Lanthanide Tags for Time-Resolved Luminescence Microscopy Displaying Improved Stability and Optical Properties

Loïc Charbonnière,[1] Raymond Ziessel,*[,1] Massimo Guardigli,[2] Aldo Roda,*[,2] Nanda Sabbatini,[2] and Michèle Cesario[3]

Laboratoire de Chimie d'Electronique et de Photonique Moléculaires, ECPM 25 rue Becquerel, BP 08, 67087 Strasbourg Cedex 2, France
Department of Pharmaceutical Sciences University of Bologna, Via Belmeloro, 40126 Bologna, Italy
Institut de Chimie des Substances Naturelles CNRS, F-91128 Gif-sur-Yvette, France

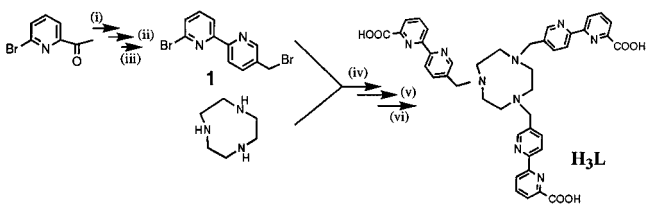
Received October 17, 2000

The use of macrocyclic[4] or metal-containing[5] compounds for clinical biochemistry and medical applications is a topical subject that has led to detailed investigation of numerous systems. In particular, lanthanide complexes are archetypal targets,[6,7] being used widely as luminescent labels for the specific analysis of certain biological materials.[8] Lanthanide-labeled biomolecules (e.g., antibodies) are employed in time-resolved (TR) fluoroimmunoassay[9] for the in vitro quantitative detection of analytes at the subpicomolar level.[10] Imaging experimental techniques, relying on fluorescent labels coupled with optical fluorescence microscopy, are now being used in vivo to localize probe molecules or gene sequences in tissue sections and in single cells.[11] Furthermore, the use of TR luminescence imaging techniques in conjunction with luminescent probes that possess unusually long-lived excited states provides a facile route by which to extend the sensitivity.

We report herein the synthesis and selected properties of lanthanide-based probes equipped with the main requisites for a TR luminescence label: namely, (i) good solubity in water at room temperature, (ii) high resistance to hydrolysis, (iii) strong absorption bands in an accessible part of the spectrum, (iv) pronounced emission from the metal center, and (v) a long-living metal-based excited state. The ligand comprises a podand incorporating dangling bipyridine arms, with each subunit bearing a negatively charged carboxylate group. The resultant lanthanide complex is stabilized owing to multiple chelating effects and by electrostatic interactions. Coordination of three tridentate N,N,COO^- units to the cation provides an overall coordination number of 9, a situation known to be highly favorable for lanthanide cations.

The target ligand was obtained from a cyclic polyamine platform and three 6-carboxylate-2,2′-bipyridine units using a multistep synthetic procedure. The pivotal starting compound **1** was synthesized via a Kröhnke protocol, followed by free-radical bromination of the product. Alkylation of 1,4,7-triazacyclononane afforded the tribranched synthon in reasonable yield. The acid-functionalized podand H_3L was obtained by an original carboalkoxylation reaction promoted by a catalytic amount of Pd^o,[12] followed by a smooth saponification reaction (Scheme I).

Scheme 1[a]



[a] Conditions: (i) I_2, pyridine, 95%; (ii) methacroleine, NH_4OAc, formamide, 68%; (iii) NBS, AIBN, CCl_4, 55%; (iv) **1** (3.2 equiv), Na_2CO_3, CH_3CN, 96%; (v) EtOH, Et_3N, CO flow, 1 atm, $[Pd(PPh_3)_2Cl_2]$ (6 mol %) 83%; (vi) NaOH, MeOH, 80 °C, protonation, 78%.

