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Fibrinogen adsorption to biomaterials

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Abstract

Fibrinogen (Fg) adsorption is an important mechanism underlying cell adhesion to biomaterials and was the major focus of the author's research career. This article summarizes our work on Fg adsorption, with citations of related work as appropriate. The molecular properties of Fg that promote adsorption and cell adhesion will be described. In addition, the adsorption behavior of Fg from buffer, binary solutions with other proteins, and blood plasma will be discussed, including the Vroman effect. Studies of platelet adhesion to surfaces preadsorbed with blood plasmas selectively deficient in Fg, vitronectin (Vn), fibronectin (Fn), or von Willebrand's factor (vWf) will be reviewed. These studies clearly showed a major role for Fg in platelet adhesion under static conditions and both Fg and vWf for adhesion from flowing suspensions, but no significant role for Vn or Fn. However, it was also shown that platelet adhesion was poorly correlated with the total amount of adsorbed Fg, but very well correlated with the binding of antibodies specific to the cell binding domains of Fg. A brief overview of nonfouling surfaces for prevention of Fg adsorption will be given. A more extensive discussion of structural changes in Fg after its adsorption is included, including changes detected with both physicochemical and biological methods. A short discussion of the state of the art of structural determination of adsorbed proteins with computational methods is also given. A final section identifies Fg adsorption as the single most important event determining the biocompatibility of implants in soft tissue and in blood.

I. Introduction

The protein fibrinogen (Fg) is a large $\alpha_2\beta_2\gamma_2$ hexameric protein that plays two major roles in limiting blood loss when tissue injury occurs, namely gelation of the blood due to formation of fibrin networks, and platelet plug formation due to Fg binding to platelets.¹ Fg has an elongated shape resembling three beads on a string, with two end nodules (65Å in diameter) and one central nodule (50Å) held together by a very thin thread (<15Å in diameter), and an overall length that is estimated at 475Å.² Fibrin is formed from Fg by limited thrombin proteolysis to remove fibrinopeptides, and the fibrin monomers then are incorporated into an extensive gel network by bonding between newly exposed polymerization sites on fibrin. Platelet aggregates form when platelets are activated by various agents, including thrombin and ADP from previously activated platelets, as these agents cause the Fg receptor GPIIb/IIIa to become capable of binding Fg. Fg has platelet binding sites at the C terminal region of each γ chain. In addition, the hexameric structure is formed when two $\alpha\beta\gamma$ monomers are bound together at the N-terminal region by disulfide crosslinks, so the C-terminal regions are far apart from each other. This structural arrangement ensures that the major platelet binding site in the C-terminal dodecapeptide of

the γ chain has free access for binding to platelets, resulting in bridging of platelets, i.e. platelet aggregation. The large size of Fg, ca. 330,000 Daltons, also causes the platelet binding sites to be fairly far away on a molecular scale, further reducing steric hindrance. Because Fg is present at a relatively high concentration (approximately 3 mg/ml) in blood plasma, it is highly likely that activated platelets will quickly encounter Fg and bind to it. Similarly, any fibrin monomers also have very good chance of encountering another fibrin monomer to facilitate fibrin gel formation. These facts help ensure that blood clotting is rapid and extensive even in the higher flow arterial system. Fg also plays a role in the immune system because monocytes and macrophages possess the so called Mac1 receptor that mediates the binding of these cells to fibrin in clots at the site of tissue injury, allowing localized phagocytic removal of any bacteria at the wound site.^{3,4}

In contrast to these highly specific, evolutionarily selected roles that Fg fulfills, its function in regard to implanted biomaterials is more or less accidental, deriving from the fact that all proteins, including Fg, are surfactants, meaning they very quickly adsorb to foreign surfaces. The surfactant nature of proteins arises partly from their large size and wide array of side chain functional groups, allowing the formation of large numbers and many types of noncovalent interactions between the protein and the surface. In addition, the marginal stability of proteins allows them to at least partially unfold from their typically compact form and thus allow further bonding interactions with the surface.

Fg thus adsorbs very rapidly to the surface of almost any biomaterial that comes in contact with biological fluids such as blood plasma or peritoneal fluids. The absorbed Fg then promotes adhesion of platelets and monocyte/macrophages, playing a key role in blood clotting and the foreign body reaction associated with biomaterial implants. The ability of Fg to promote platelet and monocyte adhesion arises from the fact that Fg has specific peptide segments in its structure that are recognized by adhesion receptors on cells. Thus, platelets have the integrin receptor GPIIb/IIIa that binds Fg⁵, while monocytes/macrophages have the integrin Mac1 adhesion receptor that binds Fg⁴. Platelet adhesion at high shear is mediated by platelet binding to vWf via the GPIb-IX-V receptor, which is not an integrin.⁶ vWf is bound to collagen in the subendothelial tissue, but becomes exposed to blood after injury, promoting platelet adhesion, activation, aggregation, and plug formation. For biomaterials, the low concentration of vWf in blood plasma is still enough to cause its adsorption to the surface^{7,8}, where it is important in promoting platelet adhesion at high shear rates, as discussed below.

Because of the importance of platelet and monocyte/macrophage interactions with biomaterials in determining their biocompatibility, and the fact that Fg adsorption plays a central role in these interactions, my laboratory and many others have studied Fg adsorption extensively. Thus, I will describe studies of Fg adsorption from buffer, binary protein solutions, and plasma, and also discuss the competitive processes with other proteins that lead to unexpected peaks in Fg adsorption i.e. the Vroman effect. (Here it is useful to note that a thorough description of many of the methods my lab developed to measure protein adsorption have been published in a single place in a book chapter⁹, with much more detail than is usually possible in a journal article.) Selective depletion studies that clearly show a major role for Fg in platelet and monocyte/macrophage adhesion will be presented. Studies

showing that platelet adhesion is poorly correlated with the total amount of adsorbed Fg but very well correlated with the binding of antibodies specific to the cell binding domains of Fg will also be presented. The ability of nonfouling surfaces to prevent Fg adsorption will be briefly overviewed, as will two fairly recent studies showing that certain nonfouling surfaces have greatly reduced foreign body reactions. A major part of this article is devoted to describing structural changes in adsorbed Fg, including postadsorptive transitions that affect detergent elutability and the infrared spectra of Fg, antibody binding, and platelet adhesion. A brief introduction to the state of the art for computational structure determination of adsorbed proteins is also given. A final section identifies Fg adsorption as the single most important event determining the biocompatibility of implants in soft tissue and in blood.

