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Novel sequence-responding fluorescent oligoDNA probe bearing a silylated pyrene molecule

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Abstract—A novel fluorescent phosphoramidite derivative of dimethylsilylated pyrene was prepared and incorporated into oligoDNA. The fluorescent oligoDNA exhibited marked fluorescent signal upon binding to the fully matched complementary DNA strand, however, the signal was strongly quenched in the single-stranded form as well as in the duplex having mismatched base pair at the terminus of the duplex-forming region.

Detecting a specific gene through the binding of a fluorescent oligonucleotide probe to a complementary gene fragment (target DNA) is becoming an increasingly popular technique for medical diagnostics and genetic studies.[1] To be a practically feasible gene-detecting tool, it is highly desirable that the probe exhibits fluorescent signal only when it binds to the target gene fragment. This would greatly simplify the detection process because of the abridgment of tedious separation and washing steps after the binding of the probe to the sample to be examined. In addition, the probe should be capable of recognizing uncomplementary dissimilarities of the target, including single-nucleotide alternations, to avoid false results. Until date, there are few reports on simple fluorescent oligonucleotide probes, with the exception of molecular beacons[2] and Quenched Autoligation probes (QUAL probes)[3] exhibiting a strong fluorescent signal upon binding to their fully matched DNA, although they remain fluorometrically silent even if they bind to DNA fragments possessing single nucleotide alternations such as SNPs.[4] Meanwhile, recent reports indicate that silylation of fluorescent aromatic compounds such as anthracene and pyrene significantly enhances their fluorescence intensity which can be attributed to the Si-associated $\sigma-\pi$ interaction.[5] During our study, while developing a highly fluorescent oligoDNA probe bearing a silylated aromatic compound, we found that an oligoDNA simply connected with novel dimethylsilylated pyrene moiety at its 5′-terminus through a phosphodiester linkage that exhibits marked fluorescent signal only under the presence of fully matched oligoDNA. The fluorescence intensity of the oligoDNA probe is also strongly influenced by the single-nucleotide alternations of the complement. As a result, the fluorescent signal in the duplex possessing a mismatched base pair close to the pyrene moiety was almost similar to that of the single-stranded probe.

Here, we would like to report on the synthesis and sequence-recognizing ability of the fluorescent oligoDNA probe in terms of fluorescence intensity, in addition to the duplex-forming ability of the probe.

Synthesis of the phosphoramidite derivative of dimethylsilylated pyrene (**5**) is summarized in Scheme 1. 1-Bromopyrene (**1**) was treated with *n*-BuLi in dry THF at −78 °C for 30 min under argon atmosphere followed by the addition of chlorodimethylsilyl chloride. The mixture was stirred under cooling (−78 °C) for 30 min and then allowed to reach room temperature to give chloromethyldimethylsilylated pyrene (**2**). The obtained compound (**2**) was reacted with sodium acetate in aqueous DMF at 100 °C for 24 h to give an acetoxy derivative (**3**), which was then treated with NaOH in aqueous methanol to produce compound **4** bearing a hydroxyl group. The quantum yields of **2** and 4 increased to 0.67 and 0.64 from 0.32 of unmodified pyrene,[5,6], respectively. The phosphitylation of **4** was achieved under normal conditions to give the fluorescent phosphoramidite derivative (**5**).[7] Although the incorporation of compound **5** to the 5′-terminus of an oligoDNA mole-

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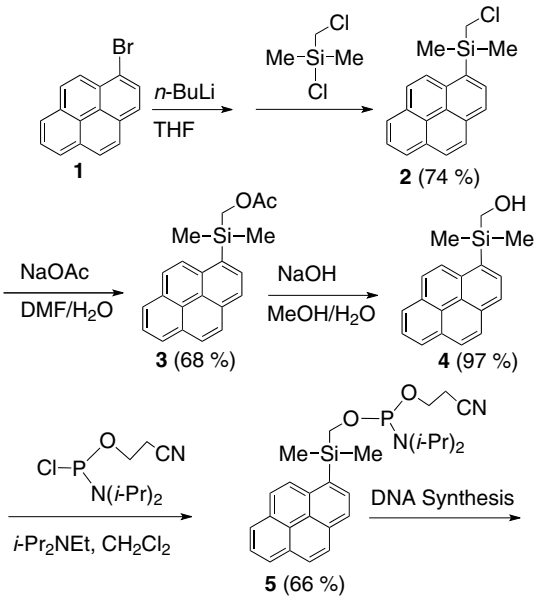

