Fluorinated surface-modifying macromolecules: modulating adhesive protein and platelet interactions on a polyether-urethane

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Abstract: Polyether-urethanes (PEUs) have been the materials of choice for the manufacture of conventional blood-contacting devices. Nevertheless, biostability and blood compatibility are still among the principal limitations in their long-term application. Studies investigating the development of protective coatings for PEUs have shown that degradation can be reduced with the use of fluorinated surface-modifying macromolecules (SMMs). It has also been hypothesized that SMM-modified PEU surfaces may exhibit improved blood compatibility because other studies have shown a modulation in fibrinogen adsorption onto these surfaces. To determine the blood compatibility of a PEU-containing fluorinated SMMs, a series of *in vitro* experiments were designed to study the pattern of protein adsorption from plasma and then to assess the nature of platelet adhesion and activation on each substrate. Western blot analysis as well as single protein studies revealed that the dominant "adhesive proteins" [fibrinogen (Fg), fibronectin (Fnc), and vitronectin (Vnc)] were adsorbed on two of the SMM-containing PEUs in lower amounts relative to unmodified base. Platelet adhesion and activation data further highlighted the differences among the various substrates. It was shown that the unmodified base had a higher number of adhered platelets relative to the SMM-modified surfaces, and that of the SMM-containing substrates, which showed the lowest levels of adhesive proteins also, exhibited significantly lower platelet densities. Close morphological examination further revealed that platelets residing on these latter substrates were not appreciably activated. Based on the current evidence, it is believed that the fluorinated SMMs demonstrate good potential for the development of surfaces with minimal thrombogenic character in *in vivo* applications.
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Key words: polyether-urethane; fluro-polymer; surface modification; additives; thrombogenesis; adhesive proteins; platelets

INTRODUCTION

It is generally accepted that one of the first events that occur during blood-material interactions is the adsorption of different plasma proteins, followed by platelet and cellular adhesion.[1] The make-up of the adsorbed protein layer on a biomaterial surface could effectively determine the types of cells that would be adhered as well as their activation state.[2,3] These events in turn are dependent on various material-related properties such as the biomaterial's surface wettability (surface energy), hydrophilicity/hydrophobicity, chemistry, charge, roughness, flexibility, and dynamics.[4] It is believed that such characteristics can influence the conformation and orientation of the adhered proteins, and thus the accessibility of moieties capable of reacting with the membrane glycoproteins of platelets.[5,6]

Of the numerous proteins present in plasma, three adhesive ones, namely fibrinogen (Fg), fibronectin (Fnc), and vitronectin (Vnc), have been implicated in platelet adhesion studies. The central role of these proteins in coagulation and their ability to promote platelet adhesion and aggregation stems from their capability to specifically bind to the platelet receptor glycoprotein (Gp) IIb-IIIa.[7–9] In studies with Biomer, a commercial polyurethane previously used in the fabrication of cardiovascular devices, it was found that the adhesion of platelets after coating with serum or "afibrinogenemic" plasma was low when compared with Biomer coated with normal plasma. Furthermore, the addition of Fg to the serum or deficient plasma restored platelet adhesion in a dose-

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dependant manner.[10] Both Vnc and Fnc are also classified as adhesive proteins and are implicated in platelet adhesion. All three of these adhesive proteins share a common arginine-glycine-aspartate (RGD) amino acid sequence, which binds to integrin receptors on the platelet surface.[11]

It has been proposed that the number of adherent platelets and their morphological appearance on the surface of biomaterials could be used as an indication of surface thrombogenicity in most *in vitro* studies. Other investigators have concluded that thrombogenesis as evaluated by platelet studies consists of three distinct events.[12] The first event is the initial adhesion of platelets to the surface, followed by platelet spreading and surface activation. The final event consists of the attachment of additional platelets and other blood elements to the already adhered platelets in order to form a thrombus.[12]

Several investigators have shown that by manipulating the surface characteristics, which define the surface energy of a polymer substrate, it is possible to influence the specific biocompatibility and biostability of materials. For instance, Vogler et al.[13] have shown that hydrophobic surfaces display a relatively low level of coagulation activation, whereas other surfaces with hydrophilic character exhibited a high coagulation activity. In another study, it was shown that the presence of fluoroalkyl groups, used as chain extenders in polyurethane, resulted in polymers that were less thrombogenic.[14] It was believed that due to fluorine's low surface energy, these groups were preferentially concentrated at the surface of the polymer and as such, reduced protein adsorption (e.g., bovine serum albumin, bovine γ-globulin, and bovine plasma fibrinogen) as well as platelet adhesion and activation.[14]

In an effort to improve thromboresistance, Ward et al.[15] had reported on the use of silicone surface-modifying additives (SMA) blended with biomedical polymers. Blending these silicone-based hydrophilic segment additives at low concentrations with a base polymer resulted in the spontaneous migration of the low-energy SMA to the air-surface interface.[15] The saturation of the SMA and its hydrophilic moieties at the surface were thought to reduce the thrombogenicity by minimizing protein denaturation or modulating protein adsorption. *In vivo* evaluation of materials containing SMAs was performed through the implantation of the Pierce-Donachy prosthetic ventricle with polyurethane blood pumping sacs and cannulas containing the SMA blends.[15] They found that the SMA copolymer blend was superior in thromboresistance character relative to the commercial polyurethane (Biomer) without accompanied loss in mechanical or physical properties.[15]

Fluorinated surface modifying macromolecules (SMMs) with a broad range of molecular weights and chemistries were synthesized and their ability to inhibit the enzyme catalyzed degradation of polyurethanes were shown.[16–18] These SMMs were linear polyurethanes with different polyol segments that were end-capped with a fluorinated alcohol. When blended with PEU, it was observed that the SMMs were able to preferentially migrate to the surface without affecting the bulk properties of the PEU and simultaneously mask hydrolyzable groups on the polyester-urethane base. Contact angle measurements revealed that the fluorine-rich surfaces had a reduced surface energy.[17] The presence of this low surface energy was further validated when fibrinogen adsorption studies revealed significantly decreased fibrinogen adsorption levels.[16] From these data, it was hypothesized that the SMM may exhibit nonthrombogenic character.

The objective of the current study was to determine if SMMs have the ability to minimize the activation of blood elements and hence reduce the thrombogenic nature of segmented polyurethanes. Experimental results are presented for the adsorption of three adhesive proteins (Fg, Fnc, and Vnc) onto a polyether-urea-urethane, in the absence of and with four different SMMs. Immunoassay and radiolabeled techniques were used to detect the presence of the selected proteins from human plasma. Subsequently, in a separate series of experiments, platelet adhesion was studied using a cone and plate device.

