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IMAGING OF METAL-COATED BIOLOGICAL SAMPLES BY SCANNING TUNNELING MICROSCOPY

Ricardo GARCIA

Departamento de Física de la Materia Condensada, C-III, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

David KELLER

Department of Chemistry and Department of Pathology, University of New Mexico, Albuquerque, New Mexico 87131, USA

John PANITZ

Department of Physics, University of New Mexico, Albuquerque, New Mexico 87131, USA

David G. BEAR

Department of Cell Biology, School of Medicine, University of New Mexico, Albuquerque, New Mexico 87131, USA

and

Carlos BUSTAMANTE *†

Department of Chemistry and Department of Pathology, University of New Mexico, Albuquerque, New Mexico 87131, USA

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A method for imaging biological samples by scanning tunneling microscopy (STM) is presented. There are two main difficulties in imaging biological samples by STM: (1) the low conductivity of biological material and (2) finding a method of reliably depositing the sample on a flat conducting surface. The first of these difficulties was solved by coating the samples with a thin film of platinum–carbon. The deposition problem was solved by a method similar to a procedure used to deposit biological molecules onto field ion microscope (FIM) tips. STM images of bacteriophage T7 and filamentous phage fd are shown. The substrate on which the samples were absorbed was atomically flat gold. The images do not show molecular detail due to the metal coating, but the gross dimensions and morphology are correct for each type of virus. Also, the surface density of virus particles increases and decreases in the way expected when the conditions of deposition are changed. These methods allow reliable and reproducible STM imaging of biological samples.

In the short time since its invention, the scanning tunneling microscope (STM) has become an important tool in surface science. The STM works by sweeping a very sharp conducting needle just a few ångströms above the surface of the sample and sensing the tiny tunneling current that flows between the sample and the needle tip [1]. The images the STM produces are three-dimensional topographs, with a lateral resolution often as good as 1–2 Å and a vertical resolution of less than one angstrom.

At present, most applications of the STM are in surface and materials science, where the samples are usually clean, solid surfaces in high vacuum. Recently, however, there has been widespread interest in applying the STM to biology [2–11]. The

* To whom correspondence should be addressed.
† 1984 Searle Scholar; 1985 Alfred P. Sloan Fellow.

high resolution of the STM and its ability to form images in aqueous environments offer the promise, ultimately, of imaging biological molecules to atomic or near-atomic resolution in their native environments. Three approaches have been used so far: (1) imaging of bare, uncoated and unstained samples adsorbed on conducting surfaces [3–7], (2) imaging of metal coated samples [8–10], and (3) imaging metal-replicas of biological samples [11]. The first approach has the best chance of yielding high resolution images, and this is the direction most efforts have taken. What has been found is that STM images of bare molecules can be obtained, but only with difficulty. Most reports are of single sightings, or of a few sightings in many attempts. The difficulty is undoubtedly due to the fact that most biological molecules are both thick and insulating, making it hard for the STM to get the current required to form an image. In fact, the mechanism by which these images are formed is not yet well understood.

Our goal is to develop a straightforward and reliable procedure for STM imaging of metal-coated biological samples. The metal coating limits resolution to the grain size of the metal layer, typically about 20–30 Å, but solves the problem of the low conductivity of biological matter and permits us to address independently the problem of sample deposition. Once biological STM images can be made routinely, it will be possible to systematically investigate ways of doing without the coating. Here we report STM images of two different viruses (bacteriophages T7 and fd) with very different morphologies. Images showing the gross morphology of each virus were obtained on a regular and routine basis, and we were able to show that the density of the particles on the surface increases and decreases as expected when the conditions of particle deposition are changed.

The method we used is an adaptation of the procedure of Panitz et al. [12] for depositing biological particles onto field-ion microscope (FIM) tips. As the substrate for the viruses, we used atomically flat Au(111) facets of gold. This substrate has also been used by Lindsay and Barris for their STM studies of DNA under water [13]. These surfaces were prepared by melting gold wire in an oxygen–acetylene flame, forming a molten sphere and allowing the sphere to resolidify. The flat Au(111) surfaces form spontaneously under these conditions. These surfaces are a nearly ideal substrate for our purposes because they are easy to prepare, highly conductive, highly adsorptive to biological samples, and extremely flat. In our experience, they are atomically flat, except for monoatomic steps, over areas of at least one square micron. This is important because a rough surface can hide the presence of the sample particles, especially when dealing with an unfamiliar sample system. The gold facets are smooth enough so that even single protein or nucleic acid molecules will be visible.

