J Incl Phenom Macrocycl Chem (2011) 71:435–444
DOI 10.1007/s10847-011-0009-4

ORIGINAL ARTICLE

Synthesis of lanthanide(III) complexes appended with a diphenylphosphinamide and their interaction with human serum albumin

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Received: 12 May 2011 / Accepted: 20 June 2011 / Published online: 12 July 2011

Abstract Two DOTA-based proligands bearing a pendant diphenylphosphinamide **4a** and **4b** were synthesised. Their Eu(III) complexes exhibit sensitised emission when excited at 270 nm via the diphenylphosphinamide chromophore. Hydration states of $q = 1.5$ were determined from excited state lifetime measurements (**Eu.4a** $k_{H_2O} = 2.14\,ms^{-1}$, $k_{D_2O} = 0.64\,ms^{-1}$; **Eu.4b** $k_{H_2O} = 2.67\,ms^{-1}$, $k_{D_2O} = 1.18\,ms^{-1}$). In the presence of human serum albumin (HSA) (0.1 mM **Eu.4a/b**, 0.67 mM HSA, pH 7.4) $q = 0.4$ for **Eu.4a** ($k_{H_2O} = 1.34\,ms^{-1}$, $k_{D_2O} = 0.75\,ms^{-1}$) and $q = 0.6$ for **Eu.4b** ($k_{H_2O} = 1.83\,ms^{-1}$, $k_{D_2O} = 1.05\,ms^{-1}$). Relaxivites (pH 7.4, 298 K, 20 MHz) of the Gd(III) complexes in the absence and presence of HSA (0.1 mM **Gd.4a/b**, 0.67 mM HSA) were: **Gd.4a** ($r_1 = 7.6\ mM^{-1}s^{-1}$ and $r_1 = 11.7\ mM^{-1}s^{-1}$) and **Gd.4b**. ($r_1 = 7.3\ mM^{-1}s^{-1}$ and $r_1 = 16.0\ mM^{-1}s^{-1}$). These relatively modest increases in r_1 are consistent with the change in inner-sphere hydration on binding to HSA shown by luminescence measurements on Eu.4a/b. Binding constants for HSA determined by the quenching of luminescence (Eu) and enhancement of relaxivity (Gd) were **Eu.4a** (27,000 $M^{-1} \pm 12\%$), **Eu.4b** (32,000 $M^{-1} \pm 14\%$), **Gd.4a** (21,000 $M^{-1} \pm 15\%$) and **Gd.4b** (26,000 $M^{-1} \pm 15\%$).

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Keywords Contrast agents · Magnetic resonance imaging · Gadolinium(III) · Europium(III) · Luminescence · Diphenylphosphinamides

Introduction

The use of gadolinium-containing contrast agents for Magnetic Resonance Imaging (MRI) has increased dramatically in recent years [1–4]. The introduction of chelates containing the highly paramagnetic Gd^{3+} ion enhances image contrast by increasing the longitudinal relaxation rate, R_1 ($1/T_1$) of surrounding water protons due to its high effective magnetic moment and long electron spin relaxation time. In order to minimize the inherent toxicity of the 'free' Gd^{3+} ion at the doses administered to patients, first generation contrast agents were developed which encase the Gd^{3+} ion in multidentate chelating ligands to render it safe for in vivo use. They are derived from diethylenetriaminepentaacetic acid (DTPA) or 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) (these are non-specific perfusion agents). Since the production of the first generation contrast agents there has been intense research into the design of probes whereby the water proton longitudinal relaxation rates are tailored to respond to changes in their biological environment, such as pH [5, 6], oxygen delivery/consumption [7], the presence of a biological molecule [8–10] or metal ions [11].

The efficacy of an MRI contrast agent is defined by its relaxivity, r_{1p}; the total paramagnetic relaxation rate enhancement of the water protons per unit concentration of the contrast agent ($mM^{-1}s^{-1}$) [12]. The design of contrast agent ligands is such that one or two coordination sites of the usually nine-coordinate Gd(III) ion are reserved for

water to bind and exchange with the bulk water. The most significant and controllable relaxation contribution with regards to contrast agent design comes from the inner-sphere. The longitudinal inner-sphere relaxation rate is expressed by Eq. 1:

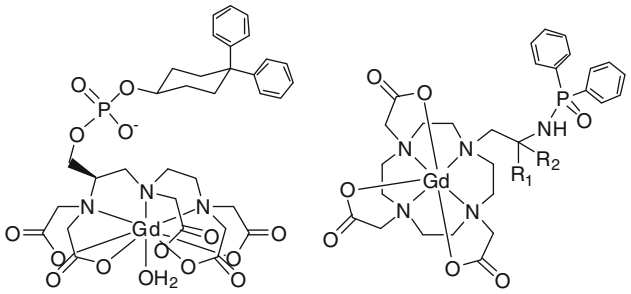
$$R_{1p}^{IS} = \frac{Cq}{55.6}\frac{1}{T_{1M} + \tau_m} \quad (1)$$

where C is the concentration of paramagnetic ion, q is the number of coordinated water molecules, T_{1M} is the longitudinal relaxation time of the inner-sphere waters and τ_m is the water exchange lifetime. According to Solomon-Bloembergen-Morgan (SBM) theory, several other factors further contribute towards the relaxivity, most notably τ_r, the rotational correlation time and T_{1e}, the electronic longitudinal relaxation time. The experimental longitudinal relaxation rate $1/T_{1M}$ depends on a correlation time τ_c that is itself a function of the correlation times τ_r, τ_m and T_{1e} in accordance to SBM theory (Eq. 2) [13, 14]. At typical clinical imaging fields (20–60 MHz) the smallest and therefore most dominant term in Eq. 2 is τ_r. As τ_c (and therefore relaxivity) for small rapidly tumbling complexes is greatly affected by τ_r, the lengthening of this parameter by reducing molecular tumbling leads to τ_r becoming comparable to both τ_m and T_{1e}. Hence, attachment of contrast agents to large, slowly tumbling proteins such as HSA lengthens τ_R and enhances relaxivity.

