Atomic Force Microscopy: Seeing Molecules of Lipid and Immunoglobulin


The atomic force microscope (AFM) can image individual molecules by raster-scanning a sharp tip over a surface. In this paper we present molecular-resolution images of immunoglobulin M (IgM) and of ultraviolet light-polymerized films of the lipid dimethyl-bis(pentacosadiynoyloxyethyl) ammonium bromide ("BRONCO"). The polar head groups of individual lipid molecules can be resolved on the surface of this and other lipid films. These lipid films also provide a good substrate for AFM imaging of DNA and of other molecules such as antibodies. Because the AFM scans surfaces, it is most often successful at imaging either molecules that can form an array on a surface or molecules that are quite firmly attached to a surface. The ability of the AFM to operate under water, buffers, and other liquids makes it possible to study biological molecules under conditions in which they are physiologically active. Imaging of the actual molecular process of fibrin polymerization shows the potential of the AFM for studying biological processes. In the six years since its invention, the AFM has excited much interest and has imaged molecules in a wide range of systems.

Additional Keyphrases: raster scanning - polymer films

The AFM images surfaces by raster-scanning a sharp tip gently over the surface at forces as small as the forces between atoms in molecules (10^{-9} to 10^{-11} N) (4). Raster scanning is the movement of a tip back and forth across a surface, which produces a topological map of the surface features. The scanning tip is attached to the end of a cantilever, which deflects as the tip scans up and down over features on the surface (Figure 1). The deflection of the cantilever is detected by the deflection of a laser beam reflected off the cantilever. The reflected laser beam shines on a two-segment photodiode, which measures the magnitude of the deflection. Especially when imaging soft biological samples, it is important for the scanning tip to maintain a constant low force on the sample surface. To do this, a feedback loop from the photodiode to a piezoelectric translator moves the sample up and down in response to small changes in cantilever deflection. This keeps the cantilever deflection nearly constant, resulting in a nearly constant force between the scanning tip and the sample surface. To image surfaces as gently and nondestructively as possible, one scans the surface at the smallest force that can be maintained without having the tip lose contact with the surface. The surface is also raster-scanned in the x and y directions by means of the piezoelectric translator. The scan area can range in size from 5 × 5 nm to 80 × 80 μm. The surface topography thus revealed is displayed continuously on a monitor.

The AFM can image individual atoms on some surfaces (e.g., 5, 6). Gold atoms 0.3 nm apart have been resolved, and the AFM has imaged the electrochemical
deposition of copper atoms on a gold surface (7, 8). Biological applications of the AFM are among the most exciting applications. The AFM can image not only in air but also under water or buffer or many other fluids. This allows us to observe cells and biological molecules at a resolution not previously possible under physiological conditions (e.g., 9–14). The AFM is at present even more useful for many industrial applications, including measurement of the residual roughness of polished optical surfaces and inspection of compact discs and diffraction gratings.

Imaging Lipid Molecules

The AFM can image molecules of lipids in Langmuir–Blodgett films at high resolution. Langmuir–Blodgett films are synthetic lipid layers, resembling those in a cell membrane, that can form at an air–water interface. The hydrophobic lipid tails align in the air, and the hydrophilic lipid headgroups remain in the water or other aqueous phase. Individual lipid molecules have been resolved with the AFM in films of many lipids, including phospholipids such as phosphatidylglycerol and phosphatidylethanolamine, and also in a mixture of a positively charged lipid and a negatively charged lipid (9, 13–15). Lipid films are an especially good biological system for imaging with the AFM. The AFM images surfaces, and lipid films are themselves a surface—unlike most biological molecules, which sit on a foreign surface in the AFM.

