

## A Novel Time-Resolved Fluoroimmunoassay Using a Macrocyclic Europium Ligand as a Label

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**A novel macrocyclic ligand for Eu<sup>3+</sup> (24,30-diphenyl-1,21:4,6:10,12:15,17-tetraetheno-8,9,19,20-tetrahydro-7H,18H-dibenzo[*b,k*]-[1,4,7,10,13,16]hexaazacyclooctadecine-8,19-diacetic acid) was used to label an anti-human chorionic gonadotropin (hCG) monoclonal antibody. The time-resolved fluoroimmunoassay of hCG using this labeled antibody showed a linear response over the range 0.1 to 10 I.U. hCG/ml.**

**Keywords** time-resolved fluoroimmunoassay; europium complex; human chorionic gonadotropin; macrocyclic chelator; chlorosulfonyl functionality

Time-resolved fluoroimmunoassay (TR-FIA) using lanthanide ions such as Eu<sup>3+</sup> as labels is receiving considerable attention because its sensitivity is comparable with radioimmunoassay<sup>1)</sup> and it has been successfully used to detect a variety of biological substances such as hormones,<sup>2)</sup> DNA,<sup>3)</sup> or viruses.<sup>4)</sup> The Eu<sup>3+</sup> chelates, reported so far for potential use in TR-FIA, include  $\beta$ -diketone derivatives,<sup>1c,5)</sup> a phenanthroline derivative,<sup>1d)</sup> a terpyridine analog,<sup>6)</sup> and several macrocycles.<sup>3b,d)</sup> Most Eu<sup>3+</sup> complexes with these ligands, however, display fluorescence quenching in aqueous media.

We recently developed a new macrocyclic ligand for Eu<sup>3+</sup>, 24,30-diphenyl-1,21:4,6:10,12:15,17-tetraetheno-8,9,19,20-tetrahydro-7H,18H-dibenzo[*b,k*]-[1,4,7,10,13,16]hexaaza-cyclooctadecine-8,19-diacetic acid<sup>7)</sup> (compound **2** in Chart 1), and demonstrated that it exhibited strong fluorescence upon complexation with Eu<sup>3+</sup> in aqueous solution. In this report we describe how ligand **2** can be used to label macromolecules and its application to TR-FIA.

### Experimental

Europium chloride hexahydrate (EuCl<sub>3</sub>·6H<sub>2</sub>O, 99.99%) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used as purchased. The anti-human chorionic gonadotropin (anti-hCG) monoclonal antibodies (HM 70 and HM 21) were obtained from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). The hCG standard was prepared in accordance with 1st IRP 75/537. <sup>1</sup>H- and <sup>13</sup>C-Nuclear magnetic resonance (NMR) spectra were measured using a Bruker AC-200P spectrometer, operating at 200 and 50 MHz, respectively, with tetramethylsilane as an internal

standard. The splitting patterns were designated as follows: s, singlet; m, multiplet; br, broad. Uncorrected fluorescence spectra were recorded on a Hitachi 650-60 spectrometer. Fluorescence spectra were measured on a Hitachi 650-60 spectrometer in 10×10 mm quartz cells. Time-resolved fluorescence measurements were performed on an Arcus 1230 fluorimeter (LKB Wallac, Turku, Finland). Infrared (IR) spectra were taken in KBr disks on a Hitachi 270-30 spectrometer.

**Preparation of the Labeling Reagent** Incorporation of chlorosulfonyl groups into ligand **2**<sup>7)</sup> to afford the labeling reagent (**1**) was carried out in a similar manner to that described by Evangelista *et al.*<sup>1e)</sup> for preparing 4,7-bis(chlorosulfonyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), an analogous but non-inclusion type ligand. Ligand **2** (60 mg) was added, in one portion, to chlorosulfonic acid (2 ml) and the solution stirred at room temperature for 16 h. The reaction mixture was poured into ice water (50 ml) and the precipitate, which formed instantly, was collected by means of suction, washed with water, and dried *in vacuo* to yield 40 mg (56%) of chlorosulfonylated compound **1** as an off-white powder. IR  $\nu_{\max}$  cm<sup>-1</sup>: 2950 (Ar), 1630 (Ar), 1460 (Ar), 1180 (SO<sub>2</sub>), 1130 (SO<sub>2</sub>), 1110, 1040, 1010, 620. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$ : 2.48 (s, 4H, CH<sub>2</sub>), 4.48 (brs, 8H, CH<sub>2</sub>), 7.36–8.66 (m, 16H, Ar). <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>)  $\delta$ : 50.85 (CH<sub>3</sub>), 122.96 (Ar), 125.46 (Ar), 126.10 (Ar), 126.91 (Ar), 128.30 (Ar), 129.02 (Ar), 138.01 (Ar), 143.40 (Ar), 148.64 (Ar), 152.20 (Ar), 152.52 (Ar).

