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Novel Method of Measuring Cantilever Deflection during an AFM Force Measurement

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Abstract

A combination of a reflection interference contrast microscope (RICM) and the atomic force microscope (AFM) was used to monitor the cantilever–surface separation distance during force measurements using the streptavidin–biotin recognition pairs. The RICM showed that the cantilever loses contact with the surface before the final rupture of the adhesive bonds is measured by the AFM detection system. This finding suggests that the immobilization of biotin by physisorbed albumin and subsequent binding of streptavidin might have created a cross-linked protein network whose cohesion is tested by the AFM cantilever with the immobilized biotin ligands.

Introduction

Direct measurement of intermolecular forces between ligand–receptor pairs of molecules has recently been made using the atomic force microscope (AFM).^{1–6} Typically, the ligand-modified AFM probe is brought in contact with a specimen surface containing immobilized receptors. Superposition of some ligands and complementary receptors results in the formation of ligand–receptor bonds whose strength is tested by withdrawing the probe away from the surface, creating a tension on the bonds. The bonds that resist the tension cause a deflection of the cantilever, resulting in an increase in the cantilever restoring force. At some restoring force, the bonds finally yield to the tension and the cantilever snaps back to its resting position. This “pull off” force is, in principle, related to the number and the strength of the tested bonds.

A majority of the AFM instruments in use today measure the deflection of the cantilever using an optical lever method: a laser beam reflected from the upper surface of the cantilever measures the cantilever angle at the position of the reflection.⁷ By assuming that the cantilever behaves as an ideal elastic beam, the measured angle change is used to find cantilever deflection, which, in turn, is used to calculate the cantilever restoring force. A plot of force vs specimen displacement, the so-called “force-displacement” plot, is a common way of presenting the results of the AFM adhesion measurements.⁷

One phenomenon, often found in the AFM measurements of ligand–receptor mediated adhesion, is the appearance of force discontinuities (colloquially referred to as “force jumps”) before the final separation between the probe and the specimen took place.^{1–4} In order to measure the force of a single ligand–receptor bond rupture, one can measure the magnitude of the final “force jump” in the force-displacement plot and from a histogram of forces find the basic force quantum and its integer multiples.^{1,2,4} Alternatively, one can calculate the force variance between many force-displacement runs performed under otherwise identical experimental conditions.⁸ A motivation for this study was the observation that the final “force jump” repeatedly occurred at probe–surface separation distances which were much larger than the total length of ligand–receptor molecules. While some of the immobilization schemes used for ligands or receptor attachments in the literature were designed to be deformable,^{2,4,5} others were quite rigid, invoking various explanations for the observed anomalies such as nonspecific receptor–probe interactions, frictional effects between the probe and the surface, and mechanics of the AFM probe–cantilever constructs.⁹ Here we describe how the combination of the atomic force microscope with a reflection interference contrast microscope (RICM) was used to determine the probe–specimen separation distances at which the “force jumps” occurred. Using a RICM, one can observe fringes which appear at cantilever–surface separation distances equal to multiples of $\frac{1}{2}$ the wave-length of the light used.^{10–14} In the AFM–RICM experiments, the interference between the light reflected by the AFM cantilever and that reflected by the flat surface of the specimen was used to provide direct information regarding the cantilever movement through space above the specimen (Figure 1a). The vertical resolution of the RICM, estimated to be as high as 1 nm,¹⁰ could, in principle, also allow for a measurement of the shape of the cantilever during the AFM experiments. The use of a RICM also facilitated leveling of the rectangular cantilever whose distal edge needed to be positioned parallel to the specimen surface. The optical microscope was used to position the cantilever edge over a suitable area of the surface and to place the optical lever laser beam at the end of the cantilever.

Experimental Section

Instrumentation

The AFM used was a modified Explorer (Topometrix Inc.). The *z*-piezo driving signal generated from a computer-controlled function generator⁶ was used to create a single movement of the probe toward and away from the specimen surface without “engaging” the two together prior to the measurement. The optical lever signal and the *z*-piezo driving signal were used to create the cantilever deflection vs piezo displacement plot. A RICM comprised an inverted light microscope (Diaphot 200, Nikon, Inc.) equipped with a 40× objective (N.A. 0.55, Leitz), a 50/50 beam splitter, a video-rate CCD camera, an image processor (Argus 20, Hamamatsu), and a video recorder (Model 6730, Panasonic). The RICM used white light (100 W Hg lamp) tightly apertured to illuminate the distal end of the rectangular AFM cantilever positioned above the surface. The interference image was filtered through a narrow band-pass filter (488 nm, 10 nm bandwidth) and recorded on the videotape.

