Lice cell imaging of lysosomal pH changes with pH responsive ratiometric lanthanide probes

David G. Smith, Brian K. McMahon, Robert Pal and David Parker*

General

Chemicals were purchased from commercial suppliers (Acros, Aldrich, Fluka, Merck) and were used without further purification unless otherwise stated. Solvents were dried using an appropriate drying agent when required (CH₃CN over CaH₂, CH₃OH over Mg(OMe)₂ and THF over Na/benzophenone). Unless otherwise mentioned, reactions were carried out under an argon atmosphere and the reaction flasks were pre-dried under reduced pressure. Ultra pure de-ionised water (<18 M Ω cm⁻¹) was used throughout. All glassware was washed with acid solution and rinsed with de-ionized, distilled water.

Spectroscopy

¹H, ¹³C spectra were recorded in commercially available deuteriated solvents on a Varian Mercury-200 (¹H at 199.975 MHz, ¹³C at 50.289 MHz), Varian Mercury-400 or Bruker Avance-400 (¹H at 399.960 MHz, ¹³C at 100.572 MHz), Varian Inova-500 (¹H at 499.772 MHz, ¹³C at 125.671 MHz) or Varian VNMRS-700 (¹H at 699.731 MHz) spectrometer. Chemical shifts are in ppm with coupling constants in Hz.

Electrospray mass spectra were recorded on a Waters Micromass LCT or Thermo-Finnigan LTQ FT instrument operating in positive or negative ion mode as stated, with methanol as the carrier solvent. Accurate mass spectra were recorded using the Thermo-Finnigan LTQ FT mass spectrometer. LC-MS analyses were performed on a Waters system comprising a 3100 Mass Detector and a 2998 Photodiode array detector.

HPLC Analysis of Eu and Tb complexes: (λ_{exc} 332 nm, λ_{em} 545 or 618 nm)

Reverse phase HPLC traces were recorded at 298 K using a Perkin Elmer system equipped with a Perkin Elmer Series 200 Pump, a Perkin Elmer Series 200 Auto-sampler

and a Perkin Elmer Series 200 fluorescence detector. A 4.6 x 150 mm 4 μ m Phenomenex Synergi Fusion RP 80Å analytical column was used. A gradient elution with a solvent system composed of H₂O + 0.1% HCOOH/ MeCN + 0.1% HCOOH was performed for a total run time of 22.4 min.

Time (min)	Solvent A (%)	Solvent B (%)	Curvature
0	95	5	0
2.4	95	5	0
15.4	0	100	1
17.4	0	100	0
19.4	95	5	1
22.4	95	5	0

Flow rate = 1 ml/min; Solvent A = $H_2O + 0.1\%$ HCOOH; Solvent B = MeCN + 0.1% HCOOH.

Representative HPLC Traces

 $[Eu.L^2]^{3+}$



Ligand and Complex Synthesis

Syntheses of the precursors for the sulphonamides L^1 and L^2 were described earlier. ⁹

3

(SS)-1,7-Bis(ethyl-*N*-acetyl-*S*-alanine)-4-[7-methoxycarbonyl-2chloromethylcarbonylmethyl-1-azaxanthone]-10-[2'-(methylsulfonylamino)ethyl]-1,4,7,10-tetraazacyclododecane, (L¹)

