Polymer brushes as active nanolayers for tunable bacteria adhesion

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A B S T R A C T
Bacterial biofilm formation on implant surfaces is a frequent reason for the failure of many biomedical devices. Polymer brushes, thin nanolayers constituted of densely grafted macromolecules, are promising candidates to use in many biomedical applications to control attachment of bacteria to a surface. In this work five different polymer brushes were synthesized and tested with respect to their ability to regulate Staphylococcus aureus adhesion. Namely, two mixed brushes (consisting of poly(ethylene glycol) and a positively charged polymer, poly(2-vinyl pyridine) or quartenized poly(2-vinyl pyridine)) are investigated along with one-component brushes of the respective polymers. Bacterial adhesion was regulated over two orders of magnitude via altering the polymer brush composition.

1. Introduction

Biomaterial-centered infection (BCI), signified by bacterial biofilm formation, is the key factor limiting utilization of biomaterials [1]. Despite the use of antibiotics and sterile conditions, a large number of patients still suffer from BCI, and the bacterial biofilm formation on implant surfaces is a frequent reason for the failure of many biomedical devices [2–4]. Therefore, considerable efforts are taken to regulate bacterial growth on the implant surface. Adhesion of microorganisms to biomedical devices (which is required for biofilm formation) is determined by the physico-chemical properties of the surface. Thus, careful design of the implant surface can decrease or even prevent BCI, thus avoiding the need for implant removal and revision surgeries.

Three types of surfaces are considered to decrease bacteria adhesion and biofilm formation [2]. Positively charged surfaces promote bacteria adhesion, but prevent biofilm formation and growth due to strong binding of the bacteria to the surface [5,6]. Furthermore, the shape of the adsorbed bacteria is distorted due to strong interactions with the surface. Low surface energy materials appear to be helpful in in vivo applications when fluctuating shear forces are present [7–9]. The third type of surface treatment involves the deposition of polymer brushes (arrays of densely end-grafted polymer chains) [10]. This method results in a significant decrease in the adhesion of microorganisms in in vitro studies [11–20]. Polymer brushes can be used to build the two above-mentioned types of surfaces by varying the nature of the polymer chains grafted to the surface. More comprehensive details regarding bacterial adhesion to the surface and the mechanisms of biofilm formation can be found elsewhere [2,21–23].

Poly(ethylene glycol) [PEG] or poly(ethylene oxide) [PEO] ultra-thin coatings with controlled properties are of great interest with respect to medical and bioengineering applications because they have been proven to prevent nonspecific adsorption of proteins [24,25]. PEG [PEO] brushes are commonly employed to reduce bacterial adhesion to surfaces. Bridgett et al. [11] used block copolymers of PEO and polypropylene oxide (PPO) of different lengths to study adhesion of S. epidermidis. Substantial reductions (up to 97%) in bacterial adhesion levels were achieved with all copolymers tested, irrespective of the PPO or PEO block lengths. Coating titanium surfaces with PEG (47 monomeric units) polylysine copolymer significantly (by 85–93%) decreased the adhesion of Staphylococcus aureus to the surfaces [13]. A self-assembled monolayer containing 6 ethylene oxide units appeared to be resistant to bacterial attachment, resulting in a 99.7% reduction of attachment for S. epidermidis and Deleya marina [14].

Hydrophilic polymer brushes (of a chemical nature other than PEG) were also explored as coatings for the prevention of biofilm formation. Surfaces grafted with zwitterionic polymers poly(sulfo betaine methacrylate) and poly(carboxybetaine methacrylate) prevent the adsorption of proteins and the adhesion of mammalian cells [26]. S. aureus and Streptococcus salivarius adhesion to a surface-grafted polyacrylamide brush was reduced by 70–92% compared with untreated silicon surfaces [19].

Positively charged surfaces show some interesting characteristics with respect to biofilm formation as well [2]. Although bacteria adhere more easily to positively charged surfaces, some microorganisms failed to grow on such surfaces. For example, Escherichia coli and...
*Pseudomonas aeruginosa* showed little growth after adhering to positively charged poly(methacrylate) surfaces [5], while *S. aureus* and *S. epidermidis* were able to grow on these surfaces. Charged surfaces have yielded promising results in *in vivo* experiments. Gottenbos et al. [27] seeded *E. coli* and *P. aeruginosa* on glass discs coated with three differently charged poly(methyl acrylate) coatings, and the discs were then implanted into rats. On 50% of all positively charged discs, viable *E. coli* were absent, while the negatively charged discs were all colonized by *E. coli*. However, *P. aeruginosa* was isolated from both positively and negatively charged discs.

