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Synthesis of ODNs Containing 4-Methylamino-1,8-naphthalimide as a Fluorescence Probe in DNA

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Abstract—Synthesis and fluorescence properties of oligodeoxynucleotides containing 4-methylamino-1,8-naphthalimide (NI) have been described. NI was successfully incorporated into DNA without significant destabilization of DNA whilst retaining its high fluorescence quantum yield. The attachment site of the NI greatly affected its property as an energy acceptor in FRET analysis.

Fluorescence resonance energy transfer (FRET) is a process by which the excited state energy of one fluorescent dye is transferred to another dye. Since the FRET efficiency is dependent upon the distance between the donor and acceptor dyes, this technique has been widely used to determine the structure[1–4] and conformational transition of nucleic acids,[5–7] and the interaction of the biomolecules even at the single molecule level.[8,9] Recently, by incorporating three dye molecules in DNA, multiple FRET resulting from irradiation at a single wavelength has been successfully applied to detect single nucleotide polymorphism.[10] Thus, the usage of several combinations of fluorescent dyes with different photochemical properties would be useful for multiple FRET analysis.[11–13] However, since fluorescence quantum yield (ϕ_F) of the dye molecules often becomes low upon attachment to DNA, the dye molecules used for DNA labeling in FRET analysis were restricted to fluorescein, *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxylrhodamine, cyanine dyes, coumarin,[14,15] and their derivatives. Here, in order to expand the dye library for FRET analysis, we have synthesized oligodeoxynucletotides (ODNs) containing 4-methylamino-1,8-naphthalimide (NI). It has been demonstrated that NI can be introduced into DNA without significant loss of the duplex stability and its high ϕ_F.

The NI group was conjugated to 5′-end of ODN by converting the *N*-(3-propanol)-4-methylamino-1,8-naphthalimide ($\mathbf{NI_{OH}}$) to the corresponding phosphoroamidite derivative (**4**, Scheme 1).[16] For incorporation of the dye at the desired position inside the DNA, Yamana et al. have reported that 2′-sugar position of ODNs is a suitable site for covalent attachment of several fluorophores.[14,17–21] According to their procedure, a nucleoside derivative containing an NI group was synthesized by the reaction of 2′-amino-2′-deoxyuridine with 2-(4-methylamino-1,8-naphthalimide)acetic acid ($\mathbf{U_{NI}}$, Scheme 2).[22] $\mathbf{U_{NI}}$ was converted to the phosphoroamidite derivative (**10**) and NI-containing ODNs were synthesized by DNA synthesizer according to the standard procedure.[23]

First, to check the duplex stability of the NI-containing ODNs, melting temperatures were measured for representative ODNs studied here (Table 1). $\mathbf{NI_{OH}}$ conjugated ODN ($\mathbf{I_{NIOH}/II}$) showed higher stability compared to the corresponding unmodified ODN (**I**/**II**). On the other hand, incorporation of $\mathbf{U_{NI}}$ ($\mathbf{I_{UNI}/II}$) resulted in slight destabilization of ODN compared with that of **I**/**II**. Thus, NI was incorporated into DNA without large disturbance of the duplex stability. Although NI has a high ϕ_F (0.8 in methanol),[24] attachment of dye to DNA often causes a significant decrease of ϕ_F. ϕ_F values for $\mathbf{I_{NIOH}/II}$ and $\mathbf{I_{UNI}/II}$ were measured to be 0.46 and 0.40, respectively. Thus, NI retained the ϕ_F upon incorporation into DNA. To obtain the information on the energy accepting properties of NI, we synthesized a series of ODNs containing NI as an energy acceptor and pyrene ($\mathbf{U_{Py}}$) as an energy donor,[25]

