LETTER

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Spatial and temporal discrimination of silica particles functionalised with luminescent lanthanide markers using time-resolved luminescence microscopy†

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Luminescent lanthanide complexes of europium and terbium grafted on amino-functionalised silica particles can easily be discriminated from conventional fluorescent markers such as fluorescein thanks to a new and simple time-resolved luminescence microscopy set-up.

The application of fluorescent markers to labelling technologies[1] has boosted research in chemistry and biology and more specifically in fields such as immunoassays and analytical detection. When applied to microscopy, it can give insight into fine details of cellular structures through the combination of high affinity interactions with constituents of the cell and increased sensitivity provided by fluorescence detection.[2] Nevertheless, fluorescence microscopy still suffers from several drawbacks like light scattering in the apparatus, background noise brought on by auto-fluorescence of the studied samples, signal losses inherent to the small Stokes' shifts of common fluorescent markers or their potential photo-instability.

In order to circumvent these drawbacks, the use of luminophores with long-lived excited states combined with gated detection has been envisaged.[3] Introduction of a delay time between the pulsed excitation of the sample and the measurement of the emitted light intensity allows for light scattering and auto-fluorescence to collapse, while the luminescence of the long-lived luminophores remains. Under these conditions, the excited state lifetime of the marker needs to be long enough, at least superior to the delay time and to the length of the excitation pulse. As a result of their extremely long luminescence lifetimes, sometimes reaching milliseconds, lanthanide complexes of europium and terbium were early postulated as good candidates for time-resolved applications and in particular, for time-resolved luminescence microscopy (TRLM).[3,4] When combined with efficient chromophores, playing the role of photon antennae,[5] they further display large Stokes' shifts, making them particularly attractive for an easy spectral discrimination, even in the conventional fluorescence mode. Although the first applications of lanthanide markers proved their potential, these pioneering experiments suffered from weak emitted signals, mainly due to technical difficulties with the apparatus. The introduction of electronically gated intensifiers and CCD (charge coupled device) cameras coupled to laser excitation was later shown to improve the quality of the signals.[6]

In this work, we demonstrate that a new and simple TRLM set-up can be obtained with a conventional fluorescence microscope using a broad band xenon flash lamp as excitation source, in conjunction with a gateable intensified CCD camera (ICCD). The performance of the set-up was tested using a new family of lanthanide complexes linked to functionalised silica particles.

Luminescent silica particles were obtained by reacting 3-aminopropyl silica gel (Sigma, 1.46 mmol N per g) with either commercially available fluorescein isothiocyanate, as a reference for a short-lived fluorescent probe, or the europium and terbium complexes [Ln(L*)],[7] as long-lived emitters (Chart 1). In a typical experiment, silica particles were suspended in 1 mL of EtOH containing 100 μL Et_3N and 2 equiv. of the luminescent label dissolved in 2 mL of a 1:1 (v/v) EtOH–DMSO mixture was added. After 2 h of vigorous stirring at room temperature, the silica particles were decanted from the solution and washed with ethanol until the washing aliquots were no longer luminescent under UV irradiation and finally thoroughly dried under reduced pressure. In a final experiment, the 2 equiv. of label were replaced by 1 equiv. each of the Eu and Tb markers.

Upon excitation ($300\ \text{nm} < \lambda_{exc} < 360\ \text{nm}$), the bipyridyl chromophores of the complexes absorb light and a subsequent energy transfer[8] led to the typical red ($\lambda_{max\ em.} = 615$ nm) and green ($\lambda_{max\ em.} = 540$ nm) luminescence for europium and terbium, respectively, characteristic of their f-f electronic transitions (see Electronic supplementary information).[9] Under the same conditions, fluorescein labelled silica particles displayed the expected yellow-green fluorescence, the intensity of which is somehow reduced as a result of the presence of the deactivating thiourea function and of an unoptimised excitation range. It is interesting to note that whatever the marker used and while the labelled particles are apparently homogeneously functionalised at the surface, fluorescence microscopy always revealed the presence of uncoated particles with intense blue fluorescence. This phenomenon is ascribed to the nature of silica itself and probably arises from defects induced by the grinding process.

