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## Surface modification with PEO-containing triblock copolymer for improved biocompatibility: *In vitro* and *ex vivo* studies

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**Abstract**—Poly(ethylene oxide) (PEO) has been frequently used to modify biomaterial surfaces for improved biocompatibility. We have used PEO–polybutadiene–PEO triblock copolymer to graft PEO to biomaterials by  $\gamma$ -irradiation for a total radiation dose of 1 Mrad. The molecular weight of PEO in the block copolymer was 5000. *In vitro* study showed that fibrinogen adsorption to Silastic<sup>®</sup>, polyethylene, and glass was reduced by 70 ~ 95% by PEO grafting. On the other hand, the reduction of fibrinogen adsorption was only 30% on expanded polytetrafluoroethylene (e-PTFE). *In vitro* platelet adhesion study showed that almost no platelets could adhere to PEO-coated Silastic<sup>®</sup>, polyethylene, and glass, while numerous platelet aggregates were found on the ePTFE. The platelet adhesion *in vitro* corresponded to the fibrinogen adsorption. When the PEO-grafted surfaces were tested *ex vivo* using a series shunt in a canine model, the effect of the grafted PEO was not noticeable. Platelet deposition on ePTFE was reduced by PEO grafting from  $8170 \pm 1030$  to  $5100 \pm 460$  platelets  $10^{-3} \mu\text{m}^2$ , but numerous thrombi were still present on the PEO-grafted surface. The numbers of platelets cumulated on Silastic<sup>®</sup>, polyethylene, and glass were  $100 \pm 80$ ,  $169 \pm 35$ , and  $24 \pm 22$  platelets  $10^{-3} \mu\text{m}^2$ , respectively. This is about 35% reduction in platelet deposition by PEO grafting. While the numbers of deposited platelets were small, the decreases were not as large as those expected from the *in vitro* study. This may be due to a number of reasons which have to be clarified in future studies, but it appears that *in vitro* platelet adhesion and fibrinogen adsorption studies may not be a valuable predictor for the *in vivo* or *ex vivo* behavior of the PEO-grafted surfaces.

**Key words:** Poly(ethylene oxide); PEO grafting surface modification; biomaterials; PEO–polybutadiene–PEO triblock copolymer; gamma-irradiation; fibrinogen adsorption; platelet adhesion.

### INTRODUCTION

Surface-induced thrombosis is one of the major complications associated with cardiovascular devices, such as vascular grafts, catheters, ventricular assist devices,

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and total artificial hearts. When biomaterials are exposed to blood, protein deposition occurs, as do activation of the blood coagulation cascade, platelet adhesion, activation, and aggregation, all of which lead to thrombus formation [1, 2]. There have been a number of attempts to create a novel surface that reduces the adverse effects of blood interaction with the material.

The seeding of the blood-contacting surfaces of cardiovascular prostheses with autologous endothelial cells to improve their biocompatibility has had little success to date [3, 4]. Systemic delivery of platelet glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists [5–7] or antibodies [8–10] has been used to inhibit platelet–vessel wall interaction and thus prevent thrombosis. While these antagonists or antibodies are effective, they have to be continuously administered parenterally due to the low bioavailability after oral administration. Local delivery of anticoagulants is known to minimize harmful side effects resulting from systemic administration. Thus, many attempts have been made to locally deliver heparin [11], nitrogen oxide donor [12], or hirudin [13]. One major limitation of this local delivery approach is that the drug reservoir is small and delivery is possible only for a short period of time. Although these approaches may not be ideal for clinical applications yet, they are useful tools in studying the mechanisms of surface-induced thrombosis.

Since the protein adsorption and platelet adhesion is a surface phenomenon, many approaches were used to modify the surface of biomaterials. One of the most commonly used approach for preparing biocompatible biomaterials has been surface modification with various macromolecules, such as albumin [2, 14, 15], albumin-binding dextran–cibacron blue adduct [2], heparin [16, 17], human thrombomodulin [18], recombinant hirudin [19], and PEO [20–25]. Grafting of heparin, thrombomodulin, or hirudin has a specific goal of inhibiting thrombin, which plays a role in thrombus formation [19, 26]. In all cases, the surface modified biomaterials prolonged the coagulation time or reduced the thrombus deposition, but none of them prevented surface-induced thrombosis. Platelet activation, which is central to thrombosis, can occur by other factors than thrombin. Thus, grafting of biomolecules with specific functions may have a limitation. PEO has been frequently used as a surface-modifying agent since PEO is known to protect the underlying substrate by nonspecifically repelling proteins and cells [27–30]. For such nonspecific steric repulsion to be effective, PEO chains have to be attached tightly to the surface (so that they remain on the surface for long-term effect), cover the surface completely (so that no empty space is exposed), and be flexible to provide entropic and osmotic repulsion.

We have been grafting PEO to biomaterial surfaces using a number of different approaches [23, 28, 31–33]. In this study, we grafted PEO to several biomaterial surfaces using PEO–polybutadiene–PEO triblock copolymer and examined their ability to prevent platelet adhesion and thrombus formation in both *in vitro* and *ex vivo* studies.

## EXPERIMENTAL

### *Preparation of biomaterials*

Biomaterials tested were glass tubing (inner diameter (i.d.) 4 mm, Dow Corning, Midland, MI, USA), silicone rubber tubing (Silastic<sup>®</sup>, i.d. 3.35 mm, o.d. 4.65 mm, Dow Corning, Midland, MI, USA), expanded polytetrafluoroethylene (ePTFE, Gore-Tex<sup>®</sup>, i.d. 4 mm, W. L. Gore and Associates, Flagstaff, AZ, USA), polyethylene (PE) tubing (Intramedic<sup>®</sup>, i.d. 3.17 mm, o.d. 3.99 mm, Becton Dickinson, Franklin Lakes, NJ, USA). Plasticized poly(vinyl chloride) (Tygon<sup>®</sup>, i.d. 3.175 mm, o.d. 6.35 mm, Norton Plastics, Akron, OH, USA) tubing was used as a connector for the test tubes. The outer surface of the ePTFE graft was sealed with adhesive silicone (NuSil<sup>®</sup>, Type A, Silicone Technology, Carpinteria, CA, USA) to prevent leakage.

