Intermediate DNA at low added salt: DNA bubbles slow the diffusion of short DNA fragments

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We report a study of DNA (150 bp fragments) conformations in very low added salt < 0.05 mM, across wide DNA concentration range 0.0015 \(\leq c \leq 8\) mM (bp). We found an intermediate DNA conformation in the region 0.05 < c < 1 mM, by means of fluorescence correlation spectroscopy (FCS) and UV-absorbance measurements. FCS detected that in this region DNA has the diffusion coefficient, \(D_p\), reduced below the values for both ssDNA coils and native dsDNA helices of similar polymerization degree \(N\). Thus, this DNA population can not be a simple mix of dsDNA and of ssDNA which results from DNA melting. Here, melting occurs due to a reduction in screening concomitant with DNA concentration being reduced, in already very low salt conditions. The intermediate DNA is rationalized through the well known concept of fluctuational openings (DNA bubbles) which we postulate to form in AT-rich portions of the sequence, without the strands coming apart. Within the bubbles, DNA is locally stretched, while the whole molecule remains rod-like due to very low salt environment. Therefore, such intermediate DNA is elongated, in comparison to dsDNA, which accounts for its reduced \(D_p\).

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I. INTRODUCTION

For transcription to proceed in a chromosome, DNA has to be separated from the histone proteins and then it has to be unwound and the strands separated to allow the access of transcription factors (enzymes). This rather complicated process has, from a physicists point of view, a lot in common with a simple, thermally induced, in vitro process of DNA melting/denaturation, where dsDNA helix completely separates into two ssDNA strands. Stability of the double stranded DNA molecule reflects the nature of the DNA as a polyelectrolyte [1, 2]. When dissolved in polar solvents polyelectrolytes dissociate into a polyon and small counterions. Electrostatic interactions and entropy of all those charges, as well as those of added salt control their phenomenology. Thus, the stability of dsDNA depends on added salt, DNA concentrations and temperature [3, 6]. The DNA melting temperature will decrease with a decrease in added salt, and also with a decrease in concentration of DNA itself, even more so if there is no added salt. The latter is due to a change in concentration of counterions. E.g., at high added salt, up to physiological levels of the order of 0.1-1 M the DNA would melt at high temperatures (80-97°C). Contrary, below 1 mM (total counterions and added salt) dsDNA may be unstable already at ambient temperature [1, 7].

These extrinsic parameters are complemented with intrinsic ones: the DNA sequence, conformation and structure. Primarily, the sequence of A-T or G-C pairs influences the melting. For example, G-C base pairs are connected with three hydrogen bonds, while the A-T pairs with only two, and thus the sequences rich in A-T pairs are less thermally stable. The stability of a Watson-Crick pair is also determined by its first neighbours, due to stacking interactions [8]. This dependence on the local sequence relates to a rather well accepted concept [9] that the denaturation proceeds from local, cooperative openings of several bp long, A-T rich sequences, called DNA bubbles, which occur well below melting temperature [10, 12]. Close to the melting temperature these fluctuations zipper along the strands and the strands separate. The bubbles indeed correlate with the actual transcription initiation sites [13]. Physically, there is about a 10 \(k_B T\) barrier for opening a bubble and the cost for breaking consecutive base-pairs is only about 0.1 \(k_B T\), which results in the so-called zippering denaturation process [14]. For the very short fragments, \(\approx 10\) bp, the transition may well be described with a two-state model. For a longer DNA molecule, partially open states may be conceived, where a part of the sequence is open and part still closed. Such molecules, if stable, may be recognized to be the intermediate DNA, nor ssDNA, neither dsDNA [15, 16].

Models for DNA denaturation start from these general concepts. Models may be simple Ising-like, starting from the original Poland-Scheraga model [18], where only the energy difference is counted for open and closed base-pairs. In more detailed models base interaction depends on the distance between the bases (Dauxois-Peyrard-Bishop type) [6]. Some models account for the entropy contribution from the more flexible ssDNA loops [14, 19].
and find a continuous (IId order) or discontinuous (I order) transition \[20, 21\]. The latter result was crucially based on taking into account the self-avoiding interactions between the various parts of the chain.

For the experimentalist, it is not straightforward to demonstrate the existence of the DNA bubbles. Certainly, experiments evaluating the basepair opening probability rates and bubble lifetimes, in any specific set of conditions for their existence, would contribute to the modeling effort and our general knowledge of DNA stability and dynamics. The information on base-pair opening was initially provided by NMR study \[11, 22\] of the imino-proton exchange, i.e. study of protons, formerly participating in hydrogen bonds, that bases exchange with D$_2$O water when a base pair opens. This NMR study actually points that the bubble opening is a very local phenomenon, occuring on the level of a single base-pair. Opening probabilities (dissociation constants) at 25°C are actually quite low: $10^{-5} - 10^{-6}$ for an AT pair and $10^{-6} - 10^{-7}$ for a GC pair. The lifetime of a base-pair is of the order of 10 ms and the rate for closing the bubble is $10^6$s$^{-1}$. Only recently these NMR studies have been complemented by other studies of bubble dynamics. An FCS study by Libchaber et al. \[23\] has found that fluctuations occur with closing rates 3-4 orders of magnitude higher than estimated by NMR, i.e. $10^3 - 10^5$s$^{-1}$. The size of the bubbles was 2 to 10 bp on a fragment only 18bp long. These authors found no contradiction with NMR rate estimates, concluding that NMR registers much smaller conformational change with respectively higher fluctuation rates. That is, they considered that the openings detected by FCS are more biologically relevant as they relate to larger, nm-scale separations.

