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Polymer Brushes Containing Sulfonated Sugar Repeat Units: Synthesis, Characterization and *In Vitro* Testing of Blood Coagulation Activation

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Abstract

A new polymer brush chemistry containing sulfonated carbohydrate repeat units has been synthesized from silicon substrates using ATRP methods and characterized both in bulk and using surface analysis. The polymer brush was designed to act as a mimic for the naturally occurring sulfonated glycosaminoglycan, heparin, commonly used for modifying blood-contacting surfaces both *in vitro* and *in vivo*. Surface analysis showed conversion of brush saccharide precursor chemistry to the desired sulfonated polymer product. The sulfonated polymer brush surface was further analyzed using three conventional *in vitro* tests for blood compatibility -- plasma recalcification times, complement activation, and thrombin generation. The sulfonated polymer brush films on silicon oxide wafers exhibited better assay performance in these blood component assays than the unsulfonated sugar functionalized polymer brush in all tests performed.

Keywords

coagulation; polymer brushes; sulfonated; sugars; *in vitro*; blood; heparin

Introduction

Polymer brushes are assemblies of macromolecules tethered by one end to a surface or interface.^{1,2} Surface attachment of the polymeric chains in close proximity to one another forces the polymer coils to adopt stretched conformations in order to minimize segment-segment interactions.³ As a result of these unique polymer architectures, these systems have found applications in tailoring surface properties,⁴ chemical gating,^{5–7} and the synthesis of inorganic nanoparticles.⁸ Increasingly, polymer brushes are being used in biotechnology applications.⁹ Recent examples include the covalent attachment of antibody Fab' fragments,¹⁰ immobilizing RNase A – with an observed 30 times more immobilized enzyme compared to SAM coatings,¹¹ functionalization of Ti surfaces for cell attachment^{12,13} and incorporating cell adhesion peptides onto hydrophilic polymer brushes for the specific adhesion of HUVEC cells.¹²

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Polymer brushes are typically prepared through either physisorption or covalent attachment. Of these approaches covalent attachment is often preferred because it overcomes the constraints of physisorption, namely thermal and solvolytic instability. Covalent attachment can be achieved using the “grafting to” or “grafting from” techniques.¹³ “Grafting to” requires the tethering of end-functional polymers to a surface using strong physical or covalent surface coupling reactions. However, this technique often suffers from low grafting density (surface coverage) and variable film thickness because of the requirement that polymer molecules must diffuse through an existing polymer layer to reach surface reactive sites. Thus steric hindrance for surface attachment increases as film density and thickness increase. As a result of these limitations, the “grafting from” strategy has become the preferred approach for polymer brush synthesis, utilizing a surface-immobilized initiator layer and subsequent *in situ* polymerization to generate the polymer brush. The advantages of using the polymer brush system with the “grafting from” strategy in conjunction with a controlled/“living” radical polymerization system include the ability to control polymer grafting density to high grafting densities, the ability to control the polymer layer thickness, and the formation of versatile polymer architecture such as block copolymers. Furthermore, in comparison to organic adlayers or self-assembled monolayers (SAMs), polymer brushes offer higher levels of functionality, more robust coatings, and post-polymerization modifications.

Many examples are described in the literature to prepare surface chemistry for blood-compatible and non-thrombogenic surfaces.¹⁴ These approaches have included the use of polymeric coatings,^{15–18} metal oxides,^{19–21} diamond-like carbon,^{22–24} naturally sourced macromolecules such as heparin,^{25–29} and living cells.³⁰ When blood contacts a foreign material, *e.g.*, a biomedical device, a complex and self-amplifying cascade of surface activation events occurs involving platelets, blood proteins, and complement factors resulting in formation of a blood clot.^{31–32} In addition to these mechanisms, an important – and often overlooked – factor is surface-induced shear stress³³ – a natural activator of platelets. Hence, surface chemistry represents one of several factors influencing blood contact activation, making this a difficult and long-standing challenge to solve in new biomaterials in blood.