II. The adsorption behavior of Fg

a. Fg adsorption from buffer

Most proteins exhibit very rapid, largely irreversible adsorption to solid surfaces. Thus, most of the collisions between the proteins and the surface result in the adsorption of the protein, and the proteins remain adsorbed even after extensive rinsing with buffered solutions. This is attributed to the fact that as protein molecules near the surface, their large size means that many interactions with the surface can occur. Since the simultaneous disruption of these many interactions is unlikely, the proteins tend to remain at the surface, as described in further detail by Latour.^{10,11} Fg adsorption to surfaces from buffer solutions to solid surfaces is thus essentially the same as for other proteins, in that it appears to reach steady state values in a relatively short time.¹²

Most common adsorption methods are similar to the one we used in my lab, where the adsorption was started by adding one volume of protein solution (with a concentration twice the desired final concentration) to an equal volume of buffer containing the adsorbing surface. Repeated pipetting was then done to get good mixing, and the adsorption was terminated by rinsing of the surface with excess buffer under flow. This type of method is incapable of high time resolution because neither the exact moment that the proteins reach the surface nor the protein concentration at the interface in the transient period are easily calculated. Thus, only rough measures of kinetics are obtained, e.g. we can say the adsorption appears to reach steady state by the time 1–2 hours has elapsed. In contrast to such rough estimates, a very high time resolution method, total internal reflection fluorescence (TIRF), and a cone and plate rheometer flow chamber to allow control of mass transfer rates, were used to show that Fg adsorption to unmodified and amino propyl silane modified quartz glass surfaces was dominated by a very fast step, with a half-life of about 5 to 20 seconds, depending on concentration.¹³ Another excellent method for kinetic studies is the thin channel flow cell and TIRF system used in Robertson's lab, which also showed high rates of protein adsorption.¹⁴ Other studies of Fg adsorption kinetics have also shown very rapid adsorption rates.¹⁵

Fg adsorption from buffer is also similar to other proteins in that a limiting or saturation value of adsorption is observed as the surface is exposed to higher and higher protein concentrations and allowed to adsorb until steady state values are reached. The “adsorption isotherm”, a plot of steady state adsorption versus bulk concentration, for Fg to polyethylene

(PE)¹⁶ or HEMA-EMA¹² reveals that the adsorption reached at higher concentrations is in the range of about 1 $\mu\text{g}/\text{cm}^2$. This value is above what one would expect for a close packed monolayer of Fg molecules laying on their side. But if the experimental value is compared instead to what is expected for a close packed monolayer with some or all of the Fg in an end-on orientation, the agreement is better. Of course, there are always considerable uncertainties about such monolayer calculations for real surfaces, where we seldom if ever know for sure how rough they are at the scale of a protein molecule, i.e. the monolayer calculations assume a completely smooth surface. In any event, it appears that steady state Fg adsorption measured after exposure of the surfaces to higher concentrations is at least approximately equal to what is expected for a fully packed monolayer.

In studies of Fg adsorption to the hydroxyethylmethacrylate-ethylmethacrylate (HEMA-EMA) series of copolymers, our group found that the isotherms were more similar to results expected for the Freundlich type of adsorption isotherm in which the surface sites are not of a single energetic type but rather a heterogeneous set.¹² Thus, the adsorption from higher bulk concentration solutions of Fg did not reach a distinct, almost constant value. Instead, the adsorption in that region still rose with concentration, but the rise was much less pronounced than observed in the lower concentration region. In the Freundlich analysis, the rise at higher concentrations is supposed to reflect the loading of lower energy sites with adsorbate, as such sites require higher protein concentration to fill.

In the HEMA-EMA-Fg studies, there was clear evidence for the effect of surface chemistry on the affinity of Fg, as the adsorption isotherms changed their slopes as the HEMA content of the copolymers changed. The analysis of these isotherms was done by extracting the constants k and b in the Freundlich formula $\Gamma = kC^b$. The exponential factor b is the slope of a log-log plot of adsorption (Γ) versus concentration (C) and is thus a measure of the strength of interaction since higher adsorption is achieved at lower concentrations if b is larger. The exponent b values are always observed to be less than 1, e.g. it was found to be 0.326 for Fg on polyethylene. The b value changed with HEMA content in the HEMA-EMA set of copolymers, from ca. 0.3 to ca. 0.5, reflecting that the concentration dependence of adsorption varied substantially in this series of copolymers.¹² In my lab, Freundlich type isotherms were also observed for Fg adsorption to polyethylene (PE) and for albumin and hemoglobin adsorption to PE, but the isotherm for γ globulin adsorption to PE was Langmuir like¹⁶. Adsorption isotherms for Fg measured by another research group were interpreted to reflect the formation of tightly packed Fg monolayers in which the Fg molecules were in an end-on orientation.¹⁵

The isotherms for other proteins adsorbing to several types of surfaces have a very distinct limiting or saturating value of adsorption achieved after exposure to higher concentration solution.^{14,17} Thus, instead of just rising much more slowly with bulk concentration in the high concentration regime as we observed for Fg on HEMA-EMA and PE, for some proteins on other surfaces there is very little or no detectable increase in adsorption as bulk concentration increases in the high concentration region of the isotherms. Such a shape is characteristic of the Langmuir isotherm, and this has led many investigators to analyze protein adsorption using the equation for this isotherm ($\Gamma = \Gamma_{\text{Max}}C/(K_d+C)$), where Γ_{Max} is the adsorption at the saturating or fully packed monolayer level, C is the bulk solution

protein concentration, and K_d is the equilibrium dissociation constant) to extract an equilibrium binding constant. But, as thoroughly discussed by Latour, the fact that protein adsorption isotherms frequently have a shape very similar to the classical Langmuir isotherm does not mean that the theoretical assumptions underlying the Langmuir isotherm apply to protein adsorption.¹¹ The Langmuir isotherm assumes reversible adsorption and an energetically identical set of adsorption sites on the surface. These assumptions do not apply to protein adsorption, so the calculated binding constants are not an estimate of the binding strength and cannot be used to calculate the free energy of adsorption.

