ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 10, pp. 1150-1154. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © D. V. Klinov, T. V. Neretina, V. V. Prokhorov, T. V. Dobrynina, K. G. Aldarov, V. V. Demin, 2009, published in Biokhimiya, 2009, Vol. 74, No. 10, pp. 1410-1415.

High-Resolution Atomic Force Microscopy of DNA

D. V. Klinov*, T. V. Neretina, V. V. Prokhorov, T. V. Dobrynina, K. G. Aldarov, and V. V. Demin

Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia; E-mail: klinov@ibch.ru; nertata1@yandex.ru

Received September 30, 2008
Revision received November 16, 2008

Abstract—A method using high resolution atomic force microscopy for imaging DNA has been elaborated. Using super-sharp probes and modified graphite as support for molecule adsorption, DNA molecule images were obtained whose resolution made possible the observation of their fine structure with repeated helical motifs. The method can be used to visualize individual spread molecules of single-stranded DNA.

DOI: 10.1134/S0006297909100113

Key words: AFM, high resolution, DNA, scanning probes, HOPG

Atomic force microscopy (AFM) has recently become a routine tool for investigations of biopolymers. The range of studied objects is very broad: from cells and viral particles to single protein and DNA molecules [1]. Thus, AFM is actively used for structural investigations of nucleic acid molecules and their complexes with proteins. For example, the AFM technique was used to study formation of *Escherichia coli* RNA polymerase complexes with DNA containing the λP_L promoter [2]. The possibility of visualizing molecular processes by AFM was shown in [3] by step-by-step analysis of the formation in real time of nonspecific DNA and RNA polymerase complexes. The application of AFM for physical DNA mapping has been described. To do this, specific regions of the macromolecule were labeled by certain markers, and analysis of their positions made it possible to obtain maps of relative location of specific sequences in the studied molecules. The ability to visualize the biotin–streptavidin complex was used to detect the position of biotin covalently bound to the first nucleotide of the oligonucleotide [4]. Earlier we elaborated and successfully used a method for mapping LTR (long terminal repeat) sequences, cloned in a cosmid vector, using specific markers (R loops) having a characteristic shape [5, 6]. The results suggest that improvement of resolution will make possible base sequence determination by AFM. Thus, in our work [7] the spatial resolution of 0.5-0.6 nm was achieved, and single defects in a polymer crystal lattice were seen.

Another interesting application of AFM in structural investigation of DNA is observation of secondary and tertiary structures of single-stranded DNA (ssDNA) and double helix melting "loops", but commercially available AFM probes do not provide sufficient resolution.

Further development of this method suggests significant enhancement of spatial resolution. The key parameter that defines resolution of this method is sharpness of the probe used for scanning. Since standard probes have point radius of curvature of about 10 nm, the measured width of single DNA molecules observed in AFM also is about 10 nm (it is known that the DNA width determined by crystallography is about 2 nm [6]). We have recently elaborated super-sharp probes (point radius of curvature about 1 nm). Application of such probes for studies of an acetylene crystal surface makes possible AFM imaging with resolution of 5-7 Å [7], and in this case individual defects in the crystal lattice can be differentiated.

In addition to spatial resolution, an important factor limiting the application of AFM for studying macromolecules is the technique used for sample preparation [8]. On one side, the biopolymer molecule should be fixed on a surface, and on the other side, surface forces emerging upon adsorption may destroy native structure of the molecule [9]. It is necessary to select a support with minimal surface forces to obtain reproducible results.

Abbreviations: AFM, atomic-force microscopy; HOPG, highly oriented pyrolitic graphite; ssDNA, single-stranded DNA.

* To whom correspondence should be addressed.

Approaches making it possible to obtain DNA images with high resolution are described in this work.

MATERIALS AND METHODS

DNA preparations. DNA preparations of Sigma (USA), in particular plasmid pSK+ 2997 and DNA of bacteriophage M13 (mp18)7300, were used in this work.

