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Unambiguous Detection of Target DNAs by Excimer−Monomer Switching Molecular Beacons

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A new class of molecular beacons were developed in which pyrene fluorophores were connected both at 3′ and 5′ ends of a single-stranded oligonucleotide. The two pyrene-based fluorophores were synthesized from the same starting material, so that the preparation of the beacons was simplified. The detection strategy of the beacons for target DNAs is based on "excimer−monomer emission switching" of the pyrene fluorophores: excimer emission of the pyrene moieties changed to monomer one when the beacons hybridized with the targets. This type of two-state mode of fluorescence allows unambiguous detection of the target DNAs because strict 1:1 correlation between the nonhybridized and the hybridized beacons can be monitored by the presence of isoemissive points of the fluorescence changes. The beacons can detect target 19-mer DNAs and can discriminate the targets from their single-nucleotide mismatches at 1 nM concentration. Advantages of the excimer−monomer switching molecular beacons were discussed in comparison with conventional ones.

Introduction

Molecular beacons are powerful tools for detecting target DNAs and RNAs, which generally consist of stem-and-loop structures in single-stranded oligonucleotides labeled with a fluorophore and a quencher at the 3′ and 5′ ends, respectively.[1,2] Before an addition of a target, the beacons produced no fluorescence emission because of the fluorescence resonance energy transfer (FRET) from the excited fluorophore to the proximal quencher.[3] Upon the addition, hybridization with the target occurred to keep the fluorophore apart from the quencher, restoring the emission. This type of "off-on" switching of fluorescence, however, may be hampered by photochemically active biomolecules existing in living cells because of the relatively long Förster distance for permitting FRET.[4] Recently, novel "fluorescence-quenched" probes were developed on the basis of collisional quenching, exciton coupling, and ground-state complex formation.[5] Nevertheless, to expand the practicability of molecular beacons, important are developments of further detection strategies, which do not employ any quencher.

Pyrene is a simple hydrocarbon aromatic molecule, having an advantage of versatility for various chemical modifications, and gives monomer and excimer emissions depending on its concentration.[6] We have previously reported synthetic macrocyclic molecules, in which two pyrene moieties were placed parallel to each other in close proximity.[7] Owing to the spatial arrangement of the pyrene rings, the macrocycles emit only excimer fluorescence. Since the formation of pyrene excimer strongly rests upon the extent of π-plane overlaps, the predominant excimer emission of the macrocycles illustrated the preciseness for our molecular design. This finding encouraged us to apply such molecular design technique to develop novel oligonucleotide probes.

Recently, several oligonucleotide probes utilizing pyrene have been described.[8] For example, Kool et al. have reported interesting DNA probes, in which the detection scheme is based on monomer−excimer fluorescence changes upon the formation of ternary complexes between the pyrene-modified two probes and target DNAs.[8b] Although the system is carefully considered, two different

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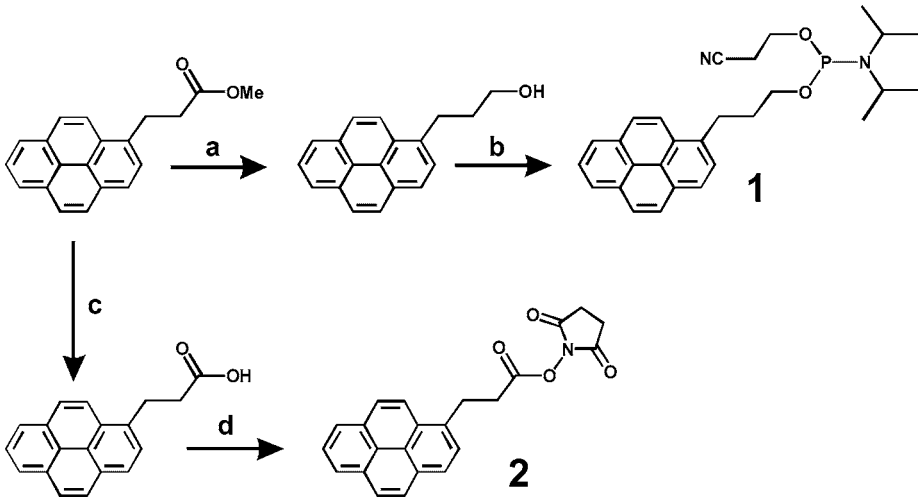
SCHEME 1. Synthetic Scheme for 1 and 2[a]



[a] Key: (a) $LiAlH_4$, THF; (b) $(i\text{-}Pr_2N)_2PO(CH_2)_2CN$, 1*H*-tetrazole, CH_3CN; (c) KOH, CH_3OH; (d) *N*,*N*′-disuccinimidyl carbonate, pyridine, CH_3CN.

