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ATOMIC FORCE MICROSCOPY OF DNA ELECTROPHORESED ONTO SILYLATED MICA

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Abstract

A new technique has been developed for electrophoresing DNA molecules from an agarose gel onto a silylated mica substrate where they can be imaged with an atomic force microscope (AFM). With a simple modification, the technique can also be used for polyacrylamide gels. This method does not require purification of samples from the gels. Using tapping mode AFM, we have observed plasmids after electrophoretic separation into two bands. Differences in conformation were observed between the plasmids in the two bands.

Key Words: Tapping mode, electrophoresis, plasmids.

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Introduction

Atomic force microscopy (AFM) (Binnig *et al.*, 1986) is a useful new tool for biology. Recently, the introduction of the "tapping mode," where the tip oscillates and gently "taps" the sample surface, has reduced lateral forces between the tip and the sample. This technique is less destructive, especially for biological samples. Unstained and unfixed samples can be observed by AFM in a gas or in a liquid environment with molecular resolution (Hansma and Hoh, 1994; Lal and John, 1994). AFM has also been used to study the conformation of plasmids adsorbed from solutions onto mica surfaces (Hansma *et al.*, 1992; Schaper *et al.*, 1993), the conformation of ligand-DNA and protein-DNA complexes (Erie *et al.*, 1994; Hansma *et al.*, 1994; Rees *et al.*, 1993; Zenhausern *et al.*, 1992) and enzymatic degradation of DNA (Bezanilla *et al.*, 1994). AFM of chromatin has given useful structural information (Fritzsche *et al.*, 1994; Martin *et al.*, 1995; Rasch *et al.*, 1993; Yang *et al.*, 1994).

Molecules and molecular complexes in solution often need to be purified through ultracentrifugation, chromatography or electrophoresis. The extraction of nucleoprotein complexes from electrophoresis gels, for example, is inefficient and potentially damaging. Recently, a new technique has been developed for transferring protein-DNA complexes directly from gels to grids for electron microscopy (Jett and Bear, 1994). We have used this technique to electrophorese DNA from agarose and polyacrylamide gels onto silylated mica and to image the molecules with tapping mode AFM in air.

The AFM has been used to study the chirality of plasmids adsorbed from a solution onto a mica substrate and imaged in propanol (Samori *et al.*, 1993). Because the AFM images samples in three dimensions, it can display the handedness of the two strands of a molecule, determining the topology of supercoiled plasmids.

With this new electrophoretic transfer technique, it is now possible for the AFM to observe the coiling of circular DNA molecules separated by agarose gel electrophoresis. This may be useful for correlations

between the theoretical conformation of DNA and images of the molecules. In this work, circular DNA molecules with different conformations were separated by agarose gel electrophoresis and directly transferred onto silylated mica substrates. This method might be useful for imaging different topoisomers of supercoiled circular DNA molecules.

Materials and Methods

DNA samples

DNA molecules, 500 base pairs (bp) long, 60 ng/μl in water, were produced by the polymerase chain reaction (PCR), using a Gene Amp PCR reagent kit obtained from Perkin Elmer Cetus (Norwalk, CT). The template was Lambda DNA, polymerase was Amplitaq DNA polymerase (Perkin Elmer Cetus) and primers were obtained from Operon Technologies (Alameda, CA). Primers and enzymes were removed using a Prep-A-Gene DNA purification kit (Bio-Rad Laboratories Inc., Hercules, CA).

Bluescript II SK (+) double-stranded plasmid DNA (2961 bp, Stratagene, La Jolla, CA), supplied in 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA) was diluted with Milli-Q purified water (Millipore System, Burlington, MA) to a final concentration of 10 to 50 ng/μl.

