J. Biomater. Sci. Polymer Edn, Vol. 11, No. 11, pp. 1121–1134 (2000)

In vitro and *in vivo* studies of PEO-grafted blood-contacting cardiovascular prostheses

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Received 21 January 2000; accepted 28 August 2000

Abstract—The initial step of thrombus formation on blood-contacting biomaterials is known to be adsorption of blood proteins followed by platelet adhesion. Poly(ethylene oxide) (PEO) has been frequently used to modify biomaterial surfaces to minimize or prevent protein adsorption and cell adhesion. PEO was grafted onto a number of biomaterials in our laboratory. Nitinol stents and glass tubes were grafted with PEO by priming the metal surface with trichlorovinylsilane(TCVS) followed by adsorption of Pluronic and γ-irradiation. Nitinol stents were also coated with Carbothane® for PEO grafting. Chemically inert polymeric biomaterials, such as Carbothane, polyethylene, silicone rubber, and expanded polytetrafluoroethylene (e-PTFE), were first adsorbed with PEO–polybutadiene–PEO (PEO–PB–PEO) triblock copolymers and then exposed to γ-irradiation for covalent grafting. For PEO grafting to Dacron® (polyethylene terephthalate), the surface was sequentially treated with PEO–PB–PEO and Pluronics® followed by γ-irradiation.

In vitro studies showed substantial reduction in fibrinogen adsorption and platelet adhesion to the PEO-grafted surfaces compared with control surfaces. Fibrinogen adsorption was reduced by 70–95% by PEO grafting on all surfaces, except for e-PTFE. The platelet adhesion corresponded to the fibrinogen adsorption. When the PEO-grafted surfaces were tested *ex vivo/in vivo,* however, the expected beneficial effect of PEO grafting was inconsistent. The beneficial effect of the PEO grafting was most pronounced on the PEO-grafted nitinol stents. Thrombus formation was reduced by more than 85% by PEO grafting on metallic stents. Only moderate improvement (i.e. 35% decrease in platelet deposition) was observed with PEO-grafted tubes of polyethylene, silicone rubber, and glass. For PEO-grafted heart valves made of Dacron, however, no effect of PEO grafting was observed at all. It appears that the extent of thrombus formation on PEO-grafted biomaterials was directly related to the extent of tissue damage during implantation surgery. Platelets can be activated and form aggregates in the bulk blood, and the formed platelet aggregates may be able to deposit on the

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PEO monolayer overcoming its repulsive property. Our studies indicate that the testing of *in vitro* platelet adhesion should include adhesion of large platelet aggregates, in addition to adhesion of individual platelets. Furthermore, the surface modification methods should be improved over the current monolayer grafting concept so that the repulsive force by the grafted PEO layers is large enough to prevent adhesion of platelet aggregates formed in the bulk blood before arriving at the biomaterial surface.

Key words: PEO; block copolymer; surface grafting; stent; biomaterial; blood; cardiovascular; platelet.

INTRODUCION

Biomaterials have been used in various clinical applications. Blood-contacting biomaterials range from coronary stent to total artificial heart. One of the most important problems associated with blood-contacting biomaterials is surface-induced thrombus formation. The sequence of thrombus formation has been well established [1]. The first event occurring after exposure of biomaterials to blood is the adsorption of blood proteins. The types and the amounts of the adsorbed proteins determine whether platelets will adhere and activate or not. Adsorption of platelet-adhesive proteins, such as fibrinogen, leads to adhesion and activation of platelets. The initial layer of activated platelets attract more platelets to form thrombus. Thrombi are embolized and the whole sequence may be repeated. Since the thrombus formation begins with the adsorption of proteins and platelets, efforts have been directed toward controlling (mainly preventing) protein adsorption and platelet adhesion. Growing endothelial cells on biomaterials appears to be the most ideal solution for making truly blood-compatible materials. Human endothelial cells, however, have been particularly difficult to grow [2, 3]. Since protein adsorption and platelet adhesion are interfacial phenomena, the surface properties of biomaterials determine the thrombogenicity. For this reason, modification of the biomaterial surfaces with protein-repulsive molecules has become an important alternative for making more blood compatible materials.