Peyrard et al. \[17, 24\] were able (by two-photon excitation of guanines) to detect when GC pairs were not paired in short ≈ 50bp DNA while temperature was raised and DNA melted. They thus obtained localized information on bubble opening and provided sequence dependent DNA melting curves. Importantly, they noted that short AT rich sequences (TATA boxes) would fluctuate and disturb both neighbouring and distant GC pairs well below the melting temperature. The fact that the opening fluctuations can have a non-local (non-single basepair) effect is related to nonlinear dynamics of DNA \[18\], but it was emphasized that this effect becomes negligible as it averages out for kbp or longer DNA sequences.

From the above it may be recognized that intermediate DNA states should exist below the melting transition, however only one group of authors has insofar managed to quantify the fraction of intermediate DNA in the population of DNA molecules. Montrichok et al. \[25\] have designed DNA constructs, ones with the TATA box at one end, and the others with TATA box (“soft” region) in the middle and GC clamps at both ends. When melting was stopped before complete denaturation, and the solution cooled quickly (quenched), strands which were completely separated were not able to find their complements and formed hairpins instead. Their fraction was quantified by simple gel electrophoresis. Fraction of the open molecules was compared (at equal temperatures) to the fraction of open basepairs in the solution, quantified by UV-absorption measurements. If the latter fraction was higher then there must have been some partly denatured, intermediate molecules before the quench. Intermediate DNA states identified from these experiments were successfully modeled by Monte Carlo simulations using the Dauxois-Peyrard-Bishop model \[10\].

One issue that arises is whether the local fluctuations influence mechanical properties of the whole DNA molecule. Early experiment, where self-diffusion coefficient of 17 kbp DNA molecules was probed in temperature by dynamic light scattering found no effect of fluctuations on DNA rigidity ( persistence length) \[26\]. Only recently it has been noted that there is the effect of fluctuations, but only on very short (10 - 90 bp) DNA fragments, which could behave as having a persistence length as low as 10-20 nm \[27, 28\], much shorter than the agreed value $L_p = 50$ nm \[29\]. Also, Tomić et al. have noted that persistence length of DNA might be reduced in some conditions \[30\]. They worked with mononucleosomal ≈ 150bp DNA fragments, which have the contour length $L_c = 50$ nm similar to the persistence length, so they are practically rodlike. Their dielectric spectroscopy technique, which provides characteristic length scales of a polyelectrolyte system, complementary to e.g. data by small angle X-ray scattering \[31\], indeed found a 50 nm length scale for fragments in pure water. However for fragments in 1 mM NaCl the characteristic length scale was 20-30 nm, lower both than $L_c$ or $L_p$. This would indicate that added salt, which does reduce the electrostatic contribution to the persistence length \[32\], has revealed $L_p$ lower than the agreed value. We may presume that the reduced $L_p$ is the consequence of partial DNA denaturation in these conditions of low salt.

In this paper, we propose that intermediate DNA is the major constituent in mononucleosomal DNA (denoted as DNA146) conformations population in the concentration range 0.05 < c < 1 mM (bp) at very low salt conditions, $c_{salt} < 0.05$mM. We infer the presence of intermediate DNA from the DNA146 polyion self-diffusion coefficient $D_p$, obtained by the fluorescence correlation spectroscopy measurements (FCS, method described in Sec.II). In Sec.III we present the $D_p$ value for 0.05 < c < 1 mM range which is below the values for both ssDNA and native dsDNA. This means that DNA conformation population there can not be a simple mix of native dsDNA helix and separated ssDNA strands. In Sec.IV we argue that an intermediate DNA conformation must be present, hydrodynamically larger than both ssDNA or dsDNA of similar polymerization degree. We suggest that under these conditions the basepair opening and closing rates have changed in such a manner to induce a variation of the diffusion properties of the whole molecule. In this sense, the intermediate DNA may be considered to be a stable form. Eventually, we analyze $D_p$ data and extract
the fraction of completely open DNA molecules, ssDNA, against partly open, intermediate DNAs and native ds-DNA. We also compare these fractions with measured fraction of open DNA base-pairs, following the procedure of Montrichok et al. [23].

II. MATERIALS & METHODS

A. Samples

We studied solutions of nucleosomal dsDNA fragments about 150 bp and 50 nm long. This DNA is denoted as DNA146, since there are 146 bp of DNA wrapped around a histone octamer in a nucleosome core particle. DNA146 pellets from the same stock as in [33] was used. The monodispersity of fragments was checked by gel electrophoresis. We also checked whether the pellets contain any added salt. DNA concentrations will be expressed as molar concentrations of basepairs in the remainder of this paper.

Several solution sets with different DNA146 and added salt concentrations were prepared, which we describe below:

set I Solutions with varying DNA concentration, in very low added salt: A set of 40 solutions covering the range of DNA concentrations $c = 0.001 - 8 \text{mM}$ was prepared by dilution with pure water of aliquots from the mother solution to reach the desired concentration. The mother solution was obtained by dissolving 10 mg of dry DNA146 in 0.55 mL pure water - the final concentration was $c = 27 \text{ mM}$. The UV-absorbance at 260 nm was measured, at 25°C for all the solutions in the set. Only then, for FCS measurements, the DNA146 solutions were fluorescently labeled by addition of synthetic 110bp DNA from a 0.5 mM stock kept in 10 mM TE buffer. This 110bp dsDNA will be further denoted DNA110*. The asterisk * denotes the fluorescent labeling by Cy5 fluorophore at one end of DNA molecule [33]. DNA110* was added to a concentration 1.5-2 $\mu\text{M}$ to achieve 20 nM Cy5 label concentration. The buffer of DNA110* stock gets diluted after addition into pure water DNA146 samples, thus introducing a minimal added ionic strength, not above 0.05mM. In this manner we regarded these samples as very low salt solutions, $c_{\text{salt}} < 0.05\text{mM}$, and labeled them appropriately. FCS results for this set are presented also in [33].

