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New Luminescent Europium(III) Chelates for DNA Labeling

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The new europium(III) chelate {2,2′,2″,2‴-{[4′-(aminobiphenyl-4-yl)-2,2′:6′,2″-terpyridine- 6,6″-diyl]bis(methylenenitrilo)}-tetrakis(acetato)} europium(III) (ATBTA-Eu^{3+}) and its 4,6-dichloro-1,3,5-triazinyl and succinimidyl derivatives (DTBTA and NHS-ATBTA, respectively) were synthesized and characterized. Both labeling complexes DTBTA-Eu^{3+} and NHS-ATBTA-Eu^{3+} are luminescent. Especially DTBTA-Eu^{3+} is strongly luminescent, with a luminescence quantum yield of 9.1%, molar extinction coefficient of 3.1×10^4 cm^{-1} M^{-1} (335 nm), and luminescence lifetime of 1.02 ms. The excitation and emission maximum wavelengths of DTBTA-Eu^{3+} are 335 and 616 nm, respectively. The complex is very stable in aqueous buffers, with a conditional formation constant log $K_{DTBTA-Eu}$ of 25.0 at pH 8, and can be conjugated to DNA and proteins. The chelates are also highly resistant to thermal decomposition, photodegradation, and ozone oxidation. These properties prove that DTBTA-Eu^{3+} is suitable as a luminescence label in DNA assays.

1. Introduction

Luminescent lanthanide chelates have been receiving increasing attention because of their applications as luminescent probes for highly sensitive time-resolved fluoroimmunoassay (TR-FIA), DNA hybridization assay, fluorescence microscopy bioimaging, and other analytical techniques.[1−4] They have specific luminescence properties that conventional organic dyes do not have, such as large Stokes shifts, sharp emission profiles, and long luminescence lifetimes. By using a luminescence lanthanide chelate as a label for microsecond time-resolved luminescence detection, we can effectively eliminate nonspecific background luminescence from the biological samples and cuvette materials and the scattering light.

The luminescent lanthanide chelates are mainly composed of Eu^{3+}, Tb^{3+}, Sm^{3+}, or Dy^{3+} ions and ligands with bipyridines,[5] terpyridines,[6] triphenylenes,[7] substituted phenyl,[8] and naphthyl groups[9] as chromophores. To design a luminescent lanthanide chelate as an analytical probe, we must fulfill the following requirements: (i) presence of a suitable absorbing group for strong luminescence with a large luminescence quantum yield and a molar extinction coefficient, in addition to a long luminescence lifetime; (ii) high thermodynamic and kinetic inertness; (iii) solubility in aqueous buffer. Recently, many luminescent lanthanide chelates have been synthesized,[10−20] and several classes of

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the chelates, such as preorganized[21] and predisposed[22] macrocyclic ligands including cryptands and cyclens, multidentate podands,[23,24] or self-assembled lanthanide triple helicates,[25] meet these requirements. Some of these chelates are applied as sensors of pH, pO_2, and selected anions such as halides[26,27] and as labeling reagents. In bioanalysis, the chelates must have a reactive substituent for coupling to targeted molecules such as DNA, proteins, and other biological molecules. In many cases, the introduction of an active binding group is not easy, and sometimes luminescence of the chelate is considerably reduced by the introduction of the binding group. Therefore, synthesis of useful luminescent lanthanide chelate labels is still a challenging theme, and until now, only a few luminescent lanthanide chelates have been developed for use in time-resolved luminescence bioassays.[28−33] The β-diketonate chelate 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro- 4″,6″-hexanedione-6″-yl)chlorosulfo-*o*-terphenyl (BHHCT)-Eu^{3+} is one of the lanthanide labels that meets most of the requirements and has been proven to give high sensitivity in protein analysis.[34−38] However, the β-diketones have low solubility in water-based buffers and low chelate stability (smaller chelate formation constant),[4] which can be a problem for DNA analysis, because phosphate oxygen has a high affinity for lanthanide ions. A terbium chelate, *N*,*N*,*N*′,*N*′-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-phenylpyridine]tetrakis(acetato) terbium(III) (BPTA-Tb^{3+}), is another label that has a high sensitivity in protein analysis.[19] For DNA analyses, however, much more stringent requirements must be fulfilled: (i) much higher chelating stability in water; (ii) thermal stability up to 90 °C, for use in PCR; (iii) stability against photodegradation; (iv) resistance to oxidation by ozone in the environment. This problem becomes serious when the label is used in chip technology,[39] in which a very small amount of the label can be very sensitive to degradation even by low levels of ozone released from the light sources of laboratory instruments or copying machines.

Polycarboxylate derivatives of the terpyridine complex of Eu^{3+} and Tb^{3+} give very stable luminescence and were used for protein labels.[15,31] In these labels, one of the carboxylate groups is used for both chelation and binding. Different from protein labeling, much attention has to be paid to the location of the binding group in DNA analysis, because labeling with a carboxylate binding group decreases the chelate stability and part of the lanthanide metal ion goes to DNA phosphate oxygen. As a result, the luminescence intensity is decreased. To avoid such an undesired effect, we must keep the binding group far from the chelation group. In the present work, new europium(III) chelates {2,2′,2″,2‴-{[4′-(aminobiphenyl-4-yl)- 2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)}-tetrakis(acetato)} europium(III) (ATBTA- Eu^{3+}) and its 4,6-dichloro-1,3,5-triazinyl and succinimidyl derivatives (DTBTA and NHS-ATBTA, respectively) were synthesized and their luminescence properties were examined. The latter has the binding group on the phenyl group and, therefore, no complex instability is observed. To evaluate the utility of the new chelate as a luminescence label, we examined the stability of DTBTA-Eu^{3+} under various conditions. The DTBTA-Eu^{3+} is very stable in various buffers, and has a conditional stability constant that is about 8 orders of magnitude higher than that of ethylenediaminetetraacetato europium(III) (EDTA-Eu^{3+}). The chelate is also highly resistant to thermal decomposition and degradation by excitation light and ozone. These facts indicate that the DTBTA-Eu^{3+} chelate is favorably useful as a new luminescence label for highly sensitive time-resolved luminescence DNA as well as protein assays.