The commonly used protein-repulsive molecules are proteins such as albumin [4, 5], polysaccharides such as heparin [6, 7] and dextran [8], synthetic polymers such as PEO [9, 10] phospholipid molecules such as phosphatidyl choline [11], and surface active molecules such as self-assembled monolayers [12]. Surface modification of biomaterials with these macromolecules makes theoretical sense, since the interfacial properties depend on the outer few layers of biomaterials. The surface modification approach has been quite successful, at least in *in vitro* experiments in prevention of protein adsorption and platelet adhesion. The *ex vivo*/*in vivo* applications of surface-modified biomaterials, however, have not met the expectations. The inability of *in vitro* results to predict the *in vivo* performance has been the bottleneck in the development of improved biomaterials, and has delayed rational design of biomaterial surfaces. We have developed a number of approaches for grafting PEO to various biomaterial surfaces, and the PEO-grafted materials were tested *in vitro*

using fibrinogen adsorption and platelet adhesion [13–19]. The PEO-grafted materials were also tested *ex vivo/in vivo* in three different laboratories using different animal models [20–22]. This paper compares the results of those studies to gain new insights into the *ex vivo/in vivo* thrombogenicity of PEO-grafted biomaterials.

SURFACE MODIFICATION OF BIOMATERIALS WITH PEO

Synthesis of PEO–PB–PEO triblock copolymers

PEO–polybutadiene–PEO (PEO–PB–PEO) triblock copolymers with various molecular weights of PEO were used for grafting of PEO to different biomaterials. PEO–PB–PEO copolymers were synthesized as described previously [16]. Briefly, poly(ethylene oxide) methyl ether (m-PEO) was azeotropically dried in benzene, and the dry m-PEO in THF (15%) was added dropwise to the hydroxyl terminated PB solution (15% w/v in tetrahydrofuran). After stirring for 48 h under dry nitrogen, the reaction product was twice precipitated from THF with hexane and dried in vacuum. The molecular weight of the PB moiety of the copolymer was 700 g mol^{-1}. The molecular weight of the PEO chain was either 350 or 5000. Notations of 'COP350' and 'COP5000' were used to indicate the copolymers with PEO molecular weights of 350 and 5000.

PEO grafting on nitinol stents

Two types of nitinol stents (HARTS® and Act-One® Progressive Angioplasty Systems, Inc., Menlo Park, CA, USA) were grafted with PEO. The stents were made of 7- or 15-mm long slotted tubes with nominal strut thickness of 0.006 in. The stents were cleaned by soaking in 1% sodium dodecylsulfate (SDS) solution with vortexing for 5 min followed by rinsing in deionized distilled water (DDW) and sonicating for 10 min in fresh DDW. The cleaned HARTS stents were silanized by immersing in a solution of 5% trichlorovinylsilane (TCVS, Aldrich, Milwaukee, WI, USA) in chloroform (analytical grade, Mallinkrodt, Paris, KY, USA) at room temperature. After 3 h they were rinsed sequentially in fresh chloroform, absolute ethanol, and DDW. The silanized stents were exposed to Pluronic® F108 (PF108) or Pluronic® F127 (PF127) at the concentration of 1 mg ml^{-1}. (PF108 and PF127 are PEO/poly(propylene oxide)/PEO triblock copolymers with the repeating monomer units of 128/54/128 and 98/67/98, respectively.) After 1 h the whole system in DDW was exposed to 0.3 Mrad γ-radiation from ^{60}Co irradiator. The samples were then washed in 1% SDS overnight and rinsed thoroughly with DDW. They were dried at 60°C and stored covered at room temperature until use.

Act-One stents were grafted with PEO using a different method. Each stent was mounted on the mandrel of a motor and rotated at 600 rpm. The 6 ml of 1% Carbothane® solution was spread onto the top of water at a rate of 1 ml min^{-1} and the stent was introduced through the polymer layer into water to form a thin porous

polymer membrane. The porous membrane was made for applications of localized drug delivery in other studies. The coated stents were then immersed in 1 mg ml^{-1} of COP5000 for 1 h before γ-irradiation for 2.5 Mrad. After irradiation stents were rinsed three times in water and collapsed in water at 70°C prior to vacuum drying.

