A single component ratiometric pH probe with long wavelength excitation of europium emission†

Robert Pal and David Parker*

Received (in Cambridge, UK) 15th November 2006, Accepted 30th November 2006
First published as an Advance Article on the web 13th December 2006
DOI: 10.1039/b616665b

Following excitation in the range 370–405 nm, the emission spectrum of a cell permeable macrocyclic Eu(III) complex incorporating an *N*-methylsulfonamide moiety changes form with pH, allowing ratiometric pH measurements in the range 6 to 8.

In seeking to define a practicable luminescent probe of pH that is suitable for usage inside living cells and may be examined by spectroscopy and microscopy, several exacting constraints need to be considered. The complex needs to be cell-permeable, kinetically stable and non-toxic; it should be addressed at an excitation wavelength above 340 nm that preferably corresponds to an available laser or LED emission (*e.g.* 355, 365 or 405 nm); it should signal local pH variation reversibly by a change in the intensity of at least two emission bands to allow a ratiometric measurement; it should possess a large Stokes shift to minimise autofluorescence and preferably possess a long excited state lifetime to allow time-resolved methods to be used.

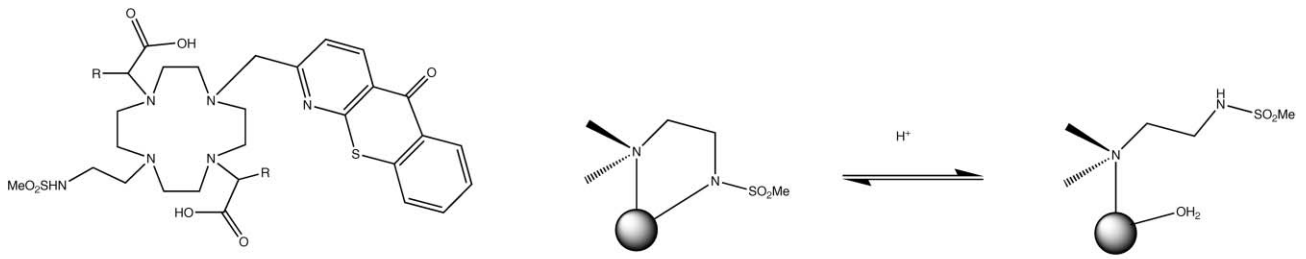
Several new approaches to luminescent pH probes are being considered including the use of functionalised nano-crystals,[1] guided by the benefits of ratiometric methodology.[2] Emissive lanthanide complexes intrinsically are particularly well suited for this purpose, and early examples of responsive europium and terbium complexes have been reported[3,4] that satisfy some of these criteria. For example, a reversible pH-dependent intramolecular sulfonamide ligation mode (Scheme 1) has been engineered into macrocyclic Eu and Tb complexes,[5] but lacked direct usage, as the distance of the sensitising moiety from the lanthanide centre was also pH-dependent, precluding ratiometric analyses.

The emission spectrum of europium(III) complexes is particularly sensitive to the local coordination environment provided by the ligand. Both the local symmetry around the metal and the charge and polarisability of the donor atoms determine the relative intensity of the emission bands from the $^5D_0$ excited state.[3a,6,7] This is most apparent in changes in the form and intensity of the hypersensitive $\Delta J = 2$ (610–630 nm) and $\Delta J = 4$ (680–710 nm) bands. These bands are very sensitive to the nature and polarisability of the axial donor ligand[7a] and the 680 nm transition in the $\Delta J = 4$ manifold has been observed for several complexes with axial N ligation.[5,8] For example, the switch from the binding of a sulfonamide N to a europium(III) centre to ligation (Scheme 1) by water or a carboxylate group, may be signalled by a diminution in the relative intensity of the 680 nm band.

