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DEPOSITION OF SUPERCOILED DNA ON MICA FOR SCANNING FORCE MICROSCOPY IMAGING

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Abstract

The deposition of DNA molecules on mica is driven and controlled by the ionic densities around DNA and close to the surface of the substrate. Dramatic improvements in the efficiency and reproducibility of DNA depositions were due to the introduction of divalent cations in the deposition solutions. The ionic distributions on DNA and on mica determine the mobility of adsorbed DNA molecules, thus letting them assume thermodynamically equilibrated conformations, or alternatively trapping them in non-equilibrated conformations upon adsorption.

With these prerequisites, mica does not seem like an inert substrate for DNA deposition for microscopy, and its properties greatly affect the efficiency of DNA deposition and the appearance of the molecules on the substrate. In our laboratory, we have some preliminary evidence that mica could also participate in DNA damage, most likely through its heavy metal impurities.

Key Words: Scanning force microscopy, atomic force microscopy, supercoiled DNA, DNA spreading techniques.

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Introduction

Freshly cleaved mica is the substrate of choice for imaging DNA with the scanning force microscope (SFM) [7, 8, 24, 34]. DNA is a polyelectrolyte with negatively charged phosphates exposed on its cylindrical surface. Mica also exposes negative charges on its planar surface. When placed in solution, these distributions of negative charges are counterbalanced by hydrated cations. It is not surprising that when DNA depositions on mica are attempted from traditional buffer solutions, mainly composed of monovalent ionic species, little adsorption of DNA on the surface occurs. Deposition of DNA from pure water (or very low ionic strength solutions) results in poor reproducibility of the specimens. Sometimes, to force adsorption, DNA in water is dried onto the substrate, leading to problems due to the coprecipitation of the residual salts along with the DNA molecules, and still to irreproducibility and non-homogeneity of the specimens. In the last few years, the situation has changed drastically and reliable protocols for DNA deposition have been made available [7, 8].

Two different basic approaches were followed: make the mica surface positively charged, or bridge the negative charges on DNA to those on mica by divalent cations. Lyubchenko *et al.* followed the former approach by chemically modifying the mica surface with aminopropyltrimethoxy silane (APTES) [5, 16, 17]. As an alternative to the above mentioned methods, layers of cationic detergents like cetylpyridinium chloride (CP) [30] or benzyldimethylammonium chloride (BAC) [29] were deposited on mica, in order to make DNA deposition a favored process. This paper deals only with the approach that uses dicovalent cation–assisted DNA deposition on bare, freshly cleaved mica. This paper is an overview of the most recent results we obtained on this topic (which are reported in detail elsewhere), and it presents a few preliminary ideas on modeling charge distributions on mica in different ionic conditions.

The paper is organized in four sections. The first reports and discusses the outlines of the most commonly used deposition protocol for imaging DNA molecules in air. The second covers the equilibration of the struc-

tures of the DNA molecules on mica. Upon their deposition, DNA molecules are transformed from three-dimensional into two-dimensional objects. Two limiting cases for the molecular mechanism of deposition can be described. The molecules are able to approach the surface, search among their accessible states, and equilibrate before being captured in a particular conformation when the sample is dried; alternatively, the chain segments of the molecules are trapped at strongly binding sites as soon as they approach and touch the surface. In this latter case, the conformations that the molecules reach on the substrate are determined by the ways they take to approach and to bind to the surface.

Rivetti *et al.* [27] have studied the magnesium assisted deposition of linear DNA molecules on mica. The shapes of the imaged molecules were analyzed using polymer chain statistics. Rivetti *et al.* [27] found deposition conditions that allow DNA molecules to equilibrate on freshly cleaved mica. In our laboratory, we addressed the problem of the equilibration of supercoiled DNA molecules by bringing into play their topology. We evaluated the time scales of molecule motions on mica under buffer.

The capability of seeing the molecular movement of DNA molecules on mica in real time opens the perspective of shifting SFM from visualization of static biological structures to the study of processes. This requires the capability of modulating the strength of attachment of the molecules to the substrate in a way that depends on the experiment to be carried out. Modeling the electrostatic forces which are brought into play during deposition processes can strongly support and drive the tailoring of the experimental conditions for the biological process we want to observe. In the third section of this paper, an approach is suggested to model distributions of the positive counterions close to the negatively charged surface of mica in different ionic conditions.

