

Fibrinogen surface distribution correlates to platelet adhesion pattern on fluorinated surface-modified polyetherurethane

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Abstract

In previous work, it had been shown that platelet adhesion could be reduced by fluorinating surfaces with oligomeric fluoropolymers, referred to as surface-modifying macromolecules (SMMs). In the current study, two in vitro blood-contacting experiments were carried out on a polyetherurethane modified with three different SMMs in order to determine if altered platelet adhesion levels could be related to the pattern of adsorbed protein and more specifically to the manner in which fibrinogen (Fg) distribution occurs at the surface. In the first experiment, the materials were placed in whole human blood and the adherent platelets were viewed with high-resolution scanning electron microscopy (SEM). In a second experiment, the materials were incubated with human plasma with the absence of platelets. The plasma contained 5% fluorescent-Fg. The materials were then viewed with a fluorescence microscope and images were collected to define the distribution of high-density fluorescent-Fg areas. The SEM and fluorescent-Fg images were imported to Image Pro Plus™ imaging software to measure the area, length and circularity and a bivariate correlation test was conducted between the two sets of data. For area and length morphology parameters, there were high and significant correlations ($r > 0.9$, $p < 0.05$) between the platelets and Fg aggregates. The data suggest that the Fg distribution may serve as a predictor of platelet morphology/activation and provides insight into the non-thrombogenic character of biomaterials containing the fluorinated SMMs.

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1. Introduction

The long-term success of blood-contacting biomaterials has been limited by the occurrence of platelet adhesion and protein adsorption. The latter events elicit the activation of the coagulation cascade and promote thrombus formation. Several strategies have been developed to improve surface properties such as chemical modification or the introduction of surface-

active additives. Novel fluorinated surface modifying macromolecules (SMMs) have been designed to enhance the blood biocompatibility of polyurethanes (PU) [1]. SMM-treated materials adsorb many cell-binding proteins including vitronectin, fibronectin and fibrinogen (Fg) [1]. Fg often adsorbs to synthetic surfaces in much higher quantities than other adhesive proteins and has been implicated as a mediator of platelet adhesion on biomaterial surfaces [2–4]. Despite the presence of adsorbed Fg, SMM-modified surfaces have been shown to reduce the platelet adhesion and activation [1]. Activation is marked by platelet change from its discoid shape to varying degrees of spread shapes; thus, activation has been assessed by measuring adherent

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platelet morphology [5–7]. Glycoprotein IIb/IIIa (GP IIb/IIIa) receptors found on the platelet surface interact with the Arg–Gly–Asp sequence on the Fg molecule and the latter interaction mediates platelet aggregation and spreading at the site of vascular injury [8]. GP IIb/IIIa has also been implicated with platelet attachment and spreading on biomaterial surfaces [9–11]. Furthermore, research suggests that the extent of platelet attachment (i.e. morphology changes) may be a more appropriate qualifier than platelet adhesion number to assess blood-material compatibility [5,12].

Sheppard and colleagues [6] studied platelet morphology with respect to Fg adsorption on polymethylmethacrylate. They found that the spread platelets were usually associated with the redistribution of adsorbed Fg. Moon et al. [13] carried out transmission electron microscopy to visualize the binding of platelets to heme-octapeptide-labelled Fg. The data showed that Fg was widely distributed over platelet aggregate surfaces. E.I.B. Peerschke examined bound Fg on stimulated platelets with a confocal scanning laser microscope and demonstrated that Fg co-localized with GPIIIa present on the platelet surface [14]. Park et al. [5] investigated the morphology characteristics of surface-induced platelet activation. They concluded that platelet shape changes occurred as a result of the surface concentration of adsorbed protein coatings, Fg and albumin in particular. Ikeda et al. [7] studied the relationship between cytoplasmic calcium on platelet activation and morphology with respect to glass surfaces preadsorbed with Fg and von Willebrand factor (vWF). They indicated that the platelet pseudopodia formation and spreading were likely mediated by the platelet receptor GPIIb/IIIa. In addition, they observed a higher positive correlation between cytoplasmic calcium and platelet morphology change on the Fg preadsorbed surfaces versus non-coated glass and vWF glass. The above-mentioned studies clearly demonstrate the importance of Fg binding and distribution in platelet activation and emphasize the necessity to consider more than just the quantity of adsorbed Fg when defining the relationship between biomaterials and thrombogenic character. Despite the highlighted importance of the Fg–platelet interaction there have been very few studies that have attempted to visually convey this Fg and platelet activation relationship.

From our previous work, the quantity of adsorbed Fg under static condition did not correlate to platelet adhesion data acquired when the SMM-treated PU surfaces were exposed to reconstituted blood under laminar flow [1]. In this new study, it was postulated that the physical distribution of the Fg adsorption, not the quantity alone, in part dictates the protein's contribution to platelet activation on these SMM modified PU surfaces. Thus, the main objective of the current study was to measure platelet morphology (area, length and

circularity/roundness) observed on the SMM-treated and non-treated surfaces and to determine if correlations were found between the latter data to the adsorbed Fg distribution patterns.