Some of the motivation for study of Fg adsorption to the HEMA-EMA series of copolymers was the hypothesis that HEMA hydrogels strong uptake of water would make them more biocompatible by virtue of low protein uptake. Fg adsorption to Silastic does decrease as more and more pHEMA is grafted to it.¹⁸ However, it was found in that early study and the later one on the HEMA-EMA series that Fg adsorption to HEMA was still quite substantial. Much better surfaces for reducing Fg adsorption were discovered many years later, as described below.

b. Fg adsorption from binary mixtures with a competing protein

By measurement of the adsorption of an ^{125}I radiolabeled protein at a single concentration in the presence of increasing concentrations of an unlabeled competing protein, the relative affinity of the two proteins for the surface can be assessed. For the case when the affinity of the two competing proteins is the same, then the adsorption of the radiolabeled protein will be reduced by 50% when the concentrations of the two proteins are the same.^{19,20} We can thus summarize results from binary competition experiments using the ratio of the competing protein required to reduce adsorption of the radiolabeled protein to 50%, R50. The R50 values of the major plasma proteins albumin and IgG against Fg were found to be approximately 10, i.e. Fg had a relative affinity for the surfaces studied which was about 10 times greater than albumin or IgG. On the other hand, the R50 value for hemoglobin against Fg was only about 0.1.¹⁶ These types of studies showed that while Fg does appear to have reasonably high surface affinity when compared to the major plasma proteins albumin and IgG, it is definitely not the most surface active protein we might expect to encounter in blood plasma.

The high surface activity of hemoglobin accounts for its presence in the adsorbed protein layer on surfaces exposed to plasma despite the fact that hemoglobin is normally present in plasma at low concentrations, presumably due to slight hemolysis that always accompanies separation of cells in the preparation of blood plasma. For example, although we found only 0.06 mg/ml hemoglobin in the plasma used in one of our studies of hemoglobin adsorption, hemoglobin adsorption was still almost as high as observed for proteins present in much greater concentrations like albumin.¹⁶ The presence of hemoglobin in adsorbates on surfaces exposed to blood plasma was discovered using the “iodogram” technique in which the adsorbates are radiolabeled in the adsorbed state and then eluted with sodium dodecyl sulfate (SDS) and subjected to analysis using SDS-polyacrylamide gel electrophoresis (PAGE).^{16,21–23} SDS PAGE separate proteins by size, and a relatively low molecular weight protein (ca. 16,000 Daltons) was found in the eluate from most surfaces exposed to blood

plasmas. This peak proved to be due to the α and β subunits of hemoglobin, which both have nearly the same molecular weight of 16,000.

c. Fg adsorption from blood plasma

1. Surface chemistry effects on the amount of Fg adsorbed from plasma—Fg adsorption from blood plasma to the HEMA-EMA series of polymers and copolymers was measured by adding small amounts of high specific activity ^{125}I radiolabeled Fg to the plasma and exposing the polymers to the plasma until steady state adsorption was achieved after 2 hours of incubation at 37° C.²⁴ It was found that Fg adsorption varied greatly among these polymers, displaying a peak of approximately 150 ng/cm² on the 50–50 HEMA-EMA copolymer. Fg adsorption was in the range of 50 ng/cm² on pHEMA or pEMA. There was some variation in absolute values of adsorption among the four series of measurements made with separate plasma lots we performed, but a peak of Fg adsorption at intermediate copolymer composition occurred in each separate plasma study. In these studies, SDS polyacrylamide gel electrophoresis was used to show that ^{125}I Fg was not being degraded during the plasma adsorption. In addition, many tests for preferential adsorption of the ^{125}I Fg compared to unlabeled Fg were reported, all of which showed that the radiolabeled form adsorbed in the same way as the unlabeled form. Because of the high amounts of Fg in plasma, very thorough rinsing protocols were developed and tested to assure there was no carryover of bulk phase protein, i.e. that the retained radioactivity represented tightly adsorbed Fg.

In this report, the adsorption of albumin, immunoglobulin G, and hemoglobin were also measured in parallel studies, using ^{125}I labeled forms of these proteins. It was found that the adsorption of each protein varied across the copolymer series in a characteristic way, e.g. albumin adsorption was minimal on the 50–50 HEMA-EMA copolymer and higher on either pHEMA or pEMA. It was concluded that the composition of the adsorbed protein layer that forms after plasma exposure is dependent on the surface chemical properties of polymers, i.e. the relative amounts of each protein will be different on each surface. In a companion study using the *in situ* radioiodination method followed by SDS PAGE analysis of the radiolabeled adsorbed proteins after elution with SDS, large variations in the composition of the adsorbed proteins among the HEMA-EMA series of copolymers was again shown.²⁵ However, this method showed the variations for 9 different proteins that formed the major peaks revealed by radioiodinating the adsorbed protein layer.

The strong effect of surface chemical composition on adsorption of Fg from plasma revealed by the early studies with the HEMA-EMA series of copolymers was greatly extended in subsequent studies in my group with many other types of polymers.^{26–34} A summary of the many early studies of protein adsorption to hydrogels and other polymers from plasma were presented in a review, where Fg adsorption from 100% plasma to 16 different polymers was reported to range from 5 to 800 ng/cm².³⁵ In general, the amount of Fg adsorption to each surface is unique, reflecting variations in affinity of each of the many plasma proteins that compete with Fg for adsorption. In this analysis, the affinity of each protein is different and dependent on surface chemistry, the end result of which is different amounts of adsorption of Fg on each surface.

2. Fg adsorption from plasma displays a displacement phenomenon called the

“Vroman effect”—When Fg adsorption from blood plasma is studied as a function of time, or after steady state adsorption is attained after exposure of surfaces to variously diluted plasma solutions, or on a surface in the regions farther and farther away from the contact point of a convex lens placed on the surface, peaks of Fg adsorption are observed, all of which are referred to as the “Vroman effect”.^{19,20,22,23,27,36–41} We can refer to these as peaks in the time, concentration, or spatial domain. In the time domain, Fg adsorption reaches a maximum after short periods of contact with plasma and decreases thereafter after longer periods of adsorption, as the initially adsorbed Fg is displaced. In the concentration domain, where adsorption from various dilutions of plasma is measured after enough time passes to establish steady state adsorption values (typically after an hour or two), the plots of Fg adsorption versus plasma concentration exhibit a peak at intermediate plasma dilutions that depend on the surface studied. Thus, the peak occurs at around 0.1% plasma for glass, but approximately 1% on polyethylene. In the spatial domain, the peak of Fg adsorption is typically observed as a ring of enhanced anti-Fg binding at a certain distance away from the contact point of a concave lens placed on the adsorbing surface.

