Prevention of protein adsorption and platelet adhesion on surfaces by PEO/PPO/PEO triblock copolymers

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Fibrinogen adsorption and platelet adhesion on to dimethyldichlorosilane-treated glass and low-density polyethylene were examined. The surfaces were treated with poly(ethylene glycol) and poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide) triblock copolymers (Pluronics®). Poly(ethylene glycol) could not prevent platelet adhesion and activation, even when the bulk concentration for adsorption was increased to 10 mg/ml. Pluronics containing 30 propylene oxide residues could not prevent platelet adhesion and activation, although the number of ethylene oxide residues varied up to 76. However, Pluronics containing 56 propylene oxide residues inhibited platelet adhesion and activation, even though the number of ethylene oxide residues was as small as 19. Fibrinogen adsorption on the Pluronic-coated surfaces was reduced by more than 95% compared to the adsorption on control surfaces. The ability of Pluronics to prevent platelet adhesion and activation was mainly dependent on the number of propylene oxide residues, rather than the number of ethylene oxide residues. The large number of propylene oxide residues was expected to result in tight interaction with hydrophobic dimethyldichlorosilane-treated glass and low-density polyethylene surfaces and thus the tight anchoring of Pluronics to the surfaces. The presence of 19 ethylene oxide residues in the hydrophilic poly(ethylene oxide) chains was sufficient to repel fibrinogen and platelets by the mechanism of steric repulsion.

Keywords: Copolymers, protein adsorption, fibrinogen, platelet adhesion, steric repulsion

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The biocompatibility of blood-contacting biomaterials is known to be determined by adsorption of plasma proteins and activation of platelets[1, 2]. Surface modifications of biomaterials appear to be beneficial in the prevention of protein adsorption and platelet adhesion[3, 4]. Surfaces treated with poly(ethylene oxide) (PEO), or poly(ethylene glycol) (PEG), have shown reduced protein adsorption and platelet adhesion[5, 6]. The protein-resistant character of PEO could be due to the lack of ionic charge and to its high hydrophilicity, which impart flexibility and mobility in the aqueous environment[7-9]. Nagaoka *et al.* showed a direct correlation between the chain length of PEO and its flexibility in aqueous medium, reaching a maximum with 100 residues of ethylene oxide (EO)[10, 11]. Terminally attached PEO can effectively repel other macromolecules from the surface by the mechanism of steric repulsion[12-14].

PEO-rich surfaces have been prepared by adsorption of high molecular weight PEO[15, 16], adsorption of PEO-containing amphiphillic copolymers[17-23], entrapment of PEO[24, 25], and covalent grafting of PEO on to the surface[26]. Merrill and Salzman[27] prepared polyurethanes with PEO soft segments and showed a significant reduction in protein adsorption and platelet adhesion. Other investigators have also shown a significant reduction in platelet deposition on PEO-grafted polyurethanes[28, 29]. Improved efficiency of haemodialysis and blood compatibility was achieved by grafting PEO on to cellulosic membranes[30].

Surface coating of PEO-containing amphipathic copolymers is a simple and versatile approach for preparing protein-resistant biomaterial surfaces. Commercially available poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide) (PEO/PPO/PEO) triblock copolymers (Pluronics®, BASF) have been used in chemical, agricultural and pharmaceutical industries as non-ionic polymeric surfactants[31]. In certain medical applications which involve blood contact, the copolymers have been found to be completely non-toxic and do not exhibit any haemolysis of red blood cells[32]. The PPO segment of the copolymer is known to adsorb on to hydrophobic surfaces by hydrophobic interactions, whilst the water-soluble PEO chains extend into the bulk aqueous medium[33-36].

In this study, we examined the relative importance of PEO and PPO chains of Pluronic copolymers in prevent-

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ing fibrinogen adsorption and platelet adhesion. Dimethyldichlorosilane-treated glass (DDS-glass) and low-density polyethylene (LDPE) were treated with PEG and PEO/PPO/PEO triblock copolymers. Surface-induced platelet activation was quantitated using two numerical parameters, circularity and spread area of activated platelets.

MATERIALS AND METHODS

Preparation and characterization of surfaces

Glass coverslips (25 × 75 mm, No. 1, Bellco, Vineland, NJ, USA) were cleaned with 2% (v/v) Isoclean (Isolab, Akron, OH, USA) for 3 h at 60°C. The cleaned coverslips were rinsed in copious amounts of deionized distilled water and dried. These clean glass coverslips were treated with 5% (v/v) dimethyldichlorosilane (Sigma, St Louis, MO, USA) in chloroform for 2 h at room temperature. DDS-glass was twice washed with chloroform and ethanol in sequence, then with deionized distilled water. DDS-glass was then dried in an oven at 60°C overnight. LDPE films (NHLBI DTB Primary reference material, ABIOMED, Danvers, MA, USA) were washed with 2% Isoclean solution for 3 h at room temperature. The films were then rinsed with deionized distilled water and air-dried.

To determine the surface fibrinogen concentration using radiolabelled fibrinogen, glass tubing (inside diameter 2.50 mm, Kimble, Vineland, NJ, USA) and LDPE tubing (inside diameter 3.17 mm, Intramedic, Parsipanny, NJ, USA) were used instead of coverslips and films, respectively. The tubing was also cleaned as described above.

Underwater contact angles were measured using a contact angle goniometer (Rame-Hart, Mountain Lakes, NJ, USA) attached with an immersion chamber[37, 38]. Air-water-surface and octane-water-surface static bubble contact angles were measured. Ten measurements on different surface regions of DDS-glass coverslips were made. Polar (γ^{p}_{sv}) and dispersive (γ^{d}_{sv}) components of the surface-free energy (γ_{sv}) and the surface-water interfacial energy (γ_{sw}) were calculated using the harmonic mean approximation method[39]. The accurate measurements of underwater contact angles on LDPE film was difficult since the film was flexible.

Preparation of polymer solutions

PEG (mol wt 10 000; Aldrich, Milwaukee, WI, USA) was dissolved in phosphate buffered saline (PBS, pH 7.4) to prepare 10 mg/ml solution. Ten different types of Pluronics were obtained from the Performance Chemical Division of BASF Corporation (Parsipanny, NJ, USA). The properties of Pluronic copolymers used in this study are listed in *Table 1*. Pluronic copolymers were dissolved in PBS to prepare solutions of appropriate concentrations. The bulk concentrations of Pluronics were varied from 0.01 to 10 mg/ml.

