Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7652–7655, August 1993
Biophysics

Masking generates contiguous segments of metal-coated and bare DNA for scanning tunneling microscope imaging

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Communicated by Calvin F. Quate, April 12, 1993 (received for review February 5, 1993)

ABSTRACT **To date, no microscopic methods are available to confirm scanning tunneling microscope (STM) images of DNA. The difficulties encountered in repeating these images may be attributed to inadequate distribution of molecules on the substrate, poor adhesion to the substrate, or the low conductivity of the molecules. However, these factors are difficult to assess in an STM experiment where they may act simultaneously. A method to isolate these factors involves partly masking the deposited molecules before coating them with a conductive film to produce adjacent segments of coated and bare DNA after the mask is removed. The coated DNA segments are conductive and mechanically stable to allow easy identification of DNA by the STM. Furthermore, the path of a molecule can be traced from a coated to an uncoated region to test STM imaging of bare DNA. Masked preparations of DNA deposited on platinum/carbon-coated mica and highly oriented pyrolytic graphite were examined with a tunneling current 1000 times lower than the usual nanoamps. The tip apparently displaces molecules adsorbed to graphite to preclude imaging whereas more stably bound DNA on platinum/carbon-coated mica appears in reversed contrast.**

Scanning tunneling micrographs have been reported for bare DNA on substrates in vacuum (1, 2), in air (3–14), and in solution (15). DNA bound to gold surfaces, either electrochemically (15) or by chemical modification of the substrate (14), can be imaged reproducibly, but the high-resolution views of DNA dried on untreated surfaces are difficult to repeat. In addition, artifacts have been reported on highly oriented pyrolytic graphite (HOPG), a commonly used substrate (16, 17). As a result, whether or not the scanning tunneling microscope (STM) can image bare DNA at atomic resolution is uncertain. Nonetheless, the potential for determining the structures of biomolecules and their complexes in aqueous environments emphasizes the importance of developing STM and similar scanning probe microscopic techniques for biology. Factors that affect STM images of DNA are the distribution of molecules on the substrate, the strength of their attachment, and their conductivity. These factors were not isolated in previous experiments, so it is difficult to understand why high-resolution STM images of DNA are elusive. However, these parameters are separable using a method that creates contiguous segments of conductively coated and bare DNA by evaporating platinum/carbon (Pt/C) through a mask placed over the DNA molecules. Metal-covered DNA segments are conductive, stably attached to the surface, and easy to identify in an STM experiment, so the deposition can be confirmed (18). More importantly, scanning adjacent bare segments determines whether bare DNA can be imaged by the STM. Scanning tunneling micrographs of DNA adsorbed on HOPG and Pt/C-coated mica show that DNA is substantially less conductive than previously indicated in STM experiments and that loosely bound molecules are swept away by the tip.

MATERIALS AND METHODS

DNA was deposited on HOPG or mica that had been coated with a thin film (6–10 nm) of Pt/C. The substrate was glow-discharged for 5 s before depositing 4 μl of λ DNA (0.75 μg/ml in 10 mM ammonium acetate) and air-drying. The mask, a perforated film of Formvar, was made by condensing vapor on a glass slide dipped in 0.05% Formvar dissolved in dichloroethane. The films were stripped from the slides onto water and picked up on transmission electron microscope grids. The holes were enlarged to a desirable diameter by etching in acetone vapor. The filmed side of the grid was placed against the substrate with DNA to act as a mask and the grid was held in place with dabs of paint. Platinum inserted into carbon rods (Balzers) was electron-beam-evaporated at a rate of 0.1–0.5 nm/s to a thickness of 2–6 nm in a vacuum of 1.75×10^{-6} millibars (1 bar = 100 kPa). The mask was removed before imaging in the scanning probe microscopes. Scanning tunneling micrographs were recorded using a NanoScope II STM (Digital Instruments, Santa Barbara, CA) using Pt/Ir tips cut from 80/20 wire (Ted Pella, Redding, CA). Scanning force micrographs were recorded with a TMX 2000 scanning probe microscope (TopoMetrix, Santa Clara, CA) using unmodified silicon nitride cantilevers.