Two commercially available blood-contacting materials both employ surface-tethered heparin to impart blood compatibility: the Corline™ heparin surface (CHS)³⁴ and Carmeda™ bioactive surface (CBAS).³⁵ As a heterogeneous sulfated, anionic, sugar macromolecule (5–25 kDa) with potent anticoagulant activity, heparin is often prescribed for systemic anticoagulation in human patients by intravenous administration.³⁶ The basic heparin sequence comprises 24 uronic acid-(1→4)-_D-glucosamine disaccharides. Of these, IdoA(2S)-(1→4)-GlcNS(6S) is the most abundant, representing up to 90% of bovine lung heparin and up to 70% of porcine intestinal mucosal heparin. Heparin binds to blood proteins through electrostatic interactions; one highly specific interaction is that with the plasma serpin (*serine protease inhibitor*) antithrombin III (ATIII). ATIII exerts functional activity by reacting with the active site of serine proteases, forming a complex devoid of enzymatic activity. In isolation, the inhibitory effect of ATIII is slow. However, upon binding to heparin the inhibitory capacity of the complex towards coagulation factors is increased by several orders of magnitude. Once the ATIII-serine protease-heparin terplex is formed, heparin then detaches, recycled and free to interact again with a new AT molecule and inactivate another serine protease. This catalytic cycle of coagulation factor inactivation is critical to hemostasis and anticoagulant therapy, and also exploited for surface coatings. It is now well known that heparin immobilized on surfaces also suppresses platelet adhesion, complement activation and protein adsorption as ancillary benefits. However, the concentration and bioactivity of immobilized heparin is often low, indeed the bioactivity has been reported to be only 16% of unbound heparin and as low as 2.6%.²⁹ Another concern is

non-adherent platelet activation producing platelet consumption (thrombocytopenia) in bulk fluid, prompted from surface contact.

Herein we report the synthesis of new polymer brushes containing sulfonated sugar residue repeats as a heparin mimic. Sulfonation has been shown to increase the blood compatibility of polymers and biomaterials.³⁷⁻⁴¹ Indeed, over-sulfation of dermatan sulfates was reported to confer an affinity for thrombin, a key component in blood coagulation control.⁴² Surface-initiated polymerization of a protected glucose residue bearing a polymerizable methacrylate group and subsequent sequential deprotection and in situ sulfonation reactions afforded sulfonated polymer brushes on silicon substrates. Model blood compatibility assays *in vitro* monitored plasma clotting time, complement activation and thrombin generation as initial screening methods for this brush.

Materials and Methods

Diacetone-D-glucose (98+%) was purchased from Alfa Aesar, silicon oxide semiconductor wafers (WaferWorld) were cleaned immediately prior to use with “piranha” solution (30/70 v/v 30% aqueous hydrogen peroxide solution/sulfuric acid) for 2 h. (Caution: “piranha solution” is very caustic and should be handled with appropriate safety precautions and care). Me₆TREN was synthesized according to literature procedures.⁴⁵⁻⁴⁶ All other chemicals were purchased from Sigma-Aldrich at the highest available purity and used as received. Human platelet poor plasma (PPP) and platelet rich plasma (PRP) were kindly provided by Prof. V. Hlady (University of Utah). Whole blood was drawn from healthy donors into a 1:6 volume of acid-citrated dextrose solution into a 60 ml syringe. The blood was then transferred to a 50 ml polypropylene centrifuge tube and centrifuged for 15 min at 1500 rpm to separate red blood cells from PRP. PRP was then aspirated off the top using a plastic transfer pipette and the red blood cells were discarded. For PPP, PRP was further centrifuged at 2100 rpm for 15 minutes and PPP collected by aspiration. PPP was used immediately or frozen at -70°C until use when it was thawed at 37°C. PRP was used immediately. Human anaphylatoxin kit cytometric bead array (CBA) assay and FUT-175 was purchased from BD Biosciences. The chromogenic reagent BIOPHEN CS-01(38) (H_D-Phe-Pip-Arg-pNa.2HCl) was purchased from Aniaira Corporation.

