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Platelet adhesion on a bioresorbable poly(propylene fumarate-co-ethylene glycol) copolymer

Laura J. Suggs, Jennifer L. West, Antonios G. Mikos*

Cox Laboratory for Biomedical Engineering, Institute of Biosciences and Bioengineering, and Department of Chemical Engineering, Rice University, 6100 South Main Street, Houston, TX 77251-1892, USA

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Abstract

Platelet adhesion and aggregation on poly(propylene fumarate-co-ethylene glycol), P(PF-co-EG), hydrogels was examined under both static and flow conditions. Adherent platelets were quantified under static conditions using both ^{111}Indium oxine-labeled platelets as well as a lactate dehydrogenase, LDH, assay. The radiolabeling assay showed a significant decrease in platelet attachment on the copolymer hydrogel films relative to the poly(propylene fumarate), PPF, homopolymer. In addition, there were reductions in adhesion resulting from the increase in poly(ethylene glycol), PEG, weight percent or molecular weight. There was good agreement between both assays under static conditions for the copolymer films. Platelet surface coverage was quantified under flow conditions in a parallel plate flow chamber using the LDH assay. There was a dramatic decrease in the number of adherent platelets on the copolymers relative to glass and silicone rubber controls. All of the copolymer surfaces showed minimal aggregation with no thrombus formation or platelet spreading as assessed qualitatively using scanning electron microscopy. These results suggest that P(PF-co-EG) is a good candidate for development as a cardiovascular implant.

Keywords: Poly(propylene fumarate); Poly(ethylene glycol); Injectable hydrogels; Biodegradable polymers; Cardiovascular implants; Tissue engineering

1. Introduction

The main clinically relevant complication of percutaneous transluminal coronary angioplasty (PTCA) is restenosis, which is experienced by 30–50% of patients [1, 2]. Restenosis, or renarrowing of the vessel, can occur acutely either from thrombosis or elastic recoil. More significantly, delayed hyperplastic narrowing results from neointimal formation due to smooth muscle cell migration, proliferation, and matrix deposition. In addition, the narrowing of the arterial lumen can result in part from vascular remodeling of the outer layers of the vessel wall [3]. Intracoronary metallic stents are currently used to preserve the vessel lumen, however, the thrombogenic nature of the materials can cause long-term problems [4]. Platelet interaction at the material surface has been shown to be the primary factor in thrombosis and subsequent thrombotic device failure [5]. Previously, we proposed a block copolymer, poly(propylene fumarate-co-ethylene glycol), P(PF-co-EG) (Fig. 1) as an alternative stenting material [6].

The components of the copolymer were chosen based on their unique properties. Poly(propylene fumarate), PPF, has unsaturated sites along the polymer backbone which are labile and can be crosslinked in situ. This injectable material can be used to fill skeletal defects of varying shape and size [7]. The PPF synthesis upon which the copolymer is based has been described by Peter et al. [8]. Poly(ethylene glycol), PEG, has been used in the development of numerous non-thrombogenic coatings [9–14]. It has unique solubility properties and is an extremely hydrophilic polymer. It is not degradable, however, low molecular weights (less than 20 000 Da) are excretable [15]. We believed that a block copolymer consisting of these two homopolymers would be injectable, bioresorbable, and appropriate for use in blood-contacting spaces. We proposed a blood-vessel barrier

* Corresponding author. Tel.: 713 285 5355; fax: 713 285 5353; e-mail: mikos@rice.edu

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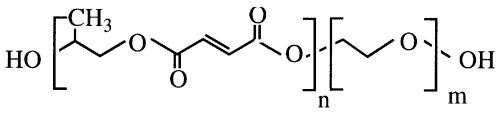

Fig. 1. Poly(propylene fumarate-co-ethylene glycol).

system which would provide a barrier to thrombosis similar to those which have been described with other polymer systems [16, 17].

In previous studies, we evaluated characteristics of the linear chain copolymer as well as swelling and mechanical properties of the crosslinked P(PF-co-EG) hydrogels [6, 18]. In addition, we investigated the degradation behavior of hydrogel films both in vitro and in vivo [19]. The copolymers degraded primarily by bulk degradation, and the in vivo implantation exhibited a normal wound healing response resulting in fibrous encapsulation with evidence of biodegradation. Based on the initial biomaterial evaluation, we were interested in examining the P(PF-co-EG) hydrogels with respect to the intended application in the vasculature.