A set of three macrocyclic ligands, L^1–L^3, has now been prepared that incorporate a suitable sulfonamide moiety, as well as a sensitising group that remains bound to the metal centre as the pH is varied. The chromophore selected was an azathiaxanthone,[6] as it allows long wavelength excitation of Eu(III) in the range 360 to 405 nm and is simple to synthesise.[9] Reaction of 2-bromomethyl-1-azathiaxanthone with the appropriate 1,7-disubstituted ester† in MeCN in the presence of $NaHCO_3$ at 70 °C led to predominant formation of the 1 : 1 adduct. Subsequent reaction with *N*-mesylaziridine, or its acyclic mesylate precursor, (MeCN, Na_2CO_3) afforded the desired ligand, and de-protection of the ester groups (TFA–CH_2Cl_2 for ^{t}Bu removal; KOH–H_2O for Me ester hydrolysis) gave the ligands L^1–L^3. Complexation of each of these ligands with $Eu(OAc)_3$ in aqueous methanol afforded the desired 1 : 1 lanthanide(III) complex. Emission spectra for the Eu(III) complexes were examined as a function of pH (0.1 M

Department of Chemistry, Durham University, South Road, Durham, UK DH1 3LE. E-mail: david.parker@durham.ac.uk

† Electronic supplementary information (ESI) available: Details of the synthesis, cell microscopy and characterization of ligands L^1–L^3 and their Eu(III) complexes, together with additional luminescence and relaxivity titrations as a function of pH in the absence/presence of anions, serum albumin or human serum solution. See DOI: 10.1039/b616665b



H_3L^1 R = H
H_5L^2 R = $CH_2CH_2CO_2H$
H_5L^3 R = $CH_2CH_2CH_2CO_2H$

Scheme 1

NaCl, 298 K) in aqueous media. For both [EuL^1] and [EuL^3], substantial and reversible changes were observed in the splitting of the ΔJ = 1 transition and the form of the ΔJ = 2 and ΔJ = 4 manifolds; new bands appeared at 627 nm and 680 nm as the pH was raised from 5 to 8. Plots of the change in the emission intensity ratio (680/587 nm) *versus* pH (Fig. 1) revealed an 80% change in this ratio (pH 4.5 to 8), and apparent protonation constants of 6.15 [EuL^3] and 6.10 for [EuL^1] were estimated. Similar values were obtained ($\pm$5%) by examining changes in the ratio of other pairs of emission bands, *e.g.* 618/627 or 612/618 nm. With [EuL^2], each of these intensity ratios was more or less invariant over the pH range 4.5 to 7, and only above pH 7.5 was the appearance of the 627 and 680 nm bands more evident. Such behaviour accords with earlier observations[5] with analogues of [EuL^2], in which intramolecular (7-ring) carboxylate ligation competed with sulfonamide chelation; this tendency is suppressed with [EuL^3] due to unfavourable 8-ring chelate formation.

Changes in complex hydration state, q, as a function of pH were assessed by measuring the radiative rate constants for excited state depopulation in H_2O and D_2O and using an established relationship to deduce the number of water molecules bound to Eu[10] (Table 1). In each case at pH 8, a q value of zero was obtained, with partial hydration only in more acidic media (q = 0.6 at pH 4.5 for [EuL^1] and [EuL^2]). With [EuL^3], the hydration state apparently remained zero over the entire pH range, with a slightly higher overall emission quantum yield (*ca.* 6%). Confirmation of this behaviour was obtained by examining the pH dependence of [GdL^3]: the measured relaxivity was 3.1 mM^{-1} s^{-1} ($\pm$0.2) over the pH range 3 to 5.5 and was reduced to 1.9 mM^{-1} s^{-1} by pH 8.5, with an apparent protonation constant of 6.2 ($\pm$0.15). Such low relaxivity values are consistent with changes only in the extent of the second sphere of hydration for a q = 0 system.

The emission behaviour of [EuL^3] was examined in the presence of putative anionic interferents and protein, in solutions of increasingly complex composition. Addition of up to 1 mM concentrations of ascorbate and urate neither perturbed the intensity of the emission spectrum nor changed the measured radiative lifetime. Spectral titrations (pH 7.4, 0.1 M NaCl, 298 K, 5 μM [complex]) with hydrogencarbonate and lactate showed that

