



Biological & Biomedical Sciences

Influence of Substrate Surface Chemistry on the Binding of DNA-RecA Protein Complexes

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Abstract

A procedure for imaging calf thymus and plasmid DNA using contact mode atomic force microscopy (AFM) is described. The stable tethering of double stranded calf thymus and plasmid DNA molecules to a mica surface was facilitated by surface modification of freshly cleaved mica with Mg^+ ions. Mica surfaces treated with spermidine are shown to result in aggregation of DNA at high treatment concentrations ($> 5 \times 10^{-2}$ mg/mL) and in little binding at low treatment concentrations ($< 5 \times 10^{-2}$ mg/mL). Improvements in tips and scanning conditions are still needed in order to achieve higher resolution.

Background and Introduction

The protein recA and its interactions with DNA have long been studied (Ogawa *et al.* 1993). This is a result of the fact that recA is a protein involved in homologous genetic recombination. This is the recombination of DNA between DNA fragments with identical or very similar sequences (Ogawa *et al.* 1993; Roca and Cox 1990; Shan *et al.* 1996). RecA is also involved in post-replicative repair of damaged DNA molecules (Ogawa *et al.* 1993; Roca and Cox 1990; Shan *et al.* 1996). RecA can bind to either single-stranded or double-stranded DNA in the presence of adenosine triphosphate (ATP), ATP γ s (which is the nonhydrolyzable form of ATP) or adenosine diphosphate (ADP). When ATP is bound to recA, the protein adopts an active conformation and has a strong affinity for DNA (Ogawa *et al.* 1993). When ADP is bound to recA, the protein adopts an inactive conformation and has a low affinity for DNA. Only when ATP or ATP γ s is bound to recA, can the protein self-assemble into nucleoprotein filaments and induce DNA strand exchange (Ogawa *et al.* 1993). An example of this strand exchange is shown in Figure 1 (<http://www.bcbp.gu.se/mbg/recomb/>).

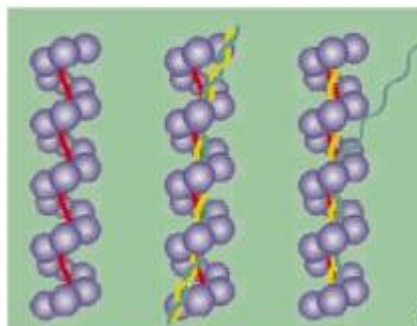


Figure 1: Schematic of homologous genetic recombination, as carried out by the recA protein. The single-stranded DNA, red, is switched with the homologous strand, green, in the double-stranded DNA.

The purple spheres are the recA proteins, which are polymerized along the single-stranded DNA, in red. The double-stranded DNA, yellow and green, then binds to the polymerized protein. Strand exchange then occurs between the red and green strands. RecA is also an essential protein in the "SOS" functions of DNA repair (Ogawa *et al.* 1993; Roca and Cox 1990; Shan *et al.* 1996). The SOS response is the coordinated activation of diverse metabolic functions in response to severe DNA damage (Ogawa *et al.* 1993; Roca and Cox 1990; Shan *et al.* 1996). RecA is able to induce its own synthesis on damaged DNA. This is done by the cleavage of a repressor protein, LexA. Once the repressor has been cleaved, all of the SOS proteins are then able to be expressed.

RecA is one of the most intensively studied of the enzymes involved in homologous recombination for many reasons. One reason is that there are large quantities of recA in the cell, it accounts for several percent of total cellular protein after induction (Ogawa *et al.* 1993). It can also be purified with relative ease, and it is possible for recA to catalyze a strand-exchange reaction *in vitro* by itself (Ogawa *et al.* 1993; Roca and Cox 1990; Shan *et al.* 1996). One of the most important reasons for studying recA is that there is a growing belief that the specific nucleoprotein structure formed by the recA proteins on the DNA strands may be a universal structure throughout all of biology (Ogawa *et al.* 1993). This suggests that recA is the ideal model protein for how homologous genetic recombination occurs in humans.

