Synthesis of photoreactive poly(ethylene glycol) and its application to the prevention of surface-induced platelet activation

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Photoreactive poly(ethylene glycol) (PEG) was synthesized by reacting 4-fluoro-3-nitrophenyl azide (FNPA) with sodium salt of PEG. The synthesized 4-azido-2-nitrophenyl PEG (ANP-PEG) was characterized by ^{1}H-NMR, IR, and UV spectroscopy. ANP-PEG was grafted to dimethyldichlorosilane-coated glass (DDS-glass) by photolysis without any premodification of the surface. The effects of various grafting factors, such as the polymer adsorption time, concentration of ANP-PEG, and UV irradiation time, on the PEG grafting efficiency were examined. The PEG-grafted DDS-glass was characterized by measuring surface free energies, surface-induced platelet activation, and the relative amount of PEG grafted on the surface using electron spectroscopy for chemical analysis (ESCA). Platelet adhesion and activation was analyzed by measuring the number and spread area of adherent platelets. The results showed that ANP-PEG had to be adsorbed onto DDS-glass for at least 12 h before photolysis for the maximum grafting efficiency. No platelets could adhere to the PEG-grafted DDS-glass, if the bulk concentration of ANP-PEG in the adsorption solution was between 1 mg/mL and 10 mg/mL. Above 10 mg/mL, platelet activation gradually increased and reached the maximum at 30 mg/mL. Our data indicate that the grafting of ANP-PEG requires careful control of the grafting conditions and that the grafted PEG can prevent surface-induced platelet activation.

INTRODUCTION

Surface-induced thrombus formation is one of the main problems in the development of blood-contacting biomaterials. Because platelet adhesion and subsequent activation are mainly responsible for the thrombus formation, ideal biomaterials would be the ones which do not allow platelet adhesion at all. One approach to make such biomaterials is to graft water-soluble polymers to the surface.[1] It has been suggested that water-soluble polymers grafted to biomaterials can prevent platelet adhesion by steric repulsion.[2] Of the many water-soluble polymers, poly(ethylene oxide) (PEO) has been used most widely.[3] Physical adsorption,[4–6] graft copolymerization,[7–10] and graft coupling[11–13] have been used for the preparation of PEO surfaces. The latter two techniques result in PEO chains covalently bound to the surface. Those

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techniques, however, require chemically active groups on the surface. This limits the application of the techniques only to certain biomaterials. In addition, the grafting procedures are usually very complicated. One approach to overcoming such problems would be to introduce photoreactive coupling agents to PEO chains.

Carbenes and nitrenes are capable of attacking various chemical bonds including carbon–hydrogen bonds upon irradiation.[14,15] Among the precursors of carbenes and nitrenes, aryl azides are chemically stable and much less susceptible to photochemical rearrangement.[14] In this study, 4-fluoro-3-nitrophenyl azide (FNPA) was chosen as a photoreactive coupling agent. Dimethyldichlorosilane-coated glass (DDS-glass) was used as a model surface for poly(ethylene glycol) (PEG) grafting, since it does not have any functional groups and is highly thrombogenic.[16,17] Thus, DDS-glass allows direct observation of the platelet-resistant effect of the grafted PEG.

This report describes the synthesis and characterization of 4-azido-2-nitrophenyl PEG (ANP-PEG) along with the grafting procedure onto DDS-glass. The PEG-grafted surfaces were characterized by platelet activation, the surface free energies, and electron spectroscopy for chemical analysis (ESCA). The efficiency of PEG grafting in the prevention of platelet adhesion and activation was quantitated by measuring the number and spread area of adherent platelets.

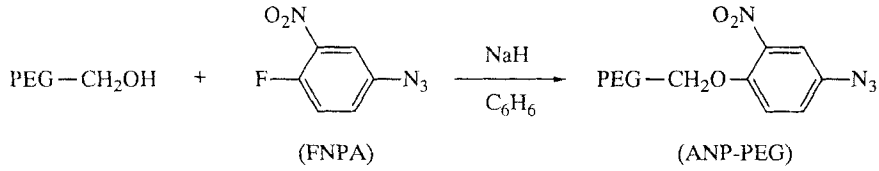
MATERIALS AND METHODS

Materials

PEG (MW = 3,400), sodium hydride (60% dispersion in mineral oil), and DDS were obtained from Aldrich Chemical Co. (Milwaukee, WI). FNPA was purchased from Sigma Chemical Co. (St. Louis, MO). Glass coverslips (22 × 22 mm, #1 thickness) were purchased from Fisher Scientific (Itasca, IL). Benzene (Aldrich, Milwaukee, WI) was dried over 4 Å molecular sieves and distilled before use.

Synthesis and characterization of ANP-PEG

FNPA has two functional groups: a photoreactive azido group and a chemically reactive fluoro group. ANP-PEG was synthesized by reacting sodium salt of PEG with FNPA as the following scheme.



PEG was dissolved in dry benzene to make the final concentration of 6 mg/mL. The solution (30 mL) was added dropwise to a suspension sodium

hydride (44 mg) in 15 mL of dry benzene. After stirring for 1 h under nitrogen, the reaction mixture was filtered through sintered glass funnel. To this solution was added 364 mg of FNPA dissolved in 10 mL of dry benzene. The reaction was continued overnight at 40°C while stirring. The crude product was precipitated by adding 600 mL of ethyl ether. This precipitation process was repeated twice and the product was dried in vacuum. All procedures were carried out under subdued red light. The yield was 85%.

A sample of ANP-PEG for the nuclear magnetic resonance was prepared in D_2O (12 mg/mL). The ^{1}H-NMR spectrum was obtained using a BRUKER WM270 NMR Spectrometer (270 MHz). Sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 (TSP-D_4) dissolved in D_2O was used as an external reference for the determination of chemical shifts. The IR spectra of PEG and ANP-PEG were obtained with a Nicolet 20SXc Fourier-Transform Infrared System after fluorolube mulls were sandwiched between KBr plates.

