Transverse fluctuations of single DNA molecules attached at both extremities to a surface

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We present a simple method to stretch DNA molecules close to a surface without any chemical modification of either the molecules or the surface. By adjusting the *p*H of the solution, only the extremities of DNA molecules are tethered to a glass coverslip made hydrophobic, while stretching is achieved using a hydrodynamic flow. These extended molecules provide a very favorable template for DNA-protein interaction studies by purely optical means. Pursuing these experiments requires first a full characterization of the thermally driven fluctuations of the tethered DNA molecules. For this purpose, these fluctuations were recorded by fluorescence microscopy and were analyzed in terms of normal modes. Our experimental results are well described by a model accounting for the nonlinear elastic behavior of the chain. Remarkably, the proximity of the molecules to a rigid surface does not alter the main features of their dynamics, and our results are in agreement with previous studies on extended DNA in viscous solutions.

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I. INTRODUCTION

Over the past several years, methods intended to manipulate single DNA molecules have been extensively developed. Most of them were aimed at studying the physical properties of these natural polymers as well as their interactions with their environment. Recently, many physicists working on DNA have shown growing interest in studying interactions between DNA and proteins such as enzymes. Advances in micromanipulation techniques have enabled the observation of enzymatic action by microscopy [1,2] and the measurement of forces exerted by individual enzymes on DNA, using, notably, magnetic beads or optical tweezers [3–5] and elongation in a flow [6]. The visualization of a single fluorescently labeled protein interacting with DNA remains, however, an experimental challenge. Only a few groups have combined micromanipulation of DNA molecules and visualization of a single labeled enzyme [7,8], but at the cost of complex experimental approaches.

In most of these experiments, the stretching of DNA molecules is first required. This usually involves either high-frequency ac fields, which allow stretched molecules to be positioned close to a surface [9], or the use of at least one bead coupled to one extremity of the molecule [3–5]. Electrostatic stretch and positioning of DNA is an efficient technique, which nevertheless requires the fabrication of vacuum-evaporated microelectrodes. Additional outermost electrodes and chemical modification of both DNA and surface are apparently necessary to ensure the success of the technique [9]. Such relatively complicated surface modifications might be a serious limitation on this method when used in experiments involving DNA and proteins. The finalization of protocols in these experiments indeed requires repetitive tests, which usually spoil the surfaces. Manipulation of DNA through surface modification should therefore avoid complex processes.

An alternative method of positioning and stretching DNA involves micrometer-sized beads, which are biochemically coupled to one or both extremities of the molecule. This technique is very fruitful but, unfortunately, the beads make the detection of a single fluorescent molecule difficult. While in epifluorescence they act as strong light scatterers, their size does not enable DNA to be set close to a surface and therefore prevents the use of evanescent wave microscopy (except with Yanagida's approach [8]).

In contrast, another technique, molecular combing, allows DNA molecules to be stretched on a surface without any chemical modification of either the molecule or the surface [10]. Recently, molecular combing was successfully applied to gene mapping using *in situ* hybridization [11] or restriction enzymes [12]. The drawbacks to this method are an overstretching of the molecules (about 150% of the initial contour length) and a strong interaction between DNA and surface, which, among other effects, inhibit the motion of enzymes along the backbone of the molecule. This technique was recently adapted to study transcription of extended DNA by a single RNA polymerase (RNAp) [13]. In this experiment, fluorescent nucleotides allowed newly synthesized RNA fragments to be detected by microscopy. Even if the dynamics of the protein were not observed, the authors convincingly demonstrated that an enzyme could work on DNA molecules stretched close to a surface. At this point, this experiment still suffers from some limitations. The mean length of molecules is difficult to adjust and it is close to the contour length of DNA. Such high stretching may alter the features of the transcription as well as those of other biological processes. Furthermore, several randomly distributed surface attachment points along combed molecules probably stopped the activity of the RNAp, and would certainly prevent the activity of other proteins.