† Electronic supplementary information (ESI) available: real colour photographs of europium and terbium functionalised silica particles obtained by conventional fluorescence microsopy. See http://www.rsc.org/suppdata/nj/b4/b402024c/

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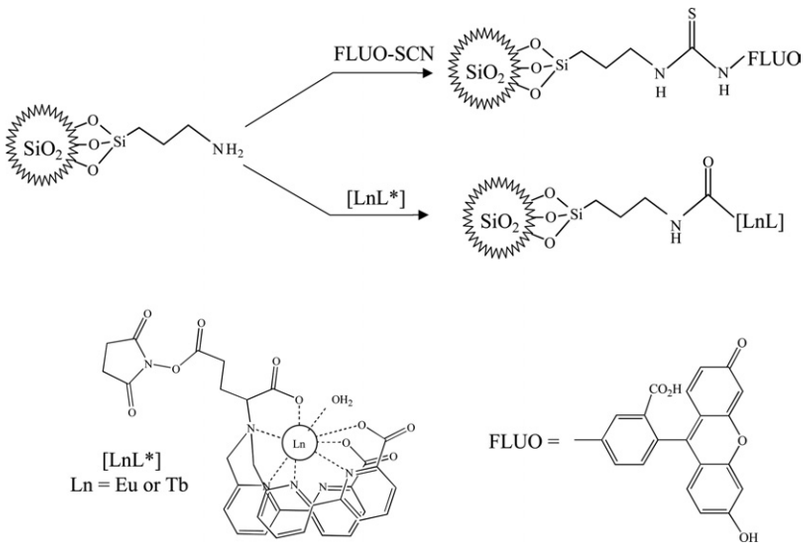

Chart 1

All samples were characterised using scanning electron microscopy (SEM) coupled with energy dispersive X-ray analysis (EDX) of the surfaces. Whatever the sample, the morphology of the particles was very similar (Fig. 1), revealing that the labelling process has no influence. Grains of irregular shapes with a mean size varying from 20 to 80 μm were observed. Thanks to EDX, it was also possible to verify the presence or absence (in the case of the starting material and fluorescein coated silica) of the different lanthanide elements at the surfaces of the particles.

The availability of the particles for use in TRLM was then tested. Fig. 2 displays TRLM images of a mixture of silica particles labelled with fluorescein and the Eu and Tb complexes. As soon as a sufficient delay time is inserted between the pulsed excitation and the integration of the signal (50 μs here), the signal emitted by the fluorescein labelled particles totally vanishes, while lanthanide-coated particles remained highly emissive.

Integration of the emitted intensity collected at fixed pixels on the CCD chip as a function of increasing delay times gave intensity decay profiles from which excited state lifetimes τ can be calculated (Fig. 3). For regions corresponding to both europium or terbium, these curves can be satisfactorily fitted with mono-exponential decays, giving lifetimes of 1.38 and 0.57 ms, respectively. This set-up safely allows for lifetime mapping for τ values superior to 20 μs and theoretically down to 4–5 μs if a correction is applied for light scattering. This feature is of particular interest if the luminescent probe is responsive to local stimuli such as the presence of anions[10] or protons[11] as it can give an indirect access to concentration mapping of the perturbing species.

The luminescence lifetimes of the lanthanide complexes in the samples are very different from those in water solutions for the corresponding unactivated precursors in which a carboxylate function replaces the *N*-hydroxysuccinimidyl ester. For Eu, the calculated value of 1.38 ms is far longer than that of the acid in water ($\tau = 0.62$ ms).[12] The replacement of the water molecule by a DMSO in the first coordination sphere of the lanthanide cation during the labelling process is likely responsible for the lifetime enhancement. Eu-centred luminescence is known to be very sensitive to the presence of inner-shell OH oscillators coordinated to the metal cation.[13] Replacement of the water molecule decreases the possibilities for non-radiative deactivation processes, thereby increasing the luminescence lifetime. The presence of DMSO in the labelled silica samples is concomitantly confirmed by EDX analysis, which revealed the presence of elevated levels of sulfur atoms.

