Critical evaluation of five emissive europium(III) complexes as optical probes: correlation of cytotoxicity, anion and protein affinity with complex structure, stability and intracellular localisation profile†

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Five structurally related europium(III) complexes of heptadentate macrocyclic ligands bearing azaxanthone or azathiaxanthone chromophores have been evaluated comparatively as responsive probes of the intracellular environment. Protein binding (HSA) and oxy-anion binding constants have been measured by titrimetric analysis, examining ratiometric emission changes for three examples, and systems exhibiting selectivity for citrate or hydrogencarbonate identified. The cytotoxicity of each ligand and complex has been assessed and correlated with the observed chemical stability profile in competitive aqueous media. The europium complex of L^2 is non-toxic, exhibits a large change in its emission spectral profile to variation in HCO_3^- (or citrate) concentration allowing ratiometric analysis, and localises in cellular mitochondria. Such features augur well for its future application as a responsive probe in microscopy to monitor local changes in $pHCO_3$.

Introduction

The pursuit of new, responsive probes for the cellular environment remains an important aspect of modern cell biology. The majority of work in this area has focused on modifications of conventional organic fluorophores, based on fluorescein, rhodamine or BODIPY core structures, and notable advances have been made in signalling changes in pH, pZn and pX amongst others.[1–3] Increasing attention has also been paid to the development of biocompatible 'quantum dots', which offer an effective means of cell tracking,[4,5] although their large molecular volume may limit attempts to direct them to particular cell organelles. Similarly, several incisive studies have addressed the utility of yellow, green, red or blue fluorescent proteins,[6,7] where applications based on fluorescence resonance energy transfer (FRET) have allowed useful conclusions to be drawn in studies probing protein–protein interactions. The extent to which these recombinant proteins may be safely regarded as a 'non-invasive' probe remains a pertinent concern.

In recent work, we have sought to develop responsive optical probes, based on our understanding and appreciation of the chemistry of emissive lanthanide complexes.[8,9] In particular, we are keen to explore systems in which the emitted light encodes information about the chemical composition of the local environment through modulation of spectral form, lifetime or circular polarisation.[9,10] Thus, systems that optically signal changes in pH,[11] hydrogencarbonate,[12,13] citrate[14] and urate[15] concentration have been developed. Each operates under the complex conditions found within the cells (protein, oxygen, variable concentrations of competing ions and quenching species), and reports on the target analyte *via* a change in the intensity of at least two emission bands, *i.e.* it provides a ratiometric analysis that is independent of probe concentration. This is a particular feature of europium(III) complexes, as the relative simplicity of the spectral emission profile has allowed reliable correlations to be made with the speciation of the europium(III) ion, relating the nature of the bound donor (*e.g.* donor atom polarisability[8a,9]) and complex constitution (*e.g.* nature of the axial donor[9,16]) to the form and relative intensity of the $\Delta J = 1$ and $\Delta J = 2$ emission bands. Alternate strategies based on the use of two (or more) different lanthanide ions in a common ligand have also been explored, examining Eu/Tb emission intensity ratios.[15,17]

We strive to develop lanthanide(III) probes that can be targeted to certain compartments within the cell.[10a,18,19] Here, we report a comparative study examining the behaviour of three structurally related lanthanide(III) complexes (Ln = Eu, Gd, Tb) that were designed to report on their local environment. Two other complexes are also introduced in which S is replaced by O in the sensitising moiety. Each is based on a heptadentate macrocyclic ligand, where the heterocyclic sensitiser is constrained to be within about 6 Å of the Ln(III) centre, *i.e.* L^1 and L^2, or is directly bound to the Ln ion, L^3. We examine their binding affinity to four representative oxy-anions (lactate, citrate, hydrogencarbonate and hydrogenphosphate), as well as to serum albumin, comparing this behaviour to their cellular toxicity and cellular localisation profile—as revealed by fluorescence microscopy analysis. Some of these aspects are then analysed with respect to their differing chemical constitution and solution structures, as revealed by analysis of NMR and emission spectral studies.

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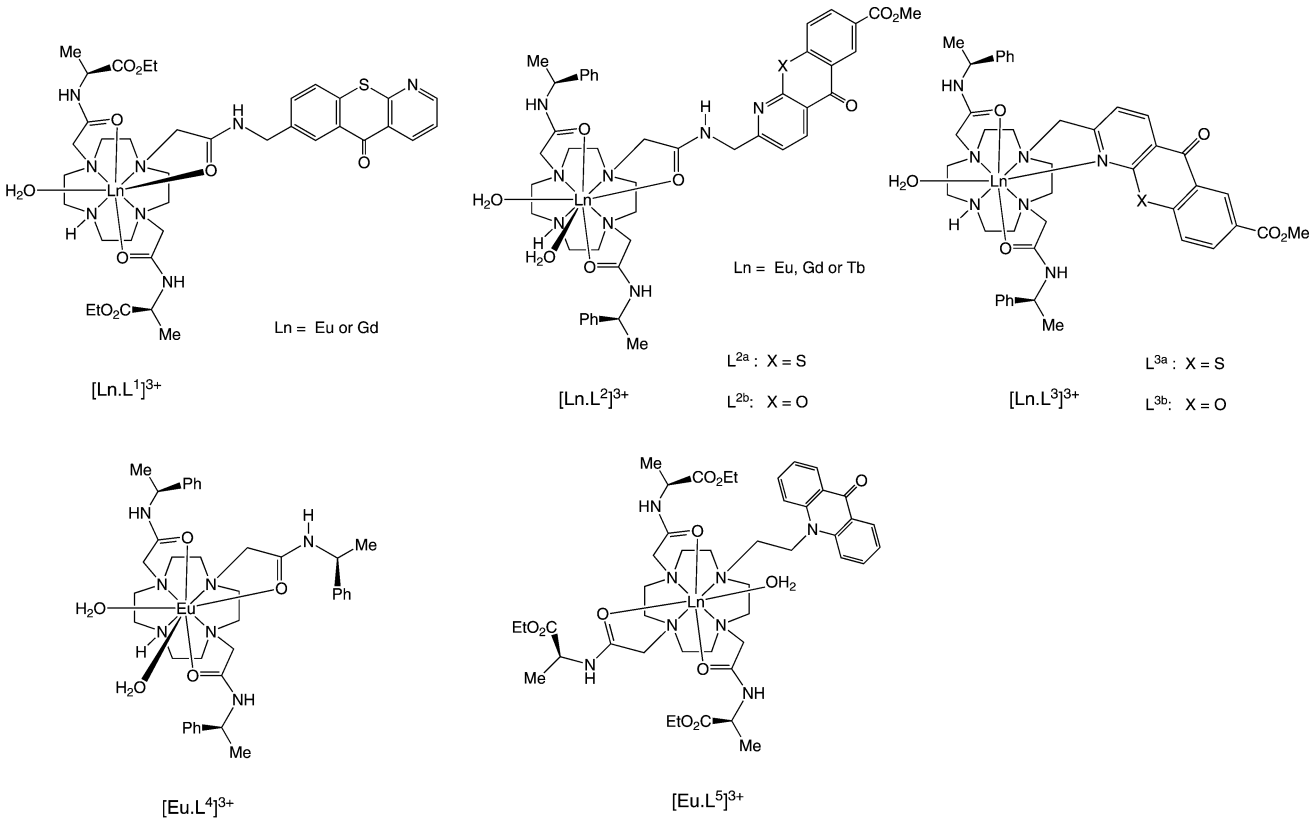
† Electronic supplementary information (ESI) available: Examples of spectral titrations for [Eu.L^n] (n = 1, 2, 3) with selected anions, protein and pH and additional microscopy images detailing the time dependence of complex localization. See DOI: 10.1039/b803895c

