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Action cross sections of two-photon excited luminescence of some Eu(III) and Tb(III) complexes†

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Four different luminescent lanthanide complexes have been studied with respect to multiphoton excitation using near-infrared femtosecond pulses. The method for measuring action cross sections of two-photon excited fluorescence in solution relative to a known standard is reviewed. Two refractive index-related corrections are necessary in this method: one for the multiphoton excitation process, the other for the collection of the emitted light. It has been found that (2,4,6-trimethoxyphenyl)dipicolinic acid and Michler's ketone are reasonable sensitisers of two-photon excited lanthanide luminescence in solution, whereas dipicolinic acid and carbostyril-124 do not give rise to any detectable two-photon excited lanthanide luminescence using modest excitation powers (<20 mW focused at the sample) in the 700–1000 nm range.

1 Introduction

Narrow emission bands and millisecond luminescence lifetimes make lanthanide ions and their complexes with photosensitising organic ligands into useful photoluminescent species for biomolecular research and medical diagnostics. Each lanthanide ion has its own characteristic emission lines, notably Eu^{3+}, Tb^{3+}, Dy^{3+} and Sm^{3+} in the visible and Nd^{3+}, Yb^{3+} and Er^{3+} in the near-infrared. The narrow emission lines allow for the simultaneous detection and imaging of several species, and pulsed excitation in combination with time-gated detection can be used to distinguish the long-lived lanthanide emission from the nanosecond fluorescence of the natural fluorophores present in biological matter. Since their coordination chemistry shows similarity to that of Ca^{2+} and Fe^{3+}, lanthanide ions, in particular Eu^{3+} and Tb^{3+}, have been considered as luminescent analogues of these ions and used to probe structure and dynamics of metal-binding biomolecules and to monitor ion transport.[1–3]

The photoluminescence of lanthanide ions is difficult to excite since their intrinsic absorption bands are narrow ($\Delta\lambda < 2$ nm) and weak ($\varepsilon < 1$ M^{-1} cm^{-1}). In most applications, lanthanide luminescence is therefore excited indirectly *via* a nearby organic chromophore whose excited states are of sufficient energy to transfer[4] the excitation efficiently and (preferably) irreversibly[5] to the lanthanide ion (see Fig. 1). Many complexes have been synthesised in which the lanthanide ion is strongly bound to a ligand containing a sensitising chromophore (for reviews see refs. 6,7). Moreover, the ligand is intended to protect the lanthanide luminescence from being quenched by molecules containing high-energy vibrations,[8–10] in particular water. These complexes have been used as luminescent labels for biological molecules in medical diagnostics[11–13] and microscopy.[14,15]

Another interesting application of luminescent lanthanide complexes is in resonance energy transfer experiments,[16–19] where the long lifetime of their excited state makes them potent energy donors, for example Tb^{3+} transferring energy to fluorescein or

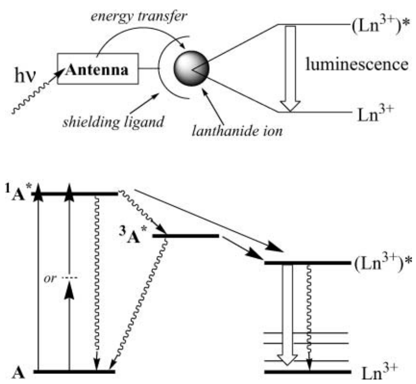

Fig. 1 Scheme and energy diagram of luminescence in lanthanide complexes.

Eu^{3+} to allophycocyanine. Such luminescence (or fluorescence) energy transfer (LRET or FRET) experiments are used to study interactions between large biological molecules, and the dynamics of these molecules.

Because of the aforementioned energetic constraints resting on the sensitising organic ligand, the visibly luminescent lanthanide complexes need excitation with ultraviolet light, which is not desirable when working with biological material, and requires special (expensive quartz) microscope optics. Visible light excitation is possible with near-infrared luminescent lanthanide ions, such as Yb^{3+}, Nd^{3+} and Er^{3+}, at the expense of greatly reduced luminescence quantum yields.[20] Moreover, these near-infrared luminescent complexes cannot easily be used as donors in LRET experiments.

An alternative way for circumventing the use of ultraviolet light is multiphoton excitation, which is becoming more and more accessible with the advent of stable and robust laser sources generating ultrashort pulses. Interestingly, two-photon excitation (predicted in 1931)[21] was first observed experimentally in a crystal containing divalent europium ions under excitation with a ruby laser.[22] There is currently an active search for molecular probes with enhanced response to multiphoton excitation

† Dedicated to Professor Dr J. W. Verhoeven, retired from the University of Amsterdam.

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(for a review see ref. 23), especially because of their applications in biological multiphoton microscopy.[24]

Little is known about the behaviour of luminescent lanthanide ions and complexes in solution with respect to multiphoton excitation, but the subject is rapidly gaining attention. Lakowicz *et al.* explored the direct multiphoton excitation of lanthanide ions in solution.[25] The laser intensities used were quite high (several hundreds of milliwatt of focused femtosecond pulse trains), and even then high concentrations of lanthanides or their complexes were necessary to achieve a detectable signal. They also observed that multiphoton excitation of lanthanide ions was enhanced in the presence of organic ligands,[26,27] implying that the multiphoton absorption cross sections of lanthanide ions are smaller than those of organic chromophores. Both two- and three-photon excitation were observed, but no cross sections of multiphoton absorption were given.

