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Synthesis of a Highly Fluorescent β-Diketone−Europium Chelate and Its Utility in Time-Resolved Fluoroimmunoassay of Serum Total Thyroxine

Feng-Bo Wu,*,† Shi-Quan Han,† Chao Zhang,‡ and You-Feng He†

Department of Isotope, China Institute of Atomic Energy, 102413, P.O. Box 275-39, Beijing, People's Republic of China, and Department of Chemistry, Tsinghua University, 100084, Beijing, People's Republic of China

A new highly fluorescent β-diketone−europium chelate was synthesized and employed as a tracer to develop a time-resolved fluoroimmunoassay (TRFIA) for detection of serum total thyroxine (T4). The tetradentate β-diketone chelator, 1,10-bis(thiophene-2′-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone (BTOT), was structurally composed of two units of thenoyltrifluoroacetone (TTA) derivatives but expressed fluorescence that was greatly enhanced, as compared to the original TTA molecules, in the presence of excess amount of Eu^{3+}. The luminescence properties of the europium chelate of BTOT were studied in aqueous solution. Chlorosulfonylation of BTOT afforded 1,10-bis(5′-chlorosulfo-thiophene-2′-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone (BCTOT), which could be coupled to proteins (i.e., streptavidin or the BSA−T4 conjugate) and used as a tracer for TRFIA. Although the BCTOT−Eu complex could be detected at a very low level ($\sim 1.07 \times 10^{-12}$ mol/L) in buffered aqueous solution (50 mmol/L Tris−HCl; pH, 8.0), the application of the chelate label in direct serum T4 TRFIA experienced a problem of matrix interference, which was probably caused by some unknown chelating components in the samples as a result of the fact that the fluorescence of the BCTOT−Eu chelate was prone to quenching or enhancement by some chelating reagents. To remove this problem, an indirect serum T4 TRFIA was proposed with the use of BCTOT−Eu-labeled streptavidin (SA) as signal generation reagent. The concentrations of T4 in 27 human serums were determined by indirect T4 TRFIA, and the assay results correlated well with those obtained by commercial Corning-CLIA ($r = 0.955$) and Wallac-DELFIA ($r = 0.965$).

Nowadays, bioanalysis based on time-resolved fluorometry (TRFIA) has become a valuable tool in clinical practices and biomedical researches. Owing to the unique luminescence properties of some lanthanide chelates (i.e., high quantum yield, long decay time, exceptionally large Stoke's shift, and narrow emission peaks), lanthanide or its chelate has been used as a label for developing a highly sensitive immunoassay,[1] DNA hybridization assay,[2] enzyme assay,[3] cellular assay, and image analysis.[4,5] In the well-established Wallac DELFIA system, a nonluminescent lanthanide chelate is used as the tracer, of which the chelated lanthanide ions (generally, Eu^{3+}) are determined by a dissociation-enhancement protocol. The effective fluorescence enhancement and the well-optimized labeling technique make the DELFIA system very successful in a variety of applications; however, the signal generation mode in DELFIA makes it vulnerable to contamination and prohibits its use in fields that require signal localization, for example, DNA sequencing, in situ immunostaining, cytofluorometry, etc. In recent years, much effort has been directed to the research of the directly luminescent lanthanide chelate for time-resolved fluorometric assays,[6] and some of these lanthanide chelates have found practical uses (e.g., BCPDA and some cage-type or polyaminopolycarboxylate chelates).[7−10] These vanguard investigations provide a facility to open up new applications for time-resolved fluorometric measurement and lay a basis for searching for other promising lanthanide chelates. However, designing a lanthanide chelate as luminescent tracer that can fulfill the requirements of bioanalysis in terms of luminescence intensity, solubility, stability, biocompatibility, and mild coupling reactivity is still a challenge.

TTA is an organic ligand commonly used for fluorometric determination of Eu^{3+}. When combined with a suitable synergic

* To whom correspondence should be addressed. E-mail: wufb@etang.com. or wufb22@21cn.com.

† China Institute of Atomic Energy.

‡ Tsinghua University.

(1) Hemmilä, I.; Dakubu, S.; Mukkala, V.; Siitari, H.; Lövgren, T. *Anal. Biochem.* **1984**, *137*, 335−343.
(2) Heinonen, P.; Iitiä, A.; Torresani, T.; Lövgren, T. *Clin. Chem.* **1997**, *43*, 1142−1150.
(3) Karp, M. T.; Suominen, A. I.; Hemmilä, I.; Mäntsälä, P. *J. Appl. Biochem.* **1983**, *5*, 399−403.
(4) Lövgren, J.; Blomberg, K. *J. Immunol. Methods* **1994**, *173*, 119−125.
(5) Seveus, L.; Väisälä, M.; Syrjänen, S.; Sandberg, M.; Kuusisto, A.; Harju, R.; Salo, J.; Hemmilä, I.; Kojola, H.; Soini, E. *Cytometry* **1992**, *13*, 329−338.
(6) Dickson, E. F. G.; Pollak A.; Diamandis, E. P. *J. Photochem. Photobiol. B: Biol.* **1995**, *27*, 3−19.
(7) Diamandis, E. P.; Morton, R. C.; Reichstein, E.; Khosravi, M. J. *Anal. Chem.* **1989**, *61*, 48−53.
(8) Mathis, G. *Clin. Chem.* 1**993**, *39*, 1953−1959.
(9) Saha, A. K.; Kross, K.; Kloszewski, E. D.; Upson, D. A.; Toner, J. L.; Snow, R. A.; Black, C. D. V.; Desai, V. C. *J. Am. Chem. Soc.* **1993**, *115*, 11032−11033.
(10) Takalo, H.; Hänninen, E.; Kankare, J. *Helv. Chim. Acta* **1993**, *76*, 877−883.

