Probing single DNA mobility with fluorescence correlation microscopy

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Fluorescence correlation spectroscopy combined with microscopy (FCSM) is used to study the mobility of DNA fragments in aqueous solution and tissue models on the single molecule level. The effective hydrodynamic radius was measured for various lengths of ds-DNA chains and obeyed the theoretically inveterate $[\text{DNA length}]^{0.5}$ relationship. Hindered diffusion of ds-DNA through the gel matrix of various densities is thought of as an extension of Kramer's problem for a flexible polymer chain. With increasing DNA length the average barrier crossing time rises as $[\text{DNA length}]^2$ and this agrees with theory predictions for polymer molecules surmounting an entropic barrier.

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INTRODUCTION

Random thermal walks of polymer macromolecules in constrained environments have a potential in addition to probing the general features of polymer diffusion to provide information about the structure and physical properties of the constraints. Generally, the mobility of flexible polymer-chain molecules varies as a power of the polymer length while the detailed mechanism of polymer diffusion remains unclear. Recently it was proven with time-lapse video microscopy that the polymer overcomes obstacles through the mechanism of entropic barriers crossing and the conventional belief of a polymer rolling over the barrier like a rigid sphere of corresponding size was not quite realistic [1]. Entropic barrier crossing is physically equivalent in many aspects to the solving of Kramer's problem for the Brownian particle escaping from a potential well [2,3] and involves the discrete cavity-to-cavity jumps and the stretching of a polymer over the barrier reducing its free energy [1]. Intuitively it is likely that the statistics of barrier crossing times would be enforced by properties of constraints and it is practical to extract this information from macroscopic translational diffusion of the whole polymer. It would be of particular interest and relevance to drug delivery related applications where liposome-mediated gene transfer after dissociation of DNA from lipid requires free DNA diffusion through the cytoplasmic interior of the cell [4]. Macromolecular fractionalization on intracellular compartments causes the strong size-dependent mobility of DNA vectors and is a serious factor limiting the efficiency of nonviral gene transfer through the cell cytoplasm. On the other hand, this would be of unambiguous benefit in bioanalytical microarrays for fast and continuous diagnostics and sorting of macromolecules. The discrimination between different molecular masses of DNA using asymmetric molecular size obstacles in such microarrays can be very efficient and fast [5], and has a potential to replace the routine gel separation, however, this requires the accurate information of DNA power-scaling mobility to quantify and predict the possible design of such devices.

In this paper we present original experimental results of the diffusion of ds-DNA fragments in water and agarose gel matrix probed by fluorescence correlation spectroscopy combined with a microscope (FCSM). The new approach has been achieved by the use of the single molecule probing technique when a focused laser beam illuminates a tiny volume where fluorescence is excited and the diffusion of a macromolecule in and out of this volume is thoroughly recorded. The excitation volume and concentration of ds-DNA are such that either one or no molecules could be present in the excitation volume concurrently. The effective hydrodynamic radius measured for various lengths of ds-DNA chains obeyed the theoretically inveterate $[\text{DNA length}]^{0.5}$ relationship in great agreement with Zimm's model. Hindered diffusion of ds-DNA in the agarose gel matrix revealed that the average crossing time is proportional to $[\text{DNA length}]^2$ in a randomly oriented three-dimensional (3D) fiber meshwork. To the best of our knowledge FCSM has not yet been applied to such studies. The previous experiments challenged theory predictions with video-fluorescence microscopy [1,6] or fluorescence recovery after photobleaching [4,8]. However, there are some issues of polymer diffusion still remaining unclear. We believe that a deeper understanding of the mechanism of polymer diffusion will launch new applications of biopolymers in different areas of science. We assume that entropic barrier crossing is the prevalent mechanism in the diffusion of long-chain DNA molecules in a constrained environment. We made an attempt to link together Kramer's problem for a polymer escaping from a potential well and the macroscopic diffusion of DNA fragments measured with FCSM.

