Luminescence-based colorimetric discrimination of single-nucleotide transversions by the combined use of the derivatives of DOTA-conjugated naphthyridine and its terbium complex

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ABSTRACT

We newly synthesized a nucleobase-binding ligand, ND-DOTA, in which 2-amino-5,7-dimethyl-1,8-naphthyridine (ND) was conjugated with 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DOTA) via an amide linker, and found that its terbium(III) complex (ND-DOTA-Tb) showed green emission based on an energy transfer from the naphthyridine moiety to Tb^{3+}. The blue emission of ND-DOTA was selectively quenched by adding abasic site-containing DNA duplexes that have pyrimidine bases opposite to the abasic site. In contrast, at the same excitation wavelength, ND-DOTA-Tb showed green emission independently of the bases opposite to the abasic site. Thus, a mixed solution of ND-DOTA and ND-DOTA-Tb enabled the luminescence-based colorimetric discrimination of single-nucleotide transversions with the naked eye at a single excitation wavelength.

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Fluorescence-based detection technologies have often been applied in the field of DNA genotyping, due to their unique advantages in setting up sensitive and simple assays. Molecular beacons represent one of the useful chemical strategies for fluorescence resonance energy transfer (FRET)-based genotyping methods.[1] The real-time polymerase chain reaction (PCR)[2] and Invader assay[3] are also genotyping assays that are based on FRET. Although they are regarded as effective approaches, there are still several problems, such as need for fluorophore-labeled DNA, the use of expensive enzymes, and the possibility of hybridization errors. Consequently, a quick, simple, and economical fluorescence-based technology for the routine detection of known mutations is highly desirable.

The recognition of nucleic acids and nucleobases using small ligands is involved in very important events in biological systems,[4] including anti-cancer reactions and the regulation of gene expression.[5] It is also known that some small ligands, for example, ethidium bromide and acridine orange, are useful staining agents for nucleic acids. However, it is difficult to construct fluorescence-based genotyping assays using these small ligands, because they have no selectivity for the nucleobases in DNA. To detect genetic disease and mutations, many studies for detection of single nucleobase have been developed.[6] Recently, the use of naphthyridine derivatives was proposed for the selective detection of nucleobases at bulge,[7] mismatch,[8] and abasic sites[9] in DNA duplexes. One of our authors (KY) and his co-workers found that naphthyridine derivatives interact with pyrimidine bases opposite to the abasic site, accompanied by strong quenching of their blue emission, and that they worked as a fluorescent probe for the detection of single nucleobases at abasic sites.[10] An abasic site is a location where a nucleobase was removed from the DNA strand; in this way, hydrophobic microenvironments are provided for ligands to recognize nucleobases through hydrogen bonding.[11] Their unique optical and nucleobase-binding properties at the abasic site have enabled the construction of a novel genotyping assay without fluorophore-labeled

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DNA, which can discriminate single-nucleotide transversions using the fluorescence quenching response. However, with this strategy, it is difficult to achieve genotyping based on the fluorescence colorimetric response, as with molecular beacons.

Here, we report a new type of genotyping method using a naphthyridine derivative functionalized with lanthanide ions. Thus, by the combined use of ND-DOTA (**1**) and ND-DOTA-Tb (**1-Tb**), the luminescence-based colorimetric discrimination of single-nucleotide transversions was accomplished with the naked eye at a single excitation wavelength.

The emissions of lanthanide complexes are characterized by very sharp and large Stokes shifts in the range of several hundreds of nanometers.[12] In order to add their functionality to the naphthyridine derivative, the chelating motif DOTA was conjugated with ND. It is known that DOTA forms highly stable complexes with a wide variety of metal ions in aqueous solutions.[13] Ligand **1** was synthesized as shown in Scheme 1. 1,4,7-Tris(*tert*-butoxy-carbonylmethyl)-1,4,7,10-tetraazacyclododecane-10-hydroxy-succinimide ester (200 mg, 0.30 mmol) was added to ND (51.7 mg, 0.30 mmol) in dichloromethane (20 ml) containing triethylamine (50 μl, 0.36 mmol) at 35 °C for 24 h. The mixture was cooled to room temperature and evaporated to dryness. The obtained residue (**2**) was dissolved in a mixture of dichloromethane (5 ml) and trifluoroacetic acid (5 ml). The mixture was stirred at room temperature for 24 h and the solvents were removed under reduced pressure. The purification of the product by reversed-phase HPLC gave **1** in 24% yield.[14]