Multiphoton excitation of Tb^{3+} bound to the protein transferrin was reported in this journal.[28] The photons, at 503 and 566 nm for two-photon excitation and at 800 nm for three-photon excitation, were absorbed by tyrosine residues in the protein and the energy was subsequently transferred to the lanthanide ion. Interestingly, the authors reported action cross sections for two-photon excitation of transferrin-sensitised Tb^{3+} luminescence (0.4 GM at 566 nm, 7.4 GM at 503 nm; 1 GM = 10^{-50} cm^4 photon^{-1} s^{-1}). These quantities are very useful for comparing the performance of different luminophores under two-photon excitation. It was also reported that the action cross section for direct two-photon excitation of Tb^{3+} luminescence using 566 nm pulses amounts to 10^{-5} GM, five orders of magnitude lower than indirect excitation *via* the tyrosine chromophores inside the transferrin protein.

Very recently, two-photon excitation of terbium chelates in the solid state has been reported[29] using nanosecond pulsed excitation in the 510–750 nm range. No two-photon absorption cross sections were given, but the complexes responded particularly well in the sub-650 nm range, which is related to the relatively high excitation energies of the ligand systems. While preparing this manuscript, Fu *et al.* reported on a very interesting europium(III) complex with optimised two-photon absorption in the 730–830 nm range accessible by femtosecond Ti:sapphire lasers (σ_2 = 157 GM at 808 nm).[30]

The focus of this paper is the study of two-photon excitation of some typical luminescent lanthanide complexes using a conventional femtosecond Ti:sapphire laser source (700–1000 nm, 76 MHz, 150 fs pulses) at modest excitation powers (< 20 mW focused at the sample). We are interested in knowing the action cross sections of two-photon excitation of these lanthanide complexes, and how these compare to conventional fluorophores.

Our choice of complexes and ligands (Fig. 2) has been guided by the ease of their preparation and synthetic accessibility, and by their *a priori* potential to be excited by two-photon excitation. Thus, we chose dipicolinic acid (DPA), a well-known ligand that forms highly luminescent complexes with Tb^{3+}. Additionally, it is an important constituent of bacterial spores, and its luminescent complexes are a means of detecting the presence of those spores.[31] The trimethoxyphenyl derivative, TMP-DPA, absorbs at longer wavelengths while inheriting from its parent compound the ability to form luminescent complexes with Tb^{3+}, Eu^{3+}, Sm^{3+} and Dy^{3+}. Its photophysical properties under one-photon excitation have been described briefly,[32,33] but its synthesis has never been adequately reported. Its electronic structure is slightly "push–pull", which may lead to an enhanced two-photon excitation cross section.

The carbostyril chromophore in cs124-DTPA sensitises the one-photon excited luminescence of Tb^{3+}.[34,35] The preparation of cs124-DTPA is based on an elegantly simple reaction between an aromatic amine (the sensitiser) and the anhydride of diethylenetriaminepentaacetic acid (a strong binding ligand for lanthanide ions).[36,37] Cs124-DTPA–Tb complexes have successfully been used in LRET experiments.[16]

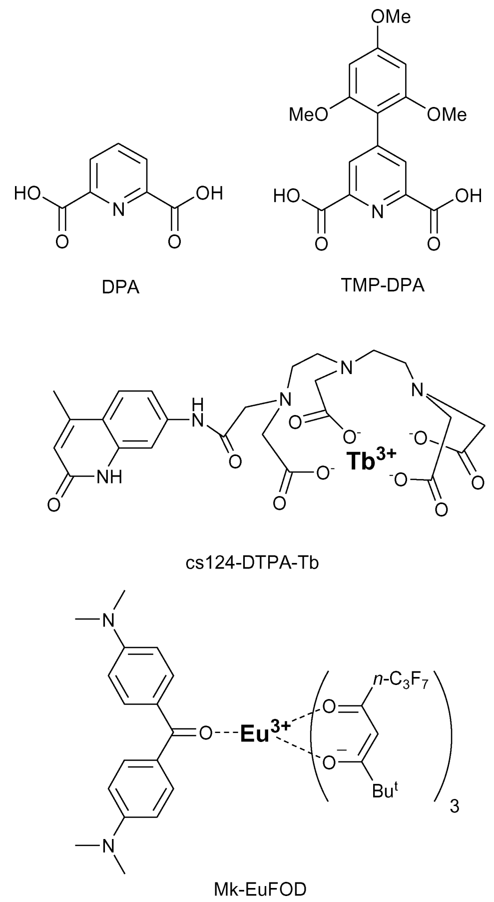

Fig. 2 Ligands and complexes selected for this study.

Michler's ketone (Mk) is a push–pull chromophore that forms luminescent complexes with tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato)europium (EuFOD) in apolar solvents. These complexes can be excited monophotonically at unusually long wavelengths for Eu^{3+} complexes (λ_{max} = 413 nm in toluene), while retaining a reasonable photoluminescence quantum yield (Φ = 0.2).[38,39] The push–pull nature of this sensitiser makes it a good candidate for efficient two-photon excitation.

The two-photon absorption cross section will be denoted σ_2 and is usually given in Göppert-Mayer units (1 GM = 10^{-50} cm^4 photon^{-1} s^{-1}). The action cross section for two-photon excited luminescence is the product ($\sigma_2\Phi$) of σ_2 and the luminescence quantum yield. We will briefly review the method for the measurement of $\sigma_2\Phi$ using a reference sample of known $\sigma_2\Phi$.[40] Both the excitation process and the detection of the emitted light will be considered.