Our method uses the deposition device shown in fig. 1. Each gold sphere on which the sample is deposited is held in a threaded pin that is then inserted into the plexiglas cover. This pin is positioned so that each gold sphere is inside one of the small wire coils. Four 10 μl droplets of sample solution are placed on a piece of clean parafilm. Deposition of the sample is begun by placing the deposition device so that each sample droplet is pulled up into the coils by surface tension; in this way, the gold spheres are immersed in solution. The adsorption time and the concentration of the sample solution control the density of particles which deposit on the substrate surface. After deposition, the sample-coated spheres are first rinsed in freshly distilled water and then in 90% alcohol. The rinsing steps are done by placing the deposition device over the top of a small plastic cup filled with water or alcohol, immersing the coils and spheres. These steps remove excess salts that would otherwise obscure the sample. The primary purpose of the deposition device is to allow transfer of the samples from one solution to the next without taking the spheres through a water–air interface, where surface tension would damage the adsorbed sample particles. The wire coils do this by holding a small droplet around each gold sphere as it is moved from solution to solution. The alcohol step dehydrates the sample and provides a low surface tension medium from which to remove the samples after the last rinsing.

After the samples are removed from the deposition device, they are allowed to dry in air and are then coated with metal. The coating is done with

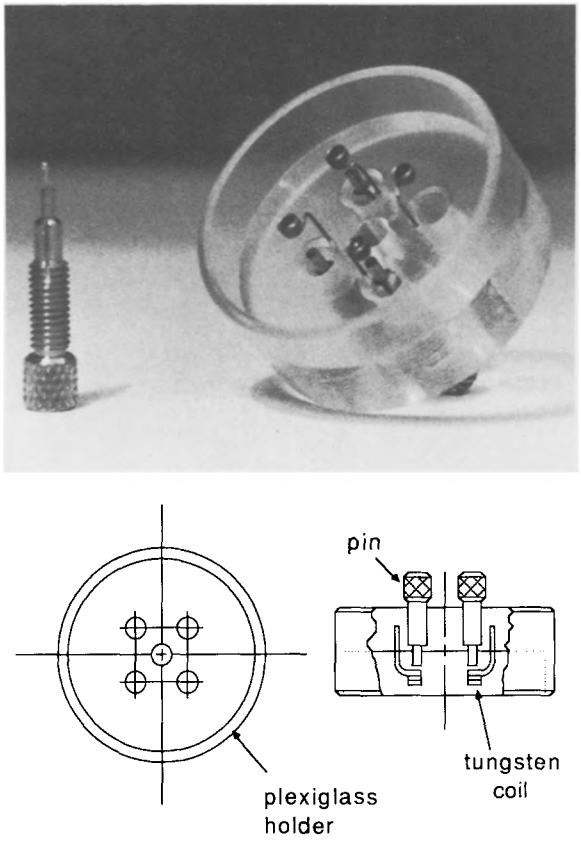

Fig. 1. The deposition device used in these studies. The purpose of the wire coils is to hold a droplet of liquid around the gold spheres as they are transferred from one solution to another.

an electron beam evaporator in a Balzers Freeze-Etch device, in high vacuum. The coating is done at room temperature. The coating material is platinum–carbon, and the coating thickness is about 25 Å as measured by a quartz crystal monitor. We have not tried to optimize the coating thickness, and it is likely that much thinner coatings will still provide enough conduction to allow reliable STM imaging.

We imaged two morphologically distinct viral particles: (1) T7, a bacteriophage with a nearly spherical head with a diameter of about 600 Å and a short tail with a length of about 200 Å [14], and (2) fd, a filamentous phage about 80 Å in diameter and 8800 Å in length [15]. For T7, the viruses were allowed to deposit on the gold spheres for 2 min at a concentration of 5×10^{11} particles per ml. The distilled water rinse was 1 min, and the alcohol rinse was 30 s. For the fd viruses, the adsorption time was 3 min at a concentration of 3×10^{12} particles per ml. The distilled water rinse was 1 min, and the alcohol rinse was 30 s. If the rinsing steps are too long, we find that it is possible to wash the virus particles entirely off the surface, no matter how concentrated the original virus solution or how long the deposition time.

A commercial STM instrument, manufactured by Digital Instruments, Inc., Santa Barbara, CA (Nanoscope I), was used in these experiments. It uses the single piezo tube design, has a maximum field of view of about one square micron, and operates in air. At present, our image display device is a storage oscilloscope; hence all images are line-scan type and have not been processed or improved in any way. All images were obtained using electrochemically etched tungsten tips.

