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Force Microscopy of Cells to Measure Bioadhesion

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I. INTRODUCTION

The direct study of molecular interactions with microscopes has been limited so far by the resolution limits of the wavelengths and the operating conditions. The traditionally used optical microscope provides a theoretical resolution of about half the wavelength of light, which is not sufficient to study effects on a molecular level. For investigations of biomolecules on this level, a field of view of some square micrometers with nanometer resolution is required. Electron microscopes, which provide much higher magnification than optical microscopes, suffer from the environmental conditions. The samples are studied in a vacuum chamber, and the samples usually have to be coated with a metal film to avoid charging by the electron beam. These conditions are far from the "real" conditions, e.g., a buffered solution (1).

A quite young technique, scanning probe microscopy (SPM), overcomes these restrictions and offers much more than the conventional micro-



scopic techniques (2,3). SPM can be utilized under any environmental conditions, in air, under liquids, and in a vacuum. It can achieve magnifications as high as $10^9 \times$, which makes it possible to visualize single atoms and atomic defects, and it measures a three-dimensional image of the surface. Moreover, other physical properties such as friction and viscoelastic, magnetic, electrical, optical, and thermal properties can be investigated simultaneously with the topography. All types and sizes of conducting and nonconducting samples can be handled with this nondestructive technique. Because of these advantages, SPM is used in material investigations, physics, semiconductor development and quality control, chemistry, and polymer studies and is gaining increasing popularity for biological applications.

SPM was invented by Binnig and Rohrer in the early 1980s (4). They presented the first scanning tunneling microscope (STM) and determined the atomic structure of a silicon surface. In 1986 they received the Nobel Prize for their work. The drawback that STM handles only conducting surfaces has been overcome with the scanning force microscope (SFM) also called the atomic force microscope (AFM), which was presented in 1986 (5). Scanning force microscopy is the technique mostly used today within the family of scanning probe techniques. SPMs are much more than magnification tools. Molecular interactions and forces can be measured and used to modify surfaces and molecules. SPM opens the way for nanotechnology, which will be one of the key technologies in the next century. The goal of this chapter is to present the basic ideas of SPM and to demonstrate the function of the SFM as a force measurement tool for bioadhesion studies. In the biological area, SFM and also STM are used for highresolution studies under "real" conditions and dynamical studies of cells, viruses, proteins, and DNA. Some references are given in Sec. IV for review purposes. The requirements for the applications of SPM for biological systems are discussed and results of adhesion measurements are presented.

II. SCANNING FORCE MICROSCOPY AND RELATED TECHNIQUES

A. Concepts of Scanning Probe Microscopy

In a scanning probe microscope, a sharp probing tip is brought either in contact with or in the immediate vicinity of a surface. The image is acquired by stirring the tip parallel to the sample surface, or alternatively the sample surface parallel to the tip, while acquiring surface properties in a rectangular array of coordinates. The actual surface properties probed by such a microscope are dependent mainly on the mechanism chosen to control the tip-to-

surface distance. For example, the STM probes the density of electronic surface states by acquiring the current between a metal tip and a conductive surface. More important in biological applications, as nonconductive surfaces can be probed as well, the AFM or SFM is sensitive to the forces acting between tip and surface. In such a microscope the tip is attached to a soft cantilever. The bending of the cantilever thus provides information about the forces exchanged between tip and surface. Thus, the AFM is the most suitable tool for studying bioadhesion forces, and this chapter will focus on this SPM mode.

A further SPM technique, scanning near-field optical microscopy (SNOM), combines the measurements of surface forces with the acquisition of optical properties, e.g., luminescence, reflectivity, transparency, and even Raman signature. A high spatial resolution of typically 50 nm is achieved by using an illuminated glass fiber as the probing tip. As there exists a great variety of surface properties and thus SPM techniques, a discussion of SNOM and other SPM techniques lies beyond the scope of this chapter and the reader is referred to Refs. 6 and 7.

B. Contact AFM: Topography Imaging

1. The Tip-Cantilever System

The heart of any scanning force microscope is the tip-cantilever system. An image of such a system is displayed in Fig. 1, which shows the front part of the cantilever with a pyramid with a height of 4 μ m pointing downward. The cantilever and pyramid are made from silicon nitride. A 3-µm-long tube of carbon is attached. The probing tip at the end of the tube remains sharp within the display limit; higher resolution images reveal that the tip end has a spherical shape with diameters typically ranging between 5 and 10 nm. The cantilever as displayed is a standard cantilever commercially available for contact imaging, i.e., imaging in the range of repulsive interatomic forces. It divides into two arms to provide lateral stiffness, each arm being about 220 µm in length and several micrometers thick. This geometry yields spring constants as low as k = 0.032 nN/nm. As a result, already small changes in force, e.g., a 0.1-nN change, a value below typical values for repulsive interatomic forces, leads to bending of a free tip-cantilever system of about 3 nm, a distance large compared with atomic corrugations. Thus, if such a tip is brought into immediate contact with a surface, the tip can generally be stirred across the surface, following surface features without destroying or otherwise altering the sample surface.