In the fourth section, one further topic relevant to the main subject of this review paper is outlined. Protocols for sample preparation and imaging conditions should ensure that damage to the structure and the chemistry of the DNA molecules is ruled out. Such damage might alter the structures and the processes that can be imaged and studied with SFM. We obtained some evidence that mica can catalyze damage of the structure of DNA molecules adsorbed on it (strand nicking). This topic was addressed by bringing again into play the topology of supercoiled DNA.

Deposition Protocols for Imaging DNA Molecules on Mica

When a drop of DNA in water is deposited on mica and blown dry, most of the molecules are swept away with the water since they are not strongly attached. Most of the DNA depositions are not reproducible. It has been reported that DNA molecules in water tend to aggregate on mica, and in order to avoid this aggregation the sample must be blown dry immediately after the deposition [5]. Low resolution imaging and the appearance of large molecular networks was reported also when DNA was deposited from a solution containing ammonium acetate [33] or from a Tris(hydroxymethyl) aminomethane (Tris) - ethylene-diamino-tetraacetic acid (EDTA) buffer [20].

It was found that divalent cations promote DNA binding to mica: this result is obtained either by pretreating mica with magnesium acetate [5, 6, 10, 33, 34], barium chloride [33], calcium acetate [33], nickel chloride [12], or by just adding them to the deposition solutions.

In particular, $MgCl_2$ was added to deposition buffers of various compositions [4, 11, 14, 25, 32, 35, 36]. In their remarkable paper on adsorption of DNA to treated and untreated mica, Bezanilla *et al.* [5] reported a quantitative comparison of the density of the adsorbed molecules on the mica surface resulting from the deposition of DNA solutions in different buffers. Four main results obtained by those authors must be highlighted.

(1) The molecular deposition density of 0.4 molecules/μm^2 obtained from a 1 μg/ml DNA solution (approximately 5×10^{-10} M) in 0.1 mM Tris, 0.01 mM EDTA buffer drops to zero when the Tris and EDTA concentrations are raised to 10 mM and 1 mM, respectively. Since Tris is a positively charged buffer, the total ionic concentration on mica is increased, and the counterion atmosphere on DNA surface is affected as well.

(2) The addition of 10 mM $MgCl_2$ to 40 mM Tris leads to a great improvement of the deposition which can be, again, accounted for on the basis of the charge densities distributions on mica and DNA.

(3) The addition of 50 mM KCl to the Tris-$MgCl_2$ solution causes the molecular deposition density to drop to a value five times smaller. The molecular deposition density critically depends on the ratio between the divalent and monovalent ions in the deposition solution. Those ions compete on the screening of the negative charges on the surfaces of both mica and DNA. This result suggests that it could be possible to modulate the strength of DNA anchoring to mica and to control its attachment and detachment simply by a careful tailoring of the charge distribution with the aid of monovalent and divalent ions only, in different molar ratios. Such an achievement would be remarkably important for further developments in the application of SFM to studies of biological processes in real time.

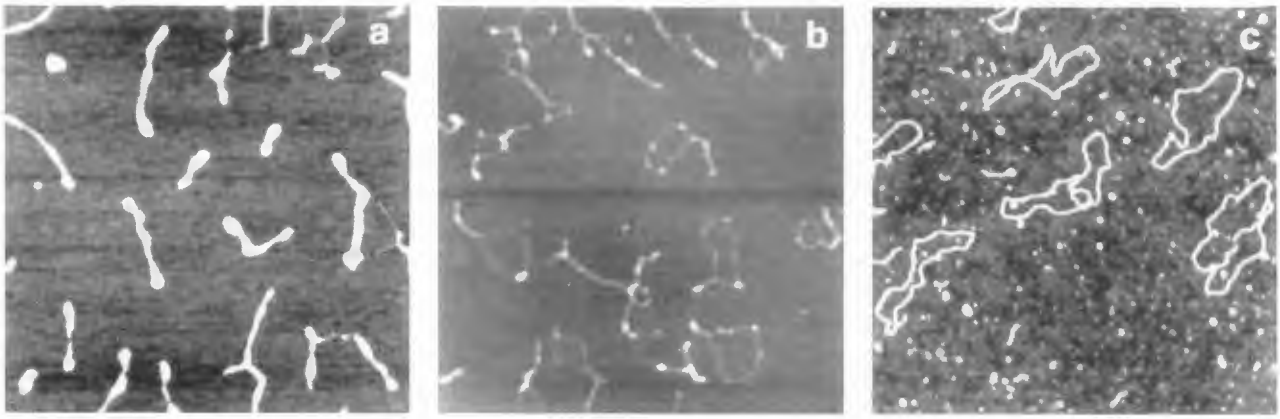

Figure 1. Single DNA molecules at three different steps of their opening process: (a) still in their tightly-close supercoiled structures, rod-like or branched; (b) with loops locally opened and variously enlarged; (c) with shapes already opened. Bar = 500 nm.

