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

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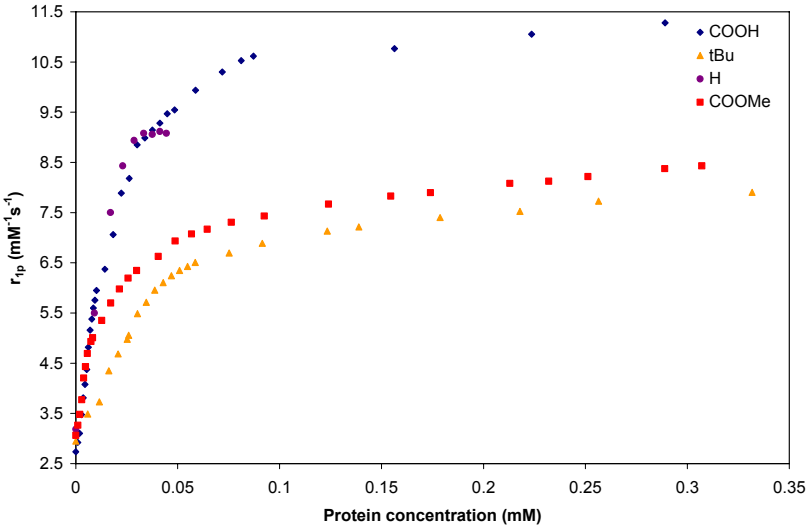
1. *Figure 1*: Variation of the relaxivity of the stated Gd complex with HSA concentration (310K, 60MHz).

2. *Figure 2*: Europium emission spectra for the stated complexes ([complex] 20μM; pH 7.4, 0.1 MHEPES, 298K) showing the intensity reduction on addition of 0.2mM human serum albumin; note the absence of change of spectral form consistent with no change in coordination environment

3. Experimental section giving synthesis, characterisation, cell culture, toxicity and microscopy details**.**

1.



Variation of relaxivity with added protein for complexes $[GdL^4]^{3+}$ (purple circles), $[GdL^1]^{3+}$ (red squares), $[GdL^2]^{3+}$ (blue squares) and $[GdL^5]^{3+}$ (yellow triangles) (310 K, H_2O, [complex] = 0.25mM).

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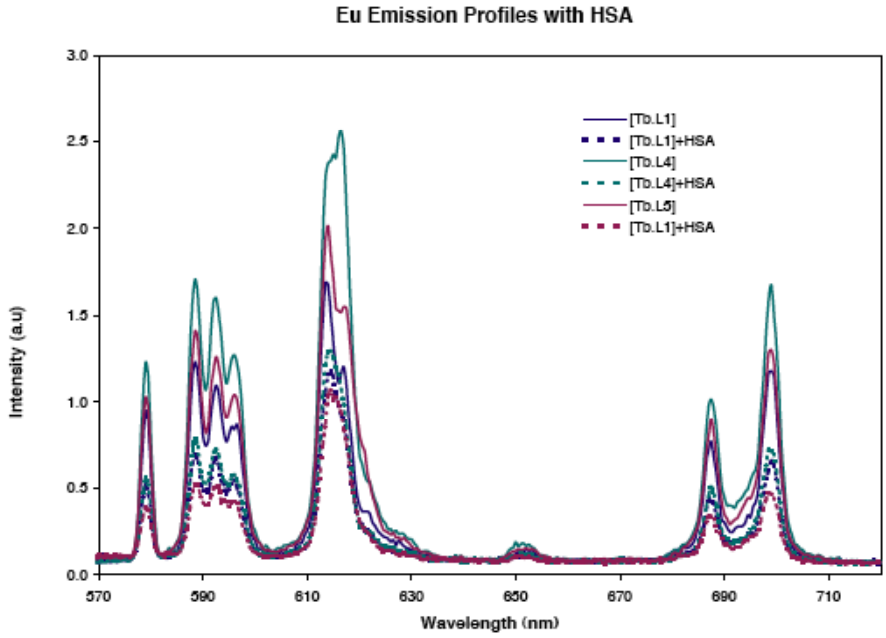
Figure 2



Europium emission spectra for the stated complexes ([complex] 20µM; pH 7.4, 0.1 MHEPES, 298K) showing the differing intensity reduction on addition of 0.2mM human serum albumin; note the absence of change of spectral form consistent with no change in the metal coordination environment.