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agent, TTA has been applied to detect very low trace amounts of Eu^{3+} (e.g., $\sim 10^{-13}$ mol/L or even less).[11−13] Chlorosulfonyl-TTA−Eu^{3+} (CTTA−Eu) has also been tried as a direct chelate label in TRFIA for analyzing BSA and cortisol.[14,15] However, the fluorescence of CTTA−Eu chelate is too weak to ensure high detection sensitivity, despite the fact that there is 1000−10000-times fluorescence enhancement after its conjugation with proteins (i.e., BSA). A more fluorescent β-diketone ligand, 1,10-bis(8′-chlorosulfodibenzothiophene-2′-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone (BCOT), has been reported by Yuan et al. The BCOT−Eu complex emits strong fluorescence in aqueous solution as a result of its tetradentate chelating ability and, therefore, is highly detectable.[16] On the basis of these works, we have synthesized a new β-diketone ligand, BTOT, which can be regarded as a combination of two units of TTA derivatives. The fluorescence features of the BTOT−Eu complex were studied with regard to its fluorescence spectra, decay time, and the chelate composition. For labeling, BCTOT was obtained by a simple chlorosulfonylation reaction of BTOT. Streptavidin (SA), BSA, and the thyroxine (T4)−BSA conjugate were labeled with BCTOT by the reaction of the sulfonyl chloride group, primarily with the amino groups of the proteins. Results show that BCTOT can form a highly luminescent complex with Eu^{3+} in buffered aqueous solution. In the presence of an excess amount of Eu^{3+}, the detection sensitivity of the conjugated BCTOT in BSA-$(BCTOT)_{12}$ is remarkably improved, as compared to the reported BCOT.

To study the utility of BCTOT−Eu chelate as a label in TRFIA, T4−BSA conjugate and streptavidin were labeled with BCTOT. On the basis of the two labeled reagents, two different formats of competitive TRFIA were constructed for detection of human serum T4. In the first T4 TRFIA (direct format), serum T4 competed with the T4−BSA-BCTOT−Eu for binding the limited amount of biotinylated anti-T4 monoclonal antibody (McAb), and the formed immunocomplex was captured by the surface streptavidin. The fluorescence of the surface immunocomplex (SA−biotin-McAb−T4−BSA-BCTOT−Eu) was determined by solid-phase fluorescence measurement. In the second T4 TRFIA (indirect format), serum T4 competed with the biotinylated T4−BSA conjugate for binding the limited amount of surface anti-T4 McAb. After the immunoreactions, the well contents were washed away, and the BCTOT−Eu-labeled SA was added to bind the surface T4−BSA-biotin. The complex formed on the well surface (McAb−T4−BSA-biotin−SA-BCTOT−Eu) was measured in the same way as that in the direct T4 TRFIA. Both the direct and indirect assays gave nice calibration curves, but severe matrix interference was observed for the direct assay format. In the meantime, the indirect T4 TRFIA was sensitive and accurate; the T4 values measured by the indirect assay showed good correlation with that obtained by T4 CLIA (Corning Co.) and T4 DELFIA (Wallac, Co.).

(11) Keelan, J. A.; France, J. T.; Barling, P. M. *Clin. Chem.* **1987**, *33*, 2292−2295.
(12) Degan, P.; Abbondandolo, A.; Montagnoli, G. *J. Biolumin. Chemilumin.* **1990**, *5*, 207−212.
(13) Xu, Y. Y.; Hemmilä, I. *Anal. Chim. Acta* **1992**, *256*, 9−16.
(14) Ci, Y. Y.; Yang, X. A.; Chang, W. B. *J. Immunol. Methods* **1995**, *179*, 233−241.
(15) Yang, X. D.; Ci, Y. Y.; Chang, W. B. *Anal. Chem.* **1994**, *66*, 2590−2594.
(16) Yuan, J.; Matsumoto, K. *J. Pharm. Biomed. Anal.* **1997**, *15*, 1397−1403.

EXPERIMENTAL SECTION

Chemicals and Buffers. Anti-T_4 McAb was obtained from Medix Biochemica Co. (Finland). Microtitration strips were products of NUNC Co. (Denmark). BSA was from Shenzhen JingMei Bioengineer Co. (Shenzhen, China). Streptavidin, biotinamidocaproate *N*-hydroxysuccinimide ester (BAC-NHS), sodium trichloroacetate (TCA-Na), 8-anilino-1-naphthalenesulfonic acid (ANS), and T4 were products of Sigma Chemical Co. (U.S.). The T4 DELFIA kit was from Perkin-Elmer-Wallac. TTA, dimethyl-octafluoroadipate, $NaOCH_3$, and 2-acetyl-thiophene were from Lancaster Synthesis Co. (U.S.). The T4−BSA conjugate was prepared by the EDC method as described in a previous work[17]

The assay buffer was 100 mmol/L Tris−HCl, pH 8.0, containing 0.5 mol/L TCA-Na, 0.1% BSA, 0.04% NaN_3, 0.9% NaCl, 0.05% Tween 20, and 1 μmol/L tri-*n*-octylphosphine oxide (TOPO). TCA-Na in the assay buffer was used to dissociate the serum T4 from its binding proteins, because the commonly used ANS and sodium salicylate exerted obvious quenching on the chelate fluorescence (see the Results and Discussion Section). The coating buffer was 100 mmol/L sodium carbonate buffer (pH 9.7) containing 0.9% NaCl and 0.04% NaN_3. The washing buffer was 10 mmol/L Tris−HCl (pH 9.0) containing 0.04% Tween-20 and 0.9% NaCl. TSA buffer for elution of the labeled reagents was 50 mmol/L Tris−HCl, pH 7.8, containing 0.9% NaCl and 0.05% NaN_3. The blocking buffer was 50 mmol/L Tris−HCl, pH 7.8, containing 1% BSA, 2.5% sucrose, 0.9% NaCl, and 0.05% NaN_3. The T4-stripped human serum was prepared by a treatment of the pooled normal serum with an anion exchange procedure through a 717 resin column (201 × 7 cm, Resin Factory of Shanghai, China). The T4 standard solutions were prepared by adding different amounts of L-T4 stock solution to the T4-free serum and calibrating with the Wallac T4 DELFIA kit. The T4 values in the seven standard solutions were 0, 20, 50, 100, 150, 300, and 600 nmol/L, respectively.