2. Materials and Methods

2.1. Synthesis of polyether-urea-urethane and SMM

The synthesis of the polyether-urea-urethane (Base PU) and the SMMs has been previously described [15–17]. Briefly, a pro-thrombogenic Base PU was synthesized with 2,4-toluene diisocyanate (TDI, Aldrich Chemical Co. Milwaukee, WI), polytetramethylene oxide (PTMO, molecular weight = 1000 Da, Dupont, Mississauga, Ont.) and ethylene diamine (ED, Aldrich Chemical Co. Milwaukee, WI) with a 2:1:1 reagent stoichiometry using a two-step prepolymer/chain extension reaction [16]. The Base PU was modified with three SMMs: PPO212L, PTMO212F and PTMO212I. The SMMs were synthesized with 1,6-hexanediisocyanate (HDI, Aldrich Chemical Co. Milwaukee, WI) and two polyols, either polypropylene oxide (PPO) or polytetramethylene oxide (PTMO) (both Mw = 1000 Da, Aldrich Chemical Co. Milwaukee, WI). Two fluoroalcohols, either BA-L or FSO-100 (Van Waters & Rogers, Montreal, PQ), end-capped the SMM molecule [15]. The BA-L was distilled into low boiling fractions (“L”, 50–55 °C, 0.025 mmHg) and intermediate boiling fractions (“I”, 60–65 °C, 0.025 mmHg) [17]. FSO-100 fluoroalcohol (F), which contains an oligo ethylene oxide unit, was distilled at 55 °C, 0.1 mmHg [15]. The SMM nomenclature is as follows; a first sequence of letters refers to the polyol (PPO or PTMO), a numerical sequence refers to the reagent stoichiometry (HDI: polyol: fluoroalcohol), and a final letter refers to the type of fluoroalcohol. For example, PTMO212I contains the PTMO polyol, with a reagent stoichiometry of 2: 1: 2 and “I” designates the intermediate fraction of the BA-L fluoroalcohol. The molecular weight, polydispersity and fluorine content were previously reported for the SMMs [1,15].

2.2. Polymer film preparation

The polymer solution contained 5% (wt/v) Base PU in DMAc (dimethylacetamide), with 5% (wt/wt) of SMM additive relative to the Base PU. To cast films for the platelet adhesion studies, 10 mL of the polymer solution was transferred to 5.0 cm × 5.0 cm Teflon® molds and placed in a 60 °C airflow oven for two days. Afterwards, the mold was placed in a 60 °C, 25 mmHg vacuum oven for 2 days. Once dried, the films were removed with tweezers and placed in covered Petri dishes until further use. Before the platelet adhesion experiments, polymer film samples were punched out into 40 mm diameter disks and incubated in plain tyrodes buffer, pH 7.4, at 4 °C overnight. For the Fg adsorption experiments, the polymers were spin-cast (500 rpm for 10 s followed by 4000 rpm for 85 s, Spincoater model P6700 series, Speciality Coating Systems Inc., Indianapolis, IN) on chromic acid-cleaned glass microscope cover slips and dried in a 60 °C, vacuum (25 mmHg) oven for 2 days.

2.3. Platelet and blood preparation

Blood was collected with a butterfly syringe from the antecubital vein of healthy volunteers (with human ethics approval from McMaster University) who were free of medication for a minimum of 10 days. Platelet preparation and radio labeling were carried out using a well-established method [18,19]. Briefly, the platelets were washed and radio labeled with ^{51}Cr label (Sodium Chromate, Perkin Elmer Inc.). Platelets prepared by this method have been shown to be viable in several studies [20,21]. The final blood suspension contained 250,000 platelets/ μL in 0.35% albumin-Tyrode's buffer (Sigma, Fraction V) and apyrase (0.3 $\mu\text{L}/\text{mL}$) with homologous red blood added at 40% hematocrit.

2.4. Platelet adhesion

A cone and plate instrument rotated at 150 rpm for 15 min was used to assess the relative differences in platelet adhesion potential for the various surfaces [1,19]. The adherent platelets were fixed with 2% glutaraldehyde solution in 0.1 sodium cacodylate solution, maintained at pH 7 overnight at 4 °C and viewed by SEM. Ten SEM images (20.5 $\mu\text{m} \times 22.5 \mu\text{m}$) were analysed for each PU material with Image Pro PlusTM software (Media Cybernetics, Silver Spring MD, USA) and the area, circularity ($\text{Perimeter}^2/(4\pi \times \text{Area})$) and maximum length were measured as percent frequency for each platelet or platelet cluster. More specifically, four levels of activation or platelet areas and lengths were grouped as per literature values [5]. The first area range represents the microparticles and may also include small surface artifact debris from sample preparation (0–0.5 μm^2), the second represents quiescent platelets (>0.5–1 μm^2), the third group represents dendritic platelets (>1–3 μm^2), and the fourth group represents fully spread, highly activated platelets (>3 μm^2). Similarly, the platelet length was divided into four groups: the first group was less than 0.8 μm , the second ranged from greater than 0.8–2 μm , the third group ranged from greater than 2–3 μm and the last group was greater than 3 μm in length. As for platelet circularity, values larger than 1 indicate deviations from round resting platelets. Four levels of circularity were also created: the first group included values of 1 (perfectly rounded), the second group represented values between 1 and 2 (small deviations from round, small pseudopods), the third group represented values between 2 and 3 (pseudopodia extension) and the last group greater than 3 (extensive pseudopodia extension, cell spreading) [5]. Histograms were generated for each morphological characteristic and material.

