Fluorescent Probes

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In-Stem Molecular Beacon Containing a Pseudo Base Pair of Threoninol Nucleotides for the Removal of Background Emission**

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A molecular beacon (MB) is an oligodeoxyribonucleotide (ODN) with a hairpin structure that is dual-labeled at the 5′ and 3′ termini with a fluorophore and a quencher.[1] MBs are widely used as efficient and convenient probes for the sequence-specific detection of target DNA and RNA.[2] In the absence of the target, hybridization of the stem region closes the MB to form a hairpin structure, and fluorescence is quenched as a result of the close proximity of the fluorophore and the quencher. In the presence of the target, however, hybridization of the loop region with the target opens the MB and moves the quencher away from the fluorophore to generate fluorescence. In general, an optimum MB has a probe length of 15–25 nucleotides (nts) with between five and seven base pairs in the stem region.[3] The use of a much longer stem would lead to a decrease in the fraction of the probes in the open state and thus a decrease in background fluorescence.[4] However, such a stem design inhibits the affinity of the loop region for the target and therefore does not favor rapid hybridization with the target. An increase in the loop length improves the affinity but reduces specificity. These limitations have prevented further improvement of the signal-to-background (signal-to-noise, S/N) ratio of conventional MBs. Therefore, efforts have been made to suppress background fluorescence in the absence of the target to enhance sensitivity.[5]

We recently developed a new base surrogate composed of a non-ribose scaffold of D-threoninol tethering dyes (threoninol nucleotide).[6] We found that hybridization of two DNA strands with such threoninol nucleotides at their center enabled interstrand clustering of the dyes to greatly stabilize the duplex. A combination of different threoninol nucleotides also stabilized the duplex. Spectroscopic analysis of the duplex revealed robust staking interactions between the dyes on the two strands. Thus, two threoninol nucleotides act as a pseudo base pair.

Herein, we propose a new MB, the in-stem molecular beacon (ISMB), in which threoninol nucleotides attached to a fluorophore and a quencher are incorporated into the stem region as pseudo base pairs, as illustrated in Figure 1 a. In the absence of target DNA, the fluorophore and quencher are stacked together in the middle of the stem region of the closed beacon (Figure 1 a, left), and background fluorescence is greatly suppressed as a result of the close stacking. In contrast, the presence of the target generates strong emission from the intercalated fluorophore (Figure 1 a, right). In this design, the stem region also recognizes the target (shared-stem MB).[7] As a result, the S/N ratio is greatly improved relative to that of conventional MBs. Furthermore, this ISMB efficiently discriminates deletion mutants from wild-type (full-match) sequences on the basis of the emission wavelength.

ISMBs containing threoninol nucleotides are based on the strong quenching of fluorescence by pseudo base pairing and emission from the intercalated fluorophore. Perylene has a high quantum yield[8] and is not easily quenched by natural bases. These fluorophore properties are preferable for ISMBs. Furthermore, perylene is known to exhibit excimer emission, which has been applied to the efficient detection of single-nucleotide polymorphisms (SNPs) and indels (insertions and deletions).[8] Thus, an MB containing perylene tethered to threoninol is applicable to the ratiometric detection of these polymorphisms (see below). Since reports of efficient quenchers for perylene are scarce, we first investigated three possible chromophores, dabcyl, azobenzene, and anthraquinone.[9] We evaluated their efficiency as perylene quenchers by using the model DNA strands $\mathbf{F_a}$ and $\mathbf{Y_b(X)}$ (Figure 1 b), which were constructed with surrogate quenchers synthesized from the corresponding phosphoramidite submonomers.

When $\mathbf{F_a}$ was hybridized with $\mathbf{Y_b(D)}$, which includes dabcyl, a typical fluorophore quencher, the emission from perylene was quenched efficiently (red line, Figure 2). The intensity of emission from single-stranded $\mathbf{F_a}$ was 15-fold that of $\mathbf{F_a/Y_b(D)}$ at 460 nm, the wavelength at which perylene has its emission maximum; similar efficient quenching was

