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A REVIEW OF SCANNING TUNNELING MICROSCOPE and ATOMIC FORCE MICROSCOPE IMAGING OF LARGE BIOLOGICAL STRUCTURES: PROBLEMS AND PROSPECTS

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Abstract

The application of the scanning tunneling microscope (STM) and the atomic force microscope (AFM) to the study of small biological molecules, such as DNA and smaller molecules, has received considerable attention in the literature. This paper reviews STM and AFM studies of larger biological structures such as bacterial membranes, bacteriophages, viruses, antibodies, etc. The problems encountered in these applications are emphasized, with particular reference to the unknown conduction mechanism, tip-sample interaction forces, and tip-sample convolution artifacts in the images. The latter problem is illustrated by new results from IgG antibody complexes attached to a bacterial sheath layer. A new conduction mechanism involving a graphite film overlayer is suggested. The future prospects are discussed, with emphasis on the unique capabilities of these microscopes compared to conventional electron microscopes.

KEY WORDS: Scanning Tunneling Microscope, Atomic Force Microscope, Biological Structures, metal coated, uncoated, tip-sample convolution, conduction mechanism, tip-sample interaction.

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Introduction

The scanning tunneling microscope (STM), invented by Binnig and Rohrer (1982), was the forerunner of a class of scanning probe microscopes (SPMs) which produce three dimensional images of a surface by probing various physical quantities near the surface. The STM probes the electron tunneling current between a sharp conducting tip and the surface. A combination of electronic and mechanical feedback is used to keep the tip-sample distance constant at about 1 nm while the tip is scanned across the surface. The atomic force microscope (AFM), Binnig et al. (1986), uses a tip attached to a very weak cantilever, to probe the small force (10^{-10} N to 10^{-7} N) between the tip and sample surface. The STM and AFM are both capable of producing images at atomic resolution. Relevant reviews of the STM and AFM were given by Hansma et al. (1988) and Rugar and Hansma (1990). Other scanning probe microscopes make use of; magnetic forces, electrostatic forces, near field thermometry, near field optical effects, near field acoustic effects, chemical potential variations (Williams and Wickramasinghe, 1991), or ionic conductance (Hansma et al, 1989) . The current status and future trends of such scanning probe microscopes were reviewed recently by Wickramasinghe (1990). The photon scanning tunneling microscope, reported recently by Tsai et al. (1990) is a promising tool for biological studies with a resolution down to about 10 nm.

The STM has become a powerful and popular tool for in-vacuum, atomic scale studies of conducting and semi-conducting surfaces. It can also operate in air or liquids, Sonnenfeld and Hansma (1986), which makes it attractive for studying biological specimens in a more natural environment than can be achieved with conventional electron microscopes (EM). This feature and the availability of several commercial STM's have lead to a recent increase of applications in the biological area.

The atomic force microscope (AFM), also called the scanning force microscope (SFM),

does not require the sample to be electrically conducting. Since most biological specimens are insulating, the AFM is potentially more useful than STM for biological studies, and a number of such studies have already been reported in the literature. The AFM is particularily well suited for studies in liquid environments both because electrical leakage is not a problem and because the liquid reduces the capillary force problem present when operating an AFM in air.

This paper reviews the results which have been obtained from STM and AFM studies of larger biological structures, with particular emphasis on the problems encountered and the future prospects. The review includes: bacterial sheaths or s-layers, bacteriophages, viruses, antibodies, antibody-host interaction, microtubules, and enzymes. We also present some new results on antibodies attached to bacterial sheath. Previous reviews were given by Edstrom et al. (1990a), Fisher (1989), Salmeron et al. (1990), Travaglini et al. (1990), Fisher et al. (1990a), and Baumeister and Zeitler (1991).

We omit the more numerous results on smaller bio-structures such as DNA, amino acids, nucleic acids, proteins and other biomolecules. These are reviewed by Allison and by Edstrom in this issue. We also omit the STM and AFM studies of Langmuir-Blodgett lipid layers.

One important goal of STM and AFM studies of biological structures is to image their surfaces in their natural state at atomic resolution. This goal is still elusive, although some worthwhile results have been obtained. Another important application may be the use of the STM or AFM to manipulate biological structures or objects on their surfaces, such as imbedding objects in membranes or pores and measuring binding strengths. The field is in its infancy and there will undoubtably be many other applications.

STM of Metal Coated Specimens.

Most biomaterials have a low electrical conductivity and must be given a conductive coating before reliable STM can be performed. This is especially true for large biological structures, and the most believable of STM results on such structures have been obtained in this way. Film roughness limits the resolution of the imaging, but in some cases the coated material has given better resolution than either STM or AFM on bare material. Another objection to coating is the unnatural vacuum-conditions to which the specimens must be subjected during the preparation procedure, as for conventional EM. For these reasons most STM studies of biological material have tried to avoid the use of coated specimens. However, coated samples are a useful complement to STM and AFM of bare biological material, and perhaps should be a mandatory part of any STM study.

