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SCANNING TUNNELING MICROSCOPY OF BIOLOGICAL MACROMOLECULAR STRUCTURES COATED WITH A CONDUCTING FILM

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Abstract

We have studied the capability of scanning tunneling microscopy (STM) to reveal the three-dimensional structure of biological macromolecular structures that have been rendered conductive by metal-coating. The sample preparation used has been derived from a well established method in transmission electron microscopy (TEM). It includes adsorption, freezing and dehydration by vacuum-sublimation, followed by metal-shadowing of the specimen. As an alternative to adsorption and coating, fluid biomaterials can be replaced by conductive freeze-fracture replica.

We give an introduction into the sample preparation of metal-coated specimens and discuss how each step can affect the structural preservation and thereby the quality of the data. Some aspects of the data acquisition and the quantitative evaluation of STM data are shown. Possible contributions of STM in the biological macromolecular research are pointed out.

KEY WORDS: Scanning tunneling microscopy, Transmission electron microscopy, biological macromolecular structures, freeze-drying, metal-coating, metal-replicas, Pt-Ir-C, tunneling tip, signal-to-noise ratio, correlation averaging

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Introduction

Scanning tunneling microscopy (STM) of biological materials is hampered by their poor intrinsic electrical conductivity. Yet an increasing number of reports prove that STM observation of bare biological macromolecules and macromolecular structures is feasible (e. g., Baro et al., 1985; Voelker et al., 1988; Amrein et al., 1989a; Guckenberger et al., 1989; Stemmer et al., 1989; Welland et al., 1989; Miles et al., 1990). However, the contrast in these STM images usually can not be attributed solely to the relief of the structures under investigation (e. g., Guckenberger et al., 1991). The electronic and elastic properties of the biomaterials contribute to the image formation in a way not yet fully elucidated. In addition, STM imaging of uncoated membranes and proteins under conventional tunneling conditions so far suffers from inconclusiveness and poor reproducibility. This obviously hampers any systematic investigation that is intended to relate the structure of a biological macromolecule or macromolecular complex to its biological function.

On the other hand, metal-coating (e. g., Travaglini et al., 1987; Amrein et al., 1988; Guckenberger et al., 1988; Blackford et al., 1988; Garcia et al., 1989, Amrein et al., 1991) or replicating the biological structures (Zasadzinski et al., 1988; Blackford et al., 1991) makes it relatively easy to obtain reproducible, trustworthy images. The sample preparation, derived from a well established method in transmission electron microscopy (TEM), includes adsorption, dehydration and metal-shadowing of the specimen. Each one of these preparation steps can considerably affect the structural resolution that is obtainable with such samples. Both, strong adsorption as well as dehydration may disrupt the macromolecular structures (e. g., Rachel et al., 1986). The conductive coating must cover the specimen homogeneously at a very small film thickness so that fine surface features are not buried.

This article is concerned with the preparation of metal-coated samples for the STM. Different specimen supports, adsorption and dehydration by vacuum subli-

mation (freeze-drying) are discussed. By freeze-drying the samples, the structural alterations due to dehydration can be minimized. Next, the manufacturing of a fine-grain, stable coating-film is detailed. When the STM measurements are carried out in air, such a coating film must not only render the sample well conductive but must also stabilize the well preserved conformation of the biological macromolecular structures.

Furthermore, general aspects of STM imaging such as the influence of the tunneling tip geometry on STM topographical images are discussed. Also, some aspects of the quantitative analysis of the STM data are presented. Finally, we attempt to point out how STM can contribute to a better understanding of the three-dimensional structure and the functional interactions of biological macromolecules.

Sample Preparation

Specimen Support

In order to perform STM of metal-coated biological macromolecular structures, they first have to be immobilized onto a smooth, solid support. A good conductivity of the support is necessary since in freeze-drying experiments usually not all regions are covered with the conducting coating film. In addition, the support must be mechanically stable to avoid an elastic response to the inevitable forces generated between the tip and the sample during scanning (Stemmer et al., 1989).

