Title: METHODS TO INCREASE THE LUMINESCENCE OF
LANTHANIDE(III) MACROCYCLIC COMPLEXES

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Methods to Increase the Luminescence of Lanthanide(III) Macrocyclic Complexes

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ABSTRACT

Previously, we have described the strong luminescence enhancing effect (cofluorescence) of yttrium(III) or gadolinium(III) on a functionalized europium(III) hexaaza macrocyclic complex, Quantum DyeTM. In the presence of Gd(III), the luminescence of these 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 these Eu-macrocycles was observed as an extremely sharp band with a maximum at 619 nm and a clearly resolved characteristic pattern. This cofluorescence has now been demonstrated with Sm(III) replacing the Eu(III) in the macrocycle. Simultaneous detection of two molecular species is now possible because the cofluorescence of the Eu(III) and Sm(III) macrocycles occurs in the same solution. Cofluorescence of the Tb(III) macrocycle has also been observed under different conditions. Thus, it will be possible to employ narrow bandwidth lanthanide luminescent tags to identify three molecular species.

Keywords: Luminescence, lanthanide, macrocycle, europium, samarium, terbium, gadolinium, cofluorescence, biomarkers.

1. INTRODUCTION

1.1. Multiple Lanthanide-Containing Luminescent Markers.

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 Melentjeva. This effect has since been the object of several investigations and has been shown to apply not only to europium but also to other luminescent lanthanides. Xu reported in a patent that the presence of yttrium(III) and other additives can greatly enhance the emission intensity of aqueous micellar solutions of certain β -diketonate complexes of samarium(III), europium(III), terbium(III) and dysprosium(III).

We have extended these studies to a class of lanthanide macrocyclic complexes that have potential applications as luminescent biomarkers for cytology and immunology. One year ago we reported [ref] that the luminescence of europium(III) complexes of six-nitrogen-donor macrocyclic ligands (Figure 1) is dramatically enhanced--by a factor of one-hundred or more--in the presence of Gd(III) ions, and to a lesser extent also of La(III) and Y(III) ions, in an aqueous micellar system. This enhancement occurs both with the unfunctionalized prototype of Figure 1 and with the functionalized analog coupled to avidin, either in an aqueous micellar solution or immobilized on a solid substrate. These results showed that gadolinium-induced cofluorescence permits the utilization of europium(III) macrocycles as luminescent markers without the need for time-gated luminescence microscopy, which at present is costly, not widely available, and often involves loss of signal or precision. The presence of gadolinium(III), besides increasing the Eu-luminescence, offers the advantage of essentially removing any Eu-macrocycles that might be present in the specimens through non-specific binding. The full utilization of flow cytometry requires the ability to detect and measure an increased number of different molecular species. Similarly, the usefulness of digital microscopy for both

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Figure 1. Schematic formula of the cationic entity of a six-nitrogen-donor macrocyclic complex, where M is any trivalent lanthanide ion. The structure shown is the unfunctionalized prototype of the Quantum Dyes.

research and clinical uses would be maximized if multiple molecular species could be detected and quantitated in the same sample. These considerations prompted the studies we report here, and our results have shown that it is possible to obtain simultaneous cofluorescence of europium and samarium macrocyclic complexes, in the same solution and utilizing the same exciting radiation. Cofluorescence-enhanced luminescence of the terbium macrocycle was also achieved under different conditions, adding to the existing palette of markers a useful new narrow-band, long-life-time species emitting in the green. These additions will permit a significant increase in the number of species that can be observed and quantitated by luminescence without the need for time-gated or other sophisticated instrumentation..

EXPERIMENTAL METHODS

1.2. Materials.

Cetyltrimethylammonium bromide (CTAB), hexamethylenetetramine, ACS Reagent (HMTA), 1,10-phenanthroline (PHEN), trioctylphosphine oxide (TOPO), 4,4,4-trifluoro-1(2-naphthyl)-1,3-butanedione (naphthoyltrifluoroacetone, HTFNA), all from Aldrich Chem. Co., and 5,5-dimethyl-1,1,1-trifluoro-2,4-hexanedione (pivaloyltrifluoroacetone, HPTFA, from Lancaster Synthesis Inc.) were checked for purity by IR and/or proton HNMR spectra and were used as received. The diketone 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione (thenoyltrifluoroacetone, HTTFA, from Aldrich) was purified by recrystallization from ethanol(charcoal)/

