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A Pyrophosphate-Responsive Gadolinium(III) MRI Contrast Agent

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Abstract: This study shows that the relaxivity and optical properties of functionalised lanthanide-DTPA-bis-amide complexes (lanthanide = Gd^{3+} and Eu^{3+}, DTPA = diethylene triamine pentaacetic acid) can be successfully modulated by addition of specific anions, without direct Ln^{3+}/anion coordination. Zinc(II)-dipicolylamine moieties, which are known to bind strongly to phosphates, were introduced in the amide "arms" of these ligands, and the interaction of the resulting Gd–Zn_2 complexes with a range of anions was screened by using indicator displacement assays (IDAs). Considerable selectivity for polyphosphorylated species (such as pyrophosphate and adenosine-5′-triphosphate (ATP)) over a range of other anions (including monophosphorylated anions) was apparent. In addition, we show that pyrophosphate modulates the relaxivity of the gadolinium(III) complex, this modulation being sufficiently large to be observed in imaging experiments. To establish the binding mode of the pyrophosphate and gain insight into the origin of the relaxometric modulation, a series of studies including UV/Vis and emission spectroscopy, luminescence lifetime measurements in H_2O and D_2O, ^{17}O and ^{31}P NMR spectroscopy and nuclear magnetic resonance dispersion (NMRD) studies were carried out.

Keywords: contrast agents · gadolinium · lanthanides · MRI · sensors

Introduction

Interest in lanthanide(III) complexes has increased dramatically in recent years as their unique magnetic and spectroscopic properties offer enormous potential for a variety of imaging applications, especially as contrast agents for nuclear magnetic resonance imaging (MRI). The current generation of contrast agents (CAs) in clinical use is able to provide invaluable anatomical information.[1] Recently, there has been increasing interest in developing responsive contrast agents, which would be able to report biological events by modulation of the relaxivity of the CAs.[2] Responsive MRI contrast agents have been reported, which respond to several different conditions, analytes or biomolecules, such as pH,[3] temperature,[4] $p(O_2)$,[5] enzyme activity[6] and metal ions.[7] As in the broader field of molecular recognition, CAs that are responsive to anionic analytes have lagged behind due to the intrinsic difficulty in binding anions selectively in aqueous media, especially in vivo. This may be attributed to several factors, especially high solvation energies, pH sensitivity and, in the case of in vivo imaging, highly competitive endogenous anions.

In spite of the above, in recent years examples of CAs that interact with anions have appeared: either responding to anion concentration,[8] or by using a change in binding affinity to endogenous anions to signal interaction with a different analyte, most notably enzymes.[9] Additionally, binding of endogenous anions has been frequently encountered as an unintended consequence of attempts to increase contrast agent relaxivity (for example by reduction of the coordination number of Gd^{3+} or by reduction of steric hindrance around free coordination sites).[10] Generally, however, these contrast agents interact with anions by direct lanthanide–

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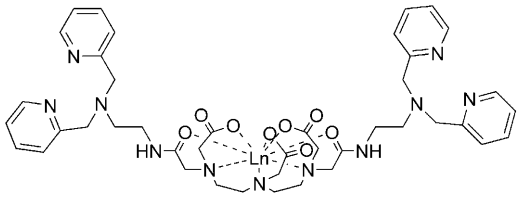
anion coordination, limiting the use of other established anion-binding moieties. As in other areas of anion recognition and sensing, a move towards modular design is desirable, in which the anion-binding moieties are separate from the "reporting" group (the lanthanide in this case), and might allow the development of anion-responsive MRI agents.

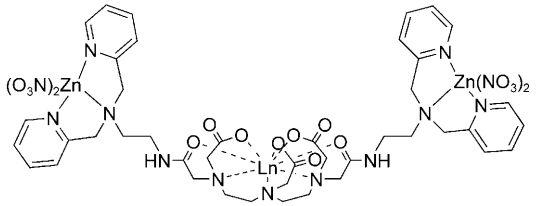
The work herein presented, involves the incorporation of known selective phosphate-binding moieties into a gadolinium(III) complex, endeavouring to achieve significant modulation of relaxivity on interaction with specific phosphorylated species, without direct gadolinium(III)–anion coordination.

Over the past few years, it has been shown that zinc(II)–dipicolylamine (Zn–DPA) complexes bind strongly and selectively to a range of phosphate species (including inorganic phosphates and phosphorylated organics such as phosphopeptides and amino acids).[11] Therefore, this type of complex was chosen as the phosphate-binding moiety to be incorporated in our lanthanide(III) complexes. These complexes have been used to develop a variety of phosphate hosts. Often, two or more Zn-DPA units are placed in either a rigid or flexible arrangement to further improve selectivity of the host for the specific phosphate guest. Several of the resulting receptors have been used as chemical sensors by using indicator displacement assays (IDAs).[11b,c,12]

The DTPA–bis(amide) complex (DTPA = diethylenetriaminepentaacetic acid) [Ln(L)] has previously been reported as a prototype zinc-sensitive optical probe[13] ($Ln = Eu^{3+}$ or Tb^{3+}) and MRI contrast agent[7c,d] ($Ln = Gd^{3+}$). The complex was designed to bind Zn^{2+} between the two converging DPA "arms", blocking water exchange and altering the optical/MRI properties of the complex. Unforeseen binding of Zn^{2+} ions by both DPA substituents independently to yield $[Gd(L)Zn_2](NO_3)_4$ was thought to be responsible for a further equilibrium, leading to the design of another generation of complexes in which this divergent binding was prevented.[7d] Herein we report a study of the interaction between the bis-zinc(II) complexes $[Ln(L)Zn_2](NO_3)_4$ (with $Ln = Gd^{3+}$ and Eu^{3+}) and different phosphate-containing anions. We show that pyrophosphate (PPi) binds strongly to these complexes resulting in a change in the relaxivity ($Ln = Gd^{3+}$) and optical properties ($Ln = Eu^{3+}$) of the lanthanide complexes.



