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Termini-free Molecular Beacon Utilizing Silylated Perylene and Anthraquinone Attached to the C-5 Position of Pyrimidine Nucleobase

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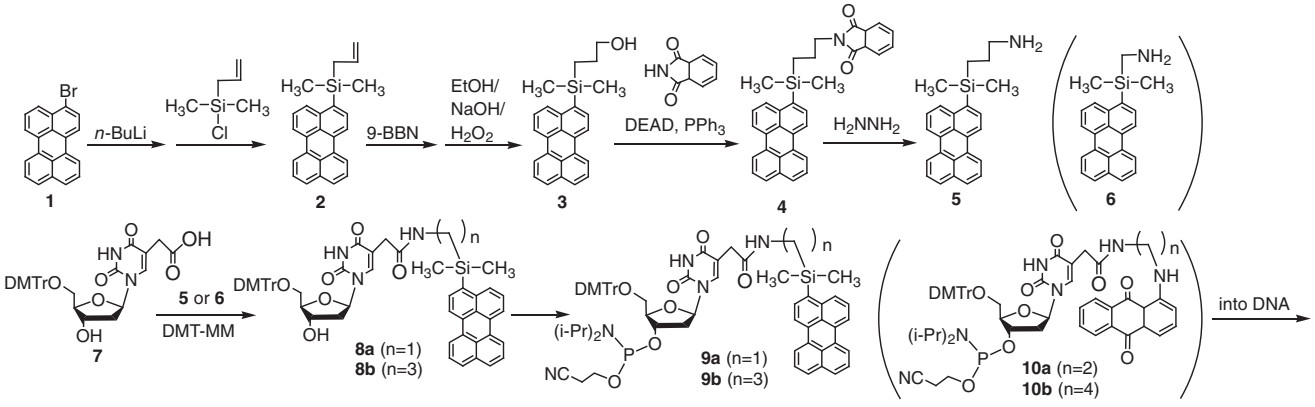
A termini-free molecular beacon type of DNA bearing a novel silylated perylene and an anthraquinone both at the C-5 position of deoxyuridine residues exhibits prominent fluorescence upon mixing with complementary oligonucleotide (24- to 50-fold increase in comparison with a single-stranded state) along with one base mismatch discrimination ability.

A molecular beacon (MB) is an oligonucleotide consisting of a partial self-complementary sequence to form a stem-loop structure along with a combination of a fluorophore at one end and a quencher at the other end.[1] Under the absence of the complementary oligonucleotide (the target), the MB forms a stem-loop structure and therefore fluorescent signal is quenched because of the close proximity of the fluorophore and the quencher. Hybridization of the MB with the target, however, brings about the recovery of the fluorescence signal due to the resolution of the stem-loop structure and separation of the fluorophore and the quencher distally. Thus, the presence of the target can be characterized by the increase in the fluorescence signal of the MB. On the other hand, insufficient fluorescence quenching of the original MB is a well-recognized drawback. This would cause poor signal-to-noise ratio (S/N ratio) and the reduction of detection limit because of the substantial background fluorescence signal even under the absence of the target. Another significant problem of the MB is that the classical MB cannot be coupled with modern microarray technology since both termini (5′- and 3′-) of the MB are connected to a fluorophore and a quencher. A number of studies have been carried out to overcome these problems.[2] In a practical point of view to apply the MB methodology for high-throughput gene analysis, development of new versatile and a straightforward technique to overcome the above problems at the same time is still highly desirable. In recent studies[3,4] to utilize novel silylated fluorescent materials[5,6] exhibiting enhanced fluorescence quantum yield along with the bathochromic shift in absorption and emission, due to the specific σ^*–π^* interaction,[7] we have found that a termini-free oligoDNA possessing a stem-loop structure[8] as well as a modified nucleotide units bearing a silylated perylene moiety (fluorophore) and an anthraquinone moiety (quencher) in the middle of the stem-region exhibited efficient fluorescence quenching (ca. 98%) in the absence of the target. In the presence of the target, however, the fluorescence signal was recovered prominently. In addition, the oligomer exhibited discrimination ability of one-base-mismatched target through the fluorescence signal.

