The minimum surface fibrinogen concentration necessary for platelet activation on dimethyldichlorosilane-coated glass

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Albumin and fibrinogen were competitively adsorbed onto dimethyldichlorosilane-coated glass (DDS-glass) and platelet activation was examined as a function of the surface fibrinogen concentration. The weight ratio of albumin to fibrinogen in the adsorption solution was varied from 10 to 700. Platelet activation was quantitated by the area and circularity of spread platelets. When the DDS-glass was coated with albumin alone, platelets were only contact adherent and could not spread at all. After competitive adsorption of fibrinogen and albumin, however, platelets were able to spread on the surface. Platelet activation increased linearly as the surface fibrinogen concentration increased up to 0.02 $\mu g/cm^2$. Platelets were able to activate fully if the surface fibrinogen concentration was 0.02 $\mu g/cm^2$ or higher, even though the surface was dominated by albumin. It appears that platelets can activate fully as long as only a small fraction (2–15%) of the surface is covered with tightly bound fibrinogen.

INTRODUCTION

Surface-induced thrombosis complicates the long-term functioning of prosthetic devices, such as prosthetic vascular grafts, artificial heart values, and hemodialysis membranes.[1–3] Thrombosis is a complex process involving predominantly platelets and selected platelet-adhesive proteins. It is generally accepted that the first event in the blood/biomaterial interaction is the adsorption of proteins onto the surface and that the nature of the adsorbed protein layer largely influences the mural thrombus formation.[4–6]

It is well known that fibrinogen-coated surfaces are thrombogenic while precoating with albumin makes all surfaces less reactive to platelets.[7,8] It is possible to inhibit acute thrombus formation for several hours or possibly for the first few days of implantation by treating surfaces with nonthrombogenic proteins such as albumin.[9–11] If the adsorption solution is heavily loaded with albumin or if the surface is exposed to this protein first, then the deposition of fibrinogen from solution is decreased and the platelet activation is reduced.[8,9,12–14] Thus, the amount of adsorbed fibrinogen has been often used as a parameter for thrombogenicity of biomaterials. The surface fibrinogen con-

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centration, however, does not always account for the variation in biocompatibility observed for many materials.[15,16] Lindon et al.[17] have shown that platelet adhesion is related to the amount of antibody-recognizable fibrinogen rather than the total amount of fibrinogen on biomaterials.

We examined the relevance of the surface fibrinogen concentration to the platelet activation. In our previous studies, we examined the ability of protein-coated surfaces to elicit platelet activation as a function of the surface concentrations of fibrinogen and albumin.[18,19] In those studies, proteins were preadsorbed from single protein solutions. In this study, we examined platelet activation on surfaces where fibrinogen and albumin were competitively adsorbed. The platelet activation was quantitated using two numeric values: spread area and circularity. Dimethyldichlorosilane-coated glass (DDS-glass) was used as a model surface for competitive protein adsorption, since albumin had the affinity to the surface high enough to effectively compete with fibrinogen for adsorption sites.[19]

EXPERIMENTAL

Surfaces

Glass coverslips (Bellco Glass, Vineland, NJ) were cleaned by soaking in 2% Isoclean (Isolab, Akron, OH) solution at 60°C for 3 h, washing extensively with distilled water, and drying at 80°C. Isoclean concentrate is composed of potassium hydroxide (<%), octylphenoxypolyethoxyethanol (2%), ethanol (<3%), and sodium xylene sulfonate (<3%) dissolved in water. Cleaned coverslips were coated with DDS by immersing in chloroform with 5% DDS for 30 min. The DDS-coated coverslips were rinsed with chloroform and ethanol in sequence twice, and finally with distilled water before drying.

The surface composition of glass and DDS-glass was measured with electron spectroscopy for chemical analysis (ESCA). Glass coverslips were sent for analysis to the National ESCA and Surface Analysis Center for Biomedical Problems of the University of Washington in Seattle, Washington. The ESCA spectra were obtained on a Surface Science Instruments X-Probe ESCA instrument.