Scheme 1. Synthesis scheme for dimethylsilylated pyrene phosphoramidite derivative (**5**).

cule was accomplished using an automated DNA synthesizer with a standard protocol, the reaction required higher concentration (0.3 M) of the amidite derivative and longer coupling period (360 min) compared with usual nucleoside phosphoramidite derivatives to achieve satisfactory result. After the assembly, the support-bound fluorescent oligoDNA was subjected to the concentrated ammonium hydroxide treatment (60 °C, 12 h) followed by reversed-phase HPLC purification. Under the condition, dimethylsilylated pyrene mosiety remained intact. This was established because of the absence of substantial by-products during HPLC analysis (see Supporting Figure 1). The yield of the fluorescent oligoDNA (**PyODN-1**,[8] 27%) complementary to the part of HIV-1 *rev* sequence[9] (**cODN-1**) was comparable to the corresponding unmodified oligoDNA (**ODN-1**, 28 %) after the purification procedure (Fig. 1). It should be noted that *rev* is responsible for nuclear export of unspliced HIV-1 mRNA.[10]

First, we performed UV-melting studies to identify the duplex-forming ability as well as the sequence-discriminating ability of **PyODN-1**, in terms of thermal stability of the duplex. The results are summarized in Table 1. The T_m value of **Duplex-2**, consisting of **PyODN-1** and **cODN-1**, was slightly higher (2 °C) compared with **Duplex-1**, which contained unmodified **ODN-1** instead of **PyODN-1**. This indicates that the fluorescent DNA has a slightly improved duplex-forming ability (affinity) to the fully matched target compared with the natural DNA. Meanwhile, the T_m values of the other duplexes consisting of **PyODN-1** and the oligomers containing single nucleotide alternation at various positions in the duplex-forming region decreased depending on the position of the alternation (mismatch). For example, theT_m values of the duplexes containing **cODN-2**or **cODN-3** bearing a mismatched nucleotide near the middle of the duplex-forming region decreased by about 9 °C compared with that of **Duplex-2**. On the other hand, the T_m value of **Duplex-5**, containing **cODN-4**, in which the mismatched nucleotide existed at the 3′-end of the duplex-forming region, decreased only about 2 °C. These results, in terms of the thermal stability of the duplexes, indicate that **PyODN-1** can recognize an imperfect complement only if the mismatched nucleotide lies near the middle of the duplex-forming region of the complement. It is well known that the base-pairing energies between fully matched and single-mismatched sequences, particularly in which a mismatch nucleotide is positioned near the end of the duplex-forming region, are usually very small. Therefore, the above results are quite understandable.

Next, we measured the fluorescent spectra of **PyODN-1** in the absence and presence of the fully matched oligoDNA (**cODN-1**) to find out whether the fluorescent signal responds to the existence of the target DNA molecule. As it is shown in Figure 2, **PyODN-1** gave very faint fluorescent signals ($\phi = 0.006$) while it existed as single-stranded form. However, this result was contrary to that seen in the previous observations, which used an analogous oligonucleotide probe bearing a simple pyrene moiety at 5′-phosphate[11] or 5′-hydroxyl group.[12] The fluorescent signal of **PyODN-1** in **Duplex-2**, how-