PEO grafting on glass tubes

Glass tubes (inner diameter (i.d.) 4 mm, Dow Corning, Midland, MI, USA) were cleaned by immersion in chromic–sulfuric acid solution overnight, rinsed with copious DDW and dried at 60°C. Glass surface was silanized by the same procedure used for nitinol stents and grafted with COP5000 [21].

PEO grafting on polyethylene, silicone rubber, and expanded polytetrafluoroethylene (e-PTFE)

Silicone rubber (Silastic®, i.d. 3.35 mm, o.d. 4.65 mm, Dow Corning, Midland, MI, USA), expanded polytetrafluoroethylene (e-PTFE, Gore-Tex®, i.d. 4 mm, W. L. Gore and Associates, Flagstaff, AZ, USA), and polyethylene (PE) (Intramedic®, i.d. 3.17 mm, o.d. 3.99 mm, Becton Dickinson, Franklin Lakes, NJ, USA) tubes were cleaned by washing with running DDW for 1 h. They were grafted with PEO using COP5000 [21]. The copolymer solution at a concentration of 1 mg ml^{-1} was introduced inside the tubing and after 2 h at room temperature, the whole system was exposed to γ-radiation for a total radiation dose of 1 Mrad [21]. The presence of the copolymer in the bulk solution during exposure to γ-radiation resulted in higher grafting efficiency compared to exposure to γ-radiation after washing bulk copolymer solution. After γ-irradiation, the grafted surface was incubated with 1% SDS solution overnight and rinsed thoroughly with DDW to remove COP5000 molecules that were not covalently linked to the biomaterial surface. The outer surface of the ePTFE graft was sealed with adhesive silicone (NuSil®, Type A, Silicone Technology, Carpinteria, CA, USA) to prevent leakage.

Albumin adsorption on plasticized poly(vinyl chloride) tubing

Plasticized poly(vinyl chloride) (Tygon®, 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 of a series shunt in acute canine *ex vivo* experiments. Tygon tubing was first cleaned with 0.1% Ivory detergent solution at room temperature and rinsed with running DDW. The inner surfaces of only Tygon tubes were exposed to albumin solution at a concentration of 10 mg ml^{-1} 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 of Dacron

Dacron® poly(ethylene terephthalate) fabric (DuPont, Wilmington, DE, USA) was cleaned by stirring in a 2% Isoclean solution (Isolab, Akron, OH, USA) at 80°C

for 2 h and rinsing in copious amount of DDW and in boiling DDW for 2 h. The clean fabric was placed in water and vacuum degassed for 1 h to displace air from the inter-fiber spaces. The degassed fabric was stirred in the COP350 solution (10 mg ml^{-1}) at room temperature for 4 h. The COP350-primed Dacron fabric was briefly rinsed in DDW, and placed in a PF108 solution at bulk concentration of 5 mg ml^{-1}. The solution was stirred at room temperature for 1 h to allow adsorption of PF108 to the priming layer. The PF108 solution containing the primed Dacron fabric was subjected to γ-radiation at room temperature for a total dose of 0.8 Mrad. After γ-irradiation, the fabric was placed in 1% SDS solution overnight to remove ungrafted molecules, rinsed with DDW several times by vortexing, and dried at 55°C in an oven [19]. The PEO-grafted Dacron fabric was assembled on the 25-mm titanium-stem of Medtronic-Hall heart valve (Medtronic Inc., Heart Valve Division). Valves with PEO-grafted sewing rings were used for an *in vivo* study [22].

In vitro *fibrinogen adsorption*

Commercial human and canine fibrinogen (Fraction I, Sigma, St. Louis, MO, USA) were purified by the method of Laki [23, 24]. Fibrinogen was radiolabeled with ^{125}I using the Iodo-Gen reagent (Pierce, Rockford, IL, USA), and the radiolabeled fibrinogen was mixed with native fibrinogen in the weight ratio of 1 : 40. The final fibrinogen concentration was 0.1 mg ml^{-1}. Fibrinogen was allowed to adsorb for 1 h at room temperature, and the surface bound radioactivity was measured on a γ-scintillation counter (Beckman, Gamma 5500B, Arlington Heights, IL, USA).