Scanning probe microscopy (SPM) is a new surface analytical tool with a potentially enormous impact on the study of biological systems, due to the near-atomic resolution attainable with these microscopes (Hansma *et al.* 1988; Lyubchenko *et al.* 1992). SPM offers the unique advantages of high-resolution imaging of DNA, RNA, and other biological molecules in the absence of stains, shadows, and labels. Furthermore, they can be operated in either air or liquid ambients (Lyubchenko *et al.* 1993). The key to successful imaging of DNA and other biomolecules such as DNA-protein complexes is the need to ensure that these molecules are both chemically stable and sufficiently tethered to the substrate surface. Although the atomic force microscope (AFM) is able to produce near-atomic resolution, its potential has been limited in microstructural studies of DNA, RNA, and other biological materials. The most immediate limitation to the practical application of AFM in studying biological complexes is sample preparation. The molecules must be attached to the substrate surface in order to avoid movement caused by lateral forces generated by the sweeping probe during scanning. If they are not adequately attached, the drift experienced by these molecules limits image resolution. An ideal tethering scheme will be able to both secure a DNA molecule to the surface, but will also allow the molecule to undergo changes in conformation without significant steric hindrance, in the presence of other reactive biomolecules. This is needed in order to study chemical reactions between proteins and DNA on a surface. A second limitation to AFM resolution may be adhesion of biological macromolecules to the scanning tip. This again, is more likely to occur if the molecules are not stably tethered to the surface. Both of these issues must be addressed in order to successfully image biological molecules using SPM.

One approach to tethering DNA is by chemically modifying a substrate surface in such a way as to increase the affinity, and therefore binding of the DNA to the surface. This technique has become the most common procedure for stably attaching DNA to mica surfaces. Many chemical modification processes have been attempted with favorable results, such as MgCl₂-

treated mica (Bezanilla *et al.* 1994), glow-discharged mica (Hansma *et al.* 1992), spermidine-treated mica (Tanigawa *et al.* 1997), and aminopropyltriethoxy silane (APTES)-treated mica (Lyubchenko *et al.* 1992). Each of these processes works by modifying the surface chemistry to be more amenable to the charge and chemical state of biomolecules.

Previous research on recA and DNA complexes has used Scanning electron microscopy (SEM), Fourier transfer infrared spectroscopy (FTIR), and X-ray crystallography to determine the difference between the active and inactive conformations (Ogawa *et al.* 1993). Our ultimate research goal is to use contact mode AFM to study the protein conformation in the presence of different nucleotides, ATP, ADP, or ATP γ s. Before this can be accomplished, we report the necessary surface modification techniques and sample treatment in order to enable us to pursue more ambitious protein conformation studies.

More information on scanning probe microscopy and recA can be found at the following websites:

Atomic Force Microscopy:

<http://www.di.com/>

<http://www.di.com/applnotes/>

RecA:

http://zebra.berkeley.edu/~vicbunny/RecA_Structure.html/

<http://www.bcbp.gu.se/mbg/recomb/>

Materials and Methods

Mg⁺ Surface Treatment

Pieces of freshly cleaved ruby mica were immersed in solutions of 33 mM magnesium acetate (Aldrich Co.) in deionized/distilled water (Hansma *et al.* 1992; Tanigawa *et al.* 1997), for periods of 12 hrs - 2.5 weeks. They were then rinsed with deionized/distilled water and dried under a gentle compressed nitrogen flow at room temperature for approximately 30 seconds. Mica pieces were stored in a nitrogen atmosphere until they were used, up to 2 weeks after treatment.

Spermidine Surface Treatment

Freshly cleaved mica pieces were submerged in 5×10^{-2} mg/mL, 2.5×10^{-2} mg/mL, 2.5×10^{-1} mg/mL, and 5×10^{-1} mg/mL spermidine (Aldrich Co.) solutions, as reported by Tanigawa *et al.* (1997). They were then allowed to incubate for 5 minutes. The pieces were subsequently rinsed with deionized/distilled water and dried under compressed nitrogen. These treated mica pieces were used immediately.

