DNA G-quadruplex detection system employing a protein fibril ligand

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Thioflavin T (ThT), a typical probe of protein fibrils, also binds human telomeric G-quadruplexes with high specificity for other DNA structures. Upon binding, ThT fluoresces brightly, making it a promising G-quadruplex probe. Because ThT was originally used for detecting protein fibrils, false-positive detection and imaging of DNA G-quadruplexes is possible in the presence of proteins, such as in living cells. We therefore developed a system for the specific discrimination of DNA G-quadruplexes from protein fibrils through measurement of fluorescence resonance energy transfer (FRET) from ThT to a DNA duplex probe. We designed a complementary DNA (cDNA) that hybridizes with the single-stranded region near the DNA G-quadruplex–forming region. Hexidium iodide (HI), a typical DNA duplex fluorescent intercalator, was utilized as an acceptor for FRET from ThT. FRET occurred only when ThT bound the G-quadruplex region and HI bound the duplex region of the cDNA. Fluorescence analysis showed that this system can detect DNA G-quadruplexes even in the presence of protein fibrils.

**Keywords:** G-quadruplex; fluorescent resonance energy transfer (FRET); thioflavin T (ThT)

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**Introduction**

G-rich DNA sequences are known to form G-quadruplexes (Fig. 1a). G-quadruplexes can be formed with four Hoogsteen base-paired coplanar guanines, a structure known as a G-quartet (Fig. 1b) [1-3]. Bioinformatics studies have identified more than 370,000 G-rich sequences in the human genome [4, 5]. The best known G-rich sequences are the telomeres at chromosome termini [6]. As telomeres are extended by the enzyme telomerase in cancer cells and are thus potential targets for anticancer agents [7-9], many G-quadruplex ligands have been developed [10-16].

G-quadruplex ligands developed as anticancer agents should not only have affinity for G-quadruplexes but also high specificity for DNA duplexes, which are the most abundant DNA structures in living cells [10]. In previous studies, we and other researchers reported that the protein fibril fluorescence indicator thioflavin T (ThT: 4-[3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl]-N,N-dimethyla
niline) (Fig. 2a) binds DNA G-quadruplexes and exhibits high specificity for other DNA structures [18, 19]. Although ThT is a potentially important fluorescent probe for G-quadruplexes, it is still not possible to detect DNA G-quadruplexes in the presence of other biomolecules, such as in the case of analyses of living cells, because ThT fluoresces upon interaction with both DNA G-quadruplexes and protein fibrils, resulting in false-positive detection and imaging of G-quadruplexes.

Here, we report the development of a G-quadruplex–specific detection system employing fluorescence resonance energy transfer (FRET) (Fig. 2b), which permits G-quadruplex detection even in the presence of protein fibrils. We focused on the flanking regions near the G-quadruplex–forming region. A synthetic complementary DNA (cDNA) sequence hybridizes to the flanking region, resulting in duplex formation. Hexidium iodide (HI) (Fig. 2a), which is a typical DNA duplex binder, was utilized as a FRET acceptor [20]. Fluorescence of ThT was observed in the presence of G-quadruplex structures. HI bound the duplex formed near the G-quadruplex–forming region. As a result, FRET from ThT to HI was observed only when HI and ThT bound the duplex and G-quadruplex regions, respectively, making it possible to detect G-quadruplexes even in the presence of protein fibrils.

**Materials and Methods**

**Sample preparation**

High-performance liquid chromatography (HPLC)-grade oligonucleotides used in this study were purchased from Sigma-Aldrich Japan (Tokyo, Japan). ThT and HI were purchased from Sigma-Aldrich Japan and used without further purification. All other chemicals were of reagent grade and obtained from Wako Pure Chemical Co., Ltd.

**UV melting curve**

UV absorbance was measured using a Shimadzu 1700 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a temperature controller. Melting curves for G-quadruplexes and duplexes were generated by measuring the UV absorbance at 295 and 260 nm, respectively, in cells with 0.1-cm path length. All measurements were carried out for a total DNA strand concentration of 20 μM in buffer containing 100 mM KCl and 50 mM MES (pH 7.0). The heating rate was 0.5°C min⁻¹ for both G-quadruplexes and duplexes. Before measurement, each sample was heated to 95°C and then gently cooled to 0°C at a rate of 0.5°C min⁻¹.
Circular dichroism (CD) spectroscopy

CD measurements for samples with a total DNA strand concentration of 20 μM were obtained using a JASCO J-820 spectropolarimeter (JASCO, Hachioji, Japan) at 25 ºC in a cuvette with a 0.1-cm path length. CD spectra were obtained by taking the average of at least three scans acquired from 200 to 350 nm. The temperature of the cell holder was regulated by a JASCO PTC-348 temperature controller, and the cuvette-holding chamber was flushed with a constant stream of dry N2 gas. Before measurement, the samples were heated to 95 ºC and then gently cooled to 25 ºC at a rate of 0.5ºC min⁻¹.

