

# Scanning Microscopy

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Volume 1996  
Number 10 *The Science of Biological Specimen  
Preparation for Microscopy*

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Article 9

5-8-1996

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### Recommended Citation

Lyubchenko, Yuri L.; Blankenship, Robert E.; Gall, Alexander A.; Lindsay, S. M.; Thiemann, Ottavio; Simpson, Larry; and Shlyakhtenko, Luda S. (1996) "Atomic Force Microscopy of DNA, Nucleoproteins and Cellular Complexes: The Use of Functionalized Substrates," *Scanning Microscopy*: Vol. 1996 : No. 10 , Article 9. Available at: <https://digitalcommons.usu.edu/microscopy/vol1996/iss10/9>

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## ATOMIC FORCE MICROSCOPY OF DNA, NUCLEOPROTEINS AND CELLULAR COMPLEXES: THE USE OF FUNCTIONALIZED SUBSTRATES

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(Received for publication August 6, 1995 and in revised form May 8, 1996)

### Abstract

Progress towards rapid and simple characterization of biomolecular samples by scanning probe microscopy is impeded mainly by limitations of the current approach to sample preparation. We are working on approaches based on chemical functionalization of mica. Treatment of mica with aminopropyltriethoxy silane (APTES) makes the surface positively charged (AP-mica) and able to hold DNA in place for imaging, even in water. We have shown that AP-mica is an appropriate substrate for numerous nucleoprotein complexes as well. The AFM images of the complex of DNA with RecA protein are stable and indicate a structural periodicity for this filament. AP-mica holds strongly such large DNA complexes as kinetoplast DNA (kDNA) and is an appropriate substrate for their imaging with AFM. We have further develop this approach for making hydrophobic substrates. Silylation of mica surface with hexamethyldisilazane (Me-mica) allowed us to get AFM images of chlorosomes, an antenna complex isolated from green photosynthetic bacteria. Me-mica may be converted into a positively charged substrate after treatment with water solutions of tetraethylammonium bromide or cetyltrimethylammonium bromide. These activated surfaces show high activity towards binding the DNA molecules.

**Key Words:** Scanning probe microscopy, AFM, SFM, mica, silylation, molecular imaging, DNA, SAM

### Introduction

Sample preparation is a crucial steps for any type of microscopy, and particularly for scanning probe microscopy (SPM). In this novel type of microscopy, which mostly include scanning tunneling microscopy (STM) and atomic force microscopy (AFM), a very sharp tip "reads" the surface profile [6, 7]. Therefore SPM imaging of molecular adsorbents requires their strong attachment to the surface in order to avoid resolution-limiting motion occasioned by the tip during scanning, which in extreme cases can result in sweeping the adsorbed molecules away from the surface. The effect of tip motion is reduced if the AFM instrument is operated in non-contact or tapping mode [see, e.g., 12, 15, 16], but even in this case the problem of sample preparation is an important one. The class of suitable substrates for AFM is rather narrow and mostly includes natural mineral mica, glass and silica surfaces. In a very limited number of cases biomolecular adsorbents bind to a bare substrate allowing their imaging with AFM. For example, images of chromatin [1] were obtained for samples prepared on cover glass. Membrane proteins can be imaged with very high resolution because on the mica surface they form a 2D array which stabilizes lateral movement of proteins during scanning [16, 18]. Note that the progress in AFM imaging of nucleic acids was made through the development of substrate preparation methods based predominantly on the polyelectrolyte character of DNA and RNA molecules (see reviews [11, 16, 25-27]). Vesenka, Bustamante and co-workers [11, 39] developed the method of ionic treatment of mica. In this approach the mica surface is treated with divalent cations (e.g.  $Mg^{2+}$ ) to increase its affinity for DNA which is held in place strongly enough to permit reliable imaging by AFM. Dramatic improvements were obtained by using specially made tips and by imaging under propanol (as reviewed in [11, 15, 16]). Other di- or trivalent cations can be used for pre treatment of mica [36]. The presence of a multivalent cation helps spread the DNA and bind it to the mica. In addition to imaging of DNA, this method of sample preparation was success-

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fully applied to studies of complexes of DNA with proteins [11, 12, 26]. Yang *et al.* [42] have developed an approach which is a modification of the well-known electron microscopic procedure. They deposited the DNA molecules onto a carbon coated mica substrate using cytochrome C spreading technique. Gold substrates can be activated by self-assembled monolayers (SAM) of thiols for reliable STM and AFM imaging of DNA [2, 3, 17]. Note that both physisorbed and chemisorbed SAM are used to make monolayer and multilayer structures with predetermined molecular architecture [38]. Non-treated cover glass can be used as the AFM substrate for binding chromatin, although the rinsing step (to remove non-bound material and salt components) should be done gently and the dried sample should be imaged immediately [1]. High resolution AFM images of chromatin were obtained by use of mica treated with spermidine [20, 43].

We [21-26] have worked out a procedure for mica modification (AP-mica) that allowed us to routinely perform visualization of DNA with AFM, achieving resolution as good as that of traditional electron microscopy (EM). The method is based on covalent attachment of aminopropyltriethoxy silane to the surface of mica, which makes it positively charged. DNA samples are deposited directly onto the modified mica (AP-mica) through self-adsorption in a *one-step procedure*. After that, DNA samples can be imaged directly with AFM. It is important to note that deposition of samples in our procedure can be performed under a wide range of environmental conditions (pH, ionic strength, temperature); this feature of AP-mica is an advantage for imaging of nucleoprotein complexes of different types [24-26]. Another important feature of AP-mica is its high stability; this often allows, for example, preparation of the substrate well in advance of deposition.

