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Manipulation of Proteins on Mica by Atomic Force Microscopy

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Abstract

The atomic force microscope was used to image adsorption of a monoclonal IgM on mica in real time. Under the smallest possible force we could achieve (<4 nN), the cantilever tip behaved as a molecular broom and was observed to orient protein aggregates in strands oriented perpendicularly to the facet of the cantilever tip. Rotating the scan direction preserved the orientational relationship, as seen by the formation of rotated strands. When the applied force was increased, the distance between the strands increased, indicating the amount of protein that can be swept depends on the applied force. The effect of scanning increased the apparent surface coverage of IgM. Manipulation of a deposited fibrinogen layer with a 4-nN repulsive force was observed only after tens of minutes, but not to the extent that strands formed, indicating a greater adhesion between the fibrinogen and mica than between IgM and mica. With an applied repulsive force of 30 nN, fibrinogen strands formed and the protein was manipulated to produce the block letter U. At a much higher repulsive force, the entire scanning area was swept clean.

Introduction

The ability of the atomic force microscope (AFM) to image nonconductors in both air and aqueous environments has enabled it to be used as a research instrument for the biological community. Already, it has been used to observe nucleic acids,^{1–4} lipid assemblies,^{2–4} proteins,^{4–10} and even entire cell surfaces.^{10–13}

The amount of AFM work in the biological area is currently small, but expanding rapidly. Unfortunately, most of the published studies merely report the observation of various structures on surfaces and do not address the issue of perturbation of the observed system by the AFM cantilever tip.

In a previous study, we had observed the adsorption of a monoclonal IgG from solution onto mica.⁶ We hypothesized that the IgG ridges were a consequence of the protein being swept across the surface by the scanning probe. Since then, we have been focusing our efforts on observing real time adsorption of proteins in a system in which perturbation by the scanning

probe is minimal. We found, however, protein manipulation so prevalent that an unperturbed observation of the adsorption process would not be easily attainable. We decided to investigate how manipulation by the cantilever tip can be used to determine interactions between the tip and protein and between protein and substrate. The results of this study demonstrate that the cantilever not only has a profound effect on adsorption processes, but also may be used to manipulate molecules into desired patterns or shapes.

To study the effects of scanning the cantilever tip on protein adsorption processes, we followed the adsorption of an IgM on mica. The rationale for using IgM in these experiments was that, due to the limited resolving power of the AFM, the protein needed to be large and have a distinct molecular shape in order to observe individual proteins. IgM has a molecular weight of 900 kDa and its tertiary structure when viewed by electron microscopy resembles a five-pointed star.¹⁴ The IgM is a mouse monoclonal antifuorescyl antibody (clone 18-2-3).^{15–17} This particular IgM is a cryoglobulin that disaggregates, producing individual pentamers, when fluorescein is bound or when the ionic strength exceeds 0.3 M. All IgM work in this study is with the fluorescein-bound, disaggregated form. The association constant for the antigen is $(2-3) \times 10^{10} \text{ M}^{-1}$, which is unusually large for an IgM.

Experimental Section

The atomic force microscope used is a commercial one (Digital Instruments) that incorporates optical beam deflection¹⁸ for sensing cantilever motion. The cantilevers (Park Scientific), with spring constants of 0.37 and 0.064 N/m, were microfabricated from silicon nitride and have a 600-Å vapor-deposited, gold–chromium layer.

To conduct the IgM adsorption experiment, mica was first imaged under pH 8.0 phosphate buffer saline (PBS) in a fluid cell. The PBS was then exchanged for a 20 µg/mL IgM solution in pH 8.0 PBS. We decided to operate at pH 8.0, the isoelectric point of the IgM clone, where adsorption of the protein would be maximum and the lateral interactions between the proteins would be the greatest, due to decreased electrostatic repulsion. A very small area was initially scanned in order to minimize the effect of the cantilever tip on the adsorbing protein while the applied force was being minimized.¹⁹ Once the force was minimized, the scan area was increased to 3000 nm by 3000 nm and the adsorption process was observed using constant-force mode (4 nN). After 12 min of continuous scanning a 3000 nm by 3000 nm area, the scan size was increased to 9700 nm by 9700 nm. Scanning of this larger area was continued in constant-height mode.

The influence of the tip on the protein adsorption process was investigated by scanning the same 1700 nm by 1700 area in the constant-height mode at three different applied forces: 8, 12, and 14 nN. IgM was allowed to adsorb on mica from a 20 µg/mL pH 8.0 PBS solution for 13.5 min. Then three images were taken consecutively, beginning at low force and ending with high force, and each was taken after three successive scans of 1700 nm by 1700 nm areas, with the first scan being from bottom to top.