hexane and stored at 4°C in a dark glass container. The complexes [Sm-macrocycle(acetate)₂](acetate), SmMac, [Eu-macrocycle(acetate)₂](acetate), EuMac, and [Tb-macrocycle(acetate)₂](acetate), TbMac, were synthesized as previously described. A high-purity sample of [Sm(macrocycle)(acetate)₂](acetate) was similarly synthesized using as starting material a Sm(III) acetate prepared from the high-purity oxide (Sm₂O₃, 99.999%, REO, from Alfa Aesar); the oxide was dissolved in 50% acetic acid with mild heating and the resulting solution was evaporated to dryness under reduced pressure to give Sm(III) acetate trihydrate as a white crystalline solid. High purity Gd(III) chloride, was obtained from the oxide (Gd₂O₃, 99.999%, REO, from Alfa Aesar) by a procedure similar to that described for Sm(III) acetate, with the substitution of 15% hydrochloric for acetic acid. All common reagents and solvents were of reagent grade and were used as received. Only high purity deionized and Micropore-filtered water was used to prepare solutions and for the final rinsing of glassware.

1.3. 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°C. Emission and excitation spectra of solutions for Figures were obtained with a SPEX 1692T spectrofluorometer (at LANL) and those for Figures 3, 4, and 8 with the SLM-8000 instrument (at VCU). Samples 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 varied as required. All experiments and measurements were performed at ambient temperature unless stated otherwise.

1.4. Stock Solutions.

(1) *Surfactant*: (a) Cetyltrimethylammonium bromide (CTAB), 1.00×10^{-3} M in water. (2) *Buffer*: (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). *Synergistic Ligands*: (a) 1,10-phenanthroline (phen), 5.50×10^{-3} M in ethanol, (b) trioctylphosphine oxide (TOPO), 5.00×10^{-2} M in ethanol, (4) *Diketones*: (a) 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione (HTTFA), (b) 4,4,4-trifluoro-1(2-naphthyl)-1,3-butanedione (naphthoyltrifluoroacetone, HTFNA), (c) 5,5-dimethyl-1,1,1-trifluoro-2,4-hexanedione (pivaloyltrifluoroacetone (HPTFA), all 1.00×10^{-2} M in ethanol, (5) *Light-emitting Complexes*: (a) SmMac(macrocycle)(acetate)₂](acetate), Sm-Mac, (b) [Eu(macrocycle)(acetate)₂](acetate), Eu-Ma, (c) and [Tb(macrocycle)(acetate)₂](acetate), all 1.0×10^{-3} M in ethanol, as primary stocks from which more dilute stock solutions were made as necessary. *Luminescence*

Enhancer: Gd(III) chloride, 1.0×10^{-3} M water.

1.5. Preparation of Solutions for Cofluorescence Studies.

A series of experiments was performed to determine the conditions of optimized cofluorescence for the SmMac and TbMac complexes, and for the SmMac/Eu-Mac mixtures. In these experiments, the concentration of the emitting complex was kept constant at a value chosen to provide a suitable range of emission intensities for screening tests. Each micellar solution also contained HMTA as the buffer, CTAB as the surfactant, either PHEN or TOPO, or both, as the synergistic ligands, HTTFA or HTFNA (for SmMac and EuMac), and HPTFA (for TbMac) as the diketone, and Gd(III) chloride as the energy transfer donor. Various final concentrations of each of these components were tested and the pH of the final solution was kept in the 5.9-6.4 range.

The detailed protocol used for the preparation of a 5-mL sample of an optimized-cofluorescence micellar solution containing SmMac (1.00×10^{-5} M) as the emitter is given here as example. All components were used as the stock solutions listed in section 2.3; volumes were measured with calibrated micropipets. In a glass vial, the following are mixed: (a) 0.080 mL of PHEN, (b) 0.050 mL of CTAB, (c) 0.800 mL of HMTA buffer (10% m/v in water, adjusted to pH 6.0 with hydrochloric acid), (d) 0.400 mL of HMTA base (10% m/v in water), (e) 0.600 mL of GdCl_3 (1×10^{-3} M in water), (f) mL of SmMac (1.0×10^{-3} M), and (g) the volume of water required to bring to total volume of the mixture to 5.00 mL after all components are added. The HTTFA (0.400 mL of a 1.00×10^{-2} M solution in ethanol) 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 15-30 min, after which time 0.080 mL of TOPO (5.00×10^{-3} M in ethanol) are added and the cloudiness of the solution becomes more pronounced. The mixture is incubated for an additional 5 min at room temperature; it is then placed in a quartz cell and its luminescence is obtained without further delay under the instrumental condition indicated in the section. The concentrations of the components in this optimized cofluorescence solution are listed in Table 1; minor variations ($\pm 5\%$) in the concentration of any component except SmMac do not affect the luminescence intensity of the solution.

