

Molecular Imaging Probes for ^{31}P Contrast



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Abstract

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^{31}P magnetic resonance has yet to realise its clinical potential principally due to the relatively low sensitivity of the ^{31}P nucleus. While ^{31}P -MRS can uniquely provide vital information regarding *in vivo* phosphorus metabolism, the long acquisition times required are incompatible with observing dynamic changes in phosphorus speciation (e.g. monitoring ATP/phosphocreatine ratios in real time).

This thesis reports how heptadentate Gd.D03A derivatives can be used to enhance ^{31}P relaxivity reducing T_1 values by more than an order of magnitude per millimolar Gd. This enables shorter repetition times markedly improving the signal to noise ratio (SNR) per unit time. These complexes bind in fast exchange with phosphate species, while linear commercial Gd complexes were found to demonstrate competitive binding of ATP to Gd.

Structural modifications of the D03A complex were found to alter the affinity for specific phosphate species, as monitored by luminescence spectroscopy of the europium complexes, and also influenced the relaxivity of the Gd complex.

In vivo, the phosphate species of interest reside intracellularly and therefore cellular uptake of these complexes was investigated, using fluorescence microscopy on luminescent complexes and NMR diffusion methods for the gadolinium complexes themselves. The complexes were found to remain extracellular or display endosomal uptake. These results indicate that these systems have clear potential as extracellular ^{31}P contrast agents. Alternative approaches will be required to achieve intracellular ^{31}P contrast.

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Abbreviations

ADP	adenosine-5'-diphosphate
AMP	adenoside-5'-monophosphate
ATP	adenosine-5'-triphosphate
a.u.	arbitrary units
BSA	bovine serum albumin
CPMG	Car-Purcell-Meiboom-Gill
cyclen	1,4,7,10-tetraazacyclododecane
DCM	dichloromethane
DI	deionised
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DO2A	1,4,7,10-tetraazacyclododecane-1,7-diacetic acid
DO3A	1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DPBS	Dulbeccos's Phosphate Buffered Saline
DTPA	diethylenetriaminepentaacetic acid
DTPA-BMA	diethylenetriaminepentaacetic acid bis(methylamide)
EDTA	ethylenediaminetetraacetic acid
ESMS	electrospray mass spectrometry
Et ₃ N	triethylamine
EtOAC	ethyl acetate

FBS	fetal bovine serum
FID	free induction decay
FLIM	fluorescence lifetime imaging
h	hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
HSA	human serum albumin
ICP-MS	inductively coupled plasma mass spectrometry
IR	inversion recovery
IR-ATR	infrared attenuated total reflection
Ln	lanthanide
MALDI	matrix-assisted laser desorption and ionisation
MeCN	acetonitrile
MeOH	methanol
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
NSF	nephrogenic systemic fibrosis
OAc	acetate
PBS	phosphate buffered saline
PCr	phosphocreatine
PCR	polymerase chain reaction
P _i	inorganic phosphate

PLIM	phosphorescence lifetime imaging
PP _i	pyrophosphate
r ₁	T ₁ relaxivity
r ₂	T ₂ relaxivity
R ₁	T ₁ relaxation rate
R ₂	T ₂ relaxation rate
R _f	retention factor
RF	radio-frequency
RP-HPLC	reverse-phase high-performance liquid chromatography
RPMI	Roswell Park Memorial Institute
SAP	square anti-prism
SBM	Soloman-Bloembergen-Morgan
T ₁	longitudinal relaxation time
T ₂	transverse relaxation time
T-Flasks	tissue-culture treated flasks
^t Bu	tert-butyl
<i>TE</i>	echo time
TFA	trifluoroacetic acid
TLC	thin layer chromatography
<i>TR</i>	repetition time
t _R	retention time
TSAP	twisted square anti-prism
SNR	signal-to-noise ratio
UV-Vis	ultraviolet-visible
XO	Xylenol Orange

Chapter 1

Introduction

1.1. Introduction

Molecular imaging agents have made a significant impact on the field of biomedical imaging, allowing biochemical detail and specificity in images beyond the capabilities of the imaging hardware.¹ The lanthanides have a broad spectrum of properties that have facilitated the application of these elements as probes in magnetic resonance imaging (MRI), optical imaging and positron-emission topography (PET).^{2–4}

Magnetic resonance spectroscopy (MRS), also known as nuclear magnetic resonance (NMR) spectroscopy, is a technique that probes the local magnetic field around a nucleus. A constant magnetic field (B_0) is applied to the sample, which aligns the nuclear spin populations. This is followed by a radio-frequency (RF) pulse perpendicular to B_0 , which transfers the magnetization away from equilibrium. As the spin populations realign with B_0 they emit a radio-frequency signal which is recorded. The frequency of this signal is dependent on the effective magnetic field at the nucleus, which is indicative of the chemical environment. MR-active nuclei must have non-zero spin in order to possess a nuclear magnetic moment—these include ^1H , ^{13}C , ^{19}F and ^{31}P (spin = $\frac{1}{2}$).

While MRS provides information regarding the chemical composition of a system regarding specific nuclei, in biological systems the main proton source is water.

Therefore, MRI uses proton relaxation times to probe biological environments. There are two different types of relaxation times: the longitudinal relaxation time (T_1) which describes the time taken for the nucleus to return to equilibrium after the applied RF pulse, and transverse relaxation time (T_2) which describes the time taken for the nuclear spins to dephase. Relaxation times are sensitive to the chemical environment of the nuclei and the relaxation times of water vary in different biological tissue, which allows tissue differentiation in images. Various relaxation-based pulse sequences in MRI can be used to generate contrast and highlight specific biological features. To further enhance contrast, relaxation times can be shortened using MRI contrast agents—such as lanthanide-based compounds.

This thesis will focus on the application of lanthanide contrast agents to ^{31}P nuclei, as opposed to ^1H , to investigate the potential of ^{31}P contrast agents as molecular probes for metabolism. The following chapter will discuss ^{31}P -MRS and the properties of ^1H contrast agents along with the anion-sensing capabilities of various lanthanide complexes.

1.2. ^{31}P Magnetic Resonance Spectroscopy in Biological Systems

1.2.1. High-energy phosphate species

High-energy phosphate species *in vivo* play a key role in metabolism. Adenosine-5'-triphosphate (ATP) provides the main energy source for all active processes in the body. Energy is stored in the high-energy phosphate bonds of ATP, and hydrolysis of each bond releases about 30 kJmol^{-1} .^{5,6} ATP is broken down into Adenosine 5'-

diphosphate (ADP) and inorganic phosphate (P_i), and ADP can be broken down further into Adenosine 5'-monophosphate (AMP) and P_i (Figure 1.1).

In high energy demanding tissues, such as the heart, brain or skeletal muscle, phosphocreatine (PCr) provides an immediate source of energy in the first few seconds of intense exercise, once ATP stores have been used up (Figure 1.1). This replenishment is very fast, but short-lived, lasting up to 10 seconds.⁷ During this time, PCr provides a source of phosphate and energy to reform ATP from ADP, which is catalysed by creatine kinase enzymes. In periods of rest, excess ATP is used to re-phosphorylate creatine and replenish PCr stores, which allows it to act as an intracellular energy shuttle.⁸ In the mitochondria, creatine is phosphorylated using ATP to form PCr, which then diffuses into the cytoplasm where it is used to regenerate ATP from ADP in the cytosol. PCr therefore plays an important role in energy homeostasis by providing a buffering system for ATP.

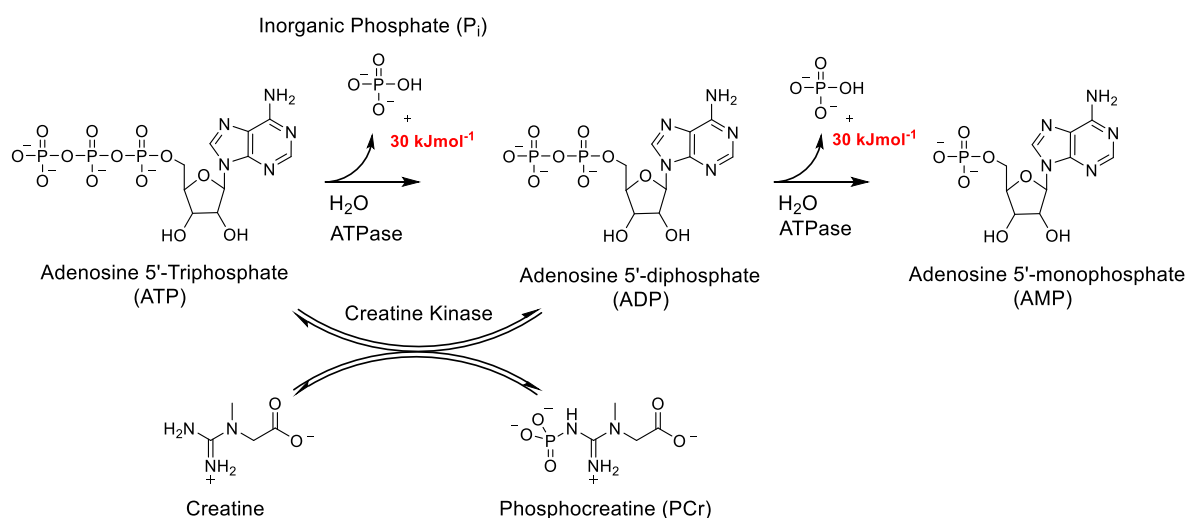


Figure 1.1: Diagram illustrating ATP hydrolysis and the phosphagen (ATP-PCr) energy system.

1.2.2. Cardiac ischaemia

In the heart, the concentration of high-energy phosphate metabolites has been shown to be a useful diagnostic marker for ischaemia.^{9–11} Ischaemia is the restriction of blood flow to tissues or organs, caused by the narrowing of arteries, which results in oxygen starvation. Cardiac ischaemia occurs in a region of the heart and causes ischaemic heart disease (coronary heart disease), which ultimately can lead to myocardial infarction and chronic heart failure.

The hypothesis that in chronic heart failure the failing heart is energy-starved has been widely studied since it was first proposed in 1939.¹² Support for this hypothesis comes from large-scale clinical trials, in which patients who received drugs which lower metabolic demand (β -blockers, angiotensin-converting enzyme inhibitors) showed a better clinical outcome compared to patients who received drugs that increase energy demand (β -receptor mimetic drugs, phosphodiesterase inhibitors).¹³ In addition, many studies have consistently measured lower intracellular concentrations of PCr and ATP in the failing heart and a lower PCr/ATP ratio.^{9,14–16} In particular, measurements of *in vivo* creatine kinase flux have shown a significant drop in the rate of flux.¹⁷ The dynamic measurement of these *in vivo* metabolite concentrations, which has allowed a better insight into myocardial energetics, has become possible with the use of ³¹P-Magnetic Resonance Spectroscopy (³¹P-MRS).

1.2.3. ³¹P-Magnetic resonance spectroscopy

³¹P-MRS was originally performed on perfused animal hearts and then *in vivo* on open-chest animals.^{18,19} The first demonstration of this technique to observe phosphorus metabolism in the human heart was in 1985.²⁰ The technique allows non-

invasive dynamic measurement of phosphate metabolism in regions of the heart, commonly by using highly sensitive surface coils for localization.

The main limitation of ^{31}P -MRS for clinical diagnosis remains the low signal-to-noise ratio (SNR) and long acquisition times associated with ^{31}P -MRS, which limit the use of this technique for the quantification of metabolites in real time. The ^{31}P nucleus is inherently less sensitive than ^1H (~6.7% of the sensitivity of ^1H), due to having a larger magnetogyric ratio.²¹ In addition, there is a lower concentration of phosphate metabolites *in vivo* compared to water protons (~10 mM, relative to 100 M for water) and the ^{31}P T_1 times of phosphate species are on the order of seconds (comparable to the T_1 of water).

More recently, technological improvements—especially the use of high-field scanners at 7 T—have significantly improved the SNR per unit time and are beginning to make ^{31}P -MRS a more clinically viable option.²² An alternative method to improve the SNR per unit time is to shorten T_1 times through the use of a contrast agent. This method is commonly used in ^1H -MRI, but to our knowledge has never been applied to ^{31}P -MRS. Application of a ^{31}P contrast agent to shorten the T_1 times of phosphate species would shorten acquisition times, so that T_1 times are no longer a limiting factor. Therefore more scans could be acquired on a shorter time scale, which would increase the SNR per unit time and in turn improve the clinical viability of ^{31}P -MRS.