The Fg peaks were found to be less when plasma deficient in high molecular weight kininogen (HMWK) was used.^{39,40,42} Thus, one frequently cited explanation of the Vroman effect is that Fg is adsorbed in higher amounts initially because of its relatively high plasma concentration, but as time progresses, or higher plasma concentrations are used in the adsorption step, or the column height of plasma under the lens becomes greater, eventually competition from HMWK is sufficient to displace the initially adsorbed Fg. In this explanation, it is assumed HMWK has much higher affinity for the surface than Fg, but because of its much lower concentration in plasma, it takes time for sufficient amounts of HMWK to interact with the surface to displace Fg.

However, because peaks in fibronectin adsorption from diluted plasma and serum and have also been observed^{43,44}, and because peaks in Fg adsorption have been observed even from mixtures with albumin or the detergent triton X-100³⁷ or for Fg mixtures with hemoglobin²⁰, a more general explanation of the Vroman effect was offered by Slack and Horbett.⁴⁵ It was shown that Fg adsorbed to surfaces underwent transitions after adsorption that gradually made it less displaceable upon subsequent exposure to competing proteins in blood plasma, and that the transition rate depended strongly on the surface chemistry of the adsorbing surface. A model based on the transition rates and the kinetics of adsorption of Fg and the competing plasma proteins resulted in the prediction of peaks in Fg adsorption that depended on time, plasma concentration, and surface chemistry. Because of the fact that the Vroman effect is modified in HMWK deficient plasma, but not eliminated, and peaks of Fg adsorption can occur from binary mixtures with other proteins or even a detergent, Slack and Horbett proposed that HMWK as well as all other competing proteins in plasma are involved in the Vroman effect. In this model, the Vroman effect is ascribed to displacement of initially adsorbed Fg by later-arriving, more surface active plasma proteins, including HMWK as well as all other proteins in plasma, and transitions in the adsorbed Fg that make it less displaceable with residence time on the surface.²³

III. Selective depletion studies establish a major role for Fg in platelet adhesion

Blood plasma contains four proteins that are known to enhance platelet adhesion when these proteins are adsorbed in pure form to a surface, namely Fg, fibronectin, vitronectin, and von Willebrand's factor (vWf). Thus, when platelets interact with a surface exposed to blood plasma, the relative importance of these proteins in promoting platelet adhesion cannot be easily discerned. For this purpose, plasmas selectively deficient in these proteins are very useful tools. In my laboratory, we found that for platelet adhesion under static conditions (i.e. no flow), only Fg was needed for platelet adhesion to polystyrene surfaces. Thus, when the surface was preadsorbed with normal plasma diluted to various degrees, a large peak in platelet adhesion occurred at intermediate plasma dilutions, but when the same experiment was done using afibrinogenemic plasma, platelet adhesion was very low to surfaces no matter what plasma dilution had been used in the preadsorption step.⁴⁶ The peak in platelet adhesion is due to the peak in Fg adsorption at intermediate plasma dilutions, i.e. it is a consequence of the Vroman effect. A peak in platelet adhesion and Fg adsorption was also observed on a polyurethane surface preadsorbed with a series of dilutions of plasma, and occurred whether platelet adhesion was measured under static or flow conditions.³⁸

In later studies, plasma was selectively depleted of fibronectin or vitronectin by passing the plasma through a chromatography column with monoclonal antibodies specific to each protein bound to the beads of the column. The depleted plasmas were then used to preadsorb the surfaces.³¹ It was found that platelet adhesion to surfaces preadsorbed with plasma missing either fibronectin or vitronectin was not significantly different from surfaces adsorbed with normal plasma. Similarly, platelet adhesion in the absence of flow to surfaces preadsorbed with plasma genetically deficient in vWf was not different than a surface adsorbed with normal plasma.

To confirm the key role of Fg adsorption in platelet adhesion, the effect of adding back various amounts of purified Fg to afibrinogenemic plasma was also studied.⁴⁶ As expected, Fg addition to afibrinogenemic plasma prior to preadsorption of the surface restored platelet adhesion. However, it was found that much less than full scale restoration of Fg was required to give maximal platelet adhesion. Instead, addition of subnormal concentrations of Fg that resulted in only about 5–10 ng/cm² of adsorbed Fg was sufficient to promote maximal platelet adhesion. Fg adsorption from plasma with normal Fg concentrations reaches approximately 100 ng/cm². We concluded from this finding that “it would seem difficult to prevent platelet adhesion on synthetic surfaces by decreasing Fg adsorption, because the data for Fg adsorption onto various substrates studied so far reveal that very few surfaces adsorb <10 ng Fg/cm².”⁴⁶

A later study of static platelet adhesion to polyurethanes (PUs) preadsorbed with normal or afibrinogenemic plasma also clearly showed the major importance of Fg in mediating platelet adhesion³⁴. When ten different PUs were preadsorbed with afibrinogenemic plasma or serum, subsequent platelet adhesion was greatly reduced in comparison to the same surface preadsorbed with normal plasma, while the absence of vWf didn't have a significant effect. Also, vWf adsorption was very low and about the same on all ten PUs but Fg

adsorption and platelet adhesion varied a lot, so the results strongly support the idea that Fg is the primary protein mediating platelet adhesion to these PUs. Four of the PUs in this study contained polyethylene oxide (PEO) chain extender in various amounts (5 to 50% by weight), and in this subset, the Fg adsorption and platelet adhesion decreased to low levels as the PEO content increased. Another group reported that PUs mixed with bulk PEO and cast on a surface greatly decreased Fg adsorption and platelet adhesion when enough PEO was mixed in.⁴⁷

In subsequent studies, we further investigated the role of Fg and vWf on platelet adhesion from washed platelet-red cell suspensions under flow conditions.^{8,48–50} All of these studies showed a major role for vWf in promoting platelet adhesion under high flow conditions. Thus, for example, platelet adhesion to surfaces preadsorbed with normal plasma is greatly increased when the shear rate is increased from 50 to 500 sec⁻¹ because of more rapid transport of the platelets to the surface at higher shear rate. In contrast, for surfaces preadsorbed with plasma missing vWf, platelet adhesion is greatly decreased when the shear rate is increased from 50 to 500 sec⁻¹.⁴⁸ Thus, despite the much greater transport rates of platelets at the higher shear and consequent increase in platelet-surface encounters, it was found that adhesion to surfaces preadsorbed with vWf deficient plasma was very low, so Fg alone is insufficient to promote adhesion at higher shear.

