



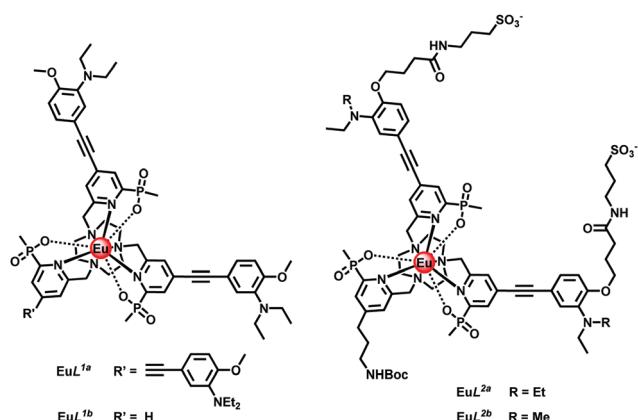
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We report the design and evaluation of pH responsive luminescent europium(III) probes that allow conjugation to targeting vectors to monitor receptor internalisation in cells. The approach adopted here can be used to tag proteins selectively and to monitor uptake into more acidic organelles, thereby enhancing the performance of time-resolved internalisation assays that require pH monitoring in real time.



During the ageing process of endosomes and phagosomes, the pH tends to reduce with time and endosomes eventually may evolve into lysosomes. The cytosolic pH of healthy cells is around 7.2, whereas endosomal pH varies from 6.5 to 5.5; in mature lysosomes the pH is typically around 4.5. Receptor internalisation and endosomal uptake¹ can be monitored provided that the species that is internalised (receptor or substrate) is labelled with a pH sensitive dye, whose emission intensity or lifetime varies significantly with pH.^{2–5}

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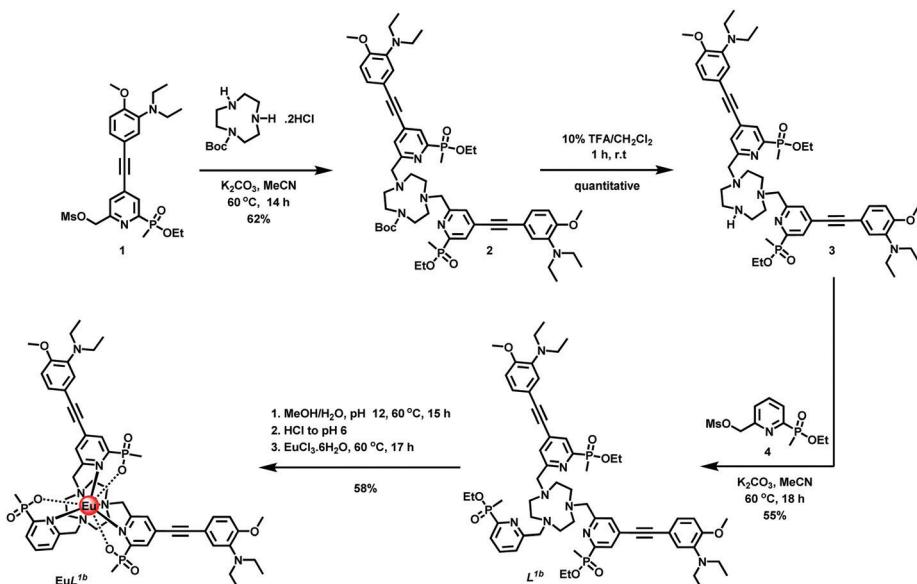
† Electronic supplementary information (ESI) available: Ligand and complex synthesis; photophysical data and spectra; cell experiments. See DOI: 10.1039/d1cc01029h

Recently, we have identified europium(III) complexes that show a pH dependent change in lifetime of a factor of 3 and an emission intensity pH switching ratio of around two orders of magnitude, using time-gated acquisition methods.⁶ Each of these Eu(III) complexes is C_3 symmetric and contains strongly absorbing alkynyl-aryl chromophores, *e.g.* \mathbf{EuL}^{1a} with a brightness of $10\,200\,M^{-1}\,cm^{-1}$ (332 nm) in acidic conditions. With a conjugated diethylamino group the pK_a value is 6.2 (295 K) and falls by one unit as a methyl group replaces one of the ethyl substituents. The absorption spectrum changes with pH because acidification perturbs the energy of the ligand centred and internal charge transfer transitions that exist in these EuroTrackerTM dyes and related series of complexes.^{7–12}

