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Design, synthesis and evaluation of ratiometric probes for hydrogencarbonate based on europium emission

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A series of cationic, zwitterionic and anionic macrocyclic europium complexes has been prepared incorporating a N or C- linked acridone chromophore that allows sensitisation of Eu emission following excitation at 390–410nm. Each of these complexes selectively binds bicarbonate at physiological pH and reversible binding is signalled by a change in the form and relative intensity of the Eu emission spectrum. Affinity for bicarbonate is regulated by overall complex charge and falls within the range required for intracellular or extracellular analyses. Monitoring the ratio of the intensity of Eu emission at up to three wavelengths, *e.g.* 618/588 or 618/702 nm allows the solution concentration of bicarbonate to be deduced in a background of competing anions such as lactate, citrate and phosphate. Preliminary screens reveal the complexes to be non-toxic to NIH-3T3 cells and to be taken inside the cell, encouraging further study.

Introduction

The bicarbonate ion is an essential component of biological systems and is vital to many cellular processes in mammals, such as intracellular pH homeostasis, kidney function and sperm maturation.[1] However, very little is known about how bicarbonate concentrations fluctuate within a cell in response to cell stimuli or stress, and such analyses are currently limited to radiochemical measurement of overall $H^{14}CO_3^-$ uptake and inferences based upon changes in intracellular pH. Information about how HCO_3^- is localised and varies within cell compartments is needed in order to understand the diverse physiological processes it may control, for example in cyclic-AMP regulation, through pH-independent, reversible binding to the soluble adenylyl cyclase enzyme.[2] Moreover, the pathological consequences of perturbing some of these physiological processes may be very significant: mis-expression of carbonic anhydrase (CA) is linked to the presence of a variety of tumour types and CA-II deficiency syndrome in humans is characterised by renal tubular acidosis, osteoporosis and mental retardation. There is, therefore, a pressing need for a direct method to allow changes in HCO_3^- concentration to be measured within a cell, and to monitor the changes in different compartments in real time.

Fluorescent probes are used to probe intracellular ionic concentrations.[3] For practical applications, they should be non-toxic and cell-permeable, susceptible to efficient excitation in the near-UV/visible region, thereby avoiding biomolecule co-excitation, and undergo spectral changes that allow ratiometric analyses to be performed, so that the concentration dependence of intensity-based systems is obviated. A limiting feature of several conventional fluorescent probes (*e.g.* for pH, pCa) is that they are sometimes prone to interference from scattered light or auto-fluorescence, as they lack large Stokes' shifts in emission. Such problems are circumvented with longer-lived probes as time-gating allows the unwanted short-lived emission to decay to zero and the probe emission can be monitored after a delay of 5 to 500 μs.[4,5] Luminescent Eu and Tb probes offer much scope in this respect, as they possess emission lifetimes in the 0.1 to 5 ms range. Most applications of luminescent lanthanide complexes in biochemistry and biology have related to the development of time-resolved immunoassays[6] or the introduction of lanthanide complexes as long-lived donors in FRET analyses.[7] However, over the past 5 years, a mechanistic approach to the development of responsive lanthanide probes has emerged,[8] coupled with a better understanding of the relationship between complex structure and both NMR and emission spectral form.[9] Considerable interest is targeted on examining complexes in which the analyte binds to the metal complex, with an affinity that is in line with the local ion concentration.

Of particular interest, therefore, have been studies examining the reversible displacement of one or both waters in diaqua complexes, associated with anion ligation to the lanthanide centre.[10] This event is signalled by changes in the intensity and form of Ln emission, allowing ratiometric analyses to be undertaken. Such methods are most appropriate in analyses of Eu emission as the absence of degeneracy of the emissive ^5D_o state leads to relatively simple spectra. The relative intensity of the magnetic-dipole allowed $\Delta J = 1$ transitions (around 590 nm) is insensitive to the associated change in coordination environment, whilst the intensity of the electric-dipole allowed $\Delta J = 2$ (near to 616 nm) and $\Delta J = 4$ (around 702 nm) manifolds changes considerably, particularly if the 'hard' axial water molecule is displaced by a more polarisable charged donor. The definition of reversible anion binding in competitive aqueous media at cationic lanthanide centres has led to suggestions that a ratiometric method can be developed, as the form of the Eu emission spectrum changes, and this is most evident in binding to the bicarbonate anion.[11] The structure of several of these 'ternary' complexes has been defined by X-ray crystallography (*e.g.* in chelated adducts with lactate, citrate, several amino acids, acetate)[12] and in solution by NMR, CD and emission studies, particularly examining Eu and Yb analogues.[13] Relative binding affinities for a range of common bioactive anions have been assessed in simple competitive analyses or in fixed interferences studies. They reveal that in the millimolar range, it is the more polarisable (higher energy HOMO) oxygen in HCO_3^- (bound as CO_3^{2-}) and various doubly charged phosphorylated anions (*e.g.* glucose-6-*O*-phosphate; 2,3-bisphosphoglycerate; AMP but *not* cAMP) which bind most avidly[10,14] These results are in line with measurements of preferred donor affinity in which the binding order follows the relative polarisability of the donor.[9] The recent reports of long wavelength sensitisation (λ_{exc} in range 370–420 nm) of Eu emission using diarylketone and acridone chromophores[15,16] prompted us to engineer such a sensitiser into a heptadentate ligand based on the 12-*N*-4

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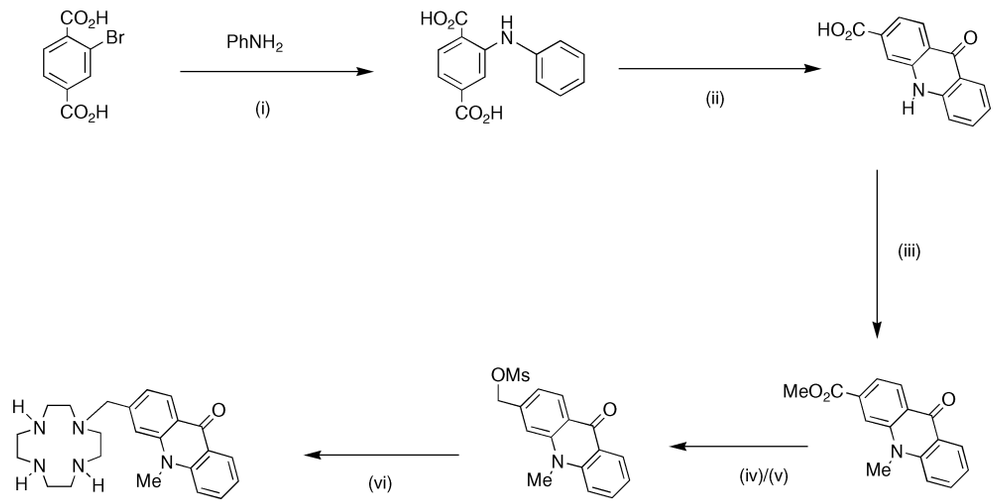


Scheme 1 *Reagents and conditions*: (i) CuBr, 2,3-butanediol; (ii) PPA/110C; (iii) MeI, K_2CO_3, acetone; (iv) $NaBH_4$, MeOH; (v) MsCl, Et_3N, CH_2Cl_2; (vi) cyclen (2 eq., $CHCl_3$).

(cyclen) ring, as complexes of such ligands were originally used in the identification of reversible anion binding.[17] Accordingly, the cationic complexes $[EuL]^{3+}$[L = L^1–L^6] were considered, in which the (*S*)-amino acid side chain confers a Δ-helicity[18] on the complex. Hydrolysis of the peripheral ester groups, then gives neutral (*e.g.* Ala) or trianionic (*e.g.* Asp, Glu) complexes, allowing ligand-based modulation of the affinity for the target anion.