Facile transitions between different types of surfaces may facilitate the study on optimization of bacterial adhesion and biofilm formation, and can be achieved by utilization of mixed brushes consisting of two (or more) different polymers. In fact, mixed polymer brushes are of special interest for the tuning of surface properties because of their ability to switch the surface properties of the coatings in response to the environment [28,29]. The responsive behavior of the mixed-polymer systems tethered to the surface is based on the phase segregation mechanism of their constituents. External stimuli such as temperature, pH, solvent medium and ionic strength of the solution result in sterical rearrangement of the polymer chain fragments and create a gradient distribution of the polymer chains in the transverse direction. The properties of the created surface will be dominated by the properties of the polymer chains which fraction prevails [30,31].

To date, only a few studies have been conducted with respect to the modification of surfaces with mixed polymer systems for the purpose of tuning protein and cell adsorption. Examples include mixed systems consisting of polystyrene/polyacrylic acid [32], polyethylene-neglycol/polyacrylic acid [33], polystyrene/poly-2-vinylpyridine [34], and polyethyleneimine/polyacrylic acid [35]. Kim et al. [36] studied platelet adhesion to a mixed monolayer consisting of a dipalmitoylphosphatidylcholine and dipalmitoyl-phosphatidylethanolamine-polyethylene glycol. The authors showed that the concentration of PEG lipid should be greater than 3% molar concentration to efficiently prevent platelet adhesion.

The purpose of this study is to investigate the influence of polymer brush structure and composition with respect to regulation of bacterial adhesion. The PEG brush is used as a “gold standard” in preventing the adhesion of microorganisms to the surface. The poly(2-vinyl pyridine) (P2VP) brush is an example of a positively charged surface [37].Quaternization of the P2VP brush (QP2VP) increases the charge on the polymer chains and makes the brush more hydrophilic at the same time. In this study, two mixed brushes (consisting of PEG and a positively charged polymer chain (P2VP or QP2VP)) are investigated along with one-component brushes. We synthesized mixed brushes, in which a non-ionic polymer bacteria-repelling PEG is grafted to the surface at a much higher grafting density than the positively charged ones. However, the degree of polymerization (chain length) of the charged, bacteria-attractive polymer is ~3 times higher, and thus the positively charged polymer chains will protrude over the PEG reference layer. These brushes cover the entire range of adhesion behavior from extreme bacterial repulsion to strong surface adhesion.

### 2. Experimental methods

#### 2.1. Materials

Highly polished single-crystal silicon wafers of (100) orientation (Semiconductor Processing Co., USA) were used as a substrate. The wafers were first cleaned in an ultrasonic bath for 30 min, placed into a hot piranha solution (3:1 concentrated sulfuric acid/30% hydrogen peroxide) for 1 h, and then rinsed several times with water of high purity (18 MΩ, Nanopure).

Glycidyl methacrylate (Aldrich, Milwaukee, USA) was radially copolymerized with oligoethylene glycol (OEGMA, Mn = 300, monomers feeding ratio 1:1, Aldrich, Milwaukee, USA) to yield PGMA-co-OEGMA. The polymerization was carried out in methyl ethyl ketone (MEK, VWR, USA) at 60 °C. AIBN (Aldrich, Milwaukee, USA) was used as an initiator. The resulting polymer was purified by multiple precipitations from MEK solution in diethyl ether. PGMA-co-OEGMA was dissolved in MEK at 0.1% w/v and thin films (~5 nm) were deposited onto the substrate by dip coating and annealed for 15 min at 110 °C.

PEG (Mn = 5,000) monomethyl ether obtained from Aldrich (Milwaukee, USA) was modified with succinic anhydride (Aldrich, Milwaukee, USA) to obtain a carboxyl end-group derivative (PEG-s). Acylation was carried out by refluxing with a large excess (ca. 20) of succinic anhydride in tetrahydrofurane (THF). PEG was purified by multiple precipitations from THF solution in diethyl ether. Carboxy-terminated P2VP (Mn = 37,000 g/mol) was obtained from Polymer Source Inc., Canada.