Sample and Cantilever Preparation

The surface, a cleaned glass coverslip (#1 1/2), was modified by physisorption of biotinylated-BSA (Pierce Chemical Co.) from a 1 mg/mL solution overnight at 37 °C. After five successive washes with large amounts of borate-buffered saline (BBS), 50 nM immunopurified streptavidin (Pierce Chemical Co.) was allowed to incubate with the surface for 30 min. The surface was washed five times in succession with BBS, and the coverslip was stored in BBS until used. For the measurements, the coverslip was placed in a custom-designed PTFE liquid cell, which fitted snugly into the stage of the microscope. The rectangular AFM cantilevers (Park Scientific Instruments) with no integral tips had dimensions of 100 μm in length, either 10 or 20 μm in width, and 0.3 μm thick. The cantilever surface was modified by physisorption of biotinylated-BSA. The measurements were performed in BBS buffer within 24 h of any surface modifications.

Video Analysis

The RISM images were analyzed on a Macintosh computer using image analysis software (Scion NIH Image, v. 1.57). A two pixel wide cross section along the cantilever length in the middle of the cantilever was selected to create a composite interference fringe image of the whole experiment. The image of fringes at this cantilever cross section was digitized for each RISM frame (captured approximately 0.07 s apart). The 500 images were combined together in chronological order to obtain the montage image shown in Figure 1b. The motion of the cantilever toward the surface is seen in Figure 1b as slanting of fringes from right to left. Interference fringes running parallel with respect to time correspond to the cantilever remaining motionless in contact with the surface (i.e. zero vertical velocity). Fringes slanting from left to right with respect to time indicate movement of the cantilever in the vertical direction away from the surface.

Results and Discussion

In the typical experiment, the biotinylated cantilever was positioned close to the specimen surface carrying the streptavidin layer. Contact of the cantilever edge carrying biotin ligands with the protein layer was initiated by moving the piezo element with the mounted cantilever toward the surface. After a given contact time has elapsed, the piezo was moved away from the surface and brought back to its original position. A typical composite interference fringe image recorded during the motion of the cantilever toward and away from the surface is shown in Figure 1b. During the approach of the cantilever toward the surface, the fringes move from right to left across the edge of the cantilever (Figure 1b, upper part, approach velocity 1210 nm/s). The contact is established when no more fringes cross the cantilever edge. Further movement of the piezo element toward the surface bends the cantilever upward, increasing the loading forces in the contact area, as illustrated as the increase in the distance between the fringes in Figure 1b. During the 8 s delay in piezo movement the fringes remain stationary (Figure 1b, middle part). In the unloading part of the cycle, the fringes move from left to right; their spacing became progressively smaller and more uniform, indicating that the cantilever becomes less bent. When the contact between the cantilever edge and the surface is lost, the fringes of first and higher order start to move across the cantilever edge (Figure 1b, lower part, withdrawing velocity 605 nm/s).

The optical lever detected force–displacement plot recorded during the same experiment is shown in Figure 1c. The laser beam of the optical lever was positioned at the distal end of the cantilever. The x-axis in Figure 1c is shown as the force computed using the nominal spring constant for the cantilever and the cantilever displacement at the positive loads during the unloading part of the experiment.

Figure 2 shows the enlarged segment of the force–displacement plot and the corresponding part of the composite interference image (contrast enhanced for better visualization of discontinuities). The combination of fringe data (Figure 2b) and the optical lever trace (Figure 2a) showed the following:

1. During the unloading, the cantilever approached the cantilever resting position, i.e. the position of zero load. Shortly after the cantilever passed through its resting position, the cantilever edge lost contact with the surface, and the fringes of higher order started to cross the edge of the cantilever.
2. The discontinuity in the fringes coincided with the final “force jump”. However, they both appeared *after* the cantilever edge lost contact with the surface. The fringes indicate that the final “force jump” occurred after the edge of the cantilever had moved approximately 330 nm away from the surface. The optical lever trace showed a change of slope from the unloading portion of the trace before the final “force jump” occurred.
3. The discontinuity seen in the fringes was observed only at the cantilever's distal end. By placing the laser beam anywhere but at the distal end of the cantilever, the “force jump” would have been underestimated, if not missed altogether.

While the vertical resolution of the RICM (estimated to be as high as 1 nm¹⁰) does not rival the vertical resolution capabilities of the AFM (approximately 0.1 nm), there are two distinct advantages which warrant the future use of the RICM in conjunction with the AFM. First, the RICM provides information as to when the cantilever contacts and leaves the surface, something the AFM trace may not accurately depict. Unlike the RICM, the AFM optical lever measures cantilever deflection, not the separation distance between the cantilever and the surface. Second, RICM data could provide a means by which the cantilever shape can be reconstructed during the AFM force measurements. Namely, the fringe intensity profile along the cantilever length, x , is of the form¹⁰

$$I(x) = I_{12} + I_{23} + 2(I_{12}I_{23})^{1/2} \cos[4\pi(n_2/\lambda)(f(x) + z) + \delta]$$

where I is the light intensity reflected from the surface–buffer (subscript ₁₂) and buffer–cantilever (subscript ₂₃) interfaces, respectively, n_2 is the buffer refractive index, λ is the wavelength of the light, $f(x)$ is the function describing the shape of the cantilever with its end at a distance z from the surface, and δ is the phase shift that occurs upon the light reflection at the buffer–cantilever interface. The RICM-derived information about the cantilever shape during the measurement could lead to an evaluation of the cantilever restoring force independent of the AFM. Note that the AFM optical lever measures an average cantilever

deflection occurring in the area illuminated by the laser beam (approximately 20 μm sized area along x); the RICM lateral resolution is on the order of a few micrometers.

