Atomic force microscopy of hydrated phosphatidylethanolamine bilayers

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ABSTRACT We present images of the polar or headgroup regions of bilayers of dimyristoyl-phosphatidylethanolamine (DMPE), deposited by Langmuir-Blodgett deposition onto mica substrates at high surface pressures and imaged under water at room temperature with the optical lever atomic force microscope. The lattice structure of DMPE is visualized with sufficient resolution that the location of individual headgroups can be determined. The forces are sufficiently small that the same area can be repeatedly imaged with a minimum of damage. The DMPE molecules in the bilayer appear to have relatively good long-range orientational order, but rather short-range and poor positional order. These results are in good agreement with x-ray measurements of unsupported lipid monolayers on the water surface, and with electron diffraction of adsorbed monolayers.

INTRODUCTION

Phospholipid monolayers and bilayers prepared by the Langmuir-Blodgett technique are best known as simplified model systems of biological membranes. The phase behavior and molecular organization of these monolayers and bilayers have been intensely studied as they are excellent tools for understanding biological processes such as adhesion and fusion, protein structure and organization, etc. Langmuir-Blodgett films also have important technical applications as insulating layers on semiconductors, substrates for biosensors, or as a well-controlled method of chemically or physically modifying solid surfaces. Recent work has shown that an L-B film can be used as a template for crystallizing proteins (Ribi et al., 1988). Hence, there have been many efforts to understand the structure of L-B monolayers and bilayers and their phase transitions.

The first data on monolayers came from measurements of surface pressure vs. molecular area isotherms. In many phospholipid systems, two distinct phase transitions exist, the first at low surface pressure (π_c), high molecular areas, which corresponds to the onset of a fluid/"gel" main transition, and the second, at high surface pressures (π_s), low molecular areas, which is still somewhat controversial (Albrecht et al., 1978; Tamm and McConnell, 1985). Recent structural studies have focussed on electron diffraction of L-B monolayers deposited on various substrates and observed in vacuum, (Fischer and Sackmann, 1984; Garoff et al., 1986) and x-ray diffraction of monolayers at the air-water interface (Kjaer et al., 1987; Helm et al., 1987; Helm, 1988). Both diffraction techniques suggest that in the high surface pressure gel phase, the lipid hydrocarbon chains possess long-range orientational order, but relatively short-range positional order. As a result, L-B films in the gel state are believed to closely resemble the so-called "hexatic" phases better known in thermotropic liquid crystals (Moncton and Pindak, 1979; Brock et al., 1989). However, both short-range and long-range order have not yet been studied in a single Langmuir-Blodgett film.

The Atomic Force Microscope (AFM) (Binnig et al., 1986) should be the ideal instrument for visualizing biological and organic surfaces at high resolution (Zasadzinski, 1989). The AFM records interatomic forces between the apex of a cantilevered spring tip and the surface of the sample. In the "constant deflection mode" of imaging, the atomic force microscope traces out contours of constant force (as opposed to the scanning tunneling microscope which traces out contours of constant electron density). The AFM is compatible with a variety of environments, including liquids like water or saline, at varied temperatures and is capable of imaging with a tracking force of only $\sim 10^{-9}$—10^{-11} Newtons under water (Drake et al., 1989). However, it is not yet known over what area the force is operating during imaging with the AFM. Lipid bilayers or other biological surfaces are incapable of withstanding such localized forces without modification. The AFM can act as a molecular "broom," sweeping objects aside as it seeks a firm base on which to scan. If the AFM is to provide molecular resolution images of biological structures, samples must be prepared so that the molecules of interest are rigidly mounted and immobilized with well-defined orientations. These requirements can be met by

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Langmuir-Blodgett deposition of bilayers onto mica substrates.

