High-resolution atomic-force microscopy of DNA: the pitch of the double helix

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Received 28 June 1995; revised version received 25 July 1995

Abstract Using a cationic lipid bilayer, we show that DNA can be reliably adsorbed to the bilayer surface for atomic force microscopy (AFM) in aqueous buffers at high resolution. The measured width of the dsDNA is close to 2 nm, and a periodic modulation on dsDNA is reproducibly detected by the AFM. The measured period is 3.4 ± 0.4 nm, in excellent agreement with the known pitch of the double helix. The right-handedness of the double helix is directly discernible in high resolution AFM images. Thus, this approach can be readily applied to the study of DNA-protein interactions, as well as sequence mapping at high resolution.

Key words: Atomic force microscopy (AFM); DNA; Double helix; Resolution; Bilayer

1. Introduction

One of the primary interests in atomic force microscopy (AFM) in biology has been the potential of high resolution imaging [1–6], and this potential was recently demonstrated with several protein specimens at 1–2 nm resolution [7–10]. However, even with significant instrumental developments, such as the tapping mode AFM [11], specially fabricated super sharp tips [12] and cryo-AFM [13], the resolution of DNA [14–19] has not been sufficient to even resolve the pitch of the double helix in aqueous media. Yet the prospect of 'reading' the sequence directly off a fragment of DNA is indeed of great interest [1–3] (for a more extensive summary of the background, see [1]), as is the capability to observe DNA-protein interactions in solution [13–20]. Although many arguments have been proposed to account for this unexpected low resolution [9,10], we believe that the main limitation at present is not technological, but technical: the ultimate resolving power of AFM has not been realized because methods for preparing high quality specimens have not been developed. In this paper, we report the use of cationic lipid bilayers as the substrate to anchor the DNA with high affinity. The uniformity of the substrate and the strong electrostatic interaction between the cationic lipid headgroups and the negatively charged phosphate groups in the DNA backbone resulted in a very stable specimen, enabling the direct resolution of the pitch and the right handed double helix under aqueous solutions at room temperature.

2. Materials and methods

Supported cationic bilayers on freshly cleaved mica surface were prepared with two methods. In the vesicle fusion method [24], small unilamellar vesicles were prepared with repeated sonification of the lipid suspension at 0.25 mg/ml in a buffer containing 20 mM NaCl or 20 mM NaCl and 5 mM NaH_2PO_4 at pH 6. Clean bilayers can be formed when a small droplet (20 μl) vesicle solution was allowed to incubate on a freshly cleaved mica surface overnight, followed by heating to 70°C for half an hour. With a Langmuir trough [25], the bilayer can be formed by two vertical transfers through the interface at a surface pressure of 35–45 mN/m. Deionized water (18 MΩ) was used for the subphase, and the lipids were dissolved in hexane/ethanol (9:1, v/v) at 1 mg/ml. The quality of the bilayer is much better with the Langmuir trough method. Both trimethyl- and dimethyl-ammonium propane (Avanti Polar Lipids, Alabaster, AL) of a hydrocarbon chain length between 14 to 18 were used and no difference was found in DNA adsorption. The quality of these bilayers was first examined by the AFM before DNA was added to the bilayer. DNA (*E. coli* plasmid of 4.36 kb [pBR322] and 6 kb [pBR325], and *Hae*III restriction fragments of ϕX174, all from Sigma, St. Louis, MO) containing solution, about 10 μl at a concentration of 20 μg/ml, either in a low ionic strength buffer (20 mM NaCl) or a buffer containing EDTA (10 mM Tris and 1 mM EDTA, pH 8), was directly added to the solution covering the cationic bilayer (to a final DNA concentration of about 1 nM). Incubation time varied between an hour to several hours. For closely packed specimens, EDTA was required (see section 3). Specimens were thoroughly washed and then imaged with the same buffer. DNA packing was further enhanced when incubated at 50°C for an hour in an EDTA containing solution. All images, with the contact mode, were obtained in a NanoScope II AFM (Digital Instruments, Santa Barbara, CA) at room temperature with a fluid cell at a scanning speed of 5–12 Hz. The cantilevers (with oxide sharpened tips) were the ones with a 0.06 N/m nominal spring constant, and the probe force was kept to the minimum (~0.1–0.2 nN). The piezo scanner was calibrated against a grid with known dimensions. All images presented are original data without filtering, and the fast scan direction is always horizontal.