Interestingly, terbium showed an inverse trend as the calculated lifetime (0.57 ms) is significantly shorter than that of the reference compound (1.48 ms).[12] Tb-based luminescence is known to be less sensitive to non-radiative deactivation processes due to OH oscillators. Nevertheless, the replacement of water is expected to lead to an increase of τ. In fact, the main deactivation process for Tb is back-energy transfer from the Tb ($^5D_4$) excited electronic state to the ligand-centred triplet excited state ($^3\pi\pi^*$). If the energy difference between these two levels is smaller than *ca.* 1850 cm^{-1} at room temperature,[14] a back-energy transfer can occur, decreasing the luminescence lifetime and efficiency. Replacement of the water molecule by a bulkier DMSO one may perturb the geometry of the complex with inherent changes in the $^3\pi\pi^*$ level. Such subtle changes are difficult to quantify but may bring about drastic changes in the Tb luminescence properties,[15] especially if the above mentioned energy gap is close to the limiting value.

Another intriguing observation is that, while fluorescence microscopy observation of the mixture of silica particles labelled with either Eu or Tb distinctively showed the corresponding red and green emissions, particles labelled with both Eu and Tb markers only showed the red emission of Eu. In this latter case, EDX analysis performed during SEM experiments confirmed the presence of both lanthanide cations on the surfaces of the particles. Measurements at various locations on the samples gave relative atomic percentages of 64 and 36, respectively, for Eu and Tb. The intensity decay profile measured by TRLM was well-fitted with a mono-exponential decay, giving a luminescence lifetime of 1.33 ms, in keeping with the τ value measured for Eu alone.

The reason for the absence of visibility of the Tb emission can be found in the solid state fluorescence spectra of the different samples (Fig. 4). For all samples, the high energy tail of the spectra (400 to 480 nm) are dominated by silica-based fluorescence emission, which vanished as soon as a 10 μs delay time is inserted (phosphorescence mode). The other emission bands are characteristic of the $^5D_0 \rightarrow {}^7F_J$ and $^5D_4 \rightarrow {}^7F_J$ ($J = 0$ to 6) electronic transitions of Eu and Tb, respectively (Fig. 4). In the case of the multiple labelling experiment, electronic transitions corresponding to Tb are weak, which explains why the observation by microscopy only revealed Eu emission, the latter corresponding to a spectral region to which human eyes are particularly sensitive.

In the absence of a determination of the luminescence quantum yields, it is not possible to know whether the Tb emission was normal or if it was decreased by the presence of Eu.