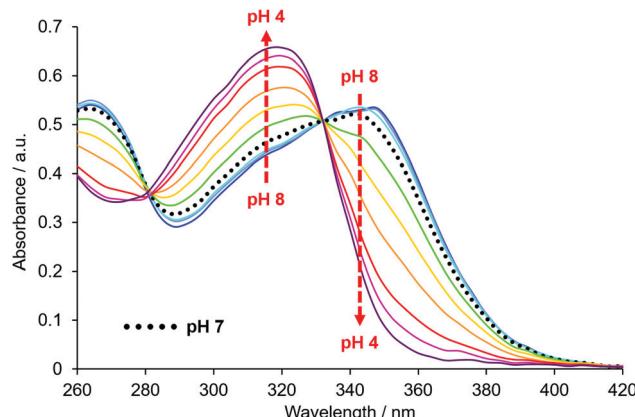
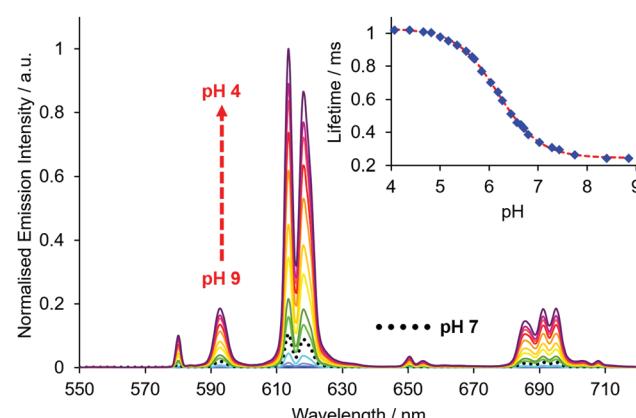
With this background in mind, we set about preparing complexes suitable for use *in cellulo* that are hydrophilic and appropriately substituted to enable conjugation to targeting vectors. Thus, in $\mathbf{EuL}^{2a/2b}$ (Scheme S2, ESI†), peripheral sulfonate groups on the two conjugated chromophores were introduced in order to minimise non-specific binding of the complex to proteins.¹² In addition, the simple pyridine group was functionalised with a primary amino-propyl group (*N*-Boc protected), in order to permit subsequent site-selective conjugation to different targeting vectors.¹³ The synthesis of the Eu(III) complexes of \mathbf{L}^{1b} and $\mathbf{L}^{2a/2b}$ was undertaken in a similar manner, (Scheme 1 and Scheme S2, ESI†), from mono-Boc-1,4,7-triazacyclononane. The syntheses involved a series of *N*-alkylation and simple deprotection steps, and the final complexes were purified by reverse phase HPLC. Under the strongly acidic conditions of the Boc removal step, protonation of the proximate dialkylamino group inhibits any unwanted acid catalysed alkyne hydration. Reaction of the mesylate $\mathbf{1}$,⁶ with mono-Boc-1,4,7-triazacyclononane gave the carbamate $\mathbf{2}$, followed by treatment with TFA in CH_2Cl_2 to yield the amine $\mathbf{3}$. Alkylation with the disubstituted pyridine mesylate $\mathbf{4}$,¹⁴ afforded the ester $\mathbf{5}$, and basic hydrolysis in aqueous methanol followed by complexation with $EuCl_3$ at pH 6 gave the desired neutral complex, \mathbf{EuL}^{1b} .

The absorption spectrum of \mathbf{EuL}^{2b} showed a pH dependence associated with reversible protonation of the dialkylamino



Scheme 1 Synthesis of complex **EuL**^{1b}

group that was characterised by the appearance of two isosbestic points at 284 and 332 nm, (Fig. 1). Similar behaviour was found with the other two complexes (Fig. S1 and S3–S5, ESI[†]). In each case, protonation switched on europium luminescence and the emission lifetime and intensity were measured as a function of pH (Fig. 2, Tables 1 and 2). Thus, with **EuL**^{2a} excitation at the isosbestic point led to an increase in lifetime from 0.24 (pH 8) to 1.02 ms (pH 4) with a 50-fold increase in emission intensity (Fig. S6, ESI[†]). Monitoring changes in emission lifetime or intensity allowed pK_a values to be estimated. In 0.1 M NaCl solution, the *N,N*-diethyl complexes had similar values around 6.2, reducing to 5.34 for the *N*-methylethyl analogue, **EuL**^{2b} (Fig. S10–S12, ESI[†]). These pK_a values are sensitive to the nature of the medium and reduced slightly in cell lysate solution, highlighting the need to calibrate the pH dependence in the medium of interest.

