Atomic Force Microscopy of Nucleoprotein Complexes

Y. L. Lyubchenko
Arizona State University, lyubchenko@phyast.la.asu.edu

B. L. Jacobs
Arizona State University

S. M. Lindsay
Arizona State University

A. Stasiak
University of Lausanne

Follow this and additional works at: https://digitalcommons.usu.edu/microscopy

Part of the Biology Commons

Recommended Citation
Available at: https://digitalcommons.usu.edu/microscopy/vol9/iss3/8

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Scanning Microscopy by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.
ATOMIC FORCE MICROSCOPY OF NUCLEOPROTEIN COMPLEXES

Y.L. Lyubchenko1,2,*, B.L. Jacobs1, S.M. Lindsay2, and A. Stasiak3

1Department of Microbiology and 2Department of Physics, Arizona State University, Tempe, AZ 85287-2701
3Lab. d’Analyse Ultrastructurale, Univ. de Lausanne, Bât. de Biologie, CH-1015 Lausanne-Dorigny, Switzerland

(Received for publication July 21, 1995 and in revised form September 26, 1995)

Abstract

Recent data on the AFM studies of nucleoprotein complexes of different types are reviewed in this paper. The first section describes the progress in the sample preparation methods for AFM studies of nucleic acids and nucleoprotein complexes. The second part of this paper reviews AFM data on studies of complexes of DNA with regulatory proteins. These studies include two different types of DNA distortion induced by proteins binding: local bending of DNA at sites of protein binding and formation of large loops due to protein-protein interactions between molecules bound to distant sites along the DNA molecules (DNA looping). The prospects for use of AFM for physical mapping of genomes are discussed in this section as well. The third part of the paper reviews data on studies of complexes of DNA with non-sequence specific binding proteins. Special emphasis is given to studies of chromatin which have resulted in progress in the understanding of structure of native chromatin fiber. In this section, novel data on AFM studies of RecA-DNA filaments and complexes of dsRNA with the dsRNA-specific protein p25 are also presented. Discussion of the substrate preparation procedures in relation to the AFM studies of nucleoprotein complexes is given in the final section.

Key Words: Atomic force microscope, DNA, RNA, nucleoprotein, RecA-DNA complex, structure, mica modification.

Introduction

The atomic force microscope (AFM, also known as scanning force microscope, SFM) and its predecessor scanning tunneling microscope (STM), belong to a novel family of scanning probe microscopes (SPM). These SPMs have enormous potential importance to structural biology and pharmacology. Both STM and AFM image surfaces by scanning them in a raster pattern with a fine tip. The tip repeats the contour of surface structure, which is depicted on a computer screen directly during scanning. These instruments offer unique advantages in their potential for structural studies of DNA, RNA and their complexes with proteins and other ligands in the absence of stains, shadows and labels. Furthermore, the instruments can be operated in air and in liquid. They are theoretically capable of resolving structural details at the level of atomic dimensions, provided the specimen is dynamically stable.

The prototype STM instrument was conceived by Binnig et al. [14], an invention for which Binnig and Rohrer were awarded the 1986 Nobel Prize in Physics. The inventors of STM realized, almost at once, the potential of their discovery for biology, producing the first image of a biological molecule obtained in absence of vacuum in 1985 [13]. The growth of activity has been furious, and was further stimulated by the invention of the AFM by Binnig, Quate and Gerber [15] and its brilliant development by the group of P. Hansma [27, 40]. The consequent deluge of papers has stimulated several recent reviews dedicated solely to biological applications of scanning probe microscopes (predominately, dealing with imaging of DNA; see, e.g., [8, 19, 20, 29, 39]) and even a book [5].

In this review, we will focus on AFM studies of nucleoprotein complexes. We will review recent data on the AFM imaging of sequence specific protein-DNA complexes, non-sequence specific nucleoprotein ensembles, particularly highlighting the problems of substrates and sample preparation procedures for different types of nucleoprotein complexes.
Figure 1. AFM image of the fragment of lambda phage (half of the genome length). The sample of lambda DNA in concentration of 0.01 $\mu$g/ml was deposited onto AP-mica, rinse thoroughly with water, vacuum-dried and imaged in air with regular Si$_3$N$_4$ tips on a NanoScope II microscope. The details of the sample preparation procedure have been published \[73, 77\].

Current progress in the sample preparation methods

Despite the fact that AFM has only been recently developed, great progress has already been made in applying AFM to biological studies. Note that an immediate practical limitation of the application of AFM and STM to structural studies of biomacromolecules is sample preparation. The macromolecules must be tethered to the substrate in order to avoid resolution limiting motion occasioned by the sweeping tip during scanning. The deformation of the specimen is another serious problem for AFM, but, in our opinion, the sample preparation procedure occupies the first place. Unfortunately, many of earlier STM papers, abstracts and conference presentations followed a simple recipe: a solution of the biomaterial of interest was dried onto a graphite substrate and imaged in ambient conditions by STM. The graphite surface is inherently rather inert (especially for hydrophilic materials like DNA) and possesses a rich array of crystallographic features \[48, 87\] so that almost any desired "molecules" can be found on the bare surface \[23\]. The operation of the AFM is, in principle, simpler; it is capable of imaging non-conducting as well as conducting surfaces and many of the early studies were promising (for example, \[29\]), but the sample preparation problem continued to be an important one.

Progress was made recently in the sample preparation technique for both STM and AFM. Electrochemical deposition for STM imaging of DNA was developed in the group of S. Lindsay several years ago \[9, 69, 70, 71, 72\]. Recent advances in electrochemical deposition under potential control permitted imaging of DNA molecules \textit{in situ} \[72\].

Another way of preventing DNA displacement by the tip is a chemical modification of the substrate and/or DNA. Criscenti \textit{et al.} \[24\] used chemical modification of DNA to improve DNA binding to a gold substrate. Heckl \textit{et al.} \[49\] modified graphite for binding phospholipids to the surface. We suggested a procedure for graphite modification \[74\], which is based on covalent binding of mercurated DNA to a sulphydryl sepharose matrix. Retention is due to the formation of very stable links between mercurated DNA bases and SH groups on the modified substrate. Gold substrates can be activated by self-assembled monolayers of thiol for reliable STM and AFM imaging of DNA \[3, 4, 50\].

Several reliable and repeatable methods of DNA sample preparation for AFM were published in recent years. Vesenka, Bustamante and co-workers \[18, 104\] applied the method of the ionic treatment of mica. In this approach, the mica surface is treated with multivalent ions (e.g., Mg$^{2+}$) to increase its affinity to DNA which is held in place strongly enough to permit reliable imaging by AFM. A similar approach with the use of solution of AlCl$_3$ was employed earlier by Weisenhorn \textit{et al.} \[108\] for imaging of short fragments of single-stranded DNA. Note that both types of mica treatment were developed for the electron microscopy (EM) sample preparation procedure \[35, 104\]. The first images produced by these methods yielded relatively poor resolution and dramatic improvements were obtained by using specially made carbonized tips and by imaging under propanol \[41, 42, 44, 68\]. Studies performed by Thundat \textit{et al.} \[100\] show that other di- and trivalent cations can be used for pre-treatment of mica. We will refer to this sample preparation approach as cation mediated deposition. Recent studies \[11, 12\] show that the pre-treatment step can be avoided, but deposition should be performed in the presence of multivalent cations.