When we studied the roles of Fg and vWf in platelet adhesion under flow conditions to protein repellant PEO-like surfaces made by radio frequency glow deposition (RFGD) treatment with tetraglyme, we found that although platelet adhesion was very low to these surfaces, it was only eliminated when the surfaces were preadsorbed with vWf deficient plasma⁴⁹. We concluded “that much of the remaining ability of our optimized tetraglymes to adhere platelets is not due mainly to Fg, but due to vWf. This suggestion is strongly supported by our findings that the enhanced adhesion to tetraglymes at higher shear rate could not only be prevented, but adhesion could be reduced to an undetectable level if the surfaces were preadsorbed with plasma that had normal amounts of Fg but were missing vWf.”

Fg adsorption is a procoagulant event because the platelets that adhere to adsorbed Fg are transformed into a different state, namely they are highly efficient catalysts for the formation of thrombin. This was shown in several studies from our group using deficient plasmas.^{51–54} The rates of formation of thrombin by surfaces was greatly increased if they had adherent platelets, in direct proportion to the number of adherent platelets. The rate of thrombin formation by non-adherent, resting platelets was found to be much lower than adherent platelets. It was also found that adsorbed vWf activated adherent platelets to a greater degree than adsorbed Fg, as the thrombin formation per platelet was greater the greater vWf adsorption was. However, whether the platelets were adherent to vWf or Fg was less influential than adhesion itself, since thrombin generation for adherent platelets on any surface was at least 60X higher than non-adherent platelets, with a modulation of ca. 2X of that activation depending on whether Fg or vWf was the adhesion protein.

Because thrombin is an extremely potent activator of platelets as well as being a catalyst for fibrin formation, platelet adhesion is a powerful feedback amplifier for blood clotting

because adhesion makes the platelet active in promoting thrombin formation. Thus, the effect of adsorbed Fg in promoting platelet adhesion is a major contributor to blood clotting by biomaterials and is the reason that clinical use of devices in contact with blood such as stents typically requires the continuous administration of powerful anticoagulants that work by inhibiting platelet aggregation.

Fg depletion has also been used to show a role in monocyte adhesion both *in vivo* and *in vitro*. Tang and Eaton used injection of an enzyme into mice to deplete their Fg and reported that “hypofibrinogenemic mice do not mount an inflammatory response to implanted PET unless the material is coated with Fg or the animals are injected with Fg before implantation. Thus, spontaneous adsorption of Fg appears to initiate the acute inflammatory response to an implanted polymer, suggesting an interesting nexus between two major iatrogenic effects of biomaterials: clotting and inflammation.”⁵⁵ However, our group showed that PEO like plasma deposited tetraglyme surfaces that adsorb very little Fg still exhibit a normal foreign body reaction to subcutaneous implants in mice.⁵⁶ Subsequent *in vitro* studies on monocyte adhesion showed that tetraglyme surfaces were complement activators⁵⁷ and led us to investigate the role of complement adsorption in monocyte adhesion. By using plasma selectively deficient in complement to preadsorb surfaces it was found that complement C3 adsorption was also involved in mediating monocyte adhesion to surfaces.⁵⁸

IV. Variations in ability of adsorbed Fg to promote platelet and monocyte adhesion

While the studies of platelet adhesion to surfaces preadsorbed with depleted plasmas clearly established a major role for Fg adsorption in mediating platelet adhesion, it was found that there was a generally poor correlation between the amount of platelet adhesion and the amount of adsorbed Fg. The reason for this appears to be that the availability of the small peptide regions that act as the adhesive motifs in Fg for the GPIIb/IIIa integrin receptor on platelets is modulated by the adsorption process so that they are not always fully available to the platelets. This conclusion was based on repeated findings that demonstrated platelet adhesion was much better correlated with the binding of monoclonal antibodies that specifically bind to the adhesion motifs in Fg.

In our first study of this type, platelet adhesion to five different types of polystyrene-based microtiter plates preadsorbed with plasma diluted to various degrees was analyzed relative to the amount of adsorbed Fg and monoclonal antibody binding to the adsorbed Fg.³⁰ The antibodies used bound to the three sites on Fg thought to be involved in platelet binding (the γ chain C-terminal dodecapeptides and the RGDF and RGDS sequences in the $\text{A}\alpha$ chains). There was no significant correlation between platelet adhesion and the absolute amount of adsorbed Fg. However, platelet adhesion was positively correlated to the ability of the adsorbed Fg to bind the three types of monoclonal antibodies. A partial least-squares model used to analyze the relative importance of these binding sites showed that the γ chain C-terminal dodecapeptide was the most important site mediating platelet adhesion, as its regression coefficient was 3 to 5 times larger than for the other sites. Another study of the relationship between Fg adsorption and platelet adhesion to a series of 10 different

polyurethanes also showed a poor correlation between Fg adsorption and platelet adhesion but a good correlation with the binding of the antibody specific to the γ chain C-terminal dodecapeptides.³⁴

Evidence for variable availability of cell binding domains in adsorbed Fg was also found in the case of monocyte adhesion to PEO-like plasma deposited tetraglyme coatings.⁵⁹ In these studies, an antibody (P2) that binds to the regions of Fg that supports adhesion of monocytes through its Mac1 receptor, the $\alpha_M\beta_2$ integrin, was used. While there was no correlation between total adsorbed Fg and adhesion observed for the tetraglyme coated materials in this study, there was a linear correlation between degree of P2 epitope exposure and monocyte adhesion. This trend was observed also on all the surfaces tested, with the correlation coefficient (R^2) value greater than 0.90 for all materials. It was concluded that for tetraglyme coatings, even though the adsorption of Fg is greatly reduced, the residual Fg is present in a more biologically active state, with a greater degree of P2 epitope exposure taking place on these surfaces. It was concluded that greatly reducing Fg adsorption by tetraglymes coating was insufficient to eliminate monocyte adhesion because the biological activity of the residual adsorbed Fg as an adhesion agent was high.