The application of FCSM in the studies of long polymer chain diffusion has certain limitations, which need to be considered *a priori*. FCSM operates with a spot size, which can be smaller than the macromolecule itself. It results in the collection of fluorescence from a partially illuminated macromolecule [7] that at the same time is undergoing rapid conformational changes. The polymer can reconfigure itself withdrawing a part of itself from the illumination volume and causing fluctuations of intensity, which are not associated with diffusive motion of the whole polymer. In our experiment the illumination volume was larger than the corre-

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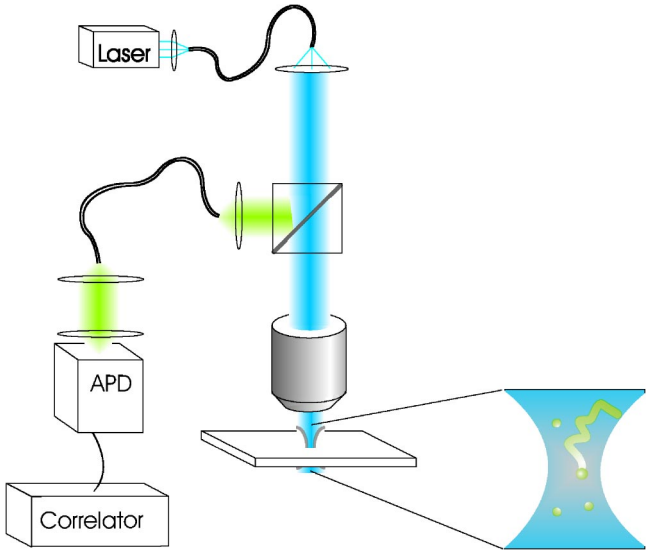

FIG. 1. (Color online). Optical scheme of experimental setup based on the up-right research microscope Nikon E800 with additional home-built detection scheme to carry out the spectroscopic measurements.

sponding radius of gyration of the longest DNA fragment, therefore, we eliminated the influence of the internal dynamics of the polymer on our results. Additionally the density of intercalating fluorescent markers might affect the persistent as well as total length of the polymer [1,6,7]. Then two originally identical polymer macromolecules with different densities of fluorescent labeling will have contrasting diffusion mobility. In order to overcome this restriction we paid particular attention to the balanced labeling density of all DNA fragments.

METHODS

The experimental setup consists of an up-right epifluorescent research microscope Nikon E800 incorporated with an additional home-built detection scheme to carry out the spectroscopic measurements. Optical scheme is shown in Fig. 1. The laser light (488 nm) was launched into the single-mode optical fiber using Gradium achromatic doublet lens (Newport). The effective lens NA matched to the NA of the fiber to ensure a stable light split and no modal noise due to environmental perturbations. The output of the fiber was expanded into the parallel beam and used to illuminate the back of the microscope objective (40X/0.60 Plan Fluor, Nikon). Fluorescence from the sample was collected through a dichroic beam splitter (Q505DLP) and a band-pass filter (HQ535/50, Chroma Technology Group) and focused on the multimode optical fiber. Then the fiber output was focused on the face of the silicon avalanche photodiode (APD) which operates in a single photon counting mode (SPCM-AQ-121, EG&G). Output of SPCM directly feeds the computer-based correlator (ALV-5000/Fast, ALV-GmbH, Langen, Germany). Correlation data is analyzed by ALV-software and SIGMAPLOT'2000 package by using Marquardt nonlinear least-square fitting routine.

For a relatively low numerical aperture of the objective (NA=0.6) the theoretical diffraction limited spot size is expected to be 1 μm. We examined the actual spot size by measuring the diffusion time of low molecular weight fluorescent dye molecules in aqueous solution. We used diluted solution of Rhodamin 6G for which the diffusion coefficient is well known and equal to $2.8 \cdot 10^{-6}$ cm^2/s. The estimated spot size was 1.5 μm with a correction factor for 3D-geometry $z/r=3.0$. Hence the corresponding detection volume was $7 \cdot 10^{-18}$ m^3 (7 fl).

