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The Vroman Effect: A Molecular Level Description of Fibrinogen Displacement

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Abstract: The molecular level details of the displacement of surface adsorbed fibrinogen from silica substrates were studied by atomic force microscopy, immunochemical assays, fluorescence microscopy, and vibrational sum frequency spectroscopy. The results showed that human plasma fibrinogen (HPF) can be readily displaced from the interface by other plasma proteins near neutral pH because the positively charged αC domains on HPF sit between the rest of the macromolecule and the underlying surface. The αC domains make weak electrostatic contact with the substrate, which is manifest by a high degree of alignment of Lys and Arg residues. Upon cycling through acidic pH, however, the αC domains are irreversibly removed from this position and the rest of the macromolecule is free to engage in stronger hydrogen bonding, van der Waals, and hydrophobic interactions with the surface. This results in a 170-fold decrease in the rate at which HPF can be displaced from the interface by other proteins in human plasma.

Introduction

The formation of biofilms on man-made surfaces affects fields ranging from food processing to biosensor design.[1,2] One particularly important topic is the adsorption of blood and related biofluids onto nascently implanted materials such as artificial hips.[3] Leo Vroman and Ann Adams first demonstrated in the late 1960s that protein adsorption from blood plasma involves a complex series of adsorption and displacement steps.[4] This phenomenon, now known as the Vroman effect,[4−7] has subsequently been shown to involve the initial adsorption from the fluid phase of abundant but weakly surface-active proteins. These early adsorbers are subsequently displaced by more strongly binding species that are present in solution at lower concentration. Despite speculation regarding possible mechanisms for this phenomenon,[8] no definitive experimental evidence has been available to explain it. This is unfortunate because a mechanistic understanding of the Vroman effect could aid in the search for biocompatible materials. Indeed, proteins are usually the first species to arrive at a nascently formed biological/artificial interface and the nature and concentration of these adsorbates strongly influence subsequent cellular recruitment, platelet adhesion, and thrombosis.[9,10]

One of the classic Vroman effects involves the displacement of HPF from a silica substrate.[11−13] Over the past few decades, it has been shown that fibrinogen displacement by other plasma proteins such as kininogen and clotting factor XII depends on numerous factors including temperature, the extent of dilution of the plasma, and the specific surface chemistry.[5,14] HPF consists of two peripheral D domains and one central E domain linked together by triple-stranded α-helical coiled coils (Figure 1).[8]

Additionally, there are two αC domains that interact with the central E domain in a pH specific manner.[16] Namely, near neutral pH, the αC domains are strongly bound to E, but upon lowering the pH below 3.5 they become reversibly detached.[8,10,16] HPF is net negatively charged at pH 7.4 with the highest concentration of negatively charge residues residing on the E and D domains. On the other hand, the αC domains, which are rich in Arg and Lys residues, are actually positively charged. The hydropathic index for HPF indicates that the E and D domains are substantially more hydrophobic than the αC domains.[8]

The work presented here employs a combination of techniques including atomic force microscopy, vibrational sum frequency spectroscopy (VSFS), immunochemical assays, and kinetic experiments to help elucidate the HPF displacement mechanism.

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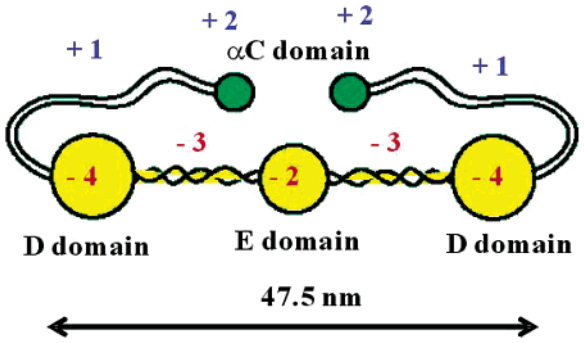