V. Prevention of Fg adsorption using nonfouling surfaces

The key role of Fg in mediating platelet and monocyte adhesion revealed by the selective depletion method led to the idea of a “design strategy” to try to improve the biocompatibility of biomaterials by making surfaces that would strongly resist the adsorption of Fg. Thus, the development of nonfouling surfaces became an important goal investigated by many research groups around the world, as reviewed in detail elsewhere.^{60–63} When it was further shown that platelet adhesion could be fully supported by amounts of Fg adsorption that were far below the amounts that adsorb to many surfaces from normal plasma⁴⁶, it was proposed that surfaces would have to be developed that had extremely low Fg adsorption. In later publications, we made more studies of Fg adsorption to tetraglyme surfaces and proposed that a design strategy for more blood compatible surfaces would be meeting a criterion for “ultra-low” Fg adsorption.^{31,49} In related studies, we showed that ultra-low adsorption of Fg would also be needed to prevent monocyte adhesion.⁶⁴ Several of our studies used radio frequency glow discharge plasma deposition of tetraglyme to form a polyethylene glycol like coating, on both flat surfaces and on the inside wall of tubing that was used as an arteriovenous shunt for evaluating blood compatibility.^{32,65,66} In our work with tetraglymes, it was found that low power deposition promoted coatings rich in ether linkages that reduced Fg adsorption and monocyte and platelet adhesion most effectively.^{49,64} Tetraglymes were also very effective in reducing activation of the intrinsic blood clotting cascade.³² Tetraglyme coatings were also found to be effective in reducing vWf adsorption.⁴⁹ However, when the ability of plasma deposited tetraglyme coatings to resist Fg adsorption was reexamined in later studies under more stringent conditions, namely using higher concentrations of Fg in buffer or with plasma that had not been diluted as much as in our earlier studies, it was found that more Fg adsorption occurred than we originally thought.^{49,58}

To date, the most effective coating strategy to reduce Fg adsorption has been the use of certain zwitterionic hydrogels that reduce Fg from plasma to below 5 ng/cm². These materials were the first ever shown to prevent the formation of a foreign body capsule after 3 months subcutaneous implantation in mice.⁶⁷ More recently, another group has reported a novel method to coat hydrogel surfaces with zwitterionic polymers that also results in great reduction of fibrosis around the surfaces after 14 days in the peritoneal cavity of mice, although Fg adsorption was not reported.⁶⁸ A recent study reported that a novel type of non-fouling coating formed by chemical vapor deposition of hydroxyl poly-p-xylylene and subsequent ring opening polymerization with an epoxide resulted in hyperbranched polyglycerol coated surfaces that strongly resisted protein adsorption and cell adhesion.⁶⁹

Although Fg adsorption is important to monocyte adhesion, it was found that tetraglyme surfaces with very low Fg adsorption that strongly resisted monocyte adhesion *in vitro* still elicited a typical foreign body reaction in subcutaneous implants in mice.⁵⁶ Subsequent studies in our laboratories showed that tetraglyme surfaces were strong complement activators.⁵⁷ Complement C3 adsorption to surfaces was then investigated and was found to promote monocyte adhesion as well.⁵⁸ Thus, it was found that monocyte adhesion to surfaces preadsorbed with complement C3 deficient plasma was decreased, and could be restored by addition of C3 to the deficient plasma prior to preadsorption of the surfaces. It appears that both C3 and Fg adsorption are involved in monocyte adhesion.

VI. Structural changes in adsorbed Fg

a. Postadsorptive transitions in Fg detected with physicochemical and biological methods

Proteins that adsorb to solid surfaces undergo conformational changes because proteins are relatively unstable and have a tendency to unfold to allow further bonding with the adsorbing surface. These changes, which I have referred to as postadsorptive transitions, can be detected with several physicochemical techniques and also by characterizing the changes in the ability of the adsorbed protein to bind to biological probes such as monoclonal antibodies or platelets.

The earliest studies of postadsorptive transitions in our group were done by measuring the ability of the detergent sodium dodecyl sulfate (SDS) and other detergents to remove adsorbed Fg or albumin from eight different surfaces as a function of time after the adsorption step.⁷⁰ Polystyrene, polyhydroxystyrene, polytetrafluoroethylene (PTFE) (Teflon), silicone rubber, polyethylene, polyvinylchloride, glass, and polyethylene terephthalate (Mylar) were the surfaces used in the study. This experimental measurement of protein binding strength was called detergent elutability. Generally, the elutability was often quite high (over 80%) if tested immediately after the protein adsorption step, although there were significant differences in initial elutability of the proteins from different surfaces. The elutability slowly declined to much lower levels if the surfaces with adsorbed proteins were allowed to soak in buffer for long periods, e.g. up to a week. These relatively slow declines in elutability could be greatly accelerated by incubating the adsorbed proteins at higher temperatures, e.g. the elutability would decrease to below 20% in one day if the surfaces with adsorbed proteins were kept at 80° C. In contrast, the decreases in elutability could also be greatly decelerated by incubating the adsorbed proteins at 4° C, e.g. the elutability of Fg

adsorbed to Teflon but then stored at 4° C remained above 90% even after one week. The strong temperature dependence was consistent with the known effects of temperature on the conformational stability of proteins, so it seemed likely that the declines in SDS elutability in the postadsorptive period was reflective of protein unfolding that allowed further contacts of the adsorbed protein with the surface that made it more and more resistant to displacement by the detergent. Elutability was also less if adsorption were done from more dilute protein solutions, an effect attributable to the availability of more unoccupied surface sites for an adsorbed protein molecule to form more bonds as it unfolds on the surface.

Good support for the idea that the postadsorptive period for adsorbed proteins involved conformational changes came from extensive studies of spectral changes in the adsorbed proteins made with the FTIR method, which is able to monitor changes in infrared frequencies related to conformation of the peptide bonds in proteins.⁷¹ Time-dependent transitions in an adsorbed layer of Fg on two different polyurethanes were studied both by use of an attenuated total reflection infrared flow cell and by SDS elutability. Elutability decreased with residence time of the adsorbed protein on the surface. The infrared spectral changes showed a good correlation with time dependent changes in the adsorbed Fg. A correlation between the center-of-gravity frequency shift of the amide II band of adsorbed Fg and the amount of Fg retained after SDS exposure was shown.

