Scanning Microscopy

Volume 4 | Number 3 Article 1

8-15-1990

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Recommended Citation

Weisenhorn, A. L.; Gaub, H. E.; Hansma, H. G.; Sinsheimer, R. L.; Kelderman, G. L.; and Hansma, P. K. (1990) "Imaging Single-Stranded DNA, Antigen-Antibody Reaction and Polymerized Langmuir-Blodgett Films with an Atomic Force Microscope," *Scanning Microscopy*: Vol. 4 : No. 3 , Article 1.
Available at: https://digitalcommons.usu.edu/microscopy/vol4/iss3/1

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This article is available in Scanning Microscopy: https://digitalcommons.usu.edu/microscopy/vol4/iss3/1

Scanning Microscopy, Vol. 4, No. 3, 1990 (Pages 511-516)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/90$3.00+.00

IMAGING SINGLE-STRANDED DNA, ANTIGEN-ANTIBODY REACTION AND POLYMERIZED LANGMUIR-BLODGETT FILMS WITH AN ATOMIC FORCE MICROSCOPE

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(Received for publication May 7, 1990, and in revised form August 15, 1990)

Abstract

The combination of an (AFM) atomic force microscope together with microfabricated cantilevers that have integrated tips opens many possibilities for imaging systems of great importance in biology. We have imaged single-stranded 25mer DNA that was adsorbed on treated mica or that was covalently bound with a crosslinker to a polymerized Langmuir-Blodgett (LB) film, the top monolayer of a bilayer system. At low magnification the AFM shows cracks between solid domains, like in an image taken with a fluorescence microscope. At higher magnification, however, the AFM reveals much finer cracks and at still higher magnification it reveals rows of individual molecules in the polymerized LB film with a spacing of 0.45 nm. We have also imaged a LB film consisting of lipids in which 4% of the lipids had hapten molecules chemically bound to the lipid headgroups. Specific antibodies can then bind to these hapten molecules and be imaged with the AFM. This points to the possibility of using the AFM to monitor selective antibody binding.

Key Words: single-stranded DNA, antigen-antibody reaction, hapten, polymerized Langmuir-Blodgett film lipid bilayers, atomic force microscope, fluorescence microscope

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Introduction

An atomic force microscope (Binnig *et al.* 1986) (AFM) can image soft surfaces of biological samples in water without destroying them (Marti *et al.* 1987, Drake *et al.* 1989, Gould *et al.* 1990a, Weisenhorn *et al.* 1990a, Egger *et al.* 1990). It was shown earlier that AFMs can also image insulating surfaces with atomic resolution (Binnig *et al.* 1987, Albrecht and Quate 1987), at 4 K (Kirk *et al.* 1988), and in vacuum (Meyer *et al.* 1988). AFMs have imaged the magnetic pattern of a thin film (Rugar *et al.* 1988, Mamin *et al.* 1988), organic films (Marti *et al.* 1988), polymeric liquid films (Mate *et al.* 1989), proteins (Weisenhorn *et al.* 1990b), membranes (Egger *et al.* 1990), DNA (Weisenhorn *et al.* 1990a), and adsorbed molecules on zeolites (Weisenhorn *et al.*1990c). The AFM's predecessor, the scanning tunneling microscope (STM) has also imaged lipids (Smith *et al.* 1987, Heckl *et al.* 1989) and DNA (Lindsay *et al.* 1989, Beebe *et al.* 1989, Lee *et al.* 1989, Dunlap and Bustamante 1989, Cricenti *et al.* 1989) with molecular resolution. High-resolution images of DNA have also shown submolecular details within the double helix.

LB films can be composed from amphiphilic molecules (*e.g.*, a hydrophobic hydrocarbon chain combined with a hydophilic headgroup) at the air-water interface and can be transferred to atomically flat surfaces like mica to form stable two dimensional quasicrystalline monolayers. Because of the large variety of possible headgroups, the molecules can be chosen specific to the experiment's need, without giving up the advantages of a flat surface. Furthermore, LB films are a model system for biological membranes.

Low magnification images of polymerized LB films, taken with both an AFM and a fluorescence microscope, show the same pattern and reveal the solid crystalline-polymerized domain structure of the LB film. The AFM was also used to image 25mer single-stranded DNA that was crosslinked to the film and to image antibody molecules that reacted with antigen molecules embedded in a LB film that consisted of lipid molecules.