The Langmuir trough has long been a standard tool in surface and colloid science for depositing chemically and physically well-defined monolayers. Langmuir-Blodgett troughs allow for the precise control of surface pressure, π, average area per molecule, A, temperature, and the lateral distribution of the molecular components at the air-water interface. These monolayers can then be quantitatively transferred to rigid substrates such as mica. Supported L-B films are remarkably robust and resistant to physical damage and wear. They are being investigated as boundary lubricants and have been shown to be undamaged at pressures >5,000 atmospheres (Briscoe and Evans, 1982).

We present images of the polar or headgroup regions of bilayers of dimyristoyl-phosphatidylethanolamine (DMPE), deposited by Langmuir-Blodgett deposition onto mica substrates at high surface pressures and imaged under water at room temperature with the optical lever AFM. The lattice structure of DMPE is visualized with sufficient resolution that the location of individual headgroups can be determined. The forces are sufficiently small that the same area can be repeatedly imaged with a minimum of damage. The DMPE molecules in the bilayer appear to have relatively good long-range orientational order along the rows, but rather short-range and poor positional order within the rows. These results are in agreement with x-ray measurements of unsupported lipid monolayers on the water surface (Helm et al., 1987; Kjaer et al., 1987), and with electron diffraction of adsorbed monolayers (Fischer and Sackmann, 1984; Garoff et al., 1986) which suggest hexatic order in LB bilayers. LB bilayer films have also been deposited onto conductive cleaved graphite surfaces for direct imaging with the STM (Hörber et al., 1988), although the interpretation of the images is controversial (Coombs, et al., 1988).

MATERIALS AND METHODS

Chromatographically pure, synthetic DMPE was purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. DMPE was dissolved at a concentration of 7 mg/ml in chloroform/methanol (3:1) and was deposited quantitatively behind the moveable barrier of a Lauda mini-trough to give directly the area per molecule. The solvent was allowed to evaporate over the course of several minutes before any measurements were taken. The surface pressure, π, was measured with a Wilhelmy balance detecting the differential force on a piece of filter paper partially submerged in the subphase. The subphase water was distilled, then treated in a Millipore Milli-Q system. A pressure/area isotherm showed that the fluid-gel coexistence pressure, π_c, occurred at 12.7 mN/m at a specific molecular area of 0.6 nm^2 and the gel phase occurred at a surface pressure, π_s, of 29.7 mN/m at a specific molecular area of 0.41 nm^2. Both of these values are consistent with previous studies (Helm, 1988).

Circular, 1 cm diam mica substrates were repeatedly cleaned in absolute ethanol and Millipore-treated water, then cleaved immediately before deposition. The mica disc was then held with tweezers and lowered into the trough at low π, high A. The surface area was decreased to ~0.40 nm^2 and a surface pressure of 40 mN/m so that the monolayer was well within the gel phase. Deposition occurred at a rate of 1 cm/min. During this process the monolayer pressure was held constant by electronic feedback-control of the film area. To deposit the second monolayer, the procedure of Tamm and McConnell (1985) was used. A monolayer-covered mica disc was gently placed onto a fresh monolayer deposited on the trough at a constant surface pressure of 40 mN/m. Then the mica disc was rapidly pushed through the interface into the water subphase. The second monolayer adsorbs with the hydrophilic heads pointed outwards. This second monolayer is only stable under water and will desorb on being retracted from water. The bilayer-coated substrate was then placed (below water) into a glass petri dish with a shallow well, with the bilayer coated side facing down. The water subphase was then aspirated out of the L-B trough. The assembly can then be removed from the trough and transferred to the AFM without exposing the bilayer to air. A water-filled cell was created in the Nanoscope II FM (Digital Instruments, Goleta, CA) AFM by placing an O-ring on top of the bilayer coated mica disc, which then is mounted on the sample stage (Drake et al., 1989).