Preparation of fibrinogen solution

Human fibrinogen (Type I, Sigma) was purified by the method of Laki[40]. Purified fibrinogen in PBS was stored in aliquots at −70°C at a concentration of about 2 mg/ml. The concentration of fibrinogen was determined by UV absorbance at 280 nm using the specific absorptivity[41, 42] of 1.506×10^3 cm^2/g. To quantitate the surface fibrinogen concentration, fibrinogen was labelled with ^{125}I (Amersham, Arlington Heights, IL, USA) using Enzymobead reagent (Bio-Rad, Rockville Center, NJ, USA).

Table 1 Properties of Pluronic® block copolymers[a]

Pluronic	Physical state	Average mol wt	EO/PO/EO	Thickness of adsorbed layer (nm)[b]
L-63	Liquid	2650	10/30/10	1.0 ± 0.4
L-64	Liquid	2900	13/30/13	-
P-65	Paste	3400	19/30/19	4.8 ± 0.6
F-68	Solid	8350	76/30/76	6.0 ± 0.6
P-103	Paste	4950	19/56/19	-
P-104	Paste	5850	28/56/28	-
P-105	Paste	6500	37/56/37	-
F-108	Solid	14 000	129/56/129	11.8 ± 1.5
P-84	Paste	4200	22/38/22	-
P-123	Paste	5750	19/69/19	-

[a]Obtained from Reference 31.
[b]The thickness of Pluronic® layer adsorbed on to polystyrene latices (diameter 0.305 μm) measured by photon correlation spectroscopy (Reference 19).

Quantitation of adsorbed fibrinogen

PEG and Pluronics were allowed to adsorb on DDS-glass and LDPE tubings for 1 h at room temperature. Non-adsorbed polymers were removed from the surface by rinsing with at least 50 ml of PBS. Fibrinogen was adsorbed at the bulk concentration of 0.1 mg/ml for 1 h at room temperature on to PEG-treated or Pluronic-treated surfaces. Excess fibrinogen was removed by rinsing with PBS. The surface fibrinogen concentration was determined by measuring the radioactivity of ^{125}I-labelled fibrinogen using a gamma counter (Gamma 5500B, Beckman, Arlington Heights, IL, USA). Eight samples from two independent experiments were used for the calculation of the surface fibrinogen concentrations.

Preparation of platelet suspension

Blood was obtained in heparinized containers (Vacutainers®, Becton-Dickinson, Rutherford, NJ, USA) from healthy adult volunteers after informed consent. All volunteers were kept free of aspirin or other drugs that might interfere with platelet functions. Heparinized blood was centrifuged at 100**g** for 10 min to prepare platelet-rich plasma (PRP). In some cases, the PRP was further centrifuged at 1000**g** for 20 min to obtain platelet-poor plasma (PPP). The final platelet suspension was obtained by diluting PRP with PPP in a ratio of 1:4. The dilution was made to avoid platelet aggregation on the surface and to evaluate the morphology of individual platelets.

Platelet adhesion and activation

A flow chamber was used to introduce polymers, fibrinogen and platelets on to the surface. The chamber consisted of DDS-glass coverslip or LDPE film, a spacer

and a microscope slide (25 × 75 mm, Fisher, Chicago, IL, USA) as described previously[43]. PEG (bulk concentration: 10 mg/ml) was introduced and allowed to adsorb for 1 h at room temperature. Platelets in diluted PRP were then allowed to spread for 1 h on PEG-treated surfaces.

DDS-glass and LDPE were treated with Pluronics at the bulk concentration of 0.1 mg/ml. After 1 h at room temperature, excess Pluronic was rinsed with PBS and platelets in plasma were allowed to spread on the Pluronic-treated surfaces. The surfaces were also treated with Pluronics and fibrinogen, either sequentially or competitively. In sequential adsorption, the surfaces were treated with Pluronics at the bulk concentration of 0.1 mg/ml for 1 h, followed by fibrinogen at the bulk concentration of 0.1 mg/ml for 1 h at room temperature. In competitive adsorption, Pluronics and fibrinogen were mixed to make the final concentration of 0.1 mg/ml each. The protein–surfactant mixture was adsorbed for 1 h at room temperature. Non-adsorbed Pluronics and fibrinogen were removed from the surface by rinsing with PBS. Platelets were allowed to spread on these surfaces for 1 h at room temperature.

Platelets which were adherent to the surface were fixed with 2% (v/v) glutaraldehyde (E.M. Grade, Polysciences, Warrington, PA, USA) in PBS for 1 h and stained with 0.1% (w/v) Coomassie Brilliant Blue (Bio-Rad) for 1.5 h at room temperature. The stained platelets were observed with a video microscope (Diaphot, Nikon, Garden City, NY, USA). The images of adherent platelets were transferred through a video processor (Colorado Video, Boulder, CO, USA) to a computer image analyser (Imaging Technology, Woburn, MA, USA). The circularity and spread area of platelets were quantitated using software obtained from Computer Imaging Applications (Madison, WI, USA).

As platelets adhere and activate on thrombogenic surfaces, the spread area increases. For a fully activated platelet, the spread area is usually above 40 μm^2. The circularity of partially activated platelets depended on the number and length of pseudopods extended from the platelets. Circularity (C) is defined by the following equation:

$$C = P^2/4\pi A$$

where P and A are the perimeter and spread area of an activated platelet, respectively. Circularity has a limiting value of 1.0 for a perfectly circular platelet and increases with the complexity of the platelet shape. For a fully activated platelet which has retracted all the pseudopods and become fully circular[43], the circularity is usually <2.0. The total number of platelets examined for each sample ranged from 30 to 80 depending on the type of surface involved.

Scanning electron microscopy

Platelets in PRP were allowed to spread for 1 h at room temperature on DDS-glass and LDPE surfaces which were treated with PEG, Pluronic F-68 (76/30/76) and Pluronic F-108 (129/56/129). Non-adsorbed polymers were removed by rinsing with PBS. Surface adherent platelets were fixed with 2% (v/v) glutaraldehyde in PBS for 2 h. The samples were then dehydrated with serial dilutions of ethanol (25–100%) and dried by critical point drying (CPD 020, Balzers, Arlington Heights, IL, USA) using CO_2 as a transitional fluid. The specimens were then sputter-coated with gold–palladium and examined with a JSM-850 (Jeol, Peabody, MA, USA) scanning electron microscope using 10 kV of accelerating voltage.

