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Quencher-Free, End-Stacking Oligonucleotides for Probing Single-Base Mismatches in DNA

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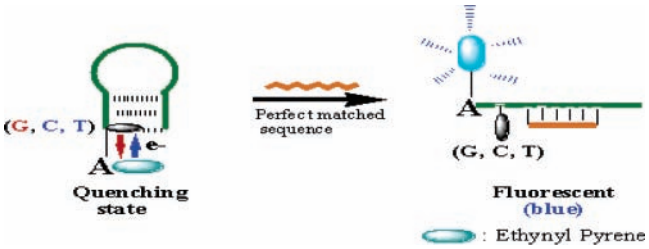
ABSTRACT



Fluorescence can be quenched through PET from the fluorophore to neighboring C, T, and G bases, but not to the A moiety. The hairpin stem stability arising from π-stacking and the PET between the pyrene-labeled 2′-deoxynucleotide units and their neighboring bases are the two main factors that affect the operation of these novel fluorescent oligonucleotides.

Highly selective methods for the detection of specific DNA sequences are becoming increasingly important for medical diagnostics, drug discovery, and genetics.[1] Growth in these areas continues to produce a strong demand for advanced biomolecular recognition probes.[2] Recently, we reported a novel type of molecular beacon (MB) that possesses only a single fluorophore in the hairpin loop.[3] Herein, we propose a conceptually new strategy for preparing modified quencher-free MBs; it is based on fluorescence quenching—through photoinduced electron transfer (PET)—of nonpolar aromatic fluorophores that stack at the termini of a hairpin stem.[4]

Although it is well-established that physicochemical interactions between DNA bases and polynuclear aromatic fluorophores strongly quench the fluorescence of the latter,[5,6] to date, no hairpin-based MB systems that take advantage of this phenomenon—through terminal base stacking—have been reported.

We chose to use pyrene and fluorene moieties as the nonpolar fluorophores because of their high quantum yields and efficient planar aromatic stacking, which we expected would enhance the thermodynamic stability of the duplex through terminal $\pi-\pi$ stacking. We used Sonogashira coupling to attach these fluorophore moieties covalently to purine and pyrimidine bases through acetylene linkers (Figure 1a).[7]

We incorporated the fluorophore-labeled phosphoramidite oligodeoxynucleotides (ODNs) by applying standard phosphoramidite methods and using a DNA synthesizer (Figure

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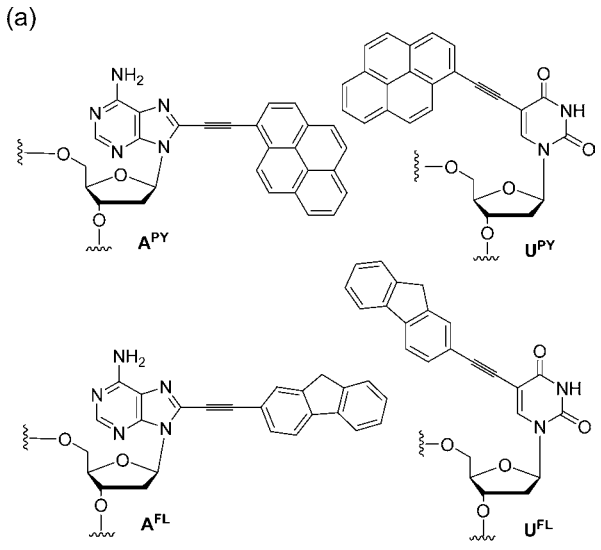

(b)

S1	5'd-$\mathbf{A^{PY}}$**G**CGAG AAGTTAGAACCTATG CTCG**C**
S2	5'd-$\mathbf{U^{PY}}$**G**CGAG AAGTTAGAACCTATG CTCG**C**
S3	5'd-GCGAG AAGTTAGAACCTATG CTCGC
S4	5'd-$\mathbf{A^{PY}}$**A**CGAG AAGTTAGAACCTATG CTCG**T**
S5	5'd-$\mathbf{A^{PY}}$**T**CGAG AAGTTAGAACCTATG CTCG**A**
S6	5'd-$\mathbf{A^{PY}}$**C**CGAG AAGTTAGAACCTATG CTCG**G**
S7	5'd-$\mathbf{A^{PY}}$**A**CGAG AAGTTAGAACCTATG CTCG**C**
S8	5'd-$\mathbf{A^{PY}}$**T**CGAG AAGTTAGAACCTATG CTCG**C**
S9	5'd-$\mathbf{A^{PY}}$**C**CGAG AAGTTAGAACCTATG CTCG**C**
S10	5'd-$\mathbf{A^{PY}}$**G**CGAG AAGTTAGAACCTATG CTCG**A**
S11	5'd-$\mathbf{A^{PY}}$**G**CGAG AAGTTAGAACCTATG CTCG**T**
S12	5'd-$\mathbf{A^{PY}}$**G**CGAG AAGTTAGAACCTATG CTCG**G**
S13	5'd-CATAGGTTCTAACTT
S14	5'd-CATAGGTACTAACTT
S15	5'd-CATAGGTGCTAACTT
S16	5'd-CATAGGTCCTAACTT

Figure 1. (a) Fluorescent nucleoside derivatives used in this study. For their syntheses, see: $\mathbf{U^{PY}}$, refs 7c and 7e; $\mathbf{A^{FL}}$ and $\mathbf{A^{PY}}$, Supporting Information; $\mathbf{U^{FL}}$, ref 3. (b) Oligonucleotide sequences.