To investigate imaging conditions that are more appropriate for poorly conducting molecules such as DNA, images were recorded using very low tunneling currents. Data in the picoampere range was collected using a custom-built low-current STM that consists of a patch clamp amplifier, CV-4 1/100 (Axon Instruments, Burlingame, CA), grafted into the Nanoscope II system. In this adaptation the sample is carried on the piezoelectric scanner and the amplifier is attached to a housing containing the tip holder (Fig. 1). This amplifier is convenient for investigating a broad current range, since it can be switched between high and low gain settings to detect currents ranging from 0.03 pA up to several nanoamps.

RESULTS AND DISCUSSION

The masking creates a dappled metal coverage of the surface as is shown in Fig. 2. Masks with closely spaced submicrometer holes create islands of Pt/C on the surface that are separated by a few hundred nanometers. This increases the probability that DNA molecules span the uncoated gaps. Such molecules are desirable, because it is easy to deduce their paths through the uncoated gap, and the segments of the molecules pinned under Pt/C islands stabilize the bare segment in between. One such molecule deposited on Pt/C-coated mica is shown in Fig. 3.

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Abbreviations: STM, scanning tunneling microscope; Pt/C, platinum/carbon; HOPG, highly oriented pyrolytic graphite.

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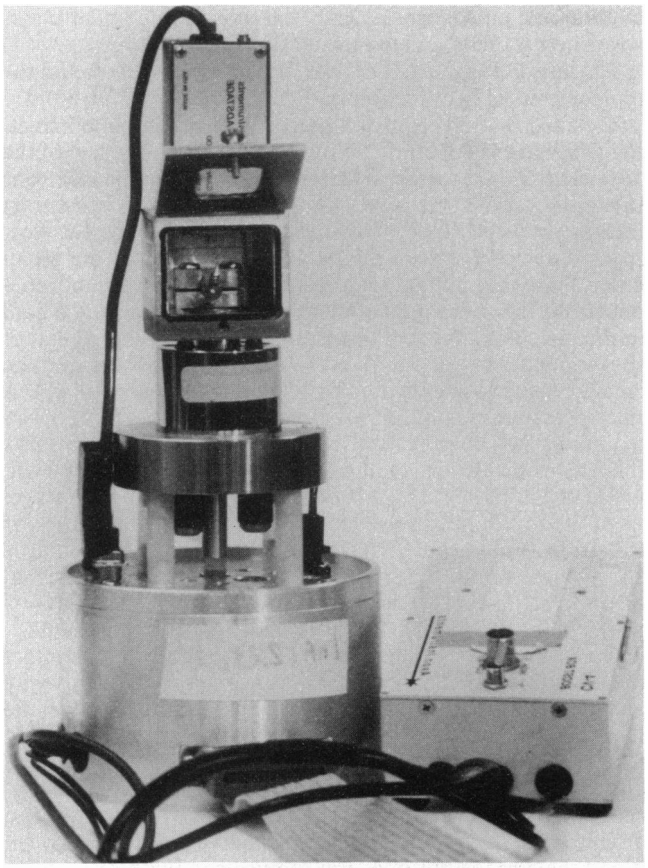

FIG. 1. In the custom-built low-current microscope, a patch clamp amplifier attached to the tip-holder housing rests on the piezoelectric scanner cradled in the microscope base. A gain control circuit stands beside the base.

In Fig. 3*A*, the path of a DNA molecule can be traced from the upper left toward the lower right. The coated segments appear as ridges, 1.1–1.7 nm tall, above and below the uncoated gap. Higher magnification of the gap area (Fig. 3*B*) reveals not a ridge but what appears to be a faint trench, 2–3

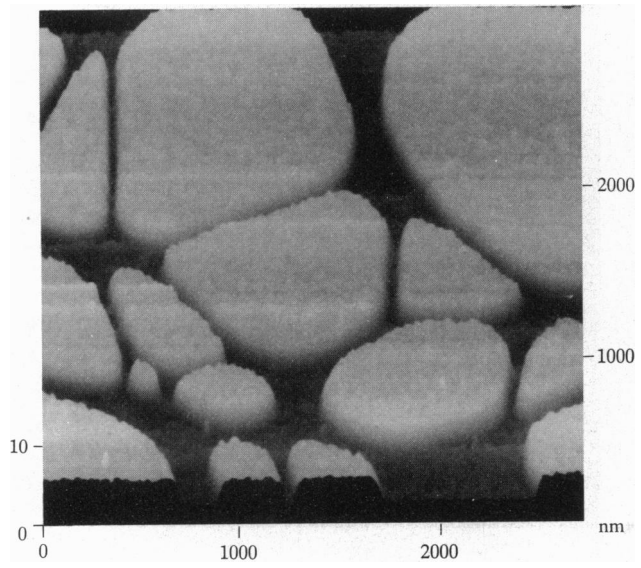

FIG. 2. STM image, viewed in perspective, shows a pattern of Pt/C islands 2 nm thick, electron-beam-evaporated onto a 6-nm-thick layer of Pt/C on mica. Tunneling current and bias voltage are 0.03 nA and 0.3 V, respectively.