The most extensive and successful STM studies of coated biological materials used freeze dried specimens coated with Pt/Ir/C films. This technique was first reported for DNA by Amrein et al. (1988), and more recently for the bacteriophage T4 type III polyheads, Amrein et al. (1989a). Wepf et al. (1991) reported a detailed study of how varying the relative amounts of platinum, iridium, and carbon in the films effects resolution and grain size. They used films of 1-2 nm thickness and were able to achieve a lateral resolution of 1.5 nm on T4 polyheads and on HPI-layer. HPI is a natural two-dimensional crystalline protein layer covering the surface of the bacterium Deinococcus radiodurans. The hexagonal symmetry structure of both these layers was readily observable, with lattice constants of 13 and 18 nm for T4 polyheads and HPI layer, respectively. They found that Pt/Ir/C films containing at least 25% carbon were three-dimensionally stable on the freeze dried macromolecular specimens even after exposure to room conditions. This technique shows considerable promise. Amrein et al. (1991) and Wepf and Amrein (1992) discuss comparative studies of STM versus SEM and TEM on Pt/Ir/C coated HPI layer and T4 polyheads. Amrein and Gross (1992) discuss the details of preparing freeze-dried, metal-coated specimens for STM imaging. Fisher (1989) also discussed the freeze-fracture technique in relation to STM.

Guckenberger et al. (1989a) reported a successful STM study of Pt/C coated HPI-layer and also of type IV collagen, although the resolution was not as good as for the Pt/Ir/C coating described above.

Our STM group has extensively studied the outer sheath layer, or S-layer, of the bacterium methanospirillum Hungatei (mH), using both Au and Pt coated specimens on highly ordered pyrolytic graphite (HOPG) substrates, Blackford et al. (1988,1989). The grain size of these coating films was larger than that for the Pt/Ir/C films discussed above, which limited the resolution to 2-3 nm. The Pt films gave considerably better resolution than the more diffusive Au films. The corrugations expected from EM studies at multiples of 3 nm running perpendicular to the sheath axis could be resolved easily. However, the rectangular symmetry structure (lattice constants 3nm by 6nm) expected on the basis of electron diffraction studies, Stewart et al. (1985), has not yet been seen in the STM images, nor has it been detected in conventional scanning electron microscopy (SEM) or transmission electron microscopy (TEM), Sprott et al. (1986). Note that this lattice structure is much smaller than for the T4 polyheads or the HPI layer discussed above.

We have also imaged the individual hoops (carbon coated) which comprise the mH sheath tubular structure, Blackford et al. (1991) and Beveridge et al. (1990). These images confirmed that the hoop heights come in multiples of the basic 3 nm corrugation of the

sheath tubular structure and also confirmed the 9 nm wall thickness of the sheath tube. The images also showed interesting three-dimensional contortions in cases where several hoops overlapped, proving the high degree of flexibility of the mH sheath material. Such information is not easily obtainable from conventional EM.

Keller et al. (1990) reported convincing STM images of Pt/C coated bacteriophage T7 and DNA strands on HOPG substrates, with a resolution of about 5 nm. Emch et al. (1990) reported STM images of the macromolecule fibronectin deposited on mica and coated with a Pt/C film. Again the resolution was about 5 nm.

Thickness Measurements.

Thickness measurements of biological structures are directly obtainable from STM images of coated samples. This is because an STM in constant current mode monitors the z-position of the tip, which follows the surface profile of the sample, thus giving the surface topology. Such thickness data can be a valuable complement to EM thickness data which, by comparison to STM, are imprecise and difficult to obtain. The metal coating is necessary since STM thicknesses from uncoated specimens are usually much smaller than expected. The latter is thought to be due to elastic deformation caused by tip-specimen interaction forces. This is discussed in more detail in the section on uncoated specimens.

Several groups have reported STM thickness measurements on purple membrane (PM). Fisher et al. (1990b) studied nitrogen-dried (or freeze-etched) and Pt/C coated PM on mica and glass substrates, respectively. They found good agreement between STM and EM thickness measurements done on the same samples. There were no significant differences in the measured PM thicknesses due to the mica or glass substrates, except for the roughness of the glass surface. They also measured the small changes in membrane thickness after papain treatment, demonstrating the utility of STM for detecting such small changes. Wang et al. (1990) have also done detailed STM thickness studies of Pt/C coated purple membrane. The PM thickness values showed very little variation with the substrate and preparation method. However the less densely packed HPI layer, which they also studied, gave thickness values which were strongly influenced by those parameters.

We have done thickness measurements of the sheath layer of methanospirillum Hungatei (mH) coated with gold or platinum and on HOPG substrates, Blackford et al. (1988,1989). The Pt coated specimens gave the best STM images and thickness values which were about 20% less than previously reported in the literature from EM studies. The Au coated specimens gave even smaller thicknesses.