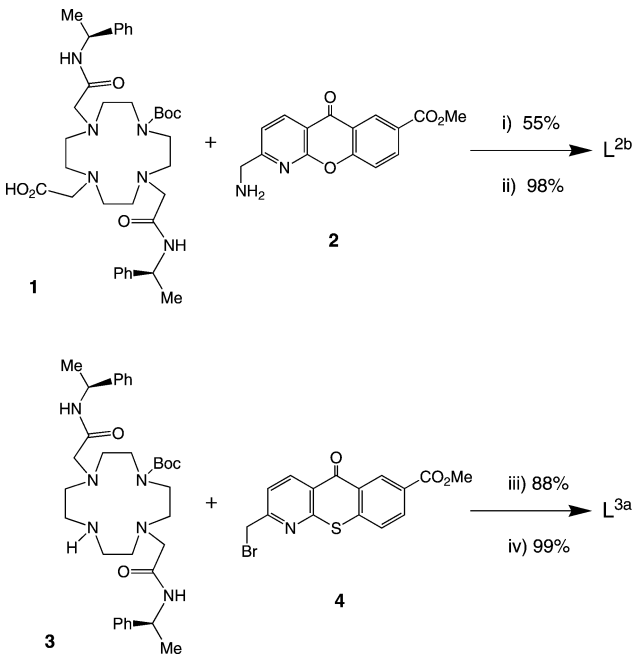


Results and discussion

Ligand and complex synthesis and characterisation

The synthesis of the ligands L^1 and L^{2a} and their Eu(III) complexes has been reported recently, in the context of their utility for two-photon excitation.[20] Similar synthetic methodology was applied for the synthesis of L^{2b} and L^3, Scheme 1. Coupling of the differentially substituted cyclen derivative, **1**, with the 2-aminomethylazaxanthone derivative **2** (prepared from the reported bromomethyl precursor[21] *via* a Gabriel reaction), using standard methods (EDC, HOBt, $CHCl_3$) followed by deprotection (TFA, CH_2Cl_2) of the ring BOC group afforded L^{2b}. A related strategy was used for the synthesis of L^3, involving BOC protection of one ring nitrogen: alkylation of the cyclen derivative, **3**, with the 2-bromomethylazathioxanthone derivative **4**[21] (K_2CO_3, MeCN) gave the N-alkylated intermediate that was purified by chromatography on neutral alumina. De-protection (TFA, CH_2Cl_2, 20 °C) afforded L^3 as its trifluoroacetate salt. Complexes of each ligand with $Eu(CF_3SO_3)_3$ were prepared in dry MeCN and then anion exchanged (Amberlite resin) to chloride to improve the water solubility of the isolated complexes. The constitution and chemical purity of the isolated complexes were confirmed by high resolution mass spectroscopic and reverse-phase HPLC analysis.

The absorption and emission spectra of $[Eu.L^1]$ in water (298 K, pH 7.4) are representative of the optical spectral characteristics for the series of complexes isolated. The azathioxanthone chromophore absorbs at 384 nm[21] (Fig. 1), and both ligand fluorescence (450 nm) and metal-based emission were observed. Measurements of the radiative lifetimes in H_2O and D_2O permit an estimate of the number of coordinated water molecules,[22] q. Values of q were typically close to unity, except for $[Ln.L^{2b}]^{3+}$ (Ln = Eu, Tb), for which a q value of 2 was estimated (Table 1). Intriguingly, $[Eu.L^{2a}]^{3+}$ appears to be a mono-aqua complex, or a mixture of mono and diaqua species, as $q = 1.3$. Presumably, the change from O to S



Scheme 1 Ligand synthesis for L^{2b} and L^{3a}: i) EDC, HOBt, Et_3N, $CHCl_3$; ii) TFA, CH_2Cl_2; iii) K_2CO_3, MeCN; iv) TFA, CH_2Cl_2.

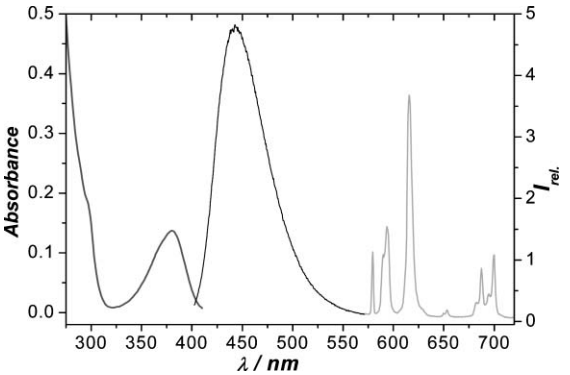


Fig. 1 Absorption (left), chromophore fluorescence (centre) and Eu(III) emission (right) spectra for $[Eu.L^1]^{3+}$ (H_2O, pH 7.4, 10 μM complex).

Table 1 Selected photophysical data[b] (H_2O, 298 K) for Eu(III) complexes of L^1, L^2, L^3

Complex	λ_{max}/nm	ε/M^{-1} cm^{-1}	τ_{H2O}/ms	τ_{D2O}/ms	q
$[Eu.L^1]Cl_3$	384	12710	0.44	0.90	0.92
$[Eu.L^{2a}]Cl_3$	372	3570	0.33	0.65	1.27
$[Eu.L^{2a}]Cl_3$[a]	340	4300	0.27	0.64	2.09
$[Eu.L^{3b}]Cl_3$	375	3790	0.32	0.62	1.33

[a] For $[Tb.L^{2b}]^{3+}$: $\tau(H_2O) = 1.17$ ms, $\tau(D_2O) = 2.07$, $q^{Tb} = 1.6$. [b] τ values are the mean of at least 3 independent measurements (±10%); q values (±20%) were estimated using the analysis reported in reference 22.

in the ligand will reduce the tendency for water to H-bond to the ligand.

In complexes with an azaxanthone sensitising moiety, absorption occurred at lower wavelength, around 340 nm, and <4% ligand-based fluorescence was observed, consistent with the faster rate of intersystem crossing in these systems.[21]

Anion binding affinity selectivity and complex structure

It is becoming more evident that the optical and NMR spectral properties of lanthanide(III) complexes, when analysed together, afford a great deal of information on the nature and symmetry of the metal ion coordination environment.[8,9,23] In particular, the nature and polarisability of the group occupying a position on or close to the principal axis of the complex primarily determine the magnitude of the second order crystal field coefficient, B_o^2, that controls the dipolar NMR shift and the degree of splitting of the $\Delta J = 1$ manifold in europium(III) emission spectra.[9a,10b,16] For lanthanide complexes of cyclen derivatives this is typically the 'axial' ligand occupying the ninth site, that caps the square anti-prismatic polyhedron. The more polarisable the axial donor atom is, the greater the relative intensity of the hypersensitive and electric-dipole allowed $\Delta J = 2$ emission band. Thus, the substitution of a 'hard' coordinated water by, for example, CO_3^{2-}, or P–O/S–O donors, has been shown to lead to emission spectra with large $\Delta J = 2/\Delta J = 1$ intensity ratios.[9a,13] Conversely, if the Eu complex remains octadentate or if the nature of the capping axial ligand in CN = 9 systems is conserved in any putative ternary adduct, then only minor variations in the form and relative intensity of the $\Delta J = 1$ and $\Delta J = 2$ manifolds may be expected.

Changes in the spectral behaviour (emission and ^{1}H-NMR) for $[Eu.L^1]$, $[Eu.L^{2a}]$ and $[Eu.L^3]$ were monitored separately under varying concentrations of the four most common bioactive oxyanions, hydrogencarbonate, HPO_4^{2-}, lactate and citrate. In each case,† the europium emission spectral form was recorded both as a function of anion concentration, and as a function of pH (298 K, 0.1 M NaCl range $3 < pH < 8$) for a fixed anion concentration (HCO_3^- 30 mM; HPO_4^{2-} 0.9 mM; lactate 2.3 mM; citrate 0.13 mM) corresponding to common extracellular anion values. With $[Eu.L^1]$, changes in spectral form were not very significant, but were still sufficient to allow the variation of the emission intensity ratio of a pair of bands (*e.g.* 617/615 nm, 700/687 nm or 594/592 nm) with anion concentration to be plotted and fitted to a 1 : 1 binding model. Apparent affinity constants could then be calculated (Table 2). The most distinctive changes (albeit *much less* than those reported for anionic adducts of related Eu(III) complexes with heptadentate ligands[12,13]) were observed following addition of $NaHCO_3$ to $[Eu.L^1]$, Fig. 2. The Eu^{3+} emission lifetimes were recorded in the presence of 20 mM $NaHCO_3$, and values recorded in H_2O ($k^{Eu} = 2.20$ ms^{-1}) and D_2O ($k^{Eu} = 1.94$ ms^{-1}) were consistent[22] with formation of a ternary adduct with no bound water molecules. The measured apparent affinity constant ($K_a^{HCO3-} = 10^{3.01}$) decreased by 10% in the pressure of a mixed anion background containing fixed concentrations of citrate (0.13 mM) lactate (2.3 mM) and HPO_4^{2-} (0.9 mM), and was unchanged (±5%) when human serum albumin (HSA, 0.7 mM) was added to this mixture. In a separate experiment, incremental addition of HSA to $[Eu.L^1]^{3+}$ in solution (0.1 M NaCl or a mixed anion background) reduced the Eu emission intensity, but not the lifetime and caused no change in the spectral form of the Eu emission, eliminating the possibility of ligation by amino acid side chains that has been observed in less sterically demanding systems.[24] Protein addition also reduced the ligand fluorescence at 445 nm, but to a lesser extent than the reduction in overall Eu emission intensity. This allowed the intensity ratio (440/616 nm) to be plotted as a function of added [HSA],† and an apparent 1 : 1 binding constant of $10^{3.10(\pm0.03)}$ was estimated for protein association.