[Ln(L)]
Ln = Gd^{3+}, Eu^{3+}



$[Ln(L)Zn_2](NO_3)_4$
Ln = Gd^{3+}, Eu^{3+}

Results and Discussion

Synthesis of complexes and characterisation of their anion binding: The [Ln(L)] and $[Ln(L)Zn_2](NO_3)_4$ complexes (with $Ln = Gd^{3+}$ and Eu^{3+}) were synthesised by adaptations of literature procedures (see the Supporting Information for details of their syntheses). Briefly, alkylation of di-(2-picolyl)amine with *N*-(2-bromoethyl)phthalimide followed by deprotection yielded amino ethyl di-(2-picolyl)amine.[14] This amine was treated with DTPA-bis(anhydride)[7c] to yield ligand H_3L which was treated in situ with the corresponding $LnCl_3$ to yield [Ln(L)] ($Ln = Gd^{3+}$ and Eu^{3+}). These neutral complexes were purified by reverse-phase flash chromatography. The $[Ln(L)Zn_2](NO_3)_4$ complexes were prepared in situ by reaction of aqueous [Ln(L)] with two equivalents of $Zn(NO_3)_2 \cdot 6H_2O$. Quantitative formation of such complexes has been established in the literature, and although in this case they may be isolated, they are extremely hygroscopic making their handling and analytical characterisation difficult.

Once the lanthanide–zinc complexes had been prepared, it was next necessary to assess their ability to bind a range of different anions. For this, indicator displacement assays were carried out by using $[Gd(L)Zn_2](NO_3)_4$, which was shown to bind to different dyes. With pyrocatechol violet (PV) a desirably strong binding, and considerable colour change upon binding, was observed: bound PV is bright blue, "free" PV is yellow-brown. Other dyes such as gallocyanin (GC) underwent less obvious colour changes on binding, but their weaker binding was interesting to contrast with that of PV. Screening of a variety of common anions for binding to the metalloreceptor in an IDA that uses PV (Figure 1) and GC (see the Supporting Information) showed a clear selectivity for polyphosphate species (e.g., pyrophosphate and adenonsine-5′-triphosphate (ATP)) over monophosphates and other anions; a degree of GC displacement by phosphorylated amino acids was also observable (see the Supporting Information).

The indicator binding stoichiometry was investigated by constructing Jobs plots of PV binding to $[Gd(L)Zn_2](NO_3)_4$ (see the Supporting Information), in which the maximum corresponded to a 1:1 stoichiometry (although considerable asymmetry hinted at a more complex system). Titrations of PV/$[Gd(L)Zn_2](NO_3)_4$ conjugates with displacing anions gave responses consistent with simple 1:1 models, however, subsequent work showed a 1:1 model to be inadequate (see

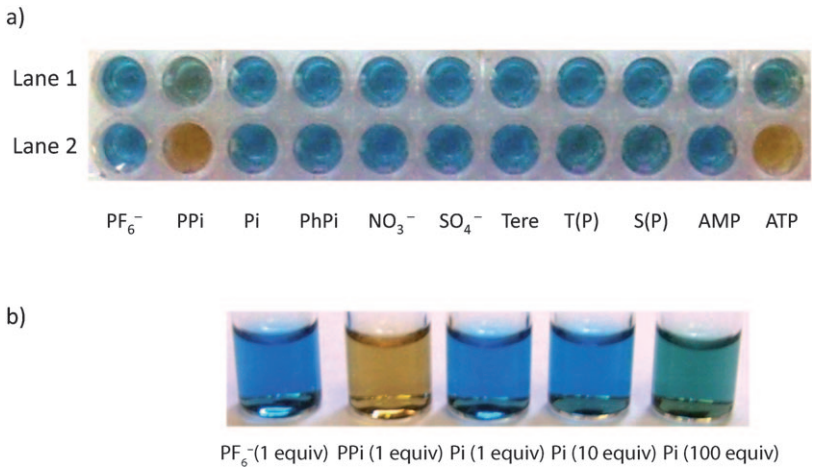


Figure 1. Indicator displacement assays that use $[Gd(L)Zn_2](NO_3)_4$, pyrocatechol violet and a range of different anions. a) Shows the changes observed when 1:1 mixture of $[Gd(L)Zn_2](NO_3)_4$ and pyrocatechol violet were mixed with one equivalent (Lane 1) and ten equivalents (Lane 2) of the corresponding anion. b) Addition of up to 100 equivalents of inorganic phosphate (Pi) to a 1:1 mixture of $[Gd(L)Zn_2](NO_3)_4$ and pyrocatechol violet does not lead to any significant colour change, whereas addition of one equivalent of pyrophosphate (PPi) induces a clear colour change from blue (bound PV) to orange (unbound/displaced PV). Concentration of $[Gd(L)Zn_2](NO_3)_4 = 0.05$ mM, 10 mM HEPES, pH 7.4, RT. Abbreviations used in this figure: PhPi = phenyl phosphate, Tere = terephthalate, T(P) = Fmoc-phosphotyrosine, S(P) = Fmoc-phosphoserine, AMP = adenosine-5′-monophosphate, ATP = adenosine-5′-triphosphate.

below) and unambiguous fitting of displacement data to a more complex model to determine binding constants was not possible.

