Hydration-Scanning Tunneling Microscopy as a Reliable Method for Imaging Biological Specimens and Hydrophilic Insulators

M. Heim
Max-Planck-Institut für Biochemie, heim@alf.biochem.mpg.de

R. Eschrich
Max-Planck-Institut für Biochemie

A. Hillebrand
Max-Planck-Institut für Biochemie

H. F. Knapp
Max-Planck-Institut für Biochemie

G. Cevc
Technische Universität München

See next page for additional authors

Recommended Citation

Hydration-Scanning Tunneling Microscopy as a Reliable Method for Imaging Biological Specimens and Hydrophilic Insulators

Authors

M. Heim, R. Eschrich, A. Hillebrand, H. F. Knapp, G. Cevc, and R. Guckenberger

HYDRATION-SCANNING TUNNELING MICROSCOPY AS A RELIABLE METHOD FOR IMAGING BIOLOGICAL SPECIMENS AND HYDROPHILIC INSULATORS

M. Heim[1*], R. Eschrich[1] A. Hillebrand[1], H. F. Knapp[1], G. Cevc[2] and R. Guckenberger[1]

[1]Max-Planck-Institut für Biochemie, Abt. Molekulare Strukturbiologie, Martinsried,
[2]Technische Universität München, Klinikum Rechts der Isar, Medizinische Biophysik, München, Germany

Abstract

The recently discovered high lateral conductivity of molecularly thin adsorbed water films enables investigation of biological specimens, and even of surfaces of hydrophilic insulators by scanning tunneling microscopy (STM). Here we demonstrate the capabilities of this method, which we call hydration-STM (HSTM), with images of various specimens taken in humid atmosphere: We obtained images of a glass coverslip, collagen molecules, tobacco mosaic virus, lipid bilayers and cryosectioned bovine achilles tendon on mica. To elucidate the physical mechanism of this conduction phenomenon we recorded current-voltage curves on hydrated mica. This revealed a basically ohmic behavior of the I-V curves without a threshold voltage to activate the current transport and indicates that electrochemistry probably does not dominate the surface conductivity. We assume that the conduction mechanism is due to structuring of water at the surface.

Key Words: Surface conductivity, structured water, thin films, surface electrochemistry, glass, collagen, bovine achilles tendon, lipid layers.

*Address for correspondence:
Manfred Heim
Max-Planck-Institut für Biochemie
Abt. Molekulare Strukturbiologie
D-82151 Martinsried
Germany

Telephone number: 49-89-85782651
FAX number: 49-89-85782641
email: heim@alf.biochem.mpg.de

Introduction

The recently discovered high electrical conductivity of surface-adsorbed water molecules (Guckenberger *et al.*, 1994; Heim *et al.*, 1995) permits scanning tunneling microscopy (STM) investigation of biological samples and even hydrophilic insulators in humid air. Water films which are only a few Å thick adsorb to hydrophilic surfaces in humid air and act as a conductive coverage of the surface to be investigated. This conductivity is exploited for imaging with the scanning tunneling microscope, a method we term hydration-STM (HSTM).

A conductive metal skin with a minimum thickness of 1-2 nm, normally necessary for studying insulators with the STM, limits resolution substantially, whereas the conductive water has the advantage of being very thin (less than 0.2 nm on average can be sufficient). Moreover, the biological specimens in HSTM experiments are present in a semihydrated state, which can be important for the biological material not to denature.

For biological applications the atomic force microscope (AFM) is the most widely used probe microscope since it does not rely on sample conductivity and is capable of measuring various mechanical properties of the sample such as friction (Mate *et al.*, 1987), elasticity (Maivald *et al.*, 1991; Radmacher *et al.*, 1993), viscosity, etc. even in an aqueous environment. However, the AFM always exerts forces upon the studied objects which can induce conformational changes in the samples. For STM studies, sharper tips can easily be manufactured and the shorter decay length of tip-sample interaction, at least in principle, allows achievement of higher resolution with the STM (Rohrer, 1990). This implies that the STM is still attractive for investigation of soft materials. In this work we describe how HSTM can be easily applied for the imaging of various biological molecules or other hydrophilic surfaces. This is demonstrated with HSTM images of glass, collagen molecules, the tobacco mosaic virus, a lipid bilayer and a cryosection of bovine achilles tendon on mica.