Figure 1 Image of a silicon nitride pyramidal tip with an attached carbon nanotip.

2. Typical Concept of a Scanning Force Microscope

To generate an image in contact mode AFM, the tip-cantilever system is brought into contact with the surface; i.e., tip and sample exchange interatomic repulsive forces. As the spring constant is small, the tip follows topographic features as the sample is moved parallel to the sample surface. Changes in topography can then be monitored by detecting the bending of the cantilever. This can be a very delicate task, considering that changes in cantilever deflection may be as small as atomic corrugations (<0.1 nm). In the development of atomic force microscopes, several approaches were used to measure the bending. Because of its reliability, versatility, and ease of use, the optical position-sensitive method is by far the most popular approach and thus will be discussed further. A schematic setup of an atomic force microscope based on the optical position-sensitive method is displayed in Fig. 2 (8). Cantilever deflection changes are monitored by acquiring the position of a laser beam reflected from the cantilever. The laser beam is generated by a laser diode placed above the cantilever. The beam is aligned on the free end of the cantilever, which is mounted with a tilt of a few degrees downward relative to the horizontal plane. The beam is reflected from the cantilever at twice this angle



Figure 2 Basic components of a force microscope (not to scale). A tip is held by a soft cantilever and touches the sample surface. The vertical tip position is acquired by an optical deflection method: a laser beam is reflected from the cantilever, and the position of the reflected beam spot in a photodiode is monitored as the photocurrent difference between the top and bottom of the photodiode. Prior to data acquisition, the instrument is aligned to yield a previously chosen value for the photocurrent difference (e.g., T - B = 0). (From Ref. 8.)

relative to the incoming beam and propagated via a mirror into a positionsensitive photodiode. Positional sensitivity is gained by dividing the diode into four quadrants, two placed at the top (labeled T in Fig. 2) and two at the bottom (labeled B). As the tip touches the surface, repulsive interatomic forces bend the tip upward. Mirror and photodiode alignment is chosen in such a way that the reflected laser beam then rests in the vicinity of the center of the four quadrants. The exact location of the beam corresponds to the force acting between tip and sample, and the approach of the tip to the surface is stopped at a preset value, called the setpoint (chosen to be zero for simplicity in Fig. 2). As the sample is moved laterally relative to the tip, changes in topography cause a shift of the laser spot at the photodiode, which is electronically accessible as a change in the photocurrent difference between top and bottom quadrants relative to the set point. For topographic imaging, the photocurrent difference serves as the input signal for a feedback loop, which controls the bending of the cantilever. In a first processing step the difference Δ between the actual photocurrent difference value and the set point is calculated. From this difference the loop tries to estimate an "improved" cantilever-to-sample position. The sample is raised or lowered to this new position, and again the photocurrent differ-



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ence value is taken. The loop is passed repeatedly until the level of the set point is reached again. Height information can then be directly obtained by monitoring the total change of the vertical sample position while scanning the sample laterally. Thus, if a fast response of the feedback loop to topographic changes is provided, the cantilever deflection and consequently the force acting between tip and sample are kept at a constant value. A topographic image is then simply obtained by acquiring the total vertical change in sample position. The information is mapped in a square array of coordinates in the sample plane and stored in a computer with from 200 \times 200 to 1000×1000 points per image. The resolution of the image is then simply determined by the distance between neighboring coordinates, as long as physical resolution boundaries are not surpassed. For display purposes the acquired height values are translated into a linear gray scale. Typically, dark gray values represent depressed regions of the sample and bright gray values are assigned to higher areas. Two main factors determine the physical resolution limit. First, the resolution limit is given by the interaction volume between tip and sample, which will be discussed in the next section. Second, the positioning accuracy of the tip relative to the sample imposes a technical limit on the best possible resolution. Of course, it is impossible to reach a positioning accuracy on or even near an atomic scale with classical mechanical components. A more suitable approach had been found in the use of piezoceramic elements. Their movement is based on the fact that they alter their shapes if placed in an electric field. As an example, a thin ceramic tube with a grounded electrode on the inside and an electrode on the outside will change in length as a function of the charge on the outer electrode. If the outer electrode is divided along the long axis of the tube, motion perpendicular to the tube axis can be generated by applying opposite charges to the electrodes. The tube expands at one side and contracts at the opposite side, resulting in bending of the tube. Thus, if four equal electrodes are placed around a tube, it is possible to bend the tube in any direction perpendicular to its long axis. Finally, the length of the tube changes if a further bias voltage is applied to the inner electrode. Thus, if a sample is placed on one end of such a piezoceramic tube, it can be positioned laterally and perpendicularly relative to its surface. In general, the positioning accuracy of these mechanical motors, called scanners, can reach values as low as 0.01 nm and thus surpasses even atomic accuracy. For large scan ranges, however, the use of tube scanners is disadvantageous. To generate a large scan area, tube scanners have to be quite long, which leads to mechanical instabilities. For this reason, scanners built from three different linear independently working piezo stacks, called tripod scanners, are favored for imaging areas larger than $10 \times 10 \ \mu$ m. In this design