RESULTS

Contact angle measurements

Contact angles were measured on control DDS-glass and DDS-glass treated with PEG, Pluronic F-68 (76/30/76) or Pluronic F-108 (129/56/129). *Table 2* shows the results of contact angle measurements and the calculated surface-free energies. The contact angles and surface-free energies of the control DDS-glass did not change significantly with the PEG treatment. The treatment of Pluronic F-68 on DDS-glass, however, caused 37% increase in the γ^p_{sv} and 12% decrease in the γ^d_{sv} of the surface-free energy. The same result was observed with DDS-glass treated with Pluronic F-108. DDS-glass became more hydrophilic by treating with Pluronic F-68 or F-108. It should be noted that the difference in the surface-free energies between Pluronic F-68- and Pluronic F-108-treated DDS-glasses was not significant. The platelet reactivity, however, was significantly different between Pluronic F-68- and F-108-treated surfaces.

Scanning electron microscopy (SEM) observations

Figure 1 shows SEM micrographs of platelets which were adherent and spread in the presence of plasma on control DDS-glass (*Figure 1a*), and DDS-glass treated with PEG (*Figure 1b*), Pluronic F-68 (*Figure 1c*) and Pluronic F-108 (*Figure 1d*). Platelets adhered and activated fully on DDS-glass. Platelets covered the surface completely and formed mural microthrombi.

Table 2 Contact angles (Θ) and surface-free energies (γ)

Surface treatment	Θ_{air} (Degree)	Θ_{octane} (Degree)	γ^p_{sv} (Dynes/cm)	γ^d_{sv} (Dynes/cm)	γ_{sv} (Dynes/cm)	γ_{sw} (Dynes/cm)
DDS-glass	75.6 ± 3.4[a]	98.3 ± 2.1	13.9	24.3	38.1	21.0
PEG-treated DDS-glass[b]	73.3 ± 3.7	93.7 ± 2.1	15.3	23.9	39.2	18.9
F-68-treated DDS-glass[b]	60.8 ± 2.9	78.3 ± 3.7	21.9	21.4	43.2	11.4
F-108-treated DDS-glass[b]	59.8 ± 2.7	76.8 ± 4.9	22.3	21.4	43.7	10.9

[a]Average ± SD.
[b]The polymer was adsorbed at the bulk concentration of 10 mg/ml for 1 h at room temperature.
The surface was washed with PBS before contact angle measurements.

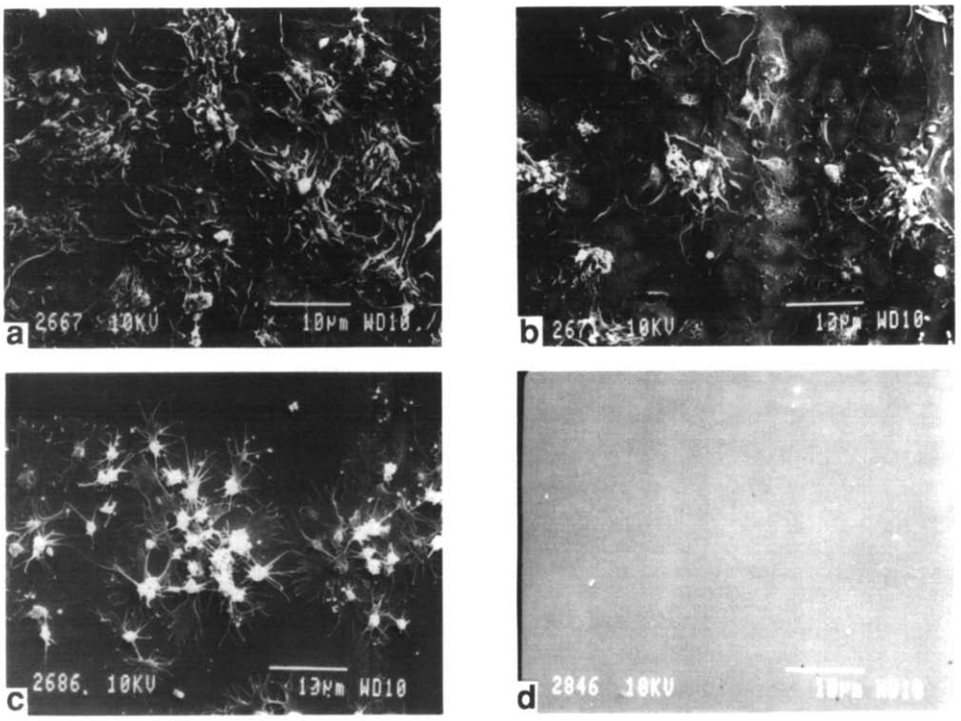

Figure 1 Scanning electron micrographs of platelets spread on **a,** control DDS-glass; **b,** DDS-glass treated with PEG; **c,** DDS-glass treated with Pluronic® F-68 (76/30/76); **d,** DDS-glass treated with Pluronic® F-108 (129/56/129). PEG and Pluronics® were adsorbed at the bulk concentration of 10 mg/ml for 1 h at room temperature.

PEG-treated DDS-glass also allowed platelets to adhere and activate in a similar way to that on control DDS-glass. It is likely that hydrophilic PEG either does not adsorb at all or cannot bind tightly to the hydrophobic DDS-glass, and thus platelets can easily displace adsorbed PEG from the surface. This will allow direct interaction of platelets with the DDS-glass. Pluronic F-68-treated DDS-glass showed platelets which were only partially activated and retained their pseudopods. Some platelets had adhered to the partially spread platelets. Although platelets were not able to activate fully, significant numbers of platelets were observed on the Pluronic F-68-treated surface. Pluronic F-108-treated DDS-glass did not, however, allow platelets to adhere at all.

Figure 2 shows SEM micrographs of platelets spread on control LDPE (*Figure 2a*) and LDPE treated with PEG (*Figure 2b*), Pluronic F-68 (*Figure 2c*) and Pluronic F-108 (*Figure 2d*). Platelet activation on the LDPE surface was similar to that on DDS-glass. Platelets activated completely on control LDPE with the formation of numerous microthrombi. The number of platelets and the extent of platelet activation on PEG-treated LDPE were similar to those on control LDPE. No preventive effect of PEG on platelet activation was observed. It appeared that platelets could easily displace PEG molecules from LDPE, or else PEG did not adsorb to the surface. On Pluronic F-68-treated LDPE, all the platelets were only contact-adherent and formed aggregates. The number of adherent platelets was significantly less than that on control LDPE. Platelets could not adhere at all on Pluronic F-108-treated LDPE. Pluronic F-108 was most effective in preventing platelet adhesion and activation on LDPE, as well as on DDS-glass.

