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I-motif and quadruplex-based device that can control a protein release or bind and release small molecule to influence biological processes

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Abstract—To expand the function of DNA machines, we constructed a non-DNA-fuel machine based on the G-quadruplex and i-motif structures within the telomere DNA sequence. Depending on the binding or non-binding of the specified form, the DNA machine is able to bind or release the telomere-binding protein TRF 1, and to release small quadruplex-binding molecules to impede progress of the polymerase. This DNA machine, driven by pH change, does not accumulate duplex DNA waste products to poison the system. These new functions undertaken by structured nucleic acids open many opportunities to create and expand the further functions and use of DNA and RNA.

1. Introduction

DNA machines based on self-assembly at a nanometer scale are currently being explored as nanomolecular devices that are capable of performing conformational changes.[1–16] Structural changes based on DNA conformation, such as B–Z transition and quadruplex–duplex exchange, can be detected by fluorescence resonance energy transfer (FRET).[17] We previously reported a device powered by the B–Z transition, switching the DNA from one structure to the other in response to temperature change.[18]

In previous studies, these DNA devices did not perform further functions, although they displayed a variety of DNA conformational changes. The effort to construct novel functional DNA nanodevices will offer new ways of delivering drugs and influencing biological processes. Dittmer and colleagues recently reported a DNA machine that can be selectively instructed to grab or release a protein, but the released protein cannot perform any further function.[19]

To create and expand the further functions and use of DNA machines, we constructed a non-DNA-fuel machine based on the G-quadruplex and i-motif structures within the telomere DNA sequence. TRF 1 is a 53-amino acid sequence that binds specifically to the duplex region of the telomere sequence, TTAGGG. The solution structure of the TRF 1–DNA complex has been elucidated by NMR.[20] TMPyP4 is a small molecule that binds selectively to the G-quadruplex structure. In the duplex form, the DNA machine binds TRF 1 and releases TMPyP4, whereas, in the G-quadruplex form, it binds TMPyP4 and releases TRF 1. In addition, the DNA conformational changes can be driven by pH variations. In previous studies, the DNA nanomachines were fuelled by complementary oligonucleotides and accumulated double-stranded DNA waste products that poison the system. Because our present system does not accumulate wasted 'fuel DNA' and produces only water, it is a relatively clean system.

The working principle of the machine is shown in Figure 1. A 22-mer single-stranded oligonucleotide I, which contains four stretches of CCC, folds into a four-stranded i-motif structure by hemiprotonated C–C$^+$-base pairs.[21,22] The second component is the complementary 22-mer DNA strand Q, which can form an intramolecular G-quadruplex structure. At pH < 5.5,

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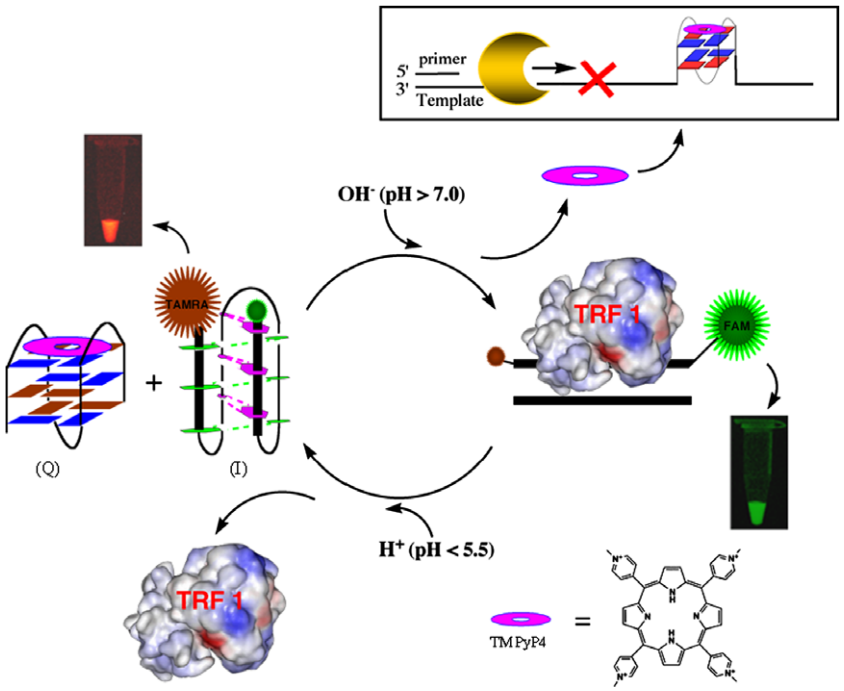