Ligand and complex synthesis

Reaction of acridone with ethylene carbonate in hot DMF in the presence of a trace of KOH[19] afforded the *N*-hydroxyethyl compound **1** and this was converted into the mesylate **2** under standard conditions (THF, Et_3N, MsCl). Addition of **2** to excess 1,4,7,10-tetraazacyclododecane (cyclen) in boiling acetonitrile gave the mono-alkylated compound **3** in reasonable yield. Reaction of **3** with three equivalents (MeCH, Cs_2CO_3) of the 2-chloroethanoyl derivatives of ethyl glycinate, alanate, β-alanate, diethyl aspartate and diethyl glutarate afforded the esters L^1–L^5, which were purified by chromatography on neutral alumina. The constitutional isomer of **3**, was also prepared from 2-bromoterephthalic acid, Scheme 1. Reaction of aniline in the presence of cuprous bromide in hot butanediol afforded 2-aminophenylterephthalic acid which cyclised in the presence of polyphosphoric acid to give acridone-3-carboxylic acid, in 81% yield over the two steps. Subsequent reaction with MeI in acetone in the presence of potassium carbonate gave the *N*-methylated methyl ester and stepwise borohydride reduction and mesylation yielded the benzylic mesylate. Alkylation of excess cyclen in chloroform afforded the C-linked macrocycle, **4**. Reaction of **4** with *N*-chloroethanoyl-ethyl alanate gave the triester L^6. Cationic complexes of $Eu(CF_3SO_3)_3$ were prepared in dry MeCN, and isolated by precipitation onto anhydrous ether. The series of zwitterionic or anionic complexes was isolated following controlled base-catalysed hydrolysis of the ester groups, of L^1–L^6, giving L^7–L^{12}, followed by complexation with $Eu(OAc)_3$ in water.

Emission and NMR spectral behaviour on anion binding

The europium emission spectra for each complex were recorded in 0.1 M MOPS buffer (0.1 mM complex pH 7.4, 295 K) in the absence and presence of excess added anions. The form of the metal based emission spectrum changed significantly, consistent with the partial or total displacement of the coordinated water molecule and the binding of the anion to the Eu centre. Representative spectra for $[EuL^7]$ – the zwitterionic complex with three peripheral Ala side-arms – in the presence of (*S*)-lactate, bicarbonate and hydrogenphosphate, (Fig. 1), were very similar to those obtained previously with the *N*-methylated complex $[EuL^{13}]$.[10a] As discussed at length in earlier articles,[9,10a] the form of the emission spectrum of the carbonate adduct is the most distinctive. It is characterised by a large $\Delta J = 2/\Delta J = 1$

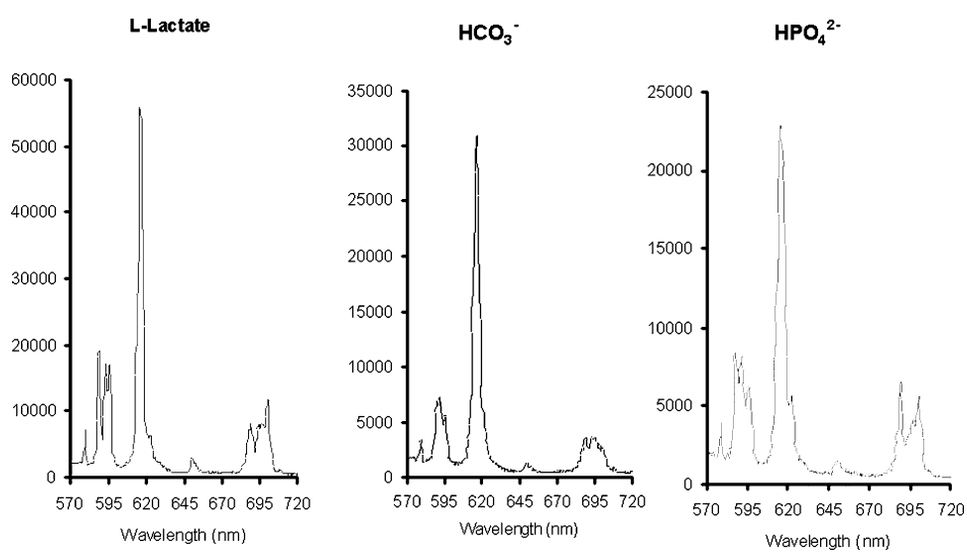
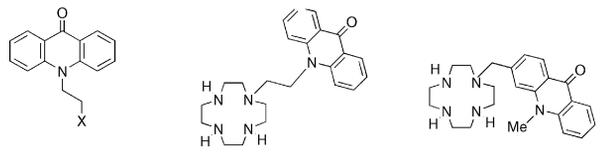


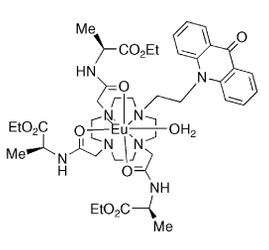
Fig. 1 Europium emission spectra for [EuL7] in the presence of a 10-fold excess of added (*S*)-lactate, hydrogencarbonate or hydrogenphosphate (pH 7.4, 0.1 M MOPS, 295 K, λ_{exc} 410 nm, 0.1 mM complex).