Mixing equimolar amounts of $LnCl_3 \cdot 6H_2O$ (Ln = Eu, Gd, and Tb) and H_3L, followed by addition of excess Et_3N, gave the analytically pure mononuclear complexes. The ^1H NMR spectrum of the Eu complex in d_6-DMSO at 300 K exhibits 13 peaks spread over 26 ppm, as a result of the paramagnetic contribution of the Eu atom. Twelve of these peaks can be assigned to the ligand while the remaining peak at 16.7 ppm corresponds to a single proton. In the presence of D_2O, this signal disappears by way of rapid exchange with deuterium. The number of unequivalent protons unambiguously points to C_3 symmetry in solution. Raising the temperature (to 370 K) or changing the solvent (D_2O) did not allow for observation of the coalescence of the different methylenic systems, proving the $\Delta \leftrightarrow \Lambda$ interconversion process to be very slow on the NMR time scale. An X-ray crystal structural determination indicates that the additional proton is encapsulated within a cavity defined by the branched triazacyclononane. High-performance ion chromatography performed in pure water, revealed the presence of 1.1 ± 0.1 equiv of Cl^- per Eu atom. Finally, elemental analysis is fully consistent with a general formulation of the type $[Eu(HL)]Cl \cdot H_2O$. Although surprising the presence of a protonated complex is in keeping with related lanthanide complexes constructed with a neutral carboxamide tripod[13] and affords good solubility in water.

The X-ray crystal structure confirms this formulation (Figure 1) and the asymmetric unit consists of one $[Eu(HL)]^+$ cation, one chloride anion, and 10 water molecules.[14] The coordination sphere around the Eu^{III} ion consists of 6 nitrogen atoms provided by the 3 bipy subunits together with 3 oxygen atoms of the carboxylate groups grafted onto the pyridine rings. These coordinating atoms form a distorted tricapped prism, with the cage-shaped compound roughly adopting a ternary symmetry with a *pseudo*-C_3 axis perpendicular to the polyamine cycle and passing through the Eu atom. A residual peak of 0.5 $eÅ^{-3}$, located at suitable distance and angle from the Nc atom of the macrocycle, can be assigned to a proton. Note there is no remaining electron density around the other two N atoms. The O-atoms that are not coordinated to the cation point outward and are linked to water molecules forming a supramolecular H-bonded network (see Supporting Information). The wrapping of the three bipyridine subunits around the Eu^{III} ion leads to formation of a triple helix, with the N,N,COO^- frames forming an angle of $121 \pm 5°$ with the mean plane defined by the macrocycle.

The photophysical properties of the lanthanide complexes of H_3L in water were constant for several days, proving their excellent stability. The intense absorption bands are attributed to $\pi-\pi^*$ transitions of the bipy moieties. The Eu^{3+} and Tb^{3+} complexes are strongly luminescent in water upon excitation in the bipy chromophores. These complexes exhibit excited-state

(1) Laboratoire de Chimie, d'Electronique et de Photonique Moléculaires. E-mail: ziessel@chimie.u-strasbg.fr.
(2) University of Bologna. E-mail: roda@alma.unibo.it.
(3) Institut de Chimie des Substances Naturelles, CNRS.
(4) Sessler, J. L.; Miller, R. A. *Biochem. Pharmacol.* **2000**, *59*, 733.
(5) Guo, Z.; Sadler, P. J. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 1512.
(6) Piguet, C.; Bünzli, J.-C. G. *Chem. Soc. Rev.* **1999**, *28*, 347.
(7) Mayer, A.; Neuenhofer, S. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1044.
(8) Chen, J.; Selvin, P. R. *J. Am. Chem. Soc.* **2000**, *122*, 657.
(9) (a) *Application of Fluorescence in Immunoassays*; Hemmilä, I. A., Ed.; Wiley: New York, 1991. (b) Mathis, G. *Clin. Chem.* **1995**, 41, 1391.
(10) Hemmilä, I.; Harju, R. In *Biological Applications of Labeling Technologies*, 2nd ed.; Emmilä, I., Ståhlberg, T., Mottram, P., Eds.; Wallac Oy and EG&G cie Pbs: Turku, 1995.
(11) Bornhop, D. J.; Hubbard, D. S.; Houlne, M. P.; Adair, C.; Kiefer, G. E.; Pence, B. C.; Morgan, D. L. *Anal. Chem.* **1999**, *71*, 2607.
(12) El-ghayoury, A.; Ziessel, R. *J. Org. Chem.* **2000**, *65*, 7757.
(13) Renaud, F.; Piguet, C.; Bernardinelli, G.; Bünzli, J.-C. G.; Hopfgartner, G. *J. Am. Chem. Soc.* **1999**, *121*, 9326.
(14) Monoclinic, *P*21/*n*, $a = 14.515$ (6) Å, $b = 21.200$ (8) Å, $c = 15.696$ (6) Å, $\beta = 102.11(4)°$, $V = 4722.5$ $Å^3$, $Z = 4$, $R = 0.033$, $wR = 0.077$.

