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Novel fluorescent oligoDNA probe bearing a multi-conjugated nucleoside with a fluorophore and a non-fluorescent intercalator as a quencher

Sinichi Kodama, Satoko Asano, Tomohisa Moriguchi, Hiroaki Sawai and Kazuo Shinozuka*

Department of Chemistry, Faculty of Engineering, Gunma University, 1-5-1 Tenjin-cho, Kiryu 376-8515, Japan

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Abstract—A set of 15mer linear oligoDNA probes bearing a modified nucleoside conjugated with a polyamine/fluorescein/anthraquinone reporting moiety were synthesized. In a single-stranded form, the fluorescence generated by the excitation of fluorescein was efficiently quenched, while marked recovery of the fluorescence was observed when the probes formed duplexes with the fully complementary strand.

Fluorescent labeling of oligonucleotides has been attracting wide attention over the last decade because of its ease of handling and relative inexpensiveness compared to the classical radio-labeling method. Many efforts have been focused on the development of a fluorescent oligonucleotide probe which is able to detect a specific oligonucleotide sequence in homogeneous solution. To fulfill this purpose, however, the probe should possess certain properties such as changing its fluorescent properties upon hybridization with its complementary strand. Specific pyrene-conjugated oligoDNA and oligoRNA probes developed by Yamana et al.[1] and a 'molecular beacon' type of probe developed by Tyagi et al.[2] are typical examples of such probes. In the latter case, particularly, the change of the fluorescent property relies on the change of the efficiency of energy transfer (ET) between two different functional groups, namely, a fluorophore (donor) and a quencher (acceptor). The groups are generally attached at both ends of a probe oligomer, independently, which is not always an easy task. In addition to this, the probe should have a stem–loop type of structure prior to hybridize the target to maintain efficient energy transfer between the two functional groups. Thus, the method also relies on the change of the secondary structure of the probe itself upon hybridization.

Meanwhile, we have previously reported the synthesis of a linear DNA probe incorporating a fluorophore (fluorescein; acceptor) and an intercalator molecule (acridine; donor) simultaneously at its 5′-terminus.[3] The probe exhibited enhanced hybridization ability due to the presence of the intercalator. Also, the fluorophore-based fluorescence induced by the excitation of the intercalator was strongly quenched upon the hybridization of the probe to its complementary strand, presumably due to the cancellation of fluorescent energy transfer (FRET) from the intercalator to the fluorophore.[3]

Along with the study to develop further a feasible oligoDNA probe, we have designed a novel oligoDNA probe. In the probe, a modified pyrimidine nucleoside bearing a fluorophore (fluorescein; donor) and a non-fluorescent intercalator (anthraquinone; quencher) molecules at the C-5 position through an appropriate polyamine molecule is incorporated in the middle of the sequence (**Probe-1** and **Probe-2**, Fig. 1). Contrary to the previous probe described above, the current probe is designed to emit a fluorescent signal upon the hybridization to its complementary strand. Through the study, we have found that the fluorescence induced by the excitation of the fluorophore of the probe was efficiently quenched in the absence of the complement. The fluores-

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* Corresponding author. Tel.: +81 277 30 1320; fax: +81 277 30 1321; e-mail: sinozuka@chem.gunma-u.ac.jp

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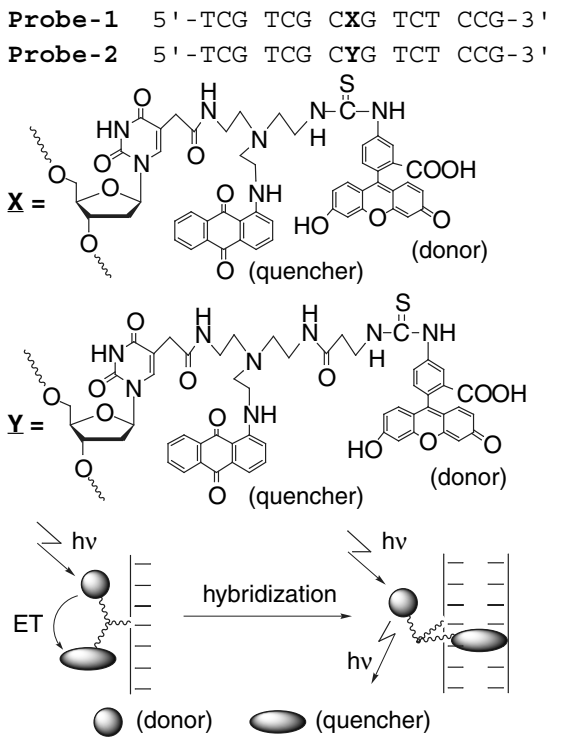