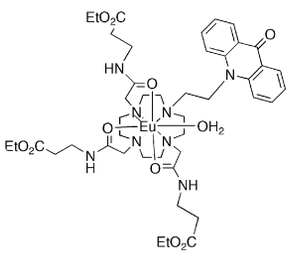
1 X = OH
2 X = OMs

3

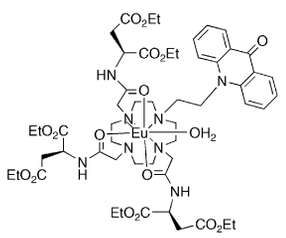
4



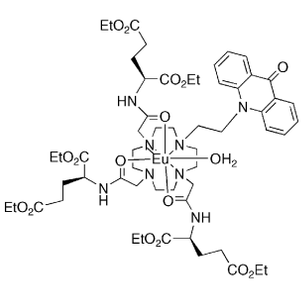
$[EuL^1]^{3+}$



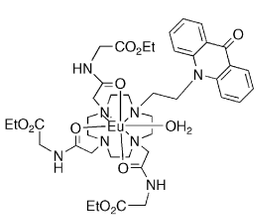
$[EuL^2]^{3+}$



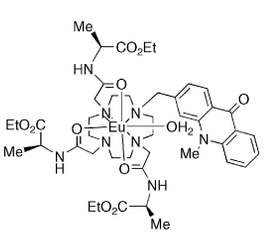
$[EuL^3]^{3-}$



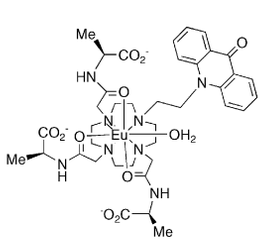
$[EuL^4]^{3+}$



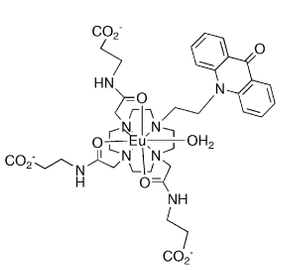
$[EuL^5]^{3+}$



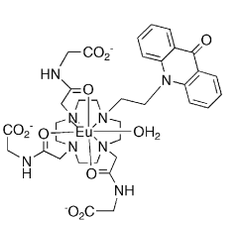
$[EuL^6]^{3-}$



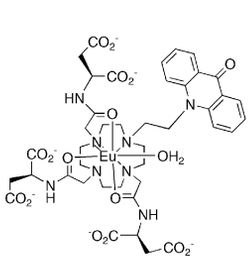
$[EuL^7]$



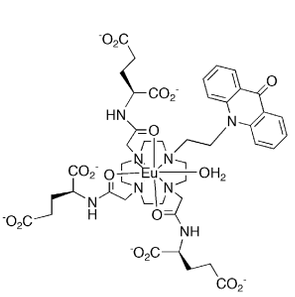
$[EuL^8]$



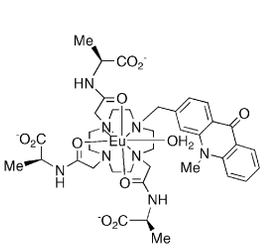
$[EuL^9]^{3-}$



$[EuL^{10}]^{3-}$



$[EuL^{11}]^{3-}$



$[EuL^{12}]$

band intensity ratio (*e.g.* 618/588 nm), a relatively small separation of the $A_2 \longleftarrow A_1$ and $E \longleftarrow A_1$ transitions in the $\Delta J = 1$ manifold around 590 nm, and a weak $\Delta J = 0$ transition at 579 nm. Proton NMR spectra (pD7, 500 MHz, 295 K) for these anion adducts were examined and were also very similar to those previously characterised with [EuL[13]], consistent with the formation of chelated (lactate/carbonate) or mono-aqua (phosphate) adducts, the structures of which had been revealed by crystallographic, chiroptical, emission lifetime and detailed NMR analyses.[12] Similarly the emission and ^{1}H-NMR analyses of spectra obtained in the presence of excess citrate, acetate, fluoride were consistent with earlier interpretations. Radiative rate constants defining the decay of the Eu emission at 592 or 618 nm were measured in H_2O and D_2O, in the absence and presence of added anion. With $[EuL^4]^{3+}$ as the aqua species for example, k_{H_2O} = 2.39 ms^{-1}, k_{D_2O} = 1.02 ms^{-1}, consistent with a hydration state of one.[20] Following addition of ≥ 10 eq. of $NaHCO_3$, the values obtained (k_{H_2O} = 1.80 ms^{-1}, k_{D_2O} = 1.18 ms^{-1}) are consistent with complete displacement of the bound water molecule and formation of a carbonate chelate. The overall quantum yields for Eu emission (295 K, H_2O) were measured for the carbonate adducts, and were 4.4% (±0.8) for [EuL[7]] and [EuL[12]] and 6% for $[EuL^3]^{3+}$.

These modest overall quantum yields are primarily limited by radiative deactivation, *i.e.* competitive fluorescence from the intermediate acridone singlet excited state. *N*-Alkyl acridones possess low-lying *n*–π* and π–π* singlet excited states, and the relative energy of the latter is particularly sensitive to solvent

polarity. Thus, for *N*-methyl acridone itself, in non-polar media E_s~ 300 kJmol^{-1}, ϕ_{fluor} = 2%, ϕ_T = 96% (77 K). In polar solvents (*e.g.* EtOH, H_2O), the π–π* transition is lowered in energy (E_s~283 kJmol^{-1}) and the rate of inter-system crossing becomes much slower relative to the rate of fluorescence (ϕ_{fluor} = 98% ϕ_T = 0.1%).[21] Thus, in the Eu complexes described herein, it is the efficiency of the rate of fluorescent emission – competing with formation of the aryl triplet state – that limits the metal based emission quantum yield.

The emission wavelength for acridone fluorescence was found to be a sensitive function of solvent polarity in the europium complexes. For example, with $[EuL^3]^{3+}$, the position of the emission wavelength maximum varied as a function of solvent polarity. Indeed a good correlation was found by plotting λ^r_{max} (acridone emission) *versus* Reichardt's empirical solvent polarity parameter, $E_T(30)$[22] (Fig. 2). Parallel variations in the form and relative intensity of 'Eu' excitation spectra were also observed, in accord with the lowering of the energy of the π–π* excited state in the more polar media. The form and relative intensity of the $\Delta J = n$ bands (n = 0, 1, 2, 3, 4) in the europium emission spectra of $[EuL^3]^+$ were also sensitive to solvent polarity, but not because of variations in the solvation of the acridone ground/excited states. The changes in this case reflect the variation in the europium coordination environment as the axial donor (the solvent) is changed.[9] This variation gives rise to a marked diminution of the splitting of the ΔJ = 1 (~590 nm) manifold accompanied by a change in the form and an increase in the relative intensity of the ΔJ = 2 transition (~620 nm) in a sequence that echoes the relative polarisability of the axial donor, *i.e.* DMSO > ROH > MeOH > THF/$CHCl_3$. For the least polar solvents, it is likely that the triflate counterion coordinates. Such a sequence has been characterised previously for related nine coordinate macrocyclic tetra-amide complexes in which the axial donor is permuted.[9]

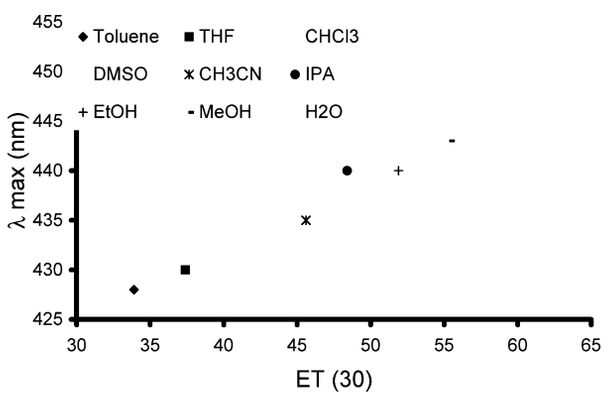


Fig. 2 Variation of the acridone fluorescence emission wavelength maximum for $[EuL^3]^{3+}(CF_3SO_3)_3$ as a function of Reichardt's empirical solvent polarity parameter, $E_T(30)$.

Affinity for bicarbonate in a simulated extracellular medium and ratiometric analysis of binding

Incremental addition of sodium bicarbonate to a solution of $[EuL^7]$ in the presence of competing anions (295 K, pH 7.4, 0.1 M MOPS; 100 mM NaCl, 0.9 mM Na_2HPO_4, 2.3 mM Na lactate, 0.13 mM potassium citrate) was monitored by following changes in the form of the europium emission spectrum, Fig. 3. An increase in the relative intensity of the ΔJ = 2 manifold was observed, (*e.g.* at 618 nm) accompanied by the formation of isoemissive points at 588 nm (ΔJ = 1) and at 702 nm (ΔJ = 4). Such changes, allow the changes in intensity ratio at 618/588 (or 618/702 nm) to be plotted as a function of added bicarbonate. This revealed an increase in the 618/588 nm ratio from an initial value of five to fifteen, following addition of 30 mM bicarbonate. The cationic complexes, *e.g.* $[EuL^1]^{3+}$, $[EuL^4]^{3+}$ and $[EuL^5]^{3+}$ bound most strongly (Fig. 4) and the anionic systems,

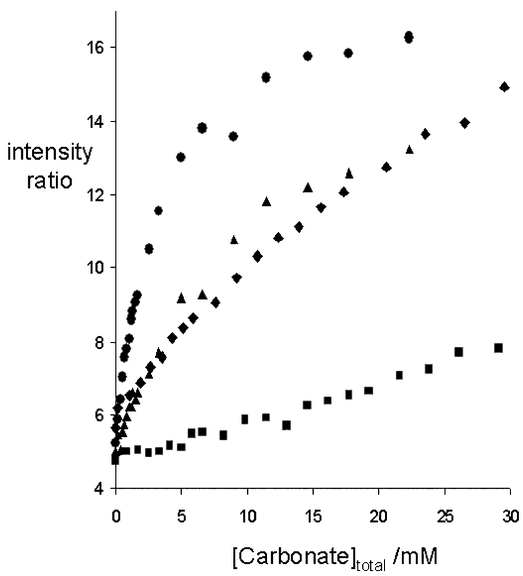


Fig. 4 Variation of the emission intensity ratio (at 618/588 nm) for $[EuL^1]^{3+}$ (●) $[EuL^8]$ (▲), $[EuL^4]^{3+}$ (◆) and $[EuL^{11}]^{3-}$ (■) as a function of added $NaHCO_3$ (pH 7.4, 0.1 M MOPS in a simulated extracellular background; 100 mM NaCl, 0.9 mM Na_2HPO_4, 2.3 mM Na lactate, 0.13 mM K citrate).

