Biomolecular Interactions Measured by Atomic Force Microscopy

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ABSTRACT Atomic force microscopy (AFM) is nowadays frequently applied to determine interaction forces between biological molecules. Starting with the detection of the first discrete unbinding forces between ligands and receptors by AFM only several years ago, measurements have become more and more quantitative. At the same time, theories have been developed to describe and understand the dynamics of the unbinding process and experimental techniques have been refined to verify this theory. In addition, the detection of molecular recognition forces has been exploited to map and image the location of binding sites. In this review we discuss the important contributions that have led to the development of this field. In addition, we emphasize the potential of chemically well-defined surface modification techniques to further improve reproducible measurements by AFM. This increased reproducibility will pave the way for a better understanding of molecular interactions in cell biology.

INTRODUCTION

The invention of the scanning tunneling microscope (STM; Binnig et al., 1983) has stimulated detailed analyses of surfaces both in material sciences and, more recently, in biology and biochemistry. It enabled the visualization of the atomic and molecular structure of surfaces. This instrument was the first in a row of newly developed scanning probe microscopes that all have in common a sharp probe that is scanned over a surface. While scanning, an interaction between probe and sample is measured in these instruments, from which an image is reconstructed.

In a second type of scanning probe microscope, the atomic force microscope (AFM; Binnig et al., 1986), the interaction force between probe and sample is exploited to maintain a constant distance between the two objects. The most important characteristic of the AFM is its ability to image objects in liquids (Marti et al., 1987), which is of special importance for biological studies because it allows visualization of biological structures in their native environment (Henderson et al., 1992). Development of imaging routines and modes of operation adapted for the measurement of the soft biological objects (Hansma et al., 1994; Putman et al., 1994) has significantly contributed to the reduction of damage done to these objects by the scanning tip. Therefore, AFM has become one of the most popular tools to image biological objects with subwavelength resolution. It should be mentioned, however, that visualization of biomolecular interaction processes in situ (Radmacher et al., 1994a; Kasas et al., 1997; van Noort et al., 1998) is not as straightforward as the imaging of fixed structures. Throughout the latter three studies, the AFM was pushed to its limits of temporal and lateral resolution. Interestingly, the imaging forces remained small enough to prevent the visible damage to the biological system studied. Also, no change in the biological function was detected in these papers.

Besides imaging surfaces, the AFM can also be exploited to obtain additional information from a sample. The interaction force between tip and sample can be measured by moving the AFM tip perpendicular to the surface while measuring the force on the tip. With these so-called force-distance curves it appeared possible to detect surface interaction forces, either continuously (Burnham and Colton, 1989; Butt, 1991) or discretely (Hoh et al., 1992; O'Shea et al., 1992). Moreover, the position accuracy and force sensitivity of an AFM probe even allowed detection of single molecular bonds (Florin et al., 1994). This method of detecting individual bonds is straightforward: the force probe, functionalized with a biomolecule, is brought into contact with a surface that is covered with the counter-molecule (see Fig. 1 *a*) and a molecular bond is formed. Upon retraction of the tip from the surface, the bond is broken and the adhesion force measured at that point represents the rupture force of the molecular pair. Although this novel application can in principle be applied to address a wide range of biological or immunological questions at the molecular level, thus far only a limited number of groups have reported the applicability of AFM for molecular interaction force measurements. Apparently, the detection of single molecular interaction forces is not as straightforward and suffers from a number of pitfalls. In this paper we will review the development of the measurement of molecular interaction forces during the past five years, illustrated with some examples of important findings instrumental for the development of the field. Because every molecular system

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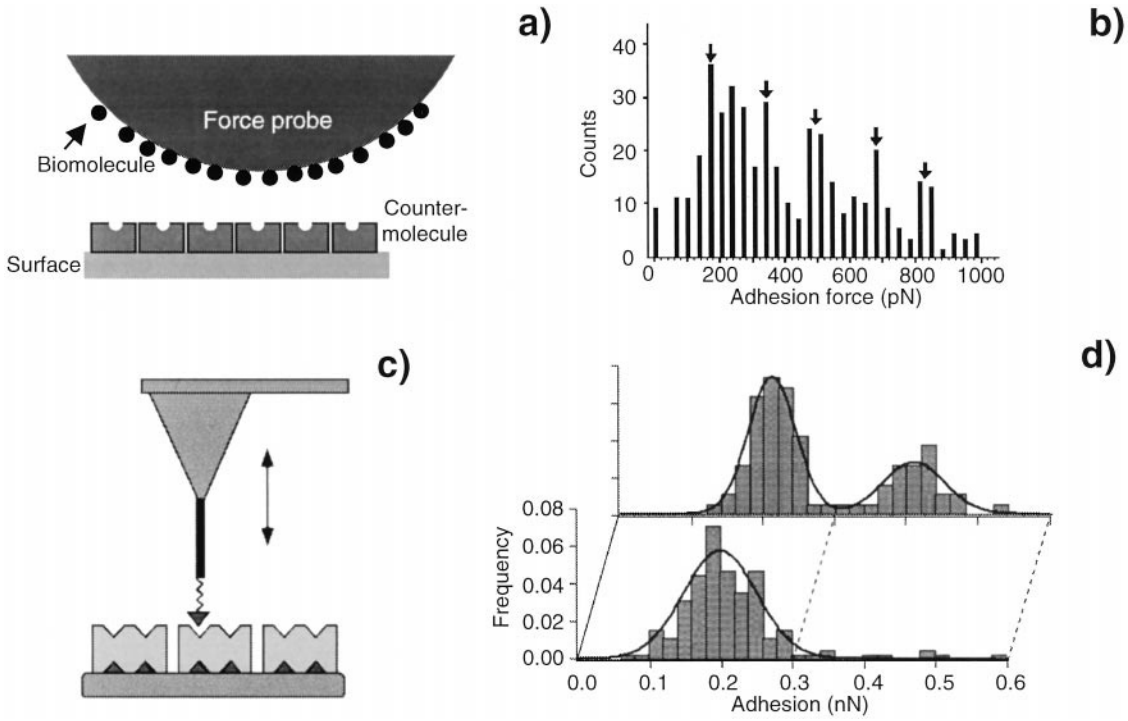

FIGURE 1 (*a*) Schematic representation of the tip-substrate configuration used in the initial studies on measurement of rupture forces of individual molecules. The force probe of the AFM was functionalized with a monolayer of the molecules of interest. The substrate was also densely packed with the counter-molecule. In the first two studies of Lee et al. (1994a) and Florin et al. (1994) the binding strength of the biotin-(strept)avidin pair was determined and the ligand (biotin) was either attached to the surface (Florin et al., 1994) or the tip (Lee et al., 1994a). (*b*) Distribution of rupture forces of the biotin-avidin pair, obtained by Florin and co-workers (1994). The histogram shows a number of peaks that have been pointed out by arrows and which denote the value of a force quantum for the molecular pair. (*c*) Diagram of the set-up that has been used by Wong and co-workers (1998). A nanotube has been glued to an AFM tip and the end of the tube has been modified covalently with a biotin molecule. This functionalized tip has been used to probe a surface that had been covered with streptavidin. (*d*) Two distributions of detected adhesion forces have been obtained with the set-up that is shown in (*c*). The lower graph shows a distribution that was obtained with a nanotube that only had a single active biotin molecule at its apex; the top graph was obtained with a nanotube that had two active biotin molecules at its apex. Panels (*c*) and (*d*) were reprinted by permission from *Nature*http://www.nature.com 394:255, Wong et al., © 1998, Macmillan Magazines Ltd; panel (*b*) was reprinted by permission from *Science*http://www.sciencemag.org 264:415–417, Florin et al., © 1994, American Association for the Advancement of Science.

has its own characteristics, there is no universal recipe to characterize single molecular systems. Binding and mechanical properties of the characterized molecules under force will depend heavily on parameters such as orientation of the molecules, degree of freedom, and affinity. Therefore, we restrict the discussion in this review to those conditions that have proven important to optimize AFM measurements of interaction forces between individual molecules. Among these are the coupling schemes of single molecules, the use of flexible cross-linkers, optimized detection schemes, and the usage of dedicated probe microscopes.