2.5. Protein adsorption of fluorescent-labeled Fg

The distribution of bound Fg was measured using fluorescently labeled Fg (FI-Fg, Alexa-488, Molecular ProbesTM, 496 nm absorbance and 520 nm emission maxima). The FI-Fg was dissolved in 0.1 M sodium bicarbonate (pH 8.3) containing 2 mM of sodium azide, separated into 200 μL aliquots (in microcentrifuge tubes) and frozen at –20 °C away from light. Prior to adsorption, Bio-beads[®] absorbents (# 152-8929 Bio-Rad Laboratories, Hercules, CA) were used as per manufacturer's instructions to separate any non-conju-

gated Alexa-488 label from the FI-Fg. Briefly, 200 μL of FI-Fg was thawed and added to 0.02 g of Bio-beads[®] absorbents and placed on an orbital shaker in a cold room for 30 min to ensure thorough mixing. The FI-Fg and the Bio-beads[®] absorbents were centrifuged at 4 °C for 5 min at 6000 rpm and transferred out of the microcentrifuge tube. With appropriate dilution, the concentration of the Fg was calculated from UV absorbance measurements (at 280 and 495 nm), which included a factor that accounted for the fluorescent intensity of the label. A 5 mm I.D. glass tube was placed on top of the polymer cast films to form a well into which 200 μL of 5% FI-Fg plasma was added. The materials were incubated for 2 h at 25 °C, pH 7.4. After which time, the surfaces were rinsed with PBS, pH 7.4, to remove any loosely bound protein. 1–2 drops of ImmunoFluore mounting media (#622701, Lot 1363F, ICN Biomedicals Inc, Aurora, OH) were added onto the surface, followed by a glass coverslip, to prevent photo bleaching and preserve the protein sample. The materials were kept at 4 °C, away from light, for a maximum of 2 days before analysis. The fluorescent images (215 $\mu\text{m} \times 160 \mu\text{m}$) were collected with a Zeiss epifluorescent microscope (40 \times fluoro in oil lens)/mercury arc 100 W lamp light source/digital camera system. The images were background subtracted with Image Pro Plus 4.5TM software. Using similar categories to those used for the platelet morphology experiments, the fluorescent images of each PU sample were exported to Image Pro PlusTM software (Media Cybernetics, Silver Spring MD, USA) and the area, circularity ($\text{perimeter}^2/(4\pi \times \text{area})$) and length were measured and grouped as percent frequency for each Fg area. The latter analysis provided information for the distribution on the surfaces. Histograms were generated for each material.

2.6. Statistics

The significant difference amongst SMM-treated and non-treated surfaces was assessed by one-factor analysis of variance (ANOVA) using the Tukey's HSD multiple comparison test to compare pairwise the mean values with a 95% confidence level. Also, Spearman's rho test evaluated the correlation between platelet (area, circularity and length) data for all the materials ($n = 40$) and the Fg (area, circularity and length) data for all the materials ($n = 40$), such that trends could be determined amongst the differently modified and non-modified materials. The platelet morphology and Fg distribution are reported with their standard error of the mean. The statistics were performed with commercially available software program, SPSS v.10.

3. Results

3.1. Platelet adhesion

SMM-treated materials reduced platelet adhesion when compared to the Base PU (Table 1). The platelet count for the Base PU was statistically higher ($p < 0.05$) than that for all of the SMM-treated materials. SEM was performed on the materials (Fig. 1). The micrographs of the Base PU indicated that the adherent platelets were spread and aggregated in clusters on the

Table 1
Platelet adhesion (platelets/mm²) on Base PU and SMM-modified PUs

Materials	Platelet adhesion + Std. Err
Base PU	2309 ± 761
PPO212L + Base PU	591 ± 135
PTMO212F + Base PU	223 ± 67
PTMO212I + Base PU	647 ± 182

Data are reported with standard error of the mean (Std. Err, $n = 12$).

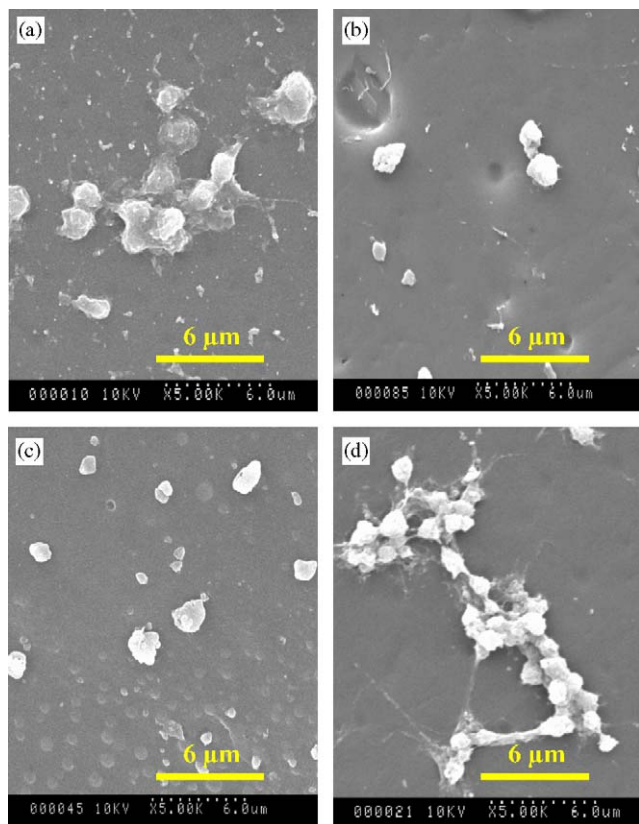


Fig. 1. SEM of adherent platelets onto different polymeric surfaces after 15 min exposure to blood at 150 rpm (37 °C). (a) Base PU, (b) PPO212L, (c) PTMO212F, and (d) PTMO212I. Image size is 20.5 μm × 22.5 μm.