Experimental

Synthesis and surface immobilization of the surface-bound ATRP initiator, (11-(2-bromo-2-methyl)propionyloxy)undecyltrichlorosilane, has been reported.⁴³ Briefly, ω-undecylenyl alcohol was esterified with an equimolar amount of 2-bromoisobutyryl bromide and then hydrosilated using trichlorosilane and Karstedt's catalyst. The product was dissolved as a 20% toluene solution and stored until use. This was deposited onto freshly cleaned (“piranha” solution) silicon oxide wafers by adding 0.2 mL of the toluene silane stock solution diluted in an additional 10 mL of toluene and heating in the presence of the wafer at 60°C for 4h. The wafers were then rinsed with fresh toluene and sonicated in toluene for 30 min before rinsing with fresh toluene, and dried under a stream of dry N₂. Purified (ASTM Research grade I 18MΩ) water was obtained from a Barnstead E-Pure water polishing system.

The protected sugar vinyl monomer 3-*O*-methacryloyl-1,2:5,6-di-*O*-isopropylidene-D-glucufuranose (MAIpGlc) was prepared following literature procedures.⁴⁴⁻⁴⁵ To a solution of 1,2 : 5,6-di-*O*-isopropylidene-D-glucufuranose (10 g, 38.4 mmol) in 50 mL of absolute pyridine, 10 mL of methacrylic anhydride (67.1 mmol) was added dropwise at room temperature. The mixture was heated at 65°C for 4 h and for another 1 h after the addition of 35 mL of water. The system was allowed to cool to room temperature overnight and then

extracted three times with 50 mL of petroleum ether. The combined extracts were washed twice with 100 mL of 5% aqueous sodium hydroxide solution and three times with 60 mL of water and dried over anhydrous magnesium sulfate. After the solvent was removed, the crude product was purified by flash silica gel chromatography with a 7:2:1 ethyl acetate : toluene : methanol mixture eluent to yield the sugar-carrying monomer as a colorless oil.

The polymerization of MAIpGlc was performed from initiator-decorated SiO₂ wafers under ATRP control.⁴⁵ MAIpGlc (3g, 0.01 moles) was dissolved in 10 mL of veratrole with 0.042g of the ligand tris[2-(dimethylamino)ethyl]amine (Me₆TREN). This solution was purged with dry N₂ for ~ 20 min. In a separate flask 0.6 mL of the soluble ATRP initiator 2-bromo-ethylisobutyrate was dissolved in 10 mL of veratrole and purged with dry N₂ for ~ 20 min. The initiator-decorated wafer was placed into a third flask and the flask purged with dry N₂ for ~ 5 min. Initiator solution (0.1 mL) was added to the flask containing the wafer using a degassed syringe. Finally, the monomer solution was added *via* degassed cannula. The polymerization solution was heated to 85°C overnight and the reaction stopped by exposure to O₂ (air), the wafer recovered and sonicated in fresh veratrole (2 × 20 min) and toluene (3 × 20 min) and dried under a stream of dry N₂.

Isopropylidene protecting groups on the resulting polymer brushes were removed through acid hydrolysis in an 80% TFA solution at RT for 30 min.⁴⁶ The wafer was then rinsed copiously in 18 MΩ DI water and sonicated in DI water (2 × 20 min) before being dried under a stream of dry N₂.

The in situ polymer brush sulfonation reaction was performed as follows: the sugar-functionalized polymer brush from the previous preparation was immersed in a 0.2M solution of SO₃.pyridine complex in anhydrous pyridine and the solution heated to 90°C overnight. After this time the solution was transferred to a beaker and neutralized with a saturated NaOH solution to a pH below 8. The wafer was collected, immersed into a saturated NaHCO₃ solution, removed, quickly rinsed with DI water and dried under a stream of dry N₂.

