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Carol E. Rabke
University of Utah

Lisa A. Wenzler
University of Utah

Thomas P. Beebe Jr.
University of Utah

Utah State University
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ELECTRON SPECTROSCOPY AND ATOMIC FORCE MICROSCOPY STUDIES OF DNA ADSORPTION ON MICA

Carol E. Rabke[1], Lisa A. Wenzler, and Thomas P. Beebe, Jr.*

Department of Chemistry and Center for Biopolymers at Interfaces,
University of Utah, Salt Lake City, UT 84112
[1]Present Address: Bausch & Lomb, Contact Lens Division, P.O. Box 450, Rochester, NY 14692-0450

Abstract

Various methods for the deposition of deoxyribonucleic acid (DNA) molecules on mica are investigated to determine their reproducibility, and to quantify their ability to bind DNA. The use of these deposition methods for sample preparation for biological scanning tunneling microscopy (STM) and atomic force microscopy (AFM) studies is discussed. Auger electron spectroscopy (AES) and electron spectroscopy for chemical analysis (ESCA) were used to investigate the quantity of DNA adsorbed. AFM images of DNA deposited using the methods investigated are presented. The combination of AFM results with AES and ESCA results provides a basic understanding of the deposition techniques studied and illustrates that electron spectroscopy can be a useful addition to studies of this nature.

**Address for correspondence:
Thomas P. Beebe, Jr.
Department of Chemistry
3416 Henry Eyring Building
University of Utah
Salt Lake City, UT 84112
            Telephone number: (801) 581-5383
            FAX number: (801) 581-8433

Introduction

Much research attention has been directed at developing a deposition method for use in scanning tunneling microscopy (STM) [2] and atomic force microscopy (AFM) [1] studies of biological molecules that is both reproducible and capable of rendering the biomolecule immobile. In this paper, established and evolving methods for depositing deoxyribonucleic acid (DNA) onto mica substrates are explored using the surface analysis techniques of Auger electron spectroscopy (AES) and electron spectroscopy for chemical analysis (ESCA).

A method for DNA deposition on coated mica substrates commonly used by electron microscopists involves "activation" of the DNA by dilute salt solutions, commonly magnesium acetate [11, 18]. It has been practiced using Ruby-B mica; however, the results presented here indicate that there is no difference between Ruby-B mica or green mica which would lead to adsorption differences. This method has been employed by researchers for biomolecule depositions for experiments on coated STM specimens [15] as well as in AFM experiments [19, 20].

Recently, a modification of this method has been re-introduced by members of the community and has been utilized by others in the field [9]. Bustamante *et al.* [3] and Vesenka *et al.* [22] have reported the binding of DNA to mica in which they implicate ion-exchange between the native potassium of the mica and the magnesium of the salt solution (33 mM magnesium acetate). Thus, since magnesium has a charge of 2^+ compared to potassium at 1^+, the DNA polyanion binds with higher affinity to the modified mica substrate. Results presented below will challenge this model.

Several aspects of these deposition techniques have been studied using electron spectroscopy. For example, experiments were completed to examine the effect of the mica cleaving technique on the chemical and structural properties of the native surface, as well as its ability to bind magnesium. In conjunction with the electron spectroscopy experiments, AFM studies were completed on the various samples.

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Experimental Methods

AES and ESCA analyses

AES and ESCA analyses were performed in an ultrahigh vacuum (UHV) surface science chamber designed and built in this laboratory [12], which has been described in detail previously [5, 14]. Briefly, it has been designed with a sample transfer interlock which allows for the transfer of samples in and out of ultrahigh vacuum in approximately ten minutes with only a momentary rise of the base pressure. The sample temperature can be varied from 77 K to 1500 K while being monitored with a chromel/alumel thermocouple attached to the sample. The current experimental capabilities of the UHV chamber, other than AES and ESCA, include: STM, ion scattering spectroscopy, temperature programmed desorption, and ion sputtering. The instrumentation and data collection are controlled using data acquisition and graphics programs written in this laboratory.

For the analyses presented in this paper, the typical chamber pressure was in the 10^{-9} Torr range, since sample contamination by residual gas was not a problem [5]. The surface of the sample was analyzed normal to the entrance axis of the hemispherical kinetic energy analyzer with the electron gun (AES) and the X-ray gun (ESCA) positioned at a 60° angle with respect to the surface normal. The AES spectra were collected in the constant relative resolution mode at a primary beam energy of 3 keV with a beam diameter of approximately 1 mm and a 5 eV peak-to-peak pass energy modulation. The ESCA spectra were collected in the pulse-counting mode at constant absolute resolution at a pass energy of 150 eV, using an Al Kα source at an anode power of 390 W. The photoelectron kinetic energy was subtracted from 1486.7 eV in order to present the data on a binding energy scale, as is customary. The X-ray source had a beam diameter of approximately 1 cm. Signal averaging and lock-in detection were employed to increase the signal-to-noise ratio.

