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Blood compatibility of surfaces with superlow protein adsorption

Zheng Zhang [a], Min Zhang [b], Shengfu Chen [a], Thomas A. Horbett [a,b,*], Buddy D. Ratner [a,b,*], Shaoyi Jiang [a,b,*]

[a] Department of Chemical Engineering, University of Washington, Seattle, WA 98195, USA
[b] Department of Bioengineering, University of Washington, Seattle, WA 98195, USA

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ABSTRACT

In this work, five self-assembled monolayers (SAMs) and three polymeric brushes with very low fibrinogen adsorption were prepared. The five SAMs are oligo(ethylene glycol) (OEG), phosphorylcholine (PC), oligo(phosphorylcholine) (OPC), and two mixed positively and negatively charged SAMs of $SO_3^-/N^+(CH_3)_3$ (SA/TMA) and $COO^-/N^+(CH_3)_3$ (CA/TMA). Three polymer brushes were prepared on gold surfaces via surface-initiated atom transfer radical polymerization (ATRP) using three monomers, sulfobetaine methacrylate (SBMA), carboxybetaine methacrylate (CBMA), and oligo(ethylene glycol) methyl ether methacrylate (OEGMA). Surface plasmon resonance (SPR) measurements show that although all of these surfaces are "nonfouling" to fibrinogen adsorption from buffer solution, their protein adsorption from undiluted human blood plasma varies widely. Polymer brushes exhibit much lower protein adsorption from plasma than any of the five SAMs tested. However, platelet adhesion measurements on plasma-preadsorbed surfaces show that all of these surfaces have very low platelet adhesion. Clotting time measurements using recalcified platelet poor plasma (PPP) incubation with the eight types of surfaces show that they do not shorten clotting times. Linear polymers of polySBMA and polyCBMA with similar molecular weights were also synthesized and characterized. In the presence of polyCBMA linear polymers, the clotting time of PPP was prolonged and increased with the concentration of the polymer, while no anticoagulant activity was observed for the polySBMA or PEG polymers. The unique anticoagulant activity of polyCBMA, as well as its high plasma protein adsorption resistance, makes polyCBMA a candidate for blood-contacting applications.

1. Introduction

For blood-contacting devices such as hemodialysis membranes, artificial blood vessels, heart valves, stents and biosensors used in contact with blood, high blood compatibility is highly desirable. The blood-material interactions that affect blood compatibility consist of a multi-step and interlinked process, including protein adsorption, and platelet adhesion and activation. These interactions strongly affect the short-term and long-term thrombotic response induced by the materials [1–3]. Historically, in the development of new blood-contacting materials, hundreds of surfaces and polymers have been evaluated in regard of one or more aspects of their interactions with a single protein such as fibrinogen, though overall blood compatibility has been assessed less often.

Protein adsorption is the first event in blood-material interactions and some proteins in blood, especially the clotting enzymes and fibrinogen, play an important role in material-associated clotting [2]. Thus, to reduce clotting, surfaces or materials with highly protein-resistant properties have been extensively investigated [4]. Several types of functional groups have been shown to be highly resistant to fibrinogen adsorption, including (1) oligoethylene glycol (OEG) or polyethylene glycol (PEG) groups, (2) zwitterionic groups such as phosphorylcholine (PC), sulfobetaine (SB), and carboxybetaine (CB), and (3) mixed positively and negatively charged self-assembled monolayers (SAMs) with balanced charges [5,6]. Furthermore, to study the interactions between polymers and blood proteins, PEG or polymers with OEG side chains such as oligo(ethylene glycol) methyl ether methacrylate (polyOEGMA) [7], zwitterionic polymers such as poly(2-methacryloyloxyethyl phosphorylcholine) (polyMPC) [8–10], poly(sulfobetaine methacrylate) (polySBMA) [11–13], and poly(carboxybetaine methacrylate) (polyCBMA) [12–14] have demonstrated high resistance to protein adsorption. PolySBMA and polyCBMA brushes exhibit fibrinogen adsorption $<0.3\ ng/cm^2$ from a single fibrinogen solution as

* Corresponding authors. Department of Chemical Engineering, University of Washington, Seattle, WA 98195, USA. Tel.: +1 206 616 6509.
E-mail addresses: horbett@uweb.engr.washington.edu (T.A. Horbett), ratner@uweb.engr.washington.edu (B.D. Ratner), sjiang@u.washington.edu (S. Jiang).

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measured by SPR. These "nonfouling" surfaces are often evaluated by protein adsorption from a single protein solution.