1b).[8] Initially, we investigated the absorption and fluorescence spectroscopic properties of the $\mathbf{A^{PY}}$-containing ODN **S1** and the $\mathbf{U^{PY}}$-containing ODN **S2**. The UV−vis spectra indicate that a strong absorption band difference appears at ca. 420 nm for ODN **S1** upon its conformational change, but not in ODN **S2** (Figure 2a,b). The absorption band arises from the ground-state interactions of the fluorophore unit. We chose to excite the system at 420 nm because of the

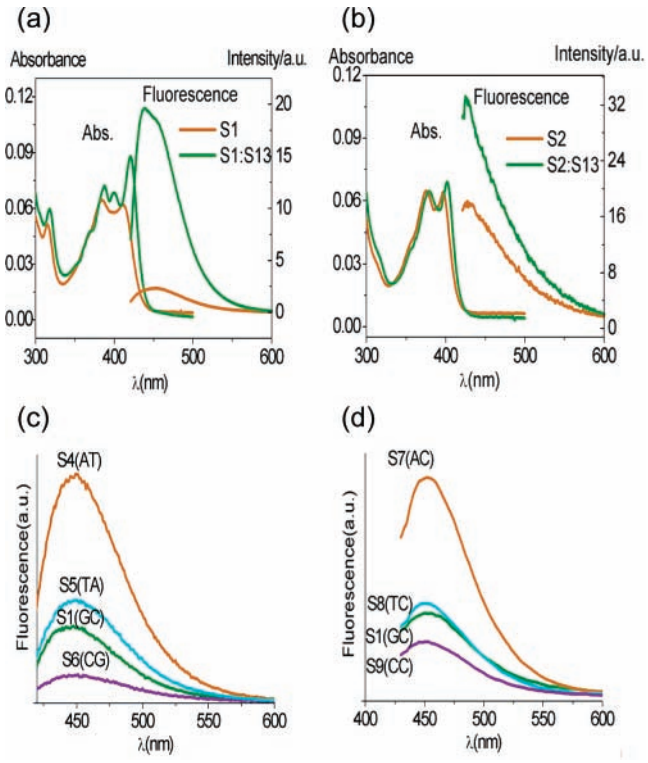

Figure 2. Absorption and emission spectroscopy of ODN **S1** (a) and ODN **S2** (b) and emission spectroscopy depending on matched NBPs (c) and mismatched NBPs and the **S1** standard (d) were recorded using 1.5 μM solutions in buffer (100 mM trizma HCl, 1 mM $MgCl_2$; pH 8) at 20 °C; 420 nm.

difference between the absorption intensities of its hairpin (closed) and matched duplex (open) states. In the resulting fluorescence spectra of ODN **S1** and ODN **S2**, we observe a sharp difference in the fluorescence of the hairpin ODN **S1** and its "fully matched" duplexes with complementary strands (ODN **S1**·**S13**), whereas the fluorescence differences of ODN **S2** were much weaker.

It is noteworthy that these spectral properties must arise from the difference between the electronic properties of the nucleobases that are linked to the ethynyl pyrene fluorophore; **A** is electron donor, whereas **U** is relatively electron acceptor.[5c] The quantum yields of $\mathbf{A^{PY}}$ are similar in both polar aprotic (acetonitrile) and polar protic solvents, such as water, and different electrostatic properties lead to the different spectroscopic properties of the ODN **S1** and ODN **S2** MB systems. The quenching properties of $\mathbf{U^{PY}}$ are known to arise from electron transfer from the pyrene moiety to the uracil base such that proton-coupled electron transfer (PCET) occurs in protic polar solvents.[6,9] This result suggests that the fluorescent nucleobase $\mathbf{U^{PY}}$ must be quenched in both the hairpin and matched duplex states in polar protic solvents.

The fluorescence quenching occurs due to two factors, namely, stable end stacking and strong electronic interaction

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between the π-stacking units. On this basis, and to determine the neighboring base (NB) effect, we designed oligonucleotides **S3**−**S12** that possess different neighboring base pairings (NBPs) for stacking with the fluorescent nucleobase in the hairpin stem (Figure 1b). In the absence of the terminal $\mathbf{A}^{\mathbf{PY}}$ unit (i.e., for ODN **S3**), the stability of the hairpin decreased dramatically (T_m = 47 °C) to a level similar to that of the mismatched NBP hairpins (Table 1). From these

Table 1. Melting Temperatures and Photophysical Properties of Pyrene-Labeled ODNs and Fluorescent Nucleosides