(4) HEPES {(N-2-hydroxyethyl) piperazine-N'-[2-ethanesulfonic acid]} buffer is more effective than Tris. The latter is positively charged in its undissociated form while the former is a zwitterion [5].

All these results made DNA deposition for SFM imaging no longer a problem. Now DNA can be reproducibly imaged in air preparing samples as follows. DNA is dissolved, in nM concentrations, in the deposition buffer (1-100 mM HEPES or Tris) containing 1-10 mM divalent cations, like Mg^{2+} or Ca^{2+}, or Ni^{2+}. A few microliters of this solution are deposited on freshly cleaved mica, and the molecules are allowed to diffuse from the solution to the substrate for 1–3 minutes. Rivetti *et al.* [27] have proved that the kinetics of deposition is governed by the diffusion. They measured the molecular surface density after different deposition times and found that the data fit the theoretical predictions for a process which is solely diffusion-controlled, and for which the molecules bind irreversibly to the substrate.

In our laboratory, after DNA deposition, the samples are either directly rinsed with water, blotted off, and blown dry with nitrogen, or immersed and equilibrated in a buffer solution before being dried [21]. The equilibration is best carried out in the dark and under argon, and all solutions used are previously bubbled with argon, in order to minimize the production of hydroxyl radicals (see **Mica catalyzes the nicking of DNA** for the explanation of this procedure). The equilibration time is chosen according to the molecules in the sample; the time scale of the equilibration of supercoiled molecules is much longer than that required for linear DNA molecules (see next section).

SFM imaging of DNA in liquid is based upon the same achievements on DNA deposition for imaging in air.

Equilibration of Supercoiled DNA Molecules on Mica

We addressed the problem of the equilibration of supercoiled molecules on mica by combining SFM with DNA topology.

Supercoiled DNA molecules in solution relax to open circular shapes when even just one of the covalent bonds along the chain is nicked, so that one strand can rotate freely about the other at the nick site, and the molecule can release the superhelical tension [3]. When DNA molecules adsorbed on a surface are nicked, they need a certain mobility from the surface to relax their supercoiled state. The relaxation of nicked supercoiled DNA molecules adsorbed on mica needs the release of two topological constraints: the looping of the two polynucleotide strands and the binding to the substrate.

On this basis, we have designed an experiment where the DNA molecules were nicked by a "flash" of hydroxyl radicals right after being deposited on mica. We have then followed their equilibration from the tightly supercoiled form to the open relaxed one, the most stable conformation for nicked molecules. In this case, the opening of the molecules depends only on the release of their binding to the substrate, thus being a measure of the mobility and diffusional properties of the molecules on mica [28]. After their deposition on mica, the deposited DNA molecules were nicked by immersing the mica

discs in a dish with approximately 50 ml of 4 mM HEPES and 1 mM $MgCl_2$ (deposition buffer), immediately adding 500 μl of H_2O_2 (3%) and irradiating for 90 seconds with three 15 W Hg/Xe lamps. The discs were removed, touched once to a 10 μl droplet of water and then immersed face-up and left for different times in a dish with the same deposition buffer. The samples were rinsed with water, blotted off and blown dry, and then imaged in air with the tapping-mode SFM. By imaging mica discs dried after different dipping times, we could follow the opening of the molecules, and evaluate the time scale of the molecular equilibration (B. Samorì *et al.*, unpublished results). The images of Figure 1 are focused on DNA molecules at three different steps of their equilibration process: (a) still in their tightly-closed supercoiled structures, rod-like or branched, (b) with loops locally opened and variously enlarged, but still supercoiled, and (c) with shapes already opened. The very compact conformation of the supercoiled molecules in the images could possibly be due to condensation conditions met during the dehydration of the specimens, in addition to the highly coiled state [31]. We have never found any condensed state when it was sure that no intact supercoiled molecules were left, regardless of the dehydration step. As of our experiments, about 20 hours were required to have completely equilibrated molecules. Rivetti *et al.* [27] had equilibration of linear molecules in about two minutes. This is most likely due to the fact that equilibration of linear molecules is a quasi-2D process, while equilibration of supercoiled molecules is a 3D process, with an activation energy which is expected to be much higher. This experiment with supercoiled molecules is bringing DNA topology into play to ensure that very minor and transient motions of single adsorbed molecules can be trapped and detected more easily than with any other physical technique or even with direct SFM imaging in solution [28].