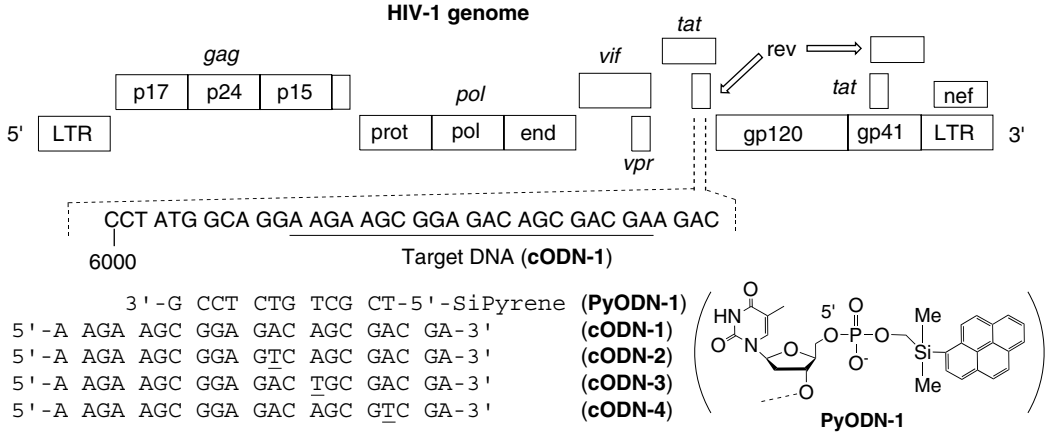

Figure 1. The sequence and structure of oligoDNA used in this study. The underlined italicized letters in **cODN-2** to **cODN-4** indicate the corresponding single-nucleotide alternations to the fully matched target DNA (**cODN-1**).

Table 1. Thermal denaturation temperatures (T_m values) for duplexes[a]

Duplex	T_m (°C)	$\Delta T_{m\text{-}1}$ (°C)[b]	$\Delta T_{m\text{-}2}$ (°C)[c]
1(ODN-1 + cODN-1)	58.4		
2(PyODN-1 + cODN-1)	60.6	+2.2	
3(PyODN-1 + cODN-2)	51.7	–6.7	–8.9
4(PyODN-1 + cODN-3)	51.3	–7.1	–9.3
5(PyODN-1 + cODN-4)	58.8	+0.4	–1.8

[a] T_m values were obtained as the maximum of the first derivative of the melting curves (A_{260} vs. temperature) in a 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM of NaCl, 1.25 μM of DNA strands indicated and were average of at least two independent measurements.

[b] $\Delta T_{m\text{-}1}$ value relative to **Duplex-1**.

[c] $\Delta T_{m\text{-}2}$ value relative to **Duplex-2**.

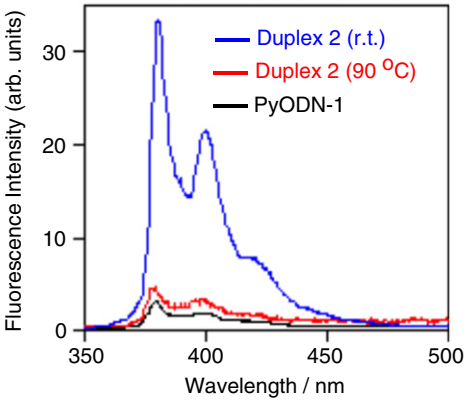

Figure 2. Fluorescent spectra of **PyODN-1** alone (black) and **Duplex-2** (**PyODN-1** + **cODN-1**) at 20 °C (red) or under heating (blue). Spectra were recorded in the thermal denaturation buffer using an excitation wavelength of 335 nm with 0.3 μM of each oligomer.

ever, markedly recovered ($\phi = 0.046$) at 20 °C after undergoing the usual annealing procedure with the fully matched target (**cODN-1**). This result was also contrary to that of previous observations.[11,12] Thus the probe seemed to emit fluorescent signals only under the formation of duplex.