The synthesis of modified perylene derivative **6** possessing (aminomethyl)dimethylsilyl function was reported previously.[9] Meanwhile, preparation of analogous compounds possessing (aminopropyl)dimethylsilyl group and incorporation of that into oligoDNA are summarized in Scheme 1. In brief, 3-bromoperylene (**1**) was coupled with allylchlorodimethylsilane and the resulting compound **2** was treated with 9-borabicyclo[3.3.1]-nonane (9-BBN) followed by a mixture of EtOH/H_2O_2/NaOH/H_2O to give compound **3** bearing hydroxy function. The reaction of **3** with phthalimide in the presence of diethyl azodicarboxylate and triphenylphosphine[10] gave the corresponding phthalimide derivative **4** which was further converted to a silylated perylene derivative **5** bearing a primary amine group by treatment with hydrazine hydrate. The silylated perylene derivatives **5** and **6** were coupled with C-5 carboxymethyl derivative of 2′-deoxyuridine[11] **7** followed by the conversion to the corresponding phosphoramidite derivatives **9**. Incorporation of phosphoramidite **9a** into DNA was carried out with a standard protocol. On the other hand, incorporation of **9b** required rather concentrated solution (0.2 M) with prolonged coupling period



Scheme 1. Synthetic route of the modified nucleoside phosphoramidite derivatives.

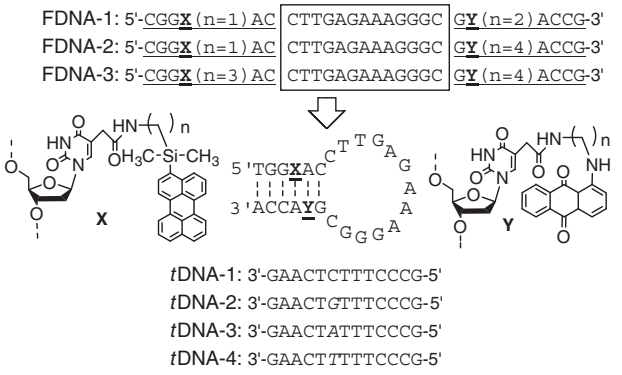


Figure 1. Sequence and structure of the modified DNA used in this study.

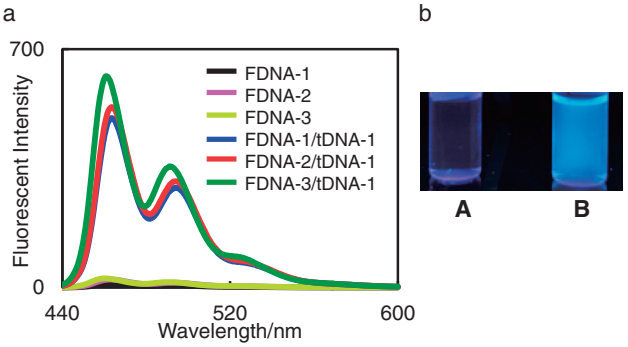


Figure 2. Fluorescent properties of FDNAs. (a) Fluorescence spectra of FDNAs and duplex with *t*DNA-1 (3.0 μM, 10 mM sodium phosphate, 100 mM NaCl, pH 7.2). (b) Photograph displaying the emission behavior at room temperature of FDNA-1 alone (**A**) and of the duplex with *t*DNA-1 (**B**) upon illumination at 302 nm.

(360 s) to achieve satisfactory coupling yield (ca. 95%). Compounds **10a** and **10b** (0.1 M) were also incorporated into the same DNA in the same manner. After the usual work-up, desirable modified DNAs (FDNA-1 to FDNA-3) were obtained with satisfactory yields. The sequence and proposed stem-loop structure of the modified DNAs are depicted in Figure 1 along with the sequence of target DNA (*t*DNA-1 to *t*DNA-4) complementary to the loop region of the modified DNAs. As it is shown in Figure 1, modified nucleotide units described above are incorporated into the stem region consisting of 6-bp units so that they are facing each other.

The fluorescence spectra of the modified DNAs under the presence and absence of target DNA were measured in near physiological conditions (100 mM NaCl) and the obtained fluorescence spectra are depicted in Figure 2. As it is clearly shown in Figure 2a, the fluorescence signal of all modified DNAs is strongly quenched in the absence of the targets. Under the same conditions, the thermal-melting properties of the FDNA-1 and the corresponding duplexes were examined. The UV-melting profiles of the duplexes showed the identical curves with the canonical duplexes, however, MB-type FDNA-1 showed no sigmoid curve (Figure 3a). Therefore, the T_m values of the FDNA estimated by fluorescence-melting experiments

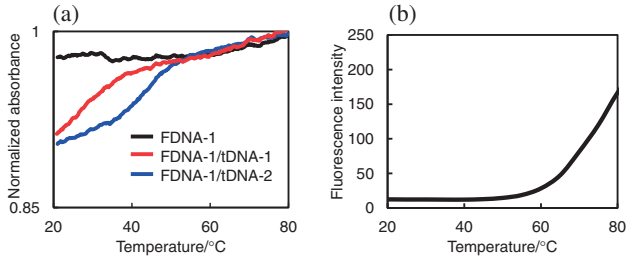


Figure 3. Thermal melting properties of FDNA-1. (a) UV melting curves of FDNA-1 and duplexes with *t*DNA-1 and -2. (b) Fluorescence melting curves of FDNA-1 (3.0 μM, 10 mM sodium phosphate, 100 mM NaCl, pH 7.2).

