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Scanning tunneling microscopy of DNA: The chemical modification of gold surfaces for immobilization of DNA

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We have investigated the coulostatic immobilization of DNA to a chemically modified gold surface. The chemical modification involves the covalent attachment of chemically polarizable groups by reaction of thiols and disulfides with clean gold surfaces. We have determined that chemically modified surfaces possessing pendant cationic groups bind DNA. In this paper we report the reliable and reproducible scanning tunneling microscopy (STM) imaging of DNA using a gold surface chemically modified with *N,N*,-dimethyl-2-mercaptoethylamine and present the first STM images of an entire genetically functional DNA molecule, the circular plasmid pBS$^+$.

I. INTRODUCTION

Scanning tunneling microscopy (STM) is a relatively new and potentially powerful method for imaging biological molecules at close to atomic resolution. With STM one can visualize biological molecules which are larger than those suitable for x-ray crystallography and at a resolution greater than transmission electron microscopy (TEM). When compared to conventional electron microscopy, STM has two important advantages that make it especially attractive to biological investigation. First, the STM image is a topograph obtained by scanning an atomically sharp tip over a conducting surface and recording movement of the tip. Therefore, unlike TEM where chemical staining is necessary to produce contrast, there is no need to sacrifice resolution through the use of stains as long as the material being imaged is conductive. Second, unlike TEM which must be performed in a vacuum, the STM can be operated in any medium including liquid, and, therefore, provides the potential to study biological structures in their natural environments. Although biological material in bulk is nonconductive, when isolated molecules are placed on a conductive surface, high-quality STM images have been obtained through a mechanism which is not yet understood.

Heterogeneous fragments of natural and synthetic DNA have been imaged on both highly oriented pyrolytic graphite (HOPG)[1–9] and gold surfaces.[10,11] Although atomic resolution has not been achieved, highly resolved images showing the helical repeat, the major and minor grooves,[2,4] base pairing,[7] and individual bases[6] have been reported. Air drying a droplet of DNA onto a HOPG or gold surface is the most common preparative technique employed to image DNA. However, only portions of DNA molecules trapped in aggregates or in lattice defects of the substrate have been imaged using this method. Imaging entire DNA molecules requires both the uniform deposition of molecules onto the substrate and the binding of these molecules with sufficient strength to resist removal by the physical and electronic forces exerted by the tunneling tip. We have frequently observed strands of plasmid DNA on gold being moved across the substrate during scanning. Successful attempts to immobilize DNA to surfaces using electrochemical methods[12,13] and covalent linking of DNA to surfaces have been reported,[14–16] but to date only fragments of DNA molecules have been imaged.

We have presented successful results of coulostatic immobilization of DNA onto chemically modified gold surfaces.[17] Our approach involved the anchoring of chemically polarizable pendant groups by reaction of surface active thiols with clean gold surfaces followed by the immobilization of nucleic acid by ion exchange. This approach is illustrated schematically in Fig. 1. Using radiolabeled DNA and monitoring the uptake onto various treated surfaces, we were able to rapidly evaluate the efficacy of each treatment. In this paper using a gold surface chemically modified with *N,N*-dimethyl-2-mercaptoethylamine, we report routine reproducible imaging of DNA, and present the first STM images of an entire circular plasmid DNA molecule.

II. EXPERIMENT

A. DNA preparation

Plasmid DNA (3204 bp, pBS$^+$ from Stratagene, La Jolla, CA) was prepared by alkaline lysis and cesium chloride-ethidium bromide equilibrium centrifugation to enrich

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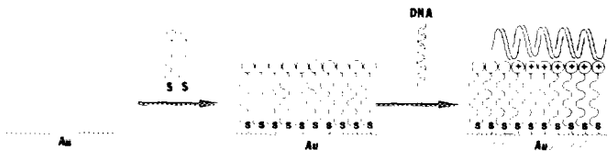

FIG. 1. Schematic of the method used for the coulostatic immobilization of DNA onto chemically modified gold surfaces.

for the supercoiled form.[18] After two ethanol precipitations, the sample was resuspended in 10 mM tris-HCl 0.1 mM EDTA (TE) at *p*H 7.5 to a final concentration of 500 μg/ml. Relaxed circular DNA was prepared by x-ray (70 000 rad) mediated single-strand nicking of supercoiled molecules. Agarose gel electrophoresis was used to differentiate supercoiled from relaxed plasmid DNA.