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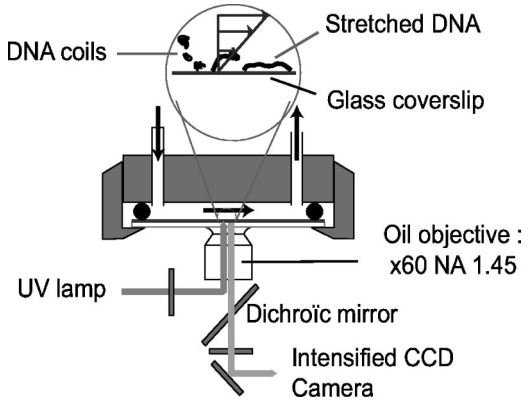

FIG. 1. Schematic representation of the experiment. DNA molecules were attached to a 22-mm-diameter glass coverslip, which formed one of the walls of a flow cell. The coverslip had been previously coated with polystyrene (5% solution in toluene) to make it hydrophobic. The gap between the two walls of the fluid chamber was about 150 μm. The cell was first incubated with 50 mM MES buffer, pH=6.0, for a few minutes. A solution of λ DNA in the same buffer was then injected with a flow rate of 2 ml/min. Fluorescent labeling of λ DNA was achieved by using the intercalating dye YOYO-1 (Molecular Probes). The pH of the solution was adjusted between 5.8 and 6.0 for optimal sticking of the DNA extremities. At lower pH, nonspecific sticking occurred along the molecules. After a few minutes, the cell was rinsed with the buffer. 1% β-mercaptoethanol, 200 μg/ml glucose oxidase, 40 μg/ml catalase, and 5 mg/ml glucose were added to the buffer to minimize photobleaching. The experiments were performed at room temperature. The chamber was placed on the top of an inverted microscope (Olympus) equipped with a X60 1.45 numerical aperture oil immersion objective. The stretched molecules were visualized using an intensified charge coupled device (CCD) camera (Pentamax, Roper Scientific).

II. EXPERIMENTAL APPROACH

Combining molecular combing and elongation in a flow, we developed a technique that allows us to tether only the two extremities of the DNA molecules to the surface. To achieve this tethering, we adjusted the pH of the solution containing DNA around 6 so that one end of a molecule would attach to a hydrophobic surface, a glass coverslip coated with polystyrene [14]. The molecule was next stretched using a hydrodynamic flow, which was maintained for a few seconds. The unbound extremity was then likely to stick to the surface (Fig. 1). Although the best results were obtained using polystyrene coated coverslips, this tethering was also achieved when using coverslips coated with PMMA (polymethylmethacrylate), PDMS (polydimethylsiloxane) or even a 150-μm-thick polystyrene sheet.

Crucially, it is possible, without untethering the molecules, to change the initial acidic morpholinoethanesulfonic (MES) acid solution for another with physiological pH, more appropriate to DNA-protein experiments (pH ranging from 7.0 to 8.0). In general, successive buffer exchanges could be done when sufficiently gentle flows were applied. The strength of the attachment of the extremities of the molecules, the absence of attachment points along the DNA, as well as the simplicity and the reproducibility of the experimental protocol, make this method particularly attractive when compared with techniques previously mentioned.

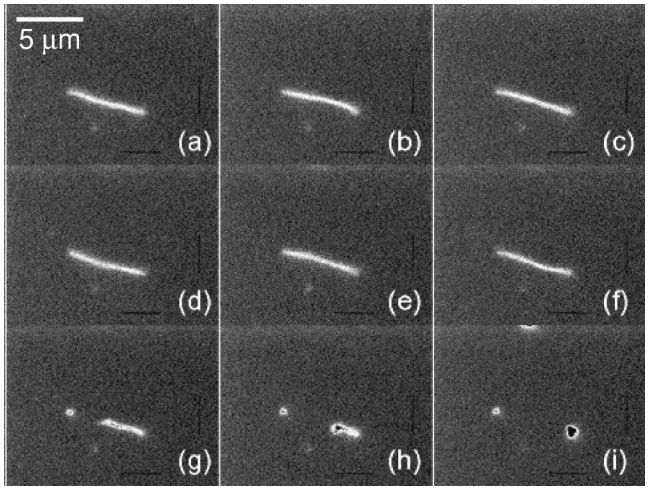

FIG. 2. (a)–(f): Thermally driven transverse fluctuations of a DNA molecule. The time between two consecutive frames is about 1 s. (g) and (h): The chain breaks, probably due to the photobleaching of the intercalating dyes and retracts because of the elasticity of the polymer. The time between these two frames is 100 ms. (i): Only the extremities of the molecule are stuck to the surface and are still visible.