ODNs	discriminaton factor[a]	T_m[b] (°C)	fluorescent nucleosides	ϕ[c]	ϕ[d]	λ_{em}[e] (nm)
S1	7.1	53	$\mathbf{A}^{\mathbf{PY}}$	0.78	0.73	413
S2	1.6	56				434
S3	none	47	$\mathbf{U}^{\mathbf{PY}}$	0.73	0.12	398
S4	1.0	47				421
S5	2.4	46				
S6	4.6	55				
S7	1.1	47				
S8	3.4	46				
S9	2.0	52				
S10	2.5	45				
S11	5.5	46				
S12	3.7	47				

[a] Discrimination factor (for details, see the Supporting Information). [b] Measured at 260 nm in 100 mM Tris-HCl buffer containing 1 mM $MgCl_2$ (1.5 μM). [c,d] Fluorescence quantum yields (Φ) measured in MeCN and MeOH, respectively, and calculated according to the procedure described in ref 11. [e] Emission wavelength.

results, we confirm that the pyrene unit stabilizes the hairpin through π-stacking with its NBP at the terminus of the stem. These experiments strongly suggest that two key factors control these novel MB systems. First, the NBs at the 5′-end play a greater role in the quenching process in the hairpin than do those at the 3′-end. The degree of quenching of the 5′ NBs follows the order **C** > **G** > **T** > **A**, irrespective of the presence of matched or mismatched NBPs in the hairpin state. In the case where **A** is the NB, no quenching occurs in the hairpin state (Figure 2c,d). The quenching of these pyrenyl residues through electron transfer to natural NBs is consistent with the results of a previous study[6] that demonstrated that pyrene is quenched through noncovalent intermolecular stacking interactions with **G**, **T**, and **C** units, but not with the **A** moiety. The discrimination factor of the A−T matching NBP (**S4**) and the A−C mismatching NBP (**S7**) which are 1.0 and 1.1, are extremely low compare to other C, T, G matching and mismatching pairs (Table 1). The formation of a stable $\mathbf{A}^{\mathbf{PY}}$/NB stacking is the other key factor for effective discrimination. Table 1 indicates that the discrimination factor between the hybridized and hairpin states is quite sensitive to the nature of the NB and to the thermodynamic stability of the end stacking interaction in the hairpin state. The **G**−**C** matching NBP (**S1**), which has a discrimination factor of 7.1, has a value of T_m of 53 °C; this value is higher than those of the mismatched NBPs **S10**−**S12** (45−47 °C).

As indicated in Figure 3, our approach makes it possible to discriminate between matched (ODN **S13**) and single-base-mismatched (ODN **S14**−**16**) sequences.[10] We have also investigated whether other types of fluorophores ($\mathbf{A}^{\mathbf{FL}}$, $\mathbf{U}^{\mathbf{FL}}$) and substitution positions can lead to systems that act as quencher-free MBs. Our results indicate that nucleosides labeled with fluorene units, which are smaller than pyrene units and have a lower π-stacking ability, are not efficient probes. In addition, the center of the stem region of a hairpin is not a desirable position for locating the fluorophore of the PET initiator (data not shown).

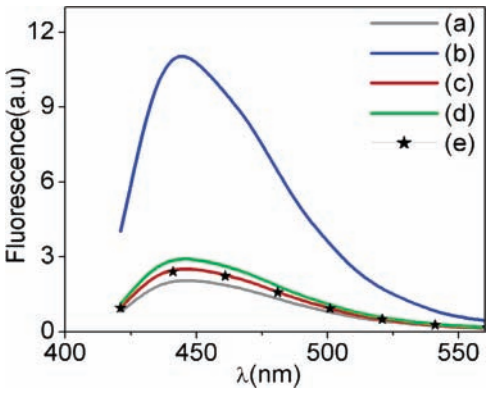

Figure 3. Fluorescence emission spectra of (a) **S1**, (b) **S1:S13**, (c) **S1:S14**, (d) **S1:S15**, and (e) **S1:S16** were recorded at 37 °C using the same conditions of Figure 2 (for details, see the Supporting Information).

In summary, the hairpin stem stability arising from π-stacking and the PET between the pyrene-labeled 2′-deoxynucleotide units and their neighboring bases are the two main factors that affect the operation of these novel MBs. The fluorescence can be quenched through PET from the fluorophore to terminal natural **C**, **T**, and **G** bases, but not to the **A** moiety. $\mathbf{A}^{\mathbf{PY}}$ can distinguish **A** from **C**, **T**, and **G** at the 5′ end of the hairpin. The ODN **S1** allows the ready detection of single nucleotide alterations and does not require the presence of an additional modified fluorescence quencher or linker unit.

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Supporting Information Available: Experimental procedures; absorption and emission spectra of ODN **S4**; energy-minimized (MM+) structures of ODN **S1** and **S2**; oligonucleotide sequences of $\mathbf{A}^{\mathbf{FL}}$ and $\mathbf{U}^{\mathbf{FL}}$; MALDI-TOF mass spectroscopic data; details of the discrimination factor and Figure 3 experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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