Charge and Counterion Distributions on Mica and on DNA

Mica

Ruby mica (muscovite) is a phyllosilicate; it is cleaved just before DNA deposition. The lamellar cleavage takes place on a tetrahedrally coordinated sheet of composition $(Si, Al)_2O_5$ which exposes a layer of basal oxygens with a structural imbalance of charge. This imbalance is due to isomorphous replacement of the cations coordinated in this tetrahedral sheet or in the octahedral layer which is located below it. Where aluminum, which occurs as Al(III), replaces Si(IV), it renders the overall charge negative by one unit. This charge imbalance is neutralized in the solid state by interlayer cations such as potassium or sodium [9]. In aqueous solution, these cations are replaced by the dominant aqueous hydrated cations. The theoretical lattice imbalance is taken as the number of surface sites per square meter $N_S = 2 \times 10^{18}$ sites/m^2 of surface, or one charged site per 46.8 Å^2 [22]. This leads to a surface charge density $\sigma = 0.34$ Cm^{-2}.

The charged surface is balanced by a region of counterions. Some of them can be considered bound, usually transiently, to the surface within the so-called **Stern layer**. They can exchange with other ions in solution, and their lifetime on the surface can be as short as 10^{-9} seconds or as long as many hours. The other counterions form an atmosphere in rapid thermal motion close to the surface.

On the basis of chapter 12 of reference [15], three main aspects related to DNA deposition can be pointed out while describing the ionic distribution on a surface like that of mica. The first is about the total ionic concentration on the surface, the second about the addition of divalent cations to the solution, and the third about the effects of pH or calcination procedures on the surface charge density.

(1) DNA is deposited on mica from a buffer solution containing different electrolytes. The total concentration of ions on the mica surface in contact with an electrolyte solution containing different types of ions i (with charge $\pm$ zi) depends solely on the surface charge density and the total ionic concentration reaches a 33 M local concentration.

The **total concentration of ions on the mica surface** is described by

$$\sum_i \rho_{0i} = \sum_i \rho_{\infty i} + \sigma^2 / 2\epsilon\epsilon_0 kT \qquad (1)$$

where ρ_{0i} is the concentration of the i th ion type on the surface; ϵ is the dielectric constant; ϵ_0 is the permittivity of free space; k is Boltzmann's constant; $\rho_{\infty i}$ is the concentration of the i th ion type in the bulk (far from the surface), which is of the order of magnitude of a few mM in the most common conditions of DNA deposition.

For a surface with $\sigma = 0.34$ Cm^{-2}:

$$\frac{\sigma^2}{2}\epsilon\epsilon_0 kT =$$

$$\frac{(0.34)^2}{2} \times 78.5 \times (8.85 \times 10^{-12}) \times (4.04 \times 10^{-21}) =$$

$$2.06 \times 10^{28}$$

which is equivalent to about 33.5 M, since 1 M = 1 mol dm^{-3} = 6.022×10^{26} molecules per m^3. The whole ion concentration on the surface is therefore determined by the surface charge density and does not depend on the bulk electrolyte concentration.

The ion distributions far from the surface are determined by the type and concentrations of the ions in solution. Cations of higher charge or present at higher concentration are more effective in screening the surface charges, causing the electric potential to drop to zero more sharply and the ion distributions to approach the bulk concentrations closer to the surface. The Gouy-Chapman theory [15] can be used to estimate the drop in potential and consequently the distribution of charges far from the surface. The calculated ionic distributions close to a surface of mica immersed in water or in a 1:1 electrolyte are plotted in Figure 2. It can be noticed that the magnitude of the negative potential close to the surface is much higher if the surface is immersed in water, where the screening is lower. The concentrations of cations close to the surface is always higher for mica immersed in a millimolar electrolyte solution than for mica in water, other than right onto the surface, where the total charge in the usual electrolyte solutions should be practically the same regardless of the electrolyte concentrations in the bulk. It could be implied that in case of a sudden drop of the electrolyte concentration in contact with a surface of mica, heavy counterions (such as, DNA) that stay very close to the surface, experience a strongly increased attraction and practically collapse onto the surface. If DNA molecules were part of those counterions close to the surface, they would be strongly bound to the surface, once the ionic strength of the solution was suddenly decreased and they were close enough not to be washed off.