Production of probes. Diamond-like needles were grown under conditions of low-temperature plasma at 13 MHz in an atmosphere of $CH_4/H_2O/Ar$ at the ratio of 10 : 5 : 85 and pressure in the reactor of 0.1 torr. A method for obtaining similar probes was described earlier [6]. Images of grown probes were obtained using a JEOL 100CX electron microscope (Japan) [7].

Support modification. Forty microliters of graphite modifying solution "GM" (Nanotuning (http://www.nanotuning.com), Russia) was applied on the surface of freshly cleaved highly oriented pyrolitic graphite (HOPG). After incubation for 10 min, the drop was blown away with compressed argon according to our previous technique [8].

Sample preparation technique. A drop of DNA solution with DNA concentration 1 μg/ml was applied onto the HOPG surface treated as described. After 5-min incubation, the drop was blown away with compressed argon.

To prepare samples of DNA adsorbed on mica surface, 10 μl of DNA solution in 1 mM CH_3COONH_4 and 5 mM $MgCl_2$ was applied onto the fresh mica chip. After adsorption for 5 min, the surface was washed with water and dried by blowing with compressed argon.

DNA visualization. A Solver P-47 device of NT-MDT Company (Russia) working in the resonance regime tapping mode was used. Scanning rate was 0.5-1.0 Hz, and amplitude of free oscillations was 2-4 nm.

RESULTS AND DISCUSSION

As mentioned above, the resolution of AFM depends on the sharpness of the scanning probe. The typical point radius of curvature of the sharpest commercially available probe (standard Micromash NSC 15 cantilevers with radius of curvature 10 nm) is about 5-10 nm, which is limiting for silicon probes because in the air the silicon surface is covered by a 4-6 nm oxide film. Attempts to grow carbon nanotubes or glue them to the probe point did not produce reproducible results because it is difficult to monitor the length of nanotubes attached to the probe point. Owing to the high length/diameter ratio, transverse oscillations emerge that significantly decrease resolution when they are used. The sharpest probes with carbon nanotubes described in [10] have radius of curvature about 4 nm. We have recently obtained super-sharp probes by growing needles of diamond-like carbon on the point of commercially available silicon probes. The radius of curvature of such needles is about 1 nm (Fig. 1) [7].

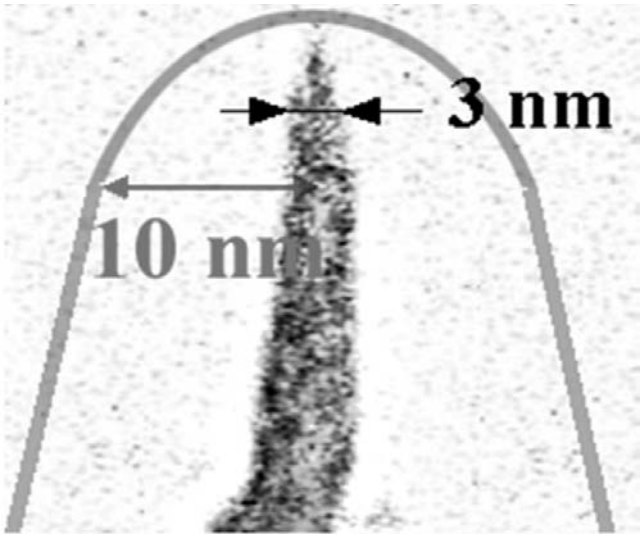

Fig. 1. Image of a super-sharp diamond-like AFM probe with radius of curvature 1 nm obtained in a transmission electron microscope. The gray line shows the shape of a standard silicon probe with radius of curvature 10 nm.