Figure 1. Molecular structure of human plasma fibrinogen (HPF). The D and E domains are formed from two sets of three polypeptide chains (Aα, Bβ, and λ) connected by 29-disulfide bonds. The N-termini of these chains form the E domain while the C-termini of the Bβ and λ chains make up the two D domains. HPF is 90 × 475 × 60 Å with a molecular weight of 340 kD.[15]

The results demonstrate that the protein's αC domains play the critical role. When the protein is adsorbed to a hydrophilic surface via these moieties, its displacement rate in the presence of human plasma is approximately ~170 times faster than when these domains are not in direct surface contact. Even more significantly, spectroscopic studies show evidence for highly aligned Arg and Lys residues interacting with the negatively charged substrate only when the αC domains make direct surface contact. The interfacial ordering of these residues appears to be the hallmark of a weak and labile electrostatic attraction between the substrate and the adsorbed macromolecule.

Experiment

Materials. Human plasma fibrinogen (>95% purity) was purchased from Sigma and its purity was verified in our laboratory using size exclusion chromatography with an Akta purifier (Amersham Bioscience). The results showed the protein to be at least 98% pure. Stock solutions of HPF were made by dissolving the macromolecule in a $NaHCO_3$/ Na_2CO_3 buffer (pH 9.0, 50 mM) at a concentration of 3.0 mg/mL. These solutions were aliquoted and stored at −80 °C. Just before use, the protein solutions were thawed and diluted in appropriate phosphate buffers. Human plasma was purchased from CBR Laboratories (Woburn, MA) and diluted to a 5% solution in water (NANOpure Ultrapure Water System, Barnstead, Dubuque, IA) before use. The water employed had a minimum resistivity of 18 MΩ·cm. Anti-fibrinogen Aα chain (Aα 529−539) antibody was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY) and labeled by Alexa Fluor-594 dye from Molecular Probes (Eugene, OR). IR grade fused silica disks, which were used in all experiments, were purchased from Quartz Plus Inc. (Brookline, NH).

Atomic Force Microscopy (AFM). AFM images were taken with a Nanoscope IIIa Multimode SPM (Digital Instruments, Santa Barbara, CA) equipped with a type "J" scanner. The silica substrates were pretreated with an $H_2 - O_2$ flame for 5 min. The pretreated substrates were then cleaned in a dichromate-sulfuric acid cleaning solution and annealed at 1050 °C for 12 h in a kiln. Finally, the disks were O_2 plasma treated immediately before use. To perform an experiment, a piece of silica was mounted onto a stainless steel disk for placement into a liquid cell. Protein solution at 0.5 μg/mL was introduced into the cell, which was equipped with an O-ring seal and allowed for exchange of the bulk solution. This concentration was chosen because higher concentrations made it difficult to keep track of the individual HPF molecules. The same concentration was therefore also used for all other experiments except VSFS. In that case, 5.0 μg/mL was employed to obtain sufficient signal. All AFM images were obtained with 100 nm long oxide-sharpened triangular probes (silicon nitride, spring constant: 0.58 N/m) in fluid tapping mode at a scan rate of 1−2.5 Hz. The drive frequency was 8−9 kHz and the drive amplitude was 0.25−0.7 V. The set point was chosen at 85−90% of the free amplitude. The only treatment applied to the images was flattening.

Vibrational Sum Frequency Spectroscopy (VSFS). VSFS experiments were performed with a passive-active mode-locked Nd:YAG laser (Continuum, Santa Clara, CA) equipped with a negative feedback loop in the oscillator cavity to provide enhanced shot-to-shot stability. The 1064 nm beam had a pulse width of 21 ps and operated at a repetition rate of 20 Hz. It was used to pump an optical parametric generator/oscillator (OPG/OPA) stage (Laser Vision, Bellevue, WA) that produced the 532 nm and tunable infrared input beams (2800 cm^{-1} to 3600 cm^{-1}) used in these experiments. All sum frequency spectra presented here have been taken with the SSP polarization combination, referring to the sum frequency, visible, and infrared beams, respectively. Each data set was normalized to spectra taken from a piece of Z-cut crystalline quartz.

