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Abstract
The initial response of blood exposed to an artificial surface is the adsorption of blood proteins that triggers a number of biological reactions such as inflammation and blood coagulation. Competitive protein adsorption plays a key role in the hemocompatibility of the surface. The synthesis of nonfouling surfaces is therefore one of the major prerequisites for devices for biomedical applications. Polysaccharides are the main components of the endothelial cell glycocalyx and have the ability to reduce nonspecific protein adsorption and cell adhesion and, therefore, are generally coupled with a wide variety of surfaces to improve their biocompatibility. We have developed a procedure for covalently binding dextran and sodium hyaluronate (HA) on silicon wafers and we have been able to achieve a high level of control over the surface properties of the coatings. In the present research effort we focus on a detailed investigation of competitive bovine serum albumin (BSA) and bovine fibrinogen (Fg) adsorption on dextran- and HA-modified silicon surfaces. Polysaccharide based biomimetic layers preferentially adsorb BSA and, in general, strongly suppress protein adsorption with respect to bare silicon and APTES-activated silicon surfaces used as control.

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Competitive adsorption of plasma proteins on polysaccharide-modified silicon surfaces

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ABSTRACT

The initial response of blood exposed to an artificial surface is the adsorption of blood proteins that triggers a number of biological reactions such as inflammation and blood coagulation. Competitive protein adsorption plays a key role in the hemocompatibility of the surface. The synthesis of nonfouling surfaces is therefore one of the major prerequisites for devices for biomedical applications. Polysaccharides are the main components of the endothelial cell glycocalyx and have the ability to reduce nonspecific protein adsorption and cell adhesion and, therefore, are generally coupled with a wide variety of surfaces to improve their biocompatibility. We have developed a procedure for covalently binding dextran and sodium hyaluronate (HA) on silicon wafers and we have been able to achieve a high level of control over the surface properties of the coatings. In the present research effort we focus on a detailed investigation of competitive bovine serum albumin (BSA) and bovine fibrinogen (Fg) adsorption on dextran- and HA-modified silicon surfaces. Polysaccharide based biomimetic layers preferentially adsorb BSA and, in general, strongly suppress protein adsorption with respect to bare silicon and APTES-activated silicon surfaces used as control.

INTRODUCTION

Polysaccharides serve as excellent coatings for devices in contact with blood since they are compatible with biological systems and have been shown to reduce protein adsorption and cell adhesion on synthetic surfaces [1]. We have developed a procedure for covalently binding dextran on silicon wafers pre-activated by amine terminated APTES and we have been able to reach a high level of control on the thickness, wettability and roughness of the coatings by varying the molecular weight, polydispersity [2, 3] and the degree of chemical oxidation of the dextrans [4]. We have also demonstrated that monodisperse, high molecular weight dextran coatings applied on microcapillary glass tubes show bubble adhesion properties almost identical to the values found for *in vivo* and *ex vivo* experiments of microvascular gas embolism [2]. More recently we have also synthesized hyaluronic acid (hyaluronan, HA) coatings on silicon wafers by using EDC/NHS grafting chemistry [5].

A key event, whenever a biomaterial is exposed to protein-containing fluids is the adsorption of proteins. The exact mechanism of how polymer structure and its chemical/physical properties influence the adsorption and activation behavior of proteins is still unknown, but it is widely assumed that the design of new biomaterials with specific functional properties and excellent biocompatibility is dependent on understanding the competitive adsorption of proteins to

artificial surfaces and the subsequent adhesion and activation of cells [6,7]. In this study, an initial investigation has been undertaken to find possible correlations between the properties of our synthetic polysaccharide coating and protein adsorption. In published reports, the characterization of protein adsorption onto surfaces has been accomplished using mainly radiolabeling [7] or fluorescence labeling [8] and immunoblotting techniques [9]. One major drawback of such methods is that they cannot be used to monitor competitive protein adsorption from complex mixtures of proteins, or they can be used to provide only semi-quantitative measures of competitive adsorption. Thus, the first goal of our study has been the development of a new method for quantifying competitive protein adsorption using a novel approach to remove all adsorbed proteins from the surface of interest. Our method involves protein displacement by CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) detergent solutions and subsequent analysis of the effluent by high performance liquid chromatography (HPLC) to separate and quantify each protein species recovered. A detailed description and discussion of such method can be found in [10]. We validated our approach studying competitive adsorption of bovine serum albumin (BSA) and bovine fibrinogen (Fg) on bare and polysaccharide coated silicon wafer surfaces previously synthesized in our laboratory. This selection was made because BSA is the most concentrated protein in plasma (ca. 40 mg/ml) [6], while Fg is the major surface protein initiating coagulation via binding to platelets and the adsorption of Fg molecules is commonly used as a thrombogenicity marker [6, 7]. In this report we studied competitive BSA and Fg adsorption on five different dextran coatings obtained varying the dextran oxidation time (0.5 h, 1h, 2 h, 4 h and 24 h) and on HA coatings. As a control, competitive BSA and Fg adsorption studies were conducted also on bare silicon wafers and amine terminated APTES coated silicon wafers.

