



STM on Wet Insulators: Electrochemistry or Tunneling?

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Science, New Series, Volume 270, Issue 5243 (Dec. 15, 1995), 1849-1852.

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same as that of bulk solvent (6), we conclude that these water molecules are positionally disordered.

Matthews *et al.* also state that as a control one would expect methyl protons that are more than 5 Å from either crystallographically identified water, or the cavity should not display NOEs to water. In this regard they cite the methyl protons of Val⁵⁸ for which NOEs to water are observed despite the fact that the γ_1 and γ_2 methyl groups are 5.1 and 7.0 Å away, respectively (in terms of C-O distances), from the nearest crystallographically observed water molecules, 10 Å and 8 Å, respectively, from the cavity, and 5.1 and 7.2 Å, respectively, from the bulk water at the protein surface. Inspection of the structure, however, reveals that the methyl groups of Val⁵⁸ are only shielded from bulk solvent by the side chains of Met⁴⁴ and Lys¹⁰³. The distance from the methyl groups of Val⁵⁸ to the N ζ H₃ group of Lys¹⁰³ is too long (>5 Å) to make an indirect mechanism involving an NOE to Lys¹⁰³(N ζ H₃) followed by chemical exchange with water likely. Hence, we suggest that the side chains of Met⁴⁴ and Lys¹⁰³ are sufficiently flexible to permit access of water to the methyl groups of Val⁵⁸. However, not all methyl groups exhibit NOEs to water. For example, no NOEs or ROEs are observed to the methyl groups of Leu⁷³ despite the fact that they happen to be on the surface of the protein. A further control is provided by a number of other systems in which these experiments have been used to study water of hydration and in which no NOEs to buried methyl groups were observed unless they happened to be in close proximity to structural water (5, 7).

Finally, Matthews *et al.* state that the closest approach that a water molecule of radius 1.4 Å within the cavity of IL-1 β can make to the C δ 2 atoms of Leu¹⁰, Leu²⁶, Leu⁶⁰, and Leu⁸⁰, respectively, are 5.3, 5.7, 5.3, and 6.0 Å, respectively. However, two additional factors need to be taken into consideration. First, the important distance as far as the NMR experiment is concerned is the distance to the methyl protons and not to the methyl carbon, which in this case will be approximately 1 Å further away from the 1.4 Å radius probe. Second, a water molecule is, strictly speaking, not spherical, and a better probe radius is around 1.2 Å (8); once again it is the distance to the protons and not the oxygen which needs to be considered. Hence, the interproton distance of closest approach is significantly less than 5 Å.

In conclusion, there is little doubt that there is positionally disordered water within the hydrophobic cavity of IL-1 β . This cavity, however, is not totally isolated from bulk solvent. As noted in our report (1), there are two small channels (1.9 \times 0.4 Å

and 1.7 \times 1.6 Å in cross-section) that could readily permit penetration of water provided that they expand transiently, and indeed IL-1 β displays inherent conformational flexibility (9). In this regard, the cavity in IL-1 β is different from those generated artificially in T4 lysozyme which are completely sealed off from solvent (10). While these cavities appear to be empty crystallographically, it remains to be tested experimentally by NMR whether positionally disordered water is present within the T4 lysozyme cavities.

The NMR and crystallographic concepts of occupancy are critical to our paper (1). The widespread failure to observe any significant electron density within non-polar cavities indicates that the occupancy is indeed low in the crystallographic sense. This may be due to one of two factors: (i) either no water is present or water is present for only a small fraction of the time; or (ii) the potential of mean force at any given point within the cavity does not have a well-defined minimum so that water molecules never return to the same position and the electron density is consequently smeared out beyond the level of detection (that is, the water is positionally disordered) (11). If the former were true, no water would have been observed by NMR as the intensities of the NOEs would have been attenuated proportionately. If the latter were true, on the other hand, water would be observed by NMR, as the NMR experiment does not require uniform ordering but is only dependent on spatial proximity, providing the lifetime of the bound water exceeds about 1 ns.

STM on Wet Insulators: Electrochemistry or Tunneling?

Reinhard Guckenberger and his co-workers (1) describe the use of a scanning tunneling microscope (STM) with high current sensitivity to image DNA on a mica surface in humid air. Because the distance between the STM tip and the sample contact was large in these experiments, current flow and imaging by direct electronic tunneling from the tip to the contact was not possible. We propose that the imaging occurs by an electrochemical mechanism similar to that which occurs in scanning electrochemical microscopy (SECM).