We used ladder DNA (BioLabs Inc.), which contained enzyme-restricted fragments ranging from 0.5 to 10 kb. The double-stranded DNA was digested to completion with the appropriate restriction enzymes, phenol extracted and equilibrated to 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Separate fragments of DNA were extracted in routine 1% agarose gel electrophoresis run and recovered using QIAquick Gel Extraction Kit (BioLabs Inc.) designed to extract and purify DNA from standard and low-melt agarose gels in TBE buffer. For the study of DNA diffusion in the gel matrix the low-melt-temperature (melting at 40 °C) agarose gel of higher concentration was mixed with particular DNA solution.

We used PicoGreen (502/523) (Molecular Probes) as an ultrasensitive fluorescent nucleic acid stain to mark our preselected DNA fragments. This fluorescent stain exhibits a high increase (about 10^3 fold) in their fluorescence quantum yields upon binding to double-stranded DNA [9]. The dye intercalates between the DNA base pairs that finally affects the total contour and persistent length of the DNA fragment [1,6–8]. The diluted solution of PicoGreen reagent (1:200 in TE buffer, at final concentration of 0.8 μM [9]) was incubated for 5 min in the dark with 500 μg/ml of ds-DNA solution, producing a total volume of 1 ml of stained molecules. All FCSM measurements were carried out on the single molecule level for concentrations of 1–3 molecules per excitation volume.

RESULTS AND DISCUSSION

The molecule of DNA is a long polymer chain of self-similar elements. The characteristic measure for such chain is its monomer number or base-pair number. Representing DNA as a flexible cylinder of diameter 2.38 nm the contour length L can be calculated by multiplication of the length of one base-pair (0.34 nm) by the total number of base-pairs N composing the molecule. For example, DNA molecule with 5 kb has a contour length 1.7 μm. Diffusing freely in solution the chain undergoes a random walk of Kuhn segments with Kuhn length $l_K=2l_p$, where $l_p=50$ nm is a persistent length of the native DNA chain [10].

If the hydrodynamic radius R_H is used to characterise the excluded volume apparently occupied by a DNA molecule, then the diffusion coefficient of the molecule as a whole is calculated from the Stokes-Einstein relation:

$$D = \frac{k_B T}{6\pi\eta R_H}, \tag{1}$$

where k_B is Boltzmann's constant, T is temperature, η is viscosity, and hydrodynamic radius (R_H) serves as a key determinant of diffusive mobility of DNA.

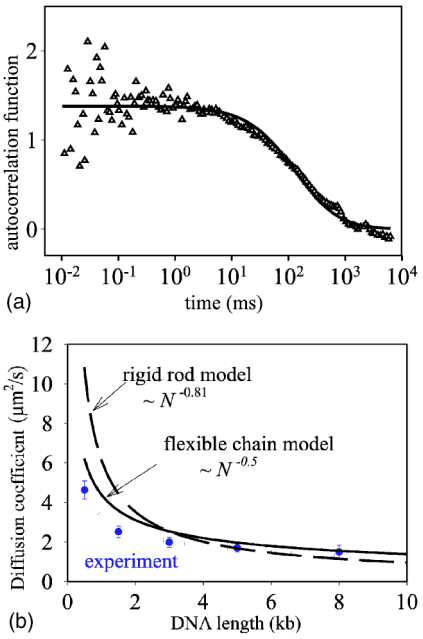

FIG. 2. (a) The experimental autocorrelation function for 5 kb DNA and its fitting showing the validity of the model used. (b) Theoretical models (solid and dashed lines) used to describe the polymer dynamics in diluted solutions and experimental data (shown by points with error bars and fitting shown by dotted line) of diffusion of DNA fragments acquired by FCS.

