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DNA height in scanning force microscopy

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Abstract

The measured height of DNA molecules adsorbed on a mica substrate by scanning probe microscopy is always less than the theoretical diameter. In this paper we show that, when imaged in ambient conditions, the molecules are usually immersed in the salt layer used to adsorb them to the substrate. This layer distorts the measurement of DNA height and is the main source of error but not the only one. We have performed different experiments to study this problem using two scanning force techniques: non-contact tapping mode in air and jumping mode in aqueous solution, where the dehydration phenomena is minimized. Height measurements of DNA in air using tapping mode reveal a height of 0.7 ± 0.2 nm. This value increases up to 1.5 ± 0.2 nm when the salt layer, in which the molecules are embedded, is removed. Jumping experiments in water give a value of 1.4 ± 0.3 nm when the maximum applied force is 300 pN and 1.8 ± 0.2 nm at very low forces, which confirms the removal of the salt layer. Still, in all our experiments, the measured height of the DNA is less than the theoretical value. Our results show that although the salt layer present is important, some sample deformation due to either the loading force of the tip or the interaction with the substrate is also present.

Keywords: Scanning force microscopy; DNA; Tip–sample interaction; Sample–surface interaction

1. Introduction

The scanning force microscope (SFM) also called atomic force microscope was invented in 1986 [1]. A few years later, a set of articles appeared showing the promising possibilities of the technique in the field of imaging biomolecules such as DNA in air and in liquids [2–9]. These early works were performed using the contact mode scanning technique. In 1994, the tapping mode was developed [10–12] and the resolution on biological material increased. Some excellent reviews were published in the following years [13–15]. SFM is a technique characterized by a high resolution in measuring the height of objects where subnanometer precision can be achieved. However, the measured height of the DNA was always less than the theoretical diameter of the molecule deduced from the Watson–Crick DNA model, which is 2 nm [16]. In the first experiments, already 10 years old, this discrepancy was attributed to sample deformation by the tip interaction

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[4,6,7], dehydration of the molecule [14] and also to salt deposition [7,17,18]. Since then, this important issue has not been the topic of much attention. Nevertheless, the low value of DNA height measured by SFM has been recurrent in the literature published up to now [2,4,6,18–24]. In all these articles, the measured height of the DNA is always less than 1 nm, and thus less than half the theoretical one.

In the present study, we intend to clarify the issue of the height of the DNA molecules as measured by SFM. Two techniques and two ambient conditions have been used: non-contact tapping mode [25,26] in air and jumping mode [27] in aqueous solution. Our experiments show that in ambient conditions the DNA molecules are embedded in a salt layer, which is used to bind the molecules to the substrate. This is a source of error in measuring the height of the DNA but it is not the only one because the complete height is not obtained even when no salt layer is present. Other reasons have been put forward: mainly, tip deformation and dehydration. We have found that, for appropriate operating parameters, contact between tip and sample is avoided [26] when measuring in ambient conditions. In this case, sample deformation by the tip must be discarded. The lower height of the DNA measured in air has been attributed to dehydration of the sample. Previous data of experiments performed in liquids indicated an increase of DNA height in water [8]. This is confirmed in our experiments performed in aqueous solution, where the molecule is fully hydrated and functional [28]. In this case, we consider that sample deformation by the tip is the reason for measuring 0.2–0.5 nm less than the theoretical height.

2. Materials and methods

2.1. DNA sample preparation

Freshly cleaved mica is commonly employed as substrate for biomolecules due to its flatness. It exposes a negatively charged surface when cleaved; therefore, divalent cations like Zn^{2+} or Mg^{2+} are used to adsorb negatively charged molecules like DNA [19]. The standard protocol consists of a final mixture of 1–10 mM $MgCl_2$ and 10–100 ng of DNA diluted in Tris-EDTA buffer placed on a freshly cleaved mica disc and extensively washed with double deionized ultrapure water. This step was found to be crucial to obtain a clean DNA sample. Finally, the sample is dried under a gentle stream of air or dry nitrogen. DNA molecules are clearly visible in samples prepared this way [23,29].

For the experiments performed in aqueous solution, samples were prepared as described above and then placed in a liquid cell filled with ultrapure water.

2.2. Non-contact tapping mode in air

All the images were obtained with a commercial SFM [30]. For the non-contact tapping mode, we have used commercial cantilevers with a nominal force constant of 1 N/m, resonance frequency 75–80 kHz and tip radius 25–35 nm [31]. The cantilever is oscillated near its resonance frequency and the normal force signal is processed to measure the amplitude and the relative phase of the oscillation. The digital control electronics establishes feedback at a certain oscillation amplitude which is slightly lower that the free resonance frequency; for more details see Refs. [25,26].