AFM Imaging

AFM imaging was done using two different microscopes in this study. The first consists of a coaxial double-tube piezo design built in this laboratory, for use in ambient imaging experiments. The design has been described previously in detail [13, 24, 26]. The AFM was controlled using custom-built feedback, scanning, and offset electronics, and images were acquired using an 80386/387 based 33 MHz AT compatible computer system equipped with a 12-bit, 150 kHz analog-to-digital converter. Images consist of 256 x 256 arrays of 12-bit data obtained in the constant force imaging mode. Image figures were photographed from the computer screen. Images were also collected under propanol using a NanoScope III AFM (Digital Instruments, Santa Barbara, CA), as described previously [8]. AFM images were obtained using triangularly shaped silicon nitride cantilevers with pyramidal tips (also obtained from Digital Instruments) and force constants of 0.12 N/m and 0.58 N/m.

Individual sample preparation

The samples for investigations of various deposition methods for STM/AFM biological sample preparation involved the use of a single-stranded 8 member oligomer with a thiolated guanine (S_1^{base}, see Schematic 1, Structure 1, personal communication from Dr. Bill Nash, Department of Geology and Geophysics, University of Utah) [4]; a single-stranded brominated poly-dA (Br_n^{base} poly-dA, see Schematic 1, Structure 2 [14]); a single-stranded 15 member oligomer with a completely sulfur-modified backbone ($S_{15}^{backbone}$, see Schematic 1, Structure 3 [14]; this oligomer is commercially available through Amersham, Inc., Arlington Heights, IL); an unmodified double-stranded 9 kb plasmid DNA, an unmodified double-stranded 2 kb plasmid DNA; and an unmodified single-stranded 24 member oligomer (the unmodified 24 base oligomer and the 2 kb and 9 kb plasmid DNA molecules were kindly donated by Dr. Robert Weiss, Department of Human Genetics, University of Utah). The substrates used for these studies were freshly-cleaved Ruby B (Plano, Germany) or green (Ashville-Schoomaker) mica. The individual preparations are detailed below.

The method involving "activation" of the DNA by Mg^{2+} was taken from the established technique for preparing DNA samples for transmission electron microscopy (TEM) [11, 18]. Throughout this paper, it is referred to as the chemical activation of DNA method. Here, the substrates used were both freshly cleaved Ruby-B mica and freshly-cleaved green mica. Droplets of the solutions to be deposited onto the mica are prepared on a hydrophobic surface, typically parafilm wax. Using this protocol, after cleaving the mica, it is placed, face down, in contact with a 5 mM magnesium acetate (MgAc) solution for approximately 2.5 minutes. Following this interaction, the mica is placed in contact with a second droplet of the DNA solution for approximately 2.5 minutes. The mica is then briefly (a few seconds) placed in contact with a third droplet of nanopure water as a rinsing step.

It has been demonstrated that it is not necessary for these to be separate steps by both the electron microscopy community (personal communication with J. Sogo, Swiss Federal Institute of Technology, Zurich Switzerland, August 1991) and the AFM community [10, 17, 23, 27]. It is a common practice for the MgAc solution to be added directly to the DNA solution before deposition. Since these samples were prepared solely for

examination by AES, ESCA, and AFM (and not scanning EM or TEM), the DNA-mica samples were left uncoated and allowed to air dry in a clean environment. Following this preparation, the mica sample was mounted onto the UHV sample holder and analyzed by AES and ESCA. If these results indicated deposition of DNA, the samples were further investigated by AFM. The following concentrations of DNA molecules were used: 9.9 μg/ml Br$_n^{base}$ poly-dA; 13 μg/ml S$_{15}^{backbone}$; 15.8 μg/ml S$_1^{base}$; unmodified 2 kb plasmid DNA in concentrations of 252 μg/ml, 102 μg/ml, and 10 μg/ml; 102 μg/ml unmodified 24-mer; and 9 kb plasmid DNA in concentrations of 100 μg/ml, 50 μg/ml, and 25 μg/ml.

For the experiments involving methods for cleaving the mica, freshly-cleaved green mica was used following the cleaving procedure first outlined by G. Borges (from a pamphlet entitled "Epitaxial Metal Films Grown on Mica", IBM Almaden Research Center, San Jose, CA, and circulated through the STM/AFM community). However, the procedure was varied by cleaving with a. no liquid, b. Nanopure® water (Milli-Q Corp.; 10 MΩ cm restivity), or c. MgAc solution. Following cleavage, the samples were either placed in 5 mM MgAc or 33 mM MgAc solutions for varying amounts of time, or directly analyzed by electron spectroscopy.

For the investigations examining the ion-exchange process on mica, either freshly-cleaved Ruby B or green mica were used as the substrates [3]. These substrates were also cleaved following the Borges procedure using Nanopure water. The freshly-cleaved samples were subjected to varying particulars of the deposition method [3] and the results of electron spectroscopy experiments were compared. The specific treatment of each sample is included with the data. The method is briefly outlined below. Following cleavage of the mica, it was sonicated for three minutes in Nanopure water. The mica was then placed in 33 mM MgAc for at least 24 hours. Following removal from the MgAc solution, the mica was sonicated for 30 minutes in Nanopure water. It should be noted that this method [3] calls for a glow-discharge step following this sonication to render the surface hydrophilic. However, it has been demonstrated by Hansma *et al.* [9] that the glow-discharge is not a rigid necessity for the DNA to bind. A 20-μl droplet of DNA was then placed on the mica for 5 minutes. The solution was wicked from the mica surface and rinsed with droplets of Nanopure water. The forms of DNA studied and their respective concentrations are the same as those listed previously. This method will be referred to as the chemical modification of substrate method in this paper. Following sample preparation, the mica was mounted onto the UHV sample holder and analyzed by AES and ESCA. If these results indicated deposition of DNA, the samples were further investigated by AFM.

