The Addition of a Second Lanthanide Ion to Increase the Luminescence of Europium(III) Macrocyclic Complexes

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ABSTRACT

At present, the microscopic visualization of luminescent labels containing lanthanide(III) ions, primarily europium(III), as light-emitting centers is best performed with time-gated instrumentation, which by virtually eliminating the background fluorescence results in an improved signal to noise ratio. However, the use of the europium(III) macrocycle, Quantum Dye™, in conjunction with the strong luminescence enhancing effect (cofluorescence) of yttrium(III) or gadolinium(III), can eliminate the need for such specialized instrumentation. In the presence of Gd(III), the luminescence of the Eu-macrocycles can be conveniently observed with conventional fluorescence instrumentation at previously unattainable low levels. The Eu(III) $^5D_0 \rightarrow {}^7F_2$ emission of the Eu-macrocycles was observed as an extremely sharp band with a maximum at 619 nm and a clearly resolved characteristic pattern. At very low Eu-macrocycle concentrations, another sharp emission was detected at 614 nm, arising from traces of Eu(III) present in even the purest commercially available gadolinium products. Discrimination of the resolved emissions of the Eu-macrocycle and Eu(III) contaminant should provide a means to further lower the limit of detection of the Eu-macrocycle.

Keywords: Luminescence, lanthanide, macrocycle, europium, gadolinium, cofluorescence.

1. INTRODUCTION

1.1 Current Limitations in the Utilization of Lanthanide-Containing Luminescent Markers.

The use of time-gated luminescence microscopy holds the promise of maximizing the detectability and quantitation of markers containing the europium(III) macrocyles, Quantum Dyes (Figure 1a), as well as other luminescent lanthanide complexes. However, at present this instrumentation is costly and not widely available; furthermore, time-gated measurements often involve loss of signal or precision. Gated image-intensifiers coupled to CCDs[1], high speed rotating choppers and ferro-electric shutters[2] have been employed for such measurements, but none of these is suitable for the clinical pathology laboratory.

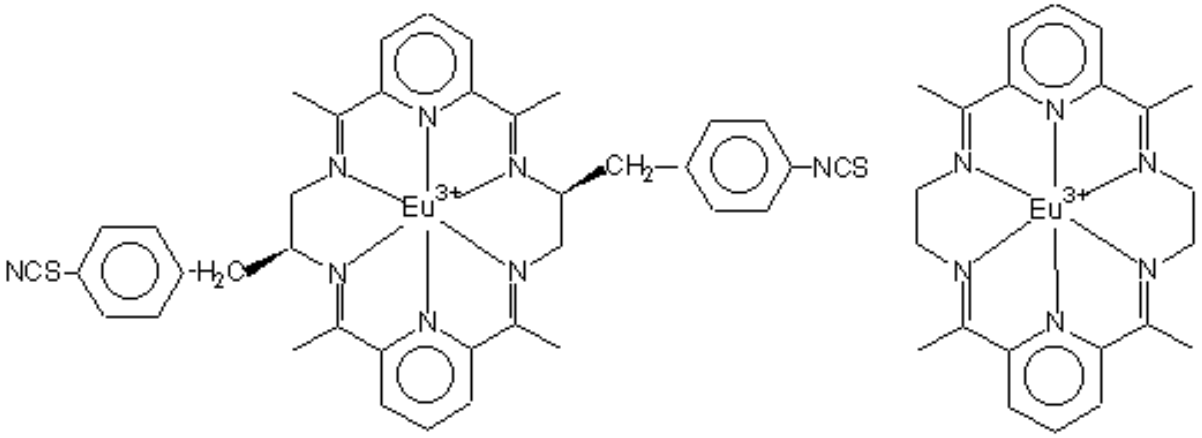

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Figure 1 Schematic formulas of: (a) One of the isomers of the cationic macrocyclic moiety present in the {Eu{macrocyle$(NCS)_2$}Cl_3 complex, Quantum Dye (other isomers differ in the relative points of attachments and steric orientation of the two NCS-functionalized side-chains). (b) The cationic moiety of the prototype complex [Eu(macrocyle)$(acetate)_2$](acetate) used as model in the cofluorescence studies reported in this paper.

Another approach for increasing the luminescence intensity, and hence the detectability, of lanthanide-containing markers is the use of microscopic phosphor particles excited with an infrared laser diode[3]. Although this technology is scientifically interesting and has the advantage of employing an inexpensive light source, it presents significant difficulties for one of its intended applications--the detection of bacteria by flow cytometry[4]. Both these lanthanide-containing phosphors and the Quantum Dyes have long excited-state lifetimes, ranging from a few hundred microseconds to more than one millisecond. For this reason we had anticipated[5], as later confirmed by Condrau et al.[6], that this feature would preclude the use of these markers in flow cytometry at reasonable flow rates.

Recently, employing the cofluorescence technology described below and in collaboration with the Los Alamos National Laboratory (Crissman et. al., unpublished results), we succeeded in obtaining strong signals in flow cytometry using the Quantum Dyes on total-protein stained cells. However, the conditions used in this work were quite different from those required for the detection of antigens present in comparatively small amounts in a dilute sample.