In vitro *platelet adhesion*

Human and canine blood were collected in heparinized tubes and the platelet-rich-plasma (PRP) was separated by centrifuging at 100 *g* for 10 min. The PRP was collected and kept in a water bath at 37°C for 30 min. The packed cell fraction was discarded. PEO-grafted samples, previously 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). Platelets were also labeled with rhodamine-phalloidin for observation by a video-intensified fluorescence microscope [25].

Porcine extracorporeal arteriovenous shunt experiment

Stent thrombosis was examined using a model based on the porcine carotid-jugular arteriovenous extracorporeal perfusion system by Badimon *et al.* [26]. Pigs weighing 24–31 kg were fasted overnight and sedated with 1% ketamine and 1% xylazine. Anesthesia was maintained with 1% isoflurane. An extracorporeal circuit

was established by cannulating the carotid artery and internal jugular vein. Body temperature was maintained at 37°C using a thermal blanket. Stents were expanded using a mandrel and mounted in a 2-mm diameter perfusion chamber [26]. A strip of porcine aorta (1.0 × 2.5 cm) with an intact internal elastic laminar was placed in contact with approximately third of the outer circumference of the stent serving as a water-tight seal between two halves of the chamber. The arterial cannula was connected to the inlet of the chamber and the outflow line returned blood to the jugular vein through a variable speed peristaltic pump (Masterflex model 7518-110 Cole-Palmer Instrument Co.). The chamber was placed in a 37°C water bath. A transit time Doppler flow probe (Transonic System Inc., Ithaca, NY, USA) was interposed after the pump to record flow. The patency of vascular catheters was maintained by injecting 20 U kg^{-1} heparin to the pigs. Such low doses of administered heparin did not have any effect on stent thrombosis in the porcine model used at the shear rates examined [27]. Pigs received 325 mg of aspirin by oral administration the day prior to the procedure.

The effects of surgical stress was minimized by allowing 60 min of a stabilization period. Then the chamber was perfused with Krebs buffer solution for 1 min. Blood flow through the circuit containing a stent sample was established at a predetermined flow rate. After a timed interval, the circuit was washed with the buffer for 30 s, and the stent was removed for analysis. New stents were inserted to the chamber after cleaning for subsequent perfusion experiments. The shear rate was approximately 1500 s^{-1}. The extent of stent thrombosis was assessed by measuring the weights of dry thrombi.

Porcine mechanical heart valve implantation study

Mechanical heart valves with PEO-grafted sewing rings were implanted into Yorkshire-cross pigs weighing 35–45 kg [22, 28]. The arterial and venous lines were connected to the components of the cardiopulmonary bypass (CPB) circuit. During CPB, systemic heparinization was maintained by 3 mg kg^{-1} intravenous injection. Once the heart was arrested, the left atrial appendage was incised and mitral valve was removed. After the prosthetic valve was inserted, the heart was rewarmed, deaired, and defibrillated. ^{111}In-labeled platelets were introduced into the blood stream after the pigs were removed from cardiopulmonary bypass circuit. Blood samples were collected at timed intervals up to 24 h to monitor the platelet radioactivity and free ^{111}In in plasma. Protamine sulfate was administered to reverse the heparin activity. At the end of each experiment, the pig was sacrificed to remove mechanical heart valve.

Acute canine ex vivo *series shunt experiment*

Adult mongrel dogs weighing 50–60 lb were used in the canine experiments [21]. The designated animal was injected with 10 ml of ^{111}In-labeled platelets 20 h prior to placement of the shunt. Each animal was fasted for 20 h prior to surgery. A series

shunt was constructed by connecting 6-cm segments of vascular grafts through Tygon spacers. Each series shunt consisted of PEO-grafted and control graft tubes randomly positioned along the shunt. Shunts were prepared 1 day before surgery and kept filled with PBS at 4°C. The animal with the implanted shunt 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. 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 raw γ-count was divided by the platelet specific activity and the graft surface area to get the number of platelets deposited in number of platelets per cm^2.

All animal experiments adhered to the principles in the guidelines of the American Physiologic Society and the Guide for the Care and Use of Laboratory Animals (NIH publication #96-23, 1996), and were approved by the Institutional Animal Use and Care Committee of the participating institutions.

