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Hemocompatibility of Chitosan/poly(acrylic acid) Grafted Polyurethane Tubing

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Abstract

The activation and adhesion of platelets or whole blood exposed to chitosan (CH) grafted surfaces is used to evaluate the hemocompatibility of biomaterials. The biomaterial surfaces are polyurethane (PU) tubes grafted with an inner poly(acrylic acid) (PAA) and an outer CH or quaternary ammonium modified CH (CH-Q) brush. The CH, CH-Q and PAA grafted layers were characterized by ellipsometry and fluorescence microscopy. Material wear tests demonstrate that CH (CH-Q) is stably grafted onto PU tubes upon exposure to saline solution for 7 days. Using quartz-crystal microbalances with dissipation (QCM-D), *in-situ* adsorption of blood plasma proteins on CH and CH-Q compared to a silicon oxide control was measured. The QCM-D results showed that the physically adsorbed plasma protein layer on CH-Q and CH surfaces is softer and more viscous than the protein layer on the SiO₂ surface. The CH-Q layer thus has the weakest interaction with plasma proteins. Whole blood and platelet adhesion was reduced by ~92% on CH-Q, which showed the weakest interaction with plasma protein but more viscous adsorbed plasma protein layer, compared to SiO₂. Last, to examine the biologic response of platelets and neutrophils to biomaterial surfaces, CH (CH-Q)/PAA, PAA and PU tubes were tested using a Chandler Loop apparatus as an ex vivo model and flow cytometry. The blood adhesion and biologic response results showed that CH and CH-Q reduced adhesion and activation of platelets and neutrophils and improved hemocompatibility relative to other surfaces (PU and PAA). Our studies demonstrated that the properties of physically adsorbed plasma protein layer on biomaterial surfaces correlates with blood coagulation on biomaterial surfaces.

Introduction

Coating a medical device provides a facile route towards imparting surfaces with complementary properties, including biocompatibility and antibacterial characteristics for biological applications. Recently, there has been much research involving coatings of biological materials on surfaces of synthetic materials, addressed at improving the materials'

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biocompatibility.¹⁻⁹ In particular, medical devices such as catheters and intravascular stents, which are inserted into a body cavity or vessel which is in contact with blood, have been of great interest to surface chemists. Synthetic polymers, which have excellent mechanical properties and are fairly biocompatible, have been used for the construction of direct blood contact devices such as catheters and cardiopulmonary bypass (CPB) circuits. However, polymer materials can induce undesirable side effects, such as blood clots and bacterial infection, which are caused in the process of device insertion.¹⁰⁻¹² A surface coating on the devices can effectively and simultaneously prevent both of these complications without changing the favorable bulk material properties. One obvious application is to short-term vascular implants such as indwelling catheters whose clinical use is limited to a few days. Such uses can still provoke biological responses resulting in serious medical complications in this time-frame.

Polysaccharides, proteins, and thrombotic inhibitors have been used as biological coating materials on the surface of polymer materials to enhance their hemocompatibility.^{1,3-6,8,13} Immobilization of biological molecules on synthetic polymer surfaces such as polymethyl methacrylate (PMMA), polyurethane (PU) and polyethylene terephthalate (PET), often uses an intermediate adhesive layer, such as polyacrylic acid (PAA), which is deposited using surface initiated radical polymerization (SIRP).^{1,5,13-15} The most commercially successful biological material for hemocompatible surface coating is heparin.^{1,16} Generally, the hemocompatibility of a surface is measured by the reduced activation of coagulation, complement and blood cells which result from direct contact of blood with artificial surfaces.^{1,2,8,9} Hemocompatibility is also evaluated by cell adhesion resulting from the direct contact of platelets, neutrophils, or whole blood with the modified surfaces.^{2-4,8} An example of this is the recent study of Finley et al., which used whole blood adhesion studies and antigen markers for platelets and neutrophils, and measured their activation by flow cytometry.⁸ They showed that a CD47 protein coating on a synthetic polymer surface diminishes adhesion and activation of platelets and neutrophils.⁸ An additional measure of hemocompatibility is human plasma protein absorption on the biomaterial; this has generally been studied since protein absorption is the first event that triggers later bioresponses, including platelet activation and blood aggregation.¹⁷⁻²² However, few research studies have determined that cell activation and adhesion from direct contact of platelets or whole blood with biomaterial surfaces correlate with the plasma protein absorption on biomaterial surfaces and the properties of the physisorbed layer.

Recently, we have been developing coatings of biological materials on surfaces of synthetic materials for improving their biocompatibility as well as imparting them with antibacterial properties. For example, we have demonstrated the immobilization of water-soluble chitosans modified with quaternary ammonium salts (CH-Q) on silicon oxide surfaces, and reported their antibacterial properties as well as biocompatible properties.^{7,15,23,24} In this study, we describe immobilization of biological material on the surfaces of polymer materials, used for biomedical devices such as blood-contacting vascular catheters, for improving their hemocompatibility. In addition, we study the interrelationship between the plasma protein absorption on biomaterial surfaces and the physisorbed layer's properties, and *activation and adhesion* from direct contact of platelets or whole blood with *biomaterial surfaces*. First, to demonstrate the generality of this approach for biomedical devices that contact blood, chitosan (CH) and CH-Q (shown in Figure 1) are immobilized on real PU tubes using PAA as an intermediate adhesive layer grown by SIRP. The coverage of CH and CH-Q grafted layers, as well as the PAA grafted layer, on PU tubes are characterized using ellipsometry and fluorescence microscopy. For biomedical applications of CH and CH-Q grafted PU tubes, material wear tests demonstrate that each tube coating is chemically stable. In order to study the plasma protein absorption on biomaterial surfaces and the physisorbed layer's properties, in-situ adsorption of blood plasma proteins on CH and CH-Q

versus silicon oxide surfaces is evaluated using a quartz-crystal microbalance with dissipation (QCM-D). Whole blood adhesion and platelet adhesion tests are performed using CH and CH-Q surfaces in order to study the interrelationship between the plasma protein adsorption and the blood coagulation on the surfaces. To examine the biological response of platelets and neutrophils to biomaterial surfaces, CH and CH-Q grafted layers as well as a PAA grafted layer on PU tubes versus unmodified PU tubing are tested using the Chandler Loop Apparatus^{8,9} as an ex vivo model. The tubes are then examined for platelet and neutrophil activation by flow cytometry. Our studies demonstrate that the properties of a physically adsorbed plasma protein layer on biomaterial surfaces correlates with blood coagulation on biomaterial surfaces. We also show that CH and CH-Q coating on PU tubing can reduce activation and adhesion of platelets and neutrophils, and improve hemocompatibility relative to other surfaces (the PAA grafted layer on PU tubes and unmodified PU tubing).