Fig. 2 shows the STM images of T7. No molecular detail is visible due to the thickness and roughness of the coating, but the dimensions of the sphere-shaped objects are correct for a somewhat flattened T7 head. The flattening of the head is apparently a dehydration and adsorption artifact and is also seen in TEM micrographs [16]. In some images, corners can be seen where facets of the icosahedral phage head join each other (figs. 2a and 2b). As the concentration of the phage solution used in the deposition is increased, the density of phages on the surface also increases, as expected. At longer deposition times, or higher phage concentration, the phages can completely cover the surface, even forming piles several phages deep. When T7 viruses are prepared for imaging in the TEM on the evaporated carbon substrates by a procedure similar to the one described here for gold, we find similar virus densities on the surfaces. This indicates that the adsorption of the viruses to gold is nearly the same as that for evaporated carbon.

Fig. 3 shows a blank surface treated exactly like the virus-containing surfaces except that, in the adsorption step, pure buffer was used instead of virus solution. In all our experiments, we found that the blank was flat and featureless, except for the roughness caused by the metal coating itself.

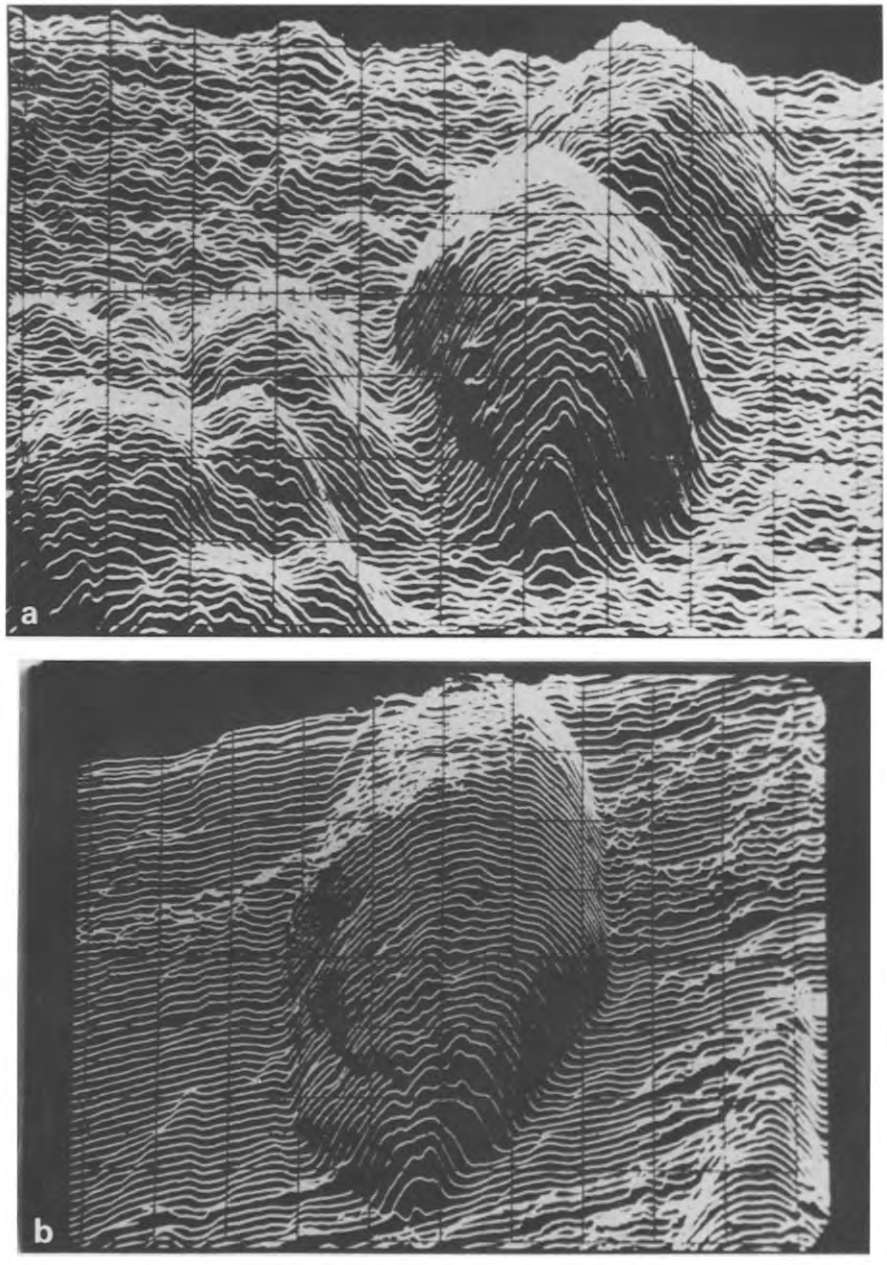

Fig. 2. STM images of T7: (a) The deposition time for this sample was 10 min at a concentration of 5×10^{13} particles/ml. This image was taken on an early sample. The long deposition time and high virus concentration was necessary because, in this preparation, extra washings were done (5 min in buffer, 4 min in alcohol). The lateral scales are 260 Å/div x and 184 Å/div y. The vertical scale is 200 Å/div. Note the sharp corners where the facets of the icosahedral head meet. (b) Deposition time 3 min, concentration 5×10^{11} particles/ml, lateral scales 216 Å/div x and 153 Å/div y. Vertical scale 165 Å/div.