A possible source of error in thickness measurements on coated specimens is that the metal film thickness on the substrate may differ from that on the specimen due to penetration of the film into the softer, more porous specimen. Some evidence for this comes from the observation that the metal film is often smoother on the specimen than on the substrate.

STM imaging of metallic replicas of biological structures is an interesting technique which has received very little attention so far. Zasadzinski et al. (1988) studied freeze-fracture replicas of DMPC bilayers. They could easily see the ripple phase and measure the period (13 nm) and amplitude (4.5 nm) with a lateral resolution of about 3 nm. Hansma et al. (1988) also discusses such studies. Blackford and Jericho (1991) reported a different technique in which the deposited specimen is overcoated with a thick metallic film, which is then peeled from the substrate. The underside of the substrate was scanned by the STM to reveal indentation-type replicas. The technique was tried on mH sheath and gave a lateral resolution of about 3 nm, which was similar to that for metal coated sheath specimens, Blackford et al. (1989). STM of metallic replicas shows some promise and deserves further study.

STM of Uncoated Specimens.

Despite the poor conductivity problem mentioned above, some STM results on uncoated biostructures have been reported. However, the conduction/contrast mechanism is still not understood even though many mechanisms have been proposed. In fact there is no totally clear evidence that either conduction, or tunneling, actually occurs through the biological material. This is especially true since possible HOPG artifacts make the many results on HOPG substrates suspect, Clemmer and Beebe (1991,1992) and Wepf and Amrein (1992). The mysterious conduction mechanism may not exist, which we discuss further in the "Problems" section. Keeping these misgivings in mind we briefly mention below some of the STM results on bare specimens.

Baro et al. (1985,1986) reported the first STM results on biological material using the virus bacteriophage ϕ29. They were able to observe several characteristic features which seemed to correlate with TEM results.

Dahn et al. (1988) reported images of fragments of mH bacterial sheath in which corrugations with the expected 3 nm minimum period could be resolved. The imaging was usually done in air, but was also tried successfully in water. We have not succeeded in imaging the complete mH sheath in the uncoated condition using STM.

Hameroff et al. (1990) studied microtubules of cytoskeletal proteins and again the expected shapes and sizes were found. In a continuation of that work Vernetti et al. (1991) found improved STM resolution by prolonged exposure of the proteins to cold temperatures prior to imaging.

Edstrom et al. (1989 and 1990a,b) and Elings et al. (1990) reported STM studies of phosphorylase kinase macro-molecules and were able to see individual molecules as well as chains of dimers. The heights of the molecules in the STM images were only about 30% of the true height, which is often a problem with STM imaging of bare biological specimens.

The above four studies all used HOPG as the substrate. Other substrates have also been used. Mantovani et al. (1989) studied the tobacco mosaic virus TM on evaporated and on sputter-coated palladium/gold substrates. They found that the virus imaged as a hump on the evaporated Pd/Au substrate but imaged as a depression on the sputter-coated Pd/Au substrates. They reasonably interpreted the humps as due to the virus getting under the thin (20 nm) evaporated Pd/Au film. The virus could not penetrate the sputtered film, and imaged as an apparent depression due to a combination of poor conductivity and elastic effects.

Guckenberger et al. (1989b) reported convincing STM images of uncoated HPI layer on Pt/C coated glass substrates. Extreme voltage and current conditions were required ($V \geq 5v$ and $I \leq .5$ pA) and it was also necessary to control the specimen hydration via humidity. Under such conditions it is unlikely that conduction was by tunneling. The resolution was similar to that for Pt/C coated HPI layer but not as good as the resolution obtained by Wepf et al. (1991) for Pt/Ir/C coating. Wang et al. (1990) did STM thickness measurements on uncoated HPI layers and found values of about 4 nm, in good agreement with those for coated HPI. More recently, Guckenberger et al. (1991) succeeded in imaging uncoated purple membrane PM also using the same extreme voltage and current values. However, the images sometimes showed negative contrast as well as positive contrast, similar to the findings of Mantovani et al. (1989) for the TM virus mentioned above. In the PM case however, the contrast was found to depend on the sharpness of the tip and Guckenberger et al. (1991) proposed a different explanation which takes the tip shape into account.

A common problem for STM imaging of bare biological material is that the specimens often do not bond well to the substrate. As a result, the lateral force of the tip pushes the specimen along the substrate and no image is obtained. One approach to avoid this problem has been to try alternative substrates to HOPG, which may produce better bonding. Epitaxial Au films on mica, Chidsey et al. (1988) and Putnam et al. (1989) for example, have been used by various groups with some success. Flame-grown Au or Pt ball crystals have also been used, Lindsay and Barris (1988b) and Schneir et al. (1988) for example. Wilson et al. (1991) used chemically modified Si surfaces. Akari et al. (1988) reported an STM study of the semiconductor WSe_2, which cleaves easily to give atomically flat surfaces. They suggested that it may be a good substrate for biological specimens, especially for higher tip biases. In a following study Fuchs et al. (1990) used WSe_2 as a substrate for Langmuir-Blodgett monolayer films. We have found that $TaSe_2$ is also a useful substrate.