FNPA (0.1 mg/mL) and ANP-PEG (1.7 mg/mL) were dissolved in methanol, placed in quartz cuvette (2-mm path length), and photolyzed with either a Blak-Ray lamp (366 nm, Model UVL-21) or a Mineralight lamp (254 nm, Model UVG-11) from a distance of 5 mm. Methanol was used as a solvent due to the insolubility of FNPA in water. Photolysis of ANP was monitored by recording the UV spectra of the FNPA solution and the ANP-PEG solution on UV-VIS Spectrophotometer (AVIV Model 14DS).

Grafting of ANP-PEG onto DDS-glass

Glass coverslips were cleaned by soaking in chromic acid overnight and washed extensively with running distilled water. They were further rinsed with deionized distilled water for 2 h at room temperature and then dried at 80°C overnight.

DDS-glass was prepared by immersing the clean coverslips in a solution of 5% DDS in chloroform for 30 min. The DDS-treated glass was rinsed with chloroform and methanol in sequence twice, and finally with water for 2 h before drying at 80°C.

Three different variables were examined to optimize the grafting efficiency of ANP-PEG onto DDS-glass. They were adsorption time, concentration of ANP-PEG solution, and irradiation time.

One milliliter of ANP-PEG in water was placed on DDS-glass for various time periods. The concentration of ANP-PEG ranged from 1 mg/mL to 100 mg/mL and the adsorption time was varied from 1 h to 24 h. The solution was irradiated at room temperature with a Blak-Ray lamp (366 nm) from a distance of 15 mm for various time ranging from 1 min to 10 min. After the irradiation, ungrafted PEG was removed by washing with water extensively.

Preirradiated ANP-PEG solution was also used for adsorption to DDS-glass. ANP-PEG preirradiated in the bulk solution before adsorption is not expected to be grafted onto the surface. Thus, the DDS-glass which was adsorbed with preirradiated ANP-PEG followed by washing will be called "PEG-treated DDS-glass." The ANP-PEG solution of 10 mg/mL was irradi-

ated for 10 min by using a Blak-Ray lamp (366 nm) before being placed on DDS-glass.

Platelet adhesion and activation

Preparation of platelet-rich plasma (PRP), perfusion chamber, and video microscopy for observing platelet activation was described in detail in our previous publication.[17]

Platelets in PRP were introduced into a perfusion chamber and allowed to adhere in the static state onto PEG-grafted, PEG-treated, and control DDS-glass at room temperature for 1 h. After the unadherent platelets were removed by washing with phosphate-buffered saline (PBS, pH 7.4), the adherent platelets were fixed with 2% glutaraldehyde in PBS for 15 min. The glutaraldehyde solution was replaced with PBS and the fixed platelets were then stained with 0.1% solution of Coomassie Brilliant Blue for 30 min. The stained platelets were observed with a video microscope and the number of adherent platelets were counted in 12 separate microscope fields for each time point. The area of platelets adherent on the surfaces were measured in 8 separated microscope fields for each time point using an image analyzer.[17]

SEM observation

Platelets on surfaces were fixed with 2% glutaraldehyde in PBS for 2 h at room temperature. The samples were dehydrated in a series of ethanol, and dried in a critical point drying apparatus (Union, Model CPD-020) using liquid CO_2 as a transition fluid. They were coated with Gold Pladium using Hummer 1 sputter coater, and examined with a JEOL JSM-840 Scanning Electron Microscope at an accelerating voltage of 10 KV.

ESCA analysis

The surface composition of PEG-grafted DDS-glass was analyzed by ESCA using a physical electronics Model 548 instrument with Mg $Ka_{1,2}$ radiation source. The photoelectron emission angle was 45°. Survey scans were carried out to determine the atomic composition of the surfaces. The high-resolution peaks for carbon 1s, oxygen 1s, and silicon 2*p* were normalized by dividing the area of each peak by the scofield cross section. The normalized values were then used to calculate the atomic ratios.

Contact angle measurements

Contact angles were measured with a Rame Hart Model A100 goniometer. Underwater contact angles of air and octane bubbles were measured to calculate the polar and dispersive components of the surface free energy and the

surface–water interfacial free energy using the harmonic mean equation.[18,19] Eight contact angles were measured for each sample and the mean value was determined from the measurements of four samples.

RESULTS

Characterization of ANP-PEG

The coupling of ANP to PEG was verified by ^{1}H-NMR, UV, and IR spectroscopy. The former two spectroscopies were used for the quantitative analysis of the ANP concentration in ANP-PEG. The ^{1}H-NMR spectrum of the synthesized ANP-PEG in Figure 1 shows well-defined signals at δ7.41 (2H) and δ7.73 (1H) resulting from the protons of the phenyl group of ANP bound to PEG. The proton peak from HDO impurity appeared at the usual position of δ4.80. The strong signal from PEG protons was seen at δ3.72. The small signals present on both sides of the PEG signal may be due to either the protons near the PEG chain ends or spinning side bands resulting from inhomogeneities in the magnetic field and in the spinning tube.[20] Since the signals from the protons of the benzene ring of PEG-bound ANP at δ7.41 was too small compared to the extremely strong signal from protons of PEG chains at δ3.72, the signal was amplified by a factor of 32. The integration of the signals gave an estimate of 55% of ANP-end in ANP-PEG. This means that on the average one PEG molecule has one ANP group because each PEG molecule has two ANP-binding sites.