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_m} + \frac{1}{T_{1e}} \quad (2)$$

There has been a vast amount of research into contrast agent development towards the imaging of blood vessels and the circulatory system [15, 16]. The utilisation of MRI for this purpose is known as magnetic resonance angiography (MRA) [17]. Human serum albumin (HSA) is the most abundant protein in the circulatory system [18, 19]. The protein is made up of 585 amino acids, has a total molecular weight of 66,400 Da and is present in human serum at a concentration of ca. 0.67 mM [20]. Receptor induced magnetisation enhancement (RIME) is achieved by the incorporation of a hydrophobic moiety into the contrast agent [21]. Conjugation of low molecular weight Gd(III) chelates to serum proteins such as HSA through non-covalent binding of such hydrophobic moieties not only increases cardiovascular retention of the contrast agent, but enhances efficiency and image intensity by reducing the molecular tumbling of the Gd(III) chelate.

A variety of hydrophobic moieties have been incorporated into contrast agents over recent years [22–25], with the most successful of these Vasovist® now in clinical use [26–31]. To varying extents, these DOTA- and DPTA-based chelates exhibit smaller increases in r_1 than theory predicts because of three main factors that diminish the relaxivity of the HSA-bound complexes: independent rotation around e.g. C–C bonds in the link between the hydrophobic group and the chelate; displacement of bound waters by endogenous anions and/or protein carboxylate residues; slowing of the water exchange rate k_{ex} in the bound complex [32–35].



Vasovist™ Gd.4(a-b)

a. R_1 = H, R_2 = Me
b. R_1 = R_2 = Me

The diphenylphosphinoyl moiety has been used as an amino protecting group in peptide synthesis as the diphenylphosphinamide (dpp) [36]. *N*-dpp aziridines provide a convenient method of introducing aminoethyl functionality into a molecule, by ring opening of the corresponding aziridine [37, 38]. This protecting group can be readily removed under acid conditions (e.g. 95% TFA). We have been using this methodology to introduce aminoethyl groups onto DO3A-based ligands; however, we discovered that there is more to this unit than simply a protecting group. It was hypothesised that the pendant hydrophobic dpp group would bind non-covalently to Human Serum Albumin (HSA). Such attachment would be expected to result in the lengthening of the rotational correlation time, τ_R, of the Gd(III) contrast agent, enhancing relaxivity, r_1, and MR image intensity. The structure of the dpp moiety, containing two phenyl groups, bears a resemblance to the pendant hydrophobic group of the commercially available blood pool contrast agent Vasovist®. Due to this similarity in structure of the dpp-containing complexes, similar HSA binding affinity was expected. We have previously reported a strategy for rigidifying the binding of hydrophobic contrast agents by incorporating a binaphthyl moiety into a DOTA-like structure; however, this agent suffered from a slow water exchange rate engendered by the amide link to the macrocycle [39]. We hoped that complexes such as **Gd.4a/b** would have faster water exchange rates, albeit with some of the rigidity sacrificed cf. our binaphthyl-based complexes. Herein we report the synthesis of these complexes and study their interaction with HSA using both luminescence (Eu) and relaxometric (Gd) methods.

Experimental section

Reagents are commercially available and were used without further purification. Human serum albumin (HSA),

product number A-1653 (fraction V powder 96–99% albumin, containing fatty acids) was purchased from Sigma-Aldrich. Dry solvents were obtained using an Innovative Technology inc. PureSolv solvent purification system.

^{1}H NMR spectra were recorded using a Bruker 300 or 400 MHz spectrometer and referenced to residual solvent peaks. All chemical shifts are recorded in ppm. Accurate mass ESMS were recorded on a Finnigan MAT 900 XLT high resolution double focusing mass spectrometer with tandem ion trap (polyethylenimine reference compound) at the EPSRC National MS Service at Swansea, UK. Electronic absorption spectra were recorded on a Shimadzu UV180 spectrometer. IR measurements were carried out using a Perkin-Elmer Spectrum One FTIR spectrometer. Concentrations of the complexes used in the studies were determined by ICP-MS using an Agilent Technologies 7500ce inductively coupled plasma mass spectrometer.

^{1}H relaxation data

Observed longitudinal water proton relaxation times (T_{1obs}) were measured on a Stelar Spinmaster spectrometer (Stelar, Mede (PV) Italy) operating at 20 MHz, by means of the standard inversion-recovery technique (16 experiments, 2 scans). A typical 90° pulse width was 3.5 μs and the reproducibility of the T_{1obs} data was ±0.5%. The paramagnetic water proton relaxation rate, R_{1p} and relaxivity, r_{1p} were determined following Eqs. 3–5, where 0.38 is the diamagnetic contribution of the bulk water molecules [40]. Complex concentrations were determined via mineralization with nitric acid or by ICP-MS.

$$R_{1obs} = \frac{1}{T_{1obs}} \tag{3}$$

$$R_{1p} = R_{1obs} - 0.38 \tag{4}$$

$$r_{1p} = R_{1p}/[\mathrm{Gd}] \tag{5}$$

Luminescence emission spectra were recorded using a Jobin-Yvon Horiba FluoroMax-P spectrometer (using DataMax for Windows v2.2). Samples were held in a 10 × 10 nm or 10 × 2 nm quartz Hellma cuvette and a cutoff filter (550 nm) was used to avoid second-order diffraction effects. Eu(III) excitation was indirect (λ_{ex} = 270 nm).