(2) DNA deposition on mica can be highly improved by **adding a divalent ion like Mg^{2+}** in a concentration of a few mM in the DNA solution. When divalent ions are present in a solution in contact with the mica surface, a high local concentration of them is reached on the surface. In the presence of monovalent ions, even with much higher bulk concentrations, the local concentration of the divalent ions remain higher. The charge density of an ion i at the surface is given by

$$\rho_{0,i} = \rho_{\infty i} e^{-zie\psi_0/kT} \qquad (2)$$

Solutions for depositing DNA commonly contain ions like Mg^{2+} and monovalent electrolytes, like Na^+; typical concentrations are 1 mM and 4 mM, respectively. The Grahame equation [15, chapter 12] allows calculation of the surface potential ψ_0 = -134 mV for a surface with σ = 0.34 Cm^{-2} and the above mentioned bulk electrolyte concentrations. From eq. (2), the following concentrations at the surface of the mica are obtained: $[Mg^{2+}]_0$ = 33 M, $[Na^+]_0$ = 0.73 M, and $[Cl^-]_0$ = 2.8 x 10^{-5} M.

The most dense ions at the surface are, of course, the counterions, and their excess concentrations over those in the bulk are:

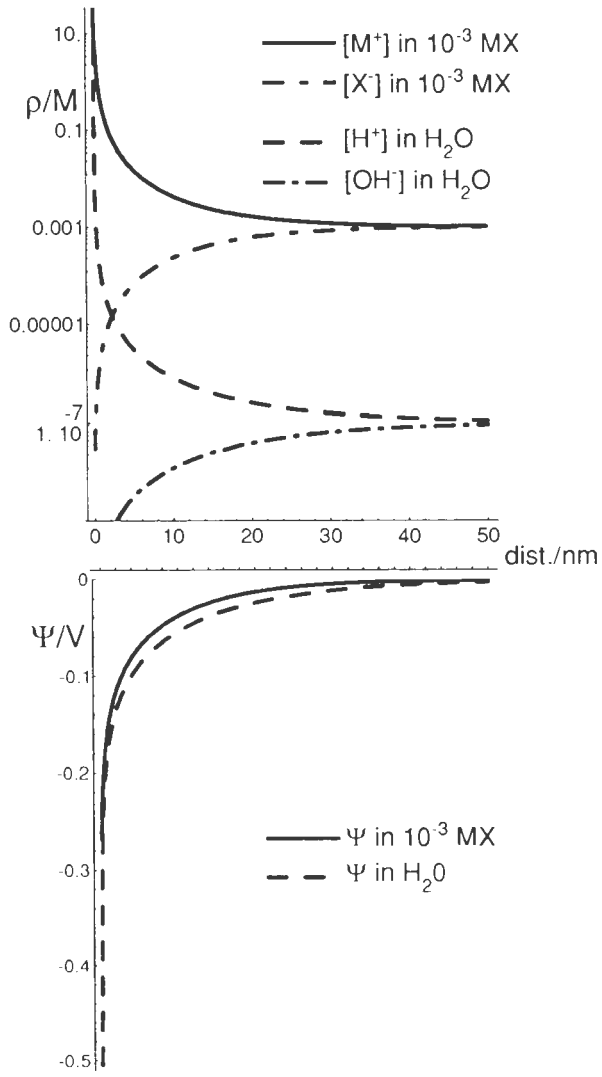

Figure 2. Plot of the ionic densities and the potentials close to the surface on mica. On the upper half of the Figure are the ionic densities of cations and anions for a 10^{-3} solution of a MX electrolyte and the ionic densities for water. On the lower half are the potentials close to a surface of mica in 10^{-3} MX or in water.

(a) dependent solely on the surface charge density,

(b) constituted almost exclusively by the divalent ions, in spite of their being less concentrated in the solution than the monovalent ions, and

(c) high enough to balance most of the surface negative charges.