Glass tubing was cleaned by soaking in chromic-sulfuric acid mixture overnight and rinsing with copious amount of running distilled, deionized water (DDW). Silastic, PE, and ePTFE grafts were cleaned by washing with running DDW for 1 h. Tygon<sup>®</sup> tubing was first cleaned with 0.1% Ivory detergent solution at room temperature and rinsed with running DDW. Tygon<sup>®</sup> tubing and all the stopcocks and fittings used in the series shunt construction were soaked in phosphate buffered saline (PBS) overnight. The inner surfaces of only Tygon<sup>®</sup> tubes were exposed to albumin solution at a concentration of 10 mg ml<sup>-1</sup> for 2 h. The bulk albumin solution was rinsed by flushing the tubes with PBS. Albumin coated tubes were kept at 4°C until use.

### *Surface modification with PEO*

PEO–PB–PEO triblock copolymer was used for grafting PEO on the biomaterials. This copolymer has been synthesized and used in our laboratory [32]. The molecular weight of the PB moiety was 750, while that of the PEO chains was 5000. For this reason, this copolymer was called 'COP5000'. For grafting of COP5000 onto Silastic<sup>®</sup>, ePTFE and PE, the copolymer solution (at a concentration ranging from 0.1 to 1 mg ml<sup>-1</sup>) was introduced inside the tubing and after 2 h at room temperature, the whole system was exposed to  $\gamma$ -radiation for a total radiation dose of 1 Mrad [32]. The presence of COP5000 in the bulk solution during exposure to  $\gamma$ -radiation resulted in higher grafting efficiency compared to exposure to  $\gamma$ -radiation after washing bulk COP5000 solution. For grafting to glass, the inner surface of glass tubing was modified with trichlorovinylsilane 2 days before introducing COP5000 [28]. After  $\gamma$ -irradiation, the grafted surface was incubated with 1% sodium dodecylsulfate (SDS) solution overnight and rinsed thoroughly with DDW to remove COP5000 molecules that were not covalently linked to the biomaterial surface. Graft material was dried in an oven at 40°C. Samples treated as above in the absence of the grafting polymer were used as controls. Pluronic F127 (PF127) was also used, instead of COP5000, in the above procedure to produce another control surface. PF127 is a block copolymer of PEO–poly(propylene oxide)(PPO)–PEO

triblock copolymer. Unlike COP5000, PF127 does not have double bonds for covalent grafting to the surface upon exposure to  $\gamma$ -irradiation.

### *Protein adsorption*

Commercial canine fibrinogen (Fraction I, Sigma, St. Louis, MO, USA) was purified by the method of Laki [34, 35]. Fibrinogen in phosphate buffered saline (PBS, pH 7.4) was radiolabeled with  $^{125}\text{I}$  using the Iodo-Gen reagent (Pierce, Rockford, IL, USA). The radiolabeled protein was purified by gel filtration over a column packed with Bio-Gel P6-DG (Bio-Rad, Exclusion limit 6 kDa), which was equilibrated with PBS and calibrated with blue dextran (Sigma, St. Louis, MO, USA). Radiolabeled fibrinogen was mixed with native protein in a 1 : 39 mass ratio to yield a  $0.1 \text{ mg ml}^{-1}$  fibrinogen solution.

PEO grafted and control graft tubes (10 cm long) were filled with PBS to hydrate the surface for 1 h prior to protein adsorption experiment. Hydrated tubes were injected with fibrinogen solution, displacing the PBS at room temperature. After 1 h of adsorption, the protein solution was displaced with PBS. Surface bound radioactivity was measured on a  $\gamma$ -scintillation counter (Beckman, Gamma 5500B, Arlington Heights, IL, USA). The raw  $\gamma$ -counts were converted to  $\mu\text{g cm}^{-2}$  values using the sample surface area and protein specific activity.

### *In vitro platelet adhesion study*

Canine blood was collected in heparinized tubes and centrifuged at 100 g for 10 min. The platelet-rich plasma (PRP) was collected and kept in a water bath at  $37^\circ\text{C}$  for 30 min. The packed cell fraction was discarded. Graft samples (approximately  $1 \text{ cm} \times 1 \text{ cm}$ ), previously degassed and hydrated in PBS for 1 h, were immersed in aliquots of the PRP. During the 1 h exposure time the test tubes containing the samples were kept on a rotator. They were rinsed with PBS to remove non-adherent platelets, fixed with 2% glutaraldehyde (Sigma, St. Louis, MO, USA) dehydrated in a graded ethanol series, and critical point dried. The dehydrated samples were examined on a scanning electron microscope (SEM) (JSM-840, Joel Inc., Tokyo, Japan).

### *Radiolabeling of platelets*

One day before the surgery date, 43 ml of blood was withdrawn from the designated dog into a syringe containing 7 ml of buffered acid citrated dextrose (pH 5.0, Squibb Diagnostics, New Brunswick, NJ, USA). The first 1 ml of blood was discarded as it may contain a large quantity of tissue thromboplastin [36]. Blood was carefully transferred into polypropylene centrifuge tubes and centrifuged at 600 g for 10 min. The platelet rich plasma (PRP) was collected and further centrifuged at 1500 g for 5 min to pellet the platelets. The platelet poor plasma (PPP) was separated and kept for later use. Platelets were washed with sterile normal saline by gently suspending them in the saline and pelleting them by centrifuging as above. They

were resuspended in a mixture of normal saline and acid citrated dextrose (1:7, pH 6.5). At this point, a 0.5-ml sample of platelet suspension was saved for platelet counting using a Cell-Dyn counter (Abbott Laboratories, Santa Clara, CA, USA).