2. Experimental

General methods

1-H and 13-C NMR spectra were recorded on a Varian Mercury 200 (1H at 199.975 MHz, 13-C at 50.289 MHz), Varian Unity 300 (1H at 299.908 MHz, 13C at 75.412 MHz), Varian VXR 400 (1H at 399.968, 13C at 100.572 MHz), or a Bruker AMX 500 spectrometer. Chemical shifts are reported relative to TMS and were referenced using the residual protio solvent resonances. Chemical shifts are reported in ppm and coupling constants in Hz. Splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). Mass spectra with electrospray ionisation (ES) were recorded on a VG Platform II (Fisons instruments), operating in positive or negative ion mode, with methanol as the carrier solvent. Accurate masses were recorded on a Thermo Finnigan LTQ instrument.

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UV/Vis absorbance spectra were recorded on a Perkin Elmer Lambda 900 UV/Vis/NIR spectrometer. Emission Spectra and Lifetimes were measured on a Perkin Elmer LS55 luminescence spectrometer and Instruments SA Fluorolog 3-11 spectrometer and DataMax v2.1 for Windows. -

The HPLC analysis and separation was carried out on a Perkin Elmer system comprising of Perkin Elmer Series 200 Pump, Perkin Elmer Series 200 Autosampler, Perkin Elmer Series 200 Diode array detector and Perkin Elmer Series 200 Fluorescence detector. GILSON-FC203B fraction collector was used in separation procedures. The stationary phase used was the Phenomenex Synergi 4μ Fusion-RP 80, and the columns used came in two different sizes; 150x4.6 mm (flow rate 1ml/min) and 250x10 mm (flow rate 5 ml/min). The gradients used are described in the Appendix. Lifetime values were measured as described in references 7a and 10. Single photon epifluorescence images were obtained using a Zeiss Axiovert 200M epifluorescence microscope with a digital camera.

Cell culture and toxicity

Three cell lines were selected for cellular studies CHO (Chinese Hamster Ovary) cells and NIH-3T3, mouse skin fibroblast (connective tissue) cells. Each line is transformed, and comprise adherent cells, which grow in a monolayer. These cell lines were cultured in a copper jacket incubator at 37°C, 20% average humidity and 5% (v/v) CO_2 in 75 cm3 plastic culture flasks. Cells for microscopy were grown on glass cover slips in 12-well plates DMEM (Dulbecco's Modified Eagle Medium), and F-12(Ham) medium were used for NIH3T3 and CHO cells respectively, each containing 10% (v/v) NCS (Newborn Calf Serum) and 1 % (v/v) penicillin-streptomycin. The HeLa (carcinoma) cells were cultured in an RMPI 1640 medium supplemented with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin in 5% CO_2. Complexes were loaded onto cells using the appropriate growth medium.

For the two-photon microscopy work, the cells were grown in 60 x 15 mm culture dishes at 2 ml/dish , and were allowed to attach overnight. The cell medium in each well was changed immediately prior to acquisition of the images. The cells were imaged in a tissue culture chamber (5% CO_2, 37^{o}C) and through a Zeiss 510 LSM

(upright configuration) confocal microscope with a femtosecond-pulsed Ti:Sapphire laser (Libra II, Coherent). The excitation beam produced by the fs laser, was tunable from 720-900 nm, (λ_{ex} = 720 nm, ~1mW), which was passed through an LSM 510 microscope with HFT 650 dichroic (Carl Zeiss, Inc.) and focused onto the cover-slip adherent cells using a 63 x oil immersion objective.

IC_{50} values were determined using the MTT assay, as described by Carmichael[11], which makes use of the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan product by the mitochondrial dehydrogenase of viable cells. This insoluble formazan was quantified spectrophotometrically upon dissolution in DMSO. Approximately 1 x 10^4 NIH-3T3 cells in 100 μL DMEM were seeded into each well of flat-bottomed 96-well plates and allowed to attach overnight. Complex solutions were added to triplicate wells to give final concentrations over a 2-log range. Following 24 h incubation, MTT (1.0 mM) was added to each well, and the plates incubated for a further 4 h. The culture medium was removed, and DMSO (150 μL) was added. The plates were shaken for 20 seconds and the absorbance measured immediately at 540 nm in a microplate reader. IC_{50} values were determined as the drug concentration required to reduce the absorbance to 50% of that in the untreated control wells, and represent the mean for data from at least three independent experiments.