Total Internal Reflection Florescence Microscopy (TIRFM). All protein solutions were prepared in phosphate buffered saline (PBS) (0.05 M NaH_2PO_4; 0.15 M NaCl) at pH 8.0. Alexa Fluor-594 dye was used to label the fibrinogen for TIRFM. To minimize the effect of the dye on the adsorption properties, the labeling degree was kept to 0.7 dyes/protein. Alexa-594 HPF was employed at a concentration of 0.5 μg/mL. Samples consisted of a silica substrate surrounded by simple poly(dimethylsiloxane) walls to prevent solutions from spilling off the surface. A cover slip could be placed over the top to prevent evaporation. To begin an experiment, Alexa-594 HPF was allowed to adsorb onto the substrate from a protein solution for 20 min. The bulk solution was then rinsed away with PBS at pH 8.0. This process and subsequent displacement experiments were monitored via total internal reflection fluorescence microscopy using a Nikon E800 fluorescence microscope equipped with a Micromax 1024 CCD camera (Princeton Instruments). All images were acquired under a 10× objective. TIRFM was performed by reflecting a 1 mW 594 nm Helium−Neon laser beam (Uniphase Manteca, CA) off the sample/solution interface using a dove prism setup which was optically coupled to the backside of the planar silica substrate through immersion oil. Displacement studies were performed by replacing the buffer with diluted human plasma, which was incubated over the surface for the duration of the experiment.

Results

Protein Displacement Kinetics. To investigate fibrinogen displacement kinetics, silica samples were coated with fluorescently labeled HPF by introducing a 0.5 μg/mL protein solution in PBS at pH 8.0 over the substrate. The solution was allowed to incubate for 20 min and then replaced with pure buffer. Next, 5% human plasma was incubated over the surface and the displacement rate of the labeled HPF was monitored as a function of time using TIRFM. As can be seen from the data, the fibrinogen was displaced from the interface as a function

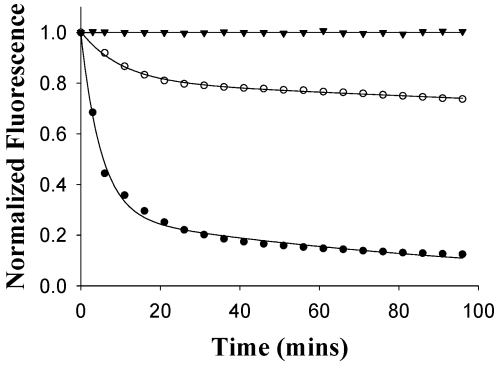

Figure 2. Displacement of Alexa 594-labeled fibrinogen from a silica surface by a 5% human plasma solution. The closed circles (●) indicate experiments with 5% human plasma after only exposing the sample to PBS at pH 8.0. The open circles (○) show displacement kinetics with 5% human plasma after pH cycling through 3.2 and back to 8.0. The inverted triangles (▼) represent a control experiment where only buffer was introduced over the HPF coated surface.

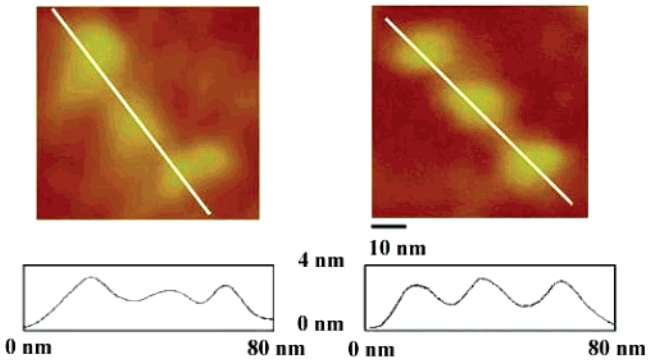

Figure 3. AFM images of a single HPF molecule adsorbed at the silica/buffer interface (a) at pH 8.0 and (b) at pH 8.0 after cycling to pH 3.2. The line profiles for height vs position (from the white lines) are shown below each image.