The first results of STM in biological application have been obtained using highly oriented pyrolitic graphite (HOPG) as a specimen support (e. g., Baro et al., 1985; Travaglini et al., 1987), and it is still widely used (e. g., Edstrom et al., 1990; Olk et al., 1991; Haggerty et al., 1991). Flakes of HOPG may be flat, on an atomic scale, over areas of micrometers, mechanically stable and well conductive. However the adsorption of biological specimens has proven to be very poor on graphite so that they are easily moved by the tip (e. g., Travaglini et al., 1987). Quite often the biomaterials form aggregates on the graphite surface, and in some cases the protein molecules have formed regular arrays. Such arrays have proven to withstand the mechanical stress caused by the scanning process and therefore could be reproducibly imaged (e. g., Miles et al., 1990; Hörber et al., 1991). However, defects and the fine structure of step edges of pure graphite may be very misleading when they resemble the expected structure of the specimen (Clemmer and Beebe., 1991). The surface of crystalline Au has been used for STM imaging of DNA (e. g., Lindsay et al., 1989), whereas its suitability as a support for membranes and proteins has yet to be investigated.

Although less flat than HOPG and Au surfaces, thin evaporated films of Pt-C deposited onto either glass or mica have proven to be suitable supports for STM that do not display the problems seen with HOPG. Adsorption of proteins and membranes onto Pt-C films is usually very efficient and they give very reproducible, high quality pictures. The maximum corrugation of Pt-C films of a nominal thickness of 5 nm amounts to approximately 2 nm and the standard deviation is 0.3 nm. The films are directly evaporated onto the freshly cleaved mica platelets or light microscope cover slips at room temperature by an electron beam-heated gun in a high vacuum apparatus (e. g., Balzers freeze-etch unit BAF 400T).

Thin carbon films, commonly used in TEM, are marginally smoother than Pt-C films (Stemmer et al., 1989). However, they do not always allow stable tunneling when directly evaporated onto mica platelets or light microscope cover slips. This is probably due to the much higher electrical resistance when compared to Pt-C films of the same nominal thickness.

Sample Deposition

On depositing the sample, a homogeneous distribution of the biological specimens, kept in a close-to-native conformation, is aimed for. Hereby, the forces between the objects and the support present a major problem. By strongly binding the biological specimen it is prevented from clustering on the support surface and it may also better withstand the forces that arise during scanning between the tip and the sample. On the other hand, the structure can be substantially distorted by strong binding forces, well known from TEM as well as from STM of biological specimens (e. g., Wang et al., 1990). The conditions, appropriate for the deposition of some specimens, may therefore represent a compromise, where the binding forces are strong enough in order to provide a sufficient distribution and, at the same time, the disruption of the specimen is kept small.

There are several methods for immobilizing the biological specimens on a solid support of which the most common is adsorption, based on van der Waals forces and forces between dipoles and charges. The strength of the adsorption depends very much on the support used. There exists wide experience from TEM on the adsorption of biological specimens to C films. Pt-C films have proven to behave basically the same. However, they tend to bind the specimen slightly stronger and thereby provide a denser distribution but also a more pronounced deformation of the macromolecular structures.

For sufficient adsorption of most biological specimens to C-films or Pt-C films, the support films have to be rendered hydrophylic by glow discharge (e. g., in a Harrick Plasma cleaner). Sometimes, however, it is worth, using hydrophobic supports in order to prevent destructive binding forces, even when the objects adsorb much less densely (Henderson et al., 1990). Glow discharge must here be omitted, and it is recommended to

use C films or Pt-C films that have been stored for several days in air. They become more and more hydrophobic with time. In some cases (e. g., DNA, recA-DNA complexes, purple membranes) bivalent cations such as magnesium acetate (≤ 10 mM) are required for sufficient adsorption in both the adsorption solution and the washing solution (Sogo et al., 1987).

Filamentous structures that do not adsorb from solution in a well spread manner (e. g., myosin) may be deposited by the spraying technique. Hereby the object solution is sprayed onto the substrate in very fine drops. Usually the solution must contain up to 50% glycerol in order to obtain a good spreading of the drops on the substrate and to prevent clustering of the molecules. The sample may be rinsed afterwards.

Dehydration of the Objects

When coating of the biological material is intended, it must be dehydrated after adsorption in order to expose the surface to metal deposition. Dehydration by simple air-drying engenders dramatic structural alterations due to the high surface tension forces of water during drying (e. g., Wildhaber et al., 1985). Air-drying artifacts can be avoided by freezing and subsequent vacuum sublimation of the water phase (freeze-drying). Polysaccharides, nucleic acids, most proteins, and even lipid bilayers are hydrophilic, i.e. they are naturally surrounded by a shell of strongly bound, well ordered water molecules. This hydration shell is essential in the establishment and stabilization of the native conformation of biological macromolecules, e. g., the correct folding of a polypeptide chain into a protein (for an introduction see Kellenberger, 1987). It has been shown by means of TEM of metal-coated protein lattices that the removal of this shell causes the protein structures to partially collapse, even when they are freeze-dried (e. g., Gross, 1987).

