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An Intensely Luminescent Polymeric Lanthanide Chelator for Multiple Fluorescence Labeling of Biomolecules.

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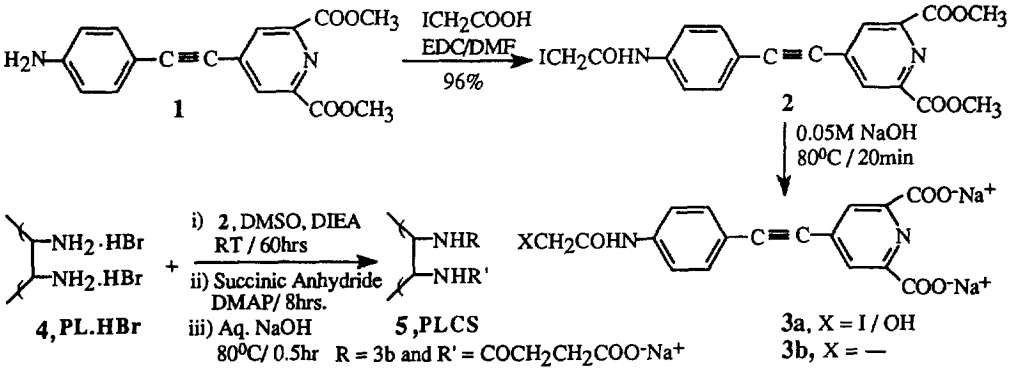
Abstract : A new bifunctional chelating agent has been synthesized and used to prepare a stable, intensely luminescent polymer, PLCS-Eu(III), with an extinction coefficient of 10^{+6} $(M\text{-}cm)^{-1}$, a very large stokes shift (Ex 337, Em 617nm), a 5nm emission bandwidth and a quantum yield of 0.6. The PLCS-Eu(III) complex was found stable towards EDTA and prolonged irradiation. It was coupled,as a coating, upon 3μm beads which in turn were coupled to oligonucleotides, for use in a nucleic acid sandwich assay. Its millisecond emission lifetime will allow it to be used for time resolved fluorescence detection. PLCS-Eu(III), a novel chelator, may be a useful new tool for array based studies of biopolymer interactions. Copyright © 1996 Elsevier Science Ltd

Recently, nonisotopic labeling methods have become a prominent technique in immunochemistry, nucleic acid hybridization and other biotechnology applications[1]. In addition to conventional fluorophores[2], aromatic lanthanide chelates have been developed for this purpose[3]. The power of these chelates is that they display a large stokes shift and a long emission lifetime, which can serve as the basis for the time resolved fluorescence method. Unfortunately, some of the commercially available reagents are nonfluorescent[4a,b], while others are either handicapped by poor quantum yield[4c] or must be excited in the UV (240-300 nm) region, which can compromise detection due to endogenous fluorescence from supports and biological chromophores[2,3].

Here, we report the synthesis of a novel bifunctional chelating agent **3a**, a polymeric chelator **5** and its application for labeling DNA molecules as the stable Eu(III) complex. Compound **1** was obtained, in better yields than reported earlier[5] with a combination of solvents in place of triethylamine alone. Compound **2** was obtained by the iodoacetylation of **1** using iodoacetic acid and EDC, in excellent yield[6] (96%). Mild base hydrolysis of **2** generated its disodium salt **3a**. As expected for other dipicolinic acids[7], we have observed maximum fluoresence at a 3:1 monomer:lanthanide ratio. The optical properties of the 3:1 Eu(III) complex with chelate **3a** (fig-1, and Table-1) reveal strong absorbance in the 330-360nm range, a very large stokes shift, with a sharp emission line at 617nm (fig.1, inset). Together, these properties are well suited for luminescence detection with a near UV laser, or broadband irradiation as from a standard UV transilluminator. Chelator **2** can be coupled to molecules containing amino or thiol groups[8]. Multiple fluorescence labeling can be achieved by first coupling chelator **2** onto a polymer backbone. As an example, Scheme-1 describes controlled (40-80%) coupling to poly-L-lysine, followed by succinylation of the remaining lysine side chains and hydrolysis of the methyl ester to yield the polymeric chelator, PLCS[9]. The pendant succinyl groups allow a route for facile coupling the PLCS reagent to biomolecules containing primary amines. Maximum fluorescence for the PLCS-Eu(III) complex is obtained at

a 3:1 ratio of **3b** chelator equivalents to lanthanide ion. The yield per bound Eu(III) is approximately 10 times greater than for the corresponding 1:1 complex (fig-2). Interestingly, when **3b** is present on the polymeric