DNA molecules were studied by the AFM technique using a super-sharp diamond-like needle grown on the point of a silicon probe (Fig. 1). Figure 2 shows AFM images of dsDNA adsorbed on a mica surface obtained by scanning using standard commercially available probes (Fig. 2a) and AFM images obtained using super-sharp probes (Fig. 2b). Scanning with a commercially available probe shows a smooth and homogeneous molecule surface. The molecule width measured by AFM is 7-10 nm. Scanning with a super-sharp probe and measurement by AFM shows a heterogeneous molecule of 4.5-5.0 nm in width and makes it possible to visualize internal structure of the molecule. High-resolution AFM images of DNA show the existence of super small zigzag-like defects (up to 5 nm) on adsorbed molecules that could not be detected by the usual probes.

The radius of curvature of super-sharp probes is several times smaller than that of standard commercially available silicon probes. This makes it possible to decrease the width of the image of DNA molecules from 10 to 3 nm when modified graphite is used as a support, which is rather close to its natural diameter of 2 nm (Fig. 3). It is possible to visualize the helical period on DNA molecules when the internal structure of DNA applied on modified graphite is observed. Images of single DNA molecules with such resolution were obtained for the first time. High-resolution AFM images of DNA were obtained [11] on which motifs corresponding to the DNA helical structure could be observed. However, these observations were carried out on liquid crystals in aqueous medium. In our

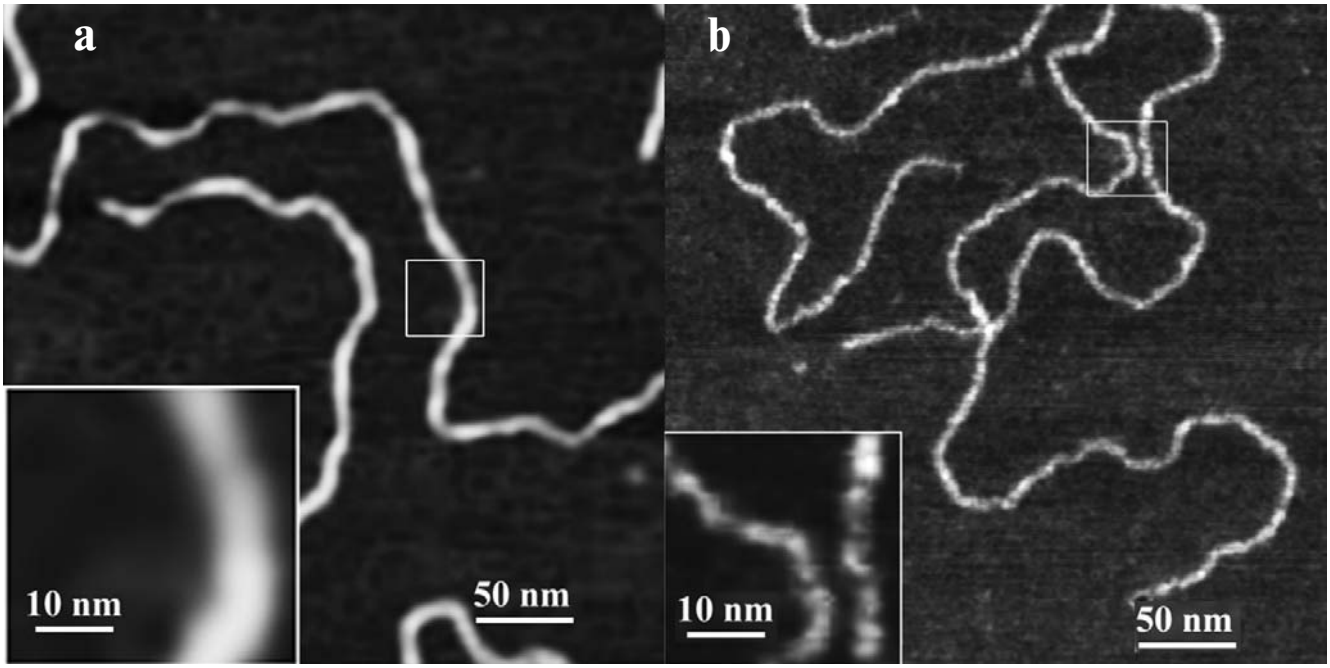

Fig. 2. AFM images of dsDNA adsorbed on a mica surface obtained by scanning using standard commercially available probes (a) and AFM images obtained using super-sharp probes (b).