Because of the higher concentrations of the divalent cations on the surface, adding small amounts of them lowers the magnitude of Ψ_0 about one hundred times more effectively than increasing the concentration of the monovalent salt. Indeed Ψ_0 is determined solely by the

divalent ion concentration, once their concentration is greater than about 3% of monovalent salt.

(3) Changes of the pH of the solution and treatments of calcination can change the surface charge density of mica [22]. Ionizable silanol groups can be formed as a result of hydrolysis of Si-O-Si in the tetrahedral layer. When metal ions are present in solution, the following surface equilibria exist on the mica basal plane:

$$SH \overset{K_a}{\rightleftharpoons} S^- + H^+$$

$$SH + M^+ \overset{K_M}{\rightleftharpoons} SM^+ + H^+$$

$$N_S = [S^-] + [SH] + [SM]$$

where S^- is a surface site, H^+ is a potential determining ion, M^+ is a metal ion, and K_a and K_M are the acid and metal ion dissociation constants.

The value of pK_a was estimated as 3.7 in reference [22] and as 6 in reference [23]. The decrease of the negative charge of mica below pH 6 is consistent with the decrease of the magnitude of the charge potential measured by an electrokinetic technique (electro-osmosis) [22]. Most of the values for pK_M range between 2 and 4 for alkali ions on layered silicate minerals [22, and references therein].

Calcination of mica with Li^+ or Mg^{2+} ions reduces N_S by one or two orders of magnitude, and at least 90% of the charge in the lattice is neutralized by the calcination procedure [22].

DNA

In a solution of a low molecular weight electrolyte, the ions can be assumed to move freely through a continuum constituted by the solvent, and in the Debye-Hückel picture, their distribution is determined by the competition between the electrostatic interaction and the free translational motion; this gives rise to an ion atmosphere of spherical symmetry. In a polyelectrolyte solution, on the other hand, the counterions are principally located along the macromolecular chains. Whatever the concentration of the polyelectrolyte solution, on the molecular level, there will be always regions of high charge concentrations, and there the interaction with the counterions will be much stronger than in the case of a comparable ordinary electrolyte solution. For DNA, like in the case of the surface of mica, we are dealing with a strong local confinement of negative charges which are counterbalanced by oppositely charged ions. Some of the counterions can be considered territorially bound to the surface of DNA, while others are in rapid thermal motion around it. The charge density of DNA is usually characterized by the non-dimensional parameter $\xi = e^2/\epsilon kTb$, where b is the average axial distance between charges in the polyion, and ϵ is the dielectric constant of the pure solvent [1, 18]. The fraction of counterions that are physically dissociated from the polyanion in solution is designated by α. An incomplete physical dissociation of counterions reduces the structural charge density ξ to the effective value $\lambda = \alpha\xi$.

Following the review by Anderson and Record [1] in parallel with what we have just done for mica, two aspects are pointed out: the first is relevant to the local counterion concentration on DNA surface, and the second to the effects of an addition of a few mM Mg^{2+} to the deposition buffer.

(1) The Manning theory applied to DNA ($\xi = 4.2$) predicts that the charge fraction is $\alpha = \xi^{-1} = 0.24$, since $\lambda = 1$ after counterion condensation in a monovalent salt solution. The territorially bound counterions (the fraction $1 - \xi^{-1} = 0.76$ per phosphate) are predicted to occupy a region extending radially from the surface of the DNA molecule (radius 10 Å) for a distance of 7 Å. In this region, the local counterion concentration is 1.2 M. The local concentration gradient is very steep, especially at a low salt concentration. Both this local counterion concentration and its spatial gradient are relatively insensitive to changes in the bulk salt concentration, as long as it is higher than the equivalent bulk concentration of the charges of the polyelectrolyte and lower than the local charge concentration at the polyion surface, i.e., lower than 1.2 M. Within the limit of infinite dilution of the electrolytes, the local molar concentration of the positive counterions on the surface retains large values, of the order of 1 M [1, 18]. The electrostatic potential near the surface of the DNA was also found to be relatively constant from about 20 mM to 0.2 M electrolyte concentration [13].

(2) When divalent ions are added, they compete with the monovalent counterions for binding to DNA. The binding constants (K) are extremely sensitive to the monovalent salt concentration. The theory predicts that ln K decreases linearly with the logarithm of the electrolyte concentration, with a slope very close to 2, which is the charge of Mg ions [1, 18]. As the bulk salt concentration approaches a 1.2 M counterion concentration in the local phase, the standard free energy of binding approaches zero and K approaches 1 M^{-1}.