Specimens. Serum samples were kindly provided by 301 Hospital (Beijing, China) with the T4 values measured by ACS-180 CLIA (Corning Co.). Twenty-seven clinic samples were analyzed by the Wallac T4 DELFIA and the present direct or indirect T4 TRFIA. Allof the patients have been diagnosed on the basis of characteristic clinical symptoms and confirmed by laboratory tests. The detailed pathologic situations of these patients were not given.

Instrumentation. The chromatographic separation system was a product of Bio-Rad Co., mainly including a model EP-1 Econo Pump and a model EM-1 Econo UV monitor. The LS-50B luminescence spectrometer, VICTOR^2 multilabel counter, 1296-003 DELFIA plateshake, and 1296-026 DELFIA platewash were products of Perkin-Elmer (U.S.). The Cary 50 mode UV−vis spectrophotometer was from Varian Co. (U,S,). The melting-point meter was from the factory of Beijing Jingmi Instrument (Beijing, China). The ^{1}H NMR spectrum was measured on a DMX-300. The elementary analysis was performed with Carlo Erba-1106 (Italy). Manual pipetting was performed with disposable plastic tips and Finnpipet (Labsystem Oy, Helsinki, Finland).

Synthesis of the β-Diketone Ligands. The synthesis route of CTTA, BTOT, and BCTOT is depicted in Figure 1. The details of the procedure are described as follows:

(17) Wu, F. B.; Xu, Y. Y.; Wang, Y. Z.; Han, S. Q. *Anal. Biochem.* **1999**, *276*, 171−176.

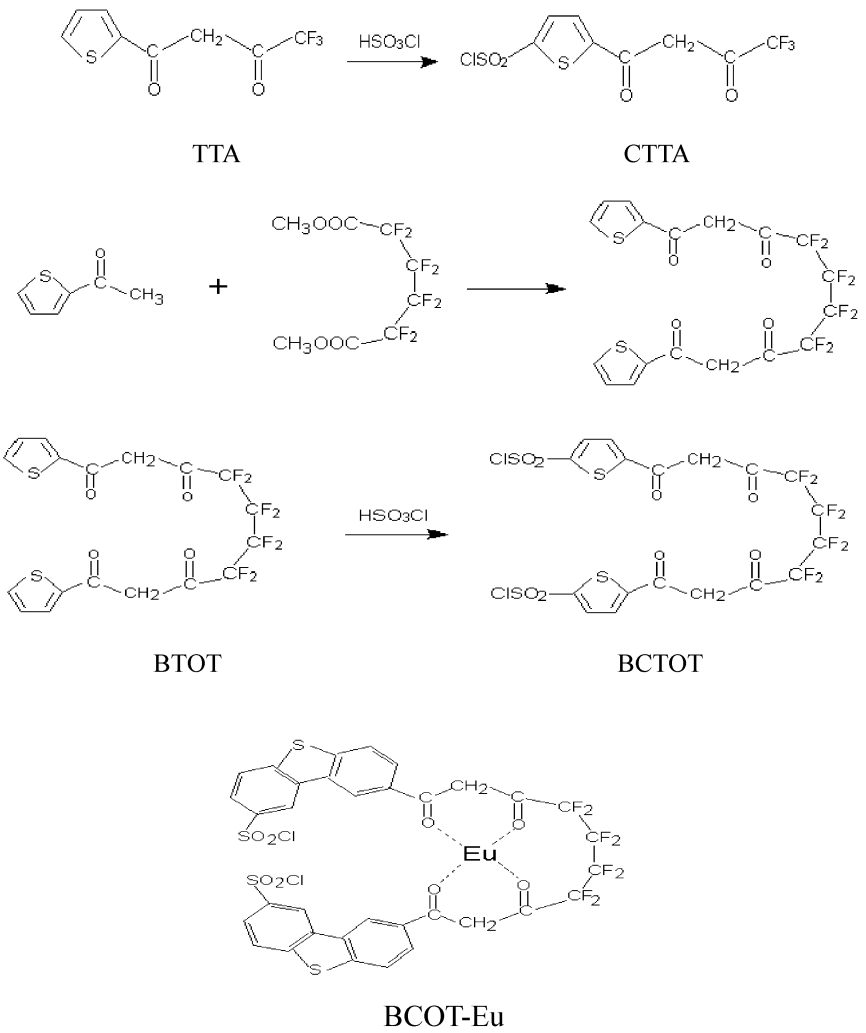

Figure 1. Synthesis routes for the three β-diketone ligands and the structure of the reported BCOT−Eu chelate.[16]

(1) Preparation of CTTA. CTTA was synthesized according to the method of Ci et al.[14] Briefly, 10 g of TTA was added in five batches in a flask containing 50 mL of $ClSO_3H$. The mixture was stirred at 55 °C for 5 h. Under continuous stirring, the solution was poured into ice water to degrade excess $ClSO_3H$. This reaction was carried out in a well-ventilated fume hood with a suitable safety shield between the reaction vessel and the operator, since the reaction of $ClSO_3H$ with water was very violent and produced toxic and corrosive fumes. After this, the precipitate was filtered and washed with a small amount of cold water. The powder obtained was recrystallized in anhydrous chloroform. A 5.1-g portion of CTTA was obtained. Anal. calc. for CTTA: C, 30.00; H, 1.25; S, 20.00. Found: C, 28.60; H, 1.30; S, 19.10.