2.3. Jumping mode in liquid

For imaging in liquids, we have used commercial cantilevers of force constants 0.06 and 0.02 N/m and a nominal tip radius of 10–15 nm [32]. Jumping mode [27], which in its working principle is very similar to pulsed force microscopy [33], was originally developed as a scanning mode to minimize shear forces. This technique basically acquires a force versus distance Z displacement curve at each point of the image. At maximum extension of the piezo element, feedback is established to control the maximum normal force (F_N) and to measure the precise height of the sample at that point. Using this method, the contact time and the applied force can be measured and controlled with high accuracy. An important feature of this method is that the tip is

moved laterally with respect to the sample when the two are out of contact, thus minimizing shear forces during lateral motion. Since contact is present in this method, the cantilevers were chosen with a small force constant to avoid damage to the sample. During the whole image process, the maximum applied force was monitored and never exceeded 300 pN. Such small forces can be obtained by imaging in liquids where the tip–sample adhesion force is almost zero. This method of measurement has been found to be non-intrusive in liquid since adhesion and shear forces are minimized [34].

3. Results and discussion

Many DNA samples prepared as described above have been analyzed. Usually, the substrate appears to be homogeneous with DNA molecules adsorbed on top of the substrate. In some cases however, *islands* and/or *holes* appear in the substrate (Fig. 1A). Since this kind of defects is not present on freshly cleaved mica, we deduce that they are composed of the ions dissolved in the buffers where the DNA is prepared. The corresponding salts are mainly $MgCl_2$ and NaCl. We now believe that these salt layers almost always

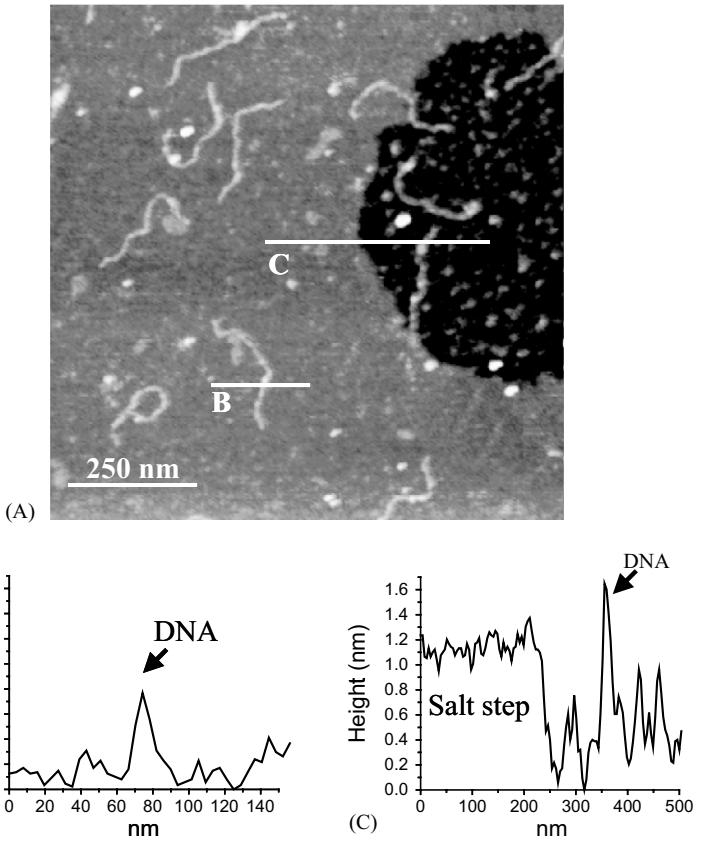


Fig. 1. Non-contact tapping mode AFM on DNA in air. (A) is a 1 μm^2 topography image of DNA adsorbed on a mica substrate using $MgCl_2$ buffer. DNA molecules can be seen embedded in the salt layer, which is clearly visible due to the *hole* that appears in the right side of the image. Two profiles are displayed in the lower part of the figure. Profile B is the one taken on a molecule which is completely embedded in the salt layer. The measured height of this molecule is 0.7 ± 0.2 nm. Profile C is the profile taken on a molecule which is partially covered by the salt layer. The height of the salt layer is 0.8 ± 0.2 nm and the height of the uncovered DNA molecule is 1.5 ± 0.2 nm.