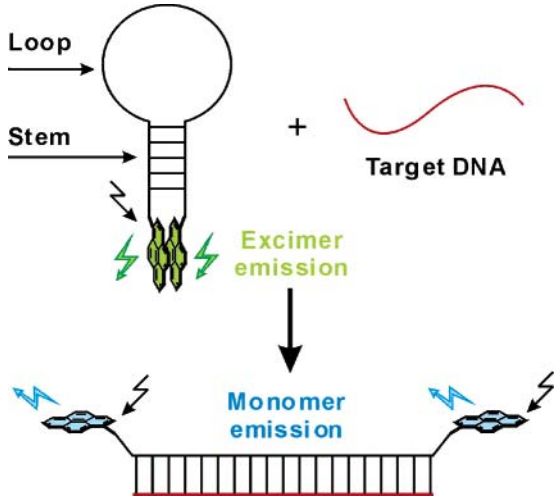


FIGURE 1. Schematic representation of excimer−monomer switching molecular beacons (EMS-MBs). EMS-MBs were dually labeled with pyrene at both 3′ and 5′ ends of single-stranded oligonucleotides with a stem-and-loop structure. In the absence of target DNAs, the stem-close-shaped EMS-MBs predominantly emit the excimer fluorescence (yellow-green). Upon hybridization with target DNAs, the EMS-MBs undergo the dynamic conformational change to emit the monomer fluorescence (pale blue).

oligonucleotide probes must be prepared accompanied with many efforts for the multistep synthesis of pyrene-modified nonnatural nucleotides. Taking into account the above points, we developed a new class of molecular beacons, excimer−monomer switching molecular beacons (EMS-MBs) (Figure 1).

Results and Discussion

Synthesis of EMS-MBs. We designed and synthesized two pyrene derivatives **1** and **2** for the modifications of the 5′ and 3′ ends of one single-stranded oligonucleotide, respectively. Both phosphoroamidite **1** and succinimidyl ester **2** could be derived from the same known intermediate, methyl 3-(1-pyrenyl)propionate (Scheme 1).[9] A single-stranded 29-mer DNA possessing a C3-alkylamino linker (3′ end) was coupled with **1** at the 5′ end on solid-phase DNA synthesis, released from the support, and purified by HPLC. The purified 5′-functionalized DNA was further tethered to **2** at the amino terminal of the 3′-linker to give the EMS-MB **3**. When purifying **3** with HPLC after the coupling reaction at 3′ end, the discrimination of **3** from other fractions was so easy as only the target fraction emitted pyrene excimer fluorescence. On the other hand, the isolation of conventional molecular beacons (FRET-MBs) is doomed to be troublesome because of the FRET. Indeed, we hardly recognized visibly the fluorescence of the HPLC fraction for a represent FRET-MB **6**, which was modified with 6-carboxyfluorescein group (6-FAM) as a fluorophore and 4-(4-dimethylaminophenylazo)benzoyl group (DABCYL) as a quencher at 5′ and 3′ ends, respectively. Thus, the synthetic procedure for EMS-MBs is simpler and more economical than FRET-MBs and other pyrene excimer probes. The sequence of **3** was selected from those of previously reported molecular beacons,[10] and the lengths of the alkyl linkers at 3′ and 5′ ends were determined by examining the molecular modeling (Figure 2A).

Thermal Profiles of EMS-MBs. The novel oligonucleotides probe **3** thus prepared must retain a stem (underlined 5-mer of **3** in Figure 2A)-and-loop (19-mer) structure before addition of target DNAs, and the helical order of the stem must be a random-coil conformation upon heating for considering the application of the beacons to real-time monitoring of PCR.[11] To show the beacons meeting these criteria, the thermal profile of **3** was investigated by electronic absorption and fluorescence analyses. Melting temperatures (T_m) of **3** and the corresponding native DNA **4** were estimated to be 59 and

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A

3 5'-(1-pyrenyl)-(CH_2)$_3$-CTGACGAGTCCTTCCACGATACCAGTCAG-($C_3NHCOC_2H_4$)-(1-pyrenyl)-3'