AP-mica, however, is only a good substrate for holding molecules bearing a negative charge. Biological objects have a broad spectrum of physical chemical characteristics; therefore, substrates with different affinities are required for strong attachment of various biological objects. Keeping in mind the positive results obtained with AP-mica, we investigated the use of chemical modification procedures to obtain surfaces with various other characteristics, such as hydrophobic and other positively charged surfaces. Hydrophobic surfaces were used for imaging of chlorosomes, an antenna complexes isolated from green photosynthetic bacteria. We have shown that a hydrophobic surface can be transformed into a positively charged one after treatment with salts carrying hydrophobic chains. These surfaces bind DNA molecules as well as AP-mica. Strongly positively charged surfaces (obtained after modifications with iodopropyltrimethoxy silane followed by surface

activation with DABCO) were used for imaging of non-membrane proteins.

## Materials and Methods

### Mica modification procedures

**AP-mica:** Modification was performed in vapors of 30-aminopropyltriethoxy silane (APTES) following the procedure described elsewhere [5, 21, 25]. Briefly, freshly cleaved strips of mica were left in the APTES atmosphere created in 2l glass desiccator containing 30  $\mu$ l of vacuum distilled APTES under ambient conditions for 2 hours.

**Me-mica:** 30  $\mu$ l of methyltrichlorosilane or hexamethyldisilazane (Aldrich, Milwaukee, WI) in a small plastic vial was placed on the bottom of a 2l-desiccator. Freshly cleaved strips of mica were mounted at the top of the desiccator and left for 2 hours for modification after filling the system with argon. Positively charged mica was obtained by incubation of Me-mica in saturated water solutions of tetraethylammonium bromide (TEAB) or cetyltrimethylammonium bromide (CTAB) for 10 min. at room temperature.

**IP-mica:** Iodopropyltrimethoxy silane (IPTMS) was synthesized as described in [9]. 10  $\mu$ l of IPTMS in a 1.5 ml plastic vial were placed on the bottom of a 100-ml glass bottle and freshly cleaved mica strips were mounted at the top of the glassware. The bottle was placed into a preheated oven (80°C) and mica modification allowed to proceed for 2 hours. N4-mica substrates were obtained by 30 min. incubation of IP-mica in 1 mM solution of diazabicyclo [2,2,2] octane (DABCO, Aldrich, Milwaukee, WI) for 30 min.

**Chlorosome samples:** *Chloroflexus aurantiacus* was grown photoheterotrophically in 1-liter batch cultures at 55°C as described in [29]. The cells were harvested by centrifugation after 3-4 days of growth and chlorosomes were isolated by the method of Gerola and Olson [14] using a 2M concentration of the chaotropic agent, NaSCN. After a 10-50% linear sucrose gradient ultracentrifugation, the chlorosome band was collected for spectroscopic studies.

**Kinetoplast DNA (kDNA):** Sample preparation was performed by a protocol described elsewhere [32, 33, 35]. Namely, kDNA was isolated from stationary phase *Leishmania tarentolae* (UC strain) cells grown in brain heart infusion medium (Difco Laboratories, Detroit, MI) supplemented with 10 mg/ml hemin. The cells were washed in SET buffer (150 mM NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 7.5), resuspended in SET at a cell density of  $1 \times 10^9$  cells/ml and lysed with 0.5 mg/ml pronase and 3% Na sarcosinate for 1 h at 60°C. The genomic DNA was fragmented by passing the lysate through an 18 gauge needle at 25 psi. The kDNA

networks, which are relatively resistant to shear forces, are pelleted by centrifugation in the SW28 rotor at 22,000 rpm for 1.5 hr at 4°C. The kDNA is resuspended in 10 mM Tris HCl (pH 7.9), 1 mM EDTA (TE buffer), and sedimented through a CsCl step gradient in an SW 28 rotor for 15 min at 20,000 rpm. The kDNA networks sedimented to the interface and were visualized by ethidium bromide staining. The band was recovered, the dye removed by n-butanol extraction, and the DNA concentrated with sec-butanol and dialyzed against TE buffer. The DNA was then extracted with phenol/chloroform, ethanol-precipitated and resuspended in TE. The network DNA was examined for integrity in the fluorescence microscope after staining with DAPI (1 mg/ml).

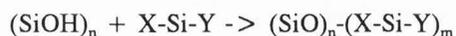
**Double-stranded RNA** samples from retrovirus containing dsRNA molecules of 11 different sizes were prepared as described elsewhere [21, 23].

**Radiolabeled assay:** Mica substrates were incubated for different times at room temperature in a solution of <sup>32</sup>P-labeled HpaII restriction fragments of pBR 322 plasmid DNA (New England BioLabs, Beverly, MA) at concentration of 0.2 ng/ml in 10 mM Tris-HCl buffer (pH 7.5), containing 20 mM NaCl, 5 mM EDTA. Ratios of bound to total DNA were calculated from the radioactivity of the rinsed mica plates and of the DNA solution before and after the binding procedure.

**Apparatus:** Imaging was carried out on a NanoScope II or a NanoScope III instruments (Digital Instruments, Inc., Santa Barbara, CA) in contact mode using commercial AFM cantilevers from Park Scientific Instruments (Sunnyvale, CA) with a nominal spring constant 0.6 N/m. Imaging in solution was performed by using a fluid cell provided by Digital Instruments. Scanning rate was 2-3 Hz, typical for scanning over 1-10 μm areas .

## Results

Our approach to substrate preparation for AFM is based on functionalization of silicon surfaces through a covalent attachment of a silane compound with a specific characteristic. The chemistry of silane coupling agents was founded by E. Plueddemann almost 50 years ago and is currently well understood [30]. A class of organofunctional silanes of the general formula X-Si-Y (I) (where Y is the organofunctional group and X is a hydrolyzable group) is widely used for bringing an organofunctional group Y to the surface. A general scheme for this reaction is as follows:



The functional groups (Y) are chosen for reactivity

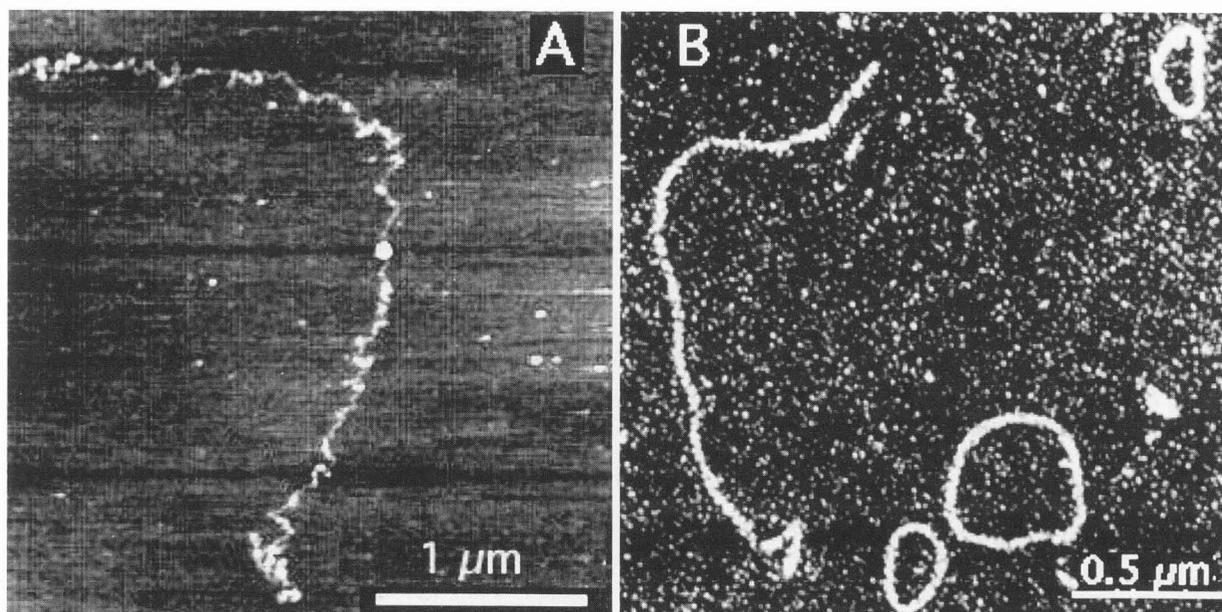
or compatibility with the biopolymer, while the hydrolyzable groups (X, (CH<sub>2</sub>O)<sub>3</sub>, for example) are responsible for covalent bonding of (I) to mineral surfaces (SiOH)<sub>n</sub>. Most commercial coupling agents are supplied as alkoxysilanes (methoxy or ethoxysilanes).

### Positively Charged AP-Mica: Imaging of kDNA

We used aminopropyltriethoxy silane (APTES) for preparing a positively charged mica surface (AP-mica, [5, 21, 25, 26]). This compound with (EtO)<sub>3</sub> as X and an aminopropyl moiety as Y attaches covalently to silicon surfaces at ambient conditions [21, 25] leaving the amino group on the surface. This group protonates in water solutions making the surface positively charged. Modification of mica in very low concentration solutions of APTES or in its vapors allowed us to obtain uniformly modified smooth AP-mica surfaces suitable for AFM imaging of DNA, RNA and bacteriophages in air and under water [21, 22, 24, 25]. Examples of images of lambda DNA and circular and linear DNA molecules coated with RecA protein are shown in Figure 1A and 1B respectively (RecA-DNA complexes were provided by Dr. A. Stasiak, Lausanne University, Switzerland). A periodic structure of RecA filament is resolved for complexes prepared on both linear and circular DNA templates. It turned out that AP-mica surface strongly holds these samples, allowing imaging in air, propanol and water (Lyubchenko *et al.*, in preparation).

We also investigated the possibility of using AP-mica as a substrate for AFM imaging of large DNA aggregates such as kinetoplast DNA (kDNA). kDNA, the mitochondria-type DNA in trypanosomes and related protozoan parasites, is unusual in both its structure and genetic function. kDNA is a giant network composed of several thousands minicircles and a few dozen of maxicircles, which are topologically interlocked (reviewed in [10]). Electron microscopic studies showed that sizes of isolated networks varied for different species between 4-6 μm for tripanosomids and 15-20 μm for *Crithidia* genus [10].

A 30 μl droplet of the kDNA sample (concentration 10 μg/ml in TE buffer (pH 7.5)) isolated from *Leishmania tarentolae* was applied onto AP-mica for 5 min, allowing the DNA molecules to adsorb, then the mica sample was rinsed with deionized water, dried in argon and used for AFM imaging. A large scale image (27 x 27 μm) obtained by using the J-scanner (maximum scanning area is 100 μm x 100 μm) is shown in Figure 2A. The majority of particles have a cap-like geometry ~3 μm in diameter with a bright rim (see, for example particle 1; cf. with EM images of kDNA particles with rims in recent paper [31]). These images are qualitatively similar to those obtained for the same sample with fluorescence microscopy [35]. A few particles with a



**Figure 1:** (A) AFM images of lambda DNA. DNA concentration is  $0.01 \mu\text{g/ml}$  in buffer containing  $0.1 \text{ mM}$  Tris-HCl,  $\text{pH}7.6$ ,  $0.01 \text{ mM}$  EDTA was deposited onto AP-mica according to the procedure described elsewhere [21-23]. (B) AFM images of RecA-DNA complexes deposited onto AP-mica from the reaction mixture for RecA protein [26].

rim are more elongated (see particle #2) and in addition to these cap-like features particles like #3, without a rim can be found. Higher resolution images of particles were obtained using a medium-size scanner ( $15 \mu\text{m}$ , D head). Imaging was done in propanol. Examples of images of individual particles are shown in Figure 2B, C and D. These particles have a clear-cut cap structure. The background on these images is due to presence in the sample of kDNA of small fragments of kDNA particles as interlocked multimers of mini circles. All particles are bound strongly to the substrate, there is no movement of particles or parts of them during repetitive scanning. This can be seen from Figures 2E and F which are consecutive zoomed images in the middle of particle in D around the rim. These images show that the network is rather dense, and it is difficult to resolve the structure of the network. The main problem is the small size of minicircles for this type of kDNA:  $0.29 \mu\text{m}$  in their length or  $92 \text{ nm}$  in diameter for perfect circles [10]. Even fragments of kDNA particles are rather tight associates of DNA minicircles (data not shown). Individual DNA strands in the kDNA particles are resolved on EM and AFM images of kDNA from *C. fasciculata*; note that this type of kDNA has DNA minicircles three times larger than our sample [10]. The study of kDNA from *L. tarentolae* with high resolution and in aqueous solutions is in progress.