Initial studies on the measurement of rupture forces by AFM

Lee and co-workers (1994a) were the first to demonstrate the capability of AFM to measure discrete and biologically specific rupture forces of molecular complexes. They chose to investigate the biotin-streptavidin system because it is one of the strongest noncovalent interactions in nature. A schematic drawing of their set-up is shown in Fig. 1 *a*. Biotin was coupled covalently to bovine serum albumin (BSA), which was attached to both the force probe (a glass microsphere glued to a cantilever) and the surface by nonspecific adsorption. Subsequently, the biotin-functionalized surface was incubated with a counter-molecule, streptavidin, resulting in a monolayer of streptavidin bound to the biotinylated BSA on the glass surface. In order to address as few individual molecular bonds as possible, a rigid surface was considered essential to minimize the contact area between tip and surface. When tip and surface were brought in contact, the biotin on the tip could interact with the streptavidin because streptavidin has four binding sites for biotin, which were not all occupied by the surface bound biotin. Upon retraction of the cantilever, the bond(s) made could be disrupted. This rupture occurs when the gradient of the cantilever potential exceeds the highest gradient in the un-

binding pathway of the ligand-receptor interaction potential (Burnham and Colton, 1989). By registering the deflection of the cantilever at rupture, the rupture force can be measured. The specificity of the interaction was demonstrated by the observation that after blocking streptavidin by free biotin, no rupture forces were measured. The interaction forces that were measured with this set-up could be attributed to the rupture of discrete bonds, though the width of the distribution of rupture forces is not small enough to attribute every unbinding event to the rupture of a single complex. Because biologically specific and nonspecific bonds can have rupture forces that lie in the same range, it is not possible to conclude specificity from a single force measurement. Repetitive measurements of a bond not only give a distribution of unbinding forces (Evans et al., 1991; Lee et al., 1994a), but can also reveal additional proof for the discreteness of the measured bond. This was shown by Hoh and co-workers (1992) for chemically specific bonds, when they demonstrated the presence of force quanta when pulling a tip from a mica surface. Florin and co-workers (1994) followed this approach to demonstrate the direct measurement of the rupture of individual biologically specific bonds. They used a slightly different biological system than Lee and co-workers (1994a) did. Sharp silicon nitride tips were covered with biotinylated BSA to which avidin was bound. Soft biotinylated agarose beads were used as a surface to mimic biochemical affinity measurements. Thus, measured forces can be compared to thermodynamic data, obtained with those techniques. Another consequence of the use of soft surfaces is that the contact area is significantly enlarged, resulting in multiple molecular bonds upon interaction of the tip with the bead. Although the use of multiple interaction sites may seem counter-intuitive, it facilitates the discrimination of quantized rupture forces and increases the precision with which such a force can be measured. Because the rupture of the tip from the surface involved rupture of many individual bounds, only the force of the final rupture was analyzed. As shown in Fig. 1 *b*, the distribution of these final rupture forces shows a number of peaks that represent an integer number of force quanta for a biotin-avidin bond. Using this graph, the unbinding force of a single biotin-avidin bond was determined as 160 pN.

These two initial publications have substantially contributed to the development of a new field of experimentation using AFM: measurement of inter and intramolecular interactions in situ. Several investigators reproduced and extended the (strept)avidin-biotin findings by determining the bond strength with discrete methods (Moy et al., 1994a, b; Chilkoti et al., 1995; Allen et al., 1996; Wong et al., 1998) or with statistical analysis (Lo et al., 1999). This was followed by the measurement of intra or intermolecular rupture forces of other biomolecules with lower affinities, including antibody-antigen and membrane receptor-ligand pairs, and flexible molecules such as titin and tenascin. Table 1 provides an overview of measurements on biomolecules carried out with AFM until now and clearly shows that this technique becomes increasingly popular to study interactions between biomolecules and to get insight into mechanical unfolding pathways.

Measurement of individual biomolecular interactions

The development of techniques to detect molecular disruption forces has evolved rapidly. In Table 1 it is shown that initially much emphasis was put on the biotin-avidin interaction, which can be considered as the primary model exploited by several groups. Although the method of Florin and co-workers (1994) is very useful in determining the unbinding forces of discrete bonds, they predominantly observed the parallel breakage of multiple bonds. This is a consequence of their tip-sample configuration, which does not allow the repetitive addressing of the same individual molecules. To really measure interactions between isolated single molecules, very strict conditions need to be fulfilled. First, the density of molecules distributed both on tip and surface should be sufficiently low to allow the formation of single molecular interactions. In AFM experiments this implies that the surface coverage of active molecules on the tip or surface should be chosen sufficiently low to allow for single molecular interactions. To verify distribution of molecules on the tip, investigators have modified flat substrates, consisting of the same material as the tip, in the same batch. Subsequently, molecular distribution is determined with conventional surface techniques, such as tapping mode AFM (Willemsen et al., 1998) or fluorescence microscopy (Hinterdorfer et al., 1996). The consequence of one functional molecule on the tip is that it has become very precious: once it has become nonfunctional or is lost from the tip apex, the tip has become useless. Loss of functionality can be caused by damage that is due to the high pressure exerted on the molecule, but can also be due to detachment of the counter-molecule from the surface, which will also effectively block the recognition site of the tip. Thus, to prevent unwanted detachment, covalent bonding of the molecules to the surface and the tip is recommended (Hinterdorfer et al., 1996; Ros et al., 1998). A striking example of a single molecular interaction, measured by using covalent coupling of tip and surface, is provided by Wong and co-workers (1998). They have glued a single nanotube to the cantilever to minimize the contact between tip and surface and to have a chemically well-defined and extremely small tip apex (see Fig. 1 *c*). The probes, which were functionalized by covalently bound biotin molecules, were used to measure the rupture force of the biotin-streptavidin bond. Fig. 1 *d* shows the histograms of unbinding forces measured with two different tips. Interestingly, they found that most tips contained only one active biotin molecule at the apex of the nanotube, resulting in histograms as

TABLE 1 AFM measurements on biomolecular forces

System	Method			
	Measurement of Disruption Force	Disruption Force versus Loading Rate	Adhesion Mode Imaging	Force Spectroscopy
Biotin-(strept)avidin	Lee et al., 1994a; Florin et al., 1994; Moy et al., 1994a, b; Chilkoti et al., 1995; Allen et al., 1996; Wong et al., 1998; Lo et al., 1999	Merkel et al., 1999*	Ludwig et al., 1997	
Antibody-antigen	Hinterdorfer et al., 1995–1997; Stuart and Hlady, 1995, 1999; Dammer et al., 1996; Allen et al., 1997; Ros et al., 1998		Willemsen et al., 1998, 1999	
Receptor-ligand				
Proteoglycans	Dammer et al., 1995			
P-selectin		Fritz et al., 1998		
Tenascin		Oberhauser et al., 1998†		Oberhauser et al., 1998
$\alpha_v\beta_3$ Integrin	Lehenkari and Horton, 1999			
VE-cadherin	Baumgartner et al., 2000	Baumgartner et al., 2000		
Acethylcholinesterase				Yingge et al., 1999
Myelin basic protein				Mueller et al., 1999
Intramolecular				
Titin	Carrion-Vazquez et al., 1999†	Rief et al., 1997b, 1998a†		Rief et al., 1997b, 1998a; Marszalek et al., 1999; Oberhauser et al., 1999; Li et al., 2000†
Bacteriorhodopsin				Oesterhelt et al., 2000†
T4 lysozyme				Yang et al., 2000†
DNA	Lee et al., 1994b; Boland and Ratner, 1995; Strunz et al., 1999	Strunz et al., 1999		Rief et al., 1999; Clausen-Schaumann et al., 2000
Shell-protein				Smith et al., 1999
Polysaccharides				Rief et al., 1997a; Li et al., 1998; Marszalek et al., 1998
Covalent bonds				Grandbois et al., 1999

*Measured with a biomembrane force probe.
†Measurement of unfolding force.

shown in the lower graph of Fig. 1 *d*. According to their findings, even when the tip expresses two active biotin molecules, it remained possible to resolve the width of the force distribution of a discrete rupture force (Fig. 1 *d*, *top graph*) with an average value of 200 pN. In addition to coating low numbers of molecules, the histograms can be used to calculate the confidence level whether a rupture event between tip and sample is indeed caused by a single molecular detachment.