Apart from the possibility of imaging insulators with the STM, the effect of the lateral conductivity of ultrathin water films is of fundamental interest itself since the physical background of the exceptionally high conductiv-

ity is not yet well understood. We hypothesize that electrical conductivity arises from a specifically ordered structure of the water molecules on the adsorbing surfaces. The influence of the surface forces could immobilize water molecules in such a way that they form a hydrogen-bond network which mediates charge transport. The surface adsorbed water would have ice-like rather than water-like properties. An indication of this was recently observed in images of surface-adsorbed water on mica, taken in humid air with the atomic force microscope (Hu *et al.*, 1995) (for a review on water structure on surfaces see Bockris and Jeng, 1990). The surface conductivity of water can probably be attributed to conformational changes in the water layer and, therefore, corresponds to well known phenomena like the increase of conductivity and the dielectric constant in frozen water (Israelachvili, 1992). However, the existence and the role of such thin layers of structured water with properties very different from bulk water is still in discussion (Israelachvili and Wennerström, 1996). We have recorded voltage-current curves on hydrated mica in order to derive more information about the properties of the conductivity of molecularly thin water films.

Experimental

All experiments were done with a home built STM described in ref. (Guckenberger *et al.*, 1988). One important feature of this device is the use of a very sensitive preamplifier (input stage, 20 GΩ feedback resistor, commercially available from Quintenz Hybridtechnik, Kramerstrasse 3, 82061 Neuried, Germany) which enables us to image with tunneling currents as low as 50 fA. The relative humidity in the humidity chamber is controlled by mixing a stream of dry nitrogen with a humid nitrogen stream which bubbles through deionized water (with 18 MΩcm resistivity from a Milli-Q plus system, Millipore Corporation, Bedford, MA). The tips used in our imaging experiments were electrochemically etched tungsten wires (Hacker *et al.*, 1992). All images were taken in the constant current mode.

The glass coverslip was cleaned first in a 2 % Hellmanex solution in an ultrasonic bath for 15 minutes and then washed in Milli-Q water at 30 °C for 15 minutes in the ultrasonic bath.

Collagen molecules were deposited on mica as follows: A small drop of collagen solution was placed on a piece of freshly cleaved mica and left to adsorb for about 1 min. The sample was then washed by placing it on top of a large drop of pure water for 5 seconds and the excess water was blotted off with a filter paper. The same procedure was also used to prepare tobacco mosaic virus (TMV) on mica. The initial TMV suspension contained 43 mg/ml TMV in sodium phosphate solution

(0.1 M; pH 7.0). The suspension was diluted 9:1 with pure water prior to deposition on the mica substrate.

The lipid bilayer consisted of DPPE (1,2-dipalmitoylphosphatidylethanolamine) as the first layer on mica and DPPC (1,2-dipalmitoylphosphatidylcholine, both from Sygena, Liestal, Switzerland) as the top layer. Both lipids were prepared with the Langmuir-Blodgett technique in a film balance. The lateral pressure of the lipids in the film balance was maintained at 40 mN/m for 45 minutes prior to the deposition. After preparation of the lipid bilayer the sample was dried as described in ref. (Heim *et al.*, 1995).

The bovine achilles tendon was embedded in glucose and frozen in liquid nitrogen. The tendon then was sectioned with a microtome and mounted on mica. To remove glucose, the sample was washed with pure water, then dyed with methylene blue to facilitate recognition in the light microscope. All samples were fixed mechanically onto a sample holder made of stainless steel, which provides electrical contact via mechanical contact to the sample side facing the tip.

The current voltage curve was recorded with a gold tip in mechanical contact with the hydrated mica surface. In this experiment the ring-shaped sample holder with an inner diameter of 6 mm was covered with gold by evaporation. The preparation of gold tips is described in (Knapp, 1996).

Results and Discussion

HSTM images of insulators and biological specimens

In this work we describe the possibilities and limitations of the newly developed hydration scanning tunneling microscopy (HSTM) method.

