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Ratiometric probes for hydrogencarbonate analysis in intracellular or extracellular environments using europium luminescence†

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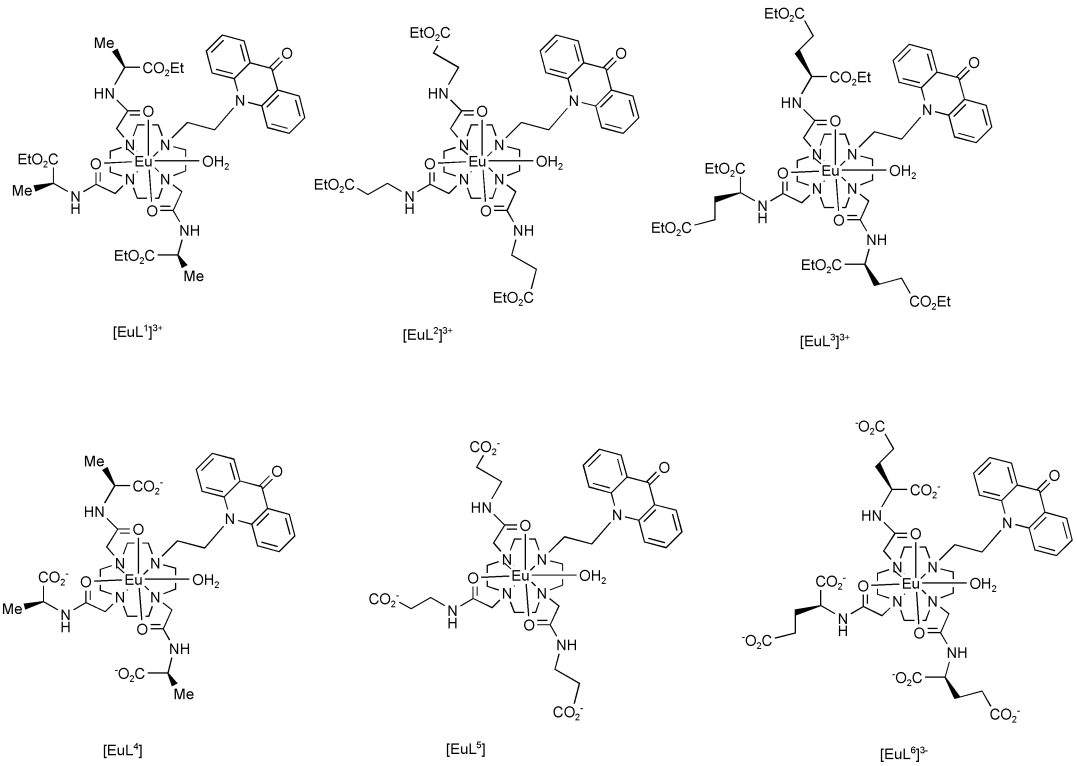
A series of six, cationic, zwitterionic and anionic Eu complexes has been examined for the analysis of hydrogencarbonate concentration in the intracellular and extracellular ranges; an anionic complex incorporating three glutarate residues and a sensitising acridone chromophore (λ_{exc} = 410 nm) exhibits a 69% change in the intensity ratio of the 618/588 nm Eu emission bands between 5 and 15 mM HCO3⁻ in a cell lysate medium.

The simple inorganic anions chloride, hydrogencarbonate and hydrogenphosphate fulfil a series of important functions inside a variety of different cell types. These functions include pH and cell volume homeostasis, fluid secretion and ion transport. The activity of these anions is regulated by a series of selective membrane transporters. For example, several transport proteins have been identified with the 'bicarbonate super-transporter family', in which either Na^+ and HCO_3^- are transported together, or HCO_3^- is exchanged for Cl^-.[1]