The Tb[3+] complex of **1** was prepared by the addition of terbium(III) chloride hexahydrate (TbCl$_3$·6H$_2$O) to an aqueous solution of **1** adjusted to pH 7.4 using 10 mM HEPES buffer. The stoichiometry of the association between **1** and Tb[3+] was determined by the molar ratio method. Figure 1 shows the change in the emission spectra of **1** upon the addition of Tb[3+]. The intensity of the metal-centered emission increased linearly up to the [Tb[3+]]/[**1**] ratio of 1.0, and then a plateau was observed up to the [Tb[3+]]/[**1**] ratio of 10.0, which clearly indicates that **1** formed a 1:1 complex with Tb[3+]. The obtained complex was characterized by UV, MS, and fluorescence spectrophotometry.[15]

Figure 2a shows the absorption, excitation, and emission spectra of **1** (10 μM) obtained at pH 7.4 and at 20 °C. When **1** was excited at 347 nm, the emission spectra of **1** displayed a broad peak in the range of 380–500 nm with a maximum at 396 nm. Figure 2b shows the absorption, excitation, and emission spectra of **1-Tb** (10 μM) under the same conditions. In the case of **1-Tb**, a large Stokes shift was observed. Thus, when **1-Tb** was excited at 332 nm, the emission peaks of **1-Tb** were observed at 489, 548, 585, and 622 nm. The emission lifetime of **1-Tb** was estimated by the analysis of the emission decay curve, and the obtained long lifetime was 1.7 ms.[16] These emission properties of **1-Tb** are explained by the process shown as follows.[17] This process is initiated by exciting the antenna chromophore from the ground state (S$_0$) to the excited singlet state (S$_1$). The formation of the excited S$_1$ state is followed by intersystem crossing (ISC) resulting in the population of its excited triplet state (T$_1$). By way of an energy transfer from the excited T$_1$ state of the antenna to the lanthanide excited state ($^5D_4$), the resulting metal-centered emission is observed as delayed lumi-
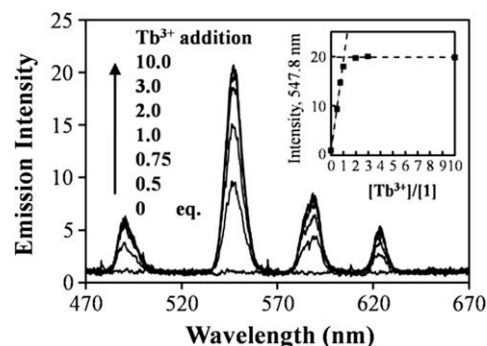

Figure 1. Emission spectra (λ_{ex} = 332 nm) of **1** (2 μM) in the presence of various concentrations of Tb[3+]: 0, 0.5, 0.75, 1.0, 2.0, 3.0, 10.0 equiv of Tb[3+] with respect to **1**. These spectra were measured at pH 7.4 (10 mM HEPES buffer) and at 20 °C. The inset shows the changes in the intensities of the $^5D_4 \rightarrow {}^7F_5$ transition peak at 547.8 nm as a function of the [Tb[3+]]/[**1**] ratio.
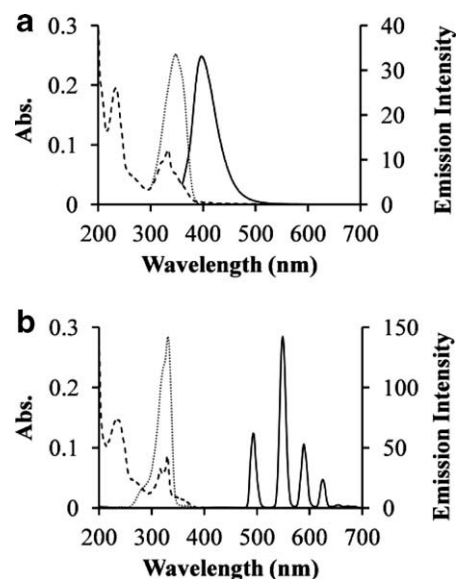
Figure 2. (a) Absorption (dashed line), excitation (dotted line, λ_{em} = 396 nm), and emission spectra (solid line, λ_{ex} = 347 nm) of **1** (10 μM). (b) Absorption (dashed line), excitation (dotted line, λ_{em} = 547 nm), and emission spectra (solid line, λ_{ex} = 332 nm) of **1-Tb** (10 μM). These spectra were measured at pH 7.4 (10 mM HEPES buffer) and 20 °C.

nescence. Thus, the characteristic emission bands of Tb[3+] in the spectra explain the ligand-to-metal energy transfer and the suitability of the naphthyridine ring as an antenna chromophore.

