

Methyl 15-iodopentadecanoate (**4**) (3.43 g, 0.0086 mol) was added to an oven dried round bottom flask, purged with argon, and then placed in an oil bath at 105 °C. Triethylphosphite (11.35 g, 0.068 mol) was added dropwise to the solution at a rate of 20 $\mu\text{L}/\text{min}$ with argon bubbling through the solution. The reaction was allowed to proceed for 18 hours. Triethylphosphite was then removed under reduced pressure (20 mTorr) to give a yellow oil. The resulting oil was purified via flash column chromatography (80:20 ethyl acetate:hexanes) to produce ethyl 15-(diethoxyphosphoryl) pentadecanoate (**5**) as a white solid (yield < 68.7%). ^1H NMR δ 1.2–1.4 (m, 26H), 1.5–1.8 (m, 7H), 2.30 (t, 2H), 3.67 (s, 3H), 4.08 (q, 4H).

15-(diethoxyphosphoryl)pentadecanoic acid (6)

Methyl 15-(diethoxyphosphoryl) pentadecanoate (**5**) (1.15 g, 0.001 mol) was added to a round bottom flask and diluted with ethanol:water (23 mL, 70:30). Sodium hydroxide (0.052 g, 0.0013 mol) was added to the reaction mixture and the reaction was allowed to proceed in an oil bath at 100 °C for 2 hours. The reaction mixture was cooled to room temperature and acidified with concentrated hydrochloric acid (0.05 mL, 0.0022 mol). Solvent was removed via rotary evaporation to give a white solid. The resulting solid was diluted with CHCl_3 and rinsed with water. The organic layers were then combined, dried over sodium sulfate, and concentrated to give 15-(diethoxyphosphoryl)pentadecanoic acid (**6**) as a white solid (yield < 64%). ^1H NMR δ 1.2–1.4 (m, 27H), 1.5–1.8 (m, 7H), 2.34 (t, 2H), 4.09 (q, 4H).

Diethyl (15-((2-(2-(2-hydroxyethoxy)ethoxy)ethyl)amino)-15-oxopentadecyl)phosphonate (7)

15-(diethoxyphosphoryl)pentadecanoic acid (**6**) (0.27 g, 0.0010 mol) was added to an oven dried round bottom flask, purged with nitrogen, and diluted with dry DCM (25 mL). 2-(2-(2-aminoethoxy)ethoxy)ethanol (**3**) (0.10 g, 0.0010 mol) and 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.16 g, 0.0015 mol) were added to the reaction mixture and the reaction was allowed to proceed for 18 hours. Excess EDC was then removed by rinsing. The organic layers were then combined, dried over sodium sulfate, and concentrated to give diethyl (15-((2-(2-(2-hydroxyethoxy)ethoxy)ethyl)amino)-15-oxopentadecyl)phosphonate (**7**) as a white solid (yield < 78%). ¹H NMR δ 1–1.5 (m, 34H), 1.5–1.8 (m, 9H), 2.17 (t, 2H), 3.4–3.8 (m, 13H), 4.08 (m, 5H).

(15-((2-(2-(2-hydroxyethoxy)ethoxy)ethyl)amino)-15-oxopentadecyl)phosphonic acid (8)

Diethyl (15-((2-(2-(2-hydroxyethoxy)ethoxy)ethyl)amino)-15-oxopentadecyl)phosphonate (**7**) (0.051 g, 0.0010 mol) was added to an oven dried round bottom flask and purged with nitrogen. Dry DCM (25 mL) and trimethylsilyl bromide (0.053 mL, 0.0040 mol) were added to the flask and the reaction was allowed to proceed for 18 hours. Water (0.053 mL, 0.0040 mol) was then added to the flask and the reaction mixture was stirred vigorously for 5 hours. The aqueous layer was then concentrated to produce a white solid. The resulting solid was purified on a silica plug (MeOH 100%) to produce (15-((2-(2-(2-hydroxyethoxy)ethoxy)ethyl)amino)-15-oxopentadecyl)phosphonic acid (**8**) as a white solid (yield < 93%). ¹H NMR was collected using deuterated methanol (CD₃OD; Cambridge Isotope Laboratories, 99.8%) δ 1–1.4 (m, 20H), 1.5–1.7 (m 4H), 1.9 (t, 2H), 3.4–3.8 (m, 13H). MALDI-TOF MS displayed a peak in the mass spectra at 476.28 *m/z* which corresponded to the molecular weight of product (**8**) plus one sodium ion.