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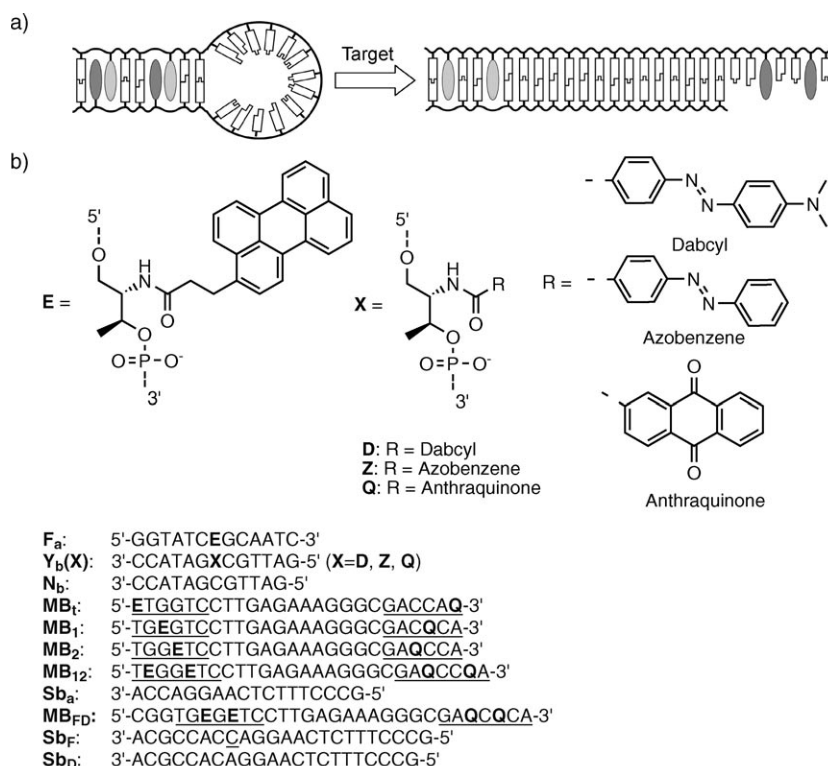

Figure 1. a) ISMB design. See the text for details. b) Structures of candidate perylene quenchers and sequences of modified ODNs. The sequences in the stem part of the MB are underlined. The base in $\mathbf{Sb_F}$ that is deleted in $\mathbf{Sb_D}$ is also underlined.

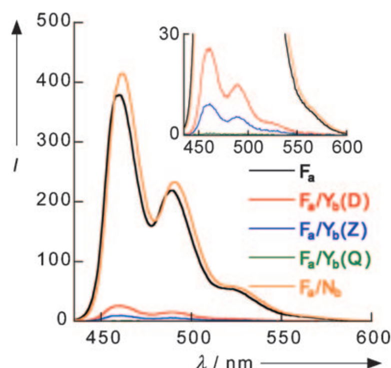

Figure 2. Fluorescence emission spectra of $\mathbf{F_a}/\mathbf{Y_b(X)}$ and single-stranded $\mathbf{F_a}$ (excitation at 425 nm). The solution conditions were as follows: [$\mathbf{F_a}$] = 1.0 μM, [$\mathbf{Y_b(X)}$] = 1.2 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), 20 °C. The corresponding UV/Vis spectra are shown in the Supporting Information.

observed with azobenzene (**Z**; blue line, Figure 2). However, anthraquinone (**Q**) showed the most effective quenching: the emission intensity of $\mathbf{F_a}$ was 500- to 1000-fold that of $\mathbf{F_a}/\mathbf{Y_b(Q)}$ (green line, Figure 2). This remarkable quenching was attributed to the "base pairing" of the fluorophore and quencher on D-threoninol: the close stacking facilitates transfer of the excited electron to the quencher.[10] Thus, rapid electron transfer from perylene to anthraquinone appears to contribute to the quenching of fluorescence.[11] A strong interaction between **E** and **Q** in the ground state might also contribute to effective quenching.[12]

In contrast with the results for $\mathbf{Y_b(X)}$, the hybridization of $\mathbf{F_a}$ with natural $\mathbf{N_b}$ did not lower the fluorescence from perylene at all (orange line in Figure 2). Rather, the emission intensity increased slightly upon intercalation between the natural base pairs.[13] This property was favorable for the design of the present ISMB, as the intercalated fluorophore does not lose its fluorescence intensity.