Polymer Brush Characterization

X-ray photoelectron spectra (XPS) were obtained with a Kratos AxisUltraDLD instrument with a monochromatic X-ray source. Survey spectra were performed in hybrid mode with a pass energy of 160 from 1200 to 0 eV. Regional scans (Na1s, O1s, C1s, S2s, Br3p and Si 2s) were performed in hybrid mode with a pass energy of 20 eV. Spectra were analyzed with Kratos Vision software and high resolution C 1s spectra were deconvoluted using a linked components procedure where each component was a fixed eV offset from the C-C,C-H peak using a GL(30) peak model and auto-fitting. The offsets were determined in part from the published C 1s spectra for glycidial methacrylate.⁴⁷ The region scan spectra were transferred to CasaXPS software (version 2.3.12) for formatting purposes only. Static aqueous contact angles were measured with a Ramé-Hart, Inc instrument with a 5 µL drop volume of ASTM grade I (ultrafiltered) water. Ellipsometry was performed using a J Woollam V-VASE spectroscopic ellipsometer (auto-angle with autocompensator, spectral range 310 nm – 1700 nm and vertical sample mounting) at 70° and 75° with default settings for Si, SiO₂, and Cauchy models with n = 1.508 for the silane initiator layer and analyzed using WVASE software.

In Vitro Blood Protein Adsorption and Activation

Plasma recalcification time (PRT): A tissue culture-polystyrene 24-well plate (BD Falcon) was used for this study. CaCl₂ solution (700 µL of 0.025 M) was placed into the bottom of a well with a sample wafer (Si/SiO₂//poly(MAGlc) or Si/SiO₂//poly(SMAGlc)). The wafers

were cut into squares of a constant area that covered the bottom of the well of the plate. This surface area was maintained for all *in vitro* assays and was chosen arbitrarily. The 24-well plate was placed into an incubator and heated at 37°C for 5 min. Platelet poor plasma (700 μ L) was added and the plate placed in a water bath heated to 37°C and the time for the fibrin gel recorded. The gel point was determined visually and defined as the time point at which the solution became stiff within the well and no longer transparent to the eye.

Complement factor cytometric bead array (CBA) assay: A tissue culture-polystyrene 24 well plate (BD Falcon) was used for this study. PPP (500 μ L) samples were placed into 9 separate wells (3 blank wells, 3 containing Si/SiO₂/poly(MAGlc) brushes and 3 containing Si/SiO₂/poly(SMAGlc) brushes). The wafers bearing the polymer brushes were cut to a constant area to cover the bottom of the well. The plate was incubated at 37°C for 1 h and then 5 μ L of Futhan-175 added to each well. The plasma was then removed and used in the complement factor CBA assay in accordance with the manufacturer recommended protocol. Data acquisition for the CBA assay was performed using an upgraded 5-color FACScan Analyzer (BD Biosciences, Mountain View, CA), employing a bench top analyzer with two lasers for fluorochrome excitation. The primary laser is a 15 mW argon (488 nm) laser and the secondary laser is a 25 mW red diode (637 nm) laser. The instrument uses seven detectors, two for light scatter (forward and 90°) and five for fluorescence. CellQuest (version 3.3) software (BD Biosciences, San Jose, CA), was used for data collection. WinMDI (version 2.9 software, J. Trotter, The Scripps Research Institute, La Jolla, CA) was used for data analysis.

Thrombin generation assay: A tissue culture-polystyrene 24-well plate (BD Falcon) was used for this study. The wells of the plate were first filled with a bovine serum albumin (BSA)-containing Tris buffer and EDTA (20 mM) overnight. PRP (500 μ L) were placed into 9 separate wells (3 blank wells, 3 containing samples of Si/SiO₂/poly(MAGlc) and 3 containing samples of Si/SiO₂/poly(SMAGlc) polymer brush). The wafers bearing the polymer brushes were cut to constant area to cover the bottom of the well. The plasma was incubated at 37°C for 15 min before 10 μ L of 0.01 M CaCl₂ solution added. Samples of the plasma were taken at 1, 3, 15, and 40 min after addition of the CaCl₂ solution and assayed for thrombin using the chromogenic reagent BIOPHEN CS-01(38) in accordance with the manufacturers protocol with added EDTA. A standard curve was prepared using samples of human thrombin (Sigma) in identical buffer solutions. Thrombin generation assay was performed using a BioTek Synergy-2 plate reader using BioTek Gen5 software (version 1.04.5).