Table 2 Affinity constants of europium(III) complexes for selected anions[a] (298 K, 0.1 M NaCl)

Complex	Citrate (pH 7.4)	Lactate (pH 6)	HCO_3^- (pH 7.4)	HPO_4^{2-} (pH 7.4)
$[Eu.L^1]^{3+}$	4.33(02)	2.67(02)	3.01(02)	3.14(02)
$[Eu.L^{2a}]^{3+}$	6.02(03)	2.73(02)	3.50(03)	3.95(03)
$[Eu.L^{3a}]^{3+}$	5.24(03)	2.98(02)	2.08(02)	2.60(03)

[a] The pH regime was selected on the basis of preliminary experiments examining the pH dependence of the observed emission profile at a fixed anion concentration (30 mM HCO_3^-, 2.3 mM lactate, 0.9 mM HPO_4^{2-}, 0.1 mM citrate with 10 μM complex), seeking a relatively 'flat' part of the spectral response/pH profile.

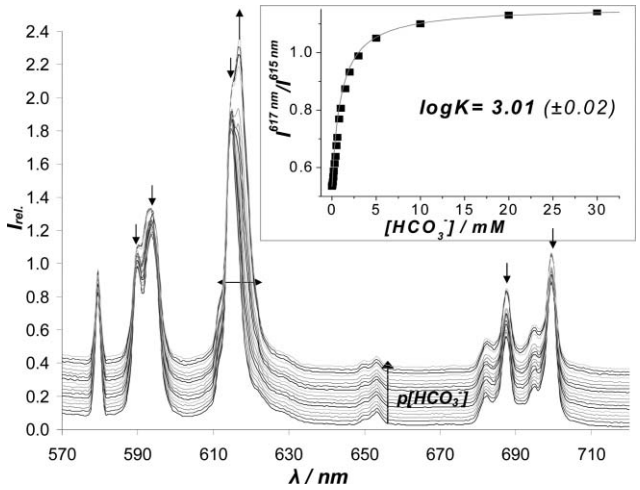


Fig. 2 Variation of the Eu emission spectrum for $[Eu.L^1]^{3+}$ following addition of sodium bicarbonate (pH = 7.4 (±0.05); [complex] = 10 μM; 298 K, λ_{exc} = 384 nm; I = 0.1 M NaCl) showing (insert) a plot of the intensity ratio (617/615 nm) with $[HCO_3^-]$, allowing calculation of the apparent anion binding constant [LV_{min} 0.535, LV_{max} 1.150].

An independent assessment of this value may be deduced by examining the variation of the relaxivity of $[Gd.L^1(OH_2)]^{3+}$ as a function of added protein. Numerous studies have vindicated the accuracy of such a method for various gadolinium(III) complexes that incorporate a lipophilic moiety.[25–27] The relaxivity of $[Gd.L^1](CF_3SO_3)_3$ was 7.6 mM^{-1} s^{-1} (310 K, 60 MHz) in water and decreased to 6.3 mM^{-1} s^{-1} in the presence of 30 mM $NaHCO_3$ (pH 7.4). In the presence of 0.25 mM HSA, these values increased to 39.2 mM^{-1} s^{-1} and 26.5 mM^{-1} s^{-1} respectively, and a titration (Fig. 3) was analysed to reveal an apparent binding constant log K = 3.07 (±0.04) M^{-1}, assuming 1 : 1 stoichiometry. This agrees well with the value for protein association determined for $[Eu.L^1]^{3+}$. The magnitude of the measured relaxivity for the protein-bound adduct suggests that one water remains bound to the Gd in the protein-bound species; this feature also accords with the Eu emission spectral study in which the Eu(III) spectral form was the same for the free and the protein-bound complex. In the presence of 30 mM $NaHCO_3$, a relatively high relaxivity was still observed.

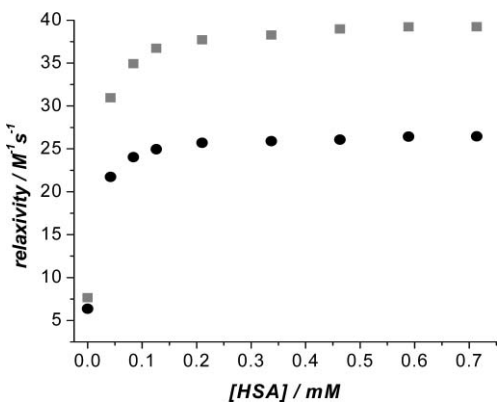


Fig. 3 Variation of the relaxivity of $[Gd.L^1]^{3+}$ with added HSA in water (squares) and in the presence of 30 mM $NaHCO_3$ (circles) (pH 7.4, 80 μM complex, I = 0.1 M NaCl, 310 K, 60 MHz).

Under these conditions the Gd complex should be >90% bound to carbonate (giving a major $q = 0$ species) as the K_a values are both about 10^3. The high relaxivity value for the ternary adduct is not unprecedented: several examples have been reported of large second-sphere contributions to relaxivity in protein conjugates of $q = 0$ Gd species.[28,29]

With $[Eu.L^{2a}]^{3+}$ and $[Eu.L^{2b}]^{3+}$, HSA addition led to a very large decrease in Eu emission intensity, whereas the emission lifetime increased from 0.26 ms to 0.35 ms, consistent with displacement of a coordinated water molecule. For $[Tb.L^{2b}]^{3+}$, HSA addition also caused a 10-fold reduction in emission intensity, suggesting that the chromophore excited state was being quenched by a change transfer process. This hypothesis was supported by parallel quenching phenomena being observed with $[Ln.L^{2a}/L^{2b}]^{3+}$ following addition of a 50-fold excess of *O*-phosphotyrosine (but not *O*-phosphoserine)—which brings an electron-rich Tyr site close to the lanthanide centre, as the phosphate group binds.

The emission and ^{1}H NMR spectral behaviour of the di-aqua complex, $[Eu.L^2]^{3+}$ (X = O, and S *i.e.* L^{2a} and L^{2b}), in the presence of anions or protein was distinctly different to $[Eu.L^1]$.† Incremental addition of $NaHCO_3$ gave rise to large changes in the emission spectral form: the size of the $\Delta J = 2/\Delta J = 1$ intensity ratio increased from 2.3 : 1 to 5 : 1, and the spectra observed resembled the behaviour of systems studied earlier, such as $[Eu.L^4]^{3+}$ and $[Eu.L^5]^{3+}$.[12,13,30] Distinctive changes in spectral form also characterised reversible binding of other anions: lactate—an increase in relative intensity of the 624 nm band; phosphate—broadening of the $\Delta J = 2$ manifold, increase in intensity at 594 nm; citrate—appearance of an isoemissive point at 698 nm, and an increase in the $\Delta J = 2/\Delta J = 1$ ratio. Overall, measured affinity constants were higher for this series (Table 2), reflecting the enthalpic and entropic benefit of displacing two water molecules in citrate–lactate and bicarbonate binding. The highest affinity constant (log K = 6.02 (±0.03)) measured was for citrate binding, consistent with optimal electrostatic attraction. In the presence of anions at their common 'extracellular' concentrations, hydrogencarbonate competes with citrate for Eu coordination, in $[Eu.L^2]^{3+}$.