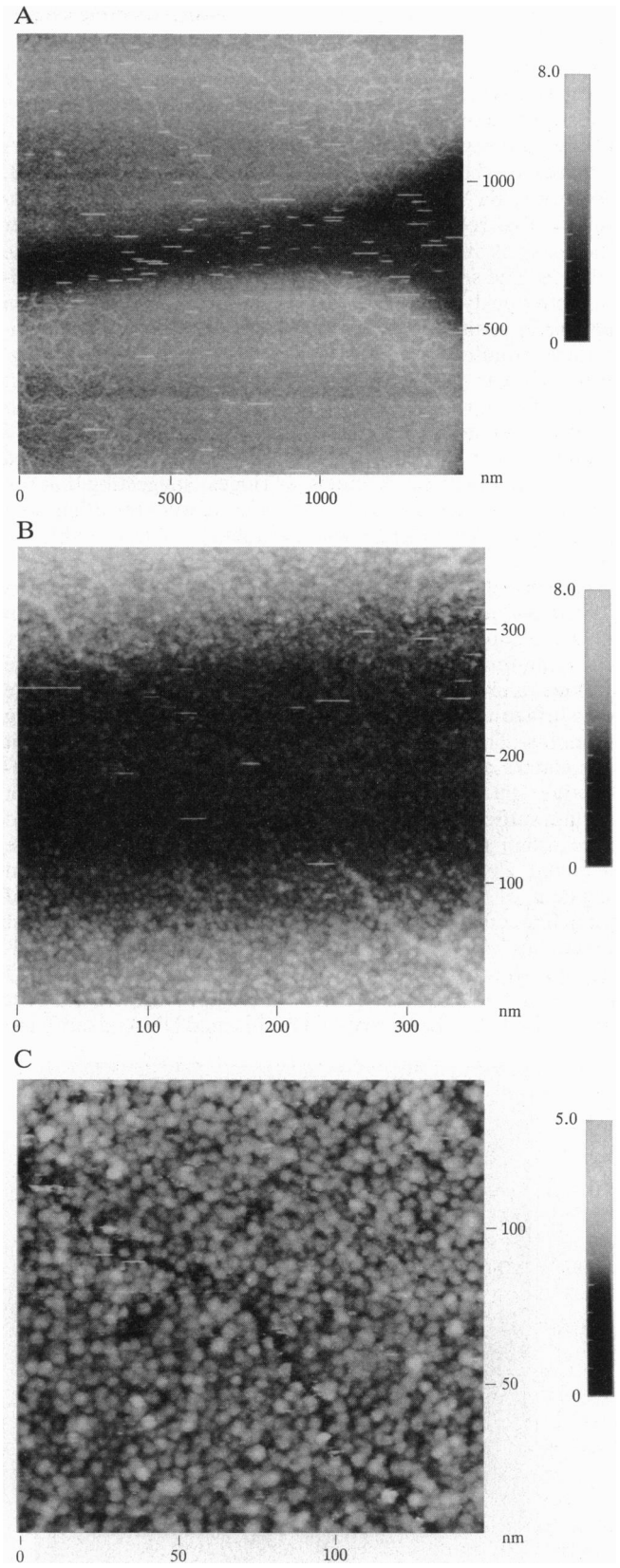

FIG. 3. (*A*) Single DNA molecule traverses a 250-nm-wide uncoated gap between the upper and lower Pt/C islands. Tunneling current and bias voltage are 0.07 pA and 0.1 V, respectively. (*B*) A ×178,000 view of the uncoated area traversed by the molecule in *A* shows what appears to be a shallow trench connecting the positively contrasted Pt/C-coated segments of the molecule on either side. (*C*) The trench, shown at ×435,000, averages 0.7 nm deep and 2.5 nm wide.