Platelet activation on Pluronic-treated surfaces

Table 3 shows the results of platelet activation on DDS-glass and LDPE which were treated with PEG and Pluronics. On control DDS-glass and LDPE, platelets were fully activated, as indicated by the low circularity values of 1.44 and 2.07 and high spread area of 47.7 and 42.3 μm^2, respectively. Platelets also adhered and activated extensively on PEG-treated DDS-glass and LDPE. The low circularity values and high spread area on PEG-treated surfaces indicated that platelets activated fully to a circular shape. The extent of platelet activation on PEG-treated surfaces was similar to those on control DDS-glass and LDPE. Platelets were only contact-adherent and could not spread, as shown by the small spread area. The spread area was only about 11 μm^2 on DDS-glass and about 17 μm^2 on LDPE. When DDS-glass and LDPE were treated with Pluronics containing 56 propylene oxide (PO) residues, platelets could not adhere at all. We noted that even 19 EO residues were enough to prevent platelet adhesion if the number of PO residues increased to 56.

Figure 3 shows the surface fibrinogen concentration as a function of the bulk Pluronic concentration used for adsorption on DDS-glass (*Figure 3a*) and LDPE (*Figure 3b*) tubing. The number of PO residues was fixed at 30 and the number of EO residues was varied from 10 to 76. The adsorption of Pluronic on surfaces was followed by the adsorption of radiolabelled fibrinogen. After Pluronics were adsorbed at 0.1 mg/ml concentration, the surface fibrinogen concentrations on DDS-glass decreased to 0.35 $\mu g/cm^2$ for L-63, 0.26 $\mu g/cm^2$ for L-64, 0.31 $\mu g/cm^2$ for P-65 and 0.20 $\mu g/cm^2$ for F-68. A similar

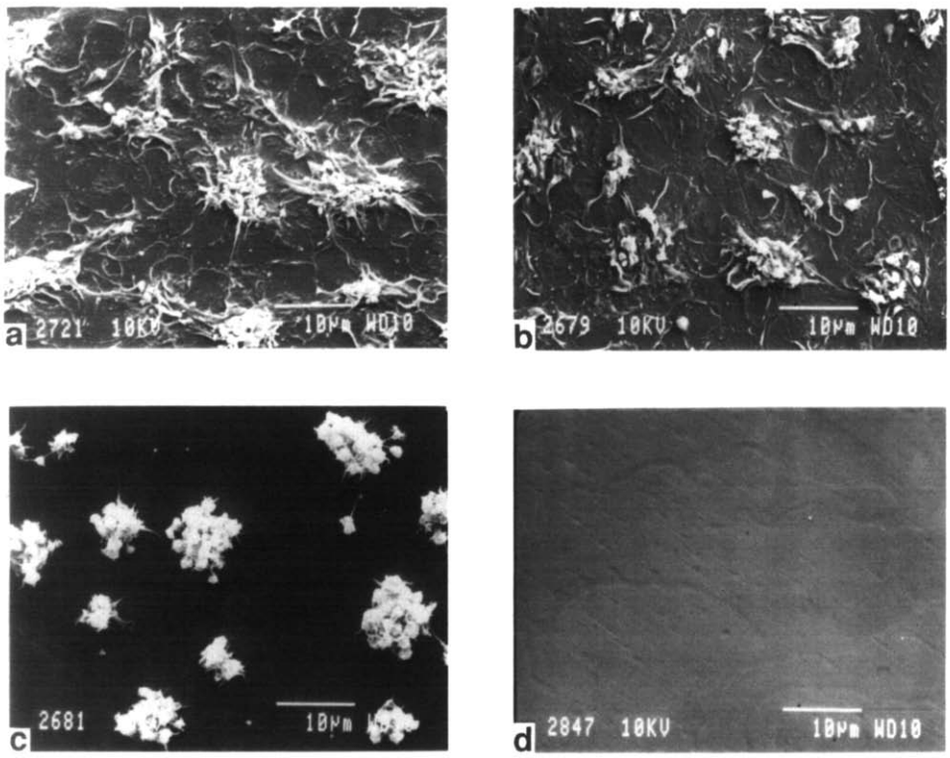

Figure 2 Scanning electron micrographs of platelets spread on **a,** control LDPE; **b,** LDPE treated with PEG; **c,** LDPE treated with Pluronic® F-68 (76/30/76); **d,** LDPE treated with Pluronic® F-108 (129/56/129). PEG and Pluronics® were adsorbed at the bulk concentration of 10 mg/ml for 1 h at room temperature.

Table 3 Platelet activation on Pluronic®-treated DDS-glass and LDPE

Surface treatment	Circularity		Spread area (μm^2)	
	DDS-glass	LDPE	DDS-glass	LDPE
Control[a]	1.44 ± 0.03	2.07 ± 0.22	47.7 ± 3.00	42.3 ± 2.65
PEG[b]	1.58 ± 0.06	1.50 ± 0.03	42.3 ± 2.26	43.6 ± 2.71
L-63 (10/30/10)[c]	1.97 ± 0.15	1.92 ± 0.08	12.5 ± 0.97	14.6 ± 1.16
L-64 (13/30/13)	1.90 ± 0.13	2.04 ± 0.09	11.3 ± 0.73	14.4 ± 1.15
P-65 (19/30/19)	1.98 ± 0.10	2.23 ± 0.14	10.3 ± 0.87	16.1 ± 1.92
F-68 (76/30/76)	2.06 ± 0.13	1.77 ± 0.06	11.8 ± 0.89	19.4 ± 1.90
P-103 (19/56/19)		NA[d]		
P-104 (28/56/28)		NA		
P-105 (37/56/37)		NA		
F-108 (129/56/129)		NA		

[a]Surfaces without pre-adsorbed Pluronics® or fibrinogen.
[b]PEG dissolved in PBS at 10 mg/ml was adsorbed for 1 h at room temperature.
[c]Pluronic® was adsorbed at the bulk concentration of 0.1 mg/ml for 1 h at room temperature.
[d]No adhesion of platelets at all.

trend was observed on LDPE at higher Pluronics concentrations. When the bulk Pluronic concentration for adsorption was increased to 5 mg/ml, the surface fibrinogen concentrations on DDS-glass and LDPE were 0.26 $\mu g/cm^2$ and 0.06 $\mu g/cm^2$ for L-63, 0.26 $\mu g/cm^2$ and 0.03 $\mu g/cm^2$ for L-64, 0.14 $\mu g/cm^2$ and 0.08 $\mu g/cm^2$ for P-65 and 0.17 $\mu g/cm^2$ and 0.02 $\mu g/cm^2$ for F-68, respectively. The effect of Pluronics on the prevention of fibrinogen adsorption was more pronounced on LDPE than on DDS-glass.