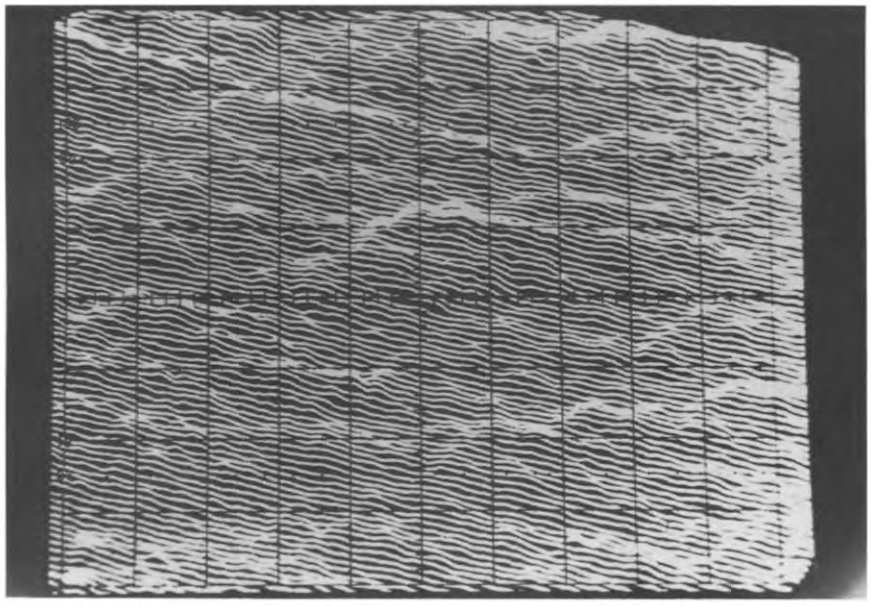

Fig. 3. STM image of a blank sample. There is surface roughness due to the coating, otherwise the surface is featureless. Scale: x-axis, 324 Å/div; y-axis 371 Å/div; z-axis 165 Å/div.

The STM images of fd viruses are shown in fig. 4. As can be seen in figs. 4a and 4b, the gross morphology is now that of a filamentous phage. However, once again, the coating prevents us from seeing any molecular detail. We have not been able to measure the full length of the fd since it is almost as long as the entire field of view of our instrument, but the diameter we see is approximately correct. In fig. 4b, the phage seems to have doubled back on itself at the top of the image. Also, two phages crossing each other can be seen at the bottom of the image. In some instances, images were obtained in which the width of the phage particle appears to be two or three times wider than it should be, although the height is about right. This distortion has been seen by other workers [5] and in our T7 images, and it is apparently caused by a blunt STM tip. By changing the tip and then re-imaging the same sample, the images can either be improved or made worse. In either case, in this intermediate range of scales (between large features of the order of a micron and small features of the order of ångströms), the shape of the tip plays a crucial role. Therefore, the tips must be very carefully selected. This points out the need for an internal imaging standard for STM, such as polystyrene beads or perhaps colloidal gold particles.