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two stacks are responsible for lateral motion and the third piezo stack works perpendicularly to the raster plane. A further advantage is that it is relatively easy to add control mechanisms to the piezo movements. Nonlinear components of the piezoceramic response to the applied voltage, such as hysteresis, are a well-known problem for large displacements. This is negligible for small imaging areas but causes large errors in length measurements on larger scales. These uncertainties can be overcome by hardware control of the piezo length during the scan process. For example, strain gages can be glued to the piezo stacks. These strain gages have negligible hysteresis and thus give precise feedback of the actual piezo lengths. The signal from these gages is processed by a feedback loop in the control electronics and used to correct piezo artifacts.

In addition to piezo artifacts, further complications may arise from unwanted movement between tip and sample such as vibrational noise or thermal expansion. Vibrational noise can range from low-frequency oscillations within a building structure up to high-frequency acoustic or electronic noise. Thermal expansion is caused by temperature changes of parts within the microscope. However, with careful site preparation, these factors can be controlled well and their disturbing influence kept to a minimum. In comparison with electron microscopes, atomic force microscopes are compact in design. The mechanical parts are often not larger than a fist, which makes vibrational damping easy and keeps thermal expansion to a minimum. Relevant to adhesion measurements are contact techniques, which are used to study the force exchange between tip and sample. Next to the forces acting vertically between tip and sample, which will be discussed in detail later in this chapter, there are further dynamic frictional forces that may have an impact on the imaging process and thus should be mentioned here. During scanning, frictional forces act antiparallel to the movement between tip and sample. In addition to surface roughness, the reactivity and chemical nature of the surface influence the frictional force and thus may be useful for the study of biologically relevant samples. The frictional force results in a distortion of the cantilever parallel to its long axis. This distortion causes a "left-right" shift of the laser spot in the photodiode in addition to the "top-bottom" motion of the spot used to contact topography imaging. Thus, by acquisition of the photocurrent difference between the left and right quadrants of the photodiode, a lateral force image is obtained at the same time as the topography is mapped in contact mode.

C. Noncontact AFM

As well as measurements in which tip and surface are in immediate contact and interatomic repulsive forces are dominant, there are further possibilities

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for acquiring images in the regime of the much weaker van der Waals forces. These forces become dominant with increasing tip-to-sample spacing above 1 nm. Unfortunately, these noncontact regions above the sample surface are technically not accessible with contact AFM as discussed so far. Because of the extremely small spring constants, the attractive forces would pull a tip attached to a contact cantilever onto the sample until these forces were again balanced by repulsive forces.

Thus, to image with the help of far-ranging attractive forces, different tip-cantilever systems and imaging techniques have to be considered. For contact-free imaging, cantilevers with spring constants above 10 nN/nm have to be used to avoid contact caused by attractive forces. However, such a stiff cantilever responds very weakly to any change in force between tip and sample, and therefore more sensitive methods of acquiring the influence of forces on the tip have to be found. Instead of simply measuring the bending of the cantilever, the tip-cantilever system is brought into oscillation at its

resonance frequency and the influence of the long-range forces on the resonance behavior is studied for positional feedback.

Thus, for noncontact operation the technical setup used for contact imaging has to be modified. The tip-cantilever system is now attached to a further piezoelectric ceramic, called a bimorph. A bimorph can be electronically excited into an oscillation of its thickness and is thus able to generate a mechanical oscillation of the cantilever. The driving frequency is typically chosen to be v_{res} , the resonance frequency of the tip-cantilever system, at which the amplitude of oscillation reaches a maximum. The value of the resonance frequency itself varies with cantilever design and environment, typically ranging from 50 to 500 kHz. In the design of a typical instrument, the cantilever oscillation leads to an oscillation in the photocurrent difference between the top and bottom of the photodiode. This oscillation is then processed by a so-called lock-in amplifier, which generates an output proportional to the amplitude of the signal. At the same time it is possible to derive information about the phase shift between the driving oscillation at the bimorph and the mechanical oscillation of the cantilever.