Yang \textit{et al.} \[114, 115\] have developed a different approach. They use a modification of the well-known EM procedure, spreading the DNA onto carbon coated mica substrate using cytochrome c that has been denatured at an air-water interface. Rather remarkably, this approach yielded high resolution with conventional (untreated) tips operated in air. Another modification of the EM procedure based on use of a cationic detergent (benzyl dimethylammonium chloride, BAC) has been developed in the group of T. Jovin \[93\].
AFM of Nucleoproteins

We have worked on a third, quite different approach [12, 73, 77, 78, 79, 80, 81]. We suggested the use of aminopropyltriethoxy silane (APTES) to functionalize the mica surface with amine groups to make the surface positively charged. We imaged DNA, dsRNA [79, 81], and kDNA (submitted). An example of the AFM image of a DNA molecule (fragment of lambda DNA) deposited onto AP-mica is shown in Figure 1. The attachment of the DNA molecules to the AP-mica is so strong that AFM images of DNA molecules under water were obtained [80, 81].

The sample preparation methods developed for imaging of nucleic acids and described above have been used in the AFM studies of nucleoprotein complexes; in sections that follow we will review the results of these studies.

AFM Imaging of Sequence Specific DNA-Protein Complexes

DNA Bending in the complex with a protein

There currently exists a substantial body of crystallographic, spectroscopic and biochemical data for a number of protein-DNA complexes indicating that selective binding of a protein to a particular DNA sequence requires not only direct interaction of specific amino acids with bases through hydrogen bonding in the recognition sites, but also a second type of recognition [47, 99, 102]. This is structural or indirect recognition, in which local sequence-dependent DNA structure can have a profound influence on protein-DNA interaction. X-ray and NMR data directly show [47, 54, 94, 99] that DNA changes its conformation at the cognate site after binding the protein. A particular aspect of conformational changes on protein binding is bending of DNA [54, 60, 94, 99, 102]. In some cases, it seems that the function of the DNA binding protein may be solely to bend DNA into a particular conformation or shape [56, 62, 85, 102, 103]. Other proteins, e.g., bacteriophage 434 repressor [58, 59, 60] and MerR repressor E. coli [7] have strong unwinding (MerR) or overwinding (434 repressor) effects on their DNA operators. Both structural deformations, bending and twisting of DNA helix provide a better contact for the proteins with DNA bases and may facilitate further direct protein-DNA interaction.

Electron microscopy (EM) is a traditional structural method, which was used for direct demonstration of DNA bending in a number of regulatory complexes [26, 63, 64]. EM has been recently successfully applied for studies of binding to DNA the eukaryotic transcription factor TBP [37]. It was shown that this protein bends the DNA about the TATA box by 80 to 90°.

AFM studies of complexes of DNA with site-specif-
In addition to the bending effect of Cro protein at specific sites, AFM provides data regarding the binding of the protein to non-specific sites on DNA [20, 30]. The authors found that Cro bends non-specific sites as well. The narrow distribution of the bend angle for specific sites can be explained by a sequence specific flexibility of the operator region facilitating the bending of DNA by Cro [76]. The observation that Cro bends DNA at non-specific sites has been discussed in terms of dynamics of diffusion of Cro along the DNA [30].

An interesting system, the complex of DNA with Fur repressor has been recently investigated [64]. Fur protein involved in the regulation of various bacterial genes related to iron transport. In the presence of divalent cations (Fe$^{2+}$, Mn$^{2+}$, or Ca$^{2+}$), Fur acts as a repressor via binding to the "iron box" at the promoter regions of Fur-regulated genes. Both AFM and EM were applied for imaging of complexes of Fur protein with the 645 base pairs (bp) DNA molecule which contains the fragment of an aerobactin operon with two contiguous Fur binding sites. It was found that protein binding does not result in DNA bending. Moreover, at high molar Fur/DNA ratios, the protein coats DNA, and this complex is much more straight than naked DNA molecule. Polymerization process starts at the Fur-binding sites. Such stiffening of DNA induced by the protein binding is rather a unique feature of complexes of DNA with regulatory proteins. Note that there is no elongation or shrinkage of DNA helix in the complex.

DNA Looping in complexes with regulatory proteins

DNA looping is caused by a protein or a complex of proteins (P1 and P2 in Fig. 2) that bind to two distant sites on a DNA molecule; schematically this phenomenon is shown in Figure 2. Owing to looping, two sites separated by a long intervening DNA region (in some cases, as long as tens to thousands of base pairs) appear to be close to each other. This seemingly simple phenomenon is central in the regulation of many biochemical transactions involving DNA. The most prominent current examples of DNA looping are in the regulation of the expression of prokaryotic and eukaryotic genes, regulation of site specific recombination, and in the regulation of DNA replication [61, 84]. DNA looping formed by protein binding at two sites separated by more than about 50 base pairs has been demonstrated by direct EM imaging in a number of systems [36, 84]. Despite its novelty, AFM has already been applied to studies of the DNA looping phenomenon as well. There are two examples when DNA looping induced by binding of eukaryotic transcription factors was demonstrated by AFM [10, 113].

Wyman et al. [113] studied looped DNA complexes with heat-shock transcription factor 2 (HSF) using both microscopic methods, EM and AFM. Two binding sites

Figure 2. DNA loop formation due to protein-protein interaction between complexes P1 and P2 specifically bound to two distant DNA regions.
for HSF were placed at the distance of ~ 900 bp, so that protein-mediated association of two HSFs resulted in formation of a rather long loop easily visualized with EM. Similar samples have been studied with AFM.

Samples were deposited onto mica in the binding buffer, rinsed with water, dried and imaged with AFM in air. As a result, the looping of intervening DNA region was also imaged by AFM. However, AFM, unlike the EM, measures the height of molecules, and this circumstance permitted determination of the stoichiometry of protein complexes at looped structures. It was found that looped complexes are formed due to interaction of two HSF trimers bound to specific sites, which is an important characteristic of looped complexes. Similar analysis can be done by EM, but it requires complicated three dimensional (3-D) reconstruction of EM images. AFM allows retrieval of this information by routine analysis of the obtained images.

Becker et al. [10] used AFM for studies of conformational changes of DNA during formation of a multi-protein eukaryotic transcription complex. Visualization was performed with the use of a nuclear extract containing the transcription pre-initiation complex (PIC). Initiation of transcription requires Jun protein which binds to a distant AP-1 site. AFM data showed that Jun protein binds specifically to AP-1 site on DNA and bends DNA. When nuclear extract is present in addition to Jun protein, looped complexes are imaged with AFM (DNA loop is indicated with an arrow in Fig. 3). These loops comprise several hundred base pairs of DNA and are not formed when either Jun or the nuclear extracts are omitted. When AP-1 site is deleted from the plasmid, AFM data not only show DNA molecules without loops, but also display no proteins binding to DNA. This suggests first, direct interaction between DNA-bound Jun proteins and pre-initiation protein complex bound to the promoter region and secondly, binding of Jun stabilizes the PIC complex. The AFM data are consistent with biochemical data obtained for other similar transcription systems [10]. This example illustrates that AFM can be applied to studies of rather complex multi-protein regulatory systems and that further research in this area will shed new light on understanding molecular mechanisms of transcription regulation.

**Targeting of sequence specific DNA sites and physical mapping**

In this section, we review applications of AFM to location of specific DNA sequences through tagging them with bulky substituents. One of the most important area of applications of these studies is direct physical mapping of specific sequences in genomes.

In the approach reported by Murray et al. [85], a bulky tag was attached to the end of a DNA molecule. The tag was a chimeric protein fusion between streptavidin and two immunoglobulin G-binding domains of staphylococcal protein A. This chimeric protein binds strongly to DNA molecules containing biotin residues at their terminals. AFM imaging showed clearly the attachment of protein tags to ends of DNA molecules. The measured height at the protein-bound end is almost twice that of the DNA, giving sufficient contrast for identification of the tag.