Synthesis of lanthanide complexes

The ligands L^1, L^4 and L^5 and their lanthanide complexes were prepared as described in the literature [5,8]

$[TbL^1]Cl_3$,

A solution of 1-(2-methyl-7-methoxycarbonyl-1-azaxanthone)-4,7,10-*tris*[(*S*)-1-(1-phenyl)ethylcarbamoylmethyl]-1,4,7,10-tetraazacyclododecane (100 mg, 0.108 mmol) and $Tb(OTf)_3$ (64 mg, 0.106 mmol) in acetonitrile (6ml) was stirred at 60 ^oC overnight. The solvent was removed uner reduced pressure and the residue re-

dissolved in the minimum volume of acetonitrile. The solution was dropped onto diethyl ether (15 ml) and the precipitate was collected by centrifugation. This procedure was repeated three times. The residue was dissolved in MeOH/H_2O mixture and the triflate counter-ions were exchanged for chloride using an Amberlite anion exchange resin. The solvent was removed to yield the product as an off-white solid (70 mg). m/z (MALDI-TOF$^+$) 1079.4 (M-2H$^+$). HPLC: t_R 8.0 min (Method A, Appendix). τ_{Tb} (H_2O) 1.53 ms.

[EuL^1]Cl$_3$

The europium complex was prepared in an analogous manner. The product was obtained as an off-white solid (190 mg). m/z (MALDI-TOF$^+$) 1073.3 (M-2H$^+$). HPLC: t_R 8.0 min (Method A, Appendix). τ_{Eu} (H_2O) 0.58 ms.

[GdL^1]Cl$_3$

The gadolinium complex was prepared in an analogous manner. The product was obtained as an off white solid (90 mg). m/z (MALDI-TOF$^+$) 1078.5 (M-2H$^+$). HRMS (MALDI-TOF$^+$) 1078.3825 ($C_{53}H_{60}GdN_8O_7$ requires 1078.3833) (M-2H$^+$), HPLC: t_R 8.0 min (Method A, Appendix).

[TbL^2]Cl$_3$,

A solution of [TbL^1]Cl$_3$ (85 mg) in the mixture of H_2O (4 ml) and MeOH (4 ml) was stirred at room temperature for 24 h, maintaining the pH at 10 by the addition of aqueous KOH solution (1M). The reaction progress was monitored by HPLC (Method A, Appendix). The pH was adjusted to 7 with HCl solution (1M). The solution was syringe filtered and the solvent was removed. The residue was purified by reverse phase HPLC (Method B, Appendix). The solvent was removed to yield the product as a white powder (30 mg). m/z (MALDI-TOF$^+$) 1065.4 (M-2H$^+$). HPLC: t_R 7.7 min (Method A, Appendix). $\tau_{\square b}$ (H_2O) 1.62 ms.

N-hydroxysuccininimide ester of [Tb.L^2]

EDC (120 mg, 0.404 mmol) and N-hydroxysuccinimde (80 mg, 0.695 mmol) were added to the stirred solution of [TbL^{1b}](150 mg, worked up reaction mixture without HPLC purification) in dry DMSO (10 ml). The reaction mixture was stirred overnight at room temperature. The reaction progress was monitored by HPLC (Method A, Appendix). The product was precipitated by dropping the DMSO solution onto dry

diethyl ether (30 ml). The residue was triturated with acetonitrile and was re-dissolved in H_2O. The solvent was removed by freeze-drying to yield the product as a white solid (100 mg). m/z (ES^+) 639.5 (M+NHS-H^{2+}), 1277.1 (M+NHS-$2H^+$). HRMS (ES^+): found 639.210 (M+NHS-H^{2+}); $C_{60}H_{67}N_{10}O_{12}{}^{159}Tb$ requires 639.209, found 1277.415 (M+NHS-$2H^+$); $C_{60}H_{66}N_{10}O_{12}{}^{159}Tb$ requires 1277.411. HPLC: t_R 8.1 min (Method A, Appendix).

The europium and gadolinium complexes were prepared in an analogous manner. The solvent was removed by freeze-drying to yield the product as a white solid (130 mg). For the Eu complex: HPLC: t_R 8.1 min (Method A, Appendix). τ_{Eu} (H_2O) 0.53 ms.

