FEBS Letters 355 (1994) 91-95
FEBS 14779

The scanning force microscopy of DNA in air and in n-propanol using new spreading agents

Achim Schaper, J.P. Pascual Starink, Thomas M. Jovin*
Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, PO Box 2841, 37018 Göttingen, Germany
Received 18 August 1994; revised version received 14 October 1994

Abstract We present recent advances in DNA specimen preparation technique for scanning force microscopy (SFM) based on spreading on mica in the presence of cationic and non-ionic detergents. Reproducible DNA imaging in air and in n-propanol has been achieved in the presence of the non-ionic detergent 2,4,6-tris(dimethylaminomethyl) phenol (DMP-30) or the cationic detergent cetylpyridinium chloride (CP) in a microdrop containing nanograms of DNA. In an alternative procedure, a microdrop of detergent is applied to the surface just prior to the DNA. Quantitative image analysis yields as the apparent molecular dimensions of the DNA a width of ~7 nm and a height of ~0.7 nm, and delineates the problems of DNA metrology by SFM.

Key words: Atomic force microscopy; Scanning tunneling microscopy; DNA; Cetylpyridinium chloride; Benzylalkonium chloride; 2,4,6-Tris(dimethylaminomethyl)phenol

1. Introduction

Scanning force microscopy (SFM) has been widely applied in the investigation of surface topography in the subnanometer-μm range. The use of SFM in the life sciences requires the immobilization of biological materials such as cells, membranes, proteins and DNA, at an air-liquid or air-solid interface (for reviews see [1-3]). Inasmuch as contrast enhancement by shadowing (as in transmission electron microscopy, TEM) or labeling with a probe (as in fluorescence microscopy) is not required, SFM offers in principle the unique potential for assessing the unperturbed ultrastructure of biomolecules under physiologically relevant conditions. Towards this goal, specimen preparation techniques need to be adapted for the requirements of SFM.

Sophisticated preparation techniques adapted from TEM have been used for spreading DNA on mica. Imaging in air by conventional contact SFM has been achieved in the presence of Mg2+ or Ca2+ [4-7], and upon spreading with cytochrome c [8], and benzylalkonium chloride (BAC) [1,2,9,10]. Several reports have demonstrated the potential for SFM of resolvated DNA in alcohol (ethanol, n-propanol) [11-16] with significant improvements in lateral resolution [4,17]. Imaging rehydrated DNA in water or buffer yields lower resolution [18] even after tethering the molecules by chemical modification of the substrate [19,20]. Imaging with the tapping mode SFM in water offers increased resolution, i.e. an apparent DNA width of 5 nm, and reduced damage of soft samples [16]. DNA specimen preparation is crucial for successful SFM imaging due to several reasons. First, molecules need to be adsorbed so as to withstand the loading force applied by the scanning probe. Second, the background should appear structureless. Third, the molecules should remain in an open configuration so as to render their substructure accessible. Fourth, a high adsorption efficiency is required for the facile acquisition of statistically relevant topographic data.

In this study we present further advances in DNA sample preparation for SFM achieved by use of the non-ionic detergent DMP-30, formerly applied in DNA specimen preparation for TEM [21], and of the cationic detergent cetylpyridinium chloride, which to our knowledge has not been previously used in microscopy. The images obtained with these detergents and with BAC used in prior studies [9,10] were compared by quantitative image analysis.

2. Experimental

2.1. DNA, substrate, detergent

The DNA was relaxed dimeric circular pUC18 DNA (5372 base pairs) exhaustively dialysed against pure water from a MilliQ filter system (Millipore, Eschborn, Germany) as described previously [9]. Freshly cleaved mica (Muskovite mica; Electron Microscopy Sciences, Fort Washington, PA) was treated by glow discharge (1 min, 0.3 mbar air) prior to DNA deposition. A 1:100 (14 mg/ml) aqueous stock dilution of semi-solid 2,4,6-tris(dimethylaminomethyl)phenol (DMP-30; Tousimis, Rockville, MD) and an aqueous stock solution (1 mg/ml) of cetylpyridinium (CP) chloride (Sigma, St. Louis, MO) were freshly prepared prior to spreading.

2.2. DNA spreading

The detergent stock solutions were further diluted to 10-3%, i.e. 10 μg/ml. 0.5 μl of the detergent dilution was added to a 10 μl drop of DNA (1 μg/ml) in MilliQ water and incubated for 5 min on Parafilm. The drop was brought into contact with the mica for 1 min. Excess solution was soaked with a filter paper and the wet mica was touched to a 50 μl water drop. The water was blotted off and the sample was air dried. Prespreading, i.e. treatment of the mica with detergent, was with 0.5 μl of the stock solution added to 10 μl of water. The drop was placed for 5 min on Parafilm, after which the mica was brought into contact with it for 1 min. Excess solution was drained and the wet mica was touched to a 10 μl drop of DNA for at least 1 min, washed with 50 μl water, and air dried. For measurements in n-propanol, the air dried sample was re-immersed in the solvent.

