10-21-1992

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Abstract

Atomic force microscopy (AFM) was used to image circular DNA adsorbed on freshly cleaved mica and mica chemically modified with Mg(II), Co(II), La(III), and Zr(IV). Images obtained on unmodified mica show coiling of DNA due to forces involved during the drying process. The coiling or super twisting appeared to be right handed and the extent of super twisting could be controlled by the drying conditions. Images of DNA observed on chemically modified surfaces show isolated open circular DNA that is free from super twisting, presumably due to strong binding of DNA on chemically modified surfaces.

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Introduction

The atomic force microscope (AFM) (Binnig et al., 1986) has attracted much attention because of its ability to obtain high resolution images on conducting as well as non-conducting surfaces. In the AFM, a sharp, pyramidal tip mounted on a cantilever is brought into close proximity to the surface where the intermolecular forces acting between the tip and the surface cause the cantilever to bend. Images of the surface are obtained by recording the cantilever deflections, as detected by a laser beam focused on the top of the cantilever tip, as the sample is scanned underneath (Sarid, 1991). The AFM can be operated in air or liquid environment and the samples can be conductors or insulators. These features make it an ideal tool for imaging and studying biomolecules such as DNA in biologically relevant media without fixation or staining. Although the AFM is capable of producing atomic resolution images of many flat surfaces, its resolution of structures is greatly influenced by the radius of the contacting probe tip. The image obtained is a convolution of tip geometry with that of the sample, and for many biological samples such as DNA, which are much smaller than the tip radius, this results in an apparent broadening of the molecular image (Thundat et al., 1992a).

Proper sample preparation has been essential to any new microscope technique. Perhaps the most common preparation technique for biological samples in scanning probe microscopy, first used to prepare specimens for the scanning tunneling microscope (STM), is drop evaporation, where a buffered droplet of the sample, such as DNA is placed on a substrate and air dried (Beebe et al., 1989; Dunlap and Bustamante, 1989; Lee et al., 1989; Driscoll et al., 1990). Although surfaces such as freshly-cleaved, highly-oriented pyrolytic graphite (HOPG) or gold evaporated on mica are routinely used, DNA molecules do not bind strongly to these substrates and are usually moved or swept away by the scanning tip. Electrochemistry has been used to deposit and immobilize DNA (Lindsay and Barris, 1988; Lindsay et al., 1989; Brown et al., 1991). Chemical means have also been employed in attempts to bind DNA to surfaces (Cricenti et al., 1989; Lyubchenko et al.,

1991; Heckl *et al.*, 1991) so that the presence of complete molecules can now be clearly observed (Allison *et al.*, 1992a).

For AFM studies, we have systematically investigated a number of surfaces for DNA deposition, such as quartz, clay, mica, nitrocellulose, silicon, lead pyrophosphate, and strontium titanate. From these studies, it was clear that mica was the best substrate. DNA can be found consistently on samples prepared by drop evaporation on freshly cleaved mica (Zenhausern *et al.*, 1992; Henderson, 1992; Thundat *et al.*, 1992a) although stress associated with drying causes super twisting of molecules. When samples are prepared with open circular DNA, only rarely are open circles observed. Modifications to increase bonding of negatively charged DNA molecules to mica substrates, therefore preventing drying artifacts and removal of DNA by tip forces, have been reported. Potassium ions on the mica surface have been replaced by magnesium ions to increase DNA adsorption (Bustamante *et al.*, 1992, Hansma *et al.*, 1992, Vesenka *et al.*, 1992). Lindsay has introduced a positive charge to a mica surface by binding 3-aminopropyltriethoxysilane, via a silane bridge, to mica and substituting methyl groups for amino protons using methyl iodine to increase DNA adhesion (Lindsay *et al.*, 1992). In this paper, we discuss artifacts introduced by the drying process on DNA adsorbed to freshly cleaved mica. We also report a systematic study of DNA adsorption on mica surfaces modified by Mg(II), Co(II), La(III), and Zr(IV). DNA adsorption on these surfaces produces reproducible images of DNA molecules free from artifacts caused by drying. In addition to topographic AFM images, we have also obtained lateral (dF/dx) and vertical (dF/dz) force modulation images of DNA adsorbed on modified mica.