Experimental

Materials

Chitosan Chitoclear® Cg-10 (Mw = 60 kDa and degree of deacetylation (DD): 87%) was supplied from Colgate-Palmolive Co., USA. N-type, (100) oriented silicon wafers (CZ silicon: dopant, Ph: 20-30 Ω resistivity) were purchased from Silicon Quest International. QCM sensor crystals, an AT-cut piezoelectric quartz crystal (14 mm in diameter and 0.3 mm thickness) coated with a 50 nm thick layer of silicon dioxide, were purchased from Biolin Scientific, Inc. Polyurethane (Nalgene 280 PUR Tubing; using a 1,4-butane-diol soft segment and 4-4'-methylene diphenyl diisocyanate [MDI] as the hard segment) was purchased from Fisher Scientific. 4% (w/v) sodium citrate tribasic solution, 3-Glycidioxypropyl-trimethoxysilane (GPTMS, 98%), hexyltrichlorosilane (HTCS, 98%), 80 wt% aqueous solution of [(2-(acryloyloxy)ethyl] trimethylammonium chloride (AETMAC), acrylic acid (98%), and HEPES (99.5%) were purchased from Aldrich Chemical. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and sulfo-NHS were purchased from Thermo and Fisher Scientific, respectively. Rhodamine Red™-X, Succinimidyl Ester (Abs/Em = 560/581 nm) and AFC-Arg-Arg-Ala-NH₂ (AFC-P; Fluorescent labeled peptide; Abs/Em = 380/500 nm; 95%) were purchased from Invitrogen and AnaSpec, Inc., USA, respectively. Ultrapure water (Millipore Direct-Q, 18 M Ω cm resistivity) was used for surface preparation. For blood related experiments, whole blood was isolated aseptically from healthy volunteers, after informed consent and following a protocol approved by the University of Pennsylvania Institutional Review Board.

Surface preparation and characterization

Silicon wafers (35 mm $\times$ 15 mm) were cleaned by immersion in piranha solution, rinsed with ultrapure water (Millipore Direct-Q, 18 M Ω cm resistivity), dried with N₂, and exposed to UV-Ozone to produce a homogeneous hydroxylated surface and to remove impurities. The HTCS monolayer on the silicon oxide surface was prepared by the vapor deposition method at 80 °C for 4 hours. The deposited samples were sonicated in toluene (2 times for 20 min.) to remove physically absorbed HTCS and impurities on the surface. The polyurethane (PU) coated surfaces were obtained by spin coating (2000 rpm) at 3 wt% solution of polyurethane in THF onto HTCS monolayer on silicon oxide surface, then dried in vacuum for one day. To coat PAA on PU tubes (35 cm length) and PU coated silicon wafers, the PU samples were initiated using O₂ plasma treatment. A plasma cleaner/Sterilizer (PDC-001, Harrick Scientific Corp. USA) and a vacuum pump with a minimum pump speed of 1.4 m³/h (0.83 ft³/min) were used. An ultimate total pressure of 200 m Torr (0.27 mbar) with pure oxygen gas and 29.6 W of power input were used for the surface initiation for 3 min., followed by radical polymerization of 30 wt% acrylic acid at 80 °C for

2 hr. under N₂. Chitosan with quaternary ammonium salts, CH-Q (see Figure 1) was prepared by the Michael reaction of chitosan with an acryl reagent (AETMAC) in water and characterized by using ¹H NMR experiments according to methods described in our previous research.²³ 51% of D-glucosamine units of chitosan (n = 0.13) are functionalized with quaternary ammonium salts. The degree of substitution of the chitosan derivative, DS, was also calculated by using ¹H NMR.²³ In order to immobilize fluorescent labeled protein (AFC-P), CH, and CH-Q on the PAA layer, EDC-mediated condensation with sulfo-NHS and HEPES^{25,26} was done on the PAA layer at RT for 1 hr. The succinimidyl ester functionalized PAA samples were immersed in 2 wt % aqueous solution of CH or CH-Q for 12 hours (in the case of AFC-P, a 5 μM solution). The immobilized samples were rinsed with ultrapure water for 1 day to remove physically adsorbed polymers and proteins, and impurities on the surface. For surface characterization, the thicknesses of dry substrate on the silicon wafer surfaces were measured by an alpha-SE ellipsometer (J.A. Woollam Co. INC. NE, USA) equipped with a wavelength range of 380 to 900 nm (70° angle of incidence). Contact angles were measured by using a 1 μL sessile drop method. For the surface characterization of CH and CH-Q tube coatings, a fluorescent label (Rhodamine-Red with succinimidyl ester, 5 μM solution for 1 hr) was used and analyzed using a fluorescence microscope. For material wear tests, the fluorescent red labeled CH and CH-Q tubes were immersed in fresh saline solutions with shaking at 100 rpm for 1, 3, and 7 days, respectively, to evaluate the stability of CH and CH-Q grafted layers on PAA/PU tubes. The fluorescent microscopy images were taken at each time point. All normalized fluorescence intensities of tube images were then averaged over a cubic region (70 × 70 μm²) from the region of fluorescence tube versus the background region using imageJ software (NIH, Bethesda, MD).