Materials and Methods

DNA on polymerized LB films:

Monolayers were formed on the surface of a home-built Langmuir trough and transferred by standard Langmuir-Blodgett technique (Agarwal 1988, Heyn, Tillman, Egger, Gaub; submitted for publication). First, a monolayer of Cd-arachidate was transferred to freshly cleaved

Figure 1 a) Scanning electron micrograph image of a Si_3N_4 microfabricated cantilever with an integrated tip (Park Scientific Instruments, Mountain View, CA). This tip touches the surface and moves up and down while the sample is raster-scanned laterally under the tip. Laser light from a laser diode is reflected off the top of the cantilever towards a two-segment photodiode, which senses the deflection of the light and thus the vertical deflection of the cantilever. This allows the topography of the surface to be imaged. All the images were taken with the samples submerged in aqueous solution. For more details see Gould *et al.* (1990b). The dimensions of the cantilever are 100 μm along the cantilever arm, 13μm across one arm, and 0.6 μm in thickness. The force constant k is 0.21 N/m, the theoretical resonance frequency is 66 kHz (Park Scientific Instruments). **b)** Detail of the integrated tip. The base area of the pyramidal tip is 4 μm × 4 μm. Figures 2a,b, 3a,b, and 4 were imaged with this kind of tip.

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Figure 2 Low magnification images of a polymerized LB film of 10,12-pentacosadiynoic acid on Cd-arachidate/mica in water, **a)** as seen by the AFM with image size 11 μm × 11 μm and step height about 9 nm. Notice the micron-size particles that appear white in the image. The smallest visible cracks have widths of less than 50 nm. **b)** The polymerized LB film as seen by the fluorescence microscope. The diameter of the image is 200 μm. The smallest visible cracks are not less than 500 nm.

mica (Asheville-Schoemaker, Newport News, VA). Second, a monolayer of the polymerizable fatty acid, 10,12-pentacosadiynoic acid (Albrecht *et al.* 1984), that was purchased from ABCR (Karlsruhe, Germany) and used after recrystallization, was compressed to about 27 mN/m at the air-water interface of the Langmuir trough, polymerized by irradiating for about 30 seconds with a high-intensity UV light (mercury Penray), and then transferred onto the monolayer of Cd-arachidate on mica by vertical dipping. A synthetic, single-stranded 25mer DNA containing 5 fluorescein labels was then covalently attached to the carboxyl groups of the polymerized monolayer with EDC [1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide] (Expert-Bezancon and Chiaruttini, 1988) as follows: An O-ring was placed on the polymerized monolayer on mica to form a small chamber for carrying out the reaction with a minimum of DNA. 110 ng DNA was incubated with the polymerized monolayer in the presence of 100 mM EDC for 4 hours at pH 6 and at room temperature. LB films were stored under water and imaged in both the fluorescence microscope and the AFM under water.

DNA on mica:

Freshly cleaved mica was installed in the AFM, rinsed with 3 mM $AlCl_3$ and then thoroughly with water. Next, a solution of single-stranded 25 mer DNA at a concentration of 0.13 μg/ml in 50 mM NaCl (Heuser 1989, Gordon and Kleinschmidt 1970) was introduced into the AFM; imaging was started one hour later, after the DNA had had time to bind to the mica.

Antigen-antibody reaction:

Quartz glass was coated with octadecyltrichlorosilane (OTS) in the following way: The glass was dried at 60°C for about one hour after cleaning. Then it was dipped into a solution of 0.1 ml OTS in 100 ml solvent consisting of 80% hexadecane ($C_{16}H_{34}$), 12% carbon tetrachloride (CCl_4), and 8% chloroform ($CHCl_3$). After the silylation reaction was completed, within about one minute, the substrate was rinsed thoroughly with $CHCl_3$ and again heated up to 120°C in order to remove non-covalently bound OTS molecules. A monolayer of the lipid DL-α-dipalmitoyl-phosphatidyl-choline (DPPC), which was used as purchased without further purification, was then compressed at the air-water interface of the Langmuir trough and transferred onto the OTS/glass, again by vertical dipping. 4% of the lipids had covalently attached hapten groups (custom synthesis) (Molecular Probes, Eugene OR). The lipid hapten consisted of the lipid hapten II described in Balakrishnan *et al.* 1982, with the modification that the chain in position 2 was marked with a NBD label for fluorescence. For the antigen-antibody reaction a 1 μg/ml solution (PBS, pH 7) of the monoclonal anti DNP antibody AN02 was prepared by standard methods (Anglister *et al.* 1984).