MATERIALS AND METHODS

Synthesis of polyether-urea-urethane

The base polymer in this work was a poly-etherurea-urethane, hereafter referred to as TDI/PTMO/ED. The polymer was synthesized using a conventional two-step prepolymer/chain extension reaction.[19] The monomers included the following: 2,4-toluene diisocyanate (TDI; obtained from Eastman Kodak, Rochester, NY), polytetramethylene oxide (PTMO; molecular weight = 1000, obtained from DuPont, Mississauga, Ontario), and ethylene diamine (ED, obtained from Aldrich Chemical Company, Milwaukee, WI). Before synthesis, TDI was vacuum distilled at 0.025 mmHg. PTMO was degassed for 24 h at 0.5 mmHg. ED was distilled under atmospheric pressure. After the preliminary preparation of the monomers, the synthesis of TDI/PTMO/ED, with a 2.1:1:1.2 stoichiometry of TDI:PTMO:ED was conducted. The detailed description of the synthesis and purification of the base PEU was described elsewhere.[19]

Synthesis of surface-modifying macromolecules

The surface-modifying macromolecules (SMMs) are fluorine containing additives that possess two segments, which

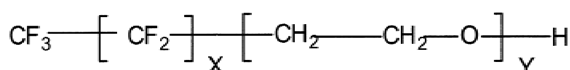
differ in their compatibility with the base polymer.[17] Detailed descriptions of the SMM synthesis procedures were given elsewhere.[17,20]

All of the SMMs were synthesized using 1,6-hexanediisocyanate (HDI; obtained from Aldrich Chemical Company, Milwaukee, WI). Two polyols were used to synthesize the four SMMs: polypropylene oxide with molecular weight of 1000 (PPO; obtained from Aldrich Chemical Company, Milwaukee, WI) and polytetramethylene oxide with molecular weight of 1000 (PTMO; DuPont, Mississauga, Ontario). Two different mono-functional fluorinated alcohols (Van Waters & Rodgers, Montreal, Quebec) were used to carry out the end-capping procedure. The two oligomeric fluoro-alcohols differed in the number of ethylene oxide units they contained, and their general structure is shown in Figure 1. HDI was vacuum distilled at 0.025 mmHg, whereas polyols (PPO and PTMO) were degassed at 60°C and 0.5 mmHg overnight. The oligomeric fluoro-alcohol material with Y = 1 (see Fig. 1) was distilled into different fractions.[17] The first fraction (recovered between 50 and 55°C and 0.025 mmHg) was designated as "L" (Low boiling fraction). The second fraction (recovered between 60 and 65°C at 0.025 mmHg) was labeled as "I" (Intermediate boiling fraction). Table I provides an explanation of the SMM nomenclature for this study. The first three series of letters refers to the polyol segment used, the numerical sequence indicates the reagent stoichiometry, and the final letter refers to the fluoro-alcohol type contained in the SMM. The designation of "F" for PTMO212F indicates the use of the second oligomeric fluoro-alcohol, which contained multiple ethylene oxide units (i.e., Y > 1). This material was purified by recovering the distillate at 55°C, 0.1 mmHg.[20]

Elemental analysis of both TDI/PTMO/ED and SMMs were obtained from Guelph Chemical Laboratories Ltd. (Guelph, Ontario). For molecular weight determination, the samples were analyzed using a gel permeation chromatography system (GPC; Waters, Mississauga, Canada) as described previously.[19]

Contact angle measurement

The surface energetics of the PEUs containing SMMs were analyzed using contact angle measurements. These measurements were carried out using a contact angle goniometer (Rame-Hart Inc., Mountain Lakes, NJ) by measuring the water drop contact angles at the material-air interface. Different polymeric surfaces were prepared by coating polymer solutions on glass slides. The solutions were prepared from TDI/PTMO/ED-SMM blends in dimethyl acetamide (DMAC, Aldrich, Milwaukee, WI) and cured according to methods previously described.[16] A total of 20 measurements was taken for each sample. Further details of the measurement were previously reported elsewhere.[16]



X = Odd number from 3-17

Y = 1 for PPO212L, PTMO212I, and PTMO322I

Y > 1 for PTMO 212F

Figure 1. Chemical structure of fluoro-alcohols.

Protein-binding studies

Polymer samples for protein studies were prepared by coating the base TDI/PTMO/ED-SMM mixture onto hollow glass tubes (5 mm in length, 4 mm OD, 2 mm ID), using 5% (w/v) polymer solutions prepared in DMAC, with the SMM content being 5% (w/w) relative to the base polymer. After solution dipping under a laminar flow hood, the tubes were blotted on a Kimwipe and then transferred to a dry Teflon plate to allow excess solution to run off. The coated tubes were dried in a convection oven at 60°C for 24 h. The coating procedure was repeated three times, with the final coat dried in a vacuum oven at 60°C for 24 h. The resulting film thickness was approximately 10 μm [measured by scanning electron microscopy (SEM)]. The day before the protein experiment, the polymer-coated glass tubes were preincubated in 0.05*M* phosphate buffer solution (PBS), pH 7.0, at 4°C.

After the equilibration period for the polymer-coated tubes in 0.05*M* PBS, the test specimens were removed from their Vacutainer vials and placed in 96-well tissue culture plates. Each polymeric group was run in triplicate, and all of the incubation experiments were conducted under sterile conditions in a laminar flow hood. The plasma pool used in both immunoblotting studies or ^{125}I-labeled fibrinogen adsorption analysis, consisted of platelet-poor acid citrate dextrose (ACD) plasma, prepared from freshly drawn healthy human blood (three donors), as described elsewhere.[21,22] A 250-μL aliquot of plasma solution was loaded into each well, covering polymer-coated glass tubes. The samples were incubated at room temperature for 3 h. After the incubation period, each polymer-coated glass tube was carefully rinsed three times with PBS buffer and then dried by blotting with a Kimwipe.