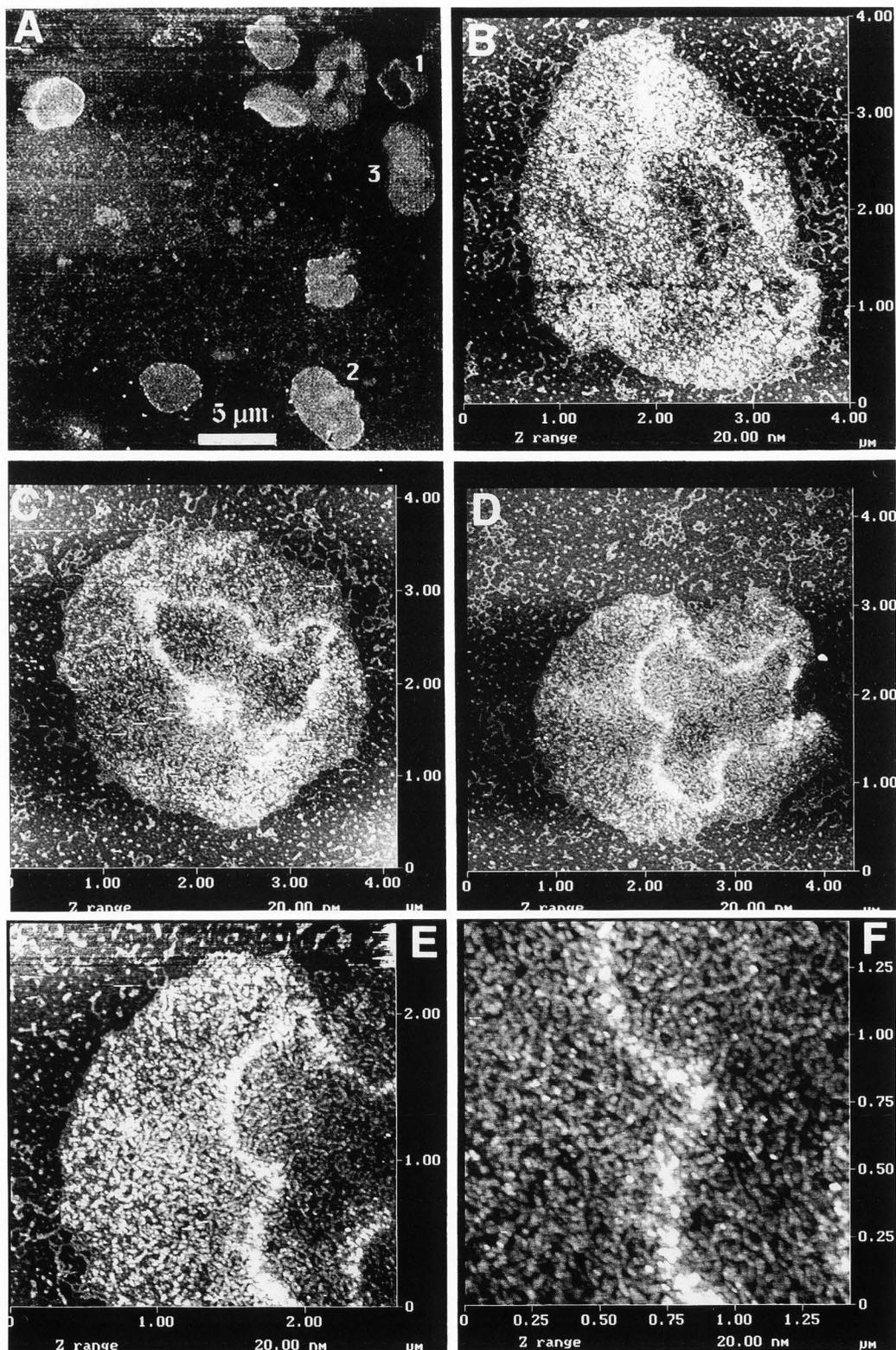
#### Preparation of Hydrophobic Mica Surface

We used two silanes for hydrophobization of mica. One of them, methyltrichlorosilane (MTCS,  $\text{Me-Si-Cl}_3$ ) belongs to a family of alkylchlorosilanes widely used for preparation of hydrophobic silicones [30, 41]. Compounds of this family were successfully used for activation of porous glass for binding mitochondria and whole blood cells [34]. Another compound, hexamethyldisilazane (HMDS,  $(\text{CH}_3)_3\text{Si-NH-Si}(\text{CH}_3)_3$ ) is also used for hydrophobization of silicones [30, 40]. Schematically the reactions of both compounds with mica surface are depicted in figure 3A and B respectively.

Both chemicals are commercially available compounds and have a rather low boiling temperature (slightly above  $100^\circ\text{C}$ ), making it possible to perform silylation of mica in gas phase at ambient conditions [21, 25]. We have found the following conditions for mica modification with both silanes:  $5 \mu\text{l}$  of chemical were placed into a  $50 \text{ ml}$  plastic vial and several pieces of mica were mounted at the top of the vial. Reaction was allowed to proceed at ambient conditions for 10 min. Then freshly cleaved mica strips were removed and investigated. A comparative study of contact angles for water for the freshly cleaved mica and methylated mica (Me-mica) indicated that surface of treated substrates became hydrophobic.