Results and Discussion

Two methods for depositing DNA on mica have been examined using electron spectroscopy: (1) the chemical activation of DNA method [11, 18], and (2) the chemical modification of substrate method [3, 22]. Both methods involve the use of MgAc. However, in the first method, it is reportedly for "activation" of the DNA, and in the second method, it is reportedly due to ion-exchange between the Mg^{2+} of the solution and the K$^+$ of the mica. The results of electron spectroscopy studies have clarified aspects of each method which are necessary for deposition of the DNA as well as provided details of the binding of the DNA to the mica.

Ruby B versus green mica

It has been suggested that only Ruby B mica should be used for depositing DNA molecules [personal communication with J. Sogo and R. Wepf, Swiss Federal Institute of Technology, Zurich Switzerland, August 1991]. However, we have compared electron spectroscopy results for both Ruby B and green mica and found no significant differences. The AES and ESCA spectra for both micas indicate, as expected, that both are composed mainly of potassium (AES 252 eV, LMM and ESCA 293 eV, 2p), silicon (AES 76 eV, LMM and ESCA 102 eV, 2p), and oxygen (AES 503 eV, KLL and ESCA 531 eV, 1s). Any differences between the two types of mica are due to small concentrations of the element responsible for the tint of the mica. For example, the green tint, that gives green mica its name, is due to iron. In Ruby B mica, the pink tint is due to lithium (personal communication with Dr. Bill Nash, Department of Geology and Geophysics, University of Utah). It is not expected or observed that these low concentration impurities play a role in the adsorption properties of the mica.

Mica cleavage experiments

The method generally used for cleaving mica was outlined by G. Borges (as mentioned above). Briefly, it involves carefully separating the layers of mica with a scalpel. Into this separation, water is applied which causes the layers to fully separate. In an effort to characterize what effect the particular liquid used during cleaving of the mica had on the chemical and structural properties of the mica surface, as well as its ability to bind magnesium, several mica samples, which differed by the choice of liquid used when cleaving the mica, were analyzed by AES. It was thought that perhaps the amount of magnesium binding would change depending upon what active sites of the mica were available for interaction. Figure 1 shows the AES spectra for green mica cleaved using Nanopure water, 5 mM MgAc, and no liquid, respectively. As illustrated by these spectra,
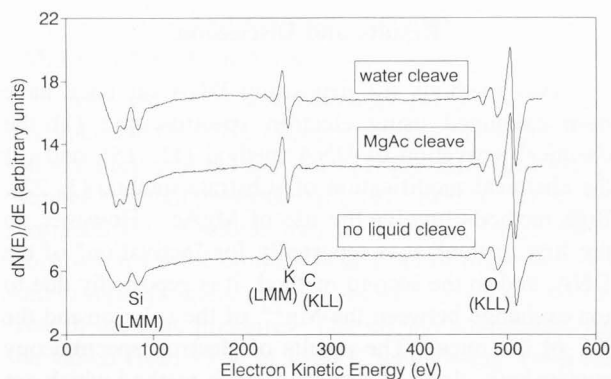
Figure 1. AES spectra of green mica, the results suggest little difference between mica cleaving methods. The following liquids were used in this study: Nanopure water, magnesium acetate, and no liquid.

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there was little difference in the three procedures. For the method using no liquid, a tearing sound was often heard when separating the layers. It was originally thought that this would lead to surface roughness. However, examination of several samples by AFM demonstrated that the surface was extremely flat (typical z-height standard deviation was 1.8 ± 0.5 nm over an image size of 1 μm x 1 μm). Each method was also investigated to determine the quantity of magnesium adsorbed by the mica. Again, no preferential binding was in evidence.

Investigations of the chemical activation of DNA method [11, 18]