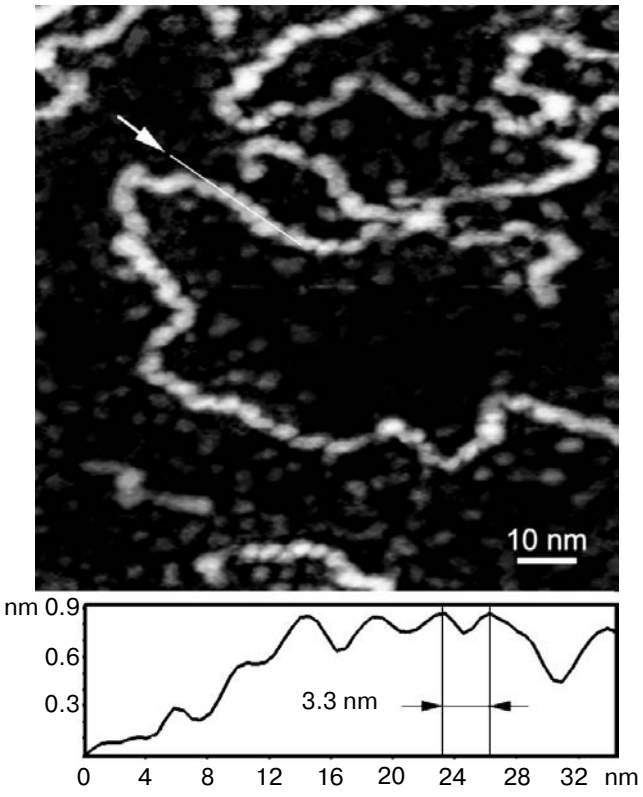

Fig. 3. AFM image of dsDNA adsorbed on graphite obtained by scanning using a super-sharp probe. A cross section along the line, indicated by the arrow on the image, is shown below. Periodicity along the AFM image of the molecule corresponds to B-form of DNA (about 3.3 nm).

case, similar resolution was obtained on individual molecules (not on crystals). Visualization of DNA molecules with such resolution and with the possibility to observe the double helix period is shown in this work for the first time.

A principal consideration in investigation of DNA and other biopolymers by AFM is the method of sample preparation and application. Initially molecules exist in aqueous solution, but for further investigation it is necessary to have dried molecules on the support surface. It is known that surface forces emerging upon adsorption and drying can significantly deteriorate the structure of DNA molecules. We have also shown that changes in the extent of surface hydrophilicity can lower the unfavorable effect of surface forces and produce dried DNA molecules with minimal distortion of their structure (in terms of molecule height measured by AFM) [7]. The standard support for biopolymer sample application is mica, but HOPG, having lamellar structure and producing an atomically even layer upon shearing, was used as well. Unlike mica, the latter is hydrophobic and thus cannot adsorb DNA. Previously, plasmochemical graphite modification was used [12]. Images of DNA molecules adsorbed on the surface of graphite modified by the commercially available modifier GM (http://www.nanotuning.com) were used in this work (Fig. 3). GM is an aqueous solution of polymers having hydrophilic (amino groups) and hydrophobic (carbohydrate) parts. When applied on a graphite surface, it forms a 0.5-0.7 nm thick film due to interaction of the polymer hydrophobic regions with the graphite surface, whereas the hydrophilic regions are ori-

AFM-measured width (nm) of DNA molecules adsorbed on mica and graphite

	Mica	Graphite
dsDNA		
Usual probes	7-12	6-10
Super-sharp probes	4.5-5	2.7-4.5
ssDNA		
Usual probes	–	5-7
Super-sharp probes	–	1.5-3

ented towards the aqueous solution. The amino groups provide positive charge of the surface, which is favorable for DNA adsorption. Upon adsorption on the support of so modified graphite, structural distortion is less pronounced. The mean height of molecules applied on mica and measured in AFM is 0.4-0.6 nm, while in the case of adsorption on graphite it is 1.2 nm. Thus, it is shown that the observed DNA molecules are narrower and higher on graphite (parameters are closer to normal DNA dimensions) and more spread on mica. Probably drying results in water film formation on the mica surface, which degrades the high resolution AFM image. The strength of interaction between the probe and mica surface exceeds by one order of magnitude the strength of interaction with graphite surface (unpublished data of the authors).