The use of lanthanide-containing phosphors as luminescent markers also has inherent limitations, in that the binding of rigid particles to cells is limited to a small contact zone and furthermore multiple binding is restricted by the mutual steric hindrance of the particles. In contrast, macromolecular carriers labeled with either conventional dyes or Quantum Dyes present minimal steric hindrance, permitting single or bivalent species to be bound at a higher concentration by cells and other substrates.

The preceding considerations point out the need for further studies aiming at the development of alternative approaches for maximizing the luminescence intensity of the Quantum Dyes. The present paper reports the results of one such study.

1.2 Enhancement of Lanthanide Luminescence by Energy-Donor Additives (Co-Fluorescence)

The luminescence enhancement, or "cofluorescence", caused by certain lanthanide(III) and lanthanide-like salts on aqueous solutions of europium(III) containing chelating β-diketonates as well as synergistic additives was first reported by Melentieva et al.[7], and has since been the object of several investigations[8,9,10,11,12,13,14] and of a recent patent. In this patent, Xu reported[15] that the luminescence of the β-diketonate complexes of samarium(III), europium(III), terbium(III) and dysprosium(III) was greatly enhanced by the presence in solution of yttrium(III) and other synergistic additives capable of acting as energy-transfer donors. A different example of the same effect was reported by Shakhverdov et al.[16], who obtained near-infrared luminescence from neodymium(III) and ytterbium(III) in aqueous media at room temperature, employing eosin as the energy transfer dye. In a model study, the concentration of eosin was maintained at 10^{-3} M for single photon excitation and at 10^{-1} M for two-photon excitation with a 1064 nm Nd(III) laser. The luminescence was solvent-dependent. In water, the excited-state lifetime was estimated to be 0.17 ms on the basis of the low quantum yield. These observations are especially remarkable because the luminescence of Nd(III) is usually quenched in the presence of water and becomes strong only in totally aprotic solvents.

Preliminary work by our group had shown[17] that the cofluorescence effect reported for the Eu(III) ion can, under appropriate conditions, be extended to the Eu-macrocycles, Quantum Dyes, and also to the prototype complex, [Eu(macrocycle)$(acetate)_2$](acetate), the cationic moiety of which is shown in Figure 1(b). In the present paper, we report a systematic study aimed at optimizing the cofluorescence effect of these compounds and also at evaluating its usefulness and limitations for practical purposes, such as conventional fluorescence microscopy utilizing multiple markers. Since the excitation spectra of the Eu-macrocycles and those of several DNA-specific dyes, including DAPI, occur in the same region of the ultraviolet, both types of compounds can be simultaneously excited at the same wavelength. The emission spectra, however, are very different-- the broad emissions of the organic dyes occurring in the blue and the very narrow emissions of the Eu-macrocycles occurring in the red. This difference allows the major europium emission at 619 nm to be unambiguously detected even when its intensity is much lower than that of the (very strong) emissions of the DNA-specific organic dyes. The cofluorescence effect described in this paper, as it significantly and selectively enhances europium luminescence, will facilitate the direct observation of Eu-macrocycle labels both in aqueous solution and in air-dried preparations.

2. EXPERIMENTAL METHODS

2.1. Materials.

Europium(III) chloride hydrate, cetyltrimethylammonium bromide, Triton X-100, hexamethylenetetramine, N[tris(hydroxymethylmethyl]glycine (Tricine), 1,10-phenanthroline, trioctylphosphine oxide, aspartic acid, 4,4,4-trifluoro-1(2-furyl)-1,3-butanedione (furoyltrifluoro-acetone) and 4.4.4-trifluoro-1(2-naphthyl)-1,3-butanedione (naphthoyltrifluoroacetone), all from Aldrich Chem.Co., were checked for purity by IR and/or ^{1}HNMR spectra and were used as received. 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione (thenoyltrifluoroacetone, from Aldrich) was purified by recrystallization from ethanol(charcoal)/hexane and stored at 4^{o}C in a dark glass container. All common reagents and solvents were of reagent grade and were used as received. Avidin (phosphate-free lyophilized powder, m.wt. ca 64,000) was obtained from Molecular Probes. Biotinylated agarose beads (4% biotin immobilized on agarose beads by epoxy activation) were obtained from Sigma. Sephadex G25 (Pharmacia) was conditioned prior to use by heating at ca. 90^{o}C for 1.5 hr in the buffer to be used for chromatography. The complexes [Eu(macrocyle)(acetate)$_2$](acetate), [Gd(macrocycle)(acetate)$_2$](acetate) and {Eu{macrocyle(NCS)$_2$}Cl$_3$ were obtained as previously described[18,19,20]; the Gd(III) complex was synthesized using as the starting material a sample of Gd(III) acetate prepared from the high-purity oxide (see below). The oxides of Gd(III), La(III) and Y(III), from Alfa Aesar, were of 99.999% purity (Eu < 0.3 ppm); a sample of the Gd(III) oxide was used to make a high-purity acetate by dissolving the oxide in hot acetic acid and evaporating the resulting solution to dryness under reduced pressure. Only high purity deionized and Micropore-filtered water was used to prepare solutions and for the final rinsing of glassware.