We worked with λ-bacteriophage DNA (λ DNA), a 48, 502 base-pair molecule with an $L=16$ μm contour length. Fluorescent labeling of λ DNA was achieved by using the intercalating dye YOYO-1 (Molecular Probes), at a ratio of ten base pairs per dye molecule. This process increases the contour length of the molecule, which is about 18 μm after staining [6]. It results in only slight changes in the initial persistence length L_p of DNA, about 50 nm [15]. We noticed that the labeling of DNA by YOYO-1 is reversible, controlled by the presence of Mg^{2+} ions. Exchanging initial buffer with a solution containing Mg^{2+} ions (concentration above 5 mM), we observed that the fluorescence vanished, and did not reappear after injection of the initial buffer. A subsequent addition of YOYO-1 in a Mg^{2+} free solution allows DNA to be stained again.

The stretched molecules were observed by fluorescence microscopy, and we recorded 45 films using an intensified CCD camera (Fig. 2). The duration of a film was typically 10 s (with a time resolution of 20 ms), often limited by photobleaching. We measured on the first image of each film the end-to-end distance R of the attached molecules. The obtained value $R=7.7\pm2.3$ μm corresponds to a relative extension of $R/L=0.43\pm0.13$. We deduced from this extension the tension experienced by the molecules, whose value can be calculated in the wormlike chain (WLC) model [16] and estimated using a polynomial approximation [17]. A relative extension of 0.5 (0.7) is associated with a tension of 0.10 pN (0.25 pN). This tension is much smaller than the force experienced by DNA during molecular combing, about 500 pN [10]. A structural transition of the molecule during the process [18,19] is then prevented. In addition, this tension is weak compared with those exerted by enzymes, which is in the pN range [20], so that this moderate stretch-

ing should not interfere with their action.

In addition, the estimated extension R/L can be compared to previous experiments in which molecules were tethered to a surface and were stretched using a shear flow [6,21,22]. In our experiments, the Weissenberg number Wi [22], which characterizes the strength of the shear flow, ranged between 20 and 50. Based on previous experimental results, it should have yielded a relative extension between 0.5 and 0.7, larger than our measured values. This discrepancy is discussed in the last part of this paper.

III. TRANSVERSE FLUCTUATIONS

Due to the interactions with the buffer, stretched molecules exhibit thermally driven fluctuations, which were visualized in real time (Fig. 2). Full characterization of these fluctuations is essential for DNA-protein interaction experiments. For instance, the relative motion of an enzyme sliding or working on DNA needs to be differentiated from the overall motion that includes fluctuations of the chain. Similar fluctuations were observed in experiments involving DNA suspended in solution [23]. In these experiments, each extremity of a molecule was attached to a polystyrene sphere trapped in a double-optical tweezer. Recorded fluctuations were analyzed in terms of normal modes and it was shown that this analysis accounted for experimental results. The dynamics properties of extended DNA in solution were further studied [24] by taking into account the effects of tension and hydrodynamics on the fundamental relaxation time of the polymer. We follow a similar analysis in this paper. The main physical differences in our experiments are (i) molecules are close to a rigid surface and (ii) the viscosity of the buffer is about that of water [25]. We deduce from a simple model, accounting for nonlinear processes involved in the dynamics of the chain, analytical expressions for both amplitudes and correlation times. We then compare our experimental results with this model and with numerical simulations.

In order to analyze transverse fluctuations of DNA molecules, we used a ''bead and spring'' model, a common way to study polymer dynamics [26]. A DNA molecule is divided into N submolecules, called ''springs'' because of their entropic elasticity, which are connected by beads. The number N is chosen so that $L \gg L_0 \gg L_p$, where $L_0 = L/N$ is the length of a spring. The beads at the two ends of the stretched molecule are fixed, separated by length R. Although in our experiment the motion of the molecule is limited to the half-plane upon the surface, we neglect here this constraint. A Langevin equation is written for each bead

$$\zeta \boldsymbol{v}_n = \boldsymbol{T}_{n-1\to n} + \boldsymbol{T}_{n+1\to n} + \boldsymbol{\mathcal{L}}_n, \tag{1}$$

where $\boldsymbol{T}_{n-1\to n}$ is the elastic force exerted by the $(n-1)th$ bead on the nth bead. In the WLC model, the modulus of this force can be expressed as $T(r) = k\, r f(r/L_0)$, where $k = 3k_BT/2L_pL_0$ is a spring constant (entropic elasticity of an ideal chain), r is the distance between two adjoining beads, and $f(r/L_0)$ is a function that accounts for the nonlinear elasticity of the chain [17,27,28]

$$f\left(\frac{r}{L_0}\right) = \frac{2}{3} + \frac{L_0}{6r}\left(\frac{1}{(1-r/L_0)^2} - 1\right) + \sum_{i=2}^{7} a_i \left(\frac{r}{L_0}\right)^i. \tag{2}$$