cover the mica substrate when DNA is prepared from a buffer solution. After drying of the preparation, the DNA molecules are trapped in this salt layer. Sometimes this layer is not complete and exposes the underneath mica as can be seen in Fig. 1A. In this case, a salt layer unambiguously covers the mica substrate. The measured height of the DNA molecules at low humidity (less than 30%) is 0.7 ± 0.2 nm (Fig. 1B) when measured with respect to the basal salt layer. This value coincides with the well-accepted value for the height of the DNA as measured by SFM [19–24]. The height of the basal salt layer as measured from Fig. 1A was 0.8 ± 0.2 nm. For molecules not covered by the salt layer, a height of 1.5 ± 0.2 nm was obtained (Fig. 1C), in good agreement with the previous values. Measured widths of the molecules are compatible with a standard tip radius of 35 nm. We estimate this tip radius by assuming a circular DNA molecule of a certain height and measuring the width of the molecules in the AFM image. From these values, the tip radius is obtained according to [35]

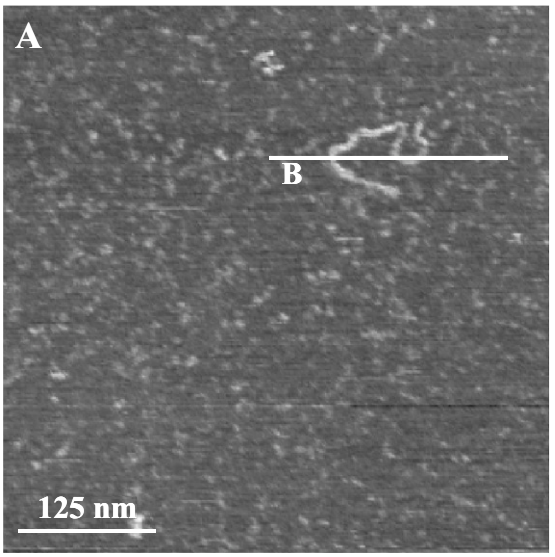
$$S = 2\sqrt{RD + \frac{D^2}{4}} \approx 2\sqrt{RD}, \quad (1)$$

where R is the tip radius, D is the measured height of the molecule, and S is the width measured at half-maximum. The approximation is valid for $D \ll R$, which is usually the case. Accordingly, higher molecules should look wider due to tip-dilation phenomena (Fig. 1C). Indeed, as can be observed in Fig. 1B, molecules appear thinner when they are immersed in the salt layer and their height is smaller than in Fig. 1C. We recall that, in this experiment, no contact between tip and sample is present; so any sample distortion by the tip can be discarded. However, the dehydration of the molecules in air could be present in this experiment.

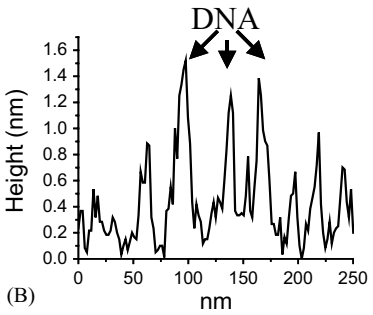
In an attempt to study the role of the dehydration of the molecules, we have performed essentially the same experiment as described above, but at high and low relative humidity. In the case of rehydration, we would expect an increase of DNA height for high relative humidity. Interestingly, we measure a lower height for the DNA at high humidity (relative humidity $>80\%$). This is probably due to an increment of the thickness of the salt layer due to swelling. Experiments done at very high relative humidity ($\sim 90\%$) were difficult because the DNA molecules started to desorb from the substrate [36]. From these results, the influence of dehydration in the DNA is not clear. For this reason, we decided to work in aqueous solution.

In these experiments, samples prepared as described above were used. When imaged in air (data not shown), the sample shows DNA molecules of a height compatible with the value measured typically by SFM (less than 1 nm). Then, the sample was placed in a liquid cell and filled with ultrapure water. Jumping mode was used to analyze the sample. Compared with the images of the same preparation acquired in air, the density of molecules is considerably lower. From this, we conclude that most of the DNA molecules which were attached to the mica when imaged in air desorb when imaged in liquid. However, some of them still remain (Fig. 2A). For these molecules, the measured height was 1.4 ± 0.3 nm at $F_N \sim 300$ pN (Fig. 2B) and 1.8 ± 0.2 nm at $F_N \sim 100$ pN, the minimum normal force we can apply. These values are similar to that obtained for DNA molecules in *holes* of the salt layer as described above. We therefore believe that, in aqueous solution, the basal salt layer is dissolved. In this experiment, the dehydration problem of the molecules is not present since these measurements are performed in water.