Materials and Methods

Sample preparation

In this study we have used pBS$^+$ plasmid DNA (3204 base pairs, from Stratagene, La Jolla, CA) prepared from cultures of *E. coli* strain MV1190 grown in 2X YT media. Cells were ruptured by alkaline lysis and the supercoiled plasmid DNA purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation, ethanol precipitated two times, and resuspended in 0.01 M Tris-HCl (pH 7.5) containing 0.001 M EDTA (TE) at a concentration of 500 μg/ml. Relaxed circular DNA was obtained by single-stranded nicking of supercoiled molecules via X-ray irradiation (70,000 rads). Linear plasmid DNA was prepared by using restriction endonuclease SmaI (New England Biolabs) at the manufacture's recommended conditions: 100 μg of plasmid was incubated with 100 units of SmaI in a 200 μl reaction mixture overnight at 25 °C. The reaction mixture was extracted with phenol, precipitated with ethanol and resuspended in TE. The purity of DNA was determined by agarose gel electrophoresis. Prior to imaging, the DNA was diluted to the desired concentration

in 0.01 M ammonium acetate (Fisher Analytical reagent grade) at pH 7.2. Further details of DNA preparations are reported elsewhere (Allison *et al.*, 1992a).

The mica (New York Mica Co., NY) substrates were either freshly cleaved unmodified, or mica modified by chemical treatments with Mg(II), Co(II), La(III) or Zr(IV) ions. The chemical modification of mica was achieved by soaking freshly cleaved mica in 10 mM solution of the corresponding ion overnight. Solutions were prepared using magnesium acetate, lanthanum chloride, cobalt chloride or zirconium chloride (ZrOCl$_2$). This process exchanges the potassium ions on mica with Mg(II), Co(II), La(III) or Zr(IV) ions. The mica was removed from the salt solution, rinsed in changes of distilled water several times, and ultrasonicated 3 times for 20 minutes in distilled water to remove excess salt on the surface. The substrates were then blown dry with nitrogen, heated in an oven for several hours at 105 °C, and stored in a desiccator until used. The optimum solution concentration and time required for ultrasonication were established by imaging the surface after modification and looking for crystallites on the surface.

On freshly-cleaved, unmodified mica, a 20 μl drop of DNA solution at a concentration of 1-3 μg/ml in 0.01 M ammonium acetate buffer was deposited and allowed to air dry prior to imaging. On chemically modified mica, 100 μl of 20 μg/ml DNA solution in 0.01 M ammonium acetate, pH 7.0 was placed on the surface for a few minutes. The DNA solution was then removed by wicking with filter paper, and the surface was then rinsed gently in a mixture of distilled water and alcohol (1:1 ratio). Samples were blown dry with nitrogen and stored overnight in a desiccator prior to imaging with the AFM. DNA adsorption onto both unmodified and chemically modified substrates were evaluated by substituting ^{32}P-labelled plasmid DNA in the sample preparation procedure and determining DNA uptake by scintillation counting as described by Allison *et al.* (1992a).