surface. The platelets on PPO212L and PTMO212F were rounded with minimal clustering and pseudopodia extension. The platelets on PTMO212I appeared in small linearly strung clusters with some pseudopodia extension, which indicates differences among the SMM treated materials. Morphological analysis of 10 SEM images was carried out on each material as the percent frequency of platelet shape change (Fig. 2). Consistently, PPO212L and PTMO212F-treated materials had statistically lower ($p < 0.05$) area = 1 μm², length = 2 μm and circularity = 2 parameters, whereas, PTMO212I and Base PU had statistically higher ($p < 0.05$) area > 3 μm², length > 3 μm and circularity > 3+ parameters. This

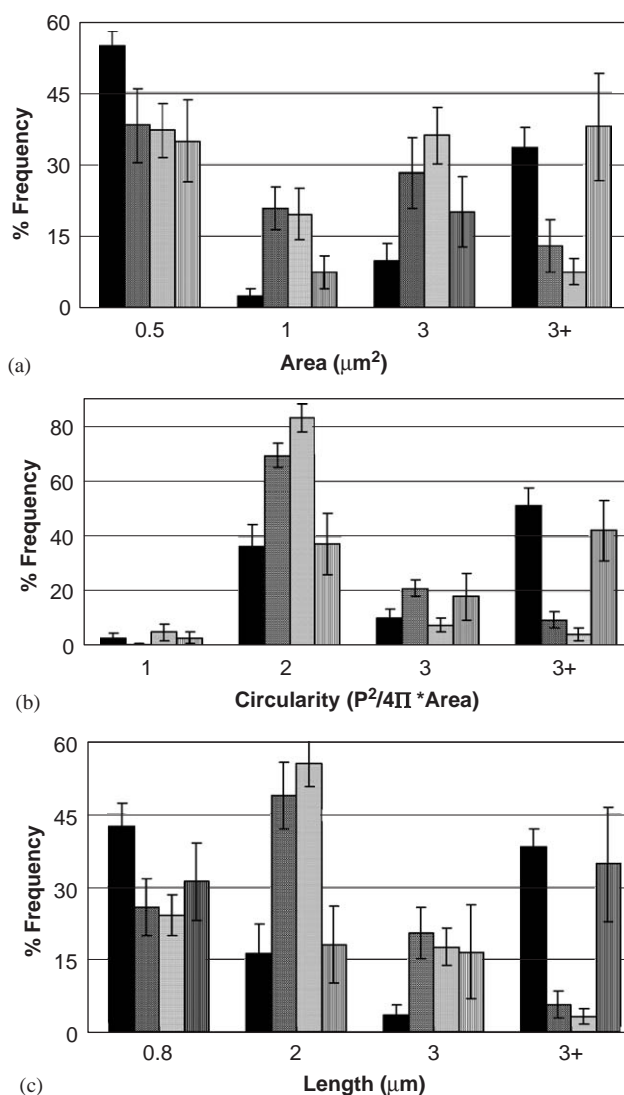


Fig. 2. Morphological analysis of SEM images (20.5 μm × 22.5 μm, $n = 40$ images from 4 experiments) of adherent platelets using Image Pro Plus™ software: (a) area, (b) circularity, and (c) Length. ■ Base PU, ■ PPO212L, ■ PTMO212F, ■ PTMO212I

indicates that the latter material surfaces have higher regions of activated platelets. Despite the low platelet attachment on the PTMO212I-treated material, the adherent platelets appear quite activated based on the measured morphological parameters. Note that it is difficult to differentiate small surface features and precipitated protein aggregates from microparticles for the shape parameter range of area < 1 μm² and length < 0.8 μm. As a result, there were no statistical differences amongst the materials for the latter characteristics.

3.2. Protein adsorption of fluorescent-labeled Fg

Examples of the FI-Fg patterns observed on the PU surfaces are illustrated in Fig. 3. After subtraction of the

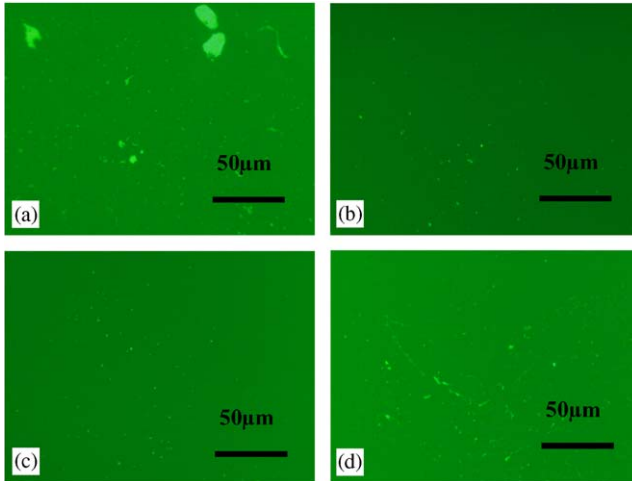


Fig. 3. Fluorescence images of (a) Base PU, (b) PPO212L, (c) PTMO212F, and (d) PTMO212I. Polymeric surfaces after 2 h exposure to human plasma (pH 7.4, 25 °C) containing 5% Alexa-488 FG ($n = 10$ images). Image size is 215 $\mu\text{m} \times 160 \mu\text{m}$.

background fluorescence, images contained isolated intense areas of fluorescence that were uniquely distributed and were measured for each surface. The fluorescent images for the Base PU displayed a patchy and heterogeneous Fg aggregate distribution in comparison with the aggregates found on the SMM-treated materials. PPO212L and PTMO212F surfaces were similar to each other and displayed very small speckled Fg areas that were uniformly distributed. PTMO212I surfaces had some speckled areas as well but also exhibited string-like patterns. Since there was only 5% FI-Fg in the plasma, the images only represent 5% of the Fg on the surface. The regions for which a detectable FI-Fg signal could not be measured are believed to contain adsorbed Fg but cannot be quantified due to the low number of the fluorescent-Fg. Despite not being able to see every Fg molecule, the FI-Fg patterns indicate how Fg is most likely organized on the surfaces.