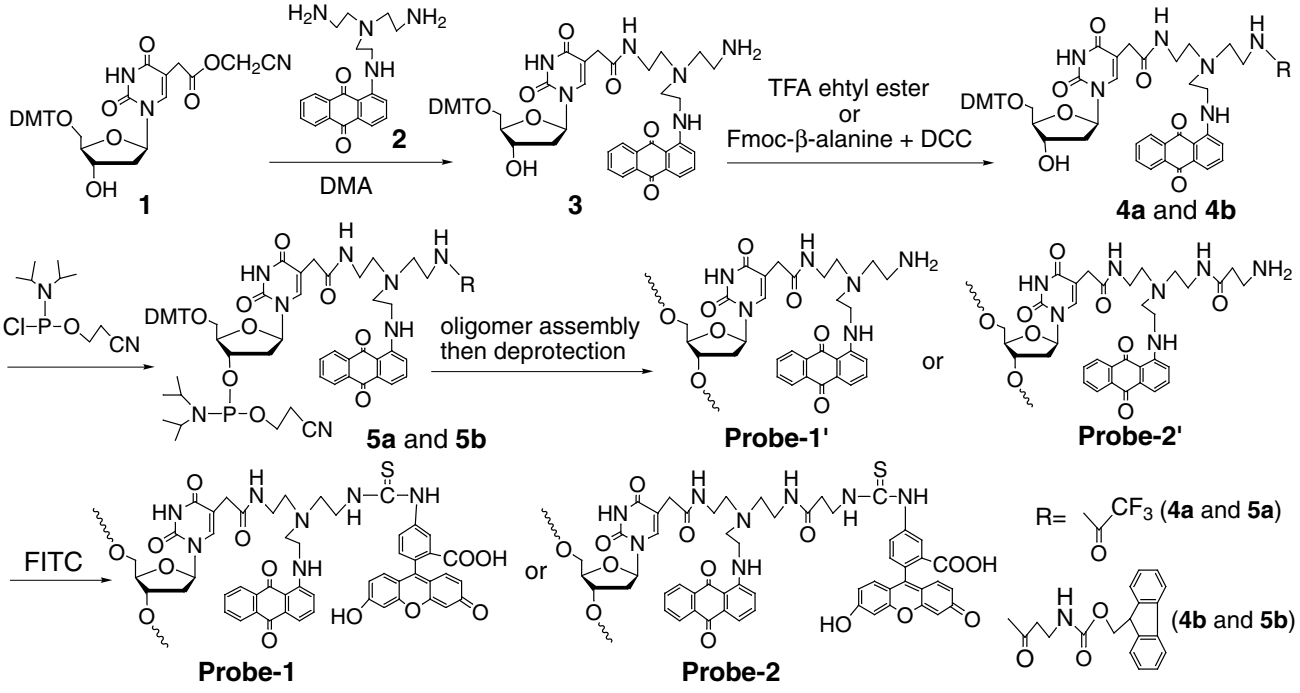
Figure 1. The structure and the sequence of multifunctional fluorescent probe bearing a fluorophore and an intercalator.

cence was, however, markedly recovered upon the formation of a double strand with its complementary strand.[4] In addition to these, the probe exhibited certain differential response with its full-matched and one-base mismatched complements on both thermal and spectroscopical properties. However, these properties depend on the structure of the linker molecule connecting the fluorophore and intercalator molecules. Here, we would like to report the synthesis and properties of a novel 15mer DNA probe conjugated with a multivalent polyamine bearing fluorescein and anthraquinone moieties.

The synthetic procedure of the probe is summarized in Scheme 1. In brief, 5′-protected 5-(cyanomethoxycarbonylmethyl)uridine[5] (**1**) was reacted with the tris(2-aminoethyl)amine derivative of anthraquinone (**2**), readily prepared by the reaction of the amine and 1-chloroanthraquinone, to give the modified nucleoside (**3**). The primary amine function of the nucleoside was reacted with either trifluoroacetic acid ethyl ester or Fmoc-protected β-alanine to give the corresponding nucleosides **4a** and **4b**, respectively. The obtained nucleosides **4** were further converted to the corresponding phosphoramidite derivatives (**5a** and **5b**) in the usual manner[6] and utilized for the oligomer synthesis with automated DNA synthesizer (ABI-392).

After the assembly, the resulting oligoDNAs were deprotected by treatment with concd NH_4OH and then purified by HPLC using reversed-phase column to give the precursors (**Probe-1′** and **Probe-2′**) of the probes. It should be noted that prolonged coupling period (350 s) was applied for **5** during the oligomer assembly. The purified precursors were dissolved in sodium carbonate buffer (pH 9.8) and reacted with FITC for 1 day at room temperature.

