# Patchwise Adsorption of Fibrinogen on Glass Surfaces and Its Implication in Platelet Adhesion[1]

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The spatial organization and intermolecular interactions of proteins adsorbed on the glass surface were investigated with a cross-linking approach. Fibrinogen and albumin were adsorbed on glass beads from single protein solutions or from a mixture of the two proteins at varying bulk concentrations. Protein molecules in molecular aggregates (patches) on the surface were cross-linked with bifunctional reagents and eluted from the surface by sonication in the presence of SDS. The eluted protein multimers were identified by gel electrophoresis. It was found that fibrinogen molecules always formed multimeric aggregates even at surface concentrations much lower than a monolayer coverage. On the other hand, albumin did not form multimers when the surface concentration was lower than that of the monolayer. The results indicate that patchwise adsorption is the unique feature of fibrinogen adsorption. The implication of patchwise fibrinogen adsorption in platelet adhesion is discussed. © 1988 Academic Press, Inc.

## INTRODUCTION

The importance of protein adsorption in surface-induced thrombosis has been well recognized and various aspects of protein adsorption at the solid–liquid interface have been studied. Typically, the surface concentration (1), thickness (2), conformation (3, 4), orientation (5), surface activation (6, 7), reversible adsorption (8, 9), mass action effect (10), competitive adsorption (11), preferential adsorption (12, 13), sequential adsorption (14, 15), two-dimensional distribution (16, 17), and surface diffusion of adsorbed protein (18) have been examined. Recent review papers on those subjects are available (19–21). One of the important aspects of protein adsorption from blood to artificial materials is that protein adsorption precedes any other events (22). It is generally accepted that the nature of the adsorbed protein layer determines platelet adhesion and activation, although the exact mechanisms are not understood. Several blood proteins, such as fibrinogen, fibronectin, and von Willebrand factor, are known to have receptors on the platelet membrane (23) and enhance platelet adhesion when preadsorbed on various surfaces (24). The enhanced platelet adhesion occurs in the presence of the same proteins in the bulk solution. Apparently the adsorbed proteins have higher interactions with platelets than their soluble counterparts.

As an attempt to understand how the adsorbed platelet-adhesive proteins overcome the interference of soluble proteins in platelet adhesion, we have examined the protein behavior at the solid–liquid interface using fibrinogen and albumin as representatives for thrombogenic and surface passivating proteins, respectively. In particular, the spatial arrangement and intermolecular interactions among the adsorbed protein molecules have been investigated with a cross-linking approach. The assumption of the approach is that adsorbed protein molecules will be cross-linked to form multimeric aggregates by bifunctional reagents, if protein molecules have lateral contacts or vertical stacking on the sur-

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face, or both. The multimer formation at the surface concentration which is lower than that of the ideal monolayer coverage indicates physical association of protein molecules in patches. On the other hand, the lack of multimer formation on the surface is assumed to indicate the presence of adsorbed protein as isolated individual molecules. Our results suggest that fibrinogen molecules always have intermolecular interactions on the surface even at a surface concentration as low as 0.02 $\mu g/cm^2$. On the other hand, albumin molecules do not form multimeric aggregates, if the surface concentration is lower than that of the monolayer. The implication of the patchwise adsorption of fibrinogen molecules in platelet adhesion is discussed.