10.1021/ja003699h CCC: $20.00
Published on Web 02/15/2001

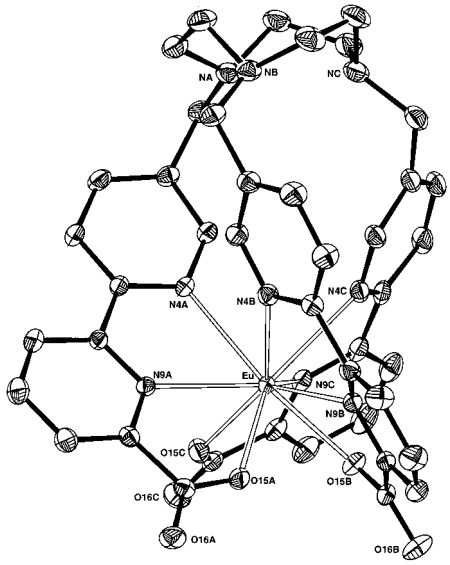


Figure 1. X-ray crystal structure of $[Eu(HL)]Cl\cdot 10H_2O$ showing an ORTEP view of the $[Eu(HL)]^+$ complex along the pseudo-C_3 axis with 30% probability thermal ellipsoïds and H atoms omitted for the sake of clarity. Selected bond lengths (Å) and angles (deg): Eu−O15B 2.367(2); Eu−O15A 2.370(2); Eu−O15C 2.388(2); Eu−N9A 2.547(2); Eu−N9C 2.558(2); Eu−N9B 2.563(2); Eu−N4A 2.635(2); Eu−N4C 2.714(2); Eu−N4B 2.727(2); O15A−Eu−N9A 64.47(7); O15C−Eu−N9C 64.71(8); O15B−Eu−N9B 64.83(8); N9A−Eu−N4A 62.69(7); N9C−Eu−N4C 61.56(8); N9B−Eu−N4B 61.42(7).

Table 1. Photophysical Properties of Lanthanide Complexes of H_3L[a]

	absorption	metal luminescence		
	λ_{max} [nm], ϵ_{max} [M^{-1} cm^{-1}]	τ^{300K} [ms]	τ^{77K} [ms]	Φ^{300K}
Eu	309, 34300	1.85 (2.95[c])	2.1 (3.2[c])	0.12 (0.18[c])
Tb	308, 33400	0.50 (0.52[c])	1.7 (2.0[c])	0.10 (0.12[c])
Gd	308, 35300	*d*	*d*	*d*

[a] Data obtained in water solution at room temperature, unless otherwise noted. [b] Upon excitation in the ligand absorption at 309 nm. [c] In D_2O solution. [d] No metal luminescence is observed.

lifetimes that are among the longest reported for such complexes in aqueous solution (Table I).[15] The emission quantum yields, measured following excitation into the organic ligand, are relatively high, due to both efficient ligand-to-metal energy transfer and inefficient nonradiative deactivations of the metal-centered excited states. Comparison of the lifetime and quantum yield measured in light and heavy water indicates that O−H vibrations make only a minor contribution to the total nonradiative deactivation. Using the Horrocks and Sudnick equation[16] it was established that for both complexes the cation coordinates less than 0.2−0.3 water molecule in solution, a value that falls to ≈0 when applying the second-sphere corrections proposed by Parker et al.[17] This confirms the excellent shielding ability of the ligand as deduced earlier from the X-ray structure. A temperature-dependent study indicates that thermally activated processes play a significant role in decay of the metal-centered emitting state in the case of the terbium but not for the other complexes. As suggested previously for Tb^{3+} complexes of related ligands,[18] such processes involve reverse energy transfer between the Tb^{3+} ($^5D_4$) emitting state and triplet states localized on the bipy fragment.

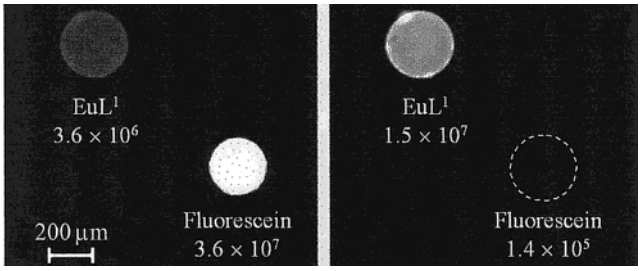


Figure 2. Microscope luminescence imaging of a model system consisting of oxirane acrylic beads (diameter ∼250 μm) containing $[Eu(HL)]^+$ or fluorescein. The left panel shows the prompt fluorescence image and the right panel the TR luminescence image. The figures represent the average luminescence intensities (expressed in photons/s per image pixel) measured for each bead.