The AFM stage with the bilayer coated mica disc was then allowed to equilibrate for several minutes to eliminate any thermal gradients that cause drift in the images. The AFM images of the bilayers were created by using an XYZ piezoelectric ceramic translator to raster scan the film laterally under a diamond tip attached to a microfabricated cantilever (Park Scientific Instruments, Mountain View, CA). Images were recorded in the constant deflection mode, i.e., feedback electronics and software were used to keep the cantilever deflection and the applied force constant. All images were taken at room temperature with an applied force of ~1–10 nN. Each image takes ~10 s to acquire. The images were then computer-processed to remove electronic noise.

RESULTS AND DISCUSSION

Fig. 1, *A* and *B* show grey scale AFM images of the polar region of a bilayer of DMPE deposited on mica. These images were taken under water at ambient temperature and pressure. The dominant features of the images are long, uniformly spaced rows roughly 0.7–0.9 nm in spacing. A modulation also can be seen along the rows, with rounded bright spots roughly every 0.5 nm. We believe that the individual bright spots along the rows correspond to the individual headgroups of the DMPE molecule. The lattice spacing is, within experimental limits, (~20%) that measured by x-ray diffraction at the air-water interface (Helm, 1988). The area per molecule in the AFM image, ~0.4 nm^2, is also in agreement with the 0.4 nm^2 area per molecule measured before deposition. The lattice spacing and symmetry are substantially different than that of the mica substrate (Drake et al., 1989) and it was possible to push through the DMPE