On the basis of these results, we designed the new ISMB by introducing the fluorophore and quencher pair into the stem region (Figure 1). We chose the survivin gene, which is highly expressed in breast cancer, as our model target.[14] The ISMB has the following advantages: 1) The fluorophore–quencher pair can be inserted at any position in the stem region; 2) tight stacking of the pair in the stem quenches fluorescence efficiently in the closed state; thus, background emission should be diminished; 3) the number of fluorophore–quencher pairs can be increased as long as the fluorophores are separated by natural base pairs. To confirm these advantages of the ISMB, the following four beacons were designed (Figure 1 b): $\mathbf{MB_1}$ and $\mathbf{MB_2}$, which contain a single perylene–anthraquinone (**E**–**Q**) pair at different positions in the middle of the stem region; $\mathbf{MB_{12}}$, which contains two **E**–**Q** pairs, each of which is separated by two intervening natural base pairs, as illustrated in Figure 1 a; and, as a control, the conventional molecular beacon $\mathbf{MB_t}$, in which the fluorophore and quencher of a single **E**–**Q** pair are located at opposite termini.

The fluorescence-intensity ratios at 460 nm for these MBs with and without the target (I_{open}/I_{closed}) are shown in Table 1 (fluorescence spectra are shown in the Supporting Information). An I_{open}/I_{closed} ratio of 3.9 was found for $\mathbf{MB_t}$, which has perylene and anthraquinone attached at its termini. This poor quenching of perylene in the closed state is due to breathing of the terminus. The introduction of an **E**–**Q** pair into the stem region led to improved discrimination, with an I_{open}/I_{closed} ratio of 26 for $\mathbf{MB_1}$. $\mathbf{MB_2}$, which has the **E**–**Q** pair at a different

Table 1: Effect of the position of the **E**–**Q** pair on MB detection.

Sequence	Relative intensity[a] without $\mathbf{Sb_a}$	Relative intensity[a] with $\mathbf{Sb_a}$	I_{open}/I_{closed}[b]
$\mathbf{MB_t}$	0.26	1.0	3.9
$\mathbf{MB_1}$	0.06	1.6	26
$\mathbf{MB_2}$	0.11	1.7	15
$\mathbf{MB_{12}}$	0.02	1.2	58

[a] Fluorescence intensity at 460 nm relative to that of $\mathbf{MB_t}/\mathbf{Sb_a}$ (excitation at 425 nm). Solution conditions: [MB] = 0.2 μM, [$\mathbf{Sb_a}$] = 0.4 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), 20 °C. [b] Ratio of the fluorescence intensity of the MB at 460 nm with (I_{open}) and without (I_{closed}) the target $\mathbf{Sb_a}$.

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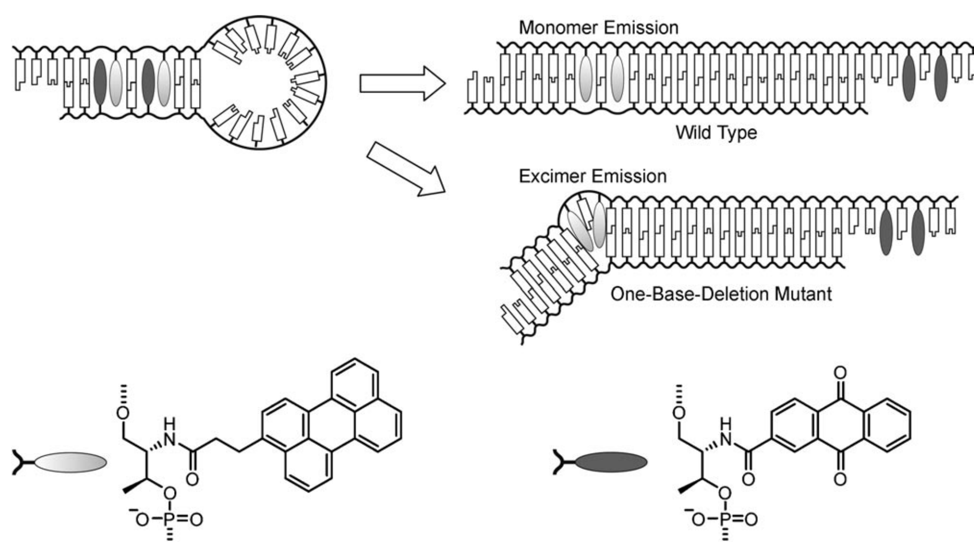

Figure 3. ISMB design for the detection of deletion polymorphisms.

position in the stem, also showed a high I_{open}/I_{closed} ratio of 15. Thus, the incorporation of an **E**–**Q** pair in the stem region appeared to increase sensitivity significantly as a result of efficient quenching. Moreover, the introduction of two **E**–**Q** pairs into the stem led to a dramatic improvement in the I_{open}/I_{closed} ratio to 58 for $\mathbf{MB_{12}}$.[15] The emission of $\mathbf{MB_{12}}$ in the closed state (without $\mathbf{Sb_a}$) was strongly quenched, and the addition of the target, $\mathbf{Sb_a}$, efficiently restored the emission of perylene. Since the two perylene moieties were separated by intervening base pairs, monomer emission was observed, but not excimer emission.[16] This property is useful for the application of the ISMB and contributes to its ability to discriminate between the wild-type (full-match) and deletion-mutant targets.