We show that our dextran and HA based biomimetic layers preferentially adsorb BSA and, in general, strongly prevent protein adsorption, suggesting that they are highly promising candidates for application on blood-contacting medical devices.

EXPERIMENTAL DETAILS

Surface preparation and characterization

The details regarding the preparation of our dextran coatings and all the different methods of surface characterization used (ellipsometry, water contact angle measurements, AFM, FTIR and micro-FTIR spectroscopy, micro-Raman spectroscopy, gas bubble adhesion tests) can be found in [2-4].

For preparing HA coatings, the etched Si surfaces were incubated in a solution comprised of Hyaluronic Acid Sodium Salt (NaHA; chain length 3-6 million; Sigma), N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC;Sigma), and N-Hydroxysuccinimide (NHS;Sigma) [5]. The concentrations of NaHA, EDC, and NHS were kept as a control- 2mg/mL NaHA and a ratio of 40:20, EDC: NHS. After the NaHA was dissolved in Millipore purified H_2O, 1 μl of Hydrochloric Acid (HCl; Fischer Scientific) was added to lower the pH of the NaHA + H_2O from ~6 to ~4. Once the NaHA was dissolved completely in water, EDC and NHS were added to bring the reaction volume to 20 ml. The surfaces, prior to incubation, were immersed in Phosphate Buffered Saline (PBS; Sigma, pH 7.4) on a shaker for ten minutes and rinsed with purified water. The immobilization reaction proceeded at room temperature on a shaker for 20

hours. Following the incubation time, all surfaces were washed with purified water and sonicated for 20 minutes in purified water.

Competitive protein adsorption studies

Bovine serum albumin, lyophilized, fatty acids- and globulin-free was obtained from Sigma Chemical Co., U.S.A., as was bovine fibrinogen (fraction I; >75% clottable protein). The proteins were used without further purification. For both proteins, solutions were prepared with the use of 40 mM phosphate-buffer saline (PBS) at pH 7.0. Chemicals used for the buffer preparation were all of analytical grade, and used without further purification. Representative physical properties of both proteins are listed in Table I

A complete description of the development and validation of a new HPLC method to study adsorption of micrograms of proteins onto the surface of materials can be found in [10]. CHAPS (Sigma C3023) was used as surfactant agent to displace BSA and Fg from bare and polysaccharide-coated silicon surfaces. This surfactant has been proven to be almost totally effective (~95%) at protein desorption from our surfaces conducting parallel experiments with Oregon Green (OG) 488 dye (Molecular Probes, Eugene, OR) labeled proteins [8,10]. Competitive protein adsorption on both control (bare and APTES functionalized silicon) and polysaccharide coated silicon surfaces was carried out in bulk BSA and Fg solutions, both with concentration of 20 μg/ml, for 1 hour on a shaker at 37°C. Solid substrates with adsorbed proteins were rinsed to remove loosely bound proteins by use of protein-free PBS. The adsorbed proteins were eluted from the surfaces by exposure to a 8 mM CHAPS solution (1 hour on a shaker at 37°C) The eluted protein samples were first dialyzed for 24 hours at 4°C, in PBS (pH ~7) replaced 4 times to remove the CHAPS and then stored at -70°C until needed, before being placed on a freeze-dryer overnight. The recovered proteins were finally identified and quantified by HPLC. The chromatography experiments were conducted on a Beckman Coulter System Gold® HPLC system (running 32 Karat™ software) comprised of a 126 pump model solvent delivery module, a 168 programmable detector module, which is a diode array UV/Vis HPLC detector set at 210 nm wavelength and a manual injector. Given that BSA and Fg have very similar pI values, but very different molecular weights (see Table I), we identified and quantified them by size exclusion chromatography (SEC), which separates biomolecules based on differences in their molecular size. We used a bonded silica Bio-Sil SEC 250 column from Bio-Rad. The eluent was a buffer with composition 0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, 0.15 M NaCl and pH 6.8, the flow rate was set at 0.6 ml/min and the injection volume was 200 μl. Calibration curves were obtained by injecting fixed volumes of standard mixtures of BSA and Fg with known concentrations (see Figure 1).