For both, the experiments in air as well as the ones in aqueous solution, our measured height of the DNA molecules is much closer to the theoretical value of 2 nm than the height reported in other works. Still, in general, the theoretical diameter for the DNA is not observed. In liquids, the height that we measure depends on the applied normal force and is almost within our experimental error at the minimum possible loading force. We therefore believe that, for precise characterization of DNA, sample deformation is an important effect that has to be taken into account [4,6,7]. To understand the deviation between the measured and the theoretical height, we propose a simple model where the DNA is



(A)



(B)

Fig. 2. Jumping mode AFM on DNA in aqueous solution. (A) is a $500 \times 500\,nm^2$ topography image of DNA in aqueous solution. One molecule can be clearly seen as well as some additional smaller features which we consider to be fragments of the salt layer that is mostly dissolved in liquid. The height profile of the molecule is displayed in (B). The height of the DNA when imaged using the jumping mode is 1.4 ± 0.3 nm at a loading force of about 300 pN.

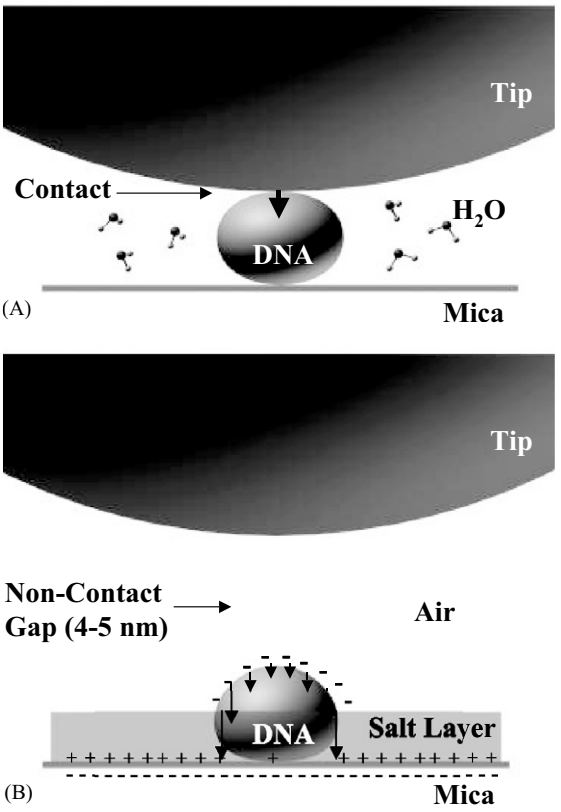


Fig. 3. Models proposed for the height reduction for the measurements in liquid (A) and in air (B). (A) attempts to sketch the situation when DNA is imaged using the jumping mode in aqueous solution. In this case, the lower measured height of the DNA is due to compression of the molecule by the tip. No salt layer is present in aqueous solution. (B) represents the situation for experiments in air. The salt layer distorts the height measurement of the DNA molecules. In addition, the molecule is also compressed due to the electrostatic interaction with the surface.

treated as an elastic continuum with a Young's modulus of about 340 MPa [37].

For the experiments in liquid, contact between tip and sample occurs, and thus some force is exerted on the DNA molecules (Fig. 3A). We will assume that Hooke's law can be applied to estimate the elastic deformation of the DNA molecule:

$$\frac{\Delta h}{h} = \frac{P}{E}, \tag{2}$$

where Δh is the deformation, h is the total height (2 nm), E is the Young's modulus of the DNA ($0.34 \times 10^9\,N/m^2$) and P is the pressure exerted by the tip on the molecule. The maximum applied force was 300 pN and if we assume a contact surface of $4\,nm^2$, then the deformation of the molecule is about 0.4 nm. For the lowest applied force, the deformation is of the order of 0.1 nm, thus negligible within our experimental error. These values are compatible with the heights of the DNA that we have measured in aqueous solution.

For the experiments performed in air, no contact between tip and sample is established. In this case, it has been argued that dehydration might cause the observed lower height of the DNA molecules. However, as discussed above, in our experiments we observe a decrease of DNA height with relative humidity. Another reason for the reduced height could be again due to mechanical deformation of the molecule induced in this case not by the tip but by attractive interaction with the mica substrate.