Results and Discussion

Polymer Brush Synthesis

The synthetic strategy used to prepare the polymer brushes in this work is shown in Scheme 1. The ATRP strategy used a SiO₂ substrate and well-characterized and often-used bromoisobutyryl-containing silane. The initial thickness of the SiO₂ and silane layer was 4 nm, and exhibited an aqueous contact angle of 74°. Polymerization of the protected sugar vinyl monomer 3-*O*-methacryloyl-1,2 :5,6-di-*O*-isopropylidene-D-glucopyranose (MALpGlc) was performed using literature precedent.⁴⁵ The group of Mueller has also used this polymer in the preparation of hyperbranched polymer brushes.⁴⁸⁻⁴⁹ Polymer brushes containing sugar units were attractive based on the results of Lindhout and coworkers who found that polysaccharide coatings greatly reduced the thrombogenicity of artificial surfaces.⁵⁰ Their work showed that while only heparin surfaces could bind AT III from flowing whole plasma, alginic acid- and chondroitin sulfate- coated surfaces (both acidic polysaccharides) also exhibited non-thrombogenic properties. The 12.5-nm thick Si/SiO₂/poly(MALpGlc) polymer brush showed a static aqueous contact angle of 63°. These findings differed to that

of Ejaz *et al* who observed aqueous contact angles near 80° after similar polymer brush synthesis, and below 20° after brush deprotection.⁴⁵ Originally the deprotection step followed the work of Ejaz *et al* and Muthukrishnan *et al*, *i.e.*, 80% formic acid treatment for 48 h.^{45,48} However, in our hands this was not successful, leading presumably to the partial cleavage of the polymer from the surface based on XPS, ellipsometry and contact angle results (data not shown). When conditions described by Yasugi and co-workers,⁴⁶ (*i.e.* 80 % TFA treatment for 30 min) were employed, a decrease in brush thickness to 10.1 nm was observed due to the loss of isopropylidene groups and good correlation between XPS observations and ideal surface composition. The deprotected brush surfaces afforded aqueous contact angles of 54° (Table 1), a value similar to aqueous contact angles reported for poly(hydroxyethylacrylate) (poly(HEMA)) polymer brushes (54°⁵¹ and 56°⁵²). We chose poly(HEMA) as a comparison due to the similar functionality present in both polymers – namely hydroxyl and ester groups. The sulfonation reaction employed followed the work of Katsuraya *et al*⁵³ and was found to yield a high degree of incorporation of sulfate as monitored by XPS spectra (*i.e.*, 5 atomic percent S 2s signal) in comparison to other procedures tested. However, ellipsometry and XPS analysis showed a decrease in polymer brush thickness to ~8 nm, suggesting that some chains were cleaved from the surface in this step. The static aqueous contact angles of the sulfonated polymer surfaces were consistently below 15° implying a very hydrophilic, almost completely wetting surface.

High-resolution XPS spectra of the surfaces prepared in this work are displayed in Figures 1-4. The ATRP initiator adlayer is shown in Figure 1, and the observed C:Br atomic % ratio of 36.5:2.2 is close to the theoretical atomic ratio of 15:1 calculated for a pure initiator monolayer. The C 1s peak was fitted with three separate peaks (C-C/C-H, C-O, and C=O) using the Kratos Vision linked components deconvolution process. The ratio of the areas of the three components is 11.3 : 1.1 : 1, which agrees with the chemical structure of the initiator (shown in Scheme 1). A large attenuation of the silicon substrate Si 2p peak after polymerization of MAIpGlc (Figure 2) supports reasonable surface coverage, *i.e.* a high grafting density has been achieved. No residual bromine peaks from the ATRP initiator were observed in the XPS spectra of the polymer brush layers -- this was expected considering the detection sensitivity of the technique (~ 0.1 % atomic abundance) and the expected Br atom remaining in the film (~ 1 atom per polymeric chain). The C:O ratio in the Si/SiO₂//poly(MAIpGlc) polymer brush is 71.8:23.6, agreeing well with the ratio of 74:25 observed by Muthukrishnan *et al.* for these films.⁴⁸ The marked broadening of the C 1s peak in the polymer film compared to the initiator adlayer reflects the greater degree of carbon bonding environments in the polymer film. The C 1s spectrum for Si/SiO₂//poly(MAIpGlc) was best fit with four separate components, where we propose the middle two components are due to differing C-O bonding environments, *i.e.* the O-C-O linkages in the sugar ring, the isopropylidene protecting groups and the acryloyloxy linkage. Combining the areas of the middle two components the ratio of the areas are 7.4 : 7.9 : 1, again in excellent agreement with the chemical structure of the repeat unit of the polymer brush (Scheme 1). The XPS spectra of the polymer brush after deprotection are shown in Figure 3. The C:O ratio in the polymer brush was observed to decrease from 64.7:26.6, reflecting the loss of the isopropylidene groups. This ratio is in agreement with the ratio of 67:31 observed by Muthukrishnan *et al.* in their study following deprotection.⁴⁸ The C 1s spectrum was fit with 4 separate components, and an increase in the contribution of the C=O component was observed (8.52 % vs. 6.16 %) compared to the protected polymer brush precursor, again reflecting the loss of the protecting groups. Interestingly, in all spectra recorded for the sulfonated polymer brush (Figure 4 shows a representative example), the Na atomic % in the polymer brush was always observed to be lower than the S 2s atomic %. These should be equivalent if the sodium sulfonate salt is produced quantitatively. This inequity is attributed to a mixture of sulfonate salt and sulfonic acids present in the brush. The atomic percentage