An experiment in which adsorbed protein was intentionally manipulated into a desired shape was conducted using fibrinogen (65 % clottable human sample, U.S. Biochemical Corp., Cleveland OH). A 100 $\mu\text{g/mL}$ solution of fibrinogen in pH 8.0 PBS was allowed to adsorb onto the mica in the fluid AFM cell for 5 min. The protein solution was then exchanged for buffer. A moderate repulsive force of 30 nN was applied and seven adjacent 2000 nm by 2000 nm areas were scanned twice each in the form of a block U. The applied force was then minimized to 4 nN and the scan area was increased to 10 μm by 10 μm for observation.

Results and Discussion

When IgM adsorption was imaged with the atomic force microscope using an initial force of approximately 4 nN, the first IgM molecules appeared as aggregates on the mica surface (Figure 1). It is not known if the IgM adsorbs in the form of aggregates existing in solution or if the cantilever tip pushes individual proteins already on the surface together to form these small aggregates. The former is possible since the protein solution was buffered to the isoelectric point of the IgM, the pH at which maximum aggregation is expected to occur. As time progressed, more aggregates appeared and they began to line up in strands oriented perpendicularly to the fast-scanning (x) direction (Figure 2).²⁰ The implications are that the adhesion between the IgM and the mica under these conditions is weak and easily disrupted by the scanning cantilever tip.

Figure 3 shows the image of IgM adsorption after 12 min (a 9700 nm by 9700 nm area captured in constant-height mode). Two partially superimposed 3000 nm by 3000 nm areas that were previously scanned are indicated in the lower right portion (area 1), The lower third of the entire 9700 nm by 9700 nm area was scanned twice (indicated as area 2), and the upper two-thirds only once (indicated as area 3). It is evident that in the less scanned areas, the IgM is more uniformly distributed over the surface and is not forced by the cantilever tip to line up in strands. Figure 4 shows that further scanning of the whole 9700 nm by 9700 nm area causes the unperturbed IgM to align in strands and apparently increases the amount of IgM on the surface.

The phenomenon of molecular alignment by AFM was observed previously with fibrin monomers in PBS on mica,⁵ with a monoclonal IgG in PBS on mica in solution,⁶ and with von Willebrand's factor on mica in air,⁹ where the applied force is much greater. Apparently, the cantilever tip behaves as a "molecular broom" that sweeps individual proteins or small protein aggregates into larger piles in the fast-scanning direction. As will be shown later, the exact orientation of the protein aggregates will depend on the orientation of the facets of the cantilever tip relative to the scanning axes. Figures 5 and 6 show how that the tip-induced aggregation depends on which scanning mode is used. The individual proteins and protein aggregates exert a force, which can be broken down into horizontal and vertical components, on the sweeping cantilever tip. When the protein pile gets too large and its interaction with the surface increases above a critical value, the vertical force exerted on the cantilever tip increases to a detectable level. In the constant-force mode (Figure 5), the piezoelectric crystal will retract to maintain constant force allowing the cantilever tip to slide over the aggregate. At this point, the cantilever tip no longer pushes the aggregate. Once past the aggregate, however, it begins the sweeping process again. In the constant-height

mode (Figure 6), the piezoelectric crystal does not respond to the cantilever deflection. Instead, the vertical force component will increase to the point where the cantilever is deflected up over the aggregate. Then, the vertical force decreases and the cantilever deflects back toward its initial position (Figure 6). These processes repeat themselves as the cantilever tip moves to the next position in the slow- (y) scanning direction.

When comparing these two scanning modes, one would expect that protein perturbation would be greater for constant-height scanning than for constant-force scanning. Assume the imaging by each mode is done at the same initial applied force. The difference in the force the protein experiences with the tip positioned above it, F , would be given by eq 1, where k is the spring constant of the

$$\Delta F = k(\Delta z - \delta) \quad (1)$$

cantilever, z is the distance the cantilever would deflect above the protein (tens of angstroms) in the constant-height mode, and δ is the minimum cantilever deflection distance the split photodiode can detect ($\sim 1 \text{ \AA}$).

The applied forces that are exerted on the cantilever are the overall forces, and they depend on the geometry and volume of the cantilever. The localized forces, however, can be very different. From the interaction potential calculations, one can conclude that the force between the apex of the cantilever tip and the substrate would have a greater repulsive contribution to the overall force than the rest of the tip would. At present, it is not known over what area this overall force is exerted. The influence of the tip at three different applied forces (i.e., at 8, 12, and 14 nN) on the protein adsorption process is shown in Figures 7–9. These three images were taken consecutively, beginning at low force and ending with high force, and each was taken after three successive scans of 1700 nm by 1700 nm areas. Two effects are evident in Figures 7–9: (a) with increasing force, the spacing between the adsorbed protein strands increases, and (b) protein strands are oriented diagonally with the respect to the scanned area.