In the group of E. Henderson [91, 92] a similar biotin-streptavidin coupling reaction was also employed, but they used streptavidin-coated colloidal gold spheres (nominally 5 nm in diameter) as bulky tags for biotinylated sites of DNA. In addition to imaging of linear DNA molecules with gold spheres attached specifically at their ends [91], circular DNA molecules labeled with gold particles were imaged by Shaiu et al. [92]. This was achieved by incorporating biotin-dUTP into a DNA plasmid in nick-translated reaction. Internal, as well as end-attached, gold particles are visualized with a high contrast. Because biotinylated nucleotides can be incorporated into replicating DNA in vivo, AFM can be used...
for studies of DNA replication, mapping of origin replication sites in particular.

In the group of T. Jovin [87], AFM was used for targeting short DNA regions adopting left-handed, Z-form structure. An anti-Z DNA antibody was used for recognition of the left-handed regions in the DNA molecule. A short DNA region with the sequence (C,G)₂₁, which can adopt a left-handed helical conformation in supercoiled DNA molecules, was inserted into a plasmid DNA which was complexed with the anti-Z DNA antibodies. Benzyldimethylalkylammonium chloride was used for spreading DNA on mica surface. Antibodies appeared as tall bright features (blobs) attached to the DNA molecule. The complex remained stable after linearizing plasmid DNA with the restriction enzyme. This allowed the authors to map the complex, which appeared to be formed specifically at the (C,G)₂₁ insert. The AFM data also show that DNA is kinked at the site of antibody binding which may be explained by deposition effects at the mica-detergent interface facilitated by a local DNA flexibility at the B-Z joints.

In its simplest form, physical mapping involves the measurements of the distance between specific sequences along the DNA molecule. The examples described above show that the AFM has sufficient resolution to map the location of bulky tags (predominantly proteins) on the DNA. It is important for mapping goals to note that DNA molecules, as long as whole lambda DNA (~50 kb), can be deposited onto surface in a rather extended form with limited number of self-intersections [77, 80]; see, for example the image of lambda DNA fragment of ~25 kb in Figure 1. In addition, the simplicity of the sample preparation procedure for AFM (usually a one-step procedure without contrasting of the sample) and fast processing of data (which are digital from the outset) are attractive features of AFM as a potential tool for physical mapping purposes.

**Imaging of Non-Sequence Specific Nucleoprotein Complexes**

In addition to studies of sequence-specific protein-DNA complexes, AFM was successfully applied to studies of a number of non-sequence specific nucleoprotein complexes, chromatin in the first place.
**AFM of Nucleoproteins**

**Imaging of chromatin**

The structural organization of chromatin is fundamental to understanding the molecular mechanism of gene replication and expression in the cell. Numerous biochemical techniques, EM, X-ray crystallography and spectroscopy were applied for resolving the structural details at different levels of the chromatin organization [110]. The basic structural unit of chromatin is the nucleosome, a particle of ~10 nm in diameter and consisting of a DNA molecule wrapped around the histone octamer. The structure of chromatin is very sensitive to ionic strength, and according to EM data, at very low ionic strength the chromatin is a "beads-on-string" fiber or an "open zig-zag structure" [51]. The situation is much more complicated as far as chromatin structure at higher ionic strength is concerned. According to Finch and Klug [31], the 30 nm chromatin fiber adopts a regular solenoid-like topology, and this model was one of the most popular chromatin models for many years. Another regular, twisted-ribbon geometry for the chromatin fiber was suggested by Woodcock et al. [111] and Bordas et al. [16]. However, these "regular" models are not consistent with recent biochemical and structural data [109]. Concerns about the regular structure organization of chromatin have been recently expressed in the work of Woodcock et al. [112].

The first AFM images of reconstituted chromatin were reported by Vesenka et al. [105]. Detailed AFM studies of reconstituted chromatin were performed by Allen et al. [1]. Histone octamers were reconstituted onto a linear 3.8 kb DNA fragment containing 18 tandem repeats of the 208-bp 5S rRNA gene, and the sample was gently deposited onto the cover glass. AFM images were taken immediately after rinsing and drying the sample. Linear chromatin fibers were resolved clearly enough (see Fig. 4A) allowing the end-to-end measurements of filaments. AFM length measurements of chromatin fibers containing different numbers of histone octamers showed that the 11-nm nucleosome core is formed by wrapping of a 146 bp DNA segment around the octamer to make 1.75 turns. The measurements of center-to-center spacing between cores (36 ± 8.8 nm) give results close to expected value (32.1 nm) assuming the precise position for all of the directly adjacent cores on the DNA template. The data obtained by Allen et al. [1] demonstrate that AFM is able to image such a delicate nucleoprotein complex as chromatin, and in some cases, AFM provides more clearly resolved images of chromatin fibers than those obtained by EM.

An AFM image of a native chromatin fiber isolated from chicken erythrocytes obtained by Allen et al. [2] is shown in Figure 4B. This image clearly demonstrates the formation of higher order structure at these salt conditions (10 mM NaCl). The progress in study of higher order chromatin structure has been recently achieved in the joint work of the groups of K. Van Holde and C. Bustamante [118]. Formation of higher order structure was induced by adding of NaCl into the chromatin solution. Chicken erythrocyte chromatin fibers were prepared at different NaCl concentrations, deposited onto mica (treated with spermidine for better binding of the chromatin filaments) and imaged with AFM. Imaging showed that chromatin fibers possessed a loose 3-D 30-nm structure even in the absence of added salt (AFM images for these conditions are similar to the image shown later in Fig. 6B). This structure slightly condenses when the concentration of NaCl is raised to 10 mM. A major change occurs when the salt concentration is increased to 80 mM. The fiber condenses to a highly compact, irregularly segmented structure, with the apparent diameter ~50% larger than the initial filament. Parallel analysis of chromatin structure by use of nuclease digestion led authors to the conclusion that the current model of chromatin as regular helical filaments (solenoidal or twisted-ribbon models) should be replaced by an irregular condensed fiber model and that previous physical and biochemical data should be carefully reexamined.

Very recently, the same group has reported high resolution AFM studies of the 3-D structure of low-salt chromatin filament [66]. Extended chromatin filaments were imaged in air with AFM operating in the tapping-mode. In this mode, the AFM tip oscillates at a very high frequency so as to touch the sample very gently on each cycle [39, 117]. This regime of AFM microscopy made it possible to reveal the 3-D organization of chromatin filaments. Experimental data were compared with computer simulations obtained for different molecular models for chromatin. This analysis led the authors to the conclusion that chromatin fibers may exist as irregular 3-D arrays of nucleosomes even at low ionic strength (similar analysis was also performed by Allen et al. [2]). Note that traditional EM typically showed extended "beads-on-a-string" or "open zig-zag" structures, and there has been concern that the EM sample preparation conditions could destroy the structure. Cryo-EM indicated a 3-D structure of the low-salt filaments, but structural details could not be resolved [66].

Recently Martin et al. [83] reported high resolution AFM data of substructural features of nucleosomes in extended chromatin fibers; the tapping mode capability was used in this paper [83] also, as by Leuba et al. [65]. Thus, application of AFM to the long-standing problem of chromatin structure resolved the controversy in numerous experimental data and made a breakthrough in this very important area of molecular biology. However, this achievement of AFM should not be overestimated. The sample preparation procedure for AFM,
AFM image of the complex of single-stranded FX174 DNA with single-stranded DNA binding protein (product of gene 32 of T4 phage). The complex prepared at specific salt conditions was fixed by glutaraldehyde and diluted with water (1:1). A droplet of solution (0.3 µl) was applied to mica, dried with compressed air, rinsed briefly with water and imaged in propanol. Micrograph courtesy of H. Hansma [39].