Close to a surface, the resonance condition changes as the tip oscillates

within the field of attractive forces. As the field decays within the oscillation amplitude, the tip is influenced by different forces during an oscillation cycle. The difference in force, more precisely expressed in terms of a force gradient $\partial F/\partial d$, enhances oscillation, resulting in a shift of the resonance frequency toward lower values. The system now behaves as if the spring constant k of the tip-cantilever system has been altered to

$$k' = k - \frac{\partial F}{\partial d}$$

This change in resonance properties is then monitored either as a decrease

in oscillation amplitude or as a change in phase signal if the tip-cantilever system is still excited at the former resonance frequency v_{res} . Thus, the approach can be stopped at a given damping value of the amplitude or change in phase signal, which then serves as a set point for positional feedback. During scanning motion a protrusion in topography further reduces the amplitude, whereas a depression causes an increase. The system is able to respond to these topography changes if the amplitude serves as a signal for the feedback loop.

Next to van der Waals forces, the contamination layer, thin film of adsorbed water and hydrocarbon molecules, has a further impact on the resonance behavior of the tip. Depending on the chosen amplitude of the tip, its influence can be controlled and even utilized for imaging.

At a relatively high amplitude, the tip typically oscillates in and out of the contamination layer, and typically it cannot be avoided that the tip touches the surface during the oscillation cycle. In this mode the tip is in periodic contact with the surface, resulting in exchanged forces in the nanonewton regime. Thus, the exchanged force has the same order of magnitude as in contact techniques. The advantage of this mode is that topography and phase information can be acquired simultaneously. Being more sensitive to small changes in force, this phase information often reveals information about the fine structure of a surface or about the contrast in adhesion forces. The disadvantage is that the periodic contact of the tip and surface has the potential of destroying either tip or surface. The development of more sensitive electronics led to the development of techniques in which smaller oscillation amplitudes can be used in the imaging process (9). In NearContact mode, oscillation amplitudes of typically 2 nm are used while the feedback loop reacts to the more sensitive change in phase signal. With this concept the tip can be held within the contamination layer while the tip touching the sample surface can be avoided. In this mode, there is no damage to tip or sample, and because of the small amplitude, extremely high resolution can be obtained. Acting forces are at least one order of magnitude smaller than those relevant to

periodic contact imaging.

Finally, if small amplitudes are applied to the cantilever and the feedback is tuned to a high sensitivity to react to small changes in force during the approach to the surface, the system can be operated in a noncontact mode, in which the surface of the contamination layer is imaged (10). This mode has the advantage of extremely low force exchange, e.g., in the piconewton regime, but due to the relatively large distance between tip and surface the resolution is somewhat limited. This, however, allows imaging of most delicate samples, which would be destroyed or otherwise influenced, especially under periodic imaging conditions. Examples of images obtained with these modes of noncontact operation are given later throughout this chapter.

III. FORCE MEASUREMENTS AND FORCE MAPPING

A. Relevant Forces in Atomic Force Microscopy

A thorough understanding of the various forces acting between tip and surface is of vital importance to the operation of an atomic force microscope. An in-depth theoretical understanding of all forces acting between such a tip and a probed surface is impossible, as a very large number of tip and sample atoms are involved within the interaction volume. Nevertheless, the natures and relative contributions of individual forces are well understood. Two contributing forces present in any system can be discussed by studying two neutral, nonpolar atoms in the gas phase. Separated by distances greater than several tens of nanometers, these atoms do not exchange any forces. As the distance between the atoms decreases, the atoms experience an attractive force, which is due to an electric dipole interaction between them. This attractive force between the two atoms is called the van der Waals force. The strength of the force is proportional to about $1/d^7$ for distances smaller than 10 nm. As soon as the electron clouds of the two atoms interact directly, the resulting repulsive forces become stronger than the weak attractive van der Waals forces. The overlap of the electron shells results in incomplete shielding of the charge of the two atomic nuclei. This leads to an exchange of repulsive Coulomb forces. In addition, according to the Pauli exclusion principle, equal electron states can overlap only if the quantum mechanical state of one of the electrons changes, i.e., is brought to a higher energy level that causes an additional repulsive force. Thus, as the interatomic distance decreases to values below 1 nm, within the range of atomic radii, the atoms exchange strong interatomic repulsive forces. These forces easily reach a level of several nanonewtons and above. Mathematically, the dependence between force and interatomic distance can be derived from the Lennard-Jones potential:

$$V(d) = -3E_{\rm Eq} \left[\left(\frac{\sigma}{d} \right)^{12} - \left(\frac{\sigma}{d} \right)^{6} \right]$$

with $E_{\rm Eq}$ being the lowest potential energy at the equilibrium distance $d_{\rm Eq} = 2^{1/6}\sigma$. Of course, the Lennard-Jones potential is a rough approach to a tip and sample system. A complete description involves many tip and sample atoms within the interaction volume. A more detailed theory of van der Waals forces (11) yields

$$F_{\rm vdw}(d) = -\frac{H}{6} \frac{R}{d^2}$$

for the van der Waals forces acting between a sphere of radius R, approximating a spherical tip, and a flat sample surface. The Hamaker constant, H, is itself a function of the refractive indices of sample, sphere, and immersion medium as well as of the absorption energies of these media. The decrease of force with distance is thus proportional to $1/d^2$, that is, slower than the distance dependence calculated from the Lennard-Jones potential. Typically, van der Waals forces are in the piconewton (1 pN = 10^{-12} N) regime; thus they are three orders of magnitude lower than the repulsive interatomic forces used for feedback in contact imaging. Depending on the nature of the sample and tip, additional forces may be exchanged between tip and sample. On the one hand, far-ranging electrostatic or magnetic forces can be moni-

tored a few hundred nanometers above a surface. On the other hand, chemical binding forces between tip and surface have to be taken into account whenever applicable—examples of such forces are discussed in Sec. V.

In an ambient air environment, surfaces are commonly covered by a thin contamination layer, which consists mainly of condensed water and hydrocarbon molecules. The absolute thickness of such a layer may reach values as high as 20 nm, with the absolute value depending on surface topography, chemical nature, and air humidity. If the tip is brought into the immediate vicinity of the surface, their contamination layers overlap and attractive capillary forces form while the system tries to minimize its total surface area. The strength of the capillary force can be calculated from thermodynamic equilibrium considerations, yielding

$$F_{\text{Capillary}}(d) = \frac{\pi R T \rho}{M} \ln \left(\frac{p}{p_s}\right) r(t - d)$$

where 2r is the diameter of the water bridge between tip and sample, which is about equal to the tip radius; R is the universal gas constant; T is the temperature; ρ and M are the mass density and the molar mass of the wetting liquid; p/p_s is the relative vapor pressure, which in ambient air is equal to the relative humidity; t is the thickness of the contamination layer; and d is the distance between tip and sample (from Ref. 12). Thus, the capillary force is proportional to the tip radius and can be quite large for a blunt tip. As a rule, assuming typical parameters, every nm of tip radius adds a nanonewton in force, so a tip with a radius of 50 nm yields a capillary force of about 50 nN. Therefore these capillary forces dominate van der Waals forces and falsify high-resolution adhesion force measurements significantly. The typical approach to solving this problem is either to operate the system in a dry gas atmosphere or to immerse the tip and sample completely in a liquid.



B. Contact AFM: Force-Distance Curves

Forces between a probing tip and a surface are accessed by force-distance curves. Such a curve displays the bending of the tip end of the cantilever versus the relative position between the free tip-cantilever system and a silicon sample. The force $F_{\text{Cantilever}}$ on the cantilever itself can then be calculated from Hooke's law:

 $F_{\text{Cantilever}} = k_{\text{Spring}} z$

where k_{Spring} is the spring constant of the cantilever (e.g., 0.032 nN/nm) and z the bending of the cantilever.

At a large tip-to-sample distance there is no force exchange and thus no influence on the cantilever (horizontal line labeled (a) in Fig. 3). As soon as the contamination layers of tip and surface overlap, or, alternatively, as soon as van der Waals forces destabilize the tip-cantilever system, the tip is rapidly pulled onto the sample surface (dip labeled (b) in Fig. 3). Thus, in liquids under ultrahigh-vacuum conditions or in a dry protective gas atmosphere, i.e., conditions in which the strong capillary forces do not appear, information about van der Waals forces can be acquired. Once tip and sample are in contact, they exchange repulsive interatomic forces. The tip follows an upward motion of the sample, which leads to an increasing deflection (labeled (c) in Fig. 3). The upward motion of the tip is reversible upon retraction of the sample (upper part of line labeled (d) in Fig. 3). It is worthwhile to mention that the curve will deviate from its original upward path if there is inelastic deformation of the sample. When the former "jump to contact' position is surpassed, the tip still remains in contact with the surface as it is now held by capillary forces (lower part of line labeled (d) in Fig. 3) or adhesion forces, which play the major role in the results discussed later in this chapter. The cantilever is bent downward until the re-



Figure 3 Schematic of a force-distance curve.

sulting force $F_{\text{Cantilever}}$ overcomes the adhesive forces. Then the cantilever snaps the tip away (line (e) in Fig. 3) from the surface until it reaches its original position (line (f) in Fig. 3).