Removal of the excess amount of FITC from the reaction mixture with Sephadex G-25 column followed by purification with reversed-phase HPLC gave the multifunctional oligoDNA probes (**Probe-1** and **Probe-2**).



Scheme 1. Synthesis of multifunctional fluorescent oligoDNA probe.

The structure of the probes was confirmed by enzymatic digestion and ESI-mass spectrometry.[7]

At first, the hybridization ability as well as the sequence specificity of the probes was examined by UV-melting experiments under near-physiological conditions (pH 7.2, 0.1 M NaCl) and the obtained T_m values are listed in Table 1.

As is shown in Table 1, the obtained T_m values indicate that the probes retain almost the same or slightly lower degree of hybridization ability compared to the parental unmodified oligoDNA (**ODN-N**) towards the full-matched complement despite the fact that they have an intercalative molecule. The magnitude of the T_m decrement in the current probes is, however, less prominent than in the case of the analogous oligoDNA probes carrying fluorescein and an intercalator (pyrene) moieties in the backbone portion. In such probes, T_m value decreased from 6 to 11 °C compared to that of the unmodified control.[4b] The results suggest that in the current probes, the anthraquinone moiety brings about the duplex-stabilizing effect by the intercalation, although, the effect is counterbalanced by the introduction of bulky fluorescein moiety, maybe due to the steric effect of the moiety. This notion could be supported by the fact that the T_m values of the corresponding precursors (**Probe-1′** and **Probe-2′**) lacking the fluorescein moiety are much higher than those of the probes (Table 1). Furthermore, the T_m value of **Probe-2** bearing an extended linker portion between the intercalator and the fluorophore is higher than that of **Probe-1**. These results indicate that the distance between the intercalator and the bulky fluorescein molecule affects the hybridization ability in the probe DNA. The factor also affects sequence discrimination ability of the probes, at least in our study. For example, the results in Table 1 revealed that both **Probe-1** and **Probe-2** retain some sequence discrimination ability since the T_m values of the probes with mismatched complement are lower than those with full-matched complement. The decrement of the T_m values of **Probe-2** with mismatched complements is, however, almost the same as that of unmodified **ODN-N** and is considerably larger than that of the **Probe-1**. The results indicate that the modified probes retain sequence discrimination ability depending on the distance between the intercalator and the fluorophore. These features make **Probe-2** more desirable than **Probe-1** since it has more feasible hybridization ability as well as sequence discrimination ability compared to **Probe-1**.

Next, the fluorescent properties of the probes were examined under the same condition as the T_m experiments. After the annealing procedure, the probes were irradiated at the wavelength corresponding to the absorption maximum of the fluorescein moiety (494 nm) in the presence or absence of complementary strands at 20 °C. The obtained fluorescence spectra are depicted in Figure 2.

As it is clearly shown in Figure 2, both probes exhibited minimal fluorescence in the absence of the complements as well as in the presence of non-complementary dT-15mer (blue and purple lines, respectively). This background emission, however, seems to be slightly higher in **Probe-1**. The observed minimal fluorescence under the conditions is presumably due to the intramolecular energy transfer from the fluorescein moiety (λ_{Em}: 515 nm) to the anthraquinone moiety (λ_{max}: 520 nm). On the other hand, marked fluorescence was detected in the presence of the full-matched complementary strand in both probes (black lines). The intensity of the observed fluorescence compared to that of the probes existing alone was, however, more prominently increased in **Probe-2**. As mentioned above, the anthraquinone moiety in the probe would intercalate to the duplex composed of the probe and the full-matched complementary strand upon the hybridization. Such

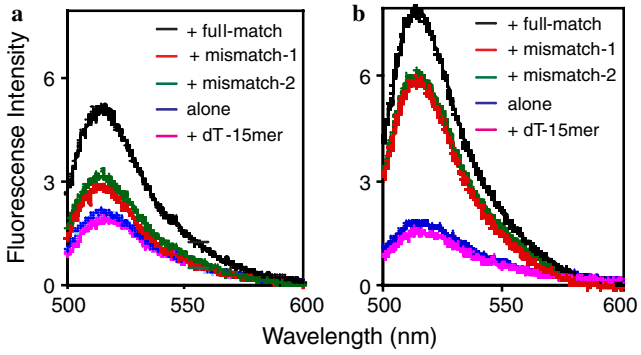


Figure 2. Fluorescent spectra of **Probe-1** (a) and **Probe-2** (b). The spectra were measured under the same conditions as the UV-melting experiments.