In the study of Lee and co-workers (1994a), however, it was demonstrated that the average value of the measured rupture force depends on the spring constant of the cantilever. According to them, this is consistent with the theory of Bell (1978), which states that the external force applied on a bond determines the lifetime of the bond. Therefore, the rupture forces measured in an interaction force experiment are highly dependent on the conditions of the experiment, indicating that interpretation of measured unbinding forces is more complicated than was generally believed.

Importance of the relation between loading rate and measured disruption forces

Evans and Ritchie (1997) have studied the strength of weak noncovalent bonds in liquids. They used Kramers' theory for reaction kinetics (Kramers, 1940; see also Hanggi et al., 1990) under the influence of force to establish a physical basis for Bell's theory (Bell, 1978) and derived a formula to predict of the dependence of force on loading rate. In a Monte Carlo simulation the dependency of rupture force on loading rate could be predicted. When the predictions of the simulations are applied to the range of loading rates that are attainable for the AFM (300–30,000 pN/s; Rief et al., 1997b; Fritz et al., 1998), an exponential dependence of the measured force on the loading rate is expected. This exponential dependence on loading rate has recently indeed been demonstrated for both inter and intramolecular unbinding forces (Rief et al., 1997b; Fritz et al., 1998; Oberhauser et al., 1998). Evans and co-workers (1995) also predicted that

for a range of loading rates, covering 12 orders of magnitude, the rupture force of biotin and avidin could not be fitted with a single exponential. Because the interaction potential of biotin and (strept)avidin contains several energy wells, as was shown in MD simulations for the biotin-streptavidin (Grubmüller et al., 1996) and biotin-avidin bond (Izrailev et al., 1997), the graph of rupture force versus the logarithm of loading rate consists of multiple sections, where one of the energy wells determines the slope of each section (Evans and Ritchie, 1997). These predictions have recently been confirmed by Merkel and co-workers (1999), who used a biomembrane force probe to measure the rupture force while varying the applied loading rate over six orders of magnitude. Although this experiment was not performed with an AFM, the implication for experiments carried out by AFM is so significant that it is discussed in this review.

In Fig. 2 the measured rupture force of a single biotin-avidin and a biotin-streptavidin bond is plotted as a function of loading rate. The measurements of streptavidin can be divided into two sections where the dependence on loading rate is quite different. The region of high unbinding forces (85–175 pN) is dominated by a barrier in the potential, located at 0.12 nm from the center of the binding pocket, and the lower regime is dominated by a barrier at 0.5 nm. In the higher rupture forces regime the graph is similar for both biotin-avidin and biotin-streptavidin interactions, but below 85 pN, the slopes deviate. These differences can be explained by comparison of the molecular dynamics of the rupture of the biotin-streptavidin (Grubmüller et al., 1996) and biotin-avidin (Izrailev et al., 1997) bonds. Interestingly, the value of the rupture force of biotin-streptavidin, as measured by the AFM experiment of Wong and co-workers (1998) and indicated with the star, is only a single point in the rupture force versus loading rate spectrum. It should be noted that in all experiments described above, a graph of rupture force versus loading rate is given. To our knowledge, no experiments have been done to prove the dependency of rupture force on loading rate unambiguously. In such experiments the spring constant of the cantilever should be varied, while maintaining the loading rate constant. If the distribution of rupture forces does not change, loading rate and rupture force are directly related.

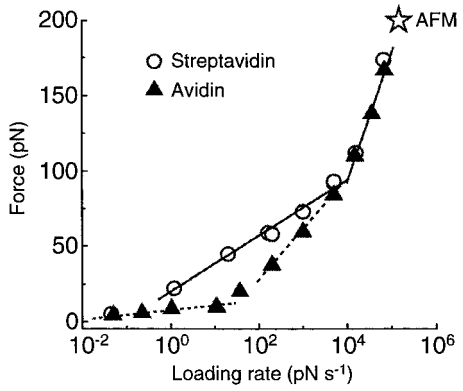

FIGURE 2 This figure shows a graph of rupture force versus loading rate, as obtained by Merkel and co-workers (1999) for the biotin-avidin (*triangles*) and the biotin-streptavidin (*circles*) system. The regions with different slopes are dominated by barriers in the energy landscape of interaction potential of the molecular complex. The slope of the curve determines the position of the energy barriers (see Merkel et al., 1999). The value of the rupture of the biotin-streptavidin, as obtained by Wong and co-workers (1998) and shown in Fig. 1 *d*, has been indicated with a star. This figure was reprinted by permission from *Nature*http://www.nature.com 395:50–53, Merkel et al., © 1999, Macmillan Magazines Ltd.

Antibody-antigen interactions

Besides biotin-(strept)avidin interactions, several other biological interactions have been investigated with the AFM. Various groups investigated antibody-antigen interactions that are important in the immune system, and which may vary considerably in affinity. A similar approach as used when measuring biotin-(strept)avidin interactions was followed. Stuart and Hlady (1995) glued a silica bead to a cantilever to create a relatively large contact area. They found that the antibody-antigen rupture forces were obscured by nonspecific interactions between functionalized tip and surface and perhaps also by a lack of molecular mobility. Dammer and co-workers (1996) followed a different approach using a self-assembled monolayer to functionalize tips and surfaces. Like Florin and co-workers (1994), distributions of rupture forces were measured to quantify unbinding forces. Hinterdorfer and co-workers (1995, 1996) were the first to determine the interaction between individual antibodies and antigens.

The latter two groups used flexible cross-linkers to couple either the antigen or the antibody to the tip and stressed that these spacers were essential in the formation of the antibody-antigen complexes. Apparently they provided the antibodies and antigens enough freedom to overcome problems of misorientation, steric hindrance, and conformational changes. Hinterdorfer and co-workers (1996) have created the largest mobility by using the longest spacer molecules (8 nm (Hinterdorfer et al., 1996) compared to 2.2 nm (Dammer et al., 1996)) and by coupling antibodies and antigens to the tip and the surface, respectively, via polyethylene glycol (PEG) spacers. The tips were functionalized at such low antibody density that, on average, only one single antibody at the tip apex had access to an antigen on the surface. Thus, single molecular antibody-antigen complexes could be examined, and an example of the rupture of such a complex is shown in Fig. 3 *a*. Here, the force on the cantilever is plotted as a function of distance between the cantilever and the surface. Approaching the surface (trace 1–5), the curve resembles the well-known force-distance curve. Upon retraction of the tip, however, the binding is disrupted in a manner characteristic for molecules linked through PEG spacers. Rather than detaching after having followed the