2. Experimental

2.1 Chemicals and characterisation methods

Terbium(III) chloride hexahydrate and EuFOD (tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato)europium) were dried and stored over activated silica gel (blue, with indicator) in a desiccator. Michler's ketone (4,4′-bis(dimethylamino)benzophenone, Mk) was purified using literature procedures.[41] Dipicolinic acid (DPA) was used as received. Solvents were generally dried and distilled prior to use. Reactions were monitored by thin-layer chromatography on Merck silica gel 60 F254 precoated aluminium sheets. Column chromatography: Merck silica gel Si 60 (40–63 mm, 230–400 mesh). NMR: ^{1}H chemical shifts (δ) are given in ppm relative to TMS as internal standard, J values in Hz, ^{13}C chemical shifts relative to the central peak of $CDCl_3$ at 77.0 ppm. High resolution mass spectra measurements and elemental analyses were performed at the Centre Régional de Mesures Physiques de l'Ouest (CRMPO, Rennes).

2.2 Synthesis of TMP-DPA

The synthesis of TMP-DPA follows the procedure described for similar compounds[42] with some modifications.

1-(2-Furyl)-3-(2,4,6-trimethoxyphenyl)propenone. This was prepared using a procedure adapted from ref. 43. To a stirred solution at 40 °C of 2,4,6-trimethoxybenzaldehyde (4.0 g, 20 mmol) and 2-acetylfuran (5.3 g, 48 mmol) in ethanol (90 ml) was added 10 ml of 10% aqueous NaOH. The formation of the yellow product was followed by TLC (R_f = 0.3; CH_2Cl_2/ethyl acetate 1 : 1). The reaction was complete within 30 min. The mixture was allowed to cool down and the crude product was collected by filtration. After recrystallisation from ethanol we obtained 4.7 g of yellow crystals (69%). ^{1}H NMR (200.13 MHz, $CDCl_3$) δ 8.31 (d, J = 16.1, 1 H), 7.75 (d, J = 16.1, 1 H), 7.61 (unresolved d, 1 H), 7.24 (d, J = 3.3, 1 H), 6.54 (unresolved m, 1 H), 6.11 (s, 2 H), 3.89 (s, 6 H), 3.84 (s, 3 H). HRMS (EI) calcd. for $C_{16}H_{16}O_5$ ($M^{+\bullet}$) m/z 288.09977, found 288.1020.

1,5-Di(2-furyl)-3-(2,4,6-trimethoxyphenyl)-1,5-pentanedione. A mixture of 1-(2-furyl)-3-(2,4,6-trimethoxyphenyl)-propenone (4.0 g, 10 mmol) and methanol (50 ml) was placed in a ice-bath and stirred. An excess of 2-acetylfuran (6.6 g, 60 mmol) was added, as well as 0.5 g of NaOH (12 mmol). The mixture was stirred for 1 h, and then heated at 50 °C for 62 h. Product formation was monitored by TLC (R_f = 0.54, CH_2Cl_2/ethyl acetate 9 : 1). After cooling down, the brown mixture was neutralised by the addition of aqueous HCl (1 M, approx. 12 ml). The slightly acidic mixture was extracted four times with CH_2Cl_2. After drying over Na_2SO_4, the organic layer was evaporated, yielding a sticky black tar. The excess of 2-acetylfuran was removed by Kugelrohr distillation and the crude product was further purified by column chromatography (silica, CH_2Cl_2). Yield: 2.9 g of an off-white powder (52%). ^{1}H NMR (200.13 MHz, $CDCl_3$) δ 7.54 (d, J = 1.8, 2 H), 7.23 (d, J = 3.5, 2 H), 6.50 (dd, J_1 = 3.5, J_2 = 1.8, 2 H), 6.05 (s, 2), 4.58 (quint, ^3J = 7.3, 1 H), 3.76 (s, 3 H, –OCH_3), 3.72 (s, 6 H, 2 × –OCH_3), 3.37 (dd, ^3J = 7.3, ^2J = 15.4, 2 H), 3.23 (dd, ^3J = 7.3, ^2J = 15.4, 2 H). ^{13}C NMR (75.48 MHz, $CDCl_3$) δ 188.7, 159.8, 159.0, 152.8, 146.0, 117.1, 111.9, 111.1, 91.0, 55.6, 55.1, 42.5, 27.8. HRMS (EI) calcd. for $C_{22}H_{22}O_7$ ($M^{+\bullet}$) m/z 398.13655, found 398.1361.

2,6-Di(2-furyl)-4-(2,4,6-trimethoxyphenyl)pyridine. A mixture of n-butanol (25 ml), 1,5-di(2-furyl)-3-(2,4,6-trimethoxyphenyl)-1,5-pentanedione (2.5 g, 6.3 mmol), and hydroxylamine hydrochloride (1.7 g, 25 mmol) was refluxed for 6 h (product R_f = 0.75, CH_2Cl_2/ethyl acetate 20 : 1). After cooling down, 35 ml of water and 35 ml of toluene were added. The mixture was basified with aqueous NaOH and extracted two more times with toluene, and once with dichloromethane. The organic layers were pooled, dried over Na_2SO_4 and evaporated. The crude product was purified by column chromatography on silica (CH_2Cl_2/heptane 3 : 1), which yielded 1.1 g of white crystals (small needles, 48%). ^{1}H NMR (200.13 MHz, $CDCl_3$) δ 7.55 (s, 2 H), 7.52 (d, J = 1.8, 2 H), 7.11 (d, J = 3.3, 2 H), 6.51 (dd, J_1 = 3.3, J_2 = 1.8, 2 H), 6.24 (s, 2 H), 3.89 (s, 3 H), 3.75 (s, 6 H). El. anal. found: C, 70.0; H, 4.97; N, 3.48; $C_{22}H_{19}NO_5$ requires C, 70.0; H, 5.07; N 3.71%. HRMS (EI) calcd. for $C_{22}H_{19}NO_5$ ($M^{+\bullet}$) m/z 377.12632, found 377.1256.