nm wide and 0.7 nm deep, connecting the flanking coated segments of the molecule. This trench has the lateral dimensions of DNA (Fig. 3*C*). The adjacent metal-coated segments form ridges protruding above the plane of the underlying substrate, so the topographically reversed (negative) contrast of the bare segment was unexpected. It could be that DNA adheres to the mask and is torn away as it is removed. However, STM images (data not shown) of masked preparations that receive an additional thin coating of Pt/C after the mask is removed show that the DNA remains on the surface. The second coating covers the entire surface including previously bare segments, and these segments then appear in positive contrast. Furthermore, in masked preparations examined by atomic force microscopy, all bare segments appear as ridges, indicating that the negative contrast observed is not an artifact of sample preparation. Fig. 4 is an atomic force micrograph of contiguous coated and uncoated segments of a DNA molecule. Both the metal-coated and bare segments of DNA appear as ridges, suggesting that the trench observed in the STM images is a feature resulting from mechanical and/or electronic interaction of DNA with the STM probe.

One possibility is that poor conductivity through the DNA molecules may cause the tip to approach them resulting in negative contrast. The tunneling current is an exponentially decaying function of the separation between the tip and the surface, and topographic images are formed by adjusting the tip–surface distance to maintain a constant current during scanning. Therefore, as the tip scans it will retract when it encounters a ridge corresponding to the metal-coated conductive DNA. However, if a bare DNA molecule cannot sustain sufficient tunneling current, the tip will extend toward it to attain the preset current. Thus bare DNA might be depicted as a trench. On one occasion a thinly coated segment of DNA was observed as a trench at 9 pA of tunneling current and a ridge at 0.09 pA. The contrast reversibly changed depending on the current setting, although some deterioration of the surface was observed presumably due to interaction with the tip. Negative contrast images have also been reported for plasmid DNA adsorbed to a modified gold surface (14) and for purple membranes deposited on Pt/C-coated mica (19).

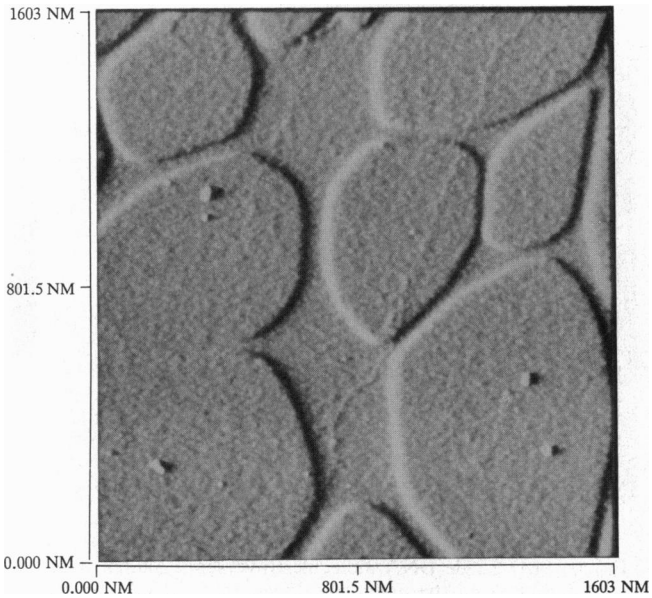

FIG. 4. Atomic force micrograph reveals the ridge of a DNA molecule covered by Pt/C extending downward across two islands from the upper left. It is also evident as a ridge in the uncoated area between two islands near the bottom. Simulated illumination is from the left.

An early STM image of DNA in vacuum also depicted the molecule in negative contrast (2). Travaglini *et al.* (3) hypothesized that electrons tunneled through the nonconducting DNA to the substrate below and that the presence of the adsorbed DNA increased the effective barrier for electrons tunneling across the gap. In numerous positive contrast images reported since, it was implicitly assumed that electrons tunneled between the tip and the surface of the molecule. This assumption requires that the electrons be conducted to the substrate below through a low-resistance path in the molecule. Yet, bulk measurements have shown that of the conductivity of DNA is several orders of magnitude smaller than those indicated by STM experiments (10, 20). A third mechanism offered to explain STM images of DNA maintains that compression of the molecule by the tip shifts the electronic levels of the molecule into resonance with those of the tip (21). In this condition, electrons may travel from the tip (or substrate depending on bias polarity) to the substrate (tip) through those modified levels. The images shown here do not discriminate between the above mechanisms, but they are evidence that the conductivity of DNA is lower than the values implicit in many STM experiments.