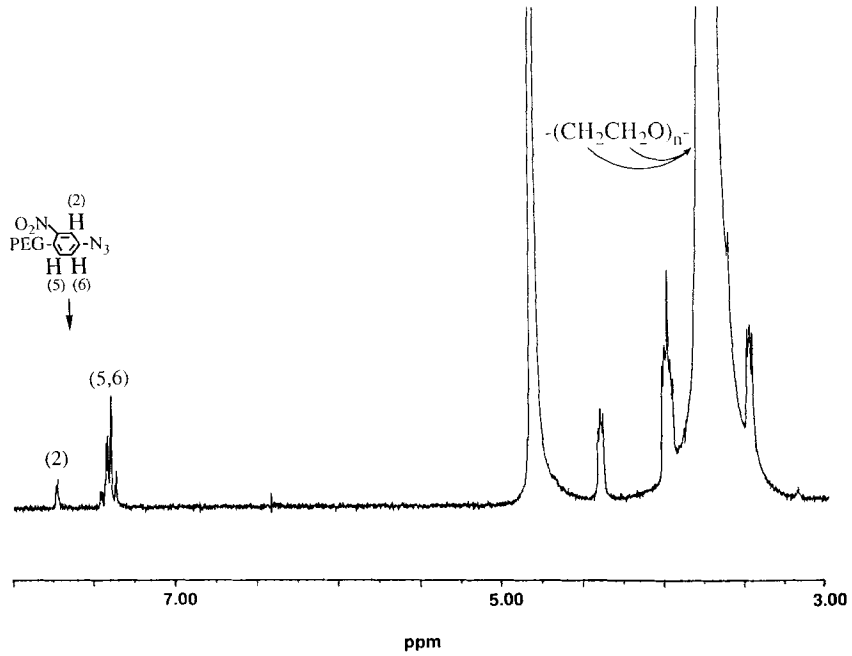


Figure 1. ^{1}H-NMR spectrum of ANP-PEG in D_2O. Signals at δ7.41 and δ7.73 are from ANP groups of ANP-PEG.

Figure 2 shows the IR spectra of PEG and ANP-PEG. The characteristic stretching band of azido group at 2117 cm^{-1} was seen on the ANP-PEG spectrum. Such a band was clearly absent on the control PEG spectrum. This indicated that azido group is still intact on the phenyl group of ANP after the reaction with PEG.

The UV spectrum of FNPA [Fig. 3(A)] showed a characteristic absorption band at 243 nm which disappeared after photolysis at 366 nm. As shown in Figure 3(A), the intensity of the FNPA band decreased gradually with an increase in irradiation time, and the absorption band almost disappeared after a 7-min exposure to the UV light. Figure 3(B), however, shows that in the case of ANP-PEG, a half of the band intensity was lost by the first 30-s exposure, and the absorption band almost disappeared after a 2-min exposure to the UV light. This indicates that photolysis proceeds more quickly after ANP is bound to PEG. As shown in Figure 3(B), ANP-PEG showed an absorption band at 250 nm. The binding of ANP to PEG caused a 7-nm shift of the peak position of the band (λ_{max}). In the spectral region between 200 and 300 nm, PEG itself did not show any absorption band. Although the λ_{max} shifted from 243 nm to 250 nm, the extinction coefficient was assumed to remain the same. The absorptivity of ANP was determined at 243 nm and used for the estimation of the ANP concentration in ANP-PEG. The calculated absorptivity of ANP was 1.93×10^3 $M^{-1} \cdot cm^{-1}$. The observed ANP/PEG-end ratio was 60% which was in good agreement with the NMR result.

ANP-PEG can also be photolyzed by UV light at 254 nm. Although the short-wave photolysis showed the same trend as the long-wave photolysis, it increased the rate of photolysis substantially. In this study 366 nm was chosen for the grafting of ANP-PEG because controlling the irradiation time and other experimental variables was easier with the long-wavelength UV light.

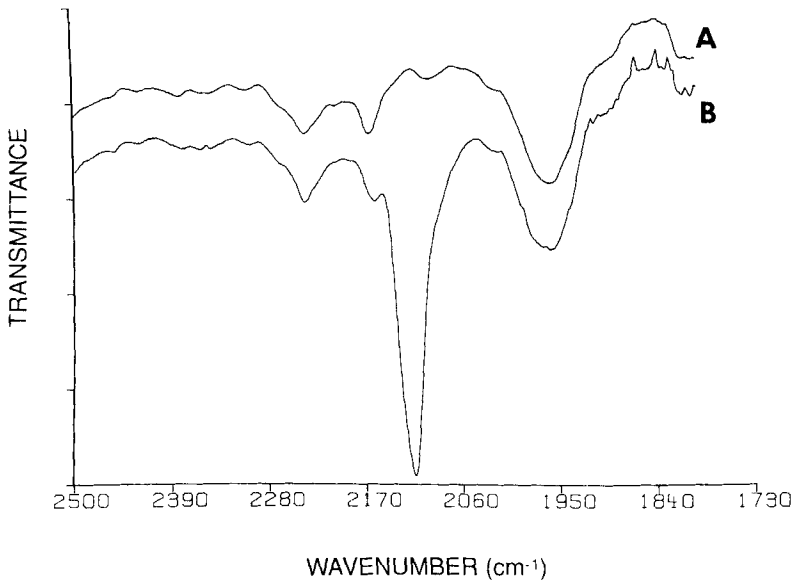


Figure 2. IR spectra of PEG (A) and ANP-PEG (B).

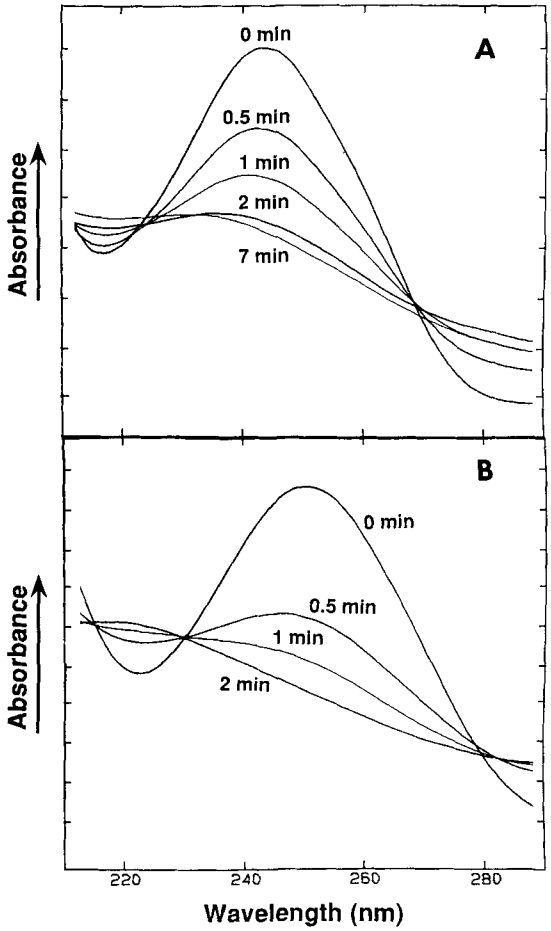


Figure 3. Photolysis of FNPA (A) and ANP-PEG (B) in methanol. Samples were irradiated with 366-nm UV light for various time periods before their UV spectra were recorded. The concentrations of FNPA and ANP-PEG were 0.1 mg/mL and 1.7 mg/mL, respectively.