The modified oligomers used in a previous study involving DNA adsorption to Au(111) substrates [14] were also used for these mica adsorption studies as well as several additional unmodified DNA molecules. Unlike the AES analyses of DNA adsorbed onto Au(111) presented in that paper [14], AES was not a reliable method for detecting deposition of DNA adsorption on mica due to sample charging problems. In contrast to those results, it should be noticed that while there is no discernible nitrogen AES signal (379 eV, KLL) in Figure 2A, the nitrogen ESCA signal (402 eV, 1s) is quite significant in Figure 2B. This difference is most likely related to conductivity and/or charging problems since mica is an insulator. In fact, in some instances, the mica exhibited visual structural changes in the region of the electron beam (cloudiness of the usually clear mica substrate) following completion of AES investigations, suggesting that the electron beam caused damage during the experiments. Thus, for determination of DNA adsorption on mica, ESCA was used. Based on ESCA nitrogen peak intensities, these results indicate that varying amounts of DNA adsorption occurred in all cases, with
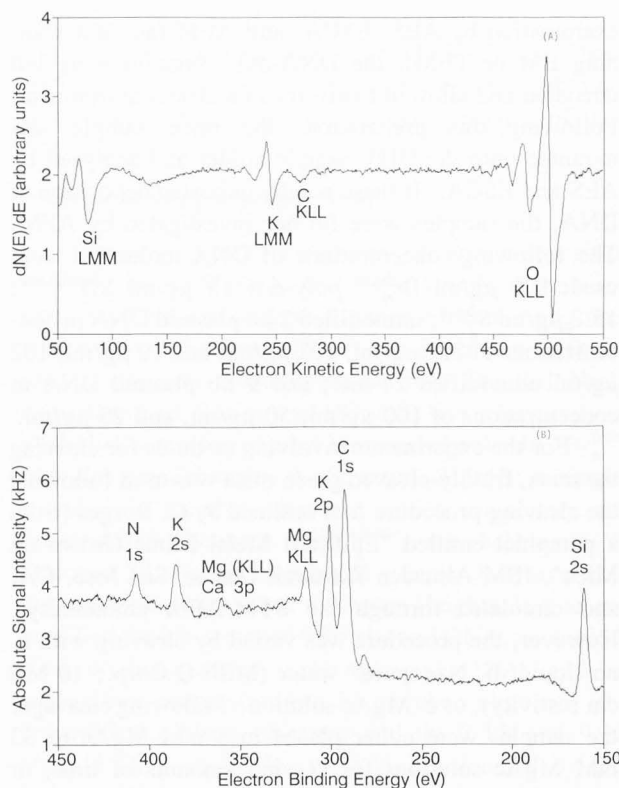

Figure 2. AES and ESCA spectra associated with the adsorption of brominated poly-dA on mica. Notice that there is no nitrogen signal in the AES but that the ESCA spectrum possess a significant nitrogen peak, indicative of DNA adsorption. **(A)** The AES spectrum collected in the range of 50-550 eV. This spectrum was signal averaged 50 times. **(B)** The ESCA spectrum collected in the range of 450-150 eV. This spectrum was signal averaged 100 times.

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the exception of the S_1^{base}. Since this particular DNA molecule is the smallest studied to date, it is quite possible that its length inhibited its ability to bind. It is interesting to note that while this particular DNA did not adsorb using this method of deposition, ESCA results indicate that it did adsorb when using the chemical modification of substrate method. The results of the other smaller DNA molecules studied utilizing the chemical activation of DNA deposition method indicated deposition only at high concentrations.

Previous solution-phase DNA studies have indicated that magnesium (II), when binding between phosphate groups, stabilizes the helical structure [16]. In contrast, if the magnesium (II) interacts with the bases, it has been shown to have a destabilizing effect [16]. Perhaps, it is one of these effects of the magnesium that aids in the binding to mica. It is interesting to note that although electron microscopy studies and STM studies,
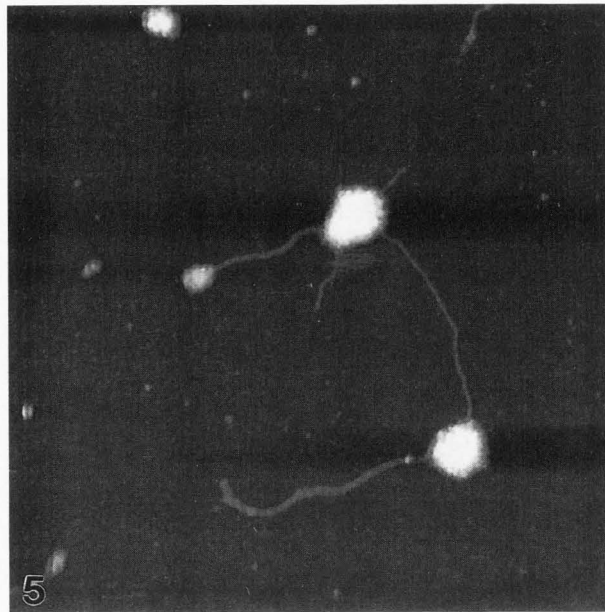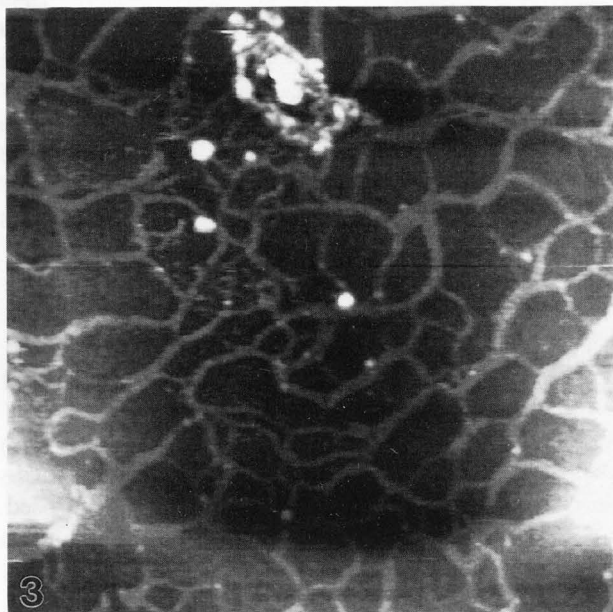
Figure 3 (left). A typical AFM image obtained using the chemical activation of DNA method for deposition of 5 μg/ml plasmid DNA (pLWGC34) on mica. Aggregation of DNA occurs across the surface with no individual molecules observed. This image measures 1.9 μm x 1.9 μm x 0.02 μm (x, y, and z) and was collected under 2-propanol.