Thin-layer chromatography (TLC, Merck Kieselgel 60, *n*-propanol: ethanol: water: pyridine: acetic acid = 10:5:1:0.1:0.1, v/v/v/v) was carried out on the hydrolyzed product that was obtained by incubating reagent **1** in water. Although a single spot was observed on TLC, it was assumed that product **1** was a mixture of isomers with chlorosulfonyl groups at different positions, as was the case with BCPDA.

**Preparation of the Labeled Antibody** Reagent **1** (10 to 150 molar excess) was mixed with 200  $\mu$ l of the anti-hCG monoclonal antibody (HM 21) in 0.1 M sodium carbonate buffer (pH 9.1). The mixture was incubated for 30 min at 24 °C with stirring. Unreacted reagents were removed using a Sephadex G-50 column (Pharmacia, Uppsala, Sweden)

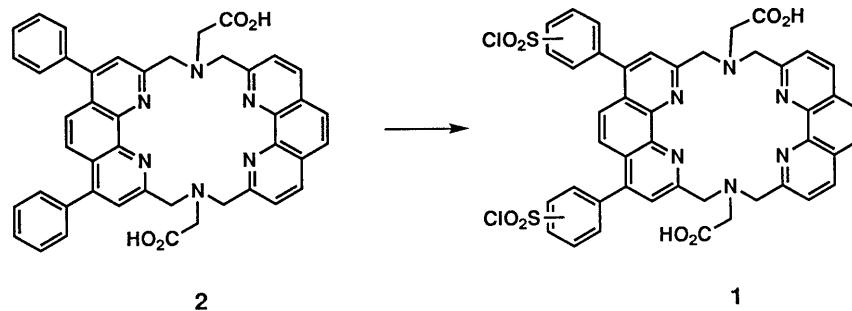


Chart 1

equilibrated with 0.1 M sodium carbonate buffer (pH 9.1). Fluorescence was observed in the void volume, fractions were pooled, concentrated using an ultrafilter unit (Advantec, Tokyo, Japan) and dialyzed against 50 mM Tris-HCl buffer (pH 7.8) containing 0.05% NaN<sub>3</sub> and 0.9% NaCl. From the absorbances at 310 and 280 nm, the concentrations of reagent **1** and antibody in the solution were calculated using the molar absorption coefficients of reagent **1** and antibody at their respective wavelengths.

**TR-FIA for hCG Using the Labeled Antibody** Two-hundred  $\mu$ l per well of the anti-hCG monoclonal antibody (HM 70, 10  $\mu$ g/ml) in 0.1 M sodium carbonate buffer (pH 9.6) was coated to the wells of 96-well microplates (Molecular Devices, Menlo Park, U.S.A.) for 1 h at 37°C. After washing with deionized water, the wells were blocked with 0.5% BSA (300  $\mu$ l/well) containing 0.05% NaN<sub>3</sub> in 0.1 M sodium carbonate buffer (pH 8.3) for 2 h at 24°C. These microplates were then stored refrigerated until required. After washing with deionized water, 100  $\mu$ l/well of both the hCG standard and assay buffer (50 mM Tris-HCl at pH 7.8 containing 0.9% NaCl, 0.5% BSA, 0.05% bovine  $\gamma$  globulin, and 0.01% Tween 40) were added to the antibody-coated wells and the plates incubated for 1 h at 24°C. After washing 5 times with saline containing 0.005% Tween 20 (wash solution), 200  $\mu$ l/well of the labeled antibody (0.25  $\mu$ g/ml) in assay buffer containing  $1 \times 10^{-5}$  M EuCl<sub>3</sub> was added to the wells and the plates incubated for 1 h at room temperature. The wells were washed with wash solution to remove unreacted labeled antibodies and then 200  $\mu$ l/well of EuCl<sub>3</sub> ( $1 \times 10^{-6}$  M) in 0.2 M Tris-acetate buffer (pH 9.0) was added to the wells. After 3 h, the time-resolved fluorescence of the wells was measured using the Arcus fluorimeter.