A systematic study of the role of polymer properties on postadsorptive changes in Fg adsorbed to 19 different polymers was undertaken.⁷² The elutability after adsorption varied widely among these 19 polymers, from 100% on polyHEMA to 40% on polystyrene. The elutability also tended to be higher on oxygen containing polymers, e.g. elutability was ca. 45% on polystyrene, but almost 90% on 75 hydroxystyrene/25styrene copolymer. Short-term transitions to a state resisting SDS elution, requiring 2 hr. or less after adsorption, were observed only on hydrophobic polymers that contained no oxygen. More gradual transitions were observed on hydrophobic polymers containing oxygen, but only after a lag time of 1–4 h.

Other studies with the SDS elution technique showed that it could also detect changes in Fg adsorbed from plasma.⁷³ In these studies, elutability was shown to depend on residence time but also on plasma concentration used during the adsorption step, i.e. it was lower for Fg adsorbed from more diluted plasma. For example, SDS elutability for Fg adsorbed polyethylene from 0.01% plasma was 60% but was close to 100% for Fg adsorbed from 10% plasma. The non-elutable portion of the protein layer formed rapidly on polystyrene, while on polyethylene elutability was high initially, followed by a gradual decrease.

Postadsorptive transitions were also studied with a much milder surfactant solution than SDS, namely the mixture of proteins in blood plasma.^{28,45,74} Using plasma as the displacing agent revealed that postadsorptive transitions in Fg to make it less displaceable occurred much more rapidly than those detectable with SDS as the displacing agent, i.e. changes in plasma displaceability were detectable within 10 min. or so after the adsorption step. It seems likely that the faster changes in adsorbed Fg detected using plasma displacement in comparison to detergent displacement reflect the fact that competing plasma proteins are

very weak surfactants in comparison to detergents, so that far fewer bonding interactions between Fg and the surface are needed to resist displacement by the plasma proteins.

The transitions in Fg adsorbed from buffer to surfaces that made it resistant to displacement upon subsequent exposure to plasma were carefully analyzed to obtain the rates of transition to a non-displaceable form, and these transition rates were used in a model of the Vroman effect, as already discussed above.⁴⁵ In another study of these transitions, radiolabeled Fg was adsorbed from blood plasma rather than buffer, prior to measuring its displacement upon exposure to plasma that did not contain radiolabeled Fg.²⁸ Fg was adsorbed to glass, Silicone rubber, and five different polyurethanes. On all surfaces, as adsorption time increased, the adsorbed Fg became increasingly resistant to displacement during the subsequent exposure to 100% plasma, but the rate of increase in resistance varied greatly with surface type. The rates of transition in Fg adsorbed from plasma were time and surface chemistry dependent, and in this regard were similar to the transitions in Fg adsorbed from buffer.

The postadsorptive transitions in adsorbed Fg detectable as decreased SDS and plasma displaceability, or as changes in infrared spectra of Fg, also cause changes in the binding of monoclonal antibodies specific to small regions of Fg.⁷⁵ The binding of ten different monoclonal anti-Fg molecules to Fg adsorbed from plasma to Biomer and other surfaces was measured. In these studies, adsorption was stopped by removing the plasma using flowing buffer to simultaneously dilute and displace the plasma, and then the adsorbed surfaces were kept in buffer for increasing postadsorptive residence time. Control experiments with ¹²⁵I radiolabeled Fg were done to assure that the adsorbed Fg remained on the surfaces during the long periods of being kept in buffer. The binding of some antibodies was very stable with respect to residence time, others rose with time, some declined with residence time and in one case the antibody binding passed through a maximum at intermediate residence time. Binding of several different antibodies that are known to recognize different peptides in the γ chain C-terminal region either did not change with residence time, increased with residence time, or else decreased more slowly than observed for platelets. Binding of the antibodies to the RGD sequences near the N-terminus of the A α chain (95–98) was very low initially but increased with residence time, while the binding to the RGD sequence near the C-terminus of the A α chain (572–575) increased slightly at short residence times but then declined substantially after longer residence times. These studies provided evidence of structural changes at many different places in the adsorbed Fg molecule, and indicated a complex rearrangement occurs that can lead to increased or decreased availability of a given region to binding with an antibody probe.⁷⁵

Fg postadsorptive residence time on the surface also leads to decreases in the ability of the adsorbed Fg to mediate platelet adhesion^{76,77}. Decreases in platelet and antibody binding and in the SDS elutability of the adsorbed Fg after it was stored in buffer were detected, although different rates of decrease were observed for each of these measures of the state of the adsorbed Fg. When the protein-coated surfaces were stored in buffered albumin solution rather than buffer, the decrease in the platelet or antibody binding to adsorbed Fg was prevented. The effect of albumin to prevent postadsorptive transitions is probably due to the occupancy of surface sites near adsorbed Fg with albumin, thus limiting the ability of the Fg

molecule to bind to additional sites. A separate study showed that Fg adsorbed from plasma to six different polyurethanes also underwent postadsorptive transitions that caused both SDS elutability and the binding of a polyclonal anti-Fg to decline over a period of days when stored in buffer, but the declines were usually greatly reduced when the adsorbed surfaces were stored in buffer that had added albumin.⁷⁸

b. Other studies of adsorbed proteins indicating structural changes after adsorption

Several studies from other groups revealing changes in the structure of adsorbed Fg should be noted. Tapping-mode atomic force microscopy was used to study the time-dependent changes in the structure of Fg under aqueous conditions following adsorption on two model surfaces: hydrophobic graphite and hydrophilic mica.⁷⁹ Spreading kinetics of Fg on the two surfaces was determined by measuring the heights of the D and E domains over a time period of approximately 2 h. On graphite, the heights of both the D and E domains decreased with time, and a spreading rate constant of approximately $4.7 \times 10^{-4} \text{ s}^{-1}$ was observed for the whole Fg molecule adsorbed on graphite. The kinetics was used to estimate values of 2.3 and 2.2 nm for the heights of the D and the E domains at the time of contact with the hydrophobic graphite substrate, both of which are significantly less than their free solution diameters.

A thorough study of the structure of Fg on surfaces having a wide range of chemical composition was performed with an extremely sensitive circular dichroism (CD) apparatus capable of measurement of CD spectra in the adsorbed state.⁸⁰ The structure of Fg adsorbed onto alkanethiol self-assembled monolayers (SAMs) on gold with CH_3 , $\text{OCH}_2\text{-CF}_3$, NH_2 , COOH , or OH terminal groups was found to vary, depending on surface hydrophobicity and solution concentration. In a subsequent report, these investigators studied the relationship between platelet adhesion and structure of the adsorbed Fg on these same surfaces⁸¹. Platelet adhesion was found to be strongly correlated with the degree of adsorption-induced unfolding of Fg ($R^2 = 0.96$) with essentially no correlation with the amount of Fg adsorbed ($R^2 = 0.04$). These results strongly support the idea that the conformation of Fg can be altered after adsorption and that conformation is a critical determinant of platelet adhesion rather than the amount of Fg adsorbed.