Glass. The first example (Fig. 1) shows the HSTM image of a glass coverslip. At a relative humidity of 68 %, the glass surface is hydrated, and the wetting film is capable of laterally transporting the tunneling current from the spot beneath the tip to the sample holder. The glass surface investigated displays holes with a diameter of approximately 20 nm. Many smaller holes can also be seen on the glass surface with a diameter of about 2.5 - 8 nm. It is noteworthy that the image is recorded with a sample bias of -0.7 V which demonstrates that imaging at low voltages, and thus, not only in the field emission regime, is possible.

A HSTM image of a plasma cleaned glass coverslip presented in (Heim *et al.*, 1996) shows basically the same structure (with a slightly reduced resolution). The big as well as the small holes are visible on the plasma cleaned and the detergent/water cleaned coverslip. This shows that plasma cleaning does not influence the glass surface on the nanometer scale. The glass shown in Fig. 1 becomes hydrophilic enough for HSTM imaging after
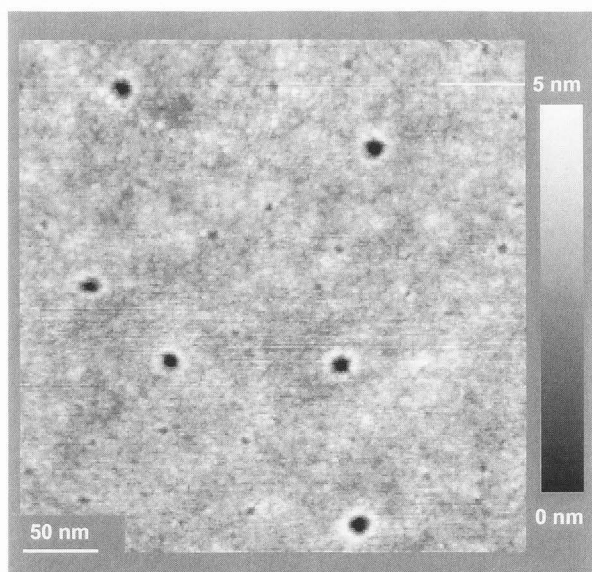
Figure 1: HSTM image of an uncoated glass coverslip taken at -0.7 V sample bias, a tunneling current of 1 pA, and at a relative humidity of 68 %. The surface adsorbed water molecules make the glass surface sufficiently conductive for STM imaging. The glass shows several holes which are about 20 nm wide and many small holes with diameters between 2.5 and 6 nm.
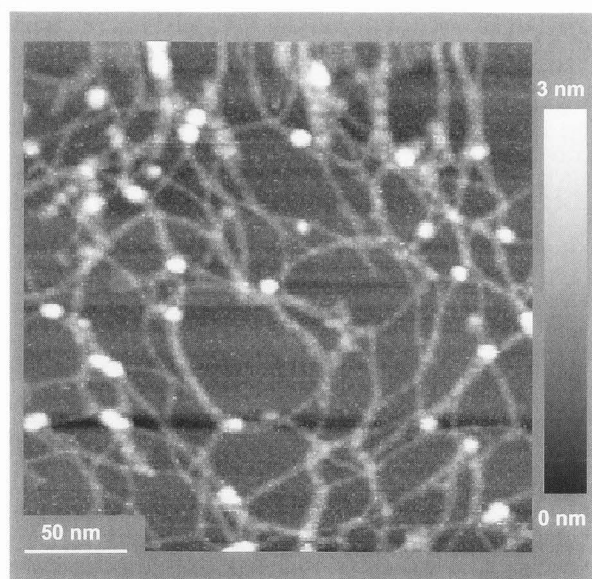

Figure 2: HSTM image of collagen IV molecules on mica. At 53 % relative humidity the uncoated sample was imaged with a sample bias of -4.5 V and 0.06 pA.

----

a conventional washing procedure, which implies that plasma cleaning is not the crucial factor for water adsorption in humid air. The important point is to thoroughly clean the surfaces in order to make them sufficiently hydrophilic.

Mica. Mica physisorbs many biological materials and is smooth on atomic scale which makes it one of the most widely used substrates for investigation of biological materials in scanning probe microscopy (SPM). Like glass, freshly cleaved mica adsorbs water molecules to its surface and therefore becomes conductive when exposed to humid air.