In this study, we investigated the adhesion and aggregation of platelets on P(PF-co-EG) hydrogels under both static and flow conditions. The effects of changes in the weight percent of the PEG component as well as the PEG molecular weight in the copolymer were examined. The relative extent of adhesion and aggregation were compared to reference materials of glass, silicone rubber, and crosslinked PPF homopolymer films. The morphology of platelets adherent to the test surfaces was examined using scanning electron microscopy (SEM) and environmental SEM (ESEM).

2. Materials and methods

2.1. Copolymer synthesis

PPF was synthesized and characterized as previously described [8]. Briefly, propylene glycol (Arcos, Pittsburgh, PA) was mixed with potassium carbonate (Fisher, Pittsburgh, PA) in a 2:1 molar excess dissolved in a double volume of chloroform to form a slurry. Fumaryl chloride (Aldrich, Milwaukee, WI) dissolved in an equal volume of chloroform (Aldrich) was then added slowly to the slurry in a molar ratio of 1:3, fumaryl chloride:propylene glycol. The reaction was performed at 25°C with vigorous stirring. The potassium carbonate was removed gravimetrically, and the PPF was then formed in a transesterification reaction at 160°C under a vacuum pressure of approximately 110 mmHg. PEG of varying molecular weights (Aldrich) was added to the reaction vessel under the same conditions to form the copolymer [6]. The resulting copolymer was precipitated using chloroform and petroleum ether (Fisher). Rotoevaporation was used to remove excess solvents. Three different copolymer formulations were fabricated from the same PPF homopolymer. The first, abbreviated as 50/5 K, consisted of 50% PEG by weight, with a PEG number average molecular weight of 4600 Da. The second, 75/5 K, consisted of 75% PEG by weight of the same molecular weight, and the third, 50/10 K, consisted of 50% PEG of molecular weight 10 500 Da. The molecular weights and copolymer compositions were determined as previously described [6].

2.2. Fabrication of test films

Glass coverslips (12 mm diameter and 22×50 mm) were used as received (Fisher). Silicone rubber films were made from medical grade silicone (A-103, Factor II, Lakeside, AZ), and the initiator was incorporated at 10% w/w as described by the manufacturer. The silicone was spread onto glass coverslips and allowed to crosslink overnight at 60°C. Copolymer hydrogel films were fabricated by first dissolving the copolymer in distilled deionized water (ddH_2O) at a 1:1 ratio. The initiator, benzoyl peroxide (BP, Aldrich), was dissolved in a vinyl monomer, *N*-vinyl pyrrolidinone (VP, Aldrich), at 1% w/v initiator in the monomer. The resulting monomer solution was then mixed with the copolymer solution at 50% v/w based on the initial weight of the copolymer, and 1 μl of *N*,*N*-dimethyl-*p*-toluidine (DMT) (Aldrich) was added. The resulting solution was poured onto a polytetrafluoroethylene surface and a glass coverslip was placed over the hardening fluid to form a film. The films were allowed to crosslink overnight at room temperature, and they were then hydrated for 24 h in ddH_2O. The films were then cut either into 12 mm diameter disks using a cork-borer or into rectangles of dimensions 22×50 mm. The PPF films were made in a similar fashion, the only difference being the exclusion of the aqueous component. All films were hydrated for 1 h in phosphate-buffered saline (PBS) at pH 7.4 prior to testing.

2.3. Contact angle measurements

Water contact angles in air of the copolymer surfaces were measured after equilibrium swelling in ddH_2O for 1–2 h at room temperature. The measurements were taken using a contact angle goniometer (Rame-Hart, Mountain Lakes, NJ).