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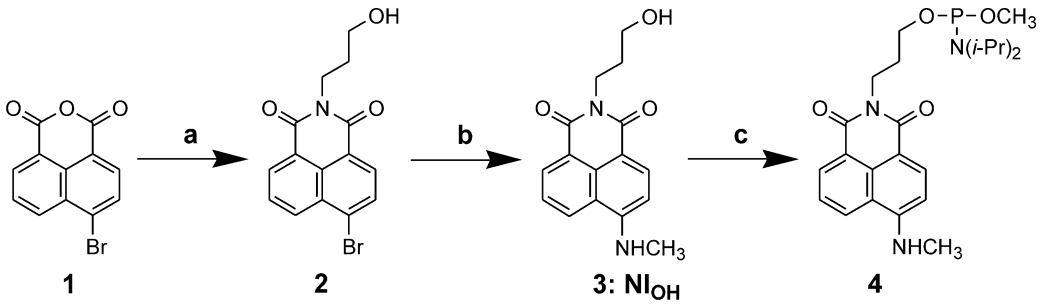
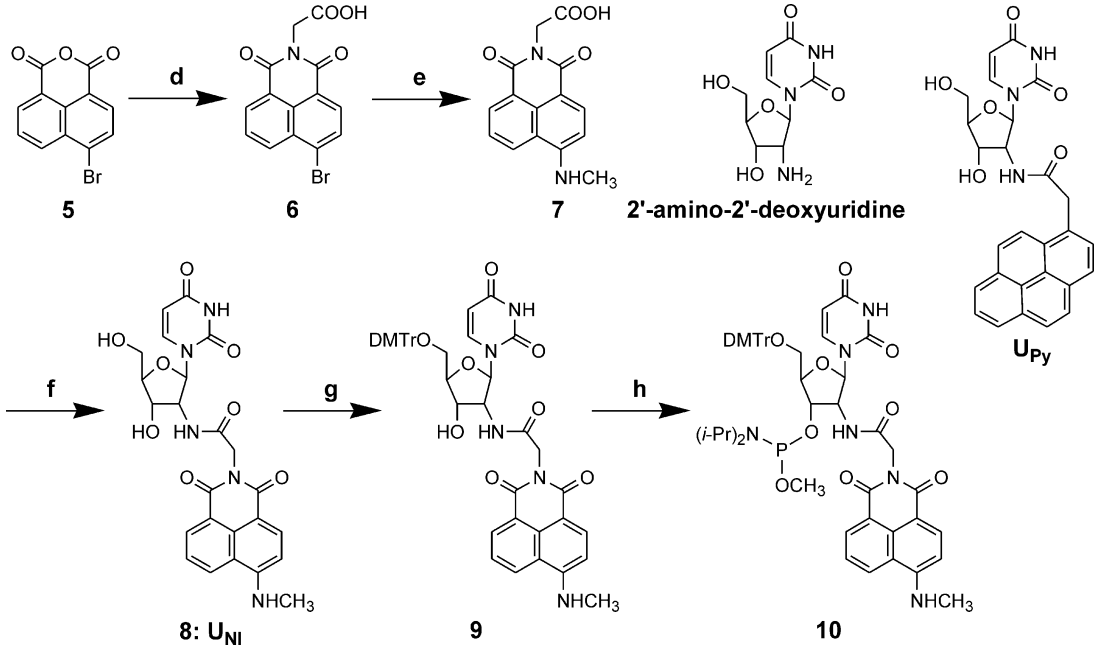


Scheme 1. (a) 3-Amino-1-propanol, EtOH, reflux, 8 h, 94%; (b) CH_3NH_2, $CuSO_4{\cdot}5H_2O$, DMA, 80 °C, 2 h, 32%; (c) P(N-*i*Pr$_2$)(OCH$_3$)Cl, DIEA, pyridine, 1.5 h.



Scheme 2. (d) Glycine, DMA, 80 °C, 17 h, 77%; (e) CH_3NH_2, $CuSO_4{\cdot}5H_2O$, DMA, 80 °C, 3 h, 47%; (f) DCC, HOBt, DMF, 20 h, 68%; (g) DMTr-Cl, pyridine, 21 h, 56%; (h) P(N-*i*Pr$_2$)(OCH$_3$)Cl, DIEA, pyridine, 16 h.

Table 1. Melting temperatures of pyrene and NI modified ODNs

ODN	T_m (°C)[a]
I/II	37.2
I$_{NIOH}$/II	40.3
I$_{UNI}$/II	36.2
I/II$_{Py1}$	41.6
I/II$_{Py4}$	44.9
I$_{NIOH}$/II$_{Py1}$	41.3
I$_{NIOH}$/II$_{Py4}$	44.3
I$_{UNI}$/II$_{Py1}$	39.0
I$_{UNI}$/II$_{Py4}$	40.8

[a]UV melting measurements were carried out in a pH 7.0 Na phosphate buffer (20 mM) at a total strand concentration of 8 μM.