DNA Attachment on treated mica

Pieces of Mg⁺-treated mica were mounted onto magnetic AFM sample discs and placed in plastic storage boxes. A solution of 2.0 ± 0.2 mg/mL calf thymus double stranded DNA (Sigma Co.) in buffer, containing 5 mM Hepes, 5 mM KCl, and 5mM MgCl₂ (Bezanilla *et al.*

1994) was pipetted onto the surface of the mica. Plasmid double stranded DNA, M13mp8 RF I DNA (Sigma Co.) was also used. When plasmid DNA was used, the concentration was 4 - 12 μL of M13 DNA as received in 100 μL of the aforementioned buffer. This solution was then pipetted onto the mica surface. The DNA-treated mica pieces were placed in a 4°C refrigerator for 10-15 minutes. They were then rinsed with distilled/deionized water and dried on ice under a gentle flow of compressed nitrogen for approximately 30 seconds.

Spermidine-treated mica was mounted onto magnetic AFM sample discs and placed into plastic storage boxes. 100 μL of 2 mg/mL solution of calf thymus DNA in the aforementioned buffer was pipetted onto the spermidine coated surface. This was allowed to incubate for 5 minutes. These samples were then rinsed with deionized/distilled water and dried under a gentle flow of compressed nitrogen for approximately 30 seconds. These were then imaged immediately.

Atomic Force Microscopy

All imaging was performed on a Nanoscope III extended Multimode AFM (Digital Instruments) in air with contact mode AFM using standard Si_2N_4 probes (Digital Instruments, NP average spring constant $k=$ **N/m) or oxide sharpened probes (Digital Instruments, NP-STT average spring constant $k=$ **N/m). The scan rates varied from 0.45-1.4 Hz, image size varied from 10 μm to 100 nm, and the z range also varied, from 8-1 nm; decreasing as the scan size decreased.

Results and Discussion

The spermidine treated surfaces did not appear optimal for DNA imaging. When 5×10^{-2} mg/mL concentrations of spermidine were used, no DNA was observed on the surface through multiple trials, as shown in Figure 2.

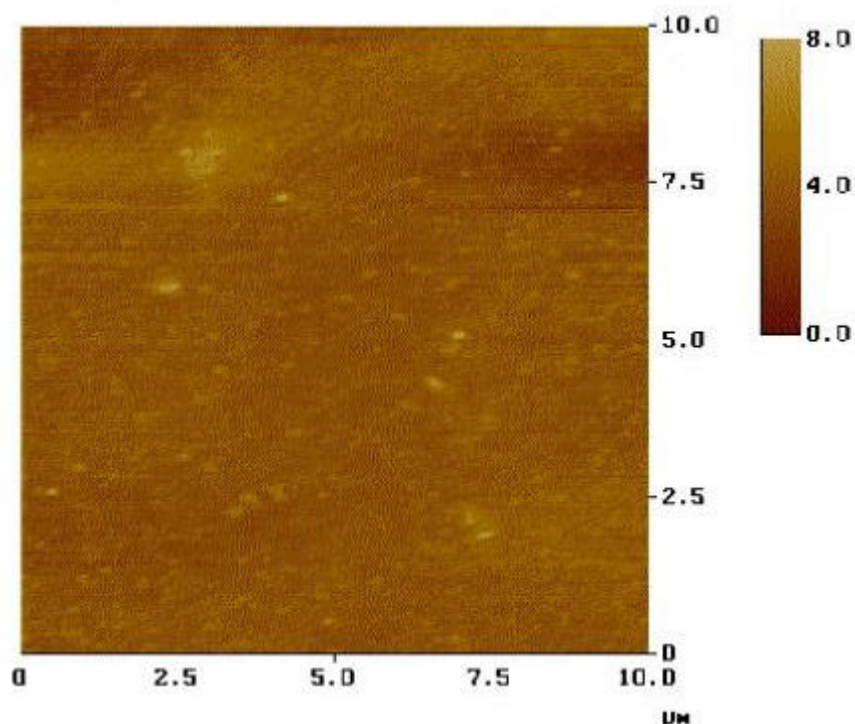


Figure 2

The concentration of spermidine was then increased by an order of magnitude to 5×10^{-1} mg/mL. In this case, there is aggregated DNA across the entire surface, as shown in Figure 3.

