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A New Tetradentate β-Diketonate−Europium Chelate That Can Be Covalently Bound to Proteins for Time-Resolved Fluoroimmunoassay

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A new chlorosulfonylated tetradentate β-diketone, 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl)chlorosulfo-*o*-terphenyl (BHHCT), was synthesized as a chelating label for Eu^{3+}. BHHCT can be covalently bound to proteins under mild conditions and forms a strongly fluorescent chelate with Eu^{3+}. Bovine serum albumin (BSA) and streptavidin (SA) were labeled with BHHCH−Eu^{3+}, and the latter was used for time-resolved fluoroimmunoassay of α-fetoprotein (AFP) in human sera. A remarkably high sensitivity was obtained, with a detection limit of 4.1×10^{-3} pg/mL, which corresponds to an improvement of about 4−5 orders of magnitude, compared to those of all conventional immunoassays including fluoroimmunoassay, enzyme immunoassay, and radioimmunoassay. The high sensitivity has been attained both by strong fluorescence of the present label and by the extremely suppressed background level brought about by the direct labeling of proteins with the β-diketone−Eu^{3+} complex. A general consideration and ideas are given for designing ideal label ligands for strongly fluorescent Eu^{3+} complexes.

Time-resolved fluoroimmunoassay (TR-FIA) using europium complexes as fluorescent labels has attracted the interest of many researchers.[1−8] There are some distinct properties of Eu^{3+} fluorescent complexes. (i) The complexes have absorption maxima in the UV region (∼330 nm) and emit strong fluorescence around 615 nm. This large Stokes shift is caused by the fact that, while the UV absorption is due to the ligand absorption, the energy emitted is due to the Eu^{3+} transition $^5D_0 \rightarrow {}^7F_2$, after the energy transfer from the ligand to Eu^{3+} via an intramolecular intersystem crossing mechanism. (ii) The emission band is very narrow, with a fwhm of ∼10 nm. (iii) The complexes have very long lifetimes, usually longer than 100 μs. These characteristics of Eu^{3+} fluorescent complexes allow a unique time-resolved fluorometry if they are used as labels for biomolecules, in which background fluorescent emission of the biological matrix can be effectively removed and a high S/N ratio is obtained.[9]

Several europium labels have thus far been developed,[4,10−14] two of which are employed in commercial immunoassay systems. In the dissociation-enhanced lanthanide fluoroimmunoassay system (Delfia, LKB Wallac, and Pharmacia systems), either isothiocyanatophenyl-EDTA−Eu^{3+} or N^1-(*p*-isothiocyanatobenzyl)diethylenetriamine-N^1,N^2,N^3,N^4-tetraacetic acid−Eu^{3+} is used as the label. However, these labels are not fluorescent, and the Eu^{3+} in the label is converted to a fluorescent complex after immunological reaction by adding β-naphthoyltrifluoroacetone (β-NTA).[4] Although the system is highly sensitive, it is vulnerable to Eu^{3+} contamination and has a high background level caused by the excess free β-NTA in the solution.[4,10] In the other system, CyberFluor, direct labeling of proteins is possible with fluorescent 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA)−Eu^{3+}. In this system, the high background in the LKB system has been removed, since no excess ligand exists in the solution, but the fluorescence of the label is much weaker than that of the β-NTA−Eu^{3+} complex in the LKB system.[15] Literature survey shows that β-diketonate ligands are suitable for efficient energy transfer from the ligand to Eu^{3+} and for high fluorescence quantum yield of the complex.[16]

Considering the above facts, an ideal ligand for Eu^{3+} should be a β-diketonate-type ligand having a group for covalent bonding