2.2. Equipment and Instruments.

All glassware was cleaned with a methanol/conc.HCl mixture (90/10 v/v), rinsed with deionized water and methanol, and dried at 60^{o}C. Atomic absorption analyses of europium were preformed on a Varian SpectraAA instrument (Eu standard from Aldrich). Emission and excitation spectra of solid samples were obtained at Virginia Commonwealth University (VCU) using a SLM-8000 photon-counting spectrofluorometer in the reflectance mode, with the incidence angle of the exciting radiation on the sample surface set at 55 degrees. Emission and excitation spectra of solutions for Figures 2, 4, 5, and 7 were obtained with the SLM-8000 instrument (at VCU) and those for Figures 3 and 6 with a SPEX 1692T spectrofluorometer (at Los Alamos). Solutions were examined in stoppered triangular quartz cuvettes, so oriented that the excitation beam entered the diagonal face at a 45 degree angle and the emitted light was collected through the bulk of the sample at 90 degrees relative to excitation. Slits (both excitation and emission) were set at 16 mm for the SLM instrument; for the SPEX instrument, slits were varied as required. All experiments and measurements were performed at ambient temperature unless stated otherwise.

2.3. Preparation of Solutions for Cofluorescence Studies.

2.3.1 Stock Solutions.

(1) *Surfactants*: (a) Cetyltrimethylammonium bromide (CTAB), 1.00x10^{-3} M in water, (b) Triton X-100, 2% m/v in water; (2) *Buffers:* (a) Hexamethylenetetramine, 10% m/v, 0.71 M in water, adjusted to pH 6.0 with HCl (HMTA buffer), (b) Hexamethylenetetramine, 10% m/v, 0.71 M in water (HMTA base), (c) Tricine 10% m/v, 0.56 M in water, adjusted to pH 6.2 with HCl; (3) *Synergistic Ligands:* (a) 1,10-phenathroline (phen), 5.50 x 10^{-3} M in ethanol, (b) trioctylphosphine oxide (TOPO), 5.00x10^{-2} M in ethanol, (4) *Diketones:* (a) 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione (HTTFA), 1.00x10^{-2} M in 5:95 ethanol-water; (b) 4,4,4-trifluoro-1(2-furyl)-1,3-butanedione), 1.00x10^{-2} M in 5:95 ethanol-water; (c) 4,4,4-trifluoro-1(2-naphthyl)-1,3-butanedione, 1.00x10^{-2} M in 5:95 ethanol-water; (5) [Eu(macrocyle)(acetate)$_2$](acetate), (Eu-Mac), 2.85x10^{-5} M in ethanol (as primary stock from which more dilute stock solutions were made as necessary), (6) *Lanthanide(III) Trichlorides:* (a) EuCl$_3$, 4.00x10^{-6} M in water, as primary stock from which more dilute solutions were made as necessary, (b) YCl$_3$, 4.00x10^{-3} M in water, (c) LaCl$_3$, 4.00x10^{-3} M in water, (d) GdCl$_3$, 4.00x10^{-3} M in water. The solutions of the Y(III), La(III) and Gd(III) chlorides were prepared by dissolving the metal oxides, M$_2$O$_3$ (5.00x10^{-4} mol), in a minimal volume of 15% hydrochloric acid with mild heating, evaporating the resulting solutions under reduced pressure, and dissolving the products in water to a total volume of 100 mL.

2.3.2 Cofluorescence Solutions for Screening.

(1) In experiments intended to determine conditions of optimized luminescence, the Eu-Mac or EuCl$_3$ concentration was kept constant (2.3x10^{-7}M); this value was chosen to provide a range of luminescence intensities suitable for measurement with the SLM-8000 instrument. Each solution also contained the following, selected from the list given in Section 2.3.1; a buffer, a sur-

factant, one or both synergistic ligands, a diketone, and a metal chloride. Various concentrations of each component were tested; the pH of the final solution was kept in the 5.9-6.4 range. (2) In experiments intended to determine the limit of luminescence detection and the luminescence time-dependence, the concentrations of Eu-Mac or $EuCl_3$ were varied as appropriate, while the composition of the solution was kept constant.