The radiolabeled DNA used was prepared by nick-translation of the above relaxed plasmid in the following reaction mixture: 35 μl DNA (500 μg/ml), 5 μl (10×) NT buffer (500 mM tris-HCl, *p*H 7.5, 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 1 mg/ml of nuclease-free bovine serum albumin), 4 μl dTAG (50 μM dTTP, 50 μM dATP, and 50 μM dGTP), 5 μl [α-^{32}P]dCTP (3000 Ci/mmol, 10 μCi/μl), and 1 μl *E. coli* DNA polymerase I (10 units/μl).[18] After 40 min at 14 °C, the sample was diluted with water to 150 and 25 μl of 3 M sodium acetate was added. After extraction with phenol: chloroform (1:1) the sample was ethanol-precipitated twice and resuspended in 35 μl TE. The radiolabeled DNA was size-fractionated by agarose gel electrophoresis and extracted from the gel utilizing the glass powder procedure[19] which allowed us to purify exclusively the relaxed circular form of the DNA.

B. DNA immobilization

Epitaxial gold surfaces 120 nm thick were prepared by electron-beam evaporation of 99.999% pure gold (Cerac Inc., Milwaukee, Wisconsin) onto freshly cleaved mica surfaces heated to a temperature of 480 °C.[20] The mica supporting the gold film was place into a hole punch (Ralmike's Tool-A-Rama, Plainfield, NJ) mica side up and 3/16-in.-diam disks were punched out.

Chemical modification of the gold surface was accomplished by immersion of the gold-coated mica disks for up to 24 h in 0.005 M aqueous or ethanol solutions containing the surface modifier to be tested, rinsed thoroughly in water, and air-dried. Disks to be imaged in the STM were floated gold side down onto 1.2 ml solutions of plasmid DNA diluted to either 5.0 or 0.25 μg/ml in 0.01 M ammonium acetate adjusted to *p*H 5.0, 7.0, or 9.8. After incubating for 3–6 h, the disks were picked up as close to the edge as possible using self-closing tweezers, and rinsed by plunging the disk ten times into each of four successive beakers filled with distilled, deionized water. Samples were then air-dried and mounted by silver paint on copper blanks prior to imaging.

The uptake of DNA by the chemically modified gold surfaces was verified by substituting ^{32}P radiolabeled pBS$^+$ plasmid DNA (0.4 μg/ml times 30 min–30 h) in the procedure described above. After rinsing, each disk was placed in a scintillation vial containing 5 ml of Insta Gel XF scintillation fluid (Packard Instruments Inc., Downers Grove, IL). The ^{32}P activity of each disk was determined by scintillation counting (2–10 min) with Beckman LS6001C scintillation counter (Beckman Instruments Inc., Fullerton, CA). 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).

C. DNA visualization

STM images were obtained with a Nanoscope II (Digital Instruments, Santa Barbara, CA) operating in the constant current mode at a tunneling current of 0.2–1.0 nA with the substrate biased 100–1000 mV positive with respect to the Pt/Ir tip. Most images were obtained at a tunneling current of 0.4 nA and a bias of 500 mV. Biases between 500 and − 500 mV did not appear to significantly affect the images. A scan rate of 4.7 Hz or less gave the best results.

III. RESULTS AND DISCUSSION

A. DNA immobilization

The chemical modification of electrode surfaces has been an active area of investigation.[21] Whitesides and co-workers[22] have shown that alkanethiols and alkanedisulfides adsorb from solution onto gold surfaces by formation of Au–S–C bonds. For long-chain alkanethiols and alkanedisulfides, adsorption onto the gold surface displaces other chemisorbed materials and results in the self-assembly of densely packed, oriented monolyers. Therefore, derivation of gold surfaces with thiols and disulfides is a particularly efficacious means for introducing functionality to the gold/solution interface.

In an earlier paper[17] we reported that gold surfaces derivatized with bifunctional alkanethiols and alkanedisulfides differentially adsorb DNA. The chemisorption of ^{32}P-radiolabeled DNA to 2-mercaptoethanol-modified gold was comparable to that observed for water-treated gold. The affinity of 3,3′-dithiobispropionic acid-modified gold for DNA was equal to or less than that observed with the water-treated gold surface. The affinity decreased with increasing solution *p*H due to the increasing ionization of the negatively charged carboxyl group. However, with surface modifiers which contained an amino end group (e.g., cystamine and *N*,*N*′-dimethyl-2-mercaptoethylamine), the affinity of DNA was equal to or greater than that observed with the water-treated gold surface. For cystamine-modified gold, the affinity increased with decreasing solution *p*H due to the increasing protonation of the amino group. However, the *N*,*N*′-dimethyl-2-mercaptoethylamine with two methyl groups on the nitrogen head group and a *p*Ka of 10.8 remained positively charged, and therefore bound DNA from *p*H 5.0 to 9.5 and was the compound selected for further study.