4-(2,4,6-Trimethoxyphenyl)-pyridine-2,6-dicarboxylic acid (TMP-DPA). 55 ml of $tert$-butanol mixed with 10 ml of water and 0.25 g (0.66 mmol) of 2,6-di(2-furyl)-4-(2,4,6-trimethoxyphenyl)pyridine was heated at 80 °C. Potassium permanganate (1.4 g, 8.8 mmol) was added in small portions over 15 min. The reaction mixture was stirred for 20 h at 80 °C, after which it was filtered while still hot. The residue and the filter were rinsed with a mixture of $tert$-butanol and 0.1 M aqueous NaOH (1 : 4) to liberate the remaining product . The filtrate was treated with $NaHSO_3$ to destroy the remaining MnO_2, and to obtain a clear solution. The $tert$-butanol was distilled off with a rotary evaporator. The TMP-DPA was precipitated by acidifying the mother liquor to pH 3. The very fine precipitate was collected by centrifugation, washed several times with water, and dried under vacuum. Yield: 110 mg of a yellowish-white powder (50%). ^{1}H NMR (200.13 MHz, MeOD) δ 8.29 (s, 2 H), 6.35 (s, 2 H), 3.87 (s, 3 H), 3.29 (s, 6 H). El. anal. found: C, 55.0; H, 4.54; N, 3.90; $C_{16}H_{15}NO_7\cdot 1H_2O$ requires C, 54.7; H, 4.88; N 3.99%. HRMS (EI) calcd. for $C_{16}H_{15}NO_7$ ($M^{+\bullet}$) m/z 333.08485, found 333.0866.

2.3 Cs124-DTPA

Cs124-DTPA[34] is a highly polar compound and is commonly purified using preparative reversed phase HPLC. Carbostyril was reacted with a five-fold excess of diethylenetriaminepentaacetic dianhydride (DTPAA) to avoid formation of doubly functionalised DTPA. Carbostyril-124 (10 mg, 0.0574 mmol) in 200 µl of DMF was added dropwise to a solution of DTPAA (100 mg, 0.287 mmol) and 40 µl of triethylamine in 4.7 ml of freshly distilled anhydrous DMF. The reaction mixture was stirred for 2 h under argon at room temperature and subsequently quenched with 5 ml of water. Complete reaction of the carbostyril was proved using UV/Vis absorption: unreacted carbostyril-124 has λ_{max} = 354 nm and 340 nm in DMF, whereas cs124-DTPA has blue-shifted and weaker absorption maxima at 351 and 336 nm (DMF). Small samples of cs124-DTPA for spectroscopic analysis were purified using analytical HPLC (MeCN/H_2O 60 : 40, Waters XTerra C_{18} column). Fluorimetric titration of cs124-DTPA with $TbCl_3\cdot 6H_2O$ in 2 mM TRIS-HCl buffer (pH 8.3) showed exclusive formation of the 1 : 1 complex cs124-DTPA–Tb, whose one-photon absorption and emission properties in agreement with previously published data.[34,44]

2.4 Optical spectroscopy

UV/Vis spectra were recorded on a Jasco V-570 double-beam spectrophotometer. Steady-state fluorescence measurements were performed at room temperature using an Edinburgh Instruments (FLS 920) spectrometer working in photon-counting mode, equipped with a quantum counter for excitation correction. Fully corrected emission spectra were obtained at λ_{ex} with $A_{\lambda ex}$ < 0.1 to minimize internal absorption. Fluorescence quantum yields were measured using standard methods on air-equilibrated samples at room temperature. Quinine bisulfate in 0.05 M H_2SO_4 (Φ_F = 0.50)[45] was used as a reference. The reported fluorescence quantum yields are within ±10%.

3 Measurement of two-photon excited fluorescence or luminescence action cross sections

We will briefly review the method for determining action cross sections described by Xu and Webb,[40] paying attention both to the properties of two-photon excitation induced by a focused femtosecond laser and to the detection of the induced fluorescence. This will clarify why femtosecond pulses are much more efficient in generating two-photon excitation than continuous wave (CW) light and which corrections are necessary to achieve a reliable measurement of TPEF cross sections using a reference sample.

In the absence of saturation, photobleaching, stimulated emission and self-quenching, the detected time-averaged two-photon excited fluorescence signal of a homogeneous solution of a dye is given by

$$\langle F(t)\rangle = \frac{1}{2}\eta_{\mathrm{det}}\Phi C\sigma_2\langle I^2_{\mathrm{exc}}(t)\rangle\int_V \mathrm{d}VS^2(r) \quad (1)$$

in which η_{det} is the detection efficiency, Φ the fluorescence quantum yield of the dye, C its concentration, and σ_2 its two-photon absorption cross section. $S(\boldsymbol{r})$ describes the spatial intensity distribution of the excitation light. $I_{\mathrm{exc}}(t)$ describes its temporal behaviour. A careful analysis of the two latter terms describing the excitation by a mode locked femtosecond laser focused through a microscope objective has been carried out by Xu and Webb.[40]

For ultrafast laser pulses, it is difficult to have experimental access to the time-average of the squared excitation intensity, $\langle I_{\mathrm{exc}}{}^2(t)\rangle$. It is more convenient to measure the time-averaged intensity $\langle I_{\mathrm{exc}}(t)\rangle$ and take its square. The ratio between the two averages $g = \langle I_{\mathrm{exc}}{}^2(t)\rangle/\langle I_{\mathrm{exc}}(t)\rangle^2$ is a measure of the second-order temporal coherence of the excitation source.

