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Letter to the editor

Flexibility of single-stranded DNA measured by single-molecule FRET

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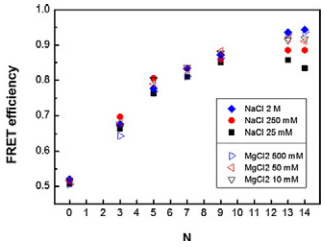
- We investigated the flexibility of ssDNA using a single-molecule technique.
- ssDNA even shorter than the persistence length was found to be flexible.
- Divalent cation is effective in screening the negative charge of the ssDNA.



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ABSTRACT

The mechanical flexibility of ssDNA is crucial for understanding the biological machinery but its characterization has been difficult due to the lack of an experimental tool that measures the structure of ssDNA in the nanometer scale. Here, we demonstrate that single-molecule FRET can be used to probe the structures of a flexible ssDNA. We designed a dsDNA with various lengths of single-stranded overhang and determined the flexibility of the single-stranded segment by measuring the FRET value. We found that three of our ssDNAs with lengths shorter than the persistence length are indeed long enough to undergo folding. Since metal ions present in solution can affect the flexibility of DNA, we employed Na^{+} and Mg^{2+} at different concentrations. We found that there is no significant effect of charge screening by metal ion when the ssDNA is less than 9 bases in length but it becomes appreciable for longer ssDNAs.

1. Introduction

Single-stranded DNA (ssDNA) plays an essential role in various DNA metabolic processes such as replication [1], transcription [2], and DNA repair [3]. Nucleic acids in the cell nucleus undergo dynamic structural changes as they transmit their genetic information. It is therefore important to understand the structural properties of ssDNA. Of particular importance here is the mechanical flexibility of ssDNA, which may even provide clues to the RNA folding structure because of the structural similarity between the RNA and DNA backbones [4].

The flexibility of ssDNA has been demonstrated by fluorescence correlation spectroscopy [5], single-molecule fluorescence resonance energy transfer (FRET) [6,7], and force spectroscopy [8–10]. The flexibility of long ssDNAs (>100 nucleotides) has been widely investigated [11], but studies on shorter ssDNAs (<15 nucleotides) are limited [6,12,13] since it is difficult to measure the structural changes in ssDNA at the correspondingly small length scale. When proteins interact with DNA, they often interact with only a small number of nucleotides (as in the case of RNA polymerase interacting with promoter DNA through a single-stranded transcription bubble formed from ~14 nucleotides [14]), the information on the flexibility of short ssDNA is very important. In the present study, we carried out direct measurement of the mechanical flexibility of ssDNA in short lengths.

DNA structures are affected by cations that can reduce electrostatic repulsion between the negatively charged phosphate groups of the DNA backbone. These ions can directly bind to DNA or change the dielectric characteristics of the medium. Divalent cations are known to be particularly effective in stabilizing the DNA structure [15]. The effect of metal ions in nucleic acid structure has been widely investigated both theoretically and experimentally [5,16]. In the present study, we

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investigated the change of ssDNA structure as we varied the concentration of monovalent and divalent cations.

Various kinds of biomolecular processes have been investigated using FRET between two fluorescent probes. The energy transfer efficiency is inversely proportional to the sixth power of the inter-probe distance and is very sensitive to the changes in distance on the length scale of 1–10 nm. For this reason, it has been widely used to examine biomolecular structures and processes, especially at the single-molecule level. However, conventional single-molecule FRET cannot distinguish changes in molecular stoichiometry from conformational changes, which can be a critical issue when studying biological processes involving assembly and disassembly of biomolecules. To overcome such limitations, the new platform of alternating laser excitation (ALEX) FRET was introduced that employs an additional excitation laser to directly probe acceptor dye [17,18]. By alternating multiple lasers rapidly, the presence of each fluorophore on the distinct molecular compartment can be identified, which yields an independent parameter for molecular stoichiometry for compositional information in addition to the FRET efficiency for spatial information. By sorting molecules in 2-D histogram along two independent parameters, i.e., FRET efficiency (E) versus stoichiometry ratio (S), molecular heterogeneities can be directly visualized along with their structural differences.

