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Zwitterionic carboxybetaine polymer surfaces and their resistance to long-term biofilm formation

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Abstract

In this work, we report a systematic study of zwitterionic poly(carboxybetaine methacrylate) (pCBMA) grafted from glass surfaces via atom transfer radical polymerization (ATRP) for their resistance to long-term bacterial biofilm formation. Results show that pCBMA-grafted surfaces are highly resistant to nonspecific protein adsorption (fibrinogen and undiluted blood plasma) at 25, 30 and 37 °C. Long-term (over 24 h) colonization of two bacterial strains (*Pseudomonas aeruginosa* PAO1 and *Pseudomonas putida* strain 239) on pCBMA surface was studied using a parallel flow cell at 25, 30 and 37 °C. Uncoated glass cover slips were chosen as the positive reference. Results show that pCBMA coatings reduced long-term biofilm formation of *P. aeruginosa* up to 240 h by 95% at 25 °C and for 64 h by 93% at 37 °C, and suppressed *P. putida* biofilm accumulation up to 192 h by 95% at 30 °C, with respect to the glass reference. The ability of pCBMA coatings to resist non-specific protein adsorption and significantly retard bacterial biofilm formation makes it a very promising material for biomedical and industrial applications.

Keywords

Biofilm; Carboxybetaine; Non-fouling; Surface; Zwitterionic materials

1. Introduction

Unintended bacterial adhesion onto surfaces and the subsequent formation of biofilm are serious problems for many biomedical and engineering applications. Antimicrobial and non-fouling coatings are the most commonly used approaches to prevent the attachment and spreading of microorganisms onto surfaces. In the antimicrobial approach, leachable biocides such as antibiotics and heavy metals [1] are either incorporated into the polymeric surface coatings and released over time, or are covalently-linked to the polymeric surface coatings [2]. Released antimicrobial agents (such as antibiotics) can, however, potentially lead to drug resistance among microorganism. Biofilm bacteria are significantly less responsive to antibiotics and antimicrobial stressors than planktonic organisms of the same species [2–5]. In fact, studies have shown that sub-lethal doses of antibiotics can exacerbate biofilm formation [6]. Other polymer coatings containing covalently-linked antimicrobial agents (such as quaternary ammonium compounds) cannot fulfill the requirements for the non-fouling

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background; bio-macromolecules and dead microorganisms can easily accumulate at surfaces and eventually block its antimicrobial functional groups [3]. To suppress the accumulation of microorganisms onto surfaces, another commonly used method is to graft surfaces with poly (ethylene glycol) (PEG) derivatives and, more recently, zwitterionic polymers. It has been shown that PEG-coated surfaces can delay biofilm formation for 24 h [4–7]. Surfaces coated with 2-methacryloyloxyethyl phosphorylcholine (MPC) or its copolymers have been shown to reduce bacterial adhesion over 90% compared to uncoated control surfaces over a 24 h period [8–10]. Our previous study demonstrated that zwitterionic poly(sulfobetaine methacrylate) (pSBMA) dramatically reduced biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* for 1 and 2 days, respectively [11]. It was also demonstrated that zwitterionic poly(carboxybetaine methacrylate) (pCBMA) highly resists short-term bacterial adhesion [12] and nonspecific protein adsorption even from undiluted blood plasma and serum [13–17]. Although existing non-fouling materials have been shown to resist biofilm formation for one or two days, none have been able to resist biofilm formation for longer periods of time.

Materials capable of resisting long-term biofilm formation in complex media while maintaining non-fouling properties are highly desirable for many applications, but their development is very challenging. The objective of this work is to systematically study the performance of ultra-low fouling pCBMA surfaces to resist long-term biofilm formation under various conditions: (1) temperature (25–37 °C), (2) type of bacterial challenge (bacterial cells were seeded for 1 h or continuously), (3) bacterial strain (*P. aeruginosa* and *Pseudomonas putida*), (4) in the presence or absence of blood plasma, and (5) zwitterionic group (pCBMA vs. pSBMA).