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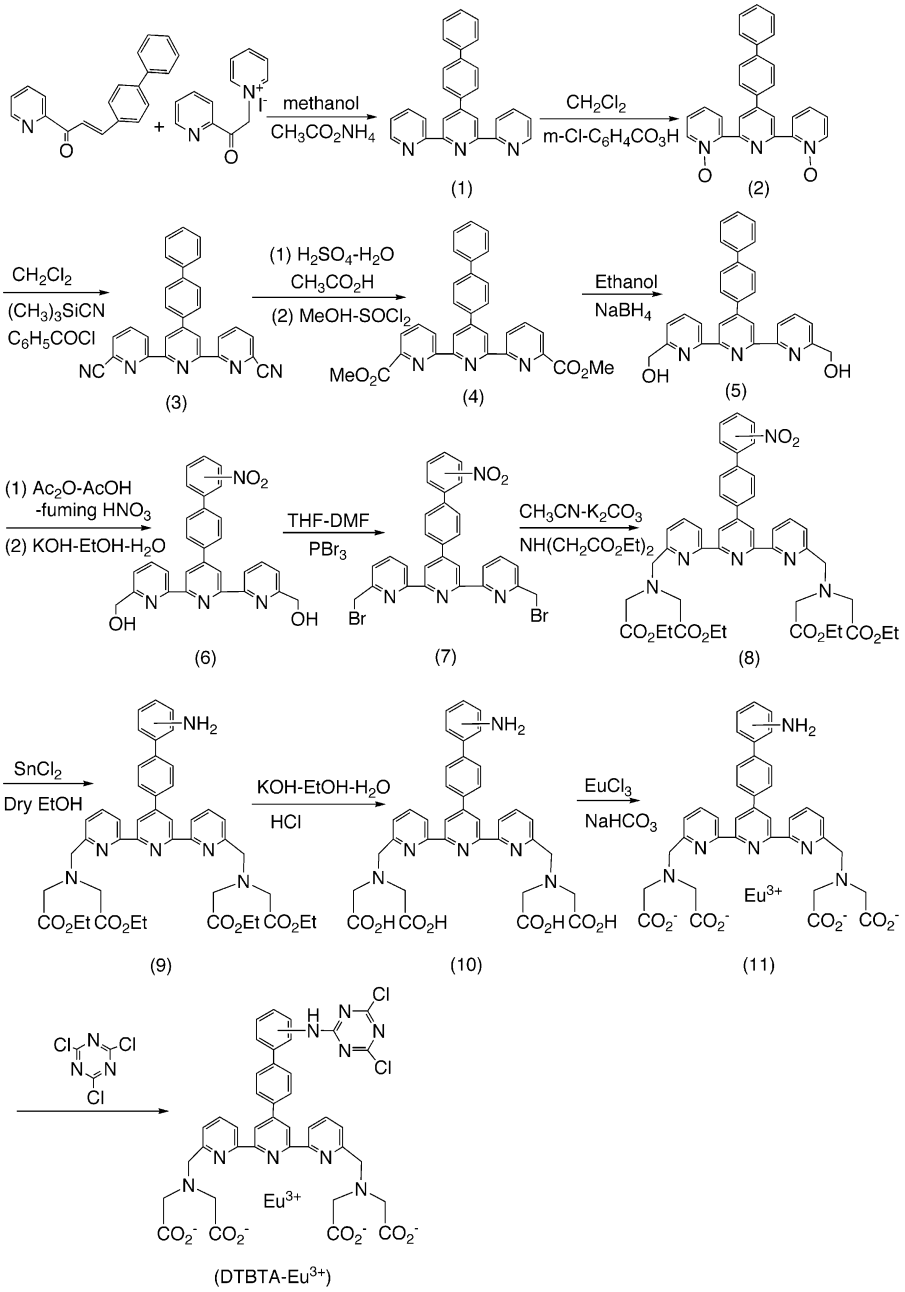
Scheme 1



2. Experimental Section

Synthesis of DTBTA-Eu^{3+}. New chelate DTBTA-Eu^{3+} was synthesized by following the twelve-step reaction shown in Scheme 1. The details of the procedure are described in the following.

(i) Synthesis of 4′-(biphenyl-4-yl)-2,2′:6′,2″-terpyridine, [(C_6H_5)-C_6H_4](C_5NH_2)(C_5NH_4)$_2$ (**1**): The starting materials of (*E*)-3-(biphenyl-4-yl)-1-(pyridin-2-yl)propenone[6] and *N*-[2-(pyridin-2-yl)-2-oxoethyl]pyridinium iodide[24] were prepared on the basis of the literature methods. A mixture of 16.30 g (50 mmol) of (*E*)-3-(biphenyl-4-yl)-1-(pyridin-2-yl)propenone, 14.26 g (50 mmol) of *N*-[2-(pyridin-2-yl)-2-oxoethyl]pyridinium iodide, and 23.10 g (300 mmol) of ammonium acetate in 500 mL of dry methanol was refluxed for 24 h. After the mixture was cooled, the precipitate was filtered and washed with cold methanol. Pure compound **1** was obtained by recrystallization from acetonitrile (10.30 g, 53.5% yield). Anal. Calcd for $C_{27}H_{19}N_3$: C, 84.13; H, 4.97; N, 10.90. Found: C, 83.98; H, 4.83; N, 10.89. ^{1}H NMR ($CDCl_3$): δ 8.80 (s, 2H), 8.75 (d, J = 4.6 Hz, 2H), 8.69 (d, J = 7.8 Hz, 2H), 8.01 (d, J = 8.6 Hz, 2H), 7.89 (t, J = 7.6 Hz, 2H), 7.75 (d, J = 8.2 Hz, 2H), 7.68 (d, J = 6.9 Hz, 2H), 7.48 (t, J = 6.9 Hz, 2H), 7.41−7.33 (m, 3H).

(ii) Synthesis of 4′-(biphenyl-4-yl)-2,2′:6′,2″-terpyridine-1,1″-dioxide (**2**): To 700 mL of CH_2Cl_2 containing 19.27 g (50 mmol) of compound **1** was added 50 g of 3-chloroperoxybenzoic acid. After being stirred for 20 h at room temperature, the solution was washed with 3 × 300 mL of 10% Na_2CO_3, dried with Na_2SO_4, and evaporated. The product was washed with acetonitrile and dried (18.60 g, 91.4% yield). ^{1}H NMR ($CDCl_3$): δ 9.29 (s, 2H), 8.38 (d, J = 6.6 Hz, 2H), 8.25 (d, J = 8.2 Hz, 2H), 7.94 (d, J = 8.6 Hz, 2H), 7.72 (d, J = 8.6 Hz, 2H), 7.66 (d, J = 6.9 Hz, 2H), 7.50−7.43 (m, 2H), 7.41−7.29 (m, 5H).

(iii) Synthesis of 4′-(biphenyl-4-yl)-2,2′:6′,2″-terpyridine-6,6″-dicarbonitrile (**3**): To 450 mL of CH_2Cl_2 containing 15.65 g (37.5 mmol) of compound **2** was added 37.20 g of Me_3SiCN (375 mmol).

After stirring the solution for 20 min, we added 150 mmol of benzoyl chloride dropwise in 20 min. After being stirred for 24 h, the mixture was evaporated to half; 900 mL of 10% K_2CO_3 was added, and the mixture was stirred for 1 h. The precipitate was filtered and washed with H_2O and cold CH_2Cl_2 (13.20 g, 80.8% yield). ^{1}H NMR (DMSO-d_6): δ 8.99 (d, J = 7.6 Hz, 2H), 8.75 (s, 2H), 8.31 (t, J = 7.9 Hz, 2H), 8.20 (d, J = 7.6 Hz, 2H), 8.10 (d, J = 7.6 Hz, 2H), 7.92 (d, J = 8.2 Hz, 2H), 7.78 (d, J = 7.6 Hz, 2H), 7.54−7.42 (m, 3H).