3. Results and discussion

Since DNA is normally negatively charged due to its phosphate groups in the backbone, divalent cations have been used to link them to the negatively charged mica surface [14–17,26–28]. Such a method, although quite successful for DNA adsorption, was not sufficient for achieving high resolution in aqueous solution [14–17]. Even when covalently linked to the substrate [18,19], the resolution was still insufficient, despite an improved specimen stability. To achieve a stable adhesion to a substrate without covalent cross-linking, a positively charged surface should be much more effective for imaging in solution, if such a substrate is clean and well behaved under the AFM tip, i.e. flat without an adhesion force [4–6]. For this purpose, we have taken the advantage of positively charged (cationic) lipids, such as trimethyl- and dimethyl-ammonium-propane (the most commonly available species), originally developed for the delivery of DNA fragments into cells for therapeutic purposes [29–31]. These lipids are cationic at neutral to low pH, and can be made into a supported bilayer on the negatively charged mica surface, either with vesicle fusion [4–6,24] or a Langmuir trough [7,8,25]. In Fig. 1a, a bilayer of dipalmitoyl-trimethyl-ammonium-propane (DPTAP) is shown where the thickness is about 4 nm as measured from the defects of the bilayer. The surface of the

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SSDI 0014-5793(95)00906-X

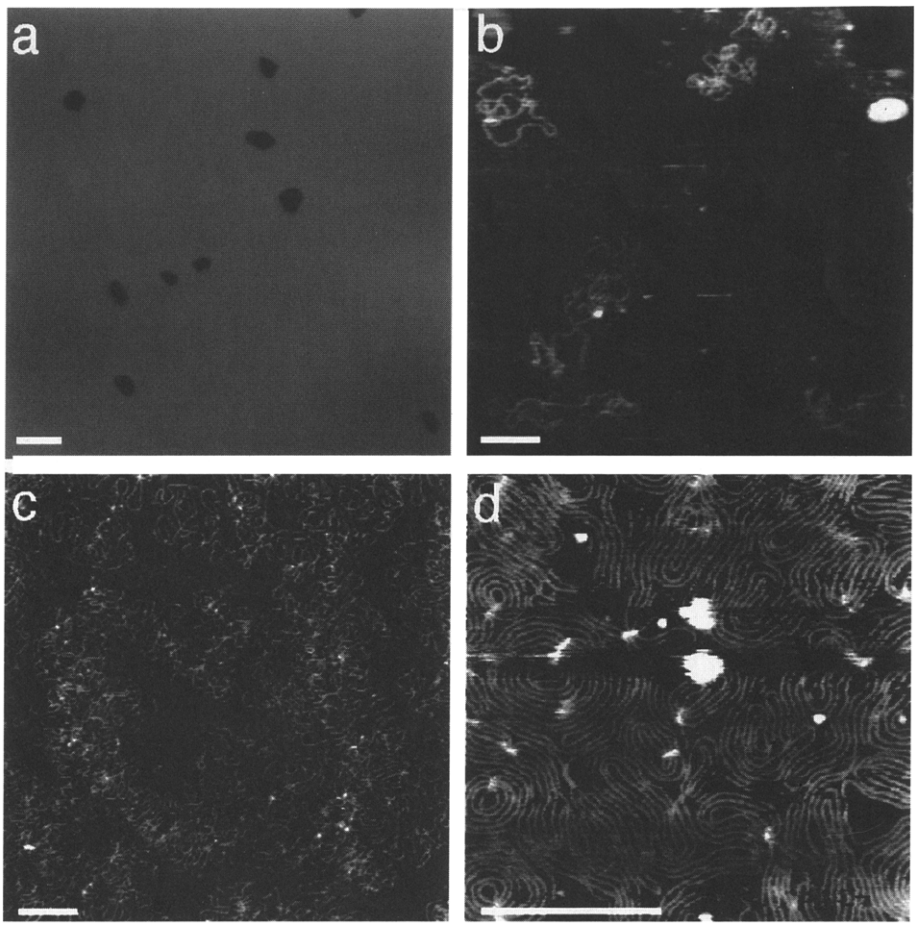

Fig. 1. (a) A typical bilayer of cationic lipids, 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP) in this case, made by a Langmuir trough at 45 mN/m. Bilayer defects are also shown (darker areas), and the thickness measured at the edge of these defects is 4 nm. With vesicle fusion (data not shown), more defects were present. The quality of the bilayer was not affected by the chain length of these lipids. At smaller scan sizes, the surface of the bilayer appeared flat without details or heterogeneity. Scale bar: 200 nm. (b) Several *E. coli* plasmids pBR322 (4.36 kb) are seen on a DPTAP bilayer. The plasmids are in a super-coiled form, and the adsorption is slight. The DNA was incubated in 20 mM NaCl for 2 h and imaged in the same buffer. The measured length of these DNA is about 1.5 μm, in agreement with the expected value for B-DNA (1.48 μm). Scale bar: 200 nm. (c) When divalent cations were chelated by 1 mM EDTA in the buffer, the adsorption of the plasmids (pBR322) was greatly increased. This observation indicates that the electrostatic interaction is the major cause of DNA adsorption. With high salt (0.5 M NaCl, 10 mM Tris and 1 mM EDTA, pH8), DNA adsorption