of sulfur S 2s (234 eV)⁵⁴ detected in the polymer brush is equivalent to one hydroxyl group per sugar residue being sulfonated. The atomic percentage of carbon in the sulfonated polymer brush is greatly reduced (43.1 % from 64.7 % in the deprotected sugar polymer brush) indicating incorporation of the SO₃ sulfonate moieties. However, due to the distinct increase in the Si 2p peak it is difficult to separate the contributions of oxygen atoms from the sulfonate generation compared to that coming from the silicon oxide surface. The C 1s peak was challenging to fit: 5 components were necessary as shown in Figure 4. The fifth peak was set to a 0.7 eV offset and reflects different contributions from C-H valency, *i.e.* CH₂ vs. CH₃.⁴⁷ The complex nature of the C 1s peak further suggests side reactions arising from the sulfonation step. Efforts are ongoing to minimize these side reactions.

In Vitro Plasma Coagulation Testing

Three *in vitro* blood protein activation tests were used as a preliminary gauge of performance of the polymer brush surface coatings as models for hemocompatibility testing. Plasma clotting time tests, complement activation and thrombin generation assays were selected as representative criteria for initial surface blood compatibility. The role of calcium in coagulation has been known as early as the late 19th century;⁵⁵ its depletion (e.g., using EDTA chelation) prevents blood coagulation as calcium is required at several stages of the coagulation cascade. Measuring the time taken for a plasma gel and blood clot to form after recalcification of anticoagulated blood in the presence of a biomaterial provides one simple assessment of blood compatibility of that material. These plasma re-calcification clotting times for the sulfonated polymer brush (292 sec, average of n=4) was significantly longer than the deprotected polymer brush surface used as a control (236 sec, average of n=3).⁵⁶⁻⁵⁸ We propose (*a priori*) that these observations result from more than differences in surface energy of the respective substrates. Indeed, Silver and co-workers demonstrated that sulfonated polyurethanes do not simply complex free calcium or interfere with factor XIIIa, but rather directly inhibit fibrin assembly.⁵⁹ Their findings indicate that the fibrin monomer complexed with the polyurethane and could not properly align to form protofibrils. In monomeric fibrin after cleavage of both fibrinopeptides FPA and FPB, the central globular domain in positively charged at physiological pH. This can plausibly bind via electrostatic interactions with the polyanion sulfonate polymer brush, prolonging the plasma recalcification time.