1.3. Gadolinium Contrast Agents

1.3.1. Contrast-enhanced MRI

MRI is widely used in medicine for full body imaging of organs, vasculature, bones and joints. Contrast enhancement for MRI began 30 years ago with the clinical introduction of the gadolinium-based contrast agent Magnevist[®] (Bayer) in 1986.²³ Gadolinium- and manganese-based contrast agents are longitudinal T_1 relaxation agents, which increase the signal intensity of water protons therefore brightening areas in the image, while transverse T_2 relaxation agents such as Superparamagnetic Iron Oxide (SPIO) nanoparticles darken the image creating negative contrast.²⁴ This thesis will focus primarily on T_1 contrast agents and the use of gadolinium.

The administration of contrast agents is widely used to enhance the identification of lesions. Differentiation and characterisation of healthy versus abnormal tissue can be improved by contrast agents, as well as facilitating the imaging of vasculature and providing dynamic information about the functionality of tissues. For example Dynamic Contrast Enhanced MRI (DCE-MRI) is used in cancer diagnosis and cardiac imaging to gain information about blood flow and vascular permeability,²⁵ while Magnetic Resonance Angiography (MRA), is used to visualise vasculature and identify aneurysms or the narrowing of arteries.²⁶

1.3.2. Relaxivity

Gadolinium (Gd) is highly paramagnetic and is primarily used as a T_1 contrast agent due to the isotropic ground state of its seven unpaired f -electrons. The relaxivity (r_1) of a contrast agent is the relaxation rate ($R_1 = 1/T_1$) per millimolar Gd – it is therefore a

concentration-independent term and can be used to compare different contrast agents. The observed relaxation of a nucleus is a combination of inner-, outer- and second-sphere effects as defined in equation 1.1 and depicted in Figure 1.2.²⁷

T_1^{dia} is the diamagnetic contribution to the T_1 time which is equivalent to the relaxation time on the molecule with no contrast agent present. The observed relaxation rate ($1/T_1$) in the presence of contrast consists of inner-sphere (IS), outer-sphere (OS) and second-sphere (SS) components. Inner-sphere relaxation requires binding of the species to the Gd centre via oxygen and is often described by the Solomon-Bloembergen-Morgan (SBM) equations (1.2-1.5).^{28,29} The magnitude of the observed relaxivity is therefore dependent on several key factors: the number of coordinated water molecules (q), water residency time (τ_m) and the mole fraction of bound to unbound water molecules (P_m). The relaxation rate ($1/T_{1m}$) of the molecule can be described by scalar (SC) and dipole-dipole (DD) contributions (equations 1.3, 1.4 and 1.5). γ_1 is the nuclear magnetogyric ratio, g is the electronic g-factor, μ_B is the Bohr magneton, S is the electron spin quantum number (For Gd^{3+} $S = 7/2$), r is the distance between the paramagnetic centre and the bound water proton. ω is the Larmor precession frequency for protons (1) and electrons (s), τ is the correlation time for dipole-dipole terms (ci, $i = 1,2$) and scalar terms (e2), $(A/\hbar)$ is the electronic nuclear hyperfine coupling constant. The scalar contribution in gadolinium complexes is generally regarded as very small, as due to the core-like f -orbitals and the two-atom distance between Gd and H, $A/\hbar$ is very small.²⁷

$$\frac{1}{T_1^{\text{obs}}} = \frac{1}{T_1^{\text{IS}}} + \frac{1}{T_1^{\text{OS}}} + \frac{1}{T_1^{\text{SS}}} + \frac{1}{T_1^{\text{dia}}} \quad (1.1)$$

$$\frac{1}{T_1^{\text{IS}}} = \frac{qP_m}{T_{1m} + \tau_m} \quad (1.2)$$

$$\frac{1}{T_{1m}} = \frac{1}{T_{1m}^{\text{DD}}} + \frac{1}{T_{1m}^{\text{SC}}} \quad (1.3)$$

$$\frac{1}{T_{1m}^{\text{DD}}} = \frac{2}{15} \frac{\gamma_1^2 g^2 \mu_B^2 (S(S+1))}{r^6} \left[\frac{3\tau_{c1}}{1 + \omega_1^2 \tau_{c1}^2} + \frac{7\tau_{c2}}{1 + \omega_1^2 \tau_{c2}^2} \right] \quad (1.4)$$

$$\frac{1}{T_{1m}^{\text{SC}}} = \frac{2}{3} (s(s+1)) \left(\frac{A}{\hbar} \right)^2 \left[\frac{\tau_{e2}}{1 + \omega_s^2 \tau_{e2}^2} \right] \quad (1.5)$$

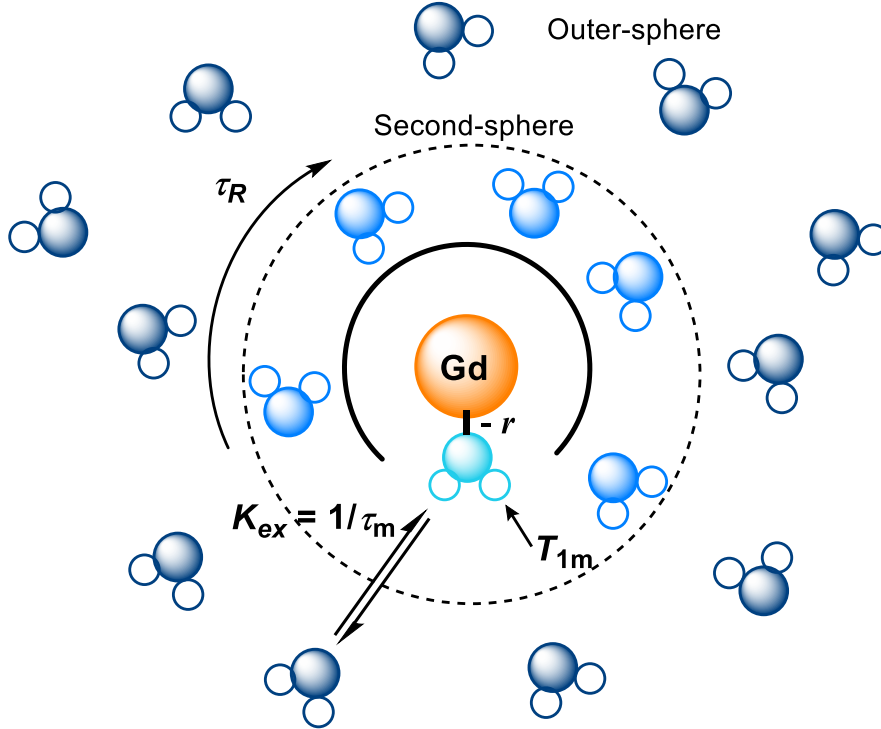


Figure 1.2: Key physical parameters which govern relaxivity in the presence of Gd contrast, with an inner-sphere water molecule bound ($q = 1$).

Outer-sphere relaxation occurs through space and increases the R_1 of diffusing neighbouring molecules through dipolar interactions. The outer-sphere relaxation is similar between molecules of a similar molecular weight containing the same metal ion, as it depends primarily on diffusion. For commercial Gd contrast agents of a similar size (Gd.DOTA, Gd.DTPA *etc.*, Figure 1.3) outer-sphere contribution is typically around 40% of the observed proton relaxivity at clinical imaging fields ($\sim 0.2\text{--}3.0$ T).³⁰

Second-sphere relaxation occurs through hydrogen bonding with the Gd complex and can also be described by the dipole-dipole SBM equation. Typically it is difficult to separate the second-sphere contribution from the inner-sphere contribution, however using $q = 0$ complexes it is possible to determine the second-sphere contribution (providing it is assumed that the second-sphere contribution is approximately the same in all complexes). Caravan *et al.* showed that the addition of phosphonate or carboxylate groups in the contrast agent can significantly enhance the second-sphere contribution resulting in a relaxivity increase of up to 88%.³¹ The second-sphere contribution to GdDTPA relaxivity has been calculated by Chen *et al.* and reported as being 25% of the observed relaxivity at 50 MHz.³² This demonstrates the significance of second-sphere relaxivity and how optimization of this parameter can benefit the development of high relaxivity contrast agents.

As equation 1.4 shows, relaxivity has a high dependence on distance between the nuclear spins (r^{-6}) and therefore increasing q —the number of inner-sphere water binding sites—can have a significant enhancement on relaxivity. However, a common problem with this is that as the inner-sphere sites are typically adjacent it allows space for endogenously available anions to also bind, such as phosphate, albumin or

carbonate.^{33,34} In turn, this lowers the relaxivity due to competition for the water binding sites. Equation 1.2 and 1.4 show that there are further methods for increasing relaxivity including altering the rotational correlation time (τ_c), which is defined as the time taken for the molecule to rotate by 1 radian. Larger molecules tumble more slowly, which increases the relaxivity, until they reach the proton Larmor frequency.³⁵ This approach has been utilised in contrast agent development to try and produce high relaxivity agents by developing dendritic molecules³⁶ or binding to proteins.³⁷ Other factors to consider include a short residency time (high water-exchange rate) of the bound water molecule in order for bulk water to be enhanced.

1.3.3. Commercial Gd contrast agents

The first gadolinium contrast agent was developed in 1984 by Weinmann *et al.* who demonstrated the improved thermodynamic stability of Gd.DTPA over Gd.EDTA in animal studies.³⁸ Gd.DTPA was approved and introduced clinically as Magnevist[®] in 1986. Following this, more contrast agents gradually emerged and there are now eight commercial, internationally available, Gd contrast agents: Gd.DOTA (Dotarem[®], 1989),³⁹ Gd.HP-DO3A (ProHance[®], 1992),⁴⁰ Gd.DTPA-BMA (Omniscan[®], 1993),⁴¹ Gd.BOPTA (MultiHance[®], 1997),⁴² Gd.DO3A-Butrol (Gadovist[®], 1998),⁴³ Gd.DTPA-BMEA(OptiMARK[®], 1999)⁴⁴ and Gd.EOB-DTPA (Primovist/Eovist[®], 2004)^{45, 23}. The structures in Figure 1.3 show that the contrast agents fall into two categories: linear and macrocyclic. Gd.DOTA was the first macrocyclic contrast agent, where the ligand is pre-organised and does not wrap around the metal ion in binding. This will be discussed in more detail in the stability Section (1.3.4).

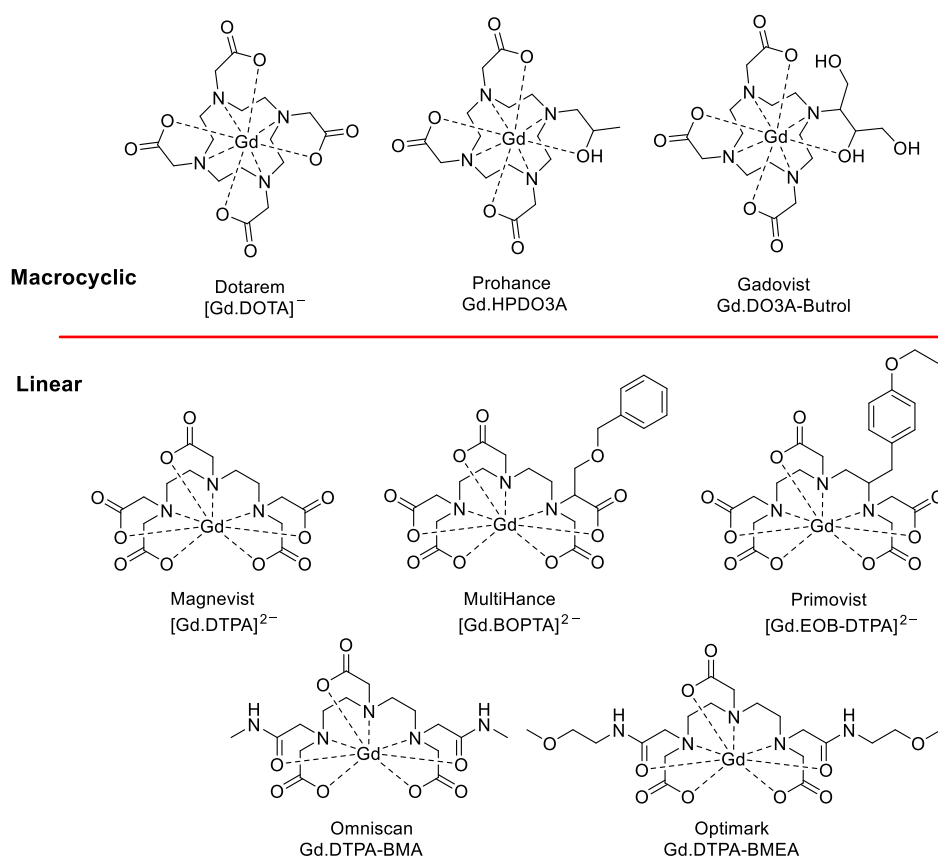


Figure 1.3: Structure of current, commercially available gadolinium contrast agents for MRI, categorized by ligand design: linear or macrocyclic.