IV. BIOLOGICAL APPLICATIONS

Microscopes are important and widespread tools in biological research in various fields, because they allow access to microscopic structures that are not directly accessible with the bare eye, such as cells or cell elements. Consequently, it is natural to apply scanning probe microscopy to biological specimens, even though this new class of microscopes was initially designed especially for applications in material science. Many SPM techniques have been used and still are in use for characterizing biological specimens, such as scanning near-field optical microscopy (SNOM) (6) or scanning tunneling microscopy (STM) (13) (a comprehensive introduction to SPM applications in biology can be found in, e.g. Ref 14). However, scanning force microscopy (SFM) is a very popular technique among scientists because it has several advantages in comparison with other SPM techniques. For example, no conductive specimen is required, and the investigations can be carried out in physiologic environments to name a few of them. Therefore, SFM is an extremely suitable and versatile technique for biological applications. Hence, the main part of this chapter focuses on SFM applications. In principle, one can identify two major fields of application for SFM in biology: investigations of biocompatible materials and imaging of surface morphologies of specimens. The former is strongly linked with materials science, because in biology the same specimen properties are of interest as in materials science, such as roughness, friction, and adhesion properties (6). Because SFM was designed to measure those properties, it is also a valuable tool for determining the corresponding properties of biological specimens. But one should keep in mind that, in contrast to materials science, the special biological environment has to be taken into account when characterizing those surfaces with SFMs. One interesting application of SFM is in the examination of molecular interactions that are manifest in forces (15). For this purpose the microscope tip is coated with organic monolayers, and the force interaction with a specially passivated surface can be measured (16). This measuring mode can be used to investigate antigen-antibody reactions (17,18). By performing these measurements in a two-dimensional manner, a mapping of the distribution of the binding partners on a surface is possible (19,20). A detailed discussion of this technique will be given in the next section.



Even if only the morphology of a specimen is of interest, the utilization of SFM is advantageous. In contrast to conventional light microscopy, which cannot provide any height information, SFM allows acquisition of the real three-dimensional geometry of a specimen with nanometer accuracy (Fig. 4). It is even superior to fairly new optical microscopes, such as confocal laser scan microscopes, which provide height information but lack sufficient spatial resolution. Microscopic techniques almost comparable with respect to resolution are scanning electron microscopy (SEM) and transmission electron microscopy (TEM). However, they do not provide any height information; they require extensive sample preparation, which probably induces artifacts; and they operate under vacuum conditions, which prevents investigation of living specimens. SFM, on the other hand, is a nondestructive technique; i.e., investigations can be performed with minimum interaction with specimens, allowing even examinations of living cells. The benefit of SPM in this field is that it enables the collection of nanoscopic surface structures of mesoscopic specimens, e.g., cells. Because the structure of a biological element is determined by its function, SPM can give deeper in-

sight into biological functionality. The following micrographs demonstrate the efficiency of SFM investigations of the biology of selected specimens.

Figure 5 shows the topography of a human carcinogenic caco-2 cell measured with SFM in the so-called noncontact mode in a liquid. This mode prevents direct contact between specimen and probe, allowing an extremely



SFM image of a chicken cell. Figure 4





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Figure 5 SFM image of a caco-2 cell (non-contact mode measurement). (Courtesy of E. Haltner and C.-M. Lehr.)

careful examination of the cell surface. Besides the coarse overall topography of the cell, fine structures on its membrane are visible (microvilli). This example shows that SFM measures the whole topography of a surface in a physiological environment without any information loss and that it is possible with appropriate image processing procedures to emphasize and visualize fine structures that are normally covered by a coarse overall specimen topography. Figures 6 and 7 depict topographies of caco cell monolayers. Because of the fairly large scan range, the dynamic range of the topography, and the utilization of the so-called contact mode, the microvilli are not visible. However, these images indicate that a topography range from micrometers to nanometers can be handled by SFM. Because of its features, e.g., zoom capabilities, the instrument's operation is comparable to the operation of conventional microscopes; that is, easy switching from a macroscopic to a nanoscopic view is feasible. Figures 8 and 9 demonstrate the resolution capabilities of SFM. Figure 8 depicts chromosomes at different magnification levels, and Fig. 9 shows pores of a cell core. Both images delineate typical high-resolution measurements with SFM, and they show that even though the achievable spatial resolution cannot be better than the finite diameter of the probe apex, state-of-the-art probes are "sharp" enough to image even the smallest structures without artifacts.