In addition, long-wave UV light was expected to be selectively absorbed by the coupling reagent without affecting the bulk properties of the polymers.[21]

Effect of adsorption time on PEG grafting

The effect of the adsorption time of ANP-PEG on the extent of PEG grafting onto DDS-glass was examined. The grafting efficiency was evaluated indirectly by determining the extent of platelet adhesion and activation on the PEG-grafted surface. Platelet adhesion and activation was evaluated by determining the number and area of the adherent platelets (Fig. 4). As the time for adsorption of ANP-PEG was increased up to 12 h, the number of platelets adherent on the surface gradually decreased [Fig. 4(A)]. Above 12 h of adsorption, PEG grafting to the surface was such that only a few platelets could

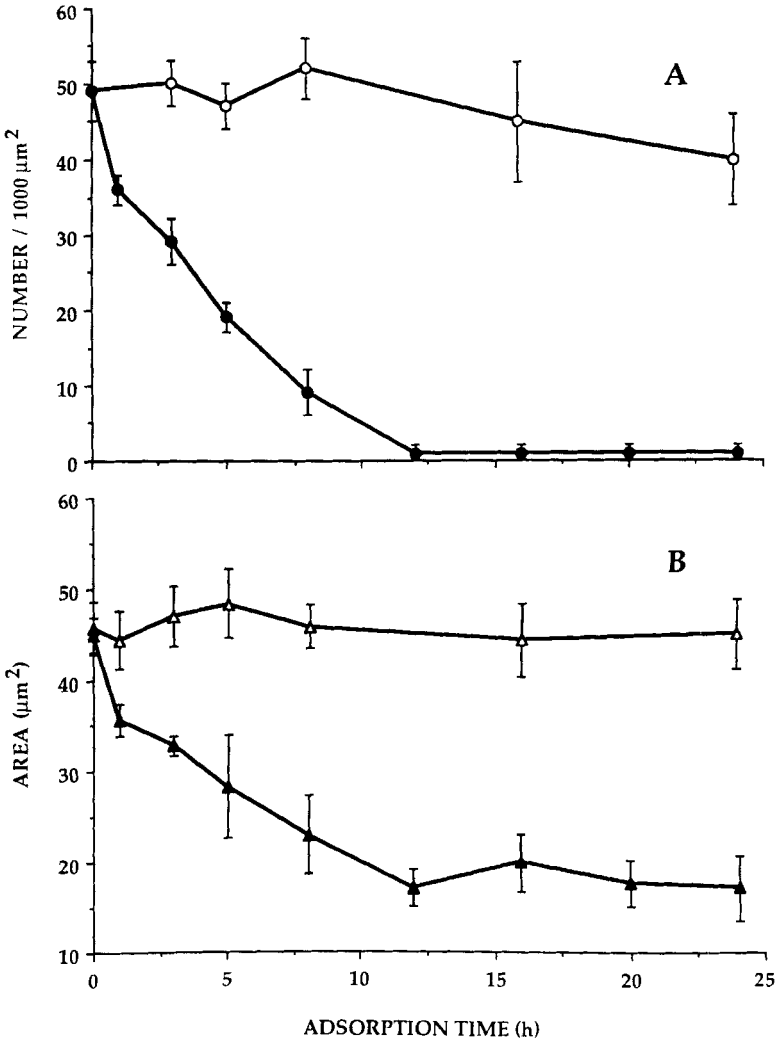


Figure 4. Changes in number (A) and area (B) of platelets adherent on PEG-grafted DDS-glass as a function of time for ANP-PEG adsorption. ANP-PEG (10 mg/mL) was adsorbed for various time periods and irradiated for 10 min at 366 nm (closed symbols). As a control, preirradiated ANP-PEG (10 mg/mL) was adsorbed for various time periods (open symbols). Platelets in PRP were allowed to adhere for 1 h at room temperature. Average ± SEM.

adhere to the DDS-glass. To examine the effect of physically adsorbed ANP-PEG on the inhibition of platelet adhesion and activation, DDS-glass was also adsorbed with preirradiated ANP-PEG. ANP-PEG in bulk solution was irradiated for 10 min before being adsorbed to DDS-glass. Preirradiated ANP-PEG solution was placed on DDS-glass up to 24 h before washing and exposure to platelets. DDS-glass which was treated with preirradiated ANP-PEG will be referred to as the "PEG-treated DDS-glass." The number of platelets adherent on the PEG-treated DDS-glass was not significantly differ-

ent from that of platelets on the control DDS-glass. Thus, it appears that the physically adsorbed PEG was removed from the surface by washing and thus cannot prevent platelet adhesion. The area of platelets also decreased as the time for ANP-PEG adsorption was increased [Fig. 4(B)]. The area of platelets spread on the control DDS-glass was larger than 40 μm^2 and the platelets were fully spread to a circular shape. As the time for ANP-PEG adsorption was increased, platelets were only partially spread and maintained many, long pseudopods. When ANP-PEG was adsorbed more than 12 h, adherent platelets could not spread at all on the surface and maintained discoidal shape. The area of adherent platelets remained below 20 μm^2. Platelets are not considered to be activated when their spread area is smaller than 20 μm^2.[17] The area of platelets spread on the PEG-treated DDS-glass was the same as that on the DDS-glass regardless of the adsorption time.