In continuous wave (CW) excitation, there is no difference between the time-average of the square and the square of the time-averaged intensity, and therefore $g = 1$. For pulsed excitation,

$$g = \frac{g_{\mathrm{p}}}{f\Delta t} \quad (2)$$

where f is the pulse repetition rate, Δt is the pulsewidth (FWHM) and g_{p} is a dimensionless constant that depends on the laser pulse shape ($g_{\mathrm{p}} = 0.664$ for a Gaussian pulse, $g_{\mathrm{p}} = 0.588$ for the sech^2 pulse generated in a femtosecond laser).

With short pulses (≈100 fs), at high repetition rates (≈100 MHz), g can easily reach ≈10^5, which means that pulsed two-photon excitation is much more efficient than CW excitation *at equal average laser power*. It also means that the generated fluorescence intensity critically depends on the laser pulse width, which may vary from wavelength to wavelength on the same laser system. Therefore, laser pulse shape and width should be carefully monitored when measuring two-photon excitation cross sections, or a reference compound should be measured under the same excitation conditions.

Precise knowledge of the actual spatial distribution of the excitation light near the focal point is also necessary. Xu and Webb[40] have found an approximate expression for the integral over all space of the excitation intensity square $\int_V \mathrm{d}VS^2(\boldsymbol{r})$ for a plane wave forming a diffraction-limited focus through a microscope objective (*i.e.* laser beam diameter entering the microscope objective much larger than its rear aperture). The final result for such experimental conditions (and used in our experiment), relates the detected time-averaged fluorescence signal $F = \langle F(t)\rangle$ to the averaged laser power $P = \langle P(t)\rangle$ by

$$F \approx \frac{1}{2}\eta_{\mathrm{det}}\Phi C\sigma_2\frac{g_{\mathrm{p}}}{f\Delta t}\frac{8n}{\pi\lambda}P^2 \quad (3)$$

where the n is the refractive index of the medium in which the excited chromophore is embedded, and λ the vacuum wavelength of the exciting light. As indicated, eqn. (3) is for a plane wave focused by diffraction-limited optics. For a focused Gaussian–Lorentzian laser beam,[46] the result is slightly modified, but only by a constant factor. The refractive index n is still present. Two-photon excitation is more efficient in media of high refractive index since the laser beam can be focused more tightly.

The detection efficiency η_{det} can be separated into different contributions:[47]

$$\eta_{\mathrm{det}} = Kn^{-2}\,\eta_{\mathrm{spectral}} \quad (4)$$

K includes all sample- and wavelength-independent factors affecting the detection efficiency (solid angle of detection, wavelength-independent reflection losses), and needs to be evaluated in case of *absolute* measurements of the two-photon absorption cross section, when a reference sample is not used. Next, the detection efficiency depends on the inverse square of the refractive index of the embedding medium (at small solid angles), in the same way as in solution fluorescence quantum yield measurements.[48]

η_{spectral} accounts for the wavelength-dependent response of the collection optics, barrier filters and the photodetector. It is a function of the normalised emission spectrum of the fluorophore $J(\lambda)$ (total area = 1) and its overlap with the quantum efficiency curve of the photomultiplier $\xi(\lambda)$ and with the product T_{optics} of the transmittance/reflectance spectra of all optical components between the sample and the detector.

$$\eta_{\mathrm{spectral}} = \int J_{\mathrm{fluo}}(\lambda)\,T_{\mathrm{optics}}(\lambda)\,\xi_{\mathrm{PMT}}(\lambda)\,\mathrm{d}\lambda \quad (5)$$

Eqn. (3) can be rearranged:

$$(\sigma_2\Phi) = \frac{2}{C}\frac{n^2}{K\eta_{\mathrm{spectral}}}\frac{f\Delta t}{g_{\mathrm{p}}}\frac{\pi\lambda}{8n}\left(\frac{F}{P^2}\right)$$

When both the two-photon excited fluorescence of a sample and that of a reference compound are measured as a function of excitation power, the ratio of the slopes (F/P^2) of the respective plots of measured fluorescence intensity *versus* laser power squared (which should be linear and go through the origin with slope F/P^2) is related to the ratio of their TPEF action cross sections (eqn. (6)).

$$\frac{(\sigma_2\Phi)}{(\sigma_2\Phi)_{\mathrm{R}}} = \frac{\eta_{\mathrm{spectral,R}}}{\eta_{\mathrm{spectral}}}\frac{n^2}{n^2_{\mathrm{R}}}\frac{C_{\mathrm{R}}}{C}\frac{n_{\mathrm{R}}}{n}\left(\frac{F}{P^2}\right)\left(\frac{F}{P^2}\right)^{-1}_{\mathrm{R}} \quad (6)$$

Note that the ratio of the refractive indices *squared* is related to the detection of the generated fluorescence (solid angle of detection depending on solvent refractive index, as in fluorescence quantum yield measurements in solution[48]), whereas the second refractive index ratio accounts for the more efficient focusing of the excitation light in more highly refracting media.