Assuming that only few of the neighbors along the chain are permitted to interact with each other, the whole chain migrates in free space as a *rigid rod* and its diffusion coefficient is calculated using the following expression [11,12]:

$$D_{RR} = \frac{k_B T}{3\pi\eta L} \ln(p + v), \tag{2}$$

where $v = 0.312 + 0.565/p + 0.1/p^2$ and p is the ratio of polymer contour length to its diameter. For our range of experimental parameters this power-scaling relationship is shown in Fig. 2 (dashed line). As the persistent length l_p restricts folding of the polymer by shorter pieces, then the diffusion of small DNA fragments could be well described within this model. For longer chains the discrepancy between the rigid rod and real molecular dynamics grows with the length of polymer [12]. A more realistic presentation of polymer permits very distant segments to interact when they become close to each other in space. The model which takes into account this sort of interactions is known as Zimm's model.

Zimm's model [13] considers the translational diffusion of the polymer as a flexible chain with power-scaling $D \sim N^{-0.5}$ or $D \sim N^{-0.6}$ for good solvent conditions. As an ideal solvent is less common in nature, the scaling argument (−0.6) is less likely to be met in rigorous experimental conditions and the diffusion coefficient is calculated by

$$D_Z = \frac{0.196 \cdot k_B T}{\eta \cdot l_K} \sqrt{\frac{300}{N}} \sim N^{-0.5}, \tag{3}$$

We extracted the diffusion coefficient of each singled out DNA fragment from experimental autocorrelation functions. In general the autocorrelation function is defined as $G(\tau) - 1 = \langle \delta I(t)\, \delta I(t+\tau) \rangle / \langle I \rangle^2$, where $\delta I(t)$ is intensity fluctuations detected by single photon counting module and the angle brackets mean time averaging. These fluctuations are created by the diffusion of molecules in a focused Gaussian beam. The measured autocorrelation function is fitted to its analytically derived solution

$$G(t) - 1 = \frac{1}{N}\left(1 + \frac{t}{\tau_d}\right)^{-1}\left(1 + \frac{t}{K^2 \tau_d}\right)^{-1/2} \tag{4}$$

where N is the number of molecules in the illuminated volume, τ_d is diffusion time, $K = z/r$ is the ratio of axial to radial dimensions of excitation volume. The diffusion coefficient is recovered using the relationship $D = r^2/4\tau_d$.

Our experimental results are shown in Fig. 2. An example of experimental autocorrelation function for 5 kb DNA is shown in Fig. 2(a). With the duration of each measurement of 300 s, the average of at least three consequent measurements was taken to yield the single point in Fig. 2(b). Standard deviation did not exceed 5%. The dotted line in Fig. 2(b) confirms the power-scaling factor (−0.5) for our experimental data in excellent agreement with the above Zimm's diffusion model (3). Estimated hydrodynamic radius R_H varied from 46 nm (0.5 kb) to 143 nm (8 kb). As it was already noted the persistent and contour lengths of the polymer increase due to the labeling of native polymer with fluorescent dye [6,7]. For example, the increase of native molecule length by a factor of 1.35 was reported in [6] when using TOTO-1 as a fluorescent dye. In our case of DNA labeled with PicoGreen dye in a ratio of 1 dye molecule per 1 bp corresponding diffusion coefficient decreased by a factor of 1.25 from that calculated using formula (3) [solid line in Fig. 2(b)].

There are some other experimental data on the power-scaling law of DNA mobility available from literature. The scaling factor (−0.72) was found in the FRAP experiment [4] for continuous sampling of the diffusion mobility of 21–6000 bp DNA fragments and (−0.68) for 367–2311 bp in the dynamic light scattering experiment [14]. We might suggest that the discrepancy of these data with Zimm's model is because the behavior of a real polymer varies from rigid rod to flexible chain when its length increases from a few tens to a few thousands of base pairs. The higher absolute value of the power-scaling argument could be a result of an attempt to fit a wider range of data by the single unified model, however, the real polymer molecule might not be well described within that model. Additionally Smith *et al.* [6] found a scaling behavior of (−0.611) for dsDNA between 4 and 300 kbp. This result matches well with the expected values for a good solvent mentioned above in Zimm's model, while for shorter DNA the scaling parameter increases as expected for the transition to rigid rod polymers.