## Results and Discussion

Since reagent **1** has the two phenyl groups apart from its coordination site, a chlorosulfonyl moiety (SO<sub>2</sub>Cl) was chosen as the functional group for anchoring to the antibody through formation of sulfonamide linkages with the amino groups. The SO<sub>2</sub>Cl group has been employed in a number of cases of labeling, including that of BCPDA, mainly because it can be readily introduced into aromatic moieties. The introduction was achieved in the same manner as for BCPDA, to afford reagent **1** in moderate yield (56%). TLC of the hydrolyzed product, which was supposed to have SO<sub>3</sub>H groups instead of SO<sub>2</sub>Cl groups, showed a single spot with an *R<sub>f</sub>* value less than that of the starting material. Although this observation basically

confirmed that the product had been formed, it was not possible to establish by NMR spectroscopy the position and number of the SO<sub>2</sub>Cl groups incorporated into the molecule.

Although the emission and excitation spectra of the Eu<sup>3+</sup> complex of reagent **1** are relatively unaffected by the reaction, as far as wavelength is concerned, the intensities of the complex in both spectra are reduced by approximately 50% (Fig. 1). The sharp bands at 619 nm are due to the <sup>5</sup>D<sub>0</sub>→<sup>7</sup>F<sub>2</sub> transition of Eu<sup>3+</sup>, at which wavelength the fluorescence lifetimes of compounds **1** and **2** were measured in the same buffer. Both compounds **1** and **2** exhibited first-order kinetics as far as their fluorescence decays after pulsed excitation are concerned. As expected, the attachment of the polar functional groups caused a reduction in the lifetime of ligand **2** from 1140  $\mu$ s to 420  $\mu$ s; this agrees well with the hypothesis that the Eu<sup>3+</sup> of the complex with the more polar reagent **1** is less shielded from bulk water.

The fluorescence of the Eu<sup>3+</sup> complex of reagent **1** is affected by the pH and the nature of the buffering species because of the presence of ionizable groups (Fig. 2). At lower pH values where the intensity is less, the nitrogen atoms of the complex tend to be protonated, rendering the molecule less potent as a ligand. The optimum pH of this complex (pH *ca.* 9) differs from that of the "unfunctionalized" **2** (pH 6). When the detection limits of the Eu<sup>3+</sup> complexes of compounds **1** and **2** were examined, each under optimized pH conditions, by dilution of  $2.5 \times 10^{-6}$  M complex solutions, complexes of up to  $1.2 \times 10^{-10}$  M (*S/N*=2) with compounds **1** (in 0.2 M Tris-acetate buffer at pH 9.0) or **2** (in 0.2 M acetate buffer at pH 5.5) could be detected. However, when the Eu<sup>3+</sup> concentration was held constant at  $2.5 \times 10^{-6}$  M, the detection limits of compounds **1** and **2** were  $1.4 \times 10^{-10}$  and  $3.8 \times 10^{-10}$  M, respectively. This indicates that the detection sensitivities depend on the conditions under which the complexes are formed.