In SECM, the faradaic current produced by an electron-transfer reaction at a small tip can be used to image electronically conductive or insulating surfaces immersed in a liquid phase (2, 3). Usually the sample is placed under a thick liquid layer, and the tip must be sheathed in an insulator (glass or wax). Difficulties in tip fabrication limit

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1 June 1995; accepted 27 June 1995

their size and hence the attainable lateral resolution. SECM measurements can also be carried out within the thin film of water that forms on the surface of a sample in air, and high resolution can be attained by using tips without insulation (that is, the usual Pt-Ir or W STM-type tips) because the tip area is defined by the small part of the tip that touches the liquid layer. We used a similar strategy to fabricate small metal structures in a Nafion film by SECM, although the attainable resolution at that time was only in the 0.5- μ m regime and imaging was not reported (4).

Our instrument can make both SECM and STM measurements with a vertical (z direction) resolution of better than 1 Å and a current sensitivity down to 50 fA with the proper filter (2). The SECM scanning head was contained in a Faraday cage and a Plexiglas box in which the relative humid-

ity (RH) of the atmosphere was controlled by a beaker of the appropriate solution (5, 6) (Fig. 1).

The mica sample was treated with one drop of a solution of 10 mM tris buffer and 1 mM EDTA (disodium salt), pH 7 (solution A), for 5 min. The sample was then dipped (1 s) in water, dried for 5 min in air, and dipped again for 1 s in water. Excess water on the surface was carefully removed with filter paper and the sample was mounted in the SECM chamber and allowed to equilibrate with the atmosphere within the box. When a W or Pt-Ir tip (biased at +3 V) approached the sample at 3 nm/s, the current remained at zero until it contacted the liquid layer, where it increased by several orders of magnitude to about 20 pA over a distance of 1 nm. Because the Au contact in these experiments was 1 to 2 mm from the tip, and direct electron tunneling through the water layer was not possible, the current must be faradaic, with electrochemical processes occurring at both tip and Au contact. As reported previously (1), this arrangement can be used for imaging (Fig. 2A). The step running diagonally from the lower left corner represents a cleavage plane for a single mica layer, ~1 nm high, and the small pieces seen on the lower plane probably represent salt residue or debris left after cleavage. A thin film (~200 nm) of Nafion

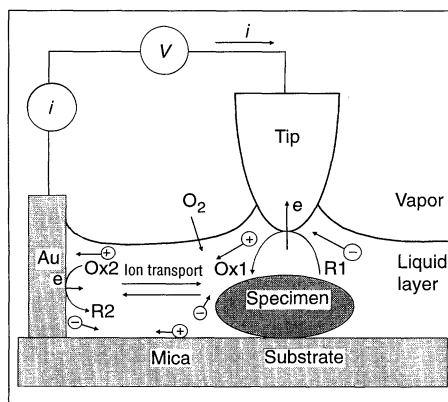


Fig. 1. Schematic diagram for the SECM chamber with controlled humidity and the electrochemical processes that control the current. The SECM tip was located ~1 to 2 mm from the Au contact. *V*, voltage bias between the tip and Au contact; *i*, current flow through the tip; R and Ox, reduced and oxidized forms of an electroactive species; ⊕ and ⊖ cations and anions in the water film. Electrochemically etched tips were prepared from either 250- μ m-diameter Pt-Ir (80 to 20%) [etchant, saturated CaCl_2 : concentrated HCl:water (60%:4%:36% by volume)] or 500- μ m W wire (etchant, 3 M NaOH). A constant humidity within the box was usually attained within 1 hour as determined by monitoring the surface conductance of the mica sample (7). Freshly cleaved mica substrates (Spruce Pine Mica, Spruce Pine, NC) were vacuum-coated with about a 500 Å thick layer of gold at one end; this served as the reference-to-counter electrode contact.

on mica imaged by this technique (Fig. 2B) shows smaller circular structures, 1 to 2 nm in diameter, consisting of a conductive central zone surrounded by a less conductive region. These structures correspond to those proposed in Gierke's cluster model for a Nafion membrane (7) in which a central hydrophilic domain consisting of backbone ions and water is surrounded by a Teflon-like hydrophobic zone. This image demonstrates that SECM can distinguish between zones of different ionic conductivity in a sample. As reported by Guckenberger *et al.* (1), images of DNA on mica could also be obtained in this configuration.