The effect of the applied force to the spacing between the strands can be explained by eq 2. As the applied force,

$$F = F_{\text{applied}} + k\Delta z \quad (2)$$

F_{applied} , increases, the force exerted on the cantilever, F , for the same height deflection of the cantilever, z , also increases. The smaller aggregates that appear in Figure 7, where F_{applied} was 8 nN, were insufficient to deflect the cantilever when larger forces are applied (Figures 8 and 9), apparently due to the smaller overall adsorption interactions between the protein aggregates and the surface. The small aggregates are swept further along in the fast-scanning direction, picking up more protein, until the growing aggregate acquired an interaction with the surface sufficient to deflect the cantilever. Consequently, the spacing between the strands must increase when the applied force increases, according to the proposed model. The surface coverage data in Table I confirm this by indicating a smaller surface coverage at increased applied force.

One noticeable difference between Figures 7–9 and Figures 1–4 is the orientation of the strands. In Figures 1–4 the strands are generally oriented vertically, while in Figures 7–9 they run diagonally across the images. As it turned out, the difference was due to the angle between the fast-scanning direction and the orientation of the pyramidal cantilever tip. In the experiment presented by Figures 1–4, the scan orientation was 0° and the front facet of the pyramid first contacted the protein (Figure 10A). When the scan orientation was 45° , as was the case in the experiment presented by Figures 7–9, the edge between two facets contacted the proteins first (Figure 10B). The facet then pushed the protein at an angle 45° from the fast-scanning direction. In this manner, the protein strands can be seen to align parallel to the plane of the pyramid facets.

Scanning over adsorbed protein aggregates in the constant-height mode can produce other artifacts as well. While traversing over a very large protein aggregate, the cantilever tip can push part of the aggregate in front of itself, which produces “streaking” in the image. This effect appears as a white horizontal line like the one visible in the left side of Figure 9. Or, after crossing over an adsorbed aggregate, the cantilever tip can also adhere to it. This will cause a temporary deflection of the cantilever toward the surface which, in turn, appears in the image as a dark horizontal line (Figures 8 and 9). As a reminder, it has to be mentioned that the cantilever tip scans back and forth over the surface in the fast- (x) scanning direction. However, the images captured by the instrument are composed from the signal derived from the forward scanning only. The forward scanning direction corresponds to the right-to-left direction in all images presented here. Thus, the protein adsorption images obtained by AFM present a dynamical picture composed from the spatially and temporally distinct interactions between the tip, protein, and surface.

The percentage of apparent protein surface coverage was calculated for each of the images as an area above background. The results are presented in Table I. As expected, Figure 2 shows a higher degree of surface coverage compared to Figure 1, which was obtained at a shorter adsorption time. In the case of Figures 3 and 4, it is not known whether the background is an image of mica or of a smooth layer of adsorbed IgM due to the lack of molecular specificity of the cantilever tip. In Figure 3, the most scanned area (area 1) had the highest apparent surface coverage, whereas the area scanned only once (area 3) had the lowest apparent surface coverage. There are two plausible explanations for this perceived increase in coverage as the number of scans over the same area is increased. One explanation is that the scanning process increases the adsorption rate. Normally, there is an unstirred layer of solution, referred to as the quiescent boundary layer, close to the surface. The rate of protein deposition is limited by the rate of diffusion of the protein through the quiescent boundary layer. During the scanning process, however, the relative motion of the cantilever tip with respect to the surface produces convective currents which can reduce the quiescent boundary layer thickness and thus increase the rate transport of IgM to the mica surface. Note that in Figure 4, the surface coverage over any reasonably sized area was 34%. Thus, within 1.5 min of scanning, the apparent surface coverage increased from 8% (area 3 in Figure 3 after 12 min of adsorption) to 34% (Figure 4 after 13.5 min of adsorption). The results suggest that, if the increase of the IgM transport rate is responsible for the sudden surface coverage increase, one can control the effect by varying the scan rate. A second

explanation is that, if the smooth background is an adsorbed IgM mono-layer, then the cantilever can push protein molecules within this layer, producing a greater corrugation. In this situation, only those molecules pushed into large mounds would be included in the coverage calculations. The coverage would reflect the extent of the molecular broom effect. If the molecular broom effect is the dominant mechanism, one concludes that the perturbation occurs very rapidly (within a few scans).