Note that the energy of such excited state, evaluated from the phosphorescence spectrum of $[Gd(HL)]^+$, is about 21 700 cm^{-1}, i.e., 1300 cm^{-1} above the Tb^{3+} emitting state.

The conditional stability constant $K_{cond,L}$ for formation of the europium complex with H_3L in water was measured at pH 7.0 (0.05 M Na_2HPO_4/NaH_2PO_4, 25 °C) by competition with EDTA,[19] followed by UV−vis absorption spectroscopy (see Supporting Information). $\log(K_{cond,L})$ was measured to be 14.3 ± 0.8 (to be compared to $\log(K_{cond,EDTA}) = 14.07$), showing the ligand to form a complex of the same order of stability as EDTA, under the same conditions. The high stability of the present lanthanide complexes is a necessary prerequisite for use in biological media. Furthermore, $[Eu(HL)]^+$ and $[Tb(HL)]^+$ complexes were found to be stable for several days in 0.1 M acetate (pH 5.8) and Tris (pH 7.9) buffers, and in the presence of up to a 1000-fold excess of Ca^{2+}, where the photophysical properties were almost identical with those obtained in pure water.

Because of the interesting photophysical properties and useful stability found for the Eu^{3+} and Tb^{3+} complexes of H_3L we used these complexes as the basis for luminescent probes for bioaffinity assays that rely on TR luminescence measurements. We carried out preliminary TR luminescence microscopy imaging experiments on a model system consisting of acrylic beads (diameter ∼250 μm) containing $[Eu(HL)]^+$ or fluorescein as an organic reference. It can be noticed that both the fluorescein- and the $[Eu(HL)]^+$-containing beads appeared in the prompt fluorescence image, while the fluorescein-containing bead, characterized by a short-lived emission, completely disappeared in the TR luminescence image. On the basis of the intensity values reported in Figure 2 it has been calculated that these systems provide for a 1000-fold increase in the Eu^{3+}/fluorescein emission ratio compared to the prompt fluorescence measurement. This suggests that suitable $[Eu(HL)]^+$ derivatives could be used as luminescent labels in TR luminescence microscopy by suppressing the sample background fluorescence. Ongoing studies are aimed at derivatizing these complexes with an anchoring function that will bind to biomolecules, with the aim to develop sensitive immunohistochemical and "in situ" hybridization methods on tissues and single cells. The availability of these luminescent labels also opens the possibility of processing micromolecular arrays based on the engineering of oligonucleotide scaffoldings.

Supporting Information Available: Experimental details for the synthesis of the ligand and complexes, characterization of all new compounds, ^1H NMR spectra, X-ray crystal structure, absorption and luminescence, and excitation spectrum of the [Eu(HL)]Cl in water and conditional stability constants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA003699H

(15) Elhabiri, M.; Scopelliti, R.; Bünzli, J.-C. G.; Piguet, C. *J. Am. Chem. Soc.* **1999**, *121*, 10747.

(16) (a) Horrocks, W. De W., Jr.; Sudnick, D. R. *J. Am. Chem. Soc.* **1979**, *101*, 334. (b) Horrocks, W. De W., Jr.; Sudnick, D. R. *Acc. Chem. Res.* **1981**, *14*, 384.

(17) Beeby, A.; Clarkson, I. M.; Dickins, R. S.; Faulkner, S.; Parker, D.; Royle, L.; De Sousa, A. S.; Williams, J. A. G.; Woods, M. *J. Chem. Soc., Perkin Trans. 2* **1999**, 493.

(18) (a) Sabbatini, N.; Guardigli, M.; Manet, I. In *Advances in Photochemistry*; Neckers, D. C., Volman, D. H., von Bünau, G., Eds.; John Wiley & Sons: New York, 1997; Vol. 23, p 213. (b) Charbonnière, L. J.; Balsiger, C.; Schenk, K.; Bünzli, J.-C. G. *J. Chem. Soc., Dalton Trans.* **1998**, 505.

(19) Wu, S. L.; Horrocks, W. D., Jr. *Anal. Chem.* **1996**, *68*, 394.