(SS)-1,7-Bis(ethyl-N-acetyl-S-alanine)-4-[7-methoxycarbonyl-2-

chloromethylcarbonylmethyl-1-azaxanthone]-1,4,7,10-tetraazacyclododecane (100mg, 123 µmol), methylsulfonate-N-methanesulfonylethylamine (28 mg, 129 µmol) and K₂CO₃ (18 mg, 129 µmol) were stirred in MeCN (3 mL), under argon, at 65 °C for 24 h. Solvent was removed under reduced pressure and the residue dissolved in DCM (5 mL) allowing the insoluble salts to be removed by centrifugation. Solvent was again removed under reduced pressure before purification by column chromatography on silica gel (DCM \rightarrow 10% MeOH) vielded an orange glassy solid (76 mg, 74.0 µmol, 60%). $\delta_{\rm H}$ $(CDCl_3, 700 \text{ MHz}) 8.93 (1H, d, J = 1.5, H^6), 8.65 (1H, d, J = 8.0, H^4), 8.36 (1H, dd, J = 1.5, H^6), 8.65 (1H, dd, J = 1.5,$ 9.0, 1.5, H^8), 7.62 (1H, d, J = 9.0, H^9), 7.59 (1H, d, J = 8.0, H^3), 4.63-4.71 (2H, m, H^{12}), 4.41-4.49 (1H, m, H¹⁹), 4.38-4.44 (1H, m, H¹⁹), 4.12-4.18 (4H, m, H²²), 4.03.4.11 (2H, m, H²⁵), 3.97 (3H, s, H¹¹), 3.85 (3H, s, H²⁷), 1.85–3.45 (24H, br, H^{15,16,24,28}), 1.40-1.49 (6H, m, H^{20}), 1.14 (6H, t, J = 7.0, H^{23}); δ_{C} (CDCl₃, 126 MHz) 176.8 (C⁵), 173.2 (C¹⁴), 172.4 (C²¹), 171.1 (C¹⁷), 165.8 (C¹⁰), 163.0 (C²), 159.7 (C^{1'}), 158.3 (C^{9'}), 138.3 (C⁴), 136.0 (C^8) , 129.4 (C^6) , 126.8 (C^7) , 121.5 $(C^{5'})$, 120.3 (C^3) , 118.9 (C^9) , 115.3 $(C^{4'})$, 61.7 (C^{22}) , 61.5 (C²²), 57.5, 55.8, 55.4, 52.6, 49.9, 48.8, 48.5, 45.1, 41.8, 40.4, 17.7 (C²⁰), 14.3 (C²³), 14.2 (C^{23}); m/z (ES⁺) 933.4181, $C_{42}H_{62}N_9O_{13}S$ requires 933.4188 [M+H]⁺.

Smith, McMahon, Pal and Parker

(SS) - 1,7 - Bis(ethyl-N-acetyl-S-alanine) - 4 - [7 - methoxycarbonyl-2-chloromethylcarbonylmethyl-1-azaxanthone] - 10 - [2' - (4 - Methoxyphenylsulfonylamino) ethyl] - 1,4,7,10 - tetraazacyclododecane, (L²)

(SS)-1,7-Bis(ethyl-N-acetyl-S-alanine)-4-[7-methoxycarbonyl-2-

chloromethylcarbonylmethyl-1-azaxanthone]-1,4,7,10-tetraazacyclododecane (100 mg, 123 µmol), 2-paramethoxyphenylsulfonate-N-methanesulfonylethylamine (50 mg, 125 μmol) and K₂CO₃ (18 mg, 129 μmol) were stirred in MeCN (3 ml), under argon, at 65 °C for 24 h. Solvent was removed under reduced pressure and the residue dissolved in DCM (5 ml) allowing the insoluble salts to be removed by centrifugation. Solvent was again removed under reduced pressure before purification by column chromatography on silica gel (DCM \rightarrow 10% MeOH) to yield an orange glassy solid (40 mg, 39 µmol, 31%). $\delta_{\rm H}$ $(CDCl_3, 700 \text{ MHz}) 8.93 (1H, d, J = 1.5, H^6), 8.65 (1H, d, J = 8.0, H^4), 8.36 (1H, dd, J = 1.5, H^6), 8.65 (1H, d, J = 1.5, H^6), 8.65$ 9.0, 1.5, H^8), 7.75 (2H, d, J = 9.0, H^{29}), 7.62 (1H, d, J = 9.0, H^9), 7.59 (1H, d, J = 8.0, H^{3}), 6.95 (2H, d, $J = 9.0, H^{28}$), 4.63-4.72 (2H, m, H^{12}), 4.42-4.50 (1H, m, H^{19}), 4.37-4.43 $(1H, m, H^{19}), 4.02-4.11 (2H, m, H^{25}), 3.97 (3H, s, H^{11}), 3.85 (3H, s, H^{31}), 1.85 - 3.45$ $(24H, br, H^{15,16,24,32}), 1.38-1.42 (3H, m, H^{20}), 1.33-1.37 (3H, m, H^{20}), 1.14 (3H, t, J = 7.0, t)$ H^{23}), 1.12 (3H, t, $J = 7.0, H^{23}$); δ_{C} (CDCl₃, 126 MHz): 176.8 (C⁵), 173.2 (C¹⁴), 172.4 (C^{21}) , 165.8 (C^{10}) , 165.2 (C^{17}) , 163.1 (C^{30}) , 163.0 (C^{2}) , 159.7 $(C^{1'})$, 158.3 $(C^{9'})$, 138.3 (C⁴), 136.0 (C⁸), 130.4 (C²⁸) 129.4 (C⁶), 129.2 (C²⁹), 126.8 (C⁷), 121.5 (C⁵), 120.3 (C³), 118.9 (C^9), 115.3 ($C^{4'}$), 114.5 (C^{27}), 61.7 (C^{22}), 61.5 (C^{22}), 57.5 (C^{24}), 55.8, 55.4, 52.6, 49.9, 48.8 (br), 48.5, 45.1, 40.4, 17.7 (C^{20}), 14.3 (C^{23}), 14.2 (C^{23}); m/z (ES⁺): 1024.459, $C_{48}H_{66}N_9O_{14}S$ requires 1024.445 [M + H]⁺.