## EXPERIMENTAL

### *Protein Preparation*

Commercially obtained human fibrinogen (Calbiochem, clottability 71%, and Sigma, Type I, clottability over 90%) was purified further by the Laki method (25). Throughout the procedure 0.1 *M* $\epsilon$-aminocaproic acid was used to prevent the activation of plasminogen and the fibrinogen solution was treated with $BaSO_4$ (40 g/g of fibrinogen) to remove prothrombin and related clotting factors (26). Fibronectin was removed by running through a gelatin column equilibrated with phosphate-buffered saline (PBS, pH 7.4) without divalent cations. The purified fibrinogen showed clottability of at least 97%. The fibronectin-free fibrinogen was reprecipitated using $\frac{1}{3}$ vol of saturated ammonium sulfate and redissolved in PBS. This fibrinogen was centrifuged to obtain clear supernate and dialyzed against PBS extensively at 4°C. The fibrinogen was frozen at −70°C in aliquots at a concentration of about 2.0 mg/ml. Human albumin (Sigma, 1× crystallized) was purified using Affi-Gel Blue (Bio-Rad). The concentration of both proteins was monitored by absorbance at 280 nm using absorptivity of 1.506 and 0.58 for 0.1% solutions of fibrinogen and albumin, respectively (27, 28).

### *Cross-Linking of Proteins in the Bulk Solution*

Proteins were cross-linked by one of the following cross-linking agents: bis(sulfosuccinimidyl)suberate (BSSS, Pierce), glutaraldehyde (Polysciences, SEM grade), and sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate (SSANPAH, Pierce). Protein solution (0.25 ml) was mixed with solution of a homobifunctional cross-linking agent (25 $\mu$l) to make the desired final concentration and incubated at room temperature for 30 min. The cross-linker solutions were prepared immediately prior to the experiment. The final concentration of the cross-linkers was varied from 0.1 to 5.0 m*M*. The cross-linking reaction was quenched by adding 50 $\mu$l of 100 m*M* asparagine in PBS and incubating for 10 min. When a photoactivatable heterobifunctional cross-linking agent SSANPAH was used, a 10 m*M* stock solution was prepared in the dark. SSANPAH was allowed to react with protein at room temperature for 30 min in the dark. After the reaction was quenched by asparagine as described above, SSANPAH was photoactivated by exposure to a 360-nm wavelength (Mineralight, UVL-21) at a distance of 10 cm for 5 min in a glass test tube. In using SSANPAH, it is important to observe the color before and after activation. It should be a bright orange which turns to orange–brown upon exposure to the 360-nm wavelength. This is a useful parameter to ensure that the experiment is properly performed. It was observed that a 5 m*M* solution of SSANPAH was stable without changing color at least for 3 h under normal room light. The color was changed to brown in a few minutes upon exposure to 360 nm of light.

### *Protein Cross-Linking on Glass Beads*

Glass beads (Polysciences, 105–150 $\mu$m) were cleaned by boiling in 5% IsoClean (IsoLab) for 2 h and washing with a copious amount of distilled deionized water (Nanopure, Barnstead). The cleaned beads were

stored at room temperature in PBS with 0.02% $NaN_3$ until use. The cleaned glass beads (12 g wet wt) were packed inside a glass column (Econo column, 2.5 × 10 cm, Bio-Rad) equipped with a three-way stopcock and washed with 30 ml of PBS. After the buffer in the column was drained to the level of the beads, 6 ml of protein solution was placed on top of the bead column and allowed to flow into the beads by draining the solution. All reagents were added in such a manner. The protein was incubated for the desired time (usually 2 h) at room temperature and flushed with at least 30 ml of PBS. Preliminary experiments showed that rinsing with 30 ml of buffer was enough to remove all the excess proteins. Cross-linking was initiated by adding 5 ml of the homobifunctional cross-linker at the desired concentration followed by incubation for 30 min. The column was then flushed with 30 ml of PBS. To ensure the complete inactivation of the added cross-linking agents, 6 ml of 0.1% asparagine solution was added and incubated for 30 min. Finally the beads were washed with 30 ml of PBS. In the case of a photoactivatable cross-linking agent, SSANPAH, these steps were performed in the dark with the aid of a safety light. To photoactivate the SSANPAH, the beads were transferred onto a watch glass and exposed to 360 nm for 15 min at 10 cm distance. The sample was agitated three times in a 5-min period. The beads were packed again into the column using PBS to aid in retuning all of the beads.