***In-Situ* adsorption studies of blood plasma proteins on CH and CH-Q surfaces using quartz crystal microbalance with dissipation (QCM-D)**

The QCM-D measurement is based on the resonance frequency change of a vibrating quartz crystal, a piezoelectric material, when mass is deposited on it. The deposited mass, Δm , has a relationship with the frequency change, Δf , according to the Sauerbrey equation,²⁷

$$\Delta m = -C \frac{\Delta f_n}{n}$$

where C is the mass sensitivity constant ($C = 17.7 \text{ ng cm}^{-2} \text{ Hz}^{-1}$ for an AT-cut, 5 MHz crystal) and n is the vibrational mode number ($n = 1, 3, 5, \dots$). In addition, the dissipation change, ΔD_n , the loss of energy stored in a vibration cycle, indicates the physical characteristics of the deposited layer such as viscosity, elasticity, and other properties.^{23,24,28,29} If ΔD_n is less than 2.0×10^{-6} , the layer is an elastic and rigid film, and the physical properties (mass and thickness) of the elastic layer can be calculated using the Sauerbrey equation.^{24,27} However, if ΔD_n is more than 2.0×10^{-6} , the layer is viscoelastic and soft. The physical properties (thickness, shear modulus, and viscosity) of the viscoelastic layer can be estimated by fitting between the QCM-D experimental data ($\Delta f_n/n$ ($n = 1, 3, 5, \dots$) and ΔD_n) and a Voigt-based viscoelastic model incorporated in the Q-Sense software Q-Tools.^{21,23,24,29}

To study protein physisorption from blood plasma on the SiO₂ (as a control), CH, and CH-Q surfaces using in-situ QCM-D, CH and CH-Q grafted onto SiO₂-coated sensor crystals were prepared according to methods described in our previous research.^{23,24} Blood plasma was prepared by centrifugation from whole blood. 50% (v/v) diluted blood plasma solution using 0.9% Sodium chloride Irrigation USP was used for each QCM experiment. An E4 QCM instrument (Q-Sense Inc., Gothenburg, Sweden) was used to monitor protein physisorption

from blood plasma on the SiO₂ (as a control), CH, and CH-Q surfaces. The liquid medium was peristaltically pumped at a rate of 50 $\mu\text{L min}^{-1}$ through a flow cell with the sensor crystal. The temperature of the system was controlled at 37 °C.

Platelet adhesion studies

To evaluate the activated platelet adhesion on surfaces, human-platelet-rich plasma (PRP) was prepared by centrifugation from citrated whole blood. Each glass, CH, or CH-Q sample was incubated with 200 μL PRP (1×10^5 cells/mL) for 1 hr at 37 °C under static stations. After the PRP was removed using a vacuum aspirator, each sample was rinsed carefully three times with PBS. For fluorescence images of adherent platelets,³⁰ calcein AM, widely used for determining cell viability, was diluted to 500 nM, first dissolved in DMSO and frozen in small (1.5 μL) aliquots at 1 mM, and then diluted using recording HBSS (pH 7.4 with 1.3 mmol/L CaCl₂, 0.9 mmol/L MgCl₂, 2 mmol/L glutamine, 0.1 g/L heparin, 5.6 mmol/L glucose, and 1% FBS). Each sample was incubated with calcein AM for 30 min at 37 °C, rinsed three times with recording HBSS, and then let sit for 15 minutes at RT before imaging. Samples were then imaged using a SensiCam QE camera (The Cooke Corp., Romulus, MI) (2 $\times$ 2 binning, 688 $\times$ 520) attached to Olympus IX70 microscope (Olympus, Melville, NY) with an Olympus LUCPlanFL N 40 $\times$ 0.6NA objective (Olympus, Melville, NY) and Photofluor light source (89 North, Burlington, VT). Computer control of the microscope was facilitated by LUDL programmable filter wheels, shutters, and focus control (Ludl Electronic Products, Hawthorne, NY) and both phase contrast and fluorescent images were collected using IPL 3.7 software (BD, Rockville, MD).

Whole blood adhesion studies

To evaluate activated blood adhesion, 18 mL of blood was drawn from a healthy donor into a syringe that already contains 2 mL of 4% (w/v) sodium citrate tribasic solution. Each glass, CH, or CH-Q sample was incubated with 200 μL blood for 1 hr at 37 °C under static stations. After the blood was removed using a vacuum aspirator, each sample was rinsed carefully twice with sterile DPBS and incubated with 4% paraformaldehyde of HBSS for 1 hr to fix blood cells. Each sample was then rinsed three times with sterile DI water and gradient-dried with 0%, 30%, 50%, 70%, 90%, and 100% (v/v) ethanol in DI water for 20 min at each step, with a final air drying step. Finally, each sample was examined with Environmental Scanning Electron Microscope - FEI 600 Quanta FEG SEM.

Chandler loop analysis

In order to evaluate the effectiveness of surface coating on tubes to improve blood compatibility (by diminishing cell activation responses initiated by blood contact with the modified tube surfaces), we employed the Chandler Loop Apparatus as an ex vivo model.^{8,9,31} This model enables evaluation of modified surfaces with respect to blood-surface interactions by flowing blood within a circular closed loop of surface modified tubing. Freshly drawn whole human blood, supplemented with sodium citrate, was either kept as control or loaded into PU, PAA/PU, CH/PAA/PU, and CH-Q/PAA/PU tubes (35 cm), connected into loops using 1 cm stainless connectors. The loops were rotated at 42.3 rpm (50 fpm; total circumference = 36 cm) at 37 °C. The blood was perfused over each surface for 1, 2, and 3 hr, respectively to simulate the effect of whole blood interaction with the each surface. Blood samples were then withdrawn for flow cytometry analysis.

Flow cytometric analysis of biomarker expression

Whole blood samples (1 mL) from the Chandler loop experiment were immediately combined with commercial fixative (100 $\mu\text{L/mL}$ Caltag Reagent A fixation medium, Invitrogen, Carlsbad, CA) and delivered to the flow cytometry facility at the University of Pennsylvania

where all analyses were performed within 24 h after arrival, ~2-6 days from time of collection. In brief, whole blood (100 μ l) was incubated at room temperature for 1 hour with the same volume of PBS and stained with the following mixture of antibodies (eBioscience, Inc. San Diego, CA): 5 μ l of FITC-conjugated anti-human CD62P (P-Selectin), 5 μ l of PE-conjugated anti-human CD18, 5 μ l of PerCP-eFluor710-conjugated anti-human CD62L (L-Selectin), and 5 μ l of APC-conjugated anti-human CD13, and evaluated by flow cytometry. Flow cytometry data collection and analysis was performed with a 4-color FACSCalibur (Becton Dickinson, San Jose, CA) using standard acquisition CellQuest software.⁹

Statistical analysis

All data were expressed as the mean $\pm$ standard deviation. The student's t-test (an unpaired t-test using GraphPad Software) was used to evaluate the data for significant between the means for specific comparisons. We accepted $P < 0.05$ as an indicator that statistically significant differences exist between the means.

