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A Highly Discriminating Quencher-Free Molecular Beacon for Probing DNA

Gil Tae Hwang, Young Jun Seo, and Byeang Hyean Kim*

National Research Laboratory, Department of Chemistry, Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Korea

Received January 13, 2004; E-mail: bhkim@postech.ac.kr

Sequence-selective DNA detection is becoming increasingly important as a tool for monitoring many biological processes and for other biotechnological applications. One recent development is molecular beacons (MBs) that are highly selective in their recognition of oligonucleotides.[1] Traditional MBs are doubly end-labeled oligonucleotides that exist in solution as stable stem-loop structures in which the fluorescence of a reporter dye attached to the 5′ end is quenched through energy transfer to a proximate quencher attached to the 3′ end. In the presence of a complementary nucleic acid, the stem opens and this event is signaled by a loss of quenching and an increase in fluorescence. While MBs have been used efficiently as biosensors, their fluorophore/quencher systems are unnecessary for sequence-selective duplex formation and are utilized only as detection tools. Herein, we describe a new strategy for designing MBs possessing only a fluorophore (i.e., no quencher); these MBs undergo DNA hybridization and can discriminate between fully matched and one-base-mismatched sequences.[2] We chose fluorene as the fluorophore because it has a good quantum yield—its absolute quantum yield is 0.54 in ethanol—and is less bulky than other commonly used fluorophores, e.g., pyrene, fluorescein, rhodamine, and coumarin derivatives.[3] To our knowledge, there have been no prior investigations concerning the conjugation of fluorene with nucleobase-containing deoxyoligonucleotides.[4] In addition, our plan was to attach a fluorene unit covalently at the C-5 position of deoxyuridine, which is a substitution that we expect will not perturb its DNA base pairing ability and so it should have very little influence on the stability of the resulting duplex DNA.[5]

The fluorene-labeled phosphoramidite was synthesized from the corresponding 5-iodo-5′-dimethoxytrityl-2′-deoxyuridine **1**.[6] Palladium-catalyzed Sonogashira coupling[7] with 2-ethynylfluorene[8] resulted in the formation of **2**, which was subsequently converted into the corresponding phosphoramidite building block **3** (Scheme 1).[9] Incorporation of this fluorene-labeled deoxyuridine into the central position of the oligodeoxyribonucleotide (ODN) **4**[10,11] was effected by standard protocols of automated DNA synthesis (Figure 1).[12]

Initially, we investigated the fluorescence intensity changes of the duplexes containing fluorene units upon hybridization of fully matched and one-base-mismatched sequences. When ODN **4** forms a hybrid with a target ODN, its fluorescence intensity is enhanced when perfectly matched base pairing exists, but is dramatically quenched when the base pairing is one-base-mismatched. It is well established that uracil and thymine nucleobases are efficient quenchers for fluorophore emission in nucleosides and single-stranded oligonucleotides.[13] Generally, the fluorescence of pyrene units is quenched by their intercalation into a duplex and their stacking with bases.[14] The absorption maxima of the single-base-mismatched duplexes, **4·5**, **4·6**, and **4·7** are slightly red-shifted relative to that of the fully matched duplex **4·8**, i.e., from 334 nm to 339, 339, and 338 nm, respectively (see Figure S1 in the Supporting Information). These results suggest that the fluorene

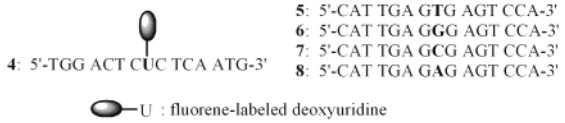


Figure 1. The sequences of synthesized oligonucleotides. Bold bases indicate the matched or mismatched bases.

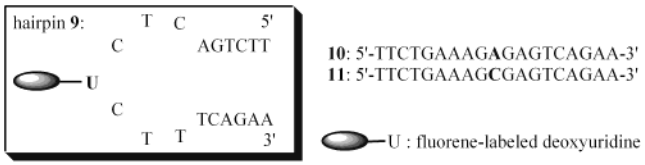
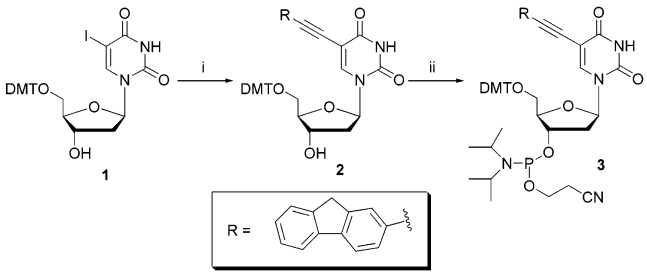


Figure 2. Oligonucleotides synthesized for the MB study. Bold letters indicate matched or mismatched bases.

Scheme 1. Synthesis of Fluorene-Labeled Nucleosides[a]



[a] Reagents and conditions: (i) 2-ethynylfluorene, $(PPh_3)_2PdCl_2$, CuI, Et_3N/THF (1:3), 45−50 °C, 2 h, 80%; (ii) 2-cyanoethyl diisopropylchlorophosphoramidite, 4-methylmorpholine, CH_2Cl_2, rt, 30 min, 74%.

units in the single-base-mismatched duplexes presumably interact with one or more of the nucleobases.[15] Further studies are necessary to confirm the effects of opposing nucleobases in the fluorescence intensity changes of the single-base-mismatched duplexes. The relative emission intensities of the single-base-mismatched duplexes **4·5**, **4·6**, and **4·7** are 12, 12, and 24%, respectively, of that of single-stranded **4** upon excitation at 340 nm. Very interestingly, the fully matched duplex **4·8** exhibits a 3.4-fold enhancement in fluorescence intensity (λ_{max} = 425 nm) relative to that of single-stranded **4**. This finding proves that the hybridization of **4** is a process that allows the distinction to be made between its target strand and a strand that contains a single mutation.