[Eu.L¹]Cl₃

Smith, McMahon, Pal and Parker

(SS)-1,7-Bis(ethyl-N-acetyl-S-alanine)-4-[7-methoxycarbonyl-2-

chloromethylcarbonylmethyl-1-azaxanthone]-10-[2'-(methylsulfonylamino)ethyl]-

1,4,7,10-tetraazacyclododecane (18.1 mg,. 17.6 µmol) was added to Eu(OTf)₃ (11.9 mg, 20.0 µmol) and dissolved in MeCN (1 ml). The reaction was stirred for 48 h at & 0 The reaction was then cooled to room temperature and the solvents removed under reduced pressure. The remaining residue was dissolved in dry MeCN (0.1 ml) and the mixture dropped onto anhydrous Et₂O (5 ml) resulting in the precipitation of the title compound as a triflate salt. The precipitate was centrifuged and dissolved in aqueous MeOH : H₂O (50 : 50, 3 ml). The pH was then adjusted carefully to 10 by addition of conc. NaOH solution to remove the excess europium as Eu(OH)₃, in a white precipitate which was removed by centrifugation. The pH was adjusted back to neutral and the mixture lyophilised to give a bright yellow solid which was loaded onto a DOWEX 1-X8(Cl) anion exchange resin. The column was eluted with water and the fractions combined and lyophilized to yield a light yellow glassy solid (7.8 mg, 7.2 µmol, 41%). m/z (ES⁺): 540.6590, C₄₂H₆₁N₉O₁₃S¹⁵¹Eu requires 540.6615 [M-H]²⁺; τ_{H2O} (pH 4) = 0.63 ms, τ_{D2O} (pH 4) = 1.40 ms, τ_{H2O} (pH 8) = 0.54 ms, τ_{H2O} (pH 8) 0.99 ms; $q_{pH4} = 0.5$, $q_{pH8} = 0.4$

[Tb.L¹]Cl₃

The complex was prepared by an analogous method as for $[Eu.L^1]Cl_3$ using L¹ (18.1 mg,. 17.6 µmol) and Tb(OTf)₃ (11.9 mg, 20.0 µmol) to yield the Tb-complex as its chloride salt (8.9 mg, 8.2 µmol, 47%). m/z (ES⁺) 544.6635, C₄₂H₆₁N₉O₁₃STb requires 544.6642 [M - H]²⁺; τ_{H20} (pH 3) = 1.24 ms, τ_{D20} (pH 3) = 1.56 ms, τ_{H20} (pH 9) = 1.58 ms, τ_{H20} (pH 9) 1.32 ms; q_{pH3} = 0.5, q_{pH9} = 0.3.