When freeze-drying protein structures at a temperature no higher than 193 K, however, a fraction of water remains bound which we refer to as the water of the hydration shell. It desorbs in distinct peaks when the specimen is warmed above about 220 K (Fig. 1). These findings, and those of many other studies (for a review see Robards and Sleytr, 1985), show that freeze-drying of the adsorbed macromolecular structures at 193 K is completed within a tolerable time (1-3 h), whilst at the same time the hydration shell is substantially maintained.

For freeze-drying, the sample is quickly frozen by dipping it into liquid nitrogen, mounted onto the specimen table under liquid nitrogen and transferred onto the precooled cold-stage (143 K) of the freeze-etch unit (e. g. Balzers BAF 400T). Freeze-drying is carried out under high vacuum conditions ($p<10^{-6}$ mbar) at 193 K for 1 to 3 h, depending on the object.

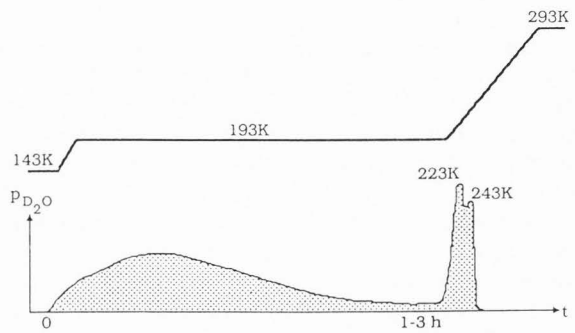

Fig. 1. The course of subliming specimen water during the freeze-drying procedure. The specimen water was substituted with D_2O allowing mass spectrometric differentiation between the specimen water (D_2O) and water vapor originating from elsewhere in the UHV freeze-etch unit. After drying at 193 K for 1-3 h, depending on the biological sample, an equilibrium at a very low sublimation rate was reached. Warming to room temperature resulted in a further sublimation of the specimen water in at least two distinctive peaks for all specimens tested. The removal of this strongly bound water, which we refer to as the water of the hydration shell, caused the biological structures to collapse partially.

Metal Coating

Metal coating of macromolecular structures for STM is primarily performed in order to render the surface uniformly conducting. When the specimens have been freeze-dried, the coating film must in addition stabilize the well preserved protein conformation during the whole period of the transfer from the sample preparation unit to the microscope and STM imaging. The coating should cover the specimen homogeneously at a very small film thickness so that fine surface features are not buried.

Up to now films evaporated by an electron beam-heated gun have provided the best results with respect to the demands outlined above. The formation of thin metal films proceeds via thermal accommodation, surface diffusion, nucleation, and crystal coalescence (e. g., Venables et al., 1984). Grains in the condensate arise due to the lateral mobility of the evaporated material on the specimen surface. If the exposed structures cause an unequal distribution of surface forces, nucleation and crystal growth occur preferentially at surface sites with higher binding energy. Therefore, at higher resolution, such preferential nucleation (decoration) can be responsible for revealing structural details as well as the actual surface relief itself (Gross et al., 1985).

Until now Pt-C has been the most commonly used evaporation material in STM of biological specimens. It reveals well-conducting, very stable coating films when applied to air-dried biological specimens. However it

does not sufficiently stabilize well preserved biological structures obtained after freeze-drying. Here the Pt-C films form large clusters and the structures underneath collapse. This is probably due to the rehydration of the sample when it is withdrawn from the vacuum system and exposed to atmospheric conditions. In high resolution shadowing for TEM such alterations of the metal films can be prevented by an additional stabilizing carbon film (Bachmann et al., 1965). Such an additional carbon coat, however, buries fine surface features when probed by a STM.