Hydration state, q, determination

Lifetimes were measured by direct (395 nm) and indirect (λ_{ex} = 270 nm) excitation of the sample with a short 40 ms pulse of light (500 pulses per point) followed by monitoring the integrated intensity of light ($\Delta J = 2$) emitted during a fixed gate time of 0.1 ms, at a delay time later. Delay times were set at 0.1 ms intervals, covering 4 or more lifetimes. Excitation and emission slits were set to 5 nm. The data is applied to the standard first order decay (Eq. 6), minimized in terms of k by iterative least-squares fitting operation using Microsoft Excel, where I_{obs} is the observed intensity, I_0 is the initial intensity, and t is the time (ms). The calculated k values obtained in H_2O and D_2O are then applied to Eq. 7 to calculate the hydration state, q [41].

$$I_{obs} = I_o e^{-kt} + offset \tag{6}$$

$$q = 1.2[(k_{H_2O} - k_{D_2O}) - 0.25] \tag{7}$$

HSA binding studies

For both luminescence and relaxivity studies, aqueous solutions were buffered to pH 7.4 using phosphate buffered saline solution (10 mM PBS, 150 mM NaCl). Titrations were carried out at fixed complex concentration (0.1 mM) with varied [HSA] at 25 °C. The Eu(III) complexes were excited indirectly, via the dpp chromophore, at λ_{ex} ~270 nm and the variations in intensity of the $\Delta J = 2$ band recorded. The concentrations of the Eu(III) solutions was determined by ICP-MS while the exact concentration of HSA was determined by electronic absorption spectroscopy. The equilibrium Eq. 8 was assumed, where K is the affinity constant and [Ln] represents the Ln(III) complex. The concentration of HSA-bound complex, [LnHSA], is determined from Eq. 9, where $[\mathrm{HSA}]_T$ and $[\mathrm{Ln}]_T$ are the total concentrations of HSA and the Ln(III) complex [23].

$$[\mathrm{HSA}] + [\mathrm{Ln}] \overset{K}{\rightleftharpoons} [\mathrm{LnHSA}] \tag{8}$$

$$[\mathrm{LnHSA}] = \frac{\left([\mathrm{HSA}]_T + [\mathrm{Ln}]_T + 1/K\right) - \sqrt{\left([\mathrm{HSA}]_T + [\mathrm{Ln}]_T + 1/K\right)^2 - 4[\mathrm{HSA}]_T[\mathrm{Ln}]_T}}{2} \tag{9}$$

Concentrations were substituted for Eu(III) emission intensity (Eq. 10), where I_{calc} is the calculated Eu(III) emission intensity (to be compared with the experimentally observed intensity), I_{Eu} is the intensity of the Eu(III) complex when in the absence of HSA (i.e. at the start of the

titration) and I_{EuHSA} is the intensity of the Eu(III) complex when entirely bound to HSA (i.e. at the end of the titration).

$$I_{calc} = \frac{1}{[\mathrm{Eu}]}\left([\mathrm{Eu}]_T I_{Eu} + (I_{EuHSA} - I_{Eu})[\mathrm{EuHSA}]\right) \quad (10)$$

Equations 9 and 10 were combined to give Eq. 11, which was minimized in terms of K. Data analysis was performed using an iterative least-squares fitting procedure, assuming a 1:1 binding stoichiometry, operating in Microsoft Excel.

$$I_{calc} = \frac{1}{[\mathrm{Eu}]_T}\left(\begin{pmatrix}(I_{Eu}\times[\mathrm{Eu}]_T)+\\(I_{EuHSA}-I_{Eu})\end{pmatrix} \times\left(\frac{([\mathrm{HSA}]_T+[\mathrm{Eu}]_T+1/K)-}{\sqrt{([\mathrm{HSA}]_T+[\mathrm{Eu}]_T+1/K)^2-4[\mathrm{HSA}]_T[\mathrm{Eu}]_T}}\right)/2\right) \quad (11)$$

Relaxometric data analysis of the Gd–dpp complexes was carried out in a similar manner to that of the Eu(III) analogues, using an iterative least-squares fitting procedure, assuming a 1:1 binding stoichiometry, operating in Microcal Origin. In order to define the concentration of the HSA-bound Gd species in terms of its relaxation rate, R_{1calc}, Eq. 12 was used, which was combined with Eq. 9 to give Eq. 13, where R_{1Gd} is the observed longitudinal relaxation rate of the Gd(III) complex in the absence of HSA (i.e. at the start of the titration) and R_{1GdHSA} is the observed longitudinal relaxation rate of the Gd(III) when entirely bound to HSA (i.e. at the end limiting asymptotic value of the titration) and $[\mathrm{HSA}]_T$ and $[\mathrm{Gd}]_T$ are the total concentrations of HSA and the Gd(III) complex.

$$R_{1calc} = \frac{1}{[\mathrm{Gd}]_T}\left([\mathrm{Gd}]_T R_{1Gd} + (R_{1GdHSA} - R_{1Gd})[\mathrm{GdHSA}]\right) \quad (12)$$

$$R_{1calc} = \frac{1}{[\mathrm{Gd}]_T}\left(\begin{pmatrix}(I_{Gd}\times[\mathrm{Gd}]_T)+\\(R_{1GdHSA}-R_{1Gd})\end{pmatrix} \times\left(\frac{([\mathrm{HSA}]_T+[\mathrm{Gd}]_T+1/K)-}{\sqrt{([\mathrm{HSA}]_T+[\mathrm{Gd}]_T+1/K)^2-4[\mathrm{HSA}]_T[\mathrm{Gd}]_T}}\right)/2\right) \quad (13)$$

Aziridines 1a/b [36] and 1,4,7-tris(methoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane 2 [43] were prepared according to literature procedures.

