Immobilization of DNA for scanning probe microscopy


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ABSTRACT Reproducible scanning tunneling microscopy and atomic force microscopy images of entire molecules of uncoated plasmid DNA chemically bound to surfaces are presented. The chemically mediated immobilization of DNA to surfaces and subsequent scanning tunneling microscopy imaging of DNA molecules demonstrate that the problem of molecular instability to forces exerted by the probe tip, inherent with scanning probe microscopes, can be prevented.

The scanning tunneling microscope (STM) and the atomic force microscope (AFM) are relatively new and potentially powerful instruments for imaging biological molecules in vacuum, air, or liquid environments. Although many reports have presented DNA images, a number of problems remain to be solved. One major problem is the instability of the DNA on its mounting surface to forces exerted by the scanning probe results in most molecules being removed from the scanned area. The usual result is that only portions of molecules trapped in adsorbate patches or surface irregularities have been imaged. One of the primary goals of sample preparation must therefore be the immobilization of DNA on the mounting surface. We propose that surfaces chemically treated to attract DNA can be used routinely to visualize DNA in STM and AFM examinations. Using a modified gold surface, we reproducibly obtain the STM images of complete DNA molecules of a genetically functional plasmid.

Fragments of natural and synthetic DNA have been imaged by STM on highly oriented pyrolytic graphite (1–11) and gold (12, 13) surfaces. However, because topographic contrast is generally used to locate these extremely fine (2-nm diameter) molecules by scanning probe techniques, the DNA mounting substrate must be extremely flat. Surfaces with atomic flatness are ideal but usually lack sites that might anchor DNA either mechanically or chemically, thereby preventing displacement or removal by the scanning probe tip. Attempts to immobilize DNA on surfaces using electrochemical methods (14, 15) and covalent linking (13, 16, 17) of DNA to surfaces have been successful, but to date only images of DNA have been reported. Evidence for improved surface binding came from examination of a surface frequently imaged with the AFM. Mica, although atomically flat, is an insulator and therefore cannot be imaged with the STM but is well suited for the AFM. By simply drying a drop of a DNA-containing buffer solution onto freshly cleaved mica, one can routinely obtain AFM images of DNA strands within minutes (18–21), perhaps stabilized by hydrogen bonding of Si-OH to DNA. Recently a technique that replaced potassium ions in the mica surface with magnesium ions, promoting stronger binding of DNA to mica resulting in improved AFM images, has been described (22–24). Since the forces between the probe and the surface are roughly comparable in STM and AFM, the AFM work gave us hope that

Plasmid DNA (3204 base pairs, pBS* from Stratagene) was extracted from Escherichia coli, concentrated by cesium chloride/ethidium bromide equilibrium centrifugation, ethanol precipitated, and resuspended in 10 mM Tris-HCl/0.1 mM EDTA (TE) at pH 7.5 to a final concentration of 500 μg/ml (27). Circular DNA was relaxed by x-ray (70,000 rads; 1 rad = 0.01 Gy) mediated single-strand nicking of supercoiled molecules. Linearized plasmid was obtained by treatment with the restriction endonuclease Sma I. Agarose gel (0.8–1% agarose) electrophoresis was used to differentiate supercoiled from relaxed plasmid and linearized DNA (27). Relaxer DNA plasmid was radiolabeled by nick-translation in the following reaction mixture: 35 μl of DNA (500 μg/ml), 5 μl (10X) of NT buffer (500 mM Tris-HCl, pH 7.5/50 mM MgCl₂/100 mM 2-mercaptoethanol/1 mg of nuclease-free bovine serum albumin per ml), 4 μl of dTAG (50 μM dTTP/50 μM dATP/50 μM dGTP), 5 μl of [α-32P]dCTP (3000 Ci/ mmol; 10 μCi/μl; 1 Ci = 37 GBq), and 1 μl of E. coli DNA polymerase 1 (10 units/μl) (27). After 40 min at 14°C, the sample was diluted with water to a total volume of 150 μl, and 25 μl of 3 M sodium acetate was added. After extraction with phenol/chloroform (1:1) to purify the DNA, the sample was ethanol-precipitated twice and resuspended in 35 μl of TE, size-fractionated by agarose gel electrophoresis, and extracted from the gel utilizing the glass powder purification procedure (28), which allowed us to purify exclusively the relaxed circular form of the plasmid.

DNA was passively mounted onto gold surfaces by placing 1.0 ml of relaxed circular pBS* plasmid DNA into a glass nebulizer (Fullam, Latham, NY). Microdroplets of the sample were deposited by spraying 10 times onto epitaxial gold surfaces maintained at a temperature of 55°C. The surfaces were incubated an additional 15 min to volatilize the buffer prior to imaging in the STM.