Further information about the mechanism of current flow was obtained from current-voltage (*i*-*V*) curves under different conditions. A rather blunt tip (radius of

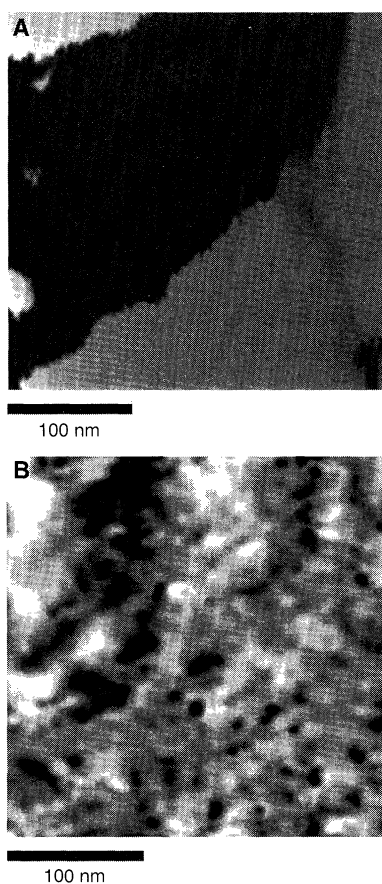


Fig. 2. (A) Image of a mica surface treated with solution A taken in humid air (80% RH and 25°C) with a sharp W tip at a reference current of 0.3 pA and a tip bias of 3 V. The tip rastering rate was 0.25 Hz. The total *z* range is 3 nm. (B) Image of a Nafion film on mica taken in humid air (100% RH at 25°C) with a sharp Pt-Ir tip at a reference current of 3 pA and a tip bias of 3 V with respect to the counter electrode. The tip rastering rate was 0.25 Hz. The image is inverted to enhance the visual effect of the domain structure; the dark region has a higher current than the lighter region. The film was spin-coated (3000 rpm with a Headway Research, Garland, TX, photoresist spinner) from an isopropanol-ethanol (4:1 by volume) solution containing 5% Nafion.

curvature ~10 μ m) was brought into contact with the sample and the piezo-feedback was switched off to avoid changes in the tip-substrate gap during the potential scan (Fig. 3). The curve for a Nafion film with a Pt-Ir tip (Fig. 3A) shows some hysteresis in the range of +1 to -1 V where picoampere currents flow. Outside this range the curves become linear and are largely independent of scan direction. The shape of the curves is consistent with an electrochemical response. Low currents flow until the applied voltage is sufficient to drive electrochemical reactions, presumably mainly water electrolysis, at the tip and Au contact. Above this region, the current is limited by the resistance of the thin film (~7.5 $\times 10^{11}$ ohms). The *i*-*V* curves with a blunt Pt-Ir tip on a mica substrate treated with solution A are more complex and depend on scan number (Fig. 3B). Thus, for scans from 0 V with the tip potential biased negative (C1 to C3), the *i*-*V* relation is fairly linear with the current increasing slightly on each scan. An initial scan of the tip toward positive poten-

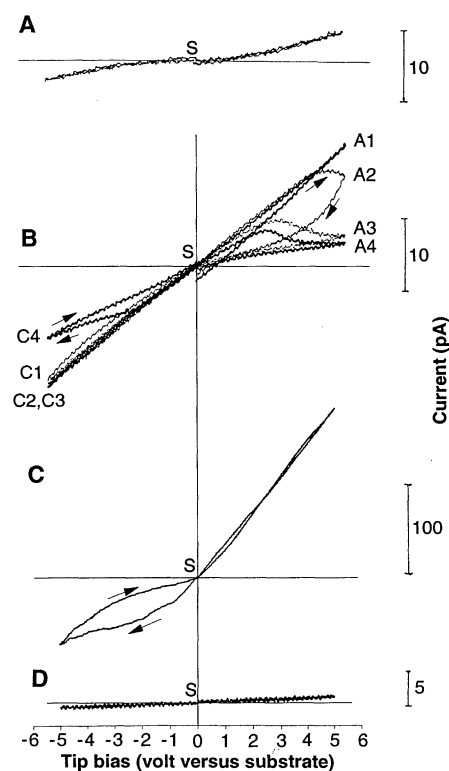


Fig. 3. Typical voltammograms in the humidity chamber at 100% RH, 25°C. Radius of tip curvature $\approx 10 \mu$ m. In all cases, the voltage was scanned from 0 V (point S) in either direction and then returned to 0 V; the scan rate was 0.2 V/s. (A) Pt-Ir tip, Nafion film (~200 nm thick) on mica. (B) Pt-Ir tip, mica substrate treated with solution A. Curves C1 to C3: first, second, and third negative scans after A4. Curve C4: first negative scan after A4. (C) W tip, mica substrate treated with solution A. (D) W tip, mica substrate treated with water only.

tials following these scans is also almost linear (A1). However, on successive scans (A2 to A4) a definite peak appears; the current decreases and the peak shifts to less-positive potentials on each scan. The voltammetric scan is restored if the tip is biased to negative potentials for about 2 min, suggesting that a species, probably H_2 , builds up around the tip at negative bias that is depleted during successive anodic tip scans. After a series of scans of the tip to positive potentials, an initial cathodic tip scan shows decreased current (C4), but gradually attains the linear behavior shown in successive cathodic scans. For purposes of imaging with a Pt-Ir tip, the current is fairly stable for negative tip bias values; with positive tip bias, oscillations frequently occurred.