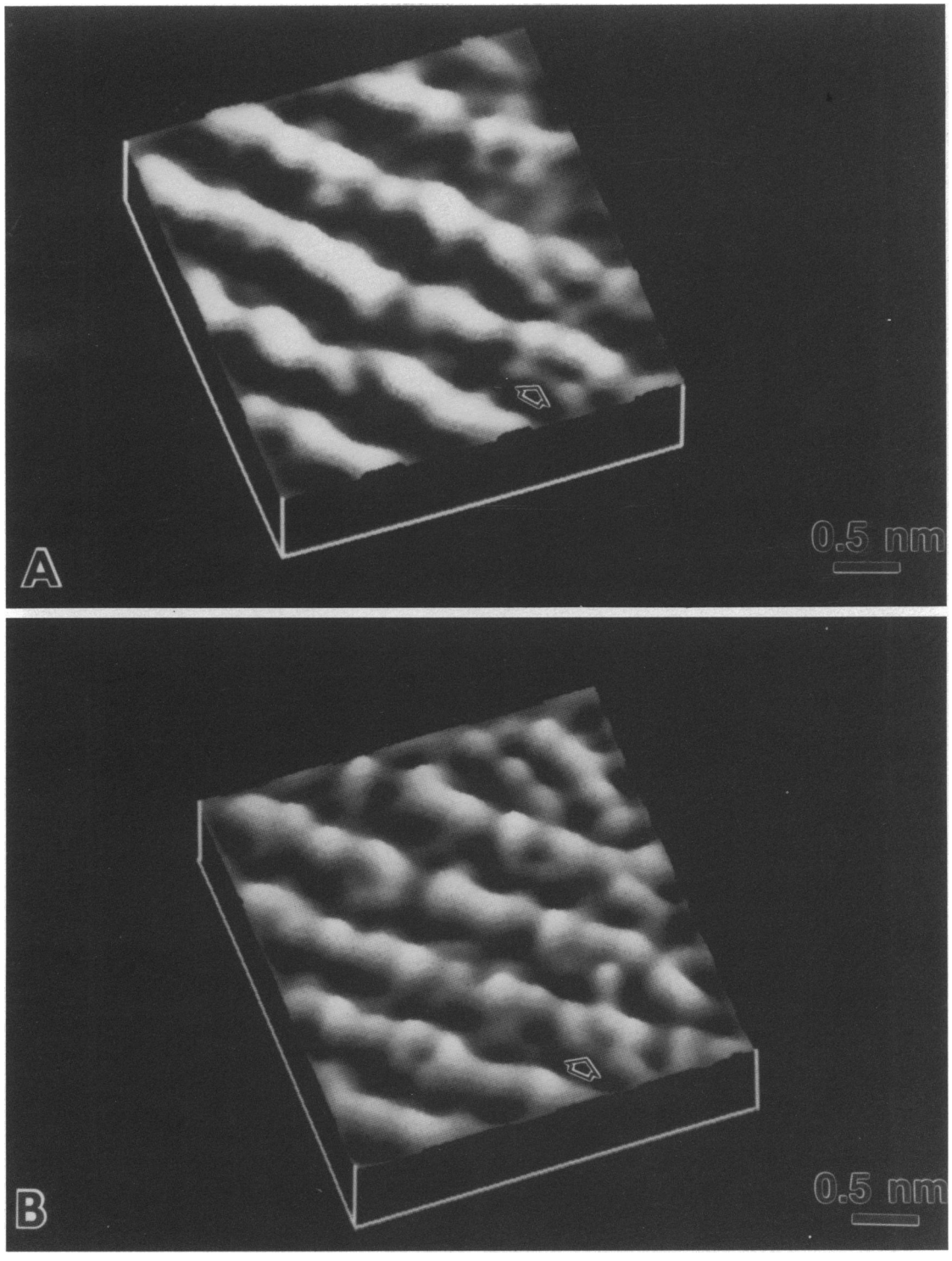

FIGURE 1 (*A, B*) Subsequent grey scale AFM images of the polar region of a bilayer of DMPE deposited by the Langmuir-Blodgett technique at a specific molecular area of 0.41 nm^2 and a surface pressure of 40 mN/m on a freshly cleaved mica substrate. The images were taken under water at ambient temperature and pressure. The long, uniformly spaced rows are roughly 0.7–0.9 nm in spacing. The modulation along the rows, with rounded bright spots roughly every 0.5 nm, correspond to the individual headgroups of the DMPE molecule. The lattice spacing is identical to that measured by x-ray diffraction at the air-water interface (Helm, 1988). The area per molecule in the AFM image is ~ 0.4 nm^2. The arrow marks similar areas on both images, which appear slightly different due to thermal drift during image acquisition (see text).

