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Long-Lived Two-Photon Excited Luminescence of Water Soluble Europium Complex: Applications in Biological Imaging using Two-Photon Scanning Microscopy.

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The unique luminescence properties of Ln^{III} ions¹ (sharp emission, large Stokes shift, insensitivity to oxygen) and particularly their long excited state lifetime ranging from μs (Yb, Nd) to ms (Eu, Tb) triggered the development of time-resolved spectroscopy or microscopy for applications in biological, environmental or clinical analysis.^{2,3} These techniques consist in the introduction of a time delay before the detection of the lanthanide luminescence in order to eliminate parasitic scattering and short-lived luminescence resulting in an enhanced signal/noise ratio. However, UV-light is needed for the metal sensitization, which is a drawback for biological applications. Indeed, these wavelengths are scattered/absorbed by the medium, which limits the investigation depth and presents some photo-toxicity for the biological samples. Two (or multi-) photon excitation, that is simultaneous absorption of two photons of half energy, is an elegant way to circumvent the use of UV-light. Following pioneering works of Webb in the 1990's,⁴ confocal biphotonic microscopy is becoming even more popular with the commercialization of tunable fs-laser source. This technique allows the use of irradiation wavelengths located in the near infra-red (700-900 nm), a spectral range where the biological media are transparent. In addition, its intrinsic confocal character strongly limits the photo-damage and gives rise to 3D resolved spectroscopy and microscopy.⁵ Whereas numerous works have been devoted to the optimization of the luminescence properties of lanthanide complexes for imaging or sensing applications,^{2,3} their two-photon sensitization is becoming an emerging field of research. The proof-of-concept of two-photon antenna effect has already been demonstrated in biological media with complexes featuring very low (generally not measured) two-photon absorption (2PA) cross-section, σ_{2PA}.⁶ On the other hand, significant σ_{2PA} values are reported in the cases of Eu^{III} complexes only stable in non aqueous solvents,⁷ not suitable for any practical applications as biological probes. In this article, we report the design of new functionalized tris-dipicolinate Eu^{III} complexes (Chart 1) that are (i) soluble and stable in aqueous media, (ii) luminescent with long excited lifetime and (iii) that present significant two-photon absorption cross-section in the biological window.

Recently, we described the two-photon antenna effect of tricationic [EuL⁰₃][OTf]₃ complex (σ_{2PA} = 96 GM at 720 nm), where L⁰ is a alkyloxyphenylacetylene functionalized pyridine-dicarboxamide ligand (Chart 1).^{7a} Its very low stability prevented any use in aqueous media and prompted us to switch to pyridine-dicarboxylic acid (DPA) analogous known to present a enough stability in water for spectroscopic studies.⁸ DPA was functionalized by extended π-system featuring weak electron donor alkoxy group (L¹ Chart 1) and the hydrosolubility was ensured by 3,4,5-tris(triethyleneglycol)phenyl moieties (HG).⁹

Ligand synthesis is reported in supporting information. The corresponding complex [Na]₃[Eu(L¹)₃], was prepared in water by mixing three equiv. of ligand in basic media with EuCl₃·6H₂O and isolated by extraction with dichloromethane. The spectroscopic characterizations are in agreement with the proposed structures (SI) and the complex is stable and soluble in organic solvents and water.

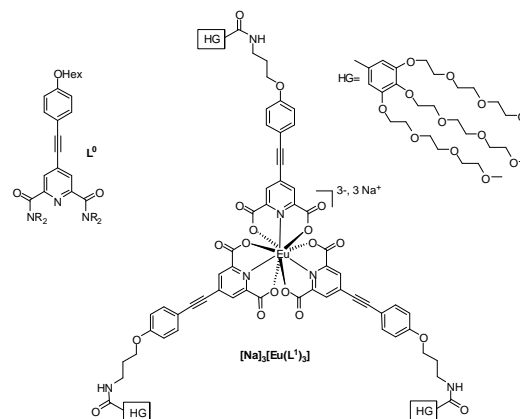


Chart 1. Structure of the ligands and related complex.