Pronounced changes in ^{1}H-NMR spectra also distinguish the different ternary anion complexes.[9a,16] With $[Eu.L^1]^{3+}$, the ^{1}H-NMR spectrum (5 mM complex, pD 7.8, 295 K, 200 MHz) of the 'aqua' complex (carefully degassed to eliminate dissolved CO_2) was compared to that measured in the presence of 30 mM $NaHCO_3$ at pD 7.8. Addition of bicarbonate caused a sharpening of the observed resonances but no major differences (±3 ppm) were observed in the resonances for the major solution species. In contrast, addition of excess $NaHCO_3$ to $[Eu.L^{2a}]$ in D_2O caused large changes in the observed ^{1}H-NMR spectrum, with some resonances moving 15 ppm (Fig. 4), consistent with the replacement of an 'axial' bound water by a more polarisable oxygen atom.[9a,10b,13,30]

This differing behaviour can be rationalised in terms of the different coordination geometries of the major solution species. With $[Eu.L^1]^{3+}$, the benzylic amide carbonyl oxygen must occupy the capping site in both the aqua complex and in the ternary anion adducts. This is reflected in the constancy of the dipolar NMR shift and the similar splitting (approximately 110 cm^{-1} for the A and E components) of the $\Delta J = 1$ transitions around 590–594 nm for each ternary species. Carbonate or lactate/citrate chelation must occur at two sites in the plane of the square

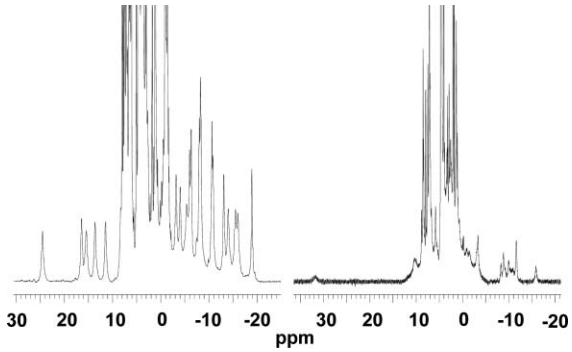


Fig. 4 ^{1}H NMR spectra for [Eu.L^{2a}]$^{3+}$ in the absence (left) and presence (right) of 30 mM $NaHCO_3$ (500 MHz, 295 K, pD 7.5, 1 mM complex).

antiprism. In contrast, with [Eu.L$^{2a/2b}$]$^{3+}$ (and for [Eu.L^4]$^{3+}$ and [Eu.L^5]$^{3+}$), a water molecule occupies the capping, 'axial' site in the 9-coordinate aqua adduct and chelation of carbonate replaces this water with an anion oxygen—consistent with the observed X-ray analyses of the diaqua [Yb.L^4] complex and of coordinated citrate, lactate and carboxylate adducts of [Eu.L^4].[10b] This changes the B_o^2 term considerably, hence changing the observed ^{1}H and Eu emission spectra.

Intermediate behaviour of [Eu.L^3]$^{3+}$

The complexes [Eu.L^{3a}]$^{3+}$ and [Eu.L^{3b}]$^{3+}$ possess a rather different structure in which the sensitiser is coordinated to the lanthanide ion *via* the pyridyl N atom. Previous work with such systems has highlighted their lower affinity for HCO_3^- and phosphate, and their tendency to bind citrate more selectively.[14] Binding affinity constants (Table 2) confirmed this trend. Changes in the form of the europium emission spectrum were very minor for binding of HCO_3^-/HPO_4^{2-} and lactate.† Addition of citrate to [Eu.L^{3a}] or [Eu.L^{3b}] was characterised by a 100% increase in the $\Delta J = 2/\Delta J = 1$ intensity ratio and by significant changes in the form and relative intensity of the $\Delta J = 0$ and $\Delta J = 1$ transitions (Fig. 5). A separate

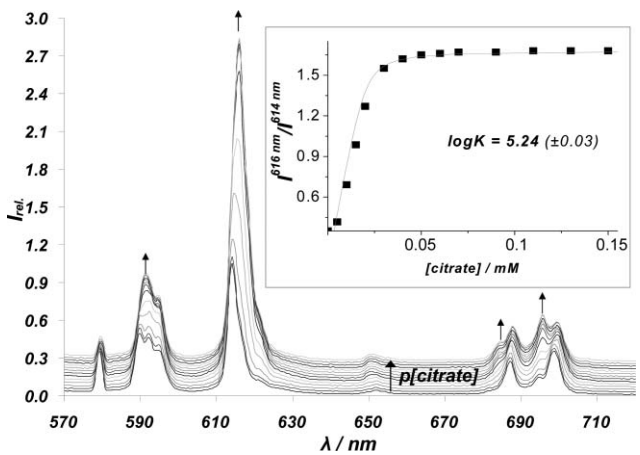


Fig. 5 Variation of the europium emission spectrum for [Eu.L^{3a}]$^{3+}$ in the presence of increasing concentrations of sodium citrate (pH 7.4, 0.1 M NaCl, 20 μM complex, 298 K, λ_{exc} 380 nm), showing (inset) the fit (line) to the observed data.

Table 3 Cell toxicity profiles (IC_{50} in NIH-3T3 cells) for selected europium complexes and ligands[a,b]

Complex or ligand	IC_{50}/μM
[Eu.L^1]$^{3+}$	>100
L^1	>150
[Eu.L^{2a}]$^{3+}$	>270
L^{2a}	>350
[Eu.L^{2b}]$^{3+}$	164(31)
L^{2b}	99.3(11.8)
[Eu.L^{3b}]$^{3+}$	173 (34)
[Eu.L^{3a}]$^{3+}$	5.62 (0.34)
L^{3a}	4.87 (1.26)
L^{3b}	180 (15)

[a] Values presented represent the mean of at least 3 independent measurements, with standard deviations in parentheses. [b] For purposes of comparison IC_{50} values were also measured for 2-methylazaxanthone (IC_{50} > 240 μM) and 2-methylazathiaxanthone (72.5{6.3} μM) and in the latter case the sulfoxide (>240 μM) and sulfone (21.1{0.2} μM) analogues, suggesting that the cytoxicity of the azathiaxanthone ligand may be linked to oxidative metabolism of this moiety.

protein titration experiment was analysed as for [Eu.L^1]$^{3+}$, and gave an apparent HSA affinity constant of 5.1 (±0.05) × 10^3 M^{-1} for a 1 : 1 binding model.

^{1}H NMR experiments with this system were constrained by the kinetic instability of the complex in solution. Over periods of hours (several days for [Eu.L^{3b}]$^{3+}$), in the presence of ≥10 mM concentrations of $NaHCO_3$ or sodium hydrogenphosphate, precipitation was observed, consistent with slow de-complexation of the Eu ion from the macrocyclic ligand. Such instability precluded more detailed solution studies of these systems.

Cellular cytotoxicity profiles

The relative cytotoxicity of the europium complexes and the free ligands were assessed in mouse skin fibroblasts (NIH-3T3 cells) using the established MTT assay.[31] This makes use of the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple 'formazan' product by the mitochondrial dehydrogenase enzymes of viable cells. The insoluble 'formazan' was quantified spectrophotometrically in a 96-well plate reader following dissolution in DMSO and an IC_{50} value recorded, following a 24 h incubation of varying concentrations of the complex with at least 10000 cells. The IC_{50} value was defined as the complex concentration required to reduce the absorbance to 50% of that in the untreated control.

The only europium complex showing significant toxicity was [Eu.L^{3a}]Cl_3 (5.6 μM), contrasting significantly with every other complex examined. The ligand, L^{3a}, was also cytotoxic (IC_{50}: 4.9 μM), and the correspondence of these values is consistent with intracellular dissociation of Eu from the complex (*vide infra* for microscopy confirmation) over the incubation period. The toxicity of L^{3a} contrasts markedly with the behaviour of L^1, L^{2b} and L^{3b}. Similar cytotoxicity profiles were observed in Chinese hamster ovarian (CHO) cells.