set II Solutions with varying DNA concentration, in 10 mM TE buffer. From $c = 27 \text{ mM}$ pure water mother solution (see above) a 0.1 mL sample was taken and diluted with 0.25 mL of 35 mM TE buffer. We used 10:1 TrisHCl:EDTA buffer set to pH7.5. Thus we obtained DNA146 starting solution with $c = 7.7 \text{ mM}$ in 10 mM TE buffer. For FCS measurements DNA110* was added to this solution, to a concentration 1.5-2 $\mu\text{M}$. After FCS measurement the sample was further diluted with appropriate volume of 10 mM TE buffer to produce a next lower DNA146 concentration sample. The procedure was repeated and 15 different DNA concentrations were measured in the range $c = 0.03 - 8 \text{mM}$. On each dilution appropriate volumes of DNA110* stock were being added to maintain the 20 nm Cy5 level.

set III Solutions with fixed DNA concentration, in varying added salt: DNA146 mother solution, $c = 7.5 \text{ mM}$ was prepared by dissolving a pellet in 10 mM NaCl. By dilution with appropriate volumes of pure water and 1mM NaCl we prepared a solution set where DNA concentration was always $c = 0.75 \text{ mM}$, while NaCl concentrations were 0.2, 0.5, 1, and 2 mM.

B. UV absorption measurements

DNA146 sample set I (varying DNA, very low salt < 0.05 mM) has been tested without dilution for UV-absorbance at 260 nm with Nanodrop (ThermoScientific). This instrument shows a linear response in absorbance for a very broad DNA concentration range, $c = 0.003 - 15 \text{ mM}$, with the sample volume of only 1-2 $\mu\text{L}$. The Nanodrop instrument was critical to get the actual UV absorbance of DNA samples without dilution.

For DNA melting experiments on set III (fixed DNA, varying salt) in 0-90 °C range we have used Agilent - HP 8452 Spectrophotometer with a Peltier based temperature control. From the temperature dependent UV-absorbance at 260 nm (hyperchromicity) curves we extracted the melting transition temperature $T_m$ and transition width dependence on total ion concentration.

C. Fluorescence correlation spectroscopy

We have used Zeiss ConfoCor II FCS instrument. Focal volume was defined by a Zeiss Plan-NeuFluar 100x/NA1.3 water immersion objective, epi-illumination was by He-Ne 632.8 nm 5mW laser, for excitation of Cy5 fluorophore. Measurements were performed at 25°C, the ambient temperature of the temperature stabilized clean-room.

Fluorescence correlation spectroscopy is used to measure the diffusion coefficient of the fluorescently labeled molecules. Number fluctuations of the molecules entering and leaving the focal volume of the instrument are registered as fluorescence variation. An autocorrelation function is calculated for the fluorescence intensity trace. This function decays exponentially with autocorrelation time $\tau_c$: 
\[ G(\tau_c) = \frac{1}{N_f} \cdot \frac{1}{1 + \frac{\tau}{\tau_p}} \cdot \frac{1}{(1 + \frac{\mu}{z_0})^2} \cdot \left(1 + T \cdot \exp\left(-\frac{\tau_c}{\tau_p}\right)\right) \]

\( N_f \) is the average number of fluorescent molecules in the focal volume, \( z_0/w_0 \) is the focal volume structure parameter and \( T \) average fraction of fluorophores in the triplet state (thus, non-fluorescing). The lifetime \( \tau_p \) of the triplet state is taken into account when fitting. The characteristic decay time \( \tau \) is the diffusion time that the fluorescent molecule takes to traverse the focal volume. The details of the procedure used to extract \( \tau \) may be found in [33].

In Fig. 1 besides the autocorrelation function experiment curve and fit for Cy5, we present the \( G(\tau_c) \) curves recorded for 2 \( \mu \)M of DNA110* found in either 0.2 mM DNA146 in very low salt \( c_{salt} \approx 0.05 \mu \)M, (set I) or 0.2 mM DNA146 in 10 mM TE buffer (set II). The experimental curves have been normalized, and thus they appear to overlap due to a rather small difference in the diffusion times. Nevertheless, the diffusion times \( \tau \) that we extracted are different, with values of 390 and 430 \( \mu \)s, respectively. To demonstrate that the fits reliably distinguish between the two experimental data sets for DNA110*, the area of the main panel, denoted with the rectangle, is shown enlarged in the inset [34]. The arrow in the inset denotes the 40 \( \mu \)s difference between the respective diffusion times obtained by fits.