2.3.3. Optimized Cofluorescence Solution Containing Gd(III).

The following is a typical protocol for the preparation of a 5-mL sample of a Gd(III)-containing optimized-cofluorescence solution, using the stock solutions listed in Section 2.3.1 (all volumes measured with calibrated micropipets). In a glass vial, the following are mixed: 0.080 mL of phen, 0.050 mL of CTAB, 0.800 mL of HMTA buffer, 0.400 mL of HMTA base, 0.600 mL of $GdCl_3$, a measured volume (V mL) of the solution containing the Eu-Mac, and the volume of water required to bring to total volume of the mixture to 5.00 mL after all components are added. Since the total volume of all fixed components is 2.410 mL, the volume of water to be added is [5.000 - (2.410 + V)] mL. The HTTFA (0.400 mL) is then added with gentle shaking and the previously clear solution becomes slightly cloudy owing to the formation of micelles. The micellar solution is allowed to stand at room temperature for 20-30 min, after which time 0.080 mL of TOPO are added and the cloudiness of the solution becomes more pronounced. The mixture is incubated for an additional 5 min; it is then placed in a quartz cell and its luminescence is obtained without further delay under the instrumental condition indicated in section 2.2. The concentrations of all components in the final cofluorescence solution are listed in Table 1; minor variations (± 5%) in the concentration of any component except the Eu-Mac do not affect the luminescence intensity of the solution. Similar protocols were used to prepare optimized cofluorescence solutions containing either Y(III) or La(III) as the energy transfer agent. In the following pages, any solution containing the first six components shown in Table 1, at the concentrations listed in the Table, will be referred to as an "optimized cofluorescence matrix".

Table 1: Concentrations of Components in the Optimized Cofluorescence Solution.

Component	Moles/L
1,10-Phenanthroline	8.80×10^{-5}
Cetyltrimethylammonium bromide	1.00×10^{-5}
Hexamethylenetetramine buffer	1.14×10^{-1}
Hexamethylenetetramine base	5.68×10^{-2}
1,1,1-trifluoro-4(2-Thienyl)-2,4-butanedione (Thenoyltrifluoro-acetone)	8.00×10^{-4}
Trioctylphosphine oxide	8.00×10^{-5}
Gd(III) chloride	1.20×10^{-4}
Eu-Macrocycle	2.30×10^{-7}

2.4. Conjugation of the Eu-Macrocycle with Avidin

2.4.1. Reagents and Quantities.

(1) Avidin, 2.5 mg, 3.9×10^{-8} mol, dissolved in 0.500 mL of sodium carbonate/bicarbonate buffer at pH 9.23. (2) Di-isothiocyanate-functionalized Eu-macrocycle[17,18] (Eu-Mac-NCS), 0.070 mL of a solution obtained by dissolving 2.4 mg, 4.0×10^{-7} mol, of Eu-Mac-NCS in 0.500 mL of DMSO. (3) Aspartic acid, 0.11 mL of a 2.1×10^{-2} M solution in water. The buffer used in the chromatography was HMTA adjusted to pH 7.6 with hydrochloric acid.

2.4.2. Procedure.

The Eu-Mac-NCS was added with gentle shaking to the avidin solution. The mixture was allowed to stand at room temperature for 60 min, after which time 0.100.mL of an aqueous solution of aspartic acid was added with gentle shaking. The mixture was allowed to stand at room temperature for an additional 15 min; it was then chromatographed through a column (17 cm height, 7 mm id) of Sephadex G25 previously equilibrated with the HMTA buffer. The Eu-Mac-thiourea-coupled avidin (Eu-Mac-Avidin) was eluted with the same buffer, using a flow-cell detector set for absorbance at 280 nm. Quantitative analysis of Eu was performed by ICP-AES (Schneider Laboratories, Richmond VA).

2.5 Preparation of Biotinylated Agarose Beads with Bound Eu-Mac-Avidin

Biotinylated agarose beads were: (1) washed three times with distilled water to remove the original phosphate buffer; (2)

soaked repeatedly in skim milk to eliminate non-specific binding. (3) The beads were then centrifuged; the supernatant was removed; and an aliquot of the Eu-Mac-Avidin solution was added as described in 2.3.2. After incubation for 5 min, the beads were washed repeatedly with the HMTA buffer to remove any unbound Eu-Mac-Avidin.

3. RESULTS AND DISCUSSION

3.1. Effect of Gd(III) on the Luminescence of the Eu-Macrocycle in Aqueous Solution.

The systematic series of experiments described below show that the luminescence of the [Eu(macrocycle)(acetate)$_2$](acetate) in an aqueous micellar solution can be enhanced by the presence of gadolinium(III) ions in association with certain synergistic additives, and that the enhancement is greatest when the solution has the optimized composition summarized in Table 1. This luminescence enhancement, or "cofluorescence", is strikingly illustrated in Figure 2, which shows the emission spectra of two solutions--one with and the other without $GdCl_3$--both measured under identical instrumental settings and containing the same concentration of Eu-Mac in the optimized-cofluorescence micellar matrix. It should be emphasized that the cofluorescence effect requires that both the Eu-Mac and the Gd(III) species be present in an aqueous micellar (slightly cloudy) solutions; the organization provided by the micellar system is essential to the energy transfer that leads to increased light emission by the Eu-Mac. Thus, cofluorescence does not occur for "true" solutions in organic solvents and the addition of ethanol or other water-miscible organic solvent to a glowing (and cloudy) cofluorescent solution instantly destroys the luminescence enhancement as it turns the solution clear. A similar phenomenon has been reported for the cofluorescence effect of the Eu(III) ion in its simple salts[15].