The observation of unintentional manipulation of adsorbed proteins by the cantilever tip naturally led us to examine whether proteins could be manipulated into desired designs. An intriguing extension of this would be to use the AFM as an instrument to construct patterned protein surfaces to be used in biosensors or organized multienzyme arrays. We needed to start with a layer of protein on the surface under a protein-free supernatant since further protein deposition would complicate our design. We found that fibrinogen had the proper surface binding affinity to conduct the experiment and provided a sufficiently stable protein layer for this work. Drake et al.⁵ showed that adsorbed fibrinogen could not be observed on mica with the AFM, probably because of the protein's weak interaction with the surface. However, when thrombin was added, causing the fibrinogen to cleave into fibrin monomers, the activated fibrinogen molecules adsorbed to the surface and the whole process was observed with the AFM. We found that we could observe the adsorbed fibrinogen without addition of thrombin. This was probably due to a somewhat higher level of impurity of our fibrinogen sample. After the fibrinogen solution was exchanged for buffer, a moderate repulsive force of 30 nN was applied and seven adjacent 2000 nm by 2000 nm areas were scanned twice each in the form of a letter U, as indicated by Figure 11. The applied force was then decreased to 4 nN while the scan area was increased to 10 μ m by 10 μ m for observation. Figure 12 shows the results: fibrinogen was uniformly distributed within the large unscanned area, but within the confines of the letter U, fibrinogen strands were oriented by the scanning procedure. The height of the strands was approximately 20–40 nm. With 4-nN applied force, the fibrinogen layer remained stable. We noticed a slight increase in protein surface roughness, but only after observing the fibrinogen for tens of minutes. When forces much larger than 30 nN were applied to the U-shaped fibrinogen layer, the entire scan area was swept clean of protein, indicating that even the largest of aggregates could not withstand the disruptive force of the cantilever tip. The swept aggregates would appear piled up just beyond the edges of the scan area in the $+x$ and $-x$ directions, demonstrating again that aggregates are pushed in the fast-scan direction.

Conclusions

It has been shown that adsorbed proteins on mica are swept into strands of aggregates oriented parallel to the plane of the cantilever tip facet. This is a manifestation of the relatively weak interaction between individual molecules or small protein aggregates and the surface of mica. The area continuously scanned by the cantilever tip had higher apparent surface coverage than the regions that were scanned fewer times. It is proposed that the cantilever tip either increases the adsorbed amount by increasing the rate of transport of protein to the surface or produces corrugations within a fully adsorbed protein layer (a “molecular broom” effect). As the applied force is increased in the constant-height scanning mode, the size of the aggregates that can be swept by the cantilever increases, which causes

the distance between the strands to increase. Researchers should therefore be wary that when studying weakly bound macromolecules at surfaces by AFM, the results will be strongly influenced by the cantilever tip and by the forces involved. Thus, AFM images of adsorbed proteins, fluid membranes, and other “soft” biological specimens may represent a system perturbed by the probe (the cantilever tip). In addition, these images are composite pictures made from the spatially and temporally distinct interactions between the tip, sample, and underlying surface.

The cantilever tip can be used to manipulate protein molecules so that desired designs can be obtained. Although this manipulation is crude, it demonstrates the potential for manipulation on a finer scale such that placement of individual proteins or protein aggregates at desired positions can be achieved. Manipulation of a deposited fibrinogen layer with a 4-nN repulsive force was observed only after tens of minutes, but not to the extent that strands formed, indicating a greater adhesion between the fibrinogen and mica than between IgM and mica. The difference between fibrinogen and IgM suggest that it is possible to use the perturbation of the protein layer caused by the AFM tip to measure the strength of interactions of proteins with surfaces. If the perturbation of the observed process by AFM could be sufficiently minimized, it might be possible to extract quantitative information regarding these interactions using the unperturbed image as a reference state.

Acknowledgments

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19. Since the force minimization was done while the IgM was adsorbing, it is not known if this reported force was between the cantilever tip and mica, the cantilever tip and IgM (if the tip was positioned above the protein), IgM and mica (if IgM had adsorbed onto the cantilever), or IgM molecules (if an IgM-coated cantilever was positioned above adsorbed IgM). These different situations would not necessarily yield the same forces.
20. The microscope scans the surface in a raster pattern with a frequency of 18 Hz in the x direction and 0.05 Hz in the y direction. The x direction is therefore referred to as the fast-scanning direction.

Figure 1.