Atomic force microscopy

AFM images were collected using a commercially available instrument (Nanoscope-II, Digital Instruments, Santa Barbara, CA). The scanning tips were Si$_3$N$_4$ cantilevers 200 μm long with a spring constant of 0.12 N/m (Nanoprobes, Digital Instruments, Santa Barbara, CA), and these tips were altered by contamination lithography in the scanning electron microscope (Keller and Chung, 1992). All images were obtained in a constant force (constant deflection) mode at 1 to 3 nN net repulsive on the cantilever and are presented here as raw data except for flattening. An additional internal force of the sample surface results from the capillary force due to water condensation at the apex of the tip and was measured by recording the force necessary to pull the cantilever from the surface. Since AFM images of DNA show contrast and width variation as a function of relative humidity (Thundat *et al.*, 1992b), the AFM was placed in a humidity controlled chamber during imaging. Humidity was measured by a VWR digital hygrometer and was kept generally constant at 20% or less during these
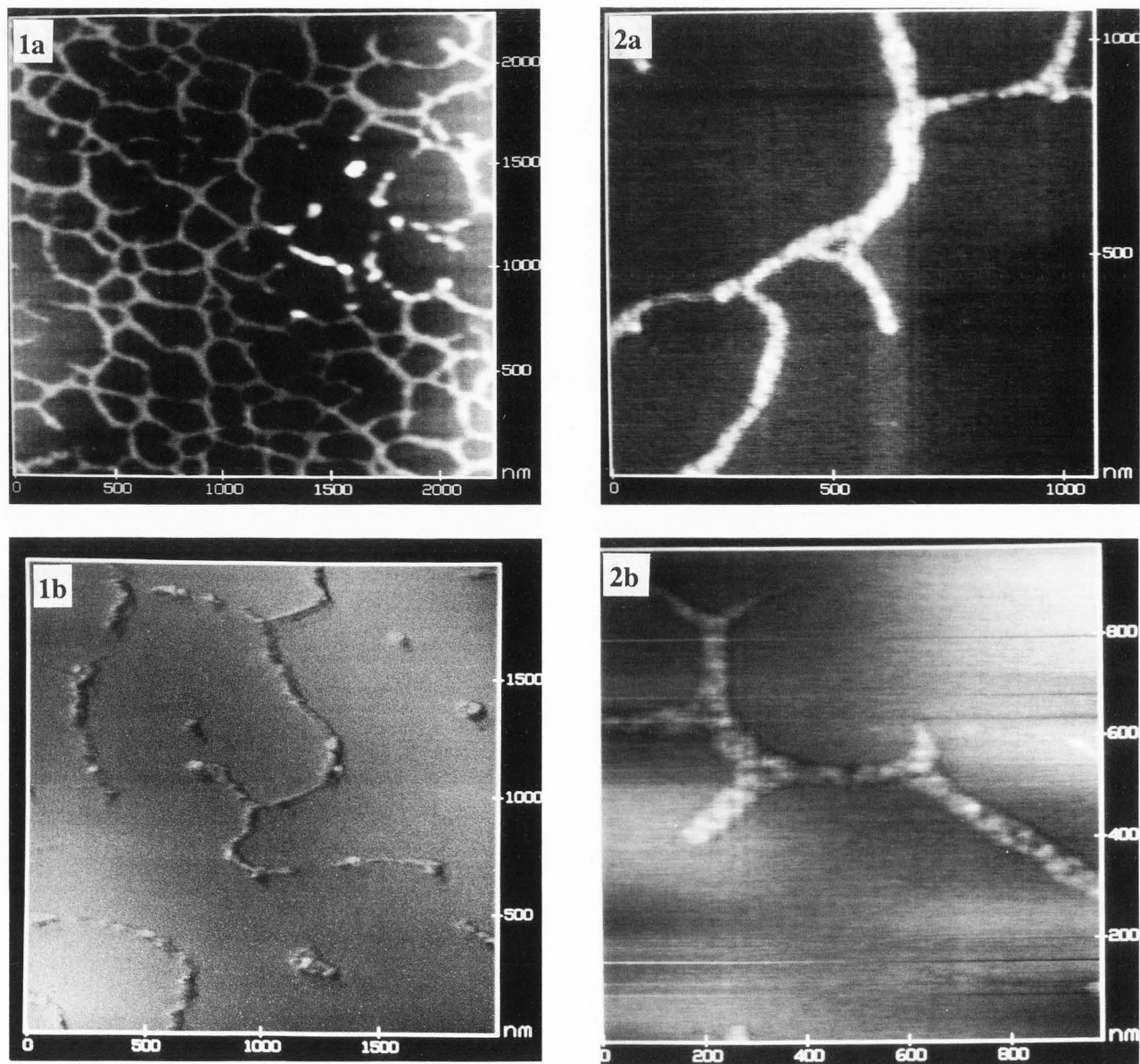
Figure 1. AFM image in dry nitrogen of network formation (a) of DNA on freshly cleaved mica observed at high concentration (10 μg/ml). The height of DNA strands above the substrate varies from 0.8 nm to 1.9 nm. Fig. **1b** shows isolated strands of DNA observed at low concentration (<3 μg/ml). Black (low) to white (high) corresponds to 10 nm. The average height of DNA above the substrate is 1.4 nm.