of time in an exponentially decaying manner (Figure 2, ●).[17] Roughly 80% was removed after 90 min. On the other hand, hardly any protein was displaced from the surface if only buffer was introduced instead of the 5% plasma solution (Figure 2, ▼). Finally, the displacement rate was probed under conditions that should allow the αC domains to be detached from the E domains and then subsequently reattached.[16] This was accomplished by replacing the pH 8.0 buffer with a similar PBS solution at pH 3.2 and then rinsing again with pH 8.0 buffer before attempting to displace the surface adsorbed HPF with 5% human plasma (Figure 2, ○). The results from this final kinetics experiment showed a severe attenuation in the amount of protein that could be displaced from the surface. The data sets taken with 5% plasma could be fit to double exponential decay curves

$$y = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \quad (1)$$

where t is time, y is the fluorescence intensity, a_1 and a_2 are coefficients between 0 and 1 such that $a_1 + a_2 = 1$, and τ_1 and τ_2 are the time constants. All data sets were repeated 5 times. Fitting the data and averaging gave the time constants and surface fractions of the fast and slow exchange components:

	first time constant (τ_1) and coefficient (a_1)	second time constant (τ_2) and coefficient (a_2)
pH 8.0 sample	5.3 ± 2.3 min (0.72 ± 0.01)	196 ± 98 min (0.28 ± 0.01)
pH cycled sample	6.7 ± 3.5 min (0.20 ± 0.02)	909 ± 372 min (0.80 ± 0.02)

As indicated, 72% of the protein from the standard pH 8.0 sample can be displaced with a time constant, τ_1, of just over 5 min. Furthermore, the pH cycled sample shows nearly the identical τ_1 value, but the surface concentration of this fraction was reduced to just 20%. There is a reasonable fraction of protein that is more difficult to remove, even on the sample that was not exposed to acidic conditions (28%). Substrate defects, trace surface impurities, differences in the orientation of the protein at the interface, as well as other factors may all potentially contribute to this slow component, τ_2. Significantly, τ_2 is substantially affected by pH cycling. Indeed, the 909 min time constant seems to reflect a restructuring of the adsorbed protein and the majority of HPF molecules (80%) now fell under the slow classification. To elucidate the origin of the pH cycling effect, atomic force microscopy, immunochemistry, and vibrational sum frequency experiments were performed.

Atomic Force Microscopy and Immunochemistry. HPF was imaged by atomic force microscopy at the silica/buffer interface. As above, a 0.5 μg/mL protein solution in PBS at pH 8.0 was flowed over a planar silica substrate, incubated for 20 min, and replaced with pure buffer before an image was obtained. Under these conditions, approximately 2.5% of the substrate was found to be covered with protein. A close-up picture of a typical fibrinogen molecule is shown in Figure 3a.

Wider field AFM images as a function of protein concentration are provided in the supplementary section of this paper. The average length of the adsorbed molecules was 59.8 ± 3.4 nm. This number is approximately 30% larger than the value obtained from electron microscope[18] mostly because of convolution with the finite-sized AFM tip and perhaps also because of a small amount of surface spreading by the protein. The three main domains of HPF (one central E domain and two D domains) were clearly identifiable. The height of the D domains averaged 2.7 ± 0.2 nm, whereas the E domain was 2.3 ± 0.4 nm. These numbers are in good agreement with previous data from hydrophilic mica surfaces.[19] At this point, the pH of the solution was lowered to pH 3.2. Images of adsorbed HPF under these conditions showed little change from the initial conditions at 8.0; however, substantial changes were noted when the pH was cycled back to 8.0 (Figure 3b). As can be seen, the central E domain was raised significantly, whereas the D domains showed only minor changes. Specifically, the heights of the D domains were still the same (2.6 ± 0.3 nm) within experimental error, whereas the E domains increased to 3.4 ± 0.4 nm. The single molecule experiment was repeated dozens of times and the ~1 nm height increase of the E domain with little change in the D domains occurred roughly 80% of the time. The other 20% of the time, little or no changes were observed in either