The AFM image shows a uniform coating of protein molecules on DNA with a "beads-on-string" filament structure, 22 nm wide and 4 nm high. The mean distance between beads is 20-25 nm. The width of the protein-DNA filament in the EM is 6 nm without any periodicity along the nucleoprotein fiber. Larger width of the AFM images suggests a convolution effect [41], but absence of periodicity along the filaments in these specific EM data is unclear; it is explained by the sample preparation procedure for EM (coating with cytochrome c and staining with uranyl acetate) [43]. The same group has recently reported AFM images of a complex of DNA with RecA protein [45] and found 25 nm periodicity for this complex. We have recently started studies on complexes of RecA protein with DNA as well.

The RecA protein was first identified over 25 years ago [22] as a consequence of its central role in conjugal recombination in E. coli. RecA is able to bind ssDNA and dsDNA molecules to form nucleoprotein filaments [25, 28, 95, 96, 97, 98]. Studies of RecA complexes formed with dsDNA showed that RecA polymerizes on DNA, forming very regular filaments with a 95 Å pitch and 110 Å diameter [95, 98]. Duplex DNA within these filaments is stretched to 150% of its protein-free length, since the average axial spacing between base pairs is increased from 3.4 Å to 5.1 Å [96, 98]. Studying the complexes of RecA with single-stranded DNA (ssDNA) demonstrated that ssDNA is also shaped by RecA into a stretched helix with very similar parameters to those for dsDNA-RecA complex [32, 57].

Examples of the AFM images of complexes of dsDNA (open circular plasmid DNA of ~1200 bp) with RecA protein are shown in Figures 6A, 6B, 6C and 6D. Striations are seen on both types of molecules, linear and circular. Figure 6C shows images of four rather perfect circles of very uniform sizes. The circumference of these circles is ~600 nm which is very close to the size expected for DNA completely coated with RecA (1200 x 0.51 nm = 612 nm). The perfect geometry of circular RecA-DNA is explained by the very high rigidity of RecA-DNA filaments. According to the EM data [28], the persistence length P of the complex is ~600 nm, that is non-strained circles formed by this type of filament should have a circumference ~2P, at least, i.e., 1200 nm.

AFM image of circular molecules in Figure 6A over a smaller region to visualize blob-like features is shown in Figure 6B. The width of filaments (20-25 nm) is consistent with the expected values because of a convolution process during imaging with tips with radius of curvature equal to ~10-20 nm [19, 20, 45, 55]. The tip convolution effect decreases the resolution of the AFM images, but this effect only cannot result in the observed 20-30 nm periodicity along the linear and circular RecA-DNA complexes on the AFM images instead of ~10 nm periodicity expected for the RecA-DNA filament found by EM [96, 98] and STM [6] studies of metal-coated samples. This discrepancy between AFM and other methods can be attributed to effects of the AFM sample preparation procedure (see Discussion with Reviewers for more details), although formation of a higher order periodic structure for the RecA-DNA filaments suggested by S. Kowalczykowski (private communication) cannot be excluded.
AFM of Nucleoproteins

Figure 6. AFM images of complexes of DNA with RecA complex. The samples were deposited onto AP-mica. A 30 µl droplet of the complex prepared in the RecA-binding buffer [98] were applied to AP-mica, rinsed after 5 minutes deposition with water thoroughly and dried with nitrogen. Images were obtained in air. The linear strand in image A may be a linear DNA (contamination of sample of plasmid DNA with bacterial chromosome) coated with RecA or self-polymerized RecA filaments. It is difficult to distinguish between the two possibilities on these images. The images were obtained in air using NanoScope II microscope (Digital Instruments, CA).

Complex of dsRNA with dsRNA-specific protein

Interaction of double stranded RNA (dsRNA) with proteins is an important event in the host defense against viral infection. dsRNA is produced during most viral infections and acts as an inducer for synthesis of interferon [65]. Interferon induces the antiviral state of the cell starting a cascade of phosphorylation events. The protein kinase PKR is one of the key proteins in this chain, and its activation requires binding to dsRNA [52]. A number of viruses, including adenovirus, influenza virus, human immunodeficiency virus type 1, reovirus and vaccinia virus [51] produce inhibitors of PKR blocking
Figure 7. AFM images of complexes of dsRNA with p25 protein (A, B and D) with different protein-RNA ratio and pure dsRNA (C). Protein concentration was 0.01 µg/ml for samples in (A) and (B) and 1 µg/ml for the sample in (D). Complexes or pure RNA samples were prepared at 0°C in the HEPES buffer solution (20 mM) containing 5 mM Mg acetate, 10% glycerol, 1 mM benzamidine, 1 mM dithiotreitol (DTT), 150 mM KCl, 0.5% nonidet-40, 6 mM ethylenediaminetetraacetic acid (EDTA). For details of specimen preparation, see [79]. The strips of AP-mica were immersed into the solution incubated at 0°C allowing the molecule to self-adsorb to the surface. After 30-40 minutes, the strips were removed from solution, rinse thoroughly with deionized water and vacuum dried. Images were obtained in air using NanoScope II microscope. p25-dsRNA complexes (thick filaments) are indicated with arrows.

the antiviral state of the cell induced by the interferon. Inmani and Jacobs [51] demonstrated that in case of reovirus, a dsRNA binding protein, σ3, inhibits PKR by competing for activator dsRNA. It was shown that the vaccinia virus inhibitor of PKR is a protein with MW = 25 kD (p25) which is characterized by its unusual bind-
ing specificity to dsRNA [21]. The p25 protein remains bound to dsRNA immobilized on the column in the presence of competing double-stranded DNA and single stranded RNA and DNA [107]. The dsRNA binding sites of p25 and the PKR inhibitory activity are located at the carboxyl terminus of the protein [21].

We performed AFM studies of p25-dsRNA complexes in order to get some characteristics of the complex formation. Freshly prepared complexes were deposited onto AP-mica at the conditions of high stability of the complex and after rinsing and drying, the samples were imaged with AFM without any additional chemical treatment of the sample. Typical AFM images of p25-dsRNA complexes prepared at very low protein concentration (0.01 µg/ml) are shown in Figure 7A (a low magnification scan over 5.5 µm area). Higher magnification image of the same sample (~1.2 µm) is shown in Figure 7B. Thin filaments of different lengths that are typical AFM images of dsRNA extracted from reovirus are seen clearly (this virus contains a segmented genome with 11 dsRNA molecules of different lengths [77, 79]). However, in addition to these thin filaments, these specimens contain bright short and thick filaments or even toroids indicated in Figures 7A and 7B by arrows. The AFM image of control pure dsRNA sample, shown in Figure 7C, does not contain these thick features that we believed are complexes of dsRNA with p25 protein. An AFM image of the sample prepared with higher concentration of the protein (1 µg/ml) is shown in Figure 7D. Thin dsRNA filaments are still present, but in a small number, and the major components of this sample are rather big aggregates.