Indium-111-oxine (Amersham, Arlington Heights, IL, USA) (700 mCi) was added to the platelet suspension dropwise and incubated at room temperature. After 30 min, the reaction mixture was centrifuged at 1500 g for 6 min to pellet the platelets. Labeled platelets were washed twice, first with acid citrated dextrose and then with the retained PPP before they were resuspended in 10 ml PPP for reinjection into the dog.

#### *Acute canine ex vivo experiment*

A series shunt was constructed by connecting 6 cm segments of vascular grafts through Tygon<sup>®</sup> spacers. Each series shunt consisted of PEO-grafted and control graft tubes randomly positioned along the shunt. The shunt had a flush port to which a PBS filled syringe was attached. The purpose of this was to flush the shunt following a predetermined period of blood contact. The flushing step was necessary to detect the radioactivity associated with adherent platelets during the subsequent counting on a  $\gamma$ -counter. The Tygon<sup>®</sup> spacer used was coated with albumin prior to construction of the shunt as described above. The shunt was prepared 1 day before surgery and kept filled with PBS at 4°C.

Adult mongrel dogs weighing 50 ~ 60 lb were used in the canine experiments. They were screened for fibrinogen concentration, hematocrit, and platelet counts, all of which were within the normal range. The designated animal was injected with 10 ml <sup>111</sup>In-platelet suspension 20 h prior to placement of the shunt. Each animal was fasted for 20 h prior to surgery, then anesthetized by intravenous injection of sodium thiopental (10 mg kg<sup>-1</sup> to effect) followed by endotracheal intubation to maintain anesthesia with isoflurane and oxygen.

The carotid artery and jugular vein were exposed and clamped. A segment of the recipient vessel was excised and the entrance and exit segments of the shunt were placed interpositionally 3 in. down into the carotid artery and jugular vein, respectively. The shunt entrance and exit sections were tied in place with a 2-0 silk suture material. After completion of both vascular anastomoses, all clamps were released and stopcocks opened for the blood to flow through the shunt. The shunt was exposed to blood for 2 h. The animal was positioned under a large field-of-view scintillation camera (Sigma 410 Radioisotope Camera, Ohio Nuclear Inc., Solon, OH, USA) fitted with a high resolution collimator and connected to a digital computer.

Separate 50 000 count images of the shunt were collected at each photopeak, digitized and stored in the computer. Data analysis was done using the Medasys Pinnacle Data Analysis system (Medasys Inc., Ann Arbor, MI, USA). Blood samples were taken at each photopeak and counted for radioactivity on a  $\gamma$ -counter (Beckman, 5500B). All animals were imaged for 2 h after the start of blood flow

through the shunt. At the end of the 2 h, the artery and vein were clamped and the shunt flushed by injecting 30 ml PBS through the flush port. This was followed by injection of 30 ml of 2% glutaraldehyde in PBS to fix the platelets and other blood cells that were adherent to the graft material surface. The shunt was disconnected and each graft sample was divided into two sections. A 4-cm segment of the graft material was counted for radioactivity on a  $\gamma$ -scintillation counter. A 2-cm segment of each graft except the glass tubes was kept in 2% glutaraldehyde until it was critical point dried and examined by SEM for cellular adhesion and activation. The raw  $\gamma$ -count was divided by the platelet specific activity and the graft surface area to get the number of platelets deposited in number of platelets  $\text{cm}^{-2}$ .

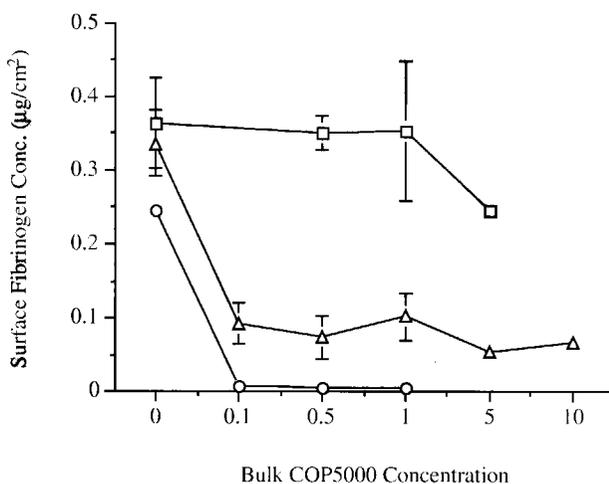
## RESULTS

### *Protein adsorption study*

The effect of the bulk concentration of COP5000 on the grafting efficiency was examined indirectly by examining the fibrinogen adsorption on surfaces grafted with different amounts of COP5000. The bulk concentration of COP5000 was varied from 0 to 10 mg/ml. As shown in Fig. 1, the surface fibrinogen concentrations were  $0.25 \pm 0.01$ ,  $0.34 \pm 0.04$ , and  $0.36 \pm 0.06 \mu\text{g cm}^{-2}$ , on the control glass, Silastic<sup>®</sup>, and ePTFE, respectively. These concentrations fall into the monolayer coverage of fibrinogen on the surface. Fibrinogen adsorption onto glass surfaces was decreased sharply as the surface was grafted with COP5000 even at the bulk concentration of  $0.1 \text{ mg ml}^{-1}$ . The surface fibrinogen concentration was decreased to  $0.01 \mu\text{g cm}^{-2}$ , which is greater than 95% reduction as compared to the control surface. Clearly, introducing vinyl groups to glass made the grafting of COP5000 more efficient [28].