14-hydroxy-3,6,9,12-tetraoxatetradecyl 4-methylbenzenesulfonate (9)

Pentaethylene glycol (68.12 g, 0.289 mol) was added to an oven dried round bottom flask and the flask was purged with argon. Tetrahydrofuran (THF) (20 mL), pyridine (20 mL, 0.296 mol) and tosyl chloride (36.18 g, 0.189 mol) were added to the flask and the reaction was allowed to proceed for 2.5 hours at room temperature. The solution was rinsed with 1 M sodium hydroxide (NaOH) (50 mL) and THF was removed under reduced pressure. The sample was then extracted into chloroform (CHCl₃). The organic layers were then combined, dried over sodium sulfate, and concentrated to give a yellow oil. The resulting oil was purified via flash column chromatography (90:10 ethyl acetate : hexanes) to produce 9.48 g (42.3%) of 14-hydroxy-3,6,9,12-tetraoxatetradecyl 4-methylbenzenesulfonate (**9**) as a clear oil. ¹H NMR δ 2.45 (s, 3H), 3.5–3.7 (m, 26H), 4.16 (t, 2H), 7.33 (d, 2H), 7.79 (d, 2H).

14-azido-3,6,9,12-tetraoxatetradecan-1-ol (10)

14-hydroxy-3,6,9,12-tetraoxatetradecyl 4-methylbenzenesulfonate (**9**) (4.74 g, 0.0121 mol) was diluted with 95% ethanol (150 mL) and sodium azide (3.32 g, 0.0255 mol) was added to the flask and the reaction was allowed to reflux for 24 hours. The solvent was reduced by ~120 mL and 1 M NaOH (25 mL) was added to the flask. The reaction was then extracted with chloroform. The organic layer was dried over sodium sulfate and concentrated, which produced 2.82 g (59%) of 14-azido-3,6,9,12-tetraoxatetradecan-1-ol (**10**) as a clear oil. ¹H NMR δ 3.39 (t, 2H), 3.6–3.8 (m, 22H).

14-amino-3,6,9,12-tetraoxatetradecan-1-ol (**11**)

14-azido-3,6,9,12-tetraoxatetradecan-1-ol (**10**) (1.19 g, 6.83 mmol) was added to an oven dried round bottom flask and the flask was purged with argon. Dry dichloromethane (DCM, 10 mL) and triphenylphosphine (1.97 g, 7.51 mmol) were added to the flask and the reaction was allowed to proceed at room temperature for 12 hours. Water (5 mL) was then added to the flask until a white precipitate formed. The organic layer was removed via rotary evaporation. The aqueous layer was rinsed twice with toluene (10 mL) and concentrated to give 14-amino-3,6,9,12-tetraoxatetradecan-1-ol (**11**) as a clear oil (yield < 96.8%). ¹H NMR δ 2.89 (t, 2H), 3.56 (t, 2H), 3.56–3.8 (m, 19H).

Diethyl (1-hydroxy-16-oxo-3,6,9,12-tetraoxa-15-azatriacontan-30-yl)phosphonate (**12**)

15-(diethoxyphosphoryl)pentadecanoic acid (**6**) (4.29 g, 0.0010 mol) was added to an oven dried round bottom flask, purged with nitrogen, and diluted with dry DCM (25 mL). 14-amino-3,6,9,12-tetraoxatetradecan-1-ol (**11**) (2.6 g, 0.0010 mol) and 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDC) (3.26 g, 0.0015 mol) were added to the reaction mixture and allowed to proceed for 18 hours. Excess EDC was then removed by rinsing. The organic layers were then combined, dried over sodium sulfate, and concentrated to give diethyl (1-hydroxy-16-oxo-3,6,9,12-tetraoxa-15-azatriacontan-30-yl)phosphonate (**12**) as a white solid (yield < 78%). ¹H NMR δ 1–1.5 (m, 38H), 1.5–1.8 (m, 15H), 2.17 (t, 2H), 3.4–3.8 (m, 21H), 4.08 (m, 5H).