The divalent counterions are bound almost entirely in the territorial mode [18]. The charge fraction α drops in the DNA from 0.24 with monovalent ions to 0.12 with divalent ions. At a very high Mg^{2+} concentration, a charge reversal has been reported [1].

A deeper knowledge of the physical-chemistry of the mica surface and of DNA can further help explain the recent improvements in DNA deposition protocols, and improve the reliability of SFM analyses of DNA structure and function.

Mica catalyzes the nicking of DNA

The experiment described in the second section of this paper shows that strand breaks (nicks) and transient desorption of supercoiled DNA molecules from the substrate are enough to release the superhelical tension and relax the supercoiled molecules to open ones. When 20 μl of a 1 μg/ml supercoiled pBR322 solution in 4 mM HEPES, 1 mM $MgCl_2$ are deposited on a disc of freshly cleaved mica, and then left immersed face-up for 24 hours in a dish with 50 ml of double-distilled water, the DNA molecules imaged after specimen drying are completely relaxed. In this case, no DNA strand breaks are intentionally induced. When, on the other hand, the same experiment is performed using only double-distilled water that had been bubbled with argon for a long time, and performing the sample wash in the dark, the relaxation of the molecules is drastically reduced (B. Samorì *et al.*, unpublished results). Low salt conditions, like those met by DNA molecules during the sample dipping in water, are expected to loosen the DNA supercoiling [2, 30].

DNA nicking is therefore very likely to be blamed for molecule relaxation; hydroxyl radicals are the main source of strand breakage in DNA [19], they are generated in water in the presence of O_2 in a Fenton-like reaction catalyzed by transition metals [26]. The flow of argon in the latter experiment had removed the oxygen from the water preventing it from generating radicals.

We have electrophoretic evidence that DNA molecules get nicked much faster than usual when they are mildly shaken in the presence of powdered mica. Evidence was also obtained that mica catalyzes the formation of hydroxyl radicals through its transition metal impurities (B. Samorì *et al.*, unpublished results).

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Discussion with Reviewers

D. Keller: Did the supercoiling relaxation time depend on how strongly nicked the DNA was? How does it depend on the ionic composition of the buffer? Do the authors have a model for DNA motions on the surface?

Authors: In solution, upon nicking a "wave" of supercoiling relaxation starts from the nick site and involves the entire molecule. In case of multiple quasi-simultaneous nicks, several relaxation waves could interest the same molecule and speed up the relaxation.

For molecules adsorbed on a surface, the speed of relaxation is thought to depend both on the number of nicks and on the strength of adhesion. Between two strongly anchored points of a DNA strand there is a closed topological domain, and the coiling of the DNA between those points could be considered independent of what is happening outside. The bigger the molecule-surface topological domains, the more effective is a nick in determining the relaxation of a molecule. The strength of adhesion and the possibility of 2D diffusion of a DNA molecule on the surface determine how big and how transient are the molecule-surface closed topological domains, thus having a profound influence on the speed of relaxation of a supercoiled molecule.

The ionic composition of the buffer is certain to have a primary role in all the phenomena under study. The possibility of part of a molecule to transiently detach from the substrate, to diffuse two-dimensionally, shifting between adjacent and equivalent binding sites, or

not to diffuse at all is certainly determined by the properties of the substrate and of the solution in contact with the substrate.

Up until now, we have not investigated the possibility of inducing a controlled number of nicks to study how the relaxation kinetics are affected; we are in the process of studying the mobility of DNA molecules on the surface.

D. Keller: Can the binding-unbinding kinetics of a single or average "binding site" be estimated? Can a time scale for movement of segments be estimated? Have the authors tried to do the same type of experiment with molecules that have a double strand cut, by watching how fast the end-to-end distance increases with time?
Authors: We already had in mind and performed this very enlightening experiment that we had designed exactly in the perspective suggested by the reviewer. The outcome of this experiment was communicated at the European Congress on Microscopy, Dublin (August, 1996), and have been accepted for publication [37].

Reviewer IV: The authors state they can trap the transient motions of single molecules. What about the forces introduced by water meniscus during drying processes?
Authors: We are currently investigating on the effect of drying processes on the shapes we observed for supercoiled molecules. Imaging in fluid could avoid problems with dehydration, but in addition to being more technically challenging, it would imply repetitive scans over the fragile hydrated supercoiled molecules with the danger of damaging and moving them. See also the **Notes added in proof**.