(2) Preparation of BTOT and BCTOT. A 4-g portion of $NaOCH_3$ was added over 10 min in several batches to a flask containing 5 g of dimethyl octafluoroadipate and 4 g of 2-acetylthiophene in 50 mL of dry ether. After a 48-h stirring at RT, the mixture was poured into 200 mL of 15% H_2SO_4 solution. The ether phase was separated and distilled at reduced pressure. The solid was recrystallized in anhydrous ethanol, filtered off, and vacuum-dried in the presence of P_2O_5. BTOT was obtained with a yield of 65%. Anal. calc. for BTOT: C, 42.69; H, 1.98; S 12.65. Found: C, 42.5; H, 1.56; S, 12.00. The product was confirmed by ^1H NMR in $CDCl_3$ with TMS as reference compound (Figure 2). The ^1H NMR spectrum shows that BTOT in $CDCl_3$ solution exists predominantly in enolic form.

Under stirring, 1.6 g of BTOT was added to a flask containing 10 mL of $ClSO_3H$. After stirring the mixture for 3 h at 40 °C, the solution was added dropwise to ice-cold water. The product BCTOT was extracted with ether. The ether was distilled to ~0.5 mL; the precipitate was filtered off and recrystallized in anhydrous ether. A grey powder of BCTOT was obtained with a yield of ~41%. The product was stored under argon in small glass bottles in a desiccator at −20 °C. No loss of coupling reactivity was detectable after one year's storage. Anal. calc. for BCTOT: C, 30.73; H, 1.14; S, 18.21. Found: C, 34.6; H, 1.08; S, 15.9.

Luminescence Properties of the β-Diketone−Eu Complex. The fluorescence intensities of the β-diketone−Eu complexes were measured using a Wallac Victor^2 multilabel counter. The measurement conditions were as follows: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 0.40 ms; window time, 0.40 ms; and cycling time, 1.0 ms. The fluorescence spectra and decay time (τ) of the BTOT−Eu complex were measured with an LS-50B luminescence spectrometer. The decay time was measured according to the equation, $\ln I(t) = \ln I(0) - t/\tau$. The buffer used for luminescence study, except as indicated, was 50 mmol/L Tris−HCl, pH 8.0, containing 0.9% NaCl. The UV absorption was determined with the

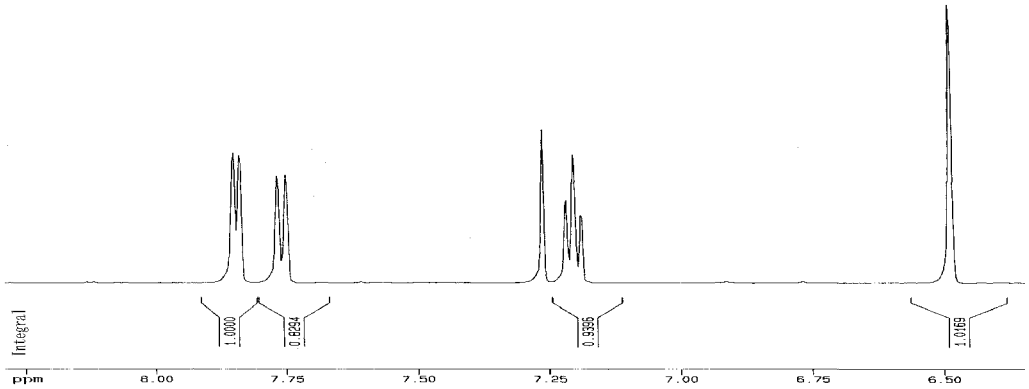

Figure 2. ^{1}H NMR spectra of BTOT.

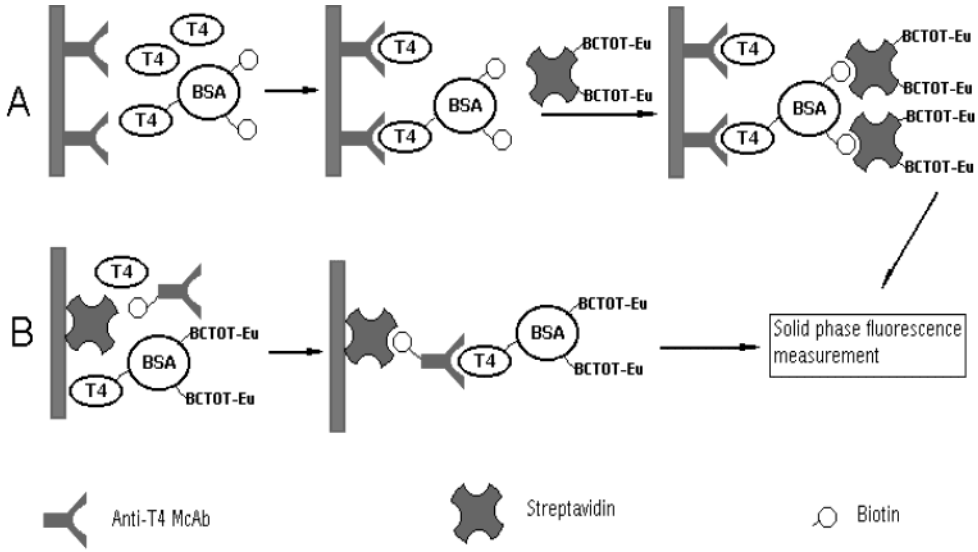

Figure 3. Principles of the indirect (A) and direct (B) competitive TRFIA of serum T4. The details of the assay procedure are given in the Experimental Section. The figure has no quantitative meaning.

UV−vis spectrophotometer (Cary 50 mode, Varian Co., U.S.).

Titration of BTOT with Eu^{3+}. A portion of 2 μmol/L of BTOT was titrated with standard Eu^{3+} solution in Tris−HCl buffer. The fluorescence intensity was measured as a function of the molar ratio of Eu^{3+}/BTOT.