Table 1: Concentrations of Components in Optimized Cofluorescence Solutions Containing Gd(III) as the Energy Transfer Donor.

Component	Moles/L
1,10-Phenanthroline, PhHEN	8.80×10^{-5}
Cetyltrimethylammonium bromide, CTAB	1.00×10^{-5}
Hexamethylenetetramine buffer, HMTA buffer	1.14×10^{-1}
Hexamethylenetetramine base, HMTA base	5.68×10^{-2}
1,1,1-trifluoro-4(2-Thienyl)-2,4-butanedione (Thenoyltrifluoro-acetone, HTTFA)	8.00×10^{-4}
Triethylphosphine oxide, TOPO	8.00×10^{-5}
Gd(III) chloride	1.20×10^{-4}
SmMac, EuMac, and TbMac	varied (see text)

2. RESULTS AND DISCUSSION

2.1. Enhancement of the Luminescence of the SmMac Complex by Gadolinium(III) in Aqueous Micellar Solutions.

A set of Gd-containing cofluorescence solutions having decreasing SmMac concentrations (1.0×10^{-5} M, 1.0×10^{-6} M, 1.0×10^{-7} M, 1.0×10^{-8} M, 5.0×10^{-9} M) were prepared according to the protocol described in Section 2.4. For comparison, a cofluorescence solution of the SmMac complex (1.0×10^{-5} M) containing all components at the concentrations of Table 1, but no gadolinium, was also prepared. The emission spectra of all solutions, obtained on a SPEX 1692T spectrofluorometer, are illustrated in Figure 2. It should be pointed out that the problem of Eu(III) contamination in the gadolinium used as energy transfer donor, discussed in Example II for EuMac, also affects the SmMac spectra in the lowest concentration range.

Figure 2. Emission spectra (excitation, 367 nm) of [Sm-macrocycle(acetate)₂](acetate) at three different concentrations, 1×10^{-5} M, 1×10^{-7} M, 5×10^{-9} M, in cofluorescence-optimized solutions with and without Gd(III). The solutions that contain gadolinium also contain "free" Eu(III) (approximately 4×10^{-11} M) as contaminant. The SmMac spectrum shows three emissions at {563}, {599}, and {644} nm, arising from the $^4G_{5/2} \rightarrow ^6H_{5/2}$, $^4G_{5/2} \rightarrow ^6H_{7/2}$, and $^4G_{5/2} \rightarrow ^6H_{9/2}$ transitions of Sm(III); the constant-intensity emission at 614 nm arises from the $^5D_0 \rightarrow ^7F_2$ transition of the Eu(III) contaminant.

results are illustrated in Figure 3 and Figure 4.

Figure 4. Excitation spectra of [Sm-macrocycle(acetate)₂](acetate) (1.0×10^{-4} M) in an ethanol solution containing HTTFA (4.0×10^{-4} M), for emission of 598.5 and 647.0 nm, respectively.

2.2. Comparison of the Luminescence Intensity of the SmMac Complex in a Gd-Containing Optimized Cofluorescence Solution and in Ethanol Solutions with Diketone Enhancers

It is well known that the emission intensity of the luminescent lanthanide(III) ions is especially strong in ethanol solutions containing certain β -diketones. It was therefore of interest to compare the emission intensities of equal concentrations of the SmMac complex in the gadolinium-containing aqueous cofluorescence solution and in ethanol solutions containing luminescence-inducing β -diketones, such as HTTFA and HTTNA. The following solutions were prepared: (a) a Gd-containing cofluorescence solution of SmMac (1.0×10^{-4} M), obtained by the protocol of Section 2.4. (b) a solution of SmMac (1.0×10^{-4} M) in anhydrous ethanol, containing 1,1,1-trifluoro-4(2-thienyl)-2,4-butanedione (HTTFA, 4.0×10^{-4} M), and (c) a solution of SmMac (1.0×10^{-4} M) in anhydrous ethanol, containing 1,1,1-trifluoro-4(2-naphthyl)-2,4-butanedione (HTFNA, 4.0×10^{-4} M). The luminescence spectra of the solutions were obtained with a SPEX 1692T spectrofluorometer and the

2.3. Simultaneous Enhancement of the Luminescence of the SmMac and EuMac Complexes by Gadolinium(III) in Aqueous Micellar Solutions.