So, we conclude that p25, when binding to dsRNA, stimulates the formation of aggregates. Moreover, the aggregation takes place at very low concentration of the protein. Results in Figure 7 indicate that thick filaments are present if the concentration of the protein is 0.01 µg/ml. The concentration of RNA in the binding experiments is 1 µg/ml. The molecular weight of the protein is 25,000, hence the molar protein/RNA ratio is one protein molecule per dsRNA molecule of 3.8 kilobase pairs (kb) in length. Extracted reovirus RNA is in a segmented form [79], so that the fragments of dsRNA fall into three classes centered around 1.3 kb, 2.1 kb and 3.7 kb with a mean size of RNA molecules of 2.4 kb [79]. Hence, in this specific sample, the mean protein/RNA ratio corresponds to roughly three molecules of the protein per two dsRNA molecules of the mean size. The appearance of thick filaments in the AFM images, even for this very diluted sample, led us to assume a very high cooperativity for p25 binding to the dsRNA template when the protein molecules prefer binding to the complex already formed instead of binding to isolated dsRNA.

![Figure 8. AFM images of the p25-dsRNA complex obtained in propanol. For the sample preparation procedure, see legend of Figure 7. The protein-dsRNA complexes are indicated with arrows.](image)

We also attempted to get additional information about the structure of p25-dsRNA complexes using the AFM at conditions that would make higher resolution possible. Images shown in Figure 7 were obtained in air when predominantly capillary effects limit the resolution at the 10 nm range at best for DNA or RNA molecules deposited onto mica [19, 20, 39, 79]. AFM imaging in liquid is a straightforward way to avoid undesirable capillary effects [39, 80, 81]. We performed imaging in propanol, thus, decreasing adhesive forces down to 1 nN [39] and improving the resolution of the AFM images. An example of high resolution AFM images of p25-dsRNA complexes is shown in Figure 8. This image shows two types of complexes. A toroid-like structure attached to a thin filament is on the left (marked 1). Molecule marked 2 is a filament with a very dense particle attached. Note, that in both molecules the width of the filaments is 3-4 nm, which is very close to the 2 nm diameter of non-hydrated double helix. So, we believe that the filaments are non-complexed parts of dsRNA molecules, and dense particles attached to their ends are p25-RNA complexes of different types. The particle marked 2 is very tall, and the probe scratches it during scanning (see streaks above the particle). Note, in these examples, molecules consist of two parts, pure RNA and the compact complex, which is a direct indication of a cooperative nature of the binding of p25 protein to dsRNA.
As we mentioned above, the biological effect of p25 protein is to inhibit the interferon-induced antiviral state of the cell by blocking the activation of dsRNA-dependent protein kinase pathway. The AFM studies revealed cooperative binding of p25 protein to dsRNA and formation of very dense nucleoprotein particles. We speculate that condensation of RNA and formation of RNA-p25 particles prevents accessibility of dsRNA to PKR, and hence, may halt the dsRNA-dependent interferon induction. Further experiments, which may help reveal more details of the structure of the p25-RNA complex and mechanisms of their formation, are in progress.

Imaging of bacterial and plant viruses

The majority of bacterial viruses (bacteriophages) and plant viruses are build rather simply: they are nucleic acid coated with a protein shell. From that point of view, they are nucleoprotein complexes. There are two examples of imaging of these viruses with AFM: (1) the tobacco mosaic virus (TMV), the single genomic RNA of which is coated with a structural protein [67]; and (2) TMV particles deposited onto mica treated with spermine reported by Vesenka et al. [106] (the AFM images were very similar to EM images obtained in parallel in this paper). Both types of images show that the virus particles have a shape of very straight rods of ~300 nm in the length. Contrast of the AFM images for the TMV particles is very high, although the height of images varies depending on the loading force and the tip sharpness. Both characteristics of these particles, straight stick-like shape and high contrast, are important features that make it attractive to use this sample as an internal standard for length measurements with AFM.

We imaged another type of viruses, bacteriophage fd deposited onto AP-mica [81]. According to EM data [34], fd phage is a rather flexible filament of 880 ± 30 nm in length and 6 nm wide with a single-stranded DNA inside the filament. These particles are stable between pH 12 and 3.5 and resistant to heating up to 70°C (K. Dunker, personal communication). The surface of the phage is highly negative at pH 7, the feature which allows us to use positively charged AP-mica as an AFM substrate for deposition of the sample. AFM images of fd phage taken in air are shown in Figure 9A. The contour length of the phage measured in air is 883 ± 33 nm, which is exactly the same as determined with EM (see above). These particles bind to the AP-mica surface very strongly, so that images under water can be obtained routinely (Fig. 9B). Moreover, binding of these particles to AP-mica is so strong that images of phage filaments in situ were obtained [81]. In these experiments, real-time attachment of the phage to the surface was observed by injecting a solution of phage particles into the AFM fluid cell and making periodic scans.
Figure 10. AFM images of circular plasmid DNA (Bluescript II SK (+)) deposited onto AP-mica by electrophoresis. The DNA sample containing mixture of molecules with different topology (open circular molecules, covalently closed relaxed molecules and supercoiled molecules with different number of supercoils) was subjected to electrophoresis at conditions allowing the separation of relaxed molecules from supercoiled ones. AP-mica strips were inserted into the gel behind the corresponding bands, and the samples from the bands were transferred onto the mica surface for 2-5 minutes electrophoresis. AFM images are of relaxed DNA (A) and of the supercoiled DNA molecule (B). Imaging was performed in air in tapping mode with NanoScope III microscope. Micrographs courtesy of I. Revenko, UCSB.

of the surface thereafter. These experiments allowed us for the first time to measure the length of the phage particles in solution. We have found that the shrinkage effect of the phage particles, if it exists, is less than 10%.

AFM Studies of Nucleoprotein Complexes and Sample Preparation Procedures

The development of sample preparation procedures is a crucial point for any type of microscopy. As soon as progress was made in the sample preparation procedures for AFM studies of DNA, these methods were applied to studies of nucleic protein complexes as well. However, one should be careful in choosing the method of sample preparation where such fragile samples as nucleoprotein complexes are concerned. The majority of complexes of DNA or RNA with proteins are stable in a rather narrow range of environmental conditions, including pH, salt concentration and temperature. The problem becomes more difficult if biochemical processes are studied.

Cation-mediated deposition onto mica is one of the most popular methods used for deposition of DNA and it was successfully used in the AFM studies of nucleoprotein complexes (see the section AFM Imaging of Sequence Specific DNA-Protein Complexes). However, two features of this method should be noted. First, efficient and quantitative binding of DNA to mica requires the use of divalent cations. In its first modification [18, 41, 104], mica was treated with magnesium acetate before deposition of DNA. Later, it was shown that deposition can be done onto bare mica, but magnesium ions should be present in the solution [12, 39].

The role of magnesium ions in binding of negatively charged DNA molecules to a negatively charged mica surface remains unclear. It was suggested in earlier works [18, 42, 104] that magnesium ions substitute for potassium ions in the mica surface, thus, creating a positively charged surface. This hypothesis has been recently disproved directly by use of electron spectroscopy measurements of surface composition [89]. The authors did not find a measurable amount of magnesium ions on the surface after treatment the mica with magnesium acetate, thus, ruling out the hypothesis of mica activation through the ionic exchange mechanism. Another possible mechanism of holding DNA with divalent cations is formation of bridges between the DNA phosphate back-
bone and the negatively charged oxide group on the mica surface. However, one cannot exclude the ion exchange process at a limited number of sites at the surfaces undetectable by Auger electron spectroscopy (AES); these sites may be primarily responsible for the adsorption of DNA on the mica surface [89]. The next step, drying of the sample, may provide more contacts between DNA and mica surface through formation of Mg bridges.