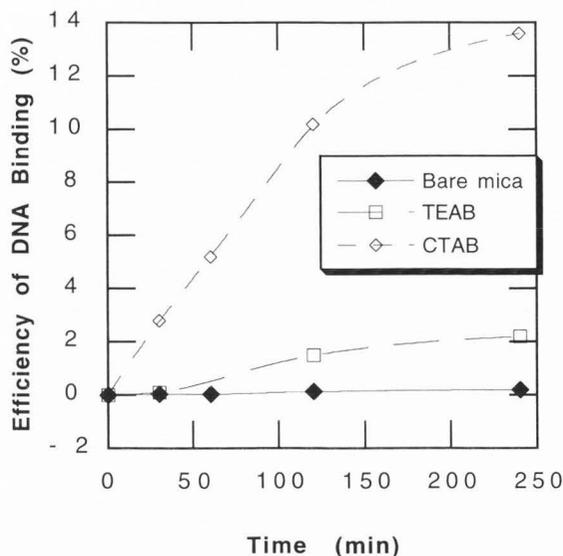
We used chlorosomes as a test sample for adhesion to Me-mica surface. Chlorosomes are intracellular structures of green photosynthetic bacteria containing

Silylated mica for AFM



**Figure 2:** AFM images of kDNA deposited onto AP-mica. from TE buffer Image (A) was obtained by use of J-scanner. Other images were obtained using D-scanner. Scanning was done in air (A) and in propanol (B-F).



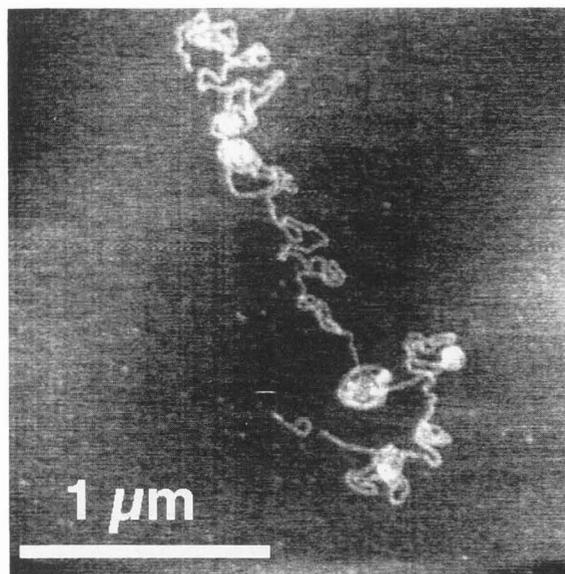


**Figure 5:** Kinetics of binding of  $^{32}\text{P}$ -labeled DNA to Me-mica treated with CTAB and TEAB. The radioactivity of samples was normalized to a total radioactivity of DNA solution before immersing the mica substrates.

linker: tetraethylammonium bromide (TEAB,  $(\text{C}_2\text{H}_5)_4\text{N}^+\text{Br}^-$ ) and cetyltrimethylammonium bromide (CTAB,  $[\text{CH}_3(\text{CH}_2)_{15}\text{N}^+(\text{CH}_3)_3]\text{Br}^-$ ). The latter contains a rather long aliphatic chain, and hence CTAB should exhibit better affinity for hydrophobic surfaces. After binding these salts, Me-mica is transformed into a positively charged surface, which should absorb DNA molecules strongly.

Me-mica strips were immersed into saturated water solutions of CTAB or TEAB for 30 min. Then wet CTAB or TEAB modified Me-mica strips were incubated in solutions of radiolabeled DNA for a specific period of time and radioactivity of rinsed and dried samples was measured. The result of DNA-binding experiments for both types of Me-mica modifications are shown in Figure 5. Freshly cleaved mica was used as a control in these experiments.

These data show that the treatment of a hydrophobic mica with TEAB or CTAB allowed us to obtain positively charged substrates which binds DNA molecules efficiently. Quantitatively the effect of DNA binding is higher for CTAB, supposedly due to its better binding to the Me-mica surface. The DNA binding efficiency of CTAB-Me-mica is at least 10 times more higher than that of TEAB-Me-mica. Note that there is practically no binding of DNA to the Me-mica. Incubation of DNA bound with modified positively charged mica in high concentrated solution of NaCl results in removal of 90% of bound DNA. These results suggest that a non-specific



**Figure 6:** AFM image of lambda DNA deposited onto CTAB-Me-mica. Pieces of CTAB-Me-mica were immersed into the DNA solution in concentration 0.1  $\mu\text{g}/\text{ml}$  dissolved in TE buffer (10 mM Tris-HCl, pH 7.6, 1mM EDTA) and incubated for 30 min. Rinsed with water and argon dried samples were imaged in air.

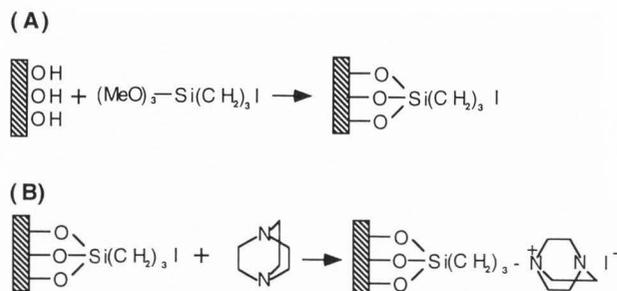
electrostatic interaction is the predominant force holding DNA molecules on the surface.

An FM image of lambda DNA deposited onto CTAB-Me-mica is shown in Figure 6. The molecule is rather convoluted (cf. Fig. 1). This happens during deposition of lambda DNA molecules onto AP-mica as well [21], although convoluted molecules are present on CTAB-Me-mica surface in larger amount in comparison with AP-mica. This may be explained by the complex character of the surface which is hydrophobic macroscopically with attached positively charged groups. Systematic comparative AFM studies of the DNA deposition onto different types of positively charged surfaces may help understand the mechanism of their adsorption onto different types of modified surfaces.

#### Chemically Reactive Mica Surface

Keeping in mind the positive results obtained with AP-mica and Me-mica [21-26], we have worked out an alternative procedure for mica modification. The key step of this procedure is the mica activation with iodopropyltrimethoxy silane,  $\text{Me}_3\text{-Si}(\text{CH}_2)_3\text{-I}$  (IPTMS). The reaction of IPTMS with mica surface is shown schematically in Fig. 7A. The chemical was synthesized according to a protocol described in [9].

