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Evidence for the optical signalling of changes in bicarbonate concentration within the mitochondrial region of living cells†

David G. Smith,[a] Ga-lai Law,[a] Benjamin S. Murray,[a] Robert Pal,[a] David Parker*[a] and Ka-Leung Wong[ab]

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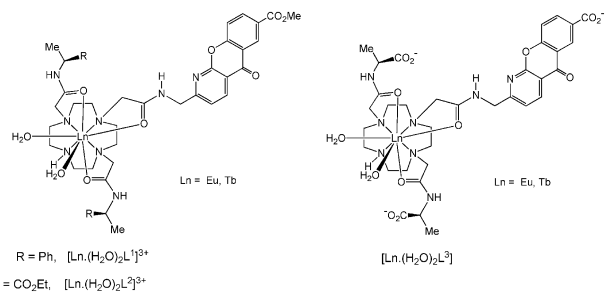
Image and spectral intensity from bicarbonate-selective europium(III) probes localised in the mitochondria of cells is modulated reversibly by variation of external pCO_2, and is suppressed by addition of the carbonic anhydrase inhibitor, acetazolomide.

A challenging objective in cellular imaging is to define optical probes that can target a cell organelle[1,2] and signal concentration changes of a key biochemical species in real time.[3,4] Progress has been made with pH and calcium-sensitive probes, for example using pH-responsive fluorophores localised in the more acidic lysosomal compartments.[2,5] Here, we report systems that serve to report changes in the local concentration of bicarbonate in the mitochondrial region of various cell types.

The production of mitochondrial carbon dioxide is a direct consequence of carbon flux through glycolysis and the citric acid (Krebs) cycle. A method is required to monitor this change in real time, using bicarbonate-selective optical probes. This could offer unprecedented insight into signalling mechanisms that regulate or are regulated through this variation.[6] For example, changes in bicarbonate concentration stimulate the activity of the enzyme adenylyl cyclase that produces cyclic-AMP. Bicarbonate levels, in turn, are regulated by the mitochondrial carbonic anhydrase enzymes. The Krebs cycle generates local CO_2, so that soluble adenylyl cyclase has been postulated as a regulator of oxidative phosphorylation, allowing respiration to keep pace with changes in nutritional availability.[6]

In recent work, we have developed families of emissive Eu and Tb complexes that report on changes in the chemical composition of the environment in various bio-fluids, through modulation of spectral form, polarisation or lifetime.[3a,4] Systems designed to report changes in pH,[7] citrate and lactate,[8] urate[9] as well as bicarbonate[10,11] have been devised. Each system allows a ratiometric analysis, by comparing band intensities either within a europium spectrum or using mixtures of Eu and Tb complexes of a common ligand.[9,12] Initial work with bicarbonate-responsive europium complexes could not be adapted for intracellular imaging, as the probes were only localised to the rather acidic endosomes and lysosomes.[10] More recently, a key structural motif in certain emissive complexes was identified that led to a predominant staining of the cellular mitochondria.[3,4] Thus, in $[Eu{\cdot}L^1]^{3+}$ it is the amide-linked azaxanthone sensitising moiety that promotes uptake of the probe into the cell by macropinocytosis and determines its subsequent trafficking to the mitochondria.[11] This complex was non-toxic to several cell types ($IC_{50} > 200\ \mu M$), and >12h post-incubation was observed by microscopy to migrate to the perinuclear lysosomes.[11] With this background in mind, we have prepared the analogues $[Ln{\cdot}L^2]^{3+}$ and $[Ln{\cdot}L^3]$, (Ln = Eu, Tb), evaluated their affinity for bioactive oxy-anions and protein and studied the changes in emitted light by microscopy, as a function of pCO_2, whilst the probes are localised to the mitochondria.