As stated above, it is known that naphthyridine derivatives strongly interact with pyrimidine bases opposite to the abasic sites in the DNA duplex, accompanied by fluorescence quenching.[9] In order to evaluate the nucleobase selectivity of the synthesized compounds, the interaction with the abasic site-containing DNA duplexes was investigated by melting-temperature (T_m) measurements and emission titration. Table 1 shows the T_m of the abasic
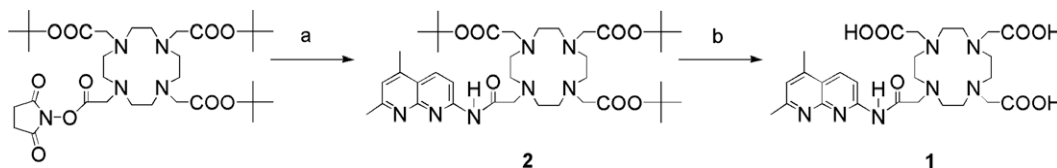
Scheme 1. Reagents and conditions: (a) 1 equiv ND, (C$_2$H$_5$)$_3$N, CH$_2$Cl$_2$, 35 °C, 24 h; (b) CF$_3$COOH, CH$_2$Cl$_2$, rt, 24 h (24% yield).

Table 1
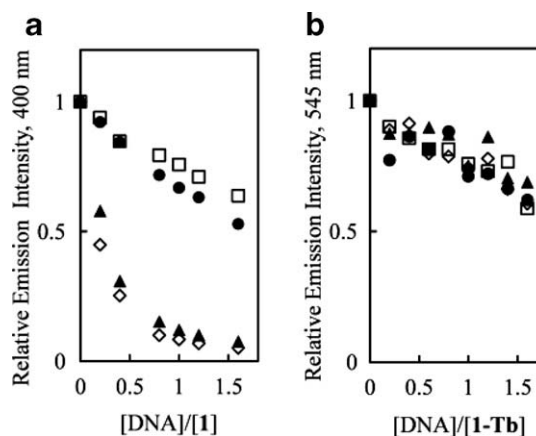T_m of the abasic site-containing DNA duplexes in the absence and presence of ND, **1**, and **1-Tb**[a]

X = abasic site Y	T_m[b]	ΔT_m		
		ND	1	1-Tb
C	23.3 (0.5)	7.7 (0.1)	5.1 (0.4)	0.4 (0.0)
G	27.0 (0.0)	−0.6 (0.2)	0.2 (0.5)	0.9 (0.3)
T	21.9 (0.2)	6.3 (0.0)	4.8 (0.5)	0.6 (0.5)
A	27.5 (0.5)	−0.3 (0.1)	0.4 (0.4)	−0.7 (0.1)

[a] The UV-melting curves were measured for the abasic site-containing DNA duplexes (5′-TCCAG**X**GCAAC-3′/5′-GTTGC**Y**CTGGA-3′; **X** = abasic site, **Y**= C, G, T, and A) (2 μM) in 10 mM PIPES buffer (pH 7.4) containing 100 mM NaCl in the presence of the compounds (5 μM).
[b] T_m values of the abasic site-containing DNA duplexes. All measurements were taken three times, and standard deviations are shown in the parentheses.