An alternative means to avoid specimen movement was reported by Jericho et al. (1989) who developed the "hopping" mode for the STM in which the tip is periodically withdrawn and reapproached during scanning, to prevent the lateral stress build-up. It was possible to move deposits around on the surface using the normal STM mode and then re-image them with the hopping mode. Successful applications of the hopping technique were reported on fragments of bare mH sheath, Jericho et al. (1990) and Beveridge et al. (1990), and on bare pepsin molecules, Jericho et al. (1990). The hopping technique does not help with the problem of poor conductivity of the specimen. The conduction mechanism was not known in these applications. We have not succeeded to image a complete mH sheath in the bare state, nor individual hoops of the bare sheath, both of which are thicker than the sheath fragments or pepsin molecules. The complete sheath often imaged as depressions, i.e. negative contrast, which is consistent with poor conductivity and the tip pushing into the sheath.

Vold (1992) reported a comparative study of the stability of adsorbates imaged with and without the STM hopping technique. He found that the hopping technique gave better images in most cases.

We recently tried the hopping technique in our efforts to obtain STM images of bare DNA on HOPG. The hopping technique compared favorably to the normal STM mode and some DNA-like structures were seen, but no truly convincing images were found. The hopping technique needs more application before definite conclusions can be reached about its usefulness.

AFM of Uncoated Specimens

The atomic force microscope does not require the specimen or substrate to be conducting, but the specimen must be adequately bound to the substrate to withstand the force of the tip. Also, the soft biological material may be deformed by the tip, causing poor resolution. Although excellent resolution has been reported for flat surfaces and small molecular structures, Egger et al. (1990) for example, the resolution on large biological structures has been poor so far. The resolution is particularily poor for individual biological specimens which do not form a regular crystalline structure.

Worcester et al. (1988) were the first to apply the AFM to a study of the purple membrane PM layer. They were barely able to resolve some features forming a 6 nm hexagonal lattice, which they identified with the cytoplasmic surfaces of trimers of bacteriophodopsin molecules. Butt et al. (1990) used

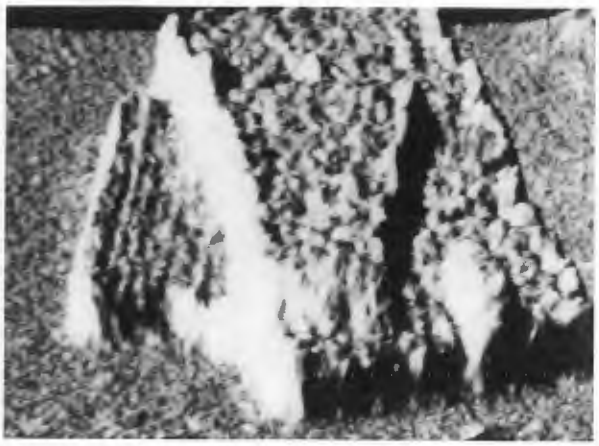

Fig.1. AFM image of two uncoated overlapping French-pressed fragments of the sheath layer of methanospirillum Hungatei (mH) on a mica substrate. The upper sheath fragment measures ≃ 320 x 480 nm² and the smallest corrugation that could be resolved on the lower fragment was ≃ 18 nm. In the upper fragment the outer sheath surface has been imaged while the lower fragment shows the smoother inner sheath surface. Scanning speed: ≃100 nm/s. Scanning force: ≃ 3 x 10^{-9} N.

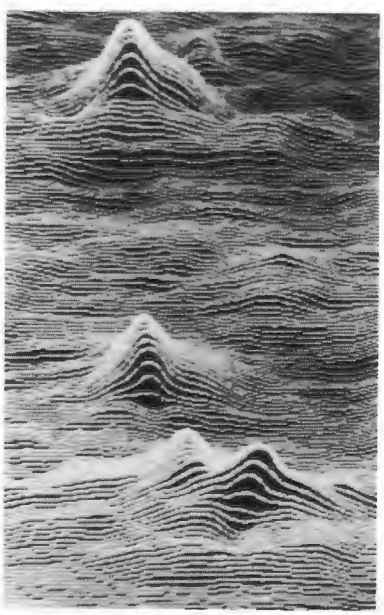

Fig.2. AFM image of four gold-labeled monoclonal IgG antibodies attached to the surface of an mH sheath on a mica substrate. In the image a gold-antibody complex is ≃ 15 nm high and several tens of nm wide. Some of the smaller (≃ 4 nm high) bumps in the image are thought to represent unlabeled antibodies.

the AFM to study purple membrane in a buffer solution. They obtained clearer images of the hexagonal symmetry, especially after strong filtering of the original data and unit cell averaging. They also observed ferritin, a ball shaped protein (12 nm diameter), bound to the surface of the purple membrane, suggesting the possibility of antibody attachment studies.

