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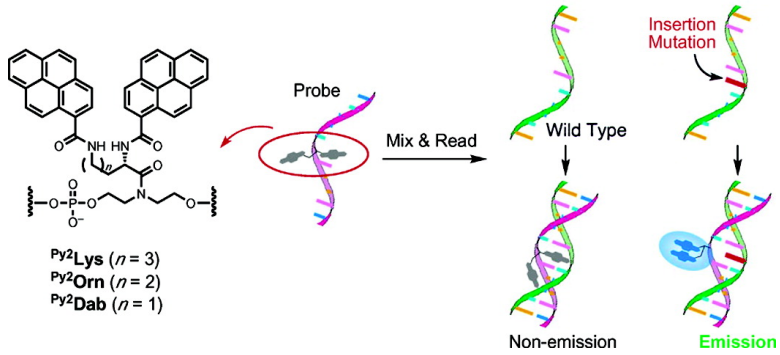

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Pyrene-Labeled Oligodeoxynucleotide Probe for Detecting Base Insertion by Excimer Fluorescence Emission

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Insertion/deletion (indel) polymorphisms occupy approximately 10% of all the polymorphisms in the human genome[1] and lead to serious gene expression errors because they often cause a translational frameshift and create premature proteins. While numerous methods for single nucleotide polymorphism (SNP) typing are available,[2] a fluorescent oligodeoxynucleotide (ODN) probe that is specific for indel detection has not been delineated. It is highly desirable to develop a new ODN probe that can easily determine indel polymorphisms at a specific site on the target DNA.

Pyrene-labeled ODN is expected to be a candidate for such an ODN probe for indel polymorphism detection. Several pyrene-labeled ODNs have so far been reported as sensitive fluorescence probes for monitoring DNA sequences and structures.[3] Excimer formation and intercalating ability of the pyrene chromophore will facilitate the detection of an extra base in target DNA. The development of pyrene-labeled ODNs suitable for the detection of extra bases will make it possible to judge the presence/absence of indel polymorphisms located at a specific site on a target DNA by simply hybridizing with target DNA (Figure 1).

Herein, we report a novel ODN probe for the facile detection of insertion polymorphisms. Our ODN probes labeled with two pyrene chromophores emit strong excimer fluorescence when hybridized with DNA containing an inserted base. This method is useful for determining the presence/absence of insertion polymorphisms located at a specific site on the target DNA.

The pyrene-linking units, ^{Py2}Lys, ^{Py2}Orn, and ^{Py2}Dab were synthesized via coupling of 2 equiv of pyrenecarboxylic acid with amino acids (lysine, ornithine, and α,γ-diaminobutyric acid, respectively) condensed with diethanolamine.[4] They were incorporated via the phosphoramidites into ODN, using a DNA synthesizer. The ODNs used in this study are summarized in Table 1.

Initially, we measured the fluorescence spectra of ^{Py2}Lys-containing ODN (**ODN(^{Py2}Lys)**) (Figure 2a). With excitation at 350 nm, the fluorescence of **ODN(^{Py2}Lys)** was negligible ($\Phi_F = 0.001$). When **ODN(^{Py2}Lys)** was hybridized with **ODN0**, which does not possess the base opposite ^{Py2}Lys, the fluorescence was still weak ($\Phi_F = 0.007$). In contrast, the fluorescence spectrum of the duplex **ODN(^{Py2}Lys)/ODN1(A)**, where A is the base opposite ^{Py2}Lys, had a strong fluorescence peak at 495 nm ($\Phi_F = 0.088$), corresponding to the fluorescence wavelength from a pyrene excimer. The fluorescence quantum yield was ca. 13 times greater than that of **ODN(^{Py2}Lys)/ODN0**. The pale blue fluorescence from the solutions of **ODN(^{Py2}Lys)/ODN1(A)** was visible with the naked eye, as shown in the inset of Figure 2a.

Next, we investigated the influence of the identity and number of inserted bases in target DNA on the fluorescence intensity of **ODN(^{Py2}Lys)** (Figure 2b, left). **ODN(^{Py2}Lys)** hybridized with the strands **ODN1(G)**, **ODN1(T)**, and **ODN1(C)** showed a strong fluorescence, as observed for the duplex **ODN(^{Py2}Lys)/ODN1(A)**.