For the Western blot analysis, adsorbed proteins were eluted from the tubes using 2% aqueous SDS (sodium dodecyl sulfate). Based on ^{125}I-experiments for Fg (described below) and albumin (data not shown), it was estimated that more than 90% of the proteins were removed from the surfaces with the 2% SDS treatment. Eluates were analyzed on 12% (w/v) resolved SDS-PAGE ready gel (BioRad, Richmond, CA). The separated proteins were transferred electrophoretically from the gel onto an Immobilon PVDF membrane (Millipore Corp., Bedford, MA). The blots were cut into 1-mm strips and blocked with 5% (w/v) solution of nonfat dry milk. The strips were incubated with antibody to one of three adhesive proteins for study and then with an alkaline phosphatase-conjugated second antibody. Polyclonal antibodies to human and rat plasma proteins [fibrinogen (Fg), fibronectin (Fnc), and vitronectin (Vnc)] and alkaline phosphatase-conjugated secondary antibodies were used in the immunoblotting studies, and their source is listed in Table II. After a 1-h incubation, the strips were developed using a combined BCIP (bromo-4-chloro-3-

TABLE I
SMM Nomenclature[a]

Example: PTM0322I	
PTMO	Poly-tetramethylene-oxide (1000), the polyether diol segment used in this SMM. An alternative polyether diol was PPO (1000).
322	Refers to the molar ratio of HDI:PTMO: fluorinated alcohol (3:2:2).
I	Designates the length or chemistry of the fluoro-alcohol component of the SMM. Other choices include "L" or "F". "F" corresponding to a fluoro-alcohol with an oligomeric polyethylene oxide chain.

[a]Adapted from Tang 1996.[16]

indolyl phosphate) and NBT (nitroblue tetrazolium) reagent, prepared as described by the supplier. All Western blot studies were repeated at least three times in order to confirm relative stain comparisons.

The fibrinogen used in the ^{125}I label protein adsorption studies was prepared in the following manner. Lyophilized human plasma Fg (Calbiochem, La Jolla, CA) was radiolabeled by Na-^{125}I (ICN, Irvine, CA) using an iodine monochloride method and then dialyzed extensively against isotonic tris-buffered saline (TBS, pH 7.4) to remove radioactive free iodide. Labeled Fg was added to pooled ACD (acid citrate dextrose) human plasma as a tracer at a concentration of 2.5 mg/mL of Fg with 5% ^{125}I-Fg. The radiolabeling methodology was followed as previously reported.[23,24] After incubation, the tubes were rinsed three times with fresh TBS and analyzed by γ-counting. The counts were converted to μg of Fg per cm^2 based on the radioactivity of known concentrations of stock solutions and the surface area of the polymer-coated glass tubes.

Platelet adhesion studies

The polymer films were prepared from the same solutions as those used in the protein adsorption studies. Ten milliliters of solution was poured into Teflon molds, and the solvent was evaporated for 48 h in a 60°C airflow oven, followed by 72 h in a vacuum oven at 60°C. The resulting polymer films were then removed from the mold and placed in 15.0 mL of PBS buffer, pH 7.0, at 4°C overnight. Polymer discs (OD = 40 mm) were prepared in accordance with the size specification of the cone and plate instrument.[26] The samples were rinsed with methanol to remove any organic surface contamination and then were immersed in Tyrode's buffer (no albumin) and allowed to equilibrate for five hours prior to the experiment.

TABLE II
List of Primary and Secondary Antibodies

Primary Antibody	Source
Fibrinogen[a]	Cappel Laboratories, Cochraneville, PA
Vitronectin[b]	Calbiochem, Behring Diagnostic, La Jolla, CA
Fibronectin[b]	Cappel Laboratories, Cochraneville, PA
Enzyme-Conjugated Secondary Antibodies	**Source**
Rabbit anti-goat IgG-alkaline phosphatase[a]	Sigma, St. Louis, MO
Goat anti-rabbit IgG-alkaline phosphatase[b]	BioRad, Richmond, CA

Isolation of platelets from freshly collected citrated human blood was conducted using methods described by Mustard et al.[27] The feasibility of such methodologies has been repeated in many studies, and the details of the procedures are given elsewhere.[26,27] The platelets were used within 2 h of preparation because it has been shown that when the platelets were prepared according to the above method and kept at 37°C, they remained responsive to adenosine diphosphate (ADP) for 4 h in the presence of fibrinogen.[27] Briefly, the platelets were radiolabeled with ^{51}Cr label and suspended at a concentration of 250,000 platelets/μL in Tyrode's buffer containing albumin (0.35%) and apyrase (1 μL/mL), with washed red cells added at 40% hematocrit.

A cone and plate device was used for the platelet adhesion studies, a detailed description of the apparatus has been previously described by Skarja et al.[26] Such a device allows for well-characterized and well-controlled fluid flow conditions and requires only a small volume of fluid.[26]

Briefly, the polymer substrates (n = 9 for each polymer group, i.e., the experiment was repeated three times with triplicate samples for each experiment), which were maintained wet from a preincubation step in Tyrode's buffer, were placed in the apparatus. The platelet/red blood cell suspension was added to the wells via a sterile syringe (1.2 mL in each well), and the cones were lowered until the cone tip touched the test material. The latter steps required about a minute. The experiment consisted of rotating the cone at a speed to generate a fluid shear rate of 150 s^{-1} for 15 min. The latter condition was selected based on research literature using this cone and plate system and indicating that it can provide for the adequate delivery of platelets to the surface, permitting effective interaction times with the materials and not introducing shear induced degradation of the platelets.[26] The cone rotation was then stopped, and cones were raised. The suspension was aspirated from the wells. The wells were rinsed with Tyrode's buffer containing 0.01*M* ethylenediamine tetra-acetic acid (EDTA; BDH Chemicals, Toronto, Ontario) to disrupt any platelet aggregate that might have formed. After disassembling the four wells, the polymer films were removed, and sample discs (26 mm in diameter) were punched out. The disks were cut so that they were remote from the periphery, because it had been previously reported that deviations in flow patterns were observed near the periphery of the apparatus.[28] The polymer samples were then placed into scintillation vials and analyzed in a gamma

counter (Minaxi, Canberra Packard, CA) or taken for microscopic evaluation by SEM, as described in other studies.[28]

Statistical analysis

For all the experiments conducted in this study, a statistical analysis of variance (ANOVA) was conducted using the Scheffé method with a confidence interval of 95%. Contact angle measurements are reported with standard deviations, and protein absorption and platelet adhesion data are reported with standard errors.

RESULTS AND DISCUSSION

Material characterization

Table III presents the physical data for the SMMs. These data are in agreement with values reported for similar SMM materials prepared by other investigators.[16,20] The fluorine content of the SMMs synthesized with the 2:1:2 stoichiometry were all similar and greater than the SMM with the 3:2:2 stoichiometry (i.e., PTMO 322I). The molecular weight values also reflected appropriately the differences in stoichiometries.