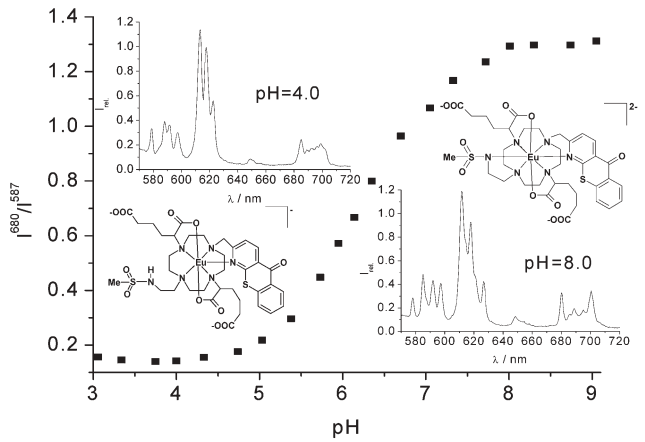


Fig. 1 pH dependence of the europium emission spectrum (λ_{exc} 384 nm, 0.1 M NaCl, 298 K) and the variation of the intensity ratio of the 680 : 587 nm bands, showing the fit to the observed data for an apparent pK = 6.15 ($\pm$0.05).

Table 1 Radiative rate constants, k ($\pm$10%), hydration states, q ($\pm$0.2) and overall absolute emission quantum yield ($\pm$15%) for Eu complexes (λ_{exc} 384 nm, 298 K)

Complex	pH	k_{H_2O}	k_{D_2O}	q^{Eu}	ϕ_{em} (%)
EuL^1	4.5	2.08	1.32	0.6	1.0
	8.0	2.43	2.10	0.1	0.9
EuL^2	3.0	1.70	0.92	0.6	1.8
	5.5	1.41	1.00	0.2	2.0
	8.0	2.13	1.87	0	1.7
EuL^3	4.5	1.36	1.21	0	6.1
	8.0	2.38	2.12	0	5.4

each anion was able to bind to the Eu centre with an apparent affinity constant, log K, of 2.75 and 4.11 respectively (see ESI for further details). The pH dependence of the emission changes with separate fixed anion concentrations (5 or 30 mM, total carbonate; 2.3 mM lactate) and in a mixed anion background (2.3 mM lactate, 30 mM HCO_3^-, 0.9 mM HPO_4^{2-}, 0.13 mM citrate) was followed and indicated that in the pH range 6 to 8, carbonate binding was competitive with the reversible intramolecular sulfonamide ligation. Similar behaviour was observed following addition of 0.7 mM human serum albumin to the mixed anion background, and in a background of 100% human serum solution. The competition from intermolecular HCO_3^- binding notwithstanding, pH dependent sulfonamide ligation was evident in serum over the pH range 6 to 8, as revealed by the appearance of the 627 and 680 nm bands. By plotting the intensity ratio of the europium emission bands spanning 621–626 nm *versus* 681–687 nm, a 250% change was obtained between pH 6 and 8.

The cellular uptake profile of the complex [EuL^3] was examined in mouse skin fibroblasts (NIH-3T3 cells), for varying incubation times from 1 to 18 h (50 and 100 μM complex concentration). The distribution of the complex within the cell was observed by fluorescence microscopy, following excitation of the chromophore at 365 or 405 nm. Azathiaxanthone fluorescence (λ_{em} 450 nm) and europium emission could be observed and the complex revealed a profile (Fig. 2) with evidence of localisation in the nucleolus that strongly resembled that exhibited by related Eu(III) complexes that possess this particular coordinated sensitising moiety.[11]

Further work is underway to assess the utility of the probe for mapping pH within the cell, by observing selected emission bands that report on the intramolecular sulfonamide pH switch.

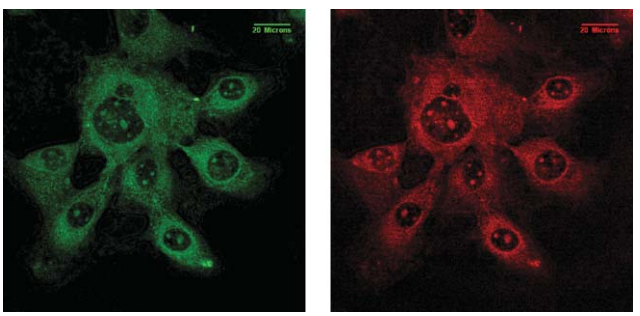


Fig. 2 Confocal fluorescent microscopy images of NIH 3T3 cells (mouse skin fibroblasts) loaded with [EuL^3] (6 h incubation, 100 μM complex concentration in the growth medium); λ_{exc} = 405 nm, observing Eu emission above 570 nm (red) and azathiaxanthone fluorescence (green) at 450 nm, revealing the localisation of the complex in the nucleolus.