Figure 2. "Super twisting" of DNA adsorbed on freshly cleaved mica in dry nitrogen. Black (low) to white (high) corresponds to 12 nm. (a) Plasmid DNA. (b) Linear plasmid DNA. The average height of DNA above the substrate is around 1.8 nm. Whenever clearly visible (as in Fig. 2b), the observed helicity of "super twisting" was always right-handed.

Results and Discussion

experiments. Since humidity effects on contrast and width have been found to be minimized when the direction of scan is rotated 180 degree, images were taken by collecting scans from left to right. Images were collected with 400 X 400 pixel information density at a scan rate of 2 Hz.

The samples prepared by depositing drops of DNA on freshly cleaved mica and air drying (without rinsing) show aggregate and network formation (Figure 1a) for concentrations above 3 μg/ml. However, at low concentration (less than 1 μg/ml) isolated DNA molecules (Figure 1b) can be found. Aggregation and net-
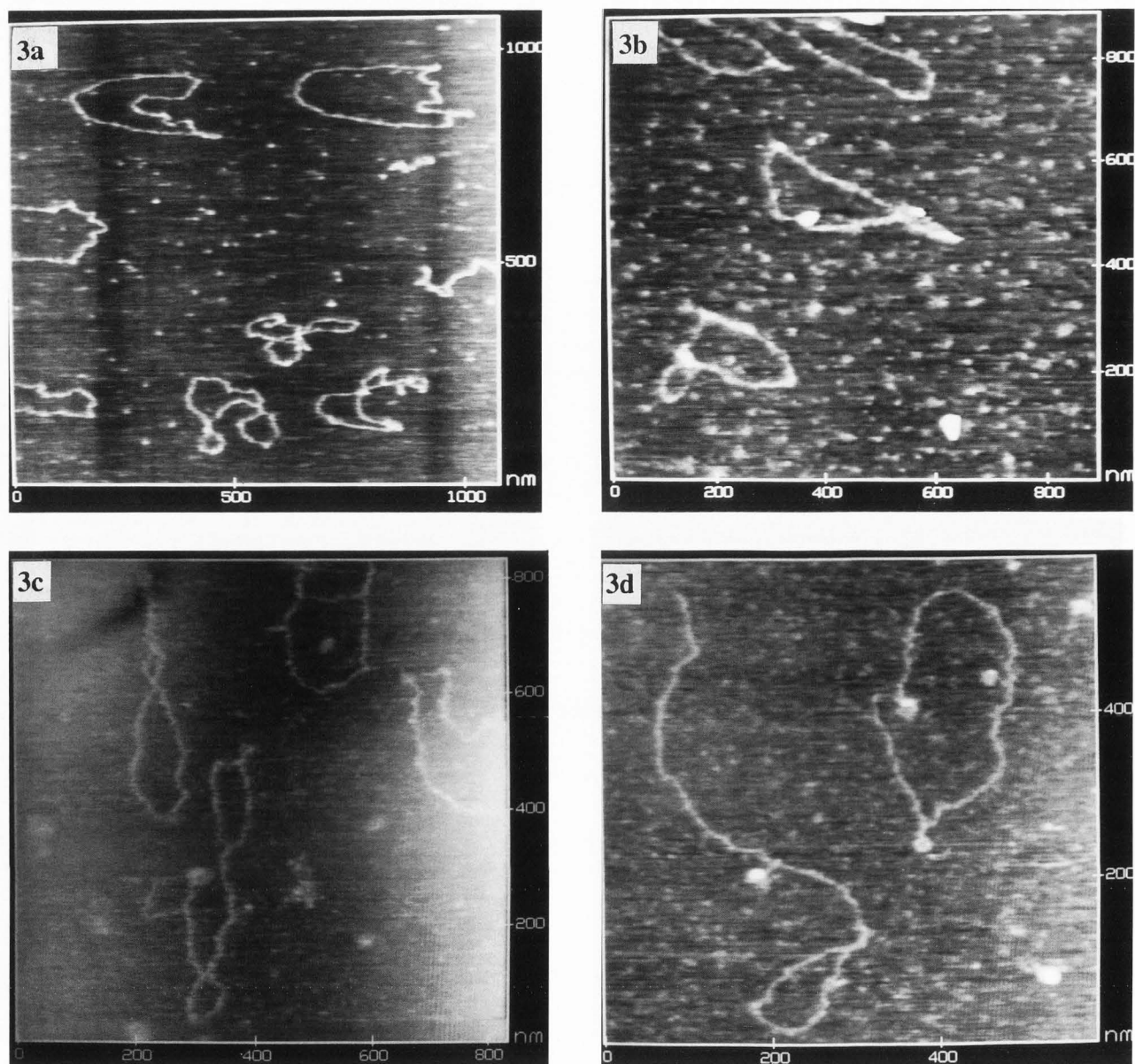
Figure 3. AFM images of DNA on chemically treated surface under dry nitrogen. Black (low) to white (high) corresponds to 10 nm. The average height of DNA above the substrate is 1.8 nm. **(a)** DNA on Mg treated mica surface. **(b)** DNA adsorbed on La treated mica surface. **(c)** and **(d)** DNA on Co treated mica surface.