IPTMS binds to SiOH groups of mica covalently similarly to APTES, but after reaction with mica leaves



**Figure 7:** Schemes of mica modification with iodopropyltrimethoxy silane (A) followed by transformation of this IP-surface into a positively charged one by treatment with DABCO (B).

a chemically reactive iodopropyl group on the surface. This is a crucial point for preparation of the AFM substrates with any desirable characteristics, because the presence of a reactive group at the IP-mica surface enables one to perform chemical reactions on the surface directly. For example, 1,4-diazabicyclo [2,2,2] octane (DABCO) can be reacted with activated IP-mica in the way schematically shown in Fig. 7B.

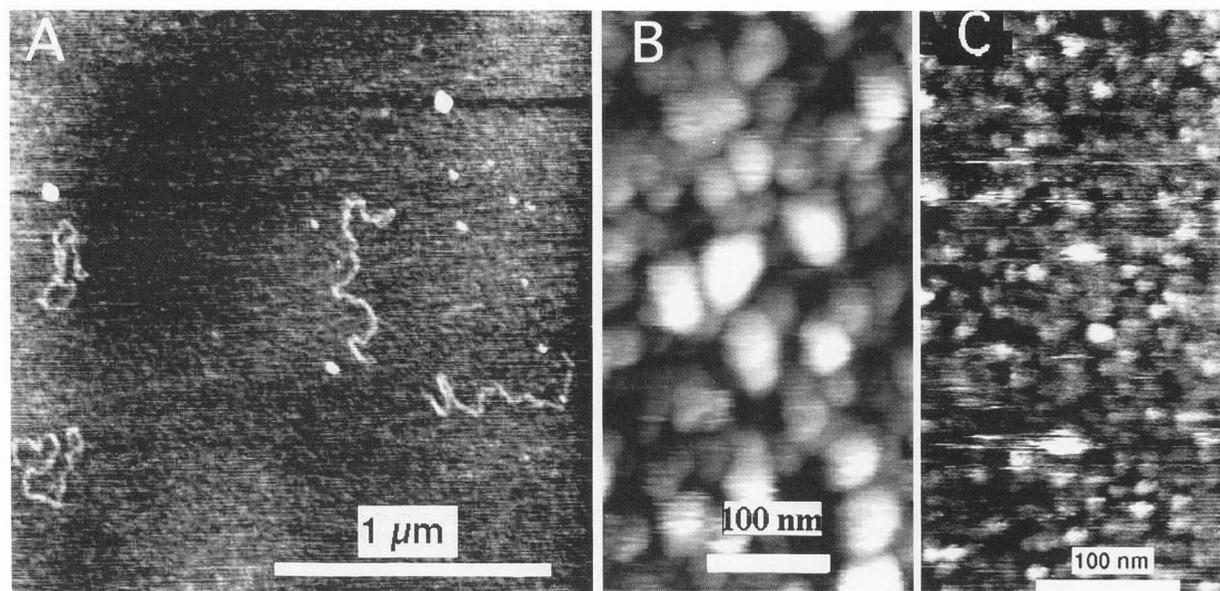
As a result of this modification quaternary amine is attached to the mica surface covalently. 4N-mica surface should have characteristics similar to those of AP-mica, but 4N-mica should be positively charged even at extremely alkalic pH values. The 4N-mica allowed us to get high quality images of DNA, dsRNA, nucleoprotein complexes and proteins. Several examples are shown in Figure 8. Plate A shows images of dsRNA molecules extracted from retroviruses [cf. 21, 23]. Similarly to molecules deposited onto AP-mica samples, RNA molecules are visualized quite easily on 4N-mica substrate as well. Another example, Fig. 8B, shows images of RNA polymerase *E. coli* (the sample was a gift of Dr. K. Severinov, Health Public Research Institute, New York). Deposition was done from solution containing 50 mM Tris-HCl, pH7.6, 40 mM KCl, 10 mM EDTA and 1mM mercaptoethanol at room temperature. As it is demonstrated by this picture, the surface is uniformly covered with sphere-like structures. Images are stable during repetitive scanning; no movement of the sample was noticed. The size of individual particles is  $28 \pm 3$  nm. RNA polymerase is a rather large complex of 5 subunits, its molecular weight is 550 kD approximately and the exact quaternary structure of RNA polymerase holoenzyme is still unknown. Our data on polymerase globule sizes are consistent with the AFM results of Bustamante *et al.* [11]. Images of CAP protein (the sample of Dr. M. Fried, Penn State University) are

shown in plate C on the scale close to that for images of RNA polymerase. Images are also very stable during repetitive scanning. In some cases individual particles can be measured. Their size is  $6.1 \pm 0.8$  nm and these data are close to crystallographic data [34], about 5 nm for a dimer, keeping in mind the convolution between scanning probe and the sample [11, 16, 21]. The images shown in this Figure were obtained in propanol with regular  $\text{Si}_3\text{N}_4$  tips. Note that similarly to AP-mica, 4N-mica retains its binding activity for several weeks.