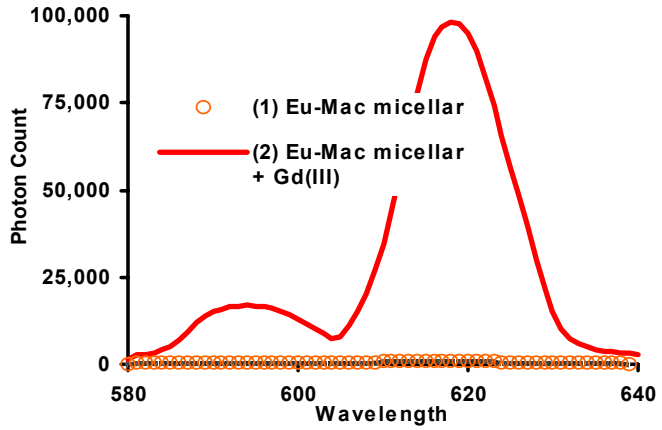

Figure 2. Emission spectra (Excitation: 375 nm) of: (1) A solution of [Eu-Macrocycle(acetate)$_2$](acetate) (2.28×10^{-7} M) in the optimized-cofluorescence matrix without Gd(III). (2) An identical solution but with Gd(III) chloride (1.2×10^{-4}M). In (2), the integrated emission intensity between 613 and 623 nm is increased over 100-fold by the addition of the Gd(III).

3.2. Limit of Detection of the Eu-Macrocycle by Gd(III)-Cofluorescence and Nature of the Emitting Species.

When considering the enhancement caused by Gd(III) on the luminescence emission of the Eu-Mac, it is necessary to keep in mind that at present even the purest commercially available gadolinium is contaminated by traces of Eu(III). This is a consequence of the fact that the two elements are obtained from the same minerals and that, being adjacent members of the Lanthanide Series, they are essentially identical in all properties except those related to the electronic configuration of their 4f electrons. The extreme similarity in the chemical behaviors and macroscopic physical properties of the two elements makes their separation from naturally occurring mixtures extremely difficult.

The $GdCl_3$ used in this work was synthesized from a gadolinium oxide containing approximately 0.3 ppm of europium by mass; accordingly, the optimized cofluorescence solution of Table 1, with 1.2×10^{-4} mol/L of $GdCl_3$, contained approximately 4×10^{-11} mol/L of Eu(III) as contaminant. The emission intensity produced by this Eu(III) contaminant under intrinsic cofluorescence conditions may be expected to be negligible, relative to that of the Eu-Mac, when the concentration of the latter is sufficiently high. At very low Eu-Mac concentrations, however, the emission of the Eu(III) contaminant may become comparable to, and eventually higher than, the emission of the Eu-Mac, thus posing a limit to its detection.

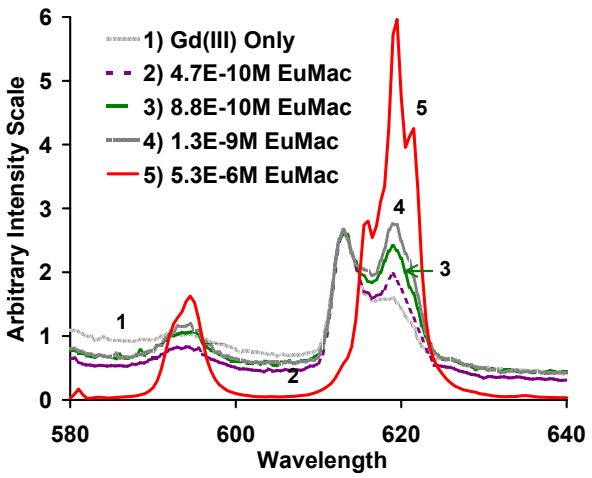

Figure 3. Emission spectra (excitation, 375 nm) of cofluorescence-optimized solutions containing "free" Eu(III) (approximately 4 x 10^{-11}M) as contaminant in the Gd(III) and the [Eu(macrocyle)(acetate)$_2$](acetate) at four different concentrations, 4.7 x 10^{-10} M, 8.8 x 10^{-10} M, 1.3 x 10^{-9} M, and 5.3 x 10^{-6} M. The peak maximum for the $^5D_0 \rightarrow {}^7F_2$ transition is 614 nm for the Eu(III) contaminant and 619 nm for the Eu-macrocycle. Because they had the same Eu(III) contaminant, spectra 1 to 4 were normalized to the same peak height at 614 nm. Spectrum 5 (the highest concentration of the Eu-macrocycle) was scaled to permit comparison of the spectra.