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work formation has been observed for linear as well as circular DNA. DNA on freshly cleaved mica is not strongly adsorbed because it can be removed by rinsing in water, buffer, or in an ethanol-water mixture. However, if freshly deposited DNA is allowed to dry completely on the surface it can be imaged under ethanol or isopropanol. DNA molecules were found to be very stable to repeated scanning at humidities below 30% while at high humidities they tend to break. Increasing the force exerted by the AFM tip or imaging at high resolution tended to break and or remove the strands (Thundat et al., 1992a), however, decreasing the humidity usually

helped to prevent the strand breaking. Sometimes decreasing the humidity had no effect. In those instances, the force-distance curves showed a large capillary force (the excess force needed to break the tip away from the contacting substrate) even under the condition of low humidity. Since these conditions could be altered spontaneously during scanning or by replacing with a new tip, tip contamination was suspected. Scanning under ethanol or isopropanol, we did not observe any significant improvement in resolution compared with imaging in dry nitrogen. However, imaging under liquid has many intrinsic advantages. When scanning in air, the tip can
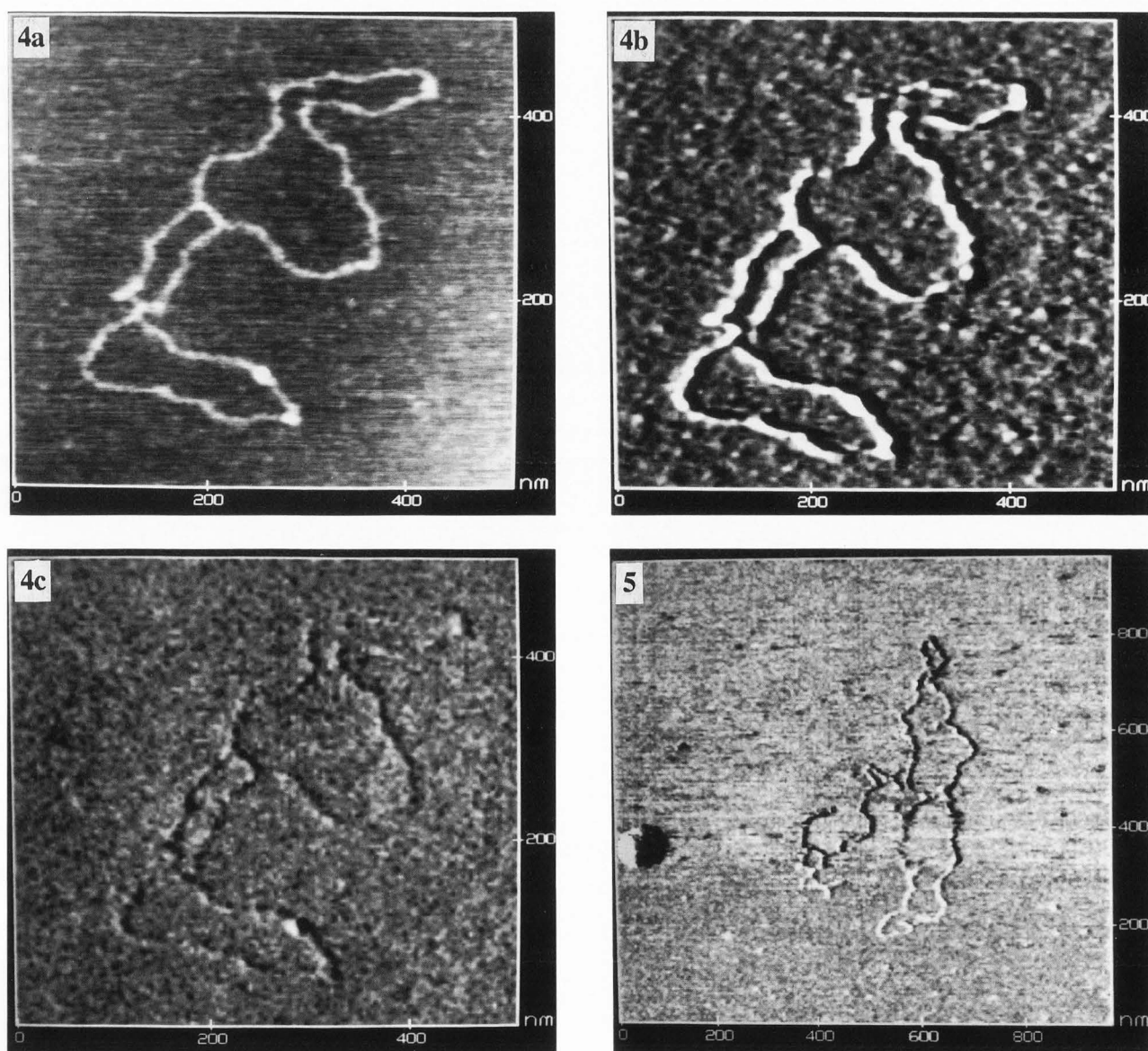
914