was entirely inhibited. Different cationic lipid head groups, i.e. dimethyl- and trimethyl-ammonium-propane, made no difference in DNA adsorption. Scale bar: 200 nm. (d) When the bilayer with the adsorbed plasmids (pBR322) was incubated at elevated temperatures (50–55°C) in the presence of EDTA, the DNA became much better packed, and the specimen surface appeared rather flat (compared with c), indicating that only a monolayer of DNA is on the bilayer surface. Only in this case, where the contact area between DNA and the positively charged bilayer surface is maximized, high resolution became possible due to the improved specimen stability. Scale bar· 200 nm. The height measurement on these specimens is much improved, generally in the range of 1.5–2 nm.

bilayer is clean and flat, perfectly suited as a substrate for adsorption of other macromolecules. When an *E. coli* plasmid DNA (pBR322, 4.36 kb) was added into the buffer containing the cationic bilayer at low ionic strength, scarce adsorption was observed (Fig. 1b) and the plasmid DNA is seen in the super coiled form. The measured length of these molecules is about 1.5 μm, in good agreement with the 4.36 kb length of the plasmid (1.48 μm for the B-form DNA). The adsorption of DNA was greatly increased when 1 mM EDTA was included in the buffer (Fig. 1c), as EDTA should have chelated all residual divalent cations, such as Ca^{2+} and Mg^{2+} [32]. It may be noted that even with 18 MΩ deionized water, μM concentrations of divalent cations, such as Ca^{2+} and Mg^{2+}, are still present. The plasmids appeared somewhat more extended with many overlaps, and it is difficult to trace out any individual plasmid, due to the high DNA density on the bilayer surface.

No qualitative difference was found in DNA adsorption with either cationic lipid (trimethyl- or dimethyl-ammonium propane), but, high ionic strength (~500 mM NaCl) totally inhibited DNA binding to the bilayer, indicating that the nature of interaction is primarily electrostatic. The height of the DNA, either with isolated molecules or at the edge of closely packed DNA patches, is between 1.5–2 nm, suggesting that the plasmids are most likely adsorbed to the surface of the bilayer without interdigitating into the hydrocarbon core. This small compression is in sharp contrast with that in air where more than 70% compression has been reported [26–28,33,34]. DNA packing was further improved when the bilayer with the adsorbed DNA (in the presence of EDTA) was incubated at about 50°C for an hour (Fig. 1d). At this temperature, the fluid state bilayer allowed the lipids to diffuse laterally, such that, along with the bound plasmids, the minimum energy state could be