The complement system contains ~30 different plasma proteins that act powerfully in the body's immuno-potential and coagulation systems, often through surface activation of pro-enzymes.⁶⁰ The complement and coagulation cascades interact significantly: Factor XIIIa and kallikrein in plasma have the capacity to trigger the complement cascade, and thrombin activates C3, C5, C6, and Factor B.⁶¹ A neoepitope-specific monoclonal antibody technology was employed in a rapid-screen bead-based flow cytometry assay for detection of complement activation products in plasma. The human anaphylatoxin bead-based CBA kit provides a platform to perform simultaneous measurements of human anaphylatoxins C3a-desArg, C4a-desArg, and C5a-desArg from a small volume sample.⁶²⁻⁶³ *In vivo* these cleaved complement peptides bind to receptors on a variety of cell types to produce chemotaxis, vasodilation, and cell activation and adhesion.⁶¹ One constraint in the quantitative measurement of anaphylatoxins is the continued activation of the plasma protein C pathways following sample collection. A broad-spectrum serine-protease inhibitor added to the plasma FUT-175 (futhan-nafamostat mesilate), after incubation in the presence of either the poly(MAGlc) surface coating (control) or the sulfonated polymer brush-modified surfaces, offers some protection from further complement activation to ensure more reliable measurements. Results from the CBA assay are presented in Figure 6. Sulfonated sugar-containing polymer brushes reduce production of complement factor products C3a, C4a and C5a with respect to the polysugar polymer brush precursor (9.2×10^4 pg/mL vs. 1.6×10^5 pg/

mL for C3a and 3.1×10^4 pg/mL vs. 3.6×10^5 pg/mL for C4a and C5a 7.5×10^3 pg/mL vs. 4.1×10^4 pg/mL). Significantly, there is almost no difference in the complement production observed after 1 h incubation with the sulfonated polymer brush substrate compared to plasma controls assayed before incubation with surfaces (data not shown). These results demonstrate that the sugar hydroxyl groups possibly strongly activate complement – as seen with polyvinyl alcohol gels, surfaces modified with hydroxylated alkyl chains or ethylene glycol units^{64,65} – and are exposed at the polymer/fluid interface. This would also imply that most hydroxyl groups on sugar residues at the polymer/fluid interface in the final SMAGlc polymer brush are preferentially sulfonated.

Thrombin activation is central to studies assessing materials blood compatibility, critically positioned in the coagulation cascade to cleave fibrinogen to fibrin, resulting in blood clot formation. Additionally, thrombin also acts in inflammation via the thrombin receptor (CD42) and the protease-activated receptors.⁶⁶ Results of thrombin generation assays are shown in Figure 7. The similar absorbance values observed from the cleaved chromogenic substrate seen among the sulfonated polymer brush, the sugar brush control and blank wafer (negative control) assays makes definitive conclusions difficult. After 40 minutes the amount of thrombin observed (calculated from a standard curve of human thrombin generation in the assay buffer) was 0.40 $\mu\text{g/mL}$ and 0.79 $\mu\text{g/mL}$ for the sulfonated polymer brush and sugar polymer brush, respectively. However, the error associated with the assay makes differences insignificant. Using similar assays, Yahapour and coworkers⁶⁷ observed a reduction in thrombin generation on titanium and glass surfaces with hydrophilic modifications compared to hydrophobic coatings, however, Tamada *et al.*⁶⁸ showed that sulfonated polyisoprene in concentrations over 10^3 $\mu\text{g/mL}$ did not inhibit thrombin, and Wu and coworkers⁶⁹ showed that the sulfated polysaccharide acharan sulfate and de-*N*-acetylated acharan sulfate did not show anticoagulant activity, while N-sulfoacharan sulfate derivatives showed substantial anti-factor IIa activity. Silver *et al.* have suggested that while sulfonated polyurethanes may interact with ATIII in a heparin-like manner, it was also possible that the polymer and ATIII can act in independent ways and concluded that sulfonated polyurethanes inhibit coagulation beyond the point where the intrinsic and extrinsic pathways overlap.⁵⁹ Interestingly, de Raucourt et al stated that sulfated dextran derivatives could directly inhibit thrombin.⁷⁰ Charef and co-workers examined thrombin inhibition by polystyrene sulfonates.⁷¹ Their findings showed that the sulfonated polymer was not a catalyst of thrombin inhibition; they concluded that the antithrombic effect was due to thrombin adsorption, even in the presence of plasma proteins.

Polymer brush systems in our studies appear to act in a similar manner to the polystyrene sulfonate: heparin-ATIII interaction is purely based on electrostatic interactions.⁷² These polymers do not possess the intricate stereochemistry, higher order polymer structures, binding sites and functionality that natural heparin does to produce more specific bioactivities, plasma protein interactions and thrombin inhibition.