(iv) Synthesis of dimethyl 4′-(biphenyl-4-yl)-2,2′:6′,2″-terpyridine-6,6″-dicarboxylate, $[(C_6H_5)C_6H_4](C_5NH_2)[(C_5NH_3)CO_2CH_3]_2$ (**4**): After a mixture of 8.17 g (20 mmol) of compound **3**, 90 mL of concentrated H_2SO_4, 90 mL of acetic acid, and 20 mL of water was stirred at 90−100 °C for 24 h, the solution was added to 800 mL of ice−water. The precipitate was filtered, washed with water and acetonitrile, and dried. To 600 mL of cooled methanol (ice−water bath) was added dropwise 24 g of thionyl chloride. After stirring the solution for 15 min at room temperature, we added the above precipitate, and the mixture was refluxed for 24 h. After evaporation, 1000 mL of $CHCl_3$ was added, and the solution was washed with 5% $NaHCO_3$ and dried with Na_2SO_4. After the solvent was evaporated, the residue was purified by column chromatography (silica gel, $CH_2Cl_2-CH_3OH$ (99:1, v/v)) and then recrystallized from toluene (4.87 g, 48.5% yield). Anal. Calcd for $C_{31}H_{23}N_3O_4$: C, 74.24; H, 4.62; N, 8.38. Found: C, 74.15; H, 4.55; N, 8.38. ^{1}H NMR ($CDCl_3$): δ 8.88 (s, 2H), 8.86 (d, J = 7.9 Hz, 2H), 8.20 (d, J = 7.6 Hz, 2H), 8.06 (d, J = 7.9 Hz, 2H), 8.00 (d, J = 7.2 Hz, 2H), 7.78 (d, J = 6.6 Hz, 2H), 7.70 (d, J = 6.9, 2H), 7.52−7.30 (m, 3H), 4.07 (s, 6H).

(v) Synthesis of 4′-(biphenyl-4-yl)-2,2′:6′,2″-terpyridine-6,6″-bis(methanol) (**5**): A mixture of 400 mL dry ethanol, 7.02 g (14 mmol) of compound **4**, and 3.02 g of $NaBH_4$ (80 mmol) was stirred at room temperature for 3 h and further refluxed for 1 h. After the solvent was evaporated, 200 mL of saturated $NaHCO_3$ was added, and the solution was heated to boiling. The cold mixture was filtered and washed with water (5.90 g, 92.0% yield). ^{1}H NMR (DMSO-d_6): δ 8.75 (s, 2H), 8.55 (d, J = 7.9 Hz, 2H), 8.07−8.00 (m, 4H), 7.92 (d, J = 8.2 Hz, 2H), 7.78 (d, J = 7.2 Hz, 2H), 7.61 (d, J = 7.9 Hz, 2H), 7.57−7.50 (m, 2H), 7.46−7.40 (m, 1H), 5.57 (t, J = 5.9 Hz, 2H), 4.74 (d, J = 4.6 Hz, 4H).

(vi) Synthesis of 4′-(nitrobiphenyl-4-yl)-2,2′:6′,2″-terpyridine-6,6″-bis(methanol) (**6**): A mixture of 40 mL of acetic anhydride and 2.67 g of compound **5** (6 mmol) was heated at 90 °C for 4 h with stirring. After acetic anhydride was evaporated, 25 mL of acetic anhydride and a mixture of 25 mL of acetic acid and 3.5 mL of fuming HNO_3 were added at 0 °C with stirring. The solution was stirred for 4 h at 0 °C and for 12 h at room temperature. To the solution was added 300 mL of water, and the mixture was stirred for 1 h at room temperature. The aqueous phase was extracted with 3 × 100 mL chloroform, and the organic phase was washed with 5% $NaHCO_3$ and dried with Na_2SO_4. After filtration and evaporation, the residue was added to a solution containing 150 mL of ethanol, 10 g of KOH, and 15 mL of water. The mixture was stirred for 20 h at room temperature, and then 300 mL of 2% NaCl was added. The precipitate was filtered and then washed with 1% HCl, 2% $NaHCO_3$, and water. After drying, compound **6** was obtained (2.73 g, 92.8% yield). ^{1}H NMR (DMSO-d_6): δ 8.73−8.71 (m, 2H), 8.53 (d, J = 7.9 Hz, 2H), 8.07−7.90 (m, 6H), 7.80−7.40 (m, 6H), 5.58 (t, J = 5.3 Hz, 2H), 4.71 (d, J = 4.6 Hz, 4H). FAB MS: 491 ($M + H^+$).

(vii) Synthesis of 6,6″-dibromomethyl 4′-(nitrobiphenyl-4-yl)-2,2′:6′,2″-terpyridine, $[(NO_2C_6H_4)C_6H_4](C_5NH_2)[(C_5NH_3)CH_2Br]_2$ (**7**): To a mixture of 300 mL of dry tetrahydrofurane (THF), 80 mL of dry *N*,*N*-dimethylformamide (DMF), and 2.70 g of compound **6** (5.5 mmol) was added 5.40 g of PBr_3 (20 mmol) with stirring. After the solution was refluxed for 10 h, THF was evaporated, and the DMF solution was added to 1 L of 5% $NaHCO_3$. The precipitate was filtered, washed with water, and dissolved in 300 mL of chloroform. The chloroform solution was washed with 2 × 100 mL of 5% NaCl and dried with Na_2SO_4. After evaporation, the residue was purified by column chromatography (silica gel, $CH_2Cl_2-CH_3OH$ (99.5:0.5, v/v)). The product was washed with hexane and dried (1.88 g, 55.5% yield). Anal. Calcd for $C_{29}H_{20}Br_2N_4O_2$: C, 56.52; H, 3.27; N, 9.09. Found: C, 56.64; H, 3.32; N, 9.10. ^{1}H NMR ($CDCl_3$): δ 8.81−8.79 (m, 2H), 8.59 (d, J, 7.9 Hz, 2H), 8.04−7.77 (m, 6H), 7.70−7.40 (m, 6H), 4.69 (s, 4H). FAB MS: 617 ($M + H^+$).

(viii) Synthesis of tetraethyl 2,2′,2″,2‴-[4′-(nitrobiphenyl-4-yl)-2,2′:6′,2″-terpyridine-6,6″diyl] bis(methylenenitrilo) tetrakis(acetate) (**8**): After a mixture of 300 mL of dry acetonitrile, 50 mL of dry THF, 1.85 g of compound **7** (3.0 mmol), 1.17 g of diethyl iminodiacetate (6.1 mmol), and 4.15 g of K_2CO_3 (30 mmol) was refluxed for 24 h with stirring, the mixture was filtered. After evaporation, the residue was dissolved in 300 mL of chloroform, and the solution was washed with 3 × 150 mL of 5% Na_2SO_4 and dried with Na_2SO_4; the solvent was then evaporated. The oily residue was washed with petroleum ether and purified by column chromatography (silica gel, ethyl acetate)(1.68 g, 67.2% yield). ^{1}H NMR ($CDCl_3$): δ 8.77−8.76 (m, 2H), 8.57 (d, J, 7.9 Hz, 2H), 8.06−7.75 (m, 6H), 7.70−7.40 (m, 6H), 4.21 (s, 4H), 4.17 (q, J = 7.3 Hz, 8H), 3.71 (s, 8H), 1.24 (t, J = 7.3 Hz, 12H).