Figure 1. Principle of the operation cycle of the pH-driven DNA nanomolecular machine. FAM (red) and TAMRA (yellow) were connected to the ends of the C-rich strand I to monitor the cycle operation. The protein TRF 1 and the small molecule TMPyP4 were bound and released by the formation of different structures. The released TMPyP4 functioned as a stabilizer of the G-quadruplex to impede progress of the polymerase. The chemical structure of TMPyP4 is shown.

strands I and Q-fold into an i-motif and G-quadruplex structure (iGS). When the pH value is raised to >7.0, strand I unfolds and hybridizes with the Q strand to form a duplex structure. Structural transition can be performed by changing the solution pH with the addition of H^+ or OH^-. In the duplex structure, the DNA binds to TRF 1, a specific double-stranded telomere DNA binding protein.[20] TMPyP4 is a G-quadruplex-binding small molecule. Increasing the pH value to >7.0 caused the human telomere sequences of I and Q to form a duplex that released the TMPyP4. The released drug might induce a significant conversion of a different G-rich region of the Rb gene, which may be sufficient to impede the progress of polymerase in the G-rich region of the template DNA.[23] In contrast, formation of iGSs that are not capable of binding to TRF 1 results in protein release.

An electrophoresis experiment was carried out to confirm the operation of the machine. As shown in Figure 2c, the DNA machine appeared to move slower at pH 7.0 than at pH 5.0. These results indicate clearly that the DNA machine binds to TRF 1 at pH 7.0 and that it forms iGS and releases TRF 1 at pH 5.0. The color changes in the gel because of the FRET were consistent with the conformation transition of the DNA machine. FRET experiments were performed to directly image the machine's operation by following distance changes with conformational alteration in the DNA device accompanying the binding and release of TRF 1 (Fig. 2). In the duplex structure (pH > 7.0), FAM emission is observed at 534 nm (green). In the iGS (pH < 5.5), FRET results in a color change from green to yellow, corresponding to TAMRA (580 nm). The operative cycling is simply reflected by two color changes. CD spectra were used to further confirm the structural transition of the DNA machine (Fig. 3). For the strands I and Q, measurement at pH < 5.0 revealed a positive band at 285–290 nm and a smaller negative band near 255 nm, which are characteristics of the formation of iGS (Fig. 3, pink dot). At pH > 7, the positive and negative bands shifted toward 275 and 240 nm, respectively, consistent with a B-form duplex DNA structure (Fig. 3, sky dot). The transition was cooperative and reversible, with a pH change between 4.5 and 8.0 (Fig. 3, blue and back dots). In the region between pH 4.5 and 8.0, the spectra intersected at isoelliptic points at about 245 and 275 nm, suggesting a transition between two different conformational states. Examination of the effect of TRF 1 and TMPyP4 on the transition pH of the DNA machine showed that the midpoints of transition in the presence of TRF 1 and TMPyP4 were at pH 6.5 and 5.8, respectively. These results suggest that these agents can influence the operating pH of the DNA machine.

In this system, the concentration of free TMPyP4 in solution is controlled by the DNA conformation. In the iGS, the TMPyP4 binds efficiently to the G-quadruplex, and its free concentration is low. With formation of the duplex at pH > 7.0, the binding of the drug to the G-quadruplex decreases, and the free TMPyP4 concentration in the solution increases. The higher concentration of drug stabilizes the G-quadruplex structure, which might result in a significant conversion of the G-rich segment to the G-quadruplex, which is sufficient to impede progress of the polymerase. To test this pos-