2.4. Platelet suspension preparation

Whole blood was drawn from healthy donors into a 1:6 volume of acid-citrated dextrose:blood (65 mM citric acid, 85 mM sodium citrate, 111 mM dextrose, pH 4.5). Washed platelets were prepared as described previously [20]. Briefly, platelet-rich plasma (PRP) was

prepared by centrifugation at $150 \times g$ for 15 min at 23°C. The plasma was drawn off and centrifuged further at $1000 \times g$ for 15 min at 23°C. This resulted in the formation of platelet-poor plasma (PPP) and a pellet. The pellet was washed and resuspended in a small amount of pH 7.0 HEPES buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 3.5 g/l bovine serum albumin, 0.1 mM $CaCl_2$, 50 U/ml sodium heparin, and 2.5 U/ml apyrase, pH 7.0). The suspension was diluted to 10 ml in the pH 7.0 buffer and allowed to rest for 45 min. Afterwards, the platelets were centrifuged again at $1000 \times g$ for 10 min. The supernatant was decanted, and the pellet was then resuspended in a pH 7.4 HEPES buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 3.5 g/l bovine serum albumin, 0.01 mM $CaCl_2$, pH 7.4). The platelet concentration was adjusted to 50×10^6 cells/ml using a Coulter counter (Coulter Electronics, Hialeah, FL) prior to the radio-labeled and LDH assays.

2.5. ^{111}Indium oxine assay

For the following experiments, 10 μCi/ml of radio-labeled indium oxine was added during the platelet washing step and incubated for 10 min at 37°C [21]. Aliquots of 50 μl of both the supernatants and platelet suspensions were used to determine both the labeling efficiency and the specific activity of the suspension. A volume of 1 ml of platelet suspension was placed on each of the 12 mm diameter film surfaces in 24-well tissue culture plates. The films were incubated with the platelet suspension for 1 h at 37°C. The films were then dip-rinsed twice in the pH 7.4 HEPES buffer. The adhesion of labeled platelets was quantified using a gamma counter (Gensys Laboratory Technologies, Elburn, IL).

2.6. Lactate dehydrogenase (LDH) assay

Platelet suspension (1 ml) was incubated on 12 mm diameter film surfaces as described for the indium oxine assay [22]. After incubation, the films were dip-rinsed twice in the pH 7.4 HEPES buffer. They were then placed into 0.5% Triton-X100 for 1 h at 25°C to lyse the adherent platelets. A volume of 1 ml of buffer was then removed, and the LDH activity was measured using an LDH endpoint concentration assay kit (Sigma Diagnostics, St. Louis, MO). This activity was compared to lysed platelet suspensions of known concentrations in order to quantify the number of platelets on each film.

2.7. Flow studies

Whole blood was drawn from healthy donors into heparinized syringes (10 U/ml blood). A parallel plate flow chamber was used in conjunction with a Harvard syringe pump as previously described [23]. The test films were mounted onto the chamber using a vacuum source with a silicone rubber gasket to separate the film from the chamber. Blood was passed over each film at an upper end arterial shear rate of 2000 s^{-1} for 2 min [28]. The films were then rinsed with PBS and placed either into a lysate buffer for LDH activity determination or a 2.5% glutaraldehyde solution (Sigma, St. Louis, MO) for fixation.

2.8. Scanning electron microscopy

Samples of each type of film were reserved for qualitative analysis via scanning electron microscopy. These films were fixed in 2.5% glutaraldehyde for 2 h immediately after the rinsing step. The glass and silicone rubber films were rinsed in PBS after fixation and serially dehydrated from water to 100% ethanol. They were then critical-point dried (Electron Microscopy Sciences, Fort Washington, PA). The dried glass and silicone films were coated with gold using a Model 3 Pelco Sputter Coater 91000 (Reading, CA). SEM images were taken using a Philips XL30 ESEM-FEG (Mahwah, NJ). The ESEM images from the polymer films were taken in wet mode after fixation without dehydration. This was done on a cold stage at 5°C at a pressure of 5 Torr in order to insure that the polymer morphology was maintained.

2.9. Statistics

Statistical analysis was performed using a student's unpaired *t*-test with a 95% confidence interval ($P < 0.05$). Eight repetitions were performed for the radio-labeled platelet assay, and four repetitions were performed for all other assays. All data are reported as means ± standard deviation (SD).

3. Results

3.1. Contact angle measurements

All of the copolymer surfaces demonstrated no advancing contact angle and were instantaneously wetted. This indicates that the copolymers are very hydrophilic and have a theoretical contact angle of zero.