To probe the problem of Eu(III) contamination, five Gd(III)-containing optimized-cofluorescence solutions were compared--one without europium added in any form and the others with increasing concentrations of the Eu-Mac. The spectra were obtained with a SPEX 1692T spectrofluorometer, programmed for very high resolution in order to detect the presence of peaks corresponding to the $^5D_0 \rightarrow {}^7F_2$ emissions of different europium species, and also to reveal the peak pattern of this transition which is characteristic of the coordination geometry of the emitter. As illustrated in Figure 3, it was established that the $^5D_0 \rightarrow {}^7F_2$ band of the Eu-Mac has maximum at 619 nm, whereas that of the Eu(III) ion present as contaminant in the Gd(III) solution has maximum at 614 nm. Thus, the high-resolution emission spectra of the two species can be unambiguously distinguished, and their intensities can be independently measured, as long as the Eu-Mac is present in concentration appreciably greater than the Eu(III) contaminant. The latter condition is dictated by the fact that the emission intensity of the cofluorescence-enhanced Eu(III) is higher than that of the Eu-Mac at the same concentration. Owing to these factors, the current limit of detection for the Eu-Mac under Gd(III)-cofluorescence conditions lies between 1.0x10^{-9} M and 5.0x10^{-10} M

The results summarized above also provide an insight on the nature of the luminescent species present in the Gd(III)-containing cofluorescence solutions. The Eu(III) contaminant is most likely present as a mixture of [Eu(TTFA)$_3$(phen)] and [Eu(TTFA)$_3$(TOPO)$_2$] species, similar to the complexes formed by these ligands with other lanthanides[21]. In turn, the Eu-macrocycle emitter is unambiguously identified as a single cationic species of formula [{Eu-macrocycle}(TTFA)$_2$]$^+$ on the basis of the following evidence (1) Only one emission corresponding to the $^5D_0 \rightarrow {}^7F_0$ transition occurs in the 580 nm region of the spectrum (see also Figure 6). (2) The entire spectrum, and in particular the pattern of the $^5D_0 \rightarrow {}^7F_2$ transition at 619 nm, is identical to that of the [Eu(macrocyle)(acetate)$_2$](acetate) in an ethanol solution containing the diketone HTTFA as the sole additive. The structure of the species present is this ethanol solution was conclusively established by ^{1}H NMR spectroscopy.

3.3. Enhancement of the Luminescence of the Eu-Macrocycle by Yttrium(III) and Lanthanum(III) in Aqueous Solutions.

The luminescence screening experiments described in Section 3.1 for Gd(III) were also carried out using the chlorides of Y(III) or La(III) as the cofluorescence agents. Each of these metal ions caused a significant increase in the luminescence of the Eu-Mac, when present in the optimized-cofluorescence matrix containing either YCl_3 or $LaCl_3$ (1.2x10^{-4} M) instead of $GdCl_3$. The luminescence enhancement caused by these ions was considerably lower than that obtained with Gd(III), as illustrated in Figure 4 for the Y(III) cofluorescence; the effect of La(III) was even lower. Furthermore, the Y (III) and La(III) chlorides used for these experiments, even though obtained from the purest commercially available oxides, still presented the problem of Eu(III) contamination previously discussed for gadolinium.

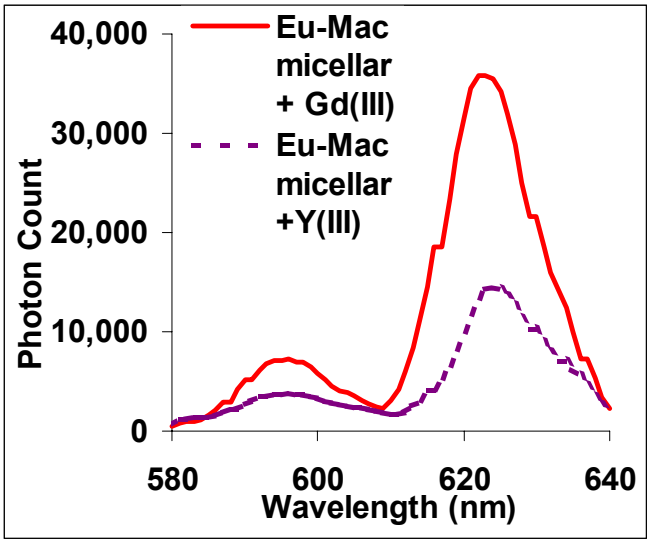

Figure 4. Emission spectra (Excitation: 375 nm) of [Eu-Macrocycle(acetate)$_2$](acetate) in two cofluorescence solutions, one containing Gd(III) and the other containing Y(III) as the donor. (All other reagents are present at the same concentrations, see Table 1). The Gd(III) provides significantly stronger, 2.6 fold, enhancement relative to Y(III).

3.4 Luminescence study of a Eu-Macrocycle-Avidin conjugate, using Gd(III) as cofluorescence agent

The Eu-Mac-Avidin conjugate described in Section [2.4] was analyzed for europium(III) luminescence by a protocol similar to that used for the prototype Eu-macrocycle. Specifically, one portion of the Eu-Mac-Avidin was used to make a micellar solution containing Gd(III) in the optimized-cofluorescence matrix. (All components of this solution had the concentrations given in Table 1 except Eu(III), which was present at 2.2×10^{-6} mol/L as determined by AA analysis.) Another portion of Eu-Mac-Avidin was used to make an identical micellar solution, except that Gd(III) was omitted. A third portion was used to make an aqueous solution containing only HTTFA. The emission spectra of the three solutions are shown in Figure 5. At the same concentration, the maximum emission intensity of the Eu-Mac-Avidin in the optimized micellar solution containing Gd(III) was more than ten times higher than that of the other solutions.