#### 2.2. Brush synthesis

To obtain polymer brushes, a “macromolecular anchoring layer” method of permanent grafting of polymers to polymeric (organic) and inorganic surfaces was employed [38–40]. To graft PEG, the PEG powder was deposited onto the surface of clean glass slides and covered with silicon wafer modified by the PGMA-co-OEGMA primary layer. The specimens were placed in a vacuum oven at 80 °C for 10 h. Unbound PEG was removed by multiple washing with toluene at 75 °C (including washing in an ultrasonic bath).

A layer (ca 30 nm) of carboxy-terminated P2VP was deposited on top of the PGMA-co-OEGMA layer (or PEG brush in case of mixed-brush synthesis) and placed into sealed tubes with water vapor for grafting. Grafting was conducted for 20–24 h at 50 °C. After grafting, the wafers were rinsed repeatedly with MEK and sonicated for 10 min to remove ungrafted polymer chains. To quaternize P2VP or PEG-P2VP, mixed-brush samples were placed into sealed tubes with 0.1 ml of benzyl bromide. The quaternization was conducted for 16 h at 65 °C. After the benzyl bromide attachment, the wafers were rinsed repeatedly with ethanol and MEK.

#### 2.3. Brushes characterization

Ellipsometry was performed with a COMPEL automatic ellipsometer (InOmTech, Inc., USA) at an angle of incidence of 70°. Original silicon wafers from the same batch and silicon wafers with a PGMA-OEGMA macromolecular anchoring layer were tested independently and used as reference samples to analyze the grafted polymer layers. Atomic force microscopy (AFM) studies were performed on Dimension 3100 and MultiMode (Digital Instruments, Inc., Santa-Barbara, USA) microscopes. We used the tapping mode to study the surface morphology in ambient air. Silicon tips with spring constants of 50 N/m were used. Imaging was conducted at scan rates in the range of 1–2 Hz. Ellipsometry and AFM were conducted after each stage of the experiments.

#### 2.4. Bacterial adhesion studies

For bacterial adhesion studies, one colony of bacteria (*S. aureus*) was taken from a stock plate and placed in 5 ml of tryptic soy broth and incubated in a test tube for 18 h at 37 °C with agitation. Bacteria in an aliquot of broth (1 ml) were centrifuged for 5 min at 1200 rpm and the supernate was then aspirated. The pellet of bacteria was resuspended in 1 ml sterilized phosphate saline buffer (PBS) and added to 49 ml PBS. At that point, the PBS solution containing bacteria was ready for the bacterial adhesion study. Samples were immersed in 5 ml of the bacterial suspension (2 × 10⁷ bacteria/ml) and shaken at 100 rpm at 37 °C. After 3 h, the samples were gently rinsed with sterile PBS three times. The viability of adhered bacteria on the surface of samples was investigated by staining with 30 μl of SYTO 9 at room temperature in the dark for 15 min and subsequently analyzed with a Leica laser confocal microscope (Leica Microsystems GmbH, Germany). The number of viable adherent *S. aureus* on each sample...
surface was counted and expressed relative to the surface area of the sample (relative fluorescence of the sample, arbitrary units).

3. Results and discussions

3.1. Synthesis and structure of polymer brush layers

In this study, one-component and mixed-polymer brushes were prepared via the “grafting to” method as described elsewhere [38,40,41]. PEG brushes were prepared via melt grafting of end-functionalized PEG at 80 °C [40]. A P2VP brush was synthesized via solvent-assisted grafting [42,43]. Mixed brushes were prepared via sequential grafting of PEG by melt grafting followed by P2VP (solvent-assisted) grafting [29]. Fig. 1 shows the results of measurements of ellipsometric thickness for the synthesized brushes. Thickness of the PEG brush in the one-component and mixed brush was 7–9 nm. Thus, the grafting density of the PEG brushes was on the level of 0.9–1.2 chains/nm² [40]. Thicknesses of the P2VP and QP2VP one-component brushes were 15 nm and 20 nm, respectively. Grafting density for the brushes was approximately 0.12 chains/nm². Based on the difference in thickness of the P2VP and QP2VP brushes, we estimated the level of P2VP monomeric unit quaternization to be 45–55%. For the mixed brushes, the ratio between bacteria-repelling PEG chains and bacteria-attracting P2VP/QP2VP chains was approximately 7.5/1. Given the molecular weights of the polymer used, densities and radii of gyration, it can be calculated that a brush regime was achieved with a layer thickness equal to 0.5 and 0.9 nm for PEG and P2VP, respectively. Thus, all layers used in this study are in the brush regime, where the distance between grafting sites is below two radii of gyration for the end-grafted macromolecule.