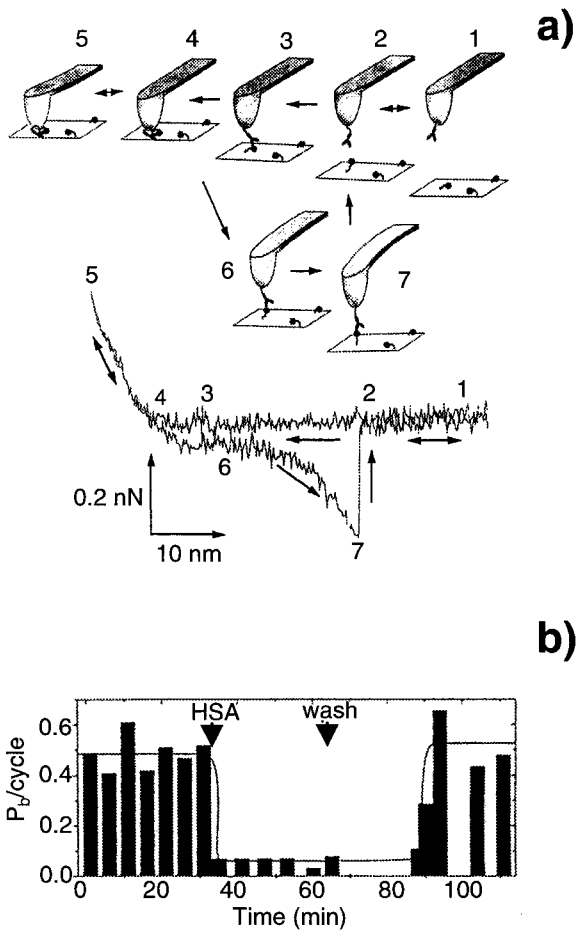

FIGURE 3 (*a*) Force-distance curve generated by Hinterdorfer and co-workers (1996). Both antibody and antigen are covalently bound to the tip and surface, respectively, via a bifunctional PEG spacer. During the approach of the tip to the surface, the line 1–5 is followed. During the retraction of the tip from the surface, the tip follows the line 6–7, indicating a delayed rupture that is attributed to the involvement of the spacer in the interaction. (*b*) Binding probability of antibody on the tip as a function of time. As indicated by the arrows, the addition of excess antigen to solution causes the binding probability to drop almost to zero. It recovers ~25 min after washing with buffer. Panels (*a*) and (*b*) are reprinted by permission from Hinterdorfer and co-workers (*Proc. Natl. Acad. Sci. USA.* 93:3477–3481, © 1996, National Academy of Sciences).

trace 5–4 for some time with the same slope until detachment is observed, the unbinding curve follows the trace 6–7. Instead of an immediate rupture a delay in detachment is observed, which is attributed to the spacer molecules that have to be stretched before they can exert force on the antibody-antigen complex. To validate the biological specificity of the antigen-antibody interaction, free antigen was added to the solution to block the binding site of the antibody on the tip. Although this is common practice to show specificity, Hinterdorfer and co-workers (1996) were the first to elegantly demonstrate the reversibility of the blocking, as shown in Fig. 3 *b*. After addition of free antigen the probability of rupturing decreased almost immediately. Washing away free antigen resulted in recovery of binding after ~25 min.

Since Hinterdorfer and co-workers (1996) were able to detect the rupture of bonds using a single antibody on the tip, they were able to perform experiments that cannot be carried out when multiple active molecules are present on the tip. For instance, they observed that both binding sites of the antibody (an antibody has two antigen binding sites) were able to bind simultaneously and independently with the same binding probability. Also, they were capable of determining the probability of a rupture event as a function of lateral position of the tip over the molecule. This demonstrates the ability of AFM to locate molecular interactions with a lateral accuracy of 1.5 nm, which is determined by the dynamic reach of the spacer molecule. This approach was the first attempt to combine the ability of the AFM to detect biological specific interactions with the imaging capability that was already known for several years, and may be used to map binding sites and binding characteristics simultaneously and in more detail.

Development of adhesion mode imaging

Rupture forces from biomolecular specific interactions can also be exploited as a contrast parameter to create images. The applicability of the adhesion force between tip and surface for contrast was first recognized by Mizes and co-workers (1991) mapping a polymer substrate. However, because the topographical and the adhesion image were measured sequentially, a shift in the images was observed that was attributed to drift. This problem can be prevented by simultaneous imaging of topography and adhesion, as was demonstrated later by van der Werf et al. (1994) and Radmacher et al. (1994b). In their experiments the tip is raster-scanned over the surface while a force-distance curve is generated for every pixel. From the force-distance curve, surface parameters such as stiffness, height, and adhesion force can be extracted by either on-line (van der Werf et al., 1994) or off-line analysis (Radmacher et al., 1994b). Incorporating a feedback on maximal force to prevent damage to the sample (van der Werf et al., 1994), Berger and co-workers (1995) were even able to image the domains in a phase-separated lipid monolayer. A topography and concomitant adhesion image are shown in Fig. 4, *a* and *b*, respectively. The topography image hardly shows any height differences, implying that surface-induced adhesion force contrast can be excluded. This is extremely important because topography-induced adhesion, caused by variation of the contact area between tip and sample, often obscures the chemically induced contrast. Therefore, the observed contrast in the adhesion image is truly chemically specific, and it was attributed to the difference in interfacial energy of the tip and acyl chain in the lipid domains. Comparison of the adhesion image, in which such chemical contrast is used to visualize the position of the domains in the layer,