## Discussion

We suggested earlier to use aminopropyltriethoxy silane (APTES) to functionalize the mica surface with amine groups which protonate at neutral pH [21-26]. The major advantage of this procedure of sample preparation is that it works in a *wide variety of ionic conditions, pH and over a wide range of temperatures*, so AP-mica may be a suitable surface for imaging DNA complexed with different types of ligands. Here we also demonstrate that such large DNA complexes as kDNA can be deposited onto AP-mica for AFM imaging. Attachment of kDNA particles is very strong. First, we did not see any movement of the sample during scanning. Moreover there is no intra-particle movement revealed by scanning over part of the kDNA particle. Secondly, concentration of kDNA particles on the surface correlates with DNA concentration used for deposition. Third, images shown in Figure 2 were obtained by drying the water-rinsed samples in a rather strong argon flow, and we did not see any orientation of kDNA particles or its fragments that should happen for weakly bound molecules. We therefore conclude that AP-mica is a good substrate for structural studies of the kDNA network. The first AFM images of kDNA were reported by Thundat *et al.* [37], who developed a special procedure, critical point mounting, for kDNA samples, because they found shrinkage of samples and orientation of DNA molecules if a regular cation-assisted preparation method developed for imaging individual DNA molecules was applied for preparation of kDNA samples. Our AFM results suggest that there is no need to modify the DNA deposition procedure as long as AP-mica is used as a substrate. Similarly to other samples prepared on AP-mica (DNA, RNA, RecA-DNA and other nucleoprotein complexes [24-26]), kDNA samples on AP-mica are stable and do not absorb any contaminants for months without special precautions for storage.

In addition to positively charged AP-mica, we have developed procedures for preparing hydrophobic mica suitable for AFM studies. We checked their ability to hold samples for AFM imaging with chlorosomes. Coverage is rather uniform, indicating uniform modifica-



**Figure 8:** AFM images of samples deposited onto N4-mica: (A) dsRNA from reovirus; (B) RNA polymerase of *E. coli* and (C) CAP protein of *E. coli*. Images were obtained in propanol in contact mode.

tion of the surface. The Me-mica surface is also relatively smooth (roughness of non-covered spots is  $0.06 \pm 0.01$  nm) so that individual hydrophobic molecules may be imaged on this substrate. Silylated porous silica and glass beads were used for binding whole cells and organelles (mitochondria, chloroplasts and microsomes) in a physiologically active state [4]. We therefore guess that hydrophobic surfaces (similar to Me-mica used in this work) may be used for AFM studies of whole cells and cell organelles. These samples are bound to hydrophobic substrates, and it is important for these studies that the binding process does not deteriorate the functional activity of the organelles: immobilized chloroplasts produced oxygen when exposed to light [4].

We also demonstrated that there is a simple means for transformation of a hydrophobic surface into a charged. We used organic salts containing alkyl groups for preparing a positively charged surface. An alkyl chain plays the role of an anchor holding the molecules at the surface while the charged group is exposed to the water solution. This type of positively charged surface allows the imaging of DNA, although large aggregates are formed at this surface in higher amount in comparison with AP-mica. The use of salts with different length of the carbohydrate chain enables one to change easily the length of the spacer between the surface and the charged group. A flexible spacer should help hold larger samples at the surface. It was shown in [4] that whole erythrocytes can be immobilized on glass beads modified with silanes with long alkyl spacer. Note that similar procedure of surface modification is usually applied for

preparation of self-assembled monolayers (SAM, [38]).

The approach discussed above can be used for transformation of a hydrophobic surface into a negatively charged one. Dodecyl sulfate (SDS) may be an appropriate candidate for that. The dodecyl chain provides strong adhesion of SDS molecules to the Me-mica surface leaving a negatively charged sulfate group exposed to the water solution. Similarly, use of non-ionic detergents allows one to obtain surfaces with hydrophilic groups attached to a hydrophobic surface through an appropriate linker.

We also developed a procedure of mica modification allowing attachment of a chemically reactive group to the surface. We used IPTMS which leaves a reactive iodopropyl group on the surface. We demonstrated the reactivity of this immobilized group by treating the IP-mica with DABCO to make the surface strongly positively charged. This capability of IP-mica is important for development of procedures of covalent attachment of samples to the surface. First, by appropriate choice of reactive group bound to the surface one can attach the molecules to the surface with a high selectivity, so that the reaction may be limited to specific sites in the molecule (for example targeting of SH groups in a protein molecule or even specific aminoacid moieties in a protein or specific groups in nucleic acids bases). Secondly, in addition to topographic studies, the AFM is becoming a very promising tool for direct measurements of intermolecular interactions [12, 13, 19, 28]. This type of scanning probe microscopy, also termed chemical force microscopy (CFM), may find a very

broad area of applications in surface science, physics, molecular and cell biology, immunology and eventually in pharmacology and medicine. An attachment of interacting components to the surface and a probe respectively is a technical, but a fundamental problem for measurements of intermolecular forces. Further studies of chemical modification of AFM probes is extremely important for routine use of AFM in this area.

### Acknowledgements

We would like to thank A. Stasiak for preparation and sending us the samples of RecA-DNA complexes, M. Fried for providing us the sample of CAP protein and K. Severinov for sending us the sample of RNA polymerase; B. Jacobs for providing us the sample of dsRNA.

This paper was supported in part by NIH grants 1R21HG-0081801A1 and 1R43GM54991-01 (SBIR, Phase I), ONR grant N00014-90-J-1455 and grant F-017-95 from the Office of the Vice President for Research at ASU.

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#### Discussion with Reviewers

**R. Lal:** The cells, both in vivo and in culture, are surrounded by hydrophilic solutions. I am not sure why they adsorb to a hydrophobic surface.

**Authors:** The structure of the shell of isolated cells is very complex. It contains both hydrophobic and hydrophilic groups. The experiments with isolated cells and organelles immobilized on the surface, which we discussed, were performed with hydrophobic glass substrates [4]. The samples adsorbed on these silylated substrates remain active.

**R. Lal:** Does the functionalized substrate change the apparent height of the isolated macromolecules?

**Authors:** Height measurements of DNA under different scanning conditions (air, propanol and water) were performed in our early work [24]. Similar to the data obtained by others (e.g., [47]) the height of DNA is substantially less than expected. The mechanism of the apparent height reduction is not understood so far, but may be a combination of both adhesion of the tip to the sample and tip-induced compression of DNA.

**J.A.R. Zasadzinski:** How is the secondary and tertiary structure of a biological macromolecule altered by tight binding to a surface? Can surfaces be systematically modified to check just how much binding is required for the various AFM modes? Clearly, the softer the hold, the less denaturation might be expected. Can tapping or other non-contact modes be brought to bear here? What is the remaining limit of resolution? Can we ever expect superior resolution to TEM?