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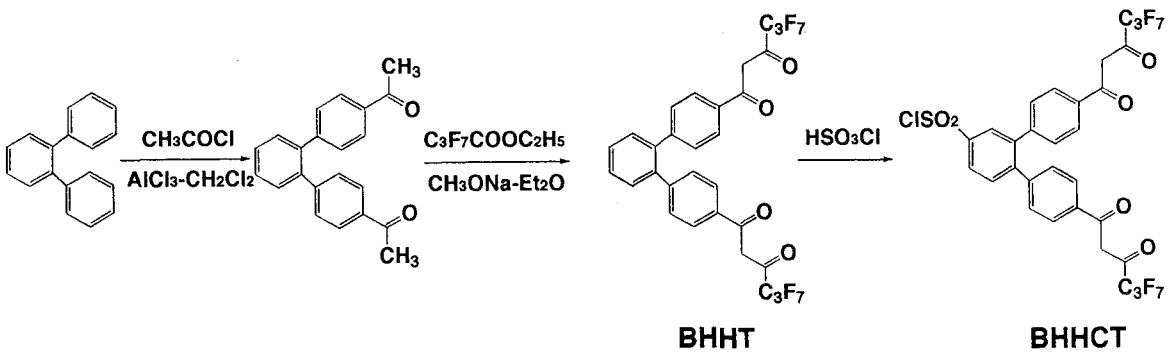

Figure 1. Synthesis of BHHT and BHHCT.

to proteins. One such ligand, 5-chlorosulfonyl-2-thenoyltrifluoroacetone (CTTA), has been prepared, but its application in immunoassay did not give any improvement of the sensitivity.[13,14] The unexpectedly small improvement is due to the low stability of the CTTA−Eu^{3+} complex and the decreased fluorescence intensity caused by the protein-binding group attached to the aromatic ring of the ligand.

In this report, synthesis of a new tetradentate β-diketone ligand, 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl)-chlorosulfo-*o*-terphenyl (BHHCT, Figure 1) is reported. The ligand forms a very stable complex with Eu^{3+} (owing to the tetradentate structure), emits very strong fluorescence when complexed with Eu^{3+} (owing to the heptafluoropropyl group on one side of the β-diketone groups),[17] and can be covalently bound to proteins. Remarkable superiority of BHHCT over conventional organic fluorescent labels and other lanthanide labels has been proved in the time-resolved fluorometric determination of bovine serum albumin (BSA) and TR-FIA of α-fetoprotein (AFP). Human AFP is one of the carcinoembryonic proteins and has been measured with various immunoassays, i.e., radioimmunoassay (RIA),[18,19] enzyme immunoassay (EIA),[19] luminescence EIA,[20] chemiluminescence EIA (CL-EIA),[21,22] latex particle immunoassay,[23] electorochemiluminescence immunoassay,[24] and TR-FIA.[25−29] These methods provide detection limits of $\sim 10^{-12}$ M (1×10^{-1} ng/mL) or higher for AFP. In the present study, detection limits of 4.6×10^{-2} pg/mL (solid-phase measurement) and 4.1×10^{-3} pg/mL (solution measurement after solid dissolution) for AFP were obtained. The latter value is 4.3 orders of magnitude lower than those of all of the previously reported immunoassays. BHHCT was also used for labeling of BSA, and the detection limit of labeled BSA was 6.5×10^{-15} M.

EXPERIMENTAL SECTION

Synthesis of BHHCT. The labeling reagent BHHCT was prepared according to the three steps in Figure 1, as described in the following.

(i) Preparation of 4,4′-Diacetyl-*o*-terphenyl. To 200 mL of CH_2Cl_2 containing 28 g (210 mmol) of anhydrous $AlCl_3$ and 16.1 g (205 mmol) of CH_3COCl was added dropwise 100 mL of CH_2Cl_2 containing 23 g (100 mmol) of *o*-terphenyl at 0 °C with stirring. The solution was further stirred at 0 °C for 30 min and then at room temperature for 24 h. After 2 h refluxing, the solution was poured into an ice−HCl solution with vigorous stirring. The CH_2Cl_2 was removed by vacuum distillation, and the residue was collected by filtration. After the precipitate was washed with water, it was recrystallized from 250 mL of 2-butanone. Pale yellow prismatic crystals were obtained (24.6 g, 78% yield). Anal. Calcd for $C_{22}H_{18}O_2$: C, 84.05; H, 5.77. Found: C, 84.06; H, 5.87. The compound was also confirmed by ^{1}H NMR.