Once strong binding to pyrophosphate had been established, the effect on relaxivity of the $[Gd(L)Zn_2](NO_3)_4$ complex on interaction was investigated. T_1 measurements showed a modulation of the complex's relaxivity on titration with pyrophosphate solutions (see Figure 2a), which was also observable in MRI images of sample tube "phantoms" (4.7 T, see Figure 2b). Negative control experiments with [Gd(L)], in the absence of zinc(II) showed little response over the same range of pyrophosphate concentration (see the Supporting Information), confirming that the zinc(II)-DPA moieties in $[Gd(L)Zn_2](NO_3)_4$ are responsible for the interaction between the complex and pyrophosphate.

The inflection in the relaxometric response of $[Gd(L)Zn_2](NO_3)_4$ to PPi (see Figure 2a) on addition of around one equivalent PPi suggests a sequential 1:1 and then 1:2 binding, as described in Equation (1) (where $K_{HG} \gg K_{HG2}$, H denoting the host, a $[Gd(L)Zn_2]$ species, and G denoting the guest, PPi).

$$[H] \overset{K_{HG}}{\rightleftharpoons} [HG] \overset{K_{HG2}}{\rightleftharpoons} [HG]_2 \quad (1)$$

To gain further insight into these equilibria, the analogous europium complex $[Eu(L)Zn_2](NO_3)_4$ was prepared and its luminescence was studied. Small changes in the emission spectrum of the complex were observed on addition of pyrophosphate (see Figure 3a). The subtle change in the form of the hypersensitive $\Delta J = 2$ transition ($\lambda = 617$ nm) is clear. On titration, it was possible to fit the variation to a stepwise 1:1/1:2 model (by using SpecFit/32, 2006, see Figure 3b), yielding formation constants $\log\beta_{HG} = 4.5 \pm 0.2$ and $\log\beta_{HG2} = 7.4 \pm 0.2$, corresponding to stepwise binding constants $\log K_{HG} = 4.5 \pm 0.2$ and $\log K_{HG2} = 2.9 \pm 0.2$. By using these values (with the presumption that there is little difference in the PPi binding of the Gd^{3+} and the Eu^{3+} complexes) it was possible to fit the T_1 data by least-squares fitting to a theoretical binding model (Figure 4). This allowed us to estimate the relaxivities (r_1) of the three different complexes proposed to be in equilibrium. The r_1 value estimated for $[Gd(L)Zn_2](NO_3)_4$ is 3.13 $mM^{-1}s^{-1}$, whereas those for the mono- and bispyrophosphate complexes are 1.97 $mM^{-1}s^{-1}$ and 4.22 $mM^{-1}s^{-1}$, respectively (all at 500 MHz).

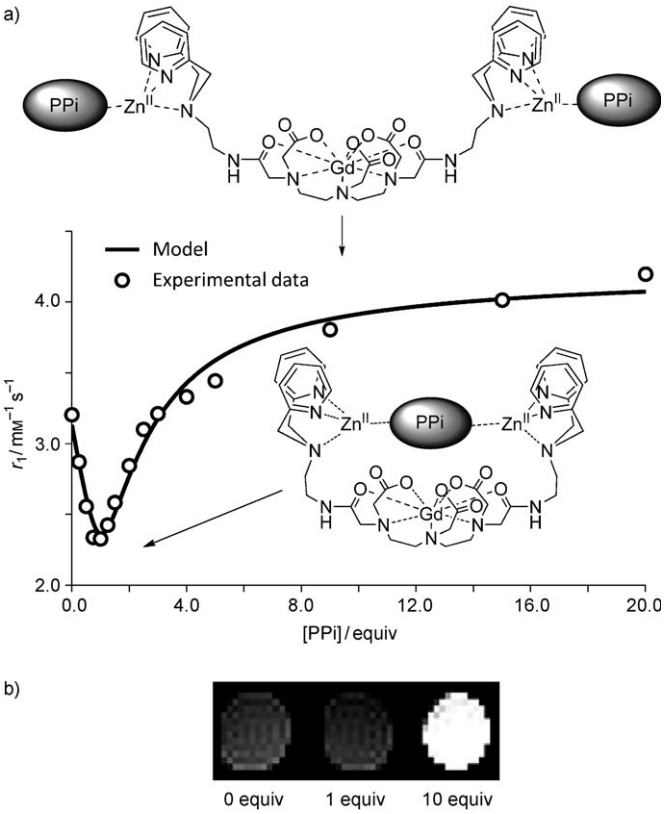


Figure 2. a) Plot of apparent relaxivity at 500 MHz of $[Gd(L)Zn_2](NO_3)_4$ as a function of the PPi concentration (with fitting of speciation model from Eu^{III} analogue titration data); $[Gd(L)Zn_2](NO_3)_4$ starting at 1.21 mM. b) Image (4.7 T, 200 MHz) of sample tubes containing 1 mM $[Gd(L)Zn_2](NO_3)_4$ and increasing [PPi]: i) 0, ii) 0.75 and iii) 8 equiv PPi (10 mM HEPES, pH 7.4, RT).