Given the low conductivity of DNA, the tip may contact the adsorbed molecules. In this case, it may tear them away along with a little of the underlying substrate. Thus the negatively contrasted molecules may be truly topographic features. To determine whether these trenches arise from electronic or topographic contrast, an atomic force microscope may be used to examine the same molecule. The atomic force microscope probe is sensitive to forces between it and the surface but does not require conductivity to trace the molecular topography. Relocating a particular molecule on a surface when switching microscopes can be tedious, but the islands generated by masking serve as large-scale guides that will make the experiment tractable.

To investigate how STM images of DNA depend on the substrate, a masking experiment using DNA on HOPG was performed. HOPG is hydrophobic and appears to bind DNA very poorly, so it may not immobilize the molecules sufficiently for imaging. However, it cleaves easily leaving an atomically flat conductive surface. Fig. 5 depicts several

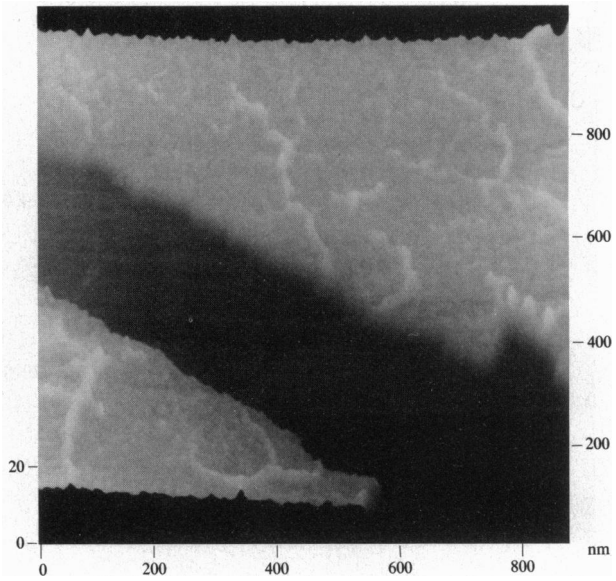

FIG. 5. Perspective view of a scanning tunneling micrograph shows several strands of DNA in the grainy Pt/C-coated areas flanking a diagonal band of clean HOPG. The tunneling current and bias voltage were 0.20 nA and 0.2 V, respectively.

DNA molecules deposited on HOPG and then masked before Pt/C evaporation. An uncoated stripe of HOPG runs diagonally from the upper left flanked by grainy Pt/C-coated areas. Several strands of DNA that appear in the coated areas can be traced to the edge of the coating, but no molecules are visible in the gap. The bare DNA in the gap appears to have been removed by the tip.

Comparison of the images of DNA deposited on Pt/C-coated mica and HOPG illustrates that mechanical fixation of the DNA on the surface is very important. The poor conductivity of the DNA obliges the tip to approach the surface possibly contacting the molecules in both preparations. Well-attached molecules appear in negative contrast on Pt/C-coated mica, but other loosely attached molecules and all of the DNA weakly adsorbed on HOPG apparently are removed by the tip. Besides providing mechanical stability, strong binding of molecules to the support may enhance their conductivity, so that sufficient current flows through them to reduce the perturbation by the probe. It should be noted that these results may depend on the particular conditions used for deposition and imaging. For example, the state of hydration of the molecules may alter their conductivity and change their contrast and these aspects have not been investigated.

In summary, DNA was deposited on two surfaces and examined by STM after partial coating with metal. Although DNA air-dried on HOPG could not be detected, negative-contrast images resulted when DNA was deposited on Pt/C-coated mica. These results were consistent for currents ranging from several nanoamps down to 0.03 pA. The effective gap resistance corresponding to the values of bias and current in Fig. 3 is 1.43×10^{12} Ω. The negative contrast displayed in this image implies a lower limit for the resistance of the deposited DNA that is three orders of magnitude higher than the average of values indicated in many previous STM images. Such poor conductivity appears to promote contact between the tip and adsorbed DNA molecules and is probably responsible for the poor reproducibility of scanning tunneling micrographs of DNA. Through the strength with which it mechanically fixes deposited molecules, the substrate determines the contrast observed (negative or none at all). Because the conditions under which DNA may be routinely imaged with the STM have not been defined, the micrometer-sized conductive regions created by masking before metal coating were essential to locate the molecules and determine their true topography. Since the metallic film supplants the conductivity of the adsorbed molecules and fixes them on the surface, this technique can serve as a control method for imaging poorly conducting molecules. Having this method at hand will simplify the interpretation of STM experiments on biomolecules.