Results and discussion

1. Immobilization and surface characterization of chitosan(CH) and quaternary ammonium modified chitosan (CH-Q) on polyurethane (PU)

Because it is difficult to directly immobilize CH and CH-Q on PU, polyacrylic acid, PAA, was used as an intermediate layer which was grafted onto the PU surface by surface initiated radical polymerization (SIRP) of acrylic acid (AA). This poly(acrylic acid) (PAA) brush was then reacted with CH (CH-Q) via succinimidyl ester functionalization of the PAA carboxylic acid groups by the well-known succinimidyl ester and amine reaction, as shown in Figure 2 (bottom left). First, the silicon wafer was rendered hydrophobic by reacting the surface oxide with hexyltrichlorosilane (HTCS). Surface characterization, Table 1, shows that the HTCS and PU layers are 1 nm and 102 nm, respectively, with contact angles of 90 and 70 deg, in reasonable agreement with the values reported in the literature.²⁶ In addition, during the subsequent reactions to attach CH (CH-Q), the PU layer remained stable without any changes in thickness. PU*, which is PU exposed to oxygen plasma, has nearly the same thickness as the original PU thickness, but becomes more hydrophilic than the PU layer (Table 1). The surface characteristics of oxygen plasma-treated PU are in good agreement with values from the literature.³² The PAA brush layer grafted to PU has a thickness of 46 nm. The PAA brush layer has a water contact angle of 29°, in reasonable agreement with values reported in the literature.³² As noted in Figure 1, CH and CH-Q have 0.87 and 0.36 mole fractions of D-glucosamine, respectively, whose primary amine functional groups can react with the succinimidyl ester on the functionalized PAA brush to form peptide bonds (i.e., $-C(=O)NH-$). The CH and CH-Q layers have thicknesses of 56 nm and 35 nm, respectively, and water contact angles (25° and 12°, respectively). The layer thicknesses and contact angles indicate that the CH and CH-Q layers are chemically grafted onto the PAA-grafted PU and the layers are hydrophilic.

2. Surface Functionalization and Characterization of PU tubes

For coating medical devices such as catheters, PU tubes (35 cm length) were surface modified using the grafting method developed for planar PU substrates shown in Figure 2. A fluorescently labeled peptide with an amine end functional group (AFC-Arg-Arg-Ala-NH₂, AFC-P) was used in order to characterize the PAA brush on the PU tubes (PAA/PU) using SIRP, and to confirm the grafting reaction between the primary amine groups of CH (CH-Q) and the succinimidyl ester functionalized PAA brush. Figure 3 shows bright field (top row) and blue fluorescent (bottom row) images of PU, PAA/PU, and AFC-P/PAA/PU tubes, respectively. A PAA/PU tube (AFC-P/PAA/PU) exposed to AFC-P via the succinimidyl ester functionalization of PAA brush fluoresces blue, compared to PU and PAA/PU tubes

(without succinimidyl ester functionalization) exposed to AFC-P (Figure 3b). The normalized fluorescence intensities of PU, PAA/PU, and AFC-P/PAA/PU tubes were 96 ± 4 , 153 ± 296 , and 2531 ± 108 , respectively (Figure 3c). AFC-P grafted onto a PAA/PU tube (AFC-P/PAA/PU) has a very high of blue fluorescence intensity compared to PU and PAA/PU tubes exposed to AFC-P. This indicates that proteins, biopolymers, and synthetic polymers, which contain amine functional groups, can be grafted onto PU tubes using PAA as an intermediate adhesive layer (Figure 2).

CH and CH-Q, which contain amine functional groups (CH: $n = 0.87$, CH-Q: $n = 0.36$, in Figure 1), were grafted onto PU tubes using a PAA brush as an intermediate adhesive layer (Figure 2). A well-known Rhodamine RedTM-X, Succinimidyl Ester (Abs/Em = 560/581 nm), which reacts with residual amine functional groups in grafted CH and CH-Q, was used as a red fluorescent label to examine the grafting CH and CH-Q layers onto PAA/PU tubes to create CH/PAA/PU and CH-Q/PAA/PU, respectively. Figure 4 shows bright field and fluorescent images of rhodamine red treated PU, PAA/PU, CH-Q/PAA/PU and CH/PAA/PU tubes. The normalized fluorescence intensities of rhodamine red treated PU, PAA/PU, CH-Q/PAA/PU and CH/PAA/PU tubes were 94 ± 13 , 96 ± 4 , 1763 ± 120 , and 3029 ± 394 , respectively (Figure 4C). The rhodamine red treated CH/PAA/PU tube has a significantly higher red fluorescence intensity, than PU and PAA. The normalized intensity of CH/PAA/PU is 1.7 $\times$ greater than that of CH-Q/PAA/PU. This result suggests that the residual amine group concentration for CH is ~ 2 times greater than CH-Q grafted layer on PAA/PU. This comparison is reasonable because the monomer fractions (n) of free CH and CH-Q polymers with amine groups are 0.87 and 0.36, respectively (i.e., ratio = 2.41, Figure 1). After the CH and CH-Q molecules react with the PAA brush, CH/PAA/PU contains more residual amine groups than CH-Q/PAA/PU, which react with the succinimidyl ester groups of the rhodamine red dye. These results suggest that fluorescent labeling can be used as a useful tag to identify grafted layers in non-planar geometries. Furthermore, the approach in Figure 2 can be extended by grafting yet another biopolymer or drug to the CH (CH-Q) layers.