ϕX174 RF1 double-stranded DNA (5386 bp, United States Biochemical, Cleveland, OH), supplied in 10 mM Tris, 1 mM EDTA, was diluted to a concentration of 40 ng/μl in Milli-Q water. ϕX174 HaeIII digest (Pharmacia Biotech, Alameda, CA), supplied in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, was diluted in water to a concentration of 50 ng/μl.

Aminopropyl mica (AP-mica)

Mica was silylated by a procedure similar to that of Lyubchenko *et al.* (1992a, 1992b, 1995). Freshly cleaved mica (10 mm x 4 mm for agarose gels and 60 mm x 4 mm for polyacrylamide gels) (Ruby Mica, New York Mica Co., New York, NY) were silylated by incubating for at least 2 hours in a 2 liter desiccator containing 50 μl of 3-aminopropyltriethoxysilane (APTES) 98%, obtained from Aldrich Chemical Co. (Milwaukee, WI). The AP-micas were not removed from the desiccator until use.

Electrophoresis

Agarose gel electrophoresis was performed using a mini-Sub Cell (Bio-Rad Laboratories) and a 15 well comb, 1 mm thick. Gels were composed of 1.2% (weight/volume; w/v) agarose (Agarose Low EEO, Fisher Scientific, Pittsburgh, PA) in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8) and 1 mg/μl ethidium bromide. Gels were 4 mm thick. Loading buffer

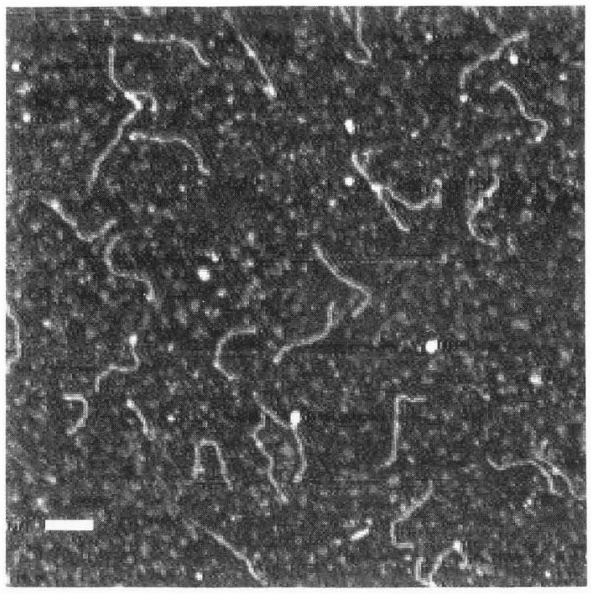

Figure 1. DNA molecules 500 bp long electrophoresed onto a silylated mica support (AP-mica); z range = 3 nm, bar = 100 nm. Width of the molecules is ~ 6 nm.

(0.25% bromophenol blue, 40% w/v sucrose in water) was added to solutions containing 25 to 100 ng of DNA. Samples were loaded into the wells and electrophoresed at 75 V (15 V/cm) for 45 to 120 minutes.

Transferring DNA from gels to AP-mica supports

Gels were illuminated to visualize the bands on a trans-illuminator with ultraviolet radiation (312 nm) and photographed with Polaroid (Cambridge, MA) type 667 film and a yellow filter. Incisions were made in the gels with a spatula at about 45° to the band and perpendicular to the gel surface. Gels were removed from the trans-illuminator and AP-micas were inserted into the wells with forceps. Gels were returned to the electrophoresis apparatus and the voltage was switched on for 2 to 5 minutes. The power was then switched off and AP-micas were left in the gels for 5 minutes. AP-micas were then removed from the gels, rinsed with a few ml of Milli-Q water and drained with a Kimwipe (Kimberly-Clark, Roswell, GA; other absorbent paper may be used interchangeably). They were attached to steel discs with double-stick tape and placed in a desiccator in the presence of P_2O_5 overnight or longer before imaging.