Fluorescence spectroscopy

Fluorescence of ThT were acquired using a Varioskan Flash (Thermo Fisher Scientific K.K., Yokohama, Japan). 96-well titer plates (Sumilon S-type plate) were purchased from Sumitomo Bakelit Co. Ltd., Tokyo, Japan. Fluorescence spectra were measured by taking the average of three scans made with 0.5-nm intervals from 460 to 650 nm. The excitation wavelength was 450 nm. Before measurement, the DNA and ThT samples were heated at 95ºC for 2 min and then gently cooled to 25ºC at 0.5ºC min⁻¹.

DNA oligonucleotides were titrated with 1 μM ThT in buffer containing 100 mM KCl and 50 mM MES-LiOH (pH 7.0). The fluorescence intensity at 485 nm (F₄₈₅) was plotted against the concentration of DNA oligonucleotide. The data were fitted with a theoretical equation (1) based on a model with n binding sites in order to evaluate the apparent dissociation constant (K_D) at 25ºC [23], where ΔF represents the change in fluorescence intensity and F₀ represents the initial F₄₈₅ value. The titration data were fitted using KaleidaGraph (Synergy Software, Reading, Pennsylvania, U.S.).

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F_{485} = \frac{\Delta F [\text{DNA}]_n}{(K_D^n + [\text{DNA}]_n) + F_0}
\]  

Results and Discussion

ThT fluorescence with G-quadruplex–forming G4-SS-DNA

ThT binding was analyzed with G4-SS-DNA, which was composed of a G-quadruplex region and a single-stranded region capable of hybridizing with a complementary sequence (see Table 1 for sequence and Fig. 2b for structure). As a control, we also used G4-DNA, which consisted of only the G-quadruplex–forming region. Figure 3a shows fluorescence spectra of 1 μM ThT with various concentrations of G4-SS-DNA in buffer containing 100 mM
KCl and 50 mM MES-LiOH (pH 7.0) at 25°C. Significant fluorescence enhancement was observed with higher concentrations of G4-SS-DNA. Because ThT fluoresces only when complexed with G-quadruplex [19], this result indicated the formation of a G4-SS-DNA G-quadruplex. Figure 3b shows the normalized fluorescence intensity of ThT at 485 nm ($F_{485}$) in the presence of various concentrations of G4-SS-DNA or G4-DNA. $F_{485}$ increased with addition of G4-SS-DNA, as was also observed for G4-DNA. As it has been reported that G4-DNA folds to form a G-quadruplex in buffer [17], these results further confirmed the formation of a G4-SS-DNA G-quadruplex.

The dissociation constant ($K_D$) at 25°C of ThT was 6.0 ± 0.2 and 2.4 ± 0.2 μM with G4-DNA and G4-SS-DNA, respectively (see Fig. 3b). Interestingly, G4-SS-DNA exhibited a slightly higher binding affinity for ThT compared with G4-DNA. The difference in the affinity of ThT for G4-SS-DNA and G4-DNA suggests that the flanking region of G4-SS-DNA affects binding with ThT. The structure of G4-SS-DNA will be discussed further below.

Structure and thermal stability of the G4-SS-DNA/cDNA complex

The cDNA was designed to bind the single-stranded flanking region near the G-quadruplex–forming region in G4-SS-DNA. Because the secondary structure of a given region in a long oligonucleotide can affect the secondary structures formed in neighboring regions, it was important to investigate whether the G4-SS-DNA/cDNA complex could fold to form both the G-quadruplex and duplex shown in Figure 2b. Figure 4a shows the normalized melting curves at 260 nm of the G4-SS-DNA/cDNA complex (red). As a control for the duplex structure, we utilized SS-DNA with the same sequence as the flanking region of the G4-SS-DNA to form a duplex with the cDNA. The UV melting curve of the SS-DNA/cDNA complex is also shown in Figure 4a (black). As the hyperchromic UV melting curve at 260 nm was indicative of a typical duplex structure [26], these data show that the G4-SS-DNA/cDNA complex formed a duplex, as was also observed with the SS-DNA/cDNA complex.

The melting temperatures of the G4-SS-DNA/cDNA and SS-DNA/cDNA complexes were 74.0 and 74.5°C, respectively, indicating that G-quadruplex formation involving the flanking region does not affect the thermal stability of the duplex.

Figure 4b shows the normalized melting curves at 295 nm of the G4-SS-DNA/cDNA complex (red) and G4-DNA only (black). Thermal denaturation of a G-quadruplex is known to produce a hypochromic UV melting curve at 295 nm [26]. Thus, these curves demonstrate that the G4-SS-DNA/cDNA complex forms a G-quadruplex, similar to G4-DNA. These data also suggest that the flanking duplex region does not significantly affect the thermal stability of the G-quadruplex. Together with the results of analyses of the thermal stability of the duplex, it is possible to conclude from these results that the duplex and G-quadruplex structures maintain their thermal stability even if the structure of the flanking region is altered. Our results thus confirm that the G4-SS-DNA/cDNA complex folds to form both a G-quadruplex and duplex, as shown in Figure 2b.