With the same probe, the measured width at the half-height of molecules adsorbed on mica exceeds that on graphite by 20% (table). Typical cross sections are shown in Fig. 4. When measured in AFM, the width of DNA molecules adsorbed on graphite is smaller (at larger height) than that of molecules adsorbed on mica, which is indicative of less pronounced molecule spreading on graphite due to less pronounced distortion of DNA secondary structure. For clear comparison of the width, the height of a DNA cross section on graphite is reduced to the DNA molecule height on mica.

As shown above, when a high-resolution probe is used, DNA molecules adsorbed both on mica and on graphite are not seen as smooth; rather, their fine structure becomes visible as heterogeneity along the molecule, while a significant part of the molecule has no such motifs when adsorbed on mica, which indicates its spreading (table).

The use of modified HOPG as a support made it possible to visualize ssDNA molecules (Fig. 5). The point is that in the case of application onto mica in the presence of Mg^{2+}, ssDNA molecules undergo condensation and form complex secondary structures, which makes it impossible to study expanded molecules, in particular measurement of their size. In solution the mica surface has negative charge owing to which adsorption of negatively charged DNA molecules requires the presence in solution of Mg^{2+} ions. When modified HOPG is used as support, the presence of Mg^{2+} is not required, and therefore the extent of ssDNA compactness is significantly lower; this makes it possible to observe individual fully expanded ssDNA molecules, and these data are shown in [8]. The use of this tech-

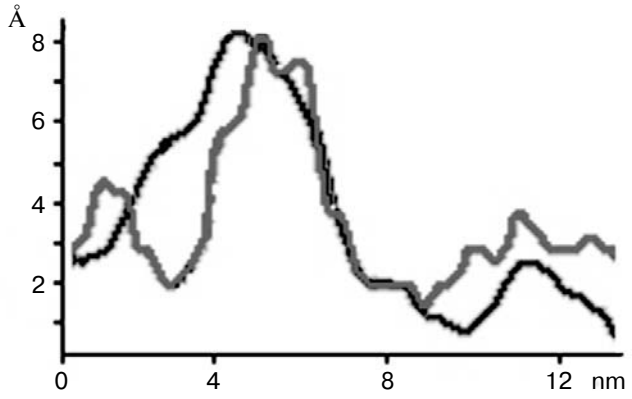

Fig. 4. Typical cross sections of dsDNA AFM images on mica (wider section) and graphite (narrower section). DNA spreading upon interaction with mica and subsequent drying is implied by the widening of the DNA AFM image on mica.