Protein adsorption from plasma is far more complicated than from a single protein solution. Since plasma is a concentrated, multi-protein solution and its adsorption is regarded as a selective and competitive process [1], "nonfouling surfaces" that are highly resistant to protein adsorption from a single-protein solution may not necessarily present a similar high efficiency in resisting protein adsorption from plasma. For example, OEG SAMs were shown to have very low fibrinogen adsorption from single protein buffer solutions, but were not as highly-resistant to protein adsorption from serum and plasma [15]. Platelet adhesion is mediated by integrins on the surface of the platelet that bind to adsorbed adhesion proteins, especially fibrinogen, and cause platelet activation. Activated platelets then accelerate thrombosis as they promote thrombin formation and platelet aggregation [16–19]. Fibrinogen in blood plasma is particularly important for platelet adhesion since it can bind to the platelet GP IIb/IIIa receptor [20]. Thus, very low fibrinogen adsorption and low platelet adhesion are considered essential to achieve improved blood compatibility [20,21]. It was found previously that less than 5–10 ng/cm^2 fibrinogen adsorption is necessary to prevent platelet attachment [22]. Later, it was found that platelet adhesion was also determined by the molecular potency of the adsorbed fibrinogen. The surfaces incorporating zwitterionic moieties, such as phosphobetaines, sulfobetaines and carboxybetaines, were shown to reduce platelet adhesion as compared to untreated samples [23–25]. However, these surfaces may not necessarily adsorb fibrinogen below the threshold of <5–10 ng/cm^2 to effectively reduce platelet adhesion and blood clotting.

In addition, consideration must be made of whether adherent platelets are activated and can promote clot formation, i.e., whether they are in the procoagulant state [26]. Just a few activated platelets may be enough to trigger the thrombotic response. On some PEG-immobilized materials, platelet reactivity in vivo was still high even though protein adsorption in vitro was low [27]. Thus, surfaces which are "nonfouling" to fibrinogen adsorption and can reduce platelet adhesion as compared to untreated surfaces may not be good enough for blood biocompatibility, and thus some active inhibition of clotting may be needed. In this regard, it is well known that some heparinized and sulfonated materials have anticoagulant activities resulting in prolongation of the blood clotting time when they are either dissolved in the solutions or coated on the blood containers [28,29]. However, at present there is a significant lack of information regarding these surfaces for their "stealth" (i.e., grafted polymers do not alter blood clotting time by themselves, such as PEG-like surfaces [30]) and anti-coagulant properties (i.e., grafted polymers can prolong blood clotting time by themselves, such as heparinized surfaces).

We report measurements of protein adsorption from blood plasma, platelet adhesion, and blood clotting times for several surfaces with very low fibrinogen adsorption prepared as grafted polymers or SAMs. The SAM surfaces include (1) OEG SAMs, (2) PC SAMs, (3) oligoPC SAMs, (4) mixed $SO_3^-/N^+(CH_3)_3$ (SA/TMA) SAMs, and (5) mixed $COO^-/N^+(CH_3)_3$ (CA/TMA) SAMs. The surface grafted polymers were made as "brushes" via surface-initiated ATRP and include polySBMA, polyCBMA, and polyOEGMA. Furthermore, we prepared linear zwitterionic polySBMA and polyCBMA polymers and evaluated their anticoagulant activities in plasma in comparison to those of polyOEGMA.

2. Experimental methods

2.1. Materials

(1-Mercaptoundec-11-yl)tetra(ethylene glycol) (OEG thiol), 11-mercapto-*N,N,N*-trimethylammonium chloride (TMA), 12-mercaptododecanoic acid (CA) and 11-mercaptoundecylsulfonic acid (SA) were purchased from Prochimia (Gdansk, Poland). Copper (I) bromide (99.999%), bromoisobutyryl bromide (BIBB 98%), *N*-(3-sulfopropyl)-*N*-(methacryloxyethyl)-*N,N*-dimethylammonium betaine (or sulfobetaine methacrylate, SBMA, 97%), poly(ethylene glycol) methyl ether methacrylate (or oligo(ethylene glycol) methyl ether methacrylate, OEGMA, average Mn 300), 11-mercapto-1-undecanol (97%), and 2,2′-bipyridine (BPY, 99%) were purchased from Sigma–Aldrich, Milwaukee, WI. Human fibrinogen and phosphate buffered saline (PBS, 138 mM NaCl, 2.7 mM KCl, pH7.4, 0.15 m) were purchased from Sigma Chemical Co. Ethanol (absolute 200 proof) was purchased from AAPER Alcohol and Chemical Co. Water used in experiments was purified using a Millipore water purification system with a minimum resistivity of 18.0 MΩ cm. Carboxybetaine methacrylate (CBMA) [12], ω-mercaptoundecyl bromoisobutyrate (Br-thiol) [11], 11-mercaptoundecylphosphorylcholine (PC thiol) [31], and 11-mercaptoundecyl-oligo-phosphorylcholine (OPC thiol) [32] were synthesized and characterized using the methods reported previously by our group.