Figure 2 (top) shows what appears to be a molecular-resolution image of the lipid dimethyl-bis(penta-cosadynojoyloxyethyl) ammonium bromide ("BRONCO"). BRONCO is a positively charged lipid with two diacetylenic hydrocarbon chains that can be polymerized by ultraviolet light. These polymerized lipid films have the advantage of being stronger than films of unpolymerizable lipids (e.g., those in cell membranes). The BRONCO sample was prepared at room temperature as follows: a BRONCO solution in chloroform was spread on the surface of a Langmuir trough by standard Langmuir–Blodgett technique, polymerized by exposure for 30 s to high-intensity ultraviolet light, and transferred by vertical dipping onto a substrate. The substrate was a monolayer of cadmium arachidate, also deposited by the Langmuir–Blodgett technique by vertical dipping onto freshly cleaved mica. After preparation, the sample was kept under water, even during imaging; thus, the images in Figure 2 show the hydrophilic surface of the BRONCO film. AFM imaging was done with a Nanoscope II AFM (Digital Instruments, Santa Barbara, CA) with an F-scanner, which has a maximum scan range of 10 × 10 μm. The cantilever was 100 μm long with an integrated tip. Scan speeds ranged from 8 to 26 Hz for scan lines in the x direction; image resolution was 400 lines × 400 points/line.

The 9 × 9 nm BRONCO image of Figure 2 (top) shows bands of molecular lattice alternating with diagonal ridges. This pattern was common to other high-resolution AFM images of the BRONCO film. A two-dimensional Fourier transform of these images gives lattice
The Langmuir moving direction 2 showed one face about two large areas where Y,'s corresponded to the Langmuir trough, where it appears that the lipid was quite dense. BRONCO samples taken from other regions on the surface of the Langmuir trough did not show as many lipid layers in the AFM.

Because ultraviolet-polymerized BRONCO molecules fluoresce, BRONCO films have also been viewed with the fluorescence microscope (16). The 300-μm images in the fluorescence microscope also show patches of lipid with a series of parallel cracks and some bright spots, but without the height information of the AFM. One impressive feature of the AFM is that it can, on some samples, give high-resolution images of areas as small as 5 × 5 nm and as large as 80 × 80 μm.

**Imaging Protein Molecules: IgM**

Figure 3 shows AFM images of IgM. The IgM was obtained by cryoprecipitation at 4 °C overnight from the serum of a patient with Waldenström macroglobuline-mia. The IgM was purified by redissolving it at 37 °C in isotonic saline (0.15 mol/L NaCl) containing sodium azide, 1 mmol/L, and cryoprecipitating it five times. IgM samples for the AFM were prepared at 37 °C by placing a freshly cleaved mica surface face down onto a 5-μL drop of IgM in isotonic saline on Parafilm. The IgM concentration was sufficient for a maximum surface coverage of 100 ng/cm². After a 15-min incubation, the samples were removed from the Parafilm and rinsed with water at 0 °C. The samples were then imaged in the AFM under ethanol. The AFM images were not stable long: typically, images with the quality shown in Figure 3 would last for three or four scans by the AFM before the molecular shapes deteriorated.

IgM is a large molecule with five major subunits, each structurally similar to a Y-shaped IgG molecule. These five subunits are arranged in a wheel shape, with the arms of the Y pointing outward and the stem of the Y pointing inward (as determined by electron microscopy). Because IgM has not been crystallized, its structure has not been determined by x-ray diffraction (20).

Figure 3 (left) shows an unfiltered AFM image of what appear to be four IgM molecules in a 200 × 200 nm field. Figure 3 (right) is a filtered image of the same four molecules, showing some submolecular detail. Molecules 1 and 3 in Figure 3 (right) have a depression in the center, appearing as a darker region, and molecule 2 appears to have about five subunits (see arrows). Electron micrographs of IgM show some fields of molecules with empty centers (21) and some fields of molecules with prominent centers (22). One model for the IgM molecule shows it as spider-like, with the arms of the Y's corresponding to the spider's legs and the stems of the Y's forming the body (20). In this model, the IgM molecules with depressed or empty centers would be lying on their backs, whereas IgM molecules with prominent centers would be standing on their arms (legs?).