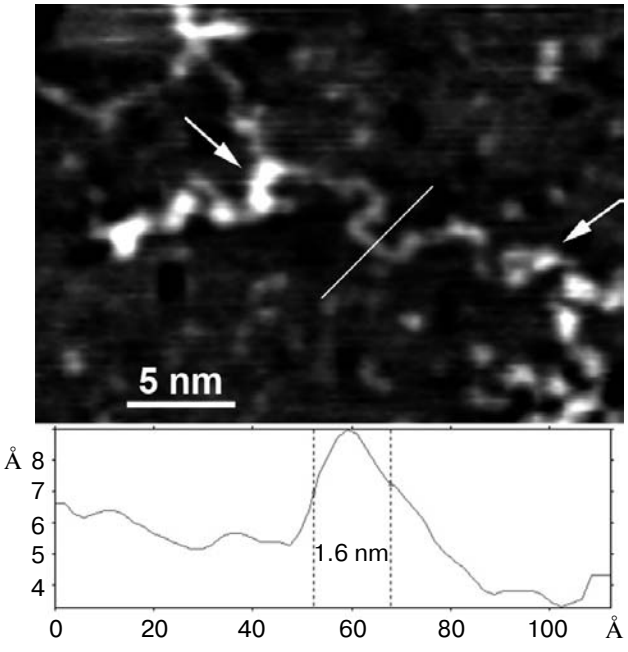

Fig. 5. An AFM image of ssDNA adsorbed on graphite, obtained by scanning using a super-sharp probe. A cross section along the line indicated on the image is shown below. Arrows point to double-stranded regions on the DNA.

nique for investigation of triplexes in liquid is also described in the same work.

We have obtained images of single-stranded molecules with individual double-stranded regions formed due to hybridization of internal parts of the molecule (Fig. 5). It is important that it is impossible to observe on mica uncondensed ssDNA.

The possibility to obtain such images is important for studying secondary structures formed by ssDNA and RNA molecules. The ssDNA width and height were measured and compared with double-stranded molecules (table). The width of single-stranded regions was about 1.5 nm, which is almost half of that of dsDNA molecules measured by the same method, which makes it possible to distinguish unambiguously single- and double-stranded structures. The possibility of visualizing single-stranded molecules and adequately measure their size can be used for studying processes of replication and transcription.

The developed technique made it possible to significantly enhance AFM resolution, which expands the possibilities for application of AFM for studies of biomolecules. The use of modified HOPG and super-sharp probes enabled observation of secondary structure of individual DNA molecules and distinguishing single- and double-stranded DNA regions, which opens the ways for application of AFM for studying processes of replication and transcription as well as mechanisms of enzymic catalysis of these processes [12].

REFERENCES

1. Uvarov, V. Yu., Ivanov, Yu. D., Romanov, A. N., Gallyamov, M. O., Kiselyova, O. I., and Yaminsky, I. V. (1996) *Biochimie*, **78**, 780-784.
2. Rees, W. A., Keller, R. W., Vesenka, J. P., Yang, G., and Bustamante, C. (1993) *Science*, **260**, 1646-1649.
3. Guthold, M., Bezanilla, M., Erie, D. A., Jenkins, B., Hansma, H. G., and Bustamante, C. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 12927-12931.
4. Murray, N., Hansma, H. G., Bezanilla, M., Sano, T., Ogletree, D. F., Kolbe, W., Smith, C. L., Cantor, C. R., Spengler, S., Hansma, P. K., and Salmeron, M. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 3811-3814.
5. Klinov, D. V. (1997) *Abstract of Candidate's Dissertation* [in Russian], Moscow Physico-Technical Institute, Moscow.
6. Klinov, D. V., Lagutina, I. V., Prokhorov, V. V., Neretina, T. V., Khil, P. P., Lebedev, Yu. B., Cherny, D. I., Demin, V. V., and Sverdlov, E. D. (1998) *Nucleic Acids Res.*, **26**, 4603-610.
7. Klinov, D., and Magonov, S. (2004) *Appl. Phys. Lett.*, **84**, 2697-2699.
8. Adamcik, J., Klinov, D. V., Witz, G., Sekatskii, S. K., and Dietler, G. (2006) *FEBS Lett.*, **580**, 5671-5675.
9. Klinov, D. V., Martynkina, L. P., Yurchenko, V. Yu., Demin, V. V., Streltsov, S. A., Gerasimov, Yu. A., and Vengerov, Yu. Yu. (2003) *Bioorg. Khim.*, **29**, 363-367.
10. Cheung, C. L., Hafner, J. H., and Lieber, C. M. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 3809-3813.
11. Mou, J., Czajkowsky, D. M., Zhang, Y., and Shao, Z. (1995) *FEBS Lett.*, **371**, 279-282.
12. Klinov, D., Dwir, B., Kapon, E., Borovok, N., Molotsky, T., and Kotlyar, A. (2007) *Nanotechnology*, **18**, 202-212.