3.2. Static adhesion studies

The number of platelets adherent to each of the film surfaces as determined by the ^{111}indium oxine assay is shown graphically in Fig. 2. PPF had the highest density of platelets adherent at 32.5 ± 8.0 platelets/cm^2. Glass had an average value of 21.0 ± 6.5 platelets/cm^2. Silicone rubber and copolymer formulations 75/5 and 50/10 K both showed significantly different values than glass for

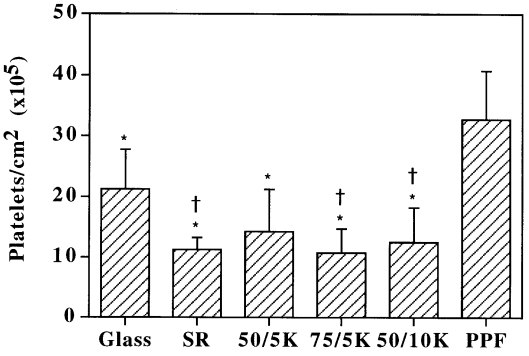

Fig. 2. Graph of platelet surface density on six different surfaces as determined by the ^{111}indium oxine assay. Error bars represent means ± SD for $n = 8$. (* = statistically different than PPF, † = statistically different than glass).

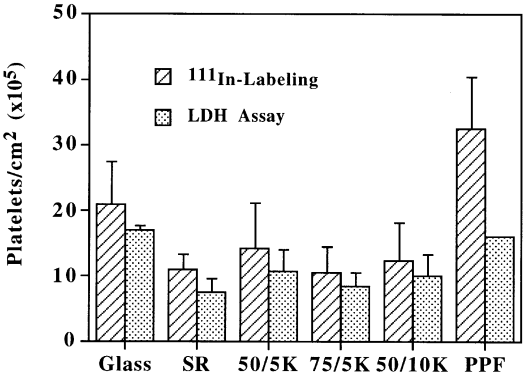

Fig. 3. Comparison graph of platelet surface density on six different surfaces as determined by both the ^{111}indium oxine assay and the LDH assay. Error bars represent means ± SD, $n = 4$ for the LDH assay. The data from the ^{111}indium oxine assay are the same as those presented in Fig. 2.

platelet surface coverage at 11.0 ± 2.2, 10.6 ± 4.0, and 12.4 ± 5.7 platelets/cm^2, with P values of 0.003, 0.002, and 0.013, respectively. Formulation 50/5 K, however, was not significantly different from glass ($P > 0.05$), and there were no significant differences among any of the three copolymer formulations ($P > 0.05$). In comparison, the results from the LDH assay were not statistically different from the results as determined from the ^{111}indium oxine assay except in the case of PPF. The results from both assays are shown together in Fig. 3.

3.3. Flow studies

The number of adherent platelets on the test films treated with whole blood under flow conditions was quantified using the LDH assay. The results from these studies are shown graphically in Fig. 4. The glass and silicone rubber films were not significantly different at surface coverages of 12.8 ± 6.5 and 17.4 ± 6.7 platelets/cm^2, respectively. The copolymer films, however, had dramatically fewer platelets on the surface. Copolymers 50/5, 75/5, and 50/10 K had surface coverage values of 1.39 ± 1.38, 0.07 ± 0.08, and 1.00 ± 0.50 platelets/cm^2, respectively.

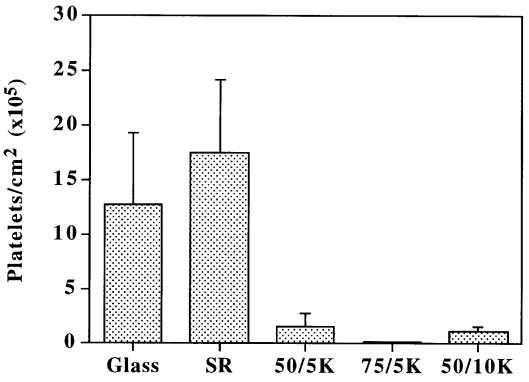

Fig. 4. Graph of platelet attachment on five different surfaces as determined by the LDH assay under flow conditions. Error bars represent means ± SD for $n = 4$.