3.2. SPM imaging of polymer brush layers

The surface morphology and smoothness of the grafted layers were determined with tapping-mode SPM. Fig. 2 displays SPM topographical images of the different brushes attached to the surface of the silicon substrate. The imaging was conducted in ambient air. In general, SPM showed that the developed grafting process resulted in complete polymer layers with surface flatness on the nanometric scale, and with roughness in the range of 0.4–1.8 nm for all surfaces. The surface of the PEG brush was covered with crystalline formations. Our previous research demonstrated that the crystals disappear when

Fig. 1. Ellipsometric thickness of the brushes used in this study. Deviation in thickness values for all samples is less than ±10%. Each color/pattern corresponds to individual polymer.

Fig. 2. AFM topographic images of the polymer brushes. Image size ~ 1 × 1 µm, vertical scale ~ 10 nm.

Fig. 3. S. aureus adsorption to the polymer brushes. Control surface — Si wafer. Y-axis is in relative fluorescence of the samples after S. aureus adsorption. Numbers below the bars indicating relative amount of S. aureus adsorbed to different brushes.
the brush contacts an aqueous environment [40]. The mixed brushes demonstrate a clear pattern caused by phase separation between PEG- and P2VP-grafted chains. The level of phase segregation in the brushes is on the level of tens of nanometers. Therefore, micrometer-size bacteria attempting to adhere to the surface are going to contact not only attractive P2VP/QP2VP chains, but also the repelling PEG macromolecules.

### 3.3. Microbial adhesion experiments

**Fig. 3** shows the amount of *S. aureus* adsorbed onto the surface of polymer brushes after 3 h of exposure to bacterial suspension. The Si wafer surface was used as a control substrate. The PEG brush exhibited a more than 2 orders-of-magnitude decrease in bacterial adsorption compared to the control. Only one surface of the positively charged QP2VP showed bacterial adsorption higher (160%) than the control. Interestingly, the P2VP brush bearing a positive charge (experimental conditions: PBS, pH = 7.4) showed a two-fold reduction of *S. aureus* adsorption. The P2VP brush showed a two-fold adsorption of *S. aureus* adsorption compared to the control Si wafer surface. The QP2VP brush exhibited bacterial adsorption that was 60% higher than the control. Two mixed brushes consisting of PEG and positively charged polymer chains (P2VP or QP2VP) were investigated along with single brushes to understand the role of addition of the bacteria repelling PEG. It appeared, that positively charged polymer brushes which potentially could inhibit biofilm formation (P2VP, PEG-P2VP and PEG-QP2VP) decrease adsorption of *S. aureus* compared to the control Si wafer surface. Modification of the surface with bacteria-repelling PEG brush and positively charged QP2VP brushes resulted in a 400-fold difference in the adsorption of *S. aureus* (from 0.005 to 2.109 au). Using a combination of PEG and QP2VP (or P2VP) chains in mixed brushes allows designing surfaces with tunable bacteria adsorption properties within more than two orders of magnitude.

### 4. Summary and conclusions

Five types of polymer brushes were synthesized and tested with respect to their ability to regulate microbial adhesion. The PEG brush was used as the “gold standard” for preventing microorganisms from anchoring to the surface, and it showed the lowest adsorption among the tested surfaces. Two positively charged brushes (P2VP and QP2VP) were prepared and tested against *S. aureus* adsorption. The P2VP brush showed a two-fold reduction of *S. aureus* adsorption compare to the control Si wafer surface. The QP2VP brush exhibited bacterial adsorption that was 60% higher than the control. Two mixed brushes consisting of PEG and positively charged polymer chains (P2VP or QP2VP) were investigated along with single brushes to understand the role of addition of the bacteria repelling PEG. It appeared, that positively charged polymer brushes which potentially could inhibit biofilm formation (P2VP, PEG-P2VP and PEG-QP2VP) decrease adsorption of *S. aureus* compared to the control Si wafer surface. Modification of the surface with bacteria-repelling PEG brush and positively charged QP2VP brushes resulted in a 400-fold difference in the adsorption of *S. aureus* (from 0.005 to 2.109 au). Using a combination of PEG and QP2VP (or P2VP) chains in mixed brushes allows designing surfaces with tunable bacteria adsorption properties within more than two orders of magnitude.

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