AFM:

The AFM, which is described in more detail elsewhere (Gould *et al.* 1990b), was operated with Si_3N_4 microfabricated cantilevers (Park Scientific Instruments, Mountain View, CA*), on which microtips are attached (see Fig. 1). These tips gave better quality images of the samples than the glued diamond tips. All the samples were imaged under water or an aqueous solution.

Results and Discussion

The AFM as well as the fluorescence microscope are able to reveal the same cracked domain structure of solid areas of a polymerized LB film (top layer of a bilayer system). The fluorescence microscope reveals the structure through optical properties of the LB film. It is well known that the polymer backbone formed from the diacetylene groups is fluorescent (Göbel *et al.* 1987). Since the diacetylene polymerization is known to be a topochemical reaction, only crystalline areas are able to polymerize. Therefore the crystalline areas are basically the same as the polymerized areas and appear bright. The AFM, that reveals the film structure through mechanical properties, is able to increase the magnification of a polymerized LB film (see Fig. 2a) compared to the fluorescence microscope (Fig. 2b). Both images show that the cracks (dark) are oriented in preferred directions. This suggests that they are parallel to one crystal axis of the LB film respectively to the polymerization direction. The areas of polymerization are shown in white. The images taken with the AFM and the fluorescence microscope are similar in their pattern. The height of the steps between areas of polymerization and cracks in the AFM image is about 9±1 nm, which makes it very likely that the domains are epitaxially oriented trilayers. This is supported by the pressure-area diagrams that give an average molecular area of 0.08 nm^2 which is about one third of what one would expect (0.22 nm^2). The fluorescence of the domains is highly polarized which means that the layers are either epitaxially oriented or only the very top layer is polymerized. It is not clear why trilayer should be more stable than a five or seven layer. Fortunately, for our imaging purposes this interesting question does not matter at all.

One big advantage in using an AFM is that one can

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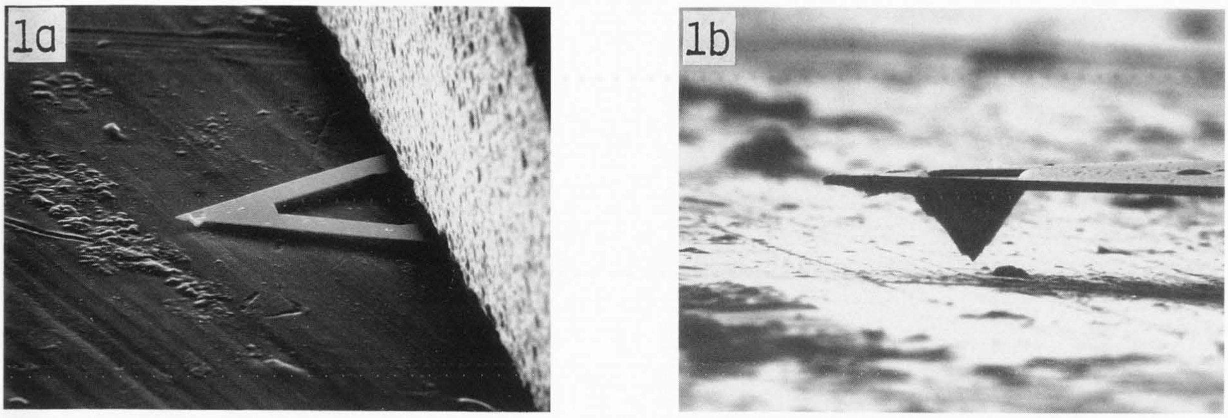
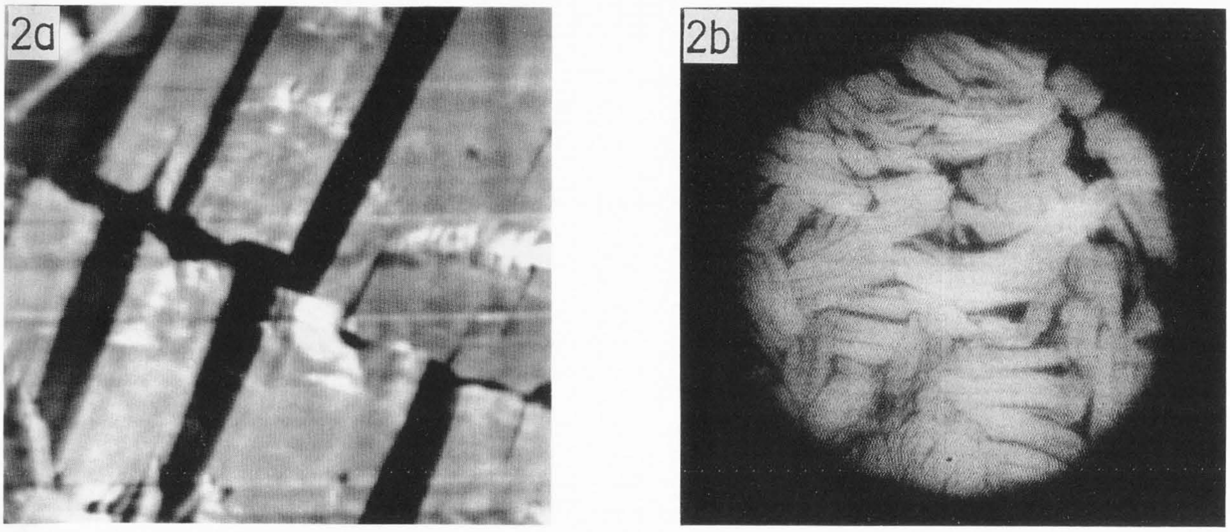
* These cantilevers and tips were developed by T.R. Albrecht and C.F. Quate and are now sold by Park Scientific Instruments.