The ligands L^2 and L^3 are analogues of the triamide, L^1, reported earlier, and were prepared similarly (ESI). In their Eu and Tb complexes they form a short series of differing hydrophobicity and steric demand. Such features were expected to modulate anion and protein affinity, allowing an *in vitro* study of the salient competitive equilibria between anions and a model protein, serum albumin (HSA). Changes in lanthanide emission were monitored as a function of added bicarbonate, HSA, citrate, lactate, phosphate and in certain cases, ATP. Distinctive limiting europium(III) emission spectra were observed in each case. For each Eu complex, the bicarbonate adduct was characterised by a $\Delta J = 2/\Delta J = 1$ intensity ratio of >4:1, compared to 2:1 in the aqua adduct. With $[Eu{\cdot}L^1]^{3+}$, lactate binding was distinguished by the emergence

[a] *Department of Chemistry, Durham University, South Road, Durham, UK DH1 3LE. E-mail: david.parker@dur.ac.uk*
[b] *Department of Chemistry, Hong Kong Baptist University, Kowloon Tong, Hong Kong*

† Electronic supplementary information (ESI) available: Cell localisation images; synthesis and characterisation of $[Ln{\cdot}L^2(H_2O)_2]^{3+}$ and $[Ln{\cdot}L^3(H_2O)_2]$, spectral titrations of lanthanide(III) complexes with selected oxy-anions. See DOI: 10.1039/c1cc11853f

Table 1 Affinity constants of lanthanide(III) complexes for selected species[a] (298 K, I = 0.1 M NaCl, pH as stated)

Complex	HCO_3^- pH 7.4	HPO_4^{2-} pH 7.4	Lactate pH 6	Citrate pH 7.4	HSA pH 7.4
$[Eu{\cdot}L^1]^{3+}$	3.85(04)	2.76(06)	3.35(07)	3.59(04)	3.17(04)
$[Tb{\cdot}L^1]^{3+}$	2.90(03)	2.95(03)	3.40(05)	4.70(03)	3.61(07)
$[Eu{\cdot}L^2]^{3+}$	3.62(07)	2.65(01)	3.61(07)	5.21(12)	nd[b]
$[Tb{\cdot}L^2]^{3+}$	3.16(03)	2.91(01)	3.72(01)	nd[b]	3.49(02)
$[Eu{\cdot}L^3]$	2.97(04)	2.81(01)	2.67(01)	3.84(04)	nd[b]
$[Tb{\cdot}L^3]$	2.85(07)	nd[b]	3.53(02)	nd[b]	3.59(01)

[a] The pH regime was selected in preliminary experiments studying the pH dependence of the emission profile at a fixed anion concentration, seeking a flat region of the spectral response/pH profile. [b] Value not determined as intensity decrease occurred without sufficient spectral modulation to allow data fitting.

of a shoulder at 624 nm and the citrate adduct showed a 70% increase in intensity in the $\Delta J = 2$ band intensity *vs.* the $\Delta J = 1$ manifold. In the lactate and citrate adducts of $[Eu{\cdot}L^2]^{3+}$ and $[Eu{\cdot}L^3]$, much smaller changes in spectral form were observed, consistent with a different constitution in the ternary adduct. A tentative assignment places the hydroxyl group, rather than the more polarisable carboxylate, in the capping axial site of the mono-capped square anti-prismatic polyhedron. The changes in the relative intensity of the hypersensitive $\Delta J = 2$ transitions (around 616 nm) *versus* the magnetic-dipole allowed, $\Delta J = 1$ transitions (around 592 nm) in most cases allowed plots of $\Delta J = 2/\Delta J = 1$ intensity ratios to be used to estimate apparent binding constants (Table 1).

Addition of serum albumin to each europium complex (20 μM) led to a large reduction in emission intensity accompanied by a change in spectral form. Measurements of complex hydration state in the free and 'bound' forms were consistent with displacement of coordinated water molecules (ESI). The large reduction in emission can be ascribed to quenching of the sensitiser triplet state by a charge transfer process from electron rich residues (*e.g.* Tyr) in the protein.[9b,13] Separate addition of Tyr or *O*-phospho-Tyr caused a similar quenching effect. Terbium emission intensity also was reduced on binding HSA but to a lesser extent; similar apparent binding constants to those found with the Eu analogues characterised this interaction (Table 1).