SPM is not only a supplement to microscopic techniques already established in biology, it widens the field of possible microscopic applications





Figure 6 SFM image of a caco-2 cell monolayer (contact mode measurement).

as well. With its high resolution and magnification capabilities, it provides the opportunity for imaging of single molecules. With SPM, it is possible to locate specific molecules within a living cell (21) or measure the shape of organic molecules. In this respect, SPM closes the gap between microscopic and molecular biology, as it allows the biologist to see single molecules and complex biological structures in the micrometer range.



Figure 7 SFM image of a caco-2 cell monolayer (contact mode measurement). (Courtesy of E. Haltner and C.-M. Lehr.)





10 µm

Figure 8 SFM image of chromosomes.

Ω.



Figure 9 SFM image of nuclear pores of Xenopus laevis.



V. BIOADHESION MEASUREMENTS WITH SFM

The ability of SFM to measure extremely weak forces makes it most suitable for investigations of binding forces between different materials. Many phenomena in biology are based on binding forces between complex organic molecules, and SFM has been used extensively for the characterization of those forces. However, this application is not restricted to molecular force interaction. The force interaction between individual particles can be measured too (6). In all experimental setups dealing with molecular interaction, a probe is chemically modified by functional groups that interact with binding partners on a surface (functionalization of probes). Depending on the chemical modification of the probe, the force interaction is specific to the chemical structure of the surface. In this respect SFM has become a chemical (material) sensitive technique. Besides the experimental efforts to measure

the forces, first attempts have been made to provide a theoretical understanding of the force interaction (22,23).

In the case of bioadhesion, the chemical modification of the probe is accomplished by coating it with a biomaterial. The choice of material is restricted only by the requirement that the binding of the material to the probe is much stronger than the binding forces present in the interaction; otherwise, the biomaterial will come off the probe and stick to the surface. But this is mainly an issue of appropriate probe preparation and is under control for various materials.

With the measurement of bioadhesion it is possible to characterize the binding forces between individual cells in a multicellular organism (24) as well as the binding characteristics of complementary strands of DNA (25) or nucleotide bases (26). In some cases SFM cannot measure the binding force between two adjacent molecules, as many probe and surface molecules are generally involved in the measurement. But by varying the experimental conditions one finds a quantization of binding forces depending on the number of molecules involved (27). This approach allows determination of the binding force between individual molecules.

This compilation reveals some of possible applications of SFM bioadhesion measurements. But this is by far not the complete spectrum of possible applications. One can expect new applications to emerge in parallel with the improvement of control in probe modification, making SFM a material-sensitive technique.

A. Biotin-Streptavidin

A prominent example of bioadhesion investigations is the characterization of binding forces between biotin and streptavidin molecules. This combi-



Experimental setup for biotin-streptavidin force interaction mea-Figure 10 surements.

nation has several advantageous features: it is robust and does not alter its binding behavior after transfer to probes or solid surfaces, and it has noncovalent binding similar to the important antibody-antigen binding, with comparable binding strength. A comprehensive description of biotin-streptavidin interactions can be found in, e.g., Ref. 28. Figure 10 depicts the principal experimental setup used for binding force measurements. On a flat surface, e.g., mica, a thin layer of bovine serum albumin (BSA) is adsorbed. BSA is necessary because it tends to adsorb nonspecifically and irreversibly to glass or mica. Biotin is then attached to BSA by covalent binding initiated by appropriate conditions. The required biotin receptor (streptavidin) has four binding sites for biotin, which allows the streptavidin to stick to the BSA-biotin complex as well as to behave as an active receptor for the biotin layer on the respective glass or probe surface. In order to perform a reference experiment the same configuration is used, but the biotin receptors are deactivated—blocked—by additional biotin (Fig. 11). This setup is used for reference purposes, because in this configuration no binding forces between biotin and streptavidin



Experimental setup for biotin-streptavidin force interaction measure-Figure 11 ments with receptor blocking.

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Figure 12 Measured force-distance curves of biotin-streptavidin binding.

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should be present. Details of the sample preparation can be found in, e.g., Refs. 29 and 30.