(ii) Preparation of 4,4′-Bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl)-*o*-terphenyl (BHHT). A solution of 70 mL of dry ether containing 3.0 g (55.5 mmol) of $NaOCH_3$, 4.84 g (20 mmol) of $C_3F_7COOC_2H_5$, and 3.14 g (10 mmol) of 4,4′-diacetyl-*o*-terphenyl was stirred at room temperature in a sealed flask for 36 h. The ether solution was poured into 100 mL of 15% H_2SO_4, after 15 min of stirring at room temperature, ether was removed by distillation. The sticky solid obtained was washed with water and dissolved in 200 mL of boiling ethanol. The solution was filtered while hot, and the filtrate was condensed to ∼20 mL and added to 200 mL of petroleum ether (30−60 °C). The solution was further stirred for 10 min at room temperature and was then filtered. The oily β-diketone residue, obtained after vacuum distillation of the filtrate, was vacuum-dried for several days, and a yellow solid was obtained (5.1 g, 72% yield). Anal. Calcd for $C_{30}H_{16}F_{14}O_4$: C, 51.00; H, 2.28. Found: C, 51.22; H, 2.61. The compound was also confirmed by ^{1}H NMR.

(iii) Preparation of 4,4′-Bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl)chlorosulfo-*o*-terphenyl (BHHCT). To 5 mL of stirred HSO_3Cl was gradually added 1.41 g (2 mmol) of BHHT. After the solution was stirred for 7 h at room temperature, it was added dropwise with stirring to 150 mL of

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ice-water. The residue was collected by centrifugation, rinsed with cold water, and twice centrifuged. The precipitate was filtered and vacuum-dried for more than 48 h. A yellow powder of BHHCT was obtained, with a yield of 75.6% (1.24 g). Anal. Calcd for $C_{30}H_{17}F_{14}O_7ClS$ (BHHCT·H_2O): C, 43.78; H, 2.08. Found: C, 43.96; H, 2.10. The compound was also confirmed by ^1H NMR and FABMS.

Labeling and Immunoassay Format. The labeling of BSA and SA with BHHCT basically followed the literature procedures,[30,31] as described in the following.

(i) Labeling of BSA. To 2 mL of 0.1 M carbonate buffer of pH 9.3 containing 10 mg (1.5×10^{-4} mmol) of BSA was added dropwise with stirring 400 μL of DMF solution containing 7.4 mg (9.0×10^{-3} mmol) of BHHCT. After 1 h of stirring at room temperature, the labeled BSA was separated from the hydrolyzed BHHCT by gel filtration chromatography on a Sephadex G-50 column. As the eluent, 0.05 M NH_4HCO_3 of pH 8.0 was used, and fractions with 1 mL of each were collected and measured with the absorption at 280 nm. The labeled BSA fractions were collected. The molar extinction coefficient (ϵ) of BHHCT at 330 nm was determined by using the unchromatographed solution appropriately diluted with 0.05 M NH_4HCO_3 of pH 8.0. The BHHCT/BSA ratio was determined by measuring the concentration of the BHHCT in the labeled BSA and calculating the ratio on the assumption that ϵ of BHHCT does not change on the labeling to BSA. The ϵ of BHHCT at 330 nm was 3.41×10^4 M^{-1} cm^{-1}, and the BHHCT/BSA ratio was 35. To the labeled BSA solution was added 0.1% of NaN_3, and the pH was adjusted to 6.2 with 1 M HCl. The solution was stored at 4 °C.