Underwater contact angle measurements were made using a contact angle goniometer (Rame-Hart, Mountain Lakes, NJ) with an immersion chamber.[20,21] Air–water–surface and octane–water–surface static bubble contact angles were measured. At least six measurements on different surface regions of each of the total five coverslips were made. Polar (r_{sv}^{p}) and disperse (r_{sv}^{d}) components of the surface energy (r_{sv}) and the surface–water interfacial energy (r_{sw}) were calculated using the harmonic mean approximation method.[22]

Platelet preparation

Blood was withdrawn from healthy adult volunteers by venous puncture after informed consent. The volunteers were kept free of aspirin throughout

the study. Twenty milliliters of blood was collected into four Vacutainers (Becton Dickinson, Rutherford, NJ) containing buffered sodium citrate. Platelet-rich plasma (PRP) was separated from whole blood after centrifugation at 100*g* for 20 min at room temperature. Platelets were isolated from plasma proteins by passage through a Sepharose CL-2B column (2.5 × 10 cm) which was preequilibrated with divalent cation-free phosphate buffered saline (PBS) (pH 7.4).[23] Previous experiments in our laboratory showed that platelets were able to activate fully on the surface in the absence of exogenous divalent cations.[18,19] The obtained platelet suspension was kept at room temperature and used in less than 30 min after separation.

Protein adsorption

Human fibrinogen (Sigma, Type I, St. Louis, MO) was purified further by the Laki method as described previously.[24] The purified fibrinogen showed clottability of at least 97%. The purified fibrinogen in PBS was frozen in aliquots at −70°C at a concentration of about 2 mg/mL. Human albumin (Sigma, 1X crystallized) was used as received. The 1X crystallized albumin contained a minor fraction of albumin multimers according to SDS-polyacrylamide gel electrophoresis. The protein concentration was measured from the absorbance at 280 nm using absorptivities of 1.506×10^3 cm^2/g and 5.8×10^2 cm^2/g for fibrinogen and albumin, respectively.[25,26]

To precoat DDS-glass with proteins and to introduce platelets, a simple perfusion chamber was assembled from a glass slide (2.54 cm × 7.62 cm), a glass coverslip (2.54 cm × 7.62 cm, number 1-1/2), and a silicone rubber spacer (0.013 cm thickness, Dow Corning, Midland, MI).[18] Protein solution was added to the chamber by replacing PBS to avoid surface–air contact. The volume of the protein solution was at least four times larger than that of PBS in the chamber. The bulk protein concentration was varied from 1 mg/mL to 20 mg/mL for albumin, and from 0.01 mg/mL to 0.1 mg/mL for fibrinogen. Proteins were adsorbed for 1 h at room temperature in the static state and the chamber was washed with PBS to remove unadsorbed protein. The surface protein concentrations were measured using radiolabeled proteins. Fibrinogen and albumin were labeled with ^{125}I (Amersham, Arlington Heights, IL) using Enzymobead reagents (Bio-Rad, Rockville Centre, NY).

Circularity and area of spread platelets

Platelets in PBS were introduced into the perfusion chamber and allowed to settle on the surface by gravity. Platelets were observed with an inverted light microscope (Nikon Diaphot, Garden City, NY) equipped with optics for interference reflection microscopy (IRM). The IRM was used to examine the platelet–surface interactions. Microscope images were projected to a video camera (Newvicon, Model 65, Dage-MTI, Michigan City, IN) and subsequently directed to a computer for image analysis. The details of video microscopy was described before.[18]

Platelets on the surface were fixed with glutaraldehyde and stained with Coomassie Brilliant Blue (CBB) solution for 1 h.[19] The area and circularity of the fixed platelets were measured using a software obtained from Computer Imaging Applications (Madison, WI). The circularity and area of at least 300 platelets from six separate experiments were measured and averaged. The circularity is defined as $P^2/4\pi A$, where A is the area and P is the perimeter of a spread platelet, respectively. The circularity has a minimum value of 1 for a perfectly circular platelet and the value becomes larger as the number and length of pseudopods increase. It should be noted that, in addition to the absolute value, the relative value of pseudopod length compared with the platelet diameter also affects the circularity. The same pseudopods may result in different circularity values depending on the size of platelets. Thus, it is important to consider the circularity in conjunction with the spread area in the evaluation of platelet activation.