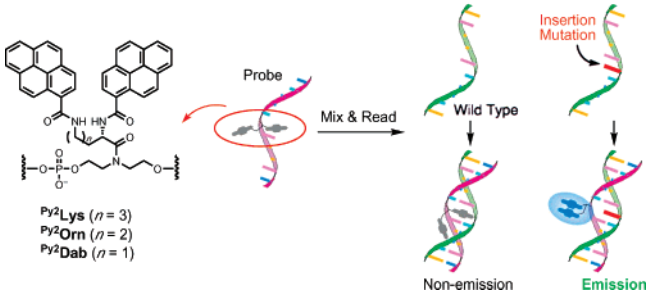

Figure 1. Schematic illustration of a new method for indel polymorphism detection using ODN probes containing two pyrene chromophores (^{Py2}Lys, ^{Py2}Orn, and ^{Py2}Dab).

Table 1. Oligodeoxynucleotides Used in This Study

	sequences (5′–3′)
ODN(X)[a]	GTG TTA AGC C**X**G CCA ATA TGT
ODN0	ACA TAT TGG CGG CTT AAC AC
ODN1(N)[b]	ACA TAT TGG C**N**G GCT TAA CAC
ODN2	ACA TAT TGG C**AA** GGC TTA ACA C
ODN3	ACA TAT TGG C**AA A**GG CTT AAC AC
ODN$_{LS}$-(^{Py2}Lys)	CTC ACT GGG GTA GGG CCC AGT **^{Py2}Lys**GT TGG GGC T
ODN$_{LS}$(WT)[c]	AGC CCC AAC ACT GGG CCC TAC CCC AGT GAG
	Ser Pro Asn Thr Gly Pro Tyr Pro Ser Glu
ODN$_{LS}$(G)[c]	AGC CCC AAC **G**AC TGG GCC CTA CCC CAG TGA G
	Ser Pro Asn Asp Trp Ala Leu Pro Gln STOP

[a] **X** = ^{Py2}Lys, ^{Py2}Orn or ^{Py2}Dab. [b] **N** = A, G, T, or C. [c] Corresponding amino acid sequences are shown in the second lines.

The fluorescence from ^{Py2}Lys-containing probe was almost constant regardless of the nature of the complementary base of ^{Py2}Lys. In contrast, the fluorescence behavior of **ODN(^{Py2}Lys)** was strongly dependent on the number of inserted bases (Figure 2b, right). With an increase in the number of bases opposite ^{Py2}Lys in the duplexes such as **ODN(^{Py2}Lys)/ODN2** and **ODN(^{Py2}Lys)/ODN3**, the fluorescence intensity gradually decreased.

The temperature dependence of the absorption maximum of **ODN(^{Py2}Lys)** hybridized with **ODN0** and **ODN1(A)** was investigated in order to determine whether the pyrene modification is involved in base-stacking (Figure 2c). At higher temperatures where the duplexes are dissociated into single strands, an absorption maximum at 347 nm was observed for both the **ODN(^{Py2}Lys)/ODN0** (T_m = 64.7 °C) and **ODN(^{Py2}Lys)/ODN1(A)** (T_m = 63.8 °C) duplexes. With temperature decrease, a red shift (ca. 6 nm) of the absorption maximum occurs in **ODN(^{Py2}Lys)/ODN0**, whereas little shift of the absorption maximum was observed for **ODN(^{Py2}Lys)/ODN1(A)**. These absorption shifts indicate that the stacking interaction between pyrene chromophores and DNA bases actually occurs in **ODN(^{Py2}Lys)/ODN0**, resulting in a very weak pyrene excimer fluorescence.

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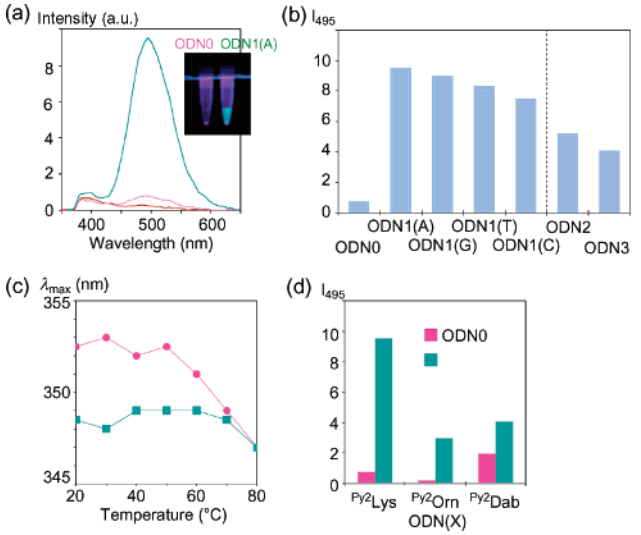