Figure 4a shows the surface fibrinogen concentration as a function of the bulk Pluronic concentration used for adsorption on DDS-glass. The number of PO residues was 56 and the number of EO residues was varied from 19 to 129. Pluronics and fibrinogen were adsorbed sequentially on to DDS-glass or LDPE. The reduction in the surface fibrinogen concentration in the presence of Pluronics containing 56 PO residues was much greater than that observed with Pluronics containing 30 PO residues (*Figure 3*). After Pluronic was adsorbed at 0.1 mg/ml concentration on DDS-glass, the adsorption of fibrinogen decreased significantly. The surface fibrinogen concentrations were 0.02 $\mu g/cm^2$ for L-103, 0.02 $\mu g/cm^2$ for P-104, 0.02 $\mu g/cm^2$ for P-105 and 0.06 $\mu g/cm^2$ for

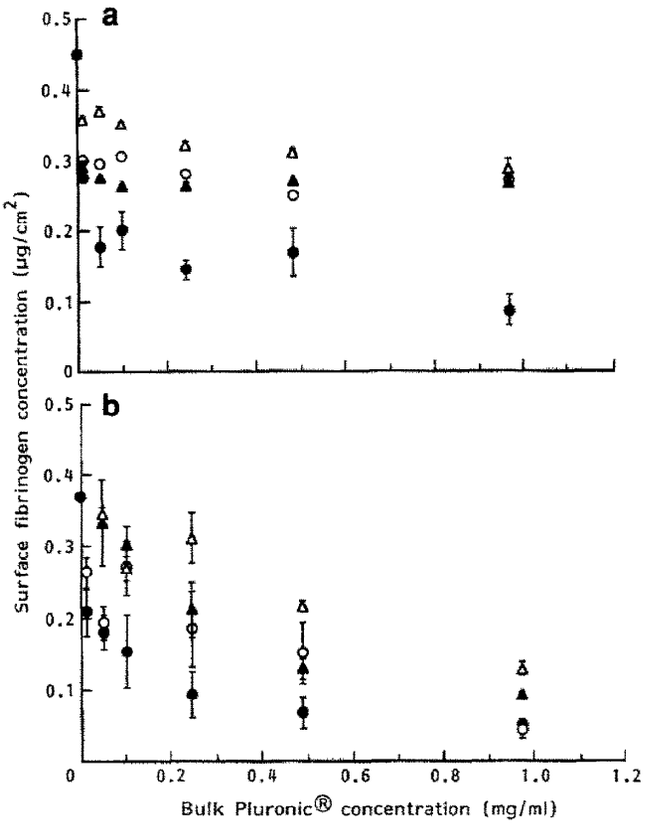

Figure 3 The surface fibrinogen concentration on **a**, DDS-glass and **b**, LDPE tubing as a function of the bulk Pluronic concentration used for adsorption. Radiolabelled fibrinogen at the bulk concentration of 0.1 mg/ml was adsorbed on Pluronic®-treated surfaces for 1 h at room temperature. The surface fibrinogen concentrations on control DDS-glass and LDPE were 0.45 and 0.37 $\mu g/cm^2$, respectively. The number of PO repeating units was 30 and the number of EO residues varied from 10 to 76. L-63 (10/30/10) (△), L-64 (13/30/13) (▲), P-65 (19/30/19) (○), F-68 (76/30/76) (●).

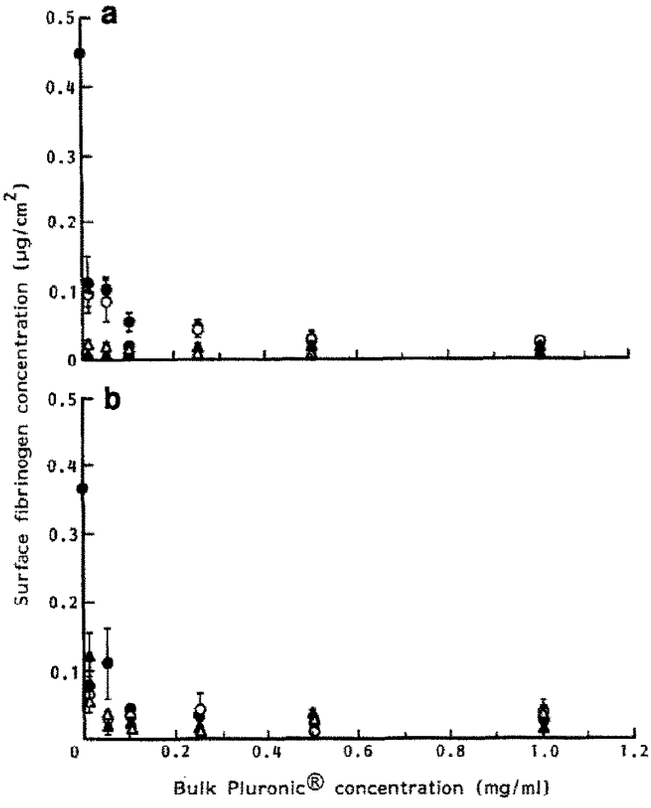

Figure 4 The surface fibrinogen concentration on **a**, DDS-glass and **b**, LDPE tubing as a function of the bulk Pluronic® concentration used for adsorption. Radiolabelled fibrinogen at bulk concentration of 0.1 mg/ml was adsorbed on Pluronic®-treated surfaces for 1 h at room temperature. The surface fibrinogen concentrations on control DDS-glass and LDPE were 0.45 and 0.37 $\mu g/cm^2$, respectively. The number of PO repeating units was 56 and the number of EO residues varied from 19 to 129. P-103 (19/56/19) (△), P-104 (28/56/28) (▲), P-105 (37/56/37) (○), F-108 (129/56/129) (●).

F-108. A more than 95% decrease in the surface fibrinogen concentration was observed, with pre-adsorption of Pluronics at the bulk concentration of only 0.1 mg/ml. When the bulk Pluronic concentration was increased to 5 mg/ml, the surface fibrinogen concentrations were 0.01 $\mu g/cm^2$ or less for all four Pluronics examined.