Another problem with the cation-assisted deposition method is its sensitivity to the composition of DNA solution. Bezanilla et al. [12] have performed direct AFM measurements of DNA molecules bound to mica during deposition from different buffer solutions. These data show that the efficiency of DNA binding to the surface is increased by a factor of 10 if HEPES-Mg buffer is used instead of Tris-Mg-KCl buffer. The mechanism of this "buffer effect" is unclear and this problem cannot be resolved until the role of Mg ions in the DNA holding at the mica surface is understood. However, despite our poor knowledge of this mechanism, the method of sample preparation based on the use of divalent cations works well for studies of nucleic acids and provides reproducible results. The limitations described above should be kept in mind if this method is used for studies of nucleoprotein complexes. For example, AFM imaging of the process of assembly of RNA polymerase-DNA complexes was studied in HEPES buffer in the presence of 10 mM MgCl₂ [38]. Due to limitations of buffer composition, the sample preparation for AFM studies of complexes of DNA with heat-shock transcription factor required a 10-fold dilution of the prepared complex with water [113].

Studies of chromatin were performed mostly on mica treated with spermidine [1, 66, 118] or baked cover glass [1, 66]; high resolution images of chromatin were obtained if activated mica was used [66, 118]. However, Leuba et al. [66] fixed chromatin samples by glutaraldehyde. This step was necessary to avoid changing the conformation of the fibers during the abrupt reduction of salt concentration which occurs during the rinsing step.

Sample preparation methods based on the EM spreading techniques [93, 114, 115] are subjected to limitations usually applied to EM procedures [24]. From that point of view, the sample preparation procedure based on the use of functionalized AP-mica [12, 73, 77, 78, 79, 80, 81] gives a great deal of flexibility. First, the samples can be deposited onto AP-mica in a broad range of ionic conditions [77, 79]. Comparative studies of DNA-binding efficiency of AP-mica and bare mica were performed by Bezanilla et al. [12]. They show that while the number of molecules imaged with AFM on bare mica varied dramatically depending on the ionic conditions and the buffer composition, AP-mica does not show this variability. For example, number of molecules per µm² is 6.4 ± 3, if the sample was deposited from pure water; and it varies between 3.9 ± 2.8 and 5.6 ± 2.5, if the sample was deposited from different buffer solutions irrespective of presence of magnesium ions. Note that Mg (or other multivalent cations) is an indispensable component for deposition of DNA onto bare mica. Moreover, the DNA molecules deposited onto bare mica from pure water aggregate, even at low concentration. The geometry of DNA molecules deposited onto AP-mica from water is typical for individual DNA molecules adsorbed onto the substrate. Second, AP-mica has amino groups exposed on the surface which have an equilibrium constant (pK) of approximately 10.6, so AP-mica retains its binding activity in a very broad range of pH; we checked this directly [79]. Third, deposition can be performed in a rather broad range of temperatures, at least between 0 and 60°C [77, 79]. This feature allowed us to perform a study of complexes of dsRNA with p25 protein (see above): the complexes were prepared at 0°C. Owing to these almost unique features of AP-mica, we managed to image a broad variety of nucleoprotein complexes.

A very promising approach, based on use of AP-mica, has been developed in the group by Hansma et al. [46]. They deposited DNA onto AP-mica during electrophoresis in agarose gel. The sample of supercoiled DNA was run in the gel at conditions which allows one to separate topologically relaxed (predominantly open circular) molecules and twisted DNA species. Then pieces of AP-mica were inserted behind these two bands, and DNA was electrophoresed for several minutes for the DNA approaching to the substrate and deposition onto the mica surface. Two examples of AFM images of DNA molecules with different topology are shown in Figure 10: Figure 10A is an image of a relaxed DNA molecule, while Figure 10B presents an image of a supercoiled molecule (a few supercoiled turns). It is interesting that possible impurities adsorbed to the surface from the gel (seen as bright blobs at the bottom of Fig. 10A) can be washed out by thorough rinsing the samples (cf. Fig. 10B). This approach opens novel prospects for direct imaging of numerous types of nucleic acids and their complexes with ligands and proteins, in particular easy degradable RNA and RNA-protein complexes. We would like to emphasize another important feature of AP-mica: the stability of the activated substrates and the prepared samples. Chemically modified AP-mica retains its activity for several months without special precautions for the substrate storage. This allows easy transportation of substrates for sample preparation. For example, complexes of DNA with RecA protein shown in Figure 6 were obtained in the following way.
AFM of Nucleoproteins

The AP-mica was shipped to A. Stasiak (Switzerland), where the samples of RecA-DNA complexes were prepared and freshly prepared complexes were deposited onto AP-mica. These samples were then shipped back to the ASU laboratory in Arizona for the AFM imaging. A similar strategy was used in our studies of chromatin. This feature of AP-mica dramatically simplifies the AFM studies of biological samples usually complicated by fast degradation of samples during shipment. Instead of shipping an unstable sample, the stable substrate can be shipped to a biochemical laboratory, so that the sample can be prepared at the site where it was obtained. After that, dried samples can be returned to the AFM laboratory without fear of their degradation. Another important feature of samples prepared on AP-mica is that they (including the RecA-DNA complexes) are stable for many months during storage at ambient conditions without special precautions. No detectable contamination appeared on the surface after several months' storage of the samples. AFM imaging in air and water solutions does not deteriorate and contaminate the samples.

As discussed above, AFM was successfully applied to studies of the protein-induced bending effect. However, the quantitative analysis of the bending effect should be done with care. In many cases, AFM images show that DNA molecules are oriented in one specific direction. Recent AFM studies of Thundat et al. [101] show that certain orientation of DNA molecules at the time of sample preparation may result in DNA stretching. If there is a kink induced by a bound protein, it may be decreased if this joint is flexible. It is clear that the softer the joint (the bend), the stronger is the straightening effect. Hence, the data obtained by the method of sample deposition in which straightening of DNA molecules occurs, may seriously distort the inherent geometry of the DNA-protein complex and a priori cannot be used for quantitative analysis. Other methods of DNA deposition based on strong binding of molecules to the substrate, and thus, excluding or minimizing the orientation effect have to be used if direct AFM imaging is to be applied for quantitation of the protein-induced bends.

Conclusions

Despite its recent invention, atomic force microscopy is becoming routine for topographic studies of biomolecular assemblies. This progress is for two major reasons. First, development of sample preparation methods has enormously expanded the field of application of AFM to structural biological studies. The progress is tremendous. Reliable images of nucleic acids appeared only three years ago, yet procedures soon became so routine that the AFM has been applied to imaging of more complex multimolecular ensembles including nucleoprotein complexes. Secondly, introduction of non-contact and tapping mode AFM is an extremely important development for studies of soft biological materials. In particular, the introduction of tapping mode was a crucial step for improving the resolution of the AFM images for a number of types of nucleic acids and nucleoprotein complexes [19, 20], and made it possible, for the first time, to follow a number of molecular processes [11, 20, 81]. However, improvement of spatial resolution for AFM imaging of molecular adsorbates is a serious problem now. Although AFM is theoretically capable of imaging individual atoms (demonstrated for imaging of crystals), a routine nanometer lateral resolution for molecular adsorbates is still a dream for AFM researchers. In our opinion, the major problem here is two-fold and is due to first, the lack of tips with a radius of curvature in nanometer range which is almost ten times smaller than the curvature of currently available tips, and second, a rather high adhesion of commercially available tips (see Discussion with Reviewers for more details). The introduction of tips which are inert and very sharp will open prospects for high resolution imaging of biological molecules and their complexes at physiological conditions making AFM rival other high resolution methods, such as X-Rays and NMR. The capability of AFM to image molecular processes with the introduction of sharp tips will open unique prospects for direct imaging of biochemical processes at molecular resolution.