(ii) Labeling of SA. To 33 mL of 0.1 M carbonate buffer of pH 9.1 containing 5 mg (8.3×10^{-5} mmol) of SA (Chemicon International Inc.) was added dropwise (25-μL drops) with stirring 0.2 mL of ethanol solution containing 4 mg (4.9×10^{-3} mmol) of BHHCT in 5 min. After the solution was stirred for 1 h at room temperature, it was dialyzed twice against 4 L of 0.1 M $NaHCO_3$ containing 0.25 g of NaN_3, first for 24 h and then for 7 h. The labeling ratio of BHHCT to SA was determined as $SA(BHHCT)_{21}$ by using the absorbance of the labeled SA solution and the molar extinction coefficient of BHHCT. To the dialyzed solution were added 50 mg of BSA and 20 mg of NaN_3, and the pH was adjusted to 6.2 with 1 M HCl. The solution was stored at −20 °C. The labeled SA solution was diluted 500-fold with 0.05 M Tris-HCl of pH 7.8 containing 1.0×10^{-7} M $EuCl_3$, 1% BSA, 0.1% NaN_3, and 0.9% NaCl, heated at 56 °C for 2 h, and was used for immunoassay.

The immunoassay format for AFP using BHHCT, shown in Figure 2, is the same as that previous report using BCPDA,[26] except that the secondary antibody was biotinylated for binding the BCPDA-labeled SA in the latter.

The 96-well polystyrene white microtiter plate (Dynatech Laboratories, Inc.) was coated with either goat anti-human AFP antibody (Nippon Bio-Test Laboratories, Inc.) or mouse monoclonal anti-human AFP antibody (Zymed Laboratories, Inc.) diluted to 2.5 and 3 μg/mL with 0.1 M carbonate buffer of pH 9.6, respectively, by incubating 100 μL of the solution first at room temperature for 1 h and then at 4 °C for 24 h. After the wells were washed twice with 0.01 M phosphate buffer containing 0.05% Tween 20 (pH 7.4, buffer 1), they were further washed with 0.01 M phosphate buffer without Tween 20 (pH 7.4, buffer 2). To each well was added 100 μL of 0.1 M $NaHCO_3$ containing 1% BSA, 0.05% NaN_3, and 2% sucrose, and the plate was incubated for 1 h at room temperature for polyclonal antibody and for 24 h at 4 °C for monoclonal antibody. The plate was washed as previously and stored at −20 °C.

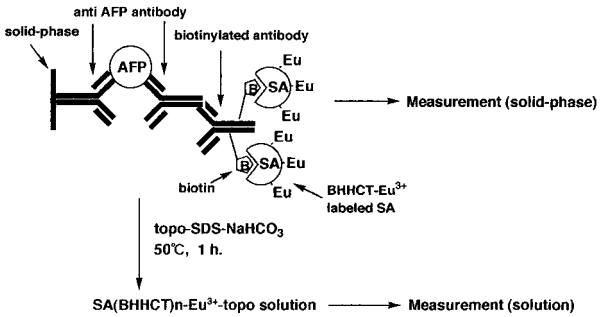

Figure 2. Schematic representation of the immunoassay system for AFP.

The standard solutions of AFP for calibration were prepared by diluting human AFP standard solution (DAKO-Immunoglobulins a/s, Denmark, Dakopatts α-1-fetoprotein standard) with 0.01 M phosphate buffer (buffer 3, pH 7.4) containing 1% BSA, 0.9% NaCl, and 0.05% NaN_3. Human serum samples were 10-fold diluted with buffer 3 and were subjected to assay.

The immunoassay procedure for AFP using BHHCT (Figure 2) is as follows. To a well coated with either goat anti-human AFP antibody or mouse monoclonal anti-human AFP antibody was added 50 μL of an AFP solution, and the solution was incubated for 1 h at 37 °C. The well was washed twice with buffer 1 and once with buffer 2. After 50 μL of rabbit anti-human AFP antibody (DAKO-Immunoglobulins a/s, affinity purified, 86 kIU/mL), diluted 500-fold with buffer 3, was incubated at 37 °C for 1 h, the well was washed twice with buffer 1 and once with buffer 2. After 50 μL of biotinylated goat anti-rabbit IgG(H+L) antibody (Vector Laboratories, Inc., affinity purified, 1.5 mg/mL), diluted 100-fold with buffer 3, was incubated at 37 °C for 1 h, the well was washed twice with physiological saline containing 0.05% Tween 20 and once with saline. After 50 μL of the $SA(BHHCT)_{21}-Eu^{3+}$ solution was incubated at 37 °C for 1 h, the well was washed 4 times with 0.1 M Tris-HCl (pH 9.1) containing 0.05% Tween 20. The plate thus prepared was subjected to solid-phase fluorometric measurement. After the solid-phase measurement, 50 μL of 0.1 M $NaHCO_3$ containing 1.0×10^{-5} M TOPO (tri-*n*-octylphosphine oxide) and 0.05% SDS (sodium dodecyl sulfate) was incubated in the well at 50 °C for 1 h, and the solution was subjected to fluorometric measurement after the solution was cooled to room temperature.