Fig. 1 pH Variation of the absorbance spectrum of **EuL**^{2b} (295 K, 0.1 M NaCl), showing isosbestic points at 284 and 332 nm.Fig. 2 pH Variation of the europium emission spectrum of **EuL**^{2a} (λ_{exc} 332 nm, 295 K, $c = 20 \mu\text{M}$, 0.1 M NaCl); the inset shows the variation of the europium emission lifetime with pH (λ_{exc} 332 nm, λ_{em} 613 nm). A pK_a value of 6.18 was estimated by non-linear least squares regression analysis.Table 1 Summary of pK_a values determined for Eu(III) complexes.^a

Complex	Conditions	pK_a
EuL ^{1a}	0.1 M NaCl	6.21 (03)
	NIH-3T3 cell lysate	5.92 (04)
EuL ^{1b}	0.1 M NaCl	6.30 (03)
	NIH-3T3 cell lysate	6.25 (04)
EuL ^{2a}	0.1 M NaCl	6.18 (03)
	NIH-3T3 cell lysate	6.00 (04)
EuL ^{2b}	0.1 M NaCl	5.34 (03)
	NIH-3T3 cell lysate	5.28 (04)

^a pK_a values given are the mean of three replicates, at 295 K.

In each case, the increases in quantum yield and brightness (Table 2) following acidification are in accord with a strongly

Table 2 Photophysical properties of the stated Eu(III) complexes (295 K, 0.1 M NaCl)

Complex	$\lambda_{\text{exc}}/\text{nm}$	$\varepsilon/\text{M}^{-1} \text{cm}^{-1}$	τ/ms^e	$\Phi/\%^e$	q	$B/\text{M}^{-1} \text{cm}^{-1}^f$
EuL^{1a}	331	60 000 ^{a,d}	0.25 ^b	0.1 ^b	0	46 ^b
			0.84 ^c	17 ^c		10 200 ^c
EuL^{1b}	328	35 000 ^a	0.34 ^b	0.2 ^a	0	70 ^b
			1.00 ^c	18 ^b		6300 ^c
EuL^{2a}	332	39 000 ^a	0.24 ^b	0.3 ^a	0	117 ^b
			1.02 ^c	16 ^b		6240 ^c
EuL^{2b}	332	39 000 ^a	0.28 ^b	0.01 ^a	0	4 ^b
			1.04 ^c	15 ^b		5850 ^c

^a Value at the isosbestic point. ^b Value at pH = 8. ^c Value at pH = 4.

^d Data from Ref. 6. ^e Errors in lifetime measurements are $\pm 10\%$ and in quantum yields $\pm 15\%$. ^f Brightness values are given at the stated excitation wavelength.

emissive and longer-lived protonated complex (see the invariance of the form of the excitation spectra with pH: Fig. S4, S7 and S13, ESI†), wherein the photo-induced electron transfer process that quenches the europium excited state is suppressed. By varying both the delay time and time window for signal acquisition, the ‘switch-on’ can be increased, as signal from the more emissive complex is favoured. For example, by using a delay time of 1.5 ms and acquiring signal for one millisecond (1500–2500 μ s acquisition period), intensity ratios for pH 4/pH 8 solutions of around 500 were found (Table 3 and Fig. 3; Fig. S8/9; Table S1, ESI†), with a progressive diminution in the apparent pK_a value as the delay time was increased.

In a proof-of-concept study for membrane receptor labelling and trafficking, the benzylguanine (BG) derivative **EuL^{2c}** was prepared from **EuL^{2a}** using established methodology (Scheme S3, ESI†).¹² The BG derivative serves to label the SNAP-tag (ST), a self-labelling suicide enzyme¹⁵ often used to study G-Protein Coupled Receptors (GPCRs). Previous studies have shown that Eu(III)¹² and Tb(III) derivatives^{16,17} can be used to label SNAP tagged receptors. Using the non-pH sensitive Lumi4-Tb complex,¹⁸ the Tag-lite technology[®] emerged and Lumi4-Tb labelled GPCR can be used to signal receptor internalisation. Therefore, such a model is well suited to test the behaviour of these new europium pH responsive probes.

The glucagon-like peptide-1 receptor (GLP-1R) is a receptor targeted for anti-diabetic drugs since it is involved in the metabolic pathway for insulin production. Tools to study its agonist-induced internalisation^{19,20} are therefore in high demand. After labelling a HEK-293 cell line that stably expresses GLP-1R-ST with 200 nM of **EuL^{2c}** at pH 7.4, the Eu time-gated luminescence was observed (Fig. 4A: 620 nm, $\Delta J = 2$ band, pH 4.5, acetate buffer; ESI†). Since labelling to non-