The irregular Fg regions associated with the Base PU and PTMO212I surfaces (Fig. 3) were similar to platelet aggregation patterns reported in Fig. 1 (i.e. large activated platelet aggregates and linear platelet streaks, respectively) for the surfaces [1]. Morphological analysis of ten fluorescent images was carried out on each material to provide an assessment of the Fg distribution (Fig. 4). PPO212L and PTMO212F had statistically higher frequencies ($p < 0.05$) of clusters with circularity = 1, whereas PTMO212I and Base PU had statistically higher ($p < 0.05$) Fg circularity > 2 . This observation suggests that Fg deposition is occurring in a much more irregular manner for the Base PU and PTMO212I PU surfaces than for the other two modified materials. As well, the Base PU and the PTMO212I-treated surfaces

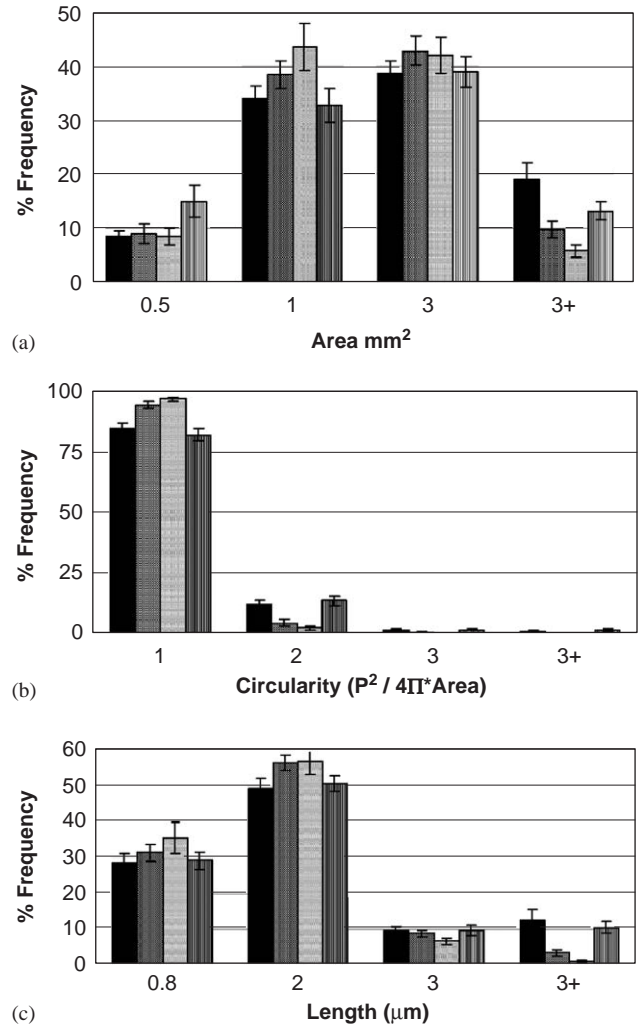


Fig. 4. Morphological analysis of fluorescent-Fg (FI-Fg) images using Image Pro Plus™ software (215 $\mu\text{m} \times 160 \mu\text{m}$, $n = 40$ images, 4 materials $\times 10$ images from 3 experiments): (a) area, (b) circularity, and (c) length. ■ Base PU, ▒ PPO212L, ▨ PTMO212F, □ PTMO212I.

are showing much larger Fg aggregates than observed on the PPO212L and PTMO212F-modified surfaces. Figs. 4a and c show a higher frequency of Fg in the “ $> 3 + \mu\text{m}^2$ ” and “ $> 3 + \mu\text{m}$ ” range, respectively, for area and length when Fg was analysed on the Base PU and the PTMO212I surfaces. When combined with the platelet data, the Fg findings suggest that platelet adhesion on these surfaces is governed not only by the presence of Fg but also by the manner in which the Fg aggregates on the surfaces. Since PTMO212I favours low platelet adhesion but yet still promotes platelet activation, it must be concluded that the SMM formulation requires more than just the presence of fluorine to yield the optimal anti-thrombogenic nature because all three of these SMMs have very similar fluorine content [15].

3.3. Platelet–Fg Correlations

To quantify the relationship between the platelet morphology and Fg distribution, a Spearman's rho bivariate correlation was carried out between the dataset for each area, circularity and length parameters of morphology. The correlation coefficients and their corresponding significance levels (i.e. $p < 0.05$ for significance) are given in Table 2. All the correlations were significant with the exception Circ 2, which represents circularity values between 1 and 2 and corresponds to small deviations from round platelets and Fg clusters, with the appearance of small platelet pseudopods. In general, the circularity correlations were the lowest when compared to the area and length correlations. The data suggest that the circularity of the Fg deposits has no influence on the shape of platelets. As well documented in the literature [8,22,23], the platelet pseudopod formation/ activation is promoted via molecular interactions that are associated with structural changes from Fg adhesive regions, occurring at the nano-scale. Such features are clearly not accounted for in the circularity parameter.

