# Time-Resolved Fluorescence of a New Europium Chelate Complex: Demonstration of Highly Sensitive Detection of Protein and DNA Samples§

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*Received July 13, 1993*

Safety and environmental considerations have made it desirable to develop nonradioactive detection technologies that offer detection limits comparable to those of radioisotopes. Although fluorometric methods are widely used for biochemical and biological analyses, disadvantages of organic fluorochromes, such as overlap of background fluorescence with emission spectra and "inner-filter effects", have resulted in less than optimum detection limits and prevented broad applications.[1] Innovative new fluorometric techniques, however, continue to reduce detection limits and expand utility into specific new applications.[2,3] The luminescence properties of europium chelates include exceptional Stokes' shifts (200–300 nM), narrow emission line spectra, long wavelength emission (615 nM), and long fluorescence lifetimes (600–1000 μs).[4] While each of these characteristics is important, the latter is key to achieving low detection limits, in that short-lived background fluorescence and scattered excitation radiation can be efficiently eliminated by measuring fluorescence emission at a delay (100–400 μs) after the excitation pulse.

A handicap of $Eu^{3+}$-based detection of bioanalytes has been the quenching of fluorescence in aqueous systems due to direct coordination of water molecules to $Eu^{3+}$ chelates and radiationless energy transfer from excited states to the aqueous solvent shell.[4c,5] This problem has been partially addressed by releasing the $Eu^{3+}$ from analyte chelates and recomplexing in low-pH micellar solutions.[6] Such systems suffer from sensitivity to exogenous $Eu^{3+}$ ion contamination, require expensive reagents, and are incompatible with most biological media.[7] Attempts to improve aqueous luminescence characteristics of $Eu^{3+}$ chelates have remained largely unsuccessful.[8] We describe a $Eu^{3+}$ complex that is relatively immune from fluorescence quenching by water and that demonstrates detection levels to those of radioisotopes in most biological systems.[9]

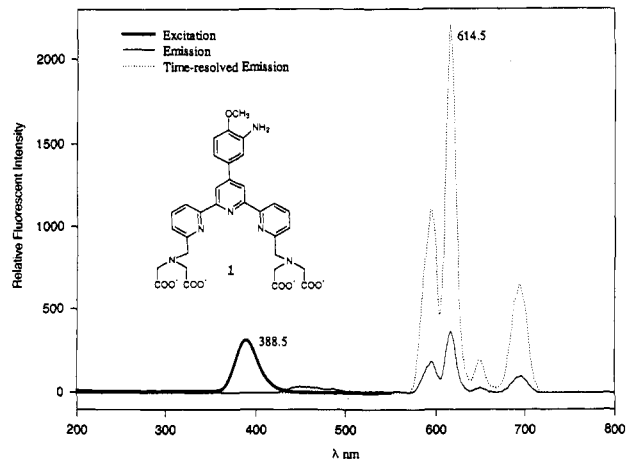


**Figure 1.** Excitation and emission spectra of TMT–$Eu^{3+}$. Measurement carried out on a Perkin-Elmer Model LS-50B fluorimeter. TMT–$Eu^{3+}$ is prepared by mixing equal volumes of $EuCl_3$ and TMT–isothiocyanate reagent at $8.3 \times 10^{-4}$ M in 0.05 M Tris–HCl, pH 7.4; cutoff filter, 430 nm, slit width, 10 nm. For time resolved spectra: delay time, 1 ms, gate time, 1 ms. Fluorescence lifetime: 1400 μs.

Bifunctional terpyridine chelates including 4′-(3-amino-4-methoxyphenyl)-6,6″-bis[*N*,*N*-bis(carboxymethyl)aminomethyl)-2,2′:6′,2″-terpyridine [trivial name, terpyridine-bis(methylenamine)tetraacetic acid (TMT)] (**1**) were designed for complexation of lanthanides.[10] In connection with efforts to develop new antibody tumor-targeting agents, we considered TMT–$Eu^{3+}$ as a probe for determination of binding and hybridization. The TMT–$Eu^{3+}$ complex has high molar absorptivity ($\epsilon$ = 26 970 at 287 nM; 15 278 at 319 nM; pH 9.0). TMT forms a nine-coordinate complex with $Eu^{3+}$, occupying all $Eu^{3+}$ coordination sites. This results in a high binding constant, allowing the chelate to retain europium ion under most conditions.[11] The aromatic terpyridyl system also provides the chelated $Eu^{3+}$ ion with a relatively hydrophobic environment necessary for maximal fluorescence and fluorescence lifetime. The excitation and emission spectra of the TMT–$Eu^{3+}$ complex (Figure 1) clearly demonstrate all of the above characteristics. It further shows a >575% enhancement in relative fluorescence intensity when a time delay is employed.