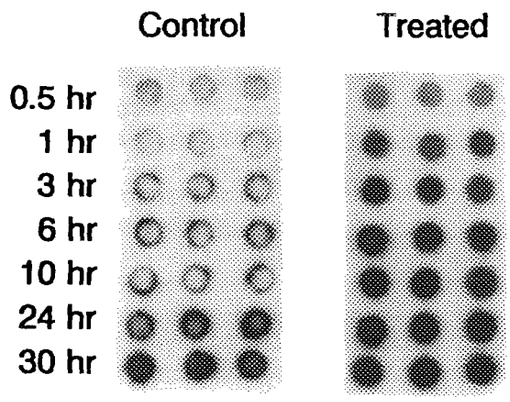

FIG. 2. Autoradiogram of gold-coated mica disks after treatment with water (control) and *N,N*-dimethyl-2-mercaptoethylamine (treated) prior to incubation on ^{32}P radiolabeled pBS$^+$ plasmid DNA (0.4 μg/ml in 0.01 M ammonium acetate *p*H 7.0) for the times indicated.

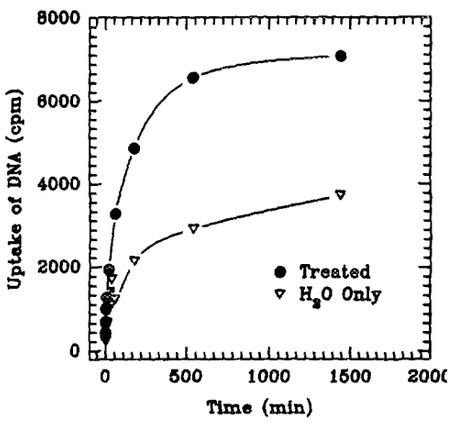

FIG. 3. Time dependence of radiolabeled DNA immobilization onto a water-treated gold surface represented by triangles and onto a *N,N'*-dimethyl-2-mercaptoethylamine-modified gold surface represented by circles. The DNA solution at a concentration of 0.4 μg/ml was buffered to *p*H 7.00 with 0.01 M ammonium acetate. Each data point is the mean cpm determined for at least four disks.

When we investigated the binding of DNA to gold-coated disks we found that flotation of the disks gold side down on the radiolabeled DNA solution was essential to ensure accuracy of the scintillation counting. When disks were immersed in the DNA solution, a wide spread in the counts was observed. We hypothesized that the anomalously high counts were 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 depicted in Fig. 2. When *N,N'*-dimethyl-2-mercaptoethylamine-treated gold disks were exposed to radiolabeled DNA for varying lengths of time, a uniform level of exposure over the surface of the disk was observed. In contrast, when disks treated with water were exposed to radiolabeled DNA solutions, 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.

The chemisorption of DNA onto the chemically induced, positively charged gold interface was found to be time dependent. Figure 3 shows the results obtained when water treated and *N,N'*-dimethyl-2-mercaptoethylamine-treated gold disks were exposed to ^{32}P DNA. The amount of DNA chemisorbed to both types of disks increased with time. At all time points, the affinity of DNA for the amine-modified disks was more than twice that of the water-treated gold disks. After approximately 6 h, the rate of DNA uptake for both surfaces were comparable. This indicated that exposure times longer than 6 h primarily resulted in increased DNA uptake at the mica lattice defect sites. This was confirmed autoradiographically.

B. DNA visualization

A STM topograph of the *N,N'*-dimethyl-2-mercaptoethylamine-modified gold surface is presented in Fig. 4(a). Plateaus separated by step edges are clearly visible, and, except for patches of globular adsorbate associated with the step edges, the images are similar to untreated gold surfaces. When *N,N'*-dimethyl-2-mercaptoethylamine-modified gold surfaces were floated for 6 h on pBS$^+$ plasmid DNA (0.01 M ammonium acetate *p*H 7.0) solution at a DNA concentration of 5.0 μg/ml and rinsed thoroughly with distilled water images [Fig. 4(b)] showed a heavy adsorbate covering the gold surface that completely obscured the flat plateaus and step edges. We presumed that