Diffusion of chain-like molecules in confined geometry, such as a porous media or gel matrix, is expected to be more complicated. Microscopic studies of gels revealed that gel meshwork creates extended cavities with junctions between

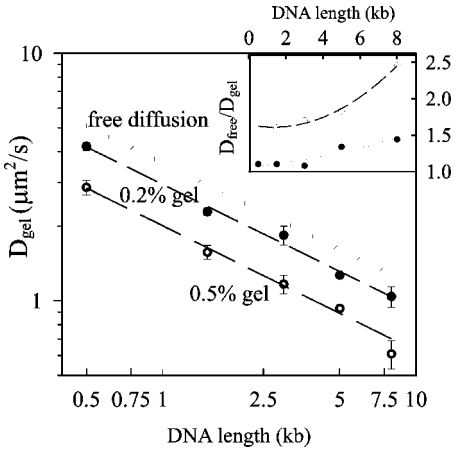

FIG. 3. Experimental log-log data for diffusion of DNA molecules in low concentration agarose gel (0.2 and 0.5%). The top-dashed line serves as a reference on data received for free DNA diffusion in aqueous solution. On the inset (top right corner) the ratio of diffusion coefficient in aqueous solution to diffusion coefficient in gel is shown as a function of polymer length for two subsequent gel concentrations.

them, where DNA molecules can diffuse freely. The transitions of a polymer molecule between the separate cavities is equivalent to crossing the entropic potential barrier and from a mathematical point of view is the generalization of Kramer's problem for a Brownian particle in a bistable potential well [2,3]. A fully mobile DNA molecule moves through the gel matrix by leaps from one cavity to another accompanied by a decrease of free energy of the polymer [1–3].

In Fig. 3 the experimental log-log data are shown for DNA diffusion in agarose gels. The volume fraction of fiber in the gel was low and all DNA fragments could diffuse freely eventually overcoming barriers formed by bunches of agarose gel fibers ($\phi=0.00195$ for 0.2% mass agarose concentration and $\phi=0.0049$ for 0.5% correspondingly). The top dash-line serves as a reference for the above data of free unrestricted DNA diffusion in aqueous solution. The fittings reveal a unique scaling factor (−0.5) for both gel compositions. It serves as an indication of the diffusion according to Rouse's model originally based on the presentation of a polymer by a set of beads connected along a chain [10]. These data are supported by previously observed [1,4,14] and reflect the fact of strong size-dependent DNA mobility in constrained environments. They also can be used to estimate various structural or functional features of gel, for example, the gel pore size [8].

In the top right corner (Fig. 3) there is an inset showing the ratio of diffusion coefficient in water to diffusion coefficient in gel as a function of DNA length. The diffusion coefficient in a constrained environment is proportional to a depth of the cavity substituting the potential well, within which diffusion is sustained and inversely proportional to average residence time in this cavity or barrier crossing time ($D_{gel} \sim a/\langle\tau\rangle$). Thus the ratio of diffusion coefficients can reveal the information about barrier crossing time when the cavity size is defined by the gel density and the length of polymer is gradually increased. If this ratio appears to be linearly proportional to DNA length it would serve as proof that the barrier crossing is favored by some free energy difference between the two sides of the barrier [2]. DNA molecule squeezing through a pore in the membrane of opposite charge can serve as an example of such a transition. If it occurs to be proportional to [DNA length]^2 then there is no free energy difference [3] that is more likely to occur in neutral gel. The fitting gives a good approximation to the parabolic law and poor to linear. In a 3D randomly oriented agarose gel matrix the crossing time is proportional to [DNA length]^2. We deduce the similar parabola-like relationship for barrier crossing time from data of [4] for diffusion of DNA fragments in cell cytoplasm, however, that parabola runs steeper.