For some medical applications, such as devices in direct contact with blood, the surface coating should be robust and strongly adhere to the PU tubes while remaining chemically and mechanically stable. To evaluate the stability of CH and CH-Q grafted layers on PAA/PU tubes, material wear tests were performed. First CH/PAA/PU and CH-Q/PAA/PU tubes were labeled using rhodamin red. PAA/PU tube was used as a control. Each tube was immersed in saline solution with shaking at 100 rpm for 1, 3, and 7 days, respectively. The fluorescent images were acquired at each time point. As shown in Figure 5, the normalized fluorescent intensities of CH and CH-Q do not vary significantly over 7 days indicating that CH and CH-Q are stably grafted onto PU tubes.

3. In-Situ adsorption studies of blood plasma proteins on CH and CH-Q surfaces

Blood plasma is a multi-component solution containing a large variety of molecules such as sugars, fats, amino acids, urea, and hundreds of distinct proteins. Protein adsorption from plasma onto biomaterials is considered to be a selective and competitive process since many different proteins are involved in the adsorption onto a surface. A layer of blood plasma proteins adsorbed onto a biomaterial surface generates a biologically active surface, which can mediate both platelet adhesion and activation of the coagulation cascade.¹⁷⁻²² In this study, protein physisorption from blood plasma on the SiO₂ (as a control), CH, and CH-Q surfaces was studied using in-situ QCM-D. After using saline solution to establish a baseline, SiO₂, CH, and CH-Q coated QCM crystal sensors were exposed to 50% plasma solutions (in figure 6). The $\Delta f_n/n$ ($n = 3$) of SiO₂ (as a control), CH, and CH-Q surfaces decreases rapidly to -120 Hz, -95 Hz, and -50 Hz, respectively; correspondingly, ΔD_n ($n = 3$) increases to 20, 60, and 40, respectively. The highest frequency change but lowest

dissipation change, shown by the SiO₂ surface, suggests that the greatest mass of proteins is physically adsorbed on the SiO₂ surface as compared to the other surfaces, but that the adsorbed protein layer is more elastic and rigid than those on the other surfaces. The thickness of physically adsorbed layer is ~40 nm, estimated from fitting to the viscoelastic model incorporated into the QTools software (in Figure 1S, shown in supporting information). After rinsing the protein layer on the SiO₂ surface with saline solution (arrows in Figure 6), $\Delta f_n/n$ ($n = 3$) increases to -45 Hz and then remains constant, while ΔD_n decreases to $\sim 2 \times 10^{-6}$. This behavior suggests that the flow of saline solution removes loosely bound species, resulting in a final protein layer that is thinner (~20 nm, estimated from fitting to the viscoelastic model, shown in Figure 1S) and more elastic than the initial physisorbed layer. Thus, the SiO₂ surface, with high rigidity and a high negative surface charge,²³ shows high protein adsorption from plasma. In addition, even after the rinsing step, the remaining stable and elastic protein layer suggests a strong physical interaction between the protein and the SiO₂ surface.

In comparison to the SiO₂ surface, the lower frequency change but higher dissipation change of the CH and the CH-Q surfaces indicate that fewer proteins are physically adsorbed on both surfaces, but that the adsorbed protein layer is softer and more viscous. After rinsing the protein layer on the CH surface with saline solution (arrow in Figure 6), $\Delta f_n/n$ ($n = 3$) increases to -60 Hz and then decreases to the original value (-95 Hz), while ΔD_n decreases to $\sim 30 \times 10^{-6}$. This suggests that exposure of saline solution removes loosely bound species and induces a viscoelasticity change in the stable protein layer, with the result being that the softness and viscosity of the final protein layer on CH is similar to the initial physisorbed layer on CH. On the contrary, after the rinsing process on the CH-Q surface (in Figure 6), $\Delta f_n/n$ ($n = 3$) increases to -0 Hz and ΔD_n decreases to ~0. This result suggests that the flow of saline solution rinses off most physisorbed species on the CH-Q surface and that the physical interaction between proteins and CH-Q surface is the weakest of all of the protein-surface interactions we measured. The CH-Q layer, with high swelling properties and a high positive surface charge at pH 7,²³ thus shows the lowest protein adsorption and the weakest lasting interaction with proteins in plasma. The CH layer, having both rigid structure and positive charge,^{24,33} shows higher protein adsorption than the CH-Q layer but lower protein adsorption than the SiO₂ surface. The physically adsorbed protein layer on both the CH-Q and CH surfaces is more viscous and softer than the layer on the SiO₂ surface. In order to study the interrelationship between the protein adsorption from plasma and the blood coagulation on the surfaces, whole blood adhesion and platelet adhesion tests were performed and are described in the following section.

4. Platelet and whole blood cell adhesion to CH and CH-Q modified surfaces

First, we evaluated platelet attachment to CH and CH-Q coated surfaces. To examine static platelet adhesion, tests were performed on glass (as a control), CH, and CH-Q surfaces by using platelet-rich plasma (PRP), and fluorescence microscopy was then used to observe and evaluate platelet adhesion. Adherent platelets were stained with Calcein AM, a cell permeant dye which is converted from a nonfluorescent substrate to a green-fluorescent product following intracellular esterase hydrolysis.³⁰ Figure 7 shows bright field (a) and fluorescent images (b) of glass (SiO₂), CH, and CH-Q surfaces after platelet incubation, respectively. The average number of adherent platelets per image on SiO₂, CH, CH-Q surfaces (image area = $111 \times 84 \mu\text{m}^2$) are 49 ± 6 , 12 ± 4 , and 4 ± 3 , respectively (Figure 7c). The smallest number of adherent platelets on the CH-Q surface indicates that platelets are significantly less activated on the CH-Q surface than on the others. Secondly, the number of adherent platelets on CH surface shows less activated on glass surface but more activated on CH-Q.