RESULTS

Surface characterization

Surface free energies of cleaned glass and DDS-glass are summarized in Table I. The hydrophilic nature of glass is indicated by the low solid–water interfacial tension (r_{sw}). The air–water–solid and octane–water–solid contact angles were increased upon DDS treatment on glass. The coating of glass with DDS rendered the surface very hydrophobic and the r_{sw} value increased significantly. The increase in the dispersion component of r_{sv} (r_{sv}^{d}) was moderate while the decrease in the polar component of r_{sv} (r_{sv}^{p}) was substantial. Thus, the increase in hydrophobicity by DDS treatment was mainly due to the decrease in the polar component of r_{sv}. A combination of high dispersion with low polar component forces are known to promote and retain more tightly bound plasma proteins.[27] Our previous data showed that albumin and fibrinogen bound more tightly onto DDS-glass than onto glass.[19]

Figure 1 shows comparative high-resolution C1s spectra for the surfaces studied. The major change in the C1s spectra with cleaning and DDS coating was a decrease in the overall intensity. Peaks in the C1s spectra near 285 eV, 287 eV, and 289 eV were observed, which were consistent with the presence of hydrocarbon, ether/hydroxyl and ester/acid species, respectively. There appeared to be only small changes in the relative amounts of the different carbon species present after the treatments. The C/Si ratio from the ESCA data has been used to measure the amount of organic material adsorbed in the

TABLE I
Contact Angles and Surface Free Energies (ergs/cm^2) of Glass and DDS-Glass

Surface	Θ_{air}	Θ_{octane}	γ_{sv}^{d}	γ_{sv}^{p}	γ_{sv}	γ_{sw}
Glass	16 ± 1	17 ± 2	21.8	48.2	70.1	0.05
DDS-glass	71 ± 3	90 ± 2	23.3	16.7	40.0	17.0

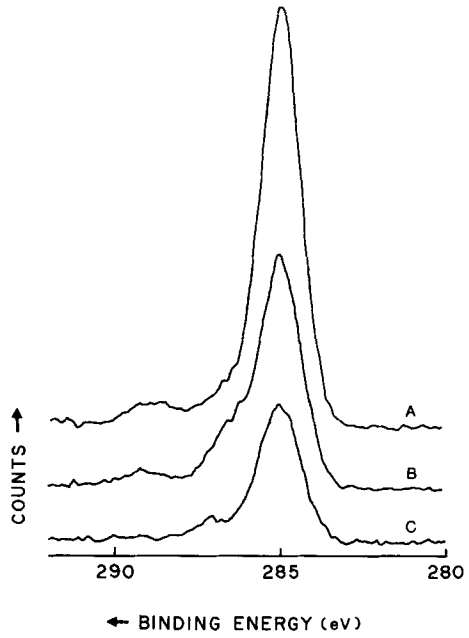

Figure 1. High-resolution C1s spectra for uncleaned (A), Isoclean-cleaned (B), and DDS-coated (C) glass coverslips.

uppermost 10–50 Å of glass surfaces.[28] The C/Si ratios of uncleaned, Isoclean-cleaned, and DDS-glass are listed in Table II. Cleaning of glass by Isoclean reduced the hydrocarbon contamination more than 60%. This suggests that the cleaning procedure removed most of the hydrocarbon contaminants held to the surface primarily by van der Waals' forces.[28] Coating of glass with DDS reduced the C/Si ratio further. Since any produced, perfectly clean surface is bound to be rapidly contaminated when exposed to air or liquid,[29] the presence of small amount of contaminant may be unavoidable. An important thing here is that the surface properties were reproducible as measured by the contact angles and platelet activation.