There is also good evidence for structural changes in adsorbed proteins from studies with other proteins. For example, albumin underwent changes upon adsorption detected by CD spectroscopy that were sometimes reversible, i.e. BSA completely regains the native structure and stability after being exchanged into the bulk phase from silica particles, but BSA desorbed from polystyrene particles had irreversible changes in structure.⁸² In another study from this group, structural stability in the adsorbed state was also measured as a function of temperature at a given wavelength. Surprisingly, the adsorbed BSA had a higher thermostability than the native protein.^{83,84} The adsorbed BSA was found to have a lower alpha-helix (50%) content than the native protein (58%), but also had considerably more disordered structure (30%) than the native protein (16%). A different group developed a novel technique, the adsorption probe, to measure the kinetics of spreading of Fg on surfaces, with the main finding being "...that fibrinogen test molecules spread from an average initial footprint of 100 nm^2 to a final footprint near 500 nm^2 per molecule on the

hydrophobic surface, with a single-exponential decay of 1735s. On a hydrophilic monolayer, the area increases from 100 to 160 nm² with a characteristic time of 6740 s.”⁸⁵

There is also supportive evidence for the role of structural transitions in proteins adsorbed at both the solid-liquid interface and the air water interface from studies of mutant proteins with altered thermodynamic stability. Lysozyme mutants with increased thermodynamic stability did not undergo a transition to a more tightly bound state (measured with detergent elutability) on hydrophobic surfaces, while for other mutants that were less stable, 90% of the protein underwent this transition. For native lysozyme, 37% of the adsorbed proteins underwent the transition to a tightly bound state.⁸⁶ For a wide range of mutants of the tryptophan synthase α subunit, several types of phenomena related to insertion of the protein at the air water interface (such as the lowering of the surface tension or the foaming power of the protein solutions) was linearly correlated with thermodynamic stability of the mutants.⁸⁷

c. Structure of adsorbed proteins: state of the art methods

A final set of comments about the structure of adsorbed proteins can serve as a short summary of the state of the art in this regard. As reviewed above, there is much evidence to support the idea that adsorbed proteins undergo changes that are likely due to changes in their structure, including changes in detergent elutability, infrared and CD spectra, and their ability to support the binding of antibodies and platelets. For a long time, indirect methods for determining the structure of proteins (such as circular dichroism) were also all that was available for solution proteins as well, but of course the advent of x-ray crystallographic structure determination led to highly detailed structural information about many proteins. However, for adsorbed proteins, a similar advance in knowledge of their detailed structure has not occurred, as in general there are no techniques to give detailed structural information for adsorbed proteins. The exception to this rule is that for small proteins like the biomineralization factor salivary phosphoprotein statherin, solid state NMR is capable of giving enough direct information so that computational refinement leads to a high degree of structure determination when these proteins interact with hard tissue minerals.⁸⁸

Because of the absence of direct methods of structure determination for adsorbed proteins, several investigators have devoted themselves to trying to adapt molecular modeling techniques for proteins in solution to attempt to predict what structures they will assume in the adsorbed state. Several reviews of this field have appeared in recent years, including two smaller reviews of recent work in this field co-authored by myself^{61,63} and a much more thorough review by one of the leading groups in this field.⁸⁹ Some of the key issues in modeling proteins discussed in the review by Ozboyaci et al are assigning appropriate force fields for the interaction of each protein group with a surface, appropriate methods for representing the effects of water molecules that are involved in protein adsorption from solutions, ways to deal with the wide range of time- and length-scales that vary from nanoseconds to days, and from nanometers to micrometers, the possibility of surface reconstruction and also changes in the proteins as they adsorb, resulting in many intermediate structural and energetic states that complicate sampling.

A fundamental problem with molecular dynamics calculations with solvent present is that more or less metastable states can be encountered. The likelihood of getting significant changes out of that state into a still lower energy state is low, so the computations reveal no changes for “long” times, and the metastable state appears to be the final state because the likelihood of the system moving on is too low to be seen in the relatively short computational time accessible. This problem was addressed explicitly in a recent publication from Latour’s group.⁹⁰ First, they showed that the conventional simulation approach resulted in predicted structures that were very similar in conformation and orientation to the initial state the protein was in when computation started, due to the inability of these calculations to evade being trapped in a metastable state. In their modified approach, the calculations are started with the protein in a wide range of configurational states and the calculations are run as if the protein were at an elevated temperature to allow the crossing of energy barriers that separate different states. They showed that their alternative approach results in the occurrence of many more distinctly different orientations and conformations. These authors also used improved force fields to account for protein-surface interactions that were developed in a prior study from this group. The measurement of the free energies of adsorption of peptides to a series of self-assembled monolayers with a wide variety of chemical properties was made using the surface plasmon resonance method. The adsorption results for 108 peptide-surface combinations were used to prepare corrected force fields for protein-surface interactions.⁹¹ In addition to showing a wider range of orientations and conformation with their improved methods, they showed that the predicted degree of secondary structural elements (helix and sheet) for the adsorbed protein made with the improved force fields was closer to the experimental value than the conventional computational approach.

VII. Summary and conclusions

The adsorption of Fg to biomaterials is rapid, substantial, and converts the surface to one that promotes the adhesion and activation of platelets that in turn promotes blood clotting. Adsorbed Fg also promotes adhesion of monocyte/macrophages involved in the foreign body reaction. Fg undergoes structural changes after adsorption that affect its potency in supporting platelet and monocyte adhesion, but these changes vary with surface type. Extremely effective nonfouling surfaces are required to lower Fg adsorption to the ultra-low range required to prevent platelet or monocyte attachment, but when this is achieved, the materials become inert in the soft tissue space, and do not elicit a foreign body reaction. It is concluded that Fg adsorption is probably the single most important event influencing the biocompatibility of biomaterials in soft tissue, and the same is likely true in blood. The author looks forward to devices with greatly improved blood compatibility based on the principle of extremely low Fg adsorption.

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