Luminescence microscopy studies

The cellular uptake profile of complexes was examined in CHO or NIH-313 cells, with complex loading concentrations varying from 10 to 100 μM, for incubation times on slide cover-slips of between

20 min and 12 h. The uptake and distribution of the complex within the cell was observed by fluorescence microscopy, examining both europium emission and (for the azathiaxanthone systems) ligand fluorescence, following excitation of the chromophore. Experimental details followed those outlined recently.[11] When optical sections throughout the cells were taken, the fluorescence could be detected in every layer, confirming that the complex was internalised and was not simply associating with the cell membrane. As a control, untreated cells were also examined but showed no luminescence in the wavelength regions examined. Images were taken at a number of points across each slide, with a similar localization profile at each position.

With $[Eu.L^1]^{3+}$ in CHO cells, there appeared to be no fluorescence in the nucleus and the appearance is most consistent with the size and distribution of late endosomes or lysosomes in the perinuclear region, especially at later time points. The relative emission intensity of ligand or Eu luminescence did not vary significantly at 30 min, 1 h and 2 h (Fig. 6), as suggested by the similar acquisition time for a constant overall brightness. This behaviour therefore resembled that reported[13] for $[Eu.L^5]^{3+}$, and the similarity of the ligand fluorescence, Eu emission and merged images is consistent with the absence of complex dissociation, at this time point.

In a separate experiment, CHO cells were incubated with $[Eu.L^1]^{3+}$ (100 μM) for 4 h. Then the growth medium was removed, the cells were washed with phosphate buffered saline (PBS) and fresh medium introduced without added europium complex. One hour later, the form of the localisation profile had changed significantly (Fig. 7). A lysosomal/endosomal profile was still manifest, but a smoother localisation throughout the cytoplasm was apparent. The brightness of the observed images was the same (±5%) as for those recorded at 4 h, suggesting that rapid egress of the complex from the cell (noted for certain cationic complexes[18] recently)—in the absence of a concentration gradient—was not occurring here. An estimate of the amount of the Eu complex within the cell can be made by measuring [Eu] by ICP-mass spectrometry, for a sample of cells that has been counted by flow cytometry. For $[Eu.L^1]^{3+}$, a 10 h loading time point was selected and 269 511 fluorescent cells were counted, sorted and collected by flow cytometry prior to ICP-MS analysis. The combined PBS washings, used after growth medium withdrawal, were also analysed by ICP-MS. The number of Eu ions within a single cell was determined to be 6.8 (± 0.4) × 10^7, or a europium concentration of 38 μM, assuming a mean cell volume of 3 000 μm^3. Thus, approximately 40% of the original extracellular complex concentration was internalised. This measurement was confirmed by comparing the absorbance values of $[Eu.L^1]$ (at 380 nm) in the growth medium, at the outset and after the 10 h incubation time. A 45% (±5%) decrease in complex concentration was measured. The concentration of $[Eu.L^1]$ in the combined PBS washings (following incubation) were also measured by ICP-MS and accounted for 7% (±1%) of the original complex concentration. Such values and microscopy observations are consistent with the behaviour of a complex that is quickly internalised, and does not rapidly leave the cell, even in the absence of an external concentration gradient. Moreover, the relative intensity of the observed ligand fluorescence

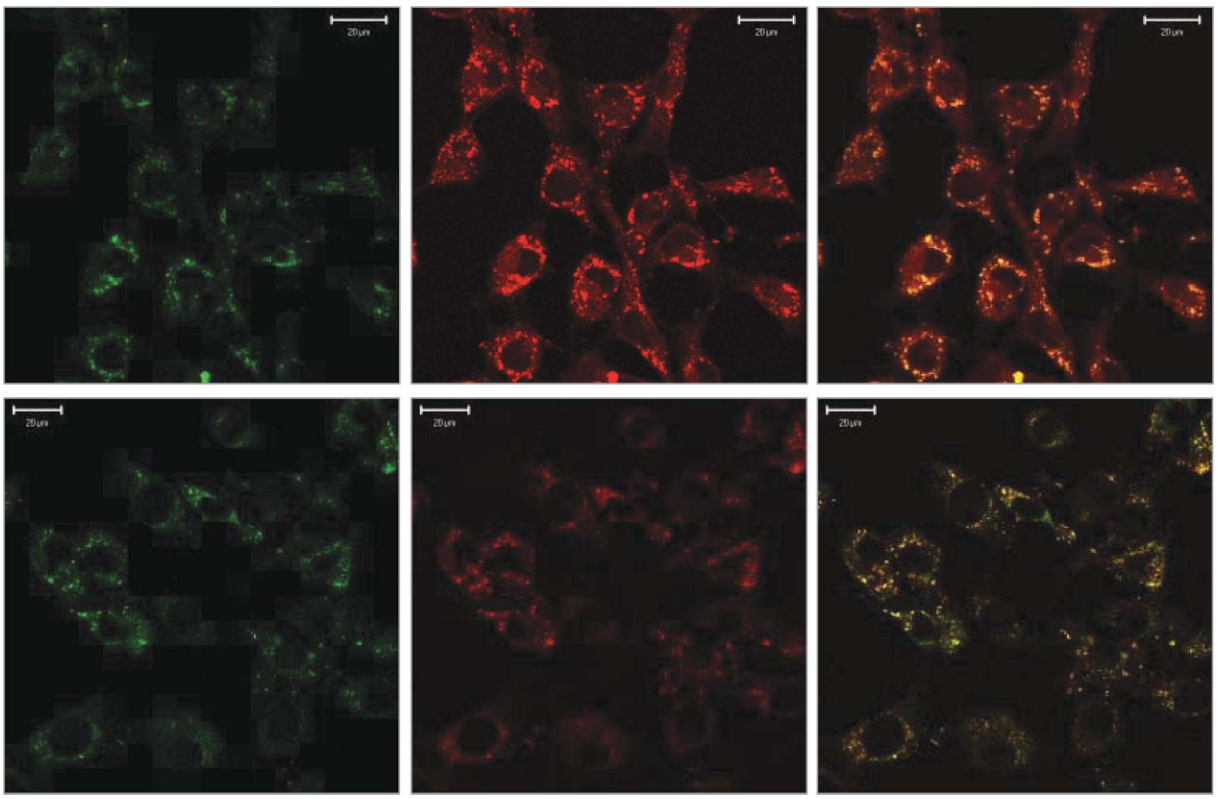


Fig. 6 Confocal microscopy images at 2 h (upper) and 4 h (lower) in CHO cells, showing the localisation of $[Eu.L^1]^{3+}$ (100 μM complex, $\lambda_{exc} = 405$ nm): left: ligand fluoresence observed using a 505–550 nm band pass filter; centre: Eu emission observed using a 590 nm line pass filter; right: merged images using Image J™ software.

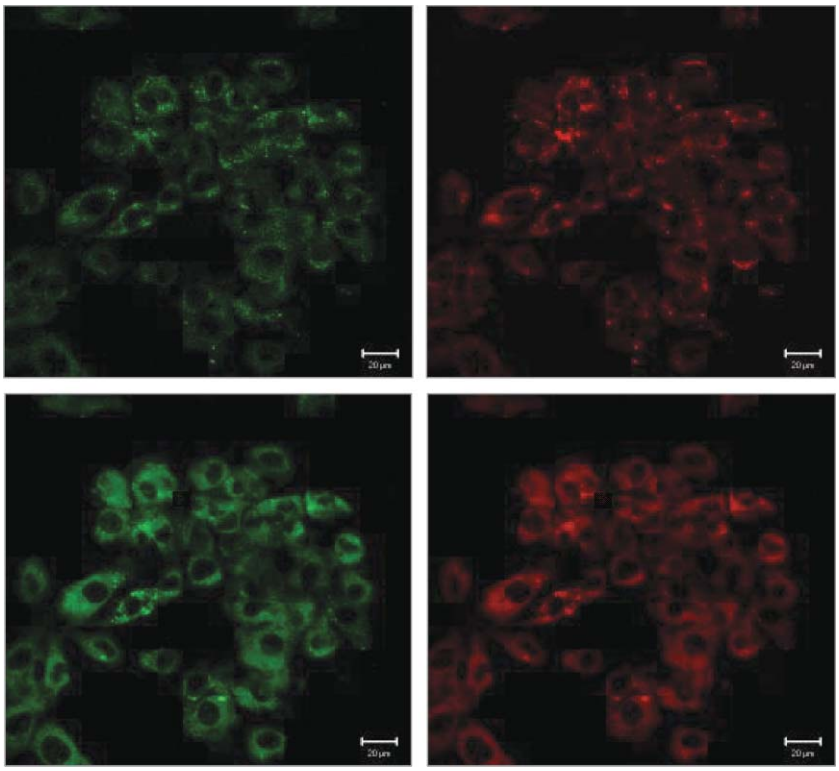


Fig. 7 Confocal microscopy images of $[Eu.L^1]^{3+}$ in CHO cells (100 μM complex, λ_{exc} 405 nm); top: after 4 h incubation in the presence of complex; bottom: 1 h later after incubation in fresh growth medium without complex (left: ligand fluorescence; right: europium emission).

to europium emission did not change significantly, consistent with retention of complex integrity.