Figure 5 (right). A typical AFM image obtained using the chemical modification of substrate method for deposition of 35 μg/ml plasmid (pLWGC34) on mica. Individual strands of DNA are observed with circular masses of bound DNA due to salt crystals. Measured lengths of individual strands of DNA are 0.81 μm $\pm$ 0.48 μm and the measured width of DNA is 0.03 μm $\pm$ 0.01 μm. Image size measures 2.8 μm x 2.8 μm x 0.035 μm (x, y, and z) and was collected under 2-propanol.
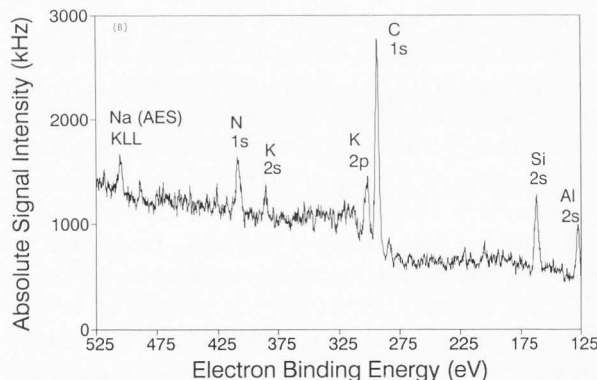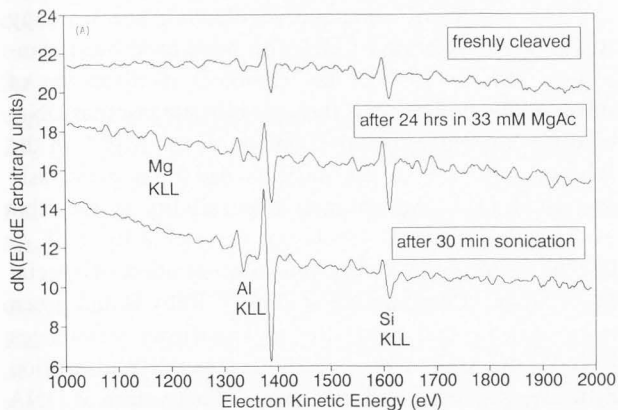

Figure 4. These figures are the AES and ESCA spectra for plasmid DNA on mica obtained for the individual steps of the chemical modification of substrate method. **(A)** These AES spectra suggest that there is no ion-exchange involved with the chemical modification of substrate method. Notice that there is little or no magnesium signal present in the final spectrum. These spectra were signal averaged 50 times. **(B)** The ESCA data illustrating confirmation of DNA deposition on mica by the N 1s peak. This spectrum was signal averaged 100 times.

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both conducted on evaporated films on mica, have confirmed this method of deposition for low DNA concentrations, the electron spectroscopy results presented here do not confirm DNA deposition below concentrations of

approximately 40 μg/ml [6, 8, 25].

In AFM experiments, DNA could be reproducibly imaged from solutions with concentrations as low as 2.5 μg/ml. Aggregation of the DNA would frequently occur

with the chemical activation of DNA method, as shown in Figure 3. This method of DNA activation relies on touching a DNA-containing droplet to the mica surface. It is likely that surface tension forces will concentrate the DNA at the air-droplet interface, so that touching a mica surface to this droplet would result in the transfer of a dense DNA film to the mica. The following method instead relies on solution-phase diffusion to drive the adsorption of DNA.

Investigations of the chemical modification of substrate method [3, 22]

The major differences between the chemical modification of substrate method and the chemical activation of DNA method (previous section) are that in the chemical modification of substrate method the mica substrates are soaked in MgAc for extended periods of time and the mica substrates are subjected to sonication before DNA deposition. It should be noted that since the initiation of this paper, an extensive AFM investigation has been completed by Thundat et al. [21] on DNA deposited onto mica treated with various ionic solutions. AES spectra were taken after each step in the deposition method. The results suggest that the initial four-minute sonication in Nanopure water had little measurable effect on the chemical composition of the mica substrate. Not unexpectedly, a greater level of carbon contamination resulted from this treatment. Further experiments have illustrated that this step is not necessary for adsorption of DNA to the mica substrate. Furthermore, if the 30 minute sonication is completed after extended soaking in 33 mM MgAc, most of the magnesium signal is removed, as illustrated in Figure 4A showing the high energy AES spectra. This suggests that there is very little ion-exchange taking place, or that the exchange can easily be reversed. This is further supported by the fact that the potassium AES peak intensity remains unchanged. However, it is interesting to note that the carbon AES peak intensity does change significantly throughout the course of this method. In some cases, a large carbon AES peak intensity was completely reduced by sonication in Nanopure water. Figure 4B is the ESCA data illustrating, by the presence of the nitrogen (1s) peak, that the deposition method did adsorb DNA.