1.3.3.1. Safety

In 2006 several studies highlighted the link between gadolinium contrast agents and nephrogenic systemic fibrosis (NSF) in patients with reduced renal function.^{46,47} NSF is a chronic, debilitating disease; the symptoms of which includes hardening of the skin, tissues and organs, and reduced movement in muscles and tendons.^{48,49} The cause of NSF in patients with kidney failure is likely to be due to poor clearance of the Gd contrast agent from the blood stream and the poor kinetic stability of some contrast agents, which allows free Gd³⁺ to be released from chelated complexes.^{50,51} Free Gd³⁺ ions are highly toxic, in part due to the similar ionic radii to Ca²⁺, which means they can compete with Ca²⁺-dependent processes such as in enzymes and ion channels, but

with a greater affinity due to the higher charge density.^{52,53} Gd^{3+} can also precipitate out in membranes forming insoluble aggregates with phosphate and other anions; Gd-phosphate deposits have indeed been found in NSF lesions.⁵⁴ For this reason highly stable chelated complexes are required, which prevent release of Gd^{3+} , but still allow fast exchange of water molecules with the Gd centre.

The highest number of NSF cases was associated with Gd.DTPA-BMA and a few cases with other linear agents,^{47,50,55} the reasons for this will be discussed further in the stability Section (1.3.4). In 2007, the FDA introduced a boxed safety warning for all Gd contrast agents following these cases of NSF.⁵⁶ Since the introduction of this warning, patients with reduced kidney function are required to be assessed before the use of Gd Contrast in MRI and any patients with compromised renal function do not receive these agents. Following this, the number of NSF cases has been significantly reduced—almost non-existent.⁵⁷

More recently, there have been a number of studies identifying the deposition of Gd in the brain of patients and animals, who currently display no symptoms or side effects.^{58,59} These findings have occurred with all Gd contrast agents, but there are significantly higher incidents with Gd.DTPA-BMA and other linear agents.⁶⁰ As of 2017, these studies have led to a review of Gd contrast agents resulting in the suspension of two contrast agents by the European Medical Agency (EMA): Omniscan[®] and Optimark[®]; and restriction of a further two agents: Multihance[®] to liver imaging, and Magnevist[®] to low concentration intra-articular.⁶¹ Thus far, the US Food and Drugs Agency (FDA) have not administered any restrictions.⁶²

1.3.4. Stability

Following the identification of NSF in patients with compromised renal function and the link with specific contrast agents, there has been concern over the stability of Gd contrast agents. In chelated Gd agents, both thermodynamic and kinetic stability must be considered, and the contribution of each of these in biological environments.

1.3.4.1. Thermodynamic stability

Thermodynamic stability has been widely used to characterise Gd complexes. It is a measure of the concentration of all species in solution at equilibrium given by the thermodynamic stability constant as shown in equation (1.6).⁶³

$$K_{Therm} = \frac{[GdL]}{[Gd^{3+}][L]} \quad (1.6)$$

For the use of Gd contrast agents at physiological pH 7.4; a more useful value is the conditional thermodynamic stability constant. This incorporates the level to which the ligand will be protonated at pH 7.4, by considering the pK_a values of the ligand as demonstrated by equation (1.7).

$$K_{Cond} = \frac{[GdL][H^+]^n}{[Gd^{3+}][H_nL]} \quad (1.7)$$

The higher the value of the stability constant, the higher concentration of GdL there is as opposed to free ligand and Gd^{3+} . The conditional stability constant will be lower than the overall stability constant as the former assumes the ligand is completely deprotonated and therefore is only relevant at very basic pH. More basic ligands, which have pK_a values around pH 7.4 and higher will be mostly protonated at pH 7.4

and therefore have a lower stability. Based on the values in Table 1.1, there is no obvious difference between the thermodynamic stabilities of macrocyclic versus linear contrast agents. The conditional stability constant for Gd.D03A shows the most significant drop as a result of incorporating pH, which shows how significant the pH can be as the thermodynamic stability is then similar to that of Gd.DTPA-BMA. However, all these complexes show a high thermodynamic stability even at pH 7.4, which has been considered sufficient for imaging agents. In light of NSF, it has been shown that thermodynamic stability does not fully describe the effects seen *in vivo* as, due to the low retention time, the complexes do not reach equilibrium and therefore kinetic stability dominates.

Table 1.1: Thermodynamic and conditional stability constants at pH 7.4 of Gd contrast agents.^{64,65}

Contrast Agent	Log K_{Therm}	Log K_{Cond}	Structure
Gd.DTPA (Magnevist [®])	22.1	17.7	Linear
Gd.DTPA-BMA (Omniscan [®])	16.9	14.9	Linear
Gd.DOTA (Dotarem [®])	24.7	17.2	Macrocyclic
Gd.HP-DO3A (ProHance [®])	23.8	17.1	Macrocyclic
Gd.D03A (Not clinically approved)	21.0 ^a	14.5 ^b	Macrocyclic

^a Reference⁶⁶ ^b Reference⁶⁷

1.3.4.2. Kinetic stability

Studies have shown that, despite high thermodynamic stability constants, the dissociation of the complexes *in vivo* does not directly follow the size of the

conditional thermodynamic stability constant.⁶⁸ Kinetic stability is an important factor in evaluating the stability of complexes in solution. The rate of formation of macrocyclic complexes is much slower than that of linear complexes, due to a two-step process to form the thermodynamically stable product. The macrocyclic ligand is pre-organised so protonation, and therefore dissociation, of one arm does not significantly alter the structure. In comparison, linear chelates are not pre-organised and the complexes form relatively quickly, by the chelating ligand wrapping around the lanthanide ion in a ‘claw-like’ fashion. As the law of microscopic reversibility states, the reverse of a reaction must be exactly the same as the formation. Therefore, linear chelates dissociate faster than macrocyclic complexes. The observed dissociation rate contains a pH dependent and independent term as demonstrated in equation (1.8) below:⁶⁵

$$K_{obs} = K_{ind} + K_{dep}[H^+] \quad (1.8)$$

The dissociation is relatively slow at neutral pH, so the majority of studies have reported the acid dissociation rate at pH 1 (0.1 M HCl), where the pH dependent term will dominate.^{63,69} Frenzel *et al.* studied the dissociation of complexes at neutral pH in human serum.⁷⁰ The values are displayed in table 1.2 alongside acid-catalysed dissociation rates. Clearly, the kinetic acid-catalysed dissociation rates give a good indication of the stability of the complexes *in vivo* and the quantity of Gd released.

Table 1.2: Kinetic stability constants (K_{obs}) and half-lives of Gd complexes at pH 1, with percentage release of Gd(III) in human serum at pH 7.4 after 15 days.

Contrast Agent	$K_{obs} (s^{-1})^a$	$t_{1/2}^a$	% Release in human serum ^b
Gd.DTPA (Magnevist [®])	1.2×10^{-3}	10 min	1.9
Gd.DTPA-BMA (Omniscan [®])	2.0×10^{-2}	35 s	20
Gd.DOTA (Dotarem [®])	2.1×10^{-5}	> 1 month	<0.1
Gd.HP-DO3A (ProHance [®])	6.3×10^{-5}	3 h	<0.1
Gd.DO3A (Not clinically approved)	2.3×10^{-3}	5 min	-

^a 0.1 N HCl at 25 °C.⁷¹ ^b 15 days in native serum at pH 7.4, 37 °C.⁷⁰

Other mechanisms which also need to be considered in the dissociation of Gd complexes are the presence of competing species *in vivo*. This includes transmetallation by endogenous anions such as Zn(II) and Cu(II). Transmetallation of linear chelates with Zn(II) or Cu(II) in phosphate buffer has been found to be the most significant for Gd.DTPA-BMA, followed by Gd.DTPA and then Gd.BOPTA.^{68,72} Muller *et al.* demonstrated that Gd.DOTA and Gd.HP-DO3A exhibit negligible transmetallation by Zn (II) ions in phosphate buffer, which demonstrates the higher stability of macrocyclic chelates.⁷³ This was consistent with results by Frenzel *et al.*⁷⁰ in human serum, which typically contains an average of 55 μ M Cu and 86 μ M Zn(II).⁶⁸ Excess inorganic phosphate was shown to have a significant destabilising effect on linear chelates in this study, significantly increasing the rate of release of Gd^{3+} although the final concentration was unaffected. Gadolinium phosphate forms as an insoluble precipitate and therefore any free Gd^{3+} present in equilibrium will precipitate out of solution. The system will equilibrate to replenish the free Gd^{3+} and

thus high levels of phosphate increases the rate at which Gd^{3+} is released.⁶³ Evidence of a Gd.DTPA phosphate intermediate has also been identified, as a potential mechanism for Gd^{3+} release.⁷⁴

Tweedle *et al.* demonstrated that Gd.DO3A has a reduced kinetic stability due to the reduced number of donor atoms to the Gd centre.⁶⁶ However, kinetic stability is not always directly related to the number of co-ordinating arms, as reported by Polasek *et al.*⁷⁵ They showed that if N-alkylation of the fourth nitrogen on the macrocyclic ring introduces steric strain, it can destabilise the system. Furthermore, Messeri *et al.* showed that heptadentate ligands could exhibit high stability provided that they were sufficiently rigidified to avoid the exchange of ligands.⁷⁶

1.4. Lanthanide Chemistry

The characteristics of the *f*-block elements are primarily characterised by their electronic structure. The *f*-electrons are highly contracted and core-like and therefore shielded by the filled 5s and 5p orbitals. As a result, the *f*-electrons do not partake in bonding, which gives rise to ionic bonding and small crystal field effects. Lanthanide contraction is also observed when moving along the series, as there is no screening of the nuclear charge Z_{eff} by the *f*-electrons. The +3 oxidation state is prevalent for all the *f*-elements, although the +2 oxidation state is available under very specific conditions with varying degrees of stability.⁷⁷ Ligand coordination is dominated mostly by steric effects and hard Lewis acid donors such as N and O are preferred. A wide range of coordination numbers exist, but the hydration number of the free ions in solution varies from 9 to 8 across the series, as a result of the lanthanide contraction.⁷⁸ Lanthanides

readily form hydrated complexes due to the high hydration energy of the small Ln^{3+} ion and with fast water exchange.⁷⁹

1.4.1. Lanthanide photophysics

$f-f$ transitions are formally forbidden by the Laporte selection rules. Ligand-field effects are relatively small in lanthanide complexes, due to the limited mixing of the orbitals, so although the transitions become partially allowed emission is relatively weak by direct excitation into the $4f$ excited states.⁸⁰ Therefore, sensitization or the *antenna effect* is typically used to excite electrons into the $4f$ excited state. The common pathway for sensitization is the absorption of light by an organic (or d -block) chromophore, which promotes electrons into the excited singlet state. Intersystem crossing transfers the electrons from the singlet state to the long-lived triplet excited state on the ligand, which allows transition to the excited $4f$ orbitals on the lanthanide (Figure 1.4). For efficient energy transfer the excited triplet state of the chromophore must lie close in energy to one of the lanthanide-centred orbitals.