All the photophysical properties of the ligand and complex were recorded in water and the stability of the complex in aqueous media is similar to that of the non functionalized parent analogous (see SI for details). L¹ presents a broad transition by UV-visible spectroscopy centred at 318 nm (25600 L.mol⁻¹ cm⁻¹) in water at pH = 5 assigned to charge transfer (CT) transition from the alkoxy donor to the pyridinic acceptor moieties.^{7a} Complexation to Eu^{III} results in a small bathochromic shift of the CT transition (λ_mmax = 332 nm (78700 L.mol⁻¹ cm⁻¹), Figure 1), the lanthanide Lewis acidity effect being partially compensated by the trianionic nature of the complex, and is accompanied by a threefold exaltation of the extinction coefficient in agreement with the 3/1 ligand-to-metal ratio. Emission spectra reveal the characteristic Eu^{III} emission profile with the very intense ⁵D₀→⁷F₂ transition upon excitation in the ligand CT transition (Figure 2). The quantum yield of the complex is good, about 15.7%, the lifetime is long (1.062 ms in water, figure S2) and remains far higher than that of other organic or endogen chromophores (few ns). The two-photon excitation spectrum was recorded, in the 700-900 nm range using a femtosecond Ti:sapphire laser source.^{7a} The 2PA process is unambiguously confirmed by the quadratic dependence (experimental coef. 1.9)

of the 613 nm band with the laser power ($\lambda_{\text{ex}} = 720$ nm, Figure S5). The 2PA spectrum matches almost perfectly the wavelength doubled one absorption spectra (1PA) with a maximum 2PA wavelength around 700 nm (Figure 1), indicating that the excited states involved in the 1PA and 2PA processes are the same. In the measured range, the maximal 2PA cross-section is significant, about 92 GM at 700 nm. It is worth underlining that this value is in the same range than that of other lanthanide compounds,^{7a-c} only stable in organic solvents and far higher than that of the water stable complexes.^{6,7c}

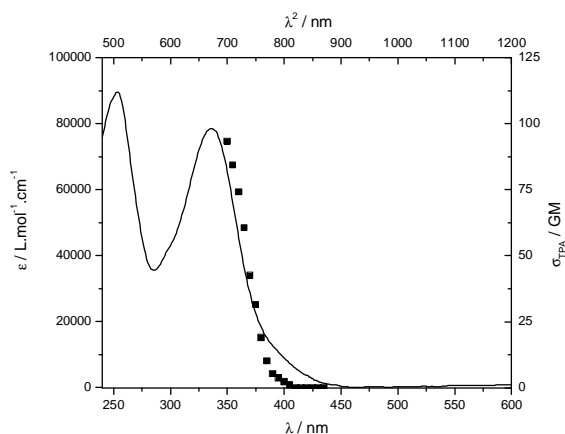


Figure 1. Absorption spectra of $[\text{Na}]_3[\text{Eu}(\text{L})_3]$ (—) in water. In upper abscissa is superimposed the two-photon excitation spectra (■).

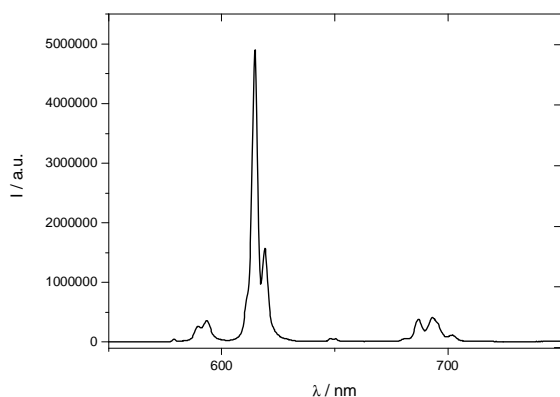


Figure 2. Emission spectra of $[\text{Na}]_3[\text{Eu}(\text{L})_3]$ in water ($\lambda_{\text{ex}} = 340$ nm, conc. $1.31 \cdot 10^{-5}$ mol.L⁻¹)

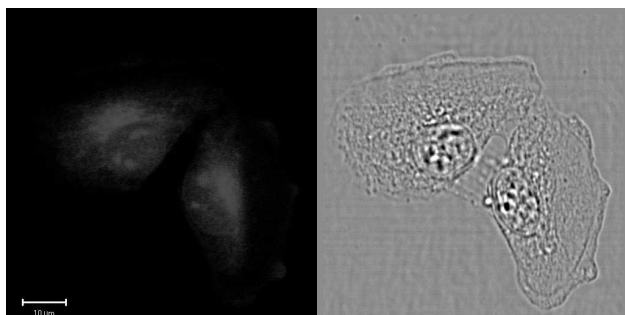


Figure 3. Two-photon excited luminescence (left, $\lambda_{\text{ex}} = 760$ nm) and phase contrast (right) images of T24 cancer cell fixed in ethanol and loaded with $[\text{Na}]_3[\text{Eu}(\text{L})_3]$. Image were taken on a Zeiss LSM510 NLO META confocal microscope equipped with a fs-Ti:sapphire laser.

