Multiphoton-Excited Luminescent Lanthanide Bioprobes: Two- and Three-Photon Cross Sections of Dipicolinate Derivatives and Binuclear Helicates

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Multiphoton excited luminescent properties of water-soluble Eu^{III} and Tb^{III} complexes with derivatives of dipicolinic acid functionalized with a polyoxyethylene pendant arm and terminal groups, $[Eu(L^{OMe})_3]^{3-}$, $[Eu(L^{NH2})_3]^{3-}$, and $[Tb(L^{OH})_3]^{3-}$, as well as of binuclear helicates with overall composition $[Ln_2(L^{CX})_3]$ (X = 2, 5) are investigated. Characteristic emission from the $^5D_0$ and $^5D_4$ excited levels of Eu^{III} and Tb^{III}, respectively, upon ≈800 nm excitation results from three-photon absorption (3PA) for $[Eu(L^{OMe})_3]^{3-}$, $[Eu(L^{NH2})_3]^{3-}$, $[Tb(L^{OH})_3]^{3-}$, and $[Ln_2(L^{C2})_3]$, while luminescence from $[Eu_2(L^{C5})_3]$ is induced by two-photon absorption (2PA) owing to its 1PA spectrum extending further into the visible. The 3PA cross sections have been determined and are the first ones reported for lanthanide complexes: (i) those of Eu^{III} and Tb^{III} bimetallic helicates $[Ln_2(L^{C2})_3]$ are 20 times larger compared to the corresponding values for tris(dipicolinates); (ii) derivatization of dipicolinic acid for Tb^{III} complexes has almost no influence on the 3PA cross section; however, for Eu^{III} complexes a ∼2 times decrease is observed. The feasibility of $[Eu_2(L^{C5})_3]$ as multiphoton luminescence bioprobe is demonstrated by two-photon scanning microscopy imaging experiments on HeLa cells incubated with this bimetallic helicate.

Introduction

Molecular multiphoton absorption (MPA), i.e., simultaneous absorption of several photons, was predicted by M. Göppert-Mayer in 1931. However, because this process requires powerful excitation sources such as lasers, the first experimental confirmation of its existence was demonstrated only in 1961 by Kaiser and Garrett on $CaF_2:Eu^{II}$.[1] The two main advantages of MPA are (i) excitation wavelengths in the near-infrared (NIR) range and (ii) nonlinear dependence of the multiphoton absorption probability on the excitation intensity. With innovative technological developments at hand, multiphoton-based processes are finding applications in many scientific and technological areas, from 3D data storage and microfabrication to confocal scanning microscopy.[2–4] The latter is considered as being one of the most promising luminescent techniques in noninvasive 3D imaging of living systems, allowing analysis of deep-lying tissues with subcellular resolution without out-of-focus background and photodamage.[5] Different types of molecular organic compounds, polymers, fullerenes, as well as metal−organic complexes exhibiting 2-, 3-, or 4-photon absorption with high MPA cross sections have been designed.[2] Lanthanide chelates are of special interest as MPA bioprobes because of their unique luminescent properties, in particular their sharp emission lines, the long excited state lifetimes, and their insensitivity to photobleaching.[6,7] In addition, recent developments in the design of lanthanide luminescent bioprobes (LLBs) allow us to optimize other important properties like water solubility, cell permeability,

noncytotoxicity, kinetic inertness, or thermodynamic stability at physiological pH, as well as to derivatize LLBs with functional groups for further coupling with biological molecules and specific organelles.[6,8–11] However, despite that the feasibility of multiphoton scanning microscopy for imaging of living cells was demonstrated with compounds possessing low MPA cross sections,[12,13] the number of investigations devoted to MPA LLBs remains scarce.[7] The first two-photon microscopy images were obtained for crystals of the Tb^{III} lysozyme derivative of tris(dipicolinate).[14] Tb^{III} macrocyclic complexes bioconjugated with argynine$_7$ or guanidinium and loaded into NIH-3T3 and HeLa cells showed two-photon excited luminescence under 720 nm excitation, however, exhibiting some cytotoxicity.[13] Two-photon microscopy images were obtained for T24 cancer cells fixed with ethanol and incubated with a derivative of Eu^{III} tris(dipicolinate).[15] Finally, a Tb^{III} complex with tripodal ligands was shown to exhibit three-photon excited luminescence under 800 nm excitation and A549, HeLa and HONE1 cells were visualized in this way.[16]