The TR-FIA measurement of AFP was performed on a Cyber-Fluor 615 fluorometer with excitation at 337.1 nm, delay time of 200 μs, and counting time of 200−600 μs. The fluorescence at 615 nm was measured. The fluorescence of the labeled BSA was measured on a ARCUS 1232 (Wallac) fluorometer with excitation at 340 nm, delay time of 0.2 ms, and window time of 0.4 ms. The excitation and emission spectra were measured on a Hitachi F-4500 fluorospectrometer. The fluorescence lifetime was measured on a Perkin Elmer LS 50B luminescence spectrometer.

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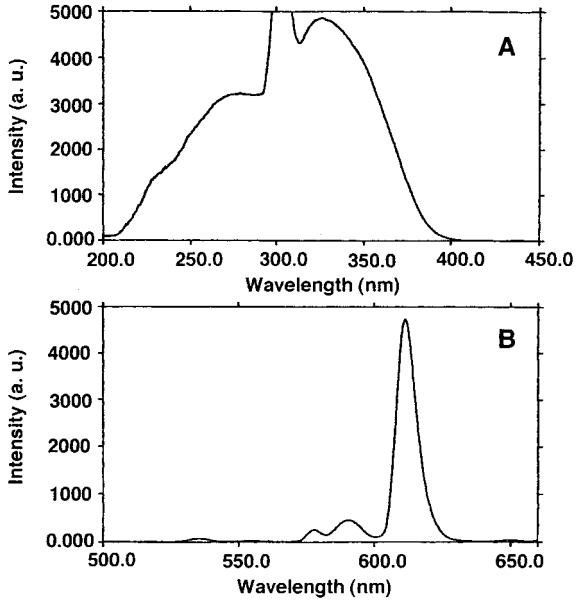

Figure 3. Excitation (A) and emission (B) spectra of BHHCT-labeled BSA in the presence of Eu^{3+}. [BSA(BHHCT)$_{35}$] = 1.8×10^{-7} M, [$EuCl_3$] = 1.0×10^{-5} M. The buffer was 0.1 M Tris-HCl of pH 8.5.

RESULTS AND DISCUSSION

Measurement of BSA and Characteristics of the Label. The labeling reaction of BHHCT with proteins involves covalent bond formation between the -SO_2Cl group of BHHCT and amino groups of proteins, producing -SO_2-NH- groups in the proteins. The excitation and emission spectra of the BHHCT-labeled BSA in Tris-HCl containing 1×10^{-5} M $EuCl_3$ are shown in Figure 3. The excitation and emission maxima are 326.0 and 611.6 nm, respectively. The lifetimes of BSA(BHHCT)$_{35}$−Eu^{3+} in 0.1 M Tris-HCl (pH 9.1) and in 1.0×10^{-5} M TOPO−0.05% SDS−0.1 M $NaHCO_3$ (pH 8.4) are 380 and 641 μs, respectively. The complex formation constant of BHHCT (bound to BSA)−Eu^{3+} was determined with the molar ratio method in 0.1 M Tris-HCl of pH 9.1 to be $\sim 2 \times 10^{10}$ M^{-1}. This large formation constant for the tetradentate ligand, compared to those of previously reported bidentate ones, is an advantage for highly sensitive detection, since, for bidentate ligands, a larger excess of Eu^{3+} is necessary for efficient complex formation, which causes a high background level. The labeling ratio of BSA(BHHCT)$_{35}$ means about one-half of the amino groups in BSA (a total of 59 amino groups) are used for the covalent bonds to BHHCT. The labeling ratio is dependent on the relative mixing ratio of the label and BSA, but the maximum ratio would be determined by the steric bulkiness and other chemical properties of the label. The relative fluorescence intensities of the Eu^{3+} complexes of TTA (2-thenoyltrifluoroacetone) and CTTA (prepared according to ref 13), as well as those of BHHT and BHHCT, were compared in ethanol at concentrations of 1.0×10^{-5} M for both ligand and $EuCl_3$. The relative intensities were 102, 29.3, 550, and 2120, respectively. It should be noted that, while the chlorosulfonyl group of CTTA decreases the fluorescence intensity of TTA, the identical group in BHHCT increases by ~4 times the intensity of BHHT. In order to determine the optimum Eu^{3+} concentration in the fluorometry, the background intensity and the Eu^{3+} complex intensity were compared as a function of Eu^{3+} concentration with the BSA-(BHHCT)$_{35}$ concentration fixed at 3.5×10^{-10} M (Figure 4). The