[Eu.L²]Cl₃

The complex was prepared by an analogous method as for $[\text{Eu.L}^1]\text{Cl}_3$ using L²(18.5 mg, 18.0µmol) and Eu(OTf)₃ (11.9 mg, 20.0 µmol) to yield the Eu-complex as its chloride salt (10.8 mg, 9.1 µmol, 52%) *m/z* (ES+): 587.6750, C₄₈H₆₅N₉O₁₃S¹⁵¹Eu requires 587.6748 [M - H]²⁺; τ_{H2O} (pH 3) = 0.48 ms, τ_{D2O} (pH 3) = 1.28 ms, τ_{H2O} (pH 9) = 1.37 ms, τ_{H2O} (pH 9) 1.96 ms; q_{pH3} = 1.0, q_{pH9} = 0. HPLC t_{r} = 10.7 min.

Smith, McMahon, Pal and Parker

$[Tb \cdot L^2]Cl_3$

The complex was prepared by an analogous method as for $[Eu.L^{1}]Cl_{3}$ using L² (10.4 mg, 9.9 µmol) and Tb(OTf)l₃ (11.9 mg, 20.0 µmol) to yield the Tb-complex as its chloride salt (6.7 mg, 5.6 µmol, 57%) *m/z* (ES⁺): 590.6763 C₄₈H₆₅N₉O₁₃STb requires 590.6774 [M - H]²⁺; τ_{H2O} (pH 3) = 1.37 ms, τ_{D2O} (pH 3) = 1.96 ms, τ_{H2O} (pH 9) = 1.72 ms, τ_{H2O} (pH 9) 1.89 ms; $q_{pH3} = 0.8$, $q_{pH9} = 0$. HPLC $t_r = 10.7$ min.

Optical Spectroscopy

UV/Vis absorbance spectra were recored on a Perkin Elmer Lambda 900 UV/Vis/NIR spectrometer using FL Winlab software). Emission spectra were recorded on an ISA Joblin-Yvon Spex Fluorolog-3 luminescent spectrometer using DataMax v2.20 software). Lifetimes were measured on a Perkin Elmer LS55 luminescence spectrometer using FL Winlab Molecular Spectroscopy Version 4.00.02 software. Each sample was contained in quartz cuvettes with a path length of 1cm. Measurements were recorded at 298 K. Generally, an integration time of 0.5 seconds, increment of 0.5 nm and excitation and emission slits of 2.5 and 1.5 nm respectively were used. Circularly polarized luminescence measurements were made using a home-built CPL spectrophotometer, based on a Spex Fluoromax-2-spectrofluorimeter at the University of Glasgow with the assistance of Dr R. D. Peacock. Measurements were undertaken in D₂O using indirect excitation at 332 nm.

Luminescence titrations and estimation of pKa values

Luminescence titrations were carried out, examining the effect of a number of different anions and protein upon the emission spectra of the complexes. Protein was added as a solid, and pH adjustments were made, if necessary, after each addition. Volume additions at each point were typically only 0.1% of the original solution. A correction for this small dilution effect was nevertheless made for each Ln(III) spectrum.

The pH of the solution was carefully controlled to the designated pH. Measurements of pH were taken at the start and end of spectral acquisition indicated that the pH fluctuated by no more than \pm 0.03 units. A further consideration took account of dissolved bicarbonate that is naturally present through the equilibrium with atmospheric CO₂. To

Smith, McMahon, Pal and Parker

minimise this uncertainty, all solutions were lowered in pH to around 5, bubbled under gentle agitation with argon for ten minutes and then increased back to the desired pH with freshly made concentrated potassium hydroxide solution under argon.

The data obtained was plotted, overlaying each spectrum obtained, so that changes to the form and intensity were easily observed. Ratiometric analysis was performed to allow protonation constants to be calculated. It is possible to take the ratio in a number of ways; by using a pair of individual emission wavelengths (such as 616 nm / 594 nm or 616 to 680 nm), by using a pair of emission bands (most commonly the $\Delta J = 2/\Delta J = 1$ ratio comparing the hypersensitive $\Delta J = 2$ band to the relatively insensitive $\Delta J = 1$ band), or alternatively by using a red/green (or europium/terbium) emission intensity ratio, selecting emission bands.