In view of the intrinsic advantages presented by double lanthanide tags[17] our laboratory has recently focused on the development of LLBs based on binuclear helicates.[8,18] In particular, $[Ln_2(L^{C2})_3]$ (Ln = Eu^{III}, Tb^{III})[19,20] and $[Eu_2(L^{C5})_3]$[21] (Scheme 1) match all requirements for luminescent bioprobes. They are thermodynamically stable, kinetically inert, display sizable luminescence, are noncytotoxic up to a concentration of 500 μM in the incubation medium, and permeate into various types of cells. Although their localization in the endoplasmic reticulum may not be ideal for investigating cellular functions, introduction of a carboxylic acid function at the extremity of the polyoxyethylene arm of H_2L^{C2} allowed easy bioconjugation

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SCHEME 1: Ditopic Hexadentate Ligands[18,21]
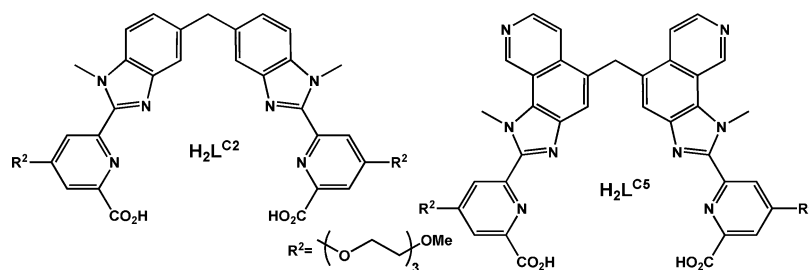
SCHEME 2: Dipicolinic Acid and Its Derivatives[23]
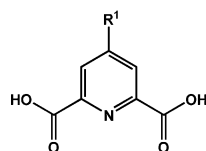
	R^1	Ligand
	H	H_2dpa
	$(OCH_2CH_2)_3OMe$	H_2L^{OMe}
	$(OCH_2CH_2)_3OH$	H_2L^{OH}
	$(OCH_2CH_2)_3NH_2$	H_2L^{NH2}

to avidin or antibodies, therefore leading to the development of dual assays of biomarkers expressed by cancerous tissues.[22] In parallel, more simple model chelates have been synthesized: tris(dipicolinates) in which the ligands bear polyoxyethylene substituents with functional terminal groups allowing coupling to biological molecules (Scheme 2, H_2L^{OH} and H_2L^{NH2}).[23]

In this work we aim at enlarging the potential uses of these compounds by shifting the excitation wavelength into the NIR range to which biomolecules are transparent. Since multiphoton excitation appears to be a convenient solution, luminescent properties of tris(dipicolinate) derivatives and binuclear helicates are investigated under 800 nm excitation from an amplified femtosecond Ti:sapphire laser system and compared to those of the parent tris(dipicolinates). Two- and three-photon cross sections are estimated for all investigated chelates and the feasibility of $[Eu_2(L^{C5})_3]$ to act as a luminescence tag under MPA for imaging HeLa cells is demonstrated.

Experimental Section

Spectra. UV−visible absorption spectra were measured on a Perkin Elmer Lambda 900 spectrometer with 1 mm quartz Suprasil cells. One-photon excited luminescence spectra were recorded on a Fluorolog FL3-22 spectrofluorometer from Horiba-Jobin-Yvon Ltd.

For multiphoton excitation a femtosecond Ti:sapphire oscillator-amplifier laser system (oscillator, Femtolasers Fusion; amplifier, Spectra Physics Spitfire; typical pulse duration, 50 fs (fwhm); repetition rate, 1 kHz) was used. The excitation light was centered at ∼800 nm with a width of 35 nm (fwhm). The resulting luminescence spectra were recorded with a 30 cm spectrograph (Andor Shamrock SR-303i) equipped with an EMCCD detector (Andor Newton) and corrected for the instrumental response functions.