SEM observation

Figure 5 shows SEM photographs of platelets adherent on DDS-glass, PEG-treated DDS-glass, and PEG-grafted DDS-glass. For the preparation of PEG-grafted DDS-glass, ANP-PEG (10 mg/mL) was allowed to adsorb on DDS-glass for 12 h and then the surface was irradiated for 10 min. As a control, DDS-glass was also adsorbed with 10 mg/mL of preirradiated ANP-PEG for 12 h. Figure 5(A) shows fully activated platelets and small thrombi on the control DDS-glass. The platelet activation on the PEG-treated DDS-glass occurred to the same extent as that on the control DDS-glass as shown in Figure 5(B). On the PEG-grafted DDS-glass, however, platelets could not adhere at all. Only occasionally, a few contact adherent, discoidal platelets were observed [Fig. 5(C)].

Effect of ANP-PEG concentration on PEG grafting

Figure 6 shows the dependence of platelet adhesion and activation on the bulk concentration of ANP-PEG used for adsorption on DDS-glass. The bulk concentration of ANP-PEG ranged from 1 mg/mL to 100 mg/mL. ANP-PEG was adsorbed onto DDS-glass for 12 h before the surface was irradiated for 10 min. As shown in Figure 6(A) platelets could not adhere onto the surface if the bulk concentration of ANP-PEG was lower than 10 mg/mL. As the concentration became higher than 10 mg/mL, the number of adherent platelets increased with the concentration and reached a plateau at the concentration of 40 mg/mL. The spread area of adherent platelets also increased as the bulk concentration of ANP-PEG was increased [Fig. 6(B)]. The spread area reached a plateau when the concentration of ANP-PEG for adsorption was 30 mg/mL.

The effect of the bulk concentration of ANP-PEG on PEG grafting onto DDS-glass was also examined by ESCA. Because the amount of nitrogen atoms in ANP-PEG is relatively small compared to that of carbon atoms in PEG, the signal of nitrogen atoms on ESCA spectra of PEG-grafted DDS-glass was not

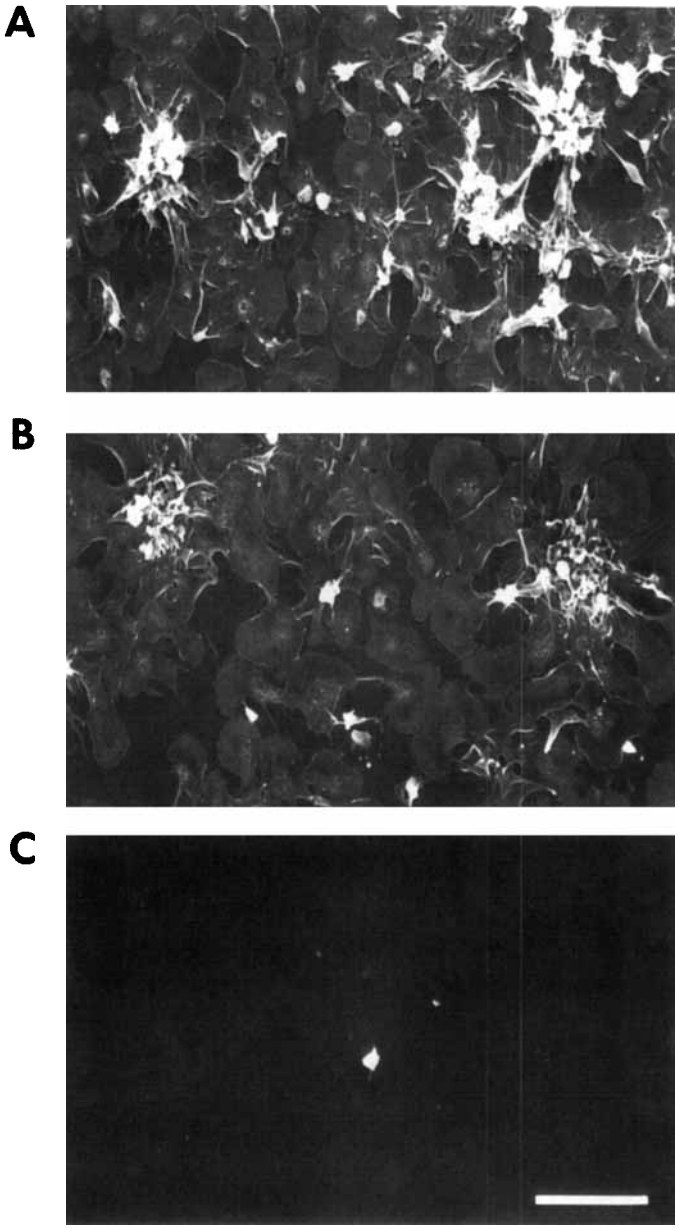


Figure 5. SEM photographs of platelets on DDS-glass (A), PEG-treated DDS-glass (B), and PEG-grafted DDS-glass (C). Platelets in PRP were allowed to interact with the surface for 1 h at room temperature (bar = 10 μm).

significant. Therefore, C1*s* spectra were chosen for the semiquantitation of the amount of PEG grafted on the surface. The C1*s* spectra of PEG-grafted DDS-glass were resolved into two distinct peaks at 285 eV and 286.5 eV, which corresponded to alkyl carbon and ether carbon, respectively. The intensity of the ether carbon was expected to be proportional to the amount of PEG grafted