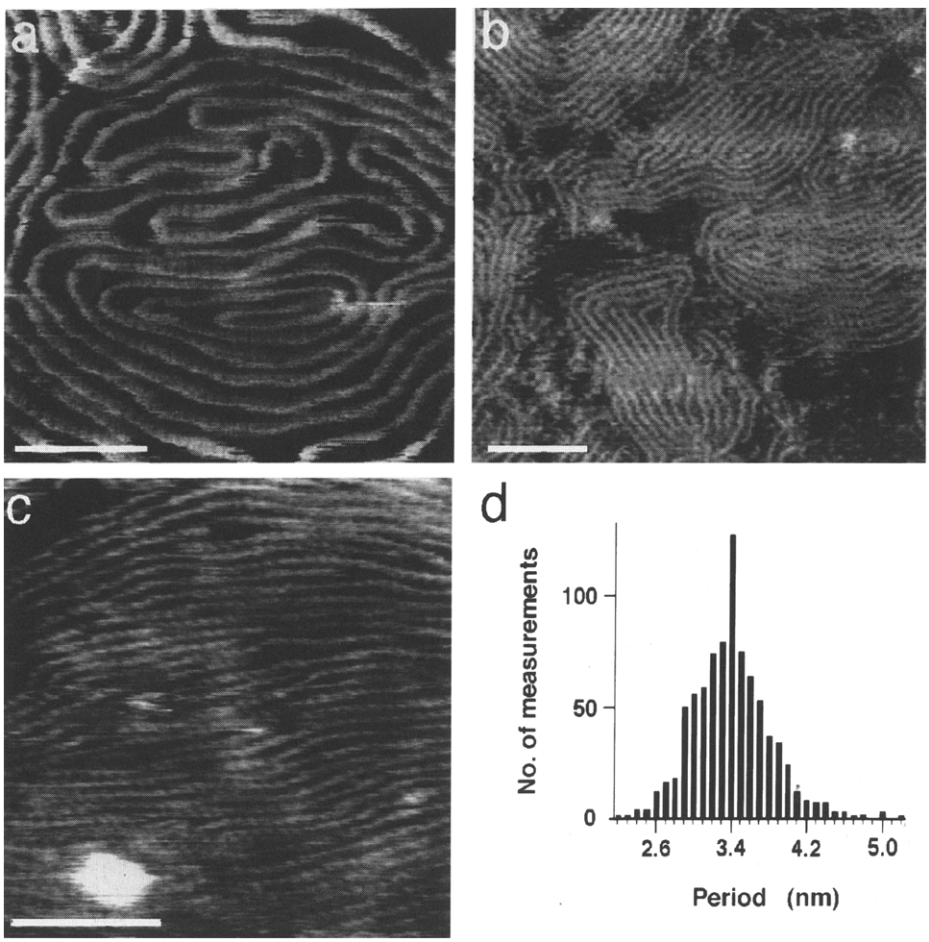

Fig. 2. Three different DNA samples are shown. (a) pBR322 (4.36 kb). (b) pBR325 (6 kb) (c) *Hae*III restriction fragments of ϕX174. Scale bar: 40 nm. All specimens were prepared with the method as in Fig. 1d. Periodic modulations are resolved with all three specimens, and were reproduced at different scales and with different specimens. The width of the DNA varied somewhat from AFM tip to tip, but most measurements gave a value below 3 nm. In c, the measured width (full width at half height) is about 2 nm, in excellent agreement with the know value for B-DNA. In d, a histogram is shown for the measurements of the modulation periodicity. All three types of specimens were included in the measurements. The mean value is 3 4 nm with a standard deviation of 0 4 nm, indicating that these modulations are indeed due to the pitch of the DNA double helix

reached Further studies are required to elucidate the exact mechanism for this phenomenon. Interestingly, the smallest inter-strand distance remained at about 5 nm. Neither salt nor temperature affected this spacing significantly.

For high resolution imaging, scarcely populated specimens were not adequate, and the plasmids were pushed around by the AFM tip. The width of the DNA in Fig. 1b and 1c was only 4–8 nm at best. However, when closely packed specimens (Fig. 2a–c) were imaged with a minimum probe force (~0.1–0.2 nN), a much better lateral resolution was achieved, due to the improved sample stability. In Fig. 2, three different types of DNA are shown: Fig. 2a and b are those of *E. coli* plasmids (4.36 kb [pBR322] and 6 kb [pBR325], respectively) and Fig. 2c is of *Hae*III restriction fragments of ϕX174. For the linear restriction fragments (where no internal strain exists), DNA packing is further improved,and the inter-strand distance is also reduced to about 4 nm (Fig. 2c). Although the width of DNA varied somewhat from image to image, depending on the AFM tip used, it was consistently below 3 nm (full width at half height). However, the height measured from these closely packed regions was normally lower than 2 nm, indicating that the finite size of the AFM tip prevented it from reaching the bilayer surface between DNA strands [1,5]. For each of the three types of DNA, quasi-periodic modulation along the DNA strands was reproducibly resolved, independent of the direction or speed of the scan and the orientation of the DNA (Fig. 2a–c). The measured periodicity, including all three types of specimens, is 3.4 ± 0.4 nm (Fig. 2d), exactly as one expects from a B-DNA [35]. Therefore, we conclude that the detected modulation is indeed the pitch of the DNA double helix. The histogram (Fig. 2d) indicates that the pitch of the double helix does not vary extensively, when DNA is adsorbed to the bilayer surface, which implies that the structure of dsDNA is not changed significantly by the electrostatic interaction with the lipids.