Weisenhorn et al. (1990) studied actin filaments on mica in a buffer solution. They could resolve monomeric subunits on individual filaments and also manipulate the filaments with the tip.

Edstrom et al. (1990a) used the AFM as well as the STM to study phosphorylase kinase macro-molecules on HOPG. The resolution was much worse for the AFM images, which presumably was due to inadequate sharpness of the AFM tip. The lack of suitably sharp AFM tips is still an important problem at this time.

Wiegräbe et al. (1991) used an AFM operating in the dynamic mode and in the attractive force regime to study the hydrated HPI layer in air. Forces as small as 10^{-12} N could be achieved with this method, but the best resolution was achieved with higher forces of about 10^{-8} N. The resolution was not as good as had been obtained previously from STM of coated HPI layer, Wepf et al. (1991), or STM of uncoated HPI layer, Guckenberger et al. (1989a).

We developed an AFM, Mulhern et al. (1991a), based on our bimorph-design STM, Blackford et al. (1987), with an optic-fibre-interference displacement sensor for the detection of the lever motion, Breen et al. (1990). AFM images of complete mH sheath on mica substrates were easily obtained, Mulhern et al. (1991b). Figure 1 shows an example of images of two partially overlapping sheath fragments. The lower one shows corrugation lines approximately 18 nm apart but is otherwise smooth, while the upper fragment has a much rougher appearance. Examination of many such fragments, as well as of complete sheath, suggested that the rougher surface is the outside of the sheath, while the smoother surface belongs to the inside part of the sheath.

We have also recently studied gold labeled IgG monoclonal antibodies attached to mH sheath as shown in Fig.(2). In this study nominally 10 nm diameter gold particles coated with protein-A molecules (≃ 5 nm diameter) were attached to the FC portion of the IgG antibodies on the sheath. (For further details see Mulhern et al, 1992). Figure 2 shows several antibody-gold complexes and possibly also a number of unlabeled antibodies. The individual complexes appeared broad and showed no structural details. The poor resolution is thought to be due to the large cone angle of the Si_3N_4 tips used, even though they were among the best tips available commercially, at the time, and could achieve atomic resolution on flat mica or HOPG. This is a serious problem

for imaging 3-D objects, which is discussed further in the section on "Tip-Sample Convolution Artifacts".

Problems

Unknown Conduction/Contrast Mechanism, STM

Most biological materials are good insulators and should not be imagible by STM unless they are thin enough (≤2 nm) to allow tunneling through the deposit to the substrate. The fact that many STM images of thicker bare biological specimens have been reported has lead to a number of proposed explanations for the apparent conduction mechanism: (a) surface conduction; (b) resonant state tunneling effects, Lindsay et al. (1990); (c) through bond tunneling; (d) surface charge relaxation effects, Heckl and Smith (1991). In addition there are some contrast mechanisms which do not require conduction: (e) variations in workfunctions Φ produced by the deposit, assuming it is thin enough to tunnel through; and (f) various tip-sample elastic interaction effects.

We believe that the evidence for conduction in biological structures is not totally convincing, especially since most of the reported images were obtained using HOPG as the substrate. It is well known that images on bare HOPG can sometimes look deceptively like various biological structures. Recently this was demonstrated dramatically by the DNA-like images reported by Clemmer and Beebe (1991) on bare HOPG, which casts doubt on the numerous DNA studies on HOPG. Wepf and Amrein (1992) and Heckl and Binnig (1992) have also reported various artifacts on HOPG.

We would like to suggest an additional conduction mechanism which does not require conduction through the biological structure. It applies to HOPG substrates and is a form of metal coating in which the specimen becomes covered by a thin graphite film conforming to the specimen shape. Tunneling occurs from the tip to the graphite film, which forms a conducting path to the substrate, Fig(3). The scanning tip itself is thought to be the means of getting the graphite film onto the specimen. There is ample opportunity for the tip to pick up a flake of graphite and drape it over the specimen, since it is usually necessary to make many scans before seeing any biological structures.

On the other hand, evidence for conduction through organic films was reported recently by Specht et al. (1991). They used a combined STM/AFM setup to simultaneously measure tunneling current and force as a function of position of the tunneling tip as it approaches and penetrates an organic film on a solid substrate. At tip voltages of about 1 V they could measure tunneling currents at thicknesses up to about 3 nm, while at 6 V currents could be detected up to several times 10 nm. The latter observation

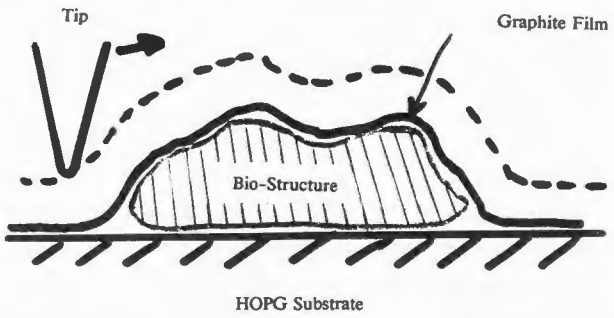

Fig.3. A proposed conduction/imaging mechanism in which a thin layer of graphite covers the biological structure, forming a conducting path to the HOPG substrate.

may be relevant to the results of Guckenberger et al. (1989b) who imaged bare HPI layer at similarily high voltages.