A typical force-distance curve of the biotin-streptavidin system is shown in Fig. 12. The solid line represents the force-distance curve with the biotin receptor active, the dashed line the force-distance curve with the receptor blocked. It can be seen that the (solid) force curve crosses the zero force line, and then at a relative displacement of the tip of approximately 80 nm an adhesion force of roughly 200 pN is present. From this adhesion force information about the bond energy can be derived, which gives information about the binding mechanism (30). Numerical calculations simulating this adhesion process have been performed and provide deeper insight into the dynamics of the adhesion and rupture process (31).

B. Interaction Between Single-Chain Fv Antibody Fragments and Corresponding Antigens

Genetically engineered single-chain Fv antibody fragments (scFvs) (32) are ideal model proteins for studying antigen-antibody interactions by force spectroscopy experiments. They can be generated against all conceivable antigenic targets, and mutants with various binding properties can be engineered. Furthermore, an scFv is the smallest part of an antibody molecule that still contains the intact antigen binding site. It is crucial for the measurements of binding forces that the attachment of antibody and antigen to their respective surfaces is so strong that the antibody-antigen binding is correctly probed and no detachment of any partner occurs. To achieve stable and directed immobilization of the scFv on a flat gold surface, the molecule was designed with a cysteine at the carboxyl terminus, i.e., at the part op-

posite to the binding site. Immobilization of the scFv fragment via the thiol group of the cystein ensures free accessibility of the binding site for antigens. To avoid denaturation of the proteins in contact with the surface, the gold was treated with mercaptoethanesulfonate, yielding a negatively charged surface (33). In these model experiment the scFv molecules used were directed against the antigen fluorescein (34). For the force spectroscopy measurements, the antigen was covalently immobilized to the silanized silicon nitride AFM tip via a poly(ethylene glycol) linker about 40 nm in length.

Surfaces with immobilized scFvs were first scanned with the antigenfunctionalized tip (Fig. 13A) in contact mode, with very low forces (F < 500 pN) in order to avoid detachment or destruction of the antibody fragments. Well-separated proteins were then chosen for series of force-distance

(A)



(B)







Figure 13 (A) AFM image of scFv antibody fragments immobilized on gold, scanned with an antigen-functionalized tip in contact mode. (B) Typical force-distance curve of an antigen-functionalized tip and a single, well-separated scFv molecule. (Courtesy of R. Ros.)

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measurements (Fig. 13B). The unbinding force, i.e., the maximum force at the moment of detachment, was taken as a measure of the binding force between the scFv fragment and the antigen.

The histogram in Fig. 14A representing the probability distribution of unbinding forces shows a single peak with a mean value of about 50 pN and a number of events where no binding forces can be observed, so-called zero events. When free antigen was added in order to block the binding sites of the scFv fragments, the number of zero events drastically increased and the peak at higher force values disappeared (Fig. 14B). These blocking experiments prove that the forces determined do indeed result from interaction between the ligand and the receptor and not from unspecific adhesion.

The power of this novel measurement technique is that it can distinguish closely related molecules (35). Comparison of the binding forces of the scFv fragment just described and a mutant that has a single amino acid exchanged within the binding pocket (34) showed 20% lower binding forces for the mutant. In order to detect such small differences, errors related to cantilever calibration must be avoided. Therefore, measurements of the wildtype and the mutant molecules were carried out with the same tip.

With the current model system it is possible to measure binding forces of a sufficient number of mutants and to correlate the values with their



Figure 14 The force values determined from 50 force-distance curves as one single molecule in histograms. Distributions for scFv fragments (A) before and (B) after blocking with free antigen. (Courtesy of R. Ros.)

thermodynamic parameters in order to obtain deeper insight into the molecular recognition processes.

VI. OUTLOOK

Scanning probe microscopes are valuable supplements to conventional microscopes as used in biology, not only with respect to bioadhesion. In terms of possible applications, we are only at the beginning of exciting developments in microscopy, especially in biology. It is very likely that one important branch of biology, medicine and pharmacy, will benefit most in the near future from SPM. Many solutions to problems in biomedical sciences are based on nanoscopic engineering, and scanning probe microscopes are tools for nanoscopic modifications and analyses. An example will support this statement. An important problem in medicine and pharmaceutical sciences is the development of biocompatible materials, and one solution seems to be the use of self-organized monolayers between the material surface and the bioactive element, i.e., the body. SPM can be used to characterize and modify those monolayers very easily. Another application is in the development of nanoscopic drug carrier systems. Such nanoparticles can be analyzed in an efficient way with SPM (36). Finally, it does not seem to be too far fetched to imagine the SPM will some day be a standard diagnostic instrument in medicine and an important tool for the development of novel drug delivery systems.

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