The cross-linking of fibrinogen on the surface using activated sialic acid residues was performed as follows. Instead of cross-linking agent, 50 m*M* sodium periodate was added to the column to react with sialic acid residues for 30 min. The excess periodate was removed by flushing with 30 ml PBS containing 0.5 *M* glucose. Subsequently 10 ml of 0.1 *M* $NaCNBH_3$ in the pH 6.0 buffer (0.1 *M* phosphate and 0.05 *M* NaCl) was added for the protein cross-linking. After 30 min, the beads were flushed with PBS and 10 ml of 10 m*M* asparagine solution was added. After another 30 min, the beads were flushed with PBS again and eluted from the surface.

### *Protein Elution from Glass Beads*

Protein was eluted from the surface according to Bohnert and Horbett (29). At the end of the protein adsorption and cross-linking, PBS was replaced with the elution buffer (0.25 *M* Tris, 0.2% SDS, pH 6.8). The glass beads were sonicated by inserting the standard tapered microtip (Heat Systems-Ultrasonics, Model W-370, sonicator cell disruptor) about 5 mm below the liquid–air interface so that it just contacted the top surface of the glass beads. Sonication was carried out at a power of "3" on the power source for 4 min in most cases. Preliminary experiments showed that SDS concentrations higher than 0.2% and sonication times longer than 4 min did not increase the protein elution efficiency further. The fluid sample was aspirated with a clean syringe and used for the analysis in SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

### *Gel Electrophoresis*

Electrophoresis samples were prepared by mixing with an equal volume of denaturant (20% glycerol, 0.2% SDS, 0.25 *M* Tris, pH 6.8) with or without mercaptoethanol. The protein reduction was achieved by heating samples at 80°C for 10 min in the presence of 5% mercaptoethanol. Electrophoresis was performed according to the method of Laemmli (30) and gels were stained with Coomassie blue R-250 (Bio-Rad). In the beginning stacking gels were discarded. Later it was found that the stacking gels presented important information on the formation of protein multimers. Thus, GelBond films (FMC) were used to preserve stacking gels. When the concentration of eluted protein was low, it was difficult to observe protein bands on the electrophoresis gels. In such cases, the eluted protein solution was concentrated using a B15 Minicon Concentrator (Amicon). As an alternative, the sample volume for electrophoresis was increased to 250 μl by repeated applications of the sample

to the same well. Right after the first protein sample (50 $\mu$l) in the well was migrated into the stacking gel, another 50 $\mu$l of the sample was added. This was repeated four times in the same manner.

### *Measurement of the Surface Protein Concentration*

The surface protein concentration was calculated using the solution depletion technique. The cleaned glass beads were dried in the 80°C oven for 24 h and exactly 10 g was weighed. The beads were then packed inside a column and soaked in 4 ml of PBS overnight before use. Subsequently an equal volume of protein solution was added and mixed well by pipetting several times. The concentrations of the protein solution before and after protein adsorption were measured from the absorbances at 280 nm. The average surface area of glass beads was estimated from the size distribution of 100 glass beads. The sizes of individual glass beads were measured using a video microscope (Nikon) and the magnification was calibrated with an objective micrometer. The total surface area calculated from the size distribution was 4,885,756 $\mu m^2/100$ beads (or 48,858 $\mu m^2$/bead). The number per milligram of glass beads was counted with the aid of a magnifying glass. One milligram of beads corresponded to 310 beads (or $3.1 \times 10^6$ beads/10 g beads). Thus, the total surface area was calculated to be 1510 $cm^2/10$ g beads. Incidentally, this is only a 5% difference from the surface area calculated using the average diameter of 128 $\mu$m which is merely the arithmetic average of the range of diameters of the glass beads.