3.4. Platelet morphology

Figure 5 shows micrographs of platelets that have adhered to both glass and silicone rubber films under static conditions. The formation of thrombi was evident on the glass coverslips. The platelets had formed large aggregates and the extension of pseudopodia was observed. In the case of the silicone rubber, however, the platelets were generally singular with the rounded shape typical of platelets in the inactivated state. Micrographs of films of PPF and copolymers 50/5, 75/5, and 50/10 K exposed to platelet suspensions are shown in Fig. 6. A greater number of platelets are apparent on PPF films than the copolymers. The platelet morphology, however, is similar on all the polymer surfaces. The platelets appear singular and rounded, without evidence of thrombus formation. Figure 7 shows micrographs of platelets on glass and silicone rubber under flow conditions. Again, thrombus formation is evident on the glass surface as well as the extension of pseudopodia. In comparison to the static assay, the platelets appear flattened and more circular. On the silicone rubber surface, even though there is no statistical difference in the number of platelets compared to the glass surface, the platelets do not appear aggregated. No adherent platelets were observed on the copolymer films tested under flow conditions.

4. Discussion

We examined platelet adhesion to P(PF-co-EG) hydrogel films under both static and flow conditions. We compared the platelet adhesion and morphology on these surfaces as well as glass, silicone rubber, and the

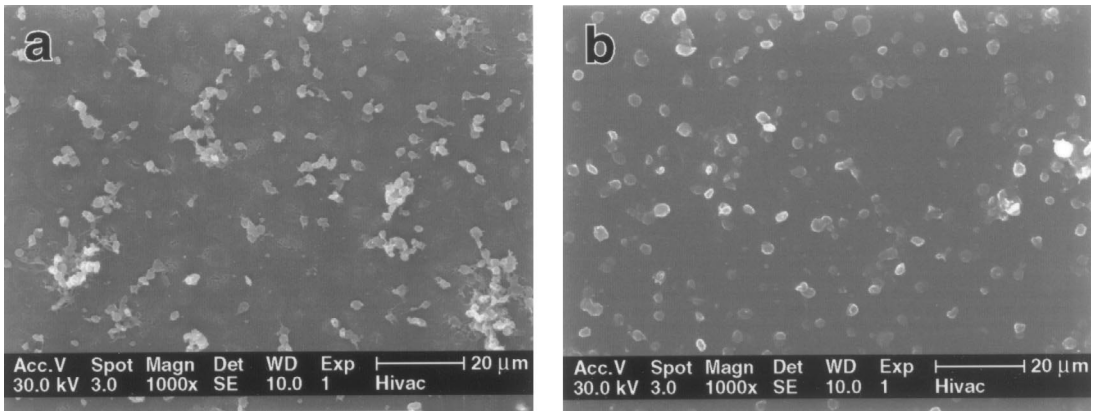

Fig. 5. SEMs of platelet surface coverage on (a) glass and (b) silicone rubber under static conditions at 1000× magnification.