(ix) Synthesis of tetraethyl 2,2′,2″,2‴-[4′-(aminobiphenyl-4-yl)-2,2′:6′,2″-terpyridine-6,6″-diyl] bis(methylenenitrilo) tetrakis-(acetate) (**9**): After a solution of 1.67 g of compound **8** (2.0 mmol) in 120 mL of dry ethanol was heated to 70 °C, 2.74 g of $SnCl_2 \cdot 2H_2O$ (12.5 mmol) was added. The mixture was stirred at 70−80 °C for 1 h. After cooling, 240 mL of water and 15.7 g of diethylenetriamine pentaacetic acid (40 mmol) were added. The mixture was neutralized with saturated $NaHCO_3$ and then extracted with 3 × 200 mL of chloroform. The organic phase was dried with Na_2SO_4 and filtered. After evaporation, the oily residue was purified by column chromatography (silica gel, ethyl acetate−methanol (98:2, v/v)). (1.01 g, 62.9% yield). ^{1}H NMR ($CDCl_3$): δ 8.77 (s, 2H), 8.57 (d, J = 7.9 Hz, 2H), 8.00 (d, J = 8.2 Hz, 2H), 7.90−7.84 (m, 2H), 7.77 (d, J = 7.9 Hz, 1H), 7.71−7.62 (m, 4H), 7.50 (t, J = 7.6 Hz, 2H), 6.91−6.80 (m, 1H), 4.21 (s, 4H), 4.17 (q, J = 7.3 Hz, 8H), 3.71 (s, 8H), 1.24 (t, J = 7.3 Hz, 12H).

(x) Synthesis of 2,2′,2″,2‴-[4′-(aminobiphenyl-4-yl)-2,2′:6′,2″-terpyridine-6,6″-diyl] bis(methylenenitrilo) tetrakis(acetic acid), $[(NH_2C_6H_4)C_6H_4](C_5NH_2)[(C_5NH_3)CH_2N(CH_2CO_2H)_2]_2$ (ATBTA, **10**): A mixture of 100 mL of ethanol, 4 g of KOH, 5 mL of H_2O, and 1.00 g of compound **9** (1.24 mmol) was stirred for 24 h at room temperature. After the solvent was evaporated, the residue was dissolved in 80 mL of water, and the solution was filtered. To the solution was added dropwise 4 M HCl to adjust the pH to ∼1, and the solution was stirred for 3 h at room temperature. The precipitate was collected by filtration and washed with 0.5% HCl. After drying, the product was added to 200 mL of acetonitrile, and the mixture was refluxed for 10 min with stirring. The precipitate was filtered and dried (0.73 g, 84.8% yield). Anal. Calcd for $C_{37}H_{34}N_6O_8$: C, 64.34; H, 4.96; N, 12.17. Found: C, 63.89; H, 5.11; N, 12.14. ^{1}H NMR (DMSO-d_6): δ 8.75 (s, 2H), 8.56 (d, J = 7.6 Hz, 2H), 8.06−8.00 (m, 4H), 7.92 (d, J = 7.9 Hz, 1H), 7.79 (d, J = 7.9 Hz, 1H), 7.67−7.43 (m, 5H), 6.82−6.66 (m, 1H), 4.13 (s, 4H), 3.59 (s, 8H).

(xi) Synthesis of {2,2′,2″,2‴-{[4′-(aminobiphenyl-4-yl)-2,2′:6′,2″-terpyridine-6,6″-diyl] bis(methylenenitrilo)}tetrakis(acetato)}-europium(III) Na(Eu{[$NH_2C_6H_4$]C_6H_4}(C_5NH_2)[(C_5NH_3)$CH_2N(CH_2CO_2)_2$]$_2$) (ATBTA-Eu^{3+}, **11**): After 138 mg of compound **10** (0.2 mmol) was added to 4 mL of water, the pH of the solution was adjusted to ~6.5 with $NaHCO_3$. To the solution was added 80.6 mg of $EuCl_3 \cdot 6H_2O$ (0.22 mmol) dissolved in 1.5 mL of water, and the pH was then adjusted to ~6.5 with $NaHCO_3$ again. After stirring the solution for 1.5 h, we adjusted the pH of the solution to ~8.5 with 1 M NaOH. The solution was further stirred for 10 min. After the precipitate was filtered off, 100 mL of acetone was added to the filtrate. The yellow precipitate of Eu^{3+} chelate **11** was isolated by centrifugation, washed with acetone, and dried. The product was used in the next step without further purification. ESI-MS: 839.5 $(M - Na^+)^-$.

(xii) Synthesis of {2,2′,2″,2‴-{4′-{[(4,6-dichloro-1,3,5-triazin-2-yl)amino]biphenyl-4-yl}- 2,2′:6′,2″-terpyridine-6,6″-diyl}bis-(methylenenitrilo)}tetrakis(acetato)} europium(III), Na(Eu{[($C_3N_3Cl_2$)NHC_6H_4]C_6H_4}(C_5NH_2)[(C_5NH_3)$CH_2N(CH_2CO_2)_2$]$_2$)·7($NaCH_3CO_2$)(H_2O) (DTBTA-Eu^{3+}): All of the above compound (**11**) was dissolved in 3 mL of 0.1 M sodium acetate at pH 4.9. To the solution were added 36.8 mg of 2,4,6-trichloro-1,3,5-triazine (0.2 mmol), 2 mL of acetone, and 2 mL of water. After stirring for 30 min at room temperature, 150 mL of acetone was added. The yellow precipitate of DTBTA-Eu^{3+} was filtered, washed with acetone, and dried (248 mg of yellow powder was obtained). The DTBTA-Eu^{3+} prepared by this method was a mixture of DTBTA-Eu^{3+} and sodium acetate, and has the approximate composition of Na[Eu^{3+} chelate]·7(NaOAc)(H_2O). Anal. Calcd for Na[Eu-($C_{40}H_{29}N_9O_8Cl_2$)]·7($C_2H_3O_2Na$)(H_2O): C, 40.49; H, 3.27; N, 7.87. Found: C, 40.55; H, 3.26; N, 7.88. ESI-MS: 986.3 $(M - Na^+)^-$, 1008.3 $(M - H^+)^-$.