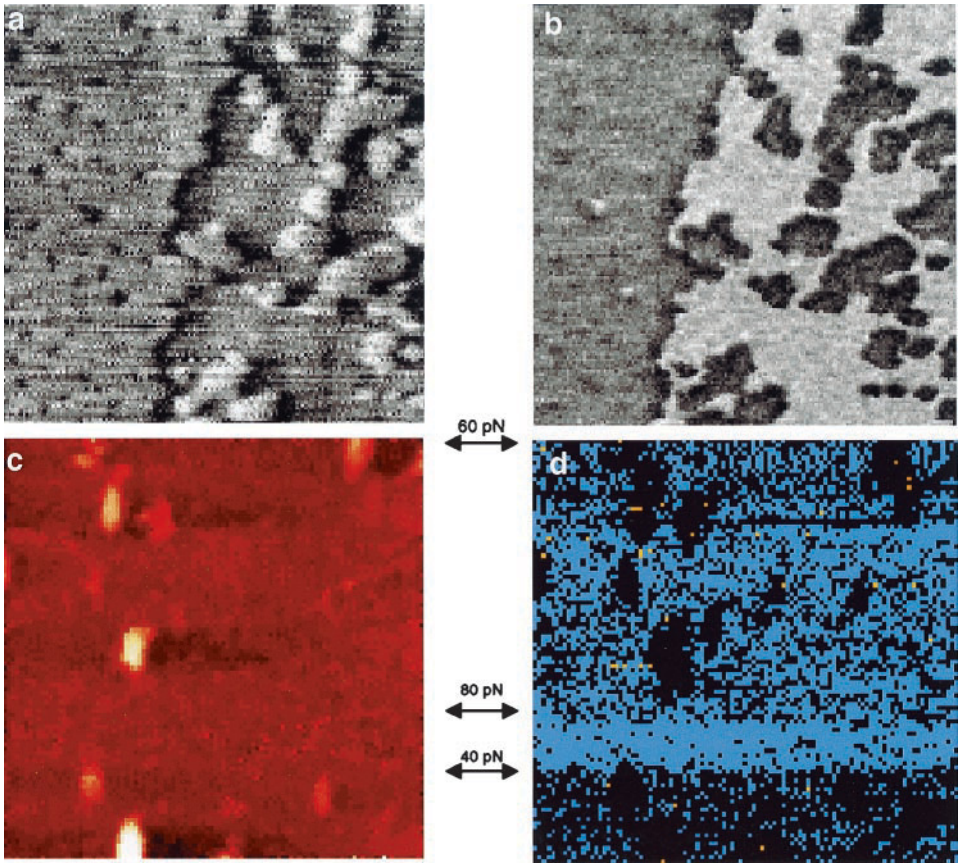

FIGURE 4 Topography (*a*) and adhesion (*b*) image of a phase-separated lipid monolayer, recorded in adhesion mode in air by Berger and co-workers (1995). The image size is 600 × 600 nm, the topography images have been high-pass filtered, and the adhesion force had the average value of 25 nN on the dark domains and 35 nN on the light domains (in *b*). Topography (*c*) and adhesion (*d*) image of a mica substrate, sparsely covered with antigen molecules. The images are recorded in adhesion mode with a tip that is functionalized with antibody via a PEG spacer. The adhesion mode image has been color-coded on the basis of the force-distance curves that were obtained on these pixels. Black pixels denote adhesion forces smaller than 30 pN, blue pixels denote adhesion forces higher than 30 pN and a rupture length smaller than 3.5 nm, and yellow pixels denote adhesion force higher than 30 pN and a rupture length larger than 3.5 nm. At the position of the arrows the maximal applied force is set to 60, 40, and 80 pN. The image size is 420 × 420 nm, the height range is 0–9 nm, and the adhesion force range is 0–300 pN. (*a*) and (*b*) are reprinted by permission from Berger et al. (*Langmuir.* 11:4188–4192, © 1995, American Chemical Society). Panels (*c*) and (*d*) are reprinted by permission from Willemsen et al. (*Biophys. J.* 76:716–724, © 1999, Biophysical Society).

with the topography image clearly demonstrates the potential of applying adhesion force as a contrast parameter.

Also, biological applications, such as the localization of surface receptors on a single living cell, might benefit from adhesion mode AFM. Because cell surface receptors are embedded in a highly flexible lipid membrane, adhesion force is expected to yield a higher contrast than the height signal derived from contact mode imaging. The high elasticity of cells (Hoh and Schoenenberger, 1994) causes a deformation of the membrane under the pressure of the tip, which may result in nonspecific adhesion to the tip and erroneous height information. For this reason, the first reports on imaging receptor molecules used a much more simplified biological system. Instead of imaging surface receptors in their native membrane, they were purified and attached to a flat and rigid surface. Ludwig and co-workers (1997) followed this method using the binding between streptavidin and biotin as a contrast mechanism for an adhesion image. To visualize the high lateral resolution of the AFM, they prepared a sample with a pattern of streptavidin and BSA, which was imaged in adhesion mode. Force-distance curves were analyzed on-line by extracting the height and the adhesion force information. Next, two concomitant images were constructed, a topography and an adhesion image, revealing the patterned structure. Blocking the surface with soluble biotin destroyed the adhesion image while the topography contrast remained.

Similarly, Willemsen and co-workers (1998, 1999) used a patterned surface to demonstrate spatially resolved molecular recognition. The simplest method to create a pattern is covering a surface sparsely enough so that individual molecules can be resolved. Thus, specific interactions are only expected on top of individual molecules. To ensure that each molecule on the surface is also probed by only a single molecule exposed by the tip, special precautions have to be taken. Following the method of Hinterdorfer and co-work-

ers (1996), PEG spacers were used to obtain tips with only a single active antibody at the apex. As the antibody was covalently coupled to the tip, it remained intact for several images, which required 10,000 force-distance curves each. Besides the high biochemical stability, the spacers also served another, perhaps even more important, purpose, which will be described below.

Modified tips are extremely sensitive to damage and/or contamination, resulting in nonspecific interactions with the surface (Willemsen et al., 1999). Previously, it was shown that spacer-involved specific interaction can be discriminated from tip-involved nonspecific interactions by analysis of the force-distance curve (Willemsen et al., 1998). Analyzing the force-distance curve for every pixel of an image, the adhesion image can be color-coded depending on the classification as no interaction, nonspecific interaction, or specific interaction (Willemsen et al., 1999), as is shown in Fig. 4, *c* and *d*. The figures show the topography and concomitant color-coded adhesion image of a mica surface that has been covered with purified cell surface adhesion molecules (intercellular adhesion molecule-1 (ICAM-1)). The topographic image (Fig. 4 *c*) shows individual ICAM-1 molecules with a resolution that is comparable to tapping mode images. In the adhesion image (Fig. 4 *d*) the nonspecific interactions are exclusively observed when the tip is located on the mica substrate, whereas the spacer-involved specific interactions are predominantly observed on top of the ICAM-1 molecules. It becomes also apparent from Fig. 4 *d* that the probability of detecting nonspecific interactions was modulated during the imaging by variations in the force applied, thus modifying the probability of the tip to snap into adhesive contact. Conceptually, the electrostatic double layer repulsion between tip and surface was used to ride the tip on, while specific interactions were still possible, since the spacer enabled the antibody to bridge the gap between tip and surface. Because cell membranes are also surrounded by an electrostatic double layer, this technique might very well be suited to prevent nonspecific interactions when measuring cells.

Ros and co-workers (1998) also used a PEG spacer molecule to functionalize AFM tips. To increase the binding probability of the antibody-antigen complex, a much longer spacer (40 nm instead of 8 nm) than in the previous studies was used. Antibodies to be probed were first selected by imaging the surface in the conventional contact mode; subsequently, the tip was moved to an antibody and force curves were measured. Hereafter, the sample was imaged again (contact mode) to reveal any damage to the sample. Raab and co-workers (1999) used the Magnetic AC mode (MAC-mode (Han et al., 1997); Molecular Imaging, Phoenix, AZ) in combination with tips that were functionalized with antibodies via PEG-spacers. This mode is similar to tapping mode, but a magnetically coated tip is oscillated by an alternating magnetic field, which enables rigid control of the tip. Using this mode, the lateral position of individual antigens on mica was determined with 3 nm resolution. Because of the specific recognition between antibodies and antigens, an extra damping of the tip with the sample occurs, and this renders additional contrast in the image. By performing proper blocking experiments, the topographical and adhesion information can be separated. The advantage of this method is that surface distributions of antigens can be characterized at high pixel frequencies (1 kHz; Raab et al., 1999).

Force spectroscopy of single molecules

The AFM can also be used to measure the elasticity of individual molecules. Rief and co-workers (1997a) demonstrated that when pulling on the polysaccharide dextran, the force-distance curve revealed multiple elastic regimes that were dominated by entropic effects, a twist in an internal bond angle, or a conformational change of the molecule. Since similar conformational changes were observed at the same force for different strands of dextran varying in length, and because the elasticity of the strands was directly proportional to length, it was concluded that individual molecules were stretched. These novel types of application of an AFM have been designated force spectroscopy. Until now, predominantly long molecules that contain repetitive domains have been examined. In dextran, all monomers composing the polymeric structure are equal and thus react in the same way to applied force, implying that changes in the length of the monomer are added and elongation of the polymer is amplified. Force spectroscopy allows reproducible detection of structural transitions that result in changes in length of the monomer as small as 0.65 Å (Rief et al., 1998b).