The emission spectra of the lanthanides are very characteristic, with sharp emission peaks resulting from the minimal mixing of the f -orbitals with those of the ligand. The ligand therefore produces relatively small changes in the emission spectra of the lanthanide, however these changes are useful for determining the effects of the ligand co-ordination sphere. In particular, hypersensitive transitions respond directly to changes in the ligand environment and this modulation of emission can be typically used to determine binding affinities.⁴

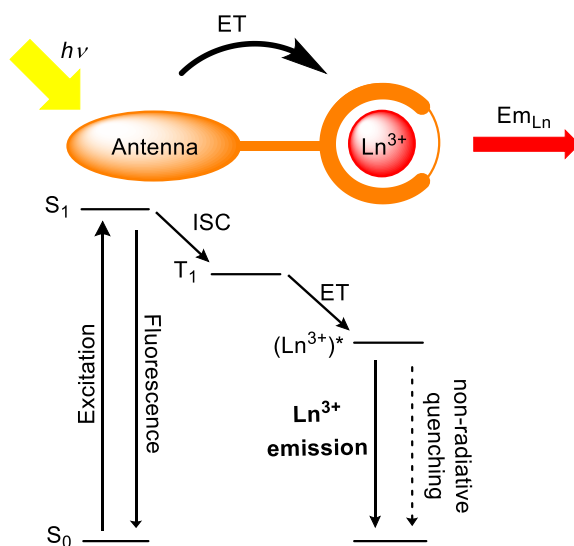


Figure 1.4: Cartoon depicting sensitization of a lanthanide complex, with a simplified Jablonski diagram of energy transfer (ET) S_0 and S_1 are the ground and excited singlet states of the chromophore, T_1 is the excited triplet state, $(Ln^{3+})^*$ is the lanthanide excited state, ISC is intersystem crossing.

As the f - f transitions are parity forbidden, this gives rise to relatively long-lived emission lifetimes of lanthanide complexes (Eu and Tb have typically millisecond emission lifetimes).⁸¹ The emission lifetimes are longer than any biological autofluorescence and therefore time-gated imaging techniques can be used to separate autofluorescence from probe emission.

Quenching of the lanthanide emission can occur by energy transfer from the excited state to stretching vibrations of surrounding solvent molecules. O-H oscillators are the most efficient at quenching lanthanide emission, due to the overlap of the vibrational states with the metal centred energy levels. The smaller the gap is between the lowest-lying lanthanide excited state and the ground level, the more efficient quenching by vibrational modes will be.⁸¹ Deuterated molecules have lower vibrational frequencies, which reduces the extent of quenching in deuterated solvents and results in longer emissive lifetimes than their protonated equivalents.

1.4.2. Paramagnetic NMR

The paramagnetic nature of most of the lanthanides characterises their NMR spectra. The lanthanide-induced shift (LIS, $\Delta\omega$) is the shift relative to the diamagnetic compound and is the sum of contact ($\Delta\omega_c$) and pseudo-contact ($\Delta\omega_{pc}$) contributions. The contact shift is a through-bond interaction generally considered a small contribution in lanthanide complexes due to the contracted nature of the f -orbitals, which reduces the overlap between the lanthanide and the respective nucleus.⁸² The pseudo-contact shift is a through-space dipole-dipole interaction, which is a result of magnetic anisotropy of the paramagnetic centre creating a local magnetic field. Therefore the pseudo-contact shift usually dominates. It can be described by equation (1.9), where C_D is a known lanthanide specific term and G is a ligand dependent term, described by equation (1.10).²⁷ θ and ϕ are the angles and r the distance relating to the paramagnetic centre, C is dependent on temperature and the nature of the complex.

$$\Delta\omega_{pc} = C_D G \quad (1.9)$$

$$G = C \left(\frac{(3\cos^2\theta - 1) + (\sin^2\phi \cos^2 2\theta)}{r^3} \right) \quad (1.10)$$

For axially symmetric complexes (such as Ln.DOTA) the equation simplifies to:

$$G = C \left(\frac{(3\cos^2\theta - 1)}{r^3} \right) \quad (1.11)$$

The C_D values for each lanthanide are dependent on the level of anisotropy; relative values at room temperature for some of the lanthanides are displayed in Table 1.3.

Table 1.3: Relative C_D values for some of the lanthanide ions at 298 K.

Ln^{3+}	C_D
Eu	4.0
Gd	0.0
Tb	-86.0
Dy	-100.0
Yb	22

From reference ⁸² Values are scaled to -100 for Dy

For a complex with a rotational axis of symmetry, the dependence of the chemical shift on the location of the nuclei of interest to the lanthanide centre is summarised in Figure 1.5. Nuclei in the blue regions ($0 < \theta < 54.7^\circ$) have the opposite chemical shift to those in the red region ($54.7 < \theta < 125^\circ$) and at 54.7° the pseudo-contact shift vanishes.

Ln.DOTA complexes and their derivatives are capable of forming four isomers (Figure 1.6)²⁷ all of which are interchangeable in solution via either rotation of the acetate arm or ring inversion. The two main geometries are the square anti-prism (SAP) and the twisted square anti-prism (TSAP), which are defined by their differences in the twist angles of 40° and 30° respectively.²⁷

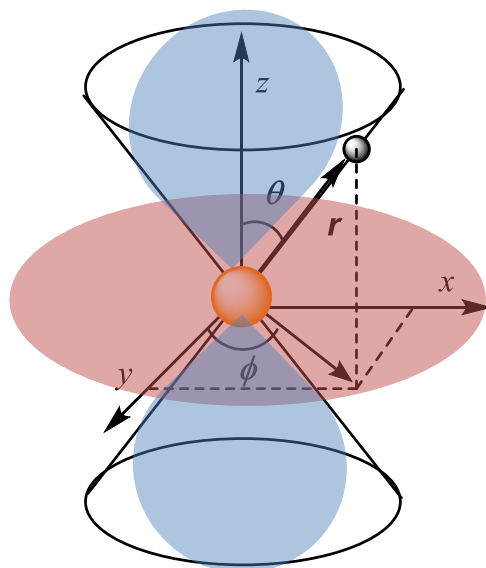


Figure 1.5: Lanthanide induced shift as a function of angle (θ , ϕ) and distance (r) of the nucleus from the lanthanide centre. Blue region and red region have opposite shifts in the NMR spectrum.

For octadentate structures, such as DOTA, the isomers are generally in slow exchange, due to the relatively high activation barrier to interconversion. The SAP isomer is the major component; however, this can be affected by solvent, temperature and changes in the structure of the complex.⁸³ Heptadentate DO3A complexes have a lower activation barrier to interconversion and therefore the SAP and TSAP isomers tend to be in fast exchange on the NMR timescale which results in broader NMR peaks.⁸⁴ The broken symmetry of DO3A complexes also makes the NMR spectra more challenging to assign, in comparison to C4 symmetric systems such as DOTA. In these axial symmetric complexes, the largest LIS of SAP axial protons is always expected to be larger than the equivalent proton on the TSAP isomer.^{85,86} This is because the SAP isomer has a smaller angle (θ) at the z axis, which results in a larger pseudo-contact shift. Additional differences in the properties of the complexes include the rate of water exchange: TSAP isomers have been found to display faster water exchange at

the Gd centre, in comparison to the SAP isomer, which could make isolation of the TSAP isomer beneficial to enhancing relaxivity.⁸⁷

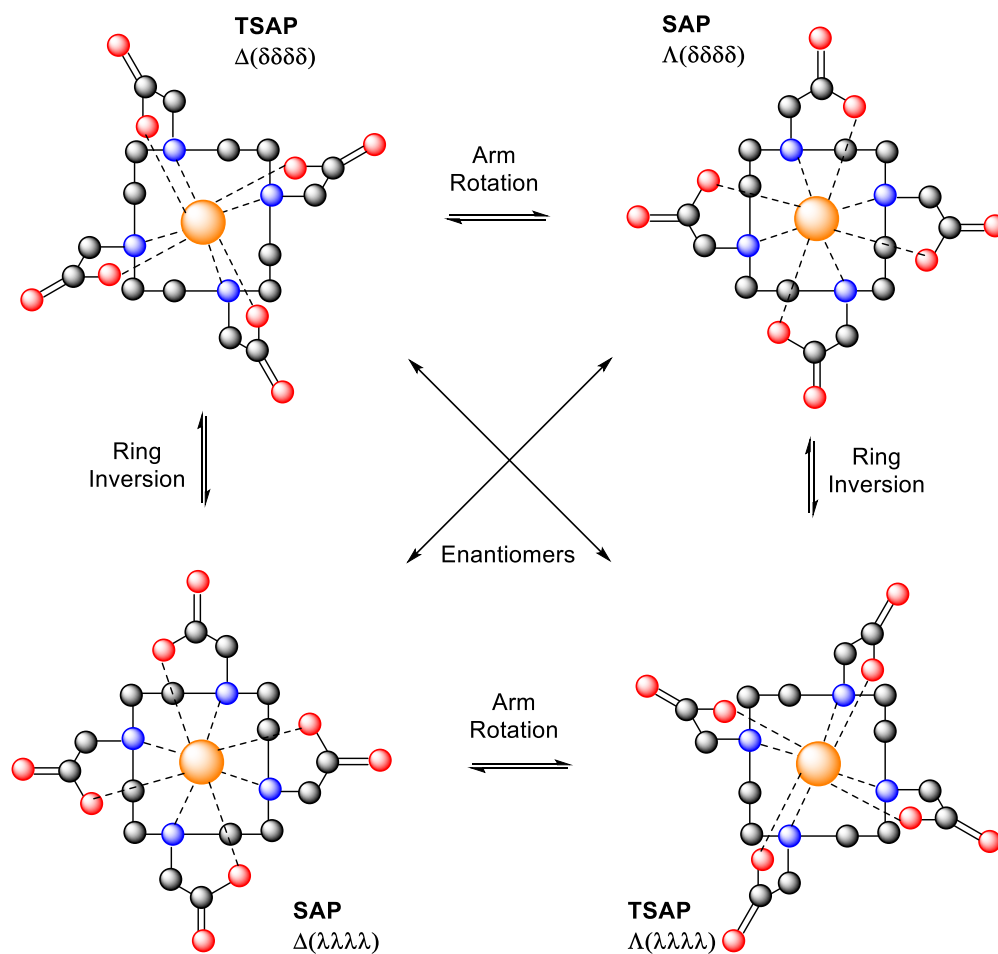


Figure 1.6: Square anti-prism (SAP) and twisted square anti-prism (TSAP) isomers and the mechanism of interconversion. Image adapted from literature.²⁷

1.5. Biological Applications of Lanthanide Complexes

1.5.1. Anion sensing

Lanthanide complexes can be exploited for their interaction with anions for a wide variety of applications. Sensing of anions can be achieved through changes in luminescence, chemical shift or relaxivity, depending on the lanthanide. The direct interaction of anions with the lanthanide centre have been studied with heptadentate DO3A scaffold complexes and reviewed by Butler *et al.*⁸⁸ Some of the key interactions relevant to this work have been summarised in figure 1.7. Most notable is that simple phosphates exhibit monodentate equatorial binding and no evidence of phosphate chelation has been observed with lanthanide complexes. However, amino acids, peptides and carboxylate species do exhibit chelation, with a preference for ring sizes of 4-6, which is likely to result in preferred binding over monodentate species.