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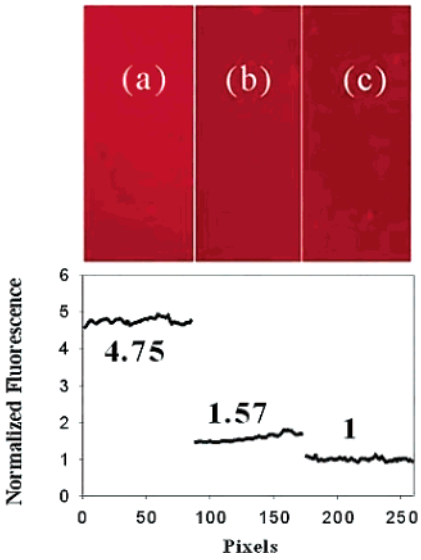

Figure 4. Fluorescence images of Alexa 594-labeled anti-fibrinogen (anti-αC domain) antibody applied to (a) an HPF coated surface after pH cycling, (b) an HPF coated surface at pH 8.0 without pH cycling and (c) an uncoated silica surface. To help inhibit nonspecific adsorption of the anti-fibrinogen antibody, 0.1 mg/mL of generic rabbit IgG was incubated over the surface before the specific binding experiments were commenced.

the D or E domains. The presence of the minority component is in good agreement with the kinetic experiments, which also showed the presence of two populations.

The AFM studies suggested that for the majority of the HPF, the αC domains migrated from the silica interface to the top of the E domain after pH cycling. To verify this conclusion, immunochemical assays were performed. HPF was deposited onto the surface of three silica samples under the same conditions used above. In the first, the pH was cycled to 3.2 and back to 8.0. A 100 μg/mL solution of dye labeled IgG raised against the αC domains (peptide sequence Aα 529−539) of HPF was introduced above the substrate for 20 min and washed away. The results showed a significant amount of binding of the IgG at the interface as indicated by the high fluorescence signal (Figure 4a). The binding experiment was repeated with the second sample without pH cycling (Figure 4b). In this case, the binding was substantially reduced. Finally, a control experiment was performed by adding the IgG over a substrate surface that was not coated with protein (Figure 4c). Subtracting the nonspecific background found in this third experiment from the data in the first two and taking the ratio of these revealed that 6.6 times as much IgG was specifically adsorbed after pH cycling than before cycling.[20] In other words, the accessibility of the αC domains to the IgG was substantially increased upon cycling. In fact, the αC domains are almost certainly at least somewhat beneath the rest of the protein upon initial HPF adsorption, since they are inaccessible to the IgG. They should also be directly bound to the E domain.[16] Once the pH was lowered to 3.2, however, these moieties became unbound from the E domain, which allowed the rest of the protein to come into direct contact with the silica surface. When the pH was subsequently raised to 8.0, these species were forced to rebind

(20) Quantitative fluorescence measurements were performed and showed that ~75 antibodies nonspecifically adsorbed per square micron. It is, however, difficult to obtain an exact count of fibrinogen molecules containing a bound IgG from fluorescence. This is because each fibrinogen contains two binding sites.

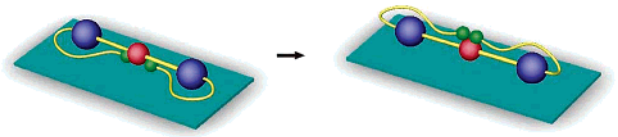

Figure 5. Proposed mechanism for interfacial HPF rearrangement upon pH cycling.