The finding that with the BSA–biotin–streptavidin system the cantilever edge separated from the surface before the final rupture of adhesive bonds was found repeatedly in many experiments using cantilevers of different geometry and differently shaped probes (i.e. edges, integral tips, and beads). A more comprehensive report on these experiments will be published in the future (M. Pierce, A. Pungor, and V. Hlady, in preparation). We recognize that each BSA molecule, whose physisorption to surfaces was used to immobilize biotin, had on average 5–9 biotin molecules and that more than one streptavidin could bind to it. Streptavidin alone can bind four biotin molecules. On the basis of the interference microscopy results, we hypothesize that this combination of multivalencies created a two-dimensional cross-linked streptavidin–biotin–BSA network on the surface: a film in which some of the streptavidin molecules still had unoccupied biotin binding sites. The contact of the biotinylated cantilever edge with the protein film brought the specific binding of biotin to these sites. Upon withdrawal of the edge, two possible scenarios might have occurred. The tension imposed on the biotin–streptavidin bonds might have caused a “peel-off” of the cross-linked protein network from the surface. One could anticipate that the immobilization of biotin via BSA physisorption was too weak to withstand the imposed tension and that the final rupture of the bonds could have occurred anywhere in the protein cross-link stretched between the surface and the cantilever edge. Note that the single biotin–avidin bond rupture force was measured in the experiments where excess biotin was present in solution, leading to the saturation of nearly all binding sites and thus blocking the formation of the protein network.² The second scenario is based on the force discontinuities observed in the molecular dynamic simulations of separation of two surfaces with densely grafted polymer chains.¹⁵ The discontinuities originated from structural rearrangements within the two polymer layers rather than from the rupture of polymer “bridges”; the final rupture distance was equal to the stretched length of the grafted polymer molecules. In order to explain large probe–surface separation distances at which the final “force jumps” occurred in our experiments, one would expect that, according to the second scenario, some unfolding of more than one protein had to occur.

In both cases, it is quite unlikely that the breakage of the stretched protein network will always occur at the specific biotin–avidin binding sites. The ubiquitous nonspecific interactions probably also contribute to the rupture forces. The combination of two or more different types of interactions (e.g. specific vs nonspecific) may smooth the force histogram to the point that the quantized rupture forces could not be resolved any more and may create problems for the variance analysis of single-bond force.⁸ Hence, the experimental system has to be designed with minimum nonspecific interactions: a problem that is equally important in the area of biomaterials, drug delivery, and biotechnology.

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Figure 1.

(a) Schematic of a combined AFM-RICM experiment. Incident microscope illumination approaches the cantilever from below. Horizontal dashed lines above the specimen surface represent the distances at which the interference fringes will appear. (b) Composite interference fringe image made of 500 cross-section images along the cantilever length captured during the force measurement; the y-axis of the montage represents elapsed time during the experiment (the beginning of the experiment is located at the top of the montage, and the end is located at the bottom); the x-axis is the distance along the projection of the

cantilever onto the surface (50 μm scale is indicated). The edge of the cantilever is at the right side of the image (marked by an arrow); the dark lines are the interference fringes. At the time when the cantilever comes in contact with the surface, the number of fringes on the cantilever becomes fixed and a change in the slope of the fringes occurs. A change in the slope of the fringes also occurs for the point in time that the cantilever leaves the surface, but the fringes begin to move off the end of the cantilever. Any discontinuity in the fringes from one frame to the next indicates a movement of the cantilever so fast that the fringes are smeared out, e.g. when the cantilever jumps from one vertical position to another in a very short instant of time (<0.07 s). One such discontinuity (marked with an arrow) occurs simultaneously with the final “force jump” of the cantilever in the force vs time trace shown in part c. (c) Optical lever detected force vs time trace of the measurement shown in part b. The time scale for parts b and c is identical.

Figure 2.

Close-up of the AFM and RICM measurements during the unloading of the cantilever from the surface. The optical lever recorded force–displacement trace (part a) and the corresponding composite interference fringe image (processed for better visualization of the fringe discontinuity, part b) are aligned to share the same time scale. The edge of the cantilever leaves the surface 0.56 s (330 nm of piezo displacement) before the cantilever distal end makes the final “jump” to its resting position.