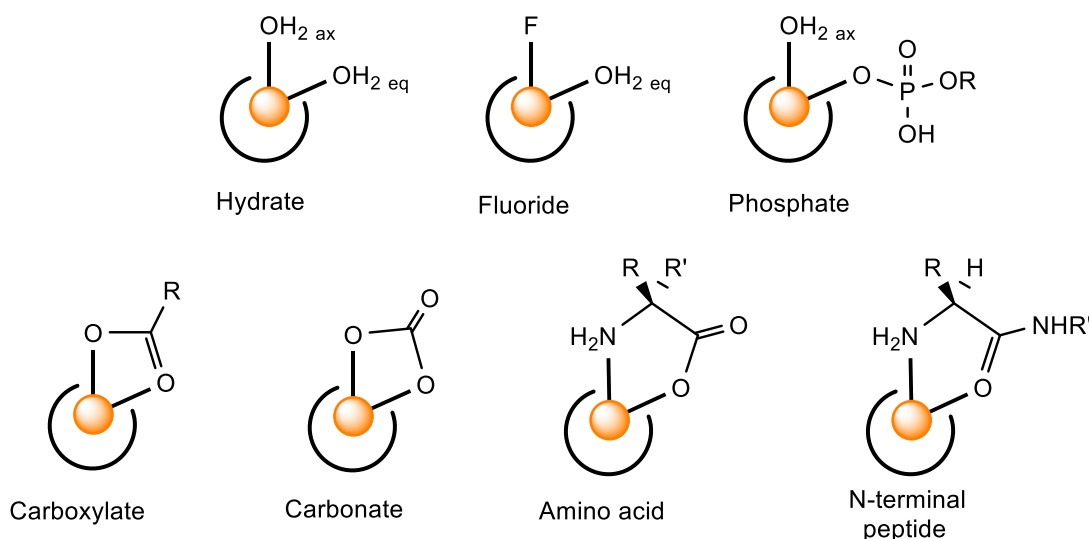


Figure 1.7: Examples of anion binding mechanisms for heptadentate DO3A-scaffold complexes, including chelating and monodentate ligands. Adapted from reference.⁸⁸

Phosphate sensing has attracted significant interest due to the key role phosphate species play in metabolism, signalling and cell differentiation. In addition, the similarity of different phosphate species has made the ability to distinguish between them by the design of specific probes challenging. Luminescence is one of the most commonly applied methods for phosphate sensing. On/off probes are one method although there is a preference for ratiometric sensing, which allows concentration-independent detection. Ratiometric probes require an internal reference, such as a static fluorescent tag or a colour change. This allows the intensity or colour change of the probe in response to the environment to be monitored, independent of the quantity of probe in that location.

For luminescent sensing, selectivity can be tweaked by adaptation of the complex to bind more strongly to specific phosphate ions or by steric limitations to the binding site. Atkinson *et al.*^{89–91} studied a series of complexes (Figure 1.8, complexes 1.1 and 1.2), which showed specificity for phosphate in the presence of other biological anions. In particular, selectivity for Tyr-OP over Ser-OP was determined in the free amino acid and in a hexapeptide model. In addition, Eu and Tb complexes displayed a high preference for phosphate binding over N-terminal peptide binding, while Yb and Tm displayed both binding modes (Figure 1.7), due to the higher charge density. The higher selectivity for Tyr-OP is attributed to the lower solvation of the anion, facilitating the activation of binding.

Butler *et al.*^{92,93} demonstrated triphosphate selectivity with europium complex 1.3 (Figure 1.8). The complex shows preferential binding to ADP and ATP over AMP, pyrophosphate (PP_i), P_i and hydrogen carbonate. Selectivity was introduced through Coulombic interactions and hydrogen bonds to the amide N-H groups on the quinoline

arms. The presence of Mg^{2+} ions was also found to enhance the selectivity of ADP over ATP, most likely due to the higher competition for Mg^{2+} with ATP.

Shuvaev *et al.*⁹⁴ demonstrated chiral selectivity of phosphate with the europium complex 1.4 (Figure 1.8). In the presence of zinc, the complex can co-ordinate ATP, ADP or AMP yielding similar intensity changes. However, upon recording the circularly polarised luminescence (CPL) spectra, it was observed that ATP and ADP/AMP yield mirror image spectra. This indicates that the opposite chiral conformer exists in each case, which allows the ratio of ADP and ATP to be determined.

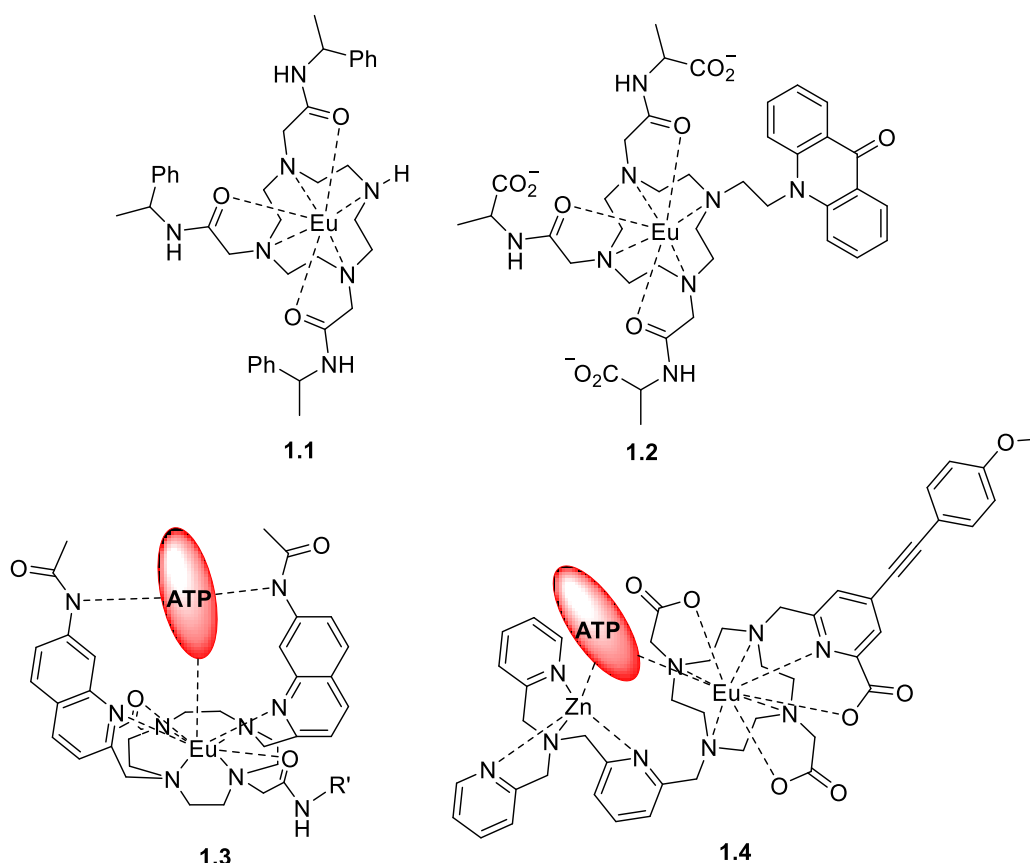


Figure 1.8: Europium complexes sensitive to specific phosphate species: 1.1 and 1.2 (Atkinson *et al.*)⁹¹

for Tyr-OP, 1.3 (Butler *et al.*)⁹² and 1.4 (Shuvaev *et al.*)⁹⁴ for ATP.

1.5.2. Responsive contrast agents

Contrast agents can also be designed to respond to changes in the environment and signal the presence of particular substrates, which can be markers of disease.⁹⁵ Methods for environmentally-responsive contrast agents for MRI tend to utilise a change in hydration number, rotational correlation time or water exchange rates, to enhance relaxivity as the result of a stimulus.

A zinc-sensitive contrast agent designed by Yu *et al.* (1.5, Figure 1.9) utilises the increase in rotational correlation time to enhance relaxivity upon binding to HSA.⁹⁶ Zn (II) is highly regulated in the body and an imbalance of Zn (II) can be a marker of diseases such as diabetes, prostate cancer or Alzheimer's. The contrast agent is only able to bind to HSA in the presence of Zn(II) and therefore acts as a zinc sensor as it elicits a significant increase in relaxivity from $6 \text{ mM}^{-1}\text{s}^{-1}$ to $50 \text{ mM}^{-1}\text{s}^{-1}$ at 0.47 T.

An example of using water-exchange rate to modulate relaxivity is presented by Surman *et al* who developed a pyrophosphate-responsive Gd contrast agent (1.6, Figure 1.9).⁹⁷ In the presence of around 1 eq. PP_i or ATP the relaxivity of the complex is reduced, despite maintaining the same hydration site. On an increase of the anion to 10 eq. the relaxivity is increased above the original relaxivity of the contrast agent at 0 eq. PP_i . The reason for this is attributed to the formation of a 1:1 complex at low anion equivalents, which limits water exchange. At high anion equivalents a 1:2 complex is formed, which increases water exchange rates at the Gd centre due to the open conformation of the 1:2 complex.

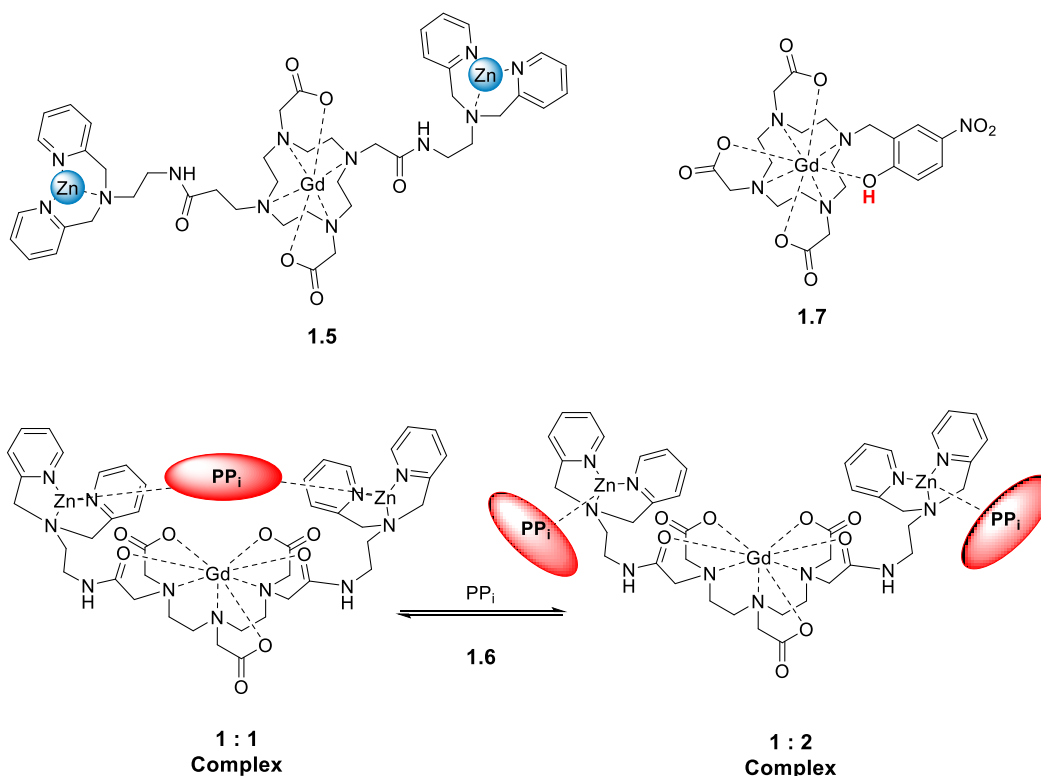


Figure 1.9: Structures of contrast agents that are responsive to different environments: 1.5 (Yu *et al.*)⁹⁶ has Zn(II) sensitivity, 1.6 (Surman *et al.*)⁹⁷ detects PP_i and 1.7 (Woods *et al.*)⁹⁸ is pH responsive.

Varying the hydration number of the Gd complex is the most commonly applied method to create responsive contrast agents, especially in pH sensing. Tissue pH is commonly used as a marker of cancer, due to increased acidity in the tumour extracellular microenvironment. One example of using the hydration site to detect pH change is presented by Woods *et al.*⁹⁸ Complex 1.7 (Figure 1.9) contains a *p*-nitrophenolate pendant arm, which co-ordinates to the Gd centre at high pH. This creates a $q = 1$ complex, but at low pH protonation of the pendant arm causes dissociation yielding a $q = 2$ complex with higher relaxivity ($7.0 \text{ mM}^{-1}\text{s}^{-1}$ versus $4.1 \text{ mM}^{-1}\text{s}^{-1}$ at 0.47 T). The *p*-nitrophenolate pendant arm has a pK_a of 7.36, which makes

it very sensitive to changes at biological pH, where the acidic tumour environment is typically around pH 6.5–7.2.⁹⁹

1.5.3. Intracellular Gd compounds

All commercial contrast agents are extracellular, but many of the biochemical pathological changes occur intracellularly. Therefore, there has been an interest in the potential of internalising responsive Gd contrast agents. There are two intracellular commercial contrast agents, which are taken up by hepatocytes and therefore used for liver-specific contrast; namely Gd.BOPTA (MultiHance[®]) and Gd.EOB-DTPA (Primovist[®]). A percentage of the contrast agent administered is taken up by hepatocytes through specific organic anion transporters and by exploiting the bilirubin pathway, while the rest is excreted through the kidneys.¹⁰⁰ This creates a hepatobiliary phase from 30 minutes–2 h after administration, where only the hepatocytes will display contrast. This allows damage to the hepatocytes or the presence of malignant cells to be clearly visualised.