In a fixed background of 0.4 mM serum albumin and 20 mM bicarbonate, the effect of added anions on observed lanthanide emission was examined. For example, with $[Eu{\cdot}L^2]^{3+}$ addition of HPO_4^{2-}, ATP, citrate or lactate (up to a 2 mM limit) led to reductions in emission intensity of $<5\%$ for phosphate and lactate, and 85 and 62% for ATP and citrate respectively. On the other hand, in the presence of 0.4 mM protein only, adding 5 to 25 mM bicarbonate to a solution of $[Eu{\cdot}L^1]^{3+}$, $[Eu{\cdot}L^2]^{3+}$ or $[Eu{\cdot}L^3]$ caused a 250% *increase* in emission intensity and up to a 25% increase in the $\Delta J = 2/\Delta J = 1$ emission intensity ratio, consistent with partial displacement of the bound protein by the added bicarbonate. With the terbium analogues, the corresponding changes in intensity were generally $\leq 20\%$. Taken together, these competition experiments suggest that binding of bicarbonate and protein to the Eu centre is competitive in this series, and is not perturbed significantly by changes in phosphate, ATP, citrate or lactate concentration. The terbium systems offer a calibration role, as changes caused by variation of anion concentration in the presence of protein were much smaller.

Incubation of each lanthanide complex (20–100 μM, 1 to 4 h) led to selective staining of the mitochondrial region of CHO, NIH-3T3, A549, MCF-7 or HeLa cells, confirmed by co-localisation studies with Mitotracker Green™. The percentage of CO_2 in the incubation chamber was lowered from 5% to 4% and 3%, and then stepped back to 5%. After each change, a period of 30 min elapsed before images were acquired by microscopy (λ_{exc} 360 nm). Incubations of $[Eu{\cdot}L^1]^{3+}$ and $[Eu{\cdot}L^2]^{3+}$ (20 or 50 μM) each gave rise to an observed image intensity (using a 550–700 nm band-pass filter to select Eu emission) that increased proportionately with the % CO_2 and changed reversibly (Fig. 1). Hyper-spectral imaging of the mitochondrial region of NIH-3T3 or HeLa cells allowed a quantification of this increase and suggested that there was no major change in the Eu emission spectral form, notwithstanding the modest spectral resolution observed. Parallel experiments examining pCO_2 sensitivity with the Tb analogues caused less than a 10% variation in observed image and Tb spectral emission intensity, summing data from pixels defining the stained mitochondrial region. Incubation of $[Eu{\cdot}L^2]^{3+}$ (20 or 50 μM) in A549 cells in the presence of 1 or 10 μM acetazolomide (a cell permeable non-specific inhibitor[14] of carbonic anhydrase activity) stepping from 3% to 5% CO_2 both lowered the overall emission intensity and suppressed the observed Eu emission intensity change; less than a 10% change was observed on cycling from 5 to 3% and then back to 5% CO_2.

The microscopy experiments suggest that the observed increase of Eu emission intensity with pCO_2 in the mitochondrial region of 3T3 or HeLa cells may be attributed to an increase in the steady-state bicarbonate concentration.