Moisture is always present on, or in, the specimens when operating an STM in air. It may play an important role in the conduction mechanism for all cases involving uncoated specimens. For example, it was very difficult to explain the STM imaging in air of thick (≃100 nm) insulating alkanes by Michel et al. (1989). However, the same alkanes could not be imaged under vacuum conditions, Dürig, 1991 (IBM Research Division, Zurich, Private Communication). One possible explanation is a surface conduction mechanism associated with a water, or contamination layer, but further experiments are needed to resolve this question.

Biological specimens are often deposited on the substrate with salt buffers in the solution, and in some cases successful STM images could only be obtained soon after deposition while moisture was still present. Amrein et al. (1989b) reported this for bare DNA-like structures and we also found it for pepsin molecules imaged with the STM hopping technique, Jericho et al. (1990).

The lack of understanding of the conduction mechanism has been a major drawback to the application of STM to uncoated biological specimens because it makes image interpretation very difficult. More experiments designed to investigate the conduction mechanism under a variety of conditions, such as those of Specht et al. (1991) and Lindsay et al. (1991), are needed.

Tip-Sample Interaction Forces

There are unavoidable forces between the tip and sample with both the STM and AFM. The force is intrinsic to position sensing with the AFM, and for an STM there is always a force-producing contamination layer when operating in air. If the forces are too large the specimen may be dislodged or damaged. Smaller forces may still deform the soft biological material and cause poor resolution.

The tunneling gap resistance of an STM provides a measure of the tip-sample force.

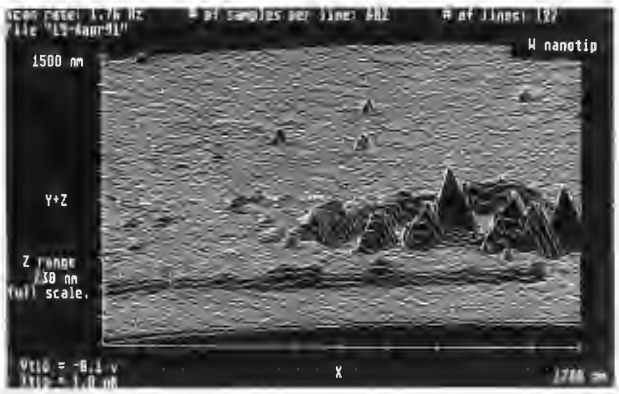

Fig.4. STM image of a carbon coated mH sheath which was damaged by previous imaging at a gap resistance of 100 MΩ. The carbon coating was ≃4 nm thick.

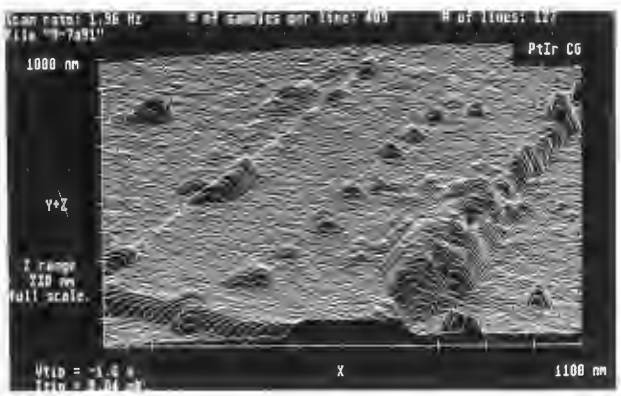

Fig.5a. An STM image of carbon-coated gold-labeled IgG antibodies attached to an mH sheath. The carbon film thickness was ≃ 4 nm.

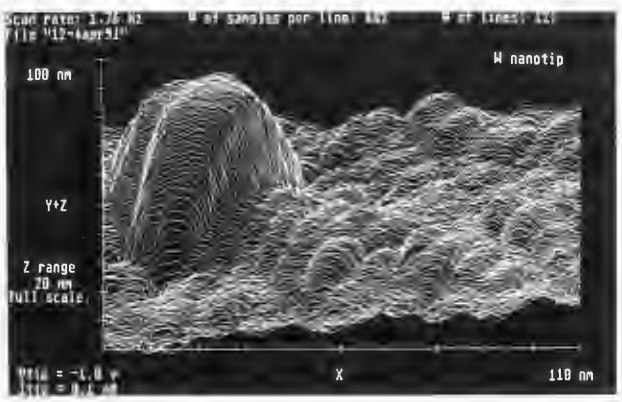

Fig.5b. A magnified view of a single antibody-gold complex imaged with an etched tungsten Nanotip. The structural detail is tip dependent, which can be seen by comparison with the image in (c) obtained with a different tip.