RESULTS

In vitro *studies*

The PEO-grafted biomaterials were tested *in vitro* for their potential to inhibit fibrinogen adsorption and platelet adhesion. The results obtained from a number of studies are listed in Table 1. Table 1 is divided into three categories based on the biomaterials used in *ex vivo/in vivo* experiments. When PEO-grafted nitinol stents were tested *in vitro*, fibrinogen adsorption was reduced for more than 88% from 0.50 ± 0.01 to 0.06 ± 0.01 $\mu g\,cm^{-2}$ [29]. On the control nitinol stents, numerous platelet aggregates covered the entire surface. Platelets on the bottoms of the aggregates were fully spread. On the PEO-grafted nitinol stents, however, only a small portion of the surface was covered with individual platelets which were only contact adherent. Apparently, platelets could not spread at all.

Several materials that could be used as vascular grafts were modified with PEO [21]. The amount of the adsorbed fibrinogen on control surfaces were 0.25 ± 0.01, 0.34 ± 0.04, 0.37 ± 0.04, and 0.36 ± 0.06 $\mu g\,cm^{-2}$ for glass, Silastic®, polyethylene, and e-PTFE, respectively. The surface fibrinogen concentrations were about the same for polymers, and only the hydrophilic glass showed lower surface fibrinogen concentration. PEO grafting reduced the surface fibrinogen concentration to less than 0.1 $\mu g\,cm^{-2}$ for Silastic and polyethylene. The fibrinogen adsorption on PEO-grafted glass was almost negligible. The reduction in fibrinogen adsorption on e-PTFE by PEO grafting was not as noticeable as on other surfaces. PEO grafting reduced the surface fibrinogen concentration to 0.25 $\mu g\,cm^{-2}$, which was still high enough to attract and activate platelets. Apparently, PEO grafting

to e-PTFE was not as effective as on other biomaterials. The results of platelet adhesion study were correlated with those of platelet adhesion study. On control glass, Silastic, and polyethylene, platelets adhered and fully activated. Platelets underwent shape change to a spread, round form, and many granules released from the activated platelets were observable. On the PEO-grafted surfaces, however, platelet adhesion was almost completely prevented. Even when a few platelets were adhered, they remained spherical and could not spread on the surface. On e-PTFE, however, platelets were able to adhere to the surface and form aggregates whether the surface was PEO-grafted or not. Many such aggregates were observed throughout the surface. The lack of the ability to prevent platelet adhesion and aggregation on PEO-grafted e-PTFE can be predicted from the lack of the ability to prevent fibrinogen adsorption. It is pointed out that the albumin-coated plasticized PVC (Tygon®) was able to prevent fibrinogen adsorption and platelet adhesion.

The surface fibrinogen concentration on Dacron® fabric was $0.59 \pm 0.04\ \mu g\ cm^{-2}$, but the true value may be different since the surface area of the fabric used for calculation was the nominal value [19]. The value, however, served as a good reference point. PEO grafting reduced the surface concentration to $0.07 \pm 0.04\ \mu g\ cm^{-2}$. This was almost 90% decrease. Platelet adhesion reflected the results of the fibrinogen adsorption. No platelets could adhere to PEO-grafted Dacron fabric, while many platelet aggregates were formed on the control Dacron fabric.

The common feature of the data listed in Table 1 is that the fibrinogen adsorption results closely reflect the platelet adhesion results. Whenever the surface fibrinogen concentration was less than $0.1\ \mu g\ cm^{-2}$, platelet adhesion was prevented. The reduction in fibrinogen adsorption of around 80% or more resulted in prevention of platelet adhesion. Please note that the surface fibrinogen concentration of $0.1\ \mu g\ cm^{-2}$ was obtained when the surface was exposed to a unary fibrinogen solution. When the PEO-grafted surface is exposed to a complex protein mixture, such as PRP, the surface fibrinogen concentration will be much smaller than the minimum surface fibrinogen concentration necessary for adhesion and activation of platelets, which was $0.02\ \mu g\ cm^{-2}$ [30].

