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Improvement of stability and luminescence properties in water of Eu(III) and Tb(III) complexes photosensitized by a bipyridine antenna

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Abstract—The convenient synthesis of a new macrocyclic octadentate chelate based on 2,2′-bipyridine chromophore and diethylenetriaminetriacetic acid core is described. In aqueous solutions, the corresponding Eu(III) and Tb(III) neutral complexes are kinetically inert and show very bright luminescence when excited with UV radiation ($\Phi = 11$ and 25% respectively). We also report the potentiality of these complexes to act as donors in delayed fluorescence resonance energy transfer (DEFRET). © 2003 Elsevier Science Ltd. All rights reserved.

Many applications of photosensitized lanthanide complexes in assays and energy transfer experiments with biological samples have validated the unique temporal and wavelength emission advantages of these systems. Thus, several europium-complex-based detection systems for heterogeneous or homogeneous time-resolved fluoroimmunoassays (Delfia, Cyber-Fluor, Trace methods) have been commercialized and are available for routine diagnostic use.[1] In this context, intense research efforts have been devoted to the design of molecular edifices that combine binding abilities and photosensitizing properties for the development of more efficient photoluminescent complexes containing Eu^{3+} and Tb^{3+} ions. However, most of the reported lanthanide complexes are either insoluble or unstable in aqueous solutions, or poorly luminescent in biological media.[2]

An optimization of the luminescent properties and kinetic stabilities of lanthanide complexes in aqueous solutions may arise from the introduction of a chromophoric unit into a macrocyclic structure bearing pendant ionizable groups. In such receptors, the chromophore may coordinate directly with the lanthanide ion thereby favoring the intramolecular energy transfer from the antenna to the metal (A.ET.E effect).[3] The presence of negatively charged oxygen donors may increase the solubility and the stability (in association with a 'macrocyclic effect') of the complexes.

The reported ligand **LH_3** (Scheme 1) is based on a triaza macrocycle acetate backbone containing an endocyclic bipyridine group and presents three main advantages: (i) it offers eight donors atoms for complexation and shielding of the lanthanide ion; (ii) the bipyridine chromophore is a widely used antenna group for photosensitizing lanthanide ions, enabling us to compare directly the optical properties of Ln(III) complexes depending on the ligand structure;[3,4] (iii) the diethylenetriaminetriacetic acid (DTTA) core was reported as an efficient chelating agent for the lanthanide ions.[5]

The synthesis of ligand **LH_3** is depicted in Scheme 1 and is based on our previously described synthetic pathway, which allows substantial variations of the heterocyclic moiety.[6] As a matter of fact, compound **1** is a useful building block for the construction of macrocyclic structures, since it contains two secondary amine groups which can be connected by suitable cross-linkers. Condensation of **1** with 6,6′-bis(bromomethyl)-2,2′-bipyridine was carried out in CH_3CN at reflux in the presence of Na_2CO_3 as a base.

This macrocyclization reaction was realized without requiring high dilution techniques and was controlled by a metal template ion effect. ^1H NMR analysis of the crude mixture evidenced the presence of only one

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Keywords: azamacrocycles; lanthanides; luminescence; bipyridine; delayed fluorescence resonance energy transfer.

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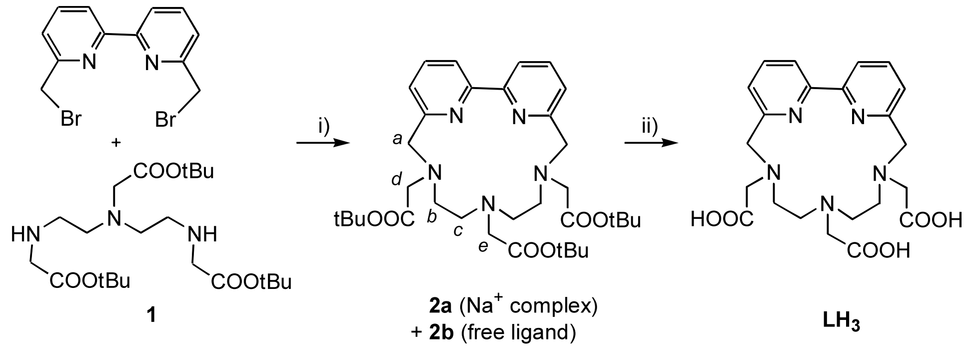

Scheme 1. *Reagents and conditions*: (i) Na_2CO_3, CH_3CN, reflux [reactants] = 2.4×10^{-3} M (**2a**+**2b**, 12%+22% yield, respectively); (ii) CF_3COOH, CH_2Cl_2 (85–90% yield).

monomeric species, characterized as the NaBr complex **2a**. Some dissociation of **2a** occurred when purification was achieved by chromatography on alumina and as a result **2a** and **2b** (free ligand) were isolated (34% overall yield). It can be noticed that these two species can be readily distinguished by their ^{1}H NMR spectra (Fig. 1). Finally, mild hydrolysis of the *tert*-butyl esters of **2a** or **2b** with trifluoroacetic acid gave the triacid **LH$_3$** in 85–90% yield.[7]