in which the distance between NI and pyrene was varied by placing A-T base pairs between pyrene and NI resulting in ca. 10–21 Å distance between **U$_{Py}$** and **NI$_{OH}$**, and 14–24 Å distance between **U$_{Py}$** and **U$_{NI}$**, respectively. The incorporation **U$_{Py}$** resulted in the increase of T_m values (**I/II$_{Py1}$** and **I/II$_{Py4}$**); thus the doubly labeled ODNs showed higher stability compared with those of unmodified ODNs (**I/II**), and their corresponding singly labeled ODNs (**I$_{NIOH}$/II** and **I$_{UNI}$/II**). Since NI has a very low molar extinction coefficient at 351 nm ($<1500\,M^{-1}\,cm^{-1}$), selective excitation of pyrene is achieved at this wavelength (Fig. 1). The emission spectra of **I$_{NIOH}$/II$_{Pyn}$** and **I$_{UNI}$/II$_{Pyn}$** ($n=1$–4), excited at 351 nm, are shown in Figure 2a and b, respectively. The fluorescence intensity of NI at 540 nm was reduced and that of pyrene at 380 nm was increased with increasing distance between two dyes, demonstrating the occurrence of the FRET between these two dyes. The efficiency of energy transfer (E) was obtained from the excitation spectrum of the NI at the given wavelength 351 nm (I_{351}) according to the equation,

$$I_{351} = \varepsilon_A + E\varepsilon_D \quad (1)$$

where ε_A and ε_D are the extinction coefficient of the energy acceptor and energy donor at 351 nm, respectively.[26] The FRET efficiencies measured for duplexes **I$_{NIOH}$/II$_{Pyn}$** and **I$_{UNI}$/II$_{Pyn}$** are shown graphically in Fig. 2c, which shows a clear dependence of E on the distance between two dyes. Interestingly, E was significantly low

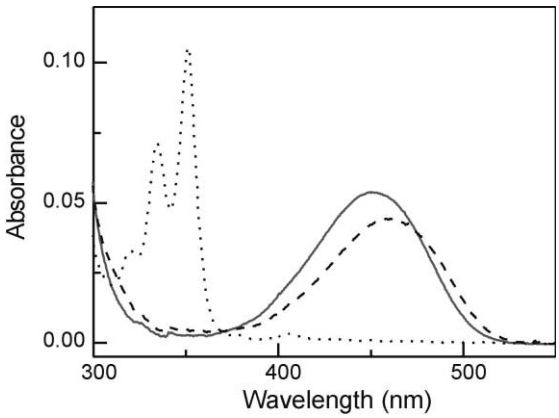


Figure 1. Absorption spectra of $\mathbf{I_{NIOH}/II}$ (solid line), $\mathbf{I_{UNI}/II}$ (dashed line), and $\mathbf{I/II_{Py1}}$ (dotted line). The measurement conditions were the same as in Table 1.

for $\mathbf{I_{UNI}/II_{Pyn}}$ compared to $\mathbf{I_{NIOH}/II_{Pyn}}$. Since the same energy donor is used in both cases, this difference is considered to rely on the properties of the energy acceptor. For the energy transfer occurring via the Förster mechanism, E is expressed as follows,

$$E = 1/\left(1 + (R/R_0)^6\right) \tag{2}$$

$$R_0 = \left(8.8 \times 10^{-28} J \phi_D n^{-4} \kappa^2\right)^{1/6} \tag{3}$$

$$J = \int \varepsilon_A(\lambda) f_D(\lambda) \lambda^4 d\lambda / \int f_D(\lambda) d\lambda \tag{4}$$

where J is the spectral overlap of the donor emission spectrum and the acceptor absorption spectrum, ϕ_D is the ϕ_F for energy donor, n is the refractive index of the medium, and κ is relative orientation of the emission dipole of the donor and the excitation dipole of the acceptor. As for the explanation of the observed low E for $\mathbf{I_{UNI}/II_{Pyn}}$, the absorption maximum for $\mathbf{I_{UNI}/II}$ (459 nm) is red-shifted by 8 nm relative to that for $\mathbf{I_{NIOH}/II}$ (451 nm), which may result in a decrease of J. However, J was found to be 1.6×10^{31} and 1.2×10^{31} nm^6 mol^{-1} for $\mathbf{I_{NIOH}/II_{Pyn}}$ and $\mathbf{I_{UNI}/II_{Pyn}}$, respectively, only partly explaining the lower E for $\mathbf{I_{UNI}/II_{Pyn}}$. As for the other factor, rotation of the NI attached at 2′-sugar position inside the DNA is likely to be restricted compared to NI labeled at the 5′-end, leading to the low κ^2 value for $\mathbf{U_{NI}}$. Thus, in this case, the difference of κ^2 may significantly contribute to this observed large difference of E between these two systems (Scheme 3).[8,27,28]

In conclusion, NI was incorporated into DNA as a fluorescence probe through two attachment procedures. It has been demonstrated that NI can be incorporated into DNA without large alternation of the duplex stability and its high ϕ_F. Though low E was observed for $\mathbf{U_{NI}}$, rotational properties of the NI may provide fruitful information on the conformational freedom of the ODN around $\mathbf{U_{NI}}$.