Preparation of DTBTA-Eu^{3+}-Labeled Streptavidine (SA). To a solution of 2 mg of SA in 1.5 mL of 0.1 M carbonate buffer at pH 9.1 was added a solution of 2.8 mg DTBTA-Eu^{3+} in 0.7 mL of 0.1 M carbonate buffer at pH 9.1. After being stirred for 2.5 h at room temperature, the labeled SA was separated by gel filtration chromatography on a Sephadex G-50 column by using 0.05 M NH_4HCO_3 as eluent. The solution of DTBTA-Eu^{3+}-labeled SA was stored at −20 °C after 15 mg of NaN_3 and 30 mg of BSA were added. The labeled SA solution was diluted 400-fold before use with 0.05 M Tris-HCl buffer at pH 7.8 containing 0.2% BSA, 0.1% NaN_3, and 0.9% NaCl.

Preparation of DTBTA-Eu^{3+}-Labeled Oligonucleotide. To a solution of 2 mM DTBTA-Eu^{3+} in 10 μL of DMSO were added a solution of 100 μM oligo DNA (5′-NH_2-$(CH_2)_6$-CCACACCGTCG-GCGCCCA-3′) in 10 μL of water and 4.0 μL of 500 mM carbonate buffer (pH 9.1). After being stirred for 24 h at room temperature, the DTBTA-Eu^{3+}-labeled oligonucleotide was isolated by RP-HPLC.

Preparation of ATBTA-Labeled Oligonucleotide. (i) Synthesis of NHS-ATBTA: To a mixture of 50 mg of disuccinimidyl adipate and 0.3 mL of triethylamine in 2 mL of dry DMF was added a solution of 10 mg of ATBTA in 2 mL of dry DMF, and the mixture was stirred for 3 h with cooling in an ice bath under an Ar atmosphere. The reaction mixture was then poured into 50 mL of cold acetone containing 0.3 mL of thionyl chloride. The precipitate formed and was isolated by centrifugation, washed with acetone and diethyl ether, and dried in vacuo. The product was used for DNA labeling without further purification.

(ii) Labeling reaction of ATBTA to oligo DNA: To a solution of 2.7 mg of NHS-ATBTA (3 mmol) in 1.0 mL of carbonate buffer at pH 9.1 was added a solution of 0.01 μmol oligo DNA (5′-NH_2-$(CH_2)_6$-CAGGAAACAGCTATGAC-3′) dissolved in 100 μL of water. After stirring for 2 h at 37 °C, the mixture was incubated overnight. The product was thermally denatured at 95 °C for 15 min and was immediately cooled with ice for 15 min. The ATBTA-labeled DNA was isolated by electrophoresis and extracted with 1 × SSC buffer. The concentration of ATBTA-labeled DNA was determined by the absorption at 260 nm and used for the titration experiment.

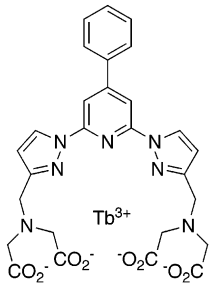


Figure 1. Luminescent terbium chelate BPTA-Tb^{3+}.

Synthesis of BPTA-Tb^{3+}. The compound (Figure 1) was prepared as reported previously.[19]

Physical Measurements. The ^{1}H NMR spectra were measured on a JEOL JNM-LA270 spectrometer. The UV−vis spectra were measured on a JASCO V-570 UV−vis spectrophotometer. The luminescence excitation and emission spectra and luminescence lifetime were measured on a Perkin−Elmer LS 50B luminescence spectrometer in the phosphorescence mode. The luminescence quantum yield (ϕ) of the DTBTA-Eu^{3+} chelate (1.5×10^{-6} M) was measured in 0.05 M borate buffer at pH 9.1 and was calculated using the equation $\phi_1 = I_1\epsilon_2C_2\phi_2/I_2\epsilon_1C_1$, with a standard quantum yield of $\phi_2 = 16.0\%$ for the Eu^{3+} chelate of *N*,*N*,*N*′,*N*′-[4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)tetrakis-(acetate) (the molar extinction coefficient at 335 nm is 14 300 cm^{-1} M^{-1}),[15] where ϵ_1 and ϵ_2 are molar extinction coefficients of the measured chelate and the standard, respectively, and C_1 and C_2 are concentrations of the chelate and the standard, respectively. The time-resolved fluoroimmunoassay (TR-FIA) was carried out with FluoroNunc 96-well microliter plates as the solid-phase carrier, and the luminescence was measured on an ARVO SX 1420 multilabel counter (Wallac) with excitation filters D340 and D320, emission filters D615 and D545, delay time 0.1 and 0.2 ms, and window time 1.5 and 0.4 ms for the Eu^{3+} and Tb^{3+} chelates, respectively.

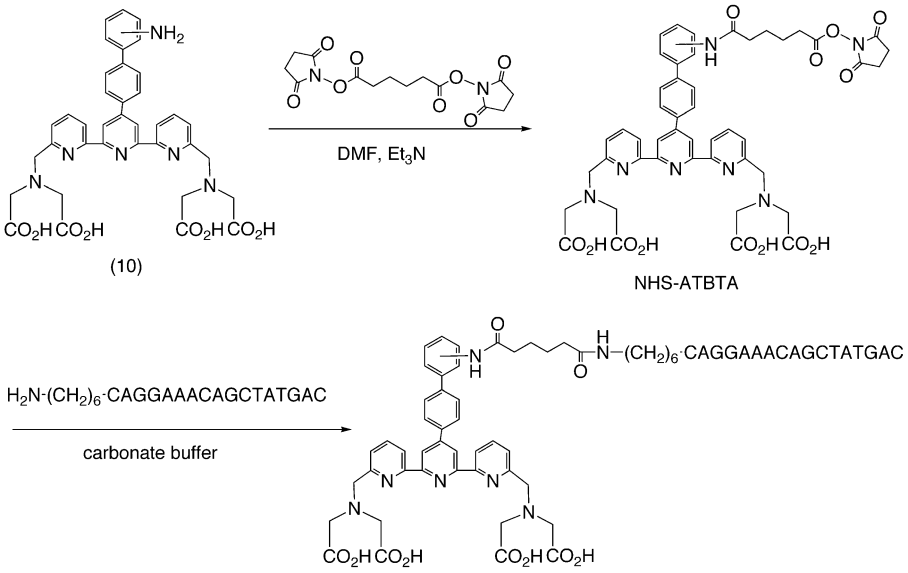
Determination of the Conditional Stability Constant of the DTBTA-Eu^{3+} Chelate. Solutions containing DTBTA-Eu^{3+} (1 nM in 15 mM Tris-HCl buffer, pH 8.0) and various concentrations of EDTA (100 nM to 100 mM) were kept at room temperature until equilibrium was reached. The conditional stability constant of DTBTA-Eu^{3+} at pH 8 was determined by using the luminescence intensity as described by Wu et al.[40]

Titration of the ATBTA-Labeled DNA with $EuCl_3$. Titration experiments were carried out in SSC buffer. Solutions containing ATBTA-labeled oligo DNA or NHS-ATBTA (600 nM) and various concentrations of $EuCl_3$ (150 nM to 420 mM) were kept at 37 °C until the equilibrium was reached, and the luminescence was measured. Similar titrations were carried out for BPTA with $TbCl_3$ for comparison.