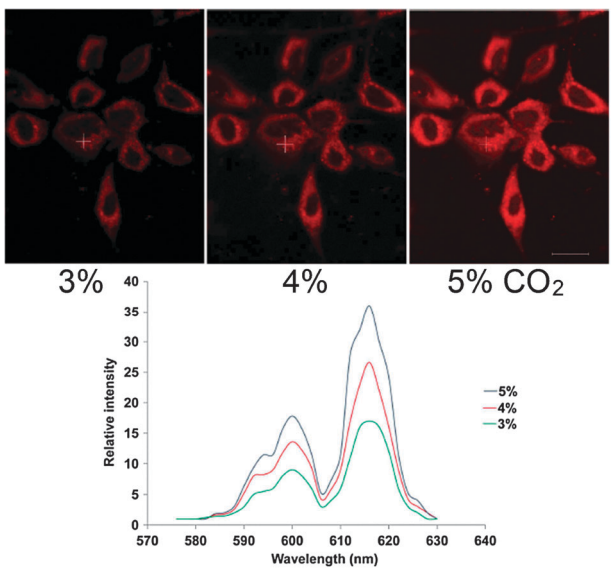


Fig. 1 *upper*: Confocal microscopy images of HeLa cells, showing the mitochondrial region stained by $[Eu{\cdot}L^1]^{3+}$ under 3, 4 and 5% CO_2 (1 h incubation, 20 μM [complex], 30 min equilibration period between images). *lower*: Variation of lanthanide emission intensity from hyper-spectral analysis of microscopy images for HeLa cells stained with $[Eu{\cdot}L^1]^{3+}$.

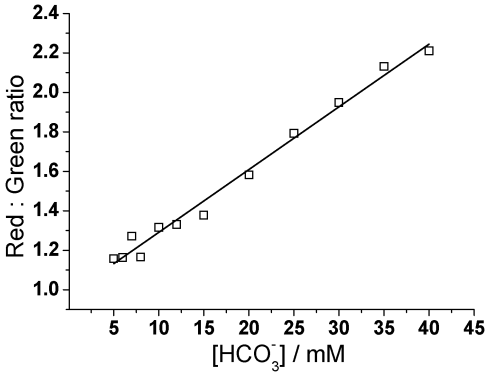


Fig. 2 Calibration curve showing the ratio of the overall $\Delta J = 2$ Eu emission band intensity (centred at 616 nm) to the $\Delta J = -1$ Tb emission band intensity (centred at 545 nm) for $[Ln{\cdot}L^1]^{3+}$ (3 : 2 mixture of complexes; 0.4 mM HSA, 0.1 M NaCl, pH 7.4, 1 mM HPO_4^{2-}, 1 mM ATP, 2 mM lactate, 0.13 mM citrate).

Increases of pCO_2 did not lead to an increase in observed emission intensity when carbonic anhydrase activity was suppressed by acetazolomide. These observations and conclusions accord with the *in vitro* control experiments. The absence of change in observed image and spectral emission intensity for the Tb analogues suggests that incubations of mixtures of $[Ln{\cdot}L^n]^{3+}$ ($n = 1, 2$; Ln = Eu, Tb), under varying pCO_2 conditions, can be used to calibrate the observed intensity variations observed by microscopy.[15] Such a procedure assumes that the Eu and Tb complexes exhibit identical cellular distributions. This is a reasonable assumption given their near-identical constitution and very similar anion and protein affinity profiles (Table 1).

Moreover, *in vitro* spectral calibration curves for $[Ln{\cdot}L^1]^{3+}$ and $[Ln{\cdot}L^2]^{3+}$ that model this change accord with this hypothesis. The variation of the Eu/Tb emission intensity ratio (3 : 2 mixture of complexes, examining bands centred at 616/545 nm, *i.e.* intensity area ratios) as a function of $[HCO_3^-]$ increased by 45% and 42% for $[Ln{\cdot}L^1]^{3+}$ and $[Ln{\cdot}L^2]^{3+}$ respectively, between 13.6 and 22.7 mM $[HCO_3^-]$ (Fig. 2). These values correspond to calculated concentrations of HCO_3^- for 3% and 5% CO_2 in the atmosphere at pH 7.4,[15] and were established in a background of 0.4 mM protein (HSA), 1 mM HPO_4^{2-}, 1 mM ATP, 2 mM lactate and 0.13 mM citrate. The linear range of these calibration curves corresponds well to the normal bicarbonate range in biological systems of 10 to 30 mM.

In summary, measurements of Eu/Tb emission intensity ratios report changes in bicarbonate concentrations both *in vitro* and *in cellulo*. As the probe localises to the mitochondrial region, changes in the steady-state bicarbonate concentration may now be able to be monitored by microscopy, as a function of perturbation to cell homeostasis.

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