On LDPE, the surface fibrinogen concentration also decreased significantly by pre-adsorption of Pluronics containing 56 PO residues, as shown in *Figure 4b*. At 0.1 mg/ml bulk Pluronic concentration, the surface fibrinogen concentrations were 0.02 $\mu g/cm^2$ for P-103, 0.03 $\mu g/cm^2$ for P-104, 0.03 $\mu g/cm^2$ for P-105 and 0.05 $\mu g/cm^2$ for F-108. Once again, a more than 90% decrease in the surface fibrinogen concentration was noted, with pre-adsorption of Pluronics only at 0.1 mg/ml concentrations. The surface fibrinogen concentrations were 0.02 $\mu g/cm^2$ or less for all four Pluronics when the bulk Pluronic concentration was 5 mg/ml. It is interesting to note that P-103 with 19 EO residues was as effective as F-108 with 129 EO residues in preventing the adsorption of fibrinogen to the surface.

The data on platelet activation after sequential adsorption of Pluronics followed by fibrinogen are shown in *Table 4*. Platelets adhered and activated on DDS-glass and LDPE which were sequentially treated with Pluronics containing 30 PO residues and fibrinogen. The extent of platelet activation on Pluronic-treated DDS-glass was significantly different from that on control DDS-glass. The average platelet circularity was 2.6, whilst the spread area was around 32 μm^2. This indicates that platelets were partially activated, with several short pseudopods. The circularity values of platelets on LDPE were <2.0 and the area was >40 μm^2 with the exception of the F-68-treated surface. The results suggest that platelets were able to spread fully to a circular shape on LDPE. *Table 4* also shows that Pluronics with 56 PO residues were able to prevent platelet adhesion completely. No platelets could adhere, regardless of number of the EO residues. Pluronic P-103 with only 19 EO residues was as effective as Pluronic F-108, which has 129 EO residues. The data on platelet adhesion agree well with the data of fibrinogen adsorption in *Figure 4*.

Table 5 shows the data of platelet activation after competitive adsorption of Pluronics and fibrinogen from a Pluronic–fibrinogen mixture. A competitive adsorption study provides information on the component, which adsorbs preferentially on to the surface. The results of platelet activation after competitive adsorption between

Table 4 Platelet activation after sequential adsorption of Pluronics® and fibrinogen on DDS-glass and LDPE

Surface treatment	Circularity		Spread area (μm^2)	
	DDS-glass	LDPE	DDS-glass	LDPE
Control[a]	1.44 ± 0.03	2.07 ± 0.22	47.7 ± 3.00	42.3 ± 2.65
Fibrinogen[b]	1.55 ± 0.12	1.61 ± 0.03	40.3 ± 2.28	43.8 ± 2.75
L-63 (10/30/10)[c]	2.32 ± 0.15	1.46 ± 0.04	31.1 ± 1.70	49.5 ± 3.57
L-64 (13/30/13)	2.56 ± 0.23	1.45 ± 0.05	32.6 ± 1.90	40.4 ± 3.00
P-65 (19/30/19)	2.31 ± 0.16	1.51 ± 0.03	34.4 ± 2.20	44.2 ± 2.92
F-68 (76/30/76)	2.99 ± 0.29	1.96 ± 0.08	29.3 ± 2.20	33.8 ± 3.20
P-103 (19/56/19)		NA[d]		
P-104 (28/56/28)		NA		
P-105 (37/56/37)		NA		
F-108 (129/56/129)		NA		

[a]Surfaces without pre-adsorbed Pluronics® or fibrinogen.
[b]Fibrinogen in PBS (0.1 mg/ml) was adsorbed for 1 h at room temperature.
[c]Pluronics® was adsorbed at the bulk concentration of 0.1 mg/ml for 1 h at room temperature.
The surface was then exposed to fibrinogen solution at the bulk concentration of 0.1 mg/ml for 1 h at room temperature.
[d]No adhesion of platelets at all.

Table 5 Platelet activation after competitive adsorption of Pluronics® and fibrinogen on DDS-glass and LDPE[a]

Surface treatment	Circularity		Spread area (μm^2)	
	DDS-glass	LDPE	DDS-glass	LDPE
Control	1.44 ± 0.03	2.07 ± 0.21	47.7 ± 3.00	42.3 ± 2.65
Fibrinogen	1.55 ± 0.12	1.61 ± 0.03	40.3 ± 2.28	43.8 ± 2.75
L-63 (10/30/10)[a]	3.38 ± 0.35	1.68 ± 0.09	26.5 ± 2.40	36.1 ± 5.38
L-64 (13/30/13)	3.30 ± 0.38	1.71 ± 0.04	26.2 ± 2.00	44.4 ± 2.52
P-65 (19/30/19)	2.74 ± 0.26	1.58 ± 0.06	33.7 ± 3.40	44.0 ± 3.28
F-68 (76/30/76)	2.97 ± 0.27	1.54 ± 0.04	28.2 ± 2.30	46.3 ± 3.07
P-103 (19/56/19)		NA[b]		
P-104 (28/56/28)		NA		
P-105 (37/56/37)		NA		
F-108 (129/56/129)		NA		

[a]The bulk concentration was 0.1 mg/ml for both Pluronics® and fibrinogen. Adsorption was carried out for 1 h at room temperature.
[b]No adhesion of platelets at all.

Pluronics containing 30 PO residues and fibrinogen were similar to those of platelet activation after sequential adsorption, given in *Table 4*. This suggests that fibrinogen was able to adsorb to the surface in the presence of Pluronics with 30 PO residues. Fibrinogen was, however, unable to elicit platelet adhesion in the presence of Pluronics with 56 PO residues. The extent of platelet activation on DDS-glass was different from that on LDPE. The circularity values of platelets on DDS-glass treated with Pluronics and fibrinogen were around 3.0 and the spread areas were around 30 μm^2. The circularity values >2.0 and rather low spread area is explained by the fact that platelets were partially spread and several short pseudopods were still extended. On LDPE, the extent of platelet activation after competitive adsorption of Pluronics and fibrinogen was complete. The circularity values were <2.0 and the spread areas were >40 μm^2. This indicates that the platelets retracted all their pseudopods and became fully spread to a circular shape. Pluronics containing 56 PO residues were effective in preventing platelet adhesion, even after the competitive adsorption with fibrinogen. The hydrophobic surfaces appeared to have a higher affinity to the 56 PO residues of Pluronics than to fibrinogen molecules.

Effects of PPO chain length on platelet activation

The results presented above suggest that the number of PO residues, rather than the number of EO residues, is more important in the prevention of fibrinogen adsorption and platelet adhesion to DDS-glass or LDPE. The effect of PPO chain length was examined in more detail by varying the number of PO residues in Pluronics from 30 to 69 while keeping the EO residues at approximately 20.