**Authors:** Generally speaking, any deposition process

should result in a deformation of a macromolecule. Even such a stable biomolecule as DNA can be stretched out during sample preparation [53]. In the case of AP-mica, DNA molecules retain their B-conformation [21]. An important observation was made in [4]. They showed that cells and cellular organelles immobilized on glass surfaces treated with silanes remain active.

The extent of surface modification with silanes can be controlled [30]. Clearly, the regime should be different for different samples and types of imaging. For example, AP-mica prepared by modification in APTES for 2 hours is sufficient for holding filamentous bacteriophage at the surface, so that imaging *in situ* can be performed [24]. However these images are quite streaky if contact mode in buffer solution is used. Our recent experiments have shown that stable images of fd phage *in situ* in buffer solution can be obtained in tapping mode AFM (Shlyakhtenko, unpublished data).

Resolution is typically higher for tapping mode AFM. For instance, double helical periodicity of DNA can be resolved [15]. There is no TEM data of DNA where this level of resolution was achieved. See below for more about other high resolution AFM data.

**J.A.R Zasadzinski:** What limits the resolution with contact mode AFM? Will non-contact modes - tapping, adhesion, etc. or lateral force provide higher resolution or other important data?

**Authors:** There are several factors, in addition to the tip geometry, that limit the resolution AFM. They are tip-sample/substrate interaction, the sample mobility and intramolecular thermal motion. Strong adhesion effects typically reduce the resolution. One type of tip-substrate adhesion is formation of a water layer between the tip and the sample while scanning is done air. For example, the capillary effect results in considerable broadening of the width DNA [20, 22]. Imaging in water in contact mode [22] or the use of tapping mode decrease the width of DNA by a factor of two. High resolution data for DNA molecules was achieved if imaging is done in propanol. Adhesion forces in propanol are much lower than in water; as a result, AFM images of DNA as thin as 2-4 nm can be obtained [15, 16, 22]. The use of tapping mode made it possible to resolve helical periodicity of DNA [15]. In cases when adhesion forces are small very high resolution can be achieved by the use of contact mode AFM. One of the recent examples is imaging of purple membrane with AFM by the group of A. Engel [48, 50]. The attachment of antibodies to C-terminal part of bacteriorhodopsin allowed the authors to obtain structural details of intracellular and extracellular sides of the membrane with subnanometer resolution. Similar high resolution AFM data for individual proteins are hard to obtain because of large thermal intramolecu-

lar motion. The development of cryo AFM in the group of Shao permitted them to get AFM images of individual IgG molecules with very high resolution [46].

**J.A.R Zasadzinski:** What are the major benefits to AFM studies of membrane proteins over cryo-electron microscopy? Are there certain systems that just cannot be done with TEM that can be examined by AFM? Are there systems in which the AFM resolution is superior to TEM?

**Authors:** Currently the resolution of AFM for membrane proteins is close to that for cryo-electron microscopy. However there is a number of advantages of AFM in comparison with cryo-EM. First, AFM topographical data can be obtained under physiological conditions. Examples include purple membrane [45], gap junctions [16], OmpF porin *E. coli* [52]. Very recently Müller *et al.* [48, 50] obtained AFM images of purple membranes in solution at subnanometer resolution. In particular they imaged individual polypeptide loops connecting transmembrane  $\alpha$ -helices of bacteriorhodopsin. Moreover the use of AFM made it possible the same group to observe reversible force-dependent structural changes of bacteriorhodopsin molecules [49]. These changes were interpreted as the bending of individual loops of the protein. Such studies cannot be done with EM. There are several examples when both AFM and TEM were applied to similar systems. In the Engel group membrane protein OmpF was imaged with AFM with subnanometer resolution which is substantially higher than early EM data obtained in the same group [52]. Additional structural information for this system were obtained from the height AFM data; such information is unavailable for EM. This advantage of AFM was critical for EM and AFM studies of complexes of DNA with the heat-shock transcription factor 2 [54]. The height AFM data led the authors to conclude that DNA looping requires trimerization of the protein.

**J.A.R Zasadzinski:** How important is the environment or the substrate in the protein conformation? Is the problem with resolution more that the proteins moving over the time scales of the images, thereby blurring the images? What are the benefits to such kinetic information that obviously cannot be determined from fixed or frozen materials?

**Authors:** Conformation of protein is quite sensitive to the environmental conditions, therefore AFM studies in solution are so attractive. Binding a protein to the surface may influence its biochemical activity, but this undesirable effect may be decreased if the protein is tethered to the surface gently enough, but sufficiently strongly to withstand the tip-sweeping effect. The use of tapping or non-contact AFM is essential in this case. For

example, lysozyme was imaged with AFM in solution.

The protein molecules remain active being bound to the mica surface, and this activity of the protein was visualized by the AFM [51]. Loose tethering of a macromolecule clearly results in lower resolution but is beneficial for imaging biochemical processes. This was shown in recent publications from the Hansma group, who imaged the process of DNA depolymerization induced by nuclease [44].

**W. Fritzsche:** Your SFM dimensions of CAP protein (ca. 6 nm) exceed hardly the crystallographic data (ca. 5 nm), in contrast to many SFM studies of small biomolecules (e.g., DNA) with stronger broadening effects. Is this low exaggeration due to a very sharp tip and/or shrinkage of the protein in alcohol, or is there another explanation?

**Authors:** The CAP protein was imaged in propanol to minimize adhesion forces [16]. At these scanning conditions we were able to get DNA and RNA molecules as thin as 3-4 nm [26], which is very close to the crystallographic sizes of DNA and RNA. We assume that the AFM probes usually have very fragile and sharp asperities, so by gentle approaching the tip at conditions of low adhesion of the tip to the sample one be able to use a sharp tip for imaging. Poor adhesion of the tip to the sample is another advantage preventing the tip contamination during scanning. We cannot exclude some shrinkage of the protein molecules at these scanning conditions in addition to the same effect induced by drying the sample.

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