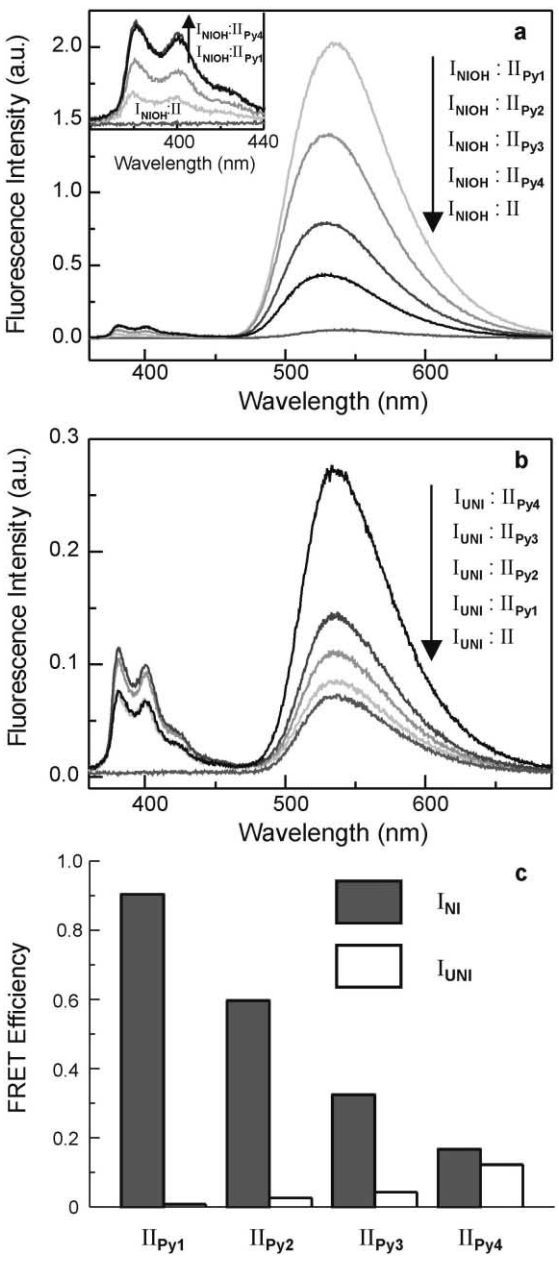


Figure 2. Fluorescence spectra ($\lambda_{ex} = 351$ nm) for (a) $\mathbf{I_{NIOH}/II}$, $\mathbf{I_{NIOH}/II_{Pyn}}$ ($n = 1$–4) and (b) $\mathbf{I_{UNI}/II}$, $\mathbf{I_{UNI}/II_{Pyn}}$ ($n = 1$–4). The measurement conditions were the same as in Table 1. (c) FRET efficiency in DNA duplexes $\mathbf{I_{NIOH}/II_{Pyn}}$ and $\mathbf{I_{UNI}/II_{Pyn}}$.

5′-AAAAAAGTGCGC-3′ **I**

5′-**NI$_{OH}$**-AAAAAAGTGCGC-3′ **I$_{NIOH}$**

5′-AAAAAAG**U$_{NI}$**GCGC-3′ **I$_{UNI}$**

3′-TTTTTTCACGCG-5′ **II**

3′-TT**U$_{Py}$**TTTCACGCG-5′ **II$_{Py1}$**

3′-TTT**U$_{Py}$**TTCACGCG-5′ **II$_{Py2}$**

3′-TTTT**U$_{Py}$**TCACGCG-5′ **II$_{Py3}$**

3′-TTTTT**U$_{Py}$**CACGCG-5′ **II$_{Py4}$**

Scheme 3.

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22. **10**: ^{1}H NMR (270 MHz, DMSO-d_6) δ 1.15 (m, 12H, NH($\underline{CH_3}$)$_2$), 3.00 (d, 3H, J = 4.1 Hz, NH$\underline{CH_3}$), 3.26 (m, 7H, N$\underline{H}$($\underline{CH_3}$)$_2$, POCH$_3$, H5′, H5″), 3.70 (s, 6H, PhOCH$_3$), 4.23 (m, 1H, H4′), 4.41 (m, 1H, H3′) 4.70 (s, 2H, C(O)CH$_2$), 4.74 (m, 1H, H2′), 5.45 (d, J = 8.1 Hz, 1H, uridine H5), 5.97 (d, J = 8.4 Hz, 1H, H1′), 6.72–8.66 (m, 21H, naphthalene, trityl, uridine H6, NHCH$_3$, C(O)NH), 11.48 (s, 1H, uridine NH). ^{31}P NMR (162 MHz, DMSO-d_6) δ 152.55, 155.65 (diastereomers). FABMS (positive ion) m/z 973 (M + 1).
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