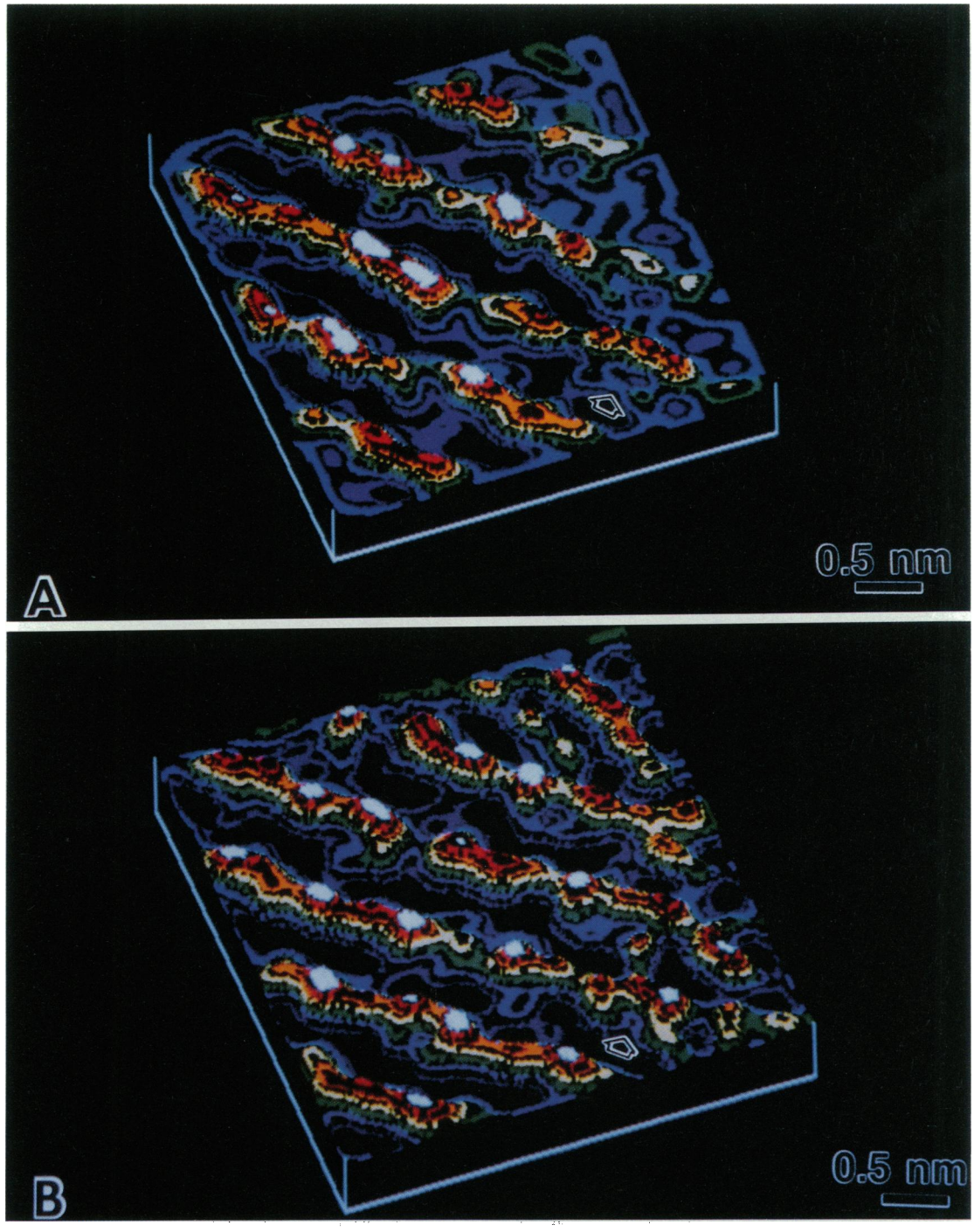

FIGURE 2 (*A, B*) Topographic AFM images of symmetric dimyristoylphosphatidylethanolamine bilayer corresponding to the grey scale images in Figs. 1, *A* and *B*, respectively. Along the rows are visible individual headgroups of DMPE separated by ~0.5 nm. Bright (white) corresponds to high points in the image, ~0.4 nm above the lowest points which are black. The organization along the rows is very imperfect, with many defects and vacancies, although the rows extend for several nanometer. This type of organization is suggestive of the hexatic phase in agreement with x-ray and electron diffraction measurements. These images were taken in water at ambient temperature. The images were stable and easily reproducible for long periods of time.

bilayer to image the mica lattice underneath by increasing the applied force on the cantilever (data not shown).

Figs. 1, *A* and *B* are subsequent traces of approximately the same area on the LB film. The images are not identical for several reasons, although the pattern of modulated rows is common to both images. Arrows point to a small patch of molecules we believe to be the same in both images. A small amount of thermal drift (tenths of a nanometer per minute) is difficult to avoid between images, which leads to small offsets between images. In addition to the offsets, depending on if the raster scan was performed top to bottom (Fig. 1 *A*) or bottom to top (Fig. 1 *B*), the lattice spacings and symmetry of the image are slightly different due to the distortion caused by the thermal drift during the ~10 s of image acquisition. Hence, it is best to average distances and symmetries over the two images to minimize errors. The images are also not expected to be identical because of the thermal motion of the DMPE molecules; bilayers are fairly fluid materials, and molecules likely move both horizontally and vertically between scans.