The apparent binding constants were calculated from the data obtained following these luminescent titrations. The equation shown below was fitted to the data, using a non-linear least squares fitting algorithm in Origin 8.1, using the solver add-in.

$$[X] = \frac{f_{K} + [EuL] * f - [EuL] * f^{2}}{1 - f}$$

Wherein,

$$f = \frac{F - F_0}{F_1 - F_0}$$

and [X] is the concentration of the anion or selected added species in solution;

K is the apparent protonation constant;

F is the ratio or intensity of the selected peak;

 F_0 is the ratio or intensity of the selected peak at the beginning of the titration;

 F_1 is the final ratio or intensity of the selected peak;

[LnL] is the total concentration of the Ln(III) complex in solution.

Details of cell culture, epifluorescence microscopy, assessment of complex toxicity using the MTT assay ^[14] of mitochondrial redox function have been reported elsewhere. ^[6,7,9]

ESI

Confocal Microscopy and Cell Spectral Imaging

Cell images and co-localisation experiments were obtained using a Leica SP5 II microscope. In order to achieve excitation with maximal probe emission, the microscope was coupled by an optical fibre to a Coherent 355nm CW (Nd:YAG) laser, operating at 12mW power. An Ar ion laser was used when commercially available organelle-specific stains (e.g. Lysotracker GreenTM) were used to corroborate cellular compartmentalization. The microscope was equipped with a triple channel imaging detector, comprising two conventional PMT systems and a HyD hybrid avalanche photodiode detector. The latter part of the detection system, when operated in the BrightRed mode, is capable of improving imaging sensitivity above 550 nm by 25%, reducing signal to noise by a factor of 5. The pinhole was always adjusted to the Airy disc size, calculated from the objective in use, using the lowest excitation wavelength. Scanning speed was adjusted to 100 Hz in a unidirectional mode, to ensure both sufficient light exposure and time to collect the emitted light from the lanthanide based optical probes. Spectral imaging on this Leica system is possible in principle with the xy λ -scan function, using the smallest allowed spectral band-pass (5nm) and step-size (3nm) settings. However, with these detection parameters, only well separated emission bands can be examined for ratiometric analysis.

The Leica SP5 systems spectral detection feature was adapted in an unconventional way, using averaged CTF (Contrast Transfer Function) calculations. This approach consists of recording not a λ -scan of the targeted probe, but carefully selecting well-separated band-pass filter settings for the desired intensity bands using an individually assigned PMT detector module. Once an optimized system setting had been established (scan speed/rate, detection window, laser power, pinhole and frame rate and size), it was conserved throughout the measurement to allow accurate detection of the changes in emitted light intensity. After the images (1024x1024 pixel size) of the selected detection bands were recorded, the CTF was calculated and averaged from 15 selected 200 x 200 pixel sized areas that displayed perfect imaging conditions regarding localization profile and an even brightness using an average of 5 independently recorded images. These CTF values were considered as the measured intensity integral values and their ratio was calculated. This allowed the variation in the measured ratio to be monitored, following changes to the cell environment. The Leica microscope has an environmental chamber

Smith, McMahon, Pal and Parker

attached for temperature and CO_2 enrichment (Life Imaging Services Brick for gas mixing, Life Imagining Services Cube for T control); standard operating conditions were 37 °C and 5% CO_2 and humidity was maintained at 10% using a bubble humidifier.

A 3:1 ratio of the europium to terbium complexes was used in the cell growth medium, typically 150 μ M and 50 μ M. This mixture was incubated with NIH-3T3 cells for one hour under 5% CO₂ to allow complex uptake within the lysosomes. At this point, nigericin was added (2 μ M) and 10 sets of spectral data were obtained immediately over the next 5 minutes, with excitation at 355 nm (12mW), examining two emission channels. Using the hybrid detector (HyD), the green terbium channel was set from 450 to 570 nm observing emission arising only from the terbium component. The red europium channel collected emission from 605 to 720 nm. To extract an intensity value for each image, 5 small areas were taken (about 200 x 200 pixels), representing a lysosomal region of the cell. The values observed in each case were averaged, giving the lanthanide intensity for that image. An intensity ratio was then calculated using the europium and terbium images to give the green/red ratio. For complexes of L², the starting Tb/Eu ratio was 1.32 at the 'normal' lysosomal pH; this was assumed to be pH 4.6(±0.1), following literature precedent. For complexes of L¹, the initial Eu/Tb ratio was 0.84 at pH 4.6.