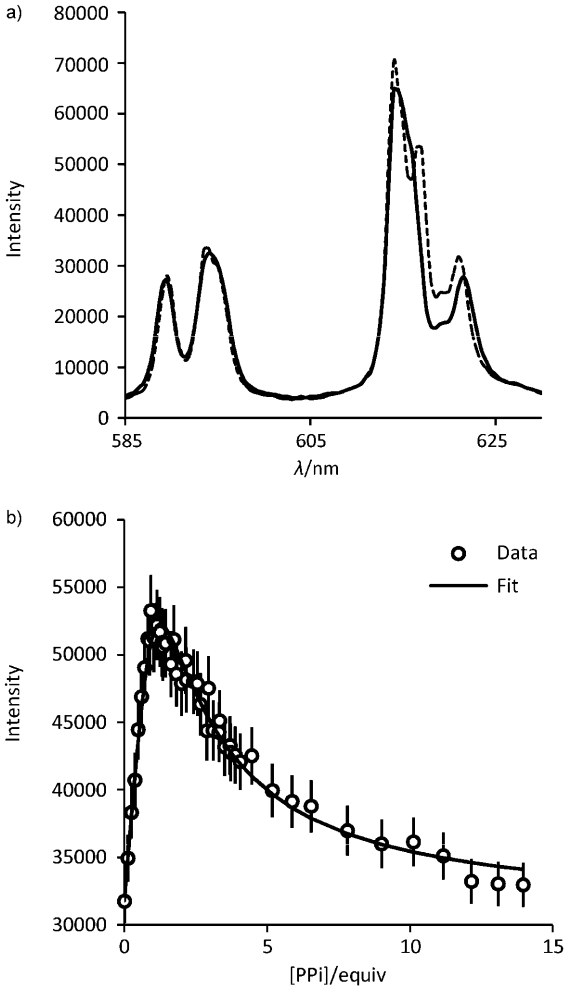


Figure 3. a) Emission spectra of $[Eu(L)Zn_2](NO_3)_4$ (1.0 mM) in the presence of zero (——) and one (-----) equivalent PPi. b) Variation of the emission intensity ($\lambda = 617$ nm) of $[Eu(L)Zn_2](NO_3)_4$ on titration with PPi (data from two titrations are presented). The error bars represent arbitrary +/− 5% error. $[Eu(L)Zn_2](NO_3)_4 = 0.8$ mM ($\lambda_{ex} = 395$ nm, 10 mM HEPES, pH 7.4, RT).

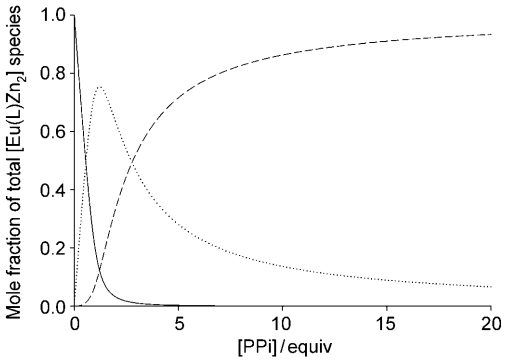


Figure 4. Calculated fractional speciation of $[Eu(L)Zn_2]$ in a model titration, either as free $[Eu(L)Zn_2]$ (——), $[Eu(L)Zn_2](PPi)$ (•••••), or $[Eu(L)Zn_2](PPi)_2$ (-----) species.

With the aim of establishing the possible mode of binding between the complexes and pyrophosphate, ^{31}P NMR spectra of PPi in the presence of 0, 0.5 and 8 equivalents

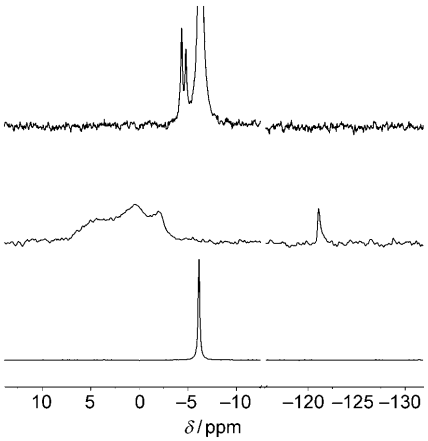


Figure 5. ^{31}P NMR spectra of a solution of PPi alone (bottom), and in solution mixed with $[Eu(L)Zn_2](NO_3)_4$: 0.5 (middle) and 8 equivalents (top) PPi compared to Eu^{3+} (400 MHz, 22 °C, 10 mM HEPES, pH 7.4).