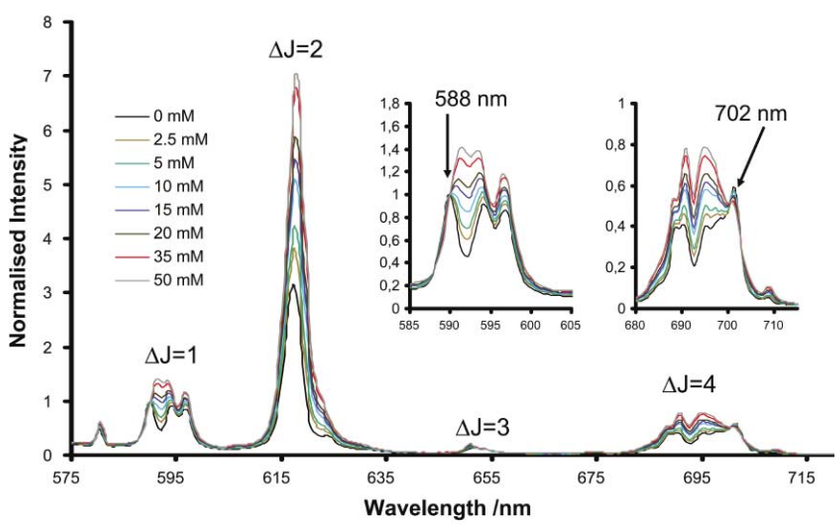


Fig. 3 Variation of the metal based emission in $[EuL^7]$ following incremental addition of $NaHCO_3$ (0.1 M MOPS, pH 7.4, 295 K, 0.1 mM complex). The spectra in the insets reveal the formation of isoemissive points at 588 nm (ΔJ = 1) and 702 nm (ΔJ = 4).

Table 1 Apparent affinity constants for complexation of HCO_3^- (295 K, 0.1 M MOPS, pH 7.4; 100 mM NaCl, 2.3 mM Na lactate, 0.9 mM Na_2HPO_4, 0.13 mM K citrate)

Europium	Complex	Log K_a
$[EuL^1]^{3+}$	(AlaOEt)	1.95
$[EuL^2]^{3+}$	(β-AlaOEt)	2.09
$[EuL^4]^{3+}$	(GluOEt)	1.69
$[EuL^5]^{3+}$	(GlyOEt)	2.27
$[EuL^7]$	(Ala)	1.49
$[EuL^8]$	(β-Ala)	1.42
$[EuL^9]$	(Gly)	1.28
$[EuL^{12}]$	(C-linked Ala)	1.48
$[EuL^{10}]^{3-}$	(Asp)	0.99
$[EuL^{11}]^{3-}$	(Glu)	0.80

e.g. $[EuL^{10}]^{3+}$ and $[EuL^9]^{3-}$ bound with the least affinity, in accord with electrostatic modulation of anion affinity following encounter with the peripheral ligand substitutents. Inspection of the form of the initial Eu emission spectrum, *i.e.* in the presence of the simulated extracellular medium, revealed that for the anionic and neutral complexes, the spectrum corresponded very closely to the lactate adduct, whereas for the cationic systems both lactate and phosphate adducts seemed to be evident.

The *apparent* affinity constant characterising reversible binding of bicarbonate in this competitive medium was measured by fitting the variation of emission intensity (*e.g.* 618 nm) with added total carbonate. A representative binding isotherm is given (Fig. 5) for the association with $[EuL^2]^{3+}$, in which the curve shows the least squares derived fit to the experimental data, based on a 1 : 1 stoichiometry, using methods previously reported [10a]. Data for a range of complexes are tabulated (Table 1) and reveal some simple trends. Affinity for bicarbonate follows the trend: cationic > neutral > anionic, consistent with Coulombic modulation of affinity for the anionic species, and within the series of cationic complexes, substitution in the side chain gave rise to complexes of lower apparent affinity (*e.g.* Gly > β-Ala > Ala > Glu-substituted systems).

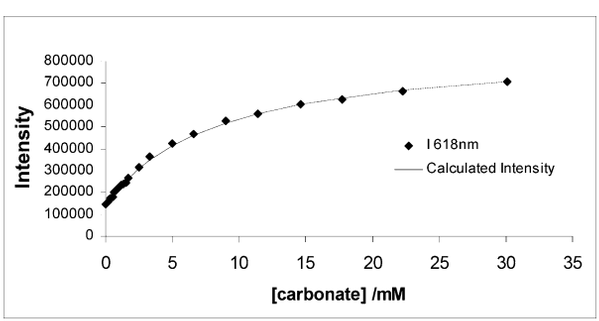


Fig. 5 Change in the intensity of the Eu emission band at 618 nm in $[EuL^2]^{3+}$ as a function of added $NaHCO_3$ showing the fit (line) to the experimental data for 1 : 1 complexation (pH 7.4, 0.1 mM MOPS, 295 K, 0.1 mM complex).

Affinity in cellular media: preliminary cell toxicity/update and microscopy

A parallel titration experiment was carried out, using a cellular lysate (pH 7.4, 0.1 M MOPS) as background medium, which contains various proteins as well as significant concentrations of lactate and various phosphoylated species, *e.g.* 2,3-diphosphoglycerate. Mouse skin fibroblasts (NIH 3T3) were sonicated and to the resultant lysate was added an 0.1 mM solution of $[EuL^7]$ or $[EuL^{11}]^{3-}$. The emission intensity ratio change (618/588 nm) was plotted as a function of added bicarbonate concentration. For $[EuL^{11}]^{3-}$, a 69% change in intensity ratio was noted between 5 and 15 mM (or 130% between 3 and 20 mM). Such a variation coincides well with the putative range of intracellular bicarbonate concentration, (Fig. 6). Indeed, potentiation of the activity of the soluble enzyme, adenylyl cyclase, that occurs on bicarbonate binding falls very clearly in this range, and has been shown to trigger the release of cyclic-AMP in mammalian cells.[23] The europium complexes discussed herein could serve as intracellular probes for bicarbonate, provided that they are non-toxic and that they are able to enter the cell. Accordingly, some preliminary toxicity and cellular uptake experiments have been carried out.

Cell toxicity was assessed in a crude screen, by incubating NIH 3T3 mouse fibroblast cells with a standard growth medium containing 1 mM complex ($[EuL^1]^{3+}$, $[EuL^7]$ and $[EuL^{11}]^{3-}$) for 3 h. Cells were washed with phosphate buffered saline (PBS) solution and were incubated (1 h) with Trypan Blue (0.1% v/v) in PBS. The number of live cells was then counted using a haemocytometer. The charged complexes $[EuL^1]^{3+}$/$[EuL^{11}]^{3-}$ showed no evidence for toxicity (same number of live cells [95 ± 2%] as control with no added complex), while the neutral complex showed only a slight toxic effect (87% live cells). In order to examine cell uptake, cells were mounted on a slide and were examined by fluorescence microscopy, exciting at 400 nm. Each complex revealed a similar distribution of staining, examining the Eu emission selectively by the use of appropriate cut-off filters. Indeed localisation and staining appeared to be independent of the period of incubation, the nature of the complex and a similar overall profile was observed when the cells were electroporated. The images obtained (Fig. 7) revealed a punctuate distribution of the complex, resembling an endosomal/lysosomal localisation. Further co-localisation studies are underway using organic fluorescent dyes whose cellular localisation profile is established. In addition, the nature and efficiency of uptake will be examined together with measurements of the ratio of the intensity of Eu emission from the localised complex, with appropriate control experiments to assess regional pH.

Conclusion

The europium complexes examined afford a new method for assessing the solution concentration of HCO_3^-, by measuring the intensity ratio of the 618/588 or 618/702 nm emission bands. Controlled modulation of the affinity for the target anion is achieved by varying the overall charge on the complex and this is most readily effected by variation of the three amino-acid derived side-chains. Such systems, therefore, allow measurements of HCO_3^- to be made in principle, either in extracellular media or in an intracellular medium. The absence of toxicity in a preliminary screen and the ability of the complexes to enter the cells augurs well for future work directed at devising practicable ratiometic imaging protocols.

Experimental

Reagents and solvents

Acetonitrile was dried over calcium hydride before use. Water and H_2O refer to high purity water with conductivity ≤0.04 μS cm^{-1}, obtained from the PURITE™ purification system. 1,4,7,10-Tetraazacyclododecane is commercially available (Strem) and was used as received.