The contact angle data for each of the TDI/PTMO/ED-SMM surfaces studied in this work are given in Table IV. It is generally accepted that for a water droplet in a water/air/surface system, the advancing contact angle is considered to be associated with the low surface energy domain of the material and is often determined by the polymer's hydrophobic groups.[28] Alternatively, the receding contact angle measurements could be used to assess the expression of the surface's hydrophilic components.[29]

A general trend observed in the contact angle data (Table IV) indicated that all of the SMM-modified surfaces, had a higher advancing contact angle than the unmodified TDI/PTMO/ED. This suggested that the presence of the fluorine component at the surface generated a low surface energy layer, which is a reflection of the increase in hydrophobic character. Tang et al.[16] confirmed the presence of the SMM molecules at the surface of polyurethanes using X-ray photoelectron spectroscopy (XPS) and reported similar increases in the contact angle values for a SMM/polyester-urea-urethane system.

TABLE III
Physical Characteristics of SMMs

Base Modifier	Wt % F	M_w (kDa)[a]	Polydispersity
PPO212L	18.0	19.2	1.20
PTMO212I	19.3	22.4	1.23
PTMO322I	11.0	35.1	1.32
PTMO212F	21.3	20.6	1.19

[a] M_w, polystyrene-equivalent weight average molecular weight.

TABLE IV
Surface-Air-Water Contact Angle Results[a]

Polymeric Material	Advancing Angle (degrees)	Receding Angle (degrees)	Hysteresis (degrees)
PEU	89.3 ± 0.4	48.9 ± 0.3	40.4 ± 0.5
PEU + PPO212L	117.1 ± 0.3	53.7 ± 0.5	63.4 ± 0.5
PEU + PTMO212I	126.8 ± 0.3	80.2 ± 0.5	46.5 ± 0.6
PEU + PTMO322I	123.0 ± 0.2	81.4 ± 0.4	41.6 ± 0.4
PEU + PTMO212F	99.7 ± 0.5	53.1 ± 0.4	46.6 ± 0.8

[a] Values are means ± SD.

It should also be pointed out that, in addition to the effect of the fluorinated compounds relative to the unmodified TDI/PTMO/ED, there were some differences in advancing contact angle values among the SMM-polymer blend themselves. Although it may be tempting to attribute these differences to the fluorine content of the SMM-polymer blends, it was found that fluorine content did not necessarily define the level of contact angle increase (cf. Tables III and IV). Tang et al. showed that although the presence of fluorine contributes to a higher advancing contact angle relative to the unmodified base, there did not seem to be a direct correlation between the relative amount of SMM contained in the base polymer and the advancing contact angles of the blends. This may be explained by the fact that surface saturation of the hydrophobic effect in these systems is achieved at about 2% SMM content.[17] In the current study, it was noted that PTMO212I had one of the highest fluorine contents (19.3%), whereas PTMO322I had the lowest fluorine content (11.0%; Table III), yet they both exhibited very similar wettabilities when blended with TDI/PTMO/ED (as measured by the contact angles, Table IV). This suggests that other structural and chemical characteristics of the SMMs contribute to the hydrophobic nature of the material surfaces. For SMM-polymer blends containing more than 2% by weight of the additive (i.e., above the saturation point, determined by Tang[16,18]), these differences are thought to be influenced by the specific chemical nature of the mid-chain component for the individual SMMs.[30]

It was interesting to note that PPO212L and PTMO212F, which contained different chemistries between the fluorine tails of the SMMs versus the soft segment chemistries of the TDI/PTMO/ED [i.e., PPO and PTMO/PEO (poly-ethylene-oxide), respectively, versus PTMO], exhibited significant differences in their advancing and receding contact angle values compared with the two SMMs containing solely PTMO chemistry between the fluorine tails (Table IV). This was despite the fact that their fluorine contents

were similar to that of PTMO212I (Table III). This further emphasizes the importance of the central segment chemistry in the SMM chain. PTMO212F, which contains a PEO spacer between its fluorinated tail and the diisocyanate of the SMM, had the lowest advancing contact angle of the four modified surfaces. The presence of the PEO group is thought to have increases the flexibility of the terminal fluorine chain and altered the manner in which these oligomeric chains interacted with the surface.

It was observed that the PPO212L blend had the highest hysteresis value among the SMMs (Table IV). This could in part be due to the (–CH_3) groups present on the PPO soft segment of this SMM. Bernett et al. have shown that the presence of pendant short alkyl groups reduces the van der Waals interaction among the polymer chains, yielding a more mobile, "liquid like" character with a lower packing density.[31,32] The presence of such segments on the surface of the polymer was believed to have allowed for relatively facile restructuring of the soft segment upon the change of media, which in turn explains the large hysteresis value. Although hysteresis has also been attributed to surface roughness,[29] this characteristic could not explain the differences observed between the SMM-modified surfaces, because morphological inspection of the polymer samples under light microscope, did not provide any evidence of such surface roughness (data not shown).

In conclusion, from the contact angle data, it can be deduced that modification of the TDI/PTMO/ED by SMMs has effectively changed the surface chemistry in a manner, which has rendered the substrates more hydrophobic (as indicated by the average advancing contact angle values). This low surface energy characteristic showed smaller but still significant differences ($p < 0.05$) from one SMM-TDI/PTMO/ED blend to another, depending on the SMM chemistry, length of the fluorine tail, and nature of the central chains and their mobility. In addition, the hysteresis phenomena as indicated above appear to be modified by such factors as chain mobility and polarity.

Protein adhesion to polymer substrates

The adsorption patterns of Fg, Fnc, and Vnc onto the five materials are shown in Figures 2, 3, and 4, respectively. All gels were repeated at least three times and the relative intensities between materials was consistent for all three proteins reported. Fg is one of the most abundant (2–3% of total plasma protein) and most important plasma proteins involved in the common pathway for blood coagulation.[33] The intact form of this protein is composed of three pairs of nonidentical but homologous polypeptide chains with molecular weights of 67, 58, and 47 kDa.[22] Figure 2 shows the typical band patterns for Fg. The band corresponding to the 67-kDa chain is present on all surfaces with relative intensities following the order of PPO212L < PTMO212F = PTMO212I < PTMO322I < PEU. The 58-kDa band represents one of the intact Fg chains. It is present in a similar intensity to the 67-kDa fragment.