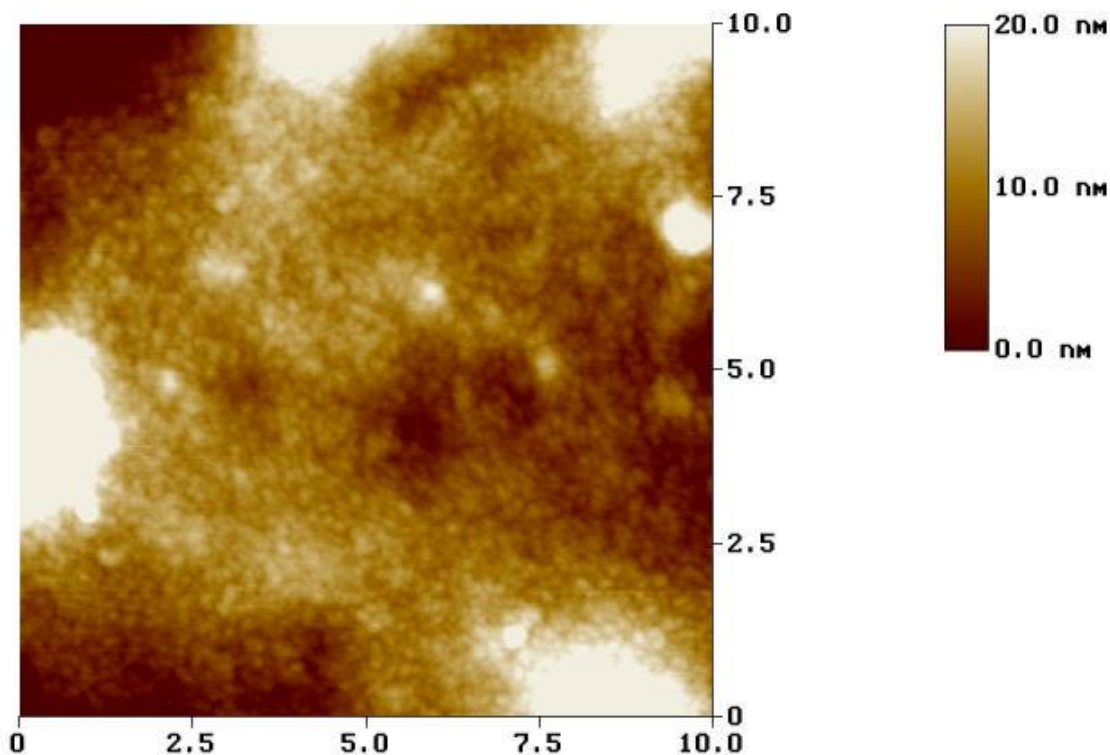


Figure 3: AFM contact mode image in air, (10 nm scan) of 5×10^{-1} mg/mL spermidine-treated mica surface after DNA exposure. DNA is aggregated on the surface with an average of 3-4 μm in diameter.

The aggregated DNA appears to be 3-4 μm in diameter, and is uniformly aggregated across the mica surface. We also tried 2.5×10^{-2} mg/mL, in which no distinct molecules were seen on the surface, they appeared to be displaced by the tip. A spermidine concentration of 2.5×10^{-1} mg/mL was also tried, this time there appeared to be more molecules on the surface, but once again they were easily displaced by the sweeping AFM tip. We were never able to observe individual DNA molecules by reproducing the spermidine treated surfaces as reported by Tanigawa *et al.* (1997).