Cell Imaging. HeLa cells grown on plastic bottom μ-dishes (ibidi GmbH) were incubated with 200 μM $[Eu_2(L^{C5})_3]$ in RPMI-1640 culture medium at 37 °C and 5% CO_2 for 12 h. The cells were washed 5 times with phosphate buffered saline (PBS) 0.01 M, pH 7.4 before measurements. Two-photon microscopy images were obtained on a laser scanning Zeiss LSM 710 NLO microscope at an upright fixed-stage Axio Examiner.Z1 stand using a water immersion W-Plan Apochromat 20×/1.0 objective and a Chameleon Ultra II Ti:sapphire laser (Coherent) as an excitation source. Excitation spectra were measured for both 167 μM solution of $[Eu_2(L^{C5})_3]$ and HeLa cells incubated with
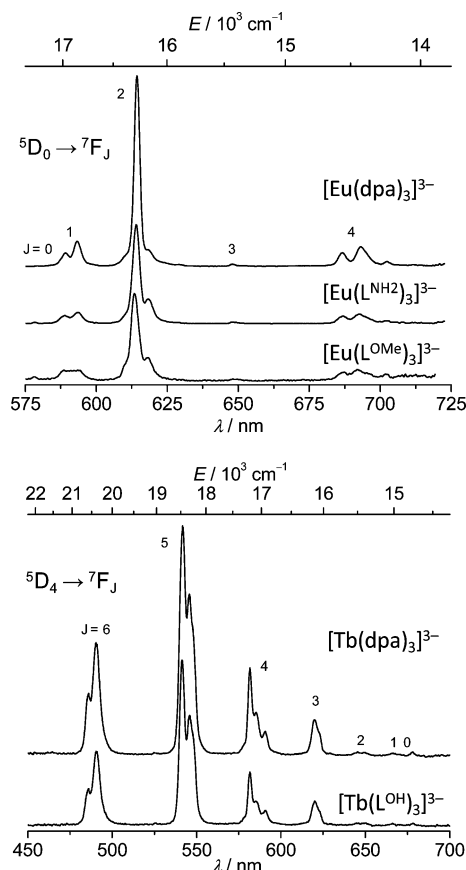

Figure 1. Three-photon excited ($\lambda_{ex} = 800$ nm) luminescence spectra for Eu[III] and Tb[III] dipicolinates and their derivatives in Tris−HCl (pH 7.4, $c = 10$ mM). Spectral intensities are relative to $[Ln(dpa)_3]^{3-}$.

200 μM $[Eu_2(L^{C5})_3]$. To record excitation spectra, the input power of the laser was kept constant and the wavelength of the laser was tuned from 690 to 840 at 5 nm intervals while integrated emission was registered in the range 570−650 nm. The resulting excitation spectra were corrected for laser power output.

Results and Discussion

Multiphoton-Excited Luminescence Properties. Under 800 nm excitation, all investigated aqueous solutions of mono- (Figure 1) and binuclear (Figure 2) chelates exhibit the characteristic red or green luminescence due to $^5D_0 \rightarrow {}^7F_J$ ($J = 0-4$) and $^5D_4 \rightarrow {}^7F_J$ ($J = 6-0$) transitions of Eu[III] and Tb[III], respectively.

The blue-shifted luminescence with respect to the excitation light evidently implies that a multiphoton absorption process is involved. This is confirmed by plotting the sensitized luminescence intensity at the maximum of the emission (Figures 3 and 4) or the total integrated luminescence intensity (Figures S1−S4,
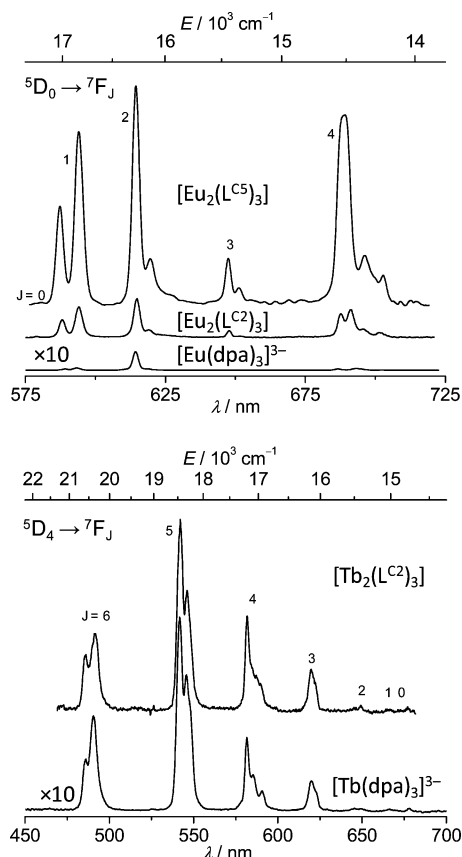
Figure 2. Three-photon ([Ln$_2$(L^{C2})$_3$]) and two-photon ([Eu$_2$(L^{C5})$_3$]) excited luminescence spectra for lanthanide helicates in comparison with Eu^{III} and Tb^{III} dipicolinates in Tris−HCl (pH 7.4, c = 167 μM). Spectral intensities are relative to [Eu$_2$(L^{C5})$_3$] and [Tb$_2$(L^{C2})$_3$], respectively.

