A pH-insensitive, ratiometric chemosensor for citrate using europium luminescence†

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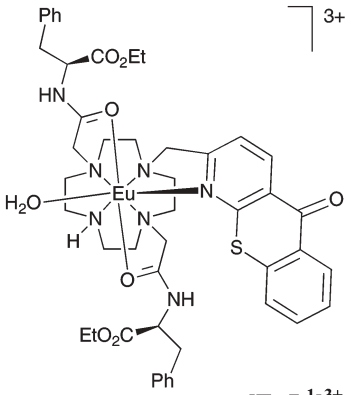
A chemoselective sensor for the citrate anion has been devised, based on a new europium complex that offers ratiometric analysis of the long-lived emission.

The citrate anion exists in all living cells. It is not only an important intermediate in the tricarboxylic acid cycle, but also a key component of fatty acid synthesis, photorespiration, the glyoxylate cycle and nitrogen metabolism.[1] Due to this metabolic significance, abnormal citrate levels have been linked to the characteristics of several diseases. For example, citrate concentration in urine can reflect renal metabolic balance.[2] Decreased urinary citrate excretion has been shown to be important in the pathogenesis of nephrocalcinosis and nephrolithiasis.[3] Recently, citrate has been selected as an *in vivo* marker for the discrimination of prostate cancer.[4] Thus, the development of robust citrate assays *in vitro* or *in vivo* is of general significance to the analytical and life sciences.

The commonest techniques for the determination of citrate involve chromatography or electroanalysis, and are most frequently applied to the analysis of beverages, food or pharmaceutical formulations.[5] NMR spectroscopy has also been applied for this purpose,[6] but low sensitivity limits its scope and range of applications. Analyses based on absorption or emission spectroscopy offer enhanced sensitivity, Anslyn[7] and Wolfbeis[8] in particular having made useful contributions based on cationic guanidinium receptors and a weakly-emissive europium(III) tetracycline complex, respectively. However, a chemoselective citrate sensor that is not compromised by pH fluctuations and affords a ratiometric method of analysis (to preclude additional calibration protocols) still needs to be developed.

In recent work we have examined the selectivity and sensitivity of a series of lanthanide-based, chromophore-sensitized luminescent probes for various anions.[9] Salient features which have guided the design of these systems included (a) an excitation wavelength for the sensitizer shifted as far to the red as possible, (b) an optimised overall emission quantum yield, (c) a complex with low toxicity and high cell permeability and (d) a system that possesses a 'ratiometric read-out' for the lanthanide luminescence. The analysis of emission intensity may be influenced not only by the nature and solvent environment of the sensitising chromophore, but also by factors such as selective compartmentalization of the probe, *e.g.* within certain intracellular organelles, instrumentation noise or sample geometry. Such limitations can be overcome through the utilization of dual wavelength luminescent probes.[10] Herein, we report the characterisation of a new ratiometric luminescent probe for citrate that takes into account these constraints.



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

A cationic europium complex, incorporating a new pyridothioxanthone sensitizer, has been prepared† in which the Eu ion is bound to seven ligand heteroatoms. In the free ligand, the chromophore (ε = 6670 L mol^{-1} cm^{-1}, λ_{abs} = 370 nm, λ_{em} = 424 nm, CH_3OH) possesses a triplet energy level of 23 700 cm^{-1} (4 : 1, EtOH : MeOH, 77 K), allowing the sensitization of both europium and terbium emission. Following europium complexation, the absorption of the sensitizer shifted to the red by 14 nm, and the weak fluorescence shifted by 17 nm in water. Such behaviour is consistent with ligation of the pyridine-N, as revealed in several related complexes.[11] Following excitation at 384 nm, europium emission was observed with an absolute quantum yield of 8.8% in water (18% in D_2O).[12] The luminescence intensity of the $\Delta J = 0$ transition (579 nm) was about one third of the intensity of the $\Delta J = 2$ manifold (centred around 616 nm).†The lifetime of the Eu $^5D_0$ excited state was 0.47 ms in D_2O and 0.30 ms in H_2O, consistent with the presence of one coordinated water molecule ($q = 1.15 \pm 20\%$).[13]

The triply-charged Eu complex is coordinatively unsaturated and may bind anions, triggering changes in the spectral form and lifetime of the europium emission.[9,14] Titration of citrate with the aqueous solution of $[EuL^1]^{3+}$ (5 μM) resulted in highly significant changes to its luminescence spectrum at very low concentrations, with the integrated band intensity of the $\Delta J = 2$ manifold increasing and the relatively sharp $\Delta J = 0$ transition decreasing. At the limit, the lifetime of emission increased to 0.36 ms ($q = 0.7$ ($\pm$0.2)). The 1 : 1 stoichiometry of complexation was confirmed

† Electronic supplementary information (ESI) available: Details of complex synthesis and emission spectra in the presence of various added anions. See http://www.rsc.org/suppdata/cc/b5/b502553b/index.sht

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by a Job plot, and an apparent binding constant of 3.3 ($\pm$0.6) $\times$ 10^6 M^{-1} was estimated by analysing the binding isotherm.†[15] This high affinity was consistent with the observation by electrospray mass spectrometry of the 1 : 1 citrate-bound species; for example at *m/z* 1743 $([EuL^1](CF_3SO_3)_3 + citrate + 4Na)^+$, 1571 $([EuL^1](CF_3SO_3)_2 + citrate + 3Na)^+$ and 1229 $([EuL^1citrate] + Na)^+$. The luminescence intensity change between the $\Delta J = 2$ and $\Delta J = 0$ transitions as a function of citrate concentration permitted a ratiometric measurement. The intensity ratio of the 616 and 579 nm bands proved particularly sensitive to variations in the concentration of citrate (the ratio limits being 3.4 and 22 respectively).