General dpp ring opening synthesis

The appropriate aziridine (**1a/b**) and **2** were dissolved in dry MeCN (**3a**) or DMF (**3b**). On addition of K_2CO_3 the solution was stirred under nitrogen for 72 h at 85 °C (**3a**) or 120 °C (**3b**). After cooling, the solution was filtered through Celite and the solvent removed under reduced pressure. The product was purified by silica column chromatography with solvent gradient elution from 100% DCM to 8% MeOH, 1% ammonia and 91% DCM.

rac-1-[2′-(Diphenylphosphinylamino)-propyl]-4,7,10-tris(methoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane, ***3a***

2 (0.311 g, 0.78 mmol), K_2CO_3 (0.11 g, 0.79 mmol) and **1a** (0.21 g, 0.82 mmol). **3a** (0.121 g, 23%) was obtained as a yellow oil. $R_f = 0.60$ (10% MeOH, 1% NH_3, 90% DCM); δ_H (300 MHz; $CDCl_3$) 1.1 (3 H, d, C*H*$_3$) 1.9–4.4 (25 H, br, NC*H*$_2$C*H*$_2$N (16 H), OCC*H*$_2$N (6 H), NC*H*$_2$CHCH$_3$ (2 H), CH$_2$C*H*CH$_3$ (1 H)), 3.7 (9 H, 2× s, CO$_2$C*H*$_3$) 7.4 (6 H, m, Ar*H*), 7.9 (4 H, m, Ar*H*); δ_C (75 MHz; $CDCl_3$) 23.5 (CH*C*H$_3$), 43.4 (CH$_2$*C*HCH$_3$), 48.4, 50.4, 51.4, 53.5 (ring CH$_2$), 51.7, 52.0, 52.5 (CO$_2$*C*H$_3$), 55.0, 55.3, 55.9 (OC*C*H$_2$N), 66.2 (NC*H*$_2$*C*H$_2$N), 129.8 (Ar*C*), 132.1 (Ar*C*), 133.3 (Ar*C*), 134.4 (Ar*C*), 172.0 (*C*O$_2$), 173.5 (*C*O$_2$), 174.3 (*C*O$_2$); δ_P (121 MHz; $CDCl_3$) 24.1; *m/z* (HR-FAB+) $[M + H]^+$ calcd for $C_{32}H_{49}N_5O_7P$; 646.33696, found; 646.33703; λ_{max} (DCM)/nm 269; ν_{max} cm^{-1} 3352 (br), 2951, 2825 (CH), 1733 (C=O), 1667, 1438, 1308, 1196 (P=O), 1112.

1-[2′-(Diphenylphosphinylamino)-2′-(methyl)-propyl]-4,7,10-tris(methoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane, ***3b***

2 (0.305 g, 0.79 mmol), **1b** (0.217 g, 0.80 mmol) and K_2CO_3 (0.112 g, 0.81 mmol). **3b** (0.102 g, 19%) was obtained as a yellow oil. $R_f = 0.70$ (10% MeOH, 1% NH_3, 90% DCM); δ_H (300 MHz; $CDCl_3$) 1.2 (6 H, br s, CH$_2$(C*H*$_3$)$_2$), 2.2–3.5 (24 H, br, NC*H*$_2$C*H*$_2$N (16 H), OCC*H*$_2$N (6 H), NC*H*$_2$C(CH$_3$)$_2$ (2 H)), 3.6, 3.6, 3.7 (9 H, 3× s, OC*H*$_3$), 7.4 (6 H, m, Ar*H*) 7.8 (4 H, m, Ar*H*); δ_C (75 MHz; $CDCl_3$) 29.0 (C(*C*H$_3$)$_2$), 38.4 (*C*(CH$_3$)$_2$), 51.0 (NCH$_2$*C*H$_2$N), 54.2, 54.8 (OC*C*H$_2$N), 69.3 (NC*H*$_2$C(CH$_3$)$_2$), 127.4 (Ar*C*), 130.5 (Ar*C*), 172.4, 172.8 (*C*O$_2$); δ_P (121 MHz; $CDCl_3$) 19.4 (s); *m/z* (HR-FAB+) $[M + H]^+$ calcd for $C_{33}H_{51}N_5O_7P$; 660.35261, found; 660.35262; λ_{max} (DCM)/nm 269; ν_{max} cm^{-1} 3370 (br), 2926, 2852 (CH), 1732 (C=O), 1655, 1378, 1438, 1215 (P=O), 1110.

General methyl ester hydrolysis

Following literature methods [5], the methyl ester protected macrocycles were dissolved in 1 M LiOH (5 mL) and heated at 80 °C for 24 h. The resulting solution was loaded

onto a Dowex 50W strong acid-cation exchange column (H^+ form), washed with water and eluted with 12% ammonia solution. The solvent was then removed under reduced pressure and the residue taken up in water and lyophilized to give the product.

rac-1-[2′-(Diphenylphosphinylamino)propyl]-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane, ***4a***

3a (0.121 g, 0.19 mmol). **4a** (0.075 g, 58%) was obtained as a white hygroscopic powder. δ_H (300 MHz; D_2O) 1.9 (3H, s, CH_3), 1.2–4.0 (25 H, br, NC*H*$_2$C*H*$_2$N (16 H), OCC*H*$_2$N (6 H), NC*H*$_2$CHCH$_3$ (2 H), CH$_2$C*H*CH$_3$ (1 H)), 7.6 (6 H, m, Ar*H*), 7.9 (4 H, m, Ar*H*); δ_C (75 MHz; D_2O) 24.7 (CH*C*H$_3$), 46.0 (CHCH$_3$), 50.9, 51.3, 51.7, 52.1, 53.0, 53.7 (N*C*H$_2$*C*H$_2$N), 58.3, 59.2 (OC*C*H$_2$N), 62.4 (N*C*H$_2$CHCH$_3$), 131.5 (Ar*C*), 134.3 (Ar*C*), 135.4 (Ar*C*), 174.0, 178.7, 180.7 (*C*O$_2$); δ_P (121 MHz; D_2O) 28.1; *m/z* (HR-FAB+) $[M + H]^+$ calcd for $C_{29}H_{43}N_5O_7P$; 604.29001, found; 604.29002; λ_{max} (H_2O)/nm 269; ν_{max} cm^{-1} 3335 (OH), 1583 (C=O), 1410, 1175 (P=O), 1094.24, 996, 862.

