SCANNING TUNNELING MICROSCOPY OF FREEZE FRACTURE
REPLICAS OF BIOMATERIALS

John T. Woodward\textsuperscript{1} and Joseph A. Zasadzinski\textsuperscript{2}

\textsuperscript{1}Department of Physics
\textsuperscript{2}Department of Chemical and Nuclear Engineering
University of California, Santa Barbara
Santa Barbara, California 93106

Abstract: Under ambient conditions STM measurements of feature heights on biological and other soft materials are often much larger than expected and vary from point to point on the surface. STM imaging of these samples is also accompanied by deformations of the surface that would not be expected from the conventional picture of noncontact STM imaging through a vacuum gap. To explain these observations we have developed a two spring model for the interaction between the tip and sample that suggests that a fluid meniscus couples the tip to the sample leading to large height amplifications and the possibility of damage to the surface. To test this theory we imaged platinum carbon replicas of cadmium arachidate multilayer Langmuir-Blodgett films under a dry nitrogen environment, exposed to humid air, and returned to a nitrogen environment. Feature heights increased significantly in the humid environment, but were reversible upon return to a dry nitrogen environment.

INTRODUCTION

With the advent of the scanning tunneling microscope (STM) in 1982 it became possible to image solid, conducting samples with atomic resolution.\textsuperscript{1} The subsequent invention of the atomic force microscope (AFM) made it possible to routinely image nonconducting surfaces as well.\textsuperscript{2} However, the local pressure applied by these probes to the sample surface during imaging has limited their usefulness in imaging soft samples. As many biological structures are too soft for direct imaging, we have modified the technique of freeze fracture replication for use with the STM.

Freeze fracture replication was originally developed as a way to observe structures with the transmission electron microscope (TEM) that could not tolerate the high vacuum inside the TEM. This includes many of the hydrated biological systems that at present are
incompatible with the scanning probe microscopes. The preparation consists of rapidly freezing the sample so as to lock in the structure or phase of interest. The sample is then fractured under high vacuum and a thin metal film is deposited on the surface. The original sample is then dissolved and the metal replica of the surface is observed in the microscope. For observation in the TEM, the metal film is deposited at an angle and structural information is obtained from the resultant shadowing. For the STM the metal is deposited vertically onto the sample in order to form a continuous metal replica of the surface for imaging.

It should be noted that this technique represents a sacrifice of what is often considered the prime motivation for using a scanning probe microscope: atomic resolution. At best we could expect each atom on the surface of the replica to have been in contact with the sample surface. In reality the replica is composed of tiny crystallites of about two nm in diameter. It is these crystallites that limit the resolution of STM on freeze fracture replicas. However, in return for our sacrifice of resolution we are able to image a class of samples that are otherwise incompatible with scanning probe microscopes. For many of these samples it is the macro-molecular structure, not the atomic scale detail, that is of interest.

We present here the techniques we have developed as a result of attempting to image two organic structures. The first is a reorganized Langmuir-Blodgett film of cadmium arachidate on mica. The reorganized films have regions that are 1, 3, 5 and 7 layers thick and have been well characterized using the AFM. As the thickness of these layers is well known from X-ray scattering they are ideal for calculating the x direction of scanning heads and determining the reproducibility of height measurements. In addition there are large (>10 μm²) flat areas between the steps that allow the smoothness of the replica to be assessed. The second sample is the ripple or Pₖ phase of dimyristoyl phosphatidylcholine (DMPC). While the ripple wavelength has been measured using X-ray scattering as well as freeze fracture TEM there are unresolved questions about the ripple amplitude, waveform, and the possibility of a smaller ripple running roughly perpendicular to the main ripple.

In our first attempts to image freeze fracture replicas with the STM we prepared the replicas in a manner similar to that used for the TEM. The replicas were 2.5 nm of platinum carbon (Pt/C) with a backing layer of 30 nm of carbon added for structural support. The replicas were mounted on 500 mesh electron microscope grids. While we were able to obtain many stable images of the ripple phase of DMPC we occasionally found images with ripple amplitudes that were several times the usual height and on a few occasions we obtained amplitudes over 100 times the typical value. Amplitudes could also change dramatically when the same area was imaged for several hours. We examined the possibility that the scanning tip might be deforming the replica, and found that if a liquid meniscus forms between the tip and replica it is possible for deformations to occur which could substantially enlarge the measured height of a surface feature. Since all of our imaging was done in air we consider it likely that a meniscus of water or other contaminant forms between the tip and sample.