To confirm that the observed distinct fluorescent signal had resulted from the formation of duplex between **PyODN-1** and the target, the solution of **Duplex-2** was heated well above the T_m. As shown in Figure 2, the fluorescent signal of **PyODN-1** at the high temperature (90 °C) was of nearly the same magnitude as that of the single-stranded form. In another set of experiments, we also observed that **PyODN-1** gave a strong fluorescent signal when simply mixing with **cODN-1** at room temperature over a short period, and the signal became as strong as the annealed duplex within 10 min (see Supporting Figure 2). These results clearly indicate that the fluorescent signal is enhanced by the formation of the duplex. Moreover, it indicates that the probe can be utilized for the rapid detection of the target sequence in solution without the need of a tedious annealing procedure. It should be noted that we did not observe a noticeable redshift for the UV-absorption maxima of the pyrene moiety after the annealing. This finding and the relatively small T_m increment ($\Delta T_{m\text{-}1}$ in Table 1) of **PyODN-1** in **Duplex-2** compared with the unmodified **ODN-1** in **Duplex-1** indicate that the silylated pyrene moiety attached to **PyODN-1** does not intercalate into the base pairs of the duplex,[13] which usually brings about a much larger increment of T_m value and severe quenching of pyrene-based fluorescence.[14,15]

The effect of single-nucleotide alternations (mismatches) in the target oligoDNA to the fluorescent signal was also examined using **cODN-2** to **4** and the results are shown in Figure 3. The fluorescent signal of the **Duplexes-3** to **5** decreased depending on the position of the mismatched nucleotide. For example, **Duplex-3** containing **cODN-2** in which a mismatched nucleotide existed near the middle of the duplex-forming region gave the most distinct fluorescent signal among all duplexes containing a mismatched base pair. The signal was, however, smaller than that of the fully matched duplex (ca. 87%). Meanwhile, the fluorescent signal decreased as the mismatched position of the targets shifted toward the end of the duplex-forming region. As a result, the signal of **Duplex-5** was only 14% that of **Duplex-2** and was comparable to that of the single-stranded **PyODN-1**. Thus, contrary to the results of the UV-melting study, **PyODN-1** can distinguish imperfect complements, particularly when the mismatched nucleotide of the complement exists at the 3′-end of the duplex-forming region. This finding would greatly facilitate the detection of the target DNA since the separation and washing processes to exclude imperfect duplexes can be avoided.

In conclusion, the easily prepared oligoDNA probe bearing a novel dimethylsilylated pyrene moiety at its 5′-terminus gives distinct fluorescent signal only when it forms duplex with the fully complementary oligoDNA in a short time period. The intensity of this signal is, however, influenced by the existence of a single-nucleotide alternations in the complement. For example, the signal is severely quenched by the existence of a single-nucleotide alternation at the 3′-end of the duplex-forming region. Although the reason to bring about the recovery of fluorescent signal of **PyODN-1** through the

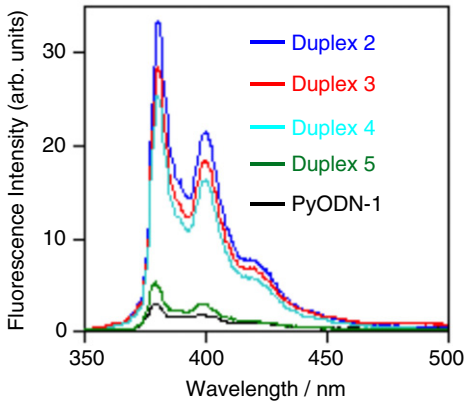

Figure 3. Fluorescent spectra of **PyODN-1** alone (black), **Duplex-2** (blue), **Duplex-3** (red, **PyODN-1** + **cODN-2**), **Duplex-4** (light blue, **PyODN-1** + **cODN-3**), and **Duplex-5** (green, **PyODN-1** + **cODN-4**). Spectra were recorded at 20 °C in the same buffer and concentration as described in Figure 2.

hybridization to the fully matched complement is not clear at this moment, the results presented here indicate that the newly devised fluorescent oligoDNA could be utilized as a highly practical probe for the distinctive detection of sequence-specific DNA fragments.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.10.012.

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