4 5'-CTGACGAGTCCTTCCACGATACCAGTCAG-3'

5 5'-TGGTATCGTGGAAGGACTC-3'

6 5'-(6-FAM)-S(CH_2)$_6$-CTGACGAGTCCTTCCACGATACCAGTCAG-($C_3OC_3H_6NHCO$)-(DABCYL)-3'

7 5'-TGGTATCGTAGAAGGACTC-3'

8 5'-TGGTATCGTTTGAAGGACTC-3'

9 5'-TGGTATCGTCGAAGGACTC-3'

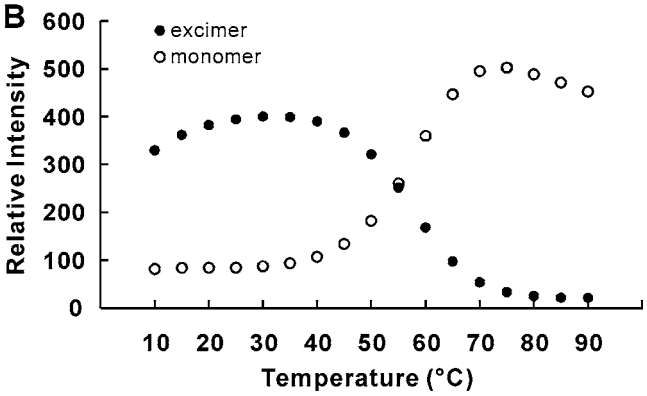

FIGURE 2. DNA sequences and a fluorescent thermal profile of EMS-MB. (A) Sequences of EMS-MB **3**, the unmodified DNA **4**, the fully complementary target DNA **5** for the loop region of **3**, FRET-MB **6** possessing a same sequence for **3**, and the single-nucleotide mismatched DNAs **7**−**9**. (B) Isotherm curves for fluorescence intensities of the excimer and the monomer emissions of **3** ([**3**], 2 μM; [$MgCl_2$], 5 mM; [KCl], 50 mM; [Tris-HCl], 20 mM, at pH 8.0) in the temperature range from 10 to 90 °C: closed circle and open circle correspond to excimer and monomer intensities, respectively. The excitation wavelength was 345 nm.

46 °C, respectively, on the basis of the electronic absorption spectra. The difference of the T_m values between **3** and **4**, both of which consist of the same sequence, suggests that two pyrene moieties of **3** would interact with each other by hydrophobic and/or π-stacking interactions and act like extra base pairs to enhance the stability of the stem-closed structures. The correlation between the T_m value and the fluorescence emission of **3** was examined by use of fluorescence spectroscopy in the temperature range from 10 to 90 °C (Figure 2B). As the temperature of a solution containing **3** was slowly raised, the excimer emission (498 nm) of **3** decreased, conversely the monomer one (382 nm) increased. Each inflection temperature (ca. 55 °C) of the isotherm curves of the fluorescence intensities was approximately identical with that of the T_m value determined by the electronic absorption spectra. These results confirmed that the switching from the excimer to the monomer emissions is due to the conformational change of **3** from the ordered state to the disordered state.[12]

Detection of Target DNAs. Abilities of **3** as molecular beacons were tested by fluorescence titration experiments in the presence of the fully complementary target DNA **5** (Figure 3A). An addition of **5** (0.1−1.0 equiv) to the solution of **3** caused a decrease in the excimer intensity (I_{498nm}) and an increase in the monomer one