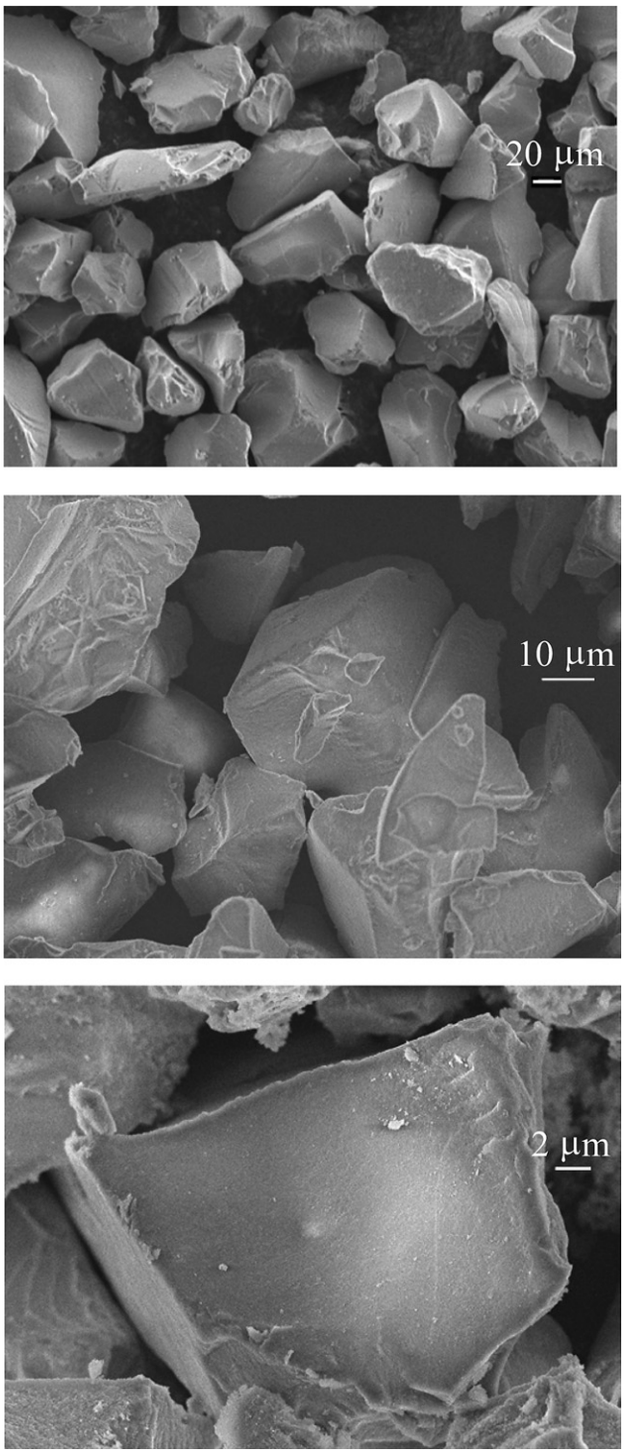

Fig. 1 SEM images of uncoated silica particles (top, × 300) and europium (middle, × 1000) and terbium (bottom, × 3500) functionalised silica particles.

Indirect evidence of the latter possibility was obtained from luminescence lifetime measurements. The decay profile corresponding to the $^5D_4 \rightarrow {}^7F_{5,6}$ transitions of Tb at 540 and 486 nm could not be fitted with mono-exponential functions. The fitting was satisfactory only if a biexponential function was used and gave lifetimes of 0.085 and 0.69 ms. Pre-exponential factors showed that the longer lifetime corresponds to *ca.* 30% of the amount of Tb species and may be attributed to an unperturbed emission, as found for silica functionalised with Tb only. In contrast, the main population (70%) of Tb species have a shorter lifetime, which can be explained by a Tb-to-Eu energy transfer.[9,16] This energy transfer is the result of the