On the basis of these results, we designed the oligonucleotide model system **9** as a new type of MB consisting of a six-base-pair stem and a seven-base loop sequence having a fluorene-labeled deoxyuridine core (Figure 2).[10,11] As target strands, we synthesized the unmodified ODN **10**, which is a fully matched sequence, and **11**, which has a one-base-mismatched sequence (mutated from A to C because this change provided the highest emission intensity for the single-base-mismatched duplexes presented in Figure 1).

Thermal denaturation studies indicate an enhanced stability for the modified hairpin **9** and the single-base-mismatched 19-mer

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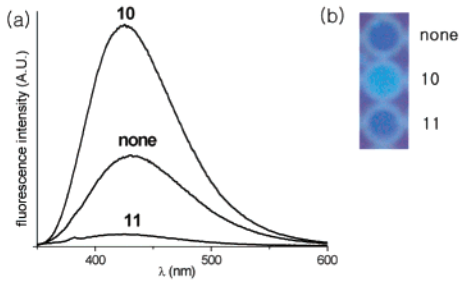

Figure 3. (a) Emission spectra recorded at 20 °C of duplexes of hairpin **9** (1.5 μM) with either **10** or **11**. Fluorescence spectra were recorded using an excitation wavelength of 340 nm. The spectrum labeled "none" is that of hairpin **9** alone. (b) Photograph displaying the emission behavior at 25 °C of hairpin **9** alone and in duplexes with unmodified ODNs **10** and **11** (2.0 μM) upon irradiation with light at 365 nm. Buffer conditions are the same as those described in Table 1.

Table 1. Thermal Melting Temperatures (T_m) of Modified and Unmodified ODN Hairpins and Their Duplexes[a]

sample	T_m (°C)	sample	T_m (°C)
9	56	**12**[b]	50
9·10	63	**12·10**	66
9·11	60	**12·11**	57

[a] Measured at 260 nm in 10 mM Tris-HCl buffer (pH 7.2) containing 100 mM NaCl and 20 mM $MgCl_2$. Estimated error is ±1 °C. [b] Compound **12** is a hairpin having unmodified sequences of **9**.

duplex **9·11** relative to the unmodified hairpin **12** and its duplex **12·11**. In contrast, however, for the hybrids of the complementary ODN, **10**, duplex **9·10** is less stable than **12·10** (Table 1); this finding suggests that the fluorene units encounter different environments in the fully matched duplex **9·10** and the single-base-mismatched duplex **9·11**.

The hybridization properties were also tested by means of fluorescence measurements (Figure 3a). The hybridization of hairpin **9** with its complementary ODN **10** and single-base-mismatched ODN **11** in solution displays a 2.2-fold enhancement and a 0.15-fold decrease, respectively, in emission intensities relative to that observed for hairpin **9**. Therefore, the total discrimination factor is 14.7 for the recognition of a single (A/C) base mismatch. The discrimination factor for this MB upon hybridization is high enough for a variety of applications. The emissive colors of these MB systems are depicted in Figure 3b.

There may be significant advantages in utilizing this type of MB over more-traditional ones. First, the fluorene-labeled 2′-deoxyuridine unit can be inserted at any position of the oligonucleotide depending on the sequence of interest. Second, the 5′ and 3′ ends can be left free to introduce other useful functionalities, e.g., for attachment to surfaces, nanoparticles, or biotin units. Third, our MB synthesis is relatively simple and inexpensive because no quencher is required. Finally, the designed MBs exhibit greater quenching behavior when meeting single-base-mismatched targets than do those of traditional MBs; in fact, traditional MB probes undergo spontaneous fluorogenic conformational changes that force the stem units apart so that fluorescence is restored only when the probes encounter a perfectly complementary target—hybridization must not occur when the target contains a mismatch.[1] In our MB system, however, even when the hairpin meets a single-base-mismatched target and duplex formation occurs, fluorescence quenching is induced; this feature produces efficient fluorescence ON/OFF systems that can be used as more-sensitive probes.

In summary, we have synthesized, by Sonogashira coupling, a fluorene-containing deoxyuridine derivative that we incorporated into the loop region of a hairpin. This hairpin functions as new type of MB that requires no fluorescence quencher unit. The fluorescence intensities of single-base-mismatched duplexes are decreased relative to that observed for the hairpin but are increased upon hybridizing with fully matched sequences. Therefore, our MBs are useful probes that distinguish between their target and one-base-mismatched (A/C) DNA sequences. Careful design and judicious selection and placement of the fluorophore will enable this general principle to be applied to the study of other MB probes.

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Supporting Information Available: Syntheses and characterization data of fluorene-labeled nucleosides **2** and **3**; absorption spectra of the duplexes; CD spectra of hairpin **9** and duplex **9·10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Similar phenomena were detected in the UV−vis spectra of the duplexes between hairpin **9** and the target DNAs **10** and **11**. The absorbance maximum of the single-base-mismatched duplex **9·11** is red-shifted slightly, from 331 to 336 nm, relative to that of the fully matched duplex **9·10**.

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