(15-((2-(2-(2-hydroxyethoxy)ethoxy)ethyl)amino)-15-oxopentadecyl)phosphonic acid (**13**)

Diethyl (1-hydroxy-16-oxo-3,6,9,12-tetraoxa-15-azatriacontan-30-yl)phosphonate (**12**) (3.84 g, 0.0010 mol) was added to an oven dried round bottom flask and purged with nitrogen. Dry DCM (100 mL) and trimethylsilyl bromide (3.39 mL, 0.0040 mol) were added to the flask and the reaction was allowed to proceed for 18 hours. Water (3.39 mL, 0.0040 mol) was then added to the flask and the reaction mixture was stirred vigorously for 5 hours. The aqueous layer was then concentrated to produce a white solid. The resulting solid was purified on a silica plug (MeOH 100%) to produce (15-((2-(2-(2-hydroxyethoxy)ethoxy)ethyl)amino)-15-oxopentadecyl)phosphonic acid (**13**) as a white solid (yield < 98%). ¹H NMR was collected using deuterated methanol (CD₃OD; Cambridge Isotope Laboratories, 99.8%) δ 1–1.4 (m, 20H), 1.5–1.7 (m 4H), 1.85 (t, 2H), 3.4–3.8 (m, 21H). MALDI-TOF MS displayed peaks in the mass spectra at 564.34 and 580.31 *m/z* which corresponded to the molecular weight of product (**13**) plus one sodium or one potassium ion, respectively.

Figure A.1.
Overall synthetic scheme for EG3PA.

Figure A.2.
Overall synthetic scheme for EG5PA.

Highlights

SS316L was modified with glycol terminated SAMs in order to reduce biofilm growth.

Antibiotics gentamicin and vancomycin were immobilized on SS316L via SAMs.

Only the antibiotic modifications reduced biofilm development on SS316L.

Figure 1.

Film molecules used to form (a) passive SAMs with hydrophobic or hydrophilic tail groups (b) SAMs that are used to link active antibiotics (gentamicin shown).

Figure 2.

(a) Representative DRIFT spectra of an EG3PA SAM with ordered alkyl chains. Peaks at 2914 cm⁻¹ and 2847 cm⁻¹ are due to the CH₂ asymmetric and symmetric stretches, respectively. (b) The phosphonic acid binding region of a COOH-PA SAM with a peak at 1079 cm⁻¹ is assigned to a group P-O bond.

Figure 3.

DRIFT spectra of COOH-PA SAMs formed on SS316L in which the carboxylic acid tail groups of the film were activated by immobilizing PFP. The light grey spectra of the PFP solid contained peaks corresponding to the bond stretching of O-H at 3550 cm^{-1} , C=C-F at 2975 cm^{-1} , and C=C at 2609 and 2470 cm^{-1} . When PFP was immobilized (black spectra), the C=C-F and C=C peaks were present, but the spectra lacked an O-H peak at 3350 cm^{-1} .

Figure 4.

DRIFT spectra of COOH-PA SAMs formed on SS316L in which **(a)** gentamicin is linked to the carboxylic acid tail group of the SAM. Solid gentamicin (grey line) yielded a peak in the spectrum corresponding to the 1° amine bend at 1631 cm^{-1} with a shoulder peak at 1550 cm^{-1} and attachment was confirmed by the shift of the shoulder to a well-defined peak at 1536 cm^{-1} indicative of amide bond formation. **(b)** Vancomycin is linked to the carboxylic acid tail groups of the SAM. Peaks attributed to the phenol rings were present for the aromatic ring ($\text{C}=\text{C}$ at 1500 cm^{-1}) and the $\text{C}-\text{O}$ stretch at 1235 cm^{-1} in both the solid and the immobilized vancomycin. The peak at 1585 cm^{-1} in the spectrum corresponds to the $\text{C}-\text{OO}^-$ stretch in vancomycin. In the bound spectrum (black line) distinct and enhanced amide peaks corresponding to the $\text{C}-\text{N}$ stretch and $\text{N}-\text{H}$ bend were present in the spectrum at 1420 and 1651 cm^{-1} .

Figure 5.

Representative mass spectrum used to determine monolayer film coverage including CHCA matrix (top) and EG5PA SAM on SS316L (bottom). Peaks at 564.34 and 580.31 (m/z) are attributed to EG5PA+Na⁺ and EG5PA+K⁺, respectively.

Figure 6. AFM images corresponding to (a) control SS316L (b) gentamicin-linked films (c) vancomycin-linked films.

Figure 7.
CLSM images representing the viability of *Staphylococcus aureus* UAMS-1 biofilms formed on modified SS316L at 24 and 48 hours.

Figure 8.