No simple probes exist that are able to measure directly changes in HCO_3^- activity. Variations of intracellular pH may be measured, of course, but it is not possible to assess their importance as they may be due to a perturbation of proton or HCO_3^- transport. A further stimulus to this work has come from the recognition of the role of HCO_3^- in stimulating soluble adenylyl cyclase (sAC) activity *in vivo* and *in vitro*, in a pH-independent manner. The hydrogencarbonate anion reversibly binds to and activates the sAC enzyme, leading to enhanced cyclic AMP production.[2] It is believed that the intracellular range for HCO_3^- concentrations lies between 5 and 15 mM, somewhat lower than the 25–30 mM concentrations typically found in mammalian extracellular fluids. Therefore, in seeking a suitable probe for measuring hydrogencarbonate concentrations, systems exhibiting an apparent dissociation constant of 5 to 30 mM are required. Furthermore, the probe should operate in the presence of endogenous anions and proteins, and be immune to pH changes.[3]

Luminescent probes have been used extensively to probe intracellular ionic concentrations. For practical applications in cell biology, they should be non-toxic, cell-permeable, susceptible to efficient excitation in the near-UV/visible region (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. Conventional fluorescence probes (*e.g.* for pH, pCa) are sometimes prone to interference from scattered light or autofluorescence, as they lack large Stokes' shifts in emission. Such problems are circumvented with longer-lived probes as time-gating allows unwanted short-lived emission to decay to zero and the probe emission can be monitored after 1 to 100 μs. Luminescent lanthanide probes offer much scope in this respect,[4–6] and the recent definition of the reversible binding of HCO_3^- in competitive aqueous media at a cationic europium

† Electronic supplementary information (ESI) available: experimental details and structures of **1** and **2**. See http://www.rsc.org/suppdata/cc/b2/b206286k/



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centre allows a ratiometric method to be developed, as the form of the Eu emission spectrum changes on binding to HCO_3^-.[7] The recent reports of long wavelength sensitisation of Eu emission using diarylketone and acridone chromophores[8,9] prompted us to incorporate such a sensitiser into the heptadentate ligands which were originally used in the identification of reversible anion binding. Accordingly, the cationic complexes $[EuL]^{3+}$[L = L^1–L^3] were prepared,[10] in which the (*S*)-Ala and (*S*)-Glu side chains confer a Δ-helicity[11] on the resultant complex. Hydrolysis of the peripheral ester groups, then gave neutral (Ala/β-Ala) and trianionic (Glu) complexes, allowing ligand-based modulation of the affinity for the target anion (see ESI†).

The limiting Eu emission spectra of each complex (0.1 mM) were recorded in the presence of a 10- to 100-fold excess of added anion (pH 7.4, 0.1 M MOPS, 295 K) allowing formation of the ternary anion complex. The spectra were very similar in form to those observed with related N-alkylated Eu complexes,[7] with the spectrum of the carbonate adduct proving the most distinctive, characterised by a 100% enhancement in the intensity ratio of the $\Delta J = 2/\Delta J = 1$ bands *e.g.* measuring the 618/594 nm ratio. The relative increase in intensity of the hypersensitive $\Delta J = 2$ transition at 618 nm may be attributed to an increase in the polarisability of the axial donor. In this context, the carbonate oxygen is more polarisable than a bound water or phosphate oxygen or a chelated lactate or citrate OH group.[12] Radiative rate constants defining the decay of the Eu emission were measured in H_2O and D_2O. With $[EuL^3]^{3+}$, for example, $k_{H_2O} = 2.39\ ms^{-1}$ and $k_{D_2O} = 1.02\ ms^{-1}$ consistent with a hydration state of one.[13] Measurements were repeated in the presence of 30 mM $NaHCO_3$ and the values obtained ($k_{H_2O} = 1.84\ ms^{-1}$, $k_{D_2O} = 1.18\ ms^{-1}$) are consistent with displacement of the inner sphere water. The overall quantum yields defining sensitised emission were also measured under these conditions, and for $[EuL^3]^{3+}$, $\phi_{H_2O} = 6\%$, $\phi_{D_2O} = 15\%$ for the carbonate bound species. Changes in Eu emission spectra were recorded for each complex in a simulated extracellular ionic background (0.1 M NaCl, 2.3 mM lactate, 0.13 mM citrate, 0.9 mM phosphate, pH 7.4, 0.1 M MOPS), following addition of hydrogencarbonate (0 to 40 mM). A representative set of binding curves is illustrated in Fig. 1, examining the 618/588 nm intensity ratio (the 618/702 bands may also be examined) as a function of added $NaHCO_3$ ($\lambda_{exc} = 410$ nm). The cationic complex $[EuL^2]^{3+}$ binds HCO_3^- more avidly than the corresponding zwitterionic complex $[EuL^5]$, whilst the anionic complex $[EuL^6]^{3-}$ bound most weakly. This pattern was repeated through the series of six complexes, with the tripositive complexes showing similar overall affinity. The most appropriate complex for following variations in $[HCO_3^-]$ in the range 20–30 mM is $[EuL^4]$, for which a 24% change in intensity ratio was observed between these limits.