Supporting Information) vs incident laser power. Linear fits of the data in double logarithmic scale give slopes in the range 2.7−2.9 for [Eu(L^{OMe})$_3$]$^{3-}$, [Eu(L^{NH2})$_3$]$^{3-}$, [Tb(L^{OH})$_3$]$^{3-}$ and the parent tris(dipicolinates), typical of three-photon excitation. This agrees with the results reported by Lakowicz et al. who have demonstrated 3PA for both [Eu(dpa)$_3$]$^{3-}$ and [Tb(dpa)$_3$]$^{3-}$ under 791 nm excitation.[24] On the other hand, upon 532 nm excitation by a femtosecond laser, different mechanisms are involved for generating luminescence of Eu^{III} and Tb^{III} tris(dipicolinates). The emission of the Eu^{III} chelate is caused by one-photon absorption through the very weak $^5D_1 \leftarrow {}^7F_1$ transition, while [Tb(dpa)$_3$]$^{3-}$ exhibits 2PA.[14]

This study shows that metal-centered luminescence in the binuclear helicates [Eu$_2$(L^{C2})$_3$] and [Tb$_2$(L^{C2})$_3$] is the result of 3PA processes, the slope of intensity vs power dependence being equal to 2.7−2.8 (Figure 4). On the other hand, the corresponding slope for [Eu$_2$(L^{C5})$_3$] is 1.97, reflecting a 2PA process. These data can be understood by examining the absorption spectra (Figures S5 and S6, Supporting Information). For the tris(dipicolinates) and their derivatives, as well as for the [Ln$_2$(L^{C2})$_3$] helicates, at least three photons are needed to sensitize luminescence with 800 nm light since their absorption spectra display a cutoff at ≈375 nm. For [Eu$_2$(L^{C5})$_3$], however, the absorption spectrum extends more into the visible and tails off at wavelength larger than 400 nm. As a matter of fact, confocal microscopy images of HeLa cells could be obtained under excitation at 405 nm.[21] In the present case, the molar absorption coefficient at 400 nm, 920 M^{-1}·cm^{-1}, is large enough to allow 2PA photoexcitation instead of 3PA, owing to the much higher probability of the former excitation mode.
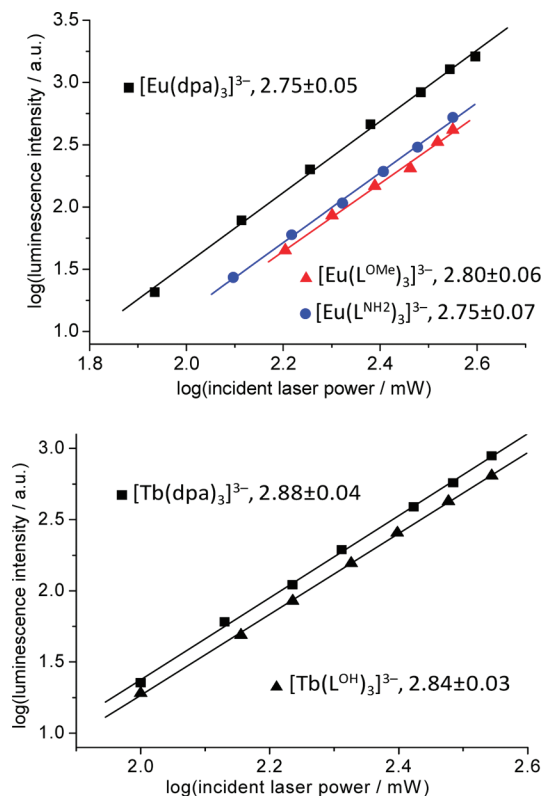