Finally, two-photon scanning microscopy experiments were carried out using T24 cancer cells fixed with ethanol at -20°C and loaded with $[\text{Na}]_3[\text{Eu}(\text{L})_3]$ in PBS solution (concentration in the sample about $2 \cdot 10^{-5}$ mol.L⁻¹.cm⁻¹, see SI for details). The images shown in figure 3 were taken on a biphotonic laser scanning microscope upon fs-760 nm irradiation ($\sigma_{2\text{PA}}(760) = 19$ GM). The red Eu luminescence was integrated in 503-695 nm spectral range. Comparison with phase contrast image clearly indicates that the complex is mainly localised in a perinuclear region and its distribution is similar to that of the endoplasmic reticulum. In addition, bright spots are observed in the nucleus indicating that the complex preferentially targeted small organelles called nucleoli, similarly to a recent study of Parker and co-workers.^{3a}

In conclusion, we report the design of a new europium complex that fulfills all the requirements for bio-imaging applications: (i) good solubility and stability in water, (ii) intense emission in the red (616 nm), (iii) long luminescence lifetime and (iv) significant two-photon absorption cross-section induced by two-photon antenna effect in the biological window. Furthermore, we describe the first two-photon scanning microscopy bio-imaging experiments using a lanthanide complex that can be considered as a new generation of molecular probes. Further studies are currently carried out (i) to study localization of the complex in the cells, (ii) to increase the molecular two-photon cross-section and water stability of the complexes and (iii) to develop biological imaging using *biphotonic time-resolved microscopy*.

Acknowledgements. The authors thank the ANR LnOnL NT05-3 42676) for financial support. Two photon microscopy facilities were partially supported by ARC and NANOBIO program.

Supporting Information Available. Experimental data, figures S1-5 are available free of charge via the Internet at <http://pub.acs.org>.

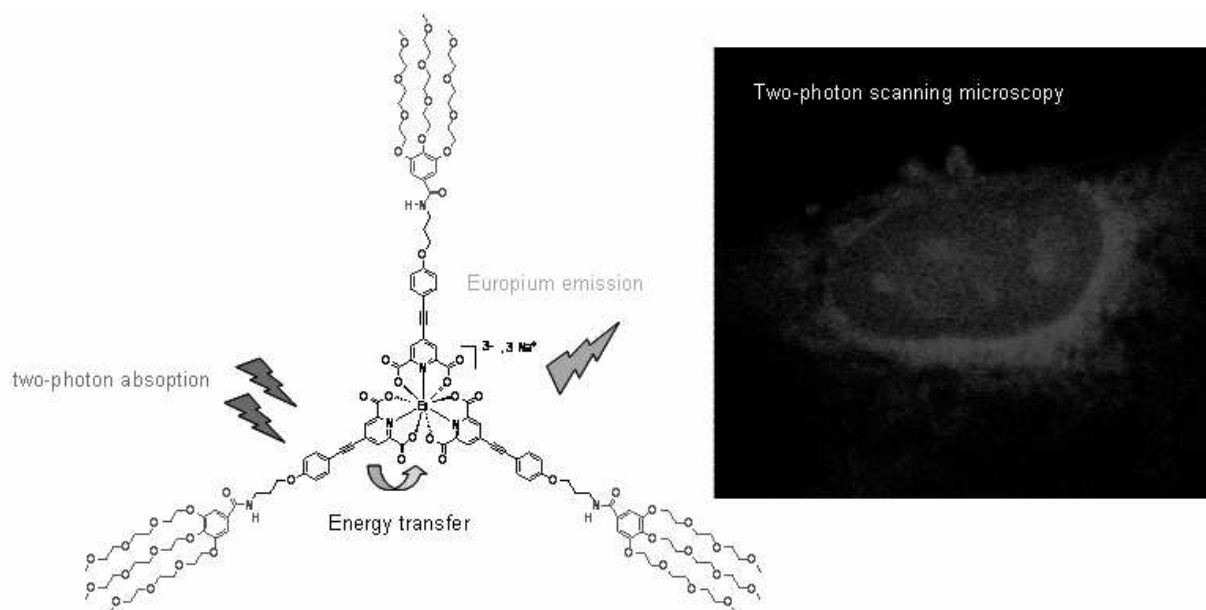
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Abstract. A new europium complex presenting good solubility and stability in water, intense emission in the red (616 nm), long luminescence lifetime and significant two-photon absorption cross-section in the biological window has been designed and successfully used for two-photon scanning microscopy bio-imaging experiments on fixed cancer cells.