We developed a model that suggests three main methods for reducing the amplifications. The first two of these are relatively simple and obvious: make the replica thicker and mount it on a substrate with smaller pores. The amplifications are reduced, but not eliminated, by adding a 100-200 nm backing of silver or gold to the replica and mounting on a fine mesh silver membrane with pores 1-3 μm in diameter. The third suggestion was to image in an inert environment. A liquid meniscus will not form between the tip and sample at a low relative humidity. To this end we built a chamber for the STM out of a belljar and a base plate that allows us to evacuate the chamber and backfill with nitrogen. Thus, we can routinely and easily image samples in an inert environment without the expense or maintenance required for an ultra high vacuum STM.

340
EXPERIMENTAL

To test whether we were able to eliminate the height amplifications, we wanted to use a sample of known height that we could replicate. We chose multilayer films of cadmium arachidate prepared by Langmuir-Blodgett deposition. By holding the films in the subphase prior to the deposition of the final monolayer the films reorganize creating a terraced surface with most levels differing by a bilayer or multiple bilayers in height. To calibrate the STM head we used both a three-layer film on mica with a half-hour wait between the second and third layers and a five-layer film with a half-hour wait between the fourth and fifth layers. Both were prepared as described in Ref. 4 and coated with Pt/C using the following procedure. The film was loaded into a Balzers BAF 400K/CL freeze fracture device and the chamber was evacuated to 10⁻⁷ mbar. We then deposited 0.8 nm of Pt/C at a 45° angle on the stationary sample and another 0.8 nm with the sample rotating. This guaranteed a good conducting path between layers and enough conductivity on the initially shadowed side to prevent tip crashes. The deposition was performed at room temperature.

We then imaged the Pt/C coated films with a Nanoscope II STM in air with several different cut platinum-iridium tips. Over 40 step heights were measured from 18 different images. The height was measured by taking bearing plots that straddled the steps using the Nanoscope software. The step height was taken to be the distance between peaks in the bearing plot with an uncertainty relative to the peak widths. The weighted mean was set equal to the known value for a bilayer step of 5.5 nm. Seventy percent of the measured values fell within eight percent of the mean. This spread of values is only slightly larger than the spread found using the AFM to measure the height of the steps directly.

We also made a replica of a five-layer film with half-hour wait between the fourth and fifth layers. The film was loaded into the Balzers freeze fracture unit and pumped down to 10⁻⁷ mbar and held at room temperature. The replica was made by depositing 2.0 nm of Pt/C with the sample rotating and then backing with 35 nm of carbon. An additional backing of 200 nm of silver was added in an evaporation chamber. The replica was removed by floating the sample on a 5% hydrofluoric acid solution overnight. The replica was then mounted on a silver membrane as described in Ref. 15. The replica was positioned in the STM and was placed under the belljar. The bell jar was pumped out using a mechanical pump with a cold trap on the line to about 0.1 torr for over an hour and backfilled with dry nitrogen. A total of 10 different images taken with three different tips gave 28 bilayer step heights. These measurements gave a weighted mean of 5.1 nm for a bilayer step, and once again 70 percent of the values fell within 8 percent of the mean. On this basis we find that there is no measurable amplification of height measurements made with the STM when imaging in an inert environment.

There has recently been some controversy over whether a meniscus that formed between a tip and sample that was covered by a continuous liquid film could apply a force to the sample. In order to demonstrate more clearly that liquid condensation is responsible for the deformations that we have observed we endeavored to change the environment in which the STM was operating while it scanned over the same object. We began by imaging the replica of the cadmium arachidate film in an inert environment as described above. A suitable feature was found and imaged for 15 to 30 minutes to ensure that it was stable. Air was then admitted to the chamber while imaging. After about 15 minutes, the chamber was flushed with nitrogen to determine if the changes observed in air were reversible.