Epitaxial gold surfaces 120 nm thick were prepared by electron beam evaporation of gold onto freshly cleaved mica surfaces heated to a temperature of 480°C (29) and cut into 0.48-cm disks. Gold surfaces were chemically modified by immersing for 24 hr in 0.005 M aqueous solutions of either cysteamine (Aldrich), which becomes 2-mercaptoethamylamine on the surface, or 2-dimethylaminoethanethiol (Aldrich), rinsed thoroughly in water, and air dried. The disks were floated, gold side down, onto 1.0-m1 solutions of plasmid

Abbreviations: STM, scanning tunneling microscope; AFM, atomic force microscope.

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DNA diluted to 0.25 μg/ml in 0.01 M ammonium acetate. After incubating for 3–6 hr, the disks were rinsed by plunging repeatedly into distilled, deionized water and air dried prior to imaging in the STM.

DNA uptake by the chemically modified gold surfaces was verified by substituting 32P-labeled pBS+ plasmid DNA in the procedure described above. Each disk was placed in a scintillation vial containing 5 ml of Insta Gel XF scintillation fluid (Packard Instruments) and assayed for radioactivity for 4 min in a Beckman LS6001C scintillation counter. Autoradiographic analysis of each disk was accomplished by removing the disk from the scintillation fluid, air-drying the disk, gluing the disk mica side down onto a piece of paper, and exposing the disks to x-ray film (Kodak).

STM and AFM images were obtained with a Nanoscope II (Digital Instruments, Santa Barbara, CA). The STM was operated in the constant current mode with the substrate biased positive with respect to the tip. STM tips were fabricated in our laboratory by shearing Pt/Ir (80/20) wire (Sigmund Cohn, Mount Vernon, NY). The AFM used commercial cantilevers (Nanoprobes, Digital Instruments) 200 μm long with a spring constant of 0.12 N/m. Images were obtained in a constant force mode (1.5–3 nN) and are presented as flattened raw data.

**RESULTS AND DISCUSSION**

The instability of DNA to forces exerted by the STM tip is illustrated in Fig. 1, where microdroplets of ammonium acetate solution containing pBS+ plasmid DNA were sprayed onto a heated monatomic gold surface to ensure uniform coverage and to volatilize the buffer. The first STM scan (Fig. 1a) shows a DNA aggregate with several DNA molecules in parallel and has a ragged appearance likely due to the interaction of DNA with the probe tip. The second scan (Fig. 1b) of this same area revealed the DNA to be mostly absent due to removal by the STM tip. In experiments where enough DNA to effectively cover the surface has been passively applied to substrates, we have found that the frequency of imaging DNA molecules is far less than expected.

When DNA is placed on mica surfaces AFM images of molecules can be easily obtained, suggesting that this insulator surface binds DNA with sufficient strength to resist removal by forces exerted by the tip. Usually the open

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**FIG. 1.** STM images of a DNA aggregate obtained from an initial scan (a) and the subsequent scan (b). The sample was prepared by spraying an aerosol solution of relaxed pBS+ plasmid DNA (5.0 μg/ml in ammonium acetate, pH 7.0) onto a heated gold surface at 55°C. Bias voltage = 100 mV, tunneling current = 0.3 nA, scan rate is 4.34 Hz, and the vertical scale (black to white shading) is 20 nm.

**FIG. 2.** AFM images of pBS+ plasmid DNA obtained by drying a 3-μl droplet (0.3 μg of DNA per ml in 0.01 M ammonium acetate) on freshly cleaved mica. The molecules are attached to the mica surface with sufficient force to resist removal by the probe. (a) Several circular plasmid molecules twisted about a centrally located open circular molecule. (b) A linearized plasmid twisted about itself to form a side branch. The coiling is readily apparent in this image but is only occasionally observed due to the bluntness of a typical probe tip.
circular plasmid molecules applied to the surface appear twisted together and poorly oriented, although occasionally an open circular molecule (Fig. 2a) associated with twisted molecules can be imaged. Although we have been able to clearly observe DNA twisting about itself (Fig. 2b) the resolution of the AFM is limited by the sharpness of the manufactured tips available and the mechanical force exerted on the sample by the tip and is not currently adequate to observe the double helix of DNA.

In Fig. 3 gold surfaces treated with surface modifiers that contain an amino end group are compared with surfaces treated with water for their potential to bind DNA. The scintillation counts clearly show that gold disks treated with either 2-mercaptopoethyamine or 2-dimethylaminoethanethiol bind DNA, at pH 5.0 and 8.0, to a greater degree than disks treated with water. For 2-mercaptopoethyamine-modified gold, the affinity increased with decreasing solution pH due to the increasing protonation of the amino group. However, the 2-dimethylaminoethanethiol, with two methyl groups on the nitrogen head group, has a $pK_a$ of 10.8 and remained positively charged and therefore bound DNA equally well at pH 5.0 and 8.0. Very likely the difference observed in DNA binding in the water control at pH 5.0 and 8.0 can be explained by deprotonation of adsorbed water at pH 8.0 with a resulting neutralization of the charge on the gold surface.