Even though area and length are also geometric parameters, larger values would translate to a higher number of molecular activation sites or a higher probability of encountering available receptor-binding sites on Fg and thus higher correlations values are observed. Overall, area and length correlations were quite strong, especially for the larger areas and lengths, $+3 \mu\text{m}^2$ and $+3 \mu\text{m}$, respectively. This indicates that higher platelet aggregation/activation is correlated to large aggregates of Fg within the distribution of Fg on the surface. This parameter may even be more important than the total amount of Fg since our previous investigations had failed to demonstrate a correlation between the amount of Fg and different SMM modified surfaces [1,24]. The FI-Fg analysis could potentially be used prior to platelet adhesion studies to identify materials, which may result in larger platelet deposi-

tion/aggregates. On a cautionary note, statistical correlation does not equal causation; thus, we cannot categorically conclude from the above findings that the larger Fg deposits are uniquely responsible for larger degrees of platelet activation. Hence, further work with a larger database of polymers would be the logical next step towards establishing a more robust correlation.

4. Discussion

Upon attachment to a foreign surface, discoid platelets may become activated and their cytoskeletal network reorganizes to change shape to varying degrees of spreading [5,25,26]. Several studies show that platelets adhere to bound Fg and are able to redistribute the Fg on the surface [5,6,11,14,26]. The current study investigated the platelet morphology on SMM and non-SMM treated PUs. Fg distribution (in the absence of platelets) was analysed separately and was tested for correlation with the platelet morphology data.

As a result of the analysis from the distributed Fg, several differences were observed in the FI-Fg pattern amongst the SMM-treated and non-treated PUs (Figs. 3 and 4). The Base PU had large Fg areas, whereas PPO212L and PTMO212F had smaller well distributed areas. Despite our knowledge that PTMO212I adsorbed similar amounts of Fg to the other materials [1,24], its surface showed string-like aggregates whereas this was not observed for the other two modified materials. The differences amongst these materials could be a result of several factors including the localized amount of protein adsorption, as well as protein conformation and orientation differences. These parameters can have an affect on the manner by which the platelets bind to the surface-bound Fg [11]. It has been reported [5] that platelets are able to fully activate and spread if the Fg surface concentration is at least $0.15 \mu\text{g}/\text{cm}^2$. In the investigators' work, all SMM-treated and non-treated materials used in the current study adsorb (from human

Table 2
Correlation data for area, circularity and length parameters of FI-Fg distribution and platelet morphology

	Area Spearman's rho		Circularity Spearman's rho		Length Spearman's rho
Area 0.5	0.763	Circ 1	0.427	Length 0.8	0.708
<i>p</i> -value	1E-06	<i>p</i> -value	0.006	<i>p</i> -value	1E-06
Area 1	0.863	Circ 2	−0.157	Length 2	0.813
<i>p</i> -value	1E-06	<i>p</i> -value	0.335	<i>p</i> -value	1E-06
Area 3	0.816	Circ 3	0.492	Length 3	0.564
<i>p</i> -value	1E-06	<i>p</i> -value	0.001	<i>p</i> -value	2E-04
Area 3+	0.947	Circ 3+	0.772	Length 3+	0.902
<i>p</i> -value	1E-06	<i>p</i> -value	1E-06	<i>p</i> -value	1E-06

Correlations are significant when $p < 0.05$.

plasma) less Fg than the above reported value [1,24]. This would imply that Fg surface concentration does not solely account for platelet variations. Fg conformation is one parameter that may mediate the adhesion pattern on the SMM modified surfaces, which may be intensified if the adsorption is leading to Fg self-assembly. The latter remains to be confirmed and is the subject of on-going research in our group.

One concern regarding the fluorescence tracer experiments may be the potential invasiveness of the Alexa-488 label. However, the investigators have found that the clottability of Fl-Fg was only minimally lowered as a result of the label, measured by a Fg clotting time assay, which measures the concentration of Fg in a solution by relating it to the clotting time of Fg, (i.e. 0.9 vs. 1.1 mg/ml using UV absorbance). This suggests that since the Fg function was not significantly altered, the structure of the protein may not have changed significantly. Many other investigators have found that fluorescent labels such as fluorescein are noninvasive in protein studies at the concentrations used in this study [27,28]. In particular, Alexa-Fg has been used in studies that have investigated the assembly of adhesive proteins by stimulated platelets [29].

The geometric analysis (area and length) indicated a high correlation ($r > 0.9$, $p < 0.05$) between platelet morphology and Fg distribution/deposition patterns despite the experimental methodology differences inherent between platelet adhesion and Fg adsorption experiments. This was particularly evident for the most highly activated area $> 3 \mu\text{m}^2$ and length $> 3 \mu\text{m}$. While the mechanisms that may define these correlations are not fully understood there is some evidence in the literature that suggests that protein conformation post-adsorption could be a key factor. Moskowitz et al. [30] investigated the conformational differences of low-density and high-density Fg adsorbed on polystyrene and found that high-density Fg behaves more like Fg in solution (i.e. less platelet active). These results may be substrate dependant, and may cause differences in protein conformation and subsequent platelet reactivity. While the findings of Moskowitz's study were contrary to what was observed on the SMM-treated and non-treated PU surfaces; they provide evidence that low-density Fg behaves differently than high-density Fg. There have not been enough experiments reported in the literature to ascertain if such a relationship is uniform in nature when the surfaces are different.

Proteins can fold, change shape and diffuse on a surface, and this may allow the exposure of latent epitopes [27,31]. Possibly the substrates that have larger Fg areas may have greater numbers or an increased probability of conformationally changed sites. Thus, it is possible that adsorbed Fg in high-density areas such as those found on the Base PU and PTMO212I-PU adopt a different conformation than the low-density areas such

as those observed for the PPO212L and PTMO212F-PU. The investigators have just begun assessing conformational states using polyclonal and monoclonal antibodies. Preliminary results with polyclonal antibodies to Fg support the above considerations.