Chromatography

Column chromatography was carried out using "gravity" silica (Merck). Cation exchange chromatography was performed using Dowex 50W 50 × 4–200 strong ion exchange resin, which had been pre-treated with 3 M HCl.

Spectroscopy

^1H NMR spectra were recorded at 65.6 MHz on a 1.53T magnet connected to a Varian VXR400 console, at 199.99 MHz on Varian Mercury-200 and at 299.91 MHz on Varian Unity-300.

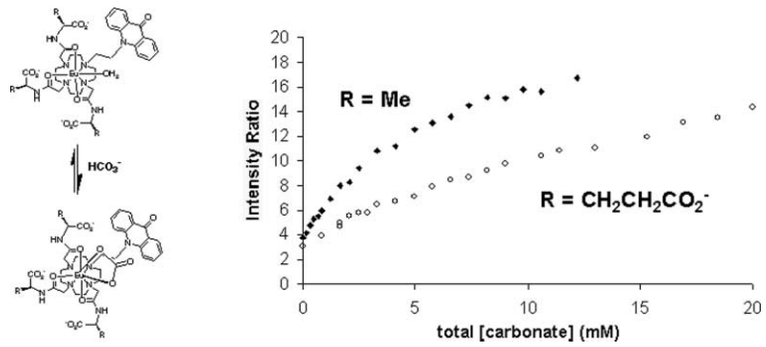


Fig. 6 Variation of the Eu emission intensity ratio (618/588 nm) with added $NaHCO_3$ in a cell lysate medium (λ_{exc} 410 nm, 0.1 M MOPS, pH 7.4, 0.1 mM complex).

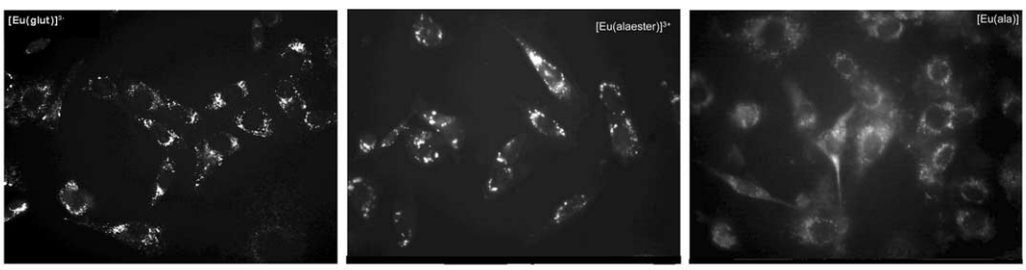


Fig. 7 Confocal fluorescence microscopy images of: $[EuL^1]^{3+}$, (left); $[EuL^7]$ and $[EuL^{11}]^{3-}$ (right), showing the localisation of each complex inside NIH 3T3 cells – the oval-shaped dark centre is the cell nucleus.

^{13}C NMR were recorded on the Varian Mercury-200 at 50.2 MHz and a Varian Unity-300 at 75.4 MHz. Mass spectra were recorded using a VG Platform II electrospray mass spectrometer with methanol or water as a carrier solvent. Accurate masses were determined at the EPSRC National MS Service at Swansea; isotope calculations for Gd and Eu complexes refer to ^{153}Eu and ^{158}Gd. Luminescence measurements for europium and terbium complexes were recorded using an Instrument SA Fluorolog 3–11 using DataMax for Windows v2.1. Second order diffraction effects were obviated by using a 375 nm cut-off filter. pH measurements were made using a Jenway 3320 pH meter (fitted with a BDH Glass + combination electrode – microsample) calibrated with pH 4, 7 and 10 buffer solutions.

Titrations

Luminescence titrations were carried out in a background of constant ionic strength and pH (0.1 M MOPS buffer, pH 7.4, 295 K) on solutions with absorbances of <0.2 at wavelengths $\geq\lambda_{ex}$ in order to avoid any errors due to the inner filter effect. Incremental addition of sodium carbonate solution was carried out using Gilson micropipettes. The luminescence spectra were recorded at each point (30–40 points per titration). Excitation wavelengths of 410 nm (sensitisation *via* acridone chromophore) or 397/355 nm (direct excitation at Eu) were used to obtain the luminescence spectra. Excitation and emission slits were 2–5 nm and 1 nm bandpass respectively. Points were recorded at 1 nm intervals with a 0.25 s integration time.

Synthesis

***N*-(2-Hydroxyethyl)-acridone, 1.** To a suspension of acridone (2.5 g, 12.8 mmol) and ethylene carbonate (2.25 g, 25.5 mmol, 2 eq.) in dry DMF (20 mL) was added a catalytic amount of potassium hydroxide. The mixture was heated at 130 °C under argon overnight. The solvent was removed under reduced pressure and the crude product was precipitated from the residual oil by adding water. The solid was filtered, washed with water and recrystallised from methanol to furnish a yellow solid, (2.36 g, 70%); mp 212–214 °C (lit.[19] 212.2–213.8 °C). δ_H (DMSO-d^6): 8.35 (2 H, dd, H_{1-8}), 7.90 (2 H, d, ArH), 7.82 (2 H, td, ArH), 7.33 (2 H, td, ArH), 5.07 (1 H, t, OH), 4.60 (2 H, t, NCH_2), 3.82 (2 H, q, CH_2OH; δ_C (DMSO-d^6): 177.2 (C=O), 142.8 (Ar C), 134.7 (Ar C), 127.3 (Ar C), 122.3 (Ar C), 121.9 (Ar C), 117.1 (Ar C), 59.0 (CH_2), 48.3 (CH_2); *m*/*z* (ES^+): 262.1 $[M + Na]^+$, 501.2 $[2M + Na]^+$, 740.3 $[3M + Na]^+$. ν_{max} (KBr): 3319 (OH), 1628, 1608 (C=O); λ_{max} (MeOH): 254, 384, 402. Found: C, 75.16; H, 5.46; N, 5.80%. $C_{15}H_{13}NO_2$ requires C, 75.30; H, 5.48; N, 5.85%.

***N*-(2-(Methylsulfonatoethyl)-acridone, 2.** To a suspension of *N*-(2-hydroxyethyl)-acridone (1.50 g, 6.46 mmol) in dry THF (25 mL) under argon was added methanesulfonyl chloride (625 µL, 8.1 mmol, 1.25 eq.) and dry triethylamine (1.25 mL, 7.9 mmol, 1.5 eq.). The mixture was stirred at room temperature until the reaction was adjudged complete by TLC analysis, completed (2 to 3 h). The solvent was removed under reduced pressure, the resulting solid dissolved in dichloromethane (20 mL), and the solution washed successively with 1% aqueous hydrochloric acid (10 mL), 50% saturated aqueous sodium bicarbonate solution (10 mL) and water (10 mL), dried ($MgSO_4$), filtered and evaporated to dryness to afford the title compound as a yellow solid, 1.96 g, 95%, which was used without further purification, mp 162–164 °C, R_f (SiO_2, 30% EtOAc in CH_2Cl_2): 0.50.

δ_H ($CDCl_3$): 8.51 (2 H, dd, *J*, H1-8), 7.71 (2 H, dd, ArH), 7.51 (2 H, d, ArH), 7.27 (2 H, t, ArH), 4.75 (2 H, t, –CH_2–OS), 4.61 (2 H, t, –CH_2–N), 2.93 (3 H, s, –CH_3); δ_C ($CDCl_3$): 177.9 (C_9); 141.7, 134.4, 128.2, 122.6, 122.0, 114.2, 64.5, 44.4, 37.8. *m*/*z* (ES^+): 318.1 $[M + H]^+$, 340.1 $[M + Na]^+$, 656.9 $[2M + Na]^+$, 974.5 $[3M + Na]^+$. ν_{max} (KBr): 3900 (Ar CH), 1596 (C=O), 1179 (SO_2); λ_{max} (MeOH): 216, 254, 382, 396.