Protein Labeling with BCTOT or CTTA. BCTOT was coupled to T4−BSA conjugate by incubating the protein with BCTOT at a molar ratio of ~200:1 (BCTOT/protein) in carbonate buffer (0.1 mol/L, pH 9.0). The reaction was allowed to proceed for 30 min at RT. The labeling of streptavidin with BCTOT was performed similarly, except the molar ratio of ligand/protein was 100:1. The labeled protein was isolated from the excess BCTOT by applying the mixture to a TSA equilibrated column of Sepharose 6B (1.0 × 40 cm) and eluting the column with the same TSA buffer at 1 mL/min. Glycine at 1 g/L was added to the purified protein-BCTOT.

For fluorescence study, BSA was labeled with BCTOT and CTTA by the same procedure as described above. The coupling ratio of the labeled BSA was determined by measuring the concentration of both the ligand and the BSA in the purified conjugation solution. The molar extinction coefficient (ξ) of the free β-diketone at 348 nm was taken as that present in the conjugation (ξ_{CTTA}: ~ 1.343×10^4 L mol^{-1} cm^{-1}; ξ_{BCTOT}: ~ 2.8×10^4 L mol^{-1} cm^{-1}). The BSA concentration was determined by measuring the absorbance at 280 nm and subtracting the absorbance arising from the coupled β-diketone via following experimental equation: C_{BSA} (g/L) = ($A_{280nm} - 0.482 \times A_{348nm}$)/0.71 for BSA-BCTOT and C_{BSA}(g/L) = ($A_{280nm} - 0.661 \times A_{348nm}$)/0.71 for BSA-CTTA.

Biotinylation of Anti-T4 McAb or T4−BSA Conjugate. Biotinylated T4−BSA conjugate or anti-T4 McAb was prepared by reaction of the proteins with BAC-NHS, followed by dialysis. The procedure detail was described previously.[18]

Serum T4 TRFIA in Two Different Formats. The principles of the two formats of T4 TRFIAs are depicted in Figure 3.

(1) Direct T4 TRFIA. Portions (25-μL) of T4 standards or serum samples were added to wells that were coated with SA.[18] A 50-μL portion of assay buffer containing 5.5 mg/L of T4−BSA-BCTOT and 10^{-6} mol/L of Eu^{3+} was added (prior to this, the T4−BSA-BCTOT and Eu^{3+} was incubated for 1 h at 50 °C), followed by the addition of 100 μL of assay buffer containing 2.5 mg/L of biotinylated anti-T4 McAb. The mixture was incubated for 4 h at RT with slow shaking. After the wells were washed four times by washing buffer, surface fluorescence of the complex (SA−biotin-McAb−T4−BSA-BCTOT−Eu) was measured on a Victor^2 multilabel counter.

(18) Wu, F. B.; He, Y. F.; Han, S. Q. *Clin. Chim. Acta* **2001**, *308*, 117−126.

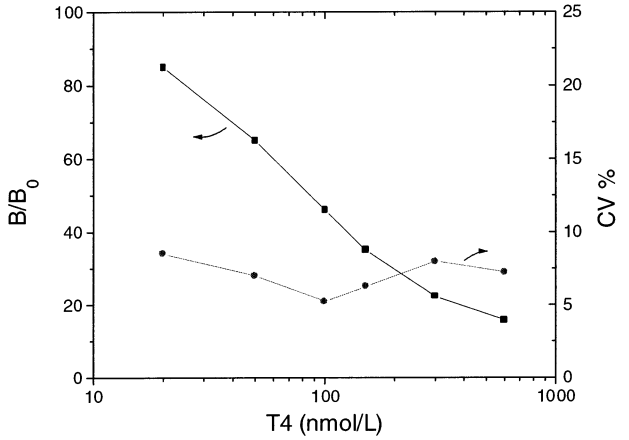

Figure 4. Typical calibration curve (solid square) and imprecision profile (solid circle, $n = 10$) of the indirect T4 TRFIA with the streptavidin-BCTOT−Eu complex as the signal generation reagent.

(2) Indirect T4 TRFIA. Portions (25-μL) of T4 standards or serum samples were added to the wells that were coated with anti-T4 McAb by passive adsorption (5 mg/L of anti-T4 McAb in coating buffer, 200 μL/well, 24 h incubation at RT. After twice washing, the microwells were blocked by incubating the well with 250 μL of the blocking buffer at RT for 6 h), then 150 μL of assay buffer containing 7.5 mg/L of T4−BSA-biotin was added, and the mixture was incubated for 4 h at RT with slow shaking. The wells were washed four times, 100 μL of assay buffer containing ~1 g/L of streptavidin-BCTOT and 10^{-6} mol/L of Eu^{3+} was added (prior to this, SA-BCTOT and Eu^{3+} was incubated for 1 h at 45 °C). After 30 min of reaction with slow shaking at RT, the wells were washed four times with washing buffer and subjected to solid-phase fluorescence measurement on a Victor^2 multilabel counter.

With both of the direct and indirect T4 TRFIAs, 27 serum samples (8 from hyperthyroid patients, 7 from hypothyroid patients, and the rest from euthyroid patients) were measured. For comparison, the T4 values in the above samples were also analyzed with the Wallac T4 DELFIA according to the instructions enclosed in the kit.

RESULTS AND DISCUSSION

Fluorescence Properties of the β-Diketone−Eu Complex. Three β-diketone ligands, CTTA, BTOT, and BCTOT, were prepared according to the routes shown in Figure 1. With regard to BTOT−Eu or BCTOT−Eu chelate, it could be anticipated that the two units of TTA derivatives could bend to proper position via the rotation of the C−C bond that links the several $-CF_2-$ groups to bind Eu^{3+} with its four oxygen atoms. Compared to the single CTTA or TTA molecules, the simultaneous coordination of Eu^{3+} by the two TTA derivatives in BTOT−Eu (or BCTOT−Eu) will favor more efficient energy absorption and transfer as well as a better protection of the Eu^{3+} from quenching water molecules. As shown in Figure 5, the fluorescence of the BTOT−Eu complex is enhanced up to ~12 000 times, as compared to that of the TTA−Eu complex. This much-strengthened fluorescence suggested that the tetradentate property of the β-diketone is an important factor for its fluorescence emission. The low limit of detection (LLD) of the BTOT−Eu complex in Tris−HCl buffer

