The nature of the sensitiser substituent determines quenching sensitivity and protein affinity and influences the design of emissive lanthanide complexes as optical probes for intracellular use†

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Introduction of different substituents at the 7-position of a sensitising azaxanthone group in a series of emissive Eu and Tb complexes can determine the intracellular uptake and distribution profile and may be linked to modulation of protein affinity.

Emissive complexes of terbium and europium are emerging as useful stains[1,2] or responsive probes of the cellular environment.[3,4] Recently, it has been found that certain amphipathic lanthanide(III) complexes show a clear tendency to be internalised within live cells, allowing a study of the factors that determine their suitability as optical probes. The emissive probes are based on ligands in which a sensitising moiety is integrated into a ligand structure, permitting an evaluation of the effect of modifying the ligand structure with a common sensitising moiety.[5] Alternatively, a common ligand may be used and the structure of the sensitising moiety perturbed in order to appreciate the key elements that may be involved in determining the rate of cellular uptake and egress and the intracellular localisation profile. The sensitising moieties in these complexes are polycyclic heteroaromatic systems, with small singlet–triplet energy gaps.[6] The nature of this chromophore may determine not only the sensitivity of the overall complex to excited state quenching,[7] but also the affinity of the complex for proteins, of which serum albumin will be the most abundant in the cell culture medium. Non-covalent binding of the lanthanide complex to such a protein is very likely to influence cell uptake kinetics and subsequent intracellular trafficking.

With this background in mind, we set out to examine the properties of the Eu and Tb complexes of 6 ligands L^1–L^5 in which only the nature of one substituent on the azaxanthone sensitiser[8] is systematically varied. Their affinity for protein is assessed comparatively and their sensitivity to dynamic quenching of the lanthanide excited state by common reductants evaluated. These properties are then compared to a preliminary assessment of how complex structure affects the cellular uptake and localisation profile and the measured cytotoxicity.

The ligands L^1–L^5 were prepared as described in recent reports[2b,7,8] and their europium, gadolinium and terbium(III) complexes synthesised following established methods. The sensitivity of the terbium(III) complexes to quenching of the long-lived lanthanide excited state by iodide, ascorbate and urate was assessed. Each of these reductants is believed to quench the Tb $^5D_4$ excited state by an electron or charge transfer process.[7] Iodide quenches by a thermally activated collisional process, whereas urate and ascorbate (common low MW intracellular reductants typically at 0.2 to 1 mM concentrations) form an exciplex with the heteroaromatic group.[7] Stern–Volmer quenching constants (K_{SV}^{-1}/mM: representing the concentration needed to reduce the observed Tb emission lifetime by 50%) were measured, (Table 1), under standard conditions (pH 7.4, 0.1 M HEPES, 10 mM NaCl). The most obvious feature relates to the relative insensitivity to quenching of $[Tb{\cdot}L^2]^{2+}$ and $[Tb{\cdot}L^{3a}]^{3+}$. With $[Tb{\cdot}L^2]^{2+}$, the aromatic carboxylic acid is ionised (pH 7.4; $pK_a \sim 4$), so the reduced electrostatic potential will disfavour encounter. However, the effect observed here for urate and ascorbate quenching is much more dramatic than that found recently involving a change of complex charge from +3 to zero or −3, by varying the nature of the three exocyclic ligand donor groups.[5] Perhaps more intriguing was the behaviour of the monomethylamide complex, $[Tb{\cdot}L^{3a}]^{3+}$, a

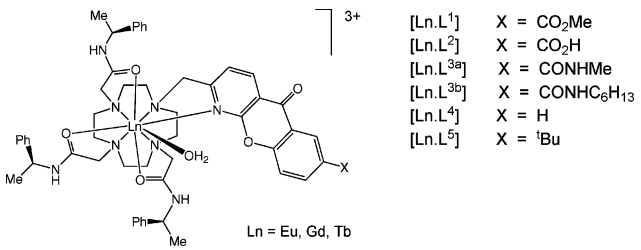

Table 1 Stern–Volmer quenching constants (K_{SV}^{-1}/mM) defining the sensitivity of selected terbium(III) complexes[a] to dynamic quenching of the metal excited state (298 K, pH 7.4, 0.1 M HEPES, 10 μM complex, 10 mM NaCl)