We previously described a DNA probe that detects deletion polymorphisms and SNPs on the strength of excimer emission.[8,17] This probe contained two pyrene or perylene moieties, which were tethered to the ODN on both sides of the target nucleotide to enable the detection of deletions or SNPs. Similar probes have been reported by other research groups.[18,19] Although these probes could discriminate efficiently between the full-match sequence and mismatch or deletion mutants, they emitted fluorescence even without the target. This emission prevented application of the probe to DNA chips owing to a significant lowering of sensitivity by this background noise. We could remove this background emission by using the ISMB design depicted in Figure 3. We synthesized $\mathbf{MB_{FD}}$ (Figure 1), which contains two **E**–**Q** pairs. In this probe, a single natural base, G, is inserted between two **E** nucleotides. The fluorescence of the probe is quenched in the absence of a target through the "base pairing" of **E** and **Q**. In the presence of wild-type (full-match) DNA, $\mathbf{Sb_F}$, the ISMB opens. Monomer emission of perylene should be observed, because the two **E** nucleotides are separated by an intervening base pair. However, excimer emission should occur upon hybridization with the one-base deletion mutant $\mathbf{Sb_D}$. Thus, a one-base deletion can be detected by analysis of the ratio between monomer and excimer emission when background emission is suppressed.

Figure 4a shows the fluorescence emission spectra of closed $\mathbf{MB_{FD}}$, $\mathbf{MB_{FD}}/\mathbf{Sb_F}$, and $\mathbf{MB_{FD}}/\mathbf{Sb_D}$. In the absence of a target, emission from the perylene moieties in $\mathbf{MB_{FD}}$ was quenched efficiently according to our design. The addition of wild-type (full-match) $\mathbf{Sb_F}$ led to a dramatic enhancement of monomer emission (Figure 4a, B). The intensity of emission at 460 nm in the presence of $\mathbf{Sb_F}$ was as high as 17-fold that in the absence of $\mathbf{Sb_F}$. This increased fluorescence could even be observed by the naked eye. Almost no fluorescence was observed from $\mathbf{MB_{FD}}$ in the absence of the target, whereas the addition of the wild-type target $\mathbf{Sb_F}$ resulted in the emission of a strong blue fluorescent light (Figure 4b, A,B). In contrast, hybridization with the deletion mutant $\mathbf{Sb_D}$ quenched monomer emission, and excimer emission appeared at around 530 nm as a result of the close proximity of the two perylene moieties (Figure 4aC). The intensity ratio between monomer and excimer emission (I_{550}/I_{461}) was 1.24, which is 31-fold that of $\mathbf{MB_{FD}}/\mathbf{Sb_F}$ (0.04). This difference could also be discrimi-

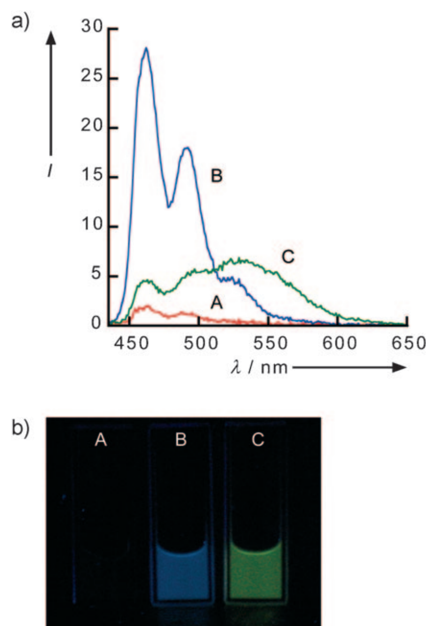

Figure 4. a) Fluorescence emission spectra and b) photographs of single-stranded $\mathbf{MB_{FD}}$ (A), $\mathbf{MB_{FD}}/\mathbf{Sb_F}$ (B), and $\mathbf{MB_{FD}}/\mathbf{Sb_D}$ (C). a) Excitation wavelength: 425 nm. $[\mathbf{MB_{FD}}]=0.2$ μM, $[\mathbf{Sb_F}]=[\mathbf{Sb_D}]=0.4$ μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), 20 °C. b) $[\mathbf{MB_{FD}}]=5.0$ μM, $[\mathbf{Sb_F}]=[\mathbf{Sb_D}]=6.0$ μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), room temperature. Cells are 1 mm thick. Fluorescence was observed over a xenon light source (400 nm).