The friction force-$\zeta \boldsymbol{v}_n$, where ζ is the friction coefficient of a submolecule, and $\boldsymbol{v}_n$ the velocity of the nth bead, is related to the random force $\boldsymbol{\mathcal{L}}_n$ through the fluctuation-dissipation theorem [26] $\langle \mathcal{L}_{n,i}(t)\mathcal{L}_{n',j}(t')\rangle = 2\zeta k_B T \delta_{ij}\delta_{nn'}\delta(t'-t)$, where i and j denote two components of $\boldsymbol{\mathcal{L}}_n$. The friction coefficient ζ has a complex dependence on the polymer's length and extension [24,29]. Because of the high stretching rate in our experiments, we use an expression for ζ, which corresponds to transverse friction over a rod of length L_0 and diameter d [26] $\zeta = 4\pi\eta L_0/\ln(L_0/d)$, where η is the viscosity of the solvent (about 10^{-3} kg m^{-1} s^{-1}) and d is the diameter of DNA (about 1 nm). In practice, the values of ζ for the coil and rod states are quite similar, and the nonlinearity of DNA elasticity has a much stronger effect than hydrodynamics on fluctuations [24,29].

To solve the set of nonlinear coupled equations (1), we assume that the mean distance R/N between two adjoining beads is large compared to the amplitudes of the transverse and longitudinal fluctuations, so that $f(r/L_0) \simeq f(R/L)$. Due to this approximation, our analysis differs slightly from that described in Ref. [24] and analytic results are then available. Equation (1) then becomes linear, and corresponds to beads connected by Hookean springs with a constant $kf(R/L)$. These equations may be solved by decomposing the motion into normal modes $NX_p(t) = \Sigma_{n=1}^{N} x_n(t)\sin(np\pi/N)$, where $x_n(t)$ denotes the transverse position of the nth bead. Each mode is characterized by its autocorrelation function [26]

$$\langle X_p(t)X_p(0)\rangle = \langle X_p^2\rangle \exp(-t/\tau_p), \tag{3}$$

where the quadratic amplitude $\langle X_p^2\rangle$ and the correlation time τ_p are given by

$$\langle X_p^2\rangle = \frac{L_p}{3\pi^2 p^2}\,\frac{L}{f(R/L)}, \tag{4}$$

$$\tau_p = \frac{8\eta L_p}{3\pi \ln(L_0/d) k_B T p^2}\,\frac{L^2}{f(R/L)}. \tag{5}$$

The $1/f(R/L)$ dependence of both the amplitude and time correlation is the expression of the nonlinear elastic (WLC) behavior of the DNA molecules. The quadratic amplitude of mode p does not depend on the number of beads and springs and decreases as $1/p^2$. The correlation time decreases equally as $1/p^2$, but depends slightly on L_0. For a given mode p, τ_p and $\langle X_p^2\rangle$ are related through $N\zeta\langle X_p^2\rangle = k_B T\tau_p/2$ as the time τ_p is needed for the friction to dissipate $k_BT/2$.

IV. EXPERIMENTAL RESULTS

This normal-mode decomposition using the sine basis has been shown to be a good approximation to describe the dynamics of an extended polymer in a solution [23] and we assumed that this was still the case in our experiments. The position of the molecule was interpolated for each frame of

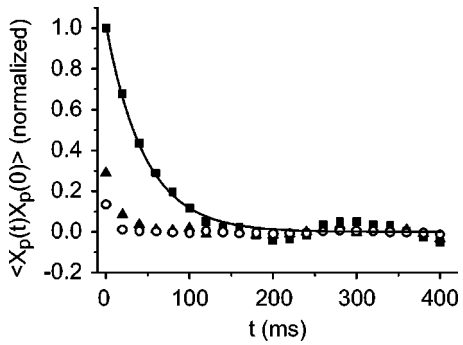

FIG. 3. Evaluation of $\langle X_p(0)X_p(t)\rangle$ for $p=1$ (■), $p=2$ (▲), and $p=3$(○) from our experimental data. Line: exponential fit for $p=1$.

the data films and the coefficient $X_p(t)$ and the related autocorrelation function were evaluated for the first three modes (Fig. 3). As predicted by the model, the shape of the autocorrelation function is exponential. For each film, we estimated τ_p by interpolating the data with exponential functions for at least the first two modes (the decay time of the third mode is on the order of the time resolution).