Stability Against Ozone. Stabilities against ozone were measured in 100 mM Tris-HCl buffer (pH 7.0). Ozone was generated by an ozone generator ON-3-2 (ROKI Engineering Co., Ltd.) and was bubbled into the solutions of DTBTA-Eu^{3+}, Cy3-dUTP, and

(40) Pfefferlé, J.-M.; Bünzli, J.-C. G. *Helv. Chim. Acta* **1989**, *72*, 1487−1494.

Scheme 2



Cy5-dUTP (10×10^{-7} M, 4.0 mL). The luminescence intensities were measured as a function of the bubbling time.

3. Results and Discussion

Syntheses of the New Chelates. The new europium chelate, DTBTA-Eu^{3+}, was synthesized with a 12-step synthetic protocol, as described in Scheme 1. To introduce a binding group to the chelate, we introduced a nitro group into compound **5**. After transformation of the hydroxyl groups to the iminodiacetate groups by a two-step reaction, the nitro group was reduced to an amino group (**8** to **9**), and the amino group was then transformed to a (4,6-dichloro-1,3,5-triazin-2-yl)amino group at the final step. Because there are three nitration sites in the biphenyl group, nitration of **5** gave a mixture of *o*- and *p*-nitro-substituted compounds (compound **6**). Pure isomers of DTBTA were isolated by RP-HPLC. The para isomer was also synthesized from 4-(4′-nitrophenyl)benzaldehyde with a protocol similar to that described in Scheme 1. DTBTA-Eu^{3+} is very reactive and easily deactivated by hydrolysis at room temperature. When DTBTA-Eu^{3+} was kept in the freezer, its activity was maintained for several months. The attempt to obtain single crystals for X-ray structural analysis was carried out with various conditions but has not succeeded.

Labeling Reaction of the New Chelates. The reactions of DTBTA-Eu^{3+} and NHS-ATBTA with streptavidine (SA) and oligo DNA, respectively, were carried out. DTBTA-Eu^{3+} is highly soluble in water and aqueous buffers. In a weakly basic carbonate buffer (pH 8.0−9.5), DTBTA-Eu^{3+} was easily conjugated to SA. The DTBTA-Eu^{3+}-labeled SA can bind to biotinylated DNA through interaction between SA and biotin. The labeling reaction of DTBTA-Eu^{3+} with oligo DNA in aqueous buffers gave no labeled DNA product because of the favorable hydrolysis of DTBTA. When the reaction was carried out in a mixture of DMSO and carbonate buffer, labeled DNA was formed. The maximum yield was obtained in 6:2:5 DMSO:carbonate buffer:H_2O. Because succinimidyl ester is one of the most widely used biolabeling groups, NHS-ATBTA with a succinimidyl group was synthesized (Scheme 2). In a weakly basic carbonate buffer, NHS-ATBTA was easily conjugated to the 5′-terminal amino group of oligo DNA. The results demonstrated that the new chelate can be easily bound to DNA both directly by succinimidyl labeling and indirectly via a SA−biotin linkage.

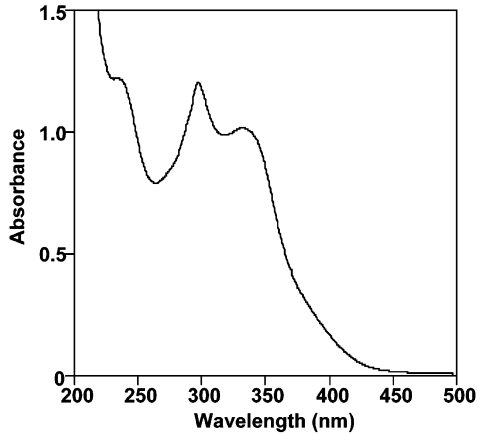


Figure 2. UV−vis spectrum of DTBTA-Eu^{3+} in 0.05 M borate buffer at pH 9.1 (3.2×10^{-5} M).

Photophysical Properties of DTBTA-Eu^{3+}. The UV−vis absorption spectrum and the excitation and the emission spectra of the DTBTA-Eu^{3+} chelate are shown in Figures 2 and 3, respectively. Its luminescence properties are shown in Table 1. The DTBTA-Eu^{3+} chelate shows very broad absorptions from 260 to 420 nm with two absorption maxima at 297 and 335 nm. The excitation and emission maxima are 335 and 616 nm, respectively. In 0.05 M borate buffer at pH 9.1, the molar extinction coefficient (335 nm), luminescence quantum yield, and luminescence lifetime of free DTBTA-Eu^{3+} chelate are 3.1×10^4 cm^{-1} M^{-1}, 9.1%, and 1.02 ms, respectively. After DTBTA-Eu^{3+} chelate is bound to SA, its luminescence intensity ($\epsilon\phi$) and lifetime decrease to ~90% cm^{-1} M^{-1} and 1.01 ms, respectively. The luminescence intensity ($\epsilon\phi$) of DTBTA-Eu^{3+} is comparable to other terpyridine chelates reported by Takalo and co-

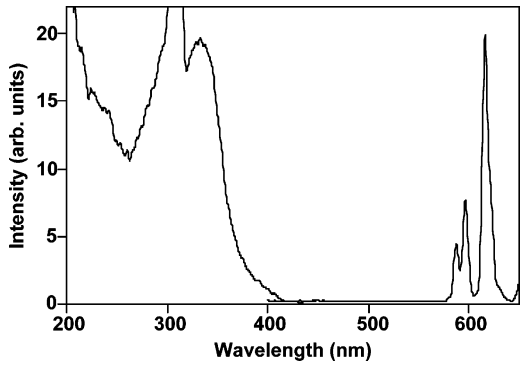


Figure 3. Excitation and emission spectra of DTBTA-Eu^{3+} in 0.05 M borate buffer at pH 9.1 (1.5×10^{-6} M). The excitation spectrum was recorded with $\lambda_{em} = 616$ nm and the emission spectrum with $\lambda_{ex} = 335$ nm. An excitation slit of 10 nm and an emission slit of 5 nm were used for the measurements.

Table 1. Luminescence Properties of DTBTA-Eu^{3+} in 0.05 M Borate Buffer at pH 9.1

chelate	$\lambda_{ex,max}$ (nm)	ϵ ($cm^{-1}M^{-1}$)	$\lambda_{em,max}$ (nm)	ϕ	$\epsilon\phi$ ($cm^{-1}M^{-1}$)	τ (ms)
DTBTA-Eu^{3+}	335	3.1×10^4	616	9.1%	2830	1.02
DTBTA-Eu^{3+} (bound to SA)	335		616	[a]	2550[a]	1.01

[a] Assuming that ϵ of DTBTA-Eu^{3+} does not change on the labeling to SA, ϕ of DTBTA-Eu^{3+} (bound to SA) is 8.2%.