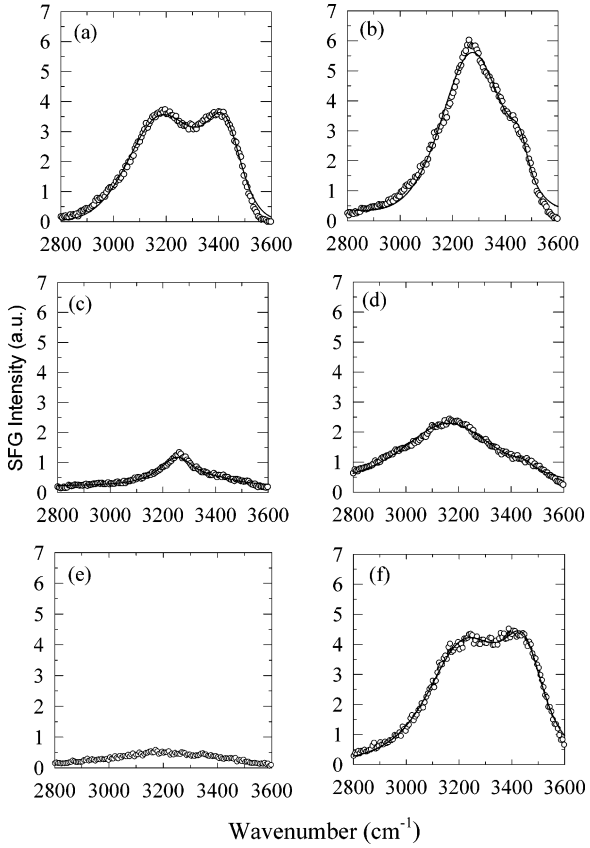

Figure 6. Sum frequency spectra of (a) a bare silica/water interface at pH 8.0, (b) an HPF coated surface at pH 8.0, (c) an HPF coated surface at pH 5.5, (d) an HPF coated surface at pH 3.2, (e) an HPF coated surface at pH 5.5 after cycling to pH 3.2, and (f) an HPF coated surface at pH 8.0 after cycling to pH 3.2

to the E domain from the top side of the molecule as the bottom was no longer accessible (Figure 5).

Vibrational Sum Frequency Spectroscopy. VSFS is a surface specific vibrational spectroscopy that can be employed to probe interfacial protein alignment and water structure even in the presence of an overwhelming contribution from bulk aqueous solution.[21−25] Experiments were carried out by first introducing protein free pH 8.0 PBS into a flow cell. An SFS spectrum of the silica/water interface in the OH stretch region of the vibrational spectrum (2800−3600 cm^{-1}) is shown in Figure 6a. The two peaks visible near 3200 and 3400 cm^{-1} correspond to water molecules with tetrahedrally coordinated structure and water with less ordered bonding, respectively.[26−28]

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The interfacial water is ordered by the charged hydrophilic silica surface. At this point, 5.0 μg/mL HPF was flowed into the cell, allowed to incubate for 20 min, and washed out with pure buffer. The surface coverage of fibrinogen was approximately 32% as judged by additional AFM measurements (see the Supporting Information). The VSFS spectrum showed dramatic changes under these conditions (Figure 6b). Specifically, the 3200 cm^{-1} feature increased in intensity and shifted upward in frequency to near 3280 cm^{-1}. Such a result is very atypical for OH stretch peaks from water at a hydrophilic interface upon the adsorption of a net negatively charged protein.[29] In fact, the charged macromolecules and their counterions should cause a suppression of the water features under these circumstances due to charge screening. This is strong evidence that an additional feature is convoluted with the water peaks.