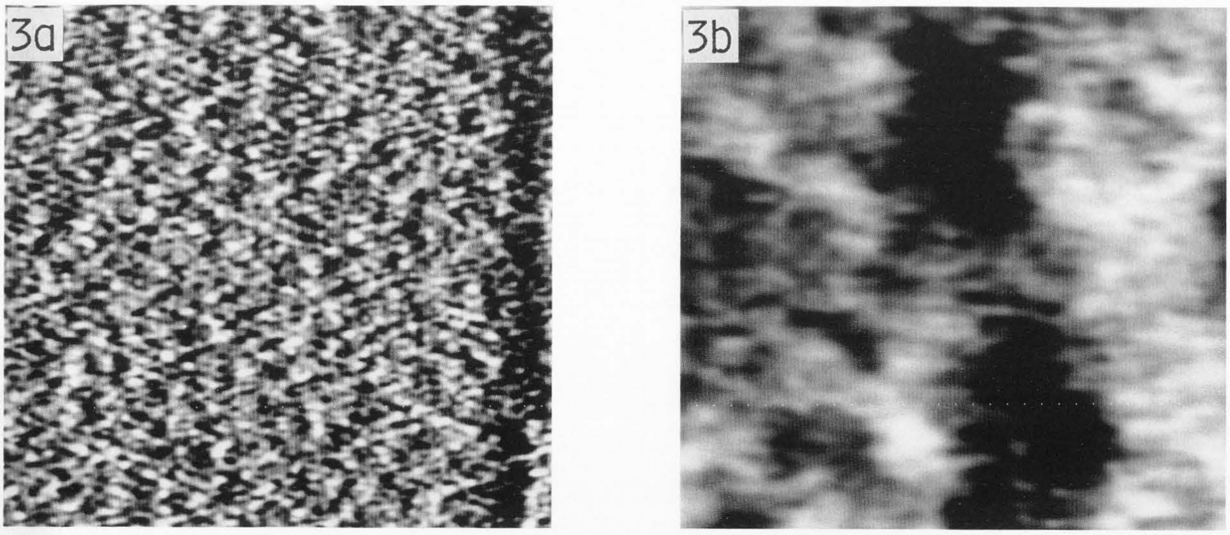


Figure 3 a) Very high magnification image of a polymerized LB film of 10,12-pentacosadiynoic fatty acid on Cd-arachidate/mica in water. Image size is 20 nm × 20 nm, image height is about 0.2 nm. b) LB film with 25mer DNA crosslinked with EDC to the fatty acids in water. Image size is 20 nm × 20 nm, image height is about 0.8 nm.