2. Materials and methods

2.1. Chemicals

Human plasma fibrinogen and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Milwaukee, WI). Human plasma was obtained from Biochemed Services (Winchester, VA). Horseradish peroxidase (HRP)-conjugated anti-fibrinogen was purchased from United States Biological (Swampscott, MA). *N*-(3-sulfopropyl)-*N*-(methacryloxyethyl)-*N,N*-dimethylammonium betaine (SBMA, 97%), dopamine hydrochloride, copper(I) bromide (99.999%), copper(II) bromide (99.999%), bromoisobutyryl bromide (BIBB, 98%), 11-mercapto-1-undecanol (97%), 2,2'-bipyridine (BPY, 99%), 0.15 M phosphate-buffered saline (PBS), (3-Aminopropyl)trimethoxysilane (APTMOs), and tetrahydrofuran (THF, HPLC grade) were purchased from Sigma–Aldrich. Ethanol (absolute 200 proof) was purchased from AAPER Alcohol and Chemical Co. Water used in the experiments was purified using a Millipore water purification system. THF for reactions and washings was dried by sodium before use.

Mercaptoundecyl bromoisobutyrate was synthesized through the reaction of bromoisobutyryl bromide and 11-mercapto-1-undecanol using a method published previously [18]. 2-Carboxy-*N,N*-dimethyl-*N*-(2'-(methacryloyloxy)ethyl)ethanaminium (carboxybetaine methacrylate, CBMA) was synthesized by the reaction of DMAEMA and β -propiolactone using a method published previously [13,16]. The ATRP initiator on glass, BrTMOS, was also synthesized using a previously published method [19].

2.2. Preparation of silane-coated surfaces

Glass cover slips (24 mm $\times$ 60 mm) from VWR were put into 20 wt% NaOH solution overnight, washed with DI water, and dried in air. Prior to silanization, the glass substrata were washed with pure ethanol, cleaned under UV light for 20 min, washed with water and pure ethanol, and dried in a stream of nitrogen. The freshly cleaned substrata were immersed in 20 ml of ethanol solution containing 0.5 g of BrTMOS for 2 h. The glass samples were then removed

from the dipping solution with forceps, slightly rinsed with ethanol, and dried with a stream of nitrogen. Substrata were kept at 100 °C for 5 h in a vacuum oven.

2.3. Preparation of thiol-coated surfaces

Electron beam evaporation under vacuum was used to coat glass samples with a layer of titanium (2 nm thick) to promote the adhesion of a subsequent layer of gold (48 nm thick). Before self assembled monolayer (SAM) preparation, the substrata were washed with pure ethanol, cleaned with a UV–ozone cleaner (Jelight, model 42) for 20 min, and then washed with water and pure ethanol. The initiator SAMs were formed by soaking gold-coated substrates in a pure ethanol solution containing 0.1 mM ω -mercaptoundecyl bromoisobutyrate [16] at room temperature for 24 h. Before polymerization, substrata were rinsed with pure ethanol, followed by THF, and dried with a stream of nitrogen.

2.4. Atom transfer radical polymerization (ATRP) of CBMA

CuBr (143 mg, 1 mmol), CuBr₂ (22 mg, 0.1 mmol) and BPY (343 mg, 2.2 mmol) and glass substrata with immobilized initiators were placed in a reaction tube within a dry box under nitrogen protection. The reaction tube was sealed with rubber septum stoppers before removal from the dry box. 16 ml of degassed methanol and 4 ml of degassed DI water with 3 g of CBMA was then transferred to the reaction tube using a syringe under nitrogen protection to obtain a final monomer concentration of 0.15 g/ml. The substratum was reacted for 24 h at 25 °C under nitrogen protection. After the reaction, the substratum was removed and rinsed with methanol and water, and kept in PBS overnight. The substratum was rinsed with water to remove unbound polymers before testing.