Pt-Ir-C films, on the other hand, have proven to remain three-dimensionally stable at macromolecular dimensions when transferred to atmospheric conditions after freeze-drying of the sample. They allow stable tunneling and yield a much finer granularity than Pt-C films both in TEM as well as in STM (Amrein et al., 1988; Amrein et al., 1989b; Amrein et al., 1991, Wepf et al., 1991). In addition, preferential nucleation is almost neglectable. However, the relative amounts of the three components has turned out to be crucial. The composition of the films changes as a function of the mass evaporated from the source electrode, a Pt-Ir cylinder (diameter 1.5 mm, 70% Ir) inserted into a graphite rod (diameter 2 mm). WDX-analysis (wavelength dispersive x-ray analysis) of the films performed in parallel with TEM and STM experiments have revealed that for three-dimensional stability a carbon content of at least 25 % (w) is required (Wepf et al., 1991). This is reproducibly achieved after preevaporating about 13.8 mg from the source electrode. For stable tunneling the metal content of the films should be no less than 60%.

A homogeneous, coherent coating, that mainly superelevates the relief of the freeze-dried biological matter, can be achieved by rotary shadowing Pt-Ir-C films at an elevation angle of 65^{o} to a calculated average film thickness of about 1.5 nm.

Metal Replicas

As an alternative to adsorption and coating, fluid, nonconductive biomaterials can be replaced by a rigid, highly conductive freeze-fracture replica (Zasadzinsky et al., 1988; Blackford et al., 1991). Metal replicas for STM are obtained by conventional freeze-fracture techniques. The sample is quickly frozen (e. g., in a Balzers, FSU 010 propan jet), transferred to a freeze-etch unit and fractured. The fractured surfaces may either be immediately replicated or freeze-etched prior to metal-coating them. Here Pt-C proves to be well suited as a coating material because the metal-film can be further stabilized with an additionally coated carbon layer (30 nm). The carbon layer also renders the replica more rigid. Finally the biomaterial is removed from the replica so that the impression of the sample surface is now accessible to the STM measurements.

Zasadzinski and co-workers have demonstrated, using the example of a naturally occurring phospholipid liquid crystal, DMPC, that such replicas may provide a better resolution when imaged in a STM when compared to TEM of the same replicas. They ascribe this gain in resolution to the fact that the STM senses only the surface layer of the replica that was in direct contact with the original fracture surface, whereas in the TEM the entire shape of the Pt crystallites determines the image. However, care must be taken when precision height measurements on metal replicas are aimed for. Surface features may appear with an amplified height due the effect of attractive forces between the tip and the flexible replica (Woodward et al., 1991). This effect may be strongly reduced by increasing the thickness of the replica and bonding them more firmly to a conductive support (Woodward et al., 1991).

STM Measurement

The properties of the tunneling tips play a crucial role for the quality of the STM images. When scanning in the constant current mode, the tunneling tip follows a surface such that the tip-sample separation is kept constant. Obviously, when strongly corrugated surfaces are scanned, the "effective" tip may become a function of the local corrugation, and hence the resulting STM topographical images are strongly dependent on the tip geometry (e. g., Chicon et al., 1987). Therefore, in order to satisfactorily resolve the surface features, the tips have to be sufficiently sharp. Furthermore, the tip-sample separation should be kept relatively large with respect to the local corrugation by choosing the tunneling parameters correspondingly (e. g., 1 pA; 1.5 V). In order to achieve tunneling currents as low as 1 pA or below, a special preamplifier, e. g., as designed for the patch clamp technique (EPC-7 probe, HEKA Elektronik P. Schulze, D-6734 Lamprecht), is required (Stemmer et al., 1989; Guckenberger et al., 1989). Under these conditions, the influence of the tip geometry remains small. On the other hand, the resolution decreases due to the larger tip-sample separation. However, this seems to be negligible in the case of metal-coated macromolecular structures, where the resolution is restricted anyway due to the coating.

Until now, tunneling tip manufacture has been still rather intuitive and tedious. We have been using tips electrochemically etched from gold, platinum/iridium or tungsten wire. The use of these materials results in tips of more or less similar quality. Mechanically cut wires usually act as multiple tips when scanning over a strongly corrugated specimen. In order to achieve good resolution, electrochemically etched or mechanically cut tips must almost always be conditioned during scanning by applying pulses to the tunneling voltage (pulse height $\geq$ 3V, pulse duration $\leq$ 1μsec). Thus, the actual

tunneling tips arise during scanning. As a rule of thumb, the sharper a tip, the less stable it is. Therefore, for optimal resolution, the tip may have to be conditioned more than once during the acquisition of a single image.