TABLE II
ESCA Analysis of Glass Coverslips

Surface	Surface Composition (Atomic Percent)			C/Si
	C	O	Si	
Uncleaned glass	35.1	37.2	19.0	1.85
Cleaned glass	18.9	45.5	26.9	0.70
DDS-glass	10.2	52.7	30.0	0.34

Competitive fibrinogen adsorption and platelet spreading

Fibrinogen was adsorbed onto DDS-glass in competition with albumin at various concentrations. Figure 2 shows examples of platelet spreading on DDS-glass after the competitive protein adsorption. The bulk concentration of fibrinogen was 0.05 mg/mL and the albumin concentration was either 2 mg/mL or 12 mg/mL. Figures 2A and 2B show the brightfilled images of fully spread and only partially spread platelets, respectively, while Figures 2C and 2D show IRM images of the same platelets. Platelets released granules while they spread and some of the released granules are seen in Figure 2A as indicated by arrows. In Figure 2B, some platelets are contact adherent and others are only partially spread with their pseudopods remaining extended (arrowheads). The center (arrows) of the poorly spread platelets remained spherical. The IRM image provides information on the platelet–surface contact sites.[18] The grey (arrowhead) and black (arrow) images in Figure 2C indicate close and focal contact sites, respectively. Overall, the ventral membrane of fully spread platelets is tightly attached to the surface. On the other hand, Figure 2D shows that platelets are only loosely attached to the surface as indicated by the white image (arrows). Only pseudopods

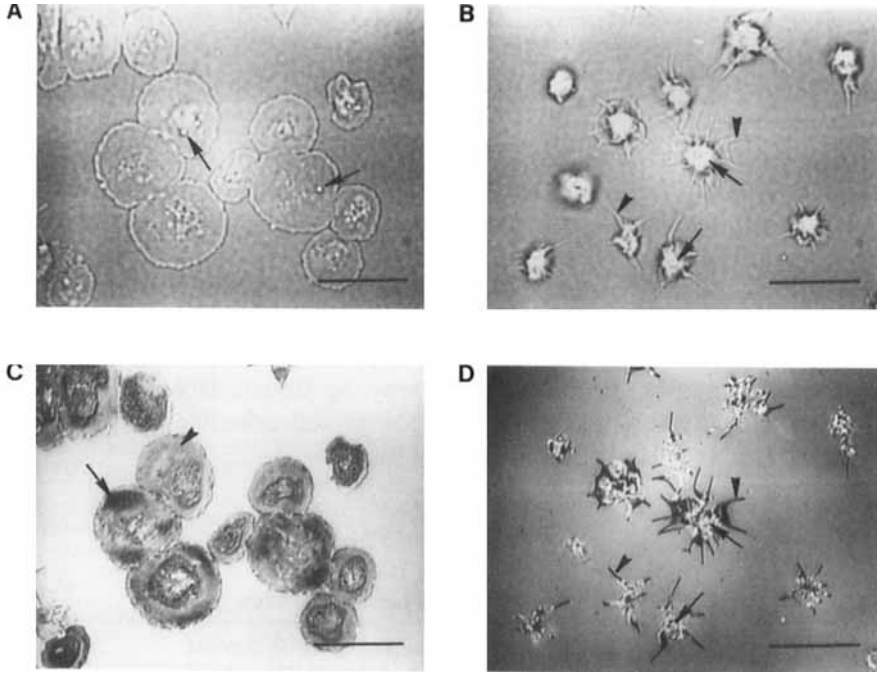

Figure 2. Brightfield (A,B) and IRM (C,D) images of platelets spread on DDS-glass after competitive adsorption of fibrinogen and albumin. The concentration of fibrinogen in the adsorption solution was 0.05 mg/mL and the albumin concentration was either 2 mg/mL (A) or 12 mg/mL (B). Proteins were preadsorbed at room temperature for 1 h. Platelets were allowed to spread for 30 min at room temperature. Bar = 10 μm.

(arrowheads) are in tight contact with the surface. The average circularity of fully spread platelets (Fig. 2A) is usually around 1.5. The average circularity of only partially spread platelets with extended pseudopods (Fig. 2B) is always larger than 2.0 and usually ranges from 3 to 5. Thus, the platelet spreading behavior can be quantitatively compared by measuring the spread area and circularity.