The diffusion time is inversely proportional to the self-diffusion coefficient \( D_p \) of the molecule. The relationship between \( \tau \) and \( D_p \) may be obtained from the measurement of \( \tau_{Cy5} \) for Cy5 fluorophore, whose diffusion coefficient is known, \( D_{Cy5} = 3.16 \cdot 10^{-10} \) m²/s [33]:

\[ D_p = D_{Cy5} \cdot \frac{\tau_{Cy5}}{\tau} \]  

The diffusion times \( D_p \) that we can obtain from \( \tau \) are for the fluorescently labeled DNA110* molecules at a relatively low concentration, diffusing in solutions of varying, but mostly higher DNA146 concentration. That is, we obtain \( D_{exp}^{Cy5} \), diffusion coefficient for DNA110* which, however, depends on DNA146 concentration \( c \). However, we assume that it is possible to rescale \( D_{exp}^{Cy5} \) to get the values for DNA146. \( D_{exp}^{Cy5} \). We remind that dsDNA persistence length \( L_p \) = 50 nm [22] is comparable to contour lengths of both DNA110* and DNA 146, 38 and 50 nm, respectively. Therefore, we expect that they assume an extended, rodlike configuration. According to Tirado et al. [36], the translational diffusion coefficient calculated for a rodlike macromolecule is given by

\[ D_{th} = \frac{kT \ln(L_c/d)}{3 \pi \eta L_c} + 0.312 \]  

Here \( L_c = N b \) is contour length, \( d \) is polyion diameter, \( \eta = 8.9 \cdot 10^{-6} \) Pas is viscosity of water (\( T = 298 \) K). With

\[ b = 0.34 \text{ nm} \text{ and } d = 2.6 \text{ nm}, \text{ the diffusion coefficient for 110bp DNA is } D_{th}^{110} = 3.98 \cdot 10^{-11} \text{ m}^2/\text{s} \text{ and for 146bp DNA is } D_{th}^{146} = 3.27 \cdot 10^{-11} \text{ m}^2/\text{s}. \text{ Stellwagen et al. [37] have reviewed the literature and shown that the expression by Tirado et al. is well applicable to experimental data obtained for DNA molecules in size from 10 to 1000 basepairs.} \]

Thus,

\[ D_{th}^{exp}(c) = \frac{D_{th}^{146}}{D_{th}^{Cy5}} \cdot D_{th}^{Cy5}(c) \]  

In this manner, fluorescence correlation spectroscopy was used to obtain the self-diffusion coefficient of DNA146, \( D_{th}^{exp}(c) \) for varying DNA (DNA110* and DNA146 combined) concentrations \( c = 0.0015 - 8 \) mM. Most importantly, we were able to distinguish and compare the values obtained for samples in very low salt (set I) and samples in 10 mM TE buffer (set II).

III. RESULTS

A. DNA melting in very low salt conditions

Classically, UV absorbance measurements at 260 nm are used to study the DNA melting, i.e. helix-coil tran-
sition [2, 3]. Since the extinction coefficient for the dsDNA, 1 O.D. = 50 µg/mL is about 40% higher than for ssDNA, 1 O.D. = 35 µg/mL, concomitantly with temperature induced DNA denaturation, occurs a 40% rise in absorbance (hyperchromicity effect).

In Fig. 2 we present thermal denaturation curves for DNA146 samples at a fixed DNA concentration c = 0.75 mM, in varying added salt, 0.2-2 mM NaCl. The measured UV absorbance rise is presented as the rise of the ratio between UV-spectrophotometrically determined concentration c_{UV} and the gravimetrically determined (during sample preparation) concentration. The absorbance was converted into c_{UV} concentrations using the extinction coefficient for the dsDNA. c_{UV}/c ratio close to 1.4 thus corresponds to fully denatured DNA and c_{UV}/c = 1.0 corresponds to the native dsDNA. Melting temperatures T_m are therefore defined at half height of the maximum c_{UV}/c rise, where half of the basepairs should be open. For DNA146 in 0.2 mM NaCl solution we note that already at ambient temperature UV-absorbance, (c_{UV}/c > 1) is enhanced. Therefore we presume that this sample was already partly denatured at ambient conditions, due to the low salt content.

In Fig. 2 we present the ratio c_{UV}/c, measured at 25°C for DNA146 dissolved in very low added salt c_{salt} < 0.05 mM (set I). At higher concentrations, above c = 1 mM c_{UV} and c seem to comply indicating that the DNA remains native. At the lowest concentrations c_{UV}/c increases to about 1.4, which easily relates to 40% hyperchromicity of denatured DNA. Therefore, in Fig. 2 dashed lines at c_{UV}/c = 1.4 and 1 are respectively labeled ssDNA and dsDNA. The scatter in the data is mostly due to errors in DNA sample preparation, and to some extent due to the very minute sample droplets applied (a cuvette free spectrophotometer is used), which may evaporate and change concentration rather quickly.

Further, we note DNA146 samples from the intermediate concentration range c = 0.4 - 0.75 mM that show c_{UV}/c ≈ 1.2, corresponding to about 20% hyperchromicity (cf. Fig. 2). These samples are thus halfway through the melting transition, at ambient temperature. Thus, for DNA146 in the c = 0.4 - 0.75 mM range we estimate the temperature where we performed measurements to be the melting temperature, T_m = 25°C.

In Fig. 3 we present the ratio c_{UV}/c for DNA146 in very low salt c_{salt} < 0.05 mM (set I). The dashed lines at c_{UV}/c = 1.4 and 1 denote fully denatured ssDNA and native dsDNA, respectively. The arrows and letter e denote the concentration range where the ratio c_{UV}/c is just below 1.2, halfway between ssDNA and dsDNA levels.

Further, we note DNA146 samples from the intermediate concentration range c = 0.4 - 0.75 mM that show c_{UV}/c ≈ 1.2, corresponding to about 20% hyperchromicity (cf. Fig. 2). These samples are thus halfway through the melting transition, at ambient temperature. Thus, for DNA146 in the c = 0.4 - 0.75 mM range we estimate the temperature where we performed measurements to be the melting temperature, T_m = 25°C.