Table 1. T_m values (°C) of the duplexes consisting of the probe oligonucleotides and their complements[c]

Probes	+ Full-matched complement	+ Mismatched complement-1[a] (ΔT_m[b])	+ Mismatched complement-2[a] (ΔT_m[b])
ODN-N	63.1	55.9 (−7.2)	56.8 (−6.3)
Probe-1′	70.0	—	—
Probe-2′	71.5	—	—
Probe-1	61.0	58.0 (−3.0)	57.9 (−3.1)
Probe-2	63.2	55.9 (−7.3)	55.3 (−7.9)

[a] Sequence of the mismatched complement is as follows in which the underlined position indicates mismatch base; mismatched complement-1, 5′-CGGAGACTGCGACGA-3′; mismatched complement-2, 5′-CGGAGACGGCGACGA-3′.

[b] ΔT_m = (T_m value with full-matched complement) − (T_m value with mismatched complement).

[c] T_m values were determined by computer fitting of the first derivative of UV-melting profile measured in 10 mM of sodium phosphate buffer (pH 7.2) containing 100 mM of NaCl and 2 μM of each oligonucleotide.

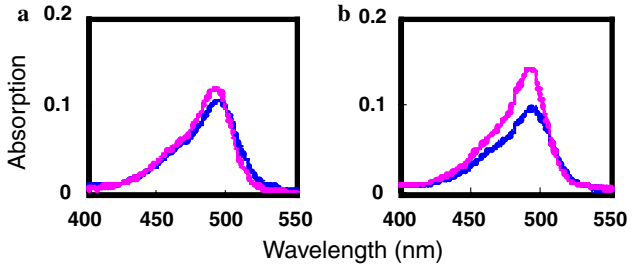


Figure 3. UV-absorption spectra of **Probe-1** (a) and **Probe-2** (b) in the absence (blue lines) and in the presence (purple lines) of the complement. The spectra were measured under the same conditions as the UV-melting experiments.

intercalation lowers the efficiency of the intramolecular energy transfer from the fluorescein moiety to the anthraquinone moiety causing the increase of the fluorescent intensity.[4a]

The influence of the sequence of the complements to the fluorescent properties was also examined. In the presence of the complement having the one-base mismatched sequence used in the T_m study, both **Probe-1** and **Probe-2** gave decreased fluorescent intensity (green and red lines, respectively) compared to those of the probes in the presence of the full-matched complementary strand. Thus, the fluorescent signal of the probes is sensitive to the sequence of the target even at 20 °C, contrary to the analogous probe reported previously.[4a] Also, the results are somewhat consistent with the results obtained in T_m studies.[8]

Interestingly, the UV-absorption spectra of the probes in the presence of the complement (purple lines) exhibited hyperchromic effect around the absorption maximum of fluorescein moiety (494 nm) as those that are shown in Figure 3. The effect is more prominent in **Probe-2** than in **Probe-1**. A slight blue shift effect around the absorption of anthraquinone moiety (520 nm) was also observed for both probes, suggesting the intercalation of anthraquinone moiety in the presence of the complement. The exact mechanism that brings about the observed hyperchromic effect is not clear at this moment. However, the phenomenon would, at least, partially be responsible for the fluorescent property of the probes, since such efficient absorption of photon by fluorescein moiety, particularly in **Probe-2**, would bring about efficient emission of the moiety, presumably.

In conclusion, we have successfully synthesized a set of novel oligoDNA probes bearing a modified nucleobase conjugated with a polyamine/fluorescein/anthraquinone reporting moiety. In the probes, **Probe-2** bearing an extended linker portion between the intercalator and the fluorophore exhibited almost the same hybridization ability as well as sequence discrimination ability as the parental unmodified oligoDNA. The probe gave a fluorescent signal depending on the presence or absence of the complement. The intensity of the signal is also sensitive to the sequence of the complement. These features are quite desirable for oligoDNA probe to realize an easy detection of the target sequence in homogeneous solution.

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6. ^{31}P NMR ($CDC1_3$, δ); 148.7 and 149.1 (doublet) for **5a** bearing the trifluorocarbonyl function. 148.6. 149.0 (doublet) for **5b** bearing the Fmoc function.
7. The average molecular weights calculated form ESI-mass spectral data are as follows. **Probe-1**; calcd mass, 5276.6; obsd mass, 5278.7; **Probe-2**; calcd mass, 5347.7; obsd mass, 5349.8.
8. The fluorescent intensity of **Probe-2** with the other one-base mismatched sequence (5′-CGGAGACAGCGGCGA-3′ where the underlined position indicates the mismatch position, T_m = 59.0 °C) was almost of the same extent as **Probe-2** and full-matched complementary strand (data not shown).