Grafting of COP5000 on Silastic<sup>®</sup> was also effective in prevention of fibrinogen adsorption, although not as effective as on glass surfaces. The surface fibrinogen concentration on control Silastic<sup>®</sup> was reduced to less than  $0.1 \mu\text{g cm}^{-2}$  even when the bulk COP5000 concentration for grafting was only  $0.1 \text{ mg ml}^{-1}$ . This was more than 70% decrease. This value practically remained the same as the bulk COP5000 concentration increased up to  $10 \text{ mg ml}^{-1}$ . The fibrinogen adsorption profile on PE was somewhere in between those of glass and Silastic<sup>®</sup>.

Figure 1 also shows the result of the fibrinogen adsorption on ePTFE. Unlike on the other surfaces, there was no decrease in fibrinogen adsorption on ePTFE even after treatment with COP5000, until the bulk concentration of COP5000 reached  $5 \text{ mg ml}^{-1}$ . The surface fibrinogen concentration decreased to  $0.25 \mu\text{g cm}^{-2}$ . This is only 30% reduction as compared to the fibrinogen adsorption on the control surface. Apparently, grafting of COP5000 by  $\gamma$ -irradiation was not efficient, and this is not surprising.



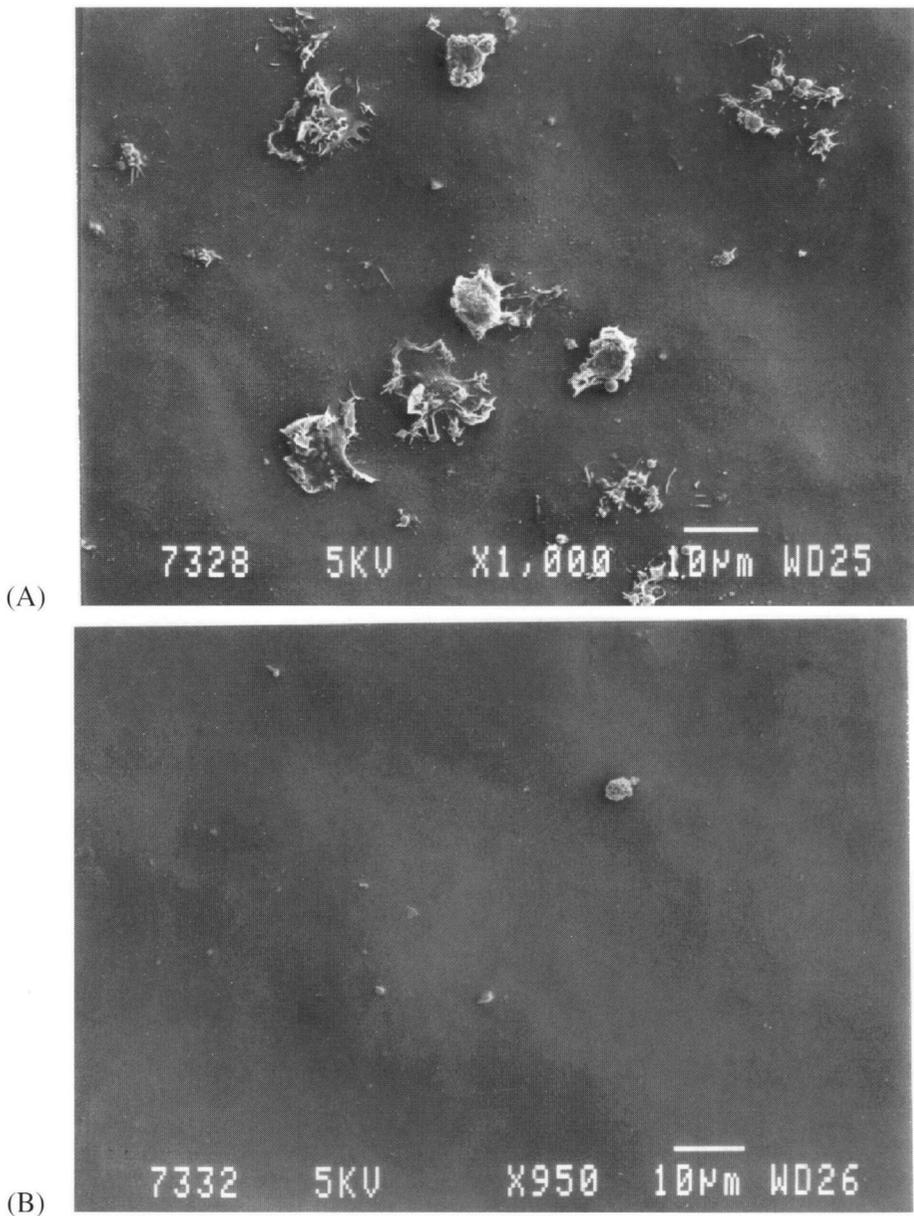
**Figure 1.** Fibrinogen concentration on ePTFE ( $\square$ ), Silastic<sup>®</sup> ( $\triangle$ ) and, silanized glass ( $\circ$ ) as a function of bulk COP5000 concentration used for grafting. Mean  $\pm$  S.D.  $n = 3$ .