2.5. Preparation of pSBMA polymer brushes using DOPA initiator

DOPA-ATRP initiator (2-Bromo-2-methyl-N-[2-(3,4-dihydroxy-phenyl)-ethyl]-propionamide) was synthesized using a previously published method [20]. The initiator was synthesized by reacting dopamine·HCl with BIBB. The product after purification was a colorless viscous liquid (1.3 g, 43%). NH₂-functionalized glass samples for use in the long-term bacterial adhesion experiments were prepared using 3-aminopropyltrimethoxysilane, following a procedure described previously [21]. Glass substrata coated with DOPA-ATRP initiator were then immersed in a 10 mM Tris–HCl buffer (pH = 8.5) with 1 mg/ml initiator for 24 h, protected from light. SBMA was grafted to the surfaces via ATRP following a method reported previously [20]. DI water and methanol were deoxygenated by passing a continuous stream of dry N₂ through the solution (15 min) at room temperature. Initiator-modified samples with BPY (210 mg, 1.34 mmol), CuBr (76.81 mg, 0.54 mmol), and CuBr₂ (12.04 mg, 0.05 mmol) were sealed in a glass tube, deoxygenated (three high-vacuum-pump/N₂ refill cycles), dissolved in methanol (10 ml), and left at room temperature under N₂. SBMA (3.75 g, 26.75 mmol) was sealed in another glass tube, deoxygenated (three high-vacuum-pump/N₂ refill cycles), dissolved in a mixture of DI water (5 ml) and methanol (10 ml), and left at room temperature under nitrogen protection. The monomer solution was then transferred into the sample tube with a syringe under nitrogen protection. After overnight polymerization, the samples were removed, washed with warm DI water (60 °C), and dried under a stream of N₂.

2.6. Film thickness measurement and surface characterization

Thickness of the polymer brushes formed on a substratum surface was measured by atomic force microscopy (AFM) in contact mode using a Dimension 3100 AFM (Digital Instruments/Veeco, Woodbury, NY) operated in air based on the previously published method [22]. Commercial Si₃N₄ cantilevers (DI) with an elastic modulus of 0.56 N/m were used. Gold-coated Si wafers were patterned using standard photolithography techniques to obtain lines of Au with a width and line spacing of ~25 nm and a measured step height of 48.2 ± 1.4 nm.

These patterned wafers were then subjected to ATRP reaction together with the regular surface plasmon resonance (SPR) sensor chips and the step height was measured again to determine the thickness of the polymer brush coating. The polymer surfaces were blown dry with filtered air before analysis. A total of 3 measurements were taken for each of 3 samples.

2.7. Measurements of protein adsorption on pCBMA-coated Au surfaces by SPR

In this study, a custom-built SPR sensor was used to measure protein adsorption on the coated substrata [23,24]. An SPR chip was attached to the base of the prism and optical contact was established using refractive index matching fluid (Cargille). A dual-channel flow cell with two independent parallel flow channels was used to contain liquid samples during experiments. A peristaltic pump (Ismatec) was used to deliver liquid samples to the two channels of the flow cell. Fibrinogen solution of 1.0 mg/ml in PBS (0.15 M, pH 7.4) or 100% human plasma was delivered to the surfaces at a flow rate of 0.05 ml/min. A surface-sensitive SPR detector was used to monitor protein-surface interactions in real time. In this work, the resulting wavelength shift was used to measure the changes in surface concentration (mass per unit area). For the SPR sensor used, a 1 nm wavelength shift at 750 nm is equivalent to ~15 ng/cm² of adsorbed protein [11].