Recently, other molecules that contain a large number of repetitive domains have been examined, such as the muscle protein titin, which contains a large number of immunoglobulin (Ig)-like domains (Rief et al., 1997b). Upon stretching of titin the force trace showed a characteristic sawtooth-like pattern (Fig. 5 *a*) that could be attributed to the unfolding of the individual Ig-like domains within titin. Because these domains have a similar tertiary structure but are not completely homogeneous, each domain will be unfolded at a defined unfolding force. As the force was exerted on the entire molecule, the domains were unfolded, starting with the weakest one, as can be observed from the increasing unfolding force (see Fig. 5, *a* and *b*). In force spectroscopy intramolecular forces are measured and thus molecules need not be detached from the tip. By careful handling they can be held between tip and surface for a long period of time, allowing repeated measurements to study the reproducibility or the dynamics of the unfolding and refolding processes of a single molecule (Rief et al., 1997b; Oberhauser et al., 1998). Therefore, force spectroscopy is an interesting novel tool to obtain dynamic information on the strength of different biological modules and will prove to

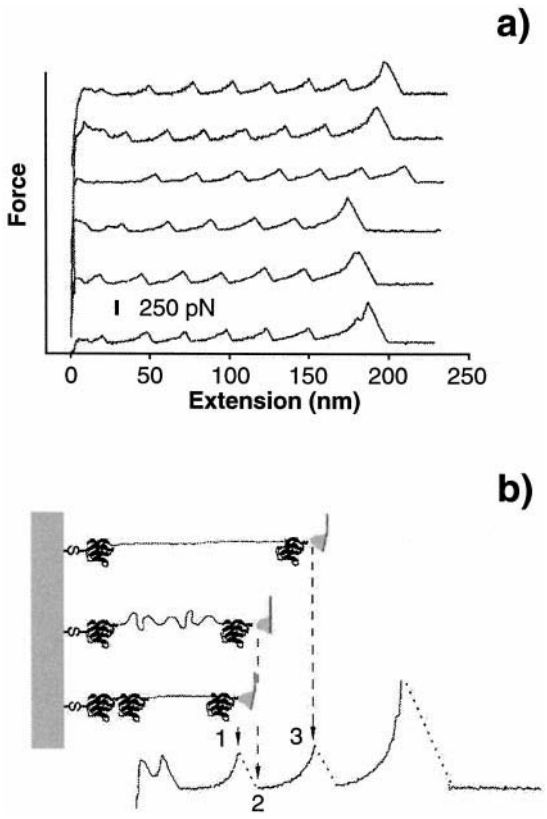

FIGURE 5 (*a*) Set of six force versus extension curves of a recombinant titin fragment, generated by Rief and co-workers (1997b). The fragment has eight immunoglobulin (Ig) domains that are unfolded under the influence of force. The peaks are equally spaced and range from 150 pN to a maximum of 300 pN. (*b*) Illustration of the interpretation of the measurement of (*a*), but now for a fragment with four Ig domains, as reported by Rief and co-workers (1997b). The titin is stretched until it unfolds (1). Subsequently, the force on the molecule has reduced and the length has increased (2). Continuation of the extending of the molecule results in the unfolding of the next domain (3). Panels (*a*) and (*b*) are reprinted by permission from *Science* http://www.sciencemag.org 276:1109–1112, Rief et al., © 1997, American Association for the Advancement of Science)

yield important information on the relationship between structure and function of single biomolecules (Oberhauser et al., 1998).

Thus far a major drawback of AFM is the lack of control when analyzing intermolecular or intramolecular interactions. In many cases the surface is just scanned until a productive (force spectroscopy) interaction is made spanning a molecule between tip and surface. Which part of the molecule is stretched is unpredictable, as the tip can land anywhere on the molecule. Also, when measuring intermolecular interactions it is extremely hard to get reproducible results because success not only depends on the possibility that molecules are oriented such and are close enough so that they can interact, but also proper solid attachment of small numbers of molecules to the apex of the tip and surface are essential. Therefore, several groups have started to develop techniques to modify tip and surface such that more reproducible measurement can be carried out.

Tip and surface modification techniques

The measurement of intramolecular forces infers high demands on the stability of the coupling between molecules and tip or surface. Whereas in the case of intermolecular force measurements the bonds between tip and molecule and between surface and counter-molecule only have to withstand rupture forces, stretching of intramolecular domains is measured at even higher forces. For instance, the conformational changes in a dextran molecule were observed at an applied force of 700 pN (independent of loading rate; Rief et al. 1997a, 1998b), which is substantially higher than the force needed to break the streptavidin-biotin complex (200 pN at a loading rate of 10^5 pN/s; see Merkel et al., 1999). To withstand these forces, rather than using self-assembly (Dammer et al., 1996), biotin and avidin (Florin et al., 1994; Lee et al., 1994a), or adsorption to attach molecules, the dextran molecule was coupled covalently to a gold substrate. Next the hydrophobic tip was allowed to interact by pushing it into the dextran polymer brush that extended in solution and dextran was allowed to bind by adsorption. Presumably due to a strong hydrophobic interaction, the dextran-tip interface could withstand forces higher than 1000 pN (Rief et al., 1997b; Marszalek et al., 1998). Grandbois and co-workers (1999), who wanted to measure the strength of a single covalent bond, managed to exert even higher forces on a single molecule. The polysaccharide amylose was covalently attached to both tip and surface by carbodiimide bonds, which could withstand forces of 2 ± 0.3 nN (loading rate of 10^4 pN/s), after which rupture occurred. Examining the rupture probability of the bonds that are involved in coupling of the molecule to the surface and of the bonds that are present in the backbone of the molecule itself, they found that a silicon-carbon bond was ruptured. Similarly, they demonstrated that sulfur-gold bonds could withstand forces up to 1.4 ± 0.3 nN before rupture occurred.

The elegant measurements on the strength of covalent bonds by Grandbois and co-workers (1999) are in accordance with and explain observations made by groups studying intermolecular interactions. Antigens or antibodies covalently bound to either tip or surface (Hinterdorfer et al., 1996; Ros et al., 1998; Willemsen et al., 1998, 1999) should according to Grandbois et al. (1999) be capable to withstand forces up to 1400 pN. Because this force is high enough to rupture at least six antibody-antigen bonds at the same time, neither antibody nor antigen will detach from the surface (Hinterdorfer et al., 1996). Therefore, both tip and surface are stable enough to obtain the thousands of force-distance curves that are necessary for imaging of reasonably sized surfaces (400×400 nm; Willemsen et al., 1998).

Force resolution

The strength of the bonds by which molecules are bound to the tip limit the largest force that can be exerted on a molecule. However, the smallest force that can be detected is determined by the spring constant of the cantilever. Because the cantilever has, according to the equipartition theorem (Hutter and Bechhoefer, 1993), on the average a thermal energy of 0.5 k_bT (with k_b the Boltzmann constant and T the temperature), the smallest force that can be detected with the commercially available silicon nitride cantilevers is in the order of 10 pN. Although averaging of the cantilever signal enables measurements of transitions that require lower forces (resolution of 3 pN for the unzipping of DNA; Rief et al., 1999), the force resolution is generally poor compared to that of magnetic or optical tweezers. This is due to the fact that smaller spring constants are used with this technique and because the measurements are performed in bulk liquid (0.4 pN; Gittes and Schmidt, 1998). By manufacturing cantilevers with lower spring constants, the force resolution may be increased, but at the same time this will lower the resonance frequency of the levers, implying that rapid movements cannot be measured anymore. If the AFM is used in adhesion mode imaging, where force measurement is combined with fast scanning of an image, high force resolution should be combined with a cantilever with a high resonance frequency. Smaller cantilevers, which have a smaller mass and thus a higher resonance frequency (up to 150 kHz in liquid; Walters et al., 1996), might solve this problem. For instance, when deflection is recorded at a bandwidth of 10 kHz, only a fraction of the thermal noise is included while fast movements of the tip can still be detected. In the study of Walters and coworkers (1996), however, the force resolution of their cantilevers is still ~10 pN (10 kHz bandwidth), caused by the relatively high spring constants of their levers. Levers with lower spring constants (thinner levers), would decrease the force resolution by almost an order of magnitude. However, it should be taken into account that the damping that the force probe has in the vicinity of the surface will limit the force resolution in the end (Gittes and Schmidt, 1998). In addition to the high force resolution, small cantilevers also help to increase the imaging velocity in force-distance mode. As has been pointed out by Willemsen and coworkers (1999) the maximal attainable pixel frequency is determined by the viscous drag of the cantilever in combination with the resonance frequency, which limits the pixel frequency to 25 Hz when imaging antibody-antigen interactions. A similar value has been reported by Ludwig and co-workers (1997) for recognition imaging of biotin and streptavidin. If quantitative measurements of adhesion forces are of minor importance, images can be obtained at much higher rates (1 kHz), using tip resonance techniques such as the MAC-mode (Raab et al., 1999). Because of their low viscous drag and a higher resonance frequency, small cantilevers should be considered for imaging tools. However, the maximal imaging velocity is limited by the association rate of the molecular pairs to be formed. Association rates, determined in AFM studies, vary between almost instantaneous for the formation of P-selectin and P-selectin glycoprotein ligand-1 (Fritz et al., 1998) to 5×10^4 M^{-1} s^{-1} for antibody-antigen bonding, which is comparable to the association rate in bulk (Hinterdorfer et al., 1996).