Ex vivo/in vivo *studies*

PEO-grafted biomaterials were tested in three different animal experiments and the results are shown in Table 2. In a porcine extracorporeal arteriovenous shunt study, the thrombus weights on the unpolished and PEO-grafted nitinol stents were 14.0 ± 3.8 ($n = 13$) and 1.0 ± 0.5 mg ($n = 6$), respectively. This is more than 90% decrease in the thrombus weight by PEO grafting. PEO-grafted stents reduced stent thrombogenicity dramatically. It is noted here that this *ex vivo* study does not stimulate underlying vascular injury of native vessels that can significantly affect the stent thrombosis.

In the canine *ex vivo* series shunt experiments, PEO-grafted shunts showed only moderate decrease in platelet deposition. Decreases in the average values of platelet deposition by PEO grafting were 37, 35, 36, and 37% for glass, Silastic®,

Table 1.
Fibrinogen adsorption and platelet adhesion on the control and PEO-grafted biomaterials

Biomaterials	Surface fibrinogen concentration ($\mu g\,cm^{-2}$)	Platelet adhesion
Nitinol stents		
Control	0.50 ± 0.01	Complete surface coverage with numerous platelet aggregates
PEO-grafted	0.06 ± 0.01	<5% surface coverage with individual platelets that are only contact adherent
Vascular grafts		
Glass		
Control	0.25 ± 0.01	Full activation of adherent platelets
PEO-grafted	0.01	No platelet adhesion
Silastic®		
Control	0.34 ± 0.04	Full activation of adherent platelets
PEO-grafted	0.07 ± 0.03	No platelet adhesion
Polyethylene		
Control	0.37 ± 0.04	Full activation of adherent platelets
PEO-grafted	0.08 ± 0.05	No platelet adhesion
e-PTFE		
Control	0.36 ± 0.06	Numerous platelet aggregates on fully spread platelets
PEO-grafted	0.25	Numerous platelet aggregates
Tygon®		
Control	0.62 ± 0.05	Numerous platelet aggregates
Albumin-coated		No platelet adhesion
Mechanical heart valves		
Dacron®		
Control	0.59 ± 0.04	Numerous platelet aggregates on fully spread platelets
PEO-grafted	0.07 ± 0.04	No platelet adhesion

Some of the data were obtained from refs [1, 19, 21, 29].

polyethylene, and e-PTFE, respectively. This relative decrease appears to be the same for all the materials tested, but there are two important points to be made. First, there is no statistically significant differences in platelet deposition between the control and PEO-grafted samples, except for on e-PTFE ($p = 0.01$). Thus, it can be said that there may not be any real beneficial impact of the PEO-grafting. Second, platelet depositions on the control glass, Silastic®, and polyethylene were only 38 ± 12, 200 ± 110, and 330 ± 110 platelets per $10^3 \mu m^2$, respectively. These values indicate that thrombus formation on the glass was negligible, and that on Silastic® and polyethylene was only moderate. Only small portions of the surfaces was covered with platelet thrombi. Platelet depositions on control and PEO-grafted e-PTFE were unacceptable. Platelet deposition at the level of 5000 platelets per

Table 2.
Thrombus formation on the control and PEO-grafted biomaterials

Biomaterials	Thrombus formation
Nitinol stents (in porcine extracorporeal arteriovenous shunt experiments)	
Control	14.0 ± 3.8 mg (the weight of thrombi formed on the stent)
PEO-grafted	1.0 ± 0.5 mg (the weight of thrombi formed on the stent)
Vascular grafts (in canine *ex vivo* series shunt experiments)	
Glass	
Control	38 ± 12 platelets per $10^3\ \mu m^2$
PEO-grafted	24 ± 22 platelets per $10^3\ \mu m^2$
Silastic®	
Control	200 ± 110 platelets per $10^3\ \mu m^2$
PEO-grafted	130 ± 90 platelets per $10^3 \mu m^2$
Polyethylene	
Control	330 ± 110 platelets per $10^3\ \mu m^2$
PEO-grafted	210 ± 45 platelets per $10^3\ \mu m^2$
e-PTFE	
Control	8170 ± 1030 platelets per $10^3\ \mu m^2$
PEO-grafted	5100 ± 460 platelets per $10^3\ \mu m^2$
Tygon®	
Control	320 ± 170 platelets per $10^3\ \mu m^2$
Albumin-coated	50 ± 10 platelets per $10\ \mu m^2$
Mechanical heart valves (in porcine mechanical heart valve implantation experiments)	
Dacron® sewing ring	
Control	$0.09 \pm 0.06\%$ (the percentage of the injected radioactivity of ^{111}In-labeled platelet)
PEO-grafted	$0.21 \pm 0.21\%$ (the percentage of the injected radioactivity of ^{111}In-labeled platelet)

Some of the data were obtained from refs [1, 21, 22].