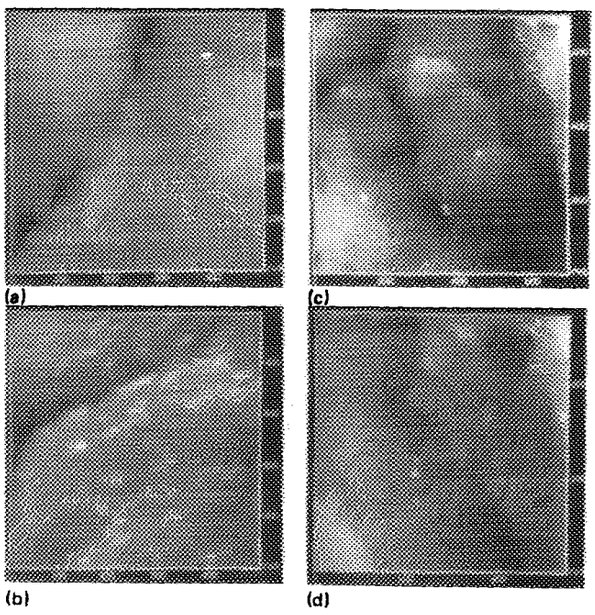

FIG. 4. STM topographs of gold-coated mica surfaces (a) after treatment with *N,N*-dimethyl-2-mercaptoethylamine, (b) after incubation of the treated surface with 5.0 μg/ml pBS$^+$ plasmid DNA in 0.01 M ammonium acetate *p*H 7.0 for 6 h, (c) after incubation of the treated surface with 0.25 μg/ml pBS$^+$ plasmid DNA in 0.01 M ammonium acetate *p*H 7.0 for 3 h, and (d) a high-resolution image showing a centrally located open circular pBS$^+$ plasmid DNA molecule. *Z* scale range for (a),(b),(c) = 20 nm, (d) = 10 nm.

the adsorbate coating the gold surface might be due to a high concentration of DNA on the surface. When the solution concentration of the DNA was reduced to 0.25 μg/ml and the treated gold surface incubated for 3 instead of 6 h, images of isolated plasmid DNA molecules were seen [Fig. 4(c)]. The DNA molecules are clearly circular although it would be difficult to trace and measure contour lengths for most of the molecules due to their twisted nature. The molecules appear to be randomly distributed over the surface and do not show a preference to congregate at step edges or on the flat plateaus. Also, the DNA can be seen to cross step edges at numerous points without affecting the image. Figure 4(d) is a higher-resolution image of plasmid DNA molecules. The centrally located molecule is an open circular molecule with a measured contour length of 0.94 μm. Since pBS$^+$ plasmid DNA contains exactly 3204 base pairs, we would expect this molecule to have a contour length of 0.815 μm if it was in the A form of DNA with a helix pitch of 2.8 nm per 11 base pairs, and a contour length of 1.08 μm if it was B form DNA with a helix pitch of 3.4 nm per 10 base pairs.[23] Since this molecule is circular and of the correct size, we propose that this is the first STM image of an entire genetically functional DNA molecule. Repeated scanning did not result in either conformational or positional changes in this molecule supporting further the utility of coulostatic immobilization.

It is interesting to note the *negative* images are obtained for the molecules immobilized on the cationic monolayers. We found that the image contrast and reproducibility was independent of bias voltage magnitude and sign. We suggest that the negative contrast may result from an increased impedance in the local region of the DNA. This has been observed for large nonconducting structures such as tobacco mosaic virus.[24] There it was suggested that the actual tip motion at the surface may have been opposite to the negative contrast observed due to mechanical stiffness of the STM. Alternatively, we suggest this increased impedance may result from our use of the volatile buffer ammonium acetate and the coulostatic binding of the DNA to the amine-modified gold surface. Since each DNA-laden disk is repetitively plunged into distilled, de-ionized water, only tightly bound DNA and ion-paired acetate is left on the cationic monolayer. We believe that the acetate ion is removed in the form of acetic acid during the course of drying. Thus, the only protonated amine that remains is that which is ion paired to the phosphates on the DNA. We envision the ion-paired phosphate-protonated amine acts as a classical double-layer capacitor and modifies the tunneling impedance in that local region. Tunneling spectroscopy experiments are currently under way to test this suggestion.

Another plausible explanation for the negative contrast may be that multilayering of the surface modifier is present on the gold surface. Upon exposure of the modified surface, the DNA molecule becomes imbedded in multilayers. To test this suggestion, we are investigating the influence of alkyl chain length on the affinity of the modified surface for DNA and the image contrast. Preliminary results have indicated that extending the alkyl chain from 2 to 11 carbons produces a modest but measurable increase in DNA binding affinity. Increasing the carbon chain length increases the ordering of the mercaptoalkylamine monolayers due to increased crystal packing forces.[22] We have determined that the binding affinity decreases by 35% when the gold surface was treated with a 1:1 mixture of 11-aminoundecanethiol and 11-mercaptoundecanol. Even though the number of amine groups at the interface is reduced to 50% by the presence of the long-chain alcohol in the monolayer, the DNA affinity does not decrease proportionately. This suggests that spacing between the cationic sites at the interface plays an important role in DNA binding. We are continuing to investigate the immobilization of DNA onto chemically modified gold surfaces and will detail our findings in due course.

IV. CONCLUSION

We have demonstrated that gold surfaces modified with bifunctional mercaptoamines and mercaptodisulfides will immobilize DNA at solution *p*H's where the amino end groups are protonated. The cationic layer holds the DNA in place by ion pairing with the negatively charged phosphate groups on the DNA backbone. This ion pairing is sufficiently strong to resist changes in the DNA's location and conformation induced by tip forces. With these chemically modified surfaces, we have observed reproducible STM topographic images of an entire DNA molecule.

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