1-[2′-(Diphenylphosphinylamino)-2′-(methyl)-propyl]-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane, ***4b***

3b (0.102 g, 0.16 mmol). **4b** (0.079 g, 71%) was obtained as a white hygroscopic powder. δ_H (300 MHz; D_2O) 1.5 (6 H, s, C*H*$_3$), 2.5–3.7 (24 H, br, NC*H*$_2$C*H*$_2$N (16 H), OCC*H*$_2$N (6 H), NC*H*$_2$C(CH$_3$)$_2$ (2 H)) 7.5 (6 H, m, Ar*H*), 7.7 (4 H, m, Ar*H*); δ_C (100 MHz; D_2O) 21.6 (*C*H$_3$), 42.8 (*C*(CH$_3$)$_2$), 48.8, 49.6, 51.8, 55.7, 56.5 (N*C*H$_2$*C*H$_2$N), 58.8, 61.2 (OC*C*H$_2$N), 66.6 (N*C*H$_2$C(CH$_3$)$_2$), 128.5 (Ar*C*), 131.4 (Ar*C*), 132.9 (Ar*C*), 137.9 (Ar*C*), 171.0, 178.1, 180.2 (*C*O$_2$); δ_P (121 MHz; D_2O) 26.4; *m/z* (ESMS+) 618 $[M + H]^+$; λ_{max} (H_2O)/nm 270; ν_{max} cm^{-1} 2970, 1738 (C=O), 1586, 1379, 1203 (P=O), 1088.

General complex synthesis

Two separate solutions of the appropriate pro-ligand and $LnCl_3 \cdot 6H_2O$ (slight excess to ensure all ligand used in complexation) in water (5 mL) were prepared and were each adjusted to pH 7 by addition of NaOH. On mixing, the pH fell to ~5 and the solution was adjusted to pH 6 using NaOH. After heating at 90 °C for 2 h, the pH was raised to 10 and the solution was filtered through a Celite plug to remove any precipitated $Ln(OH)_3$. The solution was then lowered to pH 5.5 and lyophilised. The product was extracted from the salt residue with 20% dry MeOH/DCM solution which was then removed under reduced pressure. The residue was taken up in water and lyophilised to give the Ln(dpp) complex.

Europium(III) rac-1-[2′-(diphenylphosphinylamino)propyl]-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane, ***Eu.4a***

$EuCl_3 \cdot 6H_2O$ (15.4 mg, 42.0 μmol) and **4a** (20.8 mg, 35.0 μmol). **Eu.4a** (25.7 mg, 99%) was obtained as a white hygroscopic powder. δ_H (400 MHz; D_2O; 4 °C) selected resonances: 25.7 (1H, s, NC*H*HCH$_2$N axial), 33.0 (1H, s, NC*H*HCH$_2$N axial), 34.7 (1H, s, NC*H*HCH$_2$N axial), 34.9 (1H, s, NC*H*HCH$_2$N axial), typical of a square-anti prismatic (SAP) geometry about Eu, the remaining resonances span −17.4 to 7.65 ppm; *m/z* (HR-ESMS+) $[M + H]^+$ calcd for $C_{29}H_{40}N_5O_7PEu$; 754.1872, found; 754.1876 (^{153}Eu); λ_{max} (H_2O)/nm 273; ν_{max} cm^{-1} 3368, 2984, 1594 (C=O), 1437, 1396, 1083 (P–O).

Europium(III) 1-[2′-(diphenylphosphinylamino)-2′-(methyl)-propyl]-4,7,10-tris(carboxy methyl)-1,4,7,10-tetraazacyclododecane, ***Eu.4b***

$EuCl_3 \cdot 6H_2O$ (11.2 mg, 31.0 μmol) and **4b** (14.4 mg, 23.0 μmol). **Eu.4b** (8.1 mg, 46%) was obtained as a white hygroscopic powder; δ_H (400 MHz; D_2O) 24.8 (1H, s, NC*H*HCH$_2$N axial), 26.2 (1H, s, NC*H*HCH$_2$N axial), 33.6 (1H, s, NC*H*HCH$_2$N axial), 35.6 (1H, s, NC*H*HCH$_2$N axial), typical of a square-anti prismatic geometry (SAP) about Eu, the remaining resonances span −20.4 to 9.1 ppm; *m/z* (HR-ESMS+) $[M + H]^+$ calcd for $C_{30}H_{42}N_5O_7PEu$; 768.2029, found; 768.2026 (^{153}Eu); λ_{max} (H_2O)/nm 270; ν_{max} cm^{-1} 3369, 2868, 1590 (C=O), 1389, 1085 (P–O).

Gadolinium(III) rac-1-[2′-(diphenylphosphinylamino)propyl]-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane, ***Gd.4a***

$GdCl_3 \cdot 6H_2O$ (10.7 mg, 29.0 μmol) and **4a** (12.0 mg, 20.0 μmol). **Gd.4a** (15.0 mg, 99%.) was obtained as a white hygroscopic powder. *m/z* (HR-ESMS+) $[M + H]^+$ calcd for $C_{29}H_{40}N_5O_7PGd$; 756.1886, found; 756.1885 (^{155}Gd); λ_{max} (H_2O)/nm 270; ν_{max} cm^{-1} 2819, 1587 (C=O), 1426, 1330, 1109 (P–O).