Fibrinogen was adsorbed onto DDS-glass at three fixed concentrations while the albumin concentration was varied from 1 mg/mL up to 20 mg/mL. The spread area and circularity of platelets were measured as a function of the albumin concentration in the adsorption solution (Fig. 3). As the bulk albumin concentration increased at a given fibrinogen concentration, platelet spreading was reduced and the pseudopods remained extended as indicated by the increase in the circularity value (Fig. 3). The albumin effects (decrease in area and increase in circularity) began to appear at the bulk albumin concentrations of 2 mg/mL, 5 mg/mL, and 10 mg/mL for the bulk fibrinogen concentrations of 0.01 mg/mL, 0.05 mg/mL, and 0.1 mg/mL, respectively. When the albumin concentration was very high compared to the fibrinogen

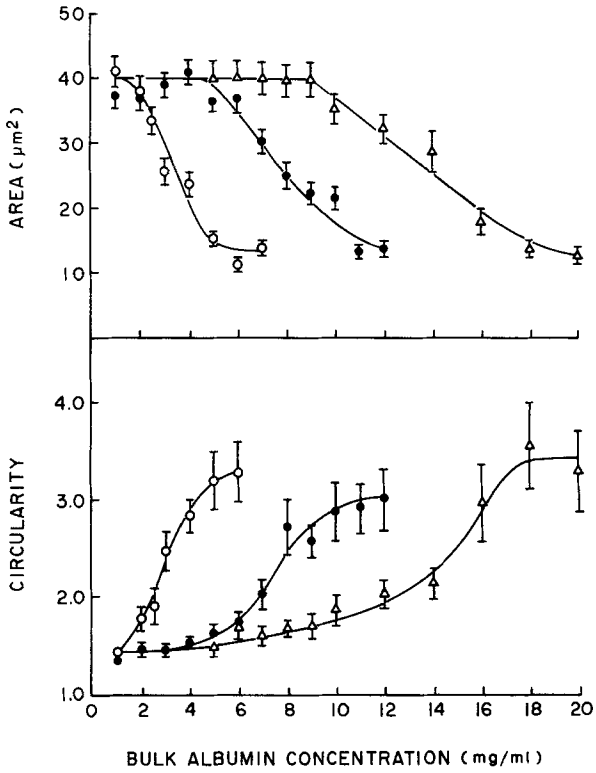

Figure 3. Changes in the area and circularity of platelets spread on protein-coated DDS-glass as a function of the bulk albumin concentration. Fibrinogen was competitively adsorbed at the bulk concentrations of 0.01 mg/mL (○), 0.05 mg/mL (●), and 0.1 mg/mL (△). DDS-glass was precoated with albumin and fibrinogen for 1 h at room temperature. Platelets were allowed to spread for 30 min at room temperature. Average ± S.E.M.

concentration, platelets were only contact adherent and could not spread at all. It should be noted that precoating of DDS-glass with only 1 mg/mL of albumin was enough to prevent platelet spreading, if fibrinogen was absent in the adsorption solution.[19] The area and the circularity of spread platelets on control DDS-glass without any precoated proteins were 40.3 μm^2 and 2.11, respectively. When DDS-glass was precoated with fibrinogen, the circularity decreased to 1.43 while the spread area remained the same.[19]

Since platelet activation on the protein-coated DDS-glass is largely dependent on the surface concentration of fibrinogen rather than the bulk concentration, we measured the surface fibrinogen concentration after competitive adsorption. Figure 4 shows the surface fibrinogen concentrations on DDS-glass as a function of the albumin concentration in the adsorption solution. When fibrinogen was adsorbed in the absence of albumin, the surface fibrinogen concentrations were 0.15 $\mu g/cm^2$, 0.33 $\mu g/cm^2$, and 0.44 $\mu g/cm^2$ for the bulk fibrinogen concentrations of 0.01 mg/mL, 0.05 mg/mL, and 0.1 mg/mL, respectively. Even when the bulk concentration of albumin is 100 times larger than that of fibrinogen in the adsorption solution, the surface fibrinogen concentration still ranges from 0.02 $\mu g/cm^2$ to 0.03 $\mu g/cm^2$.