## RESULTS

### *Optimum Concentration of Cross-Linking Agents for Multimer Formation*

The concentration of cross-linking agents was varied to determine the optimum condition for the intermolecular cross-linking of protein molecules on the surface and in the bulk solution. Fibrinogen was adsorbed on the glass beads at the bulk concentration of 0.3 mg/ml and cross-linked at varying concentrations of glutaraldehyde, BSSS, and SSANPAH. The formation of fibrinogen multimers by added cross-linkers was apparent as shown in Fig. 1. The multimeric fibrinogen band appeared at the top of the 4% separating gel as the concentration of cross-linking agents was increased to 0.5 m$M$ (lanes 3, 6, and 9). When the concentration was increased to 1 m$M$, most of the fibrinogen molecules were cross-linked (lanes 4, 7, and 10). At this concentration and above, another multimer band was seen at the top of the 3.3% stacking gel as shown in Figs. 2 and 3. Some of the formed multimers were too large to enter into the stacking gel during electrophoresis. The similar cross-linking results observed with all three reagents indicate that the multimer formation reflects the molecular association of the proteins, rather than artifacts from using a specific reagent or experimental method. The cross-linking study in the bulk solution showed essentially the same results as those on the surface, except for the fact that intramolecular

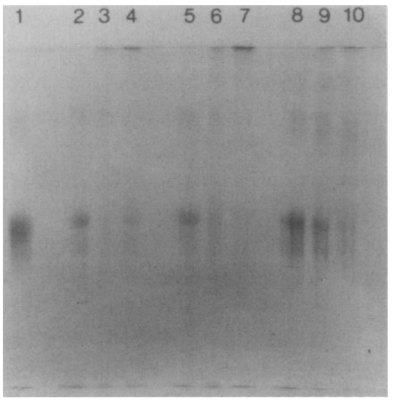


FIG. 1. SDS–polyacrylamide gel electrophoretic pattern of fibrinogen eluted from the glass surface after cross-linking with glutaraldehyde (2–4), BSSS (5–7), and SSANPAH (8–10). Lane 1 shows the fibrinogen standard. The concentrations of the cross-linkers were 0.1 m$M$ (2, 5, 8), 0.5 m$M$ (3, 6, 9), and 1.0 m$M$ (4, 7, 10). The fibrinogen multimer bands are shown at the top of the 4% separating gel.

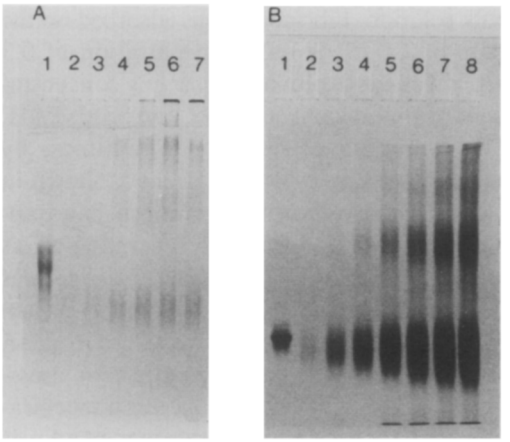


FIG. 2. SDS–polyacrylamide gel electrophoretic patterns of fibrinogen in 4% gel (A) and albumin in 7.5% gel (B). (A) Fibrinogen in the solution was cross-linked with 5 m*M* glutaraldehyde at the bulk concentrations of 0.05 mg/ml (2), 0.1 mg/ml (3), 0.3 mg/ml (4), 0.5 mg/ml (5), 1.0 mg/ml (6), and 2.0 mg/ml (7). The fibrinogen standard at the bulk concentration of 1 mg/ml is shown in lane 1 as a control. (B) Albumin was cross-linked with 5 m*M* glutaraldehyde at the bulk concentration of 0.1 mg/ml (2), 0.5 mg/ml (3), 1.0 mg/ml (4), 2.0 mg/ml (5), 3.0 mg/ml (6), 4.0 mg/ml (7), and 5.0 mg/ml (8). The albumin standard at the bulk concentration of 1 mg/ml is shown in lane 1 as a control.

cross-linking preceded intermolecular cross-linking in the bulk solution (see below).