In Fig. 3 we show DNA146 melting temperature T_m vs. total ionic concentration of the solutions. We show the values obtained from melting curves (sample set III, Fig. 2) of points a, b, c, d) alongside the just above defined T_m = 25°C for DNA146 c = 0.4 - 0.75 mM concentration range (sample set I, Fig. 3 points e). Total ionic concentration c_{tot} is a sum of all the ions present in the solution: counterions c_i = 2c and added salt anions and cations 2c_{NaCl} = c_{Na^+} + c_{Cl^-}, thus c_{tot} = c_i + 2c_{NaCl}.

As predicted by Manning [38] we obtain the linear slope dT_m/dlogc_{tot} = 28 ± 3°C per decade, which compares very well with the slope 27°C per decade found by Record [41] for T4 (170 kbp) and T7 (40 kbp) bacteriophage DNA solutions without added salt. The slope reflects the counterion condensation and should be independent of the chain size and thus invariant from the type of DNA used in the experiment, as, indeed, we observed. While the slope is similar, we note that the transition temperatures for DNA146 are about 10°C lower than for long T4 and T7 DNAs, for equal c_{tot}. We also note that the melting transitions for DNA146 are much broader, ≈20-30°C, than for T4 and T7 DNAs, where they are only 4-9°C. That the melting temperature decreases and the transition width increases for shorter DNA chain size is to be expected [39, 40].

The above determined ability of DNA146 to keep the native conformation at ambient temperature, in the very low salt (< 0.05 mM) environment, but only above a certain DNA concentration c = 1 mM, reflects the findings by Record [41] and by Tomic et al. [41]. In the following
The diffusion time was extrapolated according to Eqs. 2, 4 stranded, 110 nt long oligomer, in pure water solution. Since in buffer, DNA110* is certain we used DNA110* added to 10 mM TE buffer, without any DNA146. Since in buffer, DNA110* is certain we used DNA110* added to 10 mM TE buffer, with DNA110* being 1 mM. For DNA110* we have estimated $T_m = 25^\circ C$ (triangles).

we identify the DNA conformations present in the very low salt solutions, with the region below $c = 1$ mM being the most intriguing.

**B. DNA146 diffusion coefficient**

In Fig. 5 we present $D_{146}^{exp}$, the diffusion coefficient of DNA146 polion as a function of DNA concentration $c$ in the range 0.0015-8 mM. The coefficient values were derived from diffusion times measured by FCS, (see Materials & Methods). Both $D_{146}^{exp}(c)$ for DNA146 in very low salt $c < 0.05$ mM (set I) and for DNA146 in 10 mM TE buffer (set II) are shown. The concentration range of the FCS study encompasses the range studied by UV-spectroscopy. According to behavior of $D_{146}^{exp}(c)$ the studied concentration range has been divided into regions A,B,C,D with respective delimiting concentrations $c_{AB} = 1$ mM, $c_{BC} = 0.05$ mM and $c_{CD} = 0.006$ mM.

In Fig. 5 we also show the diffusion coefficients $D_{146}^{exp}$ and $D_{146}^{p}$, extrapolated for a DNA of a polymerization degree $N = 146$. These are extrapolated (according to Eqs. 24) from the diffusion times measured for 1.5 $\mu$M (basepair) of fluorescently labeled $N = 110$ ssDNA and native dsDNA (DNA110*), respectively. First, to get $D_{146}^{exp}$ we used a sample of fluorescently labeled, single-stranded, 110 nt long oligomer, in pure water solution. The diffusion time was extrapolated according to Eqs. 24 and we got $D_{146}^{exp} = 3.95 \cdot 10^{-11}$ m$^2$/s. Then, to get $D_{146}^{p}$ we used DNA110* added to 10 mM TE buffer, without any DNA146. Since in buffer, DNA110* is certain to keep the native, double helix conformation. Thus, $D_{146}^{p} = 3.2 \cdot 10^{-11}$ m$^2$/s is considered as the representative experimental value of the diffusion coefficient for native dsDNA, $N = 146$.

First we note that, in region A ($c = 1 - 8$ mM), the values $D_{146}^{exp}(c)$ for DNA146 both in 10 mM TE buffer and in very low salt $c < 0.05$ mM, are equal within the experimental error. This result shows that DNA is of native conformation in both environments. The variation of $D_{146}^{exp}$ is due to the 50 nm DNA146 fragments being in the semidilute regime above 1-2 mM [30]. That is, the polion diffusion slows down, due to an interchain repulsive interaction that increases with reduction of average distance between polymers, i.e. with the increase in concentration of polymers. Self-diffusion coefficient, $D_p(c)$ has been previously shown to decrease linearly with the volume fraction $\Phi$ of rod-like particles, for e.g. a mineral, boehmite, $L \approx 300$ nm, 42 and 20 bp dsDNA $L \approx 7$ nm, 43:

$$D_p(c) = D_p^0(1 - \alpha\Phi)$$

(5)

Here, $D_p^0$ is the self-diffusion coefficient measured in dilute solution. For DNA, molar concentration $c$ is related to volume fraction as $\Phi \approx 0.001c$. Proportionality factor
α depends on the aspect ratio $L/d$ of the particle [42]. For DNA146, $p \approx 20 - 25$, leading to $\alpha \approx 15$. Fit of Eq.5 to our DNA146 in TE buffer data gives $\alpha \approx 30$ (full line in Fig. 5), in a reasonable agreement with the theoretical value. Measurements at higher concentrations would be necessary to elucidate significance of this discrepancy, however this is out of scope here.