Despite the significant differences in the hydrophobic character of the SMM/polymer surfaces versus the unmodified TDI/PTMO/ED (Table IV), there was not a parallel change in Fg band intensities (Fig. 2). The PPO212L and PTMO212F blends, which exhibited hydrophilic characteristics (i.e., receding contact angles) comparable to that of the base PEU did not show similar character in terms of Fg adsorption. Other investigators have reported that Fg adsorbs preferentially onto surfaces with more hydrophobic character and that its binding strength increases with hydrophobicity.[33] However, based on the intensity of the Fg bands obtained in this study, it appeared that the base PEU and surfaces containing PTMO322I had adsorbed the highest Fg levels, though their hydrophobic character (i.e., advancing contact angles) were quite contrasting (Table IV). These observations serve to further reinforce previous observations by Wojciechowski and Brash,[34,35] which indicated that contact angle data alone could not be correlated with the process of protein binding in complex media.

In other work, Kashiwagi et al.[14] have demonstrated that the presence of low surface energy fluoro-alkyl groups on the surface of PEUs could reduce the level of adsorbed protein. Interestingly, the lowest intensity in the Fg bands was observed on the two surfaces containing PPO212L and PTMO212F. Recall that these two SMM/polymer surfaces were the most hydrophilic of the four SMM-containing surfaces (Table IV). This further puts into question studies that rationalize low protein binding based on hydrophilic/hydrophobic character alone. A particular feature of PPO212L was its contact angle hysteresis, which was the highest of all the surfaces (Table IV). Earlier in this article, it was suggested that the PPO segment was responsible in part for inferring molecular reorientation of the polymer chains at the liquid/solid interface. This segment may also be responsible for the lack of Fg bound to the substrate, because chain mobility is believed to be a contributing factor to protein binding.[4,41] Similarly, PTMO212F may have had a comparable effect resulting from the presence of the flexible hydrophilic PEO group adjacent to the fluoro-tail.

Vnc and Fnc are two other adhesive proteins that promote the attachment and spreading of a variety of cells.[36] The intact form of Vnc can be found as two subunits with molecular weights of 70 and 66 kDa.[22] Fnc is a dimeric glycoprotein, with subunit polypeptide of 200 kDa.[22] Similar to Fg, both Fnc and Vnc

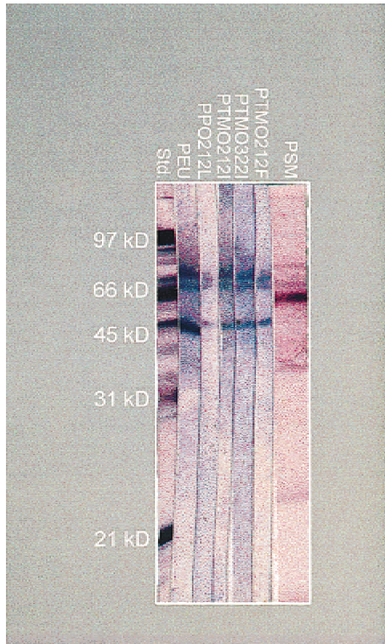


Figure 2. Immunoblots of Fg eluted form PEU and PEU-containing SMMs.

contain an RGD sequence, which is believed to mediate their binding to integrin receptors (i.e., GPIIb/IIIa) in platelets. Previous studies have revealed that Vnc typically adsorbs onto polymeric substrates in a higher amount than Fnc from plasma.[36] In other reports, it has been shown that in competitive experiments between Vnc and Fnc, Vnc adsorbed before Fnc even reached the surface.[37] It is believed that this oc-

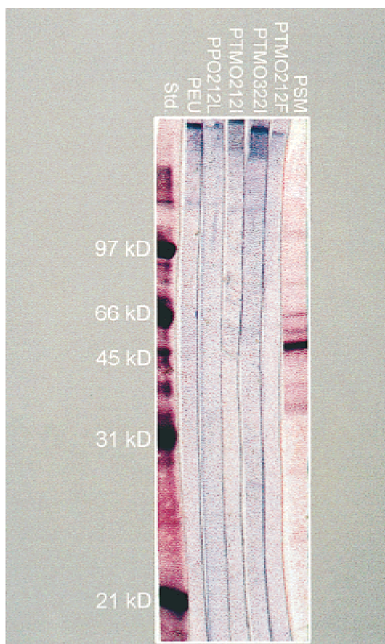


Figure 3. Immunoblots of Fnc eluted form PEU and PEU-containing SMMs.

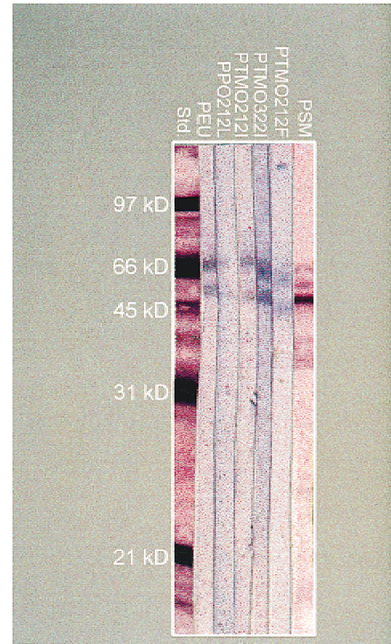


Figure 4. Immunoblots of Vnc eluted form PEU and PEU-containing SMMs.

curs because of Vnc's larger diffusivity and higher molar concentration.[37] Although it is difficult to assign a specific molecular weight to the bands associated with the Fnc data (Fig. 3), because the bands exceed the molecular weight range covered by the standard markers, it is clear that the intensity of the bands are fainter for the PPO212L and PTMO212F polymer blends when compared with the other three materials. Interestingly, this trend was also observed previously in the Fg immunoblot (Fig. 2). The band pattern in Figure 4 indicates that both subunits of the Vnc were adsorbed onto the polymer surfaces. The amount of adsorption varies, again with TDI/PTMO/ED, as well as PTMO212I and PTMO322I blends having the most intense bands, whereas the PPO212L and PTMO212F polymer blends are showing lower amounts of the two protein fragments.