Since no large difference was observed in the cross-linking efficiencies among the three cross-linking agents, experiments were done mainly using glutaraldehyde which could be easily obtained in large quantities. To ensure the intermolecular cross-linking at any protein concentration, all the subsequent experiments were done using at least 2 m*M* of cross-linking agents.

### *Cross-Linking of Protein in the Bulk Solution*

The concentrations of fibrinogen and albumin were varied from 0.05 to 5.0 mg/ml and cross-linked with 5 m*M* glutaraldehyde. Multimeric fibrinogen bands appeared at the bulk concentration of 0.3 mg/ml (Fig. 2A, lane 4). Above a fibrinogen concentration of 0.5 mg/ml, some of the formed multimers were too large to enter even into the 3.3% stacking gel. Thus, high-molecular-weight multimeric bands remained at the bottom of sample wells (Fig. 2A, lanes 5–7). It was also noted that intramolecular cross-linking preceded intermolecular cross-linking. The intramolecular cross-linking was characterized by the fibrinogen monomer band which migrated faster than the control fibrinogen. The faster migration is due to the more compact structure resulting from the intramolecular cross-linking.

The intramolecular cross-linking was also apparent with albumin as shown in Fig. 2B. The band of intramolecularly cross-linked albumin was more diffuse and migrated faster. Multimeric albumin molecules were observed above 1.0 mg/ml of albumin (Fig. 2B, lane 4). Unlike fibrinogen, however, no multimeric albumin band was observed at the bottom of the sample wells. Using a 4% separating gel, the size of albumin multimers was estimated

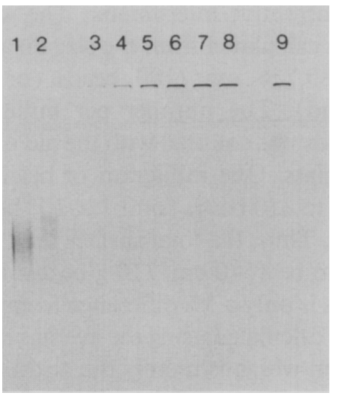


FIG. 3. SDS–polyacrylamide gel electrophoretic patterns of fibrinogen in 4% gel. Lane 1 shows the control fibrinogen which was not adsorbed onto the surface (1 mg/ml). Lane 2 shows fibrinogen eluted from the surface without cross-linking. Lanes 3–8 show fibrinogen eluted from the surface after cross-linking with 3 m*M* glutaraldehyde. Lane 9 shows fibrinogen eluted from the surface after cross-linking by activated sialic acid residues. Fibrinogen was adsorbed on the glass surface using the bulk concentrations of 0.10 mg/ml (2), 0.005 mg/ml (3), 0.01 mg/ml (4), 0.025 mg/ml (5), 0.05 mg/ml (6), 0.10 mg/ml (7), 0.30 mg/ml (8), and 0.05 mg/ml (9).

to be that of fibrinogen monomer. Thus, albumin multimers are expected to be composed of several albumin molecules. It is noted that a substantial amount of the fibrinogen and most of the albumin still remain as monomers in the bulk solution.