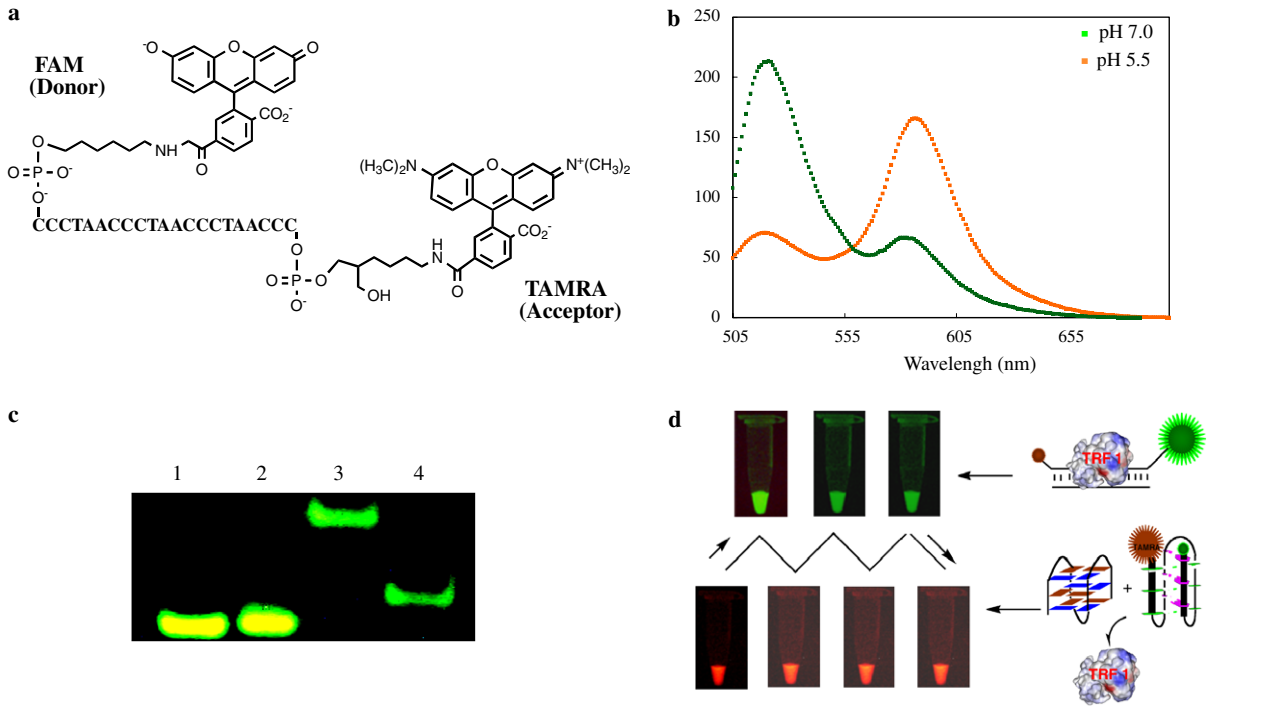

Figure 2. (a) The chemical structure of I together with FAM and TAMRA. (b) Fluorescence emission spectra of the iGS (i-motif and G-quadruplex structure) and duplex states with excitation at 490 nm in pH 7.0 and pH 5.5. (c) In-gel color change from the operation of the DNA machine. Lane 1: pH 5.0, without TRF 1; lane 2: pH 5.0, with TRF 1; lane 3: pH 7.0, with TRF 1; lane 4: pH 7.0, without TRF 1. (d) Cycling of the DNA nanomachine, as observed by the color change induced by FRET. Three cycles were recorded by digital camera.

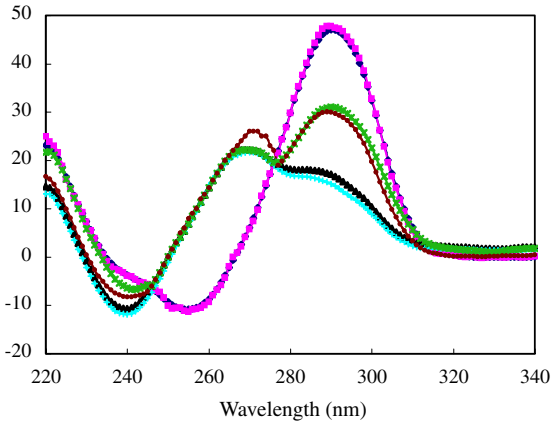

Figure 3. CD spectra of strands I and Q in different conformational states. All spectra were recorded at pHs ranging from 4.5 to 8.0 at 35 °C. The transition was induced by adjusting the sample pH by the addition of HCl or NaOH.

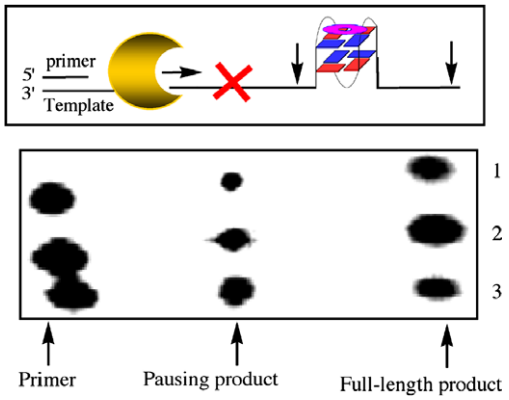

Figure 4. Blockage of polymerase-mediated DNA synthesis by TMPyP4 stabilization of the G-quadruplex on a DNA template containing a C-rich sequence (6 nM). Lane 1: without TMPyP4; lane 2: addition of G-rich segment DNA and TMPyP4 complex (12 nM); lane 3: with the DNA nanomolecular machine (12 nM) that released TMPyP4 by formation of duplex at pH > 7.0.