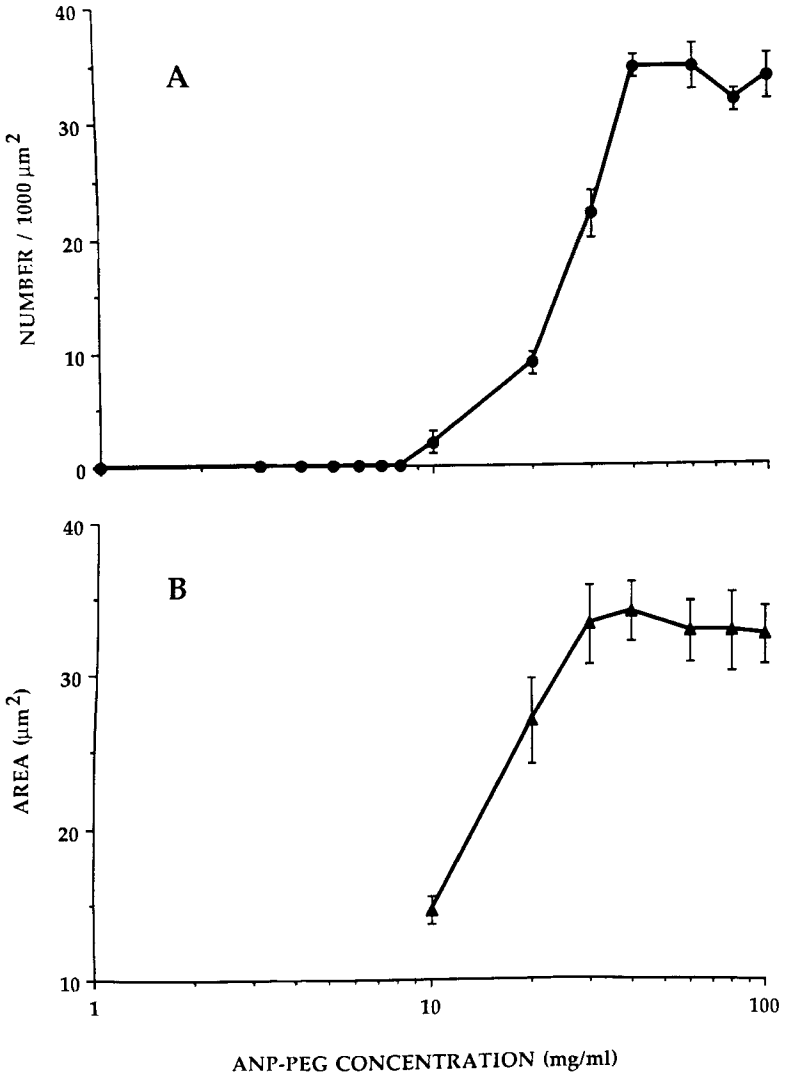


Figure 6. Changes in number (A) and area (B) of platelets adherent on PEG-grafted DDS-glass as a function of the bulk concentration of ANP-PEG used for adsorption. ANP-PEG was adsorbed for 12 h and irradiated for 10 min at 366 nm. Platelets in PRP were allowed to adhere for 1 h at room temperature. Average ± SEM.

on DDS-glass. The ether carbon/silicon atomic ratio was used to measure the relative amount of PEG grafted on the surface. The relative amount was calculated by dividing the ether carbon/silicon atomic ratio by 154, which is the number of ether carbon in the PEG molecule, and then by multiplying the value by 1000 as described by Lee et al.[22] Figure 7 shows the relative amount of PEG grafted on DDS-glass as a function of the bulk concentration of ANP-PEG used for adsorption. The grafted amount increased as the bulk concentration was increased up to 10 mg/mL. When the bulk concentration of ANP-PEG was increased further, the grafted amount decreased significantly.

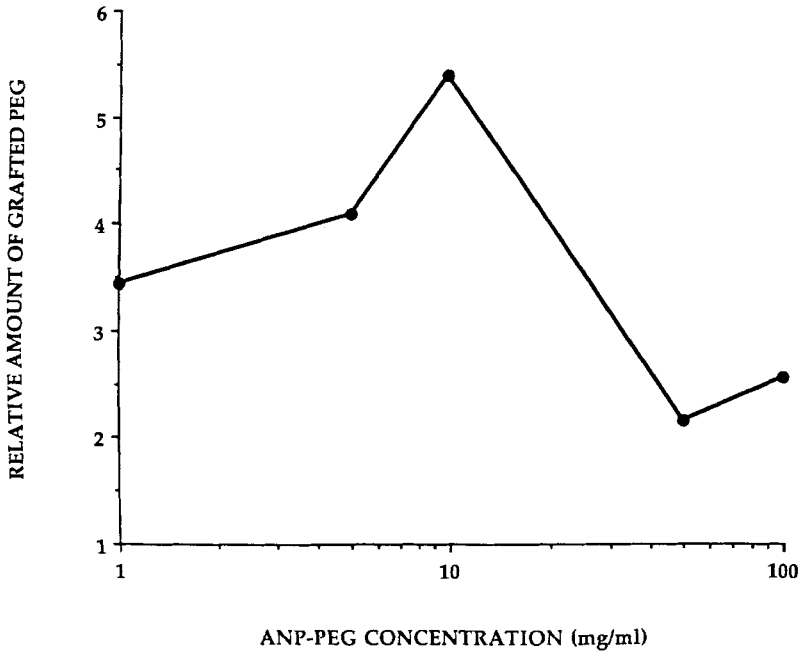


Figure 7. Relative amount of PEG grafted on DDS-glass as a function of the bulk concentration of ANP-PEG used for adsorption. ANP-PEG was adsorbed for 12 h and irradiated for 10 min at 366 nm.

The amount of PEG grafted at 50 mg/mL and 100 mg/mL of ANP-PEG was lower than that grafted at 1 mg/mL. The result in Figure 7 is in good agreement with the platelet activation result shown in Figure 6. The data in Figures 6 and 7 indicate that there is an inverse relationship between the amount of grafted PEG and platelet adhesion and activation. The grafting efficiency of ANP-PEG is clearly reduced as the bulk concentration for adsorption is increased above a certain level. Thus, higher concentration of ANP-PEG for adsorption does not necessarily result in higher grafting efficiency. It appears that ANP-PEG can be effectively grafted onto DDS-glass by photolysis at the bulk concentrations between 1 mg/mL and 10 mg/mL.