Averaging over all the films leads to the following results: $\langle X_1^2\rangle = 1.02(\pm 0.46)\times 10^{-2}\ \mu\text{m}^2$, $\langle X_2^2\rangle = 0.28(\pm 0.18)\times 10^{-2}\ \mu\text{m}^2$, $\langle X_3^2\rangle = 0.11(\pm 0.10)\times 10^{-2}\ \mu\text{m}^2$, $\tau_1 = 101 \pm 54$ ms, and $\tau_2 = 37 \pm 20$ ms. The correlation times are in good agreement with those reported for DNA molecules extended in solution [23], taking into account a factor about 20 between the viscosities involved in both experiments. The distributions of both amplitude and correlation time are broad, as illustrated for the first mode [Fig. 4(a)]. This cannot be attributed only to the dispersion of the relative extensions R/L, which would lead to a width of the distributions on the order of 20% of their mean values. This point is discussed in the last part of this paper.

For a given molecule, the ratio between two quadratic amplitudes or correlation times for different modes depends on p^2 but not on L, nor on R/L. In our experiments, the ratios $\langle X_1^2\rangle/\langle X_2^2\rangle$ and $\langle X_1^2\rangle/\langle X_3^2\rangle$ are, respectively, equal to 4.2 ± 1.0 [Fig. 4(b)] and 10.9 ± 3.4, in agreement with the expected values 4 and 9. The ratio between the times τ_1 and τ_2 is 2.9 ± 0.9, close to the expected value 4.

We compared the measured values of both amplitude and correlation time for the first mode with those expected from our bead and spring model. For $R/L=0.43$, $f(R/L)$ is approximately equal to 1.5, and the model leads to $\langle X_1^2\rangle = 2\times 10^{-2}\ \mu\text{m}^2$ and $\tau_1 \simeq 330$ ms, values that are larger than our experimental results. To account for this discrepancy, we assumed that molecules may have broken during the experiments. Direct observation of DNA molecules supports this hypothesis, which would furthermore explain the small mean extension R measured, as well as the broad distributions reported for both amplitudes and correlation times. Damage to molecules might result from residual photobleaching or during injection in the flow cell.

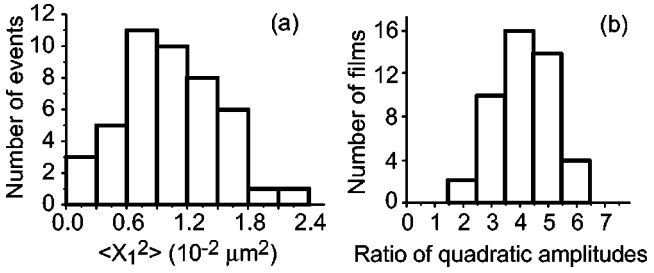

FIG. 4. Distribution of (a) amplitudes for the mode $p=1$ and (b) ratios of the quadratic amplitudes $\langle X_1^2\rangle/\langle X_2^2\rangle$.

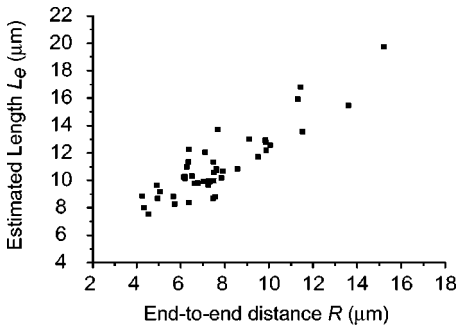

FIG. 5. ■: estimated length L_e as a function of the distance R. L_e is derived from the experimental values of the quadratic amplitude $\langle X_1^2\rangle$ and the end-to-end distance R (measured on the first image of each film). The estimated length is proportional to R, illustrating a moderately dispersed relative extension.

Under the assumption that DNA molecules may have different lengths, we determined for each of them its contour length L_e. For that purpose, we used the experimental value of the quadratic amplitude $\langle X_1^2\rangle$ and the measured end-to-end distance R. These two quantities enabled us to directly derive L_e from relation (4), assuming that our model provides a reliable expression for the quadratic amplitude of the observed fluctuations. Amplitudes are suitable for this analysis because they do not depend on the hydrodynamic parameters, unlike time correlations. Most of the contour lengths L_e were found to be smaller than 18 μm in accordance with our assumption (Fig. 5). We then deduced the relative extension R/L_e for each molecule and calculated its mean value $\overline{R/L_e} = 0.69(\pm 0.10)$. This value is larger than that calculated with a constant contour length and is consistent with those expected from estimated Weisenberg numbers. In addition, the standard deviation is smaller than previously, illustrating a good control of the strength of the shear flow in our experiments.