Below $c_{AB} = 1$ mM (basepair), $D_{146}^{exp}(c)$, when measured in 10 mM TE is practically concentration independent across almost two decades in concentration (regions B,C). These values are very close to both just above defined experimental value for 146 bp dsDNA $D_{146}^{exp} = 3.2 \cdot 10^{-11}$ m$^2$/s, and to the theoretical $D_{146}^{th} = 3.27 \cdot 10^{-11}$ m$^2$/s value, calculated according to Eq.3 for a rodlike 146 bp dsDNA, 50nm long with $d = 2.6$ nm diameter. That is, DNA146 in 10mM TE buffer keeps the native state. Also, since this is dilute regime, diffusion coefficient is constant.

On the contrary, $D_{146}^{exp}(c)$ in very low salt, deviates from the native DNA146 value, below $c_{AB} = 1$ mM (basepair). Specifically, in the intermediate concentration region B ($c = 0.05 - 1$ mM) $D_{146}^{exp} = 2.9 \cdot 10^{-11}$ m$^2$/s is rather constant, but below (beyond the experimental error) the native DNA146 value. This reduction in $D_{146}^{exp}(c)$ means that in region B, DNA polyions diffuse more slowly, than DNA polyions which keep the native conformation.

Below $c_{BC} = 0.05$ mM (basepair), in region C, $D_{146}^{exp}$ measured in very low salt starts to rise continuously and overpasses the native dsDNA146 value. Eventually, below $c_{CD} = 0.006$ mM, in region D, $D_{146}^{exp}$ finally rises sharply to come very close to $D_{146}^{th} = 3.95 \cdot 10^{-11}$ m$^2$/s, the value that we ascribe to 146 nt ssDNA. Here we note that $D_{146}^{th}$ value is higher than the theoretical value, $3 \cdot 10^{-11}$ m$^2$/s for a rodlike ssDNA that may be calculated according to Eq.3 using $b = 4.3$ nm and $d = 1.2$ nm. Further, $D_{146}^{th}$ value is lower than the experimental value $5 \cdot 10^{-11}$ m$^2$/s that may be extrapolated for 146 nt ssDNA in the high salt (10 mM or higher) [32]. In these high salt conditions ssDNA has a persistence length of the order of 1 nm and assumes a conformation of a compact, random walk coil [32] [44]. E.g., 146 nt ssDNA, which has a contour length of about 60 nm, would form a coil with a hydrodynamic radius of 3 nm. Considering the hereabove presented range of diffusion coefficients for ssDNA, we judge that ssDNA in our, very low salt conditions neither assumes the form of a compact coil, nor a rigid rod, but a rather extended coil.

IV. DISCUSSION

A. Fraction of open basepairs

As we have noted in previous Section, FCS measurements show that DNA146 in very low salt $< 0.05$ mM (sample set I) assumes native, double-helix (and rodlike) conformation, but only in region A, above DNA concentration $c_{AB} = 1$ mM. Also, we found that in region D, below $c_{CD} = 0.006$ mM DNA appears to be single-stranded, forming a rather extended coil. In this manner, we recognize the concentration of DNA itself as a parameter to induce DNA melting in very low salt environment.

Therefore we propose that our UV-absorbance data, that is, $c_{UV}/c$ ratio presented in Fig.3 may be regarded as a DNA melting curve, recorded, however vs. DNA concentration as the parameter which induces melting. We remind that UV-absorbance only provides the amount of basepairs that have opened in a given DNA sample. Therefore, analysis of $c_{UV}/c$ data should provide the fraction $f(c)$ of open basepairs in the ensemble of DNA molecules in samples from set I.

We also use the Manning free counterion fraction $\theta(c)$ data that we presented in Ref. 18, in support of $c_{UV}/c$ data. Both data sets have been obtained for sample set I. The reported $\theta(c)$ variation occurs within the range defined by the theoretical $\theta$ values for dsDNA and ssDNA. The relative variation that we obtain is also similar to the relative difference in $\theta$ values for dsDNA and ssDNA observed experimentally by other authors [3]. Thus, we consider that $\theta(c)$ curve represents DNA melting in low salt, and measures the fraction of open basepairs, as well as $c_{UV}/c$ data.

The open basepair fraction is derived in a following manner

$$f(c) = \frac{(A(c) - A_{min})}{(A_{max} - A_{min})}$$

(6)

Here $A_{min}$ and $A_{max}$ are the minimum and maximum value for the parameter being analyzed, either $A(c) = c_{UV}/c$ or $A(c) = \theta(c)$.

Fig. 6 shows a very good correlation between the open basepair fraction calculated from UV-absorbance $f(c_{UV}/c)$ and from free counterion fraction $f(\theta(c))$, across the studied DNA concentration range $c = 0.015 - 8$ mM. We judge that the $f(c)$ that we obtained is, indeed, a very good measure of fraction of open basepairs present in very low salt solutions of varying DNA146 concentrations. Both $c_{UV}/c$ and $\theta(c)$ data appear to be valid for characterization of the melting transition.

B. Intermediate DNA

The most important result of this study is that in region B the diffusion coefficient $D_{146}^{exp}$ measured for DNA146 in very low salt is diminished compared to values both for ssDNA and native dsDNA (DNA in 10 mM TE buffer). In this same region a fraction between 10 and 90% basepairs are open (cf. Fig.6 and Fig.5).

Careful consideration of the above leads to a conclusion that the DNA population in region B is NOT a simple mix of native dsDNA helix and separated ssDNA strands. If it were a mix then the measured diffusion time and the corresponding diffusion coefficient should be of some intermediate value [43], between the experimental values for native dsDNA and ssDNA, $D_{146}^{th} = 3.2 \cdot 10^{-11}$ m$^2$/s.
and $D_{ds}^{ss} = 3.95 \cdot 10^{-11} \text{m}^2/\text{s}$, respectively. However, the actual value is only $2.9 \cdot 10^{-11} \text{m}^2/\text{s}$. The DNA molecules diffuse more slowly than either a rigid-rod dsDNA or an ssDNA strand. In other words, the DNA146 conformation present in this region is distinguished by being hydrodynamically larger entity than native DNA146 helix. As this is a dilute solution regime, the interactions between the molecules are not a plausible explanation, as they are for the decrease of $D_{exp}^{ds}(c)$ above 1-2 mM, in the semidilute regime (region A, Fig.6).