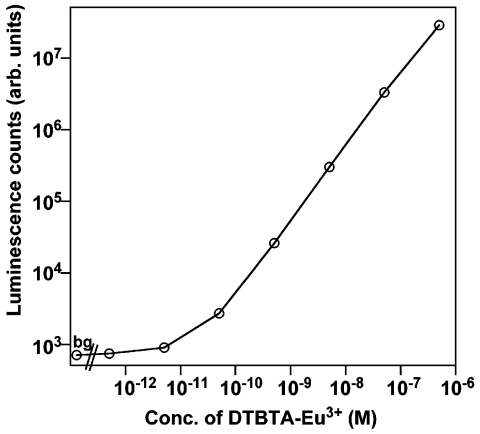


Figure 4. Diluting curve of DTBTA-Eu^{3+} in 0.05 M Tris-HCl buffer at pH 7.8. bg = background.

workers.[15,31] The luminescence intensity of DTBTA-Eu^{3+} bound to SA is almost identical to that of free DTBTA-Eu^{3+}. On the other hand, the luminescence intensity of the terbium(III) complex with terpyridine derivative, which had a binding group on the polyacetate moiety and is conjugated to an antibody, decreases to about $^1/_3$ that of the free terbium(III) complex.[15] These results suggest that the location of a binding group is important for avoiding undesired complex instabilization in the presence of biological molecules. The calibration curve of the time-resolved luminescence measurement (616 nm) of DTBTA-Eu^{3+} in 0.05 M Tris-HCl buffer at pH 8.0 is shown in Figure 4. The detection limit, calculated as the concentration corresponding to 3 × SD (standard deviation) of the background intensity, is 1.3×10^{-12} M, which is comparable to the value previously reported for BPTA-Tb^{3+}.[19]

Table 2. Relative Luminescence Intensities of DTBTA-Eu^{3+} and BPTA-Tb^{3+} in Various Buffers[a]

buffer[b]	relative luminescence intensity[c] (%) DTBTA-Eu^{3+}	BPTA-Tb^{3+}
15 mM Tris-HCl (pH 7.4)	100	100
15 mM Tris-HCl (pH 7.4), 150 mM NaCl	90	90
1 × TE (pH 8.0)	102	12
1 × SSC (pH 7.4)	88	31
1 × PBS (pH 7.4)	77	3.8

[a] [DTBTA-Eu^{3+}] = [BPTA-Tb^{3+}] = 1.0×10^{-6} M. [b] 1 × TE; 10 mM Tris-HCl containing 1.0 mM EDTA, 1 × SSC; 15 mM sodium citrate containing 150 mM NaCl, 1 × PBS; 8.0 mM Na_2HPO_4 and 2.0 mM NaH_2PO_4 containing 137 mM NaCl and 2.7 mM KCl. [c] relative to luminescence intensity compared to that in 15 mM Tris-HCl.

Thermodynamic Stability of DTBTA-Eu^{3+}. The stability of DTBTA-Eu^{3+} under various conditions was examined to demonstrate the feasibility of DTBTA-Eu^{3+} in real DNA analysis. Several common buffers used in bioassays, such as citrate, phosphate, and EDTA, have substantially high affinities for lanthanide ions. EDTA especially has a large formation constant with the europium(III) ion and yet must be added in small amounts to DNA assays to inhibit nuclease activity because of the impurity in the environment. The luminescence intensities of DTBTA-Eu^{3+} (1.0×10^{-6} M) were measured in 15 mM Tris-HCl (pH 7.4), 15 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 × TE (pH 8.0), 1 × SSC (pH 7.4), and 1 × PBS (pH 7.4). Those buffer solutions that contain tris, EDTA, citrate, and phosphorate can form a complex with the lanthanide ion. The same measurement was carried out for BPTA-Tb^{3+},[19] which has a much higher stability than β-diketonate−Eu^{3+} chelates and has already been proven to give high sensitivity in protein analysis. The tris buffer can interact with lanthanide ions, and the luminescence intensity of the lanthanide chelates in 15 mM Tris-HCl (pH 7.4) could be affected.[40] The interaction with tris buffer is much weaker than with the other buffers, so the luminescence intensities were compared with that in 15 mM Tris-HCl (pH 7.4). The results in Table 2 clearly show that DTBTA-Eu^{3+} has improved stability without serious luminescence decreases in TE, SSC, and PBS, as observed for BPTA-Tb^{3+}. The luminescence intensities of DTBTA-Eu^{3+} (1.0×10^{-9} M) in 10 mM Tris-HCl (pH 8.0) with various concentrations of EDTA were also measured (Figure 5). The intensity did not change until the EDTA concentration reached 1×10^{-3} M, which is the same concentration as in 1 × TE.

The conditional stability constants K of DTBTA-Eu^{3+} and BPTA-Tb^{3+} at pH 8.0 were determined by the competition reaction between EDTA and DTBTA for Eu^{3+} and EDTA and BPTA for Tb^{3+}. The EDTA complexes have formation stability constants of log $K_{EDTA} = 17.3$ and 17.9 for Eu^{3+} and Tb^{3+}, respectively.[41,42] On the basis of these values, we determined the log K for DTBTA-Eu^{3+} to be 25.0, whereas the log K for BPTA-Tb^{3+} was 21.5. The large conditional stability constant of DTBTA-Eu^{3+} suggests that the complex

(41) Wu, S. L.; Horrocks, W. D., Jr. *Anal. Chem.* **1996**, *68*, 394−401.
(42) Smith, R. M.; Martell, A. E. *Critical Stability Constants*; Prenum Press: New York, 1974; Vol. 1, p 204.

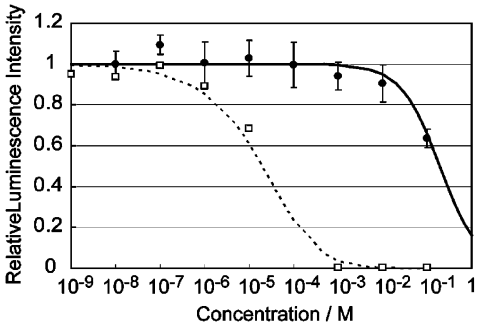


Figure 5. Luminescence intensities of DTBTA-Eu^{3+} and BPTA-Tb^{3+} in the presence of various concentrations of EDTA. The solutions are 1.0×10^{-9} M DTBTA-Eu^{3+}(●) and 1.0×10^{-8} M BPTA-Tb^{3+} (□). The solid and dashed lines represent the calculation curves at log $K_{DTBTA-Eu} = 25.0$ and log $K_{BPTA-Tb} = 21.5$, respectively.