### In vitro platelet adhesion

Since COP5000 grafted glass, Silastic<sup>®</sup>, and PE resulted in significant reduction in fibrinogen adsorption, they were evaluated *in vitro* for platelet adhesion and activation. Figure 2A shows platelet deposition on the control Silastic<sup>®</sup> surface. Platelets were fully activated and formed aggregates on the control surface. On COP5000-grafted Silastic<sup>®</sup> (Fig. 2B), almost no adherent platelets were found on this surface. PEO grafting on Silastic<sup>®</sup> using COP5000 resulted in complete prevention of platelet adhesion and activation *in vitro*. Similar results were obtained for glass and PE. On the other hand, numerous platelet aggregates were found on the ePTFE.

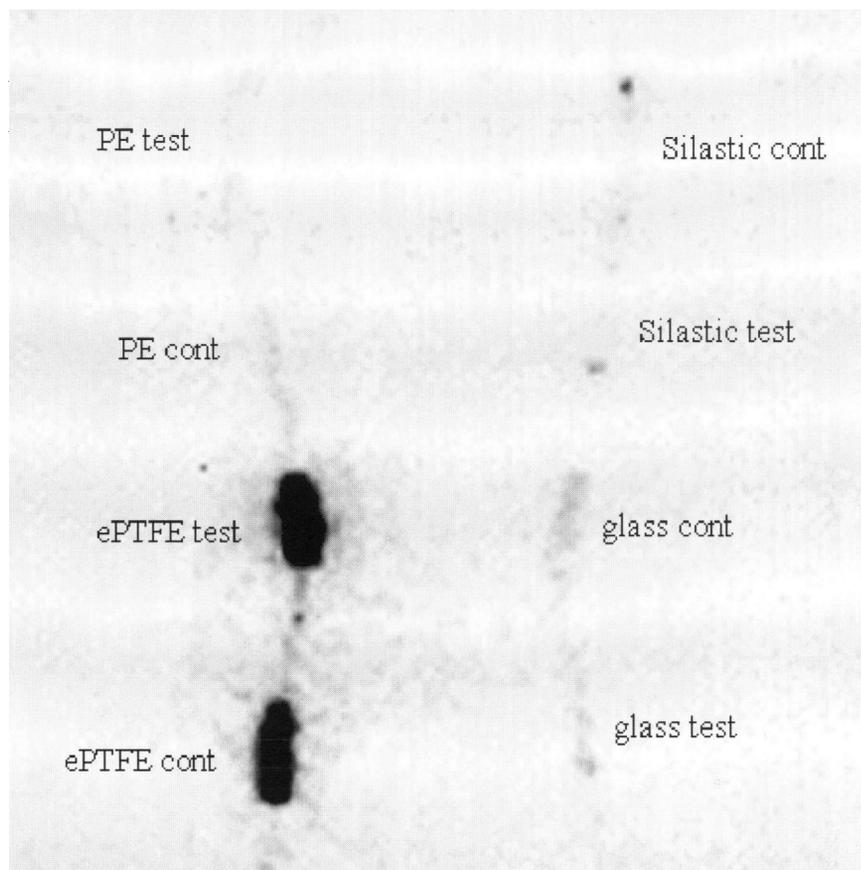
### Canine ex vivo experiment

PEO-grafted materials were tested in a canine *ex vivo* model. Serial 15-min scintiphotos were taken to monitor the deposition of platelets on the graft materials. The kinetics of platelet deposition was measured by counting the same region of interest in all the serial images. Figure 3 shows an example of scintiphotos taken during the *ex vivo* experiments. The exact numbers of adherent platelets on individual segments were calculated using the image analysis program. Those values are shown in Fig. 4. During the 2-h exposure of the graft materials to blood, the highest platelet deposition was found on the ePTFE control ( $8170 \pm 1030$  platelets  $10^{-3} \mu\text{m}^2$ ) followed by the ePTFE grafted with COP5000 ( $5100 \pm 460$  platelets  $10^{-3} \mu\text{m}^2$ ). This was not surprising since PEO-treated ePTFE did not show significant reduction in fibrinogen adsorption and platelet adhesion *in vitro*. COP5000-grafted samples of PE, Silastic<sup>®</sup>, and glass showed platelet depositions of  $169 \pm 35$ ,  $100 \pm 80$ , and  $24 \pm 22$  platelets  $10^{-3} \mu\text{m}^2$ , respectively. This is only 35%



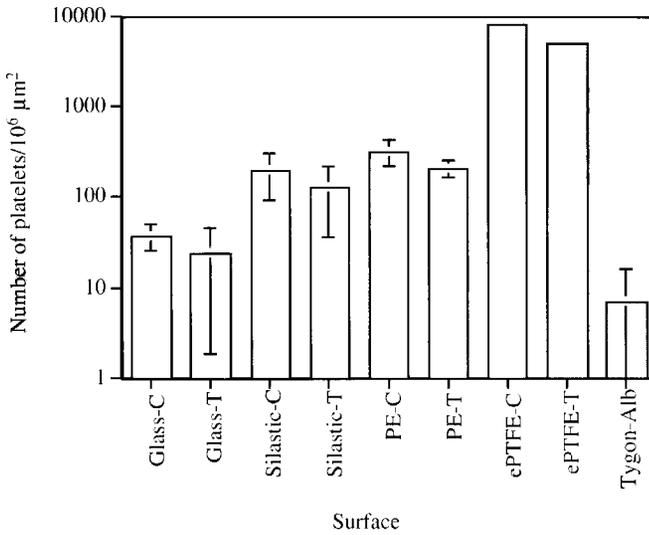
**Figure 2.** SEM pictures of control (A) and COP5000-grafted (B) Silastic<sup>®</sup>.

reduction in platelet deposition by PEO grafting. SEM examination of the inner side of tube samples showed fully spread platelets and thrombi on PEO-grafted PE and Silastic<sup>®</sup> surfaces [37]. It is interesting to observe that the albumin-coated Tygon<sup>®</sup> connector tubes showed the lowest platelet deposition ( $<10$  platelets  $10^{-3}\mu\text{m}^2$ ). It is most likely that albumin adsorbs to cover the entire surface more effectively than PEO does.



