Direct visualization of triplex DNA molecular dynamics by fluorescence resonance energy transfer and atomic force microscopy measurements

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We have detected the dynamics of 17-mer DNA triplex dissociation mechanism at the molecular level. Fluorescence resonance energy transfer (FRET) was used as an indicator of intermolecular interaction in nanometer range, whereas atomic force microscopy (AFM) was employed to address single molecule with sub-angstrom precision. The maximum rupture force of DNA triplex was found at *p*H 4.65, consistent with macroscopic observations. These results indicated that the FRET together with an AFM detection system could be used to reveal the DNA triplex interaction in nanometer scale unambiguously. © *2007 American Institute of Physics*. [DOI: 10.1063/1.2809406]

DNA triplexes have attracted considerable attention due to their possible roles played in the control of gene expression.[1,2] Gene expression can be regulated via an antigene strategy through the formation of DNA triplexes.[1,2] In this paper, we demonstrated that the reaction mechanism of DNA triplex formation and rupture could be studied by way of direct imaging methods of fluorescence resonance energy transfer[3] (FRET) and atomic force microscopy (AFM), at single molecule[4,5] levels and in variant *p*H conditions.

In order to directly monitor the DNA rupture event, a designed FRET pair of fluorophore [6-carboxyfluorescein (6-FAM, F)] and dark quencher [Dabcyl, (DQ)] was labeled on the designed DNA oligomers at 3′-end (synthesized by TriLink, Inc. at San Diego, CA) indicated as follows.

Oligo1: 5′-(biotin) $(T)_{10}$*TTCTTCTGATTCTCTCC*TTT **GGAGAGAATCAGAAGAGAA**(DQ)−3′. A 17-nucleotidyl sequence (the italic letters above) was chosen from the gene of CDC 25 of *Pnumernia carinii*.[6] Incorporating this sequence in our experiments, a hairpin duplex was designed by a corresponding complementary strand (or W strand, denoted by boldface letters) with a TTT double strand linker as shown above. Thus, oligo1 can fold back using the TTT as a loop and form an intramolecular DNA duplex composed of 17 base pairs. Although a GA pair introduced in the sequence might disturb the stability a little, it could be stabilized by its two long flanking arms composed of 7- and 8-pyrimidine motifs on either side when forming a DNA triplex. During the exploration period, we have checked its circular dichroism spectrum (data not shown) which indicated positively that the ability of triplex formation far exceeded the disrupting effect of inserting a GA pair. A single stranded decathymidylic motif $[(T)_{10}]$ with biotin is attached at the 5′-end to serve as a spacer with an anchor to the AFM probe surface. Thus, the hairpin DNA duplex will protrude (for about 7 nm) from the surface of the AFM probe. A DQ moiety was attached to the 3′-end to serve as a quencher when coupling with F in oligo2 (see below).

Oligo2: 5′-(biotin) $(T)_{10}$CCTCTCTTAGTCTTCTT(F)−3′. Oligo2 contains a 5′-biotin-$(T)_{10}$ spacer/anchor moiety and a 17-mer with a F at the 3′-end. The sequence of the 17-mer, CCTCTCTTAGTCTTCTT, was designed to form the Hoogsteen hydrogen bonds with the W strand of oligo1 under triplex formation buffer solution.[2]

Single molecular images of DNA triplex were observed in a solution environment with the MPF-3D (Asylum Research, Santa Barbara, CA) in tapping mode. The triplex formation can be visualized by AFM, as shown in Fig. 1. The lump resembles the structure of triplex that is outlined at the upper left corner according to the study of Hansma *et al.*[7] It is known that nonspecific interactions between the probe and the coverslip affect the force curve during the process of probe withdrawal from the coverslip, especially as the two are in close proximity.[5] In order to reduce these nonspecific interactions during the DNA rupture studies, we tried to enhance the specific interaction by modifying both the probe and the coverslip surface with biotin and avidin. The unbound active sites of avidin were blocked and protected with excess free biotin by the method of Wojcikiewicz *et al.*,[8] which can increase the specific interactions among DNA triplexes.