Stable DNA tethering was obtained using the Mg^+ treatment as reported from Hansma (Hansma *et al.* 1992; Tanigawa *et al.* 1997). This sample preparation involved not only treatment of the surface with Mg^+ ions, but it also included rinsing of the surfaces under a stream of water after DNA treatment to remove excess DNA molecules. This presumably leaves behind only the stably tethered molecules most suitable for imaging. The prominent features in the AFM images of the Mg^+ treated substrates are numerous regions of aggregated DNA. These appear as bright patches approximately 1-2 μm in diameter, as indicated in Figure 4.

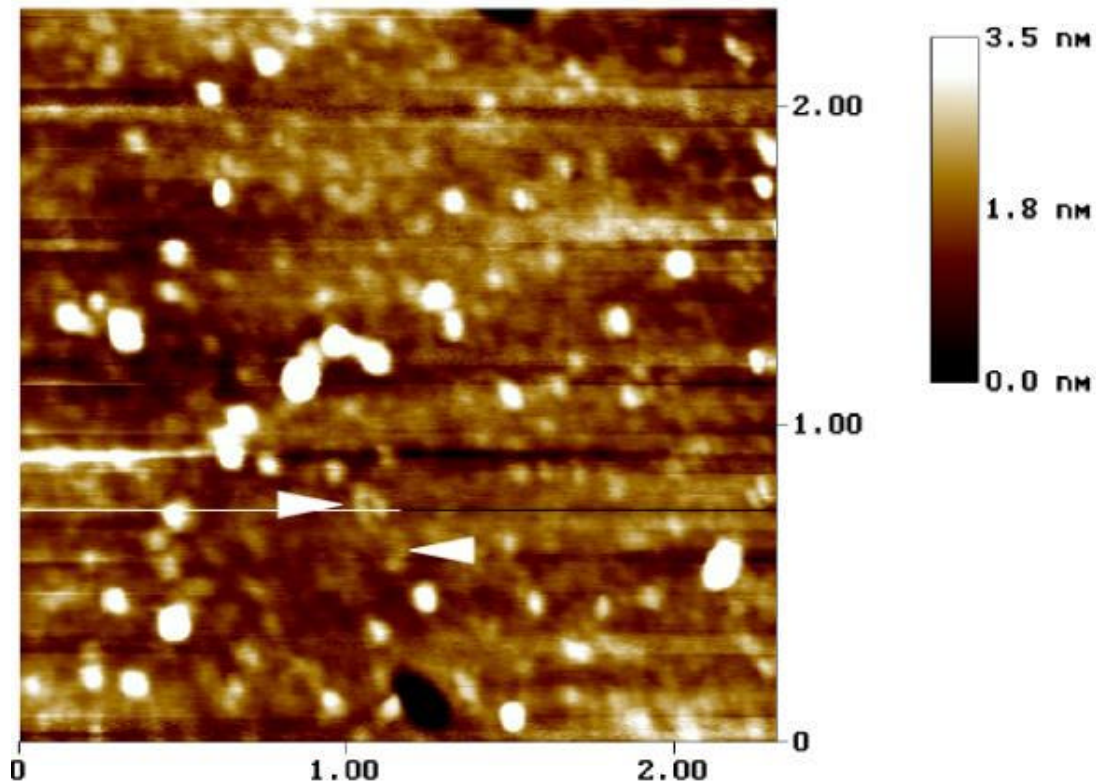


Figure 4: AFM contact mode image in air, of calf thymus DNA on Mg⁺ treated mica surface (2.25 μm scan). Individual DNA molecules are indicated with arrows.

However, single molecules can also be observed around the edges of these clusters and dispersed over the entire surface. This is also indicated in Figure 4 with an arrow. These single molecule DNA have measured lengths of 100-150 nm which is consistent with previous experimental data (Lyubchenko *et al.* 1992). We have measured widths of 8-12 nm. This is greater than the literature value of 2 nm (Lyubchenko *et al.* 1992), but is consistent with broadening that can be attributed to the minimum tip radii of the probe as previously determined experimentally (Lyubchenko *et al.* 1992).