2. Material and methods

2.1. Preparation of ssDNA sample

The scheme for our sample design is shown in Fig. 1. We purchased nucleic acid samples from Integrated DNA Technologies (Coralville, IA). HPLC-purified sample was labeled with Cy3 and Cy5 on the shorter and longer strands, respectively, as shown in Fig. 1A through the amino modifier C6 and C3, which is known to have only a slight effect on their configurations. The dye labeling sites are fixed at a specific position on each strand with a separation of 14 nucleotides between the dyes. We controlled the length of the overhang (the ssDNA segment) by varying the length of the shorter DNA from 14 to 28 nucleotides, giving the overhang a length of 14 to 0 nucleotides. If we were to label the dye on the 3′ end of the shorter DNA, it would have been difficult to see whether the overhang bends or not, especially at small N.

In our single-molecule experiment, we used a DNA concentration of 100 pM to make sure that at most only one molecule was present in the focal volume within the detection time window of 600 μs. We used a buffer that contains 50 mM pH 8 Tris, 1 mM MEA, and 5 vol.% glycerol. The salt concentration was varied from 10 mM to 2 M.

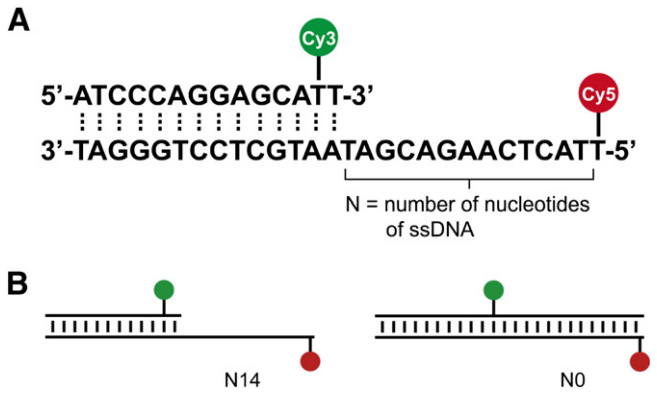

Fig. 1. (A) Sample design showing the dye labeling of two fixed sites separated by 14 nucleotides. N represents the number of nucleotides in the ssDNA segment called "overhang" and is adjusted by varying the length of the upper strand labeled with Cy3. (B) N14 (left) and N0 (right) with each dye labeling the same position.

2.2. Single molecule FRET experiments

We obtained our ALEX FRET data using a home-built fluorescence confocal setup. Two continuous-wave excitation lasers (532 nm: Samba™ 532 Cobolt; 635 nm: TECRL-25G-635, World Star Tech) were alternatingly irradiated on the diffusing sample. Alternation of two lasers was achieved using acousto-optic modulator (AOTF, AOTFnC-VIS, AA Opto-Electronic) running at 50-μs period. To avoid the temporal cross-talk, the "on" time of the AOTF was limited to 45 μs, while 5 μs remained as the "off" time. After individual lasers passed through a single-mode fiber with a diameter of 3–5 μm (P1-488-PM-FC and P1-630 PM-FC, Thorlabs), they were combined with dichroic mirrors (488/532/633rdc). Lasers were circularly polarized to efficiently excite the dyes through an objective (UplanSApo 60×/1.20 w, Olympus). Fluorescence signals from donor and acceptor were separated by a dichroic mirror (630dcxr, Chroma) and detected by two avalanche photodiodes (APDs) (SPCM-AQR-14, Perkin Elmer).

The values of E and S were calculated from the photon traces detected by APD using the following equations:

$$E = \frac{F_{D_{exc}}^{A_{em}}}{\left(F_{D_{exc}}^{A_{em}} + \gamma F_{D_{exc}}^{D_{em}}\right)}$$

$$S = \frac{F_{D_{exc}}}{\left(F_{D_{exc}} + F_{A_{exc}}\right)}$$

$$F_{D_{exc}} = F_{D_{exc}}^{A_{em}} + \gamma F_{D_{exc}}^{D_{em}},\ F_{A_{exc}} = F_{A_{exc}}^{A_{em}} + F_{A_{exc}}^{D_{em}}$$

where $\gamma = (\Phi_A \eta_A / \Phi_D \eta_D)$, Φ = quantum yield of the dye, and η = detection efficiency of the detector.