We implemented a dynamic force spectroscopy mode in the software-controlled piezodisplacement by Asylum Research and used the functionalized tip, as described previously.[8] The constant velocity approach-retract cycle was repeated 50 times in triplex formation buffer at 25 °C. The spring constants of all cantilevers were calibrated by the thermal fluctuation method[9] with an absolute uncertainty of ±10%. The measurements presented were performed with

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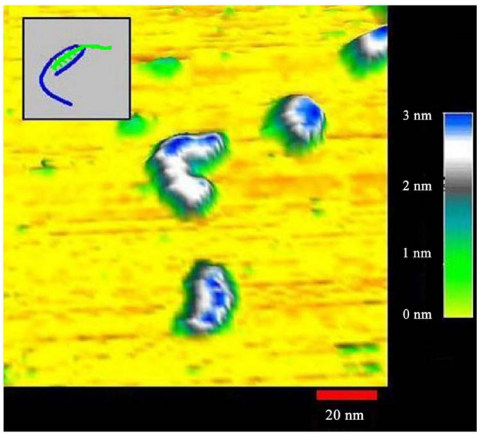

FIG. 1. (Color online) Single-molecule image and height of DNA triplex oligo 1+ oligo 2. The inset of denotes the possible conformation of DNA triplex.

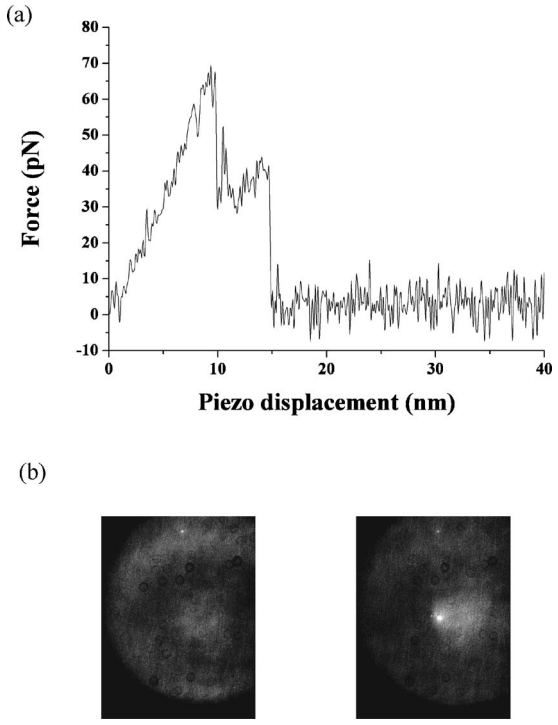

FIG. 2. (a) The rupture force curve of DNA triplex. The first rupture force at 10 nm from the contact point may be caused by the nonspecific binding between the tip and the glutaraldehyde-avidin modified coverslip surface. The last rupture force, at 15 nm from the contact point, comes from breakage of the hydrogen bonds between the Watson strand of oligo1 and oligo2 (H strand). (b) The fluorescence images before (left panel) and after (right panel) the probe withdrawal process. The left image denotes the fluorescence image observed before the probe retraction process when W and H are associated as triplex and F is quenched. It serves as a background intensity image. A brighter spot at the right denotes the fluorescence image observed after the probe retraction process.

eight different cantilevers with spring constants ranging from 4 to 7 pN/nm. The thermal fluctuation of the cantilevers was about 1.5 nm. When the DNA rupture process was finished the molecular fluorescence image of DNA triplex was taken on the Olympus IX71 total internal reflection fluorescence microscopy system (Olympus Optical Co. Ltd., Tokyo, Japan) with cool pix charge-coupled device (iXon DU-887, Andor) at −10 °C.

