Probing the stable G-quadruplex transition using quencher-free end-stacking ethynyl pyrene–adenosine†

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Pyrene-modified adenosines in the dangling positions of G-rich oligodeoxynucleotides undergo π-stacking in their G-quadruplex formation, but not in their single strands, which can be characterized by fluorescence λ_{max} changes that occur on stacking.

G-quadruplexes are tetraplex conformations of telomere DNA that directly inhibit telomerase activity in most cancer cells[1] and have the potential to control gene expression,[2] thus representing potentially important targets in drug development. Efficient probing of the G-quadruplex transition can thus provide useful insight for the design and development of telomerase inhibitors for cancer, HIV, and other diseases.[3] G-quadruplex structures comprise stacked tetrads, each of which arises from the planar association of four guanines in a cyclic Hoogsteen hydrogen bonding arrangement. Monovalent cations such as K^+ and Na^+ have been shown to stabilize G-quadruplex structures. Several researchers have adopted a structure-based design approach to the development of G-quadruplex probing systems.[4]

Fluorescent nucleoside analogs that are sensitive to the local environment are attractive candidate probes for investigating nucleic acid structure. They display strong signal changes upon structural changes in DNA, *e.g.*, the G-quadruplex and i-motif.[5] Although a broad range of substituted fluorescent dyes are now available for labeling G-quadruplexes, rapid growth in this area requires new and efficient fluorescent nucleoside analogs and advanced design systems to probe G-quadruplex structural changes.[6]

Our aim was to construct an efficient and simple probing system. Here, we propose a new conceptual strategy for probing G-quadruplexes (Fig. 1) based on stacking interactions between a guanosine and non-polar aromatic fluorophores that stack at the termini of a G-quadruplex.[7]

We used the fluorescent nucleoside $\mathbf{A^{PY}}$ developed by our research group[8] because of its high quantum yield and efficient planar aromatic stacking. We expected that pyrene-modified $\mathbf{A^{PY}}$ would enhance the thermodynamic stability of the G-quadruplex through terminal stacking, with concomitant signal change.

We incorporated the fluorophore-labeled $\mathbf{A^{PY}}$ phosphoramidite at the 5′-end of oligodeoxynucleotides (ODNs) using standard phosphoramidite methods[9] and a DNA synthesizer (Fig. 2).[10] We

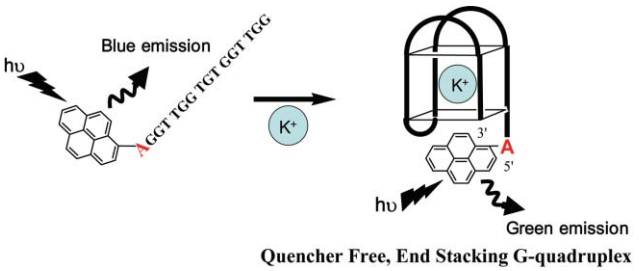

Fig. 1 Design of a quencher-free end-stacking G-quadruplex probing system.

added KCl to a solution of ODN **S1** to induce conformational change from a single strand to a G-quadruplex (anti-parallel chair type).

Initially, we investigated the circular spectroscopic properties to detect conformational changes in the $\mathbf{A^{PY}}$-containing ODN **S1** (Fig. 3A). We also measured the spectra of the natural ODNs **S2** and **S3** in order to compare with the modifier **S1** (Fig. 3B and 3C, respectively). G-quadruplex characteristic bands were identified at around 260 nm (negative) and 295 nm (positive) after adding KCl. From these CD data we can confirm that $\mathbf{A^{PY}}$ incorporation does not interfere with the conformational stability of the G-quadruplex.

Next, we studied the fluorescence for conformational change from a single strand to a G-quadruplex (Fig. 4A), which showed a dramatic emission change. The single strand showed an emission band at λ_{max} = 451 nm, which shifted to 485 nm on transition to the G-quadruplex structure. The black line indicates the single stranded ODN **S1** with no KCl. The second, red line is ODN **S1** with 40 mM KCl and the last, olive line is ODN **S1** with 400 mM

S1 5′ $\mathbf{A^{PY}}$ GGT TGG TGT GGT TGG
S2 5′ GGT TGG TGT GGT TGG
S3 5′ A GGT TGG TGT GGT TGG

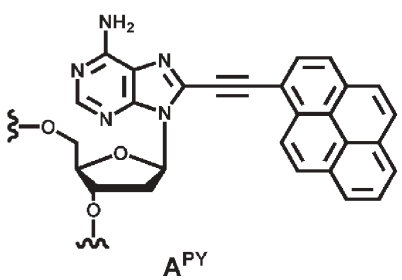

Fig. 2 Structures of $\mathbf{A^{PY}}$ and the oligonucleotide sequences used in this study.