When the data in Figure 3 were replotted as a function of the surface fibrinogen concentration of Figure 4, all the data points fell on a single master curve as shown in Figure 5. Platelets were able to spread fully when the surface fibrinogen concentration was only 0.02 $\mu g/cm^2$. At that concentration, the circularity of platelets was well below 2.0. Below 0.02 $\mu g/cm^2$, the area of spread platelets decreased and the circularity increased linearly. At about 0.005 $\mu g/cm^2$, platelets could not spread at all and remained discoid. Figure 5 indicates that platelets can spread fully as long as the surface fibrinogen con-

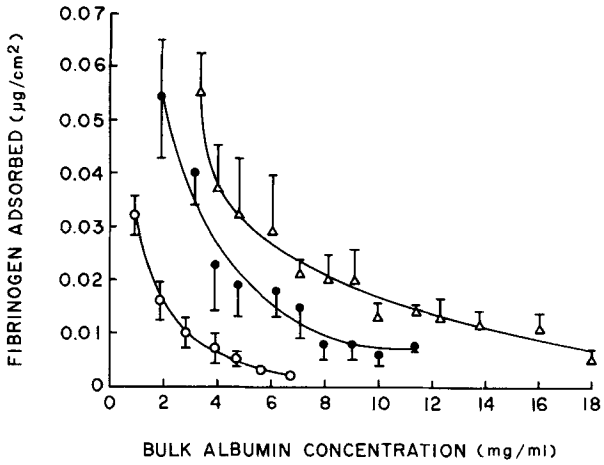

Figure 4. Surface fibrinogen concentration as a function of the bulk albumin concentration. Fibrinogen was competitively adsorbed at the bulk concentrations of 0.01 mg/mL (○), 0.05 mg/mL (●), and 0.1 mg/mL (△). DDS-glass was precoated with albumin and fibrinogen for 1 h at room temperature. Four separate experiments were done for each fibrinogen–albumin mixture. Average ± S.D.

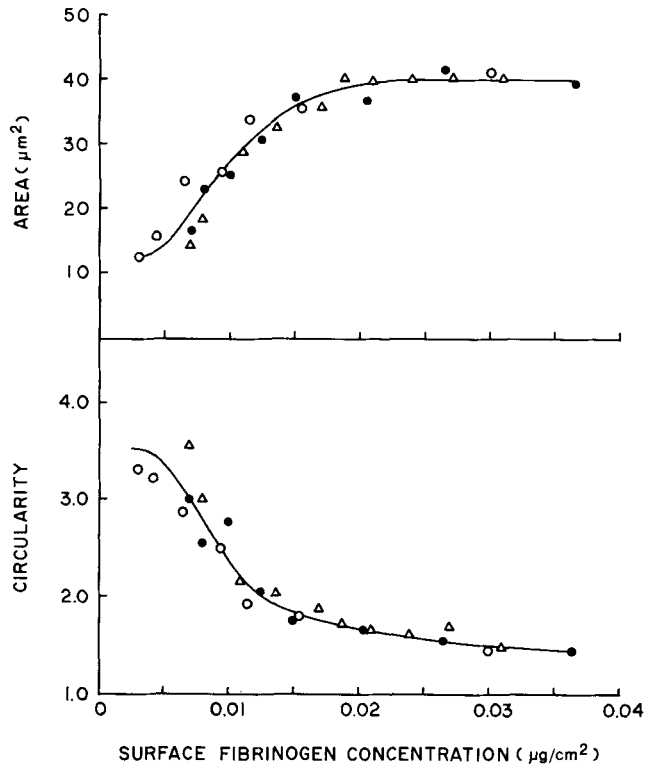

Figure 5. Changes in the area and circularity of platelets spread on protein-coated DDS-glass as a function of the surface fibrinogen concentration. Fibrinogen was competitively adsorbed at the bulk concentrations of 0.01 mg/mL (○), 0.05 mg/mL (●), and 0.1 mg/mL (△). DDS-glass was precoated with albumin and fibrinogen for 1 h at room temperature. Platelets were allowed to spread for 30 min at room temperature. Average ± S.E.M.