The i - V curves also depend on the tip material. For a blunt W tip on mica treated with solution A (Fig. 3C), the current is stable and increases almost linearly for positive tip bias, but tends to saturate and shows considerable hysteresis on scan reversal in the negative tip-bias region. Thus, with a W tip, imaging at positive bias is appropriate.

However, for mica simply immersed in water for a few hours rather than treated with solution A, an i - V curve at 100% RH at a W tip (Fig. 3D) shows only small currents across a region of ± 5 V. The current at a Pt-Ir tip at 100% RH is smaller by at least one order of magnitude for a water-treated sample compared to one treated with solution A. The current under these conditions, however, was steady and showed little hysteresis. At low RH (for example, 33%), no appreciable current (<0.1 pA) was observed in the region of ± 5 V.

Our data generally confirm the results of Guckenberger *et al.* (1) concerning the ability to image materials on insulating substrates like mica, although we propose that the measured current represents faradaic processes at tip and contact rather than tunneling (Fig 1). They also point to the importance of water and SECM-type processes in imaging insulating materials like proteins with the STM in air, even on conductive substrates, as suggested by earlier studies (8, 9). Pretreatment of the insulating substrate to provide ions in the water film and thus increase its conductivity also appears to be useful. Presumably, after treatment with solution A sufficient ions remain adsorbed on the mica surface, even after washing, to yield some conductance in the water film that forms on exposure to humid air. The electrochemical signal could arise from both capacitive charging and faradaic processes. However, charging processes are transient and cannot sustain a steady direct current. Because the observed i - V curves are perturbed by resistive drops in the solution,

one cannot identify with certainty the nature of the faradaic processes at the tip and Au contact. At Pt-Ir, candidate reactions are oxidation of water to O_2 , reduction of water or protons to H_2 , reduction of dissolved O_2 , and processes involving adsorbed species, for example, oxidation of EDTA or adventitious impurities. At W, in addition to these processes, oxidation of the W to form the oxide (WO_3), and transiently, reduction of native or electrically formed oxide, are possible. Indeed, the large and stable currents with a W tip at positive tip bias compared to Pt-Ir suggest the W oxidation reaction occurs at the tip. The faradaic processes also generate ions that can contribute to the solution conductivity. Ions in the water layer also play an important role in establishing the double layers at both electrodes and providing charge compensation for electrogenerated species and also affect the hydrophilicity of the substrate surface and the thickness and structure of the water layer that forms on it.

The resolution attained, although better than that in previous SECM studies, still is not as high as that seen in STM of ordered substrates. Constant current imaging involves the end of the tip just being maintained in contact with the thin water layer covering sample and substrate. Improved resolution should result from lower RH conditions to decrease the thickness of the water film. Resolution is also a function of the exposed tip area and perhaps could be improved by treatment of the tip with a hydrophobic agent to decrease the wetted area above the contact point.

Many previous studies said to involve STM of electronically insulating materials in air or under liquid may have, in fact, involved SECM imaging. A straightforward way to distinguish between these (with conductive substrates) would be to contrast results in vacuum or dry air (where only tunneling is possible) to those in humid air, and also to investigate tip polarity effects. For example, using this type of comparison, we have previously shown that the formation of pits in highly ordered pyrolytic graphite was probably caused by an electrochemical rather than an STM-type process (10).

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11. Supported by the Robert A. Welch Foundation and the National Science Foundation. We thank X.-H. Xu, S. Ogawa, and S. Kim for stimulating discussions and technical assistance.

21 April 1995; accepted 13 June 1995

Response: Fan and Bard describe interesting experiments that are closely related to our own study. These experiments were performed on a background different from ours, that is, with electrochemistry as opposed to STM. Their results fit with our experiments. However, we do not agree with some aspects of their data interpretation.