Collagen. Type IV collagen forms long (approximately 320 nm) triple-helical strands (approximately 1.5 nm in diameter) with a terminal globular domain. Individual collagen strands interconnect with each other either via this globular domain or via a rod-like segment at the opposite end (Timpl *et al.*, 1981). Fig. 2 shows the image of collagen IV molecules on mica imaged with the HSTM. The thinnest filaments visible in the image probably are single collagen molecules whereas the wider filaments are likely to consist of several molecules. The protrusions in the image can be attributed to the globular part of the collagen molecules, since such features appear exclusively on the filaments. The measured heights of the thinnest filaments are about 0.5 nm in contrast to their real height of 1.5 nm.

Similar to collagen, plasmid DNA can be prepared on mica and imaged with the HSTM method (Guckenberger *et al.*, 1994).

TMV. The described method also can be applied for biological specimens several nanometers thick such as tobacco mosaic virus (figure 3). The TMV virus consists of a RNA strand which is encapsulated in helically arranged proteins forming a 300 nm long cylinder. The diameter of the cylinder is 18 nm. Viruses which have lost their central RNA often break into several pieces and thus virus tubes much shorter than 300 nm can be found. The HSTM image delivers a TMV height of 15 nm which corresponds to the STM experiments of metal coated TMV (data not shown).

Lipid Bilayer. Fig. 4 shows the image of a lipid bilayer membrane on mica consisting of DPPE as the first layer and DPPC as the top layer prepared with Langmuir-Blodgett technique. This example shows that HSTM imaging works on films of organic molecules which are extended laterally over a large area. The surface consists of polar DPPC headgroups which adsorb water from the humid air and therefore produce a conductive surface. Defects are seen in the form of circular holes, which are about 6 nm deep, as expected for a DPPE/DPPC bilayer.

If only one lipid layer is transferred on mica, the surface becomes hydrophobic, since the hydrocarbon chains of the lipids then point towards the air. No water adsorbs on such a surface, and thus no surface conductivity is measured (Heim *et al.*, 1995). Examination of such specimens with the HSTM method is not possible.
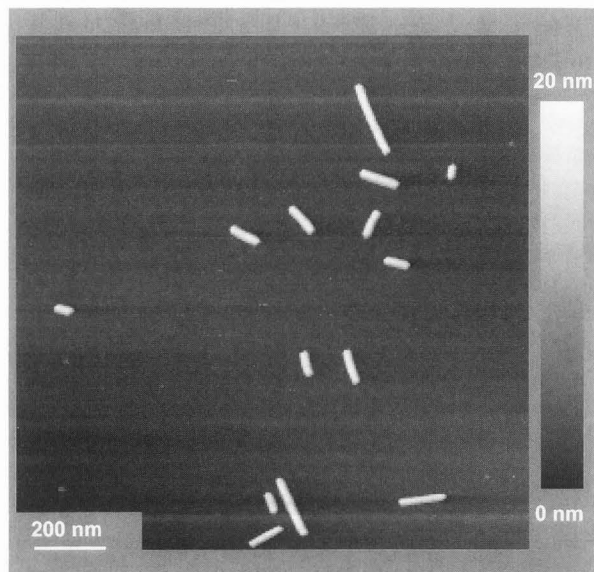
75

Figure 3: Uncoated tobacco mosaic virus on mica, imaged by HSTM at -7.2 V, 0.3 pA in the atmosphere of 72 % relative humidity. The height of the viruses measured with this HSTM method is 15 nm, which corresponds to the height value deduced from STM experiments with metal coated TMV.
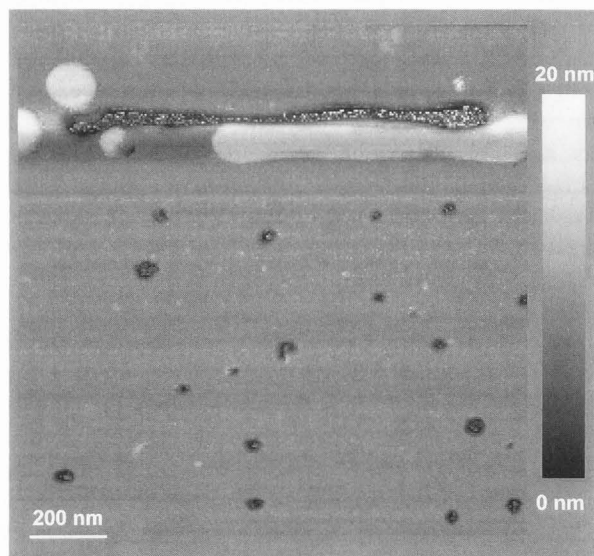