$10^3\ \mu m^2$ means formation of many, huge thrombi on the surface. Since the surface fibrinogen concentration on PEO-grafted e-PTFE is still very high ($0.25\ \mu g\ cm^{-2}$ in Table 1), formation of numerous thrombi on PEO-grafted e-PTFE is not surprising. Unlike the results observed with the nitinol stents, the effects of PEO grafting on glass, Silastic®, polyethylene, and e-PTFE were neither significant nor dramatic. It is interesting to note that the platelet deposition was almost none on albumin-coated Tygon®. Albumin may be a better choice for preventing thrombus formation on many biomaterials, and this is not surprising considering the highly beneficial effect of albumin coating in both *in vitro* and *ex vivo* experiments [1, 4, 31–34].

Platelet deposition on the PEO-grafted Dacron® sewing ring of a mechanical heart valve was not any better than that on the control surface. Platelet deposition on the control Dacron® sewing ring was $0.09 \pm 0.06\%$ of the total radioactivity of ^{111}In-labeled platelets injected into the blood. On the other hand, platelet deposition on the PEO-grafted sewing ring was $0.21 \pm 0.21\%$. Some PEO-grafted sewing

rings had much less thrombus formation, but the others showed significantly more thrombus. PEO grafting either reduced thrombus formation to an insignificant level or made the surface much worse than the control surface. These contradicting results are expected to be due to the factors other than PEO grafting, since the effect of PEO grafting, whatever it may be, should be consistent.

DISCUSSION

The *in vitro* testing of fibrinogen adsorption and platelet adhesion has been traditionally used for examining potentials of PEO-grafted biomaterials as well as biomaterials grafted with other molecules. Using the two parameters as the criteria is theoretically sound, since fibrinogen adsorption and platelet adhesion are the first events occurring upon exposure of biomaterials to blood. PEO-grafted biomaterials have been tested *in vitro* using the two parameters in our study. Fibrinogen adsorption and platelet adhesion on most of the PEO-grafted materials have been reduced by more than 90%. As shown in Table 1, PEO grafting always resulted in significant reduction of fibrinogen adsorption and platelet adhesion. Such good *in vitro* results, however, were not always translated into good *in vivo* performances of the biomaterials. As shown in Table 2, PEO-grafting reduced thrombus formation by more than 90% in a porcine extracorporeal arteriovenous shunt model. The effects of PEO grafting on glass, Silastic®, and polyethylene were not quite obvious, probably due to the low thrombogenic properties of the control surfaces. No effect of PEO grafting was observed on e-PTFE. This was mostly likely due to inadequate grafting of PEO to e-PTFE, as shown by no reduction of fibrinogen adsorption and platelet adhesion (Table 1). Animal experiments of PEO-grafted Dacron® fabric also showed no consistent, beneficial effect of PEO grafting. The comparison of the results of three different *ex vivo/in vivo* experiments strongly suggests that the *ex vivo/in vivo* performances of PEO-grafted biomaterials can be influenced by factors other than those related to PEO grafting, such as the density, chain length, and flexibility of the grafted PEO.