We followed this procedure six times and found that three of the runs showed no amplification, two of the runs showed small amplifications (less than the 8% spread in height measurements), and one run showed some dramatic changes. This is entirely
consistent with our model which predicts amplifications only when imaging over a pore and no amplification when imaging over an area that is in contact with the silver membrane. Three images and cross-sections from the run that had large amplifications are shown in Figure 1. Figure 1(a) was taken after fifteen minutes of a thirty minute continuous scan under nitrogen. Figure 1(b) was taken less than five minutes after exposure to air. The island resembling Australia has increased in height by 17% and the difference between the highest and lowest points on the image has doubled. Figure 1(c) was taken less than five minutes after we began flushing the chamber with nitrogen. The height of the island is only 8.5% above its original value. The island returned to its original height after less than half an hour of flushing with nitrogen. We attribute these changes to the formation of a meniscus between the tip and sample due to condensation from the air and the subsequent evaporation of the condensation upon return to the nitrogen environment. We therefore feel that we are able reproducibly to image replicas with an STM by imaging in an inert environment. We can make height measurements on the order of 5 nm with an accuracy of better than 10%.

The limiting factor in resolution of images of replicas is now the graininess of the replica itself. The images in Figure 1 clearly show that the replica is composed of small crystallites. This replica was deposited at room temperature and has crystallites that are about 5 nm in diameter. Figure 2 is a replica of the ripple phase of DMPC that was prepared similarly to the replicas in Figure 1 except that the sample was at -170 °C when the replica was deposited. This method formed crystallites about 1-2 nm in diameter.

Several researchers have investigated the optimal conditions for depositing replicas of Pt/C as well as tantalum-tungsten (Ta/W) for imaging in the TEM. Ruben has shown that vertically deposited Pt/C films can be made with grains as small as 0.5 nm if the films are less than 1.0 nm thick. However, these films are generally not continuous as is required for the STM. Increasing the thickness to form a continuous film increases the grain size as well. Gross et al. have made continuous films of Pt/C that are essentially grainless, but this required a freeze fracture machine capable of obtaining temperatures of -260 °C and pressures less than 10^-9 mbar both of which are beyond the capabilities of standard freeze fracture systems.

ONGOING RESEARCH

We have begun to explore the use of amorphous or 'glassy' metals to try to produce continuous, grainless films with a standard liquid nitrogen cooled freeze fracture unit. Glass-forming metals are typically binary alloys that have a deep eutectic at which the melting point is substantially less than either of the pure components. When rapidly cooled from a vapor or liquid state, as they are when deposited onto a cooled substrate during freeze fracture replication, crystallization is inhibited and they retain the amorphous structure of the liquid state. One general class of glass-forming metals is alloys of approximately 80 atomic percent transition metal and 20 atomic percent silicon or phosphorus. For example, platinum-silicon has a eutectic near 78% Pt/22% Si and palladium-silicon near 84% Pd/16% Si. Our first attempt to make an amorphous film was with an 88% titanium/12% silicon alloy. However, we were not able to get reliably stable images with the STM. We suspect that this is due to the silicon oxidizing and forming an insulating layer on the surface. The alloy also gave poor contrast in the TEM, making it difficult to assess the continuity and crystallinity of the sample using that technique. We have therefore turned our attention to the higher atomic number alloys so that we may use the STM and TEM to help optimize the quality of freeze fracture replicas.
Figure 1. (a) An STM image taken under nitrogen of a replica of a reorganized cadmium arachidate film and the height profile along the dashed line. (b) The same area after less than five minutes exposure to air. The step heights have increased and additional features are visible. (c) The same area less than five minutes after being returned to a nitrogen environment. The amplification is reduced and the additional features have disappeared.
Figure 2. An STM image of a freeze fracture replica of a 30% DMPC/70% water solution at 21 °C. The image shows the long wavelength (22 nm) A conformation of the ripple phase. This phase has a characteristic 'M' shaped ripple conformation. The individual crystallites that compose the replica are clearly resolved. The shallow valleys along the top of each ripple, however, are somewhat obscured by the crystallites.

CONCLUSION

In conclusion, we have modified the method of producing and imaging freeze fracture replicas with the STM to enable accurate and reproducible imaging. Our experiments have shown that by making replicas with thicker backing films and imaging them in an inert environment we are able to eliminate distortions due to the formation of a fluid meniscus between the tip and sample. With these improvements the current limitation on the resolution of this technique is the quality of the replicating film itself, which is an area that we are actively exploring.

ACKNOWLEDGEMENTS

We would like to acknowledge Ravi Viswanathan for his preparation of the Langmuir-Blodgett films. We are also indebted to Paul Hansma, Jacob Israelachvili, and Suzi Steinberg for helpful discussions. This work was supported by a Whitaker Foundation Biomedical Engineering Grant, the Office of Naval Research grant #N00014-90-J-1551, and National Science Foundation grant #CTS9212790.

REFERENCES