We then plotted τ_1/L_e^2 as a function of R/L_e (Fig. 6). Experimental values strongly decrease with R/L_e, demonstrating the crucial role of nonlinearities in the chain dynamics. Furthermore, our results correlate with the $1/f(R/L)$ shape predicted by our bead and spring model.

V. NUMERICAL SIMULATIONS

To estimate the pertinence of the assumption underlying our analysis (i.e., the mean distance R/N between two adjacent beads is large compared to the amplitude of the transverse and longitudinal fluctuations) as well as to partially

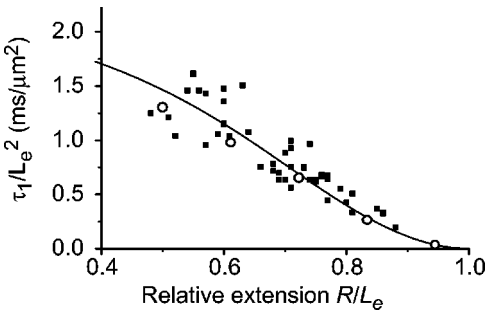

FIG. 6. ■: τ_1/L_e^2 as a function of R/L_e. The continuous line is the result of the bead and spring model. ○: numerical simulations of τ_1/L^2 as a function of R/L (calculated for $L=18$ μm). To fit our data, the same multiplicative factor 1.6 was applied to both the results of the model and the simulation. This factor is likely due to a limited knowledge of the hydrodynamic parameters, which are probably altered by the proximity to the surface.

estimate the role played by the surface, we performed simulations. They were computed using ten submolecules (we checked that simulations with a larger number of beads gave the same results). The elasticity of each submolecule was calculated using the nonlinear expression of the function $f(r/L_0)$. The surface can alter the dynamics of the molecule in two distinct ways: (i) a mechanical effect, the motion of the molecule being restricted to a half space; (ii) a hydrodynamical effect, through a modification of the properties of the fluid surrounding the molecule. While the latter, complex and beyond the scope of this paper, was not included in the simulations, we took the former into account by considering the surface as an infinite potential on which beads bounced elastically. Interestingly, we did not find it had noticeable effects on the results of the simulations, either in the amplitude or in the correlation time. As long as $R/L \geqslant 0.5$, the numerical results differed by less than 10% from the results provided by the model (Fig. 6). In the low-extension limit, which was not experimentally explored, the amplitude of the fluctuations is not small compared to R/N, and $f(r/L_0)$ cannot be approximated by $f(R/L)$.

VI. CONCLUSION

We report a simple and efficient method to attach and stretch DNA molecules to a surface without chemical modification of either the molecule or the surface. This method can be applied to various hydrophobic surfaces, such as polystyrene, PMMA, or PDMS. This last point is of particular interest, since more and more experiments are pursued in microfluidic environment involving home-made PDMS cavities. The possibility of observing several extended molecules simultaneously on the same surface could lead to parallel analyses of biological processes. Furthermore, the simplicity and the reproducibility of this method make it particularly attractive when compared with usual techniques involving ac fields or micrometer-sized beads.

We recorded the thermally driven fluctuations of the stretched molecules, which are described and analyzed in terms of normal modes. We deduced from the amplitudes of these fluctuations the length of extended molecules. We then showed that the dependence of the first mode correlation time on relative extension is in agreement with a model taking into account the nonlinearities of the chain, an essential point for understanding the transverse fluctuations of the polymers. These results are in good agreement with previous studies, where DNA molecules were extended in a viscous solution. Performing numerical simulations, we confirmed that the proximity of the molecules to a rigid surface does not significantly alter the main features of their dynamics.

This experimental technique and our analysis should be useful in the future study of DNA-protein interactions. A main feature of our system is that the free part of the tethered DNA is in average a few hundred nanometers from the coverslip. Although the DNA molecule may get closer owing to thermally driven fluctuations, we expect this proximity to be momentary and the surface interaction to be weak enough to preserve the activity of a molecular complex. Furthermore, the mean distance between DNA and the surface being on the order of an optical wavelength, our method is well adapted for the visualization by evanescent-wave microscopy of enzymes moving along DNA. This ability is specially appealing since the concentration of fluorescently labeled enzymes can be kept high enough to ensure DNA-protein interactions, while maintaining single molecule sensitivity.

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