The additional peak could be revealed by lowering the system's pH to the isoelectric point near pH 5.5,[15] which almost completely eliminated the signal from organized water molecules (Figure 6c). In this case, a small sharp feature near 3280 cm^{-1} was revealed, which can be assigned to the NH stretch of aligned primary amine moieties on Lys residues and the related NH stretches on Arg.[30] Confirmation that this was not an OH stretch peak was obtained by repeating all the experiments in $H_2{}^{18}O$ and noting that the 3280 cm^{-1} feature hardly moved while the OH peaks red shifted by $\sim$12 cm^{-1}, as expected. The spectra at pH 5.5 and 8.0 were shown to be completely reversible by pH cycling between them; however, when the pH was subsequently cycled through 3.2 (Figure 6d), both the pH 5.5 and 8.0 spectra were changed irreversibly (Figure 6, parts e and f) and the NH stretch peak disappeared.

The presence of an NH stretch upon initial protein adsorption is direct molecular level evidence that Lys and Arg residues become highly oriented at the negatively charged silica interface. Indeed, such orientation is also in excellent agreement with the idea that adsorption via the αC domains is largely electrostatic in nature. It should be noted that when the VSFS experiments were repeated at lower concentration of HPF, that the NH stretch signal became substantially weaker. Because most of the fibrinogen molecules still remained distinct from each other at 32% surface coverage, there is little reason to suspect that the NH stretch peak is the result of protein−protein interactions. Indeed, no major changes in the peak intensity were observed when very high concentrations (50 μg/mL) of HPF were employed. Under these last conditions, all protein molecules are in contact with their neighbors at the interface. Finally, it should be mentioned that no CH stretch peaks were observed in these spectra, which probably indicated that a high degree of alignment was not achieved for residues other than Arg and Lys. In fact, CH alignment from proteins[22] and polymers[31] at the silica/buffer interface has typically been found only at high pH (e.g., 9−12) where the electric field emanating from the surface was quite large. Even then, CH stretch observation was limited to cases where the macromolecules possessed a net positive charge.

Discussion

The facile displacement of HPF from a silica interface is remarkable given the protein's large size. This property is clearly related to the αC domain's ability to prevent other portions of the protein from making stronger contacts than those afforded by simple electrostatic binding between Arg/Lys residues and deprotonated surface silanols. In the somewhat analogous problem of analyte retention on chromatography columns,[32−34] the literature indicates that very long retention times on silica can be related to isolated surface silanol groups which afford strong hydrogen bonds. On the other hand, interactions with deprotonated silanols seem to be associated only with shorter retention times. In the case of proteins, strongly adsorbing species almost certainly interact with the substrate through hydrogen bonding, van der Waals, and related hydrophobic interactions.[35] The key point for fibrinogen is that these types of interfacial contact can be substantially attenuated near physiological pH by the intervention of the positively charged αC domains.

The final issue to be addressed here is the role αC domains play in HPF's function in vivo. It is well established that the cleavage of small peptides from the C-terminus of fibrinogen's α and β polypeptides by thrombin forms the active protein, fibrin.[36] Fibrin readily cross-links to form a blood clot in a process known as thrombosis. It is believed that the αC domains can perform a supporting role in maintaining a cross-linked fiber's integrity by interacting with other αC domains on neighboring molecules.[16,37] Because several of the steps in the blood clotting cascade can be accelerated by the presence of a phospholipid membrane, it is reasonable to consider whether the αC domains may also play a role in keeping fibrinogen soluble in the bloodstream until it is needed. Indeed, many cell membranes are negatively charged and might be expected to interact with the positively charged portion of HPF. Such labile interactions might help prevent fibrinogen from strongly adsorbing to cell surfaces before conversion to fibrin has commenced. It would be curious if the Vroman effect for this molecule were a consequence of such a role for the αC domains. Further experiments will be needed to test this hypothesis.

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Supporting Information Available: AFM images as a function of bulk protein concentration during incubation. This material is available free of charge via the Internet at http://pubs.acs.org.

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