2.2. Preparation of SAMs on gold surfaces

For surface plasmon resonance (SPR) analysis of protein adsorption, glass chips were first coated with an adhesion-promoting chromium layer (thickness 2 nm) and a surface plasmon-active gold layer (48 nm) by electron beam evaporation under vacuum. Before SAM preparation, the gold-coated glass substrate was cleaned by washing with ethanol and distilled water in sequence, dried with N_2, then left in an UV ozone cleaner for 20 min, followed by rinsing with distilled water and ethanol, and dried by N_2.

Gold foil with a thickness of 0.025 mm (Aldrich–Sigma, 99.99%) was cut into 10-mm-diameter circular disks. The gold disks, reusable for SAM preparation, were cleaned by soaking in cleaning solution (chromic–sulfuric acid) for more than 4 h, followed by rinsing with distilled water and ethanol, and drying by N_2.

Five SAMs with different functional groups were prepared on the gold substrates (Scheme 1): (1) OEG SAMs, (2) PC SAMs, (3) OPC SAMs, (4) mixed $SO_3^-/N^+(CH_3)_3$ (SA/TMA) SAMs, and (5) mixed $COO^-/N^+(CH_3)_3$ (CA/TMA) SAMs. To prepare OEG SAMs, the cleaned chip or disk was immediately soaked in a 0.1 mM ethanol solution of OEG thiol for 24 h to form a SAM on the gold surface. The chip or disk was rinsed in sequence with ethanol and water, and dried in a stream of N_2 [33]. Preparation of PC SAM and OPC SAM surface was reported previously by us [31,32]. Freshly cleaned chips or gold disks were dipped into a basic aqueous solution of 1% v/v NH_4OH and 0.2 mg/mL PC thiol or OPC thiol overnight. The SAMs were washed with purified water (Millipore Nanopure water) and then dried in a stream of N_2. To prepare a mixed SA/TMA SAM, the cleaned chip or gold disk was immediately soaked into a total 0.1 mM of TMA and 0.1 mM SA in PBS buffer (pH 7.4) for 24 h. A mixed CA/TMA SAM was prepared using 0.1 mM TMA and 0.1 mM CA in an aqueous solution with 1% v/v NH_4OH. Both mixed SAMs was rinsed in water, and dried in a stream of N_2 [6].

2.3. Preparation of SBMA, CBMA, and OEGMA polymer brushes on gold surfaces

Three monomers, SBMA, CBMA, and OEGMA, were grafted on the surfaces of SPR sensor chips or gold disks using surface initiated ATRP. The preparation and characterization of polySBMA [11], polyCBMA [14], polyOEGMA [34] polymer brushes were described in our previous publications. Briefly, CuBr and a gold-coated SPR chip or a gold disk with a Br-thiol SAM was placed in a nitrogen-purged reaction tube. Degassed solution with SBMA, CBMA, or OEGMA monomer, and BPY were transferred to the tube using a syringe. After reaction for more than 1 h under nitrogen, the SPR chip or gold disk was removed and rinsed with ethanol, water and PBS solution (Scheme 2). The samples were stored in PBS solutions before testing. The thickness of polySBMA brushes and polyCBMA brushes is 10–15 nm and the thickness of polyOEGMA brushes is 20–25 nm measured by ellipsometry.

2.4. Fibrinogen and plasma adsorption

Fibrinogen adsorption from a buffer solution or total protein adsorption from plasma was measured with a custom-built SPR sensor, which is based on wavelength interrogation methodology. A SPR chip was attached to the base of the prism, and optical contact was established using a refractive index matching fluid from Cargille Laboratories, NJ. A dual-channel flow cell with two independent parallel paths was used to direct liquid samples during experiments. A peristaltic pump (Ismatec) was utilized to deliver liquid samples to the two channels of the flow cell. 1 mg/mL fibrinogen in buffer solution or 100% human plasma was passed over the surface at a flow rate of 0.05 mL/min, followed by buffer solution to remove weakly adsorbed proteins until no mass loss was found from the SPR signal. A surface-sensitive SPR detector was used to monitor protein-surface interactions in real time. Protein adsorption is defined as the difference in two baselines before and after a protein solution is introduced into the SPR sensor. In this study, wavelength shift was used as a measurement of refractive index change, which was converted to surface concentration (mass per unit area) change. For the SPR sensor used in the study, a 1-nm SPR wavelength shift around 750 nm represents a surface coverage of ~15 ng/cm^2 adsorbed protein [35]. Pooled human plasma in citrate phosphate dextrose (CPD) was purchased from Biochemed Services (Winchester, VA).