sibility, a 66-mer DNA template containing a G-rich sequence was incubated in the presence of *Taq* DNA polymerase with a primer with a complementary sequence to the 3′ end of the 66-mer template. The principle of the assay is shown above the gel in Figure 4. If complex extension of the primer occurs, a full-length 66-mer product is formed. However, factors that promote and stabilize G-quadruplex formation will lead to a specific pause site on the template, resulting in the formation of a truncated product. The amount of polymerase-pausing at the G-quadruplex site is a direct measure of the degree of stabilization by TMPyP4, a G-quadruplex structure stabilizer that is released with the formation of a duplex. In the absence of TMPyP4, there was only a slight pausing of Taq polymerization at the G-quadruplex-forming site, whereas significantly greater pausing was observed at the same position when the drug was released by the formation of duplex at pH > 7.0 (Fig. 4, lanes 1 and 3). The 22-mer strand Q and TMPyP4 complex were used as parallel experiments and produced less pausing product and more full product than those produced by the DNA nanomolecular machine (Fig. 4, lane 2). These results suggest that the DNA nanodevice can

perform a further function by controlling it in a specific form.

Based on the transition of different DNA conformations, this non-DNA-fuel nanomolecular machine is able to bind and release a protein or small molecule. The device functions to control the protein concentration in solution, and performs further functions of carrying, binding, and regulating small molecules to influence biological process. Although the released TRF 1 does not influence on any biological process in the present system, pH-dependent DNA conformational changes induce the simultaneous release and binding of proteins and small molecules. Such new functions undertaken by structured nucleic acids open up many opportunities to create and expand the functions and use of DNA and RNA.

2. Experimental

2.1. Preparation of oligonucleotides

The human telomere sequences for I and Q are d(CC CTAACCCTAACCCTAACCC) and d(GGGTTAG GGTTAGGGTATGGG), I with two fluorophores, 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethylrhodamine (6-TAMRA) at the 5′ and 3′ termini as the donor and acceptor, respectively. The oligonucleotides were purchased from Proligo Japan.

2.2. Fluorescence and CD measurements

CD spectra were measured using an AVIV MODEL 62 DS/202 CD spectrophotometer. CD spectra were recorded using a 1 cm path-length cell. Solutions for CD spectra were prepared as 1 mL samples at 0.1 mM (base concentration) in the presence of 100 mM NaCl at 35 °C. The sample pH was adjusted for each experiment with HCl and NaOH, and monitored using a glass microelectrode.

The cycling of the nanomotor starts with 100 mL of 1 mM I and Q solutions containing 50 mM NaCl. The pH of the solution was cycled between 5.0 and 8.0 by alternately adding 1 M HCl or 1 M NaOH. The sample was excited using a UV illuminator and imaged with a Kodak digital camera. All images were assembled to give Figure 3.

2.3. Gel electrophoresis experiment

Non-denaturing gel electrophoresis experiments were performed with the I and Q oligonucleotides. Experiments were performed in a 50 mM MES buffer (pH 5.5), or a TBE buffer (pH 7.2). The oligonucleotides were heated at 95 °C for 5 min followed by incubation at room temperature. The samples were loaded onto a non-denaturing 15% polyacrylamide gel and run for 2 h (10 V/cm) at room temperature. The resulting gel was imaged with a Kodak digital camera as described above. The DNA-binding domain of human TRF1 was prepared as described previously.[20]

2.4. Polymerase stop assay

Labeled DNA primer d(TAATCAGCACTACATATG) (5′-labeled with Texas red) (24 nM) and template d[CCTAATCTATCTTAC(*CGGGGGGTTTTGGGCGGC*)TTACGCACTCGAATGCATATGTAGTGCTGATTA] (12 nM) were annealed in buffer (10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, with 1.5 μg/μL BSA) with 0.1 mM dNTPs by heating to 95 °C and slow cooling to room temperature. We recently demonstrated that the sequence d(*CGGGGG GTTTTGGGCGGC*) at the 5′ terminus of the Rb gene can form an antiparallel G-quadruplex that is further stabilized by TMPyP4.[23] The DNA polymerase stop experiments were carried out without TMPyP and the DNA nanomolecular machine, which released TMPyP4 by formation of a duplex at pH > 7.0. *Taq* DNA polymerase (5 U) was added and the mixture was incubated for 20 min. The polymerase extension was stopped by adding 2× stop buffer (10 mM EDTA, 10 mM NaOH, 0.1% xylene cyanole, and 0.1% bromophenol blue in formamide solution), and the solution was loaded onto a 12% denaturing polyacrylamide gel.

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