There is some evidence in the literature that suggests that adsorbed Fg may self-assemble to a conformational state similar to that of fibrin. Hu and colleagues [32] studied the interaction between Fg and phagocytes with respect to biomaterial-mediated foreign body reactions. They suggested that phagocytes might recognize the adsorbed Fg as a fibrin clot, which leads to an inflammatory response. Endenburg et al. [33] studied the platelet interaction on fibrin-coated glass from whole blood. They observed that the platelets adhered to fibrin in a band pattern of spread and dendritic platelets. Hence, in the current study, the platelets may be recognizing the larger Fg areas as a fibrin-like structure. For instance, string-like Fl-Fg on the PTMO212I, resembles the thrombin-mediated Fg conversion to fibrin, reported in other studies [34,35].

The surface morphology (i.e. roughness and/or topography) of the substrate has also been reported to account for the differences in platelet adhesion and Fg patterns [36]. This could particularly be the case for the PTMO212I modified surface. AFM height has been previously carried out on the materials and provides information on the topography of the surface [15,37]. From the AFM height images previously reported [15], the Base PU had a smooth surface with broad regions of slightly elevated features (80 nm). In general, the SMM-treated materials had greater height variation than the Base PU (100–750 nm for SMMs and 80 nm for Base PU, respectively). Interestingly, the PPO212L blend showed some broad elevations similar to the Base PU (100 vs 80 nm, respectively). While the PTMO212F-treated material contained small well-distributed “peaks” (250 nm), the PTMO212I-PU contained ridge-like features (750 nm) [15]. Fujisawa and colleagues [38] studied the relationship between cell distribution and roughness on textured surfaces. They found that white blood cells were distributed in streamwise streaks and were closely associated to areas of roughness and texture. Bai and colleagues [39] found that Fg adsorbed preferentially at the ledges or steps in an oriented long stranded fashion on a model hydrophobic surface. Hence, it seems possible that the ridge-like surface features of PTMO212I-PU (elevated white regions in the height image, left panel in Fig. 5d), which is synthesized from relatively hydrophobic components, could be responsible for the string-like Fg pattern and the platelet arrangement in the linearly clustered strings. Conversely, Bai and colleagues [39] also found that Fg assembles randomly on model hydrophilic surfaces (i.e. atomically flat mica). Even though PPO212L and PTMO212F PUs were synthesized with relatively

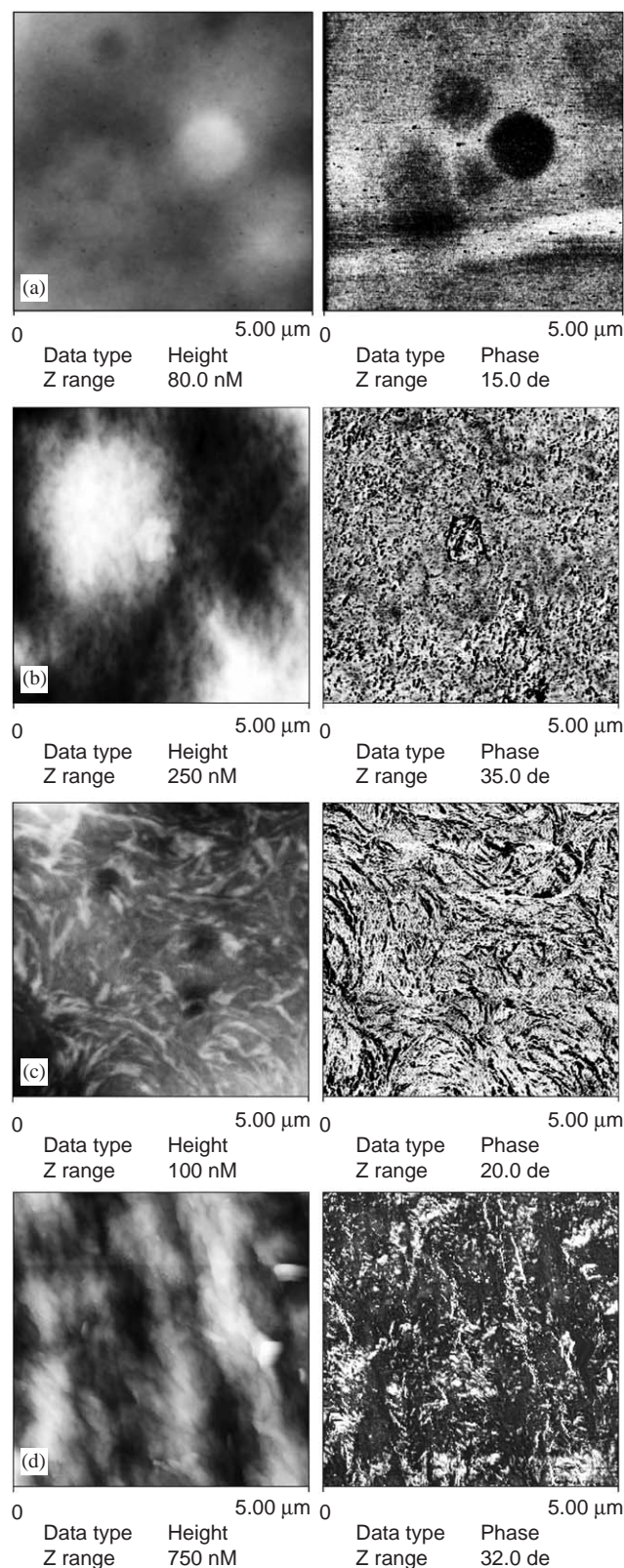


Fig. 5. AFM tapping mode phase images of (a) Base PU, (b) PPO212L, (c) PTMO212F, and (d) PTMO212I [37]. The methodology for the collection of the of AFM height data was previously reported on elsewhere [15] and the same parameters were used for the simultaneous collection of phase contrast data reported here [37]. The images on the left are height images (white is highest) and those on the right are phase images.

hydrophobic fluorine tail components, in an aqueous environment the PPO and the PEO segment in the latter materials, respectively, do contribute to a slightly more hydrophilic nature at the surface [1,15].