3.5 Time-Dependence Studies.

In the course of the experiments described in the preceding section, it was noted that the emission intensity of the cofluorescence-optimized solutions of the Eu-Mac was not constant, but often decreased slowly with time, as illustrated in Figure 6 for one sample examined over a period on 1.5 hr. This time dependence was not consistently reproducible, even for solutions of identical compositions; in some experiment, the intensity remained approximately constant, and in others it first increased slightly and then gradually decreased. A similar effect was reported[15] for the cofluorescence of the "free" Eu(III) ion. A screening of the influence of the synergistic ligands,1,10 phenanthroline and trioctylphosphine oxide, showed that they have a somewhat stabilizing effect, in the sense that the variation of the luminescence intensity with time is more erratic when either is missing from the solution. Work is in progress to find conditions leading to a time-constant cofluorescence effect.

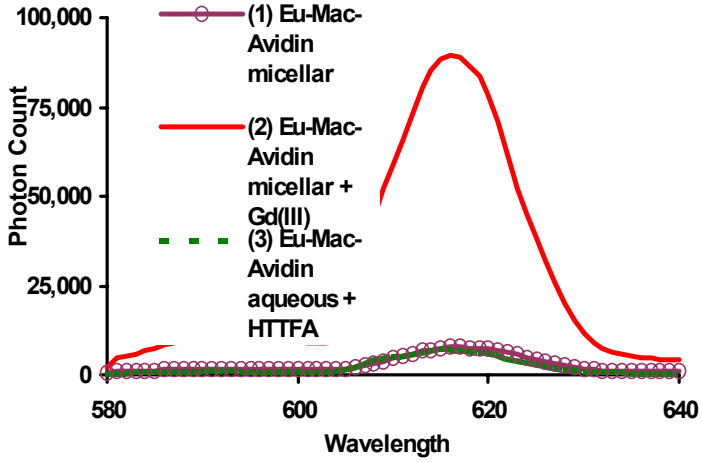

Figure 5. Emission spectra (Excitation: 365 nm) of the Eu-Mac-Avidin at a concentration of 2.2×10^{-6} mol Eu(III)/L in: (1) A cofluorescence-optimized aqueous micellar solution. (2) Identical to the preceding solution but with Gd(III) chloride (1.2×10^{-4} M. (3) An aqueous buffered solution with only HTTFA added.

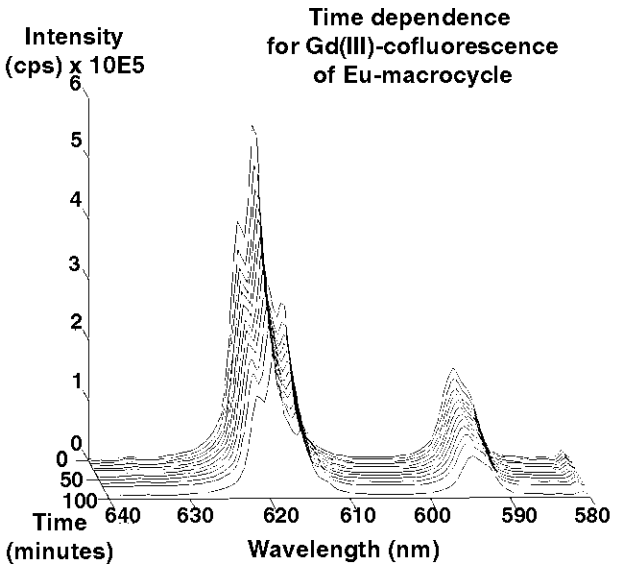

Figure 6. Time-dependence plot for the emission intensity of the [Eu(macrocyle)(acetate)$_2$](acetate) complex in a Gd-containing optimized cofluorescence solution. Only one band arising from the $^5D_0 \rightarrow {}^7F_0$ transition of the Eu-macrocycle transition occurs at ca. 580 nm, showing that only one emitting species is present. Furthermore. the peak pattern of the band corresponding to the $^5D_0 \rightarrow {}^7F_2$ transition is constant in time, even though the intensity decreases, showing that the chemical nature of the emitting species remains unchanged.

The graph of Figure 6. illustrates a significant aspect of the cofluorescence time-dependence. The intensity of the emission spectrum changes with time, but the pattern of the spectrum--the "signature" of the emitting species--remains unchanged. This shows that there is no decomposition or rearrangement of the Eu-macrocycle emitter; the variation in emission intensity is the result of the dynamic character of micellar solutions. This is further confirmed by the observation that the micellar cofluorescence solution will, over a period of one-two days, spontaneously separate into a highly luminescent precipitate and a clear, non-luminescent solution.