Figure 4: HSTM image of a lipid bilayer consisting of DPPE as the underlayer and DPPC as the top layer. The lipid layers were prepared with the Langmuir-Blodgett method (Heim *et al.*, 1995). The holes are about 6 nm deep, corresponding to the expected thickness of a DPPE/DPPC bilayer. In the upper part of the image, a piece of membrane is seen which has detached, and then came to lie near the rim of the hole. It is not clear if such a detaching of a membrane piece is tip induced or not. (Imaging parameters: +7.7 V sample bias, 0.25 pA, 50 % relative humidity.)
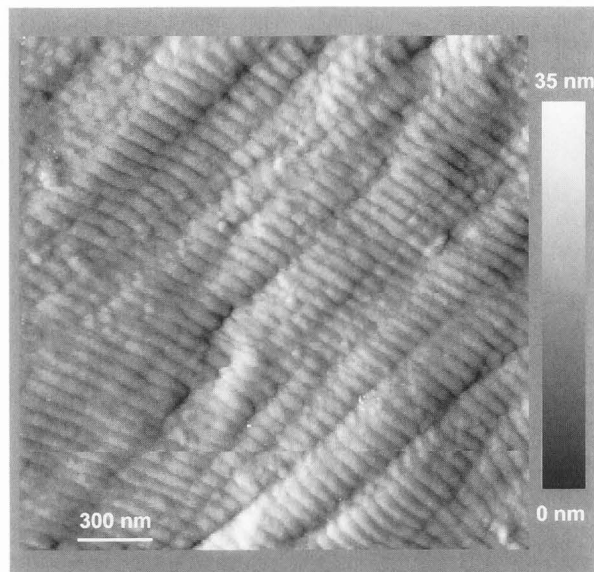

Figure 5: HSTM image of a cryosectioned bovine achilles tendon. (Imaging parameters: +7 V sample bias, 0.1 pA and 66 % relative humidity.) The sample is approximately 100 nm thick, extends over several millimeters in x-y direction, and its surface corrugation spans about 35 nm. In spite of its inhomogeneous surface topography it is hydrated uniformly enough to exhibit a sufficient conductivity for HSTM imaging. The surface reveals the d-band structure, well known from electron microscopical investigations.

Bovine tendon. Fig. 5 presents the image of a cryosectioned bovine achilles tendon which was prepared on a freshly cleaved mica surface. The well known d-band structure can be seen. Although the bovine tendon section is about 100 nm thick and shows a surface roughness of about 35 nm, it hydrates homogeneously enough to allow imaging with the STM at 66 % relative humidity. In this case, the current has to advance along the tendon surface over several millimeters until it reaches the smooth hydrated mica substrate or the conductive sample holder.

The results documented in this work show that STM on insulators is a straightforward procedure as long as the insulating surface is hydrophilic. Likewise, poorly conducting biological specimens can be imaged irrespective of their lateral and vertical size. Imaging is reproducibly possible without visible changes in the images after repeated scanning. However, during preparation, one has to ensure that the initial solution does not contain too many amphiphilic molecules since they can assemble into a monolayer covering the substrate and/or the sample surface, resulting in a hydrophobic and therefore, nonconductive system.

Independent of imaging, an aging effect of the surface-conductivity is observed on mica as well as on glass. The conductivity of mica decreases substantially within hours. This is probably due to contamination of the mica surface which reduces its surface hydrophilicity. Such contamination is directly visible in HSTM images of aged mica surfaces (several days after cleavage).

In contrast to thin filamentous biological specimens where the measured sample heights are mostly too small by a factor of up to 3 (shown for collagen and DNA), the HSTM method delivers true sample heights in the case of thick biological specimens, as demonstrated here with TMV and bovine achilles tendon. Sample heights of thin but laterally extended structures like mica steps (1 nm) are measured correctly with this method.

The imaging properties are as for all probe microscopy techniques highly dependent on the tip in use. In case of HSTM, some tips tend to build up a water meniscus between the hydrated surface and the tip. The build-up and break-down of a water meniscus leads to oscillations which prevent stable imaging (for details see (Heim *et al.*, 1996), for a discussion see (Fan and Bard, 1995) and (Guckenberger and Heim, 1995)).