Effect of irradiation time on PEG grafting

The effect of UV irradiation time on PEG grafting onto DDS-glass was also examined. ANP-PEG was adsorbed on DDS-glass for 12 h from 10 mg/mL solution and then the surface was irradiated for various time periods. The relationship between platelet activation and irradiation time after the adsorption of ANP-PEG is shown in Figure 8. The number of platelets adherent on the surface decreased sharply by irradiation of ANP-PEG for only 2 min and then decreased slowly as irradiation time was increased further [Fig. 8(A)]. Most of platelets could not adhere on the surfaces when the ANP-PEG was

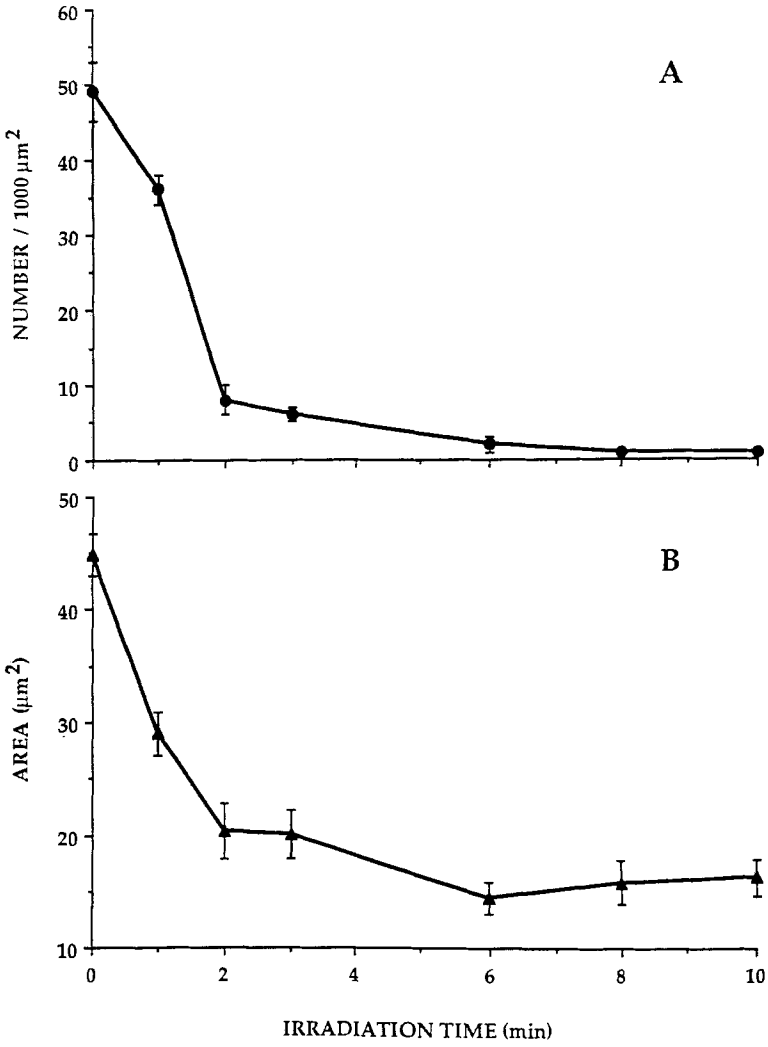


Figure 8. Changes in number (A) and area (B) of platelets adherent on PEG-grafted DDS-glass as a function of irradiation time. ANP-PEG (10 mg/mL) was adsorbed for 12 h and irradiated at 366 nm for various time periods. Platelets in PRP were allowed to adhere for 1 h at room temperature. Average ± SEM.

irradiated for 6 min or more. As shown in Figure 8(B), the area of platelets spread on DDS-glass was larger than 40 μm^2 and the platelets were fully spread. A dramatic decrease of the spread area was also observed with the first 2 min of irradiation of the adsorbed ANP-PEG. When the irradiation time was 6 min or longer, platelets were only contact adherent. Figure 8 shows that at least 6 min was required under our experimental condition for the maximum grafting of ANP-PEG on DDS-glass and subsequent prevention of platelet adhesion and activation.

Contact angle measurements

Contact angles were measured for the DDS-glass, PEG-treated DDS-glass, and PEG-grafted DDS-glass. Table I shows the result of the contact angle measurements and the calculated surface free energies. The differences in contact angles between the control DDS-glass and the PEG-treated DDS-glass were negligible. The contact angle of PEG-treated DDS-glass, however, was close to that of PEG-grafted DDS-glass, if the adsorbed PEG was not washed off before the measurement [Table I(c)]. This suggests again that PEG can not be tightly adsorbed to the hydrophobic DDS-glass and can be easily washed off, unless it is covalently linked to the surface. The PEG-grafted DDS-glass showed a lower surface–water interfacial free energy than the DDS-glass and the PEG-treated DDS-glass. Upon PEG grafting the polar component of the surface free energy (γ_{sv}^{p}) increased by 35% while the dispersive component (γ_{sv}^{d}) decreased by 15%. It is interesting to notice that the changes in surface free energy by PEG grafting is not as dramatic as the changes in platelet adhesion and activation.

DISCUSSION

Many studies suggested that grafting of water-soluble polymers to biomaterial surfaces improves the blood compatibility of the material.[1–10,23–26] To be practical, the grafting procedure should be simple and should not change the bulk properties of the materials. The use of photoreactive derivatives of water-soluble polymers can satisfy such requirements.