Quite often, specimen features are imaged twice or more on the same topographical image due to the tunneling current jumping from one "local tip" to another. Such multiple-tips frequently cause artifacts at steep and high-elevation structural features such as membrane borders and protein filaments. There is, for example, a number of early publications on STM of biological filamentous structures, where the observation of bundles are reported that almost certainly are just multiple-images of a single filament (e. g., Travaglini et al., 1987). In Fig. 2. the effect of a multiple-tip is illustrated on the example of metal-coated actin filaments.

The forces that arise between the tip and the sample during a STM measurement have proven to play an important role, even when the biological specimens have been stabilized by metal-coating. The coating film may easily be ripped off, and the structures may be pushed away by the tip (Fig. 2). In order to reduce these forces, here again a large tip-sample separation is advantageous.

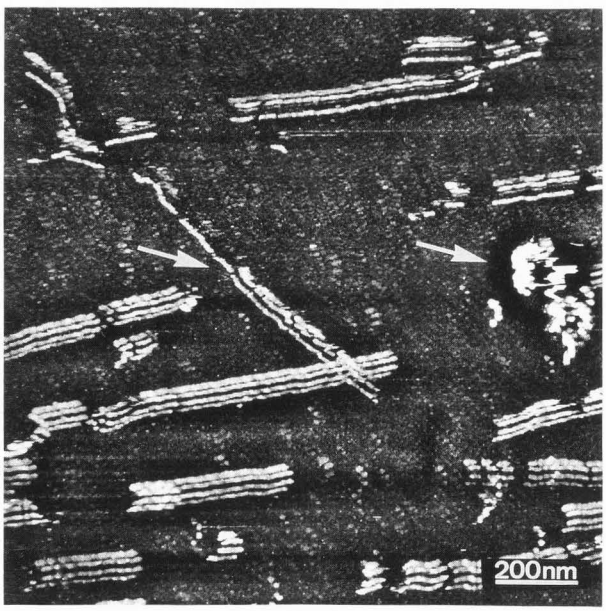

Fig. 2. STM image of metal-coated actin filaments showing both, the effect of a multiple-tip as well as the effect of strong interactions between the tunneling tip and the sample. Most of the filaments are imaged four times being indicative of a fourfold tunneling tip. Note that each filament appears four times slightly different. This is most probably due to different local geometries of the four tips. The arrows point to locations, where a strong tip-sample interaction has either caused the tunneling tip to change its shape or the metal-coating has been ripped open.

Quantitative Evaluation of STM Data

Height Measurements

STM provides very accurate data in the z-direction (i. e., in the direction perpendicular to the support). It may therefore be used as a morphometric tool to study the absolute height of adsorbed membranes and protein structures, data that are difficult to obtain with similar accuracy by other means (Amrein et al., 1989b; Fisher et al., 1990; Wang et al., 1990). First, however, the scanner must be calibrated very carefully in the z-direction, for example by using the known height on monoatomic steps of the surface of crystalline gold. For reasons discussed in the introduction of this paper, metal-coated samples are best suited for absolute height measurements. Since the average thickness of the coating film can be assumed to be the same both on the specimen surface as well as on the adjacent support, the difference in height can be taken as a measure of the thickness of the object. Before the height measurements can be carried out, any overall height ramp in the pictures has to be removed. The influence of the granularity of the coating film can be suppressed by averaging and thereby make the height measurements very accurate. For example, the thickness of metal-coated, air-dried purple membrane has been determined to be 4.3 ± 0.1 nm (Wang et al., 1990).

High precision height measurements represent important structural information. The method has been used, for example, to study how different preparation techniques affect the overall height of the metal-coated HPI-layer (Wang et al., 1990).

Digital Image Averaging and Evaluation of Data-Quality

When using the STM to determine the structure of biological macromolecules and supramolecular assemblies, it is most important to carefully evaluate the quality of the structural data obtained, i. e., to estimate to what degree topographical features revealed in a STM image may be ascribed to the actual structure of the biological specimen. The resolution of molecular details is restricted by both the shape of the tunneling tip and by noise.

There have been several attempts to reconstruct the true surface relief computationally by making assumptions on the actual tip shapes (Chicon et al., 1987; Niedermann et al., 1989). Even though these reconstruction techniques improve the fidelity of the relief data, they obviously cannot recover the structural information that is missing due to a finite tip radius.