To estimate the strain, $\Delta h/h$, within the DNA molecule, one can assume that DNA is composed of connected spheres of $R = 1\,\mathrm{nm}$ radius. In contact with the surface, each sphere is attracted by a van der Waals force [38]

$$F_{\mathrm{VdW}} \approx \frac{1}{6}\frac{AR}{D^2} = \frac{1}{6}\frac{A}{R}, \tag{3}$$

where D is the distance of the sphere from the substrate (measured from its center) and $A \approx 10^{-19}\,\mathrm{J}$ is a typical Hamaker constant. For the sphere in contact with the surface, we have $D = R$. According to Eq. (2), this force exerts a strain

$$\frac{\Delta h}{h} = \frac{P}{E} \approx \frac{F_{\mathrm{DNA}}/(\pi R^2)}{E} = \frac{1}{6\pi}\frac{A}{ER^3} \approx 0.02 \tag{4}$$

within the molecule. This is an appreciable strain but results in a deformation of only 0.04 nm and thus too low to explain the observed difference between theoretical and experimental data.

Another possible source for interaction of the DNA with the substrate is due to the electrostatic charges (Fig. 3B). In fact, on a nanometer scale, the electrostatic interaction is the one with the highest strength, as well as the longest range compared to other relevant forces. Moreover, with an effective charge density in aqueous solution of one fundamental charge e (negative) per base pair ($1e/0.17\,\mathrm{nm}$, corresponding to a charge density of about $2e/\mathrm{nm}^3$), DNA is about as highly charged as a linear polymer can be. Electrostatic forces are very high in aqueous solutions and keep molecules away one from each other. This electrostatic interaction binds the molecules to the substrate. A precise calculation of these forces for DNA molecules adsorbed on a substrate is out of the scope of the present paper. A sphere with a charge Q on a conducting surface (it has been shown that mica can be a good surface conductor due to the adsorbed water layer [39]) experiences an attractive electrostatic force

$$F_{\mathrm{el}} \approx \frac{1}{4\pi\varepsilon_0}\frac{Q^2}{4R^2}. \tag{5}$$

If a charge density as in solution is assumed, and considering again a diameter of the DNA of 2 nm, a relation analogous to Eq. (4) results in a strain of about 3 within the molecule, which is rather unphysical. Presumably when the sample is rinsed and dried, most of the charges are lost resulting in a lower attractive force. With about 1/4 of the total charge, a compression of about 0.4 nm is obtained from this simple model. We therefore believe that electrostatic forces may play a crucial role in DNA–surface interaction and that it may represent an important cause for the low height of the molecules measured in air by SFM. For our experiments performed in water, we believe that the electrostatic interaction is negligible because most of the positive charges are removed from the mica surface. Therefore, even though in water the DNA molecules are more charged, the total electrostatic interaction with the substrate is reduced considerably in aqueous solution as compared to air. In fact, this explains why most of the molecules do not remain attached to the surface: since the electrostatic interaction is the strongest interaction, it is the driving force for adhesion of the molecules to the substrate. Since this force is less in water than in air, less molecules remain adsorbed in water than in air.

4. Conclusions

A low value for the height of the DNA in SFM measurements has been a reality for the last 10 years. In this period of time, arguments like tip deformation, dehydration, and salt layers have been put forward as causes for the low height of the DNA. However, to the best of our knowledge, no clear evidences supporting any of these causes

has been presented. In this work, we clearly show that, in ambient conditions, DNA molecules are usually embedded in a salt layer. This affects the height in 0.8 ± 0.2 nm, but is not enough to recover the theoretical height. In our experiments, tip deformation is not the reason for the lower height since no contact is present in the non-contact tapping mode. Dehydration seems not to be a lowering factor either. We propose that the attractive interaction of the molecule with the substrate induces a strain that compresses the DNA molecule. Van der Waals interaction seems to be too weak for the measured effect, but electrostatic interaction is strong enough. A precise determination of the electrostatic interaction and its effect on the DNA molecules seems difficult, but would be very helpful in clarifying the important topic of DNA height in SFM measurements. In our experiments performed in aqueous solution, we find a height of 1.4 ± 0.3 nm at $F_N \sim 300$ pN and 1.8 ± 0.2 nm at $F_N \sim 100$ pN. This deformation is compatible with a simple model where the DNA is treated as an elastic continuum. Therefore, we believe that the measured height difference is mainly due to elastic deformation induced by tip–sample interaction.

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