2.8. Measurements of protein adsorption on pCBMA-coated glass slides by ELISA

The adsorption of protein on glass substrate coated with pCBMA was determined by Enzyme-Linked Immunosorbent Assay (ELISA) [25]. To measure protein adsorption, pCBMA-coated glass, unmodified glass, and tissue culture polystyrene (TCPS) samples were placed in the 24-well plate and the samples lean against walls to allow protein adsorption onto both sides of the samples. The samples were first incubated with human fibrinogen (1 mg/ml) in PBS (pH 7.4) for 1.5 h at room temperature, followed by another 5 washes with PBS. Then the samples were incubated with 1 ml of BSA in PBS (pH 7.4) for 1.5 h at room temperature, followed by another 5 washes with PBS. pCBMA-coated glass, unmodified glass, and tissue culture polystyrene (TCPS) samples were then incubated with horseradish peroxidase (HRP)-conjugated anti-fibrinogen (10 µg/ml) for 30 min in a buffer at room temperature, followed by another 5 washes with PBS. The pCBMA surface, uncoated glass and TCPS substrata were taken out and put into 24 wells plates. 800 µl of 1 mg/ml o-Phenylenediamine (OPD) in 0.1 M citrate-phosphate buffer (pH 5.0), containing 0.03% hydrogen peroxide, was added. Enzyme activity was stopped by adding an equal volume of 2 N H₂SO₄ after 15 min. The tangerine color was measured at 492 nm.

2.9. Bacterial species and culture conditions

P. aeruginosa PAO1 with a green fluorescent protein (GFP) reporter gene was used to measure biofilm formation at 25 °C and 37 °C in this work. The optimal growth temperature for *P. aeruginosa* PAO1 is 37 °C. *P. aeruginosa* were first cultured overnight at 37 °C on trypticase soy broth (TSB) (BD, USA) [7] agar plates containing 200 µg/ml carbenicillin. Cultures on agar plates can be used for two weeks, if kept at 4 °C. Several colonies from a plate were used to inoculate 25 ml of TSB (10 g/L) containing 200 µg/ml carbenicillin for *P. aeruginosa* [7]. These initial cultures were incubated at 37 °C with shaking at 100 rpm for 18 h and were then used to inoculate a second 200 ml of TSB medium.

P. putida strain 239 with a red fluorescent protein RFP expressing plasmid was also used to measure biofilm formation at 30 °C in this work, since its optimal growth temperature is 30 °C. *P. putida* were first cultured overnight at 30 °C on Luria-Bertani (LB) (BD, USA) agar plates with 10 µg/ml gentamycin. Several colonies were used to inoculate 25 ml of modified FAB medium containing 10 µg/ml gentamycin [26]. These initial cultures were incubated at 30 °C with shaking at 100 rpm for 18 h and were then used to inoculate a second culture of each species in 200 ml of modified FAB medium.

2.10. Biofilm formation assay

A parallel flow cell (BioSurface Technologies Corp, name the town Bozeman, MT) was used to quantify biofilm formation onto the pCBMA-coated substrata. Dimensions of the flow chamber were 50 mm (L), 15 mm (W), and 2.5 mm (H). The flow chamber was sterilized and cleaned using a method reported previously [7]. 24 mm × 60 mm glass cover slips coated with polymer films were placed into the flow cell, flush with the surface exposed to passing fluid.

2.10.1. Method I

P. aeruginosa cells from the second culture were washed three times with sterile PBS and subsequently re-suspended in sterile PBS at a concentration of 10^6 cells/ml. The cell suspension was delivered by peristaltic pump to the flow cell at a flow rate of 2 ml/min for 1 h. After 1 h, sterile TSB medium was delivered by peristaltic pump to the flow cell at a flow rate of 2 ml/min. The temperature of the system was maintained at 25 °C or 37 °C, depending on the demands of the experiment. At different time points, the accumulated bacteria were recorded *in situ* using a CCD-CoolSNAP camera mounted on a Nikon Eclipse 80i microscope using a 100x oil lens and epifluorescent illumination through a FITC filter. The same procedure was repeated for *P. putida* cells using sterile modified FAB medium at 30 °C; accumulated bacteria were recorded by the same microscope through a Texas Red filter.