The influence of competing, common biological anions on the complex emission profile was also assessed. In contrast to the behaviour of related diaqua complexes based on macrocyclic triamides,[14] added bicarbonate influenced the europium emission very little, while the presence of phosphate, lactate, acetate, adenosine 5′-triphosphate, adenosine 5′-diphosphate, adenosine 5′-monophosphate, and thymidine 5′-monophosphate led to decreases in the overall emission intensity with increasing anion concentration. However, for each of these anions, the emission changes did not significantly influence the intensity ratio of the 616 and 579 nm bands, which remained around 1 : 3.5 in each case.† The luminescence spectral response was measured for $[EuL^1](CF_3SO_3)_3$, monitored by titration with sodium citrate in the presence of a simulated background of extracellular anions (Fig. 1 and Fig. 2). The intensity ratio of the 616 and 579 nm bands, in the presence of competing anions, changed markedly with increasing citrate concentration. This was interpreted (Fig. 2) in terms of an apparent affinity constant of 3.7 ($\pm$0.4) $\times$ 10^3 M^{-1}, *i.e.* K_d = 0.27 mM. Values within the given error limits were obtained by integrating the total peak intensity of the appropriate transitions.

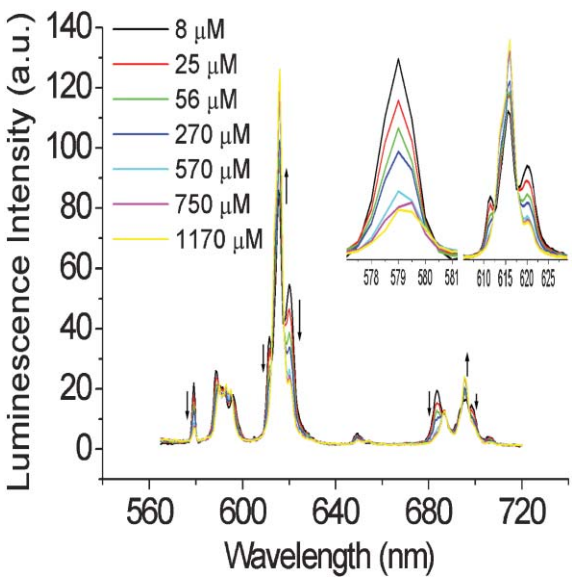

Fig. 1 The luminescence spectra of an aqueous $([EuL^1](CF_3SO_3)_3)$ solution (5 μM) upon titration with sodium citrate, including a mixed anion background: Na_2HPO_4 (0.9 mM), sodium lactate (2.3 mM), NaCl (100 mM), $KHCO_3$ (20 mM), pH = 7.4, λ_{exc} 384 nm.

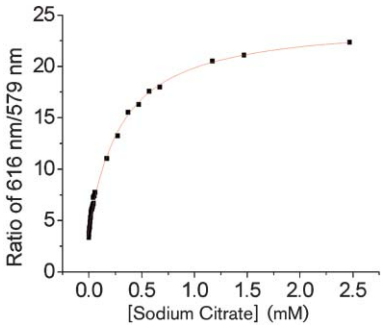

Fig. 2 The intensity ratio of the 616 and 579 nm bands for an aqueous $([EuL^1](CF_3SO_3)_3)$ solution (5 μM) upon titration with sodium citrate, including a mixed anion background: Na_2HPO_4 (0.9 mM), sodium lactate (2.3 mM), NaCl (100 mM), $KHCO_3$ (20 mM), pH = 7.4, λ_{exc} = 384 nm. The curve (red line) represents the best fit of the data using non-linear least-squares for a 1 : 1 binding model.†

The fact that citrate exhibits both a high affinity for the complex and is chemoselective, cannot simply be ascribed to a strong Coulombic interaction. Similar, limiting Eu emission spectra were also obtained in the presence of >10 mM solutions of malate and tartrate (but not succinate, maleate or fumarate), consistent with the possibility of chelation involving the α-hydroxy group and adjacent carboxylate. Such binding has been characterised in crystallographic analyses with structurally-related, macrocyclic triamide-Eu complexes (*i.e.* complexes of a heptadentate ligand) that possess a lower steric demand about the metal centre.[9]

A probe for citrate should also be free of interference from variations in pH. The overall Eu emission intensity increases as the pH of the solution rises from 4 to 6.8, and remains constant between pH 7 and 8.2. Moreover, the intensity ratio of the 616 nm and 579 nm bands does not change throughout ($\pm$5%).† Taken together, these properties render this complex a suitable candidate for the ratiometric analysis or imaging of citrate.

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