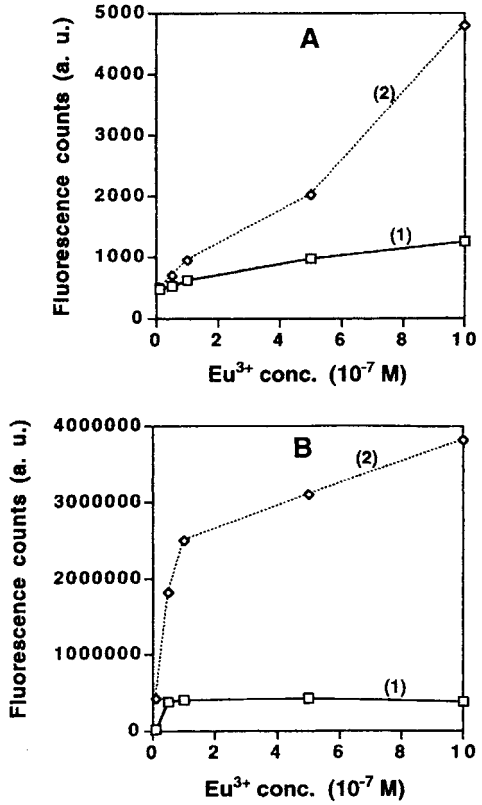

Figure 4. Effect of europium concentration on the background level and fluorescence signal intensity in time-resolved fluoromeasurement of BSA(BHHCT)$_{35}$−Eu^{3+}. (A) Background; (B) fluorescence signal when the concentration of the labeled BSA is 3.5×10^{-10} M. (1) and (2) are for the buffer solutions of 0.1 M Tris-HCl of pH 9.1 and 1.0×10^{-5} M TOPO−0.05% SDS−0.1 M $NaHCO_3$, respectively.

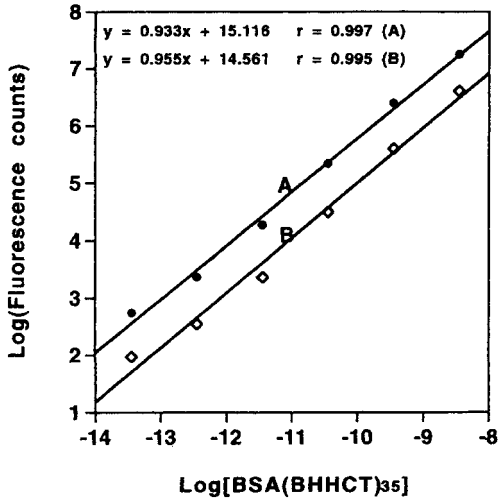

Figure 5. Log(fluorescence counts) vs log[BSA(BHHCT)$_{35}$] in the presence of 1.0×10^{-7} M $EuCl_3$. (A) 1.0×10^{-5} M TOPO−0.05% SDS−0.1 M $NaHCO_3$ solution (pH 8.4). (B) 0.1 M Tris-HCl solution of pH 9.1.