Single protein adsorption studies

The following objectives were considered in conducting the 125I-Fg adsorption studies: 1) to quantify the adsorption patterns of Fg onto TDI/PTMO/ED and the polymer-containing SMMs; and 2) to compare the adsorption profiles with the results obtained from the Western blot analysis. Brash et al.[1] have undertaken extensive studies with the adsorption of Fg onto many substrates. Because of fibrinogen's structural flexibility, the molecule can spread and adapt itself at different interfaces.[39] Data in Figure 5 demonstrate that Fg adsorption from plasma occurred on all the

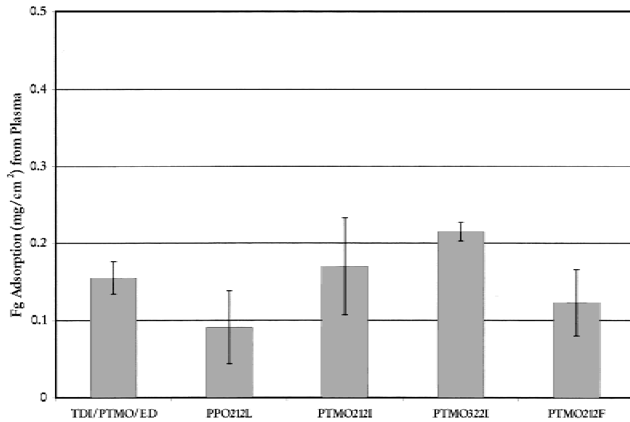


Figure 5. Adsorption of Fg from blood plasma onto different polymeric surfaces after a 3-h incubation at room temperature. Error bars are reported as standard errors.

modified and the unmodified substrates, which agreed with the earlier Western blot data. As was seen in earlier trends for the immunoblot data (Fig. 2), two of the SMM-containing surfaces (the PPO212L and PTMO212F blends) showed lower adsorption levels than the other materials. However, because of the high variability in the data, these differences were not statistically significant.

Total ^{51}Cr-platelet adhesion onto polymeric substrates

The adhesion of ^{51}Cr radiolabeled platelets from whole blood onto different polymeric surfaces is shown in Figure 6. The platelet density on unmodified TDI/PTMO/ED is statistically higher in comparison to the PEU-containing SMMs ($p < 0.05$). This difference may be related to the absence of the SMM and its fluoro-groups at the interface of the base control; however, the specific manner by which the fluorine groups may endow the polyurethane with a nonplatelet adhesive behavior is yet to be understood. Other investigators have also shown that the presence of fluorine components in PEU materials has changed the surface chemistry of the polymer and subsequently altered protein and platelet adhesion.[14] Although the latter studies have attributed such observations to the water-repelling and low surface free energy properties of the fluoroalkyl group,[14] results in the current study point to more complex phenomena.

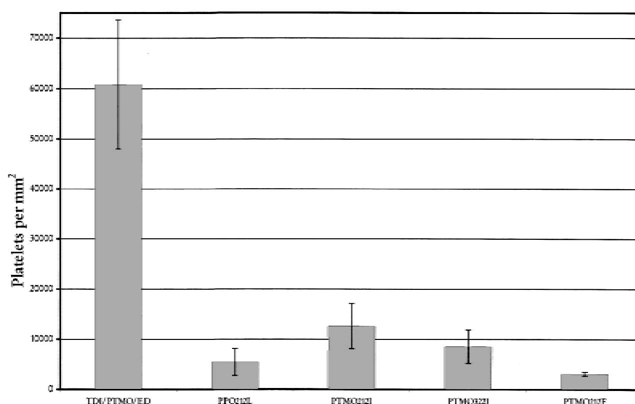


Figure 6. Platelet adhesion (averaged for n = 9) onto different polymeric surfaces after 15 min of exposure to whole blood at 150 s^{-1} (37°C). Error bars are reported as standard errors.

Statistical analysis showed that there were differences among the polymer surfaces containing SMMs themselves. It was observed that PTMO212I and PTMO322I behaved in a similar manner in terms of platelet deposition, whereas PPO212L and PTMO212F had the least amount of platelets deposited onto their surfaces. The platelet adhesion of the materials with PTMO212F and PTMO212I were significantly different from each other; however, there were no statistically significant differences between PPO212L and PTMO212F. These observations indicate that there would likely be at least two factors involved in the reduction of platelet adhesion for these SMM systems. The first factor is specifically related to the fluorine chemistry of the SMMs, and the second is likely more related to the morphological structure of the surface and the molecular dynamics of the SMM chains present at the surface of the base polymer. Recall that the adhesive proteins' binding patterns were also attributed to the latter parameter (Figs. 2–4).

PTMO212I and PTMO322I were structurally similar in many respects and different in others. Their similarities relate primarily to their identical soft segments. Because their diisocyanate:soft segment ratio, and hence molecular weight as well as fluorine content, varied (Table III), it is believed that the common soft segment is the component that might be contributing to the higher platelet adhesion levels in comparison to the other two SMMs. Whether the effect of PTMO was related to its physical state at the surface or to a specific chemical interaction of the PTMO segment with the proteins and platelets remains to be elucidated in future work.

PPO212L was one of the two SMMs that showed a low level of platelet adhesion. Grasel et al.[40] have previously suggested that, in general a PEU with PTMO as its soft segment exhibits more thrombogenic behavior than PPO-based PEUs. It was therefore hypothesized that the specific characteristics of the PPO soft segment (see discussion on contact angle data) and the manner by which it interacted with the other polymeric chains of the base polymer contributed to its unique platelet adhesion behavior. The lowest amount of platelet adhesion was seen on PTMO212F. The rationale provided to explain this observation is believed to be similar to that associated with other stud-

ies in which the presence of PEO components has been shown to significantly reduce platelet adhesion on polymeric substrates.[14] Again, this observation can be partially attributed to the highly mobile and low protein binding nature of the PEO segment at interfaces.[41]

In the current study, it is hypothesized that the parameters contributing to the particular patterns of protein adsorption (Figs. 2–5) also led to the observed trend in platelet adhesion among the SMM-containing surfaces. However, it is ultimately believed that such trends could only be explained when multiple factors (hydrophobicity, chain mobility, fluorine content, protein adsorption) are considered together and their influences are studied collectively and not individually.

Platelet morphological assessment

SEM studies were undertaken to assess the activation states of the platelets on the different substrates. It has been suggested that there is a direct correlation between the degree of platelet spreading on a polymeric surface *in vitro* and the thrombogenicity of that material evaluated *ex vivo*.[42] Goodman et al.[43] have categorized platelet adhesion and activation into five different stages, characterizing them based on different morphological changes. These steps start with inactivated platelets that have predominantly round or discoid shapes with an approximate diameter of 2 μm. As the platelets become more activated, they begin to extend their pseudopodia, and the membrane between them extends as it is pushed out by the hyaloplasm until the platelets reach their fully spread state. In the final activation state, the platelets typically have a diameter of 7–10 μm. These criteria were used to assess the activation state of the platelets on the SMM-containing PEUs in the current study. Photographs in both Figures 7 and 8 are representative of the whole sample surface for each of the materials.