**Figure 3.** Scintiphoto of the series shunt in a canine *ex vivo* system. Blood was allowed to flow for 30 min. The samples (clockwise from the bottom left) were ePTFE control, ePTFE test, PE control, PE test, Silastic<sup>®</sup> control, Silastic<sup>®</sup> test, glass control, and glass test. Test samples are those treated for PEO grafting. Each segment was connected using albumin-coated Tygon<sup>®</sup> tubing.

Figure 5 shows the SEM pictures of the blood exposed grafts. The pictures show the deposition of platelets and other blood cells on all the grafts tested. Not only platelets and blood cells deposit, fibrin networks with trapped platelets and blood cells could be observed on the ePTFE (A) and PE (B) surfaces. On PE and Silastic<sup>®</sup> (C) surfaces, fully spread platelets were clearly visible whereas on the ePTFE surface, blood cell deposition and fibrin network was so dense that the underlying platelets could not be seen. The fully spread platelets on the Silastic and PE were aggregates. The highly thrombotic nature of the ePTFE was evident

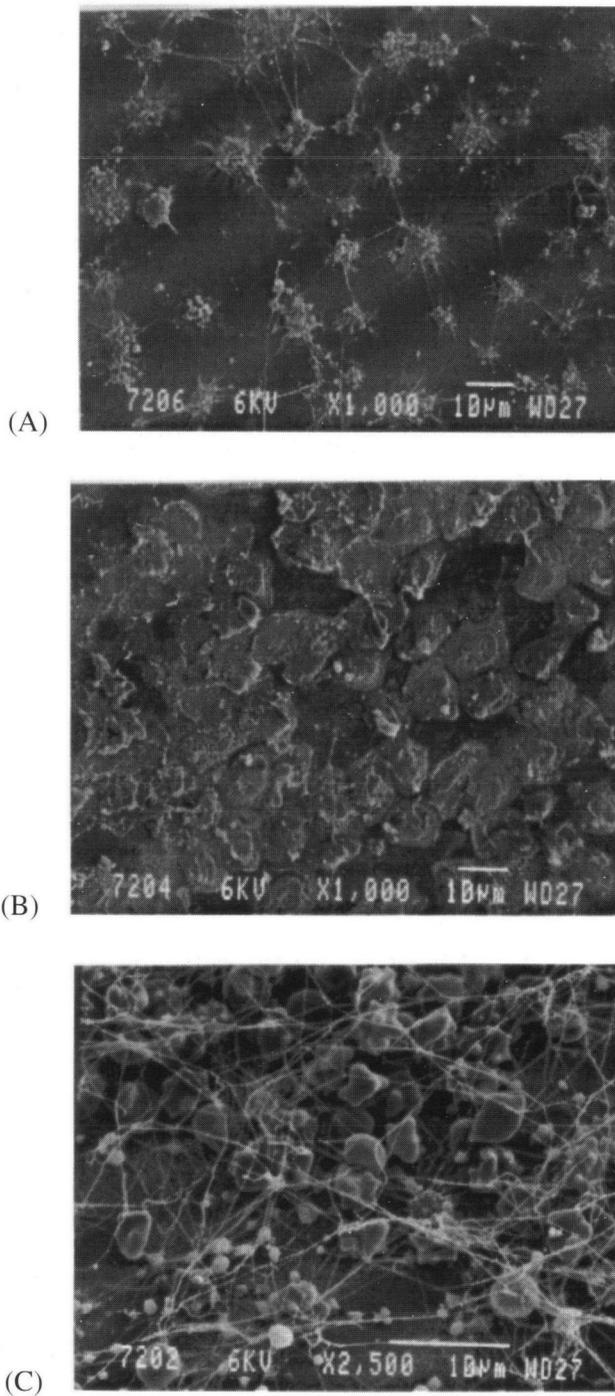


**Figure 4.** Platelet deposition on various vascular graft materials in a canine *ex vivo* series shunt model. 'C' stands for the control and 'T' stands for COP5000 grafted material. Mean  $\pm$  S.D.  $n = 2$ .