result in Figure 4 shows that the best S/N ratio is obtained at an Eu^{3+} concentration of 1.0×10^{-7} M. An Eu^{3+} concentration higher than this raises the background level and deteriorates the S/N. With this Eu^{3+} concentration, linear calibration curves for BSA (Figure 5) were obtained in Tris-HCl and SDS−TOPO−$NaHCO_3$ buffers. The detection limits (concentration corresponding to 2SD of the background intensity) of BSA were 2.4×10^{-14} and $6.5 \times$

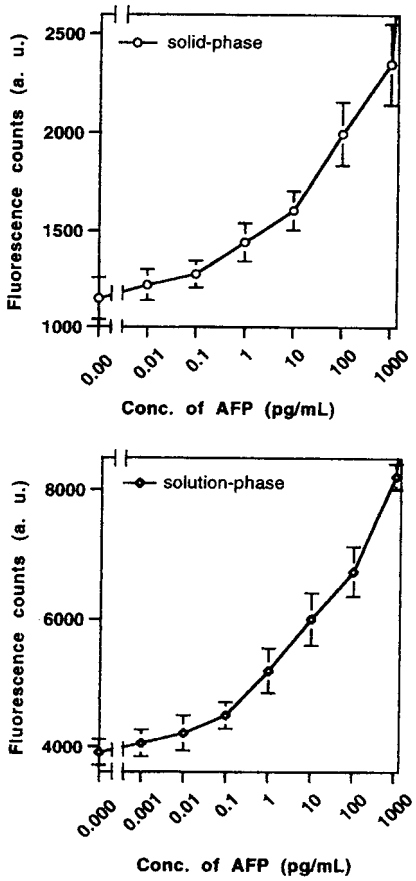

Figure 6. Calibration curves for AFP.

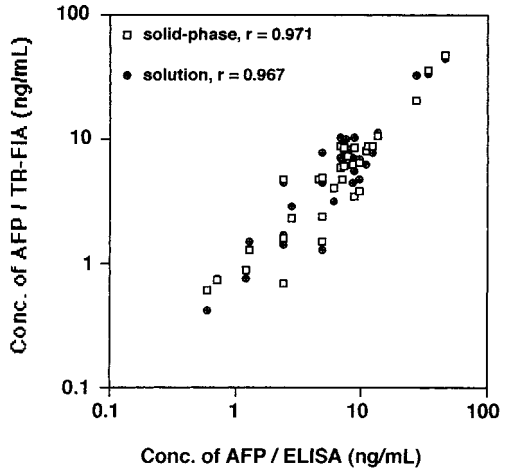

Figure 7. Correlation of AFP concentrations in 34 sera obtained by the present method and ELISA. The regression slopes are $y = 0.987x - 1.377$ for solid-phase, and $y = 0.982x - 0.686$ for solution.

10^{-15} M, respectively. These detection limits are 2−3 orders of magnitude better than that obtained with the CyberFluor system using BCPDA[30,32] and are more than 4 orders of magnitude better than that obtained with the CTTA system.[13] This marked improvement of the detection limit in the present system is due to the decreased background level as well as the strong fluorescence of the label. The background intensity in the present system is about 1/30 that of the LKB system in our comparative measurement, and this shows how the presence of excess β-diketone ligand disturbs measurements at very low concentration levels.

Measurement of AFP. The calibration curves of human AFP for solid-phase and solution-phase measurements are shown in Figure 6. The detection limits of 0.046 pg/mL for solid-phase measurement and 0.0041 pg/mL for solution-phase measurement were obtained, where the detection limit (DL), the concentration corresponding to the equation in ref 32, is $DL = (3SD \times C)/(I - I_0)$. In the equation, SD is the standard deviation of the background signal, C is the lowest measured concentration with