In order to evaluate the effects of CH and CH-Q coated layers on whole blood cell attachment, tests were performed on SiO₂ (as a control), CH, and CH-Q surfaces by using whole blood cells (WBC). Surfaces were subsequently analyzed using environmental scanning electron microscopy (SEM). Figure 8 shows SEM images of glass (SiO₂), CH, and CH-Q surfaces after whole blood cell incubation. As shown, the differences between the glass, CH and CH-Q modified surfaces were quite dramatic. On the glass surface, there was a large population of platelets (Glass (a) in Figure 8). Nearly all of the platelets were activated as shown by their spread morphology (Glass (c) in Figure 8). In addition, activated leukocytes were also observed on the glass surface (Glass (a) in Figure 8). In contrast, only a few attached platelets were observed on CH and CH-Q surfaces. Interestingly, several attached leukocytes (L) were observed on the CH surface (CH (c) in Figure 8) while no activated leukocytes were observed on CH-Q surface. These observations are consistent with our previous results, which show that CH-Q grafting can reduce macrophage adhesion and the resulting biological response (cytokine secretion by the macrophages).⁷ Overall cell adhesion results show that the number of attached platelets on CH and CH-Q surfaces was significantly reduced as compared to the glass surface. The cell adhesion results show an interrelationship with the plasma proteins adsorption results to collectively indicate that when compared to the adsorbed protein layer on SiO₂ surface, the less physisorbed and more viscous protein layer on CH-Q leads to the lower cell adhesion density from direct contact of platelets or whole blood.

5. Activation studies of platelets and neutrophils by blood contact with modified tube surfaces using flow cytometry

In order to evaluate the effectiveness of surface coating on tubes to ultimately improve blood compatibility (to diminish cell activation responses initiated by blood contact with modified tube surfaces), platelet and neutrophil activation by blood contact with the modified blood conduits for the three distinct time points were studied using flow cytometry. PU, PAA/PU, CH/PAA/PU, and CH-Q/PAA/PU tubes were exposed to whole blood using a Chandler Loop apparatus for 1 hour, 2 hours, and 3 hours. For this study, CD62P was used as a marker for platelet activation and CD62L, CD13, and CD18 were used as markers of neutrophil activation. First, the results of CD62P marker positivity for platelet activation showed a consistent pattern at 1, 2, and 3 hours of blood circulation time for all four surfaces as well as the control, as shown in Figure 9. The percentage levels of the CD62P positivity responses provoked by CH/PAA/PU are 65%, 66%, and 67% at 1, 2, or 3 hours of blood circulation time, respectively. The percentage levels of the positivity responses by CH-Q/PAA/PU are 67% (1hr), 64% (2hr), and 65% (3hr). The CD62P positivity responses provoked by CH/PAA/PU and CH-Q/PAA/PU showed no significant differences in percentage levels from one another as compared to the control (whole blood, not exposed to the tubes) at three different time points. The percentage levels of the CD62P positivity responses provoked by PU are 82%, 80%, and 76% at 1, 2, or 3 hours of blood circulation time, respectively. The positivity responses provoked by PU tubing were higher than those provoked by CH/PAA/PU and CH-Q/PAA/PU. The percentage levels of CD62P positive responses by PAA/PU had the highest values (94% (1 hr), 85% (2 hr), and 87% (3 hr)) compared with other tubes at 1, 2, or 3 hr of blood circulation time. These results demonstrate that CH and CH-Q grafting on PU tubing significantly reduced the number of circulating activated platelets in whole blood cycled through the Chandler Loop apparatus as compared to the surfaces of PU and PAA/PU tubes. For neutrophil activation, as shown in Figure 9, the percentage levels of the CD62L, CD13, and CD18 positivity responses of the control blood, which was not exposed to the blood conduits, are 19%, 20%, and 47%, respectively. The results of the CD62L, CD13, and CD18 marker positivity responses provoked by CH/PAA/PU and CH-Q/PAA/PU showed a similar and consistent pattern, and similar percentage levels to the control blood at 1, 2, and 3 hours of blood circulation time.

On the contrary, the percentage levels of the CD62L, CD13, and CD18 positivity responses provoked by PU are 43%, 68%, and 70% at 1 hours of blood circulation time, respectively. The percentage levels of the CD62L, CD13, and CD18 positivity responses provoked by PU are 34%, 69%, and 65% at 2 hours of blood circulation time, respectively. The CD62L, CD13, and CD18 marker positivity responses provoked by PU were significantly higher than those of the blood control. At 3 hr of blood circulation, the CD62L and CD18 positivity responses provoked by PU had similar levels to the control while the CD13 positivity responses by PU had still higher levels (51%) compared to the blood control (20%). These overall results of the PU tube indicate that non-modified PU tubes significantly increased the number of activated neutrophils, compared to the control, CH/PAA/PU, and CH-Q/PAA/PU tubes. In the neutrophil activation study of PAA/PU tubes (Figure 9), the CD62L, CD13, and CD18 marker positivity responses for neutrophil activation show totally different patterns compared with the blood control, CH/PAA/PU, and CH-Q/PAA/PU. The CD62L positive responses provoked by PAA/PU had the lowest levels (6%, 11%, and 7% compared with other tubes at 1, 2, or 3 hours, respectively, of blood circulation time). The CD13 positive responses by PAA/PU showed higher levels (57%) at 1 hour of blood circulation time than the blood control (20%). At 2 hours of blood circulation, the CD13 positive responses by PAA/PU had the lowest level (1%) compared with other tubes. At 3 hours of blood circulation, the CD13 positive responses by PAA/PU showed a similar level (23%) to the blood control (20%). Most interestingly, the CD18 positive responses by PAA/PU had the highest levels (99%, 98%, and 97%, respectively) compared with other tubes at 1, 2, or 3 hours of blood circulation time. Thus, the results for all markers for neutrophil activation provoked by PAA/PU did not show consistent results. Even though the CD62L positive responses showed the lowest levels of neutrophil activation compared with other tubes, CD18 positive responses by PAA/PU tube indicate that the PAA/PU coating on the tube significantly increased the number of activated neutrophils as compared to the control, CH/PAA/PU, CH-Q/PAA/PU, and PU. These flow cytometry results suggest that CH and CH-Q coating on PU tubing lead to minimal neutrophil activation effect compared to PAA coated PU and unmodified PU tubing. Overall, these cell activation results are in concurrence with whole blood and platelet adhesion studies as well as plasma proteins adsorption results. Collectively, these indicate that the weakest adsorbed plasma protein layer on CH-Q layer, which was more viscous than on SiO₂ surface, reduces activation and adhesion of whole blood and platelets on the surface.