Fan and Bard assume that the tip is in contact with a water film during STM imaging. We have done detailed measurements during tip approach (Fig. 1). The current did not show jumps as would be expected when the tip touches a water surface. On the contrary, the current increased steadily like a tunneling current between tip and a conducting surface. Only in some cases, depending on the actual tip, were jumps in the current observed, which we interpreted as contact of the tip with water. With such tips, however, it was not possible to get stable STM imaging of mica. In all our approach experiments, the tip was withdrawn far enough to ensure that no water bridge between tip and sample was present when the tip approach was started. In contrast to the interpretation given by Fan and Bard, we conclude that imaging in a tunneling mode, without contact of tip and conductive water film, is the correct description of at least our imaging conditions.

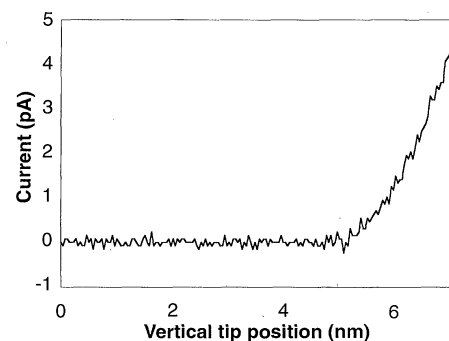


Fig. 1. Current versus change of tip position relative to the surface of freshly cleaved mica during tip approach (increasing tip position corresponds to decreasing distance). Voltage applied to the tip, -1 V; relative humidity, 61%.

The curves presented in figure 3 by Fan and Bard are taken as evidence of ordinary electrochemical processes. We agree that such processes will be involved, especially if the electrodes are partly immersed in water. However, we conclude from our experiments that other processes have to be taken into account. With platinum-carbon electrodes on mica, we have measured *i*-*V* curves that were essentially linear with only a small hysteresis (see also figure 3D in the comment of Fan and Bard). No substructure in the current curve was observed, not even in the voltage range between $\pm 2V$, where the electrochemical processes usually leave their signature. Therefore, we infer that ordinary electrochemical processes are not the only processes involved in the measured currents. From these and other experiments (1), we conclude that, in addition to ordinary ion conduction, other conduction pro-

cesses (such as proton hopping) contribute to or even dominate the observed surface conductivity.

Is it really possible to work at 100% relative humidity as implied in figures 2B and 3 of the comment? We expect that for such a humidity the thickness of surface-absorbed water films is ill-defined. In figure 2B of their comment, Fan and Bard state that they distinguish between zones of different ionic conductivity. In STM images, however, information about conductivity and topography is always superimposed and can only be separated directly if one of both quantities is constant. The image, taken in constant current mode, probably displays a nominal height contrast of 3 nm, assuming that the total *z* range is the same in figure 2, A and B. In contrast to this value of 3 nm, the decay length of the current with the tip-sample distance is about 1 nm or less, as measured by

Fan and Bard as well as plotted by us (Fig. 1). Therefore, it seems difficult to explain the nominal height range in figure 2B of the comment just by variations in conductivity. Drawing conclusions about local variations of conductivity is not as straightforward as described by Fan and Bard.

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31 May 1995; accepted 13 June 1995

18S rDNA from Lophophorates

Kenneth M. Halanych *et al.* analyze only one bryozoan for their study (1), and that species, *Plumatella repens*, is not representative of the Bryozoa as a whole (2). Thus, their conclusions are correspondingly disputable.

Plumatella repens belongs to the Phylactolaemata, an exclusively freshwater subdivision of this overwhelmingly marine phylum. The earliest fossil phylactolaemates are Cenozoic, whereas the rest of the Bryozoa (Gymnolaemata and Stenolaemata) originated in the Ordovician. Phylactolaemates produce statoblasts (asexual propagules similar in function to sponge gemmules); possess complete layers of body wall musculature, an epistome and U-shaped lophophore; and undergo budding from anatomically different body regions. Their larvae are distinct from gymnolaemate and stenolaemate larvae and are brooded differently. The differences between Phylactolaemata and other Bryozoa are so great that the burden of proof might best be placed on those who would unite these taxa within the same phylum. The results presented by Halanych *et al.* are interesting, but 18S

ribosomal evidence is incomplete until a bryozoan is studied that is more representative of the main line of bryozoan evolution.

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13 April 1995; accepted 12 August 1995

Response: Since the publication of our report, a new 18S ribosomal DNA bryozoan (ectoproct) sequence, from a species within Gymnolaemata, has been reported and analyzed (1). These results indicate that the new bryozoan taxon, *Alcyonidium gelatinosum*, is on the protostome side of the metazoan tree and within the lophotrochozoan clade. Hence molecular data supporting our

major conclusions are now available for two of the three bryozoan classes.

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15 November 1995; accepted 16 November 1995