Quencher-free Linear Beacon Systems with Dual Fluorene-labeled Deoxyuridines

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The identification of natural genetic variation in human populations is arguably one of the most important spin-offs of the effort toward the completion of the Human Genome Project.[1,2] Single nucleotide polymorphisms (SNPs) are the most abundant natural genetic variations in the human genome. To satisfy the demand for molecular diagnostic assays for identifying SNPs, Tyagi and Kramer developed fluorogenic hairpin-shaped DNA probes, called "molecular beacons in 1996.[3]
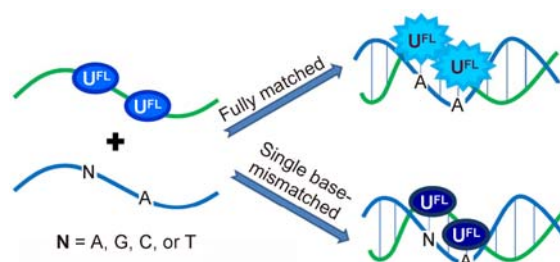
Conventional molecular beacons are single-stranded oligodeoxynucleotides (ODNs) probes that exist in solution as stable stem-and-loop structures in which a fluorophore is covalently linked to one end and a quencher is covalently linked to the other end. The stem keeps the fluorophore and quencher of the molecular beacon in extremely close proximity to each other, when they are not bound to their targets; this causes the fluorescence of the fluorophore to be quenched via the fluorescence resonance energy transfer. By contrast, the conformational change induced by binding to a target nucleic acid causes the fluorophore and the quencher to move away from each other, thereby restoring the fluorescence. Therefore, the presence of the target nucleic acid is characterized by a sharp increase in fluorescence intensity.

Recently, we reported that fluorescent hairpin oligonucleotides can function as molecular beacons even without the attachment of additional quencher moieties. Such probes can be called "quencher-free molecular beacons.[4-7] A quencher-free molecular beacon consists of only a single fluorophore (2-ethynylfluorene-labeled deoxyuridine, U^{FL}) positioned in the hairpin loop. When this probe undergoes DNA hybridization, it can discriminate between fully matched and single-base-mismatched sequences by exhibiting a strong dA-selective fluorescence. When the fluorene unit is intercalated within a DNA duplex as a result of mismatched base pairing, the fluorescence of the U^{FL} unit is quenched as a result of a photoinduced charge transfer originating from interactions with neighboring nucleobases.[6] In particular, the quencher-free molecular beacon probe having C-flanking bases (C-FBs) shows more efficient fluorescence ON/OFF behavior than do any other combinations of FBs.[6] The only drawback of this quencher-free molecular beacon system is its poor detection sensitivity due to its relatively high intrinsic emission intensity. To solve this problem, we recently positioned a quencher strand containing a deoxyguanine (dG) nucleobase, functioning as an internal quench-
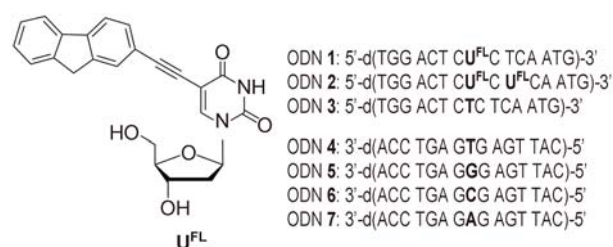
er, opposite the U^{FL} unit to reduce the intrinsic fluorescence on hybridization with a probe.[8] A short strand (five-nucleotide) containing all natural nucleotides and dG as an internal quencher was effective in reducing the intrinsic fluorescence of a linear beacon.

In another effort to eliminate this drawback, we employed an ODN containing dual U^{FL} units at specific distances (Figure 1). We observed that using this dual-labeled linear beacon system resulted in a clear improvement in the detection sensitivity for the perfectly matched target.

The modified nucleotide U^{FL} was synthesized through Sonogashira coupling of a 2-ethynylfluorene unit at the 5-position of a 2-deoxyuridine base.[4] Incorporations of the fluorene-labeled deoxyuridine U^{FL} into the central position of the ODN **1** and of the two U^{FL} units into the positions with one deoxycytosine spacer unit were effected using standard protocols of automated DNA synthesis (Figure 2).[9] The fluorescence properties of fluorophore-containing DNA are strongly dependent on the electron injection and transfer properties of the FBs. In particular, fluorescent ODNs having C-FBs produce efficient fluorescence ON/OFF systems and have higher sensitivity as probes than do any other combinations of FBs.[6] Therefore, we positioned two deoxy-

Figure 1. A linear beacon system designed in this study.