Figure 3. Three-photon excited metal-centered luminescence intensity (λ_{em} = 615 nm for Eu^{III} or 542 nm for Tb^{III}) vs incident laser power (λ_{ex} = 800 nm) for Ln^{III} dipicolinates and their derivatives in Tris−HCl (pH 7.4, c = 10 mM).
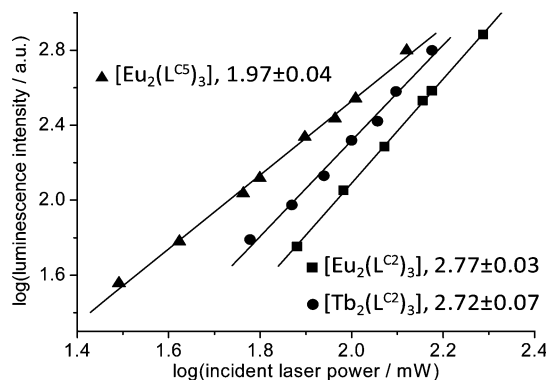

Figure 4. Multiphoton excited metal-centered luminescence intensity (λ_{em} = 615 nm for Eu^{III} or 542 nm for Tb^{III}) vs incident laser power (λ_{ex} = 800 nm) for [Ln$_2$(L^{CX})$_3$] in Tris−HCl (pH 7.4, c = 167 μM).

It is worth noting that comparison between multiphoton and one-photon excited luminescence spectra for all studied compounds shows no significant difference in their spectral shape (Figures S7−S10, Supporting Information). This is also confirmed by the relative integral luminescence intensities (Table S1, Supporting Information), indicating that the same levels are involved in one- and multiphoton excited luminescence.

Multiphoton Cross Sections. Quantitatively, the efficiency of multiphoton absorption for a given compound is determined by its MPA cross section $\sigma^{(n)}$: $\sigma^{(2)}$ (for 2PA) or $\sigma^{(3)}$ (for 3PA).[2] An absolute measurement of these values is difficult, so comparative methods with known standards are commonly used. While appropriate standards are easily available for 2PA (e.g., Rhodamine B or different Coumarins),[26] there are no reliable ones for 3PA.[2] To provide a comparison of the efficiency of 3PA-induced luminescence in the investigated compounds,

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absolute three-photon cross sections of 10 mM solutions of the parent tris(dipicolinates) in Tris−HCl (pH 7.4) were estimated by comparing the luminescence for three- and one-photon excitation. One-photon excitation was achieved with 266 nm light from third harmonic generation of the femtosecond Ti: sapphire laser. The incident beams were slightly collimated with a 20 cm CaF_2 lens resulting in a Gaussian beam profile with fwhm of 1.23 and 1.5 mm for 800 and 266 nm, respectively, at the position of the sample as measured by a beam profiler. The setup was aligned in such a way that either the 800 or 266 nm light hits the sample exactly at the same place. Only the central part of the exposure area was imaged by a 5 cm lens linked to a 100 μm optical fiber connected to the spectrograph. This setup allows one to assume constancy of the incident laser intensity over the detection area to a good approximation. Due to the high concentration of the samples, practically all 266 nm incident photons are absorbed; i.e., the number of absorbed photons per pulse and unit area is simply equal to the incoming photon flux: $p_a^{(1)} = p_{266}$.

The three-photon absorption cross section ($\sigma^{(3)}$) is defined by

$$dN = \sigma^{(3)} \cdot p(t,z)^3 \cdot c \, dz \tag{1}$$

Here dN denotes the number of absorption events along the infinitesimal length interval dz and c is the concentration, $p(t,z)$ is the time-dependent and, in general, z-dependent incident photon flux. In view of the much lower probability of 3PA compared to that for 1PA, the number of absorbed photons in the former process is sufficiently small during propagation of the laser beam through the sample, so that z dependence can be neglected. We thus use the following equation:

$$p(t) = \frac{E/h\nu}{2\pi w_{x,y}} \frac{1}{w_t\sqrt{2\pi}} e^{-t^2/2w_t^2} \tag{2}$$

for the central part of the circular, Gaussian beam profile with width $w_{x,y}$, and a laser pulse with energy E, frequency ν, and a temporal width w_t. Integration of eq 1 over the sample length l_z is trivial, and the number of excited molecules per pulse and unit area for the three-photon excitation is given by