Another focus for intracellular Gd compounds has been for cell tracking of stem cells. It is estimated that 10^7 – 10^8 Gd molecules per cell are required in order to visualise a cell by MRI.¹⁰¹ This has been achieved through red blood cell labelling by Ferrauto *et al.* where red blood cells are labelled with Gd.HP-DO3A at high enough concentrations to have significant T_1 relaxation.¹⁰² These were tracked *in vivo* using MRI. The cells were labelled by incubating with very high (100 mM) concentrations of Gd.HP-DO3A and using hypotonic swelling—a method which utilises osmotic potential of the membrane to permeabilise it.¹⁰³ Lower concentrations can also show detectable relaxivity enhancement especially when using larger macromolecules. This

was demonstrated by Wolf *et al.*, who used polyamine-substituted DTPA molecules and observed notable changes in R_1 with only 10 μM incubation.¹⁰⁴ Similarly, Allen *et al.* observed 20% shortening in T_1 by using polyarginine-DO3A complexes at 300 μM concentrations.¹⁰⁵

In fact, a common observation amongst these studies is that incubation at higher concentrations of contrast agent does not result in a significant increase in the relaxation rate observed. This was noted by Crich *et al.* who utilised glutamine transporters to internalise Gd complexes and observed a quenching effect on the observed R_1 with increasing uptake of contrast agent.¹⁰⁶ This can be explained by the compartmentalisation of Gd complexes within the cell, where water exchange rate across the membrane can limit the relaxation effects of the contrast agent on bulk water.¹⁰⁷

These complexes are normally taken up by macropinocytosis, which involves encapsulation of molecules inside membrane-bound vesicles. When the complexes are taken up using electroporation, which results in the complexes residing in the cytoplasm, the quenching effect is not observed.¹⁰⁸ The benefit of pinocytosis is that there is minimal effect on cell viability as the complexes do not come into contact with any of the important cell machinery. This is as opposed to electroporation, which is damaging to the cells and allows uptake of any compound in solution into the main compartment of the cell.

The quenching effect is one of the reasons relaxation rate is a difficult parameter by which to measure cell uptake. Therefore typically cell extraction or lysis can be used to destroy cell membranes, as well as the Gd complex, and measure relaxation rate of the solution in comparison to free Gd^{3+} .¹⁰⁹ Similarly, ICP-MS is

commonly used for quantification of cell uptake of Gd; this is a very sensitive method, but provides no localisation information unless cellular constituents are separated prior to analysis. It also does not provide any information about the integrity of the complexes and therefore any dissociation of Gd^{3+} as the method only detects the quantity of Gd. A method for studying the extent to which Gd complexes remain intact upon endosomal uptake was investigated by Gregorio *et al.* using mass spectrometry on cellular lysates (though it should be noted this is a destructive technique).¹⁰⁹ Gd.DTPA-BMA was found to exhibit considerable degradation by endosomal enzymes over time, while Gd.HP-DO3A remained intact. This indicates that the harsh acidic and enzymatic conditions of endosomes can be particularly destructive to kinetically unstable complexes. This is an important consideration in light of the safety concerns previously discussed in Section 1.3.3.1. It is also important that excretion and time inside the cell are considered, to know the experimental time-frame and also ensure that the complexes are safely removed from the cells.

1.5.4. Intracellular optical probes

There are many more examples of intracellular lanthanide probes for optical imaging rather than for MRI, due to the higher sensitivity of the agents and resolution of the technique. The uptake mechanism for these is still often micropinocytosis, but localisation of the complexes once inside the cell can be determined by fluorescence microscopy. This shows the complexes can be transported inside vesicles from encapsulated endosomes to different areas in the cell *i.e.* mitochondria, nucleus or lysosomes depending on the properties of the complex.¹¹⁰ Eurotracker[®] dyes, which were developed by Parker and co-workers,^{111,112} are a series of highly emissive

europium complexes, which includes complexes that selectively stain the mitochondria, lysosomes or endoplasmic reticulum.¹¹⁰ New *et al.* attempted to rationalise the localisation behaviour of a series of optical lanthanide complexes.¹¹³ They found that variation of the lanthanide ion from Tb to Eu did not appear to have any effect on the subcellular localisation profile. The counter-ion and charge on the complex also appeared to have no significant directional effect. The main component of the complex which appeared to drive the localisation was the nature of the chromophore and specifically the linkage of the chromophore to the macrocycle.

A degree of enantioselective sub-cellular localisation was observed for a series of complexes by Frawley *et al.*¹¹⁴ In each case, a difference in the quantity of cellular uptake of each enantiomer was observed as well as a shift between mitochondrial and lysosomal uptake. Although the reasons behind the trafficking mechanisms leading from macropinosomal encapsulation to sub-cellular localisation remain unknown, the method must involve a high level of selectivity.

1.6. Project Aims

³¹P-MRS has clearly been limited in its clinical potential by the poor SNR and long acquisition times associated with the technique. Therefore, this thesis aims to investigate the potential of a ³¹P T_1 contrast agent, based on the same approaches used for gadolinium ¹H contrast agent development. The application of a ³¹P contrast agent would shorten the T_1 relaxation time of phosphate species of interest and subsequently enhance the SNR per unit time. The previous studies on phosphate binding with lanthanide complexes have indicated that heptadentate DO3A complexes are a useful

structural scaffold for initial work. Most of these systems have been applied as optical probes for phosphate species, but to our knowledge there have been no studies utilising ^{31}P relaxivity for contrast in magnetic resonance imaging methods.

This thesis will incorporate an investigation of: the interaction of these heptadentate DO3A complexes with phosphate species, the comparison to commercial ^1H contrast agents and the impact of structural modifications on the properties of the contrast agent. In addition, the phosphate species of interest reside intracellularly and therefore a study of the cellular uptake and localisation will also be included in Chapter 5.

1.7. References

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Chapter 2

Evaluation of a ^{31}P Contrast Agent

2.1. Introduction

^1H -MRI gadolinium contrast agents generally contain octadentate ligands, which leave space for one water molecule to bind at the Gd^{3+} centre in fast exchange. Heptadentate gadolinium complexes, such as DO3A-derived scaffolds, demonstrate interaction with many biological anions and phosphate species, as discussed in Chapter 1. Therefore the initial design for a gadolinium ^{31}P contrast agent, which is discussed in this chapter, is based on a heptadentate DO3A-type ligand, which allows sufficient space for interaction of the Gd^{3+} centre with phosphate species. In order for the complex to work effectively as a contrast agent it is important that phosphate species bind in fast exchange, which will allow bulk phosphate to be relaxed. The SBM equations in Chapter 1 (Section 1.2.2), model the different contributions to the observed ^1H relaxation rate of a solution. Of these equations, the paramagnetic contribution and subsequent inner-sphere contribution induced by the Gd complex, are defined by equations 2.1 and 2.2:^{1,2}

$$\frac{1}{T_1^{\text{Para}}} = \frac{1}{T_1^{\text{IS}}} + \frac{1}{T_1^{\text{OS}}} \quad (2.1)$$

$$\frac{1}{T_1^{\text{IS}}} = \frac{qP_m}{T_{1m} + \tau_m} \quad (2.2)$$

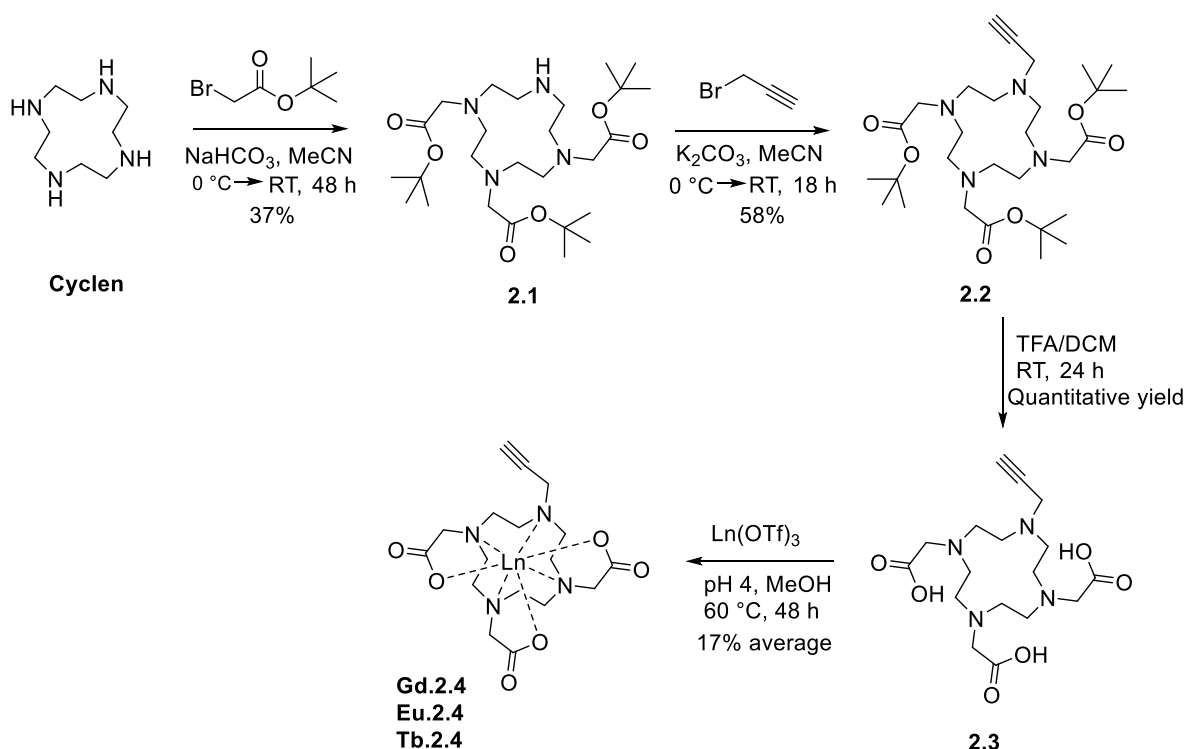
The same equations can be applied to phosphate, where q (the number of inner-sphere molecules) will be 1 for heptadentate Gd complexes, as more than one phosphate will be unable to bind simultaneously. For an ideal contrast agent, the residency time (τ_m) would need to be short (on the order of nanoseconds)³, which means weak binding of the phosphate molecules to the complex, so that bulk phosphate in solution will be relaxed. The relaxation rate of the bound phosphate molecule (T_{1m}) also needs to be short to maximise the observed relaxation rate. There are other factors which are also important to consider initially for a ^{31}P contrast agent; these include water solubility, kinetic stability and the impact of competing biological anions.

In this chapter a simple gadolinium DO3A complex is investigated as a potential ^{31}P contrast agent. This involves studying the ^{31}P and ^1H relaxivity, the binding of relevant phosphate species by luminescence studies and ^{31}P -NMR chemical shift data to assess the properties of the complex and the applicability of the complex to be used as a ^{31}P contrast agent.

2.2. Synthesis of Complex

The complex investigated as an initial ^{31}P contrast agent is Gd.pDO3A (Scheme 2.1, Gd.2.4). The advantage of pDO3A over the basic DO3A complex is that the steric bulk of a non-coordinating pendant arm on the fourth cyclen nitrogen atom will limit rotation and therefore increase kinetic stability of the gadolinium complex.^{4,5} pDO3A complexes were first published by Jauregui *et al.*⁶ for the purpose of application in ‘click’ chemistry. The synthesis of Gd.pDO3A is outlined in Scheme 2.1 and followed the procedures outlined in the literature.