Figure 2. (a) Fluorescence spectra of 2.5 μM **ODN(Py2Lys)** hybridized with 2.5 μM **ODN0** or **ODN1(A)** (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). Excitation was at 350 nm. Inset is the fluorescence image of the solutions of **ODN(Py2Lys)/ODN0** (left) and **ODN(Py2Lys)/ODN1(A)** (right) duplexes illuminated with a 365 nm transilluminator. (b) Fluorescence intensities at 495 nm from **ODN(Py2Lys)** hybridized with sequences possessing various insertion bases. The fluorescence was measured in the same way as described in a. (c) Variation of absorption maxima of **ODN(Py2Lys)/ODN0** and **ODN(Py2Lys)/ODN1(A)** with solution temperature (10 μM strand concentration, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0). (d) Intensities of fluorescence from **ODN(Py2Lys)**, **ODN(Py2Orn)**, and **ODN(Py2Dab)** hybridized with **ODN0** or **ODN1(A)**. The fluorescence was measured in the same way as described in a.

We next compared the fluorescence intensities of the pyrene-labeled ODNs consisting of different amino acids, **ODN(Py2Lys)**, **ODN(Py2Orn)**, and **ODN(Py2Dab)** (Figure 2d). **ODN(Py2Orn)** and **ODN(Py2Dab)** hybridized with **ODN1(A)** are emissive, and the fluorescence was stronger than those of the duplexes hybridized with **ODN0**. However, these fluorescence intensities are much lower than that of **ODN(Py2Lys)/ODN1(A)**. Excimer fluorescence emission was strongly affected by the length of the amino acid side chain that connects two pyrene chromophores. These experiments concluded that **ODN(Py2Lys)** is most suitable for the detection of an extra base in the target DNA among three ODN probes containing different amino acids.

The clear change in the fluorescence that depends on the presence/absence of the inserted base opposite Py2Lys is very useful for the detection of insertion polymorphisms. We tested the detection of an insertion mutation by hybridization of the Py2Lys-containing probe using the coding sequence of the epithelial sodium channel β-subunit (βENaC) associated with Liddle's syndrome, which is an autosomal dominant form of hypertension with variable clinical expression.[5] In the mutant βENaC gene, a G insertion after the 256th nucleotide of exon 13 causes a translational frameshift and a premature stop codon (Table 1).[6] We prepared the Py2Lys-containing probe **ODN$_{LS}$(Py2Lys)** and hybridized with βENaC gene sequences, a wild-type **ODN$_{LS}$(WT)**, and a G-inserted mutant **ODN$_{LS}$(G)**. The sample solutions were illuminated at 365 nm, and the fluorescence images were taken through a 380 nm filter (Figure 3). The fluorescence emission from the duplex **ODN$_{LS}$(Py2Lys)/ODN$_{LS}$(G)** was very strong and clearly distinguishable from the

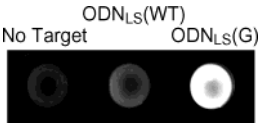

Figure 3. Detection of the G insertion in βENaC gene sequences by the fluorescence change of the Py2Lys-containing ODN probes, **ODN$_{LS}$(Py2Lys)**. **ODN$_{LS}$(WT)** and **ODN$_{LS}$(G)** (2.5 μM) were hybridized with 2.5 μM **ODN$_{LS}$(Py2Lys)** (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature, total volume 10 μL) in a 96 well microplate. Fluorescence was observed using a fluorescence imager VersaDoc Imaging System (BioRad) equipped with a 365 nm transilluminator. The image was taken through a 380 nm long pass emission filter.

poor fluorescence of the **ODN$_{LS}$(Py2Lys)/ODN$_{LS}$(WT)** duplex. The hybridization of the Py2Lys-containing ODN with a target DNA facilitates the determination of the presence/absence of insertion polymorphisms located at a specific site on the target DNA by simply mixing.

In conclusion, we have devised a facile method for the detection of insertion polymorphisms by exploiting novel pyrene-labeled ODN probes. We have designed a new pyrene-linking unit, Py2Lys, which can distinguish the presence/absence of the base opposite Py2Lys. The present method for insertion polymorphism detection using Py2Lys-containing ODN probes constitutes a very facile and accurate homogeneous assay.

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Supporting Information Available: Detailed experimental data on Py2Lys, Py2Orn, Py2Dab, and the related ODNs (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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