The time course of the complexation reaction of reagent

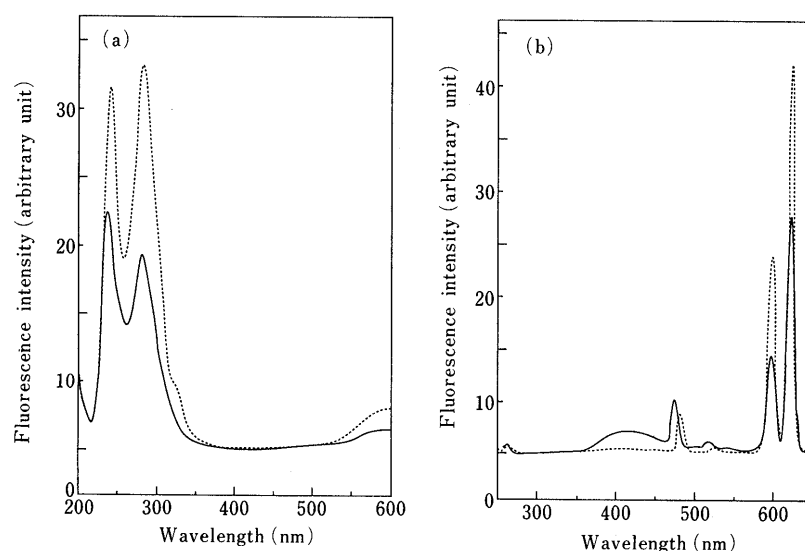


Fig. 1. Excitation (a) and Emission (b) Spectra of Eu<sup>3+</sup> Complexes of Compounds **1** and **2**

The spectra were recorded, 30 min after mixing, with compounds **1** and **2** (both at  $2.5 \times 10^{-6}$  M) and EuCl<sub>3</sub> (at  $2.5 \times 10^{-6}$  M) in 0.2 M Tris-acetate buffer (pH 9.0). The  $\lambda_{\text{ex}}$  was 236 nm (for compound **1**) or 239 nm (for compound **2**) in the emission spectra; the  $\lambda_{\text{em}}$  was 619 nm for both compounds in the excitation spectra.

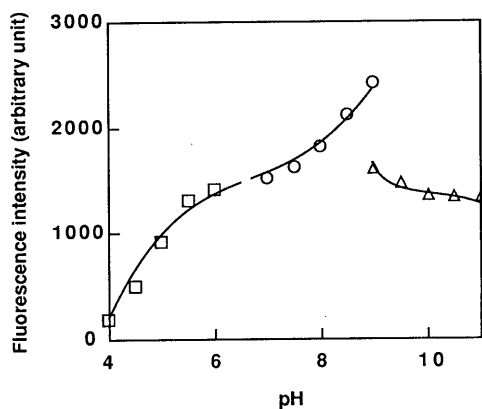


Fig. 2. pH Profile of the Fluorescence of the  $\text{Eu}^{3+}$  Complex of Reagent 1

The measurements were carried out with reagent 1 ( $2.5 \times 10^{-6} \text{ M}$ ) and  $\text{EuCl}_3$  ( $2.5 \times 10^{-6} \text{ M}$ ) in 0.2M acetate buffer ( $\square$ ), 0.2M Tris-acetate buffer ( $\circ$ ) or 0.2M carbonate buffer ( $\triangle$ ); the  $\lambda_{\text{em}}$  and  $\lambda_{\text{ex}}$  were 619 and 240 nm, respectively.

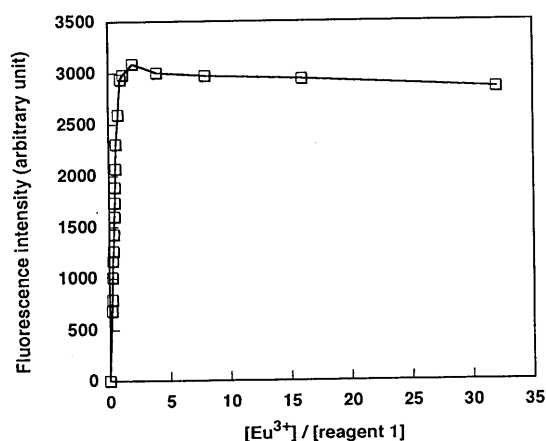


Fig. 3. Fluorescence versus the Molar Ratio of  $\text{Eu}^{3+}$ /Reagent 1

The measurements were performed with reagent 1 ( $2.5 \times 10^{-6} \text{ M}$ ) and various amount of  $\text{EuCl}_3$  in 0.2M Tris-acetate buffer at pH 9.0 and the fluorescence was measured at 619 nm ( $\lambda_{\text{ex}} = 240 \text{ nm}$ ).

1 ( $2.5 \times 10^{-6} \text{ M}$ ) with an equivalent of  $\text{Eu}^{3+}$  in 0.2M Tris-acetate buffer at pH 9.0 and  $22^\circ \text{C}$  showed that complex formation was rather slow, taking approximately 1 h for the fluorescence intensity to reach a maximum.