We thank Steven B. Smith for help in adapting the patch clamp amplifier to the microscope and Fabio Biscarini for reviewing this manuscript. These studies were supported in part by National Science Foundation Grant MCB-9118482 and U.S. Public Health Service Research Grant GM-32543 (to C.B.) and by a grant from the Lucille P. Markey Charitable Trust to the Institute of Molecular Biology. R.G. acknowledges financial support from the Ministerio de Educación y Ciencia of Spain.

1. Driscoll, R. J., Youngquist, M. G. & Baldeschwieler, J. D. (1990) *Nature (London)* **346**, 294–296.
2. Binnig, G. & Rohrer, H. (1984) in *Trends in Physics*, eds. Jauta, J. & Pantoflicek, J. (Eur. Phys. Soc., The Hague, The Netherlands), pp. 38–46.
3. Travaglini, G., Rohrer, H., Amrein, M. & Gross, H. (1987) *Surf. Sci.* **181**, 380–390.
4. Beebe, T. P., Wilson, T. E., Ogletree, F. D., Katz, J. E., Balhorn, R., Salmeron, M. B. & Siekhaus, W. J. (1989) *Science* **243**, 370–372.
5. Cricenti, A., Selci, S., Felici, A. C., Generosi, R., Gori, E., Djaczenko, W. & Chiarotti, G. (1989) *Science* **245**, 1226–1227.
6. Dunlap, D. & Bustamante, C. (1989) *Nature (London)* **342**, 204–206.
7. Arscott, P. G., Lee, G., Bloomfield, V. A. & Evans, D. F. (1990) *Nature (London)* **346**, 706.
8. Bendixen, C., Besebacher, F., Lægsgaard, E., Stensgaard, I., Thomsen, B. & Westergaard, O. (1990) *J. Vac. Sci. Technol. A* **8** (1), 703–705.
9. Coratger, R., Chahboun, A., Ajustron, F., Beauvillain, J., Erard, M. & Amalric, F. (1990) *Ultramicroscopy* **34**, 141–147.
10. Keller, D., Bustamante, C. & Keller, R. W. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5356–5360.
11. Kim, Y. & Lieber, C. M. (1991) *Scanning Microsc.* **5** (2), 311–316.
12. Vold, T. G. (1992) *Scanning Microsc.* **6** (1), 139–145.
13. Zhu, J.-d., Lin, M.-q., Xiu, L., Zhu, J.-q., Hu, J., Gu, M.-m., Xu, Y.-l., Zhang, L.-p., Huang, Z.-q., Chernov, B. K., Nechipurenko, Y. D. & Tchurikov, N. A. (1992) *Biochim. Biophys. Acta* **1115**, 239–242.
14. Allison, D. P., Bottomley, L. A., Thundat, T., Brown, G. M., Woychik, R. P., Schrick, J. J., Jacobson, K. B. & Warmack, R. J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 10129–10133.
15. Lindsay, S. M., Tao, N. J., DeRose, J. A., Oden, P. I., Lyubchenko, Y. L., Harrington, R. E. & Shlyakhtenko, L. (1992) *Biophys. J.* **61**, 1570–1584.
16. Salmeron, M., Beebe, T., Odriozola, J., Ogletree, D. F. & Siekhaus, W. (1990) *J. Vac. Sci. Technol. A* **8** (1), 635–641.
17. Clemmer, C. R. & Beebe, T. P., Jr. (1991) *Science* **251**, 640–642.
18. García, R., Jiang, Y., Schabtach, E. & Bustamante, C. (1992) *Ultramicroscopy* **42–44**, 1250–1254.
19. Guckenberger, R., Hacker, B., Hartmann, T., Scheybani, T., Wang, Z., Wiegräbe, W. & Baumeister, W. (1991) *J. Vac. Sci. Technol. B* **9** (2), 1227–1230.
20. García, R. & García, N. (1990) *Chem. Phys. Lett.* **173**, 44–50.
21. Lindsay, S. M., Sankley, O. F., Li, Y., Herbst, C. & Ruprecht, A. (1990) *J. Phys. Chem.* **94**, 4655–4660.