A 3000 nm by 3000 nm AFM constant-force image of IgM 18-2-3 deposited on mica from a 20 $\mu\text{g/mL}$ pH 8.0 PBS solution after a 1-min adsorption time using a 4-nN force. The vertical scale is height in nanometers. The dark wedge is a pit in the mica possibly formed from the force minimization process.

Figure 2.

A 3000 nm by 3000 nm AFM constant-force image of IgM 18-2-3 deposited on mica from a 20 $\mu\text{g/mL}$ pH 8.0 PBS solution after a 2.5-min adsorption time using a 4-nN force (identical area is in Figure 1). The vertical scale is height in nanometers.

Figure 3.

A 9000 nm by 9000 nm AFM constant-height image of IgM 18-2-3 deposited on mica from a 20 $\mu\text{g/mL}$ pH 8.0 PBS solution after a 12-min adsorption time using a 4-nN force. The vertical scale is force (uncalibrated). In the lower right are two superimposable 3000 nm by 3000 nm areas, previously scanned. The rest of the area was previous unscanned.

Figure 4.

A 9000 nm by 9000 nm AFM constant-height image of IgM 18-2-3 deposited on mica from a 20 $\mu\text{g/mL}$ pH 8.0 PBS solution 1.5 min after the previous image using a 4-nN force. The vertical scale is force (uncalibrated). The protein in the previously unscanned area now resembles the previously scanned areas.

Figure 5.

Schematic representation of protein manipulation on a surface by the cantilever tip in constant-force mode. (A) Tip moving in the fast-scanning direction begins sweeping the proteins across the surface, provided the vertical force exerted on the tip by the protein is small. (B) As the protein begins piling up, the interaction of the aggregate with the surface increases, producing a larger vertical force exerted on the cantilever. (C) When the vertical force becomes sufficiently large to cause cantilever deflection, the feedback system retracts the piezoelectric crystal, as indicated by the downward arrow, to maintain constant force.

(D) The piezoelectric crystal advances, as indicated by the upward arrow, when the vertical force is diminished and the sweeping process begins again.

Figure 6.

Schematic representation of protein manipulation on a surface by the cantilever tip in constant-height mode. (A) Tip moving in the fast-scanning direction begins sweeping the proteins across the surface, provided the vertical force exerted on the tip by the protein is small. (B) As the protein begins piling up, the overall interaction of the aggregate with the surface increases, producing a larger vertical force exerted on the cantilever. When the vertical force becomes sufficiently large, the cantilever deflects up over the aggregate. (C)

Once the vertical force decreases, the cantilever deflects back toward its original position.
(D) The sweeping process begins again.

Figure 7.

A 1700 nm by 1700 nm constant-height AFM image of IgM 18-2-3 on mica, using an 8-nN force. The IgM was allowed to adsorb for 13.5 min from a 20 $\mu\text{g/ml}$ solution in PBS at pH 8.0 and was then scanned three times before this image was taken.

Figure 8.

A 1700 nm by 1700 nm constant-height AFM image of IgM 18-2-3 on mica, using a 12-nN force. The IgM was allowed to adsorb for 18 min from a 20 $\mu\text{g/mL}$ solution in PBS at pH 8.0 and was then scanned three times before this image was taken.

Figure 9.

A 1700 nm by 1700 nm constant-height AFM image of IgM 18-2-3 on mica, using a 14-nN force. The IgM was allowed to adsorb for 19.5 min from a 20 $\mu\text{g/mL}$ solution in PBS at pH 8.0 and was then scanned three times before this image was taken.

Figure 10.

Schematic of orientation of the pyramidal tip to the fast-scanning direction. (A) With 0° rotation (Figures 1–4), the relative movement of one of the pyramidal facets is parallel to the fast-scanning direction (solid arrow). (B) With 45° rotation (Figures 7–9), the edge between two faces is parallel to the fast-scanning direction (solid arrow). The dashed arrows show the direction protein would be moved by the pyramidal facets.

Figure 11.
Schematic of the seven adjacent 2000 nm by 2000 nm scan areas used to produce the block letter U.

Figure 12.

A 10 μm by 10 μm constant-height AFM image of the block letter U formed by scanning seven adjacent 2000 nm by 2000 nm areas of fibrinogen adsorbed on mica using an applied 30-nN repulsive force. The vertical scale is uncalibrated force.

Table I

Percent Surface Coverage of Mica by Adsorbed IgM

image, figure	surface coverage, %	applied force, nN	image, figure	surface coverage, %	applied force, nN
1	12	4	4	34	4
2	17	4	7	42	8
3, area 1	28	4	8	34	12
3, area 2	18	4	9	8	14
3, area 3	8	4			