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nated by the naked eye: the solution of $\mathbf{MB_{FD}/Sb_D}$ emitted a green fluorescence, whereas that of $\mathbf{MB_{FD}/Sb_F}$ was blue (Figure 4b, B,C). Thus, a one-base deletion can be detected by analysis of the intensity ratio between monomer and excimer emission when background emission is suppressed.

In conclusion, a new type of molecular beacon that contains fluorophore–quencher pairs in the stem region, the ISMB, can detect a target DNA sequence with high sensitivity. In this MB design, the background emission of the probes is suppressed, which can be useful for the detection of one-base deletions. Further studies are underway to test the potential use of ISMBs as capture probes for DNA chips.[20]

Experimental Section

Synthesis of modified ODNs: Procedures for the synthesis of phosphoramidite monomers were reported previously.[8,21–23] All modified ODNs were purified by reversed-phase HPLC and characterized by MALDI-TOF MS (see the Supporting Information). No decomposition products were observed upon storage at −20°C.

Spectroscopic measurements: Fluorescence spectra were measured on a JASCO model FP-6500 instrument with a microcell. The spectrometer was equipped with programmed temperature controllers. Errors in the ratio I_{open}/I_{closed} were estimated to be within 15%. Melting curves of duplex DNA were obtained with a Shimadzu UV-1800 spectrophotometer by measurement of the change in absorbance at 260 nm versus temperature. The melting temperature (T_m) was determined from the maximum in the first derivative of the melting curve. The heating and cooling curves were both measured, and the T_m measurements obtained were within 2.0°C of each other. The temperature ramp was 0.2 or 0.5°C min^{-1}. The solution conditions were as follows: $[\mathbf{F_a}] = 1.0$ μM, $[\mathbf{Y_b(X)}] = 1.2$ μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer).

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[11] Because the absorption maxima of anthraquinone occur at much shorter wavelengths than the emission maxima of perylene (see Figure S1 in the Supporting Information), quenching due to Förster-type energy transfer can be ruled out.
[12] The melting temperature of $\mathbf{F_a/Y_b(Q)}$ was 57.5°C, which is much higher than that of the other duplexes ($\mathbf{F_a/Y_b(D)}$: 41.5°C, $\mathbf{F_a/Y_b(Z)}$: 45.5°C). Furthermore, the absorption spectra of $\mathbf{F_a/Y_b(Q)}$ changed upon hybridization. This observation indicated an excitonic interaction between perylene and anthraquinone in the ground state. See Figure S1 in the Supporting Information for absorption spectra.
[13] Although the fluorescence intensity depended on the adjacent bases, stronger fluorescence was observed for intercalated perylene than for perylene in the single strand.
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[15] The sequence specificity of the in-stem molecular beacon $\mathbf{MB_{12}}$ was comparable to that of the conventional molecular beacon $\mathbf{MB_t}$, although the response speed and stability of the duplex and hairpin were lower for $\mathbf{MB_{12}}$. The linear range of detection was about 2–200 nM with 200 nM $\mathbf{MB_{12}}$. Opening and closing of the molecular beacon were reversible. $\mathbf{MB_{12}}$ was available below 60°C, at which temperature the molecular beacon was closed in the absence of a target. See the Supporting Information for details.
[16] This result also supports the intercalation of perylene, because excimer emission should occur if the two perylene moieties were flipped out. Furthermore, the intercalated structure was substantiated by NMR spectroscopic analysis when azobenzene was used in place of perylene: X. G. Liang, H. Asanuma, H. Kashida, A. Takasu, T. Sakamoto, G. Kawai, M. Komiyama, *J. Am. Chem. Soc.* **2003**, *125*, 16408. However, the possibility that chromophores adopt an extrahelical conformation (for example, binding to the minor groove) cannot be ruled out completely.
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