Firstly, triester 2.1 was formed by reaction of cyclen with *tert*-butyl bromoacetate in acetonitrile, using sodium bicarbonate as a base. The fourth nitrogen was alkylated by addition of propargyl bromide dropwise in acetonitrile, in the presence of potassium carbonate, to form 2.2. Deprotection of the compound was achieved by stirring with TFA in DCM to give the ligand, which was precipitated as the TFA salt of 2.3. Complexation was achieved using a 1:1 ratio of the appropriate lanthanide triflate salt to ligand, in methanol at pH 4. The gadolinium complex (Gd.2.4) was made for relaxivity measurements, the europium complex (Eu.2.4) for luminescent studies and the terbium complex (Tb.2.4) for chemical shift measurements due to the larger anisotropy of terbium.



Scheme 2.1: Synthesis of pDO3A complexes.⁶

2.2.1. Desalting methods

The presence of free Ln^{3+} was determined by the Xylenol Orange test and the result observed through colour change by visual inspection. The method used follows Barge *et al.*⁷ who showed that it is sensitive enough to determine approximately $\geq 40 \mu\text{M}$ free Gd^{3+} by eye. Testing solutions of 10–40 mM Gd.pDO3A therefore allows an estimation of less than 0.1–0.4% free Gd^{3+} .

Different de-salting methods were compared for the removal of free lanthanide. It is essential that there is no free gadolinium present in the samples, as this will affect the relaxivity measurements and cause toxicity in cellular studies. A variety of methods have been used in the literature including: dialysis, size-exclusion, ion-exchange and reverse-phase HPLC.^{8,9} The complex has a relatively low molecular weight, which makes dialysis and size-exclusion chromatography inefficient due to the small size differences between triflate, free lanthanide and the complex.

Dowex[®]-50, Lewatit[®] TP-207, Amberlite[®] XAD16N ion-exchange beads were compared for removal of salts and free lanthanide. Tests were carried out using Eu.pDO3A (Eu.2.4) so that any de-complexation of europium could be determined by the appearance of the ligand in the diamagnetic region of the ^1H -NMR spectra.

Lewatit[®] TP-207 is a chelating resin, which chelates free metal ions via iminodiacetic acid groups. The Xylenol Orange test showed effective removal of free lanthanide with no colour change observed by eye after use of the chelating resin. However, ^1H -NMR spectra of the Eu complex, before and after stirring with the beads, showed the appearance of the free ligand in the NMR spectrum after exposure to the

beads. This would suggest that the beads bind to the metal ions too strongly and are able to compete with the DO3A ligand.

Dowex[®]-50 beads are a cation exchange resin, which bind positively charged ions. These beads were recommended for use with heptadentate lanthanide complexes.¹⁰ The beads were stirred initially in 5% NH_4OH to replace the proton groups with NH_4^+ so that the pH did not drop below pH 4 when the metal ions were exchanged for the cations on the beads. Stirring with these beads was found to show no appearance of free ligand in the ^1H -NMR of Eu.pDO3A while successfully removing Eu^{3+} , as determined by the Xylenol Orange test.

Amberlite[®] XAD16N works by a different mechanism, as it binds the complex as opposed to the free lanthanide ions. Therefore, it works best as a column method, where the salt is washed off with large quantities of water, followed by elution of the complex with 10–50% MeCN. This method had the advantage that anions can also be washed off as well as the cations. Xylenol Orange was used to test the water washes to determine when the complex could be eluted. No free ligand was detected in the ^1H -NMR of Eu.pDO3A after the use of Amberlite[®]. The method was found to work best with relatively low concentrations of excess lanthanide ion.

To summarise, for Ln.pDO3A the optimal desalting method proved to be Amberlite[®] XAD16N ion-exchange column, although Dowex[®]-50 can also be useful in some cases.

2.3. Relaxivity

2.3.1. ^{31}P relaxivity

The phosphate species of specific interest (as discussed in Chapter 1) are the high energy phosphate species involved in metabolism: Inorganic phosphate (P_i), Phosphocreatine (PCr) and Adenosine 5'-Triphosphate (ATP) (Figure 2.1). A model solution containing these three phosphate species (8.7 mM P_i , 5.5 mM PCr and 7.9 mM ATP) was made and buffered at pH 7.2 using phosphate buffered saline. The relaxation rate of P_i , PCr and the three phosphates of ATP— α , β and γ was measured with increasing concentrations of Gd.pDO3A (0–4 mM); the results are shown in Figure 2.2 and the relaxivity values in Table 2.1.

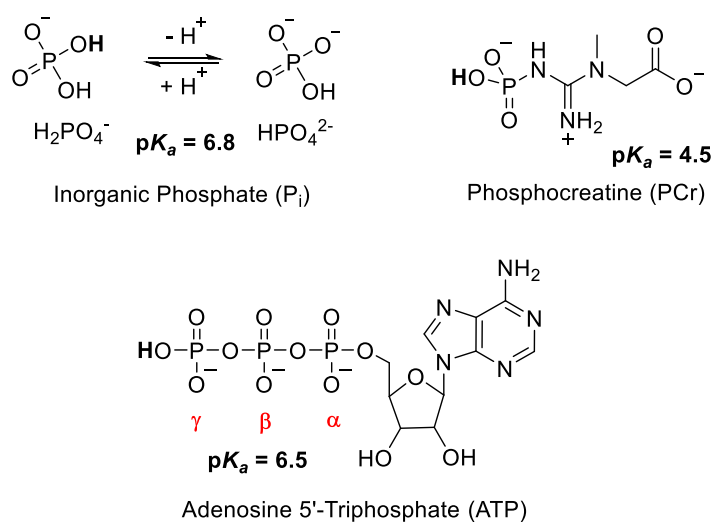


Figure 2.1: Structures of phosphate species of interest, with labelled α -, β -, γ - phosphates of ATP. pK_a values in water from literature.^{11–13}

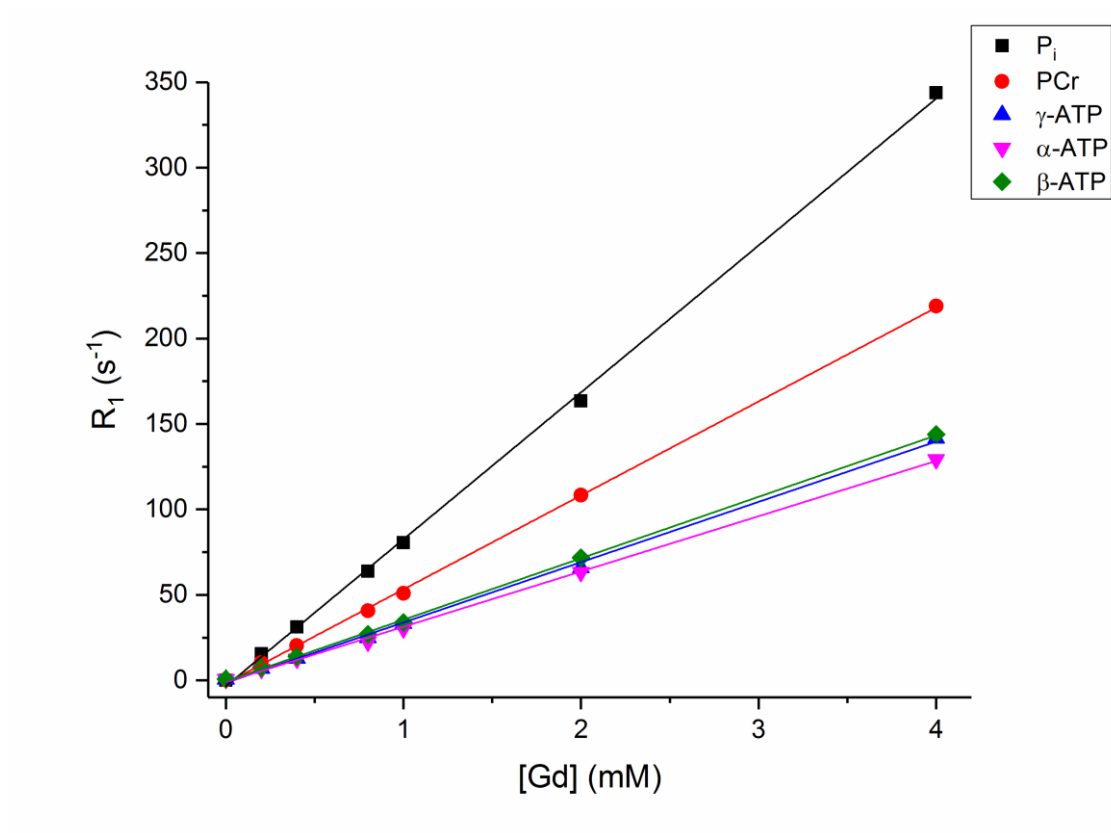


Figure 2.2: ^{31}P Relaxation rate (R_1) of P_i (■), PCr (●) and α - (▼), β - (◆), γ - (▲) ATP (8.7, 5.5 and 7.9 mM) versus concentration of Gd.pDO3A pH 7.2, 162 MHz, 298 K.

Table 2.1: ^{31}P relaxivity (r_1) of phosphate species with Gd.pDO3A , 162 MHz, pH 7.2, 298 K.

Phosphate ^a	r_1 (mMGd ⁻¹ s ⁻¹) ^b
P_i	85.96 ± 1.00
PCr	54.97 ± 0.48
α -ATP	32.35 ± 0.46
β -ATP	35.97 ± 0.35
γ -ATP	35.30 ± 0.64

^a Solution contains $[\text{PBS}] = 8.7$ mM, $[\text{PCr}] = 5.5$ mM, $[\text{ATP}] = 7.9$ mM.

^b $\pm$ Standard error of linear fit, R^2 values > 0.99 for all fits.

The ^{31}P relaxivity values in Table 2.1 show that P_i has the highest relaxivity of all the phosphate species measured. This is most likely due to the small size of phosphate, which allows it to bind in fast exchange with the Gd centre. This is followed by PCr where the relaxivity is reduced by approximately a third. The lower relaxivity may be a result of the bulky creatine moiety reducing the ease of approach to the Gd centre. All three phosphorus atoms in ATP show a lower relaxivity, which is less than half that of P_i , and may be due to the increased size of ATP over phosphocreatine. The values of all three phosphate groups of ATP are relatively similar, despite the γ -ATP being located the furthest from the adenosine moiety and therefore the least obstructed.

Previous studies on Gd.DOTA at 81 MHz demonstrated that γ -ATP showed significantly higher relaxivity than both β and α , while phosphocreatine was comparable to α -ATP.¹⁴ Gd.DOTA has no functionalisation of the fourth ring nitrogen, which means access to the Gd centre is less restricted than in Gd.pDOTA. The addition of the propargyl arm will restrict the approach of ATP to the Gd centre and this could mean that all three phosphate groups of ATP are limited in the distance of their approach by the same amount, which results in comparable ^{31}P relaxivity across the ATP molecule. Phosphocreatine displays higher relaxivity than all ATP phosphates with Gd.pDOTA; this would suggest access to the Gd centre is more restricted in Gd.DOTA. There are a few possible explanations for this: one may be that ATP is restricting access of phosphocreatine to the Gd centre with Gd.DOTA due to the closer approach of the ATP molecule. Another could be that phosphocreatine displays stronger binding to Gd.DOTA as a result of increased space at the Gd centre allowing chelation to occur and thus limiting fast exchange of phosphocreatine molecules.

2.3.2. ^1H relaxivity of water

The ^1H water relaxation rate was also measured with increasing concentrations of Gd.pDO3A in distilled water and in the phosphate solution (Figure 2.3, values in Table 2.2). The H_2O relaxivity was found to be about 16% lower in distilled water than in the phosphate solution. This is due to the competition introduced by the phosphate species for the Gd binding site, which reduces the interaction of water molecules.