$$p_a^{(3)} = \int \sigma^{(3)} \cdot p(t)^3 \cdot c \cdot l_z \, dt \tag{3}$$

where the integration in time is over one pulse. With $p(t)$ from eq 2 this yields

$$p_a^{(3)} = \frac{\sigma^{(3)} E^3 l_z}{16\sqrt{3} h^3 \pi^4 \nu^3 w_t^3 w_{x,y}^6} \tag{4}$$

for the number of excited molecules per pulse. In this experiment, the chosen pulse duration and energy were 50 fs (fwhm) and 235 μJ, respectively.

Instrumental functions are identical for one- and three-photon excitation. In addition, the overall quantum efficiency for luminescence can be assumed to be the same for the two processes; therefore, the ratio between the detected luminescence signals ($s^{(1)}$ and $s^{(3)}$) and the ratio between the number of excited molecules are identical:

$$\frac{s^{(1)}}{s^{(3)}} = \frac{p_a^{(1)}}{p_a^{(3)}} \tag{5}$$

As a result eqs 5 and 4 can be used to determine the three-photon absorption cross section $\sigma^{(3)}$.

The values of $\sigma^{(3)}$ for the other compounds were determined by comparison with $[Ln(dpa)_3]^{3-}$ using eq 6:

$$\sigma_S^{(3)} = \frac{c_R \cdot n_S \cdot F_S(\lambda) \cdot Q_R}{c_S \cdot n_R \cdot F_R(\lambda) \cdot Q_S} \cdot \sigma_R^{(3)} \tag{6}$$

where c = concentration, n = refractive index, $F(\lambda)$ = integrated luminescence intensity, and Q = quantum yield; indices R and S refer to reference and sample, respectively. The results are summarized in Table 1. Eu^{III} derivatives of tris(dipicolinates), $[Eu(L^{OMe})_3]^{3-}$ and $[Eu(L^{NH2})_3]^{3-}$, show a ∼2-fold lower cross section compared to the parent $[Eu(dpa)_3]^{3-}$, while $\sigma^{(3)}$ of $[Tb(L^{OH})_3]^{3-}$ is similar to the one of $[Tb(dpa)_3]^{3-}$. On the other hand, a 20-fold larger $\sigma^{(3)}$ is found for $[Ln_2(L^{C2})_3]$ binuclear helicates. However, the observed values remain small, not exceeding 1 GM. It should be mentioned that to our knowledge no quantitative data of 3PA cross sections for lanthanide complexes are presently available in the literature. A value of 1.9 GM is reported for $[Tb(NO_3)_3(L)]$ (L = N-[2-(bis{2-[(3-methoxybenzoyl)amino]ethyl}amino)ethyl]-3-methoxybenza-mide), but it was estimated by the comparative method assuming that $\sigma^{(3)}$ of Rhodamine B equals 1 GM.[16]

The 2PA cross section ($\sigma^{(2)}$) of $[Eu_2(L^{C5})_3]$ was determined relative to Rhodamine B[27] using the following data, Q_R = 0.45,[26,28] $\sigma_R^{(2)}$ = 120 GM,[26] Q_S = 0.09,[21] n_S (H_2O) = 1.332 99, n_R (MeOH) = 1.3288, and found to be equal to 0.75 GM upon excitation at 800 nm. This value is much lower than the highest 2PA cross-section reported to date for water-soluble lanthanide compounds: 92 GM at 700 nm for an Eu^{III} complex with a derivative of dipicolinic acid.[15] However, it is comparable with, for instance, $\sigma^{(2)}$ of Eu^{III} complexes with derivatized cyclens.[29] It is noteworthy highlighting that the two-photon cross section of $[Eu_2(L^{C5})_3]$ was measured only on the tail of the absorption band at ∼800 nm, which is far away from the maximum (350 × 2 = 700 nm); the ratio of the absorption coefficients being $\varepsilon_{350}/\varepsilon_{400}$ ∼ 87, a considerable sensibility enhancement is expected if excitation is performed at 700 nm. Thus, taking into account sufficient water solubility (∼500 μM), large thermodynamic stability, kinetic inertness, suitable luminescent properties, and very low cytotoxicity (IC_{50} > 500 μM),[21] we have explored the capability of $[Eu_2(L^{C5})_3]$ as acting as bioprobe in two-photon imaging of live cells.