Using this method of DNA deposition, 35 μg/ml DNA concentrations were the lowest that allowed reproducible observation of DNA during AFM imaging. Individual molecules were observed, but circular plasmids were denatured or supercoiled into linear features as illustrated in Figure 5. Circular masses were observed in many of the images associated with the chemical modification of substrate method. Recently, Hansma et al. [7] demonstrated that these globular masses were DNA aggregates by dissolving the aggregates into individual

DNA strands using water. They suggest that the circular aggregates of DNA were held together by salts, since the DNA polyanion did not aggregate in the absence of the counter-ions.

AES and ESCA results indicate that, by the time the mica substrate is brought into contact with the DNA solution, there is more magnesium on the surface than was present prior to the MgAc treatment. It would be difficult to conclude from this evidence that the MgAc treatment plays a significant role in any hypothesized ion-exchange process. Indeed, a number of researchers have reported DNA adsorption on mica in the absence of the MgAc step, although most also agree that the presence of magnesium seems to help in some way. Thus, it is inconclusive as to what role the magnesium plays in this method. However, these results suggest that DNA adsorption is probably not due to the previously proposed ion-exchange between magnesium and potassium in the mica substrate. It is possible that instead of a substrate role, the Mg^{2+} plays some important DNA stability role (the term "activation" has been used by Sogo [11, 18]), which allows or causes the DNA to adsorb with greater intramolecular integrity. It is also possible that an exceedingly small concentration of "special" magnesium sites, present at a level below the detection limit of AES or ESCA, are primarily responsible for the adsorption of DNA.

Conclusions

The results of comparative electron spectroscopy studies for deposition of DNA on mica have been completed. In the case of the chemical modification of substrate method [3, 22], these results suggest that DNA binding is not due to ion-exchange of the Mg^{2+} in the solution with K^+ in the mica as had been previously suggested [3]. An alternative possibility is that this exchange proceeds at levels not detectable by AES or ESCA, resulting in a very low concentration of special Mg^{2+} sites. Comparative studies of Ruby B and green mica indicate that there are no significant differences between the micas which would lead to DNA adsorption differences. Using both the chemical activation of DNA method and the chemical modification of substrate method (defined in this paper), DNA was bound to the mica substrate and AFM images illustrating the typical image qualities were presented. The surface analysis technique of ESCA was shown to be an effective method for detecting adsorbed DNA on mica.

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nal Chemistry, University of Utah, for the donation of the thiolated 8-mer and Br-DNA, Mike Christopherson for its preparation and purification, and Dr. Robert Weiss, Eccles Institute of Human Genetics, University of Utah, for the donation of the unmodified DNAs. We thank Dr. Paul L. Valint, Jr., Surface Science Core Technology Unit, Bausch and Lomb, Rochester, NY for the use of their NanoScope III.

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Discussion with Reviewers

S.M. Lindsay. The drying step appears to be crucial in all these preparations for AFM of DNA. This observation mitigates against simple charge-based arguments.

R. Wepf: How do you explain the fact that plasmids as used for the preparation for Fig. 5 are stretched to elongated non-circular molecule? Is this kind of "stress" to the molecule a major limitation of this preparation technique?

Authors: The charge-based binding argument is a postulate of others that is being examined in this paper. Yes, it is indeed true that the drying step is crucial in preparations for AFM as has been demonstrated in numerous studies. Even though the forces associated with drying have been shown to be crucial, other binding factors such as charge and ion-exchange cannot be ignored. Charge-based binding while still under water (i.e., before drying) may only assist in the initial steps of adsorption, if at all.

S.M. Lindsay: Adsorption at the surface and binding that is tight enough for SPM are not the same thing! **Authors:** We agree. However, progress is often made by combining many small advances. Thus, a confirmed ability to adsorb DNA at any level is progress at this point. Correlating coverages determined by ESCA with images can help determine what fraction of bound DNA is bound tightly enough for SPM.

S.M. Lindsay: Electrochemistry has proved a useful weapon in our work. We have focused on the STM because we reproducibly obtain better resolution than electron microscopy, a feat that has yet to be duplicated by AFM. I realize that STM is controversial, but the way to understanding is not through ignoring our results, details of which have been published elsewhere [Jeffrey *et al*. Nucl. Acids Res. **21**, 5896-5900, 1993; Jing T *et al*. Proc. Natl. Acad. Sci. USA **90**, 8934-8938, 1993].

Authors: We agree that electrochemistry has proved to be useful in biological STM studies and recognize your contributions. Your results were not ignored for any particular reason, and definitely not because we believe that STM is too controversial. AFM is most likely just as controversial! STM results were not included (yours or anyone else's) simply because we were examining

these particular deposition methods with AFM for comparison with previous AFM results. In addition to your electrochemical deposition techniques, researchers at Georgia Institute of Technology and Oak Ridge National Laboratory have illustrated that gold-thiol interactions can be applied routinely and effectively in STM studies of adsorbed DNA on gold [Bottomley *et al*. J. Vac. Sci. Tech. **A 10**, 591-595, 1992].