Current-voltage relationship of thin water films on mica

The physical mechanism for the surprisingly high conductivity of surface adsorbed water is still unclear. To shed some light on this surface conduction phenomenon we measured the current-voltage relationship of thin water films on mica.

Fig. 6 shows a representative I-V map measured at 50 % relative humidity displaying a strictly linear behavior. Nothing indicative of conventional electrochemical reactions like peaks or a threshold voltage, can be discerned in the I-V map. Scan speeds between 2 V/s and 0.1 V/s gave identical linear behavior in the I-V map. (The plot shown was recorded with 0.1 V/s). The described experiment can also be done with tungsten tips and results in similar maps.

Only in a few cases do peaks appear in the I-V maps, for example. at higher voltages, under very humid conditions or after surface treatment (more detailed experiments are ongoing). This indicates that electrochemical reactions can contribute to the surface conductivity but the major factor of conductivity is a non-electrochemical conduction phenomenon. Recent experiments revealed that the charge transfer between the tip and the sample during imaging is based on the tunneling effect and not on electrochemistry in a tip-sample water bridge. This is indicated by the exponential increase of the current when approaching or retracting the tip from the sample (Guckenberger and Heim, 1995; Heim *et al.*,
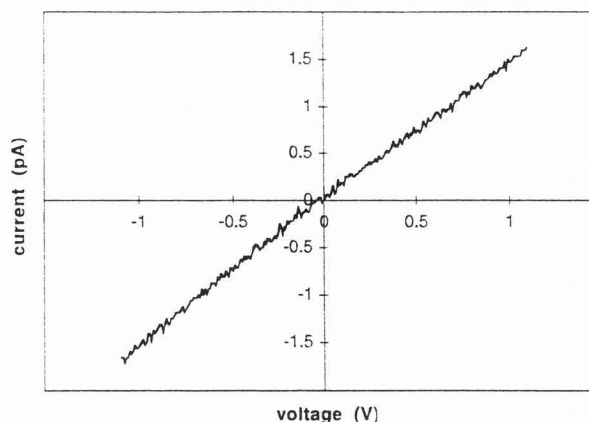

Figure 6: Voltage-current relationship on mica in air at 50 % relative humidity. The map was recorded with a gold tip in mechanical contact to the hydrated mica surface. The second electrode was a ring-shaped, gold-covered sample holder. The hysteresis between increasing and decreasing voltage scan is negligibly small. Therefore, only the curve of increasing sample bias is shown. The linear behavior indicates a non-electrochemical conduction phenomenon. The scan speed was 0.1 V/s.

1996). Thus, our results support the proposal that the conduction phenomenon is due to the surface-adsorbed water layer itself, rather than conventional ion movement.

Conclusions

The images presented in this work document that STM based on the conductivity of surface adsorbed water molecules, termed hydration-STM (HSTM), is a reliable method to image hydrophilic insulators and biological specimens, irrespective of their lateral and vertical size, or their surface roughness. Repeated imaging of the same sample areas can be done without changes in the image. The prerequisite for imaging with the hydration-STM method is that the specimens are hydrophilic, which is the case for most biological materials. The second condition for successful HSTM imaging is that the STM is able to work with low currents (below 1 pA). The specimens are exposed to humid conditions where they adsorb water from the ambient air. Thereby, the hydrated surface becomes sufficiently conductive for STM imaging.

The heights of the HSTM-imaged biological material are realistic for specimens several nanometer thick, whereas in case of thin filamentous samples, the measured heights are too low. In contrast, mica step-heights are measured correctly with HSTM.

The current-voltage relationship at 50 % relative

humidity revealed a strictly ohmic behavior of the thin water films on mica. Therefore, we conclude that a non-electrochemical conduction mechanism is responsible for the surface currents. This knowledge, and the previously published result that electronic tunneling takes place between tip and hydrated mica, leads us to assume that a specific structure of the surface water is responsible for the current transport in HSTM.

Acknowledgements

References

Bockris JOM, Jeng KT (1990) Water structure at interfaces: the present situation. Adv Colloid Interface Sci **33**: 1-54.

Fan F-RF, Bard AJ (1995) STM on wet insulators: electrochemistry or tunneling ? Technical comment. Science **270**: 1849-1851.