***N*-(2-Acridylethyl)-1,4,7,10-tetraazacyclododecane, 3.** A solution of *N*-(2-(methylsulfonyl)ethoxy)-acridone (1.96 g, 6.18 mmol) and excess cyclen (2.13 g, 12.36 mmol, 2 eq.) in dry acetonitrile (20 mL) was boiled under reflux for 36 hours. After removal of the solvent, the residue was partitioned between hydrochloric acid (2 M, 75 mL) and dichloromethane (75 mL). The acidic layer was separated, basified to pH 13.5 with concentrated aqueous sodium hydroxide solution and extracted with dichloromethane (3 × 50 mL); under these conditions the excess cyclen remains in the aqueous layer. The combined organic

extracts were dried ($MgSO_4$), filtered and evaporated to dryness to give the title compound as a hygroscopic yellow solid, 1.96 g, 80%, mp 110–112 °C (lit.[2] not reported). *m/z* (ES^+): 394.3 $[MH]^+$; 406.3 $[M + Na]^+$. δ_H ($CDCl_3$): 8.56 (2 H, dd, ArH), 7.76 (2 H, td, ArH), 7.63 (2 H, d, ArH), 7.29 (2 H, td, ArH), 4.60 (2 H, t, –C*H*$_2$–NAr), 3.00 (2 H, t, –NC*H*$_2$–CH_2–NAr), 2.9–2.6 (16 H, m, ring CH_2), 2.1 (3 H, br m, NH).; δ_C ($CDCl_3$): 178.3 (C_9), 141.9, 134.4, 128.2, 122.7, 121.7, 114.9, 52.4 (CH_2), 52.2 (CH_2), 47.3 (CH_2), 45.8 (CH_2), 45.4 (CH_2), 44.2 (CH_2); ν_{max} (KBr): 3445 (NH), 2823 (CH), 1631, 1597 (C=O); λ_{max} (H_2O) 255, 384, 400.

A typical procedure for the synthesis of an N-linked acridone ligand follows.

***N*-(2-[(*S*,*S*,*S*)-4,7,10-Tris-(1-(1,3-diethoxycarbonyl)propyl carbamoyl methyl)-1,4,7,10-tetraazacyclododecan-1-yl]ethyl)-acridone, L[3].** (*S*)-*N*-(2-Chloroethanoyl)ethyl glutamate (1.00 g, 3.57 mmol, 3.1 eq.) and caesium carbonate (1.16 g, 3.57 mmol, 3.1 eq.) were added to a solution of *N*-(2-acridylethyl)-1,4,7,10-tetraazacyclododecane (0.45 mg, 1.15 mmol) in acetonitrile (10 mL) and the mixture was boiled under reflux for 48 h. The reaction mixture was cooled, filtered, and the solvent evaporated under reduced pressure to give an oily residue that was purified by chromatography on neutral alumina (100% CH_2Cl_2 to 0.5% EtOH–CH_2Cl_2) yielding a hygroscopic yellow solid, 740 mg, 57%, mp 98–100 °C; R_f (Al_2O_3, 5% MeOH–CH_2Cl_2): 0.6. δ_H ($CDCl_3$): 8.51 (2 H, dd, ArH), 7.78 (2 H, d, NH), 7.69 (2H, t, ArH), 7.52 (2 H, d, ArH), 7.49 (1 H, d, NH), 7.24 (2 H, t, ArH), 4.50 (5 H, m, C*H*NHCO + ArNC*H*$_2$$CH_2$N), 4.01 (12 H, m, OC*H*$_2$$CH_3$), 3.12 (6 H, m, NC*H*$_2$CO), 2.95 (2 H, t, ArNC*H*$_2$-CH_2N), 2.74–2.88 (16 H, br m, ring CH_2), 3.12 (6 H, m, C*H*$_2$COO), 2.14 and 1.94 (3 H and 3 H, m and m, CHC*H*$_2$$CH_2$), 1.18 (18 H, m, OC*H*$_2$$CH_3$); δ_C ($CDCl_3$,): 177.8, 172.6, 172.5, 172.1, 171.8, 170.8, 141.7, 134.2, 127.9, 122.4, 121.4, 114.4, 61.5, 60.6, 58.2, 53.6, 53.1, 52.5, 51.5, 30.5, 27.1, 14.1, 14.0; ν_{max} (KBr): 3268, 2981, 2828 (CH), 1735, 1672 (C=O), 2598 (NH)cm^{-1}; λ_{max} (MeOH) 258, 390, 408; HRMS (*m/z*): $[M + H]^+$. Found: 1123.5927: $C_{56}H_{83}N_8O_{16}$ requires 1123.5916.

H_6L[4]. The hexaester L[3] (453 mg, 0.40 mmol) was treated with aqueous sodium hydroxide (0.5 M, 20 mL), and the solution stirred at room temperature for 4 days. After the pH was adjusted to 6 with concentrated hydrochloric acid, the solution was concentrated by one half and passed down a strong cation exchange column (DOWEX 50 W, H^+ form), eluting with 12% aqueous ammonia solution. The solvent was removed under reduced pressure, the oil dissolved in water and the solution freeze-dried to give a hygroscopic yellow solid, (400 mg, 89%), *m/z* (ES^-): 953.2 $[L^6H_5]^-$. δ_H (D_2O): 8.12 (2 H, d, ArH), 7.77 (2 H, t, ArH), 7.54 (2 H, d, ArH), 7.25 (2 H, t, ArH), 4.58 (3 H, br m, C*H*–CONH), 4.17 (2 H, t, ArNC*H*$_2$$CH_2$–), 4.11 (2 H, br s), 3.86 (6 H, m), 3.29 (16 H, m, C*H*$_2$ ring), 2.29 (2 H, t, ArNCH$_2$C*H*$_2$–), 1.82–2.33 (12 H, m, C*H*$_2$C*H*$_2$CHCO); δ_C (D_2O): 181.0, 180.6, 180.4, 179.2, 178.4, 178.0, 141.1, 135.5, 126.8, 122.6, 121.1, 115.2, 55.2, 54.6, 54.4, 50.5, 33.3, 33.1, 32.9, 28.0, 27.8. HRMS (*m/z*): $[M + H]^+$ 953.3888. Found; $C_{44}H_{58}N_8O_{16}$ requires 953.3893.

2-(Phenylamino)terephthalic acid. A mixture of 2-bromoterephthalic acid (15 g, 61.2 mmol), aniline (8.5 mL, 93 mmol, 1.5 eq.), copper(I) bromide (1.5 g.), 2,3-butanediol (70 mL) and toluene (50 mL) was heated and stirred at 120 °C for around 30 minutes with toluene being allowed to evaporate. Triethylamine (30 mL) was added, and the mixture heated at 130 °C overnight. The cooled solution was diluted with aqueous ammonia solution (0.5 M, 70 mL) and treated with activated charcoal. After filtration through Celite (washing well with water) the dark coloured solution was acidified to pH = 2 with hydrochloric acid (2 M) and extracted with 3 portions of ethylacetate (150 mL). An insoluble precipitate was filtered off and the organic layer extracted with aqueous ammonia solution (0.5 M, 100 mL). The aqueous extract was acidified with concentrated hydrochloric acid to give a yellow solid, which was collected by filtration, washed with hot water and dried, (15 g 90%), mp (sub) >180 °C. δ_H (DMSO-d_6): 12.26 (2H, br s, COO*H*), 9.61 (1H, br s, N*H*), 7.98 (1H, d, *J* 8.2, H_6), 7.74 (d, 1H, *J* 1.4, H_3), 7.40 (1H, ddd, *J* 8.2, 7.2, 1.8, $H_{3'}$), 7.25 to 7.30 (3H, m, H_5 + $H_{2'}$), 7.13 (1H, ddd, *J* 7.4, 7.2, 1.2, $H_{4'}$. δ_C (DMSO-d_6): 169.0 (C_7), 167.5 (C_8), 147.2 (C_2 or C_4), 140.1 ($C_{1'}$), 135.3 (C_2 or C_4), 131.9 (C_6), 129.2 (2 C, $C_{3'}$), 123.2 ($C_{4'}$), 121.7 (2 C, $C_{2'}$), 117.2 (C_5), 115.1 (C_3), 114.6 (C_1). *m/z* (ES^-): 256.0 $[M - H]^-$; 534.9 $[2M - 2H + Na]^-$. Found: C, 63.62; H, 4.39; N, 5.01%. $C_{14}H_{11}NO_4$ 0.5 H_2O requires: C, 63.16; H, 4.54; N, 5.26%.