Figure 4. Force modulation images of DNA adsorbed on Mg treated mica in dry nitrogen. (**a**) Topograph (constant force AFM image). (**b**) Lateral force modulation (dF/dx) image. (**c**) Vertical force modulation (dF/dz) image.

Figure 5. dF/dx image of DNA obtained at very low humidity. The change in contrast is very likely due to tip contamination from salt deposit (middle left-hand-edge) during upward scan.

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easily pick up contaminants that change the resolution and contrast drastically. More importantly, the capillary force (Israelachivilli, 1985; Thundat *et al.*, 1992b) due to the liquid bridge formed in air between the tip and the sample, can be completely avoided under solution allowing scanning with a very small repulsive force.

Although we have obtained isolated images of open, circular DNA on untreated mica (Thundat *et al.*, 1992a), the DNA in most images show some degree of "super twisting" (Figure 2a). Similar "super twisting" has also been observed with linear DNA (Figure 2b).

The helical pitch of super twisting is around 37 nm (as measured from Fig. 2b), is always right-handed, and has been observed with linear as well as circular DNA. However, it should not be confused with super coiling of DNA since it is possible to control the extent of "super twisting" by controlling the drying conditions. For example, the samples dried in high humidity (slow drying) tend to show more super twisting when compared with DNA dried faster under conditions of low humidity. "Super twisting" of DNA is probably due to the capillary forces acting on the molecules during the drying

915

process. Since the surface tension of the water is very high (72 mN/m for pure water at room temperature) (see for example, Adamson, 1990), the forces acting along the perimeter of the drop drags DNA molecules, that are strongly bonded only at few sites, over the substrate surface as the drop size decreases causing "super twisting".