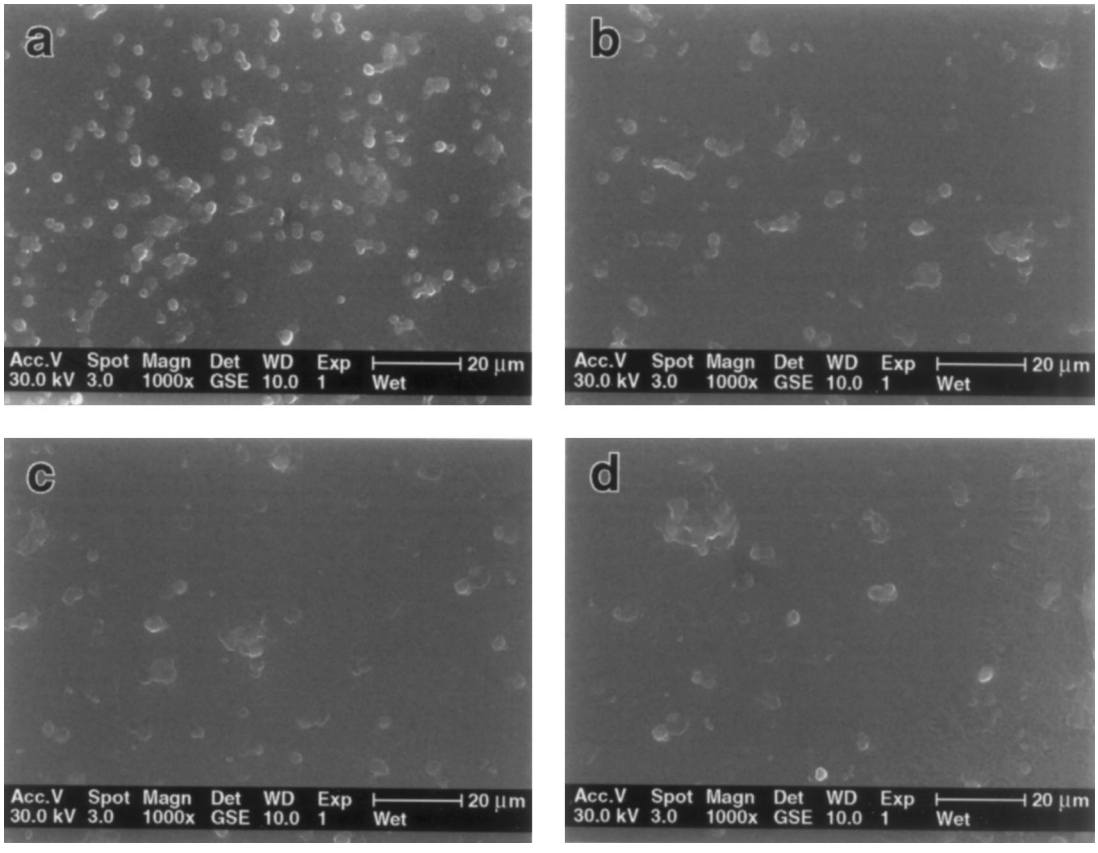

Fig. 6. ESEMs of platelet surface coverage on (a) PPF, (b) 50/5 K, (c) 75/5 K, and (d) 50/10 K under static conditions at 100× magnification.

PPF homopolymer. In addition, we altered the copolymer compositions in order to assess what effects the weight percent PEG as well as the molecular weight of PEG within the copolymer had on the resulting adhesion and morphology. It has been shown previously that increasing either the PEG content or molecular weight above a certain value decreased the adhesion on PEG copolyester hydrogels [24]. It has been suggested that increased hydrophilicity and/or the high mobility of PEG chains in water may be important in decreasing adhesion [25]. In order to correlate hydrophilicity to platelet adhesion, we also measured the water contact angles in air of the equilibrium swollen copolymer films. All copolymers were extremely hydrophilic in nature.

All of the copolymer formulations had dramatically fewer platelets adherent compared to PPF homopolymer

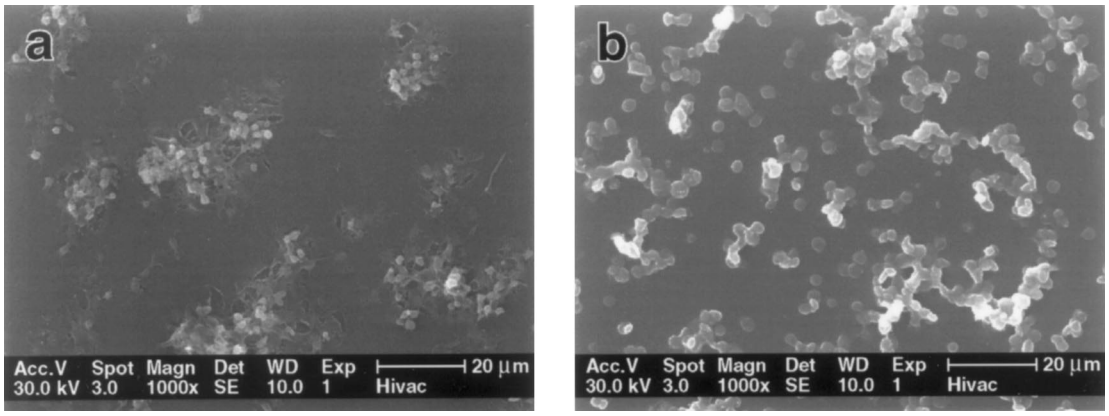

Fig. 7. SEMs of platelet surface coverage on (a) glass and (b) silicone rubber under flow conditions at 1000× magnification.