	K_{SV}^{-1}/mM		
Complex	Urate	Ascorbate	Iodide
$[Tb{\cdot}L^1]^{3+}$	0.02	0.19	5.60
$[Tb{\cdot}L^2]^{2+}$	>2[b]	>20[c]	>100[d]
$[Tb{\cdot}L^4]^{3+}$	0.04	0.37	9.20
$[Tb{\cdot}L^5]^{3+}$	0.02	0.30	10.9

[a] For $[Tb{\cdot}L^{3a}]^{3+}$, under the same conditions, the measured lifetime was 1.52 ms in the absence of added quencher and in the presence of 20 mM iodide, τ_{Tb} fell to only 1.48 ms, for 20 mM added ascorbate, $\tau_{Tb} = 0.73$ ms and for 0.2 mM added urate, $\tau_{Tb} = 1.06$ mM ($[Tb{\cdot}L^{3b}]^{3+}$ behaved similarly). [b] Following addition of 0.2 mM sodium urate, the τ_0/τ value was <5% changed, compared to a τ_0/τ value of 6.5 for $[Tb{\cdot}L^1]^{3+}$ after addition of 0.1 mM urate. [c] The τ_0/τ value increased by 20%, following addition of 5 μM sodium ascorbate (*cf.* $\tau_0/\tau = 4.5$ for $[Tb{\cdot}L^1]^{3+}$ with 1 mM added ascorbate). [d] The τ_0/τ value increased by < 10% following addition of 20 mM KI.

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complex with similar hydrophilicity around the aryl carboxamide moiety, that also resists quenching.

Addition of 0.2 mM human serum albumin (HSA) caused less than a 10% change in the measured lifetime when added to every terbium complex and in the presence of HSA quenching by urate and ascorbate was almost completely suppressed. The addition of HSA caused a reduction in the overall emission intensity of between 30 and 60% in each case. Parallel experiments were carried out with the series of Eu complexes; no change in spectral form was observed (*see ESI*) and the overall emission intensity fell by 70% for $[Eu{\cdot}L^5]^{3+}$, 60% for $[Eu{\cdot}L^2]^{2+}$ and 35% for $[Eu{\cdot}L^1]^{3+}$. Such behaviour is consistent with a modest amount of quenching of the chromophore excited state presumably *via* a charge transfer interaction with the azaxanthone, but with no perturbation of the lanthanide ion coordination environment. Further information on the interaction of the lanthanide complex with HSA may be gleaned by examining the modulation of the relaxivity of the Gd complexes with increasing protein concentration (*see ESI*).[9] The initial paramagnetic relaxivity, r_{1p}, of the Gd complexes was 3.0($\pm$0.4) mM^{-1} s^{-1}, typical of a cationic complex in which the water exchange is so slow that it quenches the inner sphere contribution, leaving only the outer and second sphere terms.[10] The variation of the measured relaxivity with added protein (range 0–0.35 mM) could not be fitted to a 1 : 1 or 2 : 1 binding isotherm, and the form of the binding curve suggested that 3 or 4 complexes were bound per protein with differing affinities. A qualitative assessment was made by comparing the concentrations of added protein needed to cause 50% of the total observed relaxivity change (0.25 mM complex) noting the limiting final relaxivities (in parentheses): $[Gd{\cdot}L^4]^{3+}$ −0.011 mM HSA ($r_{1p}^{lim} = 9.1$ mM^{-1} s^{-1}); $[Gd{\cdot}L^2]^{2+}$ −0.016 mM HSA (11.3 mM^{-1} s^{-1}); $[Gd{\cdot}L^1]^{3+}$ −0.019 mM HSA (8.3 mM^{-1} s^{-1}); $[Gd{\cdot}L^5]^{3+}$ −0.029 mM HSA (7.6 mM^{-1} s^{-1}). Thus, the protein affinity order follows the sequence given and the introduction of a substituent on the azaxanthone reduces protein affinity, with the carboxylate-substituted complex, $[Gd{\cdot}L^2]^{2+}$, the next most strongly bound, compared to the parent (R = H), $[Gd{\cdot}L^4]^{3+}$.