Aside from topographical data, the AFM can acquire information on the changes of phase angle for the cantilever probe, which yields a second image called the phase image (right side panel in Fig. 5). The latter changes in phase angle are related to the energy dissipation during tip-sample contact as a result of topography and tip-sample molecular interaction. The phase image is considered to be more sensitive to surface properties such as stiffness and chemical composition. In the absence of significant topographical differences, the oscillating force of the probe should deform softer regions more easily than harder regions of a heterogeneous surface such that phase changes between these areas can be observed. Thus far, phase data has been considered to be qualitative, at best, due to the lack of knowledge regarding tip-sample interactions [40,41]. In the instance where surface roughness is quite similar, changes in the phase image are considered to reflect some change in material structure. The phase images amongst the SMM-treated and non-treated materials are distinctly different and may represent distinct polymer domain structure distributions of the hydrophobic (i.e. fluoro segment) and hydrophilic components of the materials, or differences between hard and soft segment components of the PU (Fig. 5) [37]. A comparison of the phase data (right image, Fig. 5) for the Base PU and the PPO212L-treated material is particularly of interest given their similar topology (left image, Figs. 5a and b). The Base PU shows larger isolated phase domains whereas the PPO212L-treated PU displays well-mixed domains. The chemical nature of the domains has not been identified but it is clear that the nature of the phase structures for these materials differ dramatically.

Interestingly, the only other material to show a well-mixed phase distribution was PTMO212F-treated PU, which displayed a similar Fg distribution as the PPO212L-treated PU. It is possible that the differences in domain distribution are in part contributing to the unique Fg distribution character for the PPO212L and PTMO212F surfaces and subsequent platelet attachment. The concept of distributed phases of different chemistry has been described by Jozefowicz and Jozefonvicz [42] and has been termed as “J and J principle”. They stated that surface features or domains of a polymeric material show many different sequences of chemical function. The surface “sites” can interact in a specific manner with biological moieties such as proteins. The J and J principle supports the different platelet–Fg interactions observed on the SMM-treated materials which would have occurred as a result of their different chemical domain structure and distribution.

In considering an overall mode of action associated with the behaviour of the SMMs, with regards to both adhesion and spreading, there are potentially two distinct mechanisms dictating the platelet interactions on the PUs under study: one which controls platelet adhesion number, and a second which directs platelet morphology and distribution. The presence of the fluorinated component of the SMM additive appears to be dictating the number of adherent platelets, whereas surface roughness and domain phase distribution structure, created by the unique tri-block chemistry of the SMM in a particular Base PU polymer, seems to be dictating how Fg distributes itself and contributes to platelet activation on the surfaces.

Alternative considerations such as endogenous platelet Fg, the platelet surfaces themselves and the receptor adsorption mechanism may play a role in the observed platelet attachment on the PU surfaces. The reason why these considerations should be taken into account in future work is that platelets are able to secrete and organize adhesive proteins, such as Fg, fibronectin and von Willebrand factor, on their surface and this has implications for hemostasis and thrombosis as well as for platelet adhesion and aggregation on biomaterial surfaces [43,44]. Since recent work reported in regards to the synthesis and release of enzymes from human macrophages differentiated and re-seeded on different PU substrates showed the sensitivity of these cells to polymer chemistry [45], then the same likely follows for platelets. As well, the platelet membrane is at least 57% protein by area [46] that could bind directly to surfaces [36]. Hence the, the platelet surface can be a dominant factor. Lastly, the GPIIb/IIIa platelet receptor accounts for half of all platelet surface glycoproteins and could adhere onto the surfaces [5,47]. Both secreted endogenous Fg and adhered GPIIb/IIIa receptor mechanisms are independent of the Fg surface concentration adsorbed from blood plasma [36] and rationalizes the importance of their consideration in such studies.

5. Summary

Platelet adhesion was reduced on surfaces containing the SMM additives. This was confirmed by SEM; however, platelet morphological differences existed between the Base PU and the SMM-treated surfaces, as well amongst the different SMM-treated surfaces. There were marked differences between the adsorbed Fg distribution for the Base PU and the SMM-treated surfaces as well as among SMMs-treated surfaces themselves. The platelet adhesion morphology resembled the Fg distribution observed on the surfaces suggesting that the aggregated Fg somehow alters the manner by which platelets adhere to the surfaces. More specifically, there were high correlations between the

geometrical parameters (area and length) of platelet morphology and Fg deposition patterns. Perhaps Fg distribution rather than Fg levels alone can serve as a better predictor of platelet activation. Further investigations are required to identify how chemical components of the SMM-surfaces are directing Fg adsorption/distribution and what are the possible changes in Fg conformation that are elicited by the adsorption. With this knowledge, it may be possible to create enhanced SMM-surfaces to limit platelet adhesion and activation, thus creating improved non-thrombogenic biomaterials.

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