centration is 0.02 $\mu g/cm^2$ or above, if albumin is competitively adsorbed to the surface. The surface albumin concentrations at which the albumin effects begin to appear were measured as listed in Table III. Comparison of Figure 3 and Table III shows that albumin does not play any significant role in inhibiting platelet spreading until its surface concentration reaches about 0.19 $\mu g/cm^2$. Thus, it appears that the surface albumin concentration has to be larger than 0.19 $\mu g/cm^2$ to inhibit activation of contact adherent platelets.

DISCUSSION

In general, cells respond to changes in the nature of the substrate by striking alterations in morphology and increase their ability to spread on the surfaces suitable for adhesive contacts.[30] For platelets, spreading is a morphological manifestation of surface-induced activation and is related to the strength and nature of substrate adhesion.[31,32] We have used two parameters, area and circularity of spread platelets, to describe surface-induced platelet

TABLE III
Surface Protein Concentrations on DDS-Glass after Competitive Adsorption of Fibrinogen and Albumin[a]

Bulk Concentration (mg/mL)		Surface Concentration ($\mu g/cm^2$)	
Fibrinogen	Albumin	Fibrinogen	Albumin
0.01	2.0	0.016[b]	0.186 ± 0.003[c]
0.01	5.0	0.004	0.205 ± 0.006
0.05	5.0	0.020	0.197 ± 0.022
0.05	10.1	0.007	0.216 ± 0.017
0.10	10.1	0.017	0.197 ± 0.017
0.10	20.5		0.374 ± 0.043

[a]Proteins were adsorbed onto DDS-glass for 1 h at room temperature.
[b]The value was read from Fig. 4.
[c]Average + S. E. M. (n = 4).

activation. The two values allowed us to compare the ability of different surfaces to elicit platelet activation in a quantitative manner.[19]

This study showed that platelets could spread fully, if the surface fibrinogen concentration is 0.02 $\mu g/cm^2$ or above. The surface fibrinogen concentrations required for side-on and end-on packed monomolecular layers are calculated to be 0.14 $\mu g/cm^2$ and 0.89 $\mu g/cm^2$, respectively.[19] Thus, the surface fibrinogen concentration of 0.02 $\mu g/cm^2$ accounts for only 2% to 15% of the monolayer surface coverage depending on the fibrinogen orientation on the surface. This means that platelets can be activated fully on the albumin-dominated surface, if only 2% to 15% of the surface is covered with fibrinogen. Since albumin is expected to adsorb to the surface faster than fibrinogen, we can assume that only small number of fibrinogen molecules can adsorb in a side-on orientation, that is, a flatter adsorbed conformation. As the surface albumin concentration increases, fibrinogen molecules are likely to adsorb in an end-on orientation with fewer surface attachment.[33] Thus, the actual surface coverage by fibrinogen is expected to be much less than 15%. Fibrinogen molecules which are adsorbed in an end-on orientation may maintain their ability to interact with platelets by exposing binding sites to platelet receptors.[8,34] Grinnell and Feld made a similar observation with fibronectin.[35] The cell spreading activity and antibody reaction of fibronectin was increased when the protein was precoated on the hydrophobic surfaces in the presence of small amount of albumin. These observations support the idea that the state of the adsorbed protein is more important than its amount in influencing the behavior of adsorbed cells.[17,36]

The surface fibrinogen concentration of 0.02 $\mu g/cm^2$ corresponds to 3.6×10^{10} molecules/cm^2, since the molecular weight of fibrinogen is known to be 340,000 daltons. The surface area of platelets spread for 30 min in our experimental condition was about 40 μm^2 (Fig. 3). Thus, the total area of dorsal and ventral membranes can be assumed to be 80 μm^2. Since it is known that each activated platelet has 32,000 fibrinogen receptors,[37] the density of fibrinogen receptors on fully spread platelet membranes is

4×10^{10} molecules/cm^2. This value is approximately the same as the density of the surface fibrinogen. This simple calculation suggests that platelets can spread fully if the number of fibrinogen molecules on the surface is the same as or larger than the number of fibrinogen receptors on the platelet ventral membrane.