During the probe withdrawal, a nonspecific probe-substrate interaction can still be detected when the piezodisplacement was around 10 nm [Fig. 2(a)], owing possibly to the extension of the polyglutaraldehyde-avidin complex.[10] Unlike the wormlike-chain model of polyprotein experiments,[5] the force extension curve remains in a plateau (from 10 to 15 nm piezodisplacement) when the nonspecific interaction is relieved. This may have been caused by stretching of the glutaraldehyde-avidin-$(T)_{10}$ linker and conformational change of the ribose in DNA.[4] The stretching force is released suddenly when the probe is pulled 15 nm away from the contact point. Meanwhile, fluorescence signal can still be observed after pulling the DNA molecule for 50 times [right panel of Fig. 2(b)]. This fluorescence image showed a sudden drop of the extension force which we ascribed to a rupturing event of the DNA triplex. The rupture force histogram analysis for 50 approach-retract cycles of AFM indicated that this fluorescence image may be caused by 24 rupture molecules. Therefore, this is a fluorescence image at the molecular level. By applying a different loading rate, from 676 to 20 280 pN/s, the rupture force exerted on the DNA triplex varies accordingly. The rupture force can be revealed from the frequency histograms of the Gaussian distribution fitting at different loading rates [Figs 3(a) and 3(b)]. In the case of DNA triplex environment in *p*H 4.65, the rupture force increases from 38.1 pN (at a loading rate of 676 pN/s) to 83.9 pN (at a loading rate of 20 280 pN/s) [the red line of Fig. 3(c)]. There is a linear relation between the rupture force and the logarithm of the loading rate, indicative of a single potential well model for the DNA rupture.[11,12] Furthermore, we observed a single rupture force (i.e., from all to none) at a piezodisplacement of 15 nm during the dissociation process [Fig. 2(a)]. Therefore, we concluded that the DNA rupture, in this occasion, is a two-state process; namely, all the hydrogen bonds and the stacking force of the base pairs between the Hoogsteen and Watson strands broke at the same time (i.e., from all to none) during the DNA triplex dissociation process. The inclusion of the GA step is destabilizing and perhaps the reason for a two-state rupture. The K_{off} values of triplex DNA rupture event, derived from Evans' study,[12] were 3.13×10^{-2} and 9.38×10^{-2} s^{-1} at *p*H 4.33 and 3.48, respectively.

In order to understand the effect of *p*H on the single molecular mechanism during the triplex formation, we performed DNA triplex rupture experiments by varying *p*H values from 3.40 to 6.01 systematically. The maximum rupture force occurred in the range from *p*H 4.12 to 5.00, with magnitude between 37.6±2.2 and 40.3±1.6 pN [Fig. 3(d)]. The data points fit a Gaussian curve nicely with a maximum at *p*H 4.56. However, the rupture force declines rapidly to 17.6±1.9 pN when the *p*H is raised to 5.5, indicating that the cytosines of the Hoogsteen strand are no longer protonated and, consequently, the ability to form the hydrogen bonds with guanine of the Watson strand is reduced.[1,2]

Although the pK_a of cytosine is 4.2, the pK_a of the N_3 on cytosine base in a nucleotide chain varies with its local environment,[13] such as the dielectric constant (of interior DNA) and electrostatic potential (of neighboring bases).[14] Thus, the pK_a's of C^+ (shown by the midpoint of the association) in different sequences and chain lengths are usually measured experimentally.[1] According to previous studies,[1,14] it is not uncommon that the pK_a of N_3 on the cytosine base in a nucleotide chain may rise to 5.6, consistent with our experimental observation here. The peak at 4.56 is due to the