Table 1. Precision in the Determination of AFP in Human Sera[a]

mean (ng/mL)	SD	CV (%)
0.00046 (0.00050)[b]	0.000038 (0.000031)	8.26 (6.20)
0.0028 (0.0034)[b]	0.00017 (0.00016)	6.07 (4.70)
0.072 (0.081)[b]	0.0083 (0.0076)	11.53 (9.38)
0.81 (0.82)	0.028 (0.033)	3.53 (4.08)
1.68 (1.70)	0.12 (0.12)	7.14 (6.81)
2.26 (2.26)	0.056 (0.057)	2.49 (2.51)
3.38 (3.76)	0.22 (0.20)	6.37 (5.46)
7.58 (7.78)	0.029 (0.50)	6.38 (6.38)
9.30 (10.2)	0.44 (0.43)	4.72 (4.21)
18.4 (17.6)	0.94 (0.55)	5.14 (3.11)
39.6 (36.2)	1.53 (0.62)	3.87 (1.71)

[a] Intraassay, $n = 4$. Data in parentheses are the results of solution measurement, and others are those of solid-phase measurement. [b] Serum samples diluted with buffer 3.

Table 2. Analytical Recovery of AFP Added to Serum Samples[a]

added (ng/mL)	found (ng/mL)	recovery (%)
	3.20 (3.40)	
5.00	9.40 (8.70)	124.0 (106.0)
	7.60 (6.50)	
5.00	12.4 (12.4)	96.0 (118.0)
2.50	10.0 (9.10)	96.0 (104.0)
	16.9 (16.5)	
50.0	67.5 (67.3)	101.2 (101.6)
25.0	40.8 (37.2)	95.6 (82.8)
5.00	21.9 (20.8)	100.0 (86.0)

[a] Data in parentheses are the results of solution measurement, and others are those of solid-phase measurement.

Table 3. Sensitivity Comparison of the Immunoassay Methods for Human AFP

method	label[a]	detection limit[b]	ref
RIA	^{125}I	0.1 ng/mL	18
RIA	^{125}I	0.54 IU/mL	19
EIA	ALP	1.25 IU/mL	19
luminescence EIA	HRP-luminol	0.2 IU/mL	20
CL-EIA	ALP-1,2-dioxetanephosphate	0.03 ng/mL	21
CL-EIA	GAL-5-Br-4-Cl-3-I-β-D-galactopyranoside	0.5 ng/mL	22
TR-FIA	SNC-phenyl-EDTA−Eu^{3+}	0.1 IU/mL	25
TR-FIA	SA-BCPDA−Eu^{3+}	0.1 ng/mL	26
TR-FIA	SA-BHHCT−Eu^{3+}	0.0041 pg/mL	this work

[a] HRP, horseradish peroxidase; ALP, alkaline phosphatase; GAL, β-D-galactosidase. [b] 1 IU/mL = 1.21 ng/mL.

(32) Kropf, J.; Quitte, E.; Gressner, A. M. *Anal. Biochem*. **1991**, *197*, 258−265.

its signal intensity I, and I_0 is the background signal. The latter detection limit corresponds to 5.8×10^{-17} M, assuming $M_{AFP} =$ 70 000, and to ~1750 AFP molecules in 50 μL of sample solution. The detection limit obtained for diluted serum solutions in solid-phase measurement was ~0.1 pg/mL. The AFP concentrations in 34 serum samples were determined both by the present method and by ELISA, and the correlation of the 34 analytical values is shown in Figure 7. For ELISA measurement, a human AFP kit (Boehringer Manheim, Tokyo) was used. The dynamic range of the present method in sera is from $\sim 10^{-4}$ to 100 ng/mL. The precision and recovery of the serum analysis are summarized in Tables 1 and 2. All of the above data indicate that the use of BHHCT as a direct label gives remarkably high sensitivity for AFP in sera with low detection limits. In Table 3, the detection limits of various immunoassay systems for AFP are compared. The detection limit of ref 26 is obtained by basically the same assay format as with the present method and shows clearly how BHHCT is superior to previous labels. The tetradentate BHHCT−Eu^{3+} complex has a much larger complex formation constant, compared to those of the conventional bidentate chelates.

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