### *Cross-Linking of Protein Molecules Adsorbed on Glass Beads*

To examine the intermolecular cross-linking at varying surface concentrations, fibrinogen was adsorbed for 2 h at room temperature at the bulk concentrations ranging from 0.005 to 1.0 mg/ml and subsequently cross-linked with 3 m*M* of glutaraldehyde. Figure 3 clearly shows the fibrinogen multimeric bands at the bottom of the sample wells. In addition, other multimer bands with far less intensity are also shown at the top of the separating gel. Although the fibrinogen multimeric band formed at the bulk concentration of 0.005 mg/ml (Fig. 3, lane 3) was not as clear as others due to the small amount of fibrinogen eluted from the surface, the formation of multimeric bands is apparent. The surface fibrinogen concentration was measured using a solution depletion technique. When the bulk fibrinogen concentration was 0.1 mg/ml, the surface concentration was 0.18 ± 0.04 $\mu g/cm^2$. This value is essentially the same as that obtained by Bohnert and Horbett (29) who used radiolabeled fibrinogen as listed in Table I. The surface fibrinogen concentration was 0.07 ± 0.02 $\mu g/cm^2$, when the bulk fibrinogen concentration was 0.025 mg/ml. This was the lowest bulk concentration at which the surface fibrinogen concentration could be measured using the solution depletion technique. Below this bulk concentration, it was not easy to determine accurate and reproducible surface protein concentrations by measuring changes of the absorbance at 280 nm. It was possible, however, to calculate the highest possible surface concentrations from the total amount of fibrinogen available for adsorption and the total surface area. When the bulk concentration was 0.005 mg/ml, the highest possible surface concentration of fibrinogen was 0.02 $\mu g/cm^2$. Even at this surface concentration, fibrinogen molecules still formed multimers on the surface (Fig. 3, lane 3). The value clearly shows that the surface coverage by fibrinogen molecules is not complete, since the surface concentrations required for side-on and end-on packed monomolecular layers are calculated to be 0.18 and 0.8 $\mu g/cm^2$, respectively (28). This implies that fibrinogen molecules have lateral interactions and/or vertical stackings even when there are empty spaces. This further implies that fibrinogen molecules have a tendency to adsorb as aggregates (patches) on the surface rather than as isolated individual molecules showing homogeneous distribution. The exact size of the aggregates cannot be estimated at this time. In Fig. 3, it is also shown that fibrinogen molecules form multimers by the activated sialic acid residues in the absence of added cross-linking agents (lane 9). In the bulk solution, sialic acid activated fibrinogen formed only intramolecular cross-linking. This can explain why there was little difference in the cross-linking efficiency between cross-linking agents which have different lengths (Fig. 1). The sialic acid residues are present on the $\beta$ and $\gamma$ chains of fibrinogen which are rather compact compared to the extended $\alpha$ chain (31, 32). Thus, the multimer formation can occur only when there are intimate contacts among the adsorbed fibrinogen molecules. This again indicates that fibrinogen molecules indeed exist as patches on the surface.

The efficiency of the albumin elution was not as high as that of the fibrinogen elution.

TABLE I

Surface Fibrinogen Concentrations on the Glass Surface

| Bulk concentration (mg/ml) | Surface concentration ($\mu g/cm^2$) |
|---|---|
| 0.1 | 0.18 ± 0.04 |
| | 0.194 ± 0.024[a] |
| 0.025 | 0.07 ± 0.02 |

[a] From Ref. (29).

The concentration of eluted albumin solution was not sufficiently high to show the albumin band on the electrophoresis gels. Bands of albumin monomers and multimers were apparent only after the eluted albumin solution was concentrated at least 40 times using a B15 Minicon Concentrator. As shown in Fig. 4, the albumin multimers are seen when the bulk concentration is 1 mg/ml or higher (lanes 2 and 3), but below this bulk concentration no multimer band was seen. At the bulk albumin concentration of 0.3 mg/ml, the surface albumin concentration was $0.07 \pm 0.04$ $\mu g/cm^2$ which is below the surface concentration of the albumin monolayer (28). Unlike fibrinogen, albumin did not show multimer formation at this surface concentration (lane 1 in Fig. 4). Thus, it appears that patchwise adsorption is an unique property of fibrinogen.