Figure 2. Oligonucleotides used in this study. Bold bases indicate U^{FL} and its matched and mismatched bases.

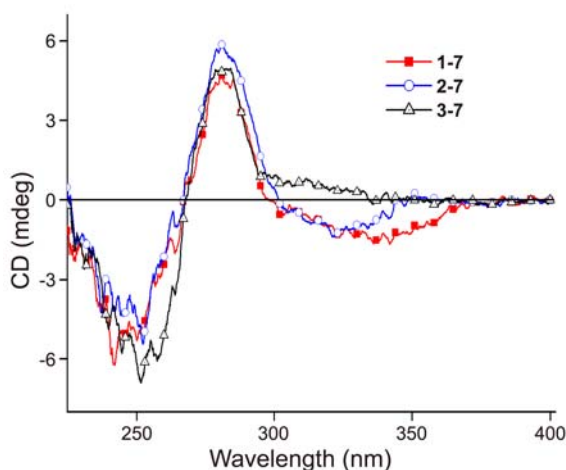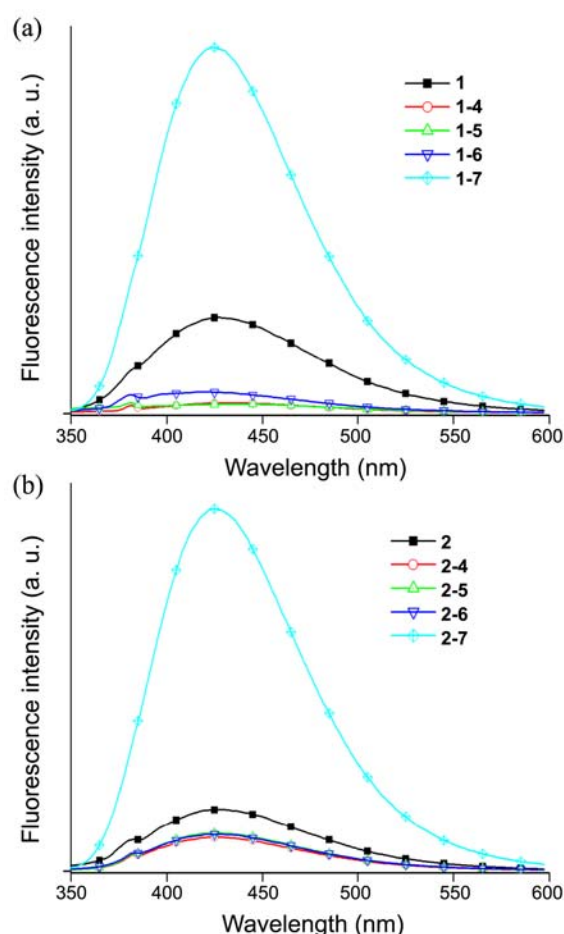**Table 1.** Thermal melting temperatures of modified and unmodified duplexes[a]

Duplexes	T_m (°C)	Duplexes	T_m (°C)	Duplexes	T_m (°C)
1·4	50.9	**2·4**	48.7	**3·4**	53.4
1·5	52.8	**2·5**	50.8	**3·5**	57.0
1·6	53.4	**2·6**	51.0	**3·6**	51.0
1·7	56.6	**2·7**	52.2	**3·7**	62.8

[a]Measured at 260 nm in 10 mM Tris-HCl buffer (pH 7.2) containing 100 mM NaCl and 20 mM MgCl$_2$. Uncertainty in values was less than 0.5 °C.

cytosines (dC) as the bases flanking the U^{FL} units.

We first determined the melting temperature (T_m) of each duplex through thermal denaturation experiments, in order to evaluate the thermodynamic stability and selectivity of U^{FL} in duplex DNA (Table 1). The modified duplexes with a correct base pair (U^{FL}:dA), **1·7** and **2·7**, had melting temperatures of 56.6 °C and 52.2 °C, respectively and were less stable than the unmodified correct duplex **3·7** in the same sequence context. Furthermore, these modified duplexes do not exhibit significant thermal selectivity relative to the mispaired duplexes between the U^{FL} unit and natural nucleotide units (dC, dG, and dT). ODN **2** in particular, having two U^{FL} units, does not differentiate strongly between its matched and mismatched nucleobases (ΔT_m: 1.2-3.5 °C). This general property suggests that the U^{FL} moiety destabilizes the natural base pairs.

To examine the effect of the U^{FL} moiety on the secondary structure of its duplexes, we recorded the circular dichroism (CD) spectra of the duplexes, **1·7**, **2·7**, and **3·7** (Figure 3). The characteristic negative and positive absorptions at 250 and 280 nm, respectively, indicate B-form DNA for the secondary structure of the modified DNA duplexes, showing that the unnatural U^{FL} nucleotide does not distort significantly.[10] Therefore, it can be inferred that U^{FL} participates selectively in a base pair with dA and maintains the normal shape of the resulting DNA duplex.