Conclusions

In this study, chitosan (CH) and chitosan modified with quaternary ammonium salts (CH-Q) were immobilized on real polyurethane (PU) tubes using poly(acrylic acid) (PAA) as an intermediate adhesive layer grown by SIRP. The coverage of CH and CH-Q grafted layers as well as PAA grafted layer on PU tubes were characterized using ellipsometry and fluorescence microscopy. Material wear tests of CH and CH-Q grafted PU tubes demonstrated that each tube coating is chemically stable. To study that the properties of the physically adsorbed plasma protein layer on the biomaterial surfaces correlates with blood coagulation on biomaterial surfaces, in-situ adsorption of blood plasma proteins on CH and CH-Q versus silicon oxide surfaces was observed using quartz-crystal microbalances with dissipation (QCM-D). The QCM-D results showed that the physically adsorbed plasma protein layer on CH-Q and CH surfaces is softer and more viscous than the protein layer on the SiO₂ surface. The CH-Q layer thus has the weakest interaction with plasma proteins. Whole blood adhesion and platelet adhesion tests showed that the number of attached platelets on the CH and CH-Q surfaces was significantly reduced as compared to the glass surface. The biological response of platelets and neutrophils to CH and CH-Q layers as well as the PAA grafted layer on the PU tubes versus unmodified PU tubing show that CH and CH-Q grafting can reduce platelet and neutrophil activation, thus improving

hemocompatibility relative to other surfaces. These results demonstrate that the plasma protein absorption on biomaterial surfaces and the physisorbed layer's properties correlate with activation and adhesion from direct contact of platelets or whole blood with biomaterial surfaces. The overall results of this study show that (1) CH-Q coating on biomaterial surfaces can improve hemocompatibility of the surfaces, (2) QCM-D can be used as an important tool for studying plasma protein absorption on biomaterial surfaces, and (3) Blood plasma protein surface absorption from blood contacting medical implants and devices, and the adsorbed layers' properties can have an important effect on the platelet and neutrophil activation and adhesion for hemocompatibility evaluation of biomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Weber N, Wendel HP, Ziemer G. *Biomaterials*. 2002; 23:429. [PubMed: 11761163]
2. Bernacca GM, Gulbransen MJ, Wilkinson R, Wheatley DJ. *Biomaterials*. 1998; 19:1151. [PubMed: 9720899]
3. Ito Y, Sisido M, Imanishi Y. *J. Biomed. Mater. Res.* 1986; 20:1157. [PubMed: 3782177]
4. Maitz MF, Sperling C, Werner C. *J. Biomed. Mater. Res. Part A*. 2010; 94A:905.
5. Kang IK, Kwon BK, Lee JH, Lee HB. *Biomaterials*. 1993; 14:787. [PubMed: 8218730]
6. Stachelek SJ, Finley MJ, Alferiev IS, Wang F, Tsai RK, Eckells EC, Tomczyk N, Connolly JM, Discher DE, Eckmann DM, Levy RJ. *Biomaterials*. 2011; 32:4317. [PubMed: 21429575]
7. Lee HS, Stachelek SJ, Tomczyk N, Finley MJ, Composto RJ, Eckmann DM. *J. Biomed. Mater. Res. Part A*. 2013; 101A:203.
8. Finley MJ, Rauova L, Alferiev IS, Weisel JW, Levy RJ, Stachelek SJ. *Biomaterials*. 2012; 33:5803. [PubMed: 22613135]
9. Eckmann DM, Tsai IY, Tomczyk N, Weisel JW, Composto RJ. *Colloids Surf. B Biointerfaces*. 2013; 108:44. [PubMed: 23524078]
10. Mermel LA. *Ann. Intern. Med.* 2000; 132:391. [PubMed: 10691590]
11. Frederick, S.; Begley, TP. *Biocompatibility of Materials in Medical Devices*. Wiley Encyclopedia of Chemical Biology. John Wiley & Sons, Inc.; 2007.
12. Braude AI, Herndon D, Davis CE. *J. Infect. Dis.* 1973; 128:S157.
13. Kang IK, Kwon OH, Lee YM, Sung YK. *Biomaterials*. 1996; 17:841. [PubMed: 8730969]
14. Huh MW, Kang IK, Lee DH, Kim WS, Lee DH, Park LS, Min KE, Seo KH. *J. Appl. Polym. Sci.* 2001; 81:2769.
15. Lee HS, Tomczyk N, Composto RJ, Eckmann DM. *PMSE Prepr.* 2012; 107:183.
16. Murugesan S, Xie J, Linhardt RJ. *Curr. Top. Med. Chem.* 2008; 8:80. [PubMed: 18289079]
17. Colman RW, Scott CF, Schmaier AH, Wachtfogel YT, Pixley RA, Edmunds J. *Ann. N. Y. Acad. Sci.* 1987; 516:253. [PubMed: 3439730]
18. Gray JJ. *Curr. Opin. Struct. Biol.* 2004; 14:110. [PubMed: 15102457]
19. Monchaux E, Vermette P. *J. Biomed. Mater. Res. Part A*. 2008; 85:1052.

20. Tsai IY, Tomczyk N, Eckmann JI, Composto RJ, Eckmann DM. Colloids Surf. B Biointerfaces. 2011; 84:241. [PubMed: 21277175]
21. Vogler EA, Siedlecki CA. Biomaterials. 2009; 30:1857. [PubMed: 19168215]
22. Vroman L. Science. 1974; 184:585. [PubMed: 4821961]
23. Lee HS, Eckmann DM, Lee D, Hickok NJ, Composto RJ. Langmuir. 2011; 27:12458. [PubMed: 21894981]
24. Lee HS, Yee MQ, Eckmann YY, Hickok NJ, Eckmann DM, Composto RJ. J. Mater. Chem. 2012; 22:19605. [PubMed: 23209343]
25. Lee HS, Tomczyk N, Composto RJ, Eckmann DM. PMSE Prepr. 2012; 107:183.
26. Tsai IY, Kuo CC, Tomczyk N, Stachelek SJ, Composto RJ, Eckmann DM. Soft Matter. 2011; 7:3599. [PubMed: 21479122]
27. Sauerbrey G. Z. Phys. 1959; 155:206.
28. Vogt BD, Lin EK, Wu W, White CC. J. Phys. Chem. B. 2004; 108:12685.
29. Hook F, Kasemo B, Nylander T, Fant C, Sott K, Elwing H. Anal. Chem. 2001; 73:5796. [PubMed: 11791547]
30. De, H.; Lindauer, K.; Lukasczyk, U.; Blum, H.; Langer, T.; Zerlin, M. Method for the detection and/or quantification of the interaction of platelets with interaction partners. WO2012038370A1. Mar 29. 2012
31. Sinn S, Scheuermann T, Deichelbohrer S, Ziemer G, Wendel H. J. Mater. Sci. Mater. Med. 2011; 22:1521. [PubMed: 21604053]
32. Choi HS, Kim YS, Zhang Y, Tang S, Myung SW, Shin BC. Surf. Coat. Tech. 2004; 182:55.
33. Liu X, Chen X, Li Y, Wang X, Peng X, Zhu W. ACS Appl. Mater. Inter. 2012; 4:5169.