Figure 7(a,b) exhibit the morphological state of platelets on the unmodified TDI/PTMO/ED. After exposure of the surfaces to blood, platelets were strongly attached, pseudopodia were formed and hyaloplasm could clearly be visualized among the pseudopodia. The level of activation was further accentuated by the size of the platelets (7–9 μm). This state corresponds to the final step of platelet activation. Figure 7(b) shows the formation of a white thrombus on the surface of the unmodified PEU, which represents highly activated platelets, forming elements on the order of 40 μm in size.[44]

The immunoblot data, as well as Fg adsorption

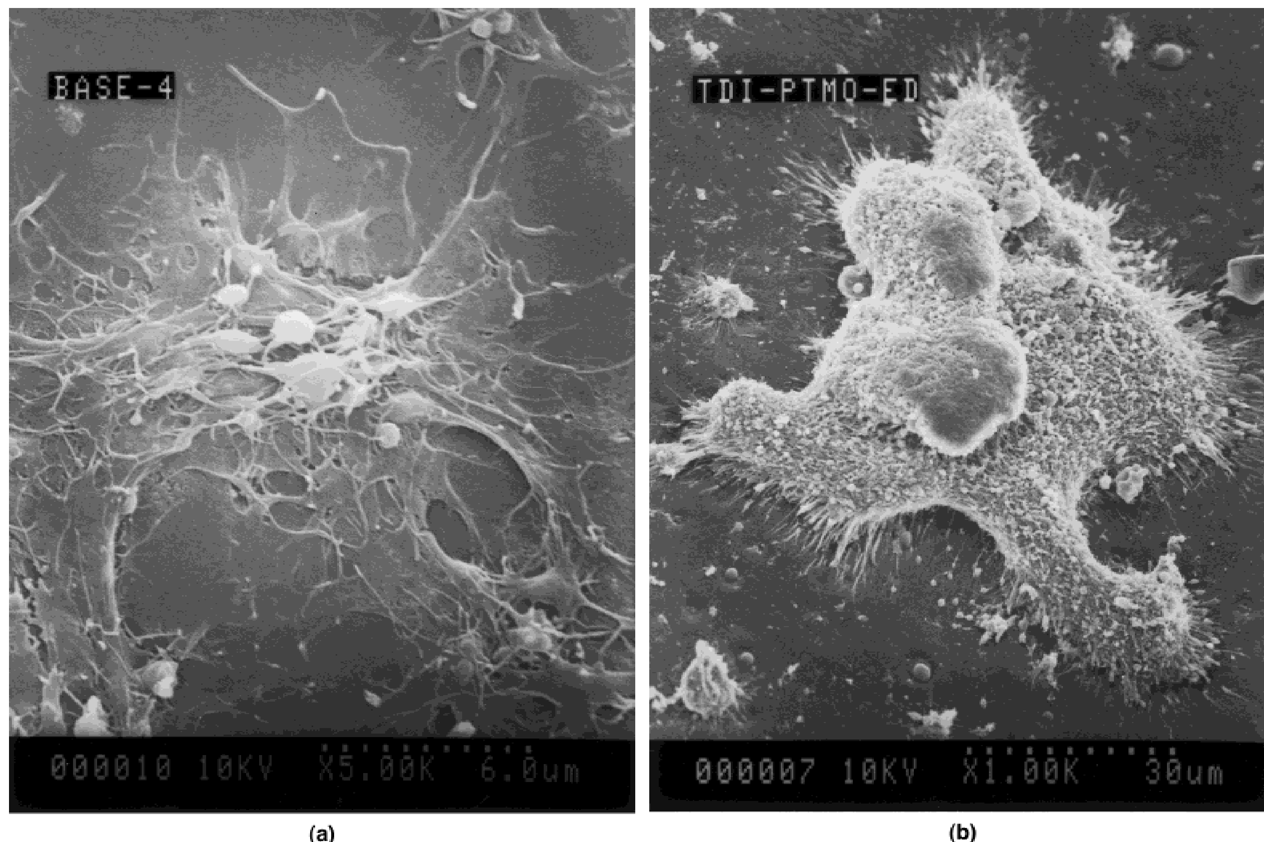


Figure 7. SEMs of platelet adhesion after 15 min of exposure to whole blood at 150 s^{-1} (37°C) onto unmodified PEU. (a) Original magnification, ×5.00K; (b) original magnification, ×1.00K.