Acridone-3-carboxylic acid. A suspension of finely powdered 2-(phenylamino)terephthalic acid (8.25 g, 32 mmol) in polyphosphoric acid (70 mL) was heated at 120 °C with vigorous stirring for *ca*. 2 to 3 hours until everything dissolved. The solution was then slowly poured into boiling water. The precipitate, which formed was collected by filtration, washed with hot water and dissolved in a mixture of methanol (100 mL) and aqueous sodium hydroxide solution (1 M, 100 mL). An insoluble black solid was filtered off whilst the solution was still hot. The filtrate was acidified with glacial acetic acid, concentrated, cooled and the solid collected by filtration, washed with water and dried under vacuum to yield a yellow solid (6.90 g, 90%) that was used without further purification; mp >260 °C. δ_H (DMSO-d_6): 13.38 (1H, br s, COO*H*), 11.97 (1H, s, N*H*), 8.31 (1H, d, *J* 8.0, H_1), 8.24 (1H, dd, *J* 8, 1.5, H_8), 8.18 (1H, d, *J* 1.5, H_4), 7.77 (1H, ddd, *J* 8, 7.5, 1.5, H_6), 7.72 (1H, dd, *J* 8.5, 1.5, H_2), 7.55 (1H, d, *J* 8.0, H_5), 7.29 (1H, ddd, *J* 8, 7.5, 1.5, H_7). δ_C (DMSO-d_6): 177.2 (C_9), 167.4 (*C*OO), 141.8 ($C_{5'}$), 141.2 ($C_{4'}$), 135.5 (C_3), 134.6 (C_6), 127.3 (C_1), 126.7 (C_8), 123.3 ($C_{1'}$), 122.2 (C_7), 121.4 ($C_{8'}$), 121.3 (C_2), 119.8 (C_4), 118.2 (C_5).

3-Methoxycarbonyl-*N*-methyl-acridone. A suspension of acridone-3-carboxylic acid (4.0 g, 16.7 mmol), methyl iodide (5.2 mL, 83.5 mmol, 5 eq.) and K_2CO_3 (11.5 g, mmol, 5 eq.) in acetone (100 mL) was boiled under reflux for 6 days. Dichloromethane (400 mL) and water (400 mL) were added and the two phases separated. The organic extract was washed with water, dried ($MgSO_4$) and filtered. Removal of the solvents under reduced pressure afforded the title compound as a yellow solid, (4.0 g, 90%); R_f (SiO_2, 2% CH_3OH in CH_2Cl_2): 0.30; mp >260 °C. ν_{max} (KBr): 1727(ester CO), 1597 (CO) cm^{-1}. λ_{max} (CH_3OH): 404 (7790), 420 (8330). δ_H ($CDCl_3$): 8.54 (1H, d, *J* 8.2, H_1); 8.49 (1H, dd, *J* 8.0, 1.8, H_8); 8.22 (1H, d, *J* 1.2, H_4); 7.82 (1H, dd, *J* 8.2, 1.2, H_2); 7.70 (1H, ddd, *J* 8.6, 7.0, 1.5, H_6); 7.50 (1H, d, *J* 8.6, H_5); 7.26 (1H, ddd, *J* 8.0, 7.0, 1.0, H_7); 3.94 (3H, s, OC*H*$_3$); 3.91 (3H, s, NC*H*$_3$) δ_C ($CDCl_3$): 178.0 (C_9); 168.8 (*C*OO); 143.1 ($C_{5'}$); 143.0 ($C_{4'}$); 134.5 (2C, C_3 + C_6); 128.4 (C_6); 128.1 (C_8); 125.2 ($C_{1'}$); 123.0 ($C_{8'}$); 121.9 (C_7); 121.4 (C_2); 117.2 (C_4); 115.1 (C_5); 52.8 (O*C*H$_3$); 34.1 (N*C*H$_3$). HRMS (ES^+), found: 290.0792; $C_{16}H_{13}NO_3Na$+ requires: 290.0793.

3-(Hydroxymethyl)-*N*-methyl-acridone. To a suspension of the ester (2.43 g, 9.10 mmol) in freshly distilled dry ethanol (50 mL) under argon was added $NaBH_4$ (690 mg, 18.2 mmol, 2 eq.). The mixture was boiled under reflux overnight, (until no more methyl ester was indicated by TLC: Al_2O_3–EtOAc), cooled and concentrated by one half. Water (20 mL) was added carefully to destroy the excess borohydride, and the mixture was concentrated again by one half. This operation was repeated twice. The resulting brown solution was extracted with dichloromethane (3 × 50 mL). At this stage, some of the product precipitated and was collected by filtration (0.72 g, 32%). The combined organic phases were dried ($MgSO_4$), filtered and evaporated to dryness. The resulting brown oil was triturated with diethyl ether and the product which precipitated was

collected by filtration as a fine yellow powder (0.5 g, 22%), mp 196–198 °C. δ_H (DMSO-d_6): 8.33 (1H, d, *J* 8.0, 1.5, H_8); 8.29 (1H, d, *J* 8.0, H_1); 7.80 to 7.86 (2H, m, H_5 + H_6); 7.76 (1H, s, H_4); 7.33 (1H, ddd, *J* 8.0, 7.5, 1.0, H_7); 7.29 (1H, d, *J* 8.0, H_2); 5.51 (1H, t, *J* 5.5, O*H*); 4.72 (2H, d, *J* 5.5, CH_2O); 3.94 (3H, s, NCH_3); δ_C (DMSO-d_6): 171.0 (C_9); 150.0 (C3); 143.1 (2C, $C_{4'}$ + $C_{5'}$); 134.6 (C_6); 127.2 (2C, C_1 + C_8); 122.4 ($C_{8'}$); 121.8 (C_7); 121.2 ($C_{1'}$); 120.3 (C_2); 116.8 (C_5); 113.5 (C_4); 63.6 (CH_2O); 34.4 (N*C*H_3). HRMS (ES^+): found: 262.0846; $C_{15}H_{13}NO_2Na$ requires: 262.0844.

***N*-(3-Acridylmethyl)-1,4,7,10-tetraazacyclododecane.** To a suspension of the alcohol **4** (470 mg, 1.96 mmol) in dry THF (15 mL) under argon was added dry triethylamine (820 μL, 5.8 mmol, 3 eq.) and methanesulfonyl chloride (305 μL, 3.9 mmol, 3 eq.). The mixture was stirred at room temperature for 2 hours. The solvent was removed under reduced pressure, the resulting solid dissolved in dichloromethane (20 mL), and the solution washed with aqueous hydrochloric acid (0.1 M, 10 mL), 50% saturated aqueous sodium bicarbonate (10 mL) and water (10 mL), dried ($MgSO_4$), filtered and evaporated to dryness to afford the mesylate as a yellow solid (574 mg, 92%) that was used without further purification. R_f (SiO_2, 5% CH_3OH in CH_2Cl_2): 0.50.δ_H ($CDCl_3$): 8.53 (1H, d, *J* 8.4, H_1), 8.50 (1H, dd, *J* 8.1, 1.8, H_8), 7.70 (1H, ddd, *J* 8.7, 7.2, 1.8, H_6), 7.54 (1H, br s, H_4), 7.50 (1H, d, *J* 8.7, H5), 7.20 to 7.30 (2H, m, H_2 + H_7), 5.34 (2H, s, CH_2O), 3.87 (3H, s, NCH_3), 2.96 (3H, s, SCH_3).