AFM imaging

Samples were imaged in air in the tapping mode with a Nanoscope III (Digital Instruments, Santa Barbara, CA), using a D scanner (ca. 10 μm x 10 μm maximum scan range) and standard 125 μm silicon cantilevers oscillated at resonant frequencies of ca. 300 kHz. Scan rates were 3 to 6 Hz.

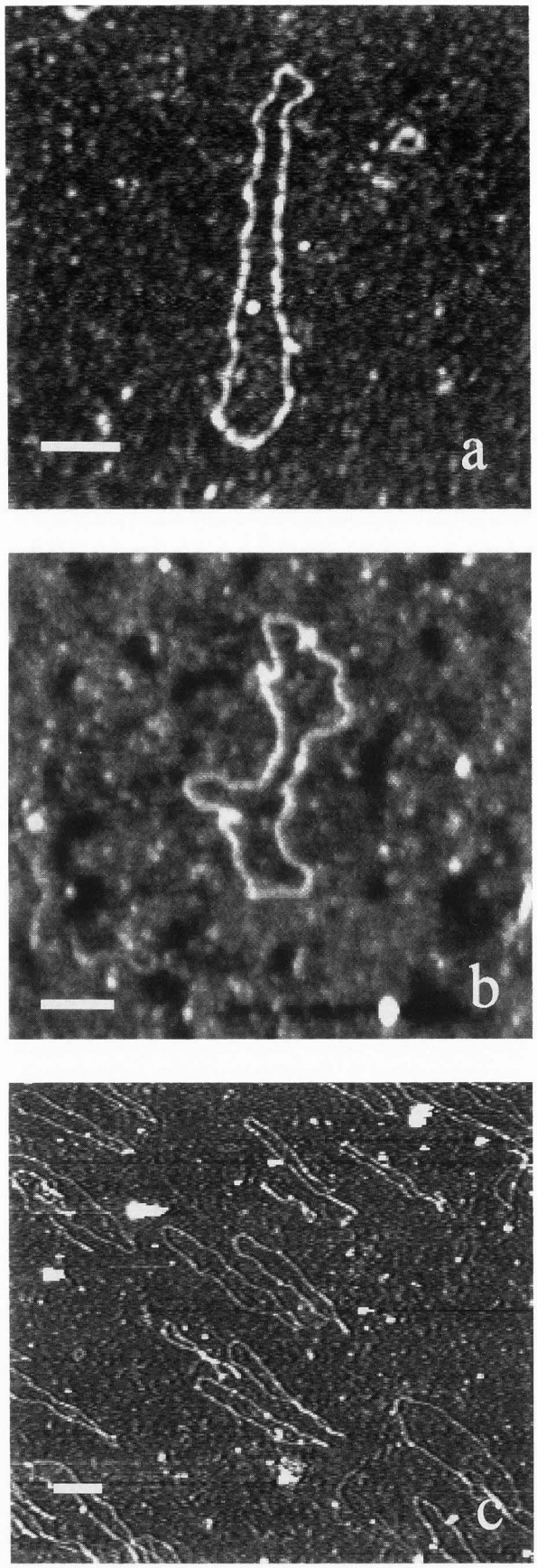

Figure 2. Bluescript plasmids electrophoresed onto AP-mica. Two bands were resolved on the agarose gels. These plasmids are from the slower-migrating band. (a) Plasmid forming a relaxed circle on the substrate; z range = 2 nm, bar = 100 nm. (b) Relaxed plasmid, not completely spread on the surface; z range = 2 nm, bar = 100 nm. (c) Plasmids oriented on the surface. Some molecules have circular conformations, other have coiled segments; z range = 5 nm, bar = 200 nm.

Results

DNA 500 bp long was used initially to develop and improve the technique, and individual molecules were observed (Fig. 1). We have tested different supports: graphite, mica and AP-mica. Molecules could only be observed when using AP-mica. Linear molecules could easily be observed.