The localisation behaviour of $[Eu.L^{2a}]^{3+}$ and $[Eu.L^{2b}]^{3+}$ was examined by microscopy in both CHO and NIH-3T3 cells. Each complex behaved similarly and revealed a profile, within 5 minutes of incubation of a 50 μM solution, that was consistent with localisation in mitochondria (Fig. 8). Co-localisation studies (4 h incubation) with Mitotracker Green™ (Invitrogen) were consistent with this hypothesis, and the merged images showed good correspondence. When longer incubation times were used, the complex appeared to migrate from the mitochondria to late endosomes/lysosomes (Fig. 9). Such behaviour accords with the idea that the rate of intracellular trafficking of these complexes by recycling vesicles may be associated with their ability to bind reversibly to a given carrier protein.

Finally, the complexes $[Eu.L^3]^{3+}$ were also studied, and exhibited similar localisation profiles. In each case, they appeared to be rapidly transported to perinuclear lysosomes. Using appropriate filter sets, the europium emission and ligand fluorescence can be distinguished easily. In this case they did not correspond, consistent with the chemical instability of the complex with respect to europium dissociation. This behaviour accords with the correspondence of ligand and complex cytotoxicity for L^{3a} and $[Eu.L^{3a}]^{3+}$.

Summary

These cellular localisation studies suggest that the Eu(III) complexes of L^{2a} and L^{2b}, and their analogues, are attractive systems for further investigation. They combine the required attributes of a selective response to a given anion or pair of anions (HCO_3^- *vs*. citrate) and show a time-dependent localisation profile that may allow the monitoring, in real time, of local changes in $pHCO_3$ selectively, within the mitochondrial region. Such studies will form the basis of future endeavours in this area.

Experimental

General details of instrumentation, cell culture, toxicity, microscopy, relaxivity and HPLC are given in the ESI.†

Ligand and complex synthesis

The synthesis of L^1 and $[Ln.L^1]$ (Ln = Eu, Gd) was carried out as described in reference 20.

$[Eu.L^1](CF_3SO_3)_3$. (*S*,*S*)-1,7-Bis(ethoxycarbonyl-2-ethylcarbamoylmethyl)-4-[7-(methylcarbamoylmethyl)-1-azathioxanthone]-1,4,7,10-tetraazacyclododecane, L^1, (25 mg, 55 μmol) was added to $Eu(CF_3SO_3)_3$ (1 eq., 33 mg) and the mixture was dissolved in dry MeCN (2 ml). The reaction was left to stir at 70 ° C for 72 h. After cooling to room temperature, the solvent was removed under reduced pressure and the remaining residue was dissolved in dry MeCN (0.1 ml) and the mixture was dropped onto anhydrous Et_2O which resulted in precipitation of the title compound as a triflate salt. The precipitate was separated by centrifugation and dissolved in H_2O (5 ml). The pH was then adjusted carefully to 10 by addition of conc. NaOH solution (in order to remove the excess Eu as $Eu(OH)_3$) resulting in a white precipitate, removed *via* a fine syringe filter. The pH was adjusted back to neutral with conc. HCl and the mixture lyophilised to give a bright yellow

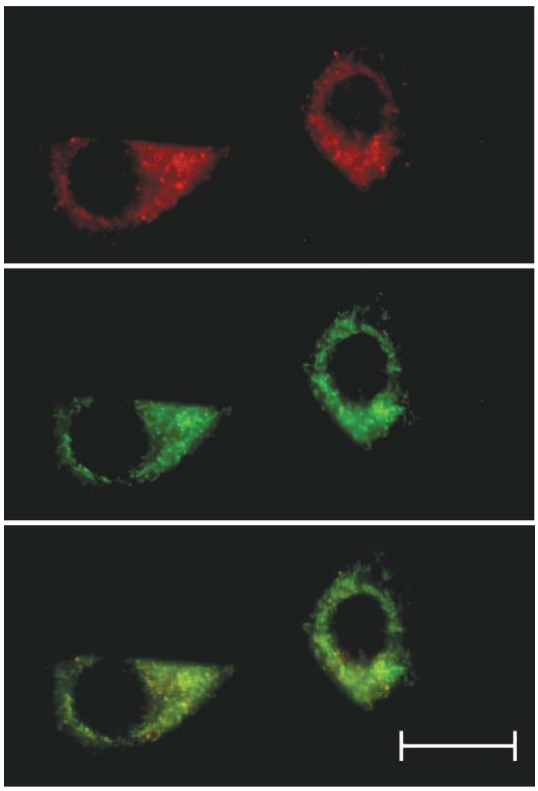


Fig. 8 Epifluoresence microscopy images for $[Eu.L^{2a}]^{3+}$ (50 μM complex, 4 h incubation) showing a mitochondrial localisation profile, as revealed by co-localisation experiments with Mitotracker Green™: upper: Eu complex; centre: Mitotracker Green™; lower: merged image (scale bar 20 microns).

solid containing approx. 2% NaCl as a result of pH adjustment (60 mg, 49 μmol). *m/z* (HRMS$^+$) 1221.2056 $(M + 2CF_3SO_3)^+$ ($C_{37}H_{53}O_8N_8S_2Eu(CF_3SO_3)_2$ requires 1221.2033); HPLC: t_R = 9.33 min; $\lambda_{max}(H_2O)$ 384 (12790 dm^3 mol^{-1} cm^{-1}); $\tau^{Eu}_{(H2O,\ pH=7.4)}$: 0.44 ms, $\tau^{Eu}_{(D2O,\ pD=7.1)}$: 0.90 ms; $q = 0.92$; $\phi^{Eu}_{(pH=7.4)} = 5.1\%$.

$[Gd.L^1](CF_3SO_3)_3$. The Gd complex was prepared as described for the europium analogue. *m/z* (HRMS$^+$) 1259.1870 $(M + 2CF_3SO_3)^+$ ($C_{37}H_{54}O_8N_8S_2Gd(CF_3SO_3)_2$ requires 1259.1859); HPLC: t_R = 9.78 min; $\lambda_{max}(H_2O)$ 384 (12790 dm^3 mol^{-1} cm^{-1}); $r_1 = 7.66\ mM^{-1}\ s^{-1}$.

Ligands L^{2a}, L^{2b} and L^3 and their lanthanide complexes. The ligand L^{2a}, 2-[(2-(4,10-bis-[((*S*)-1-phenyl-ethylcarbamoyl)methyl]-1-yl)acetylamino)-methyl]-5-oxo-5*H*-[1]benzothiopyrano[2,3-*b*]-pyridine-7-carboxylic acid methyl ester was synthesised as described in reference 20.

$[Gd.L^{2a}](OAc)_3$. A solution of 2-[(2-(4,10-bis-[((*S*)-1-phenyl-ethylcarbamoyl)methyl]1-yl)acetylamino)-methyl]-5-oxo-5*H*-[1]-benzothiopyrano[2,3-*b*]pyridine-7-carboxylic acid methyl ester (13 mg, 0.016 mmol) and $Gd(OAc)_3{\cdot}4H_2O$ (4 mg, 0.012 mmol) in aq. MeOH (1 : 1, 1 ml) was boiled under reflux under argon for 48 h. The solution was allowed to cool then solvent removed under reduced pressure. The residue was re-dissolved in water (2 ml), filtered then dried under reduced pressure leaving the desired complex as a pale yellow solid (11 mg, 0.009 mmol, 56%); MS (ES$^+$) *m/z* 525.5 $[M + CH_3CO_2]^{2+}$ 100%; HRMS (ES$^+$) *m/z* found 1050.3180 $[M + CH_3CO_2 - H]^+$ $C_{47}H_{56}O_8N_8{}^{32}SGd$ requires 1050.3198; $\lambda_{abs}(H_2O)$: 375 nm.