DNA adsorbed on mica surfaces modified by substituting potassium ions on the mica with Mg(II), Co(II), La(III), and Zr(IV) ions and washed in ethanol-water mixture, show excellent images of open circular as well as linear DNA with little indication of "super twisting." Samples prepared without rinsing in ethanol-water show network formation and "super twisting". This enhanced stability of DNA adsorption on chemically modified mica may be due to increased electrostatic interaction between DNA and the modified surface. However, rinsing in water or vigorous rinsing in ethanol-water removes all the DNA from the modified surface indicating that the DNA bonding on the surface is not strong. The drying forces associated with ethanol-water mixture may not be sufficient to displace the DNA molecules probably due to decreased surface tension of the mixture. This notion is supported by the fact that similar results can be obtained by rinsing the sample in water containing a few drops of "Photoflo" (Kodak), a surfactant. Figures 3 a-d show images of DNA recorded on mica surfaces treated with Mg(II) (Fig. 3a), La(III) (Fig.3b), and Co(II) (Fig.3c and 3d). Measured molecular lengths of the circular DNA molecules from the images were within 10% of the expected value of 1 μm. Bumps that appear in the background are probably due to salt crystals since their number and size can be varied by controlling the parameters involved in chemical treatment such as concentration of the surface modifying solution, extent of washing, ultrasonic cleaning, and drying. This background roughness was highest for Zr(IV) treated surface, and DNA images were not as clearly visible in AFM images. However, studies with ^{32}P-labeled DNA show that the DNA adsorbed on Zr(IV) treated surface is more stable against rinsing in water than Co(II), Mg(II), or La(III) treated surfaces (Table 1). The background surface roughness was least for the Mg(II) or Co(II) treated surfaces, and of all the modified surfaces, Co(II) and Mg(II) treated surfaces were found to produce superior images. From many AFM studies it appears that the DNA adsorbs uniformly on chemically modified surfaces presumably by electrostatic forces. Although the bonding of DNA to chemically-treated mica was stronger than on a freshly-cleaved surface, no satisfactory images could be obtained under water.

In addition to topographic images, we also recorded contrast changes induced by lateral modulation (dF/dx) and by vertical modulation (dF/dz) of the sample (Fig. 4). Imaging in dF/dx is sensitive to changes in lateral force exerted on the tip-cantilever system, and dF/dz images should be sensitive to variations in compressibility over the scanned area. The lateral force is strongly associated with the humidity level due to capillary forces near DNA (Thundat *et al.*, 1992b). As mentioned previously the capillary forces are also related to the condition of the tip. When the capillary force is high (greater than about 25 nN), the DNA width was observed to be broad in dF/dx imaging (Fig. 4b). This is due to the increased area of the capillary around the tip causing longer range interaction with the DNA molecule. The contrast results primarily from cantilever buckling where the tip-DNA force is strong. Figure 5 shows a much finer width for DNA strands when the capillary force was weak (<20 nN). The contrast variation seen in Fig. 5 is due to the tip picking up contaminants during its upward scan as it imaged a salt deposit (middle left-hand-side). Such contrast variation has been commonly observed when salt crystals are present in the background. The dF/dz images were of poor contrast and did not show distinction between DNA and bumps due to salt deposit. This is likely due to a uniform coverage of buffer salt on the DNA as well as the surface. Another reason for poor contrast of dF/dz is the small force constant of the cantilever which is essential in obtaining high contrast images of DNA.

Conclusions

DNA can be routinely imaged on untreated mica although it is not strongly bound. As a result of surface tension forces acting during droplet drying the strands are generally twisted around themselves. Mica that has undergone treatments by various transition metals appears to more strongly bind DNA than an untreated mica surface. The resultant images are less affected by the drying conditions and generally show open circular plasmids without "super twisting". Lateral modulation imaging shows the effects of capillary forces acting on the tip-cantilever system. The best imaging conditions consist of using a strongly binding substrate with a minimization of capillary forces.

Acknowledgments

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Table 1. Radioscintillographic results[a] demonstrating the affinity of radiolabeled pBS[+] plasmid DNA for chemically modified mica substrates.

Modified Surface[b]	pBS[+] ^{32}P Radiolabeled DNA 0.25 μg/ml in 0.01 NH$_4$OAc (pH 7.0)
H$_2$O	110 $\pm$ 19
Mg(II)	100 $\pm$ 16
Co(II)	119 $\pm$ 25
La(III)	112 $\pm$ 19
Zr(IV)	260 $\pm$ 72

[a]The values listed are the mean disintegrations per minute for 6 disks per surface treatment $\pm$ the standard deviation.