The cytotoxicity of the complexes was assessed by incubating NIH-3T3 cells (mouse skin fibroblasts) with varying concentrations of the complex for 24 h, determining toxicity using an MTT assay.[11] The IC_{50} values derived (Table 2) show the ^{t}Bu-substituted complex $[Gd{\cdot}L^5]^{3+}$ to be the most toxic under these conditions (58 μM). The cellular uptake and localisation of the terbium complexes was monitored in NIH-3T3, CHO and carcinoma (HeLa) cells by one and two photon microscopy, in the latter case following excitation at 720 nm using a Ti-sapphire laser. In both NIH-3T3 and CHO cells, $[Tb{\cdot}L^2]^{2+}$ and $[Tb{\cdot}L^{3a}]^{3+}$ were very slow to enter the cell, compared to each of the other complexes examined. Even following a 15 h incubation, microscopy images were of low intensity, (Fig. 1). The cellular localisation profile observed in each case after 18 h strongly resembles a late endosomal/lysosomal distribution that characterises the behaviour of the majority of such cationic complexes.[1,4] Two-photon microscopy was used to compare the profiles for $[Tb{\cdot}L^1]^{3+}$, $[Tb{\cdot}L^2]^{2+}$, $[Tb{\cdot}L^4]^{3+}$ and $Tb{\cdot}L^5]^{3+}$ and also the cellular uptake by studying images using varied incubation times. $[Tb{\cdot}L^1]^{3+}$ was found to be the most emissive of the series of complexes studied in both one and two-photon microscopy, and in contrast to the methylamide and carboxylate

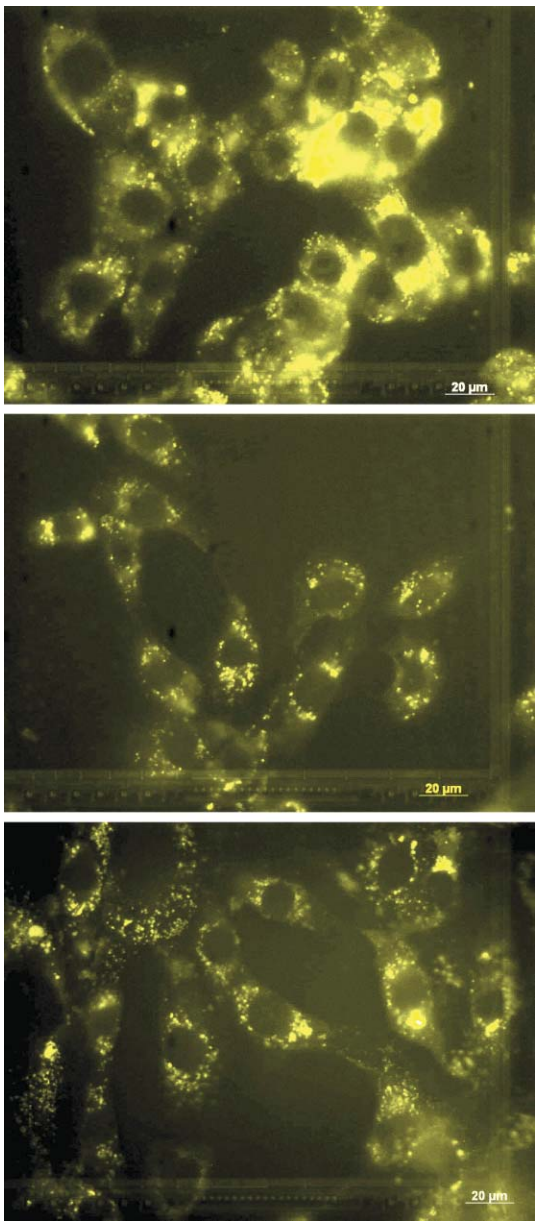

Fig. 1 Fluorescence microscopy images showing the localisation profile of $[Tb{\cdot}L^4]^{3+}$ (*upper*), $[Tb{\cdot}L^1]^{2+}$ (*centre*) and $[Tb{\cdot}L^{3b}]^{3+}$ (*lower*) in NIH-3T3 cells after an 18 h incubation, showing the predominant lysosomal distribution around the nucleus (100 μM complex).