Table I. Representative Physical Properties of Selected Proteins

protein	plasma conc mg/ml	mass Da	size nm	volume nm^3	pI	ref
BSA	40	340 000	$4 \times 3 \times 3$	271	4.7	6
fibrinogen	2-3	66 000	$47 \times 5 \times 5$	3645	4.3	6

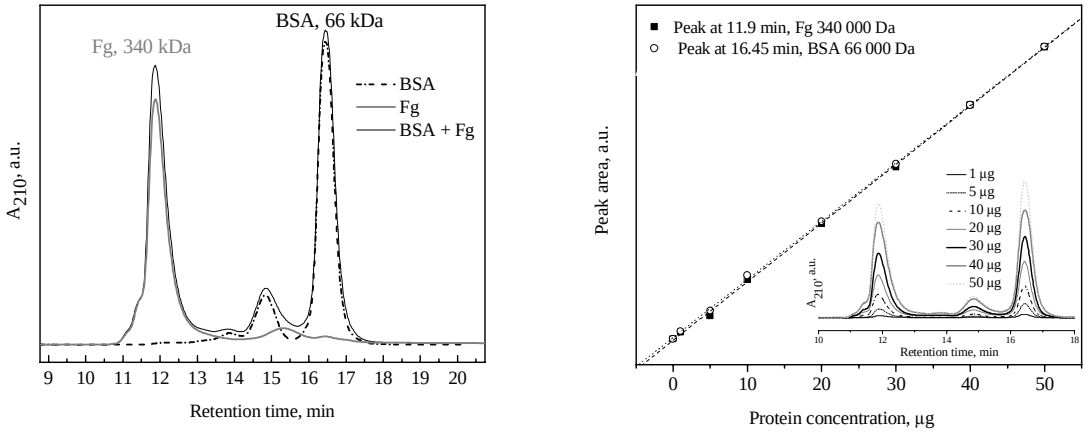

Figure 1. SEC of a standard mixture of BSA and Fg (left) and calibration curves (right). Column and conditions as described in the text.

RESULTS AND DISCUSSION

Using our newly developed HPLC method for studying competitive protein adsorption on the surface of materials [10], we tested the affinity towards serum BSA and Fg of bare and APTES-activated silicon substrates as controls and of six different polysaccharide modified silicon surfaces. The five different dextranized surfaces, having varying grafting density, have been systematically characterized [4] in an effort to correlate wettability, roughness and contact force to surface morphology. An AFM image, taken under aqueous conditions, and a section analysis of an HA coating are shown in Figure 2. HA is observed to uniformly attach and cover the silicon substrate and to extend into the aqueous solution. The section analysis shows that the surface morphology is characterized by small features, which increase the root-mean-square surface roughness (23.3 nm) and fewer large features.

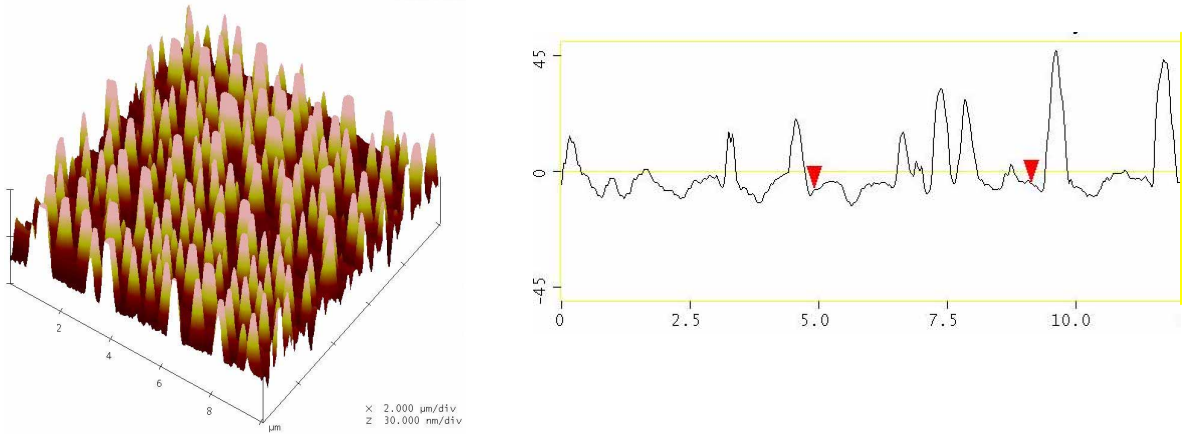

Figure 2. AFM image under aqueous conditions (left, Z scale is 60 nm) and section analysis (right) of grafted HA. Root-mean-square surface roughness 23.3 nm, maximum height 41.6 nm.