$[Eu(L)Zn_2](NO_3)_4$ were recorded (see Figure 5). The ^{31}P NMR spectrum of pyrophosphate in the absence of any added europium complex showed a single resonance at $\delta = -6.3$ ppm (Figure 5, bottom spectrum). Upon addition of a small excess of $[Eu(L)Zn_2](NO_3)_4$ to PPi ([PPi] = 0.5 equiv compared to Eu^{3+}), it was expected that practically all PPi would be coordinated in a 1:1 fashion (Figure 4). The ^{31}P NMR spectrum of this mixture gave a very broad set of signals between $\delta \approx -3.0$ and 7.0 ppm and a single resonance shifted far upfield ($\delta = -121$ ppm). In addition, the sharp and intense resonance at $\delta = -6.3$ ppm (associated to uncoordinated PPi) disappeared. These observations are consistent with the pyrophosphate being held in close proximity to the paramagnetic europium centre on convergent binding between the two Zn-DPA "arms" in a 1:1 complex (see Figure 2); the multiple resonances are indicative of a number of inequivalent conformations in equilibrium. As has been discussed extensively elsewhere,[15] in Ln-DTPA-bisamide complexes, the DTPA ligands are present as multiple isomers (*trans*, *syn*, *anti*, *cis*) in solution, and hence, the flexible amide "arms" may potentially adopt multiple conformations in each of these. It is likely that pyrophosphate binds to more than one of these isomers and therefore, several slightly different ^{31}P environments are present, giving rise to the several broad signals. The small resonance at $\delta = -121$ ppm, suggests that in one of the conformations the pyrophosphate is brought very close to, if not into contact with, the europium centre. However, as will be discussed in the next section, if direct coordination of pyrophosphate to europium occurs it can only represent a very small proportion of the species in equilibrium, because the hydration number (q) measured for this system in the presence of PPi is practically one (see Table 1) and no hydration equilibrium is observed.

Where pyrophosphate is present in a large excess ([PPi] = 8 equiv compared to Eu^{3+}) a mixture of $[Eu(L)Zn_2](PPi)_2$ and free PPi is expected (Figure 4). Indeed, this is consistent with the recorded ^{31}P NMR spectrum with one intense peak

Table 1. Measured lifetimes of $[Eu(L)Zn_2](NO_3)_4$ luminescence in the presence of 0, 0.75, and 8.00 equivalents PPi (10 mM HEPES, pH/pD 7.4, [Eu] = 1 mM, RT).

[PPi]	τ_{H_2O} [ms]	τ_{D_2O} [ms]	q_{calcd}
0.00	0.59	2.46	1.08
0.75	0.62	2.45	0.98
8.00	0.59	2.52	1.07

at $\delta = -6.3$ ppm (free pyrophosphate) and two distinct peaks at $\delta = -4.4$ and -4.8 ppm. The latter two resonances are consistent with a divergent 1:2 binding mode in which each Zn-DPA unit is bound to one pyrophosphate (see Figure 2), with one of the signals corresponding to the phosphorous bound to the zinc centre and the other corresponding to the more distant (non-coordinated) phosphorous (see Figure 2 for a schematic representation of this binding mode).

Because the majority of PPi in aqueous solution at pH 7.4 is monoprotonated $HP_2O_7^{3-}$ ($\approx$83% at RT, calculated by using the CurTiPot software[16] from pK_a values 9.32, 6.70, 2.1 and 0.91), it is potentially useful to understand whether PPi is deprotonated on binding. To endeavour to clarify the situation, an un-buffered aqueous solution of the $[Gd(L)Zn_2](NO_3)_4$ complex (pH 7.4, initial concentration of $[Gd(L)Zn_2](NO_3)_4 = 0.4$ mM) was titrated with a pH-matched un-buffered solution of PPi (pH 7.4, [PPi] = 5 mM). The pH of the titration solution was monitored by using a digital pH-meter; the results are shown in Figure 6. The

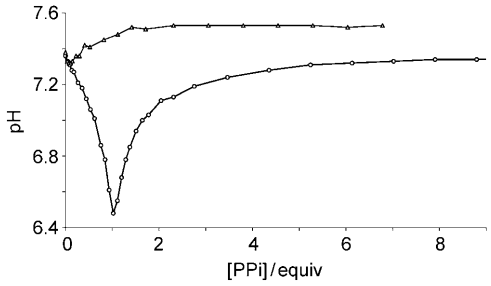


Figure 6. Changes of the pH on interaction of a complex ($[Gd(L)Zn_2](NO_3)_2$ (○) or [Gd(L)] (△)) and PPi between (pH 7.4 (un-buffered) aqueous solutions).

sharp drop in pH on addition of one equivalent of PPi clearly demonstrates the displacement of protons from PPi on 1:1 binding. That the proton displacement effect is mediated by the binding interaction with $[Gd(L)Zn_2]^{4+}$, was confirmed by a similar titration with [Gd(L)], as a negative control.

Mechanism of relaxivity modulation: To study the mechanism of this response, luminescence and absorbance spectroscopy was used to investigate whether this was mediated by changes in the Ln^{3+} hydration state (q). Luminescence lifetime measurements in H_2O and D_2O of $[Eu(L)Zn_2](NO_3)_4$ in the presence of 0, 0.75 and 8 equivalents of pyrophosphate (see Table 1 and the Supporting Information) indicate no significant change in the mean hydration state of the europium(III) centre ($q = 1$ throughout). The lack of a hydration equilibrium was corroborated by variable temperature high-resolution UV/Vis spectroscopic study in which the absorbances characteristic[17] of multiple hydration states were not observed (see the Supporting Information for further details).