Evenly distributed noise, on the other hand, may merely obscure structural information that is effectively present in the image. Sources of noise are intrinsic disorder in the specimen structure and artifacts induced by specimen preparation or metal coating. Noise stemming from the microscope itself is neglectable, since it is

typically smaller by one order of magnitude than the structural features that are resolved.

There is a number of established techniques developed for noise filtration and resolution assessment of TEM micrographs that can be applied to some extent to STM topographical images without any further adaptations. As a supposition, however, the STM topographies must contain repeated images of the investigated structure. It is then assumed that the noisy images can be aligned and summed to yield an average image. The signal-to-noise ratio of the resulting image improves by the square root of the number of averaged units. These averaging techniques rely on either Fourier filtering or alignment and spatial averaging of the repeating motifs.

The latter technique, usually referred to as "correlation averaging" (Saxton and Frank, 1977), is for several reasons well suited in the case of STM images (Amrein et al., 1989b). The cross-correlation function of two image partitions that contain a common motif at different locations displays a distinct peak at an offset position, with the offset vector corresponding to the vector by which the motif appears to be shifted form one location to another. By using this "self-detection" approach, motifs are aligned and summed to yield an average image. Besides the average unit cell, the variance in the raw data may be calculated. This yields an estimate of the accuracy with which the heights of the structural features have been measured. The ratio of the periodic (signal) to the nonperiodic (noise) portion of the averaged images can be evaluated from a pair of independent averages.

In Fig. 3, correlation averaging is illustrated using the example of freeze-dried and metal-coated polyheads (Amrein et al., 1989b). Fig. 3a. shows the STM image of the polyhead used as input data. The average capsomere morphology as shown in Fig. 3, b and c, proved to be reproducible in different experiments using different tips (Fig. 3d).