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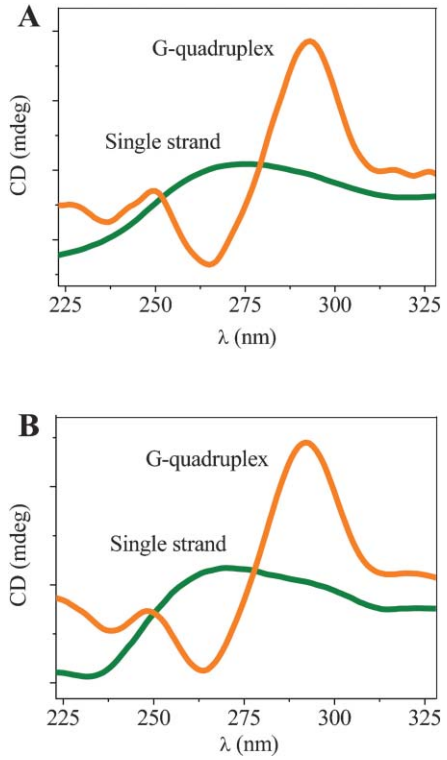

Fig. 3 (A) CD spectra of ODN **S1** (single strand) and ODN **S1** with 100 mM KCl (G-quadruplex). (B) CD spectra of ODN **S2** (single strand) and ODN **S2** with 100 mM KCl (G-quadruplex). (C) CD spectra of ODN **S3** (single strand) and ODN **S3** with 100 mM KCl (G-quadruplex). All samples were prepared at a concentration of 1.5 μM in 5 mM Tris-HCl buffer (pH 7.2) at 20 °C, under irradiation at 260 nm.

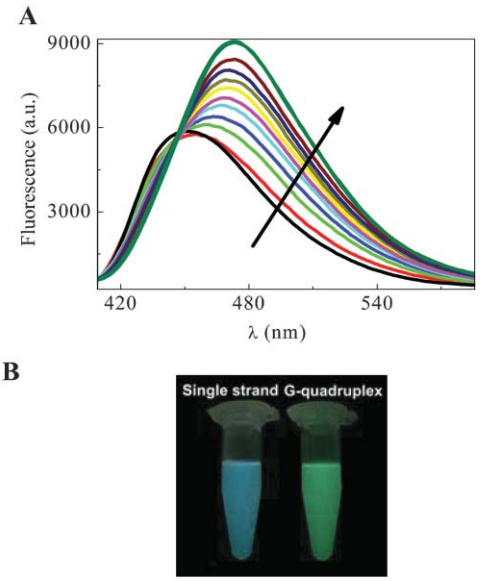

Fig. 4 (A) Fluorescence spectra of the single-stranded ODN **S1** (1.5 μM, black line) and ODN **S1** with KCl (40–400 mM, red to olive line). (B) Photographic image of single-stranded ODN **S1** and ODN **S1** with 800 mM KCl (G-quadruplex). All samples were prepared in 5 mM Tris-HCl buffer (pH 7.2) at 20 °C.

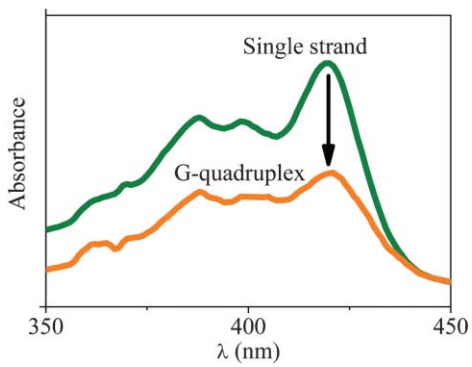

Fig. 5 Absorption spectra of single-stranded ODN **S1** and ODN **S1** with 800 mM KCl (G-quadruplex). All samples were prepared in 5 mM Tris-HCl buffer (pH 7.2) at 20 °C.

KCl. There are hyperchromic and bathochromic shifts with increasing concentration of KCl (not normalized). This emission change is shown visually as an image in Fig. 4B.

The origin of the signal change observed was clarified using UV experiments (Fig. 5). The UV spectrum of ODN **S1** showed a strong absorption band at *ca.* 420 nm for the single-strand structure. After adding KCl to form the G-quadruplex, the band at 420 nm was dramatically reduced. We believe that there are strong interactions between $\mathbf{A}^{\mathbf{PY}}$ and the neighboring base at the end position of the G-quadruplex. The decrease of intensity might be explained on the basis of the mechanism proposed by Arai and coworkers, who reported that pyrene forms an exciplex with guanosine in the stacking position.[7]

To confirm this hypothesis, we used the controlled sequence ODN **S3** again and checked its melting temperature to compare the stability. We measured T_m values in various concentrations of the ODNs **S1** and **S3** (1–20 μM). There was a marked difference in melting temperature between the natural G-quadruplex (the range of T_m: 55.9–56.4 °C) and the $\mathbf{A}^{\mathbf{PY}}$-modified G-quadruplex (the range of T_m: 57.3–59.2 °C). This increase in T_m must be due to the stacking effect between $\mathbf{A}^{\mathbf{PY}}$ and the end surface of the G-quadruplex. These results confirm that fluorescence changes upon structural ODN changes arise from hydrophobic stacking interactions between $\mathbf{A}^{\mathbf{PY}}$ and the G-quadruplex end surface. Stacking also results in higher stability of this G-quadruplex structure. Moreover, from these data we can suggest that the fluorescence change is not caused by intermolecular interaction between pyrenes but caused by intramolecular interaction between the G-quadruplex and $\mathbf{A}^{\mathbf{PY}}$.

In conclusion, we have developed a simple and efficient quencher-free end-stacking probing system to detect conformational change in G-quadruplexes. Most notably, our probe can discriminate conformational changes (from a single strand to a G-quadruplex) without using an additional quencher. The $\mathbf{A}^{\mathbf{PY}}$ was incorporated directly at the end position of a G-rich ODN without a linker. We also observed that the $\mathbf{A}^{\mathbf{PY}}$ fluorophore can induce a stable G-quadruplex upon end stacking, which is very important for inhibition of telomerase function.[11] Therefore, we believe that this quencher-free end-stacking system is very simple, economic and efficient for probing G-quadruplexes.

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