Figs. 2, *A* and *B* show a topographic map of the same areas shown in Figs. 1, *A* and *B*, respectively. The white (or bright) areas are the highest regions, ~0.5 nm higher than the lowest regions, which are black. The topographic images make it easier to see the molecules along the rows. These images also show that the DMPE packing is very imperfect, that there are a number of defects and vacancies in the lattice, although the rows of molecules extend for several nanometers. This is suggestive of hexatic ordering, although certainly not conclusive. The general features are in agreement with both x-ray and electron diffraction which suggest that LB films should exhibit hexatic ordering. It is also possible to see that the headgroups are not at a uniform height; the bilayer is rough at the nanometer scale. This molecular roughness may be important to recent theories of the so-called hydration force between bilayers in aqueous solution (Israelachvili and Wennerström, 1990). Comparing Figs. 2, *A* and *B* (at the arrow) show that some molecules change height between images, again as expected for a bilayer at room temperature in the hydrated state.

The resolution and reproducibility of our images suggest that the localized pressures most likely do not reach the level that we may have expected. A possible explanation of this was provided in a model of STM imaging by Mamin et al. (1986). Scanning electron microscopy examination of AFM or STM tips show that they are rounded, with about a 50-nm radius as shown schematically in Fig. 3. Presumably, at least a few atomic-scale projections extend irregularly from this

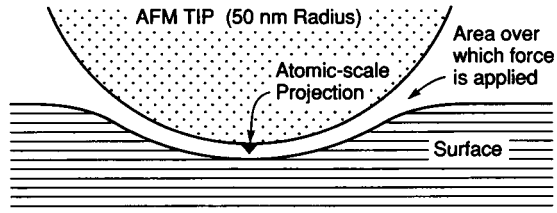

FIGURE 3 Schematic diagram of tip-surface interaction in the AFM. The AFM tip is rounded with a radius of order 50 nm. At the atomic level, small projections of atomic dimensions likely protrude from this rounded tip. When the tip is pushed into the surface, the surface smoothly deforms to follow the tip and the applied force is distributed over an area much larger than that of the atomic scale projection. Hence, the local pressures do not reach unacceptable levels. However, as the surface is homogeneous over the length scale of the tip radius (50 nm), the net interaction between the bulk of the tip and the surface is essentially constant as the tip is rastered across the surface. The AFM image is likely formed by the more localized variations in interactions between the atomic projection and the substrate. (The surface deformation and the tip roundness are exaggerated for clarity.)

generally rounded tip, otherwise resolution would be closer to the tip radius than the 0.4 nm resolution apparent in Fig. 2. When the tip is pushed into the LB film covered substrate, the surface deforms, but most likely smoothly over some fraction of the tip as shown in Fig. 3, rather than only around the atomic scale projection (the surface deformation is exaggerated for clarity). This smooth deformation will absorb much of the applied force of the AFM cantilever and distribute the force over a relatively large area of several tens or hundreds of square nanometers. The smoothly varying force over the bulk of the tip should not change much as the AFM tip is rastered across the LB film, which is homogeneous over the length scale of the tip radius. However, the local interactions probed by the atomic scale projections do change significantly as the tip is rastered across the specimen surface, and it is these changes that likely dominate the AFM image. The strong interactions between the headgroups of the DMPE molecules in aqueous solution act as a restoring force to keep the tip from puncturing the bilayer entirely.

CONCLUSIONS

High resolution imaging of biomaterials has always involved some type of compromise (high vacuum, chemical treatments, metal coating, drying, etc.) necessary to make the material to be imaged compatible with the limitations of the imaging technique (Zasadzinski, 1989). The AFM, which can image surfaces at the temperature,

state of hydration, and ionic strength at which the material is biologically active, presents these materials with the fewest compromises and with the greatest possible resolution. The Langmuir-Blodgett technique, coupled to the AFM, should make it possible to examine lipid and lipid/protein surfaces with unprecedented resolution in near physiological conditions in the near future (Weisenhorn et al., 1990). In addition, although we only have the suggestion of hexatic ordering at present, this technique provides a superb method of studying two-dimensional systems in real space.

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