Guckenberger R, Kösslinger C, Gatz R, Breu H, Levai N, Baumeister W (1988) A scanning tunneling microscope (STM) for biological applications: design and performance. Ultramicroscopy **25**: 111-122.

Guckenberger R, Heim M, Cevc G, Knapp HF, Wiegräbe W, Hillebrand A (1994) Scanning tunneling microscopy of insulators and biological specimens based on lateral conductivity of ultrathin water films. Science **266**: 1538-1540.

Guckenberger R, Heim M (1995) STM on wet insulators: electrochemistry or tunneling ? Response to technical comment. Science **270**: 1851-1852.

Hacker B, Hillebrand A, Hartmann T, Guckenberger R (1992) Preparation and characterization of tips for scanning tunneling microscopy of biological specimens. Ultramicroscopy **42**: 1514-1518.

Heim M, Cevc G, Guckenberger R, Knapp HF, Wiegräbe W (1995) Lateral electrical conductivity of mica-supported lipid bilayer membranes measured by scanning tunneling microscopy. Biophys J **69**: 489-497.

Heim M, Eschrich R, Hillebrand A, Knapp HF, Cevc G, Guckenberger R (1996) STM based on the conductivity of surface adsorbed water. Charge transfer between tip and sample via electrochemistry in a water meniscus or via tunneling? J Vac Sci Tech B **14**:1498-1502.

Hu J, Xiao XD, Ogletree DF, Salmeron M (1995) Imaging the condensation and evaporation of molecularly thin films of water with nanometer resolution. Science **268**: 267-269.

Israelachvili J (1992) Special interactions: hydrogen-bonding, hydrophobic and hydrophilic interactions. In: Intermolecular and Surface Forces. Academic Press, London.

Israelachvili J, Wennerström H (1996) Role of hydration and water structure in biological and colloidal interactions. Nature **379**: 219-225.

Knapp HF (1996) Electrochemical etching of au tips. In: Procedures in Scanning Probe Microscopies. Part One: Instrumentation. Heckl W, Guckenberger R, eds, John Wiley and Sons, Chichester, in press.

Maivald P, Butt HJ, Gould SAC, Prater CB, Drake B, Gurley JA, Elings VB, Hansma PK (1991) Using force modulation to image surface elasticities with the atomic force microscope. Nanotechnology **2**: 103-106.

Mate CM, McClelland GM, Erlandsson R, Chiang S (1987) Atomic force microscopy studies of frictional forces and force effects in scanning tunneling microscopy. Phys Rev Lett **59**: 1942.

Radmacher M, Tillmann RW, Gaub HE (1993) Imaging viscoelasticity by force modulation with the atomic force microscope. Biophys J **64**: 735-742.

Rohrer H (1990) Scanning Tunneling Microscopy - Methods and Variations. Kluwer, Dordrecht, Netherlands. pp 1-25.

Timpl R, Wiedemann H, Van Delden V, Furthmayr H, Kühn K (1981) A network model for the organization of type IV collagen molecules in basement membranes. Eur J Biochem **120**: 203-211.

Discussion with Reviewers

M. Amrein: The authors ascribe the high surface conductivity of their samples to the ordering of the water layer at the interface to the hydrophilic solid. However, it is the hydrophobic surfaces that are known to increase the ordering of the water in the proximity of the interface. Is this not somewhat contradictory?
Authors: The ordering of the water molecules has been reported on hydrophobic as well as on hydrophilic substrates (Bockris and Jeng, 1990; Toney *et al.*, 1994). On a hydrophilic substrate ordered water molecules can be present on the surface in a thin film without surrounding bulk water. At the surface of a hydrophobic substrate, it is the surrounding bulk water (together with the hydrophobic surface) which enforces the ordering. Without surrounding bulk water the water molecules would flee the interface. We measured electrical conductivity in thin surface adsorbed water layers on a hydrophilic substrate. However, such a conduction phenome-

non might also occur in ordered water layers in the vicinity of a hydrophobic surface. To measure the conductivity of ordered water on a hydrophobic substrate is more difficult since the surrounding bulk water will deliver a high background signal.

M. Amrein: It is widely believed (and proven in many cases) that upon air-drying, the surface tension of the water causes a protein sample to collapse. Do you expect your samples to be in a native state?