Lindsay et al. (30) have obtained similar results by binding 3-aminopropyltriethoxysilane to mica and substituting methyl groups for amino protons using methyl iodine and evaluating DNA uptake using $^{32}$P-radiolabeled DNA.

We found that flotation of the disks gold side down on the radiolabeled DNA solution was essential to ensure accuracy of the scintillation counting. If disks were immersed in the DNA solution, a wide spread in the counts was observed. We thought that the anomalously high counts might be a result of the inclusion of radiolabeled DNA into the partially open cleavage planes in the mica. This hypothesis is supported by the autoradiographic results shown in Fig. 3. When 2-mercaptoethyamine- or 2-dimethylaminoethanethiol-treated gold disks were exposed to radiolabeled DNA a uniform level of exposure over the surface was observed. In contrast, when disks treated with water were exposed to radiolabeled DNA solutions, only a characteristic "doughnut" image on the autoradiogram was observed. The doughnut image results from the accumulation of the $^{32}$P label at the disk edge and is consistent with the inclusion hypothesis.

Fig. 4 shows STM images obtained using 2-dimethylaminooethanethiol to chemically bind DNA to gold surfaces. Most of the STM images we have obtained (Fig. 4a, b, and d) show negative contrast (i.e., the tip apparently moves down as it traverses the DNA). However, some images show positive contrast (Fig. 4c). Considering that the feedback control of the STM adjusts the signal to the piezo transducer to keep the tunneling current constant, one would expect that DNA would present an additional tunneling barrier to this current, resulting in a downward tip motion and an increase in force over the DNA. Since for the tunneling impedance used to collect our data the tip is only about 1 nm above the gold surface, it must move up and over the DNA molecule to avoid displacing the DNA. These apparent contradictions can be reconciled by realizing that the imaging signal is a measure of transducer voltage, not of differential tip height. Flexibility in the structural elements of the STM responding to changes in force between the tip and surface can be as large as 20 nm (31). Positive and negative contrast images of DNA have also been observed in the AFM and are found to be associated with changes in ambient humidity (20–22). We found that conditions of low humidity produce the least amount of force between the probe tip and the surface, resulting in positive AFM images with improved detail and stability. Conversely, under conditions of high humidity a large capillary force exists between the probe tip and the DNA causing cantilever buckling resulting in negative images (21). The mechanism involved with tunneling through insulators and why image contrast can be either negative or positive are not well understood. Guckenberger et al. (32), using very high tunnel gap impedances (large tunnel gap), attribute contrast reversal to tip sharpness. Lindsay et al. (33) propose that the tip pressure required to achieve resonance tunneling through insulators also depends on tip geometry and therefore measured heights of insulator molecules are unreliable. However, we have observed spontaneous reversal of DNA contrast during two consecutive scans of the same area (Fig. 4c and d), which would be hard to account for by mechanical properties of the tip.

CONCLUSION

The experimental results show that it is possible to immobilize large DNA molecules on gold surfaces and image these molecules with the STM. Although theories to explain electron tunneling through insulators have been proposed, a better understanding of the mechanics of the STM and of electron tunneling in the presence of biomolecules would help direct improvements in imaging. Future experimental efforts can then be focused on improvements in resolution and investigations in aqueous environments where it may be possible to observe biochemical reactions directly in the microscope by introducing enzymes or other agents and observing their effects on DNA in situ. This capability is not conceivable in a vacuum-based electron microscope.

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FIG. 4. Unprocessed top view STM image of pBS+ plasmid, a circular DNA, mounted on 2-dimethylaminoethanethiol-derivatized gold surfaces. (a) Several molecules that are obviously circular, but in most cases contour lengths cannot be measured due to entanglements of the DNA with itself. (b) A higher magnification image of one of the more open-circular molecules taken on a subsequent scan of the surface. This molecule has a contour length of 0.94 μm, in agreement with the 1.08-μm length expected for B-form DNA of this plasmid. The apparent width of the imaged molecule is 3–5 nm; DNA should have a diameter of about 2 nm. The negative contrast indicates that the molecule appears 0.6–1.2 nm below the surface. Most of the images we have recorded show this negative contrast (a, b, and d); however, we occasionally obtain positive images (c) in which the DNA appears to be 0.6–1.2 nm above the surface. Successive images of the same area display positive-contrast DNA (c), switching to negative-contrast DNA (d). The switch in contrast was spontaneous; no operating conditions were changed. Note that the step edges of the gold in both images do not reverse contrast, and height changes in the step edges between images remain fixed at 1.27 nm (small arrowhead) and 2.64 nm (larger arrowhead). Images were obtained at voltage biases and tunneling currents set at 1000 mV, 0.37 nA (a), 500 mV, 0.21 nA (b), 500 mV, 0.4 nA (c), and 500 mV, 0.4 nA (d). The scan rate is 4.34 Hz for all images. In all images the vertical scale (black to white contrast) is 10 nm.

References