Our experimental setup follows the original design described in ref. 40. The emission is detected in epifluorescence mode, through a microscope objective (10×, NA 0.22) that also focuses the expanded excitation beam. This makes it possible to avoid the inner filter effects related to the high dye concentrations used (10^{-4} M) by focusing the laser near the cuvette window. We found that it was more convenient to use BG39 glass filters (Schott) instead of a solution of $CuSO_4$ for filtering out the remaining excitation light. The light filtering characteristics of both methods are virtually identical, as found by UV/Vis spectroscopy. The dichroic mirror (Chroma 675dcxru) transmits 700–1100 nm and reflects 350–650 nm light. The excitation intensity was measured using a biased Si photodiode, and the photoluminescence was measured simultaneously using a Hamamatsu HC125-01 multialkali photomultiplier module. The signals were digitised using a home-made computer-based data acquisition system. The present setup has only limited sensitivity in the red. Europium(III) luminescence is detected with an efficiency that is only 2% of the efficiency with which terbium(III) luminescence is detected. The limited red sensitivity is mainly due to the relatively shallow cut-off provided by the BG39 filters, and may be improved if steeper (dielectric) filters are used. For the measurement of the two-photon excitation action cross sections of Mk–EuFOD, only one BG39 filter (2 mm thickness) was used in order to enhance the red sensitivity. However, this limits the shortest excitation wavelength to 750 nm, since shorter wavelengths are not efficiently blocked.

For each wavelength and each product, including the reference (10^{-4} M fluorescein in 0.1 M aqueous NaOH), the fluorescence intensity is measured as a function of the excitation power using neutral density filters to adjust the excitation intensity. Quadratic dependence of the fluorescence intensity (F) on the excitation intensity (P) is systematically verified by assuring that F/P^2 is constant. At higher fluxes, saturation effects or—in rare cases—switching to cubic dependence as a result of three-photon excitation may be observed. Only data taken in the quadratic regime is included for calculating the action cross section of two-photon excited luminescence.

Two-photon excited emission spectra were taken using a fiber-optic Ocean Optics CCD-coupled spectrometer. The photoluminescence collected using the microscope objective was focused onto the spectrometer fiber entrance, instead of on the photomultiplier. Light throughput was not optimised in this case, and the spectra are uncorrected. Only one BG39 emission filter was used, leading to an increased sensitivity in the red, but also to leakage of excitation light into the detection system at shorter excitation wavelengths (<750 nm).

4 Results and discussion

Where possible, Tb^{3+} complexes were studied instead of the corresponding Eu^{3+} since their two-photon excited luminescence (TPEL) is much more efficiently detected in the setup used. All the ligands studied also sensitise Eu^{3+} luminescence, the Eu^{3+} complexes generally having lower luminescence quantum yields. Additionally, DPA and TMP-DPA sensitise Dy^{3+} and Sm^{3+} luminescence.[32] Since two-photon absorption is completely governed by the organic ligands, the two-photon absorption cross sections found for the Tb^{3+} complexes apply to complexes with other lanthanide ions as well.

DPA and TMP-DPA were studied as 3 : 1 complexes with Tb^{3+} because of their high luminescence quantum yields. The complexes were prepared by mixing $TbCl_3 \cdot 6H_2O$ and the ligand in a 1 : 3 ratio in 2 mM TRIS-HCl buffer (pH 8.3). Due to the high association constants between dipicolinic acid and lanthanide ions[49,50] this leads to an almost exclusive (> 95%) formation of $Tb(DPA)_3^{3-}$ and $Tb(TMP\text{-}DPA)_3^{3-}$ with little dissociation, at the concentrations used (10^{-5}–10^{-4} M).

The emission spectra (Fig. 3) of $Tb(DPA)_3^{3-}$ and $Tb(TMP\text{-}DPA)_3^{3-}$ are virtually identical, indicating that the Tb^{3+} finds itself in very similar coordination environments. $Tb(TMP\text{-}DPA)_3^{3-}$ has a broad one-photon absorption band extending

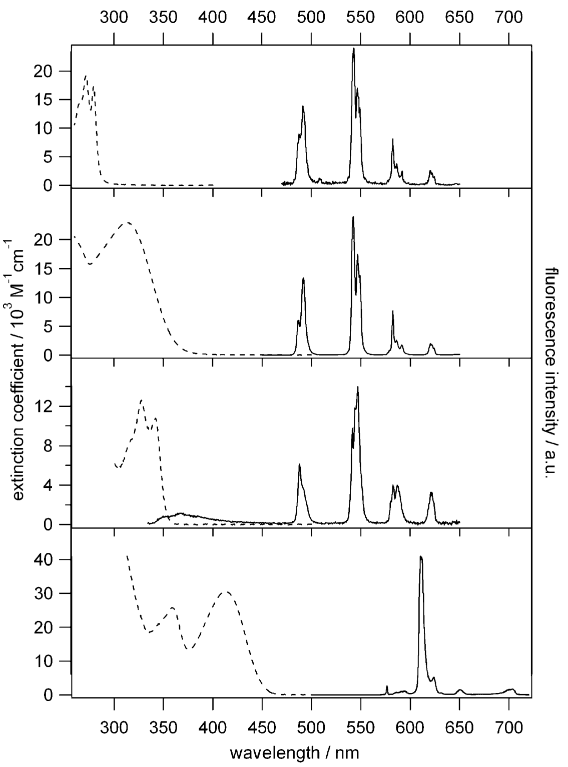

Fig. 3 One photon absorption (dashed lines) and emission (solid lines) spectra. From top to bottom: (a) $Tb(DPA)_3^{3-}$ in 2 mM TRIS buffer (pH 8.3); (b) $Tb(TMP\text{-}DPA)_3^{3-}$ in 2 mM TRIS buffer (pH 8.3); (c) cs124-DTPA–Tb in 2 mM TRIS buffer (pH 8.3); (d) Mk–EuFOD in toluene. The absorption spectrum of Mk–EuFOD was taken from ref. 39.