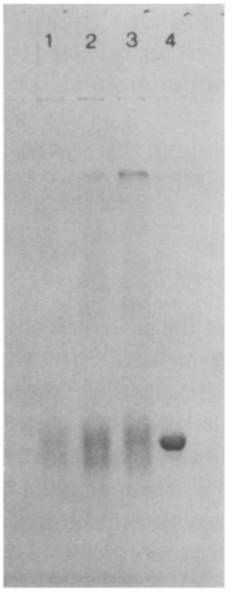


FIG. 4. SDS–polyacrylamide gel electrophoretic patterns of albumin in 7.5% gel. Albumin was adsorbed on the glass surface at the bulk concentrations of 0.3 mg/ml (1), 1.0 mg/ml (2), and 3.0 mg/ml (3). The albumin standard (1 mg/ml) is shown in lane 4. The multimeric albumin bands are clearly seen in lanes 2 and 3.

### *Competitive Adsorption of Fibrinogen and Albumin*

As shown above, fibrinogen formed multimers on the surface at the bulk concentration of 0.025 mg/ml, while albumin did not even at a bulk concentration of 0.3 mg/ml. Thus, we were interested in finding whether fibrinogen was still able to form multimers in the presence of a large excess of albumin. Fibrinogen and albumin were competitively adsorbed from the solution of which fibrinogen and albumin concentrations were 0.025 and 0.25 mg/ml, respectively. After 2 h of adsorption, proteins were cross-linked with 3 m$M$ of glutaraldehyde and eluted from the surface. The results of SDS–PAGE showed protein multimeric bands which were exactly the same as those of fibrinogen alone as shown in lane 5 of Fig. 3. Although it was not clear whether the formed multimers were pure fibrinogen aggregates or included albumin molecules, multimer formation clearly shows that protein molecules interact on the surface.

## DISCUSSION

Cross-linking offers an experimental approach for studying the spatial distribution of proteins in solution and on the surface. We have used glass bead columns to investigate patchwise adsorption of proteins. Caution is necessary, however, in applying the glass bead columns to the study of protein adsorption from plasma, since it has been shown that narrow spaces between glass beads may have preferential adsorption of certain proteins and replacement by other proteins as shown by Vroman and Adams (33). A significant finding in this study is that fibrinogen adsorbs to the surface to form patches even when the surface concentration is as low as 0.02 $\mu g/cm^2$. There are few possibilities for the patchwise fibrinogen adsorption. First, fibrinogen molecules form aggregates in the bulk solution and adsorb as aggregates. Although a small fraction of fibrinogen may exist as aggregates in the bulk solution, it cannot explain the patch formation at any bulk concentration. In addition, fibrinogen is known to be stable unless activated by thrombin (34). Brynda *et al.*, how-

ever, proposed the adsorption on carbon films of fibrinogen aggregates which were formed in the bulk solution (35). Second, fibrinogen molecules adsorb as individual molecules and diffuse on the surface to form aggregates in the experimental time scale. The notion of surface diffusion appears to be contradicted by the observation that albumin did not form multimers at the surface concentration lower than the monolayer coverage (Fig. 4, lane 1). However, we still cannot rule out the surface diffusion of adsorbed fibrinogen molecules as a possible mechanism, since there may be fundamental differences between lateral interactions among fibrinogen molecules and lateral interactions among albumin molecules on the surface. Third, individual fibrinogen molecules adsorb to form patches from the beginning. Since significant conformational changes are expected to occur upon adsorption, some of the adsorbed fibrinogen molecules may interact with bulk fibrinogen molecules reaching the surface. It is likely that more than one of the above-mentioned mechanisms can occur simultaneously.