J. Vesenka: Would the authors like to speculate on what role ambient humidity plays in the stable imaging of DNA on mica, which has been confirmed to have a profound effect by several laboratories [37, 38]?
Authors: As pointed out by many authors, high ambient humidity drastically worsens imaging contrast in SFM. More important for the scope of this paper, high ambient humidity appears to weaken the binding of dehydrated DNA molecules to mica, allowing the probe of the contact-mode SFM to damage the DNA molecules and move them around on the substrate, as has been reported in [37].

Reviewer IV: Is the catalysis of strand nicking by mica due to mica surface or by ions dissolved from mica? The idea of strand nicking stands against the number of experiments of gel electrophoresis of supercoiled DNA which are routinely done in many laboratories using water with dissolved air; please comment.

Y.L. Lyubchenko: Experiments with powdered mica are not convincing. In addition, the DNA solution in mica suspension was shaken, a procedure that can also facilitate the cleavage of trapped DNA molecules.
Authors: At this point, it is not easy for us to discriminate between the possibility that DNA is nicked as a result of dissolved metal ions or of mica itself. As we point out in the text, Fenton-like reactions can produce hydroxyl radicals in solution, with metal ions as catalyzers. It is known that layered silicates can work as ion exchangers, and the effect of local hydroxyl radicals generation could be enhanced by the localization of DNA molecules close to the source of short lived radicals. The removal of oxygen from solution seems to solve the problem, thus hinting to a Fenton-like reaction.

We do agree that the surface properties of powdered mica are different from those of a freshly cleaved lamina, yet it makes sense to us that all the above mentioned effects of DNA adsorption and ion mobilization could be strongly amplified by the powdering, and so, easier to detect. We consider standard gel-electrophoresis fairly safe for supercoiled DNA, due to the fact that there are no heavy metal ions in solution, and the radicals generation could only be very limited.

Y.L. Lyubchenko: There is an alternative explanation of the data obtained by the authors. When large volume of pure water is placed above the sample, the concentration of Mg ions decreases, stimulating the uncoiling of DNA, and eventually supercoiled molecules may look like topologically relaxed ones. Please comment.
Authors: The uncoiling of supercoiled DNA molecules placed for a long time in water bubbled with argon is limited compared to what happens during a comparable wash in normal water, letting us think that there is something more than ionic strength dependent uncoiling. See also the **Notes added in proof**.

Additional References

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Notes added in proof: Nowadays, SFM images of

nucleic acids can also be collected in fluid environments. In our experience {see our paper on Applied Physics A, (1997) **66**: S585-S589}, the shape of supercoiled DNA molecules images in fluid never resembles the ones of the first part of Figure 1: the shapes are those of intertwined molecules, with no evidence of the strong aggregation effects evident images in Figure 1. It seems at this point very likely that the shape of the molecules described here might be the result of the dehydration process that all samples need to be subjected to in order to be observed by the SFM in air. While these dehydration processes poorly affect the shapes of linear molecules [21], the superhelical tension in circular molecules makes their shapes strongly dependent on changes of the ionic strength met during the preparation of the samples. We strongly recommend to image supercoiled molecules in fluid only.

These aggregation and possible water meniscus effects (see Discussion with Reviewer IV, above) could have contributed to the poor reproducibility of the data on the ability of mica to nick DNA obtained in the great number of experiments carried out by us also after the submission of this paper. Further reasons of the observed reproducibility of the data from one sample to another and also from one area to another in the same sample could be:

(1) The ability of mica to produce hydroxyl radicals depends on Fe impurities contained. When instead of ruby mica, clintonite was immersed in a water solution of a spin trap (DMPO) OH radicals were no longer detected by electron paramagnetic resonance (EPR; unpublished results).

(2) Mica surfaces present many defects {Wicks FJ, Yoller K, Eby RK, Hawthorne FC, Henderson GS, Vrdoljak GA (1993) Can. Mineral. **31**, 541-550}, which could also make non-saturated Fe accessible to both DNA and oxygen. This could also take place wherever the cleavage has not been perfect. Our investigation on the ability of mica to catalyze DNA cleavage is not conclusive, the experiments here reported should be repeated imaging the molecules in buffer, not in air.