A series of experiments was performed to determine conditions leading to the simultaneous luminescence enhancement of the SmMac and EuMac complexes by gadolinium(III) in aqueous micellar solutions. In one group of experiments, the concentration of SmMac was kept constant at 1.0×10^{-5} M; in another group the SmMac concentration was 5.0×10^{-6} M. In both cases, various concentrations of the EuMac complex, ranging from 2.3×10^{-7} to 5.7×10^{-8} M, were investigated. The other components of these solutions were the same as those listed in Section 3.1, each at the concentration shown in Table 1. All solutions were prepared by a protocol similar to that described in Section 2.4 and their spectra were recorded on the SLM 8000 spectrofluorometer set for high resolution. Figure shows that, under Gd-mediated cofluorescence conditions, the emission intensities of both the SmMac and EuMac in the combined solutions are enhanced, and the spectra of the two complexes maintain their distinctive patterns and are individually assignable and measurable

Figure 3. Emission spectra (excitation, 367 nm) of [Sm-macrocycle(acetate)₂](acetate) (1.0×10^{-4} M) in: (a) a Gd-containing optimized cofluorescence solution, (b) an ethanol solution containing HTTFA (4.0×10^{-4} M), and (c) an ethanol solution containing HTFNA (4.0×10^{-4} M).

Figure 5. 9364629 Emission spectrum (excitation, 370 nm) of a gadolinium-induced cofluorescence solution containing 5.0×10^{-6} M [Sm-macrocycle(acetate)₂](acetate) and 1.4×10^{-7} M [Eu-macrocycle(acetate)₂](acetate); all other components as in Table 1. The SmMac and EuMac complexes were combined prior to micelle formation. The $^5D_0 \rightarrow ^7F_2$ emission of the EuMac species is well separated from the neighboring $^4G_{5/2} \rightarrow ^6H_{7/2}$, and $^4G_{5/2} \rightarrow ^6H_{9/2}$ emissions of the SmMac, so that the intensities of each emission can be measured independently.

Figure 6. 9364630 Excitation spectrum of the SmMac complex in a gadolinium-induced cofluorescence solution containing 5.0×10^{-6} M [Sm-macrocycle(acetate)₂](acetate) and 1.4×10^{-7} M [Eu-macrocycle(acetate)₂](acetate). All other components had the concentrations given in Table 1 and the SmMac and EuMac were combined prior to micelle formation. The excitation spectrum of the EuMac complex (emission at 619 nm) had nearly identical intensity. (b) Excitation spectrum of the gadolinium-induced cofluorescence solution containing 5.0×10^{-6} M SmMac and 5.7×10^{-8} M EuMac (see Figure). The same excitation spectrum is obtained for both the SmMac and the EuMac

To further explore these systems, the individual emission intensities of the SmMac and EuMac species in the SmMac/EuMac combined solutions were compared to those of parallel cofluorescence solutions, containing only SmMac or only EuMac, each at the same concentrations as the mixtures. The results of one such comparison are illustrated as example in Figure. The enhancement of the SmMac in the SmMac/EuMac mixture is the same, within the present limits of the measurements, to that of the SmMac alone. In contrast, the enhancement of the EuMac luminescence is somewhat reduced in the SmMac/EuMac mixture relative to the EuMac-only solution. The causes for this difference in behavior will be further investigated, as it may provide information on the mechanism of the energy transfer from the Gd(III) donor to the two chemically similar but electronically different acceptors/emitters.

Figure 8. 9365601 and 9365608 Composite of the gadolinium-induced cofluorescence emission spectra of two solutions, one containing only SmMac (5.0×10^{-5} M) and the other containing only EuMac (5.7×10^{-8} M). The concentrations of the macrocyclic complexes were chosen to provide approximately equal emission intensities (excitation, 370 nm).

Figure 9. 9365610

2.4. Enhancement of the Luminescence of the TbMac Complex by Gadolinium(III) in Aqueous Micellar Solutions.

The effect of Gd(III) as energy transfer donor on the luminescence intensity of the TbMac triacetate complex in aqueous micellar solutions was investigated in a series of experiments that utilized the materials listed in Section 2.1 and followed the protocol described in Section 2.4, with the substitution of TbMac for SmMac and of 1,1,1-trimethyl-5,5,5-trifluoro-2,4-pentanedione (pivaloyltrifluoroacetone, HPTFA) for HTTFA. The optimum luminescence intensity was observed when the solution contained HPTFA (8×10^{-4} M), in conjunction with the other components listed in Table 1, each at the concentration shown in that Table.