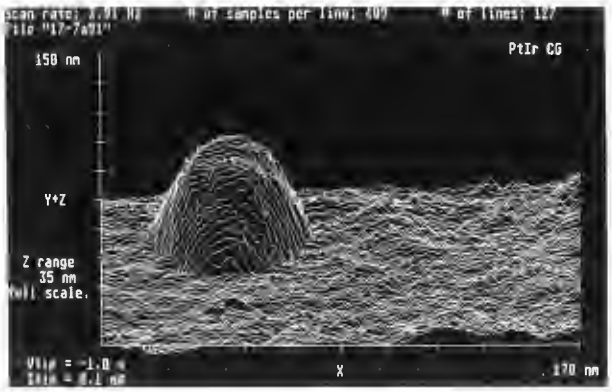

Fig.5c. A magnified view of a single antibody-gold complex imaged with an etched platinum-iridium Controlled Geometry tip.

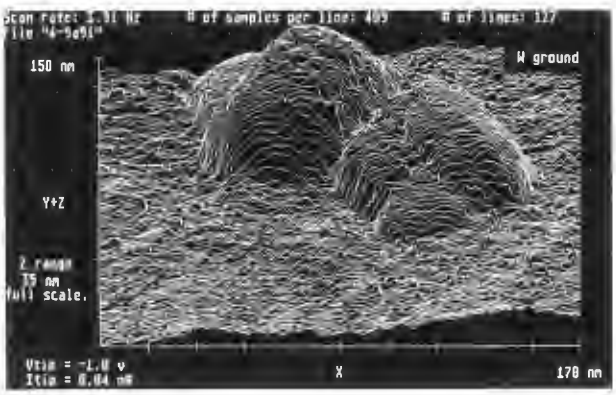

Fig.5d. A triple image of a single antibody-gold complex taken with a ground tungsten tip having three active microtips.

Salmeron et al. (1990) found that considerable damage was done to a nickel surface at 68 MΩ gap resistance and virtually no damage at 28 GΩ. Higher gap values must be used for soft biological deposits. Wilson et al. (1991) found that a layer of tRNA on oxidized silicon was dislodged at 300 GΩ, whereas it could be imaged at 900 GΩ. The conductivity of the specimen also effects the force, since poor conductivity requires a smaller tip-sample spacing for a given gap resistance.

For the AFM, the normal tip force is measured directly from the cantilever deflection. Associated with the normal force is a horizontal force which depends on both the tip and specimen shape. Weisenhorn et al. (1990) found in an AFM study of actin filaments on mica that the actin was dislodged when the normal force exceeded about 5 nN. We found in our AFM studies of mH sheath on mica that small fragments and individual hoops must be imaged at much less force than could be used with the more massive complete sheath. The horizontal force can be used to

advantage for measuring binding strengths, as reported recently by Mulhern et al. (1992) for IgG antibodies attached to mH sheath membrane.

The force of an STM tip can be measured directly by using a combined STM/AFM system. The deflection of a conducting cantilever, with a conducting tip, can be monitored during an STM scan. Salmeron et al. (1991) reported such a study on HOPG and found that the forces at typical gap resistances used in STM can have significant effects. At times the forces in STM imaging were found to be larger than in AFM imaging. These authors give an extensive general discussion of elastic/plastic deformation effects in STM/AFM. The study of Specht et al. (1991) also used a combined STM/AFM setup.

The STM tip force can not be ignored even when imaging metal coated biological specimens. The best possible resolution is obtained with thin coatings, but such films are very delicate. As an example of this, Fig.4 shows the result of trying to image a carbon coated mH sheath at a gap resistance of 100 MΩ. The carbon layer and sheath were badly damaged and dislodged on the right hand side of the sheath. Previous to this image, the same sheath had been imaged several times at 10 GΩ gap resistance without any damage. Also, subsequent images at this higher value did not cause further damage.

Tip-Sample Convolution Artifacts

An ideal STM or AFM tip has only a single atom at its apex. However, such a tip is ideal only for imaging atomically flat surfaces. If the surface has 3-D structure, then the image becomes a convolution of the 3-D shape of the sample and tip. It is easy to register the symmetry structure on a flat crystalline surface, such as HOPG, even with a relatively blunt tip. However, the apparent shape of the units (atoms in the case of HOPG) that make up the pattern in such images is usually not a reliable indicator of their true shape. This becomes a serious problem for imaging individual biological structures whose aspect ratio is similar to, or greater than, that of the tip. Also, the tip may consist of several microtips which leads to multiple image effects. These problems have been known since the beginning of STM and have been discussed by a number of authors (Amrein et al. (1989a), Fisher et al. (1990a), for example) but need to be emphasized.