The patchwise adsorption of fibrinogen may find its significance in platelet adhesion onto surfaces in the presence of bulk fibrinogen. Platelets adhere most where they find adsorbed fibrinogen on the surface (36). Platelet adhesion onto biomaterials occurs in the whole blood (37) and the extent of platelet adhesion increases significantly if fibrinogen is preadsorbed before blood exposure (15, 24). The fibrinogen concentration in plasma is known to be about 3 mg/ml (26, 38) which corresponds to $5 \times 10^{15}$ molecules/ml. The normal platelet count in the platelet-rich plasma is $2.5 \times 10^{8}$/ml (39). Since each platelet is known to have 32,000 fibrinogen receptors (40), the number of fibrinogen molecules to saturate the receptors of platelets in 1 ml plasma is $8 \times 10^{12}$/ml. Thus, even if all the platelets are activated at the same time, there are 600 times more fibrinogen molecules than that required to occupy all the fibrinogen receptors. Then, the question arises as to how platelets interact with the surface-adsorbed fibrinogen while there are excess fibrinogen molecules in the bulk solution. A related question is how a fibrinogen molecule bridges two receptors present on two different platelets (41–43) in the presence of excess fibrinogen. The answers to these questions may be provided by the fact that fibrinogen binding to platelet receptors is initially reversible until the fibrinogen binding becomes stabilized (44). The fibrinogen stabilization reaction takes about 30 min in buffer and 10 min in plasma (44). As long as the binding of individual fibrinogen to platelet receptors is reversible, it can be easily replaced by multimeric fibrinogen which has much higher and irreversible binding ability (see below).

It has been generally assumed that the surface-adsorbed fibrinogen molecules undergo conformational changes to a biochemically more active form which has high affinity to platelet receptors (45). This model assumes that receptors on the platelet surface are always available but have low affinity to soluble protein molecules. The conformational change of adsorbed fibrinogen, however, does not necessarily increase the affinity to platelet receptors. Recent studies suggest that the retention of conformational integrity of adsorbed fibrinogen (46) and preservation of recognizable platelet-binding loci on adsorbed fibrinogen molecules (47) is an important factor in platelet adhesion.

As an alternative to the conformation hypothesis, the multiple binding hypothesis emerged. The surface-adsorbed fibrinogen molecules may present continuous, multiple binding sites which substantially increase the interaction with platelet receptors (48). In this model, the change in the affinity of individual protein molecules to platelet receptors by conformational change is not a prerequisite. Using fibrinogen molecules which were polymerized with antibody molecules against the fibrinogen E domain, McManama *et al.* (49) found that the polymeric fibrinogen itself was capable of stimulating platelets without the receptor-inducing effects of either exogenous or platelet-derived ADP. It was thought that the poly-

meric fibrinogen presented continuous, multiple binding sites to platelets. Thus, even though the binding of individual fibrinogen may be reversible (43, 44), the binding of polymeric fibrinogen becomes irreversible. Based on this study, it was suggested that surface-adsorbed fibrinogen molecules might act as multivalent ligands to bind and activate platelets (47, 49). Then, it is reasonable to assume that patches of fibrinogen molecules on the surface may simultaneously present a multitude of specific binding sites for platelet membrane receptors. In fact, the multiple binding hypothesis has been used to explain the adhesion of platelets to surface-adsorbed von Willebrand factor (23, 50, 51), fibronectin (7, 52), and collagen (53).

The patchwise adsorption of fibrinogen shown in this study supports the multiple binding hypothesis rather than the conformational change hypothesis. It is likely that patches of fibrinogen on the surface can result in higher interaction with platelet receptors than monomeric soluble fibrinogen. Recent findings have shown that fibrinogen receptors on a stimulated platelet membrane cluster in patches (54–57). Hourdillé *et al.* (54) suggested that such sites might be responsible for the mediation of platelet–surface contact interactions. This polarization of the fibrinogen receptors on platelet membrane may require patches of fibrinogen molecules on the surface to maximize the interaction overcoming the effect of soluble fibrinogen. If the multiple binding hypothesis is correct, then platelet-adhesive proteins in blood, such as fibrinogen, fibronectin, and von Willebrand factor, should adsorb onto the surface to form multifunctional adhesive points. Thus, our studies are currently directed toward the identification of such multifunctional adhesive patches which may be formed upon exposure of biomaterials to the whole blood.

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