For investigation of optimal time-resolved properties of the TMT–$Eu^{3+}$ complex, we selected a single-photon-counting, time-resolved fluorimeter [excitation 325 nm (±40 nM), emission 613 nm (±3 nM)].[12] Raw fluorescence readings in aqueous systems for TMT–$Eu^{3+}$ complex (in counts/s) were >$10^4$-fold higher than those for representative commercial chelate diethylenetriaminepentaacetic acid (DTPA). Further, fluorescence emission was relatively independent of buffer media, salt concentration, pH (3–9), and the presence of free $Eu^{3+}$ ion.[13] A study of response in counts/s versus decreasing concentrations of the TMT–$Eu^{3+}$ complex revealed a lower detection limit of $3 \times 10^{-17}$ mol.

The 3-amino substituent in the phenyl ring in TMT gives it a bifunctional nature, allowing for facile conjugation to biomac-

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§ Presented at the 206th ACS National Meeting. See: *Abstracts of Papers*, 206th National Meeting of the American Chemical Society, Chicago, IL, August 22–27, 1993; American Chemical Society: Washington, DC, 1993; ORG 412.

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(10) U.S. Patents 4,637,988, **1987**; 4,837,169, **1989**.
(11) Competitive binding experiments indicate that TMT readily displaces europium ion from EDTA–Eu complex: Yuan, Lung-Chi, unpublished results.
(12) Delfia 1232 Fluorometer, Wallac Inc., Gaithersburg, MD.
(13) Thus, for example, the relative ratio of fluorescence readings in counts/s of TMT–$Eu^{3+}$ complex at pH 3.0 (citrate buffer) to that at pH 9 (borate buffer) was 1.14, indicating that Eu ion remains tightly bound to the TMT chelate even at acid pH.

0002-7863/93/1515-11032$04.00/0 

romolecules.[14] This group is readily converted to the isothiocyanate function, and the resulting molecule is stable for 3–6 months of storage at −20 °C without detectable decomposition.[15] TMT was conjugated via reaction of TMT–isothiocyanate to a 20-mer oligonucleotide containing primary amine groups at the 3′- and 5′-terminals.[16,17] The resultant molecule demonstrated no change in its binding affinity to complementary DNA as determined from melting temperature ($T_m$). The number of TMT molecules per DNA molecule was determined to be approximately 2 by titration.[18,19] TMT was conjugated to a chimeric monoclonal antibody molecule[20] in a similar fashion. The extent of substitution was controlled by the amount of TMT–isothiocyanate reagent used, reaction temperature, and duration of reaction. Titration against increasing amounts of $EuCl_3$ showed that two molecules of TMT were covalently bound to each antibody molecule. In both cases, the response versus concentration curve was linear throughout the $10^6$-fold concentration range studied. The detection limit for the DNA molecule was $1.5 \times 10^{-16}$ mol, and that for the antibody was $6 \times 10^{-16}$ mol (Figure 2). These limits should further decrease as the number of labels per molecule is increased.

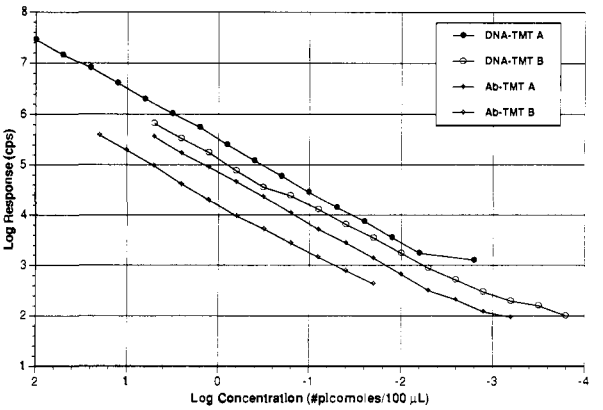


**Figure 2.** Linear response curves of TMT conjugates. Measurements were done in duplicate in 96-well microtiter plates. Background counts/s are 600–800 and are automatically subtracted from sample readings. A and B represent distinct preparations. The extent of conjugation is determined by titration and is approximately 2. Variation in the response vs concentration curves as observed is due to variation in the exact extent of TMT substitution achieved.