Figure 5 shows some recent examples of tip related problems from STM images of coated, gold-labeled IgG antibodies on mH sheath. These are the same type of antibody as used in the AFM studies previously described in the section on "AFM of Uncoated Samples". These antibody complexes are a rigorous test for the sharpness of STM tips. Figure 5a shows an STM image of carbon-coated, gold-labeled IgG antibodies attached to an mH sheath. The measured diameters are about 50 nm, which is much larger than the expected ≃30 nm, allowing for the combined thickness of the protein-A and carbon film coatings. This can be attributed to the blunt cone-like shape of the tip. Also, all antibody-complexes in a given image showed identical detailed structure. It was tempting to attribute this structural detail to the antibody-complex but it was found to be strongly influenced by the shape of the tip used. Commercially prepared etched tungsten Nanotips (Digital Instruments, Santa Barbara, CA) usually gave ellipsoidally shaped bumps with grooves along the long axis, as shown in Fig. 5b. On the other hand, commercially etched PtIr tips of the Controlled Geometry type (Materials Analytical Services, Raleigh, NC) gave a more realistic semi-spherical shape, as shown in Fig. 5c. The effect of tip shape is further demonstrated in Fig.5d which shows a triple image of a single antibody-complex taken with a mechanically ground tungsten tip having three active microtips. The structural detail is different in each image, which must be due to the tip. All antibody complexes imaged with this tip produced the same triple image as in Fig. 5d.

The lack of tip sharpness is even more of a problem for an AFM, since the tips on the best available AFM cantilevers are not yet as sharp as STM tips. This is thought to be responsible for the rather poor resolution obtained so far with the AFM on individual biological structures. Figure 2 shows an AFM image of the same IgG gold labeled antibodies as in Fig.5 and it is clear that the resolution is not as good. Also it is not possible to sharpen insulating Si_3N_4 AFM tips in-situ, which can be done with STM tips by applying high bias voltage to make carbon whiskers grow on the tip, Tiedje et al. (1988) or Amrein and Gross (1992), for example. In this regard it would be useful to have conducting AFM tips/cantilevers, and the Si ones recently developed by Nonnenmacher et al. (1991) and Wolter et al. (1992) may be suitable for this purpose. Keller and Chung (1992) recently reported that a sharp carbon whisker can be grown on a Si_3N_4 tip by placing it in the beam of a scanning electron microscope, which is similar to the procedure of Ichihashi and Matsui (1988). The resulting tip/whisker gave dramatically improved resolution and appears to be an important advance for AFM imaging of biological structures.

The imaging of individual hoops of mH sheath is also a severe test of tip sharpness, as reported by Blackford et al. (1991) for STM of coated hoops. Recently we imaged bare hoop material with the AFM and found much poorer resolution than for the STM images of coated hoops.

Prospects

It may be possible to further improve on tip shape and sharpness to reduce tip-sample

convolution artifacts in the images. However this approach is limited due to the floppiness of ultra-sharp tips, Hanrieder et al. (1992) for example, and it is likely that such tip problems will ultimately limit the usefulness of STM and AFM for topographical studies of three dimensional biostructures.

Further improvements of conductive coatings to reduce coating artifacts in the image may also be possible. The 1.5 nm resolution achieved by Wepf et al. (1991) is already very impressive. Most studies of bare material would benefit from complimentary studies on coated material since the latter are generally more reliable and easier to interpret.

Low temperature measurements to improve resolution by increasing the rigidity of the biological structures is another possibility. Vacuum drying would probably be needed to prevent ice from forming on the surface and masking detail, and/or damage to the specimen by freezing of absorbed water. Some preliminary work of this nature has been attempted by Hansma et al. (1991) but it is not yet certain how useful the technique will be.

The possibility of AFM and STM studies of biological structures in their natural liquid environments remains an attractive feature of these microscopies. Considerable work of this type has already been done on smaller organic species, Lindsay et al. (1988a,b) and Drake et al. (1989) for example. For large biological structures, it is expected that adhesion to the substrate may be a problem. Also, the structures are much softer in their hydrated state, which may aggrevate the resolution problem. However, Dahn et al. (1992) recently reported STM imaging of complete chloroplasts in solution and were able to obtain molecular scale resolution ($\leq$3 nm) on the outer membrane. In air the uncoated chloroplasts could not be imaged successfully. Recently Häberle et al. (1991) have developed an AFM set-up for holding and investigating living cells in water which shows promising results and permits the possibility of studying nanoscale biological processes in real time.

Studies of antibody-membrane interactions to determine the nature of the attachment points and the bond strengths, Mulhern et al. (1992), seems to be an area where the STM/AFM techniques will also have an advantage over electron microscopy.

The use of spectroscopic variations (eg. I vs V or dI/dV vs V) as an image contrast mechanism for detecting and identifying biological structures should become a routine compliment to topographic scans. Some recent studies of this nature were reported by Hörber et al. (1991), Allison et al. (1990) and Lindsay et al. (1991), for example.

Scanning probe microscopes have opened an entirely new field of study in biology. They should not be viewed as a replacement for conventional electron microscopes but rather as a complimentary tool with unique capabilities for doing interesting and worthwhile biological applications on the nanometer scale.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.