Two-Photon Scanning Microscopy of HeLa Cells. Selection rules, resonance enhancement, pathways of molecular transitions, and magnitudes of relevant matrix elements for 1PA and 2PA processes are different, and the extent of this difference depends on the intrinsic properties of the compound, especially on its symmetry.[2] Thus, optimal excitation wavelengths for multiphoton processes in general cannot be simply predicted from one-photon absorption or excitation spectra and have to be determined for each compound. The optimum excitation wavelength for two-photon microscopy imaging was therefore estimated from the excitation spectra recorded for both a 167 μM solution of $[Eu_2(L^{C5})_3]$ in Tris−HCl (pH 7.4) and HeLa cells incubated with 200 μM of this binuclear chelate (Figure 5; Figure S11, Supporting Information). The excitation spectrum of $[Eu_2(L^{C5})_3]$ is red-shifted in comparison with the absorption

TABLE 1: Photophysical Properties and Multiphoton Absorption Cross Sections ($\lambda_{\text{MPA}} = 800$ nm) for Lanthanide Complexes in Aqueous Solution (Tris−HCl, pH 7.4) at Room Temperature

compound	$\lambda_{\text{abs}}^{\text{max}}$/nm[a]	ε^{max}/M^{-1}·cm^{-1} [a]	$\lambda_{\text{abs}}^{\text{eff}}$/nm[b]	ε^{eff}/M^{-1}·cm^{-1} [b]	τ_{obs}/ms	Q_{1PA}/%	process	$\sigma^{(n)}$/GM[c]
Cs$_3$[Eu(dpa)$_3$]	271	13200	266	9500	1.7(1)[d]	29(2)[d]	3PA	0.011[e]
Cs$_3$[Tb(dpa)$_3$]	271	13200	266	9500	1.74(1)[d]	21(1)[d]	3PA	0.014[e]
Na$_3$[Eu(L$^{\text{OMe}}$)$_3$]	275	3800	266	5150	1.36(2)[f]	26.6(6)[f]	3PA	5.7 × 10^{-3g}
Na$_3$[Eu(L$^{\text{NH2}}$)$_3$]	274	4900	266	6150	1.43(1)[f]	28.9(9)[f]	3PA	5.7 × 10^{-3g}
Na$_3$[Tb(L$^{\text{OH}}$)$_3$]	275	3150	266	4000	0.99(1)[f]	17.8(6)[f]	3PA	0.012[g]
[Eu$_2$(L$^{\text{C2}}$)$_3$]	322	77300	266	42700	2.43(9)[h]	21(2)[h]	3PA	0.26[g]
[Tb$_2$(L$^{\text{C2}}$)$_3$]	322	77300	266	42700	0.65(2)[h]	11(2)[h]	3PA	0.28[g]
[Eu$_2$(L$^{\text{C5}}$)$_3$]	350	80300	400	920	2.30(1)[i]	9.0(9)[i]	2PA	0.75[j]

[a] Maximum of the absorption band with the lowest energy. [b] Absorption at a given wavelength. [c] For 2PA, 1 GM = 10^{-50} cm^4·s·photon^{-1}; for 3PA, 1 GM = 10^{-83} cm^6·s^2·photon^{-2}. [d] From ref 25. [e] Determined by absolute method. [f] From ref 23. [g] Corresponding Cs$_3$[Ln(dpa)$_3$] were used as standards. [h] From ref 18. [i] From ref 21. [j] Rhodamine B was used as a standard.
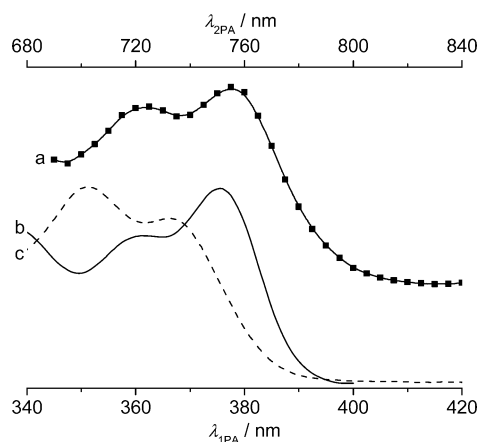