Table 1 Photophysical parameters for one-photon excitation. λ_{max}: one photon absorption maximum, ε_{max}: extinction coefficient, $\Phi_{sens.Ln}$: quantum yield for sensitised lanthanide luminescence (excitation *via* ligand, lanthanide emission only)

	λ_{max}/nm	ε_{max}/M^{-1} cm^{-1}	$\Phi_{sens.Ln}$
$Tb(DPA)_3^{3-}$	272	1.9×10^4	0.22
	280	1.7×10^4	
$Tb(TMP\text{-}DPA)_3^{3-}$	314	2.3×10^4	0.31
cs124-DTPA–Tb	327	1.3×10^4	0.24
	342	1.1×10^4	
Mk–EuFOD	413	3.1×10^4	0.17

conveniently beyond 350 nm. The extinction coefficient of this complex is still 1.5×10^3 M^{-1} cm^{-1} at 366 nm, a wavelength generated in mercury arcs used for conventional fluorescence microscopy. In contrast, the monophotonic absorption of cs124-DTPA–Tb falls off much more steeply, resulting in negligible absorbance beyond 360 nm, even though cs124-DTPA–Tb has its long wavelength absorption maximum at a longer wavelength than TMP-DPA.

Mk–EuFOD has a broad absorption band in the visible. The use of a relatively low excitation energy to sensitise Eu^{3+} emission is enabled by the lower energy needed to excite Eu^{3+} compared to Tb^{3+} and to the small gap between singlet and triplet energies of the coordinated Michler's ketone. The strong charge-transfer character of the lowest monophotonic excitation of Mk–EuFOD makes it a good candidate for two-photon excitation. Table 1 collects the main parameters for one-photon excitation of the four complexes studied.

Only in the case of $Tb(TMP\text{-}DPA)_3^{3-}$ and Mk–EuFOD was emission detected under excitation with 700–1000 nm pulses. At the low excitation powers used (< 20 mW at the sample) no emission was detected at all for $Tb(DPA)_3^{3-}$ and cs124-DTPA–Tb. The absence of two-photon excited luminescence in $Tb(DPA)_3^{3-}$ is hardly surprising since three photons of 840 nm would be necessary to excite the DPA ligands. Three-photon excitation needs roughly five to ten times as much laser power[46] as two-photon excitation (the precise value obviously depending on the cross sections of the dyes), which could not be supplied in the measurement configuration used.

The absence of two-photon excited luminescence in cs124-DTPA–Tb is somewhat disappointing. We estimate our limit of detection for 10^{-4} M Tb^{3+} complexes to lie well below 0.5 GM, which means that in the range 705–1000 nm the two-photon action cross section $\sigma_2\Phi$ of cs124-DTPA–Tb lies below this value. Piszczek *et al.*[26] used higher laser powers and observed multiphoton-excited luminescence in cs124-DTPA–Tb, whose intensity varied as the excitation intensity with an exponent of 2.4. This may be indicative of a mixture of two- and three-photon excitation, or of saturated three-photon excitation. The low two-photon absorption cross section of cs124-DTPA–Tb may be related to the fact that this chromophore has only limited push–pull character, as shown by the structured one-photon absorption spectrum with relatively narrow bands.

To confirm that the green $Tb(TMP\text{-}DPA)_3^{3-}$ and red Mk–EuFOD emission spots appearing at the focal point of the excitation light are indeed multiphoton-excited lanthanide luminescence, emission spectra were taken. The spectra in Fig. 4 show that it is indeed the lanthanide luminescence from the complexes that is two-photon excited. Although these spectra have not been corrected for the wavelength dependence of detection sensitivity, it can still be clearly seen that they are very similar, probably identical, to those obtained using one-photon excitation.

In both complexes, a quadratic dependence of the photoluminescence intensity on the incident power is observed, although the TPEL of Mk–EuFOD slightly saturates at higher laser powers. The quadratic behaviour is a good indication that we are working in an excitation regime where saturation and photobleaching are not significant. Even though the

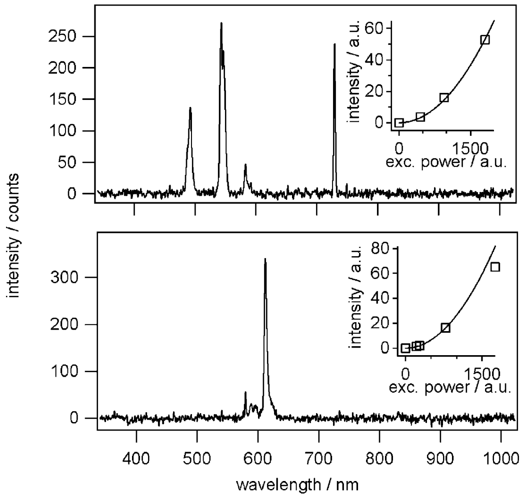

Fig. 4 Two-photon excited emission spectra (inset: excitation power dependence of luminescence intensity). Top: Tb(TMP-DPA)$_3^{3-}$, excited at 730 nm. Some excitation light leaks into the detection system. Bottom: Mk–EuFOD, excited at 830 nm.

excited-state lifetime of the lanthanide complex is much longer than the delay between pulses, a steady state is reached in which at any time only a small fraction of the complexes is in the excited state, similar to continuous wave excitation (*i.e.* the excitation may be considered to be quasi-continuous).