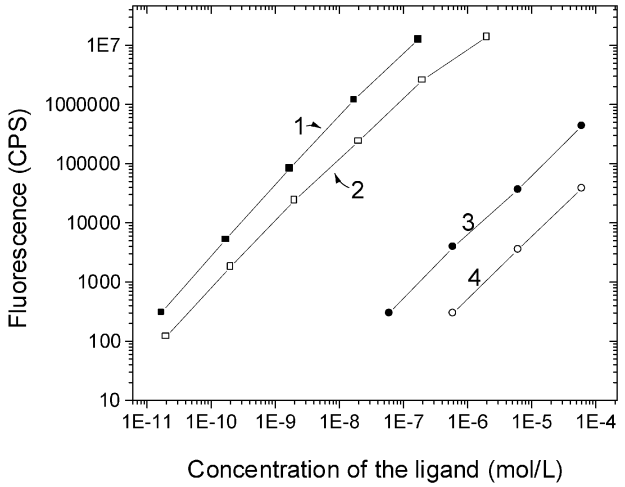

Figure 5. The fluorescence of different β-diketone−Eu complexes versus their concentrations in 50 mmol/L Tris−HCl buffer, pH 8.0. Curves 1−4 correspond to the europium complex of BCTOT in BSA-$(BCTOT)_{12}$ conjugate, BTOT, CTTA in BSA$(CTTA)_{17}$ conjugate, and TTA, respectively. No fluorescence was detected for the CTTA−Eu chelate at ~10^{-4} mol/L.

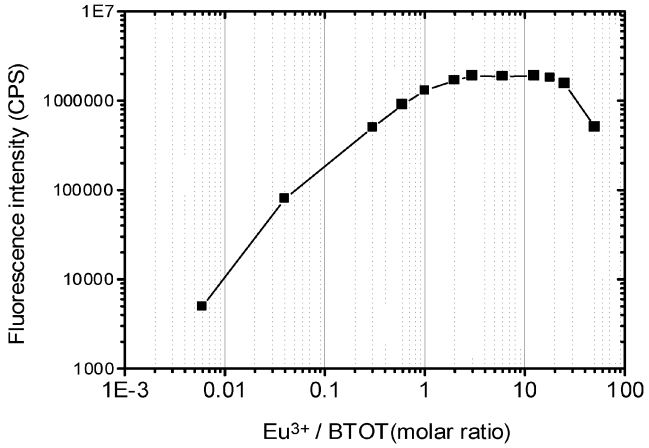

Figure 6. Fluorescence intensity of the BTOT−Eu complex at different Eu^{3+}/BTOT ratios. Each point is the mean of four determinations.

is ~8.9×10^{-12} mol/L (background, + 2 SD). The LLD of the conjugated BCTOT in BSA$(BCTOT)_{12}$ is ~1.07×10^{-12} mol/L, more than 20 times better than the reported BCOT.[16]

As shown in the titration curve (Figure 6), the maximum fluorescence was obtained at the Eu^{3+}/BTOT ratios ranged from 3 to 20, and no sharp turn was observed in the curve at the molar ratio of 0.5 or 1, so the complex formed was probably a mixture mainly involving $(BTOT)_2$−Eu and BTOT−Eu when the molar ratio of Eu^{3+}/BTOT was <20. During the initial stage of titration, $(BTOT)_2$−Eu was formed as the main component as a result of the high excess of the BTOT ligand. Because Eu^{3+} was added gradually, one $(BTOT)_2$−Eu molecule might be transformed to two BTOT−Eu molecules, and the 1:1 complex (BTOT−Eu) increased. Since the complex $(BTOT)_2$−Eu has a higher fluorescence quantum yields than BTOT−Eu, there exist two adverse effects on fluorescence intensity, namely, the increase in number and the decrease in fluorescence quantum yields for BTOT−Eu, giving rise to a fluorescence platform in the titration curve (Figure 6). However, when Eu^{3+} was in large excess to BTOT, the "pincer"

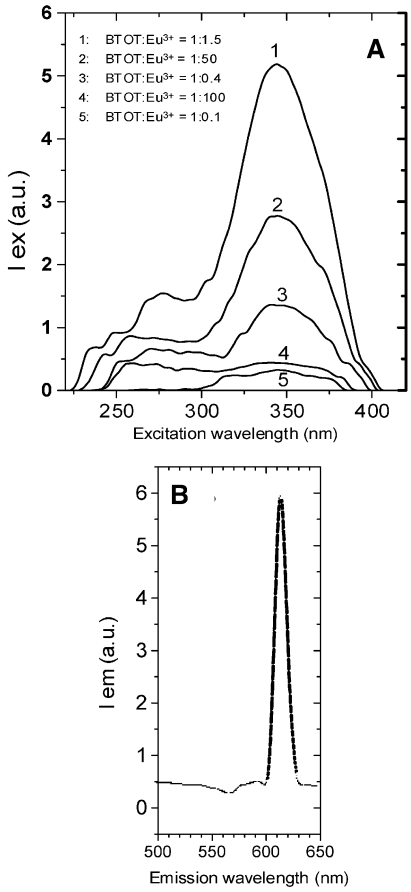

Figure 7. Excitation (A) and emision (B) spectra of the BTOT−Eu complex in 50 mmol/L Tris-HCl buffer. The excitation spectra were recorded at different Eu^{3+}/BTOT ratios. The emission spectra were obtained at a Eu^{3+}/BTOT ratio of ~1/1.5.

structure of the chelate (similar to BCOT−Eu; see Figure 1), of which two TTA derivatives in BTOT wound to bind a single central Eu, was broken. The BTOT molecule stretched out to bind two Eu^{3+}. The situation was similar to that of the free TTA−Eu chelate, leading to a decreased fluorescence quantum yield.