Figure 5a shows the surface fibrinogen concentration as a function of the bulk Pluronic concentration on DDS-glass. It is clear that the decrease in the surface fibrinogen concentration is a function of the number of PO residues of Pluronics. At 0.1 mg/ml bulk Pluronic concentration, the surface fibrinogen concentrations decreased from 0.45 $\mu g/cm^2$ to 0.31 $\mu g/cm^2$ for P-65, 0.19 $\mu g/cm^2$ for P-84, 0.02 $\mu g/cm^2$ for P-103 and 0.03 $\mu g/cm^2$ for P-123. When the bulk Pluronic concentration was 5 mg/ml, the surface fibrinogen concentrations were 0.14 $\mu g/cm^2$, 0.10 $\mu g/cm^2$, 0.01 $\mu g/cm^2$ and 0.02 $\mu g/cm^2$ for P-65, P-84, P-103 and P-123, respectively. The surface fibrinogen concentration as a function of the bulk Pluronic concentration on LDPE is shown in *Figure 5b*. The general trend in the decrease of the surface

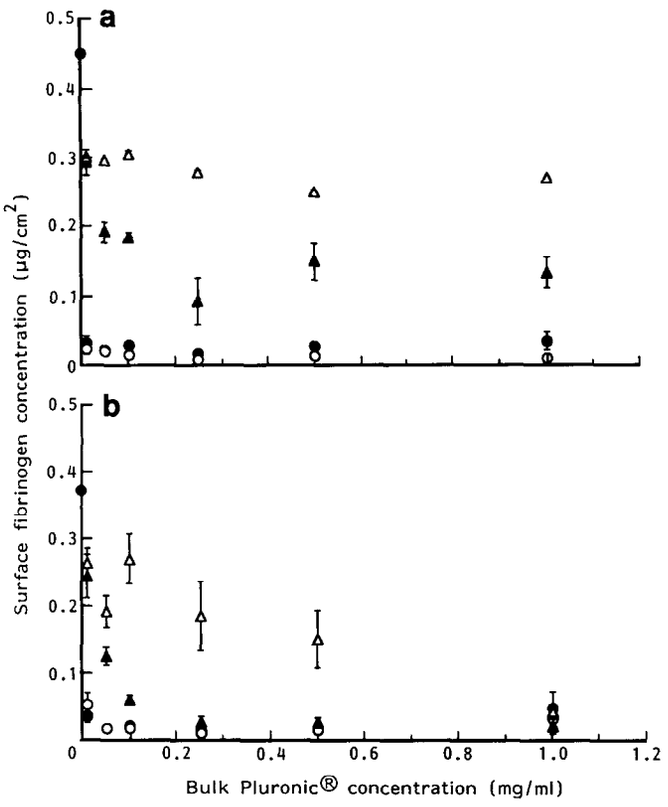

Figure 5 The surface fibrinogen concentration on **a**, DDS-glass and **b**, LDPE tubing as a function of the bulk Pluronic® concentration used for adsorption. Radiolabelled fibrinogen at the bulk concentration of 0.1 mg/ml was adsorbed on Pluronic®-treated surfaces for 1 h at room temperature. The surface fibrinogen concentrations on control DDS-glass and LDPE were 0.45 and 0.37 $\mu g/cm^2$, respectively. The number of EO residues was 19 or 22 and the number of PO residues varied from 30 to 69. P-65 (19/30/19) (△), P-84 (22/38/22) (▲), P-103 (19/56/19) (○), P-123 (19/69/19) (●).

fibrinogen concentration on LDPE was similar to that on DDS-glass. The effect of Pluronic P-65 was, however, more pronounced on LDPE than on DDS-glass. At a bulk Pluronic concentration of 0.1 mg/ml, the surface fibrinogen concentrations were 0.27 $\mu g/cm^2$ for P-65, 0.10 $\mu g/cm^2$ for P-84 and 0.02 $\mu g/cm^2$ for P-103 and P-123. At 5 mg/ml bulk Pluronic concentration, the surface fibrinogen concentrations decreased to 0.08 $\mu g/cm^2$ for P-65 and 0.02 $\mu g/cm^2$ or less for P-84, P-103 and P-123. *Figure 5* shows that as the number of PO residues increases, Pluronics become more efficient in preventing fibrinogen adsorption. Little difference, however, was observed in the prevention of fibrinogen adsorption, if the numbers of PO residues were 56 or larger.

Table 6 shows the results of platelet adhesion and activation on DDS-glass and LDPE surfaces treated with Pluronics and fibrinogen, either sequentially or competitively. Platelets were able to adhere and activate on both DDS-glass and LDPE if both surfaces were treated with Pluronic P-65. The extent of platelet activation was significantly different. The circularity of 2.31 and the spread area of 34.4 μm^2 on DDS-glass indicated that platelets had not spread fully and the pseudopods were not completely retracted. The pseudopod retraction, however, was complete on LDPE, as shown by the low circularity of 1.80. Although platelets became circular, the spread area was much smaller than that on control surface. As the PO number increased to 38, the spread areas of platelets adherent on the P-84-treated surfaces were only about 16 μm^2 or less. This suggests that platelets were only contact-adherent and could not spread. The results of competitive adsorption were similar to those of sequential adsorption. Platelets could not adhere to the surfaces treated with P-103 or P-123.

The results in *Table 6* suggest that the interaction of Pluronics with hydrophobic DDS-glass and LDPE surfaces became stronger as the number of PO residues in Pluronics increased. When the number of PO residues was >56, the interaction between Pluronic molecules and the surface becomes strong enough to withstand the external forces exerted by platelets. Once Pluronics are anchored tightly to the surface, the PEO chains with 19 EO residues are sufficient to repel fibrinogen and platelets.

DISCUSSION

We examined the effect of PEG homopolymer and Pluronics on the prevention of protein adsorption and platelet adhesion. Platelets could adhere easily and activate on DDS-glass and LDPE if the surfaces were coated with PEG homopolymer, even though the bulk PEG concentration was increased to 10 mg/ml. However, if the surfaces were coated with Pluronics, the adhesion and activation of platelets was prevented, depending on the type of Pluronic used. Thus, we examined the relative importance of PEO and PPO chain length of Pluronics to gain insight into the mechanisms of improved biocompatibility of the PEO-rich surfaces.