The structure of the G4-SS-DNA/cDNA complex was further analyzed using CD spectroscopy. Previous studies showed that a CD spectrum with positive and negative peaks around 260 and 240 nm, respectively, is indicative of a parallel type G-quadruplex conformation [27, 28]. In contrast, a CD spectrum with positive and negative peaks at around 295...
and 260 nm, respectively, is indicative of an antiparallel G-quadruplex conformation [27, 28]. Moreover, a CD spectrum with positive and negative peaks around 295 and 240 nm, respectively, is indicative of a mixed G-quadruplex conformation [27, 28]. Figure 4c shows CD spectra of the G4-SS-DNA/cDNA complex in the presence of K⁺ (red) or Li⁺ (black) ions. Positive and negative peaks around 295 and 240 nm were observed under these conditions, indicating that the G4-SS-DNA/cDNA complex assumes an antiparallel conformation. Moreover, the positive and negative peaks in the presence of Li⁺ were smaller than those observed in the presence of K⁺ ions. These results indicate that the G-quadruplex of the G4-SS-DNA/cDNA complex is unstable in the presence of Li⁺. As destabilization by Li⁺ is a signature feature of G-quadruplexes [29, 30], these results further confirm that the G4-SS-DNA/cDNA complex forms a G-quadruplex in the presence of K⁺.

Proof of concept for the G4 detection system employing ThT

Figure 5a shows fluorescence spectra of 10 μM ThT with various concentrations of HI in KCl buffer containing 10 μM G4-SS-DNA and cDNA at 25°C. The fluorescence intensity of ThT at 485 nm decreased and that of HI at 610 nm increased as a function of HI concentration, clearly demonstrating that FRET between ThT and HI occurred in the presence of the G4-SS-DNA/cDNA complex. Figure 5b shows the fluorescence intensity of HI at 610 nm (F_{610}) versus HI concentration in the presence of G4-SS-DNA and cDNA (red), G4-SS-DNA only (green), G4-SS-DNA only (blue), and in the absence of G4-SS-DNA and cDNA (black). F_{610} significantly increased as a function of HI concentration in the presence of both G4-SS-DNA and cDNA. In the presence of G4-SS-DNA only, F_{610} was almost constant, even at higher HI concentrations, indicating the absence of FRET from ThT to HI. Similarly, F_{610} did not increase significantly in the presence of cDNA only. The background fluorescence intensity of HI (ca. 50) observed in the presence of either G4-SS-DNA or cDNA may have been due to nonspecific binding of HI to single-stranded DNA. Moreover, F_{610} did not increase as a function of HI concentration in the absence of G4-SS-DNA and cDNA. These results demonstrate that FRET between ThT and HI occurs only in the presence of the G-quadruplex and duplex.

Selective detection of G-quadruplexes in the presence of protein fibrils using the ThT-HI FRET system

We also examined whether the FRET detection system proposed here can distinguish between DNA G-quadruplexes and Aβ fibrils. Figure 6a shows fluorescence spectra of 10 μM ThT with 10 μM G4-SS-DNA/cDNA complex with various concentrations of HI in buffer containing 100 mM KCl and 50 mM KH₂PO₄-KOH (pH 7.5). The fluorescence intensity of ThT at 485 nm decreased and that of HI at 610 nm increased as a function of HI concentration, demonstrating FRET between ThT and HI. We also acquired fluorescence spectra of ThT and HI with the G4-SS-DNA/cDNA complex in the presence of 5 μM Aβ in the fibril form, prepared prior to the experiment as described in the Materials and Methods [24, 25].

Figure 6b shows a plot of F_{610} versus HI concentration in the presence (red) or absence (black) of Aβ. As a control, we acquired fluorescence spectra of ThT with various concentrations of HI in buffer containing Aβ fibrils but not the DNAs (blue). In both the absence (black) and presence (red) of Aβ, the fluorescence intensity of HI increased as a function of HI concentration, although the fluorescence...
intensity of HI was slightly reduced in the presence of Aβ fibrils. This reduction in fluorescence intensity could be attributed at least in part to the binding of ThT to the Aβ fibrils. These results demonstrate that FRET between ThT and HI occurs in both the absence and presence of Aβ fibrils. Moreover, in the absence of DNAs (Fig. 6b, blue), no FRET was observed, because ThT binding to the Aβ fibrils were not able to close to HI, which binds the DNA duplex. These results demonstrate that the FRET system described here enables detection of G-quadruplexes with high specificity, even in the presence of Aβ fibrils. Use of this system with ThT may enable the detection of G-quadruplexes with high specificity in living cells, even in the presence of protein fibrils and aggregates.

**Conclusion**

Recent studies have demonstrated that ThT is a good DNA G-quadruplex probe, providing high-specificity detection of DNA duplexes. However, the use of ThT may lead to false-positive detection of DNA G-quadruplexes in living cells because ThT binds not only to DNA G-quadruplexes but also to protein fibrils. In this study, we successfully distinguished G-quadruplexes from protein fibrils using a system that detects FRET from ThT to a DNA probe hybridized to the flanking region near the G-quadruplex–forming region. It is noteworthy that this system is suitable for sequence-specific detection of both DNA and mRNA G-quadruplexes.

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**Conflict of interest**

The authors declare that they have no competing interests.

**References**