Photoreactive crosslinking agents have been used in various applications including prediction of subunit structures of molecules in cell membranes and identification surface receptors for macromolecular ligands.[27–29] They were also used for the modification of polymer surfaces.[30,31] Photoreactive derivative of PEG was used in this study to modify the surface of DDS-glass. The azido group of FNPA has a moderate inductive electron-withdrawing effect on the phenyl ring and is able to interact mesomerically with the phenyl ring.[32,33] Thus, the azido group is capable of activating both electrophilic and nucleophilic substitution. In addition, the nitro group on *ortho*-position exerts a great electron-withdrawing effect. Therefore, the fluoro group of FNPA can be displaced very easily by a nucleophilic substitution.[33] FNPA has specific binding ability to amino groups.[34] Thus, FNPA can react very easily with PEG if the hydroxyl end group of PEG is converted to amino group. This conversion, however, requires time-consuming reaction steps and the yield is not high. The conversion procedure usually requires more than 40 h of reaction and the yield of PEG with amino group is usually less than 60%.[35–38] For this reason, we used sodium salt of PEG which behaves like a nucleophilic species. The reaction developed in this study requires only a 9-h one-step reaction to prepare ANP-PEG. The yield of ANP-PEG is 85% and about 55% of PEG-ends are bound with ANP.

It was shown in Figure 3 that the substitution of fluoro groups with PEG increased the rate of photolysis. Although the mesomerical interaction of the

TABLE I
Contact Angles (θ) and Surface Free Energies (γ) of DDS-Glass, PEG-Treated DDS-Glass, and PEG-Grafted DDS-Glass

Surface	θ_{air} (degree)	θ_{octane} (degree)	γ_{sv}^{p} (dynes/cm)	γ_{sv}^{d} (dynes/cm)	γ_{sv} (dynes/cm)	γ_{sw} (dynes/cm)
DDS-glass	73 ± 2[a]	101 ± 2	12.8	33.1	45.9	24.9
PEG-treated DDS-glass[b]	73 ± 2	102 ± 2	12.5	34.6	47.1	26.0
PEG-treated DDS-glass[c]	66 ± 3	87 ± 2	18.0	27.5	45.5	16.1
PEG-grafted DDS-glass[d]	67 ± 3	89 ± 1	17.2	28.1	45.3	17.2

[a]Average ± SD.
[b]ANP-PEG (10 mg/mL) was irradiated for 10 min and then adsorbed for 12 h. The surface was washed before contact angle measurement.
[c]ANP-PEG (10 mg/mL) was irradiated for 10 min and then adsorbed for 12 h. The surface was not washed before contact angle measurement.
[d]ANP-PEG (10 mg/mL) was adsorbed for 12 h and then irradiated for 10 min. The surface was washed before contact angle measurement.

azido group with the phenyl ring is counteracted with the inductive effect of the azido group, electron release is still easy.[33] The azide of ANP which is attached to electron-donating *para*-substituent PEG appears to be less stable than that of FNPA.

Nitrenes, which can exist in either the singlet or the triplet ground state, can carry out a wide range of chemical reactions such as recombination, addition to multiple bonds, electrophilic attack, hydrogen abstraction, and direct insertion into O—H bonds, N—H bonds, and C—H bonds.[32,34] Since DDS-glass possesses only alkyl carbons, grafting to such a surface is limited to direct insertion into C—H bonds and hydrogen abstraction reaction. Direct intermolecular C—H bond insertion reactions are rare while intramolecular insertion reactions are common.[14] In addition, the photogenerated aromatic nitrenes from aromatic azides are known to be in their triplet ground state for hydrogen abstraction reaction if the photolysis is carried out in polymer matrices.[39,40] The photolysis of ANP-PEG on DDS-glass is expected to be similar to that in polymer matrices because DDS is known to form multilayers resembling a polymer matrix.[41] Therefore, hydrogen abstraction followed by coupling of radicals might be responsible for the covalent attachment of ANP-PEG to DDS-glass. It is thought that the triplet nitrene of ANP-PEG removes a hydrogen atom from the C—H bond of the DDS-glass to form an amino radical on ANP-PEG and a radical on the surface. Then, covalent bond may form by coupling the two radicals between ANP-PEG and DDS-glass. Thus, important factors in the grafting of ANP-PEG onto DDS-glass are the collision and adsorption between the ANP-end of ANP-PEG and the surface.

Figures 6 and 7 showed a relationship between grafting efficiency and the concentration of ANP-PEG solution. The inflection points in the two figures are around 10 mg/mL. Leermakers et al. have employed a dynamic scanning angle reflectometer to prove that the initial deposition rate of highly asymmetric copolymer chains onto a surface is faster than the time required for a chain to find its optimal conformations, when the surface is contacted with a certain concentration of the polymer solution. The rearrangements of the polymer chains from this thick polymer layer are very slow. On the contrary, the initial coverage at low concentration of the polymer allows for individual chain relaxation.[42] As the bulk concentration of ANP-PEG is higher than 10 mg/mL, the initial deposition rate of PEG chains onto the surface may be very high. This may lead to excess PEG concentration on the surface, and the movement of ANP-end to the surface may be limited. At low surface ANP-PEG concentrations, however, the ANP-end is expected to easily contact with the surface through individual chain relaxation. Once such a contact occurs, the ANP-end is expected to remain on the surface of DDS-glass due to the hydrophobic interaction.[43] This process may require about 12 h as shown in Figure 4.

Various water-soluble polymers, when grafted onto the surface, provide steric repulsion which does not allow adhesion of platelets.[2,7] Although the conformation of PEG grafted on DDS-glass is not known, it is highly likely that most of PEG molecules are grafted pendantly onto the surface, since on the average each PEG molecule has only one ANP-end. Nagaoka et al. showed

that PEO-grafted poly(vinyl chloride) reduces platelet adhesion significantly. Using NMR, they showed that the surface mobility of PEO chains is maximal when the number of repeating unit of PEO reaches 100.[7] The number of repeating units of PEG used in our study was about 77. The density of the PEG grafted onto the surface in our study does not seem to be very high since the surface–water interfacial free energy of the PEG-grafted DDS-glass is not very low (Table I). The PEG on the surface may inhibit adsorption of large fibrinogen molecules from plasma but allow adsorption of small albumin molecules. Thus, the surface fibrinogen concentration may not be high enough to cause platelet adhesion and activation.[16]

In summary, our study demonstrated that hydrophilic PEG can be grafted onto the inert, hydrophobic DDS-glass by a simple photolysis process, and that the PEG-grafted DDS-glass prevents platelet adhesion.

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