site-containing DNA duplexes (5′-TCCAGXGCAAC-3′/5′-GTTGCY CTGGA-3′; X = abasic site, Y = cytosine (C), guanine (G), thymine (T), and adenine (A)) (2 μM) in the absence and presence of ND (5 μM), **1** (5 μM), and **1-Tb** (5 μM) in PIPES buffer (pH 7.4, 10 mM). When ND was added to the abasic site-containing DNA duplexes that had C and T opposite to the abasic site, T_m increases (ΔT_m) of 7.7 and 6.3 were observed, respectively. When **1** was added to the abasic site-containing DNA duplexes that had C and T opposite to the abasic site, ΔT_m of 5.1 °C and 4.8 °C were observed, respectively. These results indicated that the conjugation of DOTA moiety with naphthyridine ring did not affect the nucleobase selectivity of ND, though the binding affinity of ND with pyrimidine bases slightly decreased. It is known that the nucleobase selectively of naphthyridine derivatives might depend on the hydrogen bonding pattern of nitrogen atoms on naphthyridine ring.[18,19] For example, Nakatani and co-workers reported some 2-amino-7-methyl-1,8-naphthyridine derivatives that have an alkyl-amino and acylamino linkages which showed the C selectivity[18] and G selectivity,[19] respectively, considering that the 2-amino-7-methyl-1,8-naphthyridine derivatives having acylamino linkage selectively form three hydrogen bonds with G, while those having alkylamino linkage tended to protonate at N1 position and selectively bound to C through three hydrogen bonds.[18] Thus, **1** is the first naphthyridine derivative having acylamino linkage which showed the pyrimidine base selectivity. The difference in the hydrogen bonding pattern between **1** and conventional naphthyridine derivative having acylamino linkage might be due to the electron donation effect by DOTA moiety and/or the methyl group in naphthyridine ring of ND at the C5 position. In the case of **1-Tb**, the ΔT_m values of the abasic site-containing DNA duplexes did not increase at all. These results indicate that **1** bound to the pyrimidine bases as well as to ND, while **1-Tb** did not interact with the nucleobases strongly and selectively, which is presumably due to the steric effect of the DOTA-Tb moiety and/or change of electron density in naphthyridine ring by coordinating Tb^{3+} ion.

Figure 3a shows the changes in the emission intensity of **1** (5 μM) at 400 nm excited at 347 nm upon the addition of the abasic site-containing DNA duplexes. In this experiment, 25-mer DNA sequences that correspond to codons 735–759 of exon 7 in the human *p53* gene were used.[20] In the case of the abasic site-containing DNA duplexes that have C and T opposite to the abasic site, the significant quenching of the blue emission originating from the naphthyridine ring was observed upon binding with the abasic site-containing DNA duplexes, indicating the interaction between the pyrimidine bases and the naphthyridine ring of **1**. Figure 3b shows the changes in the emission intensity of **1-Tb** (5 μM) at 545 nm upon the addition of the abasic site-containing DNA duplexes excited at 332 nm, where they showed the same tendency in the fluorescence response, and the significant quenching of the


Figure 3. Relative emission intensity of (a) **1** (5 μM) at 400 nm and (b) **1-Tb** (5 μM) at 545 nm in a titration with abasic site-containing DNA duplexes (5′-GGTGAGGATGGG**X**CTCCGGTTCATG-3′/5′-CATGAACCGGAG**Y**CCCATCCTCACC-3′; **X** = abasic site, **Y** = diamond, C; square, G; triangle, T; circle, A). These were measured in 10 mM NaCl at pH 7.4 (10 mM HEPES buffer) and at 22 °C. Excitation wavelength: (a) 347 nm, (b) 332 nm.

green emission was not observed. These data support the results obtained from the T_m measurements and indicate the selectivity for pyrimidine bases of **1** at the abasic site of the DNA duplexes. The green emission of **1-Tb** was not affected by the addition of the abasic site-containing DNA duplexes.

Under optimized experimental conditions, the combined use of **1** (5 μM) and **1-Tb** (5 μM) can be successfully utilized for the luminescence-based colorimetric discrimination of single-nucleotide transversions at a single excitation wavelength. As shown in Figure 4, the fluorescence image of a mixed solution containing **1** and **1-Tb** showed a pale blue color (leftmost), which was due to the mixed emission color of **1** (blue) and **1-Tb** (green). When the abasic site-containing DNA duplexes that contained purine bases opposite to the abasic site were added to this mixed solution, the color of the mixed solution did not change and they also showed a pale blue color. In contrast, in the case of the abasic site-containing DNA duplexes that contained pyrimidine bases opposite to the abasic site, the color of the mixed solution changed to green, because the blue emission of **1** was selectively quenched by adding the abasic site-containing DNA duplexes. Therefore, the mixed solution containing **1** and **1-Tb** can be used to analyze the pyrimidine-to-purine mutation sequences (R249S) of the cancer repression gene *p53* with the naked eye at a single excitation wavelength.