Our findings with these two different viruses show that routine imaging of biological matter by STM is feasible. The images clearly show the gross morphology, dimensions, and surface density of the viral particles. The extreme flatness and high conductivity of the gold substrate make it possible to consider STM imaging of single proteins and nucleic acids. So far we have not tried to optimize the coating procedure. It is likely that the resolution can be improved considerably by reducing the thickness and by cooling the sample during the coating process, reducing the metal grain size. The use of other coating metals (such as tungsten) may also improve resolution. By reducing the coating thickness toward zero coverage, we can now approach the ideal of an uncoated sample in a continuous manner. A careful study of the effects of metal coating in STM imaging will there-

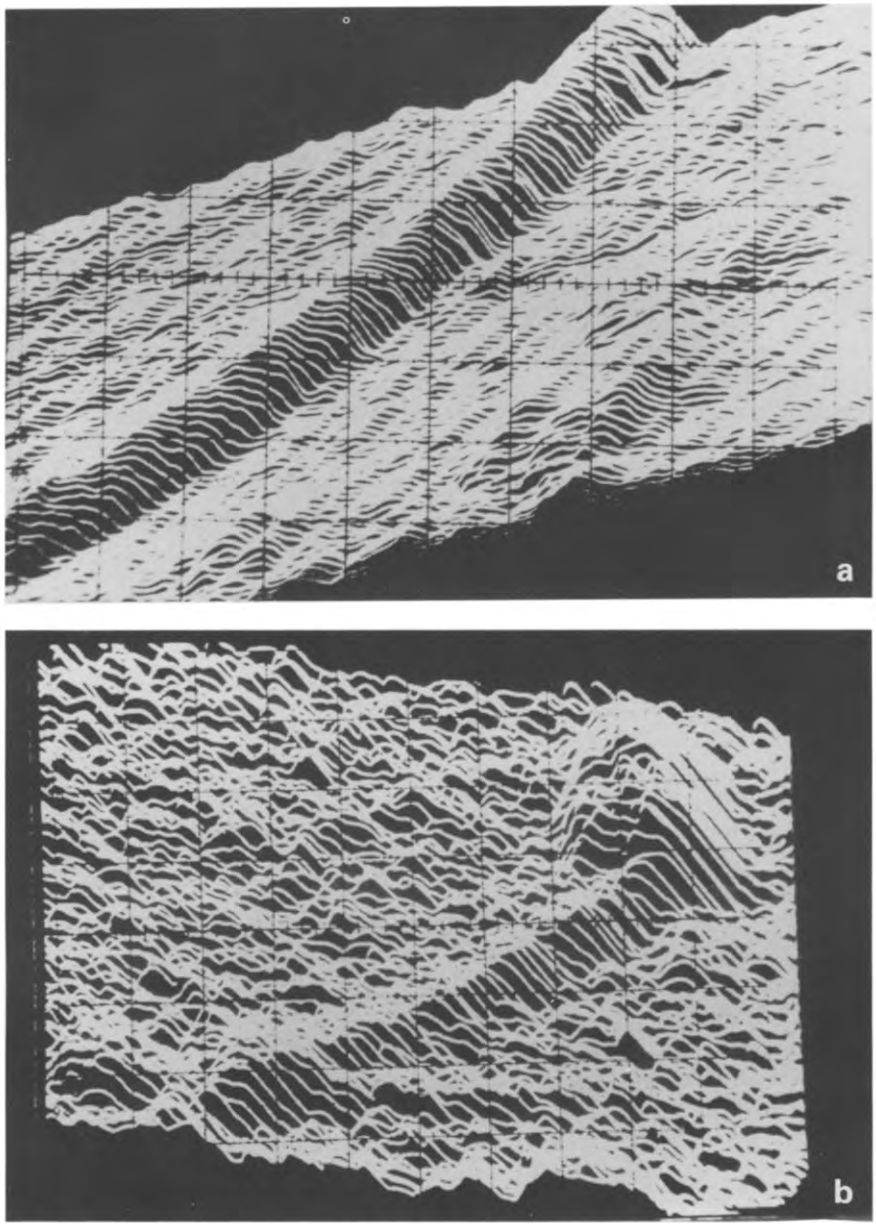

Fig. 4. STM images of fd virus. (a) Deposition time 2 min, concentrations 3×10^{12} particles/ml. Lateral scales 173 Å/div x and 265 Å/div y. Vertical scale 165 Å/div. The ends of the virus cannot be seen, but the apparent diameter is about 100 Å, in agreement with the width of a coated fd virus. (b) Deposition time 2 min, concentration 3×10^{12} particles/ml. Lateral scales 130 Å/dix x and 184 Å/div y. Vertical scale 97 Å/div. The virus seems to double back on itself at the top of the image. Two viruses cross each other at the bottom.

fore provide clues concerning the difficulties that have been encountered in STM imaging of uncoated molecules.

Finally, biological samples more than 500 Å thick may be impossible to image reproducibly without coating. In these cases, the technique presented here will render these systems accessible to STM imaging.

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