At pH 6.6, the Tb/Eu ratio was 2.9 for complexes of L^2 and the Eu/Tb ratio was 2.2 for the complexes of L^1 . Replacement of the nigercin-containing medium with fresh medium led to slow restoration of the initially observed value of the lanthanide ratio, over a period of 1.5 h. The intensity ratio did not change after incubations of 1, 4 or 24h, and the overall emission intensity (brightness) was also very similar at each of these time points. Such behaviour suggests that lysosomal localisation is fast and irreversible with no significant egress of the complex from the cell, with and without nigericin.

Using the LysoSensor Probe (5 μ M, 5 min incubation, LSBY-DND160, Invitrogen, λ_{exc} 355 nm/12mW), the same titration was performed monitoring the time dependence of neutralisation induced following addition of the potassium ionophore nigericin (2 μ M). In this case, the ratio of band intensities observed in the lysosomes corresponded to 400-485 nm ('blue) versus 495-600 nm (yellow). The final blue/yellow ratio observed also corresponded (±5%) to that induced by addition of chloroquine (100 μ M) after 5 minutes. Chloroquine (pK_a 10.1 and 8.4) is a lysosomotropic agent that is well known to

Smith, McMahon, Pal and Parker

accumulate in cellular lysosomes and it inhibits endosomal and lysosomal acidification. Typically, intra-lysosomal pH values lie in the range 6.5 to 6.7 following this treatment. In order to correlate the observed time-dependent ratiometric changes with pH for each

set of titrations following nigericin treatment, a cell lysate background was used (0.7mL). To this suspension was added the lanthanide complex or the LysoSensor probe, as above, and 2µM of nigericin was also added. The pH was adjusted to 3.9 (HCl, calibrated pH meter to observe) and the pH increased incrementally up to 7.0 by addition of aqueous sodium hydroxide solution. The same band intensity ratios assessed by microscopy, were observed spectroscopically (λ_{exc} 355 nm, Fluorolog 3) in each case, and the ratiometric variation with pH was plotted. Using the LysoSensor probe, an apparent pKa of 4.77 (±0.07) was determined, by an iterative least squares fitting algorithm operating in Origin. Limiting values of the blue/yellow ratio were 5.9 at pH 7.00 and 1.1 at pH 3.90. Parallel experiments with [Tb.L¹]³⁺ and [Eu.L¹]³⁺ in the cell lysate gave an apparent pK_a of 6.00 (±0.10), compared to a pK_a value of 5.80(±0.10) for [Tb/Eu.L²]³⁺.

Representative spectra, pH titrations and microscopy images are shown below (17 Figures).



ESI Figure 1 Calculated (left) and observed (right), accurate mass spectral data for $[Eu.L^1]^{3+}$.



ESI Figure 2 (*upper*) Europium emission spectra for $[Eu.L^1]^{3+}$ at pH 3 (left) and pH 9 (right); (*lower*) terbium emission spectra for $[Tb.L^1]^{3+}$ at pH 3 (left) and pH 9 (right).



ESI Figure 3 (*upper*) Europium emission spectra for $[Eu.L^2]^{3+}$ at pH 3 (left) and pH 9 (right); (*lower*) terbium emission spectra for $[Tb.L^2]^{3+}$ at pH 3 (left) and pH 9 (right).



ESI Figure 4 Total emission spectra (green) and circularly polarised luminescence spectra (red = pH 3, blue = pH 9) for $[Tb.L^{1}]^{3+}$ at pH 3 (*left*) and pH 9 (*right*), (295 K, *I* = 0.1 M NaCl).

Electronic Supplementary Material (ESI) for Chemical Communications This journal is o The Royal Society of Chemistry 2012

ESI



ESI Figure 5 Confocal microscopy images in NIH 3T3 cells for $[Tb.L^2]^{3+}$: (*top, observing Tb emission*), 50 µM complex, (*left*) 1h incubation, (*middle*) 2h incubation, (*right*) 4h incubation. The lower three images show the corresponding LysoTracker Red emission (50 nM).