The intracellular total carbonate concentration is believed to be in the range 5–15 mM and response profiles were studied (pH

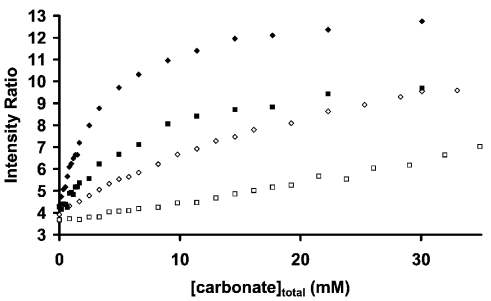

Fig. 1 Variation of the ratio of the 618/588 nm europium emission bands ($\lambda_{exc} = 410$ nm) in (◆) $[Eu(L^2)]^{3+}$, (■) $[Eu(L^5)]$, (◇) $[Eu(L^3)]^{3+}$, (□) $[Eu(L^6)]^{3-}$, as a function of added $NaHCO_3$ (pH = 7.4, 0.1 M MOPS, in a mixed anion background: 100 mM NaCl, 0.9 mM Na_2HPO_4, 2.3 mM sodium lactate, 0.13 mM sodium citrate).

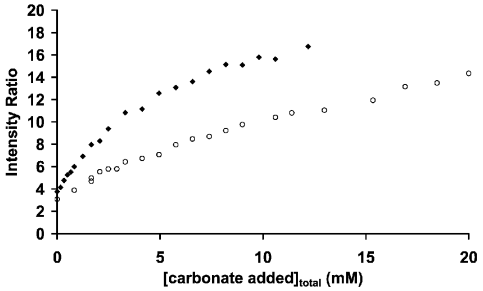

Fig. 2 Change in the ratio of emission intensity ($\lambda_{exc} = 410$ nm, $\lambda_{em} = 618$ and 588 nm) for $[Eu(L^4)]$ (◆) and $[Eu(L^6)]^{3-}$ (○), as a function of added hydrogencarbonate concentration in a lysate of NIH 3T3 fibroblast cells (pH = 7.4, 0.1 M MOPS).

7.4, 0.1 M MOPS) using a lysate of NIH 3T3 fibroblast cells (mammalian cells). In this case, the absence of significant lactate/citrate concentrations and the presence of various proteins leads to a change in the nature of the response curves. The most sensitive complex now is $[EuL^6]^{3-}$, for which a 69% change in the 618/588 nm intensity ratio was observed between 5 and 15 mM added HCO_3^- (Fig. 2). It is important to note that neither the presence of proteins (albeit partially denatured) nor the competing anion background seem to inhibit this ratiometric analytical method. Accordingly, studies are underway to assess the practical utility of such Eu complexes for cellular imaging using confocal microscopy.

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