Cutting a slit was necessary in order to easily insert the AP-micas into the gels. This probably also helped to preserve the surface of the AP-micas by minimizing scratching during the insertion. Molecules were transferred to the bottom of the AP-mica strips, about 2 mm above the end. These supports were sometimes dirty when observed by AFM, covered with contamination probably coming from the agarose gel. We tried to wash the AP-micas with Milli-Q water at room temperature or at 30°C or with propanol to remove the agarose. Rinsing with Milli-Q water was usually sufficient to obtain a clean surface. Thirty degree centigrade water was more efficient at cleaning the AP-micas, probably by softening the agarose. Propanol did not eliminate the contamination on the surface.

We have optimized the DNA deposition by varying the angle of insertion of the AP-micas and the electrophoresis time. The DNA transfer was most successful when the split was perpendicular to the surface of the gel and at an angle of 45° to the DNA band. Because of the angle of the incision, the density of the molecules on the support was position-dependent. The electrophoresis time which allows the DNA to arrive at the AP-mica depends on the rate of migration of the molecules in the gel. The electrophoresis time, t, for the molecules to just arrive at the AP-mica position is $t = l/v$, where l is the distance the band needs to travel to reach the AP-mica, and v is the speed of migration of the DNA band, measured before the AP-mica was inserted into the gel.

It is important to stop the electrophoresis before the molecules pass underneath the AP-mica strips. This effect can be observed by leaving AP-mica strips inserted into the gel for a long time while voltage is on. After a period of time, we observed a trail of DNA dyed with