Figure 5. Normalized (a) two- and (b) one-photon excitation spectra, and (c) superimposed absorption spectrum of [Eu$_2$(L$^{\text{C5}}$)$_3$] solution in Tris−HCl (pH 7.4).
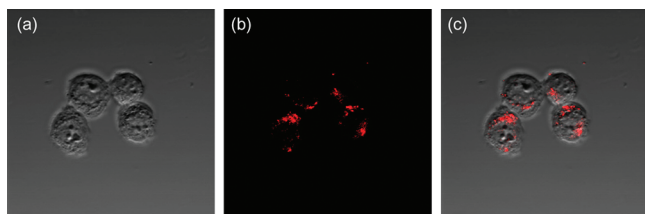

Figure 6. Two-photon microscopy images of HeLa cells incubated with 200 μM [Eu$_2$(L$^{\text{C5}}$)$_3$] in RPMI-1640 culture medium for 12 h at 37 °C, 5% CO$_2$: (a) bright field image; (b) luminescence (λ_{ex} = 750 nm, λ_{em} = 570−650 nm); (c) merged image.

spectrum, and the highest intensity is observed at 755 nm instead of the expected 700 nm; this maximum, however, coincides with the one recorded in the one-photon excitation spectrum (Figure 5). The 2PA cross section of [Eu$_2$(L$^{\text{C5}}$)$_3$] at this wavelength can be estimated to be about 12 times larger than at 800 nm, that is, around 9 GM.

Previous investigation by conventional, time-resolved and confocal microscopy showed that [Eu$_2$(L$^{\text{C5}}$)$_3$] permeates into living cells by endocytosis and localizes in secondary endosomes and lysosomes, which eventually colocalize with the endoplasmic reticulum.[21] Similarly, two-photon scanning microscopy images of HeLa cells incubated with [Eu$_2$(L$^{\text{C5}}$)$_3$] and illuminated with 750 nm light demonstrate the localization of the helicates around the cell nuclei (Figure 6).

Conclusions

Characteristic Eu$^{\text{III}}$ or Tb$^{\text{III}}$ luminescence in complexes with derivatives of dipicolinic acid and binuclear helicates can be excited by 800 nm light from femtosecond lasers through

multiphoton absorption. Extension of the absorption band into the visible range when going from [Ln$_2$(L$^{\text{C2}}$)$_3$] to [Eu$_2$(L$^{\text{C5}}$)$_3$] results in a change in the excitation mechanism from 3PA to 2PA. Three-photon cross sections are reported for the first time for lanthanide complexes. They range between 0.57 × 10^{-2} and 1.4 × 10^{-2} GM for mononuclear complexes, which is rather small, but the ligands were not optimized for MPA as were those reported previously by Maury et al.[30] For binuclear helicates with H$_2$L$^{\text{C2}}$ the 3PA cross-section is about 20-fold larger, between 0.26 and 0.27 GM. Furthermore, the 2PA cross section of [Eu$_2$(L$^{\text{C5}}$)$_3$] is much larger, being estimated to ∼9 GM at the maximum of the excitation spectrum. Building on this property, the feasibility of binuclear lanthanide helicates as multiphoton luminescent bioprobes is demonstrated by two-photon scanning microscopy experiments on HeLa cells incubated with the latter chelate. Appropriate functionalization of the ditopic ligands aiming at both increasing the MPA cross sections[30] and conjugating them to biological molecules,[22] should make the corresponding helicates excellent imaging and targeting agents.

Supporting Information Available: Two- and three-photon excited integrated luminescence vs incident laser power (Figures S1−S4), absorption spectra (Figures S5 and S6), comparison between one- and two- or three-photon excited luminescence spectra (Figures S7−S10) with analysis of relative integral intensities of $^5D_0 \rightarrow {}^7F_J$ (J = 0−4, Eu$^{\text{III}}$) and $^5D_4 \rightarrow {}^7F_J$ (J = 6−0, Tb$^{\text{III}}$) transitions (Table S1), comparison between two-photon excitation spectra of HeLa cells incubated with [Eu$_2$(L$^{\text{C5}}$)$_3$] and a solution of this Eu$^{\text{III}}$ complex in Tris−HCl at pH 7.4 (Figure S11). This material is available free of charge via the Internet at http://pubs.acs.org.

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