Figure 1.

The chemical structure of water-soluble chitosan modified with quaternary ammonium salt (CH-Q). For chitosan (CH) and CH-Q, the monomer fractions are [$n = 0.87$, $m = 0.0$, and $l = 0.13$] and [$n = 0.36$, $m = 0.51$, and $l = 0.13$], respectively.

Figure 2.

The experimental scheme for immobilization of CH (CH-Q) on PU surfaces via an intermediate polyacrylic acid brush layer. First, the substrate surface is functionalized with HTCS. Second, a PU layer is spin cast on the hydrophobic surface and then activated by a O_2 Plasma exposure. Third, the activated surface is functionalized with a polyacrylic acid brush grown by SIRP. Forth, the PAA is activated using EDC/NHS and then exposed to CH (60 kDa) or CH-Q to attach the outer brush.

Figure 3.

Bright field (a) and fluorescence microscopy (b) images of PU, PAA/PU, and AFC-P/PAA/PU tubes (left to right). Each tube was cut open for viewing. (c) Normalized fluorescence intensities from PU, PAA/PU, and AFC-P/PAA/PU tubes. Data are given as mean $\pm$ standard deviation ($n = 4$, square area = $70 \pm 70 \mu\text{m}^2$ ($\square$)). Statistical significance: *** $p < 0.0001$ versus PU and PAA/PU.

Figure 4.

Bright field (a) and fluorescence microscopy (b) images of rhodamine red treated PU, PAA/PU, CH-Q/PAA/PU, and CH/PAA/PU tubes (left to right). Each tube was cut open in order to view the inner surface. (c) Normalized fluorescence intensities from PU, PAA/PU, CH-Q/PAA/PU, and CH/PAA/PU tubes. Data are presented as mean $\pm$ standard deviation ($n = 4$, square area = $70 \pm 70 \mu\text{m}^2$ (□)). Statistical significance: *** $p < 0.001$ versus PU, PAA/PU, and CH-Q/PAA/PU.

Figure 5.

Wear testing of CH and CH-Q on PAA/PU tubes. Both tubes were labeled using rhodamine red. The PAA/PU tube is a control because the rhodamine red does not attach (cf. Figure 4b). Each tube was immersed in saline solution with shaking at 100 rpm for 1, 3, and 7 days. The fluorescent images were acquired at each time point.

Figure 6.

Plots of frequency change ($\Delta f_3/3$) and dissipation (ΔD_3) versus time after exposure of SiO₂, CH, and CH-Q coated surfaces to 50% plasma solutions, respectively, followed by exposure to saline solutions for rinsing step. * and ** denote initial exposure to plasma and saline, respectively.

Figure 7.

Bright field (a) and fluorescent images (b) of glass, CH and CH-Q coated glasses after platelet incubation. Platelets were stained with calcein, AM. (c) The Number of adherent platelets from green fluorescent images of glass, CH, and CH-Q surfaces. Data are presented as Mean $\pm$ SD (n = 5, image size = $111 \times 84 \mu\text{m}^2$). Scale bar = $10 \mu\text{m}$. Statistical significance: ***p < 0.0001 versus glass, ††p < 0.005 versus CH.

Figure 8.

Environmental scanning electron microscopy images of unmodified glass (as a control) as well as CH and CH-Q coated glasses after incubation in whole blood. Glass (a) is representative image of glass that shows extensive platelet (P) attachment and spreading onto glass surfaces leukocytes (L) were also observed on glass surface. In contrast, a few platelet (P) or leukocytes (L), erythrocyte (E) were observed on CH surface. And only few platelet was observed on CH-Q surface. Scale bars of images in row (a) and (b) equal 100 μm and 50 μm , respectively. Scale bars of Glass (c) and CH-Q (c) images equal 5 μm . Scale bar of CH (c) image is 40 μm .

Figure 9.

Flow cytometry studies of platelet and neutrophil activation on modified PU tubes using a Chandler Loop apparatus. Whole blood was circulated in PU, PAA/PU, CH/PAA/PU, and CH-Q/PAA/PU modified tubes for 1 (A), 2 (B) or 3 hours (C). As a control, blood was also set aside and not exposed to the tubes. CD62P was used to evaluate platelet activation, and CD62L, CD13, and CD18 were used to evaluate neutrophil activation. * indicates $P < 0.05$; *** indicates $P < 0.0001$ when compared to controls. Data are the mean $\pm$ standard deviation from $N = 6$ separate experiments with different donors.

Table 1
Ellipsometric thickness and contact angle of dry layers

Substrate	Total thickness (nm)	Contact angle (°, water)	Layer	Thickness (nm)
HTCS	1	90. ± 3	HTCS	1
PU/HTCS	102	70. ± 2	PU	101
PU [*] /HTCS	99 ± 1	36. ± 3	PU [*]	98
PAA/PU/HTCS	145 ± 9	29. ± 5	PAA	46
CH/PAA/PU/HTCS	201 ± 8	25. ± 7	CH	56
CH-Q/PAA/PU/HTCS	180 ± 6	12. ± 8	CH-Q	35

* O₂ plasma treated PU layer