[b]Mica surfaces were pretreated with 10 mM solutions of the chemical modifier for 24 hours ultrasonicated in water and thoroughly rinsed prior to exposure to DNA solution by floating the mica disks on the solution for 5 hours. After exposure the disks were rinsed by plunging 5 times each into three consecutive water solutions. Aqueous solutions of magnesium acetate, lanthanum chloride, cobalt chloride, or zirconium chloride were used to modify the disks.

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Discussion with Reviewers

Eric Henderson: With regard to supercoiling in general, the definitive statement that the supercoils observed are right handed seems, based on the image shown [Fig. 2], to be an overstatement.

W. Heckl: Is there any speculation for the reason that the "super twisting" is always right-handed within the framework of the explanation of its general occurrence due to the surface tension effects of retrieving water droplets while drying? Is the apparent thickness of 10-20 nm of each of the two strands which twist around the other (e.g., Fig.2b) expected to be correct for the diameter of a double strand DNA or do some proteins or buffer molecules adhere or is the excess thickness solely due to the AFM tip convolution broadening of all features?

Authors: We are referring to the DNA tightly coiling about itself as "super twisting". Fig. 2 shows this phenomenon on linear DNA and, therefore, cannot be referred to as supercoiled. The circular DNA was X-ray treated to relax the supercoiled molecules.

The image shown is a large scale image which may not clearly present the right-handedness of the DNA strands. A higher magnification image was shown in Thundat et al. (1992a) which may be more clear. We were able to determine the right- or left-handedness in only a few samples. These appeared to be loosely coiled so that the standard AFM tips were able to resolve adjacent coiled strands. This explains the observed 10-20 nm width and spacing; tightly coiled strands might approach 2 nm if the individual strands are not expanded by salt or hydration layers. Typical AFM images in our laboratory generally show 10-14 nm widths of isolated DNA molecules. This corresponds to a tip radius (assuming 2-nm diameter DNA) of 11 nm. Please see Thundat et al. (1992c, in this issue) for a detailed discussion of tip broadening.

Eric Henderson: Why were different concentrations of DNA used on unmodified and modified mica? Why was the concentration higher for the modified surface if it has tighter binding characteristics?

Authors: Unmodified mica was treated with DNA by drying a droplet of the DNA solution. This could not withstand the rinsing as could the modified mica. The modified mica was treated by diffusion of the DNA from a droplet for a specified time, followed by wicking and rinsing. The concentration of the DNA solution in the latter treatment must be higher since only a portion of the DNA diffuses to the surface.

W. Heckl: Could any alternative techniques such as X-ray photoelectron spectroscopy (XPS) for chemical composition or diffraction measurements of changes in the z-spacing of the intervening planes of mica be used to evaluate the amount and exact stoichiometric composition of the mica after intercalation/exchange of the potassium ions with Mg, Co, La, or Zr?

Authors: Yes, it would be informative to determine the precise chemical nature of the surface of treated mica, since it is active in the binding of DNA. Such an analysis has been used to characterize covalently bound phospholipids on graphite (Heckl et al., 1989).

S. M. Lindsay: What do you estimate your resolution to be and what factors control it?

Authors: We have measured a typical radius of curvature for our tips (from Digital Instruments, Inc.) to be about 11 nm (Thundat et al., 1992c). This would produce an apparent DNA width of 13 nm (without absorbed salt layers). We consistently observe 14 ± 2 nm. We have made and used sharpened tips to get widths less that 10 nm. The full width of the strands shown in Fig. 3d, for example, is 14 ± 2 nm. This is typical for DNA imaged in dry air. Widths for images taken under higher humidity conditions are larger (Thundat et al., 1992b). There are several effects from the increased humidity: **1)** The capillary bridge causes an increased tip interaction area. **2)** The resulting higher vertical force increases the lateral (shearing) force and causes some instabilities. Cantilever buckling anomalies are also observed. **3)** An increased rate of tip contamination is observed.

S. M. Lindsay: You say that in Fig. 2b, isolated DNA molecules are seen. I can see isolated blobs. Can you tell that these are isolated molecules (as opposed to isolated aggregates)?

Authors: It is difficult to determine from Fig. 2b whether the white areas are aggregates or individual strands. Other images over smaller areas show what appear to be circular strands twisted about each other (Allison et al., 1992b). Some aggregation is usually seen but is less likely on treated mica.