Using the protocol embodied in eqn. (6), action cross section spectra of two-photon excitation were measured for Tb(TMP-DPA)$_3^{3-}$ in TRIS buffer and for Mk–EuFOD in toluene (Fig. 5). Tb(TMP-DPA)$_3^{3-}$ responds mainly at the short-wavelength side of the excitation window. This is a result of the fact that its first electronic transition has a one-photon energy of 320 nm. The first two-photon excitation maximum can be expected to be around 640 nm, and thus the experimental excitation window only gives access to the long-wavelength tail of this first two-photon absorption band.

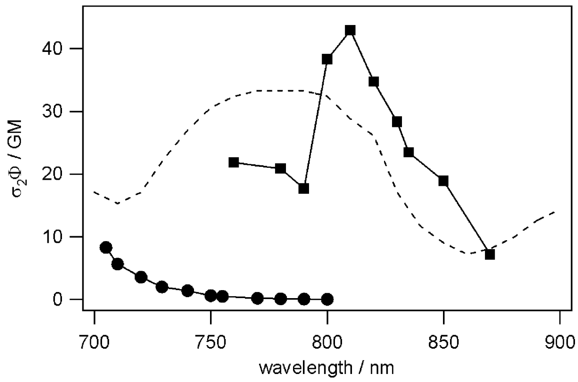

Fig. 5 Action cross section spectra for two-photon excited luminescence. Circles: Tb(TMP-DPA)$_3^{3-}$ (2 × 10^{-4} M in 2 mM TRIS buffer pH 8.3), squares: Mk–EuFOD (10^{-4} M Michler's ketone + 10^{-3} M EuFOD in toluene). A solution containing only EuFOD does not generate any detectable multiphoton-excited luminescence. The dashed line is the spectrum of fluorescein in 0.1 M NaOH,[40] shown here for comparison.

The first electronic transition of Mk–EuFOD is at a photon energy of 413 nm. A two-photon excitation maximum is indeed found near twice this wavelength. The measured values of $\sigma_2\Phi$ of Mk–EuFOD are comparable to that of fluorescein. Since Mk–EuFOD has a lower fluorescence quantum yield, its two-photon absorption cross section of 253 GM (see Table 2) is significantly higher than that of fluorescein (σ_2^{max} = 38 GM). It is known that chromophores with strong push–pull character have relatively intense two-photon absorption bands at twice

Table 2 Photophysical parameters for two-photon excitation. λ_{max}^{TPEL}: two-photon excitation maximum, σ_2: two-photon absorption cross section, $\sigma_2\Phi$: action cross section for two-photon excited lanthanide luminescence, n.d.: no two-photon excited luminescence detected in the 705–900 nm range. 1 GM = 10^{-50} cm^4 photon^{-1} s^{-1}

	λ_{max}^{TPEL}/nm	σ_2/GM	$\sigma_2\Phi$/GM
Tb(DPA)$_3^{3-}$	—	—	n.d.
Tb(TMP-DPA)$_3^{3-}$	≤705^a	≥26^a	≥8^a
cs124-DTPA–Tb	—	—	n.d.
Mk–EuFOD	810	253	43

^{a} Maximum not yet reached.

the one-photon absorption wavelength. The recently reported TPEL-optimised Eu^{3+} complex,[30] constructed on the basis of a push–pull chromophore is a striking example.

Although push–pull type chromophores indeed have their strongest two-photon absorption bands at twice the wavelength of the first one-photon absorption band, this is not a general feature of all chromophores. For example, the most intense two-photon absorption band of fluorescein peaks at 780 nm, a shorter wavelength than expected on the basis of the position of its one-photon absorption maximum (490 nm). The electronic transition of fluorescein at 390 nm is very weak in one-photon absorption, but relatively strong in two-photon absorption. Its transition near 490 nm is very strong in one-photon, but weaker in two-photon absorption.

5 Conclusion

Several lanthanide complexes have been investigated with respect to their luminescence under two-photon excitation by measuring two-photon excitation and (two-photon excited) emission spectra. In the measurement of the action cross sections $\sigma_2\Phi$ relative to a reference in a different solvent, two corrections for the index of refraction are needed (see eqn. (6)), one for the excitation process, the other for the detection of the emission. This fact has not been emphasized in the two-photon excited fluorescence literature.

If lanthanide complexes are to be used as luminescent labels in (multiphoton) scanning laser microscopy, it should be noted that the unquenched luminescence lifetimes of the complexes (100–2000 μs) are significantly longer than usual pixel times (the time that is spent measuring the fluorescence intensity for each point). Depending on the mode of detection (with or without pinhole, descanned or non-descanned) this may lead to either a reduced detected signal or to a blurred image, if the scanning is too fast. This effect might perhaps be used to perform a sort of luminescence lifetime imaging, where several images are acquired at different scan rates.

It is possible to find luminescent lanthanide complexes that have a reasonable response to two-photon excitation. The values of $\sigma_2\Phi$ of Tb(TMP-DPA)$_3^{3-}$ are relatively low, but measurable and still much higher than most endogenous chromophores[24] found in biological material. Mk–EuFOD demonstrates that two-photon absorption can be optimised in lanthanide complexes, although it would be desirable to find a water-soluble equivalent for biological applications.

Luminescent lanthanide complexes suitable for two-photon excitation might be identified by looking for complexes with: (1) relatively broad structureless one-photon absorption bands, related to transitions with push–pull character; (2) one-photon absorption at >350 nm, since the longest wavelength two-photon absorption should be at >700 nm for use with Ti:sapphire lasers and (3) good overall luminescence quantum yield and photostability. The third criterion also applies to single-photon excitable lanthanide complexes.

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