T. Thundat: Would the authors like to speculate that the observed increase in adhesion of DNA on sonicated mica is due to increased surface roughness? As for samples prepared with Mg added to the solution, might the increased adhesion be due to electrostatic interaction? **Authors:** The idea of increased surface roughness aiding in adhesion is often discussed in studies of this nature. However, we have not found the method of mica cleavage to have an effect on DNA binding.

T. Thundat: The authors could strengthen the paper by including a discussion on the height and width of DNA observed in their experiments.

Authors: We recognize that your papers often include a section devoted entirely to the matter which you address here. Although we present the width and length of the DNA illustrated in Figure 5, we did not extensively address this issue since we were solely interested in determining differences between deposition methods. Analysis of DNA deposited on chemically modified mica had an average width of 74 $\pm$ 44 nm and height of 1.7 $\pm$ 0.9 nm (data obtained from 16 measurements taken from 3 different images) while analysis of the chemically modified DNA had averages of 40 $\pm$ 9 nm and 1.3 $\pm$ 0.3 nm, respectively (data obtained from 11 measurements taken from 3 different images).

R.L. McCarley: Why was AES chosen to evaluate the surface of the mica which had been modified with magnesium acetate? From a reader's point of view, the authors have shown AES to be problematic in the discussion concerning DNA adsorption. It would seem that ESCA would be the preferred method of analysis for the data shown in Figure 4A, data collected to investigate very small changes in the ion composition of the mica surface. I feel that the authors should comment on this point. If nothing else, the authors should display the AES spectra including the potassium peak for the various treatments.

Authors: The AES spectra in the potassium region all looked similar to Figure 2A, with no differences observed (and therefore are not shown). Mg peak intensity was very low in ESCA, regardless of the preparation; AES proved to be more illustrative for detection of Mg.

R.L. McCarley: Although I am certain that the authors have previously shown AFM images of DNA adsorbed on untreated mica in other publications, it would be of help to have an image of DNA deposited in the same manner as here, but with no mica or DNA modification. It is clear that the chemical modification of DNA method and the mica modification method yield DNA images which are similar, but it is not clear what differences exist between the untreated mica/untreated DNA method and those discussed here. This would give a more fair comparison of the effects of magnesium treatment on the morphology of the deposited DNA.

Authors: The problem with depositing DNA on untreated mica or using untreated DNA is that the DNA has to rely on drying forces to bind to the surface. Thus, it is quite difficult to find the DNA, or when one does find it, the force of the cantilever brushes it out of the field of view. I am unaware of any one in the literature who did not employ some method of deposition similar to these studied here. Some of the earliest DNA images taken by AFM were made by Hansma and co-workers [Hansma HG *et al.* J. Vac. Sci. Tech. **B 9**, 1282-1284, 1991; Weisenhorn AL *et al.* Langmuir **7**, 8-12, 1991] who used Langmuir-Blodgett films to adhere the molecules for investigation.

R.L. McCarley: A small discussion of what the authors believe to have happened during the sonication step would be appreciated. This is a commonly used technique for cleaning surfaces such as Si and Au with solvents like propanol, and any information on surface damage by sonication would certainly be helpful. Also, it would be informative if a similar discussion were included which addressed the possible causes of increased mica surface roughness upon exposure to the magnesium acetate solution. Is dissolution occurring during the magnesium treatment?

Authors: We believe that it is unlikely that dissolution of the mica would occur during the overnight soaking in magnesium acetate. We do not know of any studies directly addressing this issue in the literature.

Reviewer VI: This paper contains many good experimental ideas, but lacks control experiments, data correlation, and quality AFM images to make conclusive statements. For example, no mention is made whether the AFM Figure 4 was examined by ESCA afterwards. If this had been done, the authors could have correlated DNA density from AFM images with the ESCA spectra (both N and Mg peaks). If it is true, as the authors claim, that ESCA might be insensitive to these levels of Mg, different DNA/Mg loads could have been examined to determine at what surface concentration the Mg sensitivity drops off. Since the thesis is "Is Mg effective in binding DNA to mica?", the authors could have used control experiments that compared the DNA spreads with other cationic treatments such as sodium, cobalt or barium to see if these treatments affect DNA density.

Authors: We disagree that this paper lacks control experiments, data correlation, and quality AFM images from which to make conclusive statements. The data presented in this paper come from over 50 experiments that were completed to address differences between the two preparation techniques. Control experiments were included in these studies. They may have been different than the particular controls that you would have chosen. The experiments that you suggest would be both informative and interesting to study. While the many different ions that you propose to study may be interesting, they significantly broaden the scope of this paper beyond our interest. The thesis you suggest "Is Mg effective in binding DNA to mica" was not exactly what we wished to examine. We were interested in comparing the two AFM preparation techniques and to determine, if possible, what role the Mg was playing in these binding processes. Perhaps, the experiments you suggest could be explored in the future because they are valid issues. When samples were prepared, several were prepared at once. Thus, samples from the same preparation lot were both imaged by AFM and analyzed by ESCA, as stated in the experimental section of the paper. We did modify the concentrations of DNA and correlate which levels DNA could be identified by both AFM and ESCA.