Once we were able to obtain reproducible AFM images of calf thymus DNA, we then attempted the same procedure on plasmid, M13mp8 RF I, DNA. Plasmid DNA was used, since plasmid DNA and single stranded poly dT, are the types of DNA that will subsequently be used in the experiments involving DNA-recA protein interactions. Single plasmid DNA molecules were also observed on the mica surface, as shown in Figure 5.

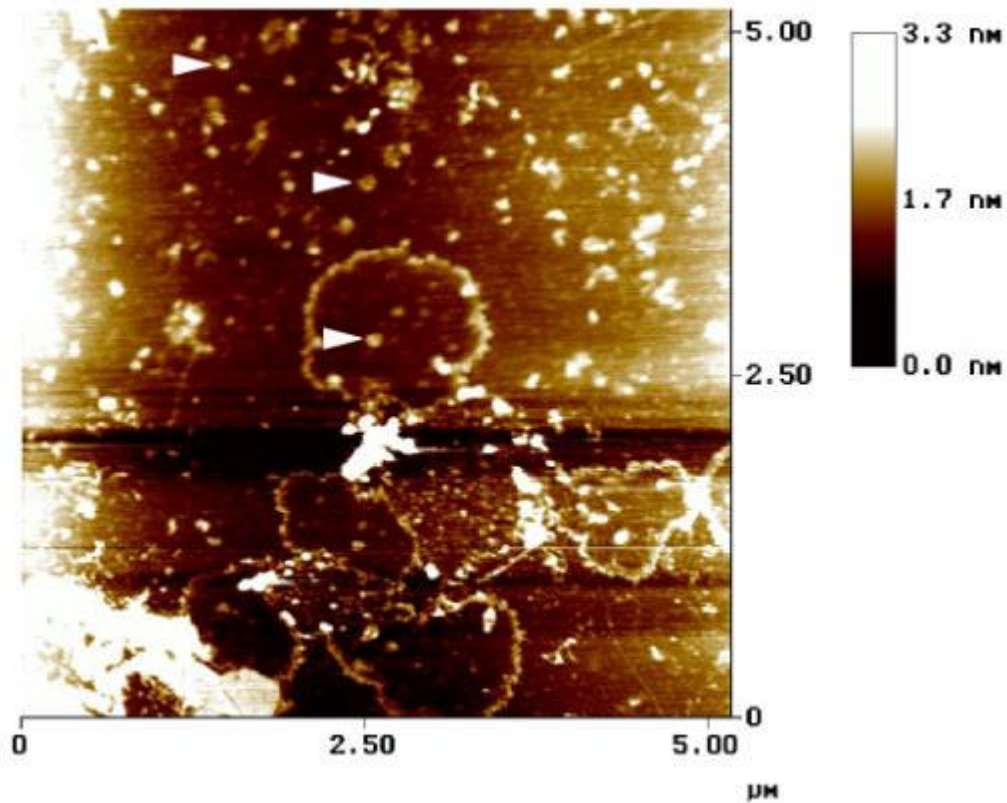


Figure 5: AFM contact mode image in air of plasmid, M13mp8 RF I, DNA on Mg⁺ treated surface (2.5 μm scan). Individual DNA molecules are indicated with arrows.

The DNA was sufficiently bound to the surface, and able to withstand multiple higher resolution scans in contact mode. The plasmid DNA molecules can easily be seen, as the circular features, approximately 80-90 nm in diameter as indicated in Figure 6.