From forces to energies

As has already been pointed out, rupture forces directly depend on the loading rate and do not reveal the entire shape of the interaction potential of the interacting molecules. Merkel and co-workers (1999) demonstrated that it is possible to measure the height and the position of the energy barriers in the unbinding path of a molecular complex by measuring the rupture force as a function of loading rate of the biotin-avidin interaction. However, as has already been pointed out by Izrailev et al. (1997), the amount of information about the thermodynamic interaction potential that can be obtained from rupture experiments is rather limited.

Cleveland and co-workers (1995) showed that AFM can also be used in a different way to obtain information about interaction potentials. They used the Boltzmann distribution to reconstruct a potential from the probability distribution of the position of a tip that was brought close to a calcite surface (Fig. 6 *a*). Instead of operating the AFM in force-distance mode, the tip was brought so close to the surface that it began to jump between metastable energy states. The deflection of the cantilever, representing the position of the tip, was used to reconstruct the potential by analyzing the probability distribution of tip position using the Boltzmann distribution (see Cleveland et al., 1995 for details). The wells in the potential, which are only separated by barriers with a height in the order of k_bT, are due to the sum of the interaction potential between tip and surface and the harmonic cantilever potential. The relative position of these two potential wells depends on the distance between the base of the cantilever and the surface (Willemsen et al., 1999). Unfortunately, because the cantilever is brought to the surface in a rather undefined way, it was not possible to obtain the tip-sample interaction potential. Nevertheless, the work of Cleveland and co-workers (1995) demonstrates the capability of the AFM to measure potentials with a resolution in energy that is lower than the thermal energy k_bT and a resolution in distance as low as 0.15 nm. To demonstrate that this information is of direct relevance when measuring the interaction potentials of biological objects, we have plotted the interaction potential that resulted from a molecular dynamics simulation of the unbinding of the biotin-avidin complex (Fig. 6 *b*, obtained from Evans and Ritchie, 1997; and a reference therein). Comparing Fig. 6, *a* and *b*, it is clear that both the energy resolution and the distance

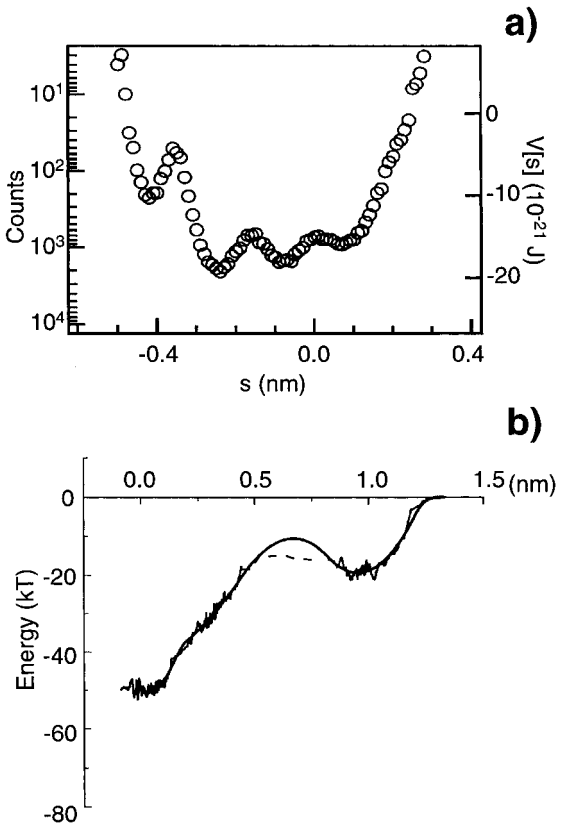

FIGURE 6 (*a*) Histogram of tip position when an AFM is brought in the vicinity of a calcite surface, as has been reported by Cleveland and co-workers (1995). By normalizing the histogram and using the Boltzmann distribution, the histograms can be converted into energies (right axis; see Cleveland et al. (1995) for details). The potential that is obtained has four energy minima that are caused by the tip-surface interaction potential and the cantilever potential. (*b*) Interaction potential for the force-driven dissociation of the biotin-avidin complex, as reported by Evans and Ritchie (1997). The instantaneous energies in the molecular dynamics computation have been coarse-grained averaged (*thin line*). The thick line denotes the smooth polynomial fit to this curve. The dashed segments in the thin curve represent jumps with few statistics. The *y* axis is calibrated in units of k_bT ($1\ k_bT$ is equal to 4.1×10^{-21} J at room temperature). Panel (*a*) is reprinted by permission from Cleveland et al. (1995); (*b*) is reprinted by permission from the Biophysical Society (Evans and Ritchie, *Biophys. J.* 72:1541–1555, © 1997, Biophysical Society).

resolution of the AFM are sufficient to resolve the wells that are observed in Fig. 6 *b*.

DISCUSSION AND CONCLUSIONS

In this review we have summarized the advances in the detection of molecular interaction forces and energies that have been reported during the past five years. From the earliest experiments, where tip and surface are covered with monolayers of ligands and receptors (Lee et al., 1994a; Florin et al., 1994), the surface modification techniques have been refined to allow the detection of the interaction forces between individual molecules (Wong et al., 1998). The addressing of individual molecules with a functionalized force probe has contributed to the detailed understanding of the mechanics and dynamics involved in complex formation and disruption of individual ligand-receptor pairs. Reaction kinetics of molecular interactions that had been formulated over 50 years ago (Kramers, 1940), could now be applied to the theory of rupture of single molecular bonds under application of an external force (Evans and Ritchie, 1997). Conceptually, the external force tilts the ligand-receptor interaction potential, and as a result the activation barrier from the bound to the free state is lowered. For weak bindings, activation energies are relatively low (order of 50 k_bT) and thermal fluctuations in the energy of the cantilever can induce early rupture of the complex. As a consequence, rupture force is not determined by the shape of the interaction potential only, but also depends on the time scale of the experiment in relation to important statistical parameters like attempt frequency (Zangwill, 1988) and temperature. For the measurement of rupture forces, it was predicted (Evans and Ritchie, 1997) and demonstrated (Merkel et al., 1999) that these forces are exponentially dependent on loading rate and that parameters that describe the dependency reveal information about the interaction potential.