Figure 3. CD spectra of DNA duplexes. All spectra were recorded at 10 °C using 4.5 μM solutions of the samples in 10 mM Tris-HCl buffer (pH 7.2; 100 mM NaCl, 20 mM MgCl$_2$).

Figure 4. Emission spectra of duplexes with either matched target ODN **7** or mismatched target ODN **4-6**. (a) ODN **1**; (b) ODN **2**. All spectra were recorded at 20 °C using 1.5 μM solutions of the samples in 10 mM Tris-HCl buffer (pH 7.2; 100 mM NaCl, 20 mM MgCl$_2$), with irradiation at 340 nm.

We also tested the hybridization properties through fluorescence measurements. First, we measured the fluorescence spectra of the single-stranded ODN **1** and the double-stranded DNAs **1·4-1·7**, each at a concentration of 1.5 μM in a Tris-HCl buffer (pH 7.2) containing 100 mM NaCl and 20 mM MgCl$_2$ (Figure 4(a)), with excitation at a wavelength of 340 nm. The fluorescence excitation spectra of the single strand and its duplexes exhibited a strong emission peak at *ca.* 430 nm. The fluorescence intensities after hybridization with single-base-mismatched targets (ODN **1**·ODNs **4-6**) were dramatically quenched relative to that of the single-strand ODN **1** (Table 2). From the relative fluorescence ratios between the fully matched and single-base-mismatched duplexes, it was inferred that the fluorescence intensity of the matched duplex **1·7** was enhanced 28-, 28-, and 14-fold relative to those of the single-base-mismatched duplexes **1·4-1·6**, respectively. Although these ratios are sufficiently high to allow the recognition of a single base mismatch, the linear beacon system presented herein exhibits low detection sensitivity because of its intrinsically high emission intensity; the fluorescence intensity of the fully matched duplex was

Table 2. Relative fluorescence intensities upon hybridization between a probe and its targets[a]

Probes	Fluorescence intensity[b]	Probes	Fluorescence intensity[b]
1	1	2	1
1·4	0.12	**2·4**	0.52
1·5	0.12	**2·5**	0.58
1·6	0.24	**2·6**	0.57
1·7	3.4	**2·7**	5.2

[a]All experiments were conducted using the same concentration (1.5 μM) of DNA in 10 mM Tris-HCl buffer (pH 7.2; 100 mM NaCl, 20 mM MgCl$_2$), with excitation at 340 nm. [b]Ratio of relative change in fluorescence intensity (350-600 nm) upon hybridization compared to nonhybridized ODN **1** or **2**.

enhanced only 3.4-fold relative to that of single-strand **1** (Table 2).

To minimize the intrinsic fluorescence of the current linear beacon system, we examined the fluorescence properties of the DNA probe **2**, having two **U**^{FL} units with one deoxycytosine spacer. Surprisingly, ODN **2** showed a 1.47-fold decrease in the fluorescence intensity relative to that of ODN **1**, which indicates that an additional **U**^{FL} moiety modifies the electronic interactions with the FBs or the secondary structure of the single-stranded ODN **2**, mediating the fluorescence quenching.

Next, we measured the fluorescence spectra of the hybrids ODN **2·**ODNs **4-7** to examine the effect of the additional **U**^{FL} unit on the fluorescence intensity (Figure 4(b)). Surprisingly, the emission intensity of the matched duplex **2·7** improved 5.2-fold relative to that of single-strand **2** (Table 2), which represents an increased fluorescence intensity ratio as compared to that for ODN **1** upon hybridization with a matched target DNA. This result strongly indicates that the additional **U**^{FL} moiety minimizes the intrinsic fluorescence of ODN **2**, allowing for enhanced detection sensitivity as a DNA probe.

The florescence intensities of the single-base-mismatched duplexes between ODN **2** and ODNs **4-6** were dramatically quenched relative to that of the perfectly matched duplex **2·7** and single-strand ODN **2** (Table 2). The fluorescence intensity of the matched duplex **2·7** improved 10-, 9.0-, and 9.1-fold relative to those of the single-base-mismatched duplexes **2·4-2·6**, respectively. Although the discrimination factors showed an approximate two-fold decrease relative to that of ODN **1**, the ODN **2** probe would be extremely useful for SNP typing owing to the decreased intrinsic fluorescence intensity, displaying a highly dA-selective fluorescence emission.