The fluorescence lifetime of BTOT−Eu complex was measured with the LS-50B luminescence spectrometer in the same Tris−HCl buffer. The decay of the chelate fluorescence exhibited typical first-order kinetics after pulsed excitation. The fluorescence lifetime of the BTOT−Eu complex at different Eu^{3+}/BTOT ratios (1/10 to 100/1) was ranged from 328 to 339 μs, obviously longer than that of the reported BCOT−Eu complex (225 to 240 μs).[16]

The excitation maximums of theBTOT−Eu complex were near 345 nm (Figure 7A). The excitation spectra of the complex at different Eu^{3+}/BTOT ratios were of similar shapes. However, the excitation efficiency was strongly decreased when the Eu^{3+}/BTOT ratio was too high or too low, for example, at a value of 1:100 or 1:0.1. The most effective excitation was obtained at the Eu^{3+}/BTOT ratio of near ~1/2. As anticipated, a sharp emission peak appeared at ~612 nm, corresponding to $^5D_0-^7F_2$ transition of Eu^{3+} (Figure 7B).

For convenience in synthesis, a chlorosulfonyl group (−SO_2-Cl) was introduced into BTOT and used as the functional group for labeling −NH_2-containing substances. Results in our experiments show that the −SO_2Cl group is very effective for protein labeling. However, the high reactivity of the −SO_2Cl group and the presence of two −SO_2Cl groups in the BCTOT molecule make it tend to cross-link different proteins and lead to polymer formation. The relatively large distance between the two −SO_2Cl groups in BCTOT intensifies this possibility. Accordingly, the coupling conditions (e.g., the reaction time, the volume, and the concentration of both the protein and ligand) need to be carefully controlled to avoid severe cross-linking between different proteins (or within the same protein) that may cause denaturation or even aggregation of the labeled proteins. In our experiments, BSA and streptavidin showed a stronger resistance to this negative effect than IgG does; we assume that this fact is associated with the relatively lower molecular weight and higher −NH_2 density in BSA or streptavidin molecules.

To assess the stability of the chelate fluorescence, the fluorescence originating from the surface complex (i.e., SA−biotin-McAb−T4−BSA-BCTOT−Eu) was measured at different times after washing the wells at the end of the T4 assay. Unexpectedly, the fluorescence was rather steady, and drying the surface complex in a natural way by exposing it to air led to only a minor signal increase. Therefore, the fluorescence of the surface complex can be detected without drying the surface as a result of the insignificant dependence of the fluorescence on the surface humidity.

TRFIA of Serum T4 in Two Different Formats. (1) Direct T4 TRFIA. We have recently developed a direct T4 TRFIA based on DELFIA technique and the use of surface streptavidin as the separation agent.[18] In this work, we performed the T4 TRFIA in a similar way, except that the highly fluorescent T4−BSA-BCTOT−Eu was used as the tracer in place of the original T4−BSA−Eu. A good calibration curve for the direct T4 TRFIA was obtained, with the B_1/B_0 and B_6/B_0 values at 75 and 18%, respectively. However, the measurements of T4 in clinical serum samples in this assay format deviated greatly from that determined by the commercial CLIA or DELFIA and did not reflect the actual thyroid status. Of the 27 tested serum samples, 21 samples gave obviously increased T4 values, and 1 sample gave a value <0 nmol/L. Only five samples had T4 values approaching that determined by CLIA or DELFIA, as listed in Table 1.

(2) Indirect T4 TRFIA. In light of the above problem, another indirect T4 TRFIA was carried out to avoid the direct contact of the tracer with serum samples. A typical calibration curve and imprecise profile of the indirect method is shown in Figure 4. The T4 concentrations measured by the indirect T4 TRFIA correlated well with that obtained by the Wallac T4 DELFIA and Corning T4 CLIA. The correlation equations were $Y_{\text{indirect,TRFIA}} = -20 + 1.21X_{\text{Corning,CLIA}}$ ($r = 0.955$) and $Y_{\text{indirect,TRFIA}} = 3.43 + 0.96X_{\text{Wallac,DELFIA}}$ ($r = 0.965$). The recovery of the indirect T4 TRFIA was between 97 and 117%, determined by analyzing three clinical samples (T4: 58, 129, 221 nmol/L) before and after addition of exogenous T4. The intra-assay ($n = 12$) and interassay ($n = 8$) imprecision (CV%) obtained by measuring the same three serums were in the range of 4.1−7.5% and 4.8−12.3%, respectively. The relatively higher CV values were related to the solid-phase fluorescence measurement mode, which sets a stricter requirement for the uniformity of the microwell surface, because only a small area of the surface is illustrated. The LLD of the indirect T4 TRFIA was 6.7 nmol/L, calculated as the concentration corresponding to the mean of 12 determinations of zero standard

Table 1. Comparison of the Serum T4 Values Determined by Four Different T4 Immunoassays

	T4 values, nmol/L			
sample[a]	present direct assay[b]	present indirect assay[b]	CLIA	T4 DELFIA[b]
1	235	53	64	51
2	163	45	57	57
3	287	35	46	37
4	129	62	57	66
5	53	48	65	49
6	88	53	68	60
7	169	59	45	58
8	251	147	169	126
9	>300	69	75	67
10	237	98	120	88
11	276	98	112	103
12	109	83	79	101
13	>300	132	169	153
14	168	139	118	147
15	267	122	150	136
16	129	89	105	97
17	215	95	69	77
18	141	138	112	153
19	<0	76	89	89
20	>300	189	156	177
21	>300	226	178	197
22	>300	251	219	298
23	268	226	199	176
24	>300	>300	258	>300
25	>300	168	147	190
26	259	182	159	170
27	233	203	179	180

[a] Samples numbered 1−7, 8−19, and 20−27 were from hypothyroid, euthyroid, and hyperthyroid patients, respectively. [b] Values were the mean of replicate measurements.

minus 2 SD. This sensitivity is enough for clinical analysis of serum T4.