3. Results and discussion

In ALEX FRET, a two-dimensional E–S diagram represents different subgroups of sample that has a heterogeneous distribution in chemical composition and molecular structure. Fig. 2A shows such a two-dimensional E–S diagram with four distinct (E, S) domains that may represent possible configurations of species anticipated from our sample. Both singly labeled "impurities" that result from partial bleaching of fluorophores or failure of hybridization can be readily distinguished from doubly labeled species that have an intermediate S value. The latter may further branch into those flexible enough to bring the two fluorophores together to yield a large E value and those not flexible enough. The minimum E value is expected to occur for N0 (i.e., double-stranded DNA or dsDNA). Since the persistence length of dsDNA is 50 nm [19], the 15-bp segment between the fluorophores should remain straight at a length of ~5 nm. Since Forster radius of Cy3–Cy5 pair is also ~5 nm, the minimum E value expected from our experiments is ~0.5. Those with ssDNA overhang should have a higher E value since the two fluorophores can be brought closer due to its flexibility.

Fig. 2B shows two representative single-molecule ALEX FRET data for N14 (left) and N0 (right) collected from a large number (>1000) of DNA molecules at 2 M NaCl. The value of E was determined by fitting the peak at S ~ 0.5 to a Gaussian distribution and taking its center value. The E value of N14 was 0.944, representing a strongly curved structure of DNA while that of N0 was 0.541, indicating that dsDNA indeed has a rigid structure.

Averaged FRET efficiencies for different lengths of overhang obtained under different ion concentrations are given in Fig. 3. Any salt effect observed here is due to its effect on the DNA structure since the characteristics of dyes are rarely influenced by salt conditions [6].

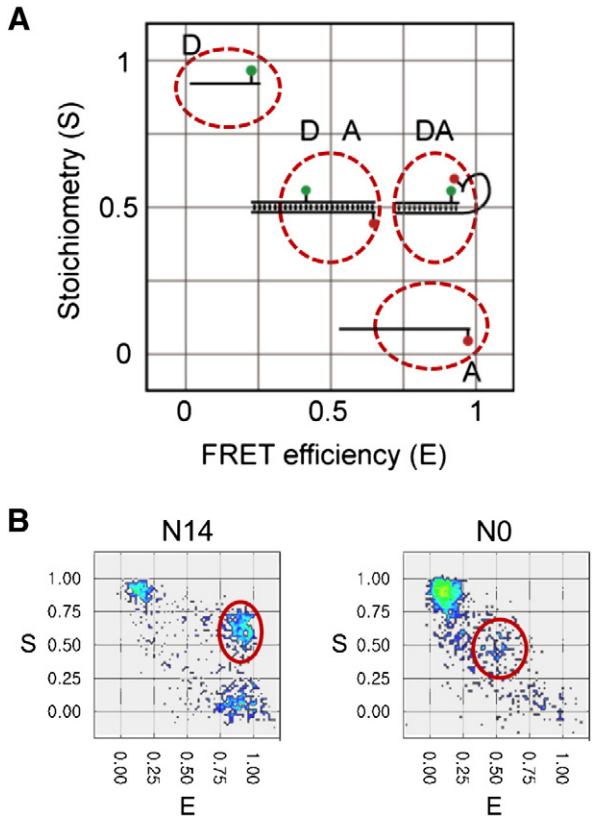

Fig. 2. (A) Two-dimensional E–S diagram representing different domains of (E, S) values anticipated for four different subgroups of our sample: donor-only impurities with S ~ 1, acceptor-only impurities with S ~ 0, and doubly labeled species (S ~ 0.5) that are more flexible (high E) or less so (low E). (B) Experimental E–S diagrams for N14 (left) and N0 (right) obtained at 2 M NaCl. Red circles represent the doubly labeled species with S ~ 0.5 that are the subject of our interest.