2.10.2. Method II

In method II, cells were continuously produced using a continuously-fed, well mixed constant volume suspended cell reactor (*i.e.*, chemostat). Once at steady-state, a portion of the cells produced in the chemostat were delivered in a once-through mode to the flow cell system. The parallel flow cell, the chemostat containing 500 ml of 10 g/l TSB medium, and a 10 L container with 10 g/l TSB medium were sterilized by autoclaving. The chemostat containing 500 ml of sterile TSB medium was inoculated with 0.5 ml of *P. aeruginosa* culture grown overnight. The chemostat was maintained at 25 °C or 37 °C with a dilution rate (defined as nutrient feed rate/reactor volume) of 0.24 h^{-1} for 24 h to allow the population of bacteria to reach a steady-state. Once at steady-state, the bacterial suspension was then pumped through the parallel flow chambers with pCBMA-2 and glass samples at a rate of 2 ml/min. At different time points, the accumulated bacteria were recorded *in situ* as above. The same procedure was repeated for *P. putida* using the sterile modified FAB medium at 30 °C, and the accumulated bacteria were recorded by the same microscope through a Texas Red filter.

3. Results and discussion

In this work, pCBMA and pSBMA polymer brushes were prepared via atom transfer radical polymerization (ATRP), following the procedure reported previously [19]. Our prior study showed that polymer film thickness could dramatically influence the ability of surface coatings to resist biofilm formation [11] and protein adsorption from 100% human plasma [22]. The optimized polymer film thickness was previously determined to be between 20 nm and 30 nm for pCBMA surface (unpublished data). Atomic force microscopy (AFM) was used to determine the film thickness [22] of the pCBMA-coated surface used in this study to be 29.0 ± 1.3 nm. Protein adsorption from 100% human blood plasma and from single fibrinogen solution on substrata was first measured by an SPR sensor. Fig. 1 shows the change in SPR signal due to protein adsorption as a function of time on pCBMA surface. Protein adsorption is defined as the wavelength shift before protein injection and after buffer wash. The wavelength shift after protein injection is mainly due to the change in bulk refractive index. At 25 °C (Fig. 1a), 30 °C (Fig. 1b), and 37 °C (Fig. 1c), pCBMA surface is highly resistant to non-specific fibrinogen adsorption and 100% human plasma (*i.e.*, 0.3 ng/cm^2 , which is the detection limit of the SPR sensor). The adsorption of fibrinogen on glass substrate coated with pCBMA was determined by ELISA to be 7.1% and 2.3% relative to unmodified glass and

tissue culture polystyrene (TCPS) substrates, respectively. Protein adsorption onto pCBMA coated onto gold was observed to be lower than on pCBMA coated onto glass due to the higher packing density of ATRP initiators on gold [27].

In this study, a strain of *P. aeruginosa* PAO1 with GFP and a strain of *P. putida* with RFP were used to quantify biofilm formation on pCBMA surface under different conditions. *P. aeruginosa* is a major pathogen in the chronic pulmonary infections of patients with cystic fibrosis and also a major colonizing bacterium on various biomedical implants (contact lenses, intraocular lens, catheters). *P. aeruginosa* is able to grow over a wide range of temperatures, from common environmental temperatures to 42 °C, with an optimum temperature at 37 °C. Biofilm formation of *P. aeruginosa* on pCBMA surface was quantified here at 25 °C and 37 °C. *P. putida*, whose optimum temperature for biofilm formation is 30 °C, was used to test the resistance of pCBMA surface to biofilm formation at 30 °C. Unmodified glass was used as a control surface. Biofilm formation was quantified *in situ* using a parallel flow cell system.

Fig. 2 shows a series of representative qualitative images of *P. aeruginosa* and *P. putida* accumulated on pCBMA surface and unmodified glass at different temperatures. In these growth-mode experiments (Method I), pCBMA surface can effectively resist biofilm formation for 10 days at 25 °C, 8 days at 30 °C and 2.5 days at 37 °C.

Bacterial cells were seeded via two methods. In Method I, a bacterial cell suspension (10^6 cells/ml) was pumped through the flow cell at a flow rate of 2 ml/min for 1 h, allowing bacterial cells to attach to the surface. After 1 h, sterile medium was pumped into the flow cell at a rate of 2 ml/min and at different temperatures to allow any adherent cells to grow on the surface and to wash away loosely attached cells. In Method 2, a chemostat operated at different temperatures at a dilution rate of 0.24 h^{-1} was used to generate bacterial suspension ($\sim 10^8$ cells/ml), that was pumped through the parallel flow cells with pCBMA and glass samples at a rate of 2 ml/min. In this method, the surface is continuously challenged with a high concentration of viable bacteria. Biofilm formation on the pCBMA surface was compared side-by-side with glass. Because the purpose of this study is to test surface resistance to bacterial accumulation, experiments on the glass surface were stopped once the control glass surface was completely covered with bacteria, while tests on the pCBMA surface continued.