Acknowledgments

We would like to thank H. Hansma (University of California, Santa Barbara), I. Revenko (University of California, Santa Barbara), M. Allen (University of California, Davis) and A. Nikroo (General Atomics) for kindly providing us their results; S.C. Kowalczykowski for the stimulating discussion of RecA-DNA data; and L.S. Shlyakhtenko for a critical review of the manuscript. This paper was supported in part by the NIH grant 1R21HG-0081801A1, ONR grant N00014-90-J-1455 (SML, YLL), NIH grant CA48654 (BLJ), grant F-017-95 from the Office of the Vice President for Research, ASU (YLL) and grant 32-42158.94 from the Swiss National Science Foundation (AS).

References


[32]. Flory J, Tsang SS, Muniappa K (1984) Isola-
Biomolecular Stereodynamics. Sarrna RH, Sarrna MH

atomic force microscope. In: Ninth Conversation in

Cleveland JP (1995) Bending and motion of DNA in the


repressor forms loops with linear DNA carrying two suitably spaced lac operators. EMBO J. 6: 1481-1491.


AFM of Nucleoproteins

Electron spectroscopy and atomic force microscopy studies of DNA adsorption on mica. Scanning Microsc. 8: 471-480.


[118]. Zlatanova J, Leuba SH, Yang G, Bustamante
Y.L. Lyubchenko, B.L. Jacobs, S.L. Lindsay and A. Stasiak


Discussion with Reviewers

H.G. Hansma: There is a controversy in the literature about DNA bend angles in Jun-Fos complexes as measured, I think, EM and X-ray crystallography. Do you know where AFM results stand in this controversy?

Authors: Molecular modeling of complexes of these proteins with B-DNA suggest that bending of DNA helix at the recognition region provides much more contacts between DNA bases and amino acids of the protein [A2, A6]. Gel electrophoresis data of Kerppola and Curran (circular permutation and phasing analysis) and EM data of Wang and Griffith [A14]. A significant distortion of DNA helix in crystallographic experiments was found only for the complex of the fragment GCN4 of the protein of a similar type with CRE binding site [A2]. The bending angle is less than that for Fos-Jun determined by EM [A14]. However, the discrepancy between EM, electrophoresis on one side and crystallographic data on another side can be explained by a number of factors. First, typically short peptides used for X-ray experiments may lack some of the residues needed for DNA bending (there is no EM or electrophoresis data for exactly the same DNA-protein complexes). Second, crystal packing forces may resist DNA bending. Third, crystallization conditions may decrease the bend angle. For instance, recent data from the group of S. Harvey have shown that dehydrating agents used for crystallization of DNA oligonucleotides decreased dramatically intrinsic DNA curvature at the A-tracts [A12]. Becker et al. [10] found that Jun bends DNA at AP-1 site and according to these AFM data the bend angle is 36°. Although this magnitude is much less than that for heterodimer Jun-Fos (EM and electrophoresis data), but this finding is consistent with both EM and electrophoresis results.

H.G. Hansma: Thresher and Griffith [A13] have published beautiful EM images showing 8-9 nm helix periodicity for DNA complexes with various single-stranded binding proteins.

Authors: The results on p32 protein were discussed according to the paper [43] where the authors cited a rather old EM work [A1]. Many recent EM data for other ssDNA binding proteins show well-resolved periodicity. For example, the very recent high resolution EM data of Olah et al. [A9] for complexes of ssDNA with gp5 protein (which is similar to p32) even allowed the authors to built a molecular model for this type of complex.

W. Fritzsche: You suggest that tip convolution causes the difference between the observed (20-30 nm) and the literature (~10 nm) periodicity of RecA-DNA complexes. How do you explain such an effect?

Authors: The difference between the lateral periodicity for RecA-DNA filaments obtained by AFM and other methods (EM, STM, X-ray) is unclear for us. It cannot be due to the tip convolution effect only. It could be the effect of the sample preparation procedure (deposition onto mica or AP-mica surfaces followed by drying of the sample). However, it is not a simple shortening or elongation of the filament, because our measurements of sizes of circular complexes are consistent with the conventional model for the RecA-DNA filament, namely 5.1 Å per base-pair rise. We assume that the sample preparation procedure (interaction with the surface of the substrate and drying of the sample bound to the substrate) may result in re-arrangement of the protein molecules which are not in a direct contact with the substrate. The hypothesis about local mobility of RecA protein molecules is supported by our recent AFM data on imaging of the same RecA-DNA samples in propanol. We did not resolve any periodicity along the filament at all: the circular filaments were devoid of any lateral periodicity, but conserved their lengths and height.

H.G. Hansma: The author seems strongly prejudiced in favor of AP-mica and against mica. The AP-mica that the author sent to us did not bind DNA after it was 4 weeks old, in contrast to the claim of several months without special precautions. The shelf life of mica is certainly much longer than that of AP-mica. AP-mica is much more difficult to prepare than fresh-split mica; a number of good DNA-AFM laboratories have not gotten the AP-mica method to work at all, and those that do use it, find that conditions must be carefully controlled. Also, much more is known about the structure of the mica surface than that of the AP-mica surface.

Authors: The experience of storage of AP-mica is mostly based on the experiments performed at ASU, Tempe, which is situated in an area with rather low humidity (Arizona). Because the humidity is very critical for preparation of AP-mica [73], we cannot exclude that shorter life-time of the sample obtained from us was because of humidity effect. This is a very important information for us, and experiments for measurements of the life-time of AP-mica samples stored at different relative humidity are in progress. We know that AP-mica samples which were used for our experiments with RecA-DNA complexes (samples were prepared in Switzerland on the AP-mica shipped from Arizona) were good for a month at least including 2 weeks shipment by the surface mail in tubes sealed by parafilm.

The procedure of AP-mica preparation requires the
use of a rather fresh chemical (we recommend to use vacuum-distilled APTES) and treatment of mica should be performed in the atmosphere of dry gas (we recommend argon). The chemistry of APTES and similar silanes was described in many papers and even books [A11], so precautions we mentioned are explained by very high reactivity of silanes and their fast reaction with water vapors. Following these recommendations leads to almost 100% reproducibility of AP-mica preparation (L. Bottomley, private communication).

Although the structure of AP-mica is unknown, the mechanism of DNA binding is clear: predominately electrostatic interaction [77]. This effect provides quantitative, reproducible binding of DNA to the surface. The mechanism of DNA binding effect and the structure of cation-modified mica surface are still unknown. In addition, there is a limitation for use of buffer for deposition. We described advantages and disadvantages of both methods in detail in text so that every investigator may make a choice.

A. Schaper: How is this functionalization of the freshly cleaved mica surface with APTES? From other studies, it is known that the mica surface is chemically inert and has to be activated, e.g., by glow discharge, prior chemical modification [A10].