The final step, namely the determination of the complete interaction potential of ligand and receptor, has not been made yet. Cleveland and co-workers (1995) have demonstrated that the AFM can, in principle, be used to detect the complete shape of interaction potentials. Their method, using thermal fluctuations of the energy of the cantilever, is sensitive enough to resolve the energy minima in the simulated interaction potential of biotin and avidin (Evans and Ritchie, 1997). The range of energy differences that can be determined with this novel method, however, is too small. Although only a seven-times higher energy range would be required ($50\ k_bT$ compared to $7\ k_bT \approx 1229 \times 10^{-21}$ J); see Fig. 6, *a* and *b*), it is impossible to measure the interaction potential with the new method. This is due to the fact that an additional energy difference of $43\ k_bT$ would require e^{43} times longer the measurement time (implying 900 million years of measurement time). Therefore, there is a big challenge to develop novel techniques, exploiting thermal fluctuation of tip energy, that are able to determine the large energy differences that occur in the interaction potentials of ligands and receptors.

The coupling of biomolecules to tips and surfaces has proven to be an essential factor in the measurement of both inter and intramolecular forces. Although the use of nonspecific adsorption and self-assembly as coupling mechanisms allow the detection of discrete rupture forces, the measurement of interaction force has benefited from covalent coupling. With this bonding procedure the molecules are bound firmly to the substrate and tip and as a consequence, they are not removed from the surface during the rupture of the ligand-receptor complex. This is especially important for measurements using adhesion mode AFM,

where the detection of molecular interactions is combined with image construction, as this mode of operation requires a force-distance curve for each pixel of an image.

Various groups have reported that the use of flexible cross-linkers to covalently couple molecules to tips and substrates facilitates the formation of antibody-antigen complexes (Hinterdorfer et al., 1995–1997; Dammer et al., 1996; Ros et al., 1998; Willemsen et al., 1998, 1999). These spacers give both antibody and antigen the flexibility to diffuse freely in a volume that is only restricted by the dynamic length of the spacer (Hinterdorfer et al., 1996), implying that both antibody and antigen can explore various conformations. The lower restrictions to the freedom of the antibody resemble the physiological relevant conformation, and the detected probability of biological recognition is of the same order as in bulk measurements. The spatially resolved detection of individual antigens by an antibody functionalized tip (Willemsen et al., 1998) has benefited from this advantage because force-distance curves are generated at relatively high pixel rates and interaction time is low. A second advantage of the use of spacers is that the tip has already detached from the surface before the rupture of antibody-antigen complex. Not only does this facilitate the analysis of the measured force-distance curves (Hinterdorfer et al., 1996), but also the lateral movement of the tip during a force-distance curve (Dammer et al., 1996) will not interfere with the detection of the rupture force. The final advantage is a reduction of the sensitivity to lateral drift in the detection of rupture forces: if a molecule is rigidly bound to the tip, a lateral movement of the tip can cause an unwanted and undetectable rupture event. If spacers are used, a lateral movement of the tip, smaller than the dynamical length of spacer and antibody, will not influence the rupture force but only the distance at which rupture occurs. A disadvantage of the use of spacers is that the dynamic reach of the spacer causes a convolution in the adhesion image (Willemsen et al., 1998). This could be a disadvantage in experiments where the epitope on an antigen is mapped.

If spacers are used in experiments that investigate the dependence of unbinding force on loading rate, special precautions have to be taken. As reported by Evans and Ritchie (1997), the dissociation rate of a bond is increased under force because the ligand-receptor interaction potential is tilted and the activation energy between the bound and the free state is lowered. Willemsen and co-workers (1999) have refined this concept for measurements with an AFM, showing that a shift of the harmonic cantilever potential with respect to the tip-substrate interaction potential reduces the activation energy. In the case where a spacer is stretched between tip and molecule, however, entropy also plays a role. In an energetic picture of the unbinding process, this means that the potential the tip feels would have degenerate energy states, causing equations for the dissociation rate of a bond to become complicated. Rief and co-workers (1998b) have simplified the problem by combining the description of polymer extension under force (worm-like chain model; Marko and Siggia, 1995) with the dissociation of bonds under force (Evans and Ritchie, 1997). Using a Monte Carlo simulation they could fit the extension curves that they had measured one year earlier (Rief et al., 1997a, b). This was soon followed by Evans and Ritchie (1999), who refined their own model of bond strength such that it could be applied to bonds that are connected through flexible linkages. They demonstrated that the combination of the stiffness of both force probe and flexible linker determines the dependency of the unbinding force on the loading rate.

The generation and detection of forces on individual molecules has reached fundamental limits. On one hand, the lower limit is determined by the Brownian motion of the force probe and the time resolution that is required in the experiment, but this is sufficient to allow the detection of the disruption force of even individual strands of DNA (Rief et al., 1999). On the other hand, the weakest chain in the system of tip, molecule(s), and substrate determines the highest force that can be exerted. In the case of rupture force measurements, the biomolecular bond determines the force and in the case of a system with strictly covalent bonds, the weakest of these bonds determines the rupture force (Grandbois et al., 1999).

The AFM set-ups that are used to detect and localize biomolecular interactions are quite diverse. For the measurement of the rupture forces of complexes only, the AFM need not to be pushed to its limits with respect to both hardware and software requirements. For these measurements, a force-distance curve needs to be generated and recorded at a fixed position. Because force-distance curve generation is one of the basic features of commercially available atomic force microscopes, they need not be adapted for the measurement of rupture forces. However, for novel applications of the AFM that require new measurement routines, a high stability of the set-up, or high control of the movement of the tip, most frequently home-built instruments are used. For force spectroscopy studies (Rief et al., 1997b), for instance, an AFM was constructed that could only move in the vertical direction to have the best control over the distance between tip and surface. Another application that requires a special purpose AFM is the adhesion mode imaging of specific molecular interactions. With these measurements force-distance curve generation is combined with image construction, implying that both hardware and software routines needed to be adapted for the generation, recording, and processing of the relevant data (Ludwig et al., 1997; Willemsen et al., 1999). Yet another application that used a modified AFM is the measurement of an interaction potential by the use of thermal fluctuations. Here, improvement of the sensitivity of the beam deflection system that is used to detect the position of the tip was needed, combined with a high stability of the

AFM set-up (Cleveland et al., 1995). In all these studies it was demonstrated that a detailed knowledge of the physical mechanisms in the AFM is required to operate it at the limit of stability, resolution, and control.

In this review we have shown that the AFM has become a versatile instrument to stretch and disrupt individual molecules and molecular complexes. However, thus far, the AFM force measurements on individual molecules have only been reported on rather isolated systems that hardly resemble the ones in the physiological situation. If the instrument is to be exploited to solve, for instance, immunological questions that require a cascade of molecular reactions and interactions, it should be adapted for the detection of biomolecular interactions in systems containing multiple molecular components. Here it is essential that the resolution of the AFM is high enough to resolve individual molecules, so that cooperative processes between homogeneous and heterogeneous sets of molecules can be discerned.

Another disadvantage of the force measurements with an AFM is that force is generated in only one direction. Especially in the case of rupture force measurements, this might not be the energetically mostly preferred way to separate complexes. Although the use of flexible cross-linkers also allows rotational degrees of freedom that might be favorable in the disruption process, it would be extremely interesting to be able to generate and measure the lateral and torsional forces in a directed way to see the effect on the rupture forces.

Compared to other nanoscopic force measurement devices, such as optical and magnetic trapping and micropipettes, the AFM has the disadvantage that it is a surface technique, implying that the hydrodynamic damping is higher than in bulk liquid, and thus the force sensitivity is intrinsically lower than those other techniques (Gittes and Schmidt, 1998). However, most rupture force experiments are performed on molecular complexes that are formed in or on top of a biological surface, e.g., the cell membrane. Here, surface effects, like discrete layering of fluid, electrostatic, and van der Waals attraction, might influence the functioning of the biological system, and the AFM might be the most suitable technique to study these effects after all.

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