The effect of temperature on the biofilm formation of *P. aeruginosa* PAO1 on pCBMA surface was investigated at 25 °C and 37 °C, using Method I. Results for the long-term biofilm formation of *P. aeruginosa* at 25 °C and 37 °C are reported in Fig. 3a and Fig. 4, respectively. Significant delay in biofilm formation of *P. aeruginosa* was observed on pCBMA surface at both temperatures, as compared to unmodified glass at the same temperatures. At 25 °C, the glass surface is completely covered by *P. aeruginosa* after 48 h, while after 10 days the bacterial density on the surface is less than 5% of that accumulated on glass surface at 48 h. At the optimal growth temperature of 37 °C for *P. aeruginosa*, the glass surface showed much faster accumulation of *P. aeruginosa* comparing to that at 25 °C; the glass surface was completely covered by *P. aeruginosa* within 15 h. On pCBMA surface, however, the bacterial density after 64 h, is less than 7% of that on glass surface at 15 h.

The effect of bacterial seeding densities on biofilm formation was studied with two methods (Method I and Method II) described above. Method I was used to mimic the situation (such as catheter insertion) wherein bacterial cells were initially attached to the pCBMA and glass surface and then proliferate on those surfaces, with no further fluid phase cell challenge. Method II was employed to simulate conditions (such as pipeline or filter system in water treatment) in which pCBMA and glass surfaces are continuously challenged with bacteria from the mobile phase. At 25 °C in Method I, the glass surface is completely covered by *P. aeruginosa* after 48 h while after 10 days the bacterial surface density on the pCBMA surface

is less than 5% of that accumulated on glass surface at 48 h. In Method II, a faster formation of biofilm (Fig. 3b) was observed within 24 h on glass. After 4 days, the bacterial density on the pCBMA surface was less than 9% of that accumulated on glass surface at 24 h.

Biofilm formation in practical biomedical applications occurs at 37 °C and in the presence of blood. Therefore, the pCBMA surface in this study was incubated with 100% human plasma for 10 min, and biofilm formation on protein exposed plasma-treated pCBMA surface was then studied using Method I. It was observed that biofilm formation on plasma-treated pCBMA surface (Fig. 4) was accelerated on the protein-exposed pCBMA coated samples as compared to that on the unexposed pCBMA surface. After 64 h, the bacterial density on the protein-exposed pCBMA coated samples is 34% of that accumulated on glass surface at 15 h. After 64 h the bacterial density on unexposed pCBMA surface is less than 7% of that accumulated on glass surface at 15 h. It has been reported that the attachment of peptide sequences derived from blood proteins promote the attachment of *P. aeruginosa* [7] and that blood proteins interact with *P. aeruginosa* during bacterial invasion into tissue [28,29]. The accelerated accumulation of *P. aeruginosa* on plasma-treated surface is caused by the deposition of blood protein on the pCBMA-coated glass surface. Protein adsorption on gold coated with pCBMA is less than 0.3 ng/cm² (the detection limit of the SPR sensor), which is equivalent to ~0.1% of adsorbed proteins on a hydrophobic methyl-terminated SAM. However, protein adsorption on glass coated with pCBMA was 2.3% with respect to that on a hydrophobic TCPS. Higher protein adsorption on the pCBMA-coated glass than on the pCBMA-coated Au from 100% plasma is expected. It is expected that even longer resistance to biofilm formation can be achieved if pCBMA coatings can be further improved on glass surfaces.