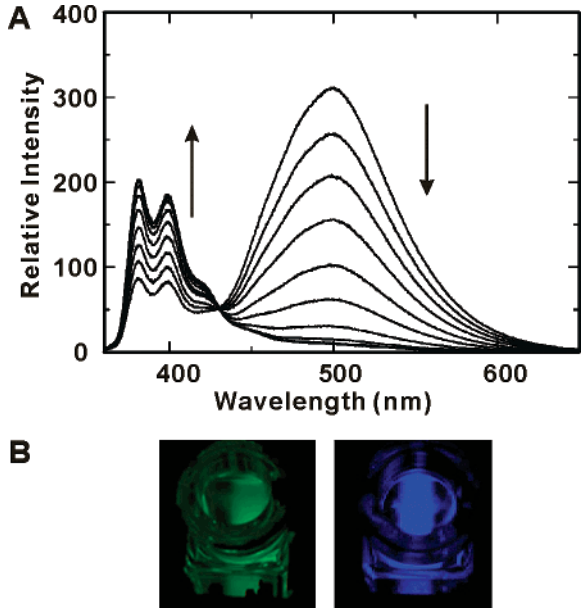

FIGURE 3. (A) Fluorescence titration spectra of **3** ([**3**], 200 nM; [$MgCl_2$], 5 mM; [KCl], 50 mM; [Tris-HCl], 20 mM, at pH 8.0) in the presence of a fully complementary target DNA **5** (0.1−1.0 equiv). (B) A visual discrimination of the color change for **3** (2 μM). Yellow-green (left) and pale blue (right) fluorescences were observed in the absence and presence of 1 equiv of **5**, respectively. These solutions in quartz cells were irradiated by monochromatic light (345 nm) on the spectrofluorometer and photographed with a digital camera.

(I_{382nm}). Noteworthy is that ca. 1 equiv of target **5** was enough for completing the switching of the emission and that the excess of **5** led to no further spectral changes at the concentration in Figure 3A. The spectral changes could be clearly followed up at a sufficiently low concentration of 1 nM of **3** with a standard fluorescence spectrometer.[13] Since this sensitivity is similar to that of FRET-MB **6** for fully restoring the emission of the fluorophores, the EMS-MB **3** is thought to be a useful DNA probe. A visual discrimination of the change is also possible with relatively a high concentration of **3**. The probe **3** alone emits yellow-green fluorescence from pyrene excimer, while upon the addition of **5**, the color of the fluorescence turns into pale blue from monomeric pyrene under irradiation of 345 nm light (Figure 3B).

In Figure 3A, the ratio of I_{382nm}/I_{498nm} was estimated to be 0.2 in the absence of **5**, while the value went up to 20 upon the addition. The variance by a factor of 20/0.2 = 100 is higher than the FRET-MB **6**, in which the ratio of I_{on}/I_{off} was approximated to be 5.2 at the emission maximum under the same conditions.[13] The presence of the substantial background fluorescence for **6** in the absence of a target may result in the reducing S/N. The well-defined behavior of **3** was ensured on CD spectra of **3**, comparing with that of native DNA **4** by following experiments. The spectra were recorded before and after the annealing (heating followed by cooling) of the solution of **3** containing 1 equiv of **5** (Figure 4). These two spectra were nearly the same not only to each other but also to that of **4** containing **5** in the wavelength region where DNA absorbs. This finding indicates that the switching

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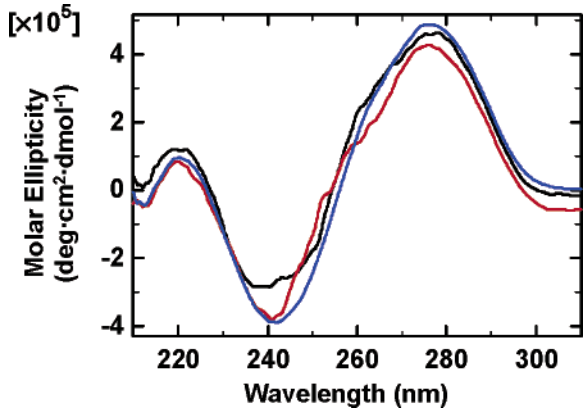

FIGURE 4. CD spectra of an equimolar mixture of **3** and **5** before (black line) and after (red line) annealing and a mixture of **4** and **5** (blue line) under the same condition without annealing.

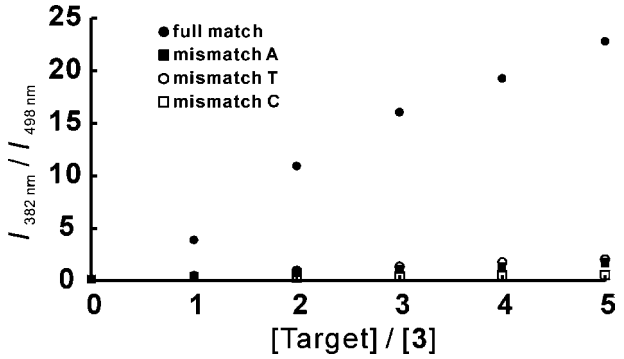

FIGURE 5. Discrimination of complementary target DNAs from their single-nucleotide mismatches. A ratio of $I_{382\ nm}/I_{498\ nm}$ for **3** ([**3**], 5 nM; [$MgCl_2$], 5 mM; [KCl], 50 mM; [Tris-HCl], 20 mM, at pH 8.0 including 20% DMF) was plotted as a function of [target]/[**3**]. Closed circle, closed square, open circle, and open square correspond to the full-match **5** and the single-nucleotide mismatches **7**, **8**, and **9**, respectively.

is only due to the hybridization with the target but not to other accidental factors.