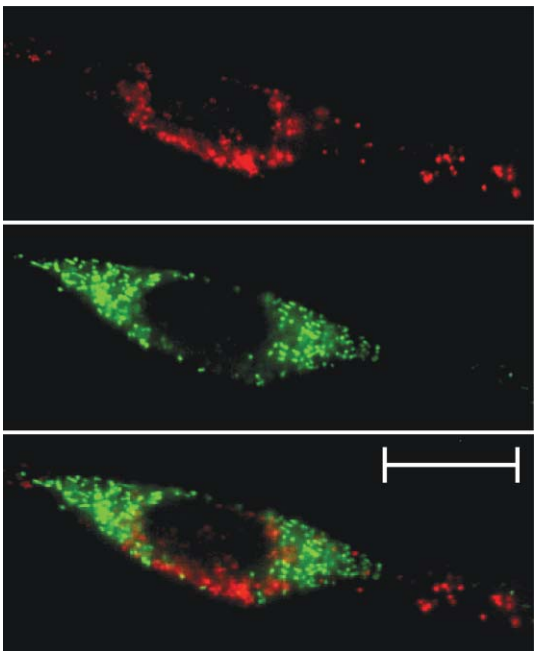


Fig. 9 Microscopy images for $[Eu.L^{2a}]^{3+}$ (50 μM complex, 24 h incubation time), upper: Eu emission; centre: Mitotracker Green™; lower: merged image, consistent with migration of the complex from the mitochondria to the perinuclear endosomes/lysosomes (scale bar 20 microns).

7-Methoxycarbonyl-2-phthalimidomethyl-1-azaxanthone. 7-Methoxycarbonyl-2-bromomethyl-1-azaxanthone (0.061 g, 0.175 mmol; synthesised as described in reference 21) and potassium phthalimide (0.130 g, 0.702 mmol) in DMF (5 ml) were stirred, under argon, for 12 h at room temperature. The solution was poured into ice water (500 ml), the yellow/orange precipitate being isolated by centrifugation. The filtrate was further cooled in the fridge yielding further precipitate which was also isolated. The solids were combined and recrystallised from DCM–ether to yield the product as a yellow/orange crystalline solid (0.065 g, 0.157 mmol, 90%); Mp >250 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.96 (d, 1H, J = 2.0, H_O), 8.68 (d, 1H, J = 8.0 Hz, H_K), 8.39 (dd, 1H, J = 9.0, 2.0 Hz, H_D), 7.93 (dd, 2H, J = 5.5, 3.0 Hz, H_R), 7.79 (dd, 2H, J = 5.5, 3.0 Hz, H_S), 7.60 (d, 1H, J = 9.0 Hz, H_E), 7.42 (d, 1H, J = 8.0 Hz, H_J), 5.14 (s, 2H, H_I), 3.97 (s, 3H, H_A); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.0 (1C, C_M), 168.2 (2C, C_P), 165.9 (1C, C_B), 162.1 (1C, C_H), 160.3 (1C, C_G), 158.5 (1C, C_F), 138.8 (1C, C_K), 136.5 (1C, C_D), 134.7 (2C, C_S), 132.4 (2C, C_Q), 129.5 (1C, C_O), 127.2 (1C, C_C), 124.1 (2C, C_R), 121.6 (1C, C_N), 119.5 (1C, C_J), 119.3 (1C, C_E), 116.1 (1C, C_L), 52.9 (1C, C_A), 43.1 (1C, C_I); MS (ES$^+$) *m/z* 437.3 $[M + Na]^+$ 100%, 851.2 $[2M + Na]^+$ 50%; HRMS (ES$^+$) *m/z* found 437.0741 $[M + Na]^+$ $C_{23}H_{14}O_6N_2Na$ requires 437.0744. $C_{23}H_{14}N_2O_6{\cdot}1/6CH_2Cl_2$ (%): calc. C 64.93 H 3.37 N 6.54%; Found: C, 65.09; H, 3.34; N, 6.48%; $C_{23}H_{14}N_2O_6{\cdot}1/6CH_2Cl_2$ requires: C, 64.93; H, 3.37; N, 6.54%.

7-Methoxycarbonyl-2-aminomethyl-1-azaxanthone, 2. A solution of 7-methoxycarbonyl-2-phthalimidomethyl-1-azaxanthone (0.061 g, 0.147 mmol) and $NH_2NH_2.H_2O$ (0.0144 ml, 0.297 mmol) in DCM–MeOH (50 : 50, 8 ml) was heated at 50 °C followed

by further additions of $NH_2NH_2{\cdot}H_2O$ (0.0144 ml, 0.297 mmol) at 3 h intervals until TLC analysis showed that all starting 7-methoxycarbonyl-2-phthalimidomethyl-1-azaxanthone had been consumed. The clear yellow solution was allowed to cool followed by the addition of conc. $HCl_{(aq)}$ until a pH of 2 was reached. The resultant solution containing a white precipitate was heated at 50 °C for 2 h then allowed to cool to room temperature, the solution was filtered followed by the drying of the yellow filtrate under vacuum to yield a yellow powder. Immediately prior to use, the solid was dissolved in H_2O containing one eq. NaOMe then extracted with dichloromethane (2 × 5 ml), the organic phase was dried under reduced pressure to leave the title product as a pale green solid (0.029 g, 0.102 mmol, 69%); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.00 (d, 1H, J = 2.0 Hz, H_O), 8.69 (d, 1H, J = 8.0 Hz, H_K), 8.43 (dd, 1H, J = 9.0, 2.5 Hz, H_D), 7.60 (d, 1H, J = 8.5 Hz, H_E), 7.52 (d, 1H, J = 8.0 Hz, H_J), 4.16 (s, 2H, H_I), 3.99 (s, 3H, H_A), 1.84 (s br, 2H, NH_2); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.2 (1C, C_M), 169.3 (1C, C_H), 166.0 (1C, C_B), 160.4 (1C, C_G), 158.5 (1C, C_F), 138.1 (1C, C_K), 136.2 (1C, C_D), 129.3 (1C, C_O), 127.0 (1C, C_N), 121.5 (1C, C_C), 119.4 (1C, C_J), 119.0 (1C, C_E), 115.3 (1C, C_L), 52.7 (1C, C_A), 45.0 (1C, C_I); MS (ES$^+$) m/z 285.2 [M + H]$^+$ 100%; HRMS (ES$^+$) m/z found 285.0869 [M + H]$^+$ $C_{15}H_{13}O_4N_2$ requires 285.0870.