The lack of correlation between *in vitro* results and *ex vivo/in vivo* performances is not easy to understand. One way of finding the answer for this question may be to compare the experimental conditions between *in vitro* and *ex vivo/in vivo* experiments. *In vitro* experiments usually do not include shear flow, but this does not seem to be a factor, since the thrombus formation on the nitinol stents was reduced by PEO grafting even when they were tested under shear flow condition in pigs. This also eliminates the possibility of any potential effects of blood shear flow on the grafted PEO molecules, such as conformational change and subsequent repulsive property of the grafted PEO molecules. One important difference in experimental conditions is the presence of anticoagulants in most *in vitro* experiments. In *ex vivo/in vivo* experiments, use of anticoagulants varies from laboratory to laboratory. Since anticoagulants have significant effects on platelet activation, it is something to be examined further in more detail. In clinical

practices, surface-induced thrombosis can be minimized by using antiplatelet agents. For example, the stent thrombosis rate after intracoronary stenting is very low with the use of antiplatelet agents [35, 36]. The acute stent thrombosis under high shear flow conditions was also inhibited in a dose-dependent manner by monoclonal antibody to platelet GP IIb/IIIa receptor [37, 38] or GP IIb/IIIa antagonists [39]. These observations suggest that the biomaterial surface may not be the sole origin for the generation of mural thrombi. In addition to the surface-induced platelet activation leading to thrombus formation, it is highly likely that thrombi formed in the bulk blood adsorb to the surface.

The surface-grafted PEO monolayers can effectively prevent adsorption of proteins and individual platelets, as shown in Table 1 and by numerous experiments in the literature. The PEO monolayer, however, may not exert enough repulsive force to prevent adhesion of platelet aggregates that are formed in the bulk blood. The attractive interaction between platelet aggregates and the surface, which increases linearly with the size of aggregates, may be too large for the grafted PEO monolayer. In our previous study, we have shown that PEO chains can have attraction with hydrophobic surfaces [40, 41]. Others have also shown that PEO has affinity, even though weak, to the hydrophobic surface [42]. Platelet aggregates, due to their large size, can push the grafted PEO layer to the surface resulting in attractive interactions between collapsed PEO chains and the hydrophobic surface.

It is not too difficult to find out the sources of platelet aggregation in the bulk blood. Many mediators of platelet activation and aggregation accumulate at sites of vascular injury, including thromboxane A_2, serotonin, ADP, platelet activating factor, thrombin, oxygen-derived free radicals, and tissue factor [43]. Tissue injury has been related to thrombogenicity of implanted stents [44, 45]. Tissue damage resulting from surgical incision is a factor that has not been compared when the data of animal experiments are compared. The absence of any beneficial effect observed with mechanical heart valve in Table 2 may be a direct result of the tissue damage causing platelet aggregate formation in the blood. This can explain the platelet deposition to PEO-grafted sewing ring of a mechanical heart valve with such a large deviation. When the tissue damage is significant, it can override the effect of PEO grafting. Otherwise, we can still have the beneficial effect of PEO grafting. Excellent and moderate effect of PEO grafting was observed with nitinol stents and polymer shunts in Table 2 can be explained by the less tissue damage during the experimental procedures. The best method of testing biocompatibility of biomaterials is known to be testing them in *ex vivo/in vivo* experiments. However, if the surgical procedures are not controlled, i.e. if the tissue damages are not controlled, the results may vary and this makes comparison of the *ex vivo/in vivo* data difficult. The use of anticoagulants and/or antiplatelet agents also has to be carefully controlled to evaluate the pure material effect on thrombosis. Different agents inhibit platelet activation differently. Aspirin prevents platelet aggregation by inhibiting the cyclo-oxygenase enzyme, while ticlopidine interferes with ADP-dependent platelet activation [46]. Each of these agents can be effective in *ex vivo/in*

vivo studies only in response to specific aggregatory agents dependent on these mediators. Thus, depending on anticoagulants and/or antiplatelet agents used, the experimental results may be different.

Our hypothesis that the adhesion of platelet aggregates formed in the bulk blood is responsible for causing thrombus formation on PEO-grafted biomaterials surfaces, can explain the discrepancies between *in vitro* results and *ex vivo*/*in vivo* performances of many surface modified biomaterials. This hypothesis also provides a new insight into rational development of biomaterials. Biomaterials modified with PEO, albumin, heparin, and others have to be tested using platelet aggregates rather than individual platelets. This may more closely represent the situation in animal experiments. The surface modification approaches that have been applied to date need to be changed so that the repulsive forces of the grafted layer should be high enough to prevent adhesion of platelet aggregates. Theoretical calculations of the attractive interaction energies between the biomaterial surface and platelets/platelet aggregates would also provide valuable information for the development of better biomaterials.

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