The weight ratio of albumin/fibrinogen in the adsorption solution used in our study ranged from 10 to 700. Figure 3 shows that platelets can spread fully if the albumin/fibrinogen ratio in the adsorption solution is about 100 or less. Only about 1% of fibrinogen in the adsorption solution is enough to create a surface environment which elicits full spreading of platelets. The fibrinogen concentration in plasma is known to be about 3 mg/mL,[38] and the total serum proteins (that is, nonclottable proteins in plasma) is known to be 73 mg/mL.[39] Thus, the ratio of nonthrombogenic proteins versus fibrinogen is about 25. This ratio becomes even smaller, if other platelet-adhesive proteins remaining in the serum, such as fibronectin and von Willebrand factor, are also considered. This may partly explain why platelets in PRP or blood can still adhere and spread on biomaterials even in the presence of high concentrations of inert proteins such as albumin. The adsorption process of a protein is known to be strongly influenced by the surface activities of other proteins present in solution.[40] In addition, the extent of conformational changes of adsorbed proteins depends on the surface properties.[41,42] Thus, the exact ratio of serum proteins/fibrinogen in the adsorption solution necessary for platelet activation will depend on the type of proteins present in the adsorption solution and the surface properties. Brash and ten Hove[43] and Horbett[44] measured the adsorption of fibrinogen from plasma to various solid surfaces, such as glass and polyethylene. Their data showed that the surface fibrinogen concentrations were larger than 0.02 μg/cm^2 even when the whole plasma was used as an adsorption medium. Thus, it is not surprising to see thrombus formation on those surfaces.[9]

Once the surface fibrinogen concentration is above a certain level, the extent of platelet activation appears to remain the same. This seems true whether fibrinogen is adsorbed from a single or binary protein solution. Thus, the surface which adsorb larger amount of fibrinogen is not necessarily a more thrombogenic surface. The extent of changes in fibrinogen conformation and bioactivity may depend on the material surface properties and the presence of other proteins.[15–17] Therefore, comparison of the amount of adsorbed fibrinogen on different biomaterials is not expected to provide a decisive information on the potential thrombogenicity of the biomaterials. The amount of albumin on the surface is also not expected to provide any conclusive information on the surface thrombogenicity. Unless albumin completely covers the surface, platelets can still recognize the surface. In addition, platelets can displace adsorbed albumin, if albumin–surface interaction is not strong.[18,19] Thus, protein adsorption data alone bear only a very crude relationship to thrombogenic potential of biomaterials.

The fact that biomaterials must ultimately be used clinically leads us to a question as to whether *in vitro* tests have any predictive values in assessing

the *in vivo* performance of any device. It has been suggested that mechanisms governing short-term interactions of blood with biomaterials (minutes to hours) are only partially related to the long-term (months to years) compatibility of prosthetic vascular grafts and are unlikely to provide valid predictive information.[45] As suggested by Vroman, however, the main purpose of *in vitro* studies is to understand how chain reactions of events are set in motion by certain surface properties rather than to predict *in vivo* performance of biomaterials.[46] In addition, the absence of a direct correlation between the *in vitro* or short-term *in vivo* studies and the long-term behavior of vascular implants may reflect the present inadequacy of the short-term model rather than any lack of relationship between short-term and long-term events. For example, the short-term models, especially *in vitro* studies, do not account for the changes in the composition of the initially adsorbed protein layer. The initially adsorbed proteins can be displaced by other proteins[43,47,48] or degraded by enzymes released from damaged cells.[49] Short-term models have to incorporate these changes that may occur *in vivo* during long-term blood–surface contacts. Thus, accelerated test methods need to be developed for the accurate prediction of the long-term blood–surface interactions.

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