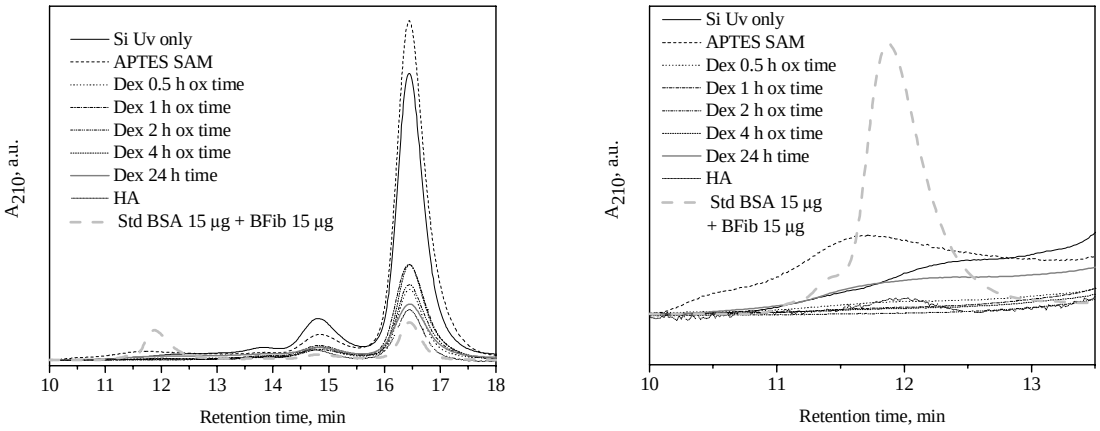

Figure 3. SEC of mixtures of BSA and Fg displaced from different solid substrates (for details see the text). Column and conditions as in Figure 1.

The competitive BSA and Fg adsorption data, reported as chromatograms of the displaced proteins in Figure 3 and converted into protein surface density in Figure 4, show that all the six polysaccharide-modified silicon surfaces strongly suppress protein adsorption with respect to bare silicon and APTES-activated silicon surfaces used as controls. Dextranized surfaces suppress total protein adsorption from 65% (1 h oxidation time) to 75% (0.5 h oxidation time) with respect to bare hydrophilic silicon and from 72% (1 h) to 80% (0.5 h) with respect to hydrophobic APTES-grafted silicon wafers. The HA coating is even more effective at protein rejection, lowering adsorption to 84% with respect to silicon and 88% with respect to APTES. Dextranized and HA surfaces strongly preferentially adsorb BSA with respect to Fg. BSA represents a percentage of the total adsorbed amount which goes from 69% (24 h oxidation dextran) to 93% (2 h oxidation dextran). This is even more outstanding if we consider that the adsorption took place from mixtures of BSA and Fg at the same concentration (20 µg/ml).

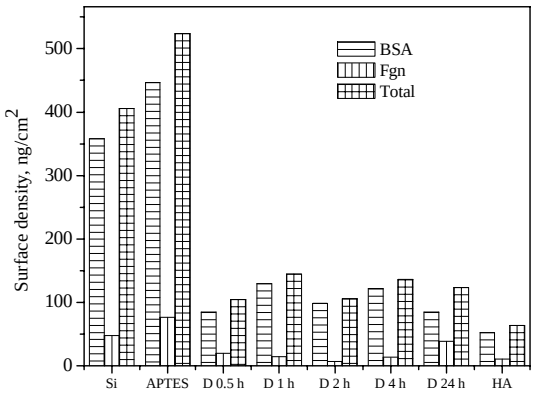

Figure 4. Protein surface density on bare and APTES-activated silicon substrates as controls and on six different polysaccharide-modified silicon surfaces. For details see the text.

Polymers capable of preferentially adsorbing BSA are thought to be less thrombogenic than those enriched in Fg, because BSA is inert to platelets, while fibrinogen enhances adhesion and aggregation of platelets [11]. This initial investigation thus suggests our polysaccharide-based coatings are highly promising candidates for applications on blood-contacting medical devices, especially fibrinogen-inert materials for the venous system [11].

CONCLUSION

In this research effort, we investigated the efficacy of dextran and HA based biomimetic films on silicon substrates in controlling nonspecific protein interactions. BSA and Fg competitive adsorption studies on unmodified and polysaccharide-modified silicon surfaces indicated that these polysaccharide films are very efficient in reducing protein adsorption. Therefore, we expect them to show reduced cell adhesion and perform favorably in actual biological environments.

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