In conclusion, the unique advantages of time-resolved $Eu^{3+}$ fluorescence have been combined with those of the TMT chelator to create a very sensitive aqueous nonradioactive detection system. The results described demonstrate promise for general utility of this technology in a variety of biological applications. Additional results including synthesis and crystal structure of TMT–$Eu^{3+}$ and applications to flow-cytometry will be reported separately.

**Acknowledgment.** We thank Liz Wanner for the peptide–TMT standard and Mark Sardaro for DNA synthesis.

**Supplementary Material Available:** Graphical data for peptide– and DNA–TMT–$Eu^{3+}$ titrations and concentration vs response of TMT–$Eu^{3+}$ alone (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(14) For a review on biomolecule conjugation and characterization, see: Brinkley, M. *Bioconjugate Chem.* **1992**, *3*, 2–13.

(15) The TMT–NCS reagent was prepared in multigram scale by the chemical development group, Sterling Winthrop Pharmaceutical Research Division.

(16) Amine synthons are commercially available (Clonetech, Palo Alto, CA) for rapid utilization in automated DNA synthesis.

(17) General procedure for preparation of TMT conjugates. (a) The DNA–TMT conjugates used in this study were prepared by mixing a solution of the amine-modified DNA molecule (300 nmol) with an excess of TMT–isothiocyanate (50 equiv, 12 mg) at pH 9.0 (carbonate/bicarbonate buffer) for 2 h at 37 °C and then overnight at room temperature. The reaction was quenched by the addition of a 1 M ethanolamine solution and then purified by Sephadex G-25 column chromatography using UV detection at 280 nm. The fractions containing DNA were pooled and characterized by 12% PAGE. (b) The antibody–TMT conjugate was prepared by the same procedure except a 10-fold molar excess of TMT–isothiocyanate reagent was used and the reaction was conducted for 1–3 h at 4 °C. The conjugate was purified by size-exclusion chromatography, and the purified conjugate was characterized by amino acid analysis, peptide mapping, SDS–PAGE, and size-exclusion HPLC (data not shown). The immunoreactivity was analyzed by competitive ELISA and flow cytometry and was >82%.

(18) General procedure for titration and labeling of TMT conjugates with europium. A known amount of conjugate was titrated against increasing amounts of $EuCl_3$ in an aqueous buffer. The conjugate, typically 1 µL from a dilute sample containing 1–30 pmol was added to wells in duplicate in a Costar EIA/RIA 96-well plate containing a precalculated amount of Tris–HCl buffer (pH 7.4). The buffer volume was derived by subtracting the volume in microliters of aqueous $EuCl_3$ (typically $10^{-4}$–$10^{-6}$ M in Tris–HCl buffer) from 99 µL. Thus total volume in each well was fixed at 100 µL. Aqueous $EuCl_3$ is added last. The plate was covered with a lid and shaken at low speed for 1 h. The time-resolved fluorescence was then measured using a Delfia 1232 time-resolved fluorimeter (Wallac Inc.). Averaged data in counts/s were plotted against increasing concentration of $EuCl_3$, and the concentration at which fluorescence emission started decreasing was considered the end-point of titration. For preparation of standard curves, the conjugates were labeled with a little over 2-fold molar excess of $EuCl_3$.

(19) A hexapeptide containing a single TMT molecule was titrated as a control.

(20) The preparation, characterization, and properties of the antibody used in this study are described: Robinson, R. R.; Chartier, J., Jr.; Chang, P. C.; Horwitz, A. H.; Better, M. *Hum. Antibod. Hybridomas* **1991**, *2*, 84–93.