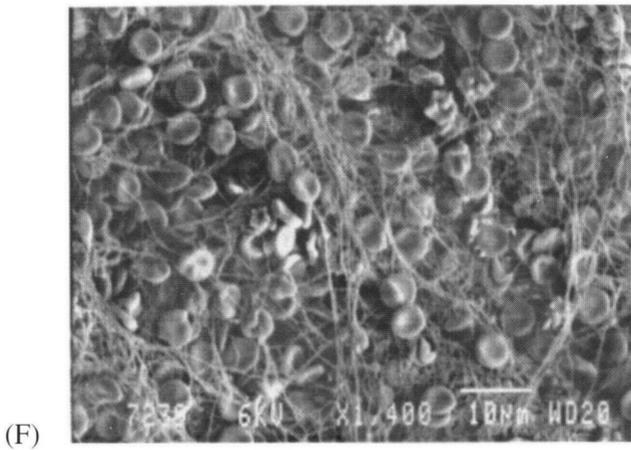
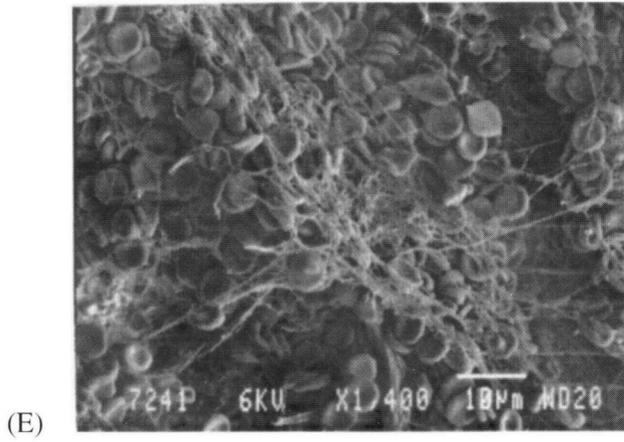
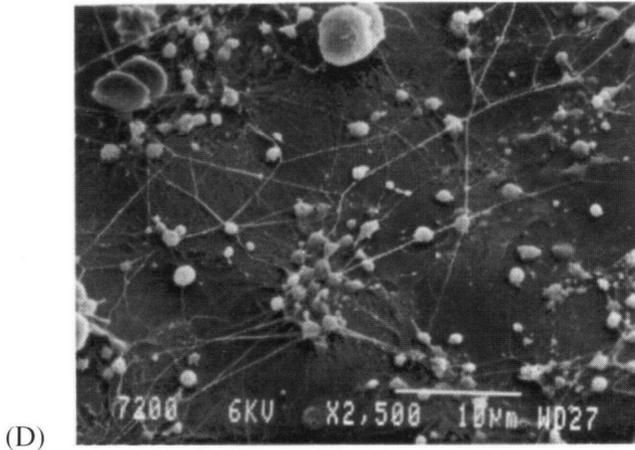
both from the SEM observation and the radioactivity count. Overall, the Silastic<sup>®</sup> surface was better than both the ePTFE and the PE surfaces in thrombus formation. The SEM results show no dramatic difference between the PEO grafted and control graft materials in terms of platelet adhesion and activation.

## DISCUSSION

The copolymer COP5000 is a triblock amphiphilic copolymer of the form PEO–PB–PEO. The hydrophobic PB segment causes the spontaneous adsorption of this copolymer on hydrophobic surfaces. The PEO chain, because of its hydrophilicity, extends from the surface providing steric repulsion to the approaching protein molecules and cells thereby preventing their adsorption. Vinylsilane coated glass presents a hydrophobic surface that is rich in double bonds. The copolymer molecules adsorb on this hydrophobic surface spontaneously. A double bond is particularly sensitive to radiation induced reaction [32]. Irradiation of the double bonds both on the glass surface and the PB generates free radicals which react with one another forming a covalent linkage between the surface and the copolymer. Adsorption of the hydrophobic PB segment on hydrophobic surfaces in an aqueous solution enhances the intimate contacts of double bonds with the surface. This intimate contact ensures the reaction between the free radicals generated and the surface molecules. The efficiency of grafting of COP5000 on the graft surfaces was assessed indirectly by measuring fibrinogen adsorption. Vinylsilane coated glass grafting was the most efficient one, followed by Silastic<sup>®</sup> and PE. Expanded PTFE did not react with the COP5000 as shown by the lack of reduction of



**Figure 5.** SEM pictures showing platelet deposition on control (A, C, and E) and COP5000-grafted (B, D, and F) surfaces. Silastic<sup>®</sup> (A and B), PE (C and D), and ePTFE (E and F).



**Figure 5.** (Continued).

fibrinogen adsorption. Vinyl groups are susceptible to  $\gamma$ -irradiation and hence when a COP5000 adsorbed vinyl surface is exposed to  $\gamma$ -irradiation, both the surface vinyl groups and the double bonds of the PB ionize resulting in significant reaction. By comparison, the saturated hydrocarbon of the methyl groups on the surface of Silastic<sup>®</sup> is not expected to ionize easily. Expanded PTFE contains C–C and C–F bonds which are much stronger bonds than a C–H bond [38]. It would be very difficult to break C–F bonds and form C–C bonds between the ePTFE and the COP5000. Hence grafting of COP5000 on ePTFE by  $\gamma$ -irradiation was very inefficient as seen from the fibrinogen adsorption results.

One of the major difficulties in studying biomaterials is that there is no correlation between *in vitro* data and *ex vivo* or *in vivo* results [39]. One of the reasons may be that *in vitro* models of thrombosis are so far removed from reality and due to their nature can generate many artifacts [40]. It has been generally assumed that PEO is sufficiently 'compatible' to serve as the masking agent for many underlying, non-leaching materials, and this hypothesis has been backed by numerous partially successful tests of PEO-modified surfaces and biologically active proteins in contact with blood or its components [22]. The lurking danger here is that the relatively benign surface in static conditions fails in actual use, as historically exemplified by platelet consumption by the clean hydrogel surfaces [41]. We, as well as many others in the field, have noticed that the surfaces which prevent fibrinogen adsorption and platelet adhesion *in vitro* still elicit thrombus formation in *ex vivo* experiments. Thus, it appears that the use of *in vivo* or *ex vivo* models of experimental thrombosis in animals is an obligatory step to the understanding of mechanisms involved in thrombogenesis as well as in the evaluation of anti-thrombotic therapeutics [40]. For these reasons, it is necessary to use models as representative as possible of the human pathological condition [40]. Besides these theoretical requirements, practical needs have also to be fulfilled. Thus, factors such as accessibility of the models, adaptation to the type of the technique to different animal models, and cost play an important role in choosing a model for the study of surface-induced thrombosis.