In conclusion, we have developed a new linear beacon probe. We successfully minimized the fluorescence of the probe itself by introducing an additional **U**^{FL} moiety that alters electronic interactions with the FBs or the secondary structure of the ODN **2** probe, resulting in fluorescence quenching. Because this probe exhibits a drastic change in fluorescence intensity when it hybridizes with its fully matched target sequence, we expect this type of beacon system to provide important information for SNP typing.

Experimental Section

Synthesis of Oligonucleotides. A fluorene-labeled phosphoramidite was synthesized as described.[4] ODNs were prepared using the β-cyanoethylphosphoramidite method on controlled pore glass supports (1 μmol) with a POLYGEN Basic 10-Column DNA synthesizer and standard methods.[10] After automated synthesis, the oligonucleotides were cleaved from the solid support and deprotected through treatment with 30% aqueous NH$_4$OH (1.0 mL) for 10 h at 55 °C. The crude products from the automated ODN synthesis were lyophilized and diluted with distilled water (1 mL); they were then purified using high-performance liquid chromatography (HPLC; Grace VyDAC^{TM} C18 column, 250 × 10 mm; pore size: 120 Å). The HPLC mobile phase was held isocratically for 10 min using 5% acetonitrile/0.1 M triethylammonium acetate (TEAA; pH 7.0) at a flow rate of 2.5 mL/min. The gradient was then increased linearly over 10 min from 5% acetonitrile/0.1 M TEAA to 50% acetonitrile/0.1 M TEAA at the same flow rate. The fractions containing the purified ODN were pooled and lyophilized. The concentrations of the ODNs were determined through measurements of UV-vis absorptions. For characterization, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra of the ODNs were recorded using a PE Biosystems Voyager System 4095 spectrometer operated in the positive-ion mode with a 1:1 mixture of 3-hydroxypicolinic acid (0.35 M) and ammonium citrate (0.1 M) as the matrix; the accelerating voltage was 25 kV.

Melting Temperature (T_m) Measurements. The unnatural and natural nucleosides were incorporated into the complementary ODNs 5'-d(TGGACTC**X**C**Y**CAATG)-3' and 3'-d(ACCTGAG**N**GAGTTAC)-5' at the positions labeled **X**, **Y**, and **N**. In UV melting experiments, the absorption of each sample [1.5 μM duplex in 100 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl$_2$] was monitored at 260 nm from 5 to 90 °C at a heating rate of 1 °C/min, using a Cary 100 Conc UV-vis spectrophotometer equipped with a temperature controller. Melting temperatures were determined using a derivative method and Cary Win UV thermal application software.

UV and Fluorescence Measurements. ODN solutions were prepared as described for the T_m measurements. Fluorescence spectra were obtained using a Hitachi F4500 Spectrofluorometer, with a cell path length of 1 cm and excitation at 340 nm. The excitation and emission bandwidth was 1 nm.

Circular Dichroism (CD) Spectroscopy. The mixture of ODNs was equilibrated by cooling to 10 °C; after 30 min the CD spectra were recorded using a JASCO J-715 CD spectropolarimeter. The temperature was controlled using a JASCO PTC-348WI temperature controller.

References

1. Altshuler, D.; Pollara, V. J.; Cowles, C. R.; Van Etten, W. J.; Baldwin, J.; Linton, L.; Lander, E. S. *Nature* **2000**, *407*, 513.
2. The International SNP Map Working Group *Nature* **2001**, *409*, 928.
3. (a) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303. (b) Tyagi, S.; Bratu, D.; Kramer, F. R. *Nat. Biotechnol.* **1998**, *16*, 49.
4. Hwang, G. T.; Seo, Y. J.; Kim, B. H. *J. Am. Chem. Soc.* **2004**, *126*, 6528.
5. Venkatesan, N.; Seo, Y. J.; Bang, E. K.; Park, S. M.; Lee, Y. S.; Kim, B. H. *Bull. Korean Chem. Soc.* **2006**, *27*, 613.
6. Ryu, J. H.; Seo, Y. J.; Hwang, G. T.; Lee, J. Y.; Kim, B. H. *Tetrahedron* **2007**, *63*, 3538.
7. Venkatesan, N.; Seo, Y. J.; Kim, B. H. *Chem. Soc. Rev.* **2008**, *37*, 648.
8. Lee, Y.-A.; Hwang, G. T. *Bull. Korean Chem. Soc.* **2010**, *31*, 2011.
9. Gait, M. J. *Oligonucleotide Synthesis: A Practical Approach*; IRL Press: Washington, DC, 1984.
10. Baase, W. A.; Johnson, W. C., Jr. *Nucleic Acids Res.* **1979**, *6*, 797.