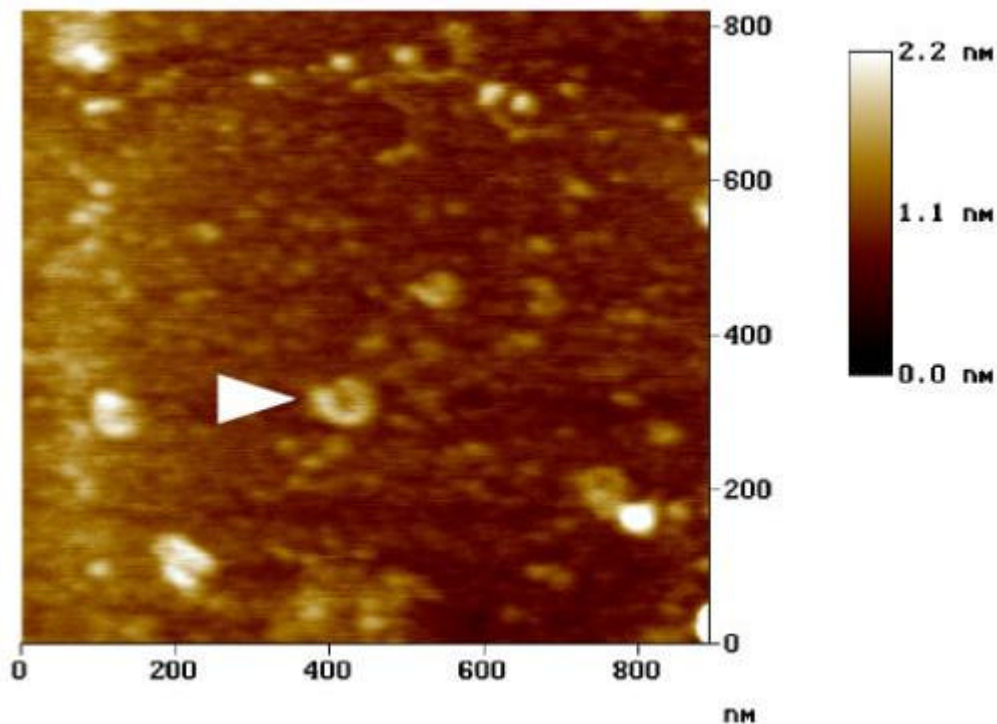


Figure 6: AFM contact mode image, in air (800 nm scan) of individual plasmid DNA on the Mg⁺-treated mica surface. Diameter of plasmids is about 80-90 nm.

The width of these features is approximately 10-15 nm. Again, this is wider than expected, but may be attributed to the relatively large radius of curvature of even the oxide-sharpened tips. Higher resolution images could be obtained by using tips that have smaller radii of curvature such as electron beam deposited tips (Guthold *et al.* 1999) or nanotube probes (Wong *et al.* 1998).

Conclusions

Images of DNA have been obtained at relatively high resolutions using AFM on chemically modified mica surfaces. These molecules have been successfully tethered to the surface by treating the substrate with Mg⁺ ions. DNA is stable for repeated scanning, and minimal drift occurred in the imaging of these molecules. It appears that both the surface modification and the sharpness of the tips were the limiting factors in image resolution, and through this work a surface modifying procedure has been found that is amenable to our research. The surface modification and sample procedure still require testing for the DNA-recA complexes, but we believe that since this procedure works for DNA, then a DNA-protein complex should have similar surface-molecule interactions. It is also obvious that in order to achieve higher resolution images, other scanning techniques will need to be applied. Some of the techniques to be used in the future will include scanning in liquid; and sharper tips that have a smaller radius of curvature and experience less tip contamination by surface molecules.

Acknowledgements

The authors would like to thank the Materials Research Society Undergraduate Materials Research Initiative (MRS-UMRI), the National Science Foundation Research Experiences for

Undergraduates (Grant# 97-31912), and the National Science Foundation (Grant# MCB 9733566) for support of this project.

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Journal of Young Investigators. 2001. Issue Three.
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JYI is supported by: The National Science Foundation, The Burroughs Wellcome Fund, Glaxo Wellcome Inc., Science Magazine, Science's Next Wave, Swarthmore College, Duke University, Georgetown University and many others.

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