$[TbL^{3a}]Cl_3$

Methylamine (5 µl, 33% wt in absolute ethanol, 0.040 mmol) was added to a stirred solution of the Tb N-hydroxysuccinimidyl complex (20 mg) in dry DMF (2 ml). The reaction mixture was stirred at room temperature under an argon atmosphere overnight and the reaction progress was monitored by HPLC. The reaction mixture was dropped onto diethyl ether (2 ml) and the product was collected by centrifugation. The residue was re-dissolved in H_2O and solvent removed by freeze-drying to yield the product as a white solid (10 mg). m/z (MALDI-TOF^+) 1078.4 (M-$2H^+$). HPLC: t_R 7.52 min (Method A, Appendix). τ_{Tb} (H_2O) 1.52 ms.

*$[TbL^{3b}]Cl_3$*The hexylamide complex was prepared in an analogous manner. The crude product was purified by HPLC (Method A, Appendix). The solvent was removed by freeze-drying to obtain the product as a white solid (8 mg). m/z (MALDI-TOF^+) 1148.4 (M-$2H^+$). HRMS (MALDI-TOF^+) 1148.4770 ($C_{58}H_{71}N_9O_6Tb$ requires: 1148.4775) (M-$2H^+$). HPLC: t_R 8.7 min (Method A, Appendix). τ_{Tb} (H_2O) 1.55 m

$[EuL^{3b}]Cl_3$,

The europium complex was prepared in an analogous manner to the terbium hexylamide derviative. The product was obtained as a white solid (9 mg). HPLC: t_R 8.68 min (Method A, Appendix).

Appendix 1: HPLC Conditions

Method A (Basic analytical gradient)

Solvent A: H_2O/0.1% HCOOH (TFA or no added acid)

Solvent B: ACN/0.1% HCOOH (TFA or no added acid)

Flow: 1 ml/min

Time (min)	Solvent A (%)	Solvent B (%)	Curvature
0	100	0	0
15	0	100	1
20	0	100	0
25	100	0	-3
27	100	0	0

Method B

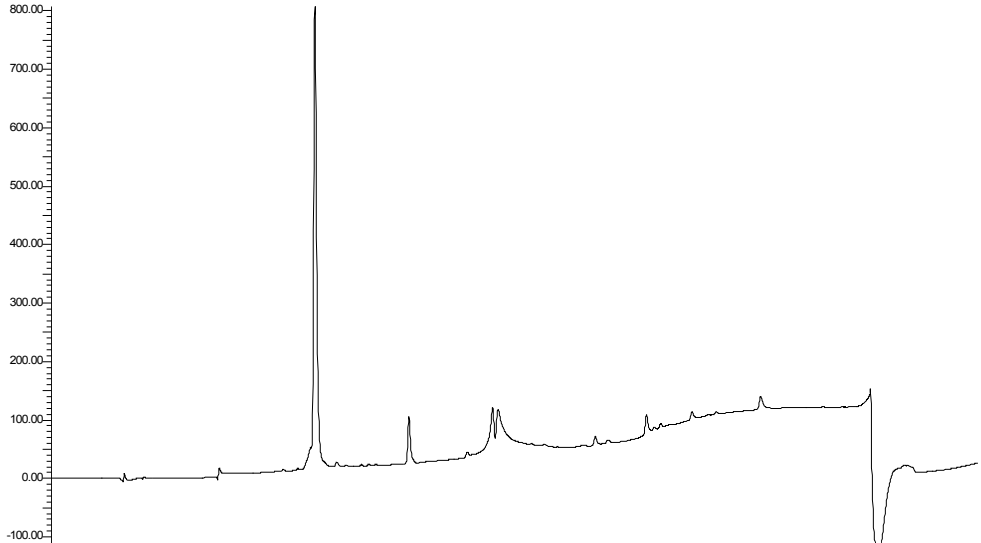
Solvent A: H_2O/0.1% HCOOH

Solvent B: ACN/0.1% HCOOH

Flow: 5 ml/min

Time (min)	Solvent A (%)	Solvent B (%)	Curvature
0	100	0	0
11	50	100	1
13	0	100	-3
16	0	100	0
16	100	0	-3
22	100	0	0

Chromatogram of [Tb.L^2] (λ_{em} 340 nm)



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Chromatogram of [Tb.L^{3a}]