Variable-temperature transverse and longitudinal ^{17}O relaxation rate and chemical shift measurements were made at 11.7 T in aqueous solutions of $[Gd(L)Zn_2](NO_3)_4$ with 0, 0.75 and 10 equivalents of pyrophosphate (with respect to the complex). The ^{17}O relaxation rates were analysed according to the Solomon–Bloembergen–Morgan theory, yielding the parameters shown in Table 2. The resulting ex-

Table 2. Parameters obtained from the analysis of ^{17}O NMR data for the $[Gd(L)Zn_2](NO_3)_4$ complex with no PPi (A), 0.75 (B) and 10 equivalents of PPi (C) in water. Reported data for monoaqua complexes of [Gd(dota)] and [Gd(dtpa)] are shown for comparison[19] (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid).

	A	B	C	[Gd(dtpa)]	[Gd(dota)]
k_{ex}^{298}[a] [$10^6 s^{-1}$]	0.28 ± 0.02	0.17 ± 0.01	0.31 ± 0.02	3.3 ± 0.2	4.1 ± 0.2
$\Delta H^{\#}$ [kJ mol^{-1}]	25.5 ± 0.2	20 ± 0.3	19.6 ± 0.2	51.6 ± 1.4	49.8 ± 1.5
τ_R^{298}[b] [$10^{-12} s^{-1}$]	570 ± 15	430 ± 12	683 ± 11	103 ± 10[c]	90 ± 15[c]
E_r [kJ mol^{-1}]	25.3 ± 0.8	19.4 ± 0.3	24.2 ± 0.3	–	–

[a] Exchange rate. [b] Rotational correlation time. [c] The τ_R values are calculated from a simultaneous fit of ^{17}O NMR and nuclear magnetic resonance dispersion (NMRD) data.

change rate, k_{ex}^{298}, for the complex in the presence of 0.75 equivalents PPi is notably lower than that with no PPi or where it is in large excess, which are similar, and of a similar magnitude than those reported for other Gd^{3+}–DTPA–bis(amide) complexes (generally, $0.22 \times 10^{-6} s^{-1} < k_{ex}^{298} < 0.45 \times 10^{-6} s^{-1}$).[18] This supports the hypothesis of a 1:1 $[Gd(L)Zn_2](PPi)$ species predominating in the presence of 0.75 equivalents PPi, in which the convergent binding of PPi hinders water exchange without displacing coordinated water, and of removal of this hindrance on divergent 1:2 binding (on addition of a large excess of PPi).

The values calculated for the rotational correlation time, τ_R^{298}, vary rather unusually: reduction on addition of 0.75 equivalents PPi, followed by an increase on addition of a further excess. The τ_R^{298} in the presence of excess PPi being considerably larger than that of the complex alone might be rationalised by the bulk of the putative $[Gd(L)Zn_2](PPi)_2$ species, and the rigidity conferred by electrostatic repulsion of the two "arms". The decrease in τ_R^{298} on binding one equivalent of PPi (unusual on increase of M_w) might be the consequence of the formation of a more compact and more spherical species. There might also be an extended hydrogen-bonding network around the less contracted complexes (those without PPi or with an excess of PPi), resulting in

more water molecules being held around the complexes. This would give rise to a longer τ_R^{298} for these species, and the less spherical character of the species would also lead to a greater outer-sphere contribution to relaxivity (especially for the larger 1:2 complexes).

To further investigate the possible role of changes in τ_R^{298}, ^{1}H NMRD profiles were also obtained for the same three systems ($[Gd(L)Zn_2](NO_3)_4$ with 0, 0.75 and 10 equiv) at 298 K in the proton Larmor frequency range of 0.01–500 MHz (see the Supporting Information). Due to the large number of variables to be adjusted simultaneously, lack of independent access to data on electronic properties and relatively small variations in water proton relaxivity, it is not possible to unambiguously fit the NMRD and ^{17}O NMR data to give reliable values for the electronic and rotational parameters. Qualitatively, the shape of the profiles observed does not vary dramatically on addition of PPi, however this is not inconsistent with relatively small changes in τ_R^{298} contributing to the modest degree of r_1 modulation.

Taken together, these studies rule out hydration equilibria and suggest that both changes in rotational correlation time and the exchange rate of Gd^{3+}-coordinated water play a role in mediating the observed relaxivity response. Because second and outer-sphere relaxivity is not easily defined or studied, it is likely that a greater solvent-accessible surface area and greater possibility for hydrogen-bonding interaction also contribute to them, hence, the greater r_1 with ten equivalents of PPi compared to the value with no PPi added. Similarly, contraction of the complex's solvent-accessible surface area may also play a role in the reduction of r_1 on binding one equivalent PPi.