A closer examination of Fig. 2c suggests that the handedness of dsDNA might be resolved. This was indeed confirmed when higher resolution images were obtained with very clean specimens. In fact, the right handed double helix was resolved with all three types of DNA samples (Fig. 3). It may be noted that, although the pitch angle varied from image to image, which was due to the drift of the AFM, the right handedness remained clearly discernible in all these images. To our knowledge, this is the highest resolution ever achieved on DNA by any direct imaging technique [36]. These results indicate that perhaps, we

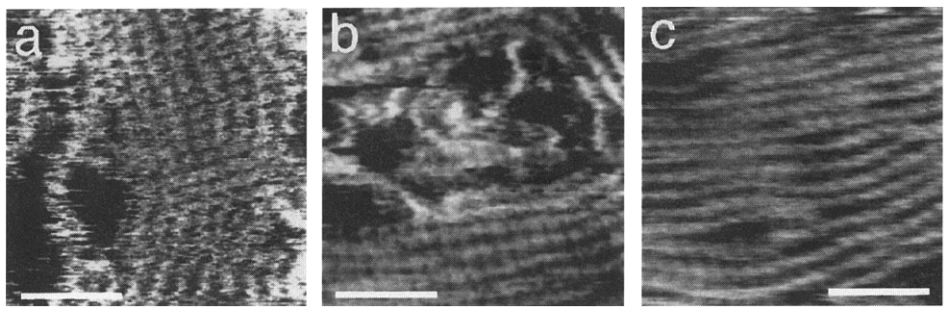

Fig. 3. The right handedness of the double helix is also resolved by the AFM. These images were obtained from the same samples as in Fig. 2, but with a different scale and/or different scan direction. These results show the excellent stability of these samples and the reproducibility of AFM. (a) pBR322 (4.36 kb). (b) pBR325 (6 kb). (c) *Hae*III restriction fragments of ϕX174. Scale bar: 20 nm. Occasionally, even the minor grooves were recognizable (see b). It is also seen that the handedness is better resolved when the DNA strand is parallel with the fast scan direction (see c). This is because the instrumental drift is more noticeable in the slow scan direction.

have not reached the ultimate resolution of AFM, for when suitable specimens are pepared, a much better performance may be obtained, as demonstrated here. Since the DNA-substrate interaction is primarily electrostatic and can be modulated by divalent cations and high ionic strength, this approach should be preferred for the study of DNA–protein interactions at a high spatial resolution.

When this approach was applied to thermally denatured *Hae*III restriction fragments of ϕX174, sufficient adsorption was also achieved (data not shown), even though the sample was prepared at a high temperature (95°C). The width of the single stranded DNA was below 2 nm with many bends, but the quality of the specimen was not as good as those with dsDNA. The exceedingly high temperature used (up to 4 h) may have played some role in the degraded specimen quality. This result opens the possibility of directly imaging the secondary structures of various RNA molecules, and of resolving individual bases if the methodology, including a more stable AFM and the preparation of specimens of oriented single stranded nucleic acids, is further refined. Obviously, once small labels for each base are developed, the latter can then be applied to DNA and RNA sequencing.

Acknowledgements: We thank Dr. R. Nakamoto for helpful discussions, and Dr. Jie Yang for his preliminary experiments on the preparation of cationic lipid bilayers when he was associated with this laboratory. We also thank Drs. A.V. and A P Somlyo for encouragement. The work is supported by grants from National Science Foundation (BIR-9115655) and National Institutes of Health (RO1-RR07720 and PO1-HL48807).

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