In conclusion, we newly synthesized a nucleobase-binding macrocyclic ligand. The characteristic emission of its terbium(III) complex indicated a ligand-to-metal energy transfer and the suitability of naphthyridine as an antenna. The experimental results obtained
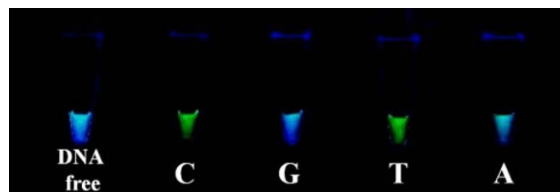
Figure 4. Colorimetric discrimination of single-nucleotide transversions using a mixed solution containing **1** and **1-Tb**. Fluorescence images of the mixed solution containing **1** (5 μM) and **1-Tb** (5 μM) in the presence and absence of the abasic site-containing DNA duplexes (10 μM) (5′-GGTGAGGATGGG**X**CTCCGGTTCATG-3′/5′-CATGAACCGGAG**Y**CCCATCCTCACC-3′; **X** = abasic site, left to right: DNA free, **Y** = C, G, T, and A) in water were obtained. These were measured in 10 mM NaCl at pH 7.4 (10 mM HEPES buffer) and at 22 °C. The samples were excited with a UV lamp at 310 nm.

from the T_m measurements and the emission titration of **1** and **1-Tb** with the abasic site-containing DNA duplexes indicate that **1** bound to the pyrimidine bases opposite to the abasic site, accompanied by the strong quenching of the blue emission arising from the naphthyridine ring, while **1-Tb** showed a green metal-centered emission independent of the bases opposite to the abasic site. A mixed solution of **1** and **1-Tb** can be used in the analysis of single-nucleotide transversions of the cancer repression gene *p53* R249S based on the fluorescent color change from pale blue (wild type; G) to green (mutation type; T) observable with the naked eye at a single excitation wavelength. Recently, several terbium complexes of DOTA derivatives with antenna moiety have been synthesized and used as a functional probe for the detection of metal ion and enzyme in cell.[21] In addition, it was revealed that some naphthyridine derivatives worked as a fluorescent staining reagent for the cytoplasm and nucleus of cell.[22] Therefore, **1-Tb** might have a potential as a novel cell staining reagent and it will be investigated in the future paper with the effect of the spacer length between naphthyridine moiety and DOTA moiety.

Acknowledgment

Supplementary data

Supplementary data (General information on, characterization for synthesized compounds and the experimental details of the emission decay curve) associated with this Letter can be found, in the online version, at doi:10.1016/j.tetlet.2009.02.152.

References and notes

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14. Selected data for 2-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododec-1-methylcarbonylamino]-5,7-dimethyl-1,8-naphthyridine **1**: UV (H_2O): λ_{max} 334; fluorescence (H_2O, pH 7.4) λ_{em} 491, retention time = 13 min (HPLC analysis: column, Inertsil ODS-3 JET Column 20 I.D mm × 50 mm (GL Sciences); eluent, a 40-min linear gradient from 0% to 100% solvent B (solvent A, H_2O, 0.1% TFA; solvent B, acetonitrile); flow rate, 5.0 ml/min; UV (H_2O), 328 nm and 238 nm. ^{1}H NMR (D_2O, 270 MHz): d 2.79 (s, 6H), 2.9–4.0 (m, 24H), 7.55 (s, 1H), 8.25 (d, 1H, *J* = 7.2 Hz), 8.59 (d, 1H, *J* =7.2 Hz); MS (ES$^+$): *m/z* calcd for [M+H]$^+$ ($C_{26}H_{38}N_7O_7{}^+$): 560.30, found: 560.32; Calcd for $C_{26}H_{37}N_7O_7 \cdot 4H_2O \cdot 2.9CF_3COOH$: C, 39.69; H, 5.02; N, 10.19. Found: C, 39.73; H, 4.95; N, 10.23.
15. Selected data for **1-Tb**: UV (H_2O): λ_{max} 332; luminescence (H_2O, pH 7.4) λ_{em} 491, 547, 587, 621; MS (ES$^+$): *m/z* calcd for [M+H]$^+$ ($C_{26}H_{37}N_7O_7Tb^+$): 716.20, found: 716.17.
16. The experimental details about the analysis of emission decay curve were described in Supplementary data.
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