Table 1. Ralative fluorescence intensity ratios of FDNAs

Oligomer	Target	$I_{complex}/I_{single}$[a]	I_{mis}/I_{full}[b]
FDNA-1	*t*DNA-1	49.1	—
FDNA-2	*t*DNA-1	23.6	—
FDNA-3	*t*DNA-1	23.7	—
FDNA-1	*t*DNA-1	—	1
FDNA-1	*t*DNA-2	—	0.30
FDNA-1	*t*DNA-3	—	0.35
FDNA-1	*t*DNA-4	—	0.17

[a]Ratio of fluorescence intensity of duplexes ($I_{complex}$) with that of single strand (I_{single}). [b]Ratio of fluorescence intensity of mismatched duplexes (I_{mis}) with that of matched strand (I_{full}).

was around 71 °C indicating that the FDNAs form stem-loop structure (Figure 3b). It is well known that the C-5 position of pyrimidine nucleobase is facing the major-groove of nucleic acid duplex and, therefore, both the fluorophore and the quencher molecules are expected to be located in the groove. These indicate that the observed fluorescence quenching could be brought by the interaction of the fluorophore and the quencher in rather hydrophilic major-groove since the molecules are quite hydrophobic in nature. Fluorescence intensity shown in Figure 2a also indicates that the efficiency of the quenching depends on the length of the linker (alkyl) portion connecting the fluorophore and/or the quencher to the nucleobase since the signal of FDNA-1 possessing the shorter linker {**X**($n = 1$), **Y**($n = 2$)} is quenched more effectively than those (FDNA-2 and FDNA-3) possessing longer linker portion.

Meanwhile, prominent recovery of the fluorescence signal (24- to 50-folds) in the presence of the complementary DNA (*t*DNA-1) was observed for all modified DNAs as those are also depicted in Figure 2a. It should be noted that the fluorescence quantum yield of all FDNAs under these conditions is about 0.5. Thus, the change of the fluorescence signal in the presence and the absence of the target was easily recognized by the naked eye as it is shown in Figure 2b. The observed phenomenon is presumably due to the formation of a double helical complex between the loop-region of the modified DNAs and *t*DNA-1 causing the opening of the stem and relocation of the fluorophore and the quencher to a distant position. The fluorescence intensity ratios under the presence and the absence of the target DNA ($I_{complex}/I_{single}$) at 462 nm (emission maxima) are listed in Table 1. Among the modified DNAs studied, FDNA-1

Table 2. Sequence discrimination ability of FDNA-1 under lower salt concentration (50 mM NaCl)

Oligomer	Target	$I_{complex}/I_{single}$[a]	I_{mis}/I_{full}[b]
FDNA-1	*t*DNA-1	16.1	1
FDNA-1	*t*DNA-2	3.0	0.19
FDNA-1	*t*DNA-3	6.9	0.43
FDNA-1	*t*DNA-4	3.0	0.19

[a]Ratio of fluorescence intensity of duplexes ($I_{complex}$) with that of single strand (I_{single}). [b]Ratio of fluorescence intensity of mismatched duplexes (I_{mis}) with that of matched strand (I_{full}).

exhibited the most impressive result showing the $I_{complex}/I_{single}$ ratio of 49. This means that in the absence of the target, about 98% of the fluorescence signal of the complex was quenched and the number is close to the recently reported analogous MB type of probe possessing two unmodified perylene moieties and two anthraquinone moieties connected to non-nucleosidic scaffold, namely D-threoninol.[12]

Since FDNA-1 exhibited drastic fluorescence-increment effect in the presence of the complementary DNA, we further examined discrimination ability of FDNA-1 to the one-base-mismatched target through the fluorescence signal using imperfectly complementary strands (*t*DNA-2 to *t*DNA-4) possessing one-base substitution to the native target DNA (*t*DNA-1) and the obtained fluorescence intensity ratios between the full matched complex (I_{full}) and the mismatched complex (I_{mis}) are also listed in Table 2. Under certain conditions (50 mM NaCl), FDNA-1 exhibited varied I_{mis}/I_{full} ratio depending on the mismatched base in the complements. According to the results listed in Table 1, FDNA-1 recognizes G/T mismatch more nicely than other mismatches despite the fact that G/T mismatch base pair is expected to form the most stable base pair of all possible mismatch base pairs.

In conclusion, we developed the novel termini-free molecular beacon type of DNA bearing a novel silylated perylene and an anthraquinone both at C-5 position of deoxyuridine residues. These probe DNAs showed low noise emission for the discrimination of the target DNA. Therefore, we anticipate that these DNA probes will provide a practical tool capable of simple visible detection.

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