Authors: Since our sample preparation was very similar to an air drying procedure we expect that the surface tension of the water will exert forces to the samples. Another effect of drying any biological specimen is the loss of structural water. However, this surface water which contributes to the stability of biological macromolecules is preserved when imaging in humid atmosphere. Conditions of high humidity in some cases even suffice to preserve biochemical activity, as it is true for bacteriorhodopsin in purple membrane.

A. Quist: How close to the surface can you go without building up a water meniscus between the tip and the surface? You describe that a water meniscus is absent; can you relate the presence/absence of a water meniscus between tip and sample to the absence/presence of a specific structure of the surface adsorbed water?

Authors: To evaluate the distance between the tip and a hydrated sample surface is quite difficult and we can only indirectly estimate this distance. In experiments where the tip was approached, and subsequently retracted from a hydrated mica surface while recording the electrical current, we found that a water meniscus between tip and sample could be pulled to a length of only about 1 nm (Heim *et al.*, 1996). This indicates that the tip can approach the sample to a distance of probably 1 nm or less without the build-up of a water meniscus.

We can only speculate about the structure of the water in case of the presence of a water meniscus.

The water molecules are probably bound quite strongly to a hydrophilic substrate, decreasing the tendency of the surface adsorbed water to build up a water meniscus even when the tip approaches within molecular distances. The forces between substrate and water molecules would on the one hand, induce the ordering of the water (and thus induce the conductivity) and on the other hand, prevent the build-up of a water meniscus between the tip and surface.

A. Quist: The collagen is measured at one third of its true height. Is collagen (or other small molecules) "buried" partially in the water layer, resulting in a lower height measurement?

Authors: If the collagen molecules were partially buried in the water layer (without any flattening of the molecules itself) the measured height would be reduced by no more than the thickness of the water layer on the surrounding mica (less than 0.5 nm according to (Beaglehole *et al.*, 1991) and our measurements). The height of thin filamentous molecules like collagen or DNA is found to be reduced by more than 0.5 nm (which is found also in most AFM experiments). This indicates that the measured height artifacts cannot arise from the water layers alone. One possibility is that the conductivity on the collagen molecules is reduced, resulting in a decreased tip-sample distance on the collagen causing reduced height values of the molecules. Another reason for the reduced heights could be real flattening of the samples due to the influence of surface forces. This interpretation is supported by the observation that thin but laterally extended biological objects (e.g., the lipid layers) do not show such a dramatic height artifact. Such layers can not be so easily flattened since the lipid molecules are stabilized by neighboring molecules. However, it is difficult to believe that helical structures like DNA and collagen are flattened so dramatically. Further experiments are necessary to understand the background of the observed height artifacts.

A. Quist: Different images are taken with very different biases, (+ and -). Why? Is there a difference for each sample?

Authors : It is possible to image all samples at all biases with a good quality. While doing the experiments we often changed the bias as well as the current without finding preferences for the different samples. As a general tendency, imaging seems to be more stable at positive sample bias (Heim *et al.*, 1996).

A. Quist: Can any of the holes observed on glass be due to patchy adsorption of water on the surface?

Authors: The holes greater than ten nanometers in diameter and several nanometers deep, do not arise from patchy adsorbed water. If there were no holes at these places, the tip would approach several nanometers to the glass, hit the glass surface and break. Furthermore, these holes appear in AFM images of the samples as well as in STM images of metal coated coverslips.

The smaller (2 - 5 nm in diameter) holes, which appear to be only 1 - 2 nm deep are not visible in AFM images, and at least in principle, could arise from holes in the water layer. At such places the conductivity is reduced or even nonexistent leaving a hole in the HSTM image. Nevertheless, we think that the small holes are real topographical holes since we have never found such phenomena on other substrates like mica or thermally oxidized silicon wafers.

Additional References

Beaglehole D, Radlinska EZ, Ninham BW, Christenson HK (1991) Inadequacy of lifshitz theory for thin liquid films. Phys Rev Lett **66**: 2084-2087.

Toney MF, Howard JN, Richer J, Borges GL, Gordon JG, Melroy OR, Wiesler DG, Yee D, Sorensen LB (1994) Voltage-depending ordering of water molecules at an electrode-electrolyte interface. Nature **368**: 444-446.