Conclusion

The $[Ln(L)Zn_2](NO_3)_4$ complexes herein presented, have been shown by IDAs to bind polyanionic species, especially polyphosphates, such as pyrophosphate, with a great degree of selectivity over a range of other anions (including monophosphates). Significant modulation in the relaxivity of $[Gd(L)Zn_2](NO_3)_4$ has been shown on addition of pyrophosphate. The behaviour of the system may be explained by the stepwise formation of 1:1 and 1:2 complexes between $[Gd(L)Zn_2](NO_3)_4$ and PPi bringing about changes in rotation dynamics and accessibility of both the inner and outer coordination spheres of the complex to the bulk water, and hence the efficiency of relaxation enhancement. With this, the main motivation for carrying out these studies, namely, to show that relaxivity can be modulated by binding of anions outside of the inner coordination sphere of gadolinium, has been accomplished. It should, however, be noted, that the interaction of $[Gd(L)Zn_2](NO_3)_4$ with other polyphosphates such as ATP (as revealed by IDA), is likely to prevent the use of this compound as a contrast agent responsive to a particular polyphosphate in a cellular environment (due to lack of specificity). Further work would be required to improve the selectivity of the complex if it is to be used as contrast agent in a competitive environment such as that encountered in the cell.

Experimental Section

All synthetic procedures including the description of general instrumentation used for characterisation are described in detail in the Supporting Information.

Indicator displacement assays (IDA): The anion-binding ability of $[Gd(L)Zn_2](NO_3)_4$ was screened by examining the colour (naked eye) and spectroscopic (UV/Vis) changes on addition of salts to a 1:1 mixture of the gadolinium–lanthanide complex and the selected indicator molecule (i.e., one previously shown to change colour on interaction with the complex). In each well of a 96-well plate a solution was prepared (in 10 mM HEPES buffer, pH 7.4) containing: $[Gd(L)Zn_2(NO_3)_4]$ (0.05 mM), an indicator (either pyrocatechol violet (PV) or gallocyanine, (GC), 0.05 mM) and a salt (at either 0.05 mM and 1 equiv, or at 0.5 mM and 10 equiv, all were Bu_4N^+, H_4N^+ or Na^+ salts, see Figure 1 for the studied anions). Absorption spectra of the solutions were measured in 96-well plates by using a UV/Vis spectrometer at room temperature (21 °C).

Relaxometric titration of complex $[Gd(L)Zn_2](NO_3)_4$ with pyrophosphate at 500 MHz: The modulation of $[Gd(L)Zn_2](NO_3)_4$ relaxivity on binding pyrophosphate was studied by measuring relaxivity of these complex on titration with pyrophosphate. Pyrophosphate in deuterated aqueous buffer solutions was added stepwise to a solution of $[Gd(L)Zn_2](NO_3)_4$ in the same deuterated buffer (starting concentration = 1.21 mM), and the T_1 relaxation time was measured. These measurements were performed by using a Bruker DRX-500 NMR spectrometer in a temperature-controlled environment (20 °C); an inversion-recovery pulse sequence (with the correct delay for a 90° pulse determined specifically for each measurement) was applied to determine the T_1 relaxation time of the residual H_2O present in the solution, by using Bruker Topspin software version 3.0a, 2009, for non-linear regression of the data produced; relaxivity was calculated by using the following Equation (2):

$$1/T_{1\,obs} = 1/T_{1\,dia} - r_1[Gd] \quad (2)$$

subtracting the diamagnetic contribution to T_1, and accounting for [Gd]. A correction to take into account the difference of viscosities of H_2O and D_2O was then used to allow direct comparison with other relaxivity data,[20] by using Equation (3):

$$r_1(H_2O) = r_1(D_2O) \times (\eta_{H_2O}/\eta_{D_2O}) \quad (3)$$

(the values $\eta_{H_2O} = 1.01\ MPa\,s^{-1}$ and $\eta_{D_2O} = 1.25\ MPa\,s^{-1}$ at 20 °C were used).

MRI imaging experiments: Imaging was performed on a 4.7 T (200 MHz) Varian Direct Drive system by using VnmrJ version 2.3A software (Varian, Palo Alto, CA, USA). A quadrature volume-coil was used, with an outer diameter of 66 mm and an internal diameter of 38 mm. Aqueous solutions (typically 1 mM) were scanned in 250μl PCR tubes in a Perspex holder placed inside the RF coil. For T_1 measurements of the samples [Gd] = 1.0 mM was acquired by using saturation recovery, a series of spin-echo scans were run with an echo of the following parameters: TE = 10.97 ms, FOV = 40 × 40 mm, matrix = 256 × 256, 2 mm thick coronal slice and varying TR = 0.1, 0.3, 0.5, 0.7, 1.0, 3.0, 5.0, 7.0, 10.0 and 15.0 ms. Details of image analysis can be found in the Supporting Information.

$[Eu(L)Zn_2]$ luminescence lifetime measurements for determination of q: Luminescence lifetime measurements of $[Eu(L)Zn_2](NO_3)_4$ in D_2O and H_2O solution (10 mM HEPES, pD 7.4 and pH 7.4, respectively) and in the presence of 0, 0.75 and 8 equivalents PPi were recorded by using a Jobin Yvon Horiba FluoroMax-P spectrometer (with DataMax for Windows v2.2 software). Samples were held in a 10 × 10 mm quartz cuvette and a cutoff filter (550 nm) was used to avoid second-order diffraction effects. Lifetimes were measured by direct excitation (λ = 395 nm) with a short 40 ms pulse of light (500 pulses per point) followed by monitoring

the intensity of emission ($\lambda = 595$ nm, $\Delta J = 1$) emitted during a fixed gate time of 0.1 ms, at a delay time later. Delay times were set at 0.1 ms intervals; excitation and emission slits were both set to 5 nm.