Wilk et al. have also found a diffusion coefficient to be reduced for DNA fragments (20 bp) in low salt, 0.01 mM NaCl in comparison to high salt, 0.2 M NaCl solutions and for a rather similar DNA concentration range. However, they have neglected in their analysis the fact that DNA denatures in the conditions of very low salt, which we have carefully analyzed in the previous subsection. To account for the reduction in diffusion coefficient, they have proposed that the hydrodynamic volume of the DNA molecule is enlarged, by the amount of the Debye screening length $\kappa^{-1}$, that is, the contour length $L_c$ becomes $L_c + \kappa^{-1}$, and the diameter becomes $d + \kappa^{-1}$. We consider this scenario to be inappropriate. Already for DNA146 in 10 mM TE buffer (and a rather small $\kappa^{-1} = 1 \text{nm}$), the value of the diffusion coefficient $D_{exp}^{ds}$ thus calculated (using Eq.3) would deviate for 10% from the experimental result we got. We remind, we get the discrepancy of only 1-2% between our experimental $D_{146}^{ds}$ result and theory, if we do not use a correction like Wilk et al.

Theoretically, the diffusion coefficient for rodlike molecules (see Eq.3) is linearly dependent on the contour length of the molecule, and only logarithmically on its diameter. Thus, the most plausible mechanism for $D_{exp}^{ds}(c)$ becoming reduced in region B would be if DNA contour length increased about 10%, leading to a 10% decrease in diffusion coefficient. Now, we note that dsDNA monomer size is 0.34 nm while for ssDNA it is 0.43 nm, 20% longer. When a sequence of several basepairs in a dsDNA molecule breaks hydrogen bonds and unstacks (DNA bubble), then each strand locally assumes an ssDNA coil conformation. Consequently the molecule would elongate in the region of the bubble. The molecules being elongated by 10% - half the maximum 20% - should then have half of the basepairs open. Simply, half of the sequence would be DNA bubbles and half native helix - an intermediate DNA would form. We assume that the fraying, separating ends of a dsDNA molecule would have a similar effect as DNA bubbles, on slowing the intermediate DNA diffusion.

Remaining issue would be whether these elongated, intermediate DNA molecules really maintain rodlike shape, otherwise application of Eq.3 would not be justified. We remind that, in these very low salt conditions, the charges along the DNA backbones repel strongly due to the lack of Debye screening and it is very plausible that this allows the intermediate DNA molecule to remain extended, rodlike in shape, despite having defects of lower intrinsic rigidity. We provide an illustration above the data panel of Fig.6.

### C. Fractions of DNA conformations

For the analysis of fractions of conformations in the population of DNA146 molecules, we will postulate that intermediate DNA146 is of a rodlike form and is characterized with about 50% basepairs open, and that the only other conformations that may appear in here analyzed solutions are fully separated ssDNA coils and rodlike native dsDNA helices. In other words, we do not expect DNA intermediate states with varying proportions of open base-pairs to coexist along the melting transition. Accordingly, we will analyze the diffusion coefficient data, and find how the fractions of dsDNA helices, intermediate DNA and strands of ssDNA vary with the DNA concentration, in very low salt solution. Fig.6 Eventually, we will provide arguments to support this proposition.

First, we compare diffusion coefficient data obtained for DNA146 in very low salt (set I) and in buffer (set II) in regions A and B. We denote these $D_{146}^{exp}(c)$ and $D_{146}^{ds}(c)$, respectively. Specifically, $D_{146}^{exp}(c)$, being obtained for DNA in buffer is thus the value for native helices, where the concentration dependence is due to samples above $c = 1 \text{mM}$ being in the semi-dilute regime. Variation in $D_{exp}^{ds}(c)$ is also due to this influence, but, as DNA concentration is lowered below about 1-2 mM, $D_{exp}^{ds}(c)$ deviates from $D_{146}^{ds}(c)$, showing that intermediate DNA starts to appear in the molecular population. The difference between $D_{146}^{exp}(c)$ and $D_{146}^{ds}(c)$ stabilizes in the lower part of region B, $c = 0.05 - 0.3 \text{mM}$, indicating that only intermediate DNA remained in the population. We remind that the diffusion coefficient value $D_{146}^{exp} = 2.9 \cdot 10^{-11} \text{m}^2/\text{s}$ in this region is lower than values either for ds-
DNA helices ($D_{146}^{ds} = 3.2 \cdot 10^{-11}$ m$^2$/s) or ssDNA coils, and therefore can be representative only of intermediate DNA. Thus, the fraction of intermediate DNA $im(c)$ as compared with fraction $1 - im(c)$ of native dsDNA across A and B regions is calculated in the following manner

$$im(c) = \frac{(D_{146}^{exp}(c) - D_{146}^{ds}(c))}{(D_{146}^{im} - D_{146}^{ds})} \quad (7)$$

In Fig. 7 it is apparent that as DNA concentration is lowered dsDNA helices (open circles) give way to intermediate DNA (black circles).