Scheme-1 : Synthesis of bifunctional chelator **3a** and PLCS **5**

backbone, the quantum yield of the 3:1 complex is found to be twice that obtained with the 3:1 complex with free monomer **3a**(Table-1). As seen in fig-2 (inset) the PLCS -Eu(III) complex is stable for at least 12 hours subsequent to addition of a 10^{+5} mole excess of Na_4EDTA. Under similar conditions the **3a**:Eu(III) complex dissociates upon addition of only one EDTA equivalent per lanthanide.The enhanced stability of the PLCS-Eu(III) complex towards competition by chelators or a high concentration of monovalent ion is in agreement with earlier results[10] for such chelates, and shows that the complex can be used as a chemically stable dye entity. Because the 3:1 PLCS complex has an emission lifetime in the millisecond range (Table-1), it can be used as such a fluorescent dye in context of both standard and time resolved fluorescence immunoassays [11]. In order to demonstrate the ease with which the PLCS-Eu(III) complex can be used as a fluorescence label, we have coupled it to

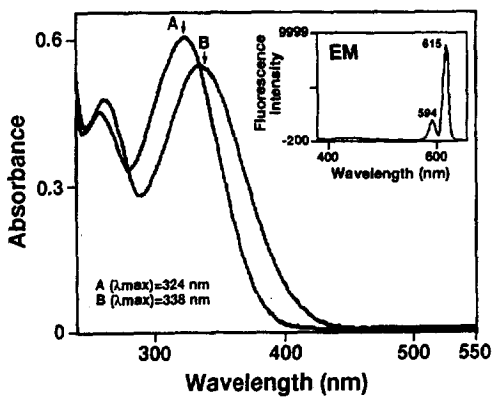


Figure-1

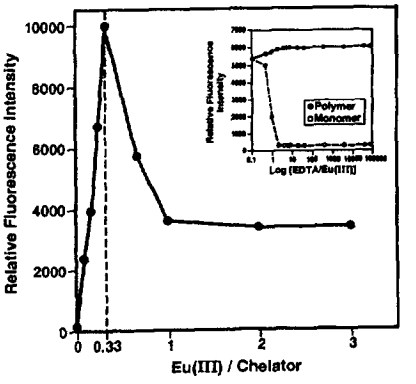


Figure-2

Figure-1 : Optical Properties of the Monomer : Absorption spectra of 3a as a sodium salt **(A), or** as the Eu(III) complex at a 3:1 stoichiometry (B). **Inset :** Emission spectrum of B, exciting at 337 nm. **Figure-2 : Effect of Stoichiometry and Competitors on Eu(III) Fluorescence :** 1uM of **3a**, on PLCS, was titrated with increasing mole ratio of $EuCl_3$ in 0.1M Tris-HCl, pH 7.8. The dotted line at 0.33 corresponds to a ratio of 3 monomer side chain equivalents per Eu(III). **Inset :** Up to 10^{+5} mole equivalents of Na_4EDTA was added to 0.25 uM Eu(III) as the 3:1 complex with PLCS (●) or as the 3:1 complex with **3a** monomer (O).

Table-1 : Optical Properties of Chelates :

Property	Solvent	Monomer	Polymer
Solubility (as Na-salt)	H_2O, pH 8-9	6mg/mL	2-3mg/mL
Extinction Coefficient[12]	0.1M Tris, pH 7.8	30,288 $M^{-1}cm^{-1}$	0.6-1.2x$10^6M^{-1}cm^{-1}$
λ Max (Na salt)	0.1M Tris, pH 7.8	326 nm	324 nm
λ Max (3:1 Eu complex)	0.1M, Tris,pH 7.8	338 nm	334-338 nm
λ Excitation/Emission	0.1M Tris, pH 7.8	334nm/ 617nm	337 nm/ 617 nm
Fluorescence Lifetime[13]	0.1M Tris, pH 7.8	---	0.58 ms, 2 ms
QuantumYield/Solution[14]	0.1M Tris, pH 7.8	0.32	0.60
Quantum Yield/Surface[15]	0.1M Tris, pH 7.8	-- -	0.70

polylysine modified 3μm polystyrene beads[16a]. 3'-amine modified-oligonucleotides were then coupled to the beads via the unused succinyl groups on PLCS[16b] (Scheme-2). Labelled beads were purified by repeated centrifugation, then assessed for the extent of Eu(III) and oligomer coupling (Table-2).

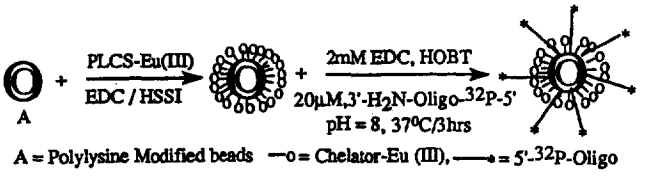


Table-2 : Labelled Beads

Chelator groups / bead = 450x10^6
Eu(III) Molecules/bead = 150x10^6
Number of Oligos/bead = 1.6x10^5

Scheme-2 : Synthesis of 3 um Beads-PLCS-Eu(III) Complex and Beads-PLCS-Eu(III)-Oligos Complex

Figure-3 displays a photograph of luminescence obtained from microspots dried onto Whatman filter paper, irradiated with a long UV transilluminator. PLCS-Eu(III) coated beads (panels C and D) and the beads which had been subsequently coupled to oligonucleotides (panels E and F) not only remain luminescent upon drying, but also remain insensitive to repeated 0.5M EDTA(pH 7.8) washings (Table-3). Such PLCS-Eu(III)-oligomer beads, and other approaches to nucleic acid coupling are currently being used to detect duplex formation on solid supports. The results will be reported in due course.