Conclusions

A new polymer brush containing sulfonated sugar repeat residues has been synthesized from a silicon oxide surface using ATRP methods. The polymerization yields a dense polymer surface coverage based on the attenuation of Si substrate peaks in XPS spectra and thickness yields from ellipsometry. Sulfonation yields seem to be highest at the ambient surface of the brush due to the in situ solution-phase sulfonation reaction. In vitro testing of the polymer brush-coated surfaces compared to the unsulfonated sugar polymer brush controls showed improvements in conventional metrics for in vitro blood compatibility in terms of plasma recalcification clotting times and complement activation. However, thrombin generation assays could not distinguish either surface chemistry.

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Scheme 1.

Synthetic ATRP strategy for the preparation of Si/SiO₂//poly(SMAGlc) polymer brush. (i) 10/3 v/w monomer solution in veratrole, DP_{theo} = 200, 12 h, 85 °C; (ii) 80% TFA solution, 30 min, RT; (iii) 0.2 M SO₃.py solution in anhydrous pyridine, 2 h, 85°C, sat. NaHCO₃ solution.

Figure 1. High resolution XPS regional spectra for the ATRP initiator adlayer on SiO₂ wafer showing peaks for (a) O 1s, (b) C 1s, (c) Si 2p, and (d) Br 3p.

Figure 2.
High resolution XPS region spectra for the Si/SiO₂/poly(MAIPGlc) brush preparation showing peaks for (a) O 1s, (b) C 1s, and (c) Si 2p.

Figure 3. High-resolution XPS region spectra for the Si/SiO₂//poly(MAGlc)wafer showing peaks for (a) O 1s, (b) C 1s, and (c) Si 2p.

Figure 4. High-resolution XPS region spectra for the Si/SiO₂//poly(MAGlc) brush surface showing peaks for (a) O 1s, (b) C 1s, (c) S 2s, and (d) Si 2p.

Figure 5.

PPP re-calcification times in a 1:1 v/v mixture of a 0.025M CaCl_2 solution in a TCPS 24-well plate at 37°C for Si/SiO₂/poly(MAGlc) polymer brush (MAGLc) and a Si/SiO₂/poly(SMAGlc) polymer brush (SMAGlc). Error bars represent twice the standard deviation between samples (n = 3 for virgin wafers and n = 4 for brush-coated wafers).

Figure 6.

CBA-based anaphylatoxin assay for C4a, C3a, and C5a complement fragment production after PPP incubation for 1 h at 37°C in a TCPS well containing a wafer bearing a Si/SiO₂//poly(MAGlc) polymer brush (white bars) and a wafer bearing a Si/SiO₂//poly(SMAGlc) polymer brush (grey bars). Error bars represent twice the standard deviation between samples (n =3).

Figure 7. Colorimetric thrombin generation assay on both polymer brush surfaces using PRP and the chromogenic reagent BIOPHEN CS-01(38). Data points represent the average of $n=3$ and error bars are twice the standard deviation. Sample optical absorbance is measured at 405 nm.

Table 1

Polymer brush comparisons: contact angles ($n = 2, \pm 1^\circ$) were determined with static 5 μL drops of 18 M Ω DI water. Film thickness ($d \pm 1$ nm) determined using ellipsometry. Refractive indices (n) for Si and SiO₂ were used as default settings in a thin film Cauchy model. Elemental brush compositions determined using XPS were obtained from high-resolution XPS region scans ($n = 3$).

Surface	$\theta_{\text{(aq, static)}}^\circ$	d (nm)	elemental composition (%)
Si/SiO ₂ //initiator	74	4.0	O:C:Si:Br = 20.7 : 36.5 : 40.6 : 2.2
Si/SiO ₂ //poly(MAIPGlc)	63	12.5	O:C:Si = 23.6 : 71.8 : 4.6
Si/SiO ₂ //poly(MAGlc)	52	10.1	O:C:Si = 28.6 : 64.7 : 6.8
Si/SiO ₂ //poly(SMAGlc)	11	7.9	O:C:S:Na:Si = 29.9 : 43.1 : 6.1 : 3.0 : 18.0