not only get images comparable to the low magnification images of a fluorescence microscope, but more detailed information at high magnification. Figure 3a shows a high-magnification AFM image of the polymerized LB. Individual rows going in the +159° direction (measured from the positive x-axis counterclockwise) are spaced by 0.45±0.05 nm as determined by Fourier transformation. This is approximately the spacing one would expect for the carboxyl headgroups in a crystalline monolayer, such as a LB film, provided the chains are slightly tilted. We have resolved individual headgroups of lipids previously with the AFM (Weisenhorn *et al.* 1990a), using synthetic saturated lipids in a crystalline bilayer. This region of the polymerized LB film in Fig. 3a is quite flat. The surface roughness is less than 0.2 nm. In contrast, Fig. 3b shows the AFM image of a much rougher surface (0.8 nm). Single-stranded 25mer DNA was covalently crosslinked to the polymerized LB film with EDC. The width of single strands of DNA is somewhat less than one nanometer, though it appears wider in the AFM due to the width of the AFM tip. The spacing of nucleotide bases in single-stranded DNA ranges from 0.3 nm to 0.7 nm. There is some evidence for this structure in Fig. 3b, especially along the upper left hand edge, where a short strand can be seen that is 1.2-1.4 nm wide with bands (nucleotide bases?) spaced approximately 0.5 nm apart. Although the image is really too crowded with DNA, we have recently been able to identify both fluorescein labels and a few of the nucleotide bases in another AFM image of the same polymerized LB film (Hansma *et al.* submitted for publication).

Figure 4 shows the same single-stranded DNA adsorbed on mica that had been rinsed with $AlCl_3$ solution. Al^{+3} displaces K^+ from the surface of mica and should chelate the DNA to the mica (Heuser 1989, Gordon and Kleinschmidt 1970). We were excited to see both the lattice of the mica substrate (lower right in Fig. 4) and the DNA so clearly in this image. The height of the DNA in Fig. 4 is comparable to the thickness of single-stranded DNA. The lengths of the DNA segments are shorter than one would expect for a 25mer DNA; perhaps DNA on a hard mica substrate is cut by the AFM tip. AFM images of mica rinsed with salt solutions show clean mica images similar to the lower right corner of Fig. 4.

The DNA in Fig. 4 also moved around while being imaged because the applied force (order of 10^{-9} N) was still too big compared with the binding force between the DNA and the mica. In fact, the DNA-free square in the lower right, was scanned previously several times before the image (Fig. 4) in a slightly different area was taken. Clearly the DNA had been removed by the previous scanning. The shading effect (left side of strands black, right side white) is caused by an on-line high pass filter, that suppresses structure of long periods and doesn't alter structure of short periods (cut off ≈1.3 nm).

LB films of lipids can also be used for antigen presentation in antigen-antibody reactions. Figure 5a shows an image of DPPC on OTS/glass. Four percent of the lipids were replaced by hapten lipids, having an antigen-site in the headgroup. However, they cannot be resolved in Fig. 5a because the hapten lipid has about the size of pure lipids and because glass was used as substrate. We never were able to resolve individual headgroups when glass was used as a substrate, probably due to the roughness of the glass (at least 0.3 nm), which can be seen in Fig. 5a. In spite of the roughness we were able to clearly see antibodies at the surface, 40 min after a 1 μg/ml solution was introduced into the AFM (Fig. 5b). The apparent size of the antibodies is between 25 nm x 8 nm and 35 nm x 12 nm. The height is roughly half a nanometer. These observed dimensions are again too big; the total length of an antibody is about 16 nm and the thickness at the base is about 3-4 nm. We have previously resolved submolecular structure of fragments of antibodies (Egger *et al.* 1990), that were densely packed and covalently bound to a lipid LB film. In the experiment presented here, the antibodies have a much bigger mobility and flexibility which might explain that the apparent size is too big and why submolecular resolution is not achieved. We also observed circles of depression of about 50 nm radius, about 15 min after having the antibody solution introduced. We don't know if this is something specific to this reaction or due to the fact that glass was used as substrate.