2-[(2-(7-*tert*-Butoxycarbonyl-4,10-bis-[((*S*)-1-phenyl-ethylcarbamoyl)-methyl]-1,4,7,10-tetraaza-cyclododec-1-yl)-acetylamino)-methyl]-10-oxo-10*H*-9-oxa-1-aza-anthracene-6-carboxylic acid methyl ester. 7-Carboxymethyl-4,10-bis-[((*S*)-1-phenyl-ethylcarbamoyl)-methyl]-1,4,7,10-tetraaza-cyclododecane-1-carboxylic acid *tert*-butyl ester, **1**, (0.128 g, 0.196 mmol; synthesised as described in reference 20), EDC·HCl (0.037 g, 0.193 mmol), $HOBt{\cdot}H_2O$ (10 mg) and NEt_3 (0.082 ml, 0.588 mmol) in chloroform (20 ml) were stirred for 20 min at room temperature to form a yellow tinged solution. 7-Methoxycarbonyl-2-aminomethyl-1-azaxanthone (0.042 g, 0.148 mmol) in chloroform (3 ml) was added to form an orange solution that was left to stir for 12 h at room temperature. The resulting yellow solution was extracted with sat. aqueous $NaHCO_3$ solution (2 × 15 ml) then H_2O (10 ml). The organic phase was dried and solvent removed under reduced pressure to leave an orange foam. The desired product was purified *via* alumina column chromatography (using a graduated solvent system starting from 100% DCM with 0.1% MeOH increments every 100 ml) yielding a glassy orange solid, mp 70–72 °C (0.073 g, 0.079 mmol, 53%); R_F (alumina, DCM–MeOH, 99 : 1): 0.16; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.95 (d, 1H, J = 2.5 Hz, H_O), 8.60 (d, 1H, J = 8.0 Hz, H_K), 8.39 (dd, 1H, J = 8.5, 2.0 Hz, H_D), 8.00 (s br, 1H, linker amide NH), 7.58 (d, 1H, J = 8.5 Hz, H_E), 7.32 (d, 1H, J = 6.5 Hz, H_J), 7.19–7.25 (m, 10H, amide arm Ar), 5.08 (m, 2H, arm CH), 4.54 (m, 2H, H_I), 3.97 (s, 3H, H_A), 2.40–3.16 (m br, 22H, cyclen CH_2 and amide arms CH_2CO), 1.43 (d, 6H, J = 6.5 Hz, amide arms CH_3), 1.37–1.41 (m br, 9H, ^{t}Bu CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.8 (1C, C_M), 171.4 (1C, linker amide C=O), 170.4 (2C br, amide arm C=O), 165.8 (1C, C_B), 163.9 (1C, C_H), 160.1 (1C, C_G), 158.3 (1C, C_F), 156.0 (1C, ^{t}Boc C=O), 143.7, 143.1 (2C br, amide arm $Ar_{(q)}$), 138.3 (1C, C_K), 136.5 (1C, C_D), 129.4 (1C, C_O), 126.5–128.8 (10C, amide arm Ar), 127.5 (1C, C_N), 121.6 (1C, C_C), 120.1 (1C, C_J), 119.0 (1C, C_E), 115.6 (1C, C_L), 80.3 (1C, ^{t}Boc$_{(q)}$), 60.0, 59.4, 54.6, 53.4 (8C, cyclen CH_2), 52.8 (1C, C_A), 48.7 (2C, amide arms CH), 47.8 (3C, amide arms and linker CH_2CO), 44.6 (1C, C_I), 28.7 (3C, ^{t}Boc CH_3), 21.9, 21.5 (2C, amide arms CH_3); MS (ES$^+$) m/z 919.7 [M + H]$^+$ 100%; HRMS (ES$^+$) m/z found 919.4726 [M + H]$^+$ $C_{50}H_{63}O_9N_8$ requires 919.4713.

2-[(2-(4,10-Bis-[((*S*)-1-phenyl-ethylcarbamoyl)-methyl]-1,4,7,10-tetraaza-cyclododec-1-yl)-acetylamino)-methyl]-10-oxo-10*H*-9-oxa-1-aza-anthracene-6-carboxylic acid methyl ester, L^{2b}. 1-[(2-(7-*tert*-Butoxycarbonyl-4,10-bis-[((*S*)-1-phenyl-ethylcarbamoyl)-methyl]-1,4,7,10-tetraaza-cyclododec-1-yl)-acetylamino)-methyl]-10-oxo-10*H*-9-oxa-1-aza-anthracene-6-carboxylic acid methyl ester (72 mg, 0.078 mmol) in DCM–TFA (50 : 50, 4 cm^3) was stirred in a sealed flask for 12 h yielding a yellow solution. Solvent was then removed under reduced pressure to yield the desired product as its TFA salt as a glassy yellow solid in quantitative yield; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.39 (s, 1H, linker NH), 8.86 (d, 1H, J = 1.5 Hz, H_O), 8.56 (d, 1H, J = 8.0 Hz, H_K), 8.34 (dd, 1H, J = 8.5 Hz 1.5 Hz, H_D), 8.10 (s br, 1H, arm NH), 7.87 (s br, 1H, arm NH), 7.54 (d, 1H, J = 8.5 Hz, H_E), 7.36 (d, 1H, J = 8.0 Hz, H_J), 7.15–7.23 (m br, 10H, arm Ar), 4.85–4.95 (m br, 2H, arm CH), 4.59 (m br, 2H, H_I), 3.94 (s, 3H, H_A), 3.00–3.66 (m br, 22H, cyclen, arm and linker CH_2), 1.40 (s br, 6H, arm CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.9 (1C, C_M), 171.1, 170.1 (3C, amide arm C=O and linker arm C=O), 165.9 (1C, C_B), 163.6 (1C, C_H), 161.6 (q, 1C, TFA C=O), 160.1 (1C, C_G), 158.3 (1C, C_F), 143.6 (2C, arm $Ar_{(q)}$), 138.5 (1C, C_K), 136.5 (1C, C_D), 129.3 (1C, C_O), 128.9, 127.7, 126.5, 126.4 (10C, arm Ar), 127.1 (1C, C_N), 121.5 (1C, C_C), 119.4 (1C, C_J), 119.2 (1C, C_E), 115.7 (1C, C_L), 115.4 (q, 1C, TFA CF_3), 55.4, 53.8, 52.4 (br, 8C, cyclen CH_2), 52.9 (1C, C_A), 49.8 (br, 2C, arm CH), 45.0 (br, 1C, C_I), 43.5 (br, 3C, arm and linker CH_2), 22.2, 21.9 (br, 2C, amide arm CH_3); MS (ES$^+$) m/z 819.5 [M + H]$^+$ 100%, 841.5 [M + Na]$^+$ 40%; HRMS (ES$^+$) m/z found 819.4198 [M + H]$^+$ $C_{45}H_{55}O_7N_8$ requires 819.4188.

[Eu.L^{2b}]Cl$_3$. A solution of 2-[(2-(4,10-bis-[((*S*)-1-phenyl-ethylcarbamoyl)-methyl]-1,4,7,10-tetraaza-cyclododec-1-yl)-acetylamino)-methyl]-10-oxo-10*H*-9-oxa-1-aza-anthracene-6-carboxylic acid methyl ester as its TFA salt (46 mg, 0.049 mmol) and $Eu(OTf)_3$ (26 mg, 0.043 mmol) in MeCN (1 cm^3) was boiled under reflux under argon, in Schlenk apparatus, for 48 h. Solvent was removed under reduced pressure to leave a orange glassy solid. DCM (5 cm^3) was added to the solid which was sonicated for 5 min, solvent was then decanted leaving a orange residue. The sonication process was repeated followed by drying of the remaining residue, under reduced pressure, to yield the triflate salt of the desired product as a fine cinder coloured powder (50 mg, 0.034 mmol, 79%). The solid was made water soluble by the exchange of triflate anions for chloride anions using DOWEX 1 × 8 200–400 mesh chloride ion exchange resin. The procedure involved the dissolving the solid in MeOH (1 ml) followed by the addition of H_2O (1 ml); this complex solution was added to a mixture of the resin (0.2 g) in H_2O–MeOH (50 : 50, 5 ml) then stirred for 2 h. The resin was then removed by filtration followed by the drying of the solution under reduced pressure to yield the complex in quantitative yield; ^{1}H NMR (as trichloride salt, D_2O, 500 MHz, partial data and assignment) δ 25.1 (1H, NH), 16.9 (1H, H_{ax}), 15.2 (1H, H_{ax}), 14.0 (1H, H_{ax}), 11.7 (1H, H_{ax}); MS (ES$^+$) m/z 508.3 [M + HCO_2]$^{2+}$ 100%, 1015.2 [M + HCO_2 − H]$^+$ 10%; HRMS (ES$^+$) m/z found 1027.3359 [M + CH_3CO_2 − H]$^+$

$C_{47}H_{56}O_9N_8{}^{151}Eu$ requires 1027.3363; $\lambda_{abs}(H_2O)$: 333 nm; $\tau_{(H2O)}$: 0.38 ms, $\tau_{(D2O)}$: 0.60 ms; $q = 0.68$.

[Tb.L^{2b}]Cl$_3$. This was prepared in an analogous manner to the Eu complex above. MS (ES$^+$) m/z 511.3 $[M + HCO_2]^{2+}$ 100%, 1021.2 $[M + HCO_2 - H]^+$ 25%; HRMS (ES$^+$ + NH_4OAc) m/z found 1035.3409 $[M + CH_3CO_2 - H]^+$ $C_{47}H_{56}O_9N_8{}^{159}Tb$ requires 1035.3418; $\lambda_{abs}(H_2O)$: 332 nm; $\tau_{(H2O)}$: 1.17 ms, $\tau_{(D2O)}$: 2.07 ms; $q = 1.56$.

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