Both *in vivo* and *ex vivo* techniques have been widely used in the evaluation of the blood compatibility of biomaterials [42–44]. They involve the use of experimental animals. In the *in vivo* tests, a biomaterial is placed in the animal's body at a position where it is in contact with blood. After a certain period of time, the biomaterial is explanted and analyzed for thrombosis and related phenomena. The primary goal of *in vivo* tests is to determine patency of grafts in the body. This is an 'endpoint' evaluation and it does not show the dynamics of the processes of thrombosis and embolization. *Ex vivo* tests involve the use of arterio-venous extracorporeal shunts that contain the graft materials to be tested connected in series [44]. The series shunt allowed researchers to evaluate a number of graft materials in a single run so that comparative examination of test and control samples is possible. Both platelet deposition, and protein adsorption have been measured quantitatively in these shunts using radiolabeled platelets and protein,

and qualitatively through microscopic observations. The dynamics of platelet deposition, thrombus formation, and embolization can be easily monitored using the *ex vivo* arterio-venous shunt model.

The *in vitro* platelet experiments have shown that COP5000 grafting on Silastic® prevented platelet adhesion completely. Although the control surface itself was not very bad in terms of platelet adhesion *in vitro*, the effect of COP5000 grafting was clear. When the graft materials were tested in *ex vivo*, platelet adhesion, aggregation and thrombosis were observed both on PEO grafted and the non-grafted surfaces. The discrepancy between the *in vitro* and *ex vivo* results was quite obvious. There are distinct differences between the *in vitro* and *ex vivo* test systems. In the *in vitro* system, blood is anticoagulated. Anticoagulants are known to affect the interaction of platelets with biomaterials. As shown by these and other investigators, platelets are more reactive towards artificial surface in the absence of anticoagulants than in their presence. Schultz *et al.* observed that thrombus formation on the Dacron surface coincided with the disappearance of heparin from the system of a heparinized dog [45]. Also the amount of thrombus was less after a dose of heparin. Another factor that affects platelet adhesion on to material surfaces is temperature [46]. *In vitro* tests are routinely carried out at temperatures other than 37°C. Absolom *et al.* examined the effects of temperature on platelet adhesion and activation on films of low density polyethylene, and polystyrene. They concluded that for a given substrate surface, platelet adhesion decreases with decreasing temperature and that platelet activation follows the same pattern.

Our *in vitro* evaluation of platelet adhesion was carried out in the absence of shear. The graft sample was incubated with the PRP and the test tube containing the sample was rotated. The shear force this movement produced was minimal. In the *ex vivo* experiment, however, blood was allowed to flow through the small diameter shunt for up to 2 h. The graft material surface was subjected to high shear force. High shear enhances platelet adhesion and activation [47]. Also, shear created turbulence and impact of blood cells with one another and the edges of the graft materials result in lysis of blood cells causing the release of platelet activating chemicals such as ADP, serotonin, and epinephrine [48]. Also, the connection edges in our series shunt serve as a point of stagnation of blood. They may cause back flow and eddies current which contribute to the behavior of platelets observed in our *ex vivo* experiments. This may explain the lack of correlation between the *in vitro* and the *ex vivo* results obtained in this work.

The reaction of platelets with artificial surfaces has been modeled as a diffusion controlled process [49] where the platelet diffusivity is a function of blood flow rate. Leonard *et al.* examined the platelet reactivity towards five different materials exposed to blood at equal flow rate and exposure time. They found that upon exposure to blood at the same flow rate for the same time, the five materials tested showed no difference in the amount of platelet deposited on their surfaces. If in fact diffusion is controlling the reactivity of platelets with artificial surfaces,

no difference would be expected among materials that showed difference when examined under no flow condition.

The involvement of complement activation in platelet adhesion and the subsequent thrombus formation has been suggested [50]. C5a, a product of complement activation, is known to induce the generation of both tissue factor and platelet activating factor in leukocytes [51]. These substances activate the coagulation system and platelets. This promotes platelet adhesion and activation leading to thrombus formation. PEO grafted surfaces have been shown to activate the complement system. Although this activation is rather moderate, it may be enough to attract a sufficient number of leukocytes and cause them to release tissue and platelet activating factors. The *in vitro* platelet rich plasma is devoid of leukocytes and hence the leukocyte mediated thrombus formation is not expected in this case. In *ex vivo* experiments, however, graft materials were exposed to whole blood and upon complement activation, the leukocytes may play a role in the observed platelet adhesion and thrombosis on all the graft materials.

There is another basic difference between the *in vitro* and *ex vivo* experiments. In the *in vitro* model, the number of platelets that can act on a surface are finite, whereas *in vivo* the platelet pool that acts on a small surface may be considered infinite [44]. Constant exposure of the graft surface to a new population of platelets every time the surface sees blood may result in increased platelet adhesion and thrombus formation.

## CONCLUSION

Our *ex vivo* studies on COP5000-grafted biomaterials did not reveal any significant difference between the grafted samples and the non-grafted controls. This is contrasted with the significant differences observed between the two groups of samples in *in vitro* studies. Careful comparison of experimental conditions between *in vitro* and *ex vivo* experiments is expected to provide key factors causing thrombus formation on the PEO-grafted surfaces. Two obvious differences between *in vitro* and *ex vivo* experiments are the presence of anticoagulant and the lack of flow in *in vitro* experiments. The observation that platelet activation and thrombus formation on the control surfaces even in *in vitro* studies removes the anticoagulant from the prime factor. The flow of blood in *ex vivo* experiments is expected to influence the grafted PEO layer and the surface in ways that we do not understand. In addition, the flow of blood may activate certain proteins and cells allowing them to overcome the resistance of the grafted PEO layer. It may be that our PEO grafting technique (and most others in the literature) may result in surface coverage high enough for prevention of platelet adhesion in *in vitro* experiments, but not high enough for flowing blood in *ex vivo* experiments. The surface-grafted PEO chains may be simply ineffective when subjected to the complex flow and shear condition in *ex vivo* model. Improving correlation between *in vitro* and *ex vivo/in vivo* studies

may require exhaustive experiments testing all the possible grafting conditions and grafting techniques.

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