J. Vesenka: Figure 3 would be recognized by researchers in the field as having very high residual salt concentration. It would be much more interesting to see ESCA spectra from cleaner DNA spreads.

Authors: Figure 3 was prepared exactly as the referenced literature details. We were addressing the issue of reproducibility of literature preparations. Thus, if this is a common problem with preparations of this type, ways to correct the problem should have been listed in the original publication (they were not). It should be further noted that this image was characteristic of the preparation, not rarity. The surface spectroscopy techniques that were employed were pushed to their limits to analyze these particular samples. The spectra were reproducible. Further, both AES and ESCA spectra have been duplicated by a contracted commercial company.

O. Johari: In the **Discussion**, the authors state: "Indeed, a number of researchers have reported DNA adsorption on mica in the absence of the MgAc step, although most also agree that the presence of magnesium seems to help in some way." Please provide a reference.

Authors: The fact that people can prepare DNA spreads on mica completely without the Mg was first made known to one of us (TPB) at the Second Annual Workshop on STM and AFM in Molecular Biology held at Royaumont Abbey, France in April, 1991; only those who were in attendance received the proceedings. Those present who acknowledged this fact were Helen Hansma, Jane Frommer, Jean-Claude Poulin, Eric Leniewska, and especially Etienne Delain, who showed and published several excellent AFM images of DNA at that meeting.

R. Wepf: After roughly half a century of electron microscopy studies [Williams and Wyckoff. Science **101**: 594-596, 1945] on single particles (e.g., Latex spheres, viruses e.g., TMV, different proteins and DNA) adsorption onto any kind of substrate is still a process which is not well understood. Especially in the case of DNA, where cleaved mica crystals and their atomic flat fracture faces expose negative charges as the DNA molecule itself (polyanion) does. The presented paper gives some interesting features about two substrates and techniques frequently used for DNA studies. The so called "chemical activation" of DNA and the "chemical modification" of the substrate, which *per se* is a spin off of the previous method.

The results published in literature with the first method have clearly proven that DNA can be easily adsorbed in a relaxed and stress free way, which is of special interest when studying conformational arrangement of DNA and/or DNA-Protein complexes. The sample preparation based on standard replica technique to visualize DNA molecules in TEM down to 200 bp is published *in extendo* [18]. The results so far published by the method of Hansma *et al.* [7-9] and Bustamante *et al.* [3] have not reached the quality and reproducibility of the standard replica technique for TEM. Therefore, it is of interest for the STM and AFM community to compare these two methods and find reasons why the later technique causes so many problems, even though such adsorbed DNA images were published in respected journals.

The comparative study would have gained in value if TEM images from the same samples as studied by AFM in Figures 3 and 5 would have been added (using the same samples after propanol treatment and making standard replicas for TEM). Such a comparative study would also allow the authors to give any statistical figures about adsorption density and reproducibility, since in TEM it is very easy to have an overview over a large area. The major difference between both imaging techniques is that for AFM the samples are imaged in a liquid (e.g., propanol) whereas TEM samples must be completely dried. This may be the reason why very few molecules are found in AFM preparations. It would have been of interest if AFM images of a TEM samples would have been collected to discuss the striking difference between these techniques.

Nevertheless, the comparison of the two different mica types and the effect of different treatments with MgAc by AES and ESCA is an important result which helps to clarify the different interpretations and some mysterious models of adsorption to these substrates. In particular, it has weakened the model of exchanging K^+ in freshly cleaved mica faces by Mg^{++}, since no significant Mg-signal after the sonification procedure of the modification technique was found in ESCA measurements. Also, it has proven that there is no significant difference in the overall chemical composition of "Ruby B" and "Green" mica. The limitation of both analytical methods is that both techniques reveal an averaged signal over the area covered by the probe size (1 mm in AES and 1 cm in ESCA). These methods, therefore, do not allow to detect local variations of the composition and hence local differences in charge, which could effect DNA adsorption without or in presence of Mg-ions. From TEM studies, it is well known that local differences on a support play an important role for adsorption of any kind of sample [e.g., de Murcia and Koller. Biol. Cell **40**, 165-174, 1981, on effects of substrate treatments to adsorption; Sogo *et al.* J. Microsc. **104**, 187-198, 1975, specially for DNA; Dubochet *et al.* in Advances in Optical and Electron Microscopy (Academic Press, London), 107-135, 1982, for adsorption in general]. Such difference can produce stress to the DNA molecules during adsorption leading to plasmids which are stretched into linear molecules or DNA molecules adsorbed in a straight and stretched fashion. Another artefact can be the formation of compact aggregates which was also found and discribed in Figure 3.

Authors: Thank you for your comments. It certainly would be nice to do the comparative study Dr. Wepf is suggesting.

R. Wepf: A citation, as e.g., Amrein *et al.* [Science **240**, 514-516, 1988; and Science **243**, 1708-1711] should be added, both publications have for the first time adapted the "activation" technique established by Sogo *et al.* [18] for STM imaging DNA and RecA-DNA complexes on uncoated and coated mica.

Authors: Thank you.