ESI Figure 6 Confocal microscopy images showing the lysosomal localisation of $[Ln.L^2]^{3+}$ in NIH 3T3 cells after a 1h incubation (pH 4.6) : (*left*) $[Eu.L^2]^{3+}$ 150 µM; (*middle*) $[Tb.L^2]^{3+}$ 50 µM; merged image (*right*) establishing correspondence with a Pearson coefficient of 0.85.



ESI Figure 7 Confocal microscopy images observing the lysosomes of NIH 3T3 cells (1h incubation) at pH 6.6 : (*left*) $[Eu.L^2]^{3+}$ 150 μ M; (*middle*) $[Tb.L^2]^{3+}$ 50 μ M; (*right*) merged image establishing correspondence, with a Pearson coefficient of 0.91.



ESI Figure 8 Variation of lysosomal pH with time, immediately following treatment with nigericin (2 μ M) in NIH-3T3 cells, monitoring the Eu/Tb band intensity ratio for [Ln.L¹]³⁺ compared to the blue/yellow fluorescence intensity ratio for the LysoSensor DND160.



ESI Figure 9 Comparison of the pH dependence of the Eu/Tb emission band intensity ratio for $[Ln.L^1]^{3+}$, in a cell lysate background (pK_a 6.0) and as derived from microscopy image intensity measurements in lysosomes, highlighting the need for *in situ* calibration.



ESI Figure 10 Variation of the emission intensity ratio of Eu (605 to 630 nm) vs Tb emission (534-550 nm) with pH for $[Ln.L^{1}]^{3+}$ (295K, 0.1 M NaCl). The line shows the fit for an apparent pK_a of 5.7 (±0.1).



ESI Figure 11 Variation of lysosomal pH with time following treatment with nigericin (2 μ M) in NIH 3T3 cells, comparing the Tb/Eu ratio changes for [Ln.L²]³⁺ (*purple*) and the Eu/Tb ratio changes (*orange*) for [Ln.L¹]³⁺.



ESI Figure 12 2D Plot showing the variation of lysosomal pH, with the Tb/Eu ratio changes for $[Ln.L^2]^{3+}$ (black squares) and the Eu/Tb ratio changes for $[Ln.L^1]^{3+}$ (red circles), following treatment with nigericin (2µM) in NIH 3T3 cells.



ESI Figure 13 Variation of the total emission spectrum for $[Ln.L^1]^{3+}$ showing the intensity ratio of Eu (605 to 630 nm) vs Tb emission (534-550 nm) with pH for $[Ln.L^1]^{3+}$ (295K, cell lysate + 0.2 mM nigericin). The line shows the fit for an apparent pK_a of 6.0 (±0.1), using a Modified Lorenzian function.



ESI Figure 14 Variation of the time-gated emission spectrum with pH for $[Ln.L^2]^{3+}$ in a cell lysate background (λ_{exc} 365 nm; t_g 10 µs; 295K, cell lysate + 0.2 µM nigericin) from pH 4.15 to 7.10.



ESI Figure 15 Calibration of the pH response of the LysoSensor dye, LSBY TBD160 in a cell lysate background, showing the fit (line) of the ratio of emission bands with pH ($pK_a = 4.77 (\pm 0.07)$).





ESI Figure 17 Variation of the Ln(III) emission spectrum vs pH in a background of 0.4mM HSA, 2.3 mM lactate, 0.9 mM phosphate, 0.13 mM citrate and 30 mM bicarbonate (H₂O, 40 μ M complex, 298 K, *I* = 0.1 M NaCl, λ_{exc} = 332 nm). (*top left*) [Eu.L¹]³⁺ at pH 3.2, 6.7 and 8.7; (*top right*) [Eu.L²]³⁺ at pH 3.1, 6.4 and 7.9; (*bottom left*) [Tb.L¹]³⁺ at pH 8.4, 6.9 and 3.0; and (*bottom right*) [Tb.L²]³⁺ at pH 3.4, 6.5 and 8.3.