Gadolinium(III) 1-[2′-(diphenylphosphinylamino)-2′-(methyl)-propyl]-4,7,10-tris(carbo-xymethyl)-1,4,7,10-tetraazacyclododecane, ***Gd.4b***

$GdCl_3 \cdot 6H_2O$ (19.5 mg, 52.5 μmol) and **4b** (21.7 mg, 35.0 μmol). **Gd.4b** (17.3 mg, 64%) was obtained as a white hygroscopic powder. *m/z* HR-ESMS + $[M + H]^+$ calcd for $C_{30}H_{42}N_5O_7PGd$; 773.2057, found; 773.2060 (^{158}Gd); λ_{max} (H_2O)/nm 277; ν_{max} cm^{-1} 2628, 1575 (C=O), 1436, 1402, 1187, 1128, 1054 (P–O).

Results and discussion

The proligands **4a** and **4b** were synthesised via ring opening of the aziridine precursors (**1a/b**) with **2** followed by base hydrolysis of the methyl esters. Complexation was achieved in water using the appropriate $LnCl_3$ salt. Complex formation was verified by ESMS (and ^1H NMR spectroscopy of the Eu(III) analogues) (Scheme 1).

Luminescence studies

The luminescence emission spectrum of **Eu.4a** at pH 7.4 (Fig. 1—solid line) is typical in shape and form of Eu(III) complexes (the **Eu.4b** spectrum, not shown, is near identical). The peaks correspond to the $\Delta J = 0$ to $\Delta J = 4$ transitions, i.e. from the $^5D_0$ excited state to the ^7F_J ground state of Eu(III) where J = 0, 1, 2, 3, 4. The spectrum was obtained by indirect excitation via the dpp chromophore ($\lambda_{ex} = 270$ nm); this sensitised emission is much more intense than that obtained via direct excitation. As O–H oscillators can quench the excited state of Eu(III) complexes [41, 42], the hydration state of **Eu.4a/b** could be

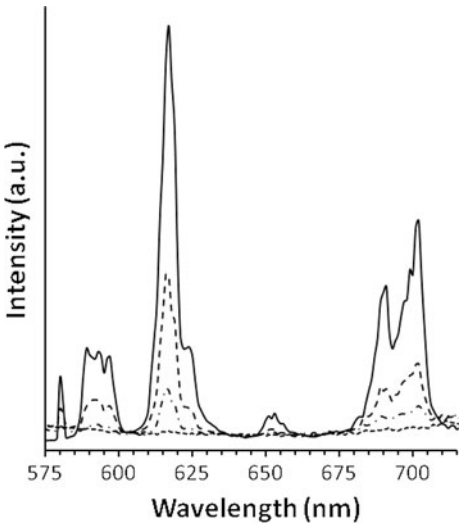
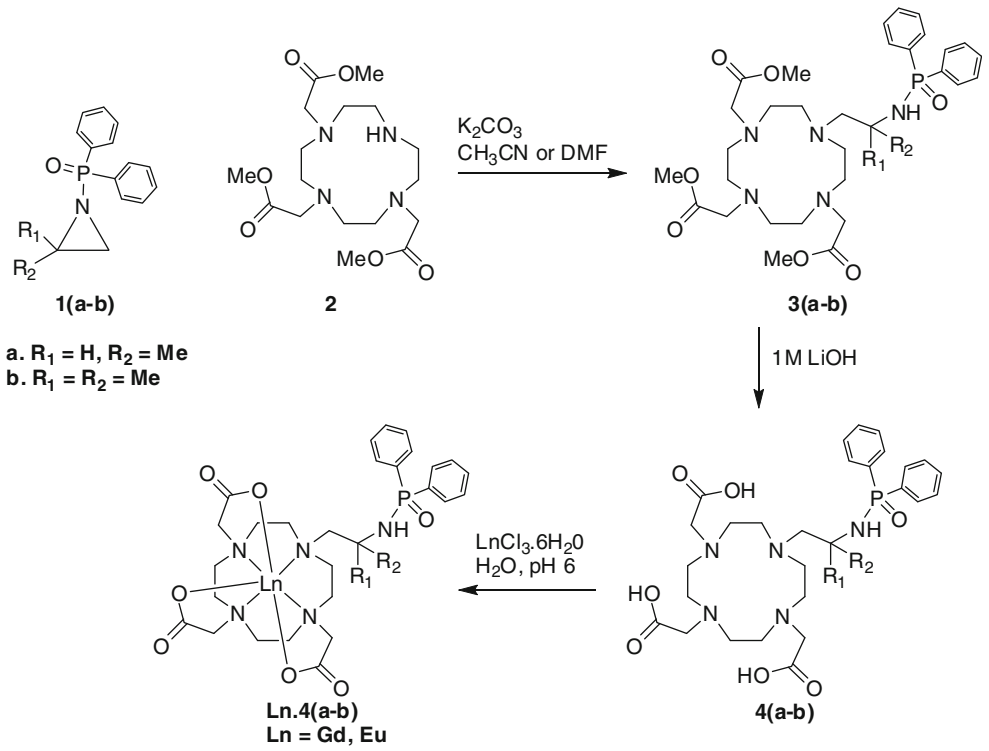


Fig. 1 Emission spectra of **Eu.4a** (0.1 mM, pH 7.4) in the absence (*solid line*) and presence of HSA (*dashed line* 0.16 mM, *dot-dash line* 0.32 mM, *dotted line* 1.6 mM)



Scheme 1 Synthesis of proligands and complexes

determined by exploiting the differing quenching ability of O–H cf. O-D oscillators. The difference in rate constants (measured in H_2O and D_2O) for depopulation of the excited state, enable q, the hydration state (number of inner-sphere waters) to be determined for the Eu(III) complexes (Eq. 7). These values can be reliably extrapolated to the analogous Gd(III) complexes [2]. At pH 7.4 both **Eu.4a** ($k_{H_2O} = 2.14\,ms^{-1}$, $k_{D_2O} = 0.64\,ms^{-1}$) and **Eu.4b** ($k_{H_2O} = 2.67\,ms^{-1}$, $k_{D_2O} = 1.18\,ms^{-1}$) gave a value of $q = 1.5$. Fractional q values such as this are indicative of the presence of a $q = 2$ species in equilibrium with a $q = 1$ species. A high value of q should be reflected in a high relaxivity for the corresponding Gd(III) complexes.