(a) COMSTAT analysis of control and modified SS316L coupons indicating the average thickness of live bacteria within the biofilm (b) COMSTAT analysis of control and modified SS316L coupons indicating the biomass of live bacteria within the biofilm (c) average colony forming units of bacteria within biofilms formed on SS316L. The marker of significance * was used to indicate $p < 0.05$ level of significance.

Figure 9.

SEM images representing the viability of *Staphylococcus aureus* UAMS-1 biofilms formed on modified SS316L at 24 and 48 hours.

Table 1**Center Column**

Peaks in the DRIFT spectra of SAMs formed on SS316L are assigned to ν_{CH_2} asymmetric, ν_{CH_2} symmetric present after solvent sonication. Right Column: average water contact angles ($\theta \pm$ standard deviation) for unmodified SS316L (control) and SAM modified surfaces.

Coupon	ν_{CH_2} asymm, ν_{CH_2} symm	Contact angle (θ)
Control	n/a	68 ± 9
ODPA	2915, 2848	97 ± 4
OCA	2914, 2847	113 ± 9
EG3PA	2914, 2847	83 ± 7
EG5PA	2915, 2849	68 ± 4
COOH-PA	2915, 2848	73 ± 7

Table 2

Peaks in the DRIFT spectra of SAMs on SS316L used to determine binding mode with the surface.

Film molecule	Peak (cm ⁻¹)	Corresponding stretch	Binding mode
ODPA	1262, 1030	P=O, P-O group stretches	bidentate
OCA	1585	C-OO ⁻	bidentate
EG3PA	1079	P-O group stretches	tridentate
EG5PA	1075	P-O group stretches	tridentate
COOH-PA	1050	P-O group stretches	tridentate

Table 4

Peaks found in the m/z spectrum corresponding to films formed on SS316L that were deionized and evaluated using MALDI-TOF MS.

Film molecule	m/z peak	Corresponding molecular weight
ODPA	335.70	ODPA+ H ⁺
EG3PA	476.23	EG3PA + Na ⁺
EG5PA	564.34	EG5PA + Na ⁺
	580.31	EG5PA + K ⁺
COOH-PA	335.04	COOH-PA·+
	359.13	COOH-PA+Na ⁺

Table 5

Average colony forming units ($\pm$ standard error) of *S. aureus* UAMS-1 bacteria within the biofilms formed on modified SS316L coupons. Shaded areas represent a significant difference from the control SS316L at the $p < 0.05$ level of significance for $N=9$.

Sample	2 hours	6 hours	24 hours	48 hours	72 hours
Control	8.02E+03 $\pm$ 2.12E+03	1.64E+06 $\pm$ 4.53E+05	4.03E+06 $\pm$ 1.19E+06	2.87E+06 $\pm$ 2.44E+05	4.68E+06 $\pm$ 6.22E+05
ODPA	7.51E+03 $\pm$ 1.78E+03	1.35E+06 $\pm$ 2.95E+05	5.63E+06 $\pm$ 2.50E+06	6.76E+05 $\pm$ 1.08E+05	4.06E+06 $\pm$ 7.21E+05
OCA	7.71E+03 $\pm$ 2.65E+03	1.09E+06 $\pm$ 2.29E+05	3.93E+06 $\pm$ 1.02E+06	7.60E+05 $\pm$ 1.41E+05	4.36E+06 $\pm$ 9.42E+05
EG3PA	1.75E+04 $\pm$ 7.18E+03	1.21E+06 $\pm$ 2.45E+05	5.69E+06 $\pm$ 1.58E+06	9.81E+05 $\pm$ 2.64E+05	4.87E+06 $\pm$ 8.05E+05
EG5PA	6.58E+03 $\pm$ 6.43E+02	1.14E+06 $\pm$ 2.68E+05	3.53E+06 $\pm$ 6.17E+05	9.17E+05 $\pm$ 3.01E+05	7.21E+06 $\pm$ 1.56E+06
Gentamicin	2.21E+02 $\pm$ 4.13E+01	1.00E+01 $\pm$ 5.00E+00	9.42E+05 $\pm$ 3.54E+04	1.64E+06 $\pm$ 2.63E+05	1.24E+07 $\pm$ 1.85E+06
Vancomycin	7.28E+03 $\pm$ 7.97E+02	4.86E+02 $\pm$ 6.52E+01	5.72E+02 $\pm$ 3.63E+02	5.78E+01 $\pm$ 3.39E+01	2.51E+06 $\pm$ 1.12E+06