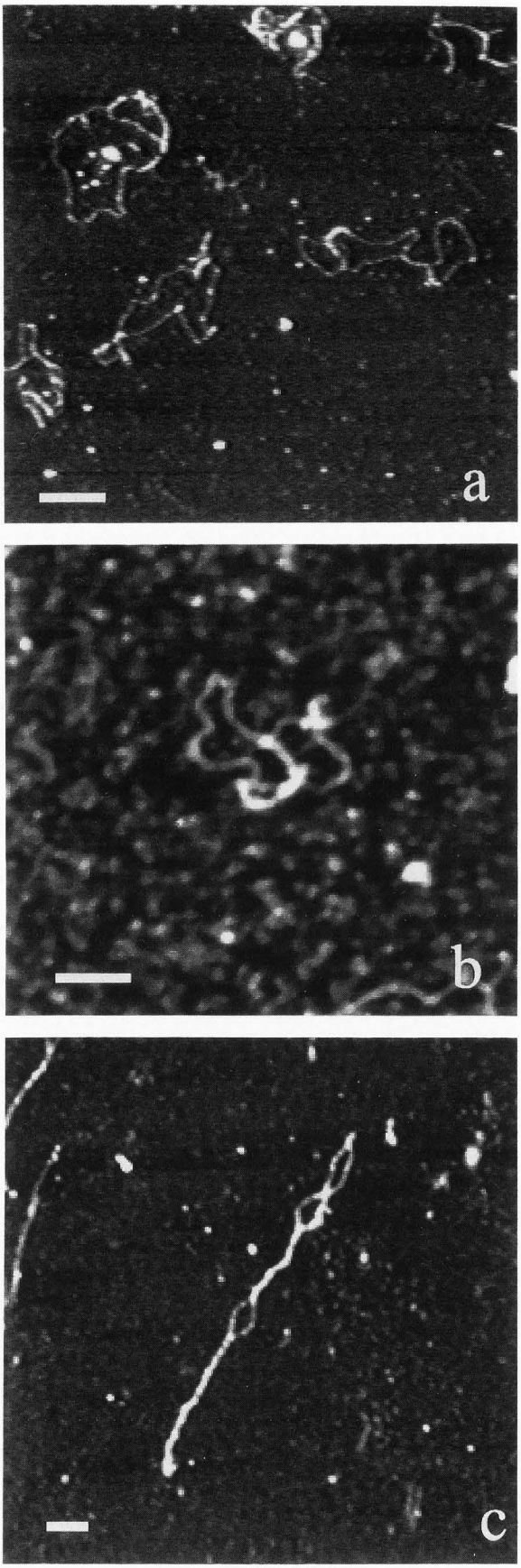

Figure 3. Circular DNA molecules electrophoresed onto AP-mica strips. These images are from the faster-migrating band of agarose gels in which two bands were resolved. Molecules are folded on themselves and never completely relaxed. (**a**) Bluescript plasmids coiled on the surface; z range = 3 nm, bar = 200 nm. (**b**) Bluescript plasmid obtained from the same gel as plasmid in Figure 2b. The plasmid here is completely folded and the molecular contour cannot be determined; z range = 2 nm, bar = 100 nm. (**c**) ϕX174 RF1 DNA: molecule is tightly wound so that it looks like a filament of 880 nm in length, with 3 small loops; z range = 6 nm, bar = 100 nm.

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ethidium bromide, extending well beyond the AP-mica strip.

ϕX174 and Bluescript DNA molecules were easily collected on the AP-mica supports, but the molecular density was not always reproducible. The transfer process was sometimes inefficient, with only a few molecules being observed. It could also be too efficient, with a dense network of molecules covering the AP-mica. It sometimes took a long time to find the molecules on the support, since their position is never exactly defined on the supports and the scanned areas are proportionally small in comparison with the AP-mica surface. Broken molecules and molecular fragments of different lengths were often observed.

Two bands were usually observed on the gel, which means that 2 groups of conformations were separated. These conformations are predicted to be supercoils and relaxed circles, which is consistent with the AFM results. Three bands were also frequently observed. The third band may be broken (linearized) DNA, which typically migrates between supercoiled and relaxed circular DNA. Intensity of the bands, as observed by ethidium bromide staining, always increased from the slower-migrating to the faster-migrating bands. We have imaged plasmids transferred from the gels displaying two bands. A difference in shape of the molecules was observed. Plasmids obtained from the slower-migrating band were sometimes folded but were often spread and looked like rings on the AP-micas (Figs. 2a and 2b). They sometimes formed loops, and the two strands entwined so that part of the molecules looked like a rope (Fig. 2c). Molecules were sometimes oriented on the surface (Fig. 2c).

Circular DNA molecules transferred from the fast band were folded on themselves (Figs. 3a and 3b) and were rarely well-spread. Parts of the molecules formed loops or ropes and molecules sometimes looked like linear filaments with small loops (Fig. 3c).

Polyacrylamide gels

DNA separated by polyacrylamide gel electrophoresis (PAGE) can be indirectly electrophoresed onto AP-

mica. DNA bands cannot be electrophoresed directly from polyacrylamide gels onto AP-mica because polyacrylamide gels have glass plates on both surfaces.

To electrophorese DNA from polyacrylamide gels onto AP-mica, the following technique was used: duplicate samples of DNA were separated by PAGE. A piece of the gel containing one sample was stained with ethidium bromide. The unstained lane of the polyacrylamide gel was laid across an agarose gel, perpendicular to the direction of current flow, and the DNA bands were electrophoresed from the polyacrylamide gel on the agarose gel onto the AP-mica, which was inserted into slits in the gel located at the positions of the DNA bands, as determined by comparison with the stained piece of the polyacrylamide gel.

Using this technique, ϕX174 Hae III digest DNA fragments were easily collected on the AP-mica supports, and we have observed pieces of different sizes separated in the same lane in the polyacrylamide gel (data not shown). It was easier to find the molecules on the support than in the case of the agarose gels, since molecules were collected on a small spot which could be located with a mark written on the back of the AP-mica strips when those were inserted into the gels.