The experiment in which the fluorescence intensity was measured as a function of the molar ratio of  $\text{Eu}^{3+}$ /reagent 1 clearly demonstrated that the complex was 1:1  $\text{Eu}^{3+}$ /reagent, as was ligand 2 (Fig. 3). The figure also demonstrates that, in the region of  $[\text{Eu}^{3+}]/[\text{reagent 1}] < 1$ , no further complex formation of  $\text{Eu}^{3+}$  takes place with more than 1 eq of reagent. Once the complex has been formed, it is so stable that  $\text{Eu}^{3+}$  is not replaced by adding excess EDTA. This feature is an additional advantage as far as its use as a label in TR-FIA is concerned.

Labeling of the anti-hCG antibody with reagent 1 was carried out at pH 9.1 and the labeled antibody was purified by gel-filtration chromatography. The conjugate was eluted in the void volume following monitoring at 280 nm; the elution profile corresponded to that monitored by fluorescence (in the presence of  $\text{Eu}^{3+}$ ) and by immunoreactivity towards hCG. No products that might be derived from crosslinking of the antibodies were

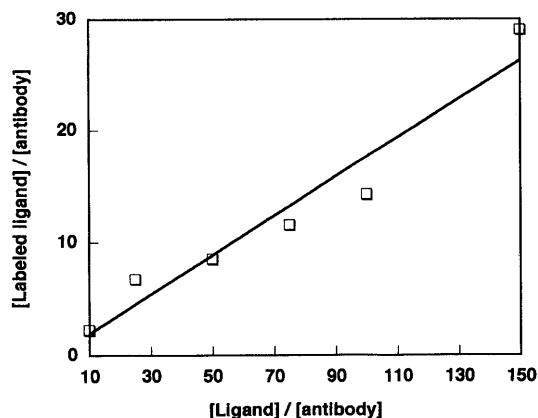


Fig. 4. Labeling Efficiency of Reagent 1 with Respect to Anti-hCG Antibody as a Function of the Molar Ratio of the Reagent/Antibody

The labeling procedure is described under Experimental.

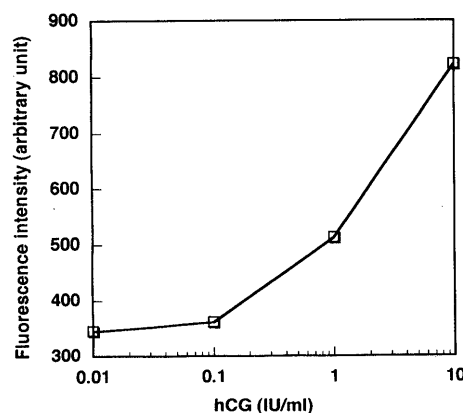


Fig. 5. TR-FIA of hCG Using Reagent 1

The experimental procedure is described under Experimental.

observed. When the molar ratio of reagent 1 per antibody was increased in the labeling reaction, up to 30 molecules of the reagent per antibody could be incorporated, as determined by the absorbance at 280 and 310 nm (Fig. 4). Although the fluorescence intensity of the labeled antibody increases with the number of reagents incorporated, maximum immunoreactivity is obtained when roughly 10 molecules of the reagent are labeled (data not given). The labeling ability of the reagent shown in Fig. 4 thus prompted us to use a 75 molar excess of the reagent for labeling.

The TR-FIA of hCG was carried out with this labeled antibody, with the complex formation being performed after the labeling because we were afraid that the chlorosulfonyl moieties might not survive the complexation step. Thus,  $\text{Eu}^{3+}$  and the second antibody labeled with the reagent were added to each well where the anti-hCG antibody had been coated and reacted with various amounts of hCG. The time-resolved fluorescence of each well was measured after these immunoreactions. The dose-response curve was obtained over the range 0.01 to 10 I.U. hCG/ml, with relatively high background fluorescence (Fig. 5). A more sensitive assay will be possible if the background fluorescence can be reduced. To do this, we believe that the presence of a suitable

spacer between antibody and reagent would effectively remove any interference from the antibody. Although the TR-FIA needs to be more sensitive for practical use, the present method using the macrocyclic  $\text{Eu}^{3+}$  ligand **1** is of potential value as a novel TR-FIA.

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