The Eu(III) and Tb(III) neutral complexes of the macrocycle **LH$_3$** were prepared by the addition of the lanthanide chloride salt (1 equiv.) to the aqueous solution of ligand. This solution was then adjusted at a final concentration of 1×10^{-5} M in borate buffer (pH 8.6). Eu·**L** and Tb·**L** complexes were characterized in water solutions by UV, MS and ligand-sensitized Ln^{3+} luminescence technique.[8]

Table 1. Luminescence lifetimes (ms) and quantum yields (%) of Eu·**L** and Tb·**L** complexes in aerated borate buffer (pH 8.6) solutions at 300 K[a]

	τ_{H_2O}	τ_{D_2O}[b]	Φ_{H_2O}	Φ_{D_2O}
Eu·L	0.64	1.90 (1.90)	11	25
Tb·L	1.19	1.64 (2.70)	25	36

[a] Measured by excitation from the lowest-energy ligand-centered absorption band (λ = 320 nm).
[b] Data at 77 K in parentheses.

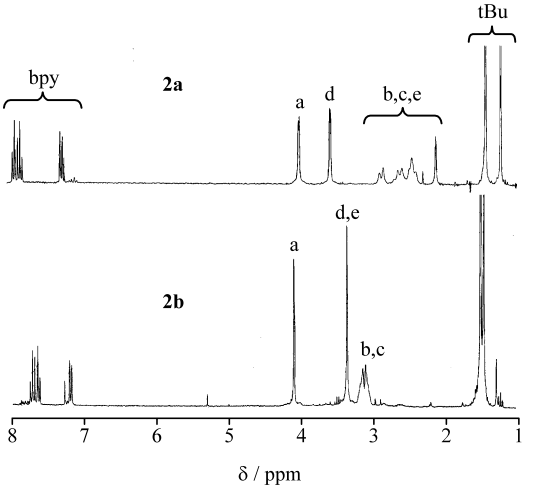

Figure 1. ^{1}H NMR spectra of compounds **2a** and **2b** in $CDCl_3$ at 250 MHz.

Photoexcitation of these complexes from the lowest energy absorption of the bipyridine antenna at 320 nm gives rise to intense and long lived green (Tb·**L**, λ_{em} = 545 nm) or red (Eu·**L**, λ_{em} = 617 nm) luminescence. Table 1 lists the overall quantum yields of sensitized emission (Φ) and the lifetimes (τ) of the metal excited states determined under various experimental conditions. The Φ values are among the largest reported in aqueous solutions for either Eu^{3+} or Tb^{3+} complexes containing one or more 2,2′-bipyridine chromophore.[3,4] Comparison of the quantum yield and lifetime determined in H_2O and D_2O solutions indicates that a non radiative energy transfer occurs from the excited states of the lanthanide to the OH stretching vibrations of coordinated water molecules. The use of the empirical Horrocks's equations[9] reveals that the average number of coordinated water molecules is n = 1.1 for Eu·**L** and 1 for Tb·**L**. By comparison with other Tb^{3+} complexes of bipyridine ligands, no large increase in the lifetime is observed for Tb·**L** when the temperature is lowered to 77 K. This indicates that non-radiative deactivation by back-energy transfer from the $^5D_4$ emitting state to the bipyridine lowest triplet state is rather inefficient in Tb·**L**. As a matter of fact, the energy gap ($^3E_{00}$–$^5D_4$) measured as usual from the ligand phosphorescence spectrum of Gd·**L** (ca. 2000 cm^{-1}) is slightly above the minimum value of 1850 cm^{-1} proposed by Latva et al. to prevent such a reversible process.[10] From Table 1, it is clear that the lifetime of the europium level does not depend on temperature, showing that no upper-lying excited state of other configuration (particularly LMCT states) is thermally accessible from the $^5D_0$ emitting state.

Eu·**L** and Tb·**L** are also efficient luminescent emitters at physiological pH (0.05 M HEPES buffer pH 7.4) where their photophysical characteristics (τ, Φ) are similar with those observed at pH 8.6. Moreover, these complexes are highly resistant to dissociation in water