Authors: Additional treatment of the mica was used for hydrophobization of mica surface through complete coating of the surface with hydrophobic silane compounds (octamethylcyclotetrasiloxane (I) in the paper cited above). However, hydrophobic self-assembling layer of hydrolyzed octadecyltriethoxysilane (II) can be formed on non-treated mica [A7]. In both papers, similar macroscopic physical chemical methods were used for the analysis of surface characteristics, but no microscopic studies of the surface structure have been done. Formation of monolayer of (II) on non-treated mica indicates that reactive groups on bare mica do exist, but use of different silane agents may require different concentration of these hydroxyl groups and/or their availability for a chosen chemical. For example, hydrophobic chlorosilanes, which are hydrolyzed very fast, cannot form a monolayer on bare mica. The active groups on the mica surface can be formed owing to the contact with the water molecules in air during the cleavage step similar to the mechanism suggested for freshly broken glass [A3]. We developed a procedure for preparation of functionalized mica surface (positively charged surface) suitable for microscopic studies. The main goal of this procedure was to preserve the mica surface as flat as possible. We believe that concentration of AP-groups on the surface, which are formed after reaction of APTES with active groups on mica surface is sufficient for preparation of positively charged surface which holds strongly DNA molecules as small as hundreds base pairs. Note that use of APTES in high concentration (close to that used in the paper of Parker et al. [A10]) result in formation of a very rough surface, unsuitable for microscopic studies [73, 77].

Z. Shao: The authors showed that for fd phage, shrinkage due to drying was very small, contrary to what is expected. Can the authors offer some explanation? Is it possible that the substrate prevented such shrinkage? Authors: The absence of shrinkage is also valid for double stranded DNA molecules as well. Our measurements of the length of lambda phage DNA molecules are consistent with B-form DNA geometry bound to AP-mica. Similar results were obtained by M. Allen (private communication) who measured the length of a circular DNA plasmid. The strong interaction of the molecules electrostatically bound to the substrate may be one reason. Another explanation is based on rather unusual characteristics of mica and glass surfaces. They retain a water film at the surface which persists in vacuum, requiring heating to remove it [A3]. The formation of water layers on mica surface has been studied recently by Hu et al. [A5]. The presence of water layers on mica surfaces may prevent dehydration of samples and help them retain their geometry in hydrated state.

Z. Shao: The authors stated that when the sample was prepared with AP-mica, it does not absorb any contaminants for months. What are the possible reasons for such a phenomenon? In fact, as we found, supported bilayers in solution could accumulate surface contaminants over time.

Authors: We do not have a firm explanation of this unexpected feature of AP-mica, but suggest that it may be also explained by formation of water layer on the mica surface mentioned above. According to Hu et al. [A5], formation of water film is a reversible process, and film is detected with AFM at rather low humidity. The AFM tip easily penetrates through this film, so images of mica lattice can be obtained. The formation of a penetrable film above the AP-mica surface was observed by J. Graham (Park Scientific Instruments, CA, private communication), when the samples of dsRNA molecules deposited onto AP-mica were studied by a non-contact mode AFM. The formation of a such "liquid" film above the sample prevents its contamination and does not require specific precautions for storage of the sample. It is interesting to note that coating the sample in propanol does not result in accumulation of contaminant...
after imaging of the sample in propanol.

**H.G. Hansma:** It is stated that samples on mica were fixed with glutaraldehyde to avoid conformation changes induced by water rinses. Water rinses are of course necessary with AP-mica, too.

**Authors:** Glutaraldehyde was used to prevent chromatin decondensation after deposition of the sample to the substrate (spermidine-treated mica is the best substrate for this specific sample). Whether or not the glutaraldehyde treatment is needed if AP-mica is used as a substrate for chromatin, we cannot give an answer. We did not study the chromatin structure in details, although our early preliminary data showed AP-mica binds chromatin (extracted from chicken erythrocytes) very strongly.

**H.G. Hansma:** What is the sensitivity of the AES detection of Mg? Some DNA can bind to mica even in the absence of Mg, so the AES evidence does not seem convincing. Sample preps on mica typically use DNA in Mg-buffers, where Mg is clearly present.

**Authors:** Rabke et al. [89] that we cited, is the only paper where the mechanism of DNA binding to bare mica in the presence of Mg was studied. The authors claim that there is no substitution of Mg ions for K ions in the mica surface: potassium AES peak intensity remains unchanged. Mg ions were detected if DNA was deposited onto bare mica in the presence of Mg acetate. There were no reported experiments on surface analysis for the case when DNA was deposited from the solution with no Mg acetate in the buffer, but it is well known that the reproducibility of this method of sample preparation is very poor with very low DNA concentration on the surface [11]. The ability of bare mica to bind DNA in solution containing no Mg ions added may be explained by the presence of divalent cations bound to the DNA molecules. Ca and Mg cations usually present in water in a small amount, and they are trapped by DNA molecules during storage [A8]. At the same time, chelating of divalent cations by EDTA prevents binding of DNA to bare mica [12].

**Z. Shao:** Since height measurement in AFM is very inaccurate, which has been documented by many groups, including the authors’ group, structural inference based on height measurements (topology) can be ambiguous and unreliable. Local elastic property could be very different which can lead to wrong conclusions, if not carefully examined under various conditions. Please comment on this important point.

**Authors:** The height measurements are crucial for topographic AFM studies of any type of surfaces. Local elastic property of samples is one problem for biological AFM studies. Introduction of AFM instruments operating in tapping mode allows one to decrease the undesirable effect of the scanning tip on the sample. Freezing the samples followed by AFM imaging at low temperatures is another possibility in avoiding the deformation of the sample. An important contribution in developing reliable instrument capable to operate at low temperature has been recently made in the reviewers’ laboratory [A4]. In our opinion, there is another problem in correct interpretation of the height measurements, namely local interaction of the tip with the surface. The AFM measures not topography, but local forces (repulsion and adhesion) which depends on the local chemistry of the surface and the tip. Strong adhesion of the tip to one spot of the surface in comparison with repulsion with another part of the surface would be recorded by the AFM as a bump in the first case and a hole in another case although both features may belong to completely flat parts of the surface. This artifact of AFM imaging has been recently demonstrated directly by Zenhausern et al. [A15]. Therefore, development of neutral tips is another problem for topographic AFM studies. From this point of view, all commercially available AFM tips (which are made of silicon or silicon nitride and hence are far from being neutral) should be replaced by tips that are much less reactive. Unfortunately, this important area of AFM instrumentation has not been developed yet and requires very intensive studies. Introduction of carbonized tips [55] is one possibility; this allowed several groups to get high quality AFM images [19, 20, 39], but this invention has been not commercialized so far. In addition, adhesion characteristics of these tips, which are presumably more inert than silicon or silicon nitride materials, needs careful studies. Introduction of carbon nanotubes is another extremely promising approach. The inert character of the material is one advantage of nanotubes as a potential candidate for a novel type of AFM tips.

**A. Schaper:** What is the authors’ opinion about the perspectives of stable AFM imaging of nucleoprotein complexes in water/buffer and is this goal already achievable with (one of) the preparation methods presented in this review?

**Authors:** We have already published images of fd bacteriophage (which is a nucleoprotein complex) in water and in Tris-buffer solution deposited onto AP-mica [81]. Even images of fd phage in situ have been obtained (AP-mica was the substrate). It is also possible to image RecA-DNA complexes deposited onto AP-mica in water as well (our unpublished data). A capability of Mg-assisted binding technique for imaging complexes of RNA-polymerase with DNA in situ (HEPES buffer) has been demonstrated by Guthold et al. [38]. Thus, both methods can be used for AFM imaging of protein-DNA com-
plexes in solutions, but details of the sample preparation procedure are probably dependent on specific characteristics of the system.

A. Schaper: In Introduction, the authors make the statement that AFM has the potential of atomic resolution “provided the specimen is dynamically stable”. What is the meaning of “dynamic stability”?

Authors: This means low thermal mobility of atoms of the specimen in comparison with their interatomic distance.

Additional References


AFM of Nucleoproteins