Figure 1 shows that there is a marked change in the intensity of the luminescence on addition of HSA. The spectra shown for 0.16, 0.32 and 1.6 mM HSA show a decrease in Eu(III) emission intensity on binding to HSA. In the presence of the protein, the diphenylphosphinamide moiety binds to hydrophobic sites in HSA and the Eu(III) luminescence is quenched (no Eu(III) emission is seen at 1.6 mM HSA). The intense emission in the absence of HSA is a result of energy transfer from the dpp triplet excited state to the ^5D_o excited state of Eu(III). The decrease in sensitization (or quenching) observed upon addition of HSA is likely to originate from an energy transfer mechanism, most likely involving the solitary tryptophan residue in the protein found in subdomain IIA (site I), a typical site for binding of MRI contrast agents [26–31]. Addition of HSA also causes a change in hydration state of the Eu(III) complexes; under physiological conditions in the presence of HSA (0.1 mM **Eu.4a/b**, 0.67 mM HSA, pH 7.4) both **Eu.4a** ($k_{H_2O} = 1.34\,ms^{-1}$, $k_{D_2O} = 0.75\,ms^{-1}$) and **Eu.4b** ($k_{H_2O} = 1.83\,ms^{-1}$, $k_{D_2O} = 1.05\,ms^{-1}$) show a significant reduction in inner-sphere hydration ($q = 0.4$ and $q = 0.6$ respectively). Predominantly $q = 2$ complexes (such as **Eu.4a/b** in the absence of HSA) are susceptible to displacement of inner-sphere waters by competitive binding of endogenous serum anions such as carbonate or protein carboxylic acid residues [44]. This decrease in emission intensity on increasing HSA concentrations can be used to determine binding constants for **Eu.4a/b** to HSA. Eu(III) emission spectra were recorded (0.1 mM complex) with varying concentrations of HSA. The complexes were excited indirectly, via the chromophore, at $\lambda_{ex} \sim 270$ nm and the variations in intensity of the $\Delta J = 2$ band (615 nm) were plotted *vs.* [HSA] (Fig. 2). The titrations of **Eu.4a** and **Eu.4b** were carried out at pH 7.4 (phosphate buffered saline). The decrease in emission intensity as HSA concentration is increased, fits to a 1:1 binding isotherm; for related complexes it is common to observe only one strong affinity site on HSA [22–31].

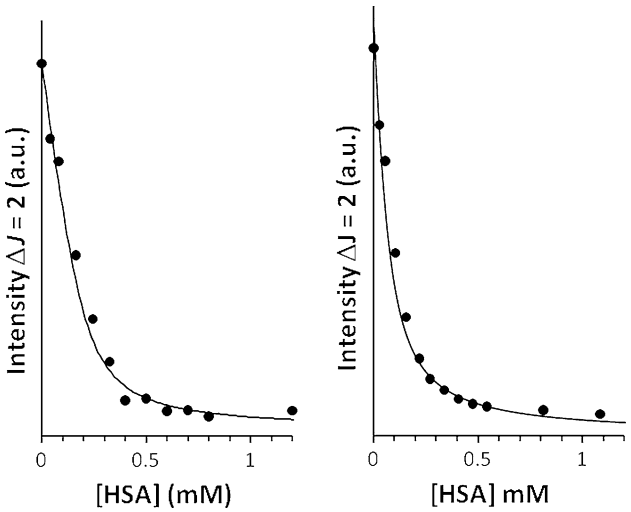


Fig. 2 Plot of $\Delta J = 2$ Eu(III) emission intensity versus [HSA] for **Eu.4a** (*left*) and **Eu.4b** (*right*) showing the experimental data (*solid circles*) and the calculated data (*line*). ~0.2 mM **Eu.4(a–b)**, 0.1 M NaCl, pH 7.4 (10 mM PBS), 25 °C

The binding constants obtained by this method for **Eu.4a** (27,000 $M^{-1} \pm 12\%$) and **Eu.4b** (32,000 $M^{-1} \pm 14\%$) are similar and show a fairly high affinity of these complexes for HSA, indeed slightly higher than that of the benchmark HSA-binding contrast agent Vasovist® (~11,000 M^{-1}) [29, 45]. These values suggest that at imaging concentrations (0.1 mM **Eu.4a/b**, 0.67 mM HSA) the complexes would be >90% bound to HSA (at 298 K). The values for the two complexes are too similar (within the errors of this method of determination) for any conclusions to be drawn with respect to the slightly higher value for **Eu.4b**.