Figure 2 (top) showed lipid molecules with a spacing of 0.7 nm. Figure 3 (right) shows protein molecules where the resolution is only about 25 nm. Why is there so much less resolution on the protein molecules than on the lipid molecules? Perhaps the isolated protein molecules show much less detail because of thermal motion or because the AFM tip pushes the surface of the molecule around more than in the crystallized lipid film.

IgM has been imaged with the transmission electron...
microscope. Negatively stained IgM molecules in the electron microscope appear smaller (20–30 nm in diameter) and thinner than IgM molecules in the AFM, and the five subunits or even the 10 "arms" of IgM can be more easily identified with the electron microscope (18–20). Negative staining tends to decrease the apparent size of molecules, whereas the AFM broadens molecules by an amount approximating the diameter of the AFM tip.

Discussion

In conclusion, what are the strengths and weaknesses of the AFM for biomedical applications? The minimum limit for sensitivity or detection is a single molecule or a subunit of that molecule, which makes the AFM one of the most sensitive instruments known. Current instrument problems include drift, in which features slowly move through the field of view because of thermal drift, and horizontal streaks, as in Figure 3. These problems are being minimized with improvements in instrumentation and image-processing techniques. The AFM tip interacts with the sample in ways that are sometimes damaging and not yet well characterized.

Another important consideration in AFM imaging is the substrate on which the sample sits. Mica is a favorite substrate for many applications, because clean planar surfaces can be prepared by simply peeling off the top mica layer with a piece of tape. However, many biological molecules do not stick well to mica because of its extreme flatness and negative surface charge. Glass is also used as a substrate, but in the AFM it shows hills that can be confused with biological molecules.

Under good working conditions, the AFM can image samples quickly and routinely. The time for a single scan is about 10 s, so a sample can be imaged in detail in less than an hour. Samples can be changed in about 15 min, allowing several samples to be run in the course of a morning or an afternoon. It is not usually necessary to have elaborate sample-preparation techniques. Because the AFM images the surface of the sample itself, the sample does not need to be embedded or sectioned. There is much room, however, for new and innovative research in finding better ways of firmly attaching samples to flat substrates.

This is a very new field. The AFM was invented only six years ago, and its use to investigate biological samples is even more recent. We have already come a long way in imaging proteins, lipids, and nucleic acids in ways that have never been seen before.

What future directions might one envision for the AFM? The AFM is already being modified for combined optical and AFM imaging of samples, allowing one to scan ever smaller samples with the AFM and to scan selected areas of a sample, e.g., an individual cell or a specific region on the cell surface. Electrochemistry is a promising new area of AFM research; with electrodes in the fluid cell of the AFM, it is possible to observe the changes in each step of a complete electrochemical cycle at atomic resolution (8). An AFM may be able to sequence DNA many orders of magnitude faster than conventional techniques (21). A dedicated AFM could be a versatile, yet expensive, biosensor. It could scan a surface that had an affinity for a particular type of molecule until it detected an individual molecule of this type. The AFM may be able to engrave ultra-fine patterns on surfaces (22). The movie of fibrin polymerization (summarized in reference 3) has aroused much excitement; with improved data-collection instrumentation, we look forward to making and seeing more such movies, especially of processes occurring at liquid–solid interfaces. For an instrument in its infancy, the AFM has performed well and shows much promise.

We gratefully acknowledge the expert technical assistance of Eloise Martzen and Gregory Kelderman and the helpful discus-
sions of Scot A. Gould, Srin Manne, and Craig Prater. This work was supported by an IBM Manufacturing Fellowship (A.W.), the Deutsche Forschungsgemeinschaft (H.E.G.), NSF Grants DIR-9018846 and DMR-89-17194 (H.G.H., P.K.H.), and the Office of Naval Research (P.K.H.).

References

CLINICAL CHEMISTRY, Vol. 37, No. 9, 1991 1501