Discussion

This technique is similar to the technique for electrophoretically transferring DNA onto DEAE (diethylaminoethyl)-cellulose (Sambrook *et al.*, 1989). The advantage of this technique is that molecules are directly collected onto a surface flat enough for AFM imaging. All the steps for eluting the molecules from the cellulose membranes are avoided. This simplifies the technique and eliminates all the chemical treatment that is necessary to detach the molecules from the DEAE-cellulose, such as low and high-salt buffer treatment, extraction by phenol:chloroform and ethanol precipitation.

We were unable to adsorb molecules from agarose gels onto bare mica. This is consistent with previous data which showed that DNA in solution needed divalent or trivalent cations to adsorb well onto bare mica. For example, DNA cannot be adsorbed onto bare mica from a buffer of 10 mM Tris, 1 mM EDTA (Bezanilla *et al.*, 1995), which is close to the composition of the electrophoresis buffer. DNA in a variety of buffers adsorbs well to AP-mica. Therefore, AP-mica is preferable for this application because the composition of the buffer seems to be less important for the adsorption of DNA than onto a bare mica support.

Mica is an insulator. Because graphite is a conductor, we thought the DNA might migrate more easily toward a conducting graphite support. It was difficult to obtain blocks of graphite thick enough to be manipulated and inserted into the gels with tweezers. The surfaces of these slabs also displayed many steps and fractures that could easily be confused with DNA, as has also been seen by scanning tunneling microscopy (Clemmer and Beebe, 1991; Heckl and Binnig, 1992). Although we made a number of attempts, we did not succeed in obtaining clear images of molecules on graphite.

Supercoiled molecules are more compact than relaxed circular molecules and their mobility in the gel is faster (Stryer, 1988). Our results are in agreement with this. Molecules extracted from the faster bands appeared more folded and twisted than molecules collected from the slower bands, where they were more relaxed. It is also reasonable that the fastest band on gels stained strongest with ethidium bromide, since commercial circular DNAs are primarily supercoiled. A typical batch of Bluescript DNA from Stratagene, for example, is certified to be ca. 90% supercoiled.

Conclusion

This technique is simple and fast and combines the advantages of AFM with those of agarose or polyacrylamide gel electrophoresis. We anticipate that it will be useful for investigating conformations of supercoiled molecules with different linking numbers and should also be useful for imaging nucleoproteins and protein-DNA complexes separated by gel electrophoresis.

Acknowledgments

We thank John Carbon for bringing to our attention the paper of Jett and Bear, Louise Clarke for helpful discussions, Mary Baum for expert technical assistance, Roger Proksch for critical reading and preparation of the manuscript, and Kerry Kim for preparing the 500 bp DNA by PCR. This research was supported by the National Science Foundation grant # MCB-9317466 and Digital Instruments.

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Discussion with Reviewers

Y. Lyubchenko: Typically, the separation between bands for individual topoisomers in high resolution gel electrophoresis is comparable with the width of the band. It would be a problem to deposit individual topoisimers from this type of gel if the incision in the gel is made at 45°.

Authors: Closely spaced bands could be deposited onto the same piece of mica and imaged successively in the AFM.

J. Vesenka: Can you speculate as to why only AP-mica was used, especially considering the perfection of the cationic treatment of mica by one of the authors?

Authors: When mica is rinsed with Ni(II), Co(II), or Zn(II) [Hansma HG, Laney DE (1996) DNA binding to mica correlates with cationic radius: Assay by atomic force microscopy. Biophys J **70**: 1933-1939], DNA molecules in Mg(II) buffers bind weakly to the surface and can be imaged in motion. We tried electrophoresing DNA onto Ni(II)-mica as a result of your suggestion but could not find any DNA in the AFM. We have not yet found an ionic treatment for mica that allows DNA to bind in the presence of EDTA.

F. Zenhausern: The resolution of the images is in good agreement with many results obtained earlier by Hansma's group and others when imaging is done with supertips.

Authors: New commercial tips are at least as sharp as supertips in our hands and have replaced supertips for most applications.