We thank the EC networks of excellence, EMIL (503659) and DIMI (512146) and the Royal Society for support.

Notes and references

1 P. T. Snee, R. C. Somers, G. Nair, J. P. Zimmer, M. G. Bawendi and D. G. Nocera, *J. Am. Chem. Soc.*, 2006, **128**, 13320 discusses a ratiometric pH sensor ($pK_a \sim 8.5$) for a fluorophore modifier nanocrystal emitting in the red.

2 T. J. Rink, R. Y. Tsien and T. Pozzan, *J. Cell Biol.*, 1982, **95**, 189; G. Grynkiewicz, M. Poenie and R. Y. Tsien, *J. Biol. Chem.*, 1985, **260**, 3440; O. S. Wolfbeis, *J. Mater. Chem.*, 2005, **15**, 2657.

3 (*a*) D. Parker, *Chem. Soc. Rev.*, 2004, **33**, 156; (*b*) T. Gunnlaugsson and J. P. Leonard, *Chem. Commun.*, 2005, 3114; (*c*) D. Parker, J. A. G. Williams and P. K. Senanayake, *J. Chem. Soc., Perkin Trans. 2*, 1998, 2129; (*d*) C. P. McCoy, F. Stomeo, S. E. Plush and T. Gunnlaugsson, *Chem. Mater.*, 2006, **18**, 4336.

4 (*a*) S. Blair, M. P. Lowe, C. E. Mathieu, D. Parker, P. K. Senanayake and R. Kataky, *Inorg. Chem.*, 2001, **40**, 5860; M. Woods and A. D. Sherry, *Inorg. Chem.*, 2003, **42**, 4401; (*b*) D. Parker, S. Pandya and J. Yu, *Dalton Trans.*, 2006, 2757.

5 M. P. Lowe, D. Parker, O. Reany, S. Aime, M. Botta, G. Castellano, E. Gianolio and R. Pagliarin, *J. Am. Chem. Soc.*, 2001, **123**, 7601; M. P. Lowe and D. Parker, *Chem. Commun.*, 2000, 707.

6 R. A. Poole, F. Kielar, S. L. Richardson, P. A. Stenson and D. Parker, *Chem. Commun.*, 2006, 4084; D. Parker and J. Yu, *Chem. Commun.*, 2005, 3141.

7 (*a*) R. S. Dickins, D. Parker, J. I. Bruce and D. J. Tozer, *Dalton Trans.*, 2003, 1264; (*b*) O. L. Malta, H. J. Batista and L. D. Carlos, *Chem. Phys.*, 2002, **282**, 21.

8 (*a*) S. Quici, G. Marzanni, M. Cavazipni, P. C. Anelli, M. Botta, E. Gianolio, G. Accorsi, N. Armaroli and F. Barigelletti, *Inorg. Chem.*, 2002, **41**, 2777; (*b*) G. Bobba, J. C. Frias and D. Parker, *Chem. Commun.*, 2002, 890.

9 P. A. Atkinson, K. S. Findlay, F. Kielar, R. Pal, D. Parker, R. A. Poole, H. Puschmann, S. L. Richardson, P. A. Stenson, A. L. Thompson and J. Yu, *Org. Biomol. Chem.*, 2006, **4**, 1707.

10 (*a*) R. S. Dickins, D. Parker, A. S. de Sousa and J. A. G. Williams, *Chem. Commun.*, 1996, 697; (*b*) A. Beeby, I. M. Clarkson, R. S. Dickins, S. Faulkner, L. Royle, D. Parker, A. S. de Sousa, J. A. G. Williams and M. Woods, *J. Chem. Soc., Perkin Trans. 2*, 1999, 493.

11 J. Yu, R. Pal, D. Parker, R. Poole and M. J. Cann, *J. Am. Chem. Soc.*, 2006, **128**, 2294.