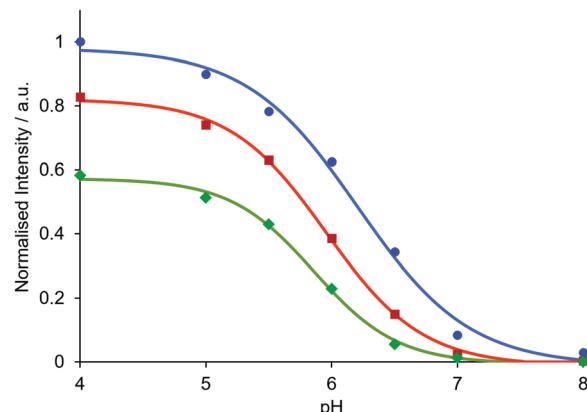


Fig. 3 Relative emission intensity of **EuL^{1b}** (λ_{em} 613 nm) as a function of pH for different time periods of signal acquisition (blue = 60–460 μ s, red = 1000–2000 μ s, green = 1500–2500 μ s). Data are normalised to a 60–460 μ s time window at pH 4. Measurements were made in aqueous solutions of NH₄OAc (pH 4 and 5), MES (pH 5.5, 6, 6.5), HEPES (pH 7) and NH₄HCO₃ (pH 8) buffers ($c = 20 \mu\text{M}$, 0.1 M buffer in 0.1 M NaCl).

transfected HEK-293 cells is negligible under the same conditions, this demonstrates that labelling of the GLP-1R with **EuL^{2c}** on the cell surface is specific, and the compound does not accumulate in endosomal particles, as this would give rise to an

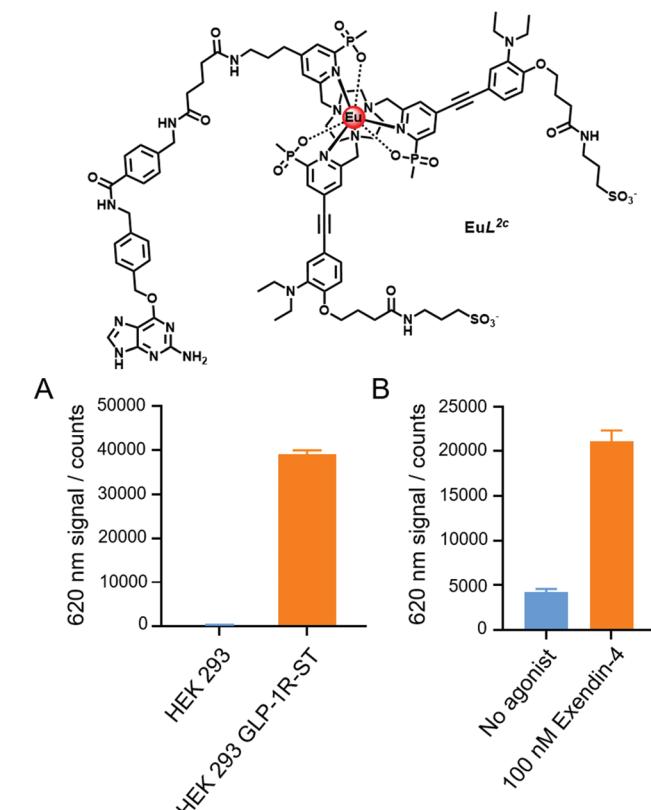


Fig. 4 Time gated measurements of the Eu $\Delta J = 2$ band intensity of: (A) GLP-1R-ST receptor labelling, using **EuL^{2c}** (200 nM) on non-transfected and GLP-1R-ST expressing HEK293 cells; (B) GLP-1R-ST receptor internalisation, 1 h after addition of 100 nM of the peptide agonist, Exendin-4, versus a control with no agonist added (see ESI† for experimental details).

Table 3 Ratios of emission intensities (I_{rel} = ‘switch-on’ factors) for **EuL^{1b}**/**EuL^{2a}** for differing time gate periods, showing the effect on the apparent pK_a values (295 K, $c = 20 \mu\text{M}$, 0.1 M NaCl; buffers, NH₄OAc (0.1 M, pH 4), NH₄HCO₃ (0.1 M, pH 8))

	60–460 μ s	1000–2000 μ s	1500–2500 μ s
I_{rel} : pH 4/pH 8	34/32	266/227	527/465
Apparent pK_a	6.21/6.57	5.96/6.26	5.86/6.16



observable luminescence signal. As has been shown for related Eu complexes,¹² the introduction of peripheral anionic sulphonate groups in the probe structure suppresses any non-specific labelling to cells.

To follow receptor internalisation by monitoring endosomal acidification, 100 nM of the GLP-1R peptide agonist Exendin-4 was added to the EuL^{2c} labelled receptors at pH 7.4. Measuring the time gated luminescence at 620 nm after a 1 h incubation revealed a 5-fold increase in luminescence intensity, as a result of both receptor internalisation and the subsequent endosomal acidification (Fig. 4B). Further pharmacological studies are underway on GLP-1R and other membrane receptors to reveal whether these promising europium pH probes have a bright future for studying receptor internalisation and its time dependence, both *in vitro* and in live cell assays.

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Conflicts of interest

Authors state that there are no conflicts to declare.

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