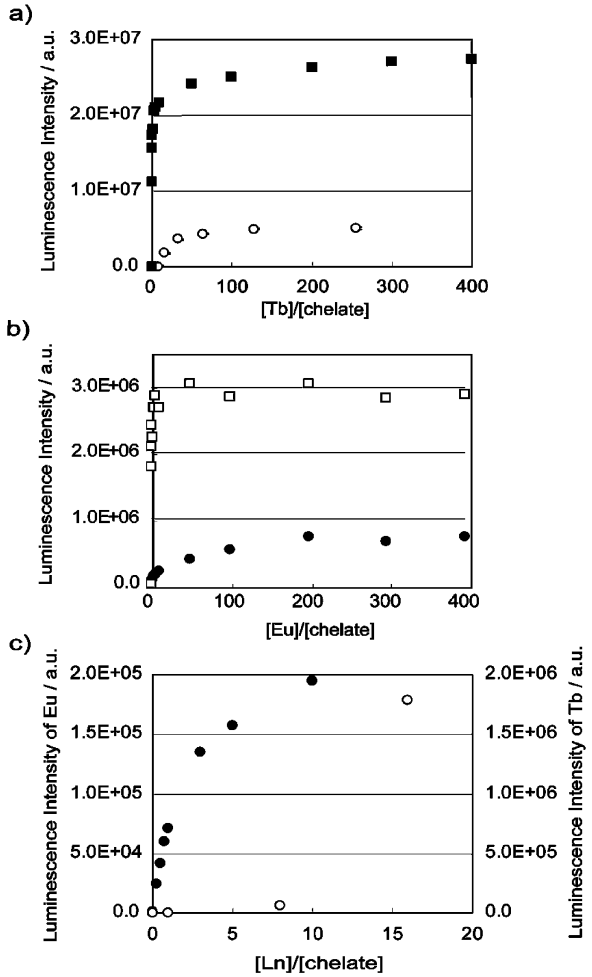


Figure 6. Time-resolved fluorometric titration of the free chelates and the oligo DNA (17 bases)-bound chelates with lanthanide ions. Solvent: 1 × SSC, [chelate] = 600 nM. (a) ATBTA-labeled DNA (●) and ATBTA (□) with $EuCl_3$; (b) BPTA-labeled DNA (○) and BPTA (■) with $TbCl_3$; (c) low-concentration regions of the titration curves for ATBTA-labeled DNA with Eu^{3+} (●) and BPTA-labeled DNA with Tb^{3+}(○).

would be a suitable label for DNA as well as for protein. To see the effect of the bound DNA on the chelate formation, we carried out titration experiments for ATBTA- and BPTA-labeled oligo DNAs. Luminescence intensity increased with increasing lanthanide ion concentration up to [Ln]/[chelate] = 200 (Figure 6). In both cases, much larger amounts of

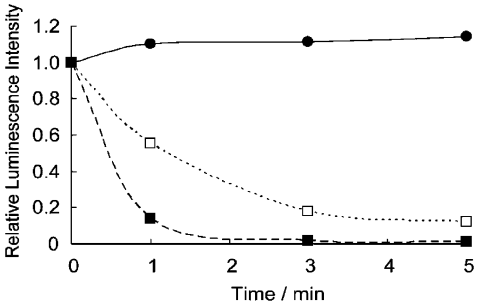


Figure 7. Degradation by ozone oxidation of DTBTA-Eu^{3+} and Cy dyes in 100 mM Tris-HCl buffer (pH 7.0). [Fluorophores] = 1.0×10^{-7} M. DTBTA-Eu^{3+} (●), Cy3-dUTP (□), Cy5-dUTP (■).

lanthanide ions were needed for DNA-bound lanthanide chelates than for the free chelate in order for saturation, and the saturated luminescence intensities of both DNA-bound lanthanide chelates were smaller than those of the free chelates. It is probably due to the smaller luminescence quantum yield of the DNA-bound lanthanide chelate. The significant difference between ATBTA-DNA and BPTA-DNA is that the luminescence is increased even in the low-europium-concentration region for ATBTA-DNA, whereas the intensity did not increase until [$TbCl_3$]/[BPTA-DNA] = 10. This might be due to the Tb^{3+} specific property of its coordination to DNA guanine.[43,44]

Thermal Stability of DTBTA-Eu^{3+}. In DNA assays, labeled samples are heated, for instance in PCR and thermal denaturation, and therefore high thermal stability is an important requirement for label reagents. The thermal stability of DTBTA-Eu^{3+} was examined by monitoring the UV−vis spectrum. The 10 mM Tris-HCl (pH 8.0) solution of DTBTA-Eu^{3+} was heated to 95 °C for 30 min, and after the solution was cooled to 4 °C, the UV−vis spectrum was measured, which was almost identical with that before heating. The photodegradation of labels is another issue to evaluate. The DTBTA-Eu^{3+} in 15 mM tris-HCl (pH 7.4) containing 150 mM NaCl was irradiated with UV light at 327 nm, and the intensity of the emission at 615 nm was monitored. The intensity did not decrease for at least 1 h, whereas a 20% decrease was observed for the common organic label fluorescein isothiocyanate (FITC) under the same conditions except that visible light (488 nm) was irradiated. The result shows that DTBTA-Eu^{3+} is suitable for long-term measurement. In the current DNA microarrays, the organic labels Cy3 and Cy5 are most widely used. For these organic labels, degradation by ozone is a problem. A low level of ozone in the air released from Xe lamps and other instruments degrade the dyes of conjugated olefins. The degradation lowers the reproducibility of the analysis, and the results are affected by the cleanness of the environment. To examine the stability against ozone, we exposed the solutions of DTBTA-Eu^{3+}, Cy3-dUTP, and Cy5-dUTP to ozone and monitored their luminescence intensities. During ozone exposure, the fluorescence of Cy3 and Cy5 decreased, whereas the luminescence of DTBTA-Eu^{3+} increased slightly

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(Figure 7). These results clearly show the superior stability of DTBTA-Eu^{3+} over organic dyes.

4. Conclusion

New amino-reactive and luminescent Eu^{3+} chelates DTBTA-Eu^{3+} and NHS-ATBTA-Eu^{3+} were synthesized, and it was demonstrated that they are easily attached to streptavidine and oligo DNA in weakly basic carbonate buffer. The previous problem of BPTA-Tb^{3+}, that the luminescence intensity was very much dependent on the buffer, was obviated with DTBTA-Eu^{3+}. This is probably due to the change in the position of the binding group from one of the carboxylates in the coordination site (BPTA) to the noncoordination site (DTBTA). Compared to the reported β-diketone type Eu^{3+} luminescence labels,[34] the new Eu^{3+} chelate labels have the advantages of higher solubility in water-based buffers, easier attachment to biomolecules, longer luminescence lifetimes, and longer excitation wavelength range (longer excitation wavelength is a considerably important factor for laser-induced luminescence measurements). Especially because of the very large conditional stability constant of the new Eu^{3+} chelates, the label can be used as a stable and sensitive label for DNA. The time-resolved fluorometric detection on microarrays using DTBTA-Eu^{3+} is now underway and will be reported separately.

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