3.6 Solid-State Studies

To explore the extension of the Gd(III)-cofluorescence effect to solid samples, the following experiments were performed. A strip of electrophoresis film (Sepraphore III, Gelman Sciences) was spotted with 0.010 mL of the cofluorescence-optimized aqueous micellar solution of Eu-Mac (2.3×10^{-7} M) discussed in Section 3.1. The outline of the wet spot was marked in pencil to determine the area and its emission spectrum was recorded by reflectance. The strip was allowed to dry at room temperature, and the spectrum of the dry spot was measured again. The results of this experiment, illustrated in Figure 7, showed that the cofluorescence effect for the Eu-Mac, once established in solution, is maintained in solid samples. Under the conditions described here, the Eu-Mac was easily detected at a surface density of ca. 10^{-12} moles/cm^2 (Figure 7). A second strip was similarly spotted with 0.010 mL of an ethanol solution of $EuCl_3$ (2.3×10^{-7} M) containing only the diketone HTTFA (8.0×10^{-4} M). The spectrum of the spot was recorded before the solvent had evaporated; the emission intensity was found to be much lower than that obtained from the aqueous cofluorescence solution (Figure 7); no luminescence was detected from this strip when dry.

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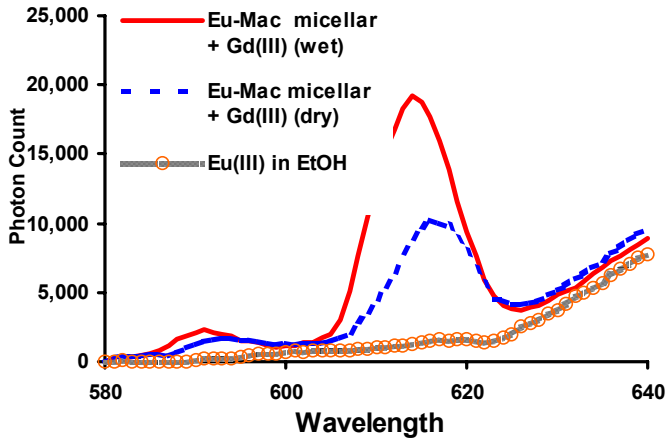

Figure 7. Reflectance emission spectra (Excitation: 375 nm) of wet and dry spots obtained from a cofluorescence-optimized aqueous micellar solution of [Eu-Mac(acetate)$_2$](acetate) (2.3×10^{-7} M), and of a wet spot from an ethanol solution of Eu(III) (2.3×10^{-7} M) with only HTTFA added. Spectra were recorded under identical instrumental settings and the background from the paper was subtracted; however, the reflectance behavior of the paper changes upon drying. The rise in the curve above 630 nm is due to scattering from the paper.

In a second set of experiments, the biotinylated agarose beads with bound Eu-Mac-Avidin (Section 3.4) were tested as follows: (1) One portion of beads was treated with the optimized cofluorescence matrix containing no Gd(III); these beads showed no luminescence upon irradiation at 360 nm. (2) Another portion of beads was treated with the optimized cofluorescence matrix containing $1.2x10^{-4}$mol/L of Gd(III). These beads immediately displayed strong luminescence upon irradiation at 360 nm; the intensity remained unchanged for over one week when the beads were stored in the mother liquor in a refrigerator. The beads were then centrifuged, the supernatant was removed, and the beads were allowed to dry in air at room temperature. The dry beads still showed strong luminescence. (3) A third portion of beads was similarly treated with the Gd(III) optimized cofluorescence solution; the glowing beads were centrifuged and the supernatant was removed. A solution of HTTFA in ethanol was then added. The previously strong luminescence nearly disappeared.

These results showed that the organization required for luminescence enhancement can be established around the Eu-macrocycle even when this is coupled to Avidin and linked to a solid support, provided the optimized Gd(III) cofluorescence solution is present. Once formed, the organization and the resulting luminescence enhancement persist even when the aqueous solvent is removed by evaporation. Luminescence is lost, however, upon addition of ethanol containing the HTTFA diketone enhancer, because the ethanol dissolves all components of the system and thus disrupts the organization.

4. CONCLUSIONS

This work has shown that the major enhancement produced by Gd(III), and to a lesser extent also by Y(III) and La(III), on the luminescence intensity of aqueous micellar solution of the prototype Eu-macrocycle and of the Eu-macrocycle marker conjugated to avidin permits the detection of these species at unprecedentedly low levels without the need for specialized instrumentation. A similar effect was observed for air-dried preparations suitable for fluorescence microscopy. Although the traces of Eu(III) present in even the highest-purity gadolinium currently available could limit some of the proposed measurements, the use of Gd(III) as cofluorescence agent does provide a way to visualize labeled materials in aqueous solution, such as electrophoretigrams, and to greatly improve the sensitivity of conventional fluorescence microscopy. Work is in progress to find conditions leading to a cofluorescence-enhanced as well as time-constant emission intensity of the Eu-macrocycle and related species.

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