Quenching Studies. ANS, EDTA, and sodium salicylate have different extents of the quenching effect on the fluorescence of the BCTOT−Eu complex. To evaluate this effect quantitatively, different amounts of ANS, EDTA, or sodium salicylate were added to Tris−HCl buffer containing ∼0.0015 μmol/L of BCTOT−Eu. As shown in Figure 8, >65% of the total fluorescence was quenched by ∼0.1 g/L of EDTA, but the fluorescence was not noticably decreased with an increase in the EDTA concentration. In the meantime, the chelate fluorescence was completely quenched by ANS at a concentration of ∼3 g/L. Sodium salicylate expressed an obvious quenching effect, but not as much as that of ANS and EDTA. The fluorescence quenching by TCA-Na was inconsiderable. The quenching mechanisms for the different reagents may be different, probably by extraction of the Eu^{3+} (e.g., by EDTA) or by additional coordination of the reagent with the central ion that will cause energy leaking through the high-energy N−H or O−H stretching (e.g., by ANS or salicylate). On the contrary, about 7-fold enhancement of the chelate fluorescence was achieved by TOPO at ∼100 μmol/L. Two factors may have played a role in the fluorescence enhancement: the replacement of water molecules from the inner sphere of the chelate by TOPO and the mediating action for energy transfer (from TOPO to β-diketone).[1,19] On the basis of the above observations, it would be reasonable to believe that some other untested compounds

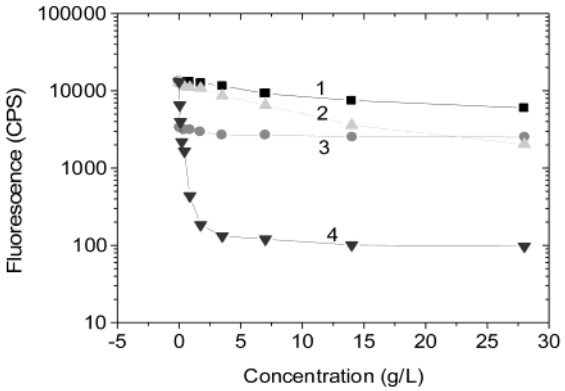

Figure 8. Fluorescence quenching caused by four reagents. Curves 1−4 were obtained by using TCA-Na, sodium salicylate, EDTA, and ANS, respectively. After the addition of different amounts of these quenching reagents in Tris−HCl buffer containing ∼0.002 μmol/L of the BCTOT−Eu chelate, the mixture was incubated for 25 min with shaking before fluorescence measurement.

may exert a similar quenching or enhancing effect on the BCTOT−Eu fluorescence. This possibility can be used to explain the matrix interference encountered in our direct T4 TRFIA, because various ligands originating from the sample (e.g., some endogenous molecules or circulating drugs) may act in ways similar to ANS, DTPA, salicylate, or TOPO does. To remove this limitation, designing an indirect assay as the described indirect T4 TRFIA is a workable strategy. However, a more basic route for this may depend on the design of a chelate that is robust enough to resist interference, for example, the extraction of the chelated ions by other ligands, the replacement of the central ion by other ions, and the extra coordination that may quench or enhance the specific signals. To this end, a nonadentate chelate is preferable because of its efficiency in preventing direct contact of the central ion with other ligands. Besides, incorporating the fluorescent chelate into latex particles represents another feasible way to obviate such a problem. A bonus benefit might be the greatly enhanced fluorescence caused by the hydrophobic environment in the beads and the amount of fluorescent chelate enclosed in a single bead. In such a case, inner-filter quenching will not function because of the very long Stokes' shift of the chelate fluorescence.

In the work of Ci et al., CTTA was used as label to develop competitive TRFIA of BSA or cortisol.[14,15] It was reported that the relatively low stability of the CTTA−Eu chelate could be compensated by increasing the Eu^{3+} concentration and by incorporating the probe into the protein molecule.[14] However, no data showed that the CTTA label used in these works was kinetically stable enough to withstand the matrix interference as observed in our work, since the BSA TRFIA had not been used to detect analyte of different samples. For the urine cortisol TRFIA, an extraction step to draw cortisol from the urine to dichloromethane prior to the assay was required, and this may exclude most of the interfering species in the urine, the composition of which is much less complicated than serum. Furthermore, the dissociation step used to release the surface immunocomplex to solution for fluorescence measurement in the two TRFIAs may also swamp the interference. Neither the BSA nor cortisol TRFIA was compared with the other accepted method. For these reasons, it is very likely that the matrix interference has been neglected in

(19) Hu, J. M.; Chen, G. Q.; Zeng, Y. *Fenxi Huaxue.* **1990**, *18*, 875−877 (in Chinese).

these works, because it is distinct that the tetradentate BCTOT−Eu chelate is more stable than the didentate CTTA−Eu.

CONCLUSIONS

In conclusion, the BCTOT−Eu chelate described in this work is highly fluorescent in aqueous solution, simple to synthesize, highly hydrophilic, and has an acceptable chemical stability. Although there is still much work to be done to further explore the features and usage of this chelate, preliminary results in this study show that this new europium chelate can be used as a suitable tracer for developing a sensitive indirect bioanalytical assay. The problem of ligand contamination in the direct T4 assay is noteworthy, because it is also possibly present in other lanthanide chelate label-based analyses. Unlike the DELFIA system, which requires a dissociation-enhancement step, the fluorescence of BCTOT−Eu chelate can be detected directly from the surface in a very steady state; therefore, this chelate may be applied in areas that require a spatial signal discrimination. It is also interesting to use this type of label to establish sensitive immunochromatography for rapid and quantitative detection of many biomolecules, provided that suitable membrane materials with a very low background can be obtained.[20]

(20) Wu, F. B. *J. Immunol.* **1997**, *13* (Special), 54−59 (in Chinese).

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SUPPORTING INFORMATION AVAILABLE

Experimental details of the verification of the high CV values. This material is available free of charge via the Internet at http://pubs.acs.org.

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