as determined by the ^{111}indium oxine-labeled platelet adhesion assay under static conditions. This demonstrates the efficacy of PEG copolymerization in prevention of platelet adhesion. Although there were some differences in the platelet adhesion among the three copolymers under static conditions, there were no measurable differences in surface contact angles among the copolymer hydrogels; all three formulations were shown to be extremely hydrophilic. As evidenced by the ^{111}indium oxine assay, however, increasing either the weight percent or the molecular weight of PEG caused a significant decrease in adhesion compared to glass. This suggests that mechanisms other than hydrophilicity have an important effect on platelet adhesion such as steric hindrance or surface free energy. It is important also to note that only two different PEG weight percentages and molecular weights were examined. Although, the weight percentages were chosen based on the limits of the copolymer fabrication technique and the molecular weights were chosen based on previous work [19], they may not demonstrate the range of effects that PEG can elicit on platelet adhesion.

The LDH assay was compared with the ^{111}indium oxine assay in order to develop a non-radioactive, reliable assay to measure platelet attachment in the high volume flow studies. In this assay it was assumed that the composition of thrombus on the material surfaces would be primarily composed of platelets with very few white blood cells. The LDH assay has previously been shown to be an effective method for counting adhered platelets, as compared to the radioactively-labeled indium technique [22]. In our studies, we have shown that the LDH assay does not give statistically different values from the ^{111}indium oxine assay except on the PPF surfaces. We believe one or both of the following events may be occurring in the case of PPF: (1) The adhered platelets may be activated and release their LDH prior to lysis. (2) The LDH enzyme may become adsorbed and/or inactivated on the polymer surface. In order to examine event 1, we performed the LDH assay on the supernatants of all the films immediately after incubation. There were no statistical differences among any of the film surfaces and the values were very low compared to the values obtained using lysed platelets. (Data not shown.) There is some morphological evidence for platelet disruption at the PPF surface, as shown by debris evident in the scanning electron micrographs (Fig. 6). This shows that platelet activation is probably not the single cause for the discrepancy in the case of PPF.

The flow studies were quantified using the LDH assay on glass, silicone rubber, and the three copolymer formulations. Two important differences prohibit direct correlations to be made between the static and flow studies. First, there are proteins present in whole blood that are not present in the buffered platelet suspension. These proteins, when adsorbed to materials, determine the extent of adhesion and aggregation, and they may affect the test surfaces differently [26, 27]. Second, flow itself affects platelet adhesion and the number of platelets may be surface dependent [28]. Changes in relative total adherent platelets, however, can be demonstrated. The differences between the reference materials and the copolymer films were more pronounced in the studies conducted under flow conditions, and in fact, the number of platelets was not statistically different from zero for all three copolymers. This suggests that the platelet adhesion to the copolymers in the static case was relatively weak, and that in a more physiologically relevant environment, the platelets may detach readily.

The measurement of platelet attachment does not differentiate between platelets which have adhered and those which have aggregated. Morphology was therefore examined qualitatively using SEM and ESEM. Under static conditions, glass was the only surface which showed aggregation as evidenced by thrombus formation. Similar aggregates were seen in the flow study; however, the platelets exhibited the more rounded, flattened shape typical of a highly activated platelet [29].

Morphological differences may also serve to explain the relatively large surface coverage of platelets on silicone rubber under flow conditions. On other surfaces, no changes were evident in the platelet morphology between static and flow as were seen on glass. This indicates that the platelets were not highly activated, and the silicone rubber was relatively less thrombogenic than glass [30]. These promising results indicate that the P(PF-co-EG) materials may have successful use in vascular applications where prevention of thrombosis is critical.

5. Conclusions

P(PF-co-EG) hydrogel films showed decreased platelet attachment compared to the PPF homopolymer under static adhesion conditions. Increasing weight percent PEG or PEG molecular weight caused a significant decrease in attachment as determined by ^{111}indium oxine-labeled platelets. An enzymatic assay based on lactate dehydrogenase (LDH) was shown to be an effective and reproducible method for counting platelets on the copolymer surfaces. This assay was used to examine platelet adhesion in a flow system using whole blood under physiological shear conditions. The platelets exhibited reduced surface coverage relative to the reference materials of glass and silicone rubber under the conditions of the flow study. In addition, platelet morphology was examined for both studies and morphological changes were only apparent on the glass surfaces. The platelets adherent on the copolymer films were rounded and there was no evidence of thrombus formation.

Acknowledgements

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