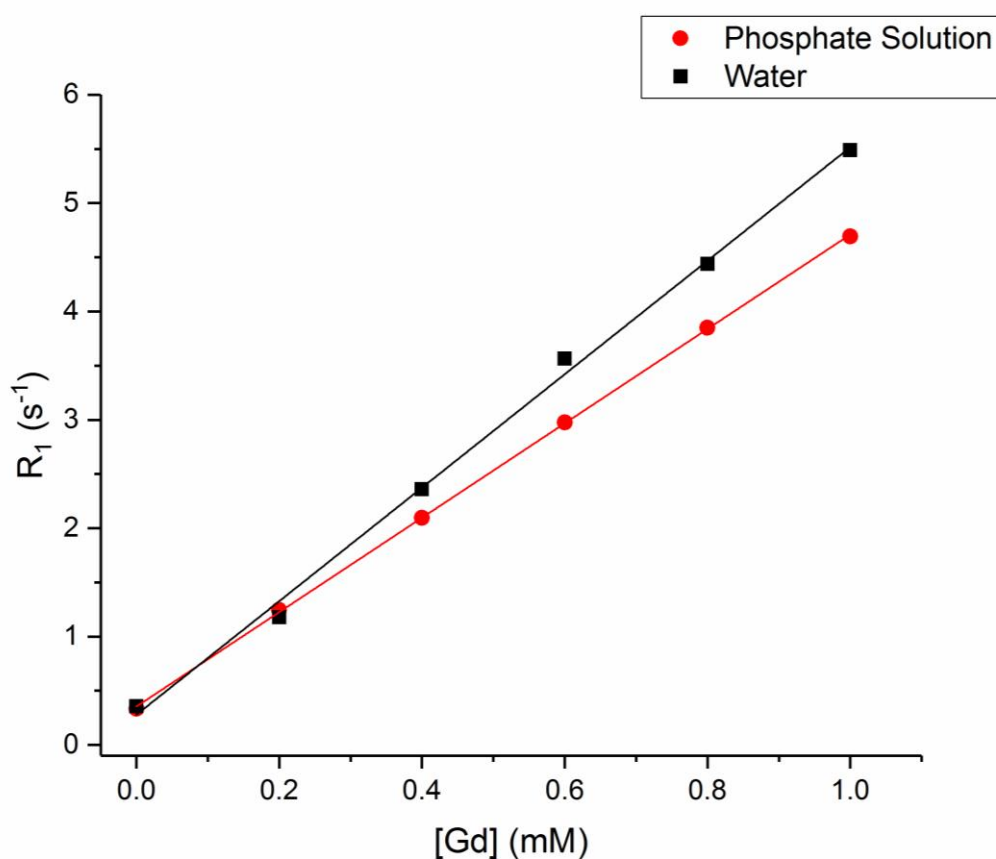


Figure 2.3: ^1H water relaxation rate (R_1) in pure water (■) or phosphate solution (●) (8.7 mM PBS, 5.5 mM PCr and 7.9 mM ATP) versus concentration of Gd.pDO3A pH 7.2, 400 MHz, 298 K. Error bars are within the size of the data points.

Table 2.2: H_2O ^1H relaxivity with Gd.pDO3A at 400 MHz, pH 7, 298 K.

Solution	r_1 (mMGd $^{-1}\text{s}^{-1}$) ^b
Water	5.24 ± 0.13
Phosphate solution ^a	4.36 ± 0.02

^a Phosphate solution = [PBS] = 8.7 mM, [PCr] = 5.5 mM, [ATP] = 7.9 mM.^b $\pm$ Standard error of linear fit, R^2 values > 0.99 for all fits.

This reduction in relaxation rate is relatively small, considering that phosphate species are expected to act as monodentate ligands and therefore would reduce the hydration number (q) from 2 to 1.^{15,16} Gd.DOTA, as a $q = 1$ complex, was found to have a relaxivity of 3.59 ± 0.07 mMGd $^{-1}\text{s}^{-1}$ in water measured under the same conditions. Therefore the relaxivity of Gd.pDO3A in the presence of phosphate species is not, as would be expected, similar to the value for Gd.DOTA, it is higher.

Higher relaxivity than anticipated for a $q = 1$ complex has been previously observed for both phosphate and fluoride in cationic triamide Gd complexes, where the relaxivity change in the presence of phosphate is almost negligible.^{17,18} This was attributed to the ability of phosphate to form a hydrogen bonded network with water. This significantly increases the contribution of second-sphere relaxation to the water relaxivity and results in a minimal decrease in the overall observed relaxivity, despite the loss of one inner-sphere water. In the case of Gd.pDO3A, the slightly higher relaxivity than expected may be a result of a weaker hydrogen-bonded network, as phosphate will be bound more weakly than in a cationic complex and therefore in faster exchange. In order for a significant contribution from the second-sphere water, the residency time of the hydrogen bonds (and therefore of the phosphate) must be

longer than the diffusion correlation time of the outer-sphere solvent molecules.¹⁹ It is also possible that as the phosphate species are in fast exchange with the complex, water is not entirely prevented from interacting at the second hydration site.

In addition to this, not all water molecules are necessarily equal. It is well established that the water exchange kinetics vary between SAP and TSAP isomers, with the TSAP isomer displaying the faster water exchange.^{20,21} It has also been reported that the water exchange rate for axial and equatorial water can differ.¹⁷ Upon binding of anions in the equatorial position, the water exchange rate was found to be higher than in the free complex. This was attributed to the difference in the water exchange rates of axial and equatorial water where $K_{\text{ex}}(\text{axial}) > K_{\text{ex}}(\text{equatorial})$. In the presence of the anion the observed water exchange rate corresponded to axial water, as opposed to an average of axial and equatorial. Slower water exchange will limit the relaxation rate of the bulk solution, as demonstrated by the SBM equations at the start of this chapter. Phosphate has been shown to bind equatorially to heptadentate lanthanide complexes in previous studies.¹⁶ Therefore, an alternative explanation for the reduction in relaxivity being smaller than expected, could be that the water molecules were not equivalent and axial water has a slightly larger contribution to relaxivity. However, as the single inner-sphere water molecule in Gd.DOTA is also axial, it would suggest that axial water exchange rate is lower in Gd.DOTA than in Gd.pDO3A.

2.3.3. Interaction with albumin

In vivo, human serum albumin (HSA) is one of the main plasma proteins in the blood – with an average concentration of 35-50 mg/mL.²² Previous work has shown that heptadentate DO3A complexes can interact with human serum albumin and enhance relaxivity due to the increase in rotation correlation time.^{16,23} This is in spite of the formation of a $q = 0$ ternary complex which negates the contribution of inner-sphere water. Therefore we looked at the interaction of Gd.pDO3A with bovine serum albumin (BSA), which is structurally comparable to HSA, to see whether phosphate relaxation was still observed in the presence of BSA. The ^1H water relaxation rate with 1 mM Gd.pDO3A in FBS and the phosphate solution are compared in Table 2.3 below, along with the ^{31}P relaxation rate of P_i in FBS and phosphate solution.

Table 2.3: ^{31}P and ^1H relaxation rate (R_1) of P_i and water with 1 mM Gd.pDO3A in FBS versus in phosphate solution, 9.4 T, 298 K.

Solution	r_1 (mMGd $^{-1}\text{s}^{-1}$)	
	^{31}P	^1H
FBS and PBS ^a	11.48 $\pm$ 0.57	2.09 $\pm$ 0.08
Phosphate solution ^b	85.96 $\pm$ 1.00	4.35 $\pm$ 0.03

^a 90% FBS + 10 mM PBS.

^b Phosphate solution = [PBS] = 8.7 mM, [PCr] = 5.5 mM, [ATP] = 7.9 mM.

The ^{31}P relaxation rate is approximately eight times lower in the presence of BSA, which is consistent with stronger binding of BSA to Gd.pDO3A and therefore a significant reduction in the accessibility of the Gd centre for phosphate interaction.

The ^1H water relaxation rate is approximately halved in FBS compared to the phosphate solution and about 60% lower compared to pure water ($5.21 \text{ mM Gd}^{-1}\text{s}^{-1}$). This result is consistent with the formation of a $q = 0$ ternary complex, but does not correlate with the previous enhancement in relaxivity observed as a result of the increased molecular weight.²³ This can be explained by the measurement of the relaxation times at high field strengths (9.4 T). Previous measurements have been at lower field strengths (0.47 T and 2.1 T). At high field strengths an intermediate rotational correlation time is optimal ($\sim 1 \text{ ns}$), as slower rotation times result in a significant drop off in relaxivity at increasing field strengths.³ Complexes bound to albumin will have a rotation correlation time of around 10 ns. Caravan *et al.* demonstrated that at 9.4 T the relaxivity of a complex with 10 ns rotation time will be equivalent to that of a complex with 0.1 ns rotation time (typical for a small Gd.DOTA size molecule).³ The ^1H relaxivity observed in FBS therefore correlates with that of a $q = 0$ ternary complex, as the enhancement in rotational correlation time has a negligible effect on the observed relaxivity at 9.4 T. Therefore the ^1H relaxivity value of $2.09 \pm 0.08 \text{ mM Gd}^{-1}\text{s}^{-1}$ for Gd.pDO3A in FBS can be attributed to second-sphere and outer-sphere interactions with water molecules only.

The reduced ^{31}P relaxivity observed for P_i with Gd.pDO3A in FBS may still contain an inner-sphere relaxivity contribution. Phosphate will be able to bind more strongly to Gd.pDO3A than water does and therefore would be able to compete with FBS for the Gd hydration sites. However, this cannot be concluded without the relevant association constants or exchange rates. Yet as the initial ^{31}P relaxivity of

Gd.pDO3A is so high, the reduced enhancement remains very effective as the relaxation times will still be 10 times shorter than native T_1 times.

2.4. Photophysical Characterisation

2.4.1. Europium and phosphate binding

In order to study the binding of these phosphate species of interest with Gd.pDO3A, the europium analogue (Eu.pDO3A) was used to monitor the change in luminescence upon binding of the phosphate anion. Europium has a very distinctive luminescence spectrum with minimal overlapping of the emission bands and minimal crystal-field splitting, which makes changes in the spectrum easy to monitor. Figure 2.4 shows the europium spectrum of Eu.pDO3A with direct excitation of europium at 394 nm and the corresponding transitions labelled. The $\Delta J = 2$ ($^5\text{D}_0 \rightarrow ^7\text{F}_2$) transition in particular is hypersensitive and therefore notable intensity changes occur upon binding events to Eu or changes in the co-ordination sphere.

Modulation of the emission intensity of Eu.pDO3A was monitored with PBS (P_i , Figure 2.5), ATP (Figure 2.6) and PCr (Figure 2.7). Fluorescence emission spectra are shown for P_i and ATP, while PCr was monitored using phosphorescence emission as a result of the increasing fluorescent emission from the anion. For ATP, this was not found to significantly affect the transitions of interest at $\Delta J = 2$ and even $\Delta J = 1$, but higher equivalents of PCr were necessary to determine the binding due to the lower affinity.

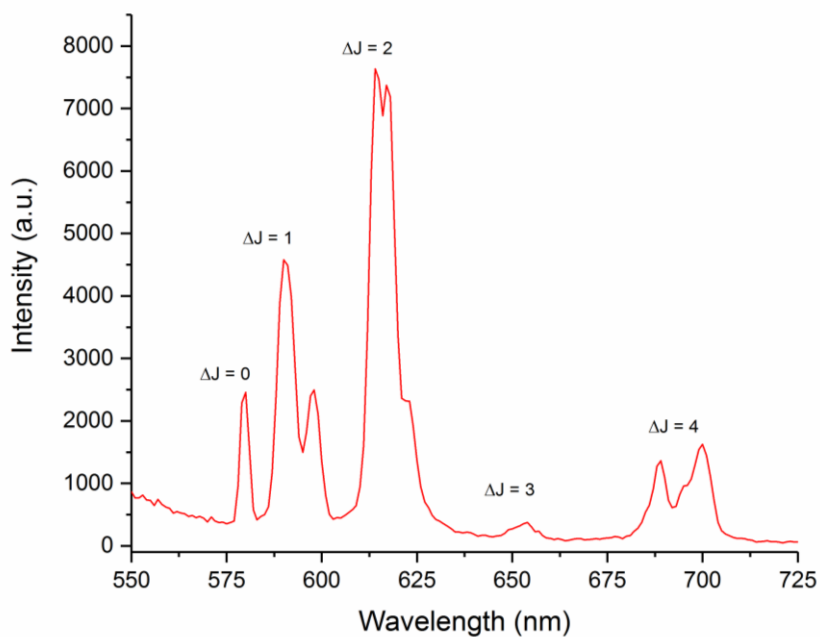


Figure 2.4: Fluorescence emission spectrum of Eu.pDO3A in 10 mM HEPES with labelled transitions.

$\lambda_{\text{ex}} = 394 \text{ nm}$, pH 7.4.

