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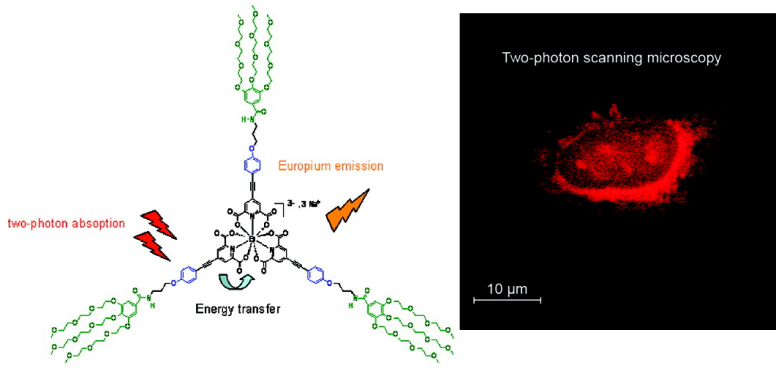

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Long-Lived Two-Photon Excited Luminescence of Water-Soluble Europium Complex: Applications in Biological Imaging Using Two-Photon Scanning Microscopy

Alexandre Picot,[†] Anthony D'Aléo,[†] Patrice L. Baldeck,[‡] Alexei Grichine,[§] Alain Duperray,[§] Chantal Andraud,*[,†] and Olivier Maury*[,†]

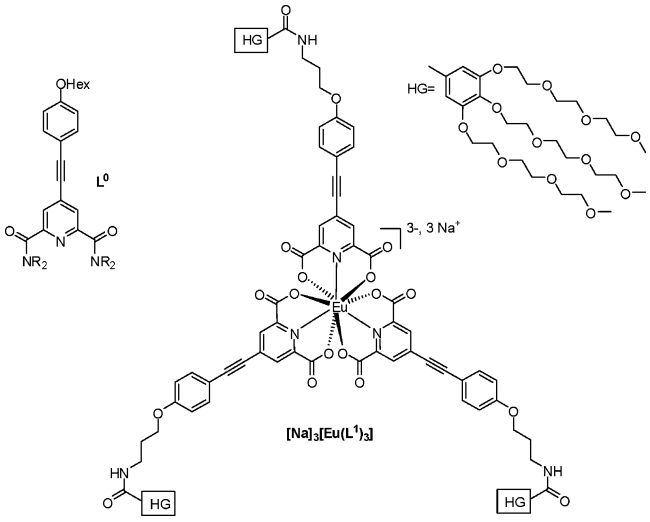
Université de Lyon, Laboratoire de Chimie, CNRS-ENS Lyon, 46 allée d'Italie 69364 Lyon, France, Laboratoire de Spectrométrie Physique, Université Joseph Fourier, 38402 Saint Martin d'Hères, France, and INSERM U823 and CHU Grenoble-EFS, Institut d'oncologie/développement Albert Bonniot, Université Joseph Fourier-Grenoble I, 38042 Grenoble, France

Received September 13, 2007; E-mail: olivier.maury@ens-lyon.f

The unique luminescence properties of Ln^{III} ions[1] (sharp emission, large Stokes shift, insensitivity to oxygen) and particularly their long excited state lifetime ranging from microseconds (Yb, Nd) to milliseconds (Eu, Tb) triggered the development of time-resolved spectroscopy or microscopy for applications in biological, environmental, or clinical analysis.[2,3] These techniques consist of 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 phototoxicity 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 1990s,[4] confocal biphotonic microscopy is becoming even more popular with the commercialization of a tunable femtosecond laser source. This technique allows the use of irradiation wavelengths located in the near-infrared (700−900 nm), a spectral range where the biological media are transparent. In addition, its intrinsic confocal character strongly limits the photodamage 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 a two-photon antenna effect has already been demonstrated in biological media with complexes featuring a 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 nonaqueous solvents,[7] not suitable for any practical applications as biological probes. In this paper, we report the design of new functionalized tris-dipicolinate Eu^{III} complex (Chart 1) that (i) is soluble and stable in aqueous media, (ii) is luminescent with long excited lifetime, and (iii) presents a significant two-photon absorption cross-section in the biological window.

Recently, we described the two-photon antenna effect of a tricationic $[Eu\mathbf{L^0}_3][OTf]_3$ complex (σ_{2PA} = 96 GM at 720 nm), where $\mathbf{L^0}$ 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) analogously known to present a sufficient stability in water for spectroscopic studies.[8] DPA was functionalized by an extended π-system featuring a weak electron donor alkoxy group ($\mathbf{L^1}$, 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 $[Na]_3[Eu(\mathbf{L^1})_3]$ was prepared in water by mixing 3 equiv of ligand in basic media with $EuCl_3{\cdot}6H_2O$ and isolated by extraction with dichloromethane. The spectroscopic characterizations are in agreement with the proposed structures (Supporting Information), 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 nonfunctionalized parent analogous (see Supporting Information for details). $\mathbf{L^1}$ presents a broad transition by UV−visible spectroscopy centered at 318 nm (25 600 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$) in water at pH = 5 assigned to a 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_{max} = 332 nm (78 700 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$) Figure 1), with the lanthanide Lewis acidity effect being partially compensated by the tris-anionic nature of the complex. Furthermore, it 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 $^5D_0 \rightarrow {}^7F_2$ transition upon excitation in the ligand CT transition (Figure 2). The quantum yield of the complex is good, about 15.7%, and the lifetime is long (1.062 ms in water, figure S2) remaining 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 coefficient = 1.9) of the 613 nm band with

[†] CNRS-ENS Lyon.
[‡] Laboratoire de Spectrométrie Physique, Université Joseph Fourier.
[§] INSERM, U823 Institut d'oncologie/development Albert Bonniot, Grenoble.