2.3. Instrumentation

The samples were scanned with a NanoScopeIII-contact SFM (Digital Instruments (DI), Santa Barbara, CA). Measurements were done in air (relative humidity 15-60%) at room temperature (18-20°C) or in n-propanol using the fluid cell from DI. Imaging was with a J-scanner with a 135×135(x,y)×5(z) μm scan range. We used microfabricated Si-tips integrated into triangular cantilevers with typical force constants in the range of ~0.06 N/m (Ultralevers; Park Scientific Instruments (PSI), Sunnyvale, CA). Images were obtained in the topographic (isoforce) mode. The loading force of the tip was always minimized by adjusting the setpoint of the optical readout signal to a minimum value, which

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SSDI 0014-5793(94)00166-4
typically corresponds to a cantilever bending force as low as 1 nN. The total load of the tip is estimated to be 1–2 orders of magnitude higher in air, but appreciably reduced in fluid [22]. Images (512 x 512 pixels) were taken at an 6 Hz scan frequency.

2.4. Image analysis

Images were processed with the NanoScope software including the operations of plane fitting, flattening, and geometric filtering. Further image analysis was performed on a SPARCstation 10 (Sun Microsystems Corp., Sunnyvale, CA) with the image processing package SCIL-Image (University of Amsterdam; Technical University of Delft, NL). The DNA contour length was obtained from the contour line of connected pixels (skeleton) after refinement using an optimal path algorithm. Height and width data were collected from cross-sections perpendicular to the skeleton. Mean values were from least squares fits. A more detailed analysis of the data and the image processing algorithms will be published elsewhere.

3. Results and discussion

Representative SFM images of circular relaxed pUC18 DNA in air after the DMP-30 and CP spreading are shown Fig. 1a,b. The molecules appeared distributed in a monolayer over the mica surface; contaminations were rarely observed. The differences in the adsorbed molecules in Fig. 1a and b reflect the fluctuations found with single preparations. Molecules were tightly adsorbed, permitting stable SFM imaging. Minimization of the loading force was essential since the topographic contrast decreased drastically at higher loads (data not shown). Usually, the surface of the mica support, with or without DNA and prespreading, appeared amorphous with a mean roughness of ~0.1 nm/μm² and with a corrugation peak value of ~1 nm. Prespreading of the detergent (see section 2.2) potentiated DNA adsorption as a monolayer and yielded a surface morphology similar to that in Fig. 1. The results achieved with these procedures suggest that a thin film of DMP-30 or CP surfactant forms at the air–liquid interface and is transferred by microdrop spreading in a manner similar to that postulated for the BAC procedure reported earlier [9,23]. DNA adsorption is very efficient due to this surface modification, probably due to a significant degree to the positive surface charge density generated by the reagents. A virtue of prespreading is that direct contact of the DNA and the detergent in bulk phase is minimized, i.e. restricted to a strong interaction at the liquid-solid boundary, and thus favours prespreading for sensitive molecules such as protein–DNA complexes. The glow discharge treatment of the mica substrate improves the stability of the detergent film which on the other hand increases the amount of unfolded DNA molecules. It is not absolutely required. The mechanism by which plasma modification of mica takes place is unclear (see e.g. [24,25]). Most probably, removal of cations and adsorbrates from the mica cleavage plane is involved.

3.1. Statistical data analysis

Open molecules lacking branching or overlaps were selected from different samples imaged with different tips for contour length measurements. In Fig. 2a,b a typical image of a single open molecule is presented from which the length, height, and the width were computed. The results of the global statistical data analysis collected from a representative set of molecules are summarized in Table 1 (top). For comparison, the data are supplemented with results from samples prepared by BAC [9]. The contour length of the pUC18 after DMP-30 and CP

Table 1

<table>
<thead>
<tr>
<th></th>
<th>CP</th>
<th>DMP-30</th>
<th>BAC</th>
</tr>
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<tbody>
<tr>
<td>Contour length (μm)</td>
<td>1.92 ± 0.09</td>
<td>1.83 ± 0.11</td>
<td>1.82 ± 0.12</td>
</tr>
<tr>
<td>Height (nm)</td>
<td>0.76 ± 0.21</td>
<td>0.65 ± 0.17</td>
<td>0.72 ± 0.16</td>
</tr>
<tr>
<td>Width (nm)</td>
<td>7.0 ± 1.2</td>
<td>7.6 ± 1.2</td>
<td>14.2 ± 2.4</td>
</tr>
</tbody>
</table>

Samples were air dried after detergent spreading.

*Global statistical analysis of the DNA topography from different samples and tips. Mean value ± S.D. Contour length measurements based on 30 molecules (CP, 12; DMP-30, 12; BAC, 6 closed curves). For width and height statistics data from hardware zooms were included collected along a total contour length of 33 μm (CP), 40 μm (DMP-30), and 16 μm (BAC).