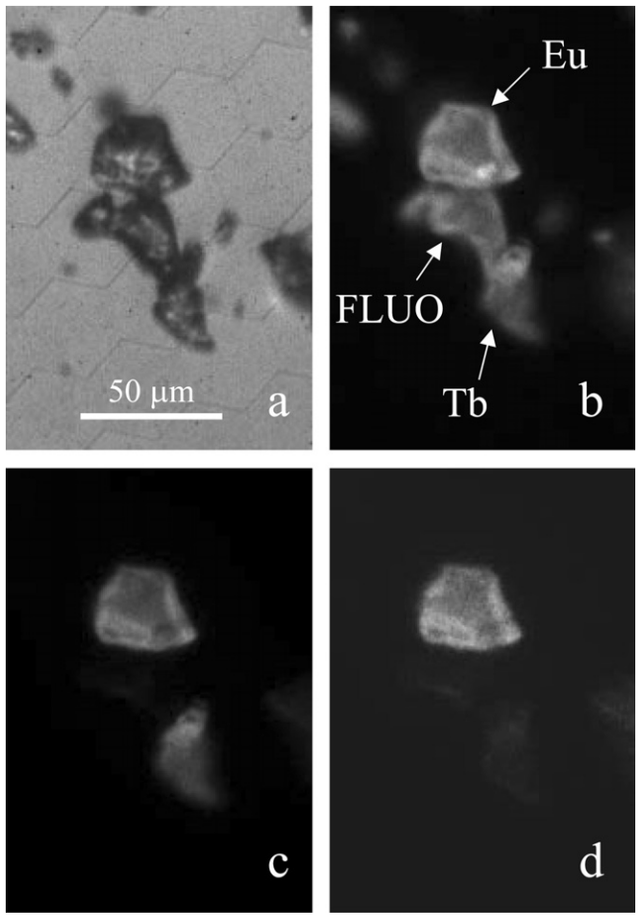

Fig. 2 Microscope images of a mixture of silica particles stained with fluorescein and the lanthanide complexes recorded in (a) transmittance, (b) prompt fluorescence and (c,d) time-resolved luminescence modes with delays of (c) 50 µs and (d) 3 ms.

overlap between the Tb emission spectrum and the Eu absorption spectrum in the region corresponding to f-f electronic transitions. In a rough approximation, the europium absorption spectrum can be regarded as corresponding to its emission spectrum, as a result of the weak Stokes' shifts of inner shell f-f transitions. This overlapping can then be observed in Fig. 4 for wavelengths superior to 575 nm, where $^5D_4 \rightarrow {}^7F_J$ ($J < 5$) electronic transitions of Tb are superimposed with transitions of europium. As a result of the statistical distribution of Tb

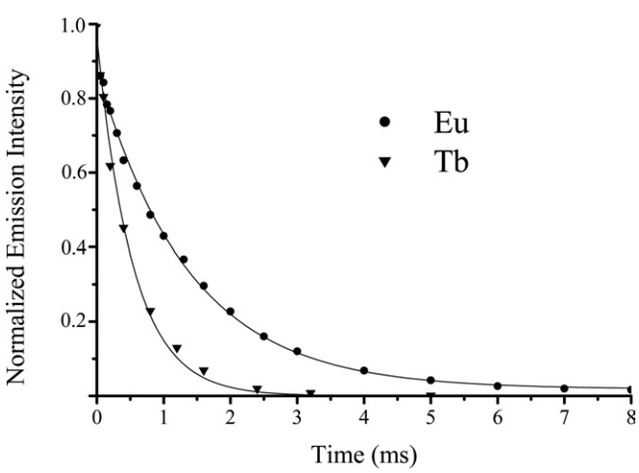

Fig. 3 Evolution of the normalised emitted intensity as a function of time for Eu or Tb labelled silica particles as measured on the CCD. Full lines represent their mono-exponential fitting curves.

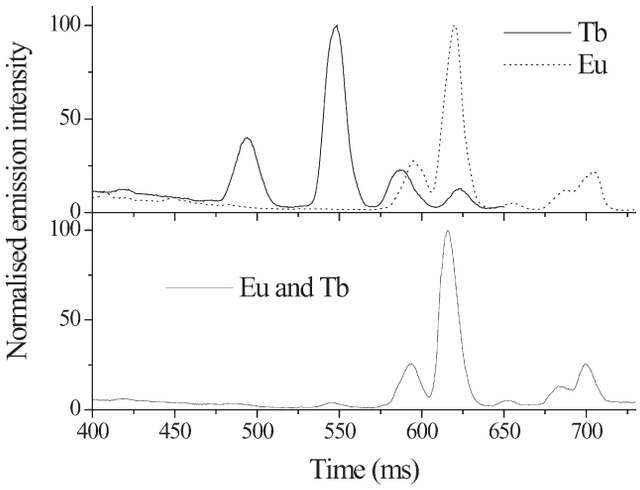

Fig. 4 Room temperature solid state emission spectra of the silica particles labelled with Eu or Tb (top) or with both Eu and Tb (bottom).