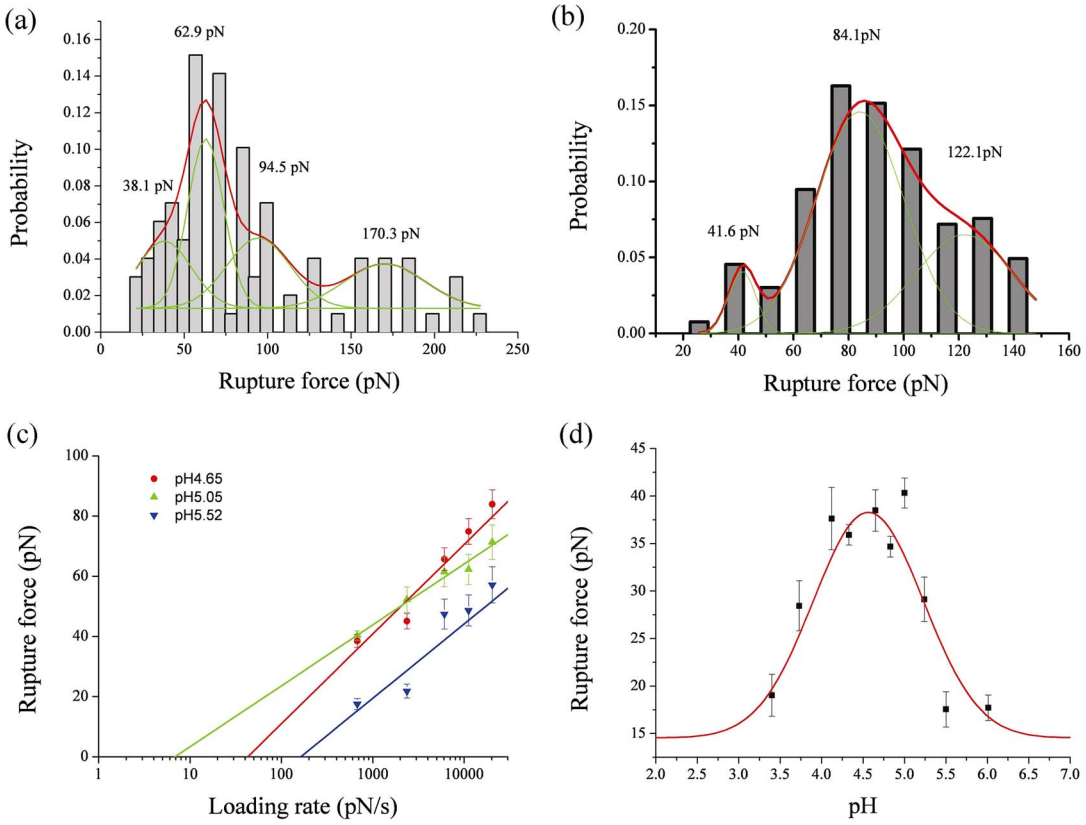

FIG. 3. (Color online) Frequency histograms of rupture forces for DNA triplex at (a) the loading rates of 676 pN/s (n=309 rupture events) and (b) 2366 pN/s (n=261 rupture events). Red lines: The Gaussian distribution fitting of the rupture forces. Green lines: The deconvolution of the Gaussian distribution which revealed the rupture force of the single molecule. (c) The loading rate vs the rupture forces of DNA triplex as a function of pH. The red, green, and blue dots (with least square fittings) denote the rupture forces in pH 4.65, 5.05, and 5.52 buffer conditions, respectively. (d) The rupture forces of DNA triplex (black squares with standard error bars) and their Guassian distribution fittings in different pH environments. The loading rate is 676 pN/s.

formation of a fully protonated triplex, this means that the pK_a of cytosine is higher than 5.6 and consistent with NMR observations of the literature.[1] One should also pay attention to the underlying DNA duplex stability.[15] Although the formation of DNA duplex may be less favorable in a lower pH environment, however, weaker duplexes may yield stronger triplexes. Therefore, we observed the strongest triplex formation at pH 4.56.

The rupture force decreases to 19.0±2.2 pN at pH 3.40, which means that the overall hydrogen bonds of the triplex are weakened in a low pH environment. The baseline of Gaussian distribution curve fitting is around 14.57 pN. This indicates that the DNA triplex may dissociate spontaneously at a pH level lower than 2.5 or higher than 6.5. The formation mechanism of DNA triplex, revealed here by AFM at the single-molecule level, is consistent with our previous macroscopic results[1,2] that both protonated cytosine and pH environment play vital roles in the DNA triplex formation.

In summary, we have designed a pair of specific nucleotide sequences, with which a 17-mer DNA triplex can be formed. By combining AFM and FRET techniques the dynamics of its dissociation mechanism has been revealed at the single-molecular level. DNA dynamics and the related molecular mechanism of other sequences, at the single-molecule level, are under investigation following similar design and methods.

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