The non-fouling performance of pCBMA surface was further investigated using *P. putida* with an optimal growth temperature at 30 °C for biofilm formation. Results are reported in Fig. 5a (Method I) and Fig. 5b (Method II). Significant delay in biofilm formation of *P. putida* was observed on pCBMA surface, as compared to that on the unmodified glasses tested by the same method side-by-side. In Method I, the glass surface is covered by *P. putida* after 96 h, while after 8 days the bacterial density on the pCBMA-coated glass surface was less than 5% of that accumulated on glass surface at 96 h. In Method II, a faster formation of biofilm was observed within 72 h on glass, as expected. After 7 days the bacterial density on the pCBMA-coated glass surface was less than 2% of that accumulated on the glass surface at 72 h.

Finally, another zwitterionic polymer surface, pSBMA surface, was also evaluated and compared with pCBMA surface. In this work, pSBMA brushes were grafted from a glass surface coated with 3,4-Dihydroxyphenyl-L-alanine (DOPA) initiators. The pSBMA surfaces were then exposed to *P. aeruginosa* PAO1 with GFP at 25 °C, in a Method I protocol. Results (Fig. 6) show that pSBMA coated surfaces can dramatically reduce biofilm formation over 9 days, where accumulated bacterial cells on pSBMA surface are less than 1 percent of that accumulated on the unmodified glass at 38 h. The glass surface is nearly covered by *P. aeruginosa* within 38 h. The ability of pSBMA to resist biofilm formation is comparable to that of pCBMA.

4. Conclusions

In this work, we report a systematic study of zwitterionic pCBMA grafted from glass surfaces via ATRP for their resistance to long-term bacterial biofilm formation. Results show that pCBMA coatings reduced long-term biofilm formation of *P. aeruginosa* up to 240 h by 95% at 25 °C and up to 64 h by 93% at 37 °C, and suppress *P. putida* biofilm accumulation up to 192 h by 95% at 30 °C, with respect to the glass reference. Zwitterionic pCBMA brushes grafted from gold surfaces via ATRP strongly resist non-specific protein adsorption from 100% plasma at 25 °C, 30 °C and 37 °C. This new non-fouling polymer surface has great potential to prolong

the performance and increase the efficiency of devices for biomedical and industrial applications.

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Appendix

Figures with essential colour discrimination. The majority of figures in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.05.058.

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Fig. 1. Representative SPR sensorgrams showing the adsorption of 1 mg/ml human fibrinogen in PBS buffer and 100% human plasma on the gold surfaces grafted with pCBMA via ATRP at (a) 25 °C, (b) 30 °C, and (c) 37 °C.

Fig. 2.

Representative fluorescence microscopy graphs of biofilm formation on pCBMA surface tested by Method I at different temperatures (a) *P. aeruginosa* on unmodified glass for 48 h at 25 °C, (b) *P. aeruginosa* on pCBMA surface for 240 h at 25 °C, (c) *P. putida* on unmodified glass for 96 h at 30 °C, (d) *P. putida* on pCBMA for 192 h at 30 °C, (e) *P. aeruginosa* on unmodified glass for 15 h at 37 °C, (f) *P. aeruginosa* on pCBMA surface for 64 h at 37 °C.

Fig. 3. *P. aeruginosa* accumulation on pCBMA surface (■) and glass (•) surfaces as a function of time at 25 °C (a) tested with Method I and (b) tested with Method II; reported as the mean $\pm$ SD ($n = 20$).

Fig. 4. *P. aeruginosa* accumulation on pCBMA surface (■) and pCBMA treated with 100% plasma (□) and glass (●) surfaces as a function of time at 37 °C tested with Method I; reported as the mean $\pm$ SD ($n = 20$).

Fig. 5. *P. putida* accumulation on pCBMA surface (■) and glass (●) surfaces as a function of time at 30 °C (a) tested with Method I and (b) tested with Method II; reported as the mean $\pm$ SD ($n = 20$).

Fig. 6. *P. aeruginosa* accumulation on pSBMA surface (■) and glass (●) surfaces as a function of time at 25 °C tested with Method I; reported as the mean $\pm$ SD ($n = 20$).