Another notable point of the EMS-MB is that an isoemissive point was observed in the fluorescence titration at 430 nm (Figure 3A). The isoemissive point demonstrates the presence of *only two* fluorescent species in the mixture, i.e., only the stem-and-loop and the hybridized structures. This makes **3** be unambiguous for probing the target DNAs because an output format of the EMS-MB **3** is more comprehensive than those of normal FRET-MBs. The output pattern of FRET-MBs is [*0*,*1*]: *0* means no fluorescence, while *1* means natural fluorescence. Sometimes, *0* is subjected to serious problems owing that *0* signal might be induced by unpredictable accidents, e.g., the attendance of other quenchers, the removal of fluorophores, etc., in living cells. The output pattern [*1*,*1′*,*0*] of **3** has an advantage that unambiguous detections could be monitored, in which *1* and *1′* signals represent the excimer and monomer emissions, respectively, and *0* signal can be proved to be a false signal resulting from the unexpected factors.

Discrimination of Target DNAs from Single-Nucleotide Mismatches. We examined how single-nucleotide mismatches on complementary sequences had influences on detection abilities of **3** (Figure 5).[14] Each single-nucleotide mismatch was placed at the middle of the entirely complementary DNA **5** (the right base is guanine) (Figure 2A). An excess of single-nucleotide mismatched DNAs **7**−**9** hardly caused fluorescence intensity changes (I_{382nm}/I_{498nm}) of **3**, while the full-match **5** clearly induced the excimer−monomer switching at 5 nM concentration of **3**. This type of enhanced specificity for discriminating the target DNAs from their single-nucleotide mismatches was also seen in FRET-MBs that possessed a 15-nucleotide loop and 5-nucleotide stems.[12] Thus, the EMS-MB **3** can discriminate the target DNA from single-nucleotide mismatches on the 19-mer sequence as sensitively as the FRET-MB **6**.[10,15] The probability that a particular 19-mer sequence will appear on some genomes is $1/4^{19}$, meaning only once in 2.7×10^{11} base sequence. Recent advances for fluorescence-based technologies showed the possibility for single-molecule detection of fluorophores, especially using fluorescence lifetime measurements, since there is a large difference in fluorescence lifetime between pyrene excimer and monomer.[16] Thus, when combining the single-molecule detection methodology, the EMS-MB will be expected to selectively detect a single target sequence from any genome existing on the earth without PCR amplifications.

Conclusions

We developed an excimer−monomer switching molecular beacon, EMS-MB that has the advantages of synthetic simplicity, high S/N, and unambiguous detectability as compared with conventional FRET-MBs. The EMS-MB can respond to 1 equiv of complementary 19-mer DNAs and discriminate the targets from their single-nucleotide mismatches as sensitively as FRET-MBs. To fully sophisticate EMS-MBs, we are studying the following points under way: (1) varying both lengths of stem-and-loop sequences, (2) utilizing other fluorophores forming excimers, (3) optimizing the linker lengths between fluorophores and terminal bases, and (4) improving pyrene moieties into more potent fluorophores from viewpoints of absorption maxima, fluorescence intensity, fluorescence quantum yield, etc. Furthermore, utilization

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of fluorescence lifetime and fluorescence detected circular dichroism (FDCD)[17] might expand the possibility of the EMS-MBs because these spectroscopic methods are more reflective on molecular dynamics than electronic absorption and fluorescence spectroscopies at the lower concentration range. Apart from DNA detection, EMS-MBs have an interesting possibility for developing DNA-based nanotechnologies such as molecular logic gates and computers because of their inherent output pattern,[18] and such an approach is also now under way.

Supporting Information Available: All Experimental Sections and fluorescence titration spectra of the EMS-MB **3** (1 nM) and the FRET-MB **6** (200 nM). This material is available free of charge via the Internet at http://pubs.acs.org.

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