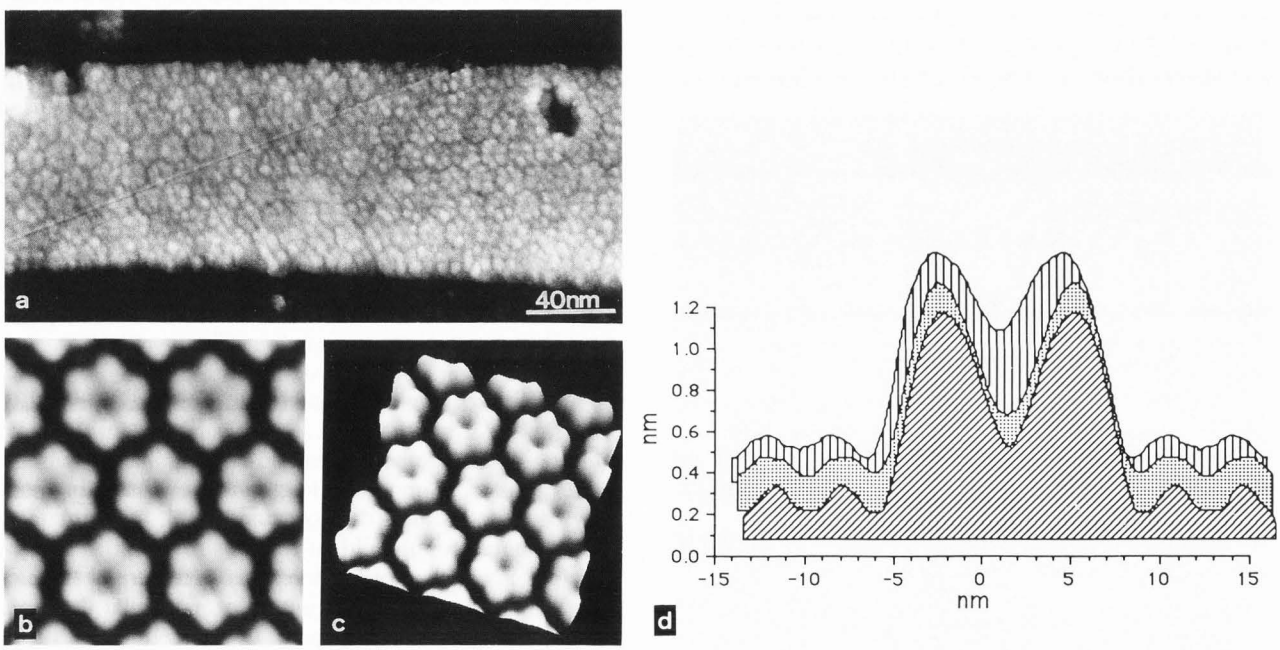

Fig. 3. The outer surface morphology of a bacteriophage T4 polyhead, revealed after correlation averaging. Polyheads, morphologically aberrant capsids of a mutated bacteriophage, are composed of capsomeres arranged on a near-hexagonal lattice which is folded into a cylinder (Steven et al. 1976). The used polyheads have a lattice constant of 13 nm. Fig. 3a. shows the STM image of the polyhead used as input data. The height difference between the support film and the top of the relief of the polyhead amounts to about 7 nm. Note that the capsomeres can already be distinguished as hexamers in the raw data. The averaged capsomere morphology is shown in (b) in a top-view and in (c) in a surface-view representation. Fig. 3d. shows a comparison of the averaged capsomere structures from three different experiments acquired with three different tunneling tips. The data are shown as crossections through the center of capsomeres. The two data sets denoted with dots and tilted lines, respectively, are almost identical whereas the capsomere shaded with "vertical lines" reveals less well resolved protomers. The latter may be due to a "flatter" tip.

STM in Molecular Structure Research

When investigating the structure of biological macromolecules or their supramolecular assemblies, STM has become an alternative to transmission electron microscopy (TEM) or X-ray diffraction analysis. In many cases X-ray diffraction allows the determination of a structure down to atomic resolution. However, it requires perfectly ordered, three-dimensional crystals that are quite often not attainable. TEM, on the other hand, is a very powerful technique for determining the overall size and shape of biological macromolecules that cannot be crystallized in three dimensions. In the following section, we discuss how the STM can provide complementary structural information to the TEM data. As an example, TEM and STM data of a similarly prepared protein monolayer, the HPI-layer, are compared (Fig. 4).

Any transmission electron micrograph is basically a two-dimensional projection image and the three-dimensional structure of the object has to be reconstructed. Three-dimensional reconstructions are obtained by tomographic techniques (i. e., by combining the information from a series of projections with the specimen tilted at different angles with respect to the electron beam) or by surface relief reconstruction (e. g., Guckenberger, 1985) using micrographs of unidirectionally shadowed specimens (Fig. 4b). However, the resolution that is obtained in the z direction is usually considerably lower than the lateral resolution. In contrast, the STM provides direct information about the specimen surface profile, and hence thickness, with high accuracy.

The quality of three-dimensional reconstructions from TEM data strongly depends on the degree to which the structural information is obscured by noise in the raw data (i. e., signal-to-noise ratio). In order to attain resolution at a molecular level, the signal-to-noise ratio usually has to be increased by averaging over redundant structural information (Fig. 4b). Consequently, subnanometer resolution has only been obtained with large, highly ordered two-dimensional crystals. In contrast, the signal-to-noise ratio of raw STM data is usually significantly higher than in TEM micrographs of the same structure and the z dimension needs not to be reconstructed (Fig. 4a). Therefore in contrast to TEM, STM may reveal the architecture of biological structures where the signal-to-noise ratio cannot be increased considerably by averaging.