The affinity of PEO homopolymers adsorbed on to hydrophobic surfaces depends on the molecular weight of the polymer. Only high molecular weight PEO (mol wt >100 000) are known to adsorb effectively on to hydrophobic surfaces[44]. PEG-10 000 used in our study was not effective in preventing platelet adhesion, as the low molecular weight PEG could not interact strongly with hydrophobic surfaces. PEG either remains in the bulk aqueous solution or interacts weakly with the hydrophobic surfaces. Platelets were able to displace weakly-bound PEG molecules from the surfaces. Luckham[45] measured the steric repulsion forces between mica surfaces coated with PEO homopolymers (mol wt 160 000) or PEO/poly(methyl methacrylate) (PMMA) copolymers. Steric repulsion due to PEO-PMMA copolymers with only 17 EO residues was stronger than that of PEO homopolymers. When PEG molecules are terminally grafted on the surface, even small molecular weight PEG is effective in steric repulsion.

We employed Pluronics to anchor PEO chains terminally to the hydrophobic surfaces. Pluronics are known to anchor on hydrophobic surfaces by PPO segment, whilst the PEO chains are extended into the bulk aqueous solution[35, 36]. The strength of the hydrophobic interaction is a function of the chain length of the PPO segment in the copolymer. When DDS-glass or LDPE was adsorbed with Pluronics containing 30 PO residues, platelets were still able to adhere to the surface. This is probably because Pluronics with 30 PO residues

Table 6 Effects of PPO chain length on platelet activation on DDS-glass and LDPE[a]

Surface treatment	Circularity		Spread area (μm^2)	
	DDS-glass	LDPE	DDS-glass	LDPE
Control	1.44 ± 0.03	2.07 ± 0.22	47.7 ± 3.00	42.3 ± 2.65
Fibrinogen	1.55 ± 0.12	1.61 ± 0.03	40.3 ± 2.28	43.8 ± 2.75
Sequential adsorption[b]				
P-65 (19/30/19)	2.31 ± 0.16	1.80 ± 0.19	34.4 ± 2.20	33.8 ± 3.74
P-84 (22/38/22)	3.67 ± 0.78	1.86 ± 0.08	16.1 ± 2.70	11.0 ± 2.98
P-103 (19/56/19)		NA[c]		
P-123 (19/69/19)		NA		
Competitive adsorption[d]				
P-65 (19/30/19)	2.74 ± 0.26	1.80 ± 0.09	33.7 ± 3.40	35.4 ± 3.25
P-84 (22/38/22)	4.36 ± 0.50	1.79 ± 0.12	21.7 ± 1.80	16.9 ± 4.43
P-103 (19/56/19)		NA		
P-123 (19/69/19)		NA		

[a]The bulk concentration was 0.1 mg/ml for both Pluronics® and fibrinogen.
[b]Pluronics® was adsorbed for 1 h at room temperature followed by fibrinogen adsorption for 1 h at room temperature.
[c]No platelet adhesion at all.
[d]Pluronics® and fibrinogen were mixed before adsorption. The mixture was adsorbed for 1 h at room temperature.

could not anchor tightly to hydrophobic DDS-glass and LDPE surfaces. The surface fibrinogen concentrations on DDS-glass and LDPE tubing treated with Pluronic containing 30 PO residues were significantly higher than those on surfaces treated with equivalent concentrations of Pluronics containing 56 PO residues (compare *Figures 3* and *4*).

The extent of platelet activation after sequential and competitive adsorption was dependent on the type of surface examined. Platelets were partially activated on DDS-glass, but were completely activated on LDPE. This is probably due to the fact that DDS-glass is more hydrophobic than LDPE, so Pluronics with 30 PO residues were able to bind more tightly to DDS-glass than to LDPE. As seen from the surface fibrinogen concentration data, there was no correlation between the number of EO residues in Pluronics containing 30 PO residues and the extent of platelet activation on either DDS-glass or LDPE. Platelets could not adhere at all on surfaces treated with only 0.1 mg/ml Pluronics containing 56 PO residues, although the number of EO residues varied from 19 to 129. The results of competitive adsorption in *Table 5* suggest that Pluronics with 56 PO residues have higher affinity to the surface than does fibrinogen.

Lee *et al.*[20-23] examined the protein-resistant characteristics of Pluronic-coated DDS-glass and LDPE using Pluronics with 29 or 30 PO residues. They suggested that longer PPO chain length would lead to self-aggregation of the Pluronic and might cause weak binding to the surface. Our results show, however, that longer PPO chain length was more effective in anchoring Pluronics to the surface. Tan and co-workers[18,19] also prepared protein-resistant surfaces by adsorption of Pluronics to polystyrene latices. Pluronic F-108 (129/56/129) was most effective in repelling fibrinogen and other proteins from the surface. Pluronic F-108 was also effective in preventing adhesion of cells on octadecyl glass[17]. On a Pluronic F-108-treated surface, between 97.0% and 99.5% of the initially adherent cells were removed by applying a shear stress of 0.03 N/m^2. On control surfaces, however, nearly all adherent cells remained on the surface at this shear stress.

Significant emphasis has been directed towards the correlation between the repulsive ability of PEO surfaces and the chain length of PEO. Nagaoka *et al.*[10,11] measured the mobility of PEO chains using the peak width of ^{13}C NMR signal. They found that increasing the PEO chain length to 100 residues resulted in the increased peak width of ^{13}C NMR signal, indicating high flexibility. The adsorption of blood proteins and platelet adhesion was minimized by randomly grafting PEO chains with 100 EO residues on to a methyl methacrylate backbone. Our results, however, showed that as long as the Pluronic copolymers are tightly bound to the hydrophobic surfaces, 19 EO residues were sufficient for the prevention of fibrinogen adsorption and platelet adhesion. Prime and Whitesides[46] showed that as long as PEO is bound tightly to a gold surface with PEO-alkanethiols only, 6 EO residues were sufficient in preventing protein adsorption. Our study suggests that the tight anchoring of Pluronic copolymers to the hydrophobic surfaces requires a PPO segment with more than 38 PO residues.

In summary, the adsorption of PEG homopolymer was not effective in preventing platelet adhesion and activation. The hydrophilic PEG molecules cannot bind tightly to the hydrophobic DDS-glass or to LDPE. Pluronics with 30 PO residues were not effective in preventing platelet adhesion, but were effective in preventing activation of adherent platelets. Platelets could not adhere at all on surfaces treated with Pluronic containing 56 PO residues. Pluronic P-103 (19/56/19) was as effective in repelling fibrinogen and platelets as was Pluronic F-108 (129/56/129). It appears, therefore, that tight binding to the surface is more important than the length of PEO segment. As long as strong binding to the surface is achieved, PEO can effectively prevent fibrinogen adsorption and platelet adhesion by steric repulsion. Strong binding to the surface can be achieved, either by hydrophobic interactions or through covalent bonding. Our current efforts are directed towards the covalent grafting of PEG to various biomaterials, including inert polymers such as polyethylene and polypropylene.

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