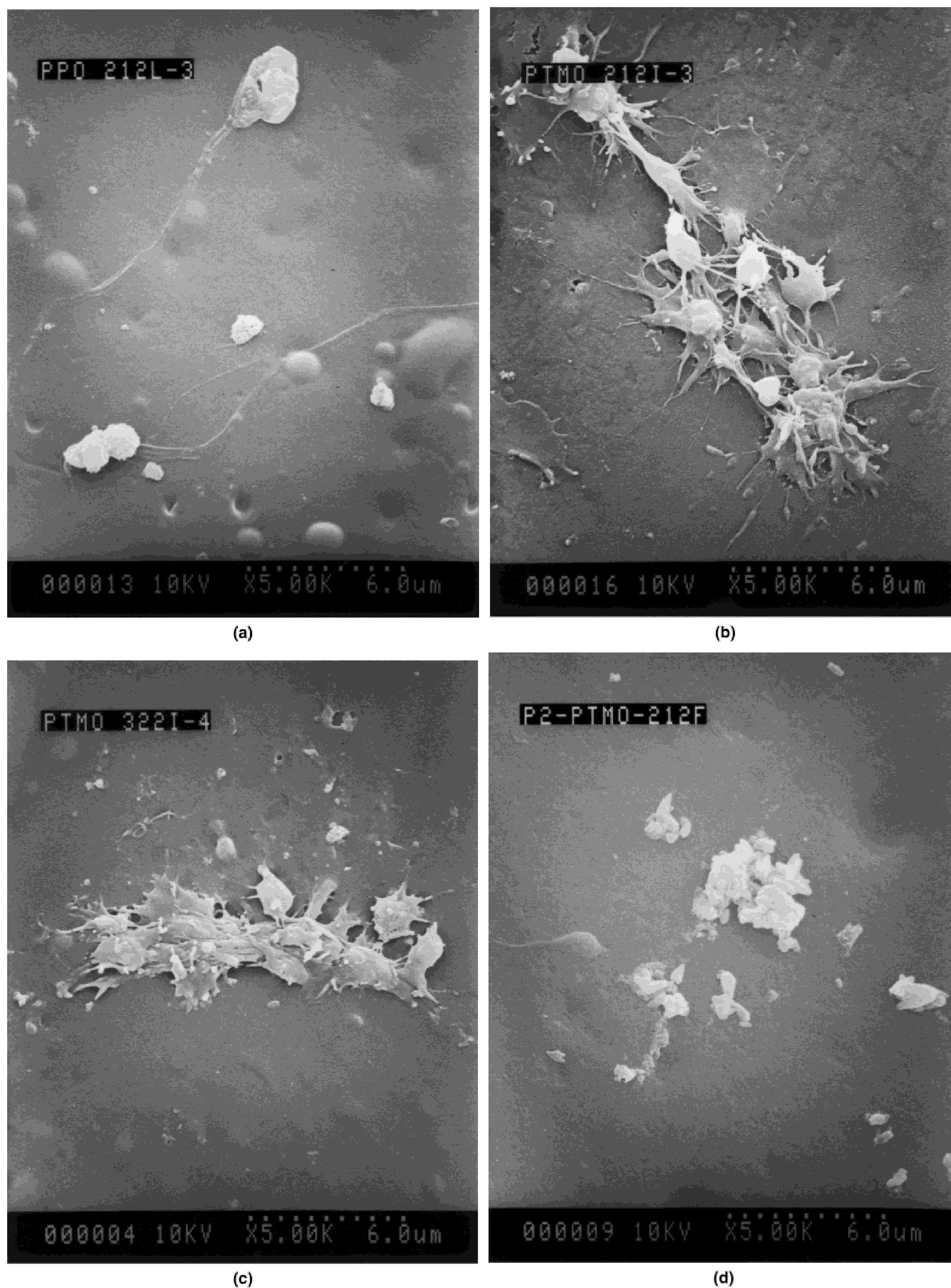


Figure 8. SEMs of platelet adhesion (original magnification, ×5.00K) after 15 min of exposure to whole blood at 150 s^{-1} (37°C) onto (a) PPO212L; (b) PTMO212I; (c) PTMO322I; (d) PTMO212F.

analysis of the base polyurethane, certainly suggested the presence of adhesive proteins that would permit the binding of platelets (Figs. 2–5). These proteins contain the amino acid sequence (RGD, or Arg-Gly-Asp) that interacts with the GPIIb/IIIa receptor on the surface of platelets.[2,5] In particular, two RGD sites have been reported on Fg (E domain Aα 95–97 and E domain γ 572–575).[45] The presence of such extensive platelet binding on TDI/PTMO/ED suggests that the conformational state of the protein was appropriate for binding.[46] It has also been shown that the postadsorptive conformational changes of the three proteins may either prevent or inhibit the platelet adhesion, reducing both the binding of polyclonal antibodies and SDS elutibility of the proteins.[47] Because these alterations are believed to be surface-dependent phenomena,[45] the unmodified TDI/PTMO/ED surface chemistry not only allowed for the adhesion of the platelets on the surface, but also allowed the binding of the proteins to occur in a manner that specifically exposed their RGD sequence to the platelets through an active adhesion process.[48]

Figure 8(a) shows the micrograph of platelet adhesion onto the PPO212L blend. It was observed that, although platelets were present on the surface, they appeared to be relatively passive (i.e., low activation). It is believed that a more rigorous washing procedure in the postexperimental stages might have removed these platelets from the surface. Their morphological shape was characterized by a lack of pseudopodia. The size of the platelets further confirmed their nonactivated state (~ 2 μm).[43] Again, this low active state was in accordance with the immunoblotting data presented earlier, in which the PPO212L blot exhibited very faint bands for the three adhesive proteins (Figs. 2–4). Although these proteins were adsorbed, the nature of their conformational states is unknown. It is therefore hypothesized that, because of the particular chemical and physical structures (e.g., diisocyanate:soft segment ratio, mobility of the side chains, hydrophilicity, surface charges) of the SMMs at the interfaces, proteins might have either become denatured or configured themselves in such manner that "masked" their RGD sequence from the platelets, and hence a low platelet density and absence of activation was observed.

Because of the already established similarities between PTMO212I and PTMO322I, these SMMs were analyzed together. The micrographs in Figure 8(b,c) revealed that platelets in both groups were activated to the same extent. This agreed well with the data presented in Figure 6, which showed more radiolabeled platelets on PTMO322I and PTMO212I versus the other two SMM-modified substrates. The platelets were, however, less activated than on the base PEU. The pseudopodia were beginning to bud out; however, the hyloplasms were not as extended as they were for the unmodified TDI/PTMO/ED.

Figure 8(d) shows an SEM micrograph of adhered platelets on the PTMO212F surface. There were very few platelets on the substrate, and there was no indication of pseudopodia. The platelets appeared as clustered with submicron fragments. The submicron particles may be evidence of platelet fragments that have been reported on by others[49] and will need to be further investigated. As in the case of the PPO212L-containing material, it was believed that a more aggressive washing technique might have been able to remove these platelet clusters from the surface. The results were again in accordance with the platelet adhesion data presented in Figure 6. Although immunoblotting data indicated evidence of moderate amounts of the adhesive proteins on this surface, it is suggested that the presence of the PEO segment adjacent to the fluorine tail, among other factors, may have rendered this particular SMM more flexible, thus allowing more mobility between the fluoro-tail and the central component of the SMM. These factors might have contributed to postadsorptive protein conformational changes, which subsequently influenced platelet adhesion. Confirmation of this will be sought in future work by specifically assessing the availability of protein adhesion sites such as the RGD sequence.

CONCLUSION

This study has shown that novel fluorinated SMMs, which were previously reported on with respect to their ability to reduce polyurethane hydrolysis by enzymes,[16–18] also exhibit significant reductions in platelet adhesion and activation. It was concluded that platelet adhesion and activation onto the unmodified TDI/PTMO/ED was significantly different from the SMM-containing materials. These differences were such that the unmodified surfaces were more thrombogenic than surface-modified substrates. The contact angle data indicated that the surface chemistries of all the SMM-containing substrates have been effectively altered in a manner that yielded more hydrophobic surfaces (as indicated by the elevated advancing contact angles) relative to the unmodified PEU substrate. Variability in surface energetics was also exhibited among the four SMM-containing PEUs, because receding contact angles and contact angle hysteresis were quite different. Hence, the nature of the protein-platelet interactions on the polymer surfaces was influenced factors by such as the overall surface chemistry, the SMM's chain mobility, fluorine content, and diisocyanate:soft segment ratio, and the morphological surface features induced by the SMMs.[30]

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