A set of optimized cofluorescence solutions containing different concentrations of the TbMac complex (1.0×10^{-4} M, 1.0×10^{-5} M, 1.0×10^{-6} M) were prepared, and their emission spectra were obtained with a SPEX 1692T spectrofluorometer. For comparison, a solution of TbMac (1.0×10^{-4} M) in ethanol containing only HPTFA (4.0×10^{-4} M) was also prepared and examined under the same conditions. Figure 10 shows the emission spectra of TbMac in the ethanol/HPTFA solution and in the optimized cofluorescence solutions containing Gd(III), illustrating that the luminescence of TbMac is greatly enhanced by the presence of Gd(III) in an aqueous micellar system.

Figure 10. Emission spectra (excitation, 319 nm) of: (a) an ethanol solution of TbMac, 1.0×10^{-4} M with only HPTFA (8×10^{-4} M), (b) (c) and (d) Gd-containing cofluorescence-optimized solutions of TbMac, 1.0×10^{-4} M, 1.0×10^{-5} M, 1.0×10^{-6} M, respectively. All other compo-

This comparatively long wave excitation of a Tb(III) complex should permit the use of some commercial fluorescence objectives rather than requiring very expensive quartz objectives.

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Methods to Increase the Luminescence of Lanthanide(III) Macrocyclic Complexes

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Comparison of Dyes

Organic

short lifetimes
10 nanoseconds

small Stokes shift

- multiple colors

broad band

- water insensitive

no enhancement

autofluorescence interferes

Rare earth

- long lifetimes
100 usec to 1 ms

- large Stokes shift
> 100 nm

few colors

- narrow band
FWHM $\approx$ 10–15 nm

water quenches

- activated by GdCl_3

- autofluorescence a non-issue

Simultaneous detection of Sm and Eu biomarkers

1. Same excitation wavelength at 370 nm
2. Same micellar solution preparation conditions
3. Same activation ion: Gd(III)
4. Same macrocyclic ligand
5. "Plug 'n Shine"

Sample Preparation Protocol

Concentrations of Components in Optimized Cofluorescence Solutions Containing Gd(III) as the Energy Transfer Donor.

Step	Component	Concentration (M)
1	1,10-Phenanthroline, PhHEN	8.80×10^{-5}
1	Cetyltrimethylammonium bromide, CTAB	1.00×10^{-5}
1	Hexamethylenetetramine buffer, HMTA buffer	1.14×10^{-1}
1	Hexamethylenetetramine base, HMTA base	5.68×10^{-2}
1	Gd(III) chloride	1.20×10^{-4}
1	SmMac, EuMac, and TbMac	1×10^{-4} to 5×10^{-9}
2 let stand 15-30 minutes	1,1,1-trifluoro-4(2-Thienyl)-2,4-butanedione (Thenoyltrifluoro-acetone, HTTFA)	8.00×10^{-4}
3 let stand 5 minutes	Trioctylphosphine oxide, TOPO	8.00×10^{-5}

Macrocyclic Ligand

Schematic formula of the cationic entity of a six-nitrogen-donor macrocyclic complex, where M is any trivalent lanthanide ion. The structure shown is the unfunctionalized prototype of the Quantum Dyes.

Dual Emission

Both SmMac and EuMac enhanced versus no Gd(III) added
by how much?

Distinct patterns identical to when excited alone.

Sm intensity same as when excited alone

Eu intensity decreased

TbMac emission

Modified protocol for this rare earth

UV light harvesting ligand is HPTFA

Excitation at shorter wavelength than EuMac/SmMac

Different detection limits

**Excitation Spectrum (Em. 599 nm) of SmMac/EuMac
Combined Cofluorescence Solution with Gd (III)**

Excitation Spectra of SmMac

Ethanol Solution with HTTFA

Emission Spectra of SmMac in Different Media

Emission Spectrum (Ex. 370 nm) of Combined SmMac/EuMac Cofluorescence Solution with Gd(III)

Emission Spectra (Ex. 370 nm) of SmMac and EuMac
Individual Cofluorescence Solutions with Gd (III)

**Emission Spectrum (Ex. 370 nm) of SmMac/EuMac
Combined Cofluorescence Solution with Gd (III)**

Emission Spectra (Ex. 319 nm) of TbMac and HPTFA

Emission Spectra of SmMac Cofluorescence Solutions at Different Concentrations