Single molecule statistics. The molecules selected from the data set were representative for the optimal topographic contrast achievable at present with the detergent spreading procedures.

Fig. 1. SFM micrograph of air-dried circular relaxed pUC18 dimer after spreading with (a) DMP-30, and (b) CP. Top view.
spreading was \( \sim 1.9 \) \( \mu \text{m} \), in agreement with the BAC data reported earlier and thus consistent with the length expected for the pUC18 in a B-DNA conformation. The DNA height (above the substrate) was computed to be \( \sim 0.7 \) \text{nm} (Table 1, 2nd row) for all three spreading agents, a value lower than the theoretical DNA diameter (\( \sim 2 \) \text{nm}). The apparent DNA width (Table 1, 3rd row) decreased from \( \sim 14 \) \text{nm} for BAC to \( \sim 7 \) \text{nm} for DMP-30 and CP. Thus, there appears to be a structural broadening due to BAC/DNA association in addition to the inherent effects of tip-sample convolution (see below). A structural broadening of the DNA width (\( \sim 5 \) \text{nm}) after BAC spreading was also indicated from TEM measurements [26].

The imaging conditions (e.g. noise, thermal drift, tip adsorbates and tip load) are of manifest importance in the height and width measurement, as evidenced by subnanometer differences in these parameters derived from scanning of different molecules or during repeated scanning of the same molecule from frame to frame. As a consequence, a non-random distribution of the height and width pixel values collected for the global statistics in Table 1 appeared among the population of molecules. In Table 1 (lower) the statistics for a single molecule are presented. The selected molecules, of which the SFM micrographs after CP and DMP-30 spreading are shown in Fig. 2a and b, matched the criteria of maximal height and narrow width corresponding to optimal imaging conditions, i.e. a sharp probe and minimal depletion of the surface material. The latter process is dependent on adsorption and air drying effects, as well as on the loading force of the tip.

Modulations along the longitudinal axis of the DNA are perceived in Fig. 2c and d. We tried to reveal any periodicity in the height data by means of distance autocorrelations but the corrugations appeared randomly distributed along the entire contour. Only within selected areas of the substructure could a modulation period of \( \sim 7 \) \text{nm} be discerned, possibly reflecting the limit of spatial resolution imposed by the tip. The Si tip diameter is assumed to have been \( \leq 10 \) \text{nm} (specified by the supplier, PSI, from electron microscopy data) and defined the point spread detection limit of the probe. Since one presumes that an additional broadening at steeper surface structures appears due to the lateral interactions of the conical tip body [2,6], the point spread detection limit cannot be achieved even with the subtle nanometer surface corrugations inherent to DNA.

Fig. 2. SFM imaging of single relaxed pUC18 dimers. (a,b) Single molecule after DMP-30 and CP spreading. (c,d) Software zoom of the DNA substructure in a and b, respectively.
DNA imaging in n-propanol

3.2. DNA imaging in n-propanol

An SFM micrograph of DNA spread with CP after air drying and reimmersion in n-propanol is shown in Fig. 3a. In Fig. 3b, the image is zoomed so as to demonstrate the DNA substructure. In contrast to the BAC spreading, with which the surface appeared unstable in the SFM under the same conditions, imaging was achieved and the density of adsorbed DNA remained constant. The DNA torus appeared in a frayed morphology and the apparent width varied between 5 and 20 nm, possibly reflecting structural mobility of the non-adsorbed regions of the DNA helices. As a consequence, the height of the DNA was ~0.6 nm and thus within the lower limits given by the molecule in the dried state. We surmise that the compliance of the molecule was primarily responsible for the reduced resolution. A detailed investigation would require viscoelastic measurements and/or cantilevers of higher sensitivity [27]. Up to now we have not found experimental conditions for imaging in n-propanol without predrying, an important goal on the way towards SFM imaging of DNA in an aqueous environment. Other groups reported that drying following adsorption was essential for imaging DNA on mica after re-exposure to water or n-propanol [14,15]. Thus, it has been suggested that tethering of DNA occurs during the drying process. The effect of drying might be to embed the molecule in a co-precipitate of inert salt and DNA [28], which would exhibit reduced solubility after re-immersion. The background spots that appear occasionally, presumably arising from inert salt, support this view [14,17]. The detergent spreading is performed without excess inert salt and we conceive that the surface–molecule interaction is more specific and in general advantageous for imaging air-dried specimens. We note in conclusion that detergent spreading with DMP-30 and CP is also very effective for the SFM imaging of proteins and protein–DNA complexes, as will be shown in subsequent publications.

Acknowledgements: This research was supported by the Max Planck Society, the German Research Council (DFG) (Jo 1059), and the European Community Human Capital and Mobility Program (project FRHCRXCT930177).

References