On lowering DNA concentration dsDNA disappears from population already in region B and only intermediate DNA and separated strands of ssDNA could remain in solution. The variation of $D_{146}^{exp}(c)$ across B, C, D regions may only be due to varying proportion of intermediate DNA and ssDNA coils, as they have different diffusion coefficients: $D_{146}^{im} = 2.9 \cdot 10^{-11}$ and $D_{146}^{ss} = 3.95 \cdot 10^{-11}$, respectively. Here DNA is in dilute regime and the diffusion coefficient of either conformation is not expected to depend on concentration. Thus, the fraction of intermediate DNA $im'(c)$ as compared with fraction $1 - im'(c)$ of separated ssDNA strands across B, C, D regions is calculated in the following manner

$$im'(c) = \frac{(D_{146}^{exp}(c) - D_{146}^{im})}{(D_{146}^{ss} - D_{146}^{im})} \quad (8)$$

In Fig. 7 it is apparent that as DNA concentration is lowered intermediate DNA (black squares) separates into ssDNA coils (open squares), and in region D only ssDNA remains in solution.

Finally, we note that $im'(c)$ is complementary with $im(c)$ data, and both indicate the same region $c = 0.05 - 0.3$ mM where intermediate DNA is prevalent in the population. Interestingly, the fraction of open base-pairs in this region is about 50%, see Fig. 8. This supports our initial proposition that we do not expect DNAs with varying proportions of open base-pairs.

Half of the basepairs being open for intermediate DNA relates to the fact that DNA that we used has a quite random sequence and equal content of G-C and A-T pairs. Since A-T pairs have smaller binding energy [8, 12], it could be that only these break in the reported conditions and thus contribute to formation of intermediate DNA as a step in DNA melting process.

Here, we remind that the counterion concentration is the only parameter that changes for this DNA and the reduction of screening due to decreasing concentration leads to DNA melting. Apparently, this parameter variation is mild enough to allow for preferential breaking of A-T pairs, or, which is rather equivalent [17, 24], formation of bubbles in AT-rich portions of the sequence. That is, across our range of counterion concentrations 1-0.01 mM the Debye screening length $\kappa^{-1}$ varies from about 10 up to about 100 nm. The corresponding exponential factor $\exp(-\kappa r)$ in the screened Debye-Hückel potential at $r = 2$ nm, characteristic distance of two repulsing phosphate charges would concomittantly change for about 20%. In other words, if the Coulombic repulsion at 1-2 mM counterion concentration was barely enough to break any basepairs, the reduction in screening at lower concentrations would enhance the repulsion for 20% and start to break A-T pairs but not yet G-C pairs which are bound 50-100% stronger.

Considering the above illustration we note that Chen and Prohofsky [2] calculate that below 1 mM added salt phosphate-phosphate force becomes insensitive to the salt concentration, and conclude that the effective poten-
tial has become a simple Coulomb potential. However, this is a long range potential and contributes to the energy balance of the intermediate molecule specifically in the open, ssDNA sections, where the interaction occurs not only between complementary bases, but among at least several neighboring bases. That is, disrupted bond force constant is not zero. We emphasize that this renders the calculation of free energy of such an intermediate state nontrivial. Phosphate-phosphate contribution is non-additive, depends on DNA concentration, and on the size of the bubbles. As such it is not tractable and not predicted by present models for thermally induced DNA melting, where Coulomb potential was neglected, being screened (at most, Coulomb potential has only renormalized the hydrogen bonds).

D. Concluding remarks

In our scenario the intermediate DNA with about half basepairs open coexists with dsDNA helices or ssDNA coils in very low salt environment. We suggest that the low DNA concentration and very low salt conditions of this study allowed for DNA to open preferably along AT-rich portions of the sequence, without the strands separating further. These local lesions reflect on hydrodynamic properties of the whole molecule, i.e. its diffusion coefficient has been reduced measurably below the value for the original dsDNA helix. However, whether these DNA lesions remain stably open or it is a dynamical situation where the bubbles open and close often enough to change the diffusion properties may not be judged from our result. Experiments capable of measuring the fluctuation rates at a given site within the sequence, as those mentioned in the Introduction (optical spectroscopy of G bases, FRET, FCS) should be performed on DNA fragments in the very low added salt conditions. Only in this manner the nature of intermediate DNA could be judged and then also the stability mechanism would be more easily inferred. An interesting scenario would be the critical slowing of fluctuations, a concept known from the studies of glass forming systems [40]. Finally, our finding of intermediate DNA provides another novel twist. That is, to explain the diffusion properties of intermediate DNA in very low salt, the ssDNA loops within such a molecule have to be extended, and the molecule has to keep the rodlike form. This may be perceived contrary to the usual notion that the fluctuational openings, for DNA in an added salt environment, remove the molecule away from the rigid rod form [20, 21, 27].

V. SUMMARY

In this work we have revealed the presence of novel intermediate DNA conformation at low DNA concentrations (c < 1 mM basepair) in very low salt (< 0.05 mM) solutions at ambient temperature. The diffusion coefficient for DNA146 below 1 mM and above 0.05 mM (in DNA basepair) occurs to be below the values for both native dsDNA helices and ssDNA strands of equal polymerization degree. Thus the population in the intermediate regime is not a mixture of these two conformations. Intermediate DNA molecules, consisting of dsDNA segments and ssDNA loops would have appropriate hydrodynamical properties to explain for the diffusion data. The ssDNA loops are possibly due to DNA fluctuational dynamical properties to explain for the diffusion data. The ssDNA loops are possibly due to DNA fluctuational openings (bubbles) that open and close at such rates, that they may be considered to have become stabilized since they influence the diffusion of the whole molecule. We suggest that the especially long-range Coulombic repulsion in the very low salt conditions of our experiment might allow for this effective stability.

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