Relaxivity studies

The relaxivities (298 K, 20 MHz) of the Gd(III) complexes were recorded at pH 7.4: **Gd.4a** $r_1 = 7.6\ mM^{-1}s^{-1}$ and **Gd.4b.** $r_1 = 7.3\ mM^{-1}s^{-1}$. These values are higher than typical $q = 1$ complexes such as $[GdDOTA]^-$ ($r_1 = 4.1\ mM^{-1}s^{-1}$) [46] and indicative of a predominantly $q = 2$ species (in equilibrium with a $q = 1$ species); this is consistent with the hydration state data obtained for the Eu(III) analogues. In the presence of HSA (0.1 mM **Gd.4a/b**, 0.67 mM HSA) the relaxivities were: **Gd.4a** $r_1 = 11.7\ mM^{-1}s^{-1}$ and **Gd.4b.** $r_1 = 16.0\ mM^{-1}s^{-1}$. These increased relaxivities are disappointing given the apparently high binding affinities these complexes have for HSA; however, given the luminescence data on the Eu(III) analogues these lower than hoped for values are to be expected. It was noted that the q-values of **Eu.4a** and **Eu.4b** were both significantly lowered on binding to HSA (from $q = 1.5$ to $q = 0.4$ and 0.6 for **Eu.4a** and **Eu.4b**

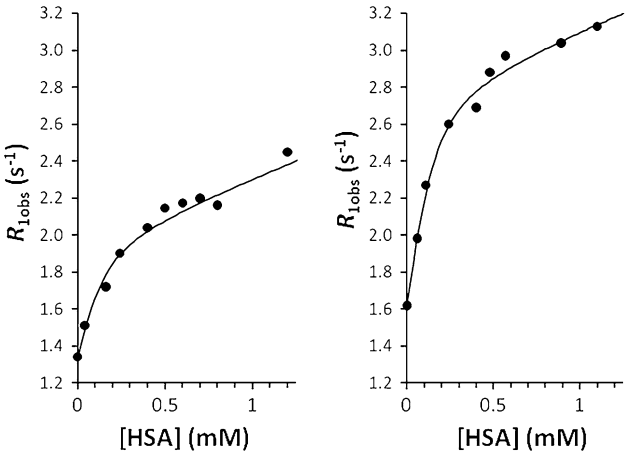


Fig. 3 Plot of the observed longitudinal relaxation rate, R_{1obs}, versus [HSA] for **Gd.4a** (*left*) and **Gd.4b** (*right*) showing the experimental data (*solid circles*) and the calculated data (*line*). ~0.2 mM **Gd.4(a–b)**, 0.1 M NaCl, pH 7.4 (10 mM PBS), 20 MHz, 25 °C

respectively). This displacement of inner-sphere waters by, presumably, carboxylate residues within the HSA binding site is a common problem for neutral $q = 2$ complexes [44]. Vasovist® ($q = 1$) has r_1 ~35 $mM^{-1}s^{-1}$ under similar conditions (298 K, pH 7.4, 20 MHz) [47]. When MRI contrast agents bind to large slowly tumbling proteins such as HSA, relaxivity is enhanced; however, lower than expected relaxivities are often a result of several effects: lowering of hydration state by competitive binding of endogenous anions, rotational freedom within the bound agent (i.e. about C–C bonds, thus the motion of the contrast agent is not effectively coupled to the motion of the protein), and slowing of the water exchange rate. A combination of these factors (particularly the first two) is likely to explain the lower than hoped for relaxivities of HSA-bound **Gd.4a/b**. It is interesting to note that a higher relaxivity was observed for HSA-bound **Gd.4b.** *cf.* **Gd.4a** ($r_1 = 16.0$ $mM^{-1}s^{-1}$ cf. $r_1 = 11.7$ $mM^{-1}s^{-1}$); this may be tentatively assigned to the fact that there is additional steric bulk in **Gd.4b** (two methyl groups in the link to the phosphinamide) leading to less free rotation of the bound complex.

Relaxivity versus [HSA] titrations were carried out for **Gd.4a/b** in the same manner as for the Eu(III) complexes, the difference being that relaxation rate measurements were used instead of luminescent emission. As expected, the observed relaxation rates increased upon addition of HSA. This is because of the increased rotational correlation time, τ_R, of the protein bound complex (Fig. 3).

The data analysis was carried out in a similar manner to that of the Eu(III) analogues. The affinity constants calculated from the relaxivity data are, as expected, similar to those calculated from luminescence: **Gd.4a** (21,000 M^{-1} ± 15%) and **Gd.4b** (26,000 M^{-1} ± 15%), again showing a relatively high affinity for HSA. At imaging concentrations (0.1 mM **Gd.4a/b**, 0.67 mM HSA) the complexes would be >90% bound to HSA (at 298 K).

Conclusion

Eu(III) and Gd(III) complexes bearing pendant diphenylphosphinate residues have been prepared which display a hydration state $q = 1.5$ at pH 7.4, reflected in the relatively high relaxivities that were observed for **Gd.4a/b**. The hydrophobicity of the dpp moiety enables non-covalent binding to HSA. This binding event can be monitored using luminescence (Eu) and relaxometry (Gd) giving relatively high binding affinities, whereby >90% of the agents would be non-covalently bound to HSA under physiologically relevant concentrations of complex and HSA. Unfortunately the expected relaxivity gains were disappointing; this can be attributed primarily to displacement of inner-sphere waters on binding to the protein. This lowering of hydration state was shown by luminescent lifetime measurements on the Eu(III) analogues.

As the binding affinities of the complexes was shown to be favourably high, we are seeking to maintain this high affinity whilst suppressing the reduction in hydration state (caused by endogenous anion binding) by incorporating negatively charged substituents in the alpha position of the carboxylate arms [8, 9]. We are also investigating further, the binding of **Ln.4a**. The compound synthesised in this report was a racemic mixture of the R and S enantiomer (at $CH_2CH(CH_3)NHP$). Although in previous work using binaphthyl-derived DOTA-based complexes we saw no discrimination between R and S binaphthyl units, it is possible that the R and S-enantiomers of **Ln.4a** have different binding affinites (e.g. the S-enantiomer of Eovist® has been shown to bind to HSA with a greater affinity than the R-enantiomer) [48].

Acknowledgments We thank the EPSRC National Mass Spectrometry Service Centre, Swansea for high resolution ESMS.

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