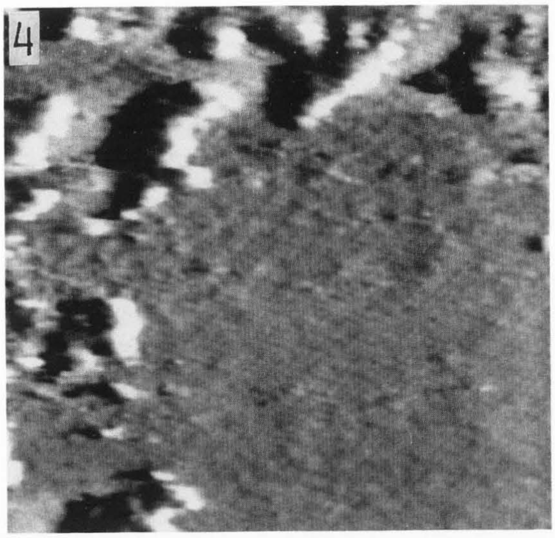


Figure 4 25 mer DNA adsorbed on mica that was previously treated with $AlCl_3$ and then imaged in water. Image size is 32 nm × 32 nm, image height is about 0.6 nm. Note that the mica structure can clearly be seen in the lower right corner.

We have shown that the combination of an AFM and LB films can be useful for imaging single-stranded DNA and antigen-antibody reaction. The AFM can also resolve rows of molecules in the polymerized LB film as well as its cracks in the solid domain structure.

Acknowledgment

We would like to thank L. Coutts for synthesizing the DNA, B. Göttgens for purifying the fatty acids, S.A.C. Gould for helping with image processing, Digital Instruments for their generous support, M. Kirk from Park Scientific Instruments, T.R. Albrecht and C.F. Quate from Stanford University for supplying the microfabricated cantilevers. This research was also supported by an IBM Manufacturing Fellowship (A.L.W.), the Deutsche Forschungsgemeinschaft (H.E.G.), a National Science Foundation-Solid State Physics Grant DMR89-17164 (H.G.H., G.L.K.,P.K.H.), BRSG S07 RR07099-24 Biomedical Research Support Grant Program, Division of Research Resources, National Institute of Health (R.L.S.), and the Office of Naval Research (P.K.H.).

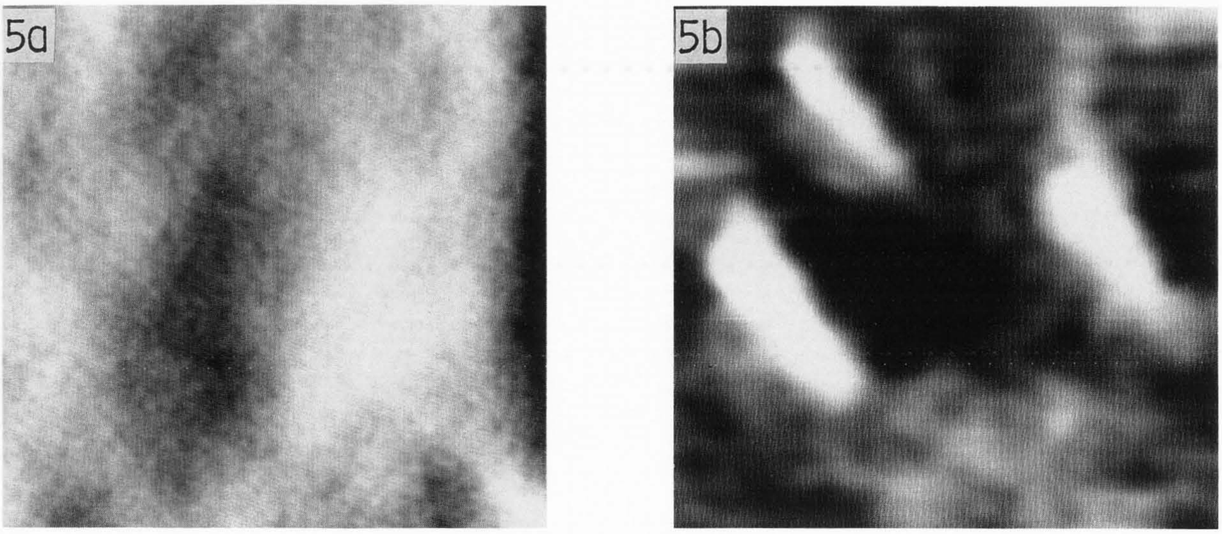


Figure 5 DPPC with 4% hapten on OTS/glass in water. Image sizes are 90 nm × 90 nm. **a)** Before adding a solution of antibodies. Image height is about 0.3 nm. **b)** After adding a 1 μg/ml solution of antibodies. Image height is about 0.9 nm. Both images were taken with a diamond tip that was glued onto a microfabricated cantilever.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.