Fig. 3 shows that, for instance, N0 and N3 have different FRET values. The E value of N3 is much higher than that of N0 even though the fluorophores are labeled on the same positions in N0 and N3. It indicates that sufficient flexibility is introduced with only 3 nucleotides of ssDNA. Since ssDNA may fluctuate freely during the detection time window of 600 μs, we use the contour length of ssDNA that represents its length at maximum physically possible extension. The contour length of N3 is estimated to be 1.89 nm since the unit length of each base is well known to be 0.63 nm [20,21]. This shows clearly that a ssDNA shorter than its persistence length can still bend. (The persistence length of ssDNA was reported from 2 to 5 nm, depending highly upon the ion concentration in the buffer [6].)

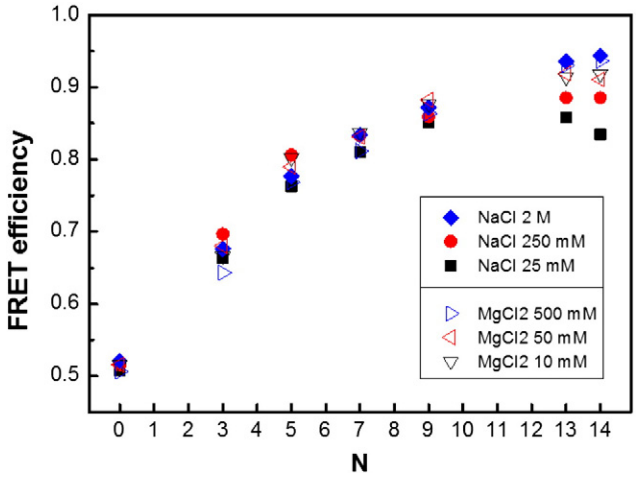

Fig. 3. Average FRET efficiency values for different lengths of ssDNA (N = 0–14) in various concentrations of NaCl and $MgCl_2$.

DNA structure is affected by cations that screen the negatively charged phosphate backbone, perhaps more strongly for ssDNA than for dsDNA because of the higher flexibility of the former. In this study, we also examined the difference in the salt effect between monovalent and divalent cations by employing NaCl and $MgCl_2$.

In the case of Na^+ cation, our result shows that, for smaller N, there is little dependence of E on the cation concentration since the ssDNA structure is still not very flexible. For larger N (N > 9), however, E is significantly influenced by the cation concentration, increasing as the latter increases.

At an intermediate concentration of NaCl (250 mM), the E values of N9, N13, and N14 are all comparable and seem to have reached a plateau, indicating that the flexibility does not increase even for longer overhangs at this cation concentration, which may not be sufficient to screen all negative charges. When we use a higher concentration (2 M) of Na^+, however, the E value increases beyond the plateau since the flexibility of the overhang is enhanced again by further screening of the excess negative charges. By also looking at the data for the lowest Na^+ concentration (25 mM), we can see that it takes a certain amount of cations to sufficiently screen the negative charges of the backbone to make it flexible enough.

In the case of $MgCl_2$, we observed little concentration dependence. Since the E values obtained for various concentrations of Mg^{2+} are also comparable to that for 2 M Na^+, we think that the ssDNA is made flexible enough by the divalent cation even in its low concentration. This indicates that a divalent cation has a much stronger screening effect toward neutralization of the negative charges on the DNA backbone.

4. Conclusion

We examined the flexibility of a short ssDNA overhang ranging in length from 0 to 14 nucleotides at various concentrations of NaCl and $MgCl_2$. The distance between the fluorophores measured by single-molecule ALEX FRET yielded information about the bending structure of ssDNA and its flexibility. We discovered that sufficient flexibility is introduced with only 3 nucleotides of ssDNA and that a ssDNA shorter than its persistence length can still bend. The flexibility of ssDNA is little affected by cations when its length is short but becomes increasingly affected by cation concentration as its length is increased. The screening effect was much greater for a divalent cation than a monovalent one.

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