A solution of the mesylate (390 mg, 1.23 mmol) in dry chloroform (15 mL) was added dropwise over 3 hours to a solution of cyclen in excess (867 mg, 5.04 mmol, 2 eq.) in dry chloroform (30 mL). The solution was stirred at room temperature for a further 24 hours before adding hydrochloric acid (0.1 M, 80 mL). The acidic layer was separated, filtered and the pH raised to 13 by adding concentrated aqueous potassium hydroxide solution. The mixture was re-extracted with chloroform (3 × 50 mL), the organic phase dried (K_2CO_3), filtered and evaporated to dryness to afford a very hygroscopic pale yellow solid, (360 mg, 74%). δ_H ($CDCl_3$): 8.50 (1H, dd, *J* 8.0, 1.4, H_8), 8.43 (1H, d, *J* 8.2, H_1), 7.65 (1H, ddd, *J* 8.6, 7.0, 1.4, H_6), 7.58 (1H, br s, H_4), 7.44 (1H, d, *J* 8.6, H_6), 7.13 to 7.26 (2H, m, H_2 + H_7), 3.86 (3H, s, NCH_3), 2.73 (2H, s, NCH_2-Ar), 2.51 to 2.82 (16H, br m, ring CH_2); δ_C ($CDCl_3$): 178.1 (C_9), 142.0 (C3); 141.1 + 141.0 (2C, $C_{4'}$ + $C_{5'}$), 134.6 (C_6), 127.2 (2C, C_1 + C_8), 122.4 ($C_{8'}$), 121.8 (C_7), 121.6 ($C_{1'}$), 120.5 (C_2), 116.4 (C_5), 114.2 (C_4), 59.1 (NCH_2Ar), 52.4 (NCH_2), 52.2 (NCH_2), 47.3 (NCH_2), 45.9 (NCH_2), 34.0 (NMe); ν_{max} (KBr): 3445 (NH), 2823 (CH), 1631, 1596 (C=O); *m*/*z* (ES^+): 394.3 $[M + H]^+$; 406.3 $[M + Na]^+$.

L^6. A mixture of the monoalkylated cyclen (200 mg, 0.63 mmol), 2-chloroethanoyl alanine ethyl ester (402 mg, 2.08 mmol, 3.3 eq.) and caesium carbonate (634 mg, 1.95 mmol, 3.1 eq.) in dry acetonitrile (10 mL) was boiled under reflux for 2 days. A white solid was filtered off, washed well with acetonitrile and the filtrate and combined washings evaporated to dryness. The resulting orange oil was dissolved in dichloromethane and the product purified by column chromatography (SiO_2, 5% triethylamine in CH_2Cl_2), to yield a yellow solid, 332 mg, 61%. R_f (SiO_2, 5% Et_3N in CH_2Cl_2): 0.1; *m*/*z* (ES^+): 865 $[MH]^+$, 887 [M + Na] +. Found: 887.4642. $C_{44}H_{64}N_8O_{10}Na$ requires 887.4643.

L^{12}. A suspension of the triester (260 mg, 0.3 mmol) in 0.05 M aqueous potassium hydroxide (40 mL) was stirred at room temperature for 2 days. The pH of the resulting clear solution was adjusted to 6 with dilute hydrochloric acid and the volume reduced to one third. The solution was loaded onto a cation exchange column (DOWEX 50 W 4, H^+ form). The column was eluted with water, and the product eluted with 12% aqueous ammonia. The solvent was removed under reduced pressure and the resulting brown oil dissolved in water and freeze-dried to yield a hygroscopic yellow solid, (110 mg, 46%). *m*/*z* (ES) : 778 $[MH]^+$; Found : 778.3648 $C_{38}H_{50}N_8O_{10}$ requires: 778.3650.

Europium(III) complexes

A typical procedure for Eu(III) complex formation with neutral ligands follows methods previously reported, with representative characterization data.

$[Eu(L^4)](CF_3SO_3)_3$. Europium trifluoromethanesulfonate (51 mg, 0.09 mmol) and L^3 (80 mg, 0.07 mmol) were dissolved in acetonitrile (5 mL) and the solution was boiled under reflux for 18 h. The solution was cooled, filtered and added dropwise with stirring to cold diethyl ether (50 mL). A yellow solid precipitated which was recovered by centrifugation and re-precipitated by following the same procedure twice more to yield a very hygroscopic yellow solid. This was dissolved in water (and the minimum amount of methanol) and the solution freeze-dried, to give a bright yellow solid (88 mg, 72%). *m*/*z* (ES^+): 646.4 $[M + (OH)]^{2+}$; ν_{max} (KBr): 1734, 1718, 1700, 1685, 1654, 1637 (C=O); λ_{max} (H_2O) 258, 390, 408.

$[Eu(L^6)](CF_3SO_3)_3$. *m*/*z* (ES^+): 508.7, 509.7 $[EuL - H]^{2+}$, 1214 $(EuL + 2CF_3SO_3]^+$. Found: 1315.2992 $C_{46}H_{66}N_8O_{16}S_2F_6Eu$ requires: 1315.2998 $[EuLX_2]^+$.

Eu(III) complexes of anionic ligands were prepared using the lanthanide acetate salt.

$[Eu(L^{11}H_3)]$. A solution of europium acetate (20 mg, 0.1 mmol) in water (2 mL) was added to a solution of H_6L^{11} (100 mg, 0.10 mmol) in water (3 mL). A solid precipitated. The mixture was heated under reflux for 18 h, cooled down and filtered. The clear filtrate was freeze-dried to yield a very hygroscopic yellow solid, (100 mg, 85%). δ_H (D_2O, pD 5.5, partial analysis): 28.0, 19.8, 17.1, 12.2 (each 1H, H_{ax}), 9.8(1H, $H_{eq.}$), 9.0–7.3 (8H, br mult, ArH); 2.1, 0.4, −0.5 (12H, br s + s + s, CMe + CHC), −1.0, −2.1, −4.1, −6.0, −7.9, −10.0, −10.8, −11.8, −13.0, −14.1, −14.7, −15.8, −16.7 (br s, $H_{ax'}$ + NCH + $CH_{eq'}$, 19H); ν_{max} (KBr): 1773, 1734, 1717, 1685, 1654, 1637 (C=O); λ_{max} (H_2O) 258, 390, 408.

$[Eu(L^{12})]$. *m*/*z* (ES^-): 928.4, 930.4, 931.3 $[EuL]^-$; *m*/*z* (ES^+): 953.4 $[NaEuL]^+$. Found: 953.268 $C_{38}H_{49}N_8O_{10}EuNa$ requires (15 − Eu): 953.2682; δ_H (D_2O; pD6, partial analysis of aqua complex): 30.6, 21.4, 19,2, 13.8 (CH_{ax}); 10.4 (2H, br s), 8.2, 7.6 (11H, br s + br s, ArH + CH_{eq}); 0.3 + 0.0 (s + s, 9H, CMe); −1.0 (1H), −1.8 (3H, s), −5.4 (2H, br s), −6.3 (1H), −7.8 (1H), −11.3, −11.8, −12.3, −13.3, −14.7, −15.3, −16.3 (each 1H, CH_{ax} + NCHCO).

Cell Culture and imaging

NIH 3T3 mouse fibroblast cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) foetal calf serum, 1 × non-essential amino acids, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Cells were passaged prior to confluence using 500 U trypsin and 180 μg ml^{-1} ethylenediaminetetraacetic acid tetrasodium salt in DMEM.

For cell loading experiments, cells were grown to approximately 70% confluence on a sterile coverslip, washed twice with 1× phosphate buffered saline (PBS) or 1× tris buffered saline, and incubated with growth medium containing a 1 mM concentration of the appropriate indicator. Transfection of the cells was performed using a 1 mM concentration of the indicator using standard protocols. Loaded cells were mounted on a slide and imaged using a Zeiss Axiovert 135 inverted fluorescent microscope (excitation wavelength, 400 nm).

Toxicity was assessed by incubating indicator loaded cells with 0.1% (v/v) Trypan Blue in 1x PBS and counting live cells in a haemocytometer.

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