We presented experimental data of DNA mobility in water and gel matrix. Polymer translocations were measured with FCSM on the single molecule level. Diffusion rate greatly depends on DNA length. We suggest that both theoretical models governing polymer diffusion in aqueous solution—rigid rod and flexible chain—might be effective for description of native fragments of DNA molecules resolving the controversy of published experimental data. However, for our range of DNA fragments the Zimm's model gives a universal description of polymer translocations. As the longer DNA fragments rapidly became immobile in the cell cytoplasm our data covers most of the practical range of polymer lengths applicable to gene therapy. FCSM can also evaluate the mobility of very short fragments of DNA, which are due to fast diffusion out of reach by low temporal resolution methods like FRAP or real-time video-microscopy. Our future concern is to access the diffusion of short DNA fragments with contour length of a few times greater than its persistent length, which are likely to distort the traditional understanding of polymer diffusion.

We examined the case of low obstacle concentration, which is essential for many biotechnological applications [4,15,16]. The obstructed diffusion of DNA molecules in agarose gel matrix preserves the dynamical scaling of Rouse's model while the microscopic crossing time is proportional to [DNA length]^2 and the diffusion is sustained within cavities of greater size than the gyration radii of molecules with random jumps between them.

FCSM shows to be an excellent instrument to probe the diffusion of macromolecules of larger size than the excitation volume. Although the application of the method to test long-chain polymers is overcomplicated by their folding dynamics [7], FCSM has a great advantage over other single-molecule techniques in studies of spherically shaped macromolecules such as liposomes with potential capability, for example, to quantify drug delivery in native physiological conditions.

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[1] D. Nykypanchuk, H. H. Strey, and D. A. Hoagland, Science **297**, 987 (2002).
[2] P. J. Park and W. Sung, J. Chem. Phys. **111**, 5259 (1999); M. Muthukumar, Phys. Rev. Lett. **86**, 3188 (2001).
[3] K. L. Sebastian and A. K. R. Paul, Phys. Rev. E **62**, 927 (2000).
[4] G. L. Lukacs *et al.*, J. Biol. Chem. **275**, 1625 (2000).
[5] T. A. J. Duke and R. H. Austin, Phys. Rev. Lett. **80**, 1552 (1998); G. W. Slater, H. L. Guo, and G. I. Nixon, *ibid.* **78**, 1170 (1997); L. Huang *et al.*, *ibid.* **89**, 178301 (2002).
[6] D. E. Smith, T. T. Perkins, and S. Chu, Macromolecules **29**, 1372 (1996).
[7] D. Lumma, S. Keller, T. Vilgis, and J. O. Radler, Phys. Rev. Lett. **90**, 218301 (2003).
[8] D. A. Berk, F. Yuan, M. Leunig, and R. K. Jain, Biophys. J. **65**, 2428 (1993); A. Pluen, P. A. Nett, R. K. Jain, and D. A. Berk, *ibid.* **77**, 542 (1999).
[9] V. L. Singer, L. J. Jones, S. T. Yue, and R. P. Haugland, Anal. Biochem. **249**, 228 (1997).
[10] M. Doi and S. F. Edwards, *The Theory of Polymer Dynamics* (Clarendon Press, Oxford University Press, Oxford, New York, 1986).
[11] M. M. Tirado and J. Garcia de la Torre, J. Chem. Phys. **73**, 1986 (1980).
[12] T. Kral *et al.*, Biophys. Chem. **95**, 135 (2002).
[13] B. H. Zimm, J. Chem. Phys. **94**, 2187 (1956).
[14] S. Sorlie and R. Pecora, Macromolecules **23**(2), 487 (1990).
[15] H. Qian, E. L. Elson, and C. Frieden, Biophys. J. **63**, 1000 (1992); S. Tatarkova, C. Lloyd, and D. Berk, Quantum Electron. **33**, 357 (2003).
[16] A. Pluen *et al.*, Proc. Natl. Acad. Sci. U.S.A. **98**, 4628 (2001).