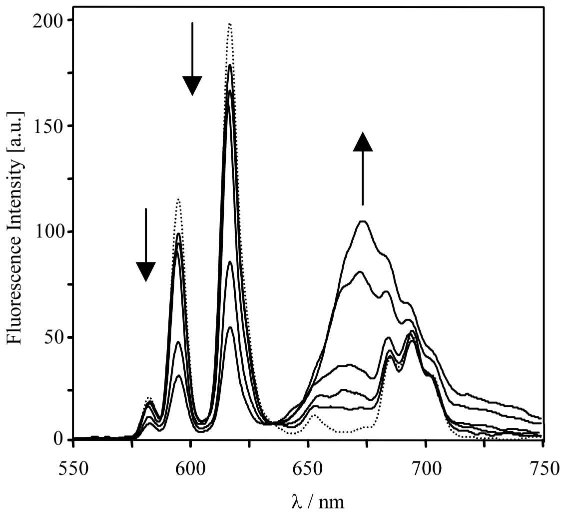

Figure 2. Diffusion-enhanced energy transfer from Eu·**L** to Cy-5. Eu·**L** (2 μM dashed line) was mixed with increasing concentration of Cy-5 (0.15, 0.3, 0.5, 2 and 4 μM) in 50 mM borate buffer, pH 8.6. These spectra were obtained using excitation wavelength of 320 nm, delay time 0.1 ms, gate time 0.4 ms and slit width 5 nm.

(examined pH range 6.6–8.6), in the presence of cations (Na^+, Ca^{2+}, 100-fold excess) or in biological medium (HEPES buffer:human serum, 2:1). In these experiments, no decrease of the luminescence intensity of Eu·**L** and Tb·**L** was observed after several days at room temperature. When the Eu·**L** complex was challenged with a fivefold excess of DOTA at pH 7.4,[11] a conditional stability constant was measured to be 10^{18} by using the Verhoeven's method.[12]

Next, we investigated the potentiality of these lanthanide chelates to act as donors in delayed fluorescence resonance energy transfer (DEFRET).[13] With this aim in view, we used the diffusion-enhanced energy transfer method, in which a freely diffusing lanthanide donor (D) transfers energy to an organic acceptor fluorophore (A).[14] The results for the experiments between Eu·**L** as the energy donor ($\lambda_{em} = 617$ nm) and an organic dye Cy-5[15] as the energy acceptor ($\lambda_{abs} = 647$ nm) are shown in Figure 2.

Addition of Cy-5 decreases the donor emission intensity while the acceptor emission intensity ($\lambda = 670$ nm) increases, as a result of the sensitized emission of Cy-5 via the energy transfer. The excitation spectrum corresponding to the acceptor emission (Fig. 3) shows clearly that the emitting state of Cy-5 is populated through absorption of the bipyridine chromophore, i.e. from the europium donor.

In this system, the energy transfer was moreover evidenced by the coincidence of the luminescence lifetimes at 617 and 670 nm. As a matter of fact, when engaged in the FRET process, the long-lived Eu·**L** complex induces a long-lived emission from the Cy-5 acceptor. In the absence of lanthanide donor, the Cy-5 lifetime is ~1 ns.[16] In the presence of this process and, for instance, for a [D]/[A] ratio of 1, the Cy-5 displays a lifetime of 300 μs. Finally, for this donor–acceptor pair and by using standart equation,[17] we calculate that the critical transfer distance (R_0) at which 50% of the energy is transferred is 59.5 Å (72.3 Å in D_2O). These R_0 values are relatively high with regard to those generally observed for conventional donor–acceptor pairs[18] and compare well with those reported for a polycarboxylate chelate of europium (Eu.DTPA-Cs124) which is used in DEFRET techniques in conjunction with Cy-5.[19]

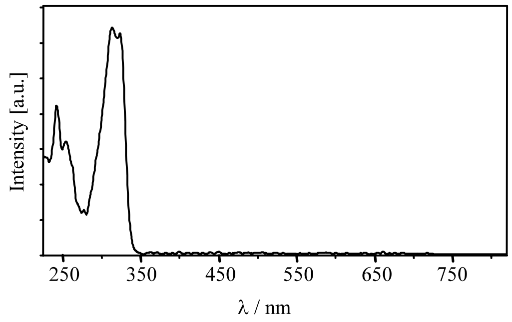

Figure 3. Excitation spectrum under the same conditions as those indicated in Figure 2 ([D] = [A] = 2 μM). The excitation spectrum is recorded at an observation wavelength of 670 nm.

In conclusion, the described macrocyclic complexes fulfill the requirements to be used as luminescent bioprobes: a good hydrophylicity and a high kinetic inertness in aqueous solutions, a long luminescence lifetime and an efficient cation emission. Moreover, the results reported here open up the possibility to use these chelates as donors in DEFRET techniques, which permits to extend fluorescence detection time scale from the nanoseconds to the hundred microseconds range. Further improvement of the ligand structure may arise from the elimination of the water molecule coordinated to the metal ion. Replacement of acetate arms by sterically encumbered phosphinate groups is in progress.

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8. Selected data for [Eu·**L**]: UV (H_2O): λ_{max} (ε) 321 (9200); Luminescence (H_2O, pH 8.6) λ_{em} 582 (3.8), 595 (31.4), 617 (100), 652 (6), 694 (81.5); MS (ES+): *m/z* 608.1 $[M+H]^+$ (100%), 630.1 $[M+Na]^+$ (25%), 646.2 $[M+K]^+$ (15%).
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