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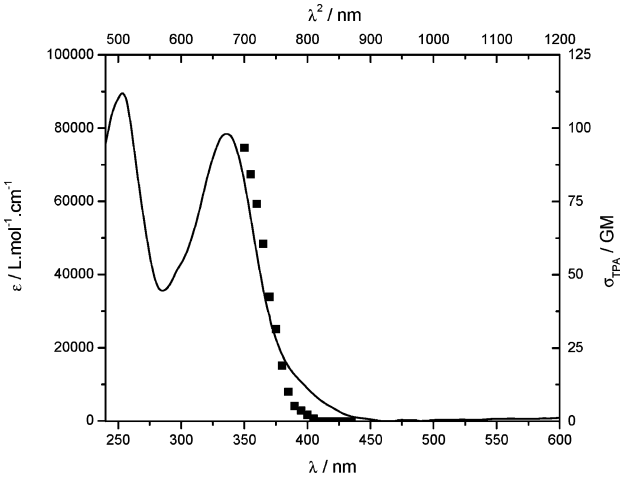

Figure 1. Absorption spectra of $[Na]_3[Eu(\mathbf{L^1})_3]$ (—) in water. In upper abscissa is superimposed the two-photon excitation spectra (■).

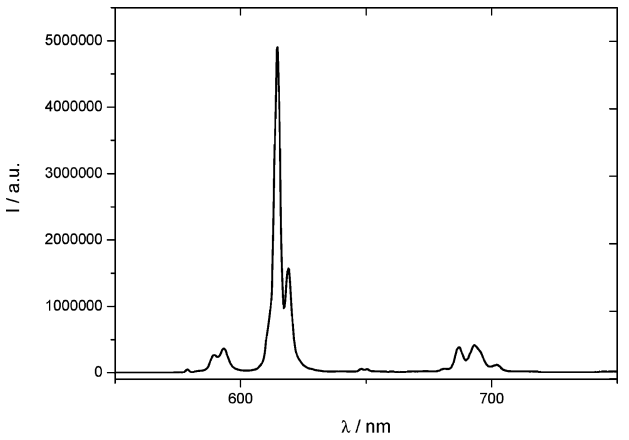

Figure 2. Emission spectra of $[Na]_3[Eu(\mathbf{L^1})_3]$ in water (λ_{ex} = 340 nm, concn = 1.31×10^{-5} mol·L^{-1}).

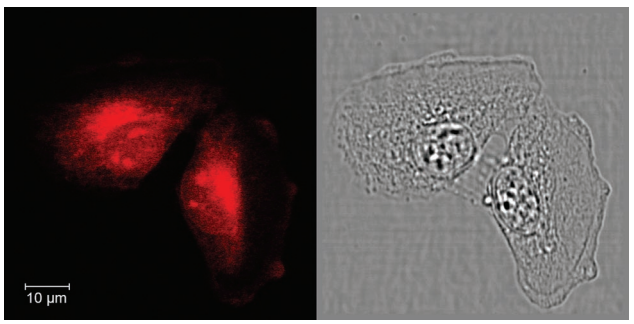

Figure 3. Two-photon excited luminescence (left, λ_{ex} = 760 nm) and phase contrast (right) images of T24 cancer cell fixed in ethanol and loaded with $[Na]_3[Eu(\mathbf{L^1})_3]$. Images were taken on a Zeiss LSM510 NLO META confocal microscope equipped with a femtosecond Ti:sapphire laser.

the laser power (λ_{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 as that of other lanthanide compounds,[7a−c] only stable in organic solvents and far higher than that of the water-stable complexes.[6,7c]

Finally, two-photon scanning microscopy experiments were carried out using T24 cancer cells fixed with ethanol at −20 °C and loaded with $[Na]_3[Eu(\mathbf{L^1})_3]$ in PBS solution (concentration in the sample about 2×10^{-5} mol·L^{-1}·cm^{-1}; see Supporting Information for details). The images shown in Figure 3 were taken on a biphotonic laser scanning microscope upon femtosecond 760 nm irradiation ($\sigma_{2PA}(760)$ = 19 GM). The red Eu luminescence was integrated in the 503−695 nm spectral range. Comparison with a phase contrast image clearly indicates that the complex is mainly localized 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 bioimaging applications: (i) good solubility and stability in water, (ii) intense emission in the red (613 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 bioimaging experiments using a lanthanide complex that can be considered as a new generation of molecular probes. Further studies are currently being 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*.

Acknowledgment. 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 and Figures S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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