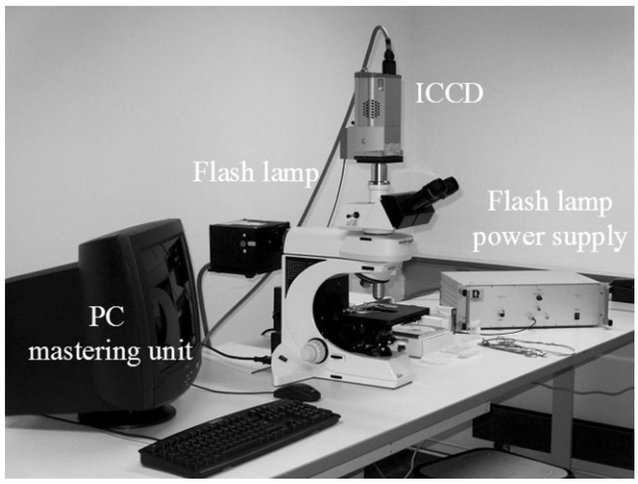

Fig. 5 Time-resolved microscopy set-up.

and Eu atoms at the surface, this lifetime must be understood as a mean value for the Tb atoms that are subject to energy transfer. The efficiency η of this energy transfer can be calculated by the relation:[9]

$$\eta = 1 - \tau/\tau_0$$

where τ and τ_0 refer to the lifetimes in the presence of the energy acceptor (Eu) and in the pure sample, respectively. Taking the lifetime measured for pure Tb functionalised silica particles as the τ_0 value (0.57 ms), the efficiency of the energy transfer to europium amounts to 85%.

The principal findings of the studies disclosed in this letter are as follows. We have developed a new and simple TRLM set-up that easily gives access to lifetime mapping with samples containing long-lived luminophores ($\tau > 20$ μs) such as europium and terbium complexes. Specific labelling of silica particles with fluorescein, europium or terbium luminophores allowed us to validate the concept. Furthermore, simultaneous labelling with Eu and Tb reveals a very efficient Tb-to-Eu energy transfer process. Our interest is currently directed toward the covalent grafting of these lanthanide complexes to specific biological matter for microscopy applications.[12] Among those, one can envisage multiple labelling patterns with the idea to put into play both spectral[17] and temporal resolution or time-resolved resonant energy transfer experiments.[18]

Experimental

Conventional fluorescence microscopy images were obtained on a Leica DMLB fluorescence microscope equipped with a continuous 100 W mercury lamp and were recorded with a Hewlett Packard digital camera. Time-resolved luminescence microscopy images were obtained on the same microscope (Fig. 5), with the following changes. The continuous mercury lamp was replaced by a xenon flash lamp (FlashMic system, Rapp Opto Electronic, Hamburg, Germany) delivering pulses of varying full width at half height (FWHH) from 4 to 400 μs. In all experiments described here a FWHH of 4 μs was used. The numerical camera was replaced by an ICCD camera (model DH734-18F-03, Andor Technology, Belfast, Northern Ireland) integrating a digital delay generator allowing for 5 ns optical gating. The ICCD was fitted with a C-mount adaptor to the binocular of the microscope and was monitored by a personal computer. Triggering of the flash lamp was mastered by the ICCD and delivered with a 5 V TTL output to the opto-coupler of the lamp. In a typical experiment, images were recorded on the CCD chip for a duration of 1 s, during which 10 illumination cycles were performed. Within a cycle, the flash is triggered by the DDG, a variable delay, δ, is then implemented before the intensifier is turned on for a period of time w (typically 5 ms). At time $\delta + w$, the intensifier is turned off until the beginning of the next cycle. For prompt fluorescence measurements, δ is set to 0.

Fluorescence emission spectra of the samples were recorded on a Perkin Elmer LS 50B spectrofluorimeter equipped with a solid sample holder. Spectra were recorded in the phosphorescence mode of acquisition using 10 ms integration windows and a 0 μs delay time for fluorescence acquisition. Excitation was performed at 308 nm (maximum of absorption of the complexes) with a cut-off filter at 350 nm.

Scanning electron microscopy coupled with energy dispersive X-ray analysis was performed on a JEOL JSM 840 apparatus.

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