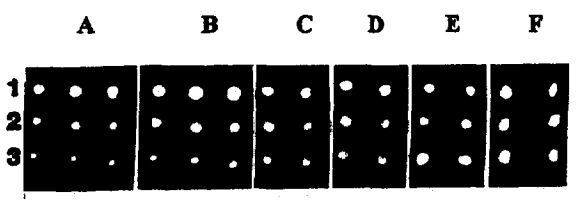


Figure-3

Table-3 : Quantitation of Bead Microspots

Lane	A	B	C	D
1	0.72	0.78	0.39	0.49
2	0.44	0.49	0.41	0.44
3	0.23	0.27	0.35	0.46

Figure-3 : Fluorescence of PLCS-Eu(III) (Panels A and B),Beads- PLCS-Eu(III) (Panels C and D) and Beads-PLCS-Eu(III)-Oligos (Panels E and F) : 1 uL droplets dried on Whatman filter paper and data obtained before (A,C and E) and after 0.5M Na_4EDTA washing (B, D and F) respectively. **Table-3** : Numbers in columns A and B are averages of triplicates of 0.2,0.1, 0.05 nmoles of Eu(III) (Panels A and B, rows 1-3) and in C and D are averages for spots in Panels C/ D respectively and correpond to 0.1nmoles of Eu(III). Similar results were obtained for microspots in panels E and F.

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6. Recrystalized from MeOH, m.p. 230-233^{0}C. ^{1}H NMR(DMSO-d_6): δ 11.3 (s, 1H, CONH);8.4(s,2H, DPA); 7.8(2xd, 4H, Ar-H);4.4(s, 2H, ICH_2); 4.1(s, 6H, 2x$COOCH_3$). ^{13}C NMR (DMSO-d_6) : δ 164, 163, 148, 138, 134, 133, 129, 119, 112, 96, 85, 53 and 42. High Resolution Mass: Calculated for $C_{19}H_{15}IN_2O_5$: 478.002574 and Observed : 478.003060, at 12000 ppm resolution.
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8. A detailed investigation of DNA target labelling and fluorescence detection of hybridization to solid surfaces will be reported elsewhere.
9. 10,000 MW poly-L-lysine.HBr(15μmol as lysyl units) was reacted with **2** (30μmol) in dry DMSO (1mL) and DIEA(250μmol), followed by succinylation with succinic anhydride(200μmol) and DMAP (30μmol). The resulting product was hydrolysed in 0.1M NaOH, followed by purification via dialysis (Spectrapor-6 tubing) and Centricon-3 concentration.The degree of modification of the lysyl groups was monitored by a TNBS test for primary amines as reported by Means, G.E.; Feeney, R.E. (1971), *Chemical Modifications of Proteins*, Holden-Day Inc. San Francisco, p217.
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12. Extinction coefficient of the monomer has been determined gravitometrically, and that of the PLCS polymer based upon a standard yield of 20-40 **3b** equivalents per poly-L-lysine, as determined in 8 and assuming that the extinction coefficient of the monomer is not altered in the polymer.
13. Emission lifetime of the 3:1 PLCS:Eu(III) complex has been measured with a home-built device, based on a chopped Ar-laser (for details see Lamture, J. B.; Zhou, Z; Kumar, A.S.; Wensel, T. G. *Inorganic Chem.* **1995**, *34*, 864), generously provided by Dr. Wensel. Decay was adequately fit as the sum of two components, 0.58 and 2ms, amounting to 0.6 and 0.4 of the overall amplitude, respectively.
14. Quantum yield in solution has been measured at the absorption and emission maxima of the chelator, on a Hitachi F-2000 fluorimeter, for excitation at 337 nm. Ethidium bromide/ duplex DNA of equal absorption at 334 nm was used as a standard. Ethidium bromide, when complexed with duplex DNA, has a quantum yield of 0.8. (source : Molecular Probes).
15. Solid surface quantum yield was obtained by measuring the luminescence of 1uL wet / dried microspots of the PLCS-Eu(III) and Ethidium bromide-DNA duplex deposited on polyethylene film and illuminated with a broadband long wavelength transilluminator. The luminescence densities were quantified on a BioRad 620 densitometer by comparing the yield of dried and undried microdroplets.
16 a) Eu(III) molecules / bead were determined by quantifying fluorescence intensities of one uL spots of the labelled beads (fig-3, panels C and D) against a known concentration of PLCS-Eu(III) as the 3:1 complex (fig.-3 panels A and B). Data were quantifed on a BioRad 620 densitometer. Chelators per bead were known from ref.8. b) The number of oligos(5'-GGAGGGATC-N) per bead were determined by use of ^{32}P-labeled oligomers at known specific activity. Quantitation was perfomed via scintillation counting. Beads per unit volume were determined on a Hausser Scientific Neubauer Hemacytometer-3520.

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