Lifetimes are obtained by iterative least-squares fitting (Microsoft Excel Solver of the data obtained to a standard first order decay: $I_{obs} = I_0 e^{-kt}$ + offset minimising in terms of the emission decay constant (k), where I_{obs} is the observed intensity at time t, and I_0 is the initial intensity. The luminescence lifetimes quoted (τ_{H_2O} and τ_{D_2O}) are the reciprocal of the respective decay constants (i.e., $\tau_{H_2O} = 1/k_{H_2O}$). The hydration state (q) is then calculated from the emission decay constants obtained in H_2O and D_2O solutions by using Equation (4):

$$q_{Eu} = 1.2[(k_{H_2O} - k_{D_2O}) - 0.25 - 0.15] \quad (4)$$

where the correction of 2×0.075 for two amide N–H oscillators adjacent to the Eu^{III} is included, as established by Parker et al.[21]

NMRD profile: The ^1H NMRD profiles were recorded on a Stelar smartracer FFC fast-field-cycling relaxometer covering magnetic fields from 2.35×10^{-4} to 0.25 T, which corresponds to a proton Larmor frequency range of 0.01–10 MHz. The relaxivity at highest fields was recorded by using a Bruker WP80 with the spinmaster smartracer PC NMR console at variable field from 20 to 80 MHz. The temperature was controlled by a VTC90 temperature control unit and fixed by a gas flow. Samples were solutions of $[Gd(L)Zn_2](NO_3)_4$ (1 mM) in the presence of the required amount of PPi (pH 7.4, 10 mM HEPES). The relaxivity at 200 MHz was recorded by using a 4.7 T Varian Direct Drive system as described for imaging experiments (see above). Relaxivity at 500 MHz was measured in D_2O buffer solutions by using a Bruker DRX-500 spectrometer, operating and calculating relaxivity at described for relaxometric titrations (see above—T_1 titrations with PPi).

^{17}O NMR analyses of $[Gd(L)Zn_2](NO_3)_4$ on interaction with PPi: The transverse and longitudinal ^{17}O relaxation rates ($1/T_{1,2}$) and the chemical shifts were measured in the aqueous solution of $[Gd(L)Zn_2](NO_3)_4$ (20 mM) in the presence of 0, 0.75 and 10 equivalents PPi in the temperature range of 276–348 K, on a Bruker Avance 500 (11.75 T, 67.8 MHz) spectrometer. The temperature was calculated according to previous calibration with ethylene glycol and methanol.[22] An acidified water solution was used as reference ($HClO_4$, pH 3.3). Longitudinal ^{17}O relaxation times (T_1) were measured by the inversion-recovery pulse sequence,[23] and the transverse relaxation times (T_2) were obtained by the Carr–Purcell–Meiboom–Gill spin-echo technique.[24] The samples were sealed in glass spheres fitted into 10 mm NMR tubes, to eliminate susceptibility corrections to the chemical shifts.[25] To improve sensitivity in the ^{17}O NMR experiments, ^{17}O-enriched water (10% $H_2{}^{17}O$, CortecNet) was added to the solutions to yield around 2% ^{17}O enrichment. The ^{17}O NMR data have been evaluated according to the Solomon–Bloembergen–Morgan theory of paramagnetic relaxation.[17] The analysis of ^{17}O NMR data was performed by using Micromath Scientist[26]; the hydration number $q = 1$ was fixed having been independently confirmed.

High-resolution UV/Vis spectroscopy: UV/Vis spectra of the $^5D_0 \rightarrow {}^7F_0$ transitions of Eu^{III} complexes were obtained with a PerkinElmer Lambda 19 spectrometer in the region $\lambda = 577$–581 nm with data steps of 0.01 nm. A constant temperature was maintained by using a liquid heated/cooled cell with a 10 cm path length, and spectra were obtained at 298 and 338 K. Aqueous solutions of $[Eu(L)Zn_2](NO_3)_4$ were made up to approximately 0.02 M concentrations, and the pH adjusted to 7.4 with small additions of aqueous NaOH/HCl; spectra were recorded in the presence of 0, 0.75 and 8 equivalents PPi; data from multiple acquisitions over some time (typically around 20 h) was combined by using the visualisation program running on a MATLAB platform version 6.5. The recorded spectra can be found in the Supporting Information.

pH-metric binding titration: An un-buffered aqueous solution of a complex (either $[Gd(L)Zn_2](NO_3)_4$ or $[Gd(L)]$, 5 mM solution, $\approx$12 mL) was made up, and its pH adjusted to 7.4 by using aqueous HCl/NaOH. A similar solution was made up of tetrasodium pyrophosphate (also un-buffered, pH adjusted to 7.4). Aliquots (typically 50 μL) of the pyrophosphate solution were added to the stirring solution of the Gd^{III} complex by using a pipette, with a pH reading taken between each addition by using an electronic pH meter (typically allowing 2 min for equilibration).

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