Table 2 IC_{50} values (NIH-3T3 cells, 24 h incubation) for selected lanthanide complexes[a,b]

Complex	IC_{50}/μM
$[Tb{\cdot}L^1]^{3+}$	77(8)
$[Gd{\cdot}L^2]^{2+}$	148(3)
$[Tb{\cdot}L^{3a}]^{3+}$	>200[c]
$[Tb{\cdot}L^4]^{3+}$	>200
$[Gd{\cdot}L^5]^{3+}$	58(0.3)

[a] Values are the mean of at least three independent values, with standard deviations in parentheses. [b] On the chloride salts, using the method reported in reference 11 ('MTM' assay). [c] For the C_{12} amide analogue in the same cell line, the IC_{50} value is 8 μM, owing to membrane destabilisation leading to necrotic cell death.[2b]

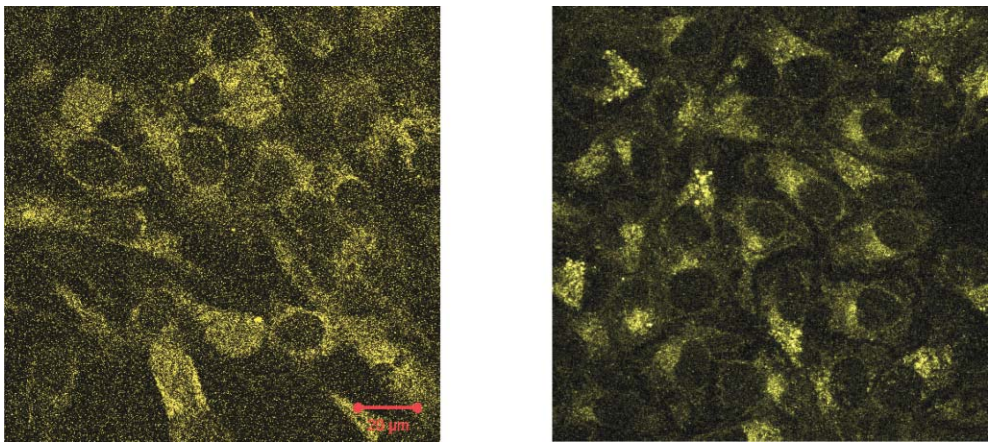

Fig. 2 Two-photon luminescence microscopy images (λ_{exc} 720 nm, 50 μM complex) showing terbium emission in NIH-3T3 cells from $[Tb{\cdot}L^1]^{3+}$ after 2 minutes (*left*), and after 5 h (*right*).

substituted complexes (>1 h incubation time before images could be obtained), was found to enter the cells in under 2 minutes (Fig. 2). The images reveal a combination of punctuate cytosolic and perinuclear endosomal distributions over the different time periods examined (2 min–5 h); similar final profiles were observed for the other complexes. The *tert*-butyl substituted complex, $[Tb{\cdot}L^5]^{3+}$ gave a similar final localisation profile, but after 6–10 h in HeLa cells, the cells began to increase markedly in volume, suggesting some degree of membrane destabilisation—in accord with its greater cytotoxicity.

In summary, the nature of the substituent on the heterocyclic sensitising group has a key role in defining the sensitivity of a lanthanide complex to excited state quenching and also strongly influences protein affinity. The sensitising moiety is a key recognition element in determining how readily the complex is taken up; by varying the 7-substituent, ingress is observed to be either very fast (R = CO_2Me) or very slow (R = CO_2^- or CONHMe) The latter property is likely to play an important role in both determining cytotoxicity and controlling the kinetics of cellular uptake and distribution.

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References

1 (*a*) H. C. Manning, S. M. Smith, M. Sexton, M. Haviland, M. F. Bai, K. Cederquist, N. Stella and D. J. Bornhop, *Bioconjugate Chem.*, 2006, **17**, 735; (*b*) A. S. Chauvin, C. Vandevyver, S. Comby and J.-C. G. Bunzli, *Chem. Commun.*, 2007, 1716; (*c*) D. Parker, S. Pandya and J. Yu, *Dalton Trans.*, 2006, 2757.

2 For recent examples of multi-photon microscopy to study lanthanide(III) probes of Eu systems: (*a*) A. Picot, A. D'Ako, P. L. Baldeck, A. Grichine, A. Duperray, C. Andraud and O. Maury, *J. Am. Chem. Soc.*, 2008, **130**, 1532; G.-L. Law, K.-L. Wong, C. W.-Y. Man, W.-T. Wong, S.-W. Tsao, M. H.-W. Lam and P. K.-S. Lam, *J. Am. Chem. Soc.*, 2008, **130**, 3714; (*b*) Tb complexes: F. Kielar, A. Congreve, G.-L. Law, E. J. New, D. Parker, K.-L. Wong, P. Castrenos and J. de Mendoza, *Chem. Commun.*, 2008, DOI: 10.1039/b803864c.

3 R. Pal and D. Parker, *Chem. Commun.*, 2007, 474; R. Pal and D. Parker, *Org. Biomol. Chem.*, 2008, **6**, 1120.

4 J. Yu, D. Parker, R. Pal, R. A. Poole and M. J. Cann, *J. Am. Chem. Soc.*, 2006, **128**, 2294; Y. Bretonnière, M. J. Cann, D. Parker and R. J. Slater, *Org. Biomol. Chem.*, 2004, **2**, 1624.

5 R. A. Poole, C. P. Montgomery, E. J. New, A. Congreve and D. Parker, *Org. Biomol. Chem.*, 2007, **5**, 2055.

6 (*a*) G. Mathis, *Clin. Chem. (Washington, D. C.)*, 1993, **39**, 1953; G. Mathis, *Clin. Chem. (Washington, D. C.)*, 1995, **41**, 1391; I. Hemmila and V. K. Mukkala, *Crit. Rev. Clin. Lab. Sci.*, 2001, **38**, 441; (*b*) J.-C. G. Bunzli and C. Piguet, *Chem. Soc. Rev.*, 2005, **34**, 1048; (*c*) G. Vereb, E. Jares Erjman, P. R. Selvin and T. M. Jouin, *Biophys. J.*, 1998, **74**, 2210.

7 F. Kielar, C. P. Montgomery, E. J. New, D. Parker, R. A. Poole, S. L. Richardson and P. A. Stenson, *Org. Biomol. Chem.*, 2007, **5**, 2975.

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

9 S. Aime, M. Botta, M. Fasano, G. S. Crich and E. Terreno, *JBIC, J. Biol. Inorg. Chem.*, 1996, **1**, 312; P. Caravan, N. J. Cloutier, M. T. Greenfield, S. A. McDermid, S. U. Dunham, J. W. M. Bulte, J. C. Amedio, R. J. Looby, R. M. Supkowski, W. D. Horrocks, T. J. McMurry and R. B. Lauffer, *J. Am. Chem. Soc.*, 2002, **124**, 3152; S. Aime, E. Gianolio, E. Terreno, R. Pagliarin, G. B. Giovenzano, M. P. Lowe, D. Parker, M. Sisti, M. Palmisano and M. Botta, *JBIC, J. Biol. Inorg. Chem.*, 2000, **5**, 488.

10 A. L. Thompson, D. Parker, D. A. Fulton, J. A. K. Howard, S. U. Pandya, H. Puschmann, K. Senanayake, P. A. Stenson, A. Badari, M. Botta, S. Avedano and S. Aime, *Dalton Trans.*, 2006, 5605.

11 J. Carmichael, W. G. DeGraff, A. F. Gazdar, J. D. Mina and J. B. Mitchell, *Cancer Res.*, 1987, **47**, 936.