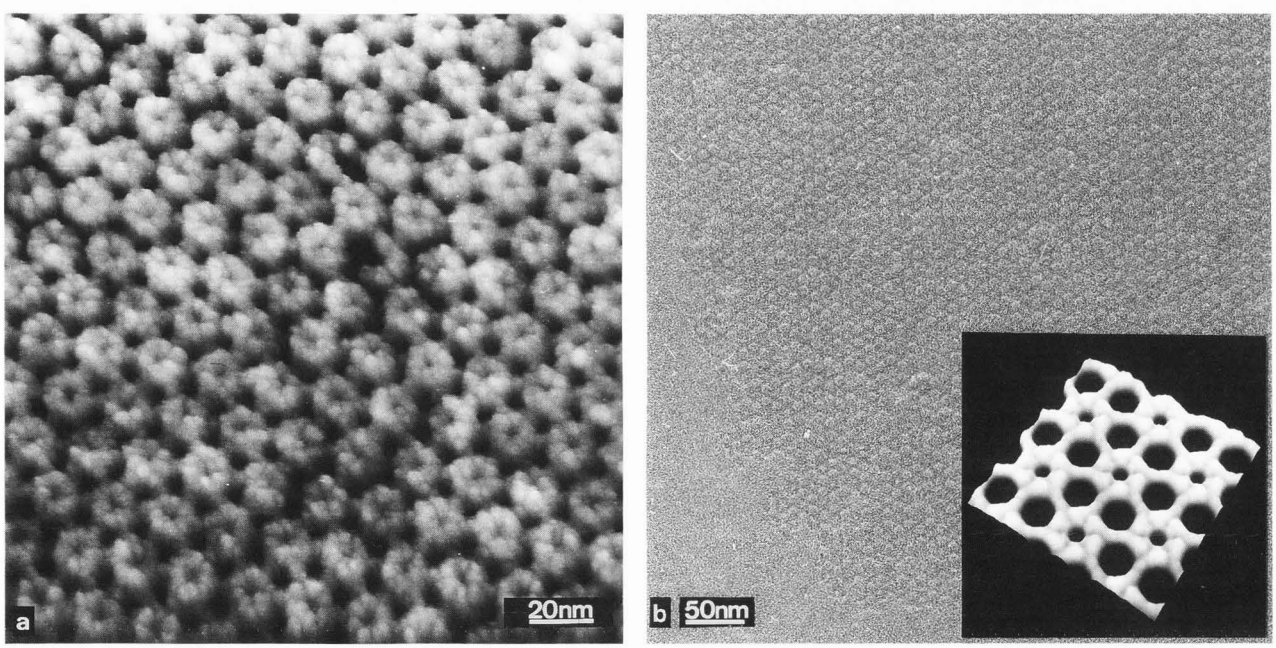

Fig. 4. The outer surface of the HPI layer, a regular protein monolayer found outermost in the cell wall of the bacterium Deinococcus radiodurans. (a) STM raw data, (b) TEM raw data of freeze-dried and metal-coated HPI layer.
The HPI layer has a prominent surface structure with p6 symmetry and a lattice constant of 18 nm (Baumeister et al., 1986). The overall-height of the HPI layer amounts to about 5 nm in the STM (a). Most of the structural features that are directly observed by STM (a) are obscured by noise in the TEM data (b). This is because the ratio of the periodic (signal) to the nonperiodic (noise) portion of the STM raw data is at least twice as high as for the raw data obtained from TEM of a similarly prepared sample. However, in the case of TEM data, correlation averaging followed by surface relief reconstruction reveals these structures with a slightly better resolution than STM. The inset in (b) shows a TEM relief reconstruction.

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Discussion with Reviewers

O. Marti: What physical effect limits the resolution of the STM to a value slightly worse than that of the TEM?
Authors: The structural resolution that can be obtained with an STM on biological macromolecules is not principally worse than that of the TEM. It depends on both the radius of the tunnelling tip as well as the geometry of the object itself. The less corrugated the objects are, the less important becomes the actual tip shape (e.g., near-atomic resolution has been obtained on monolayers of organic ascorbates). Furthermore, in order to attain resolution at a molecular level by TEM, the signal to-noise ratio has to be increased by averaging. In contrast, STM may reveal the molecular architecture of a biological structure better than TEM, where the signal-to-noise ratio cannot be increased considerably. This may even be the case on a strongly corrugated object, where the resolution of the STM is clearly limited by the tip radius.

O. Marti: You describe a tip formation procedure by applying voltage pulses. This procedure modifies the tip, but what happens to the sample?
Authors: Occasionally, the voltage pulses produce holes in the sample surface. It is important to note that the described tip shaping procedure cannot be applied on each type of sample. In the case of pure carbon films, for example, the tip becomes merely contaminated when applying pulses.

O. Marti: In your paper you describe the correlation averaging method. Can this method be used to get rid of ghost images by multiple tips?

Authors: It is possible to detect accurately the locations of ghost images by cross-correlation. One then could subtract the artifactual portions from the image. However, the sample surface is not imaged at locations, where ghost images of a large object appear and the lacking information cannot be reconstructed by any means.

O. Marti: You note that there are changes to the sample after freeze-drying and metal-coating steps. Do you know of an attempt to circumvent this problem by using a low temperature STM *in situ* in the freeze-drying machine?

Authors: Usually the samples are warmed up to room temperature and withdrawn from the vacuum system after freeze-drying and metal coating. You state correctly that these steps may cause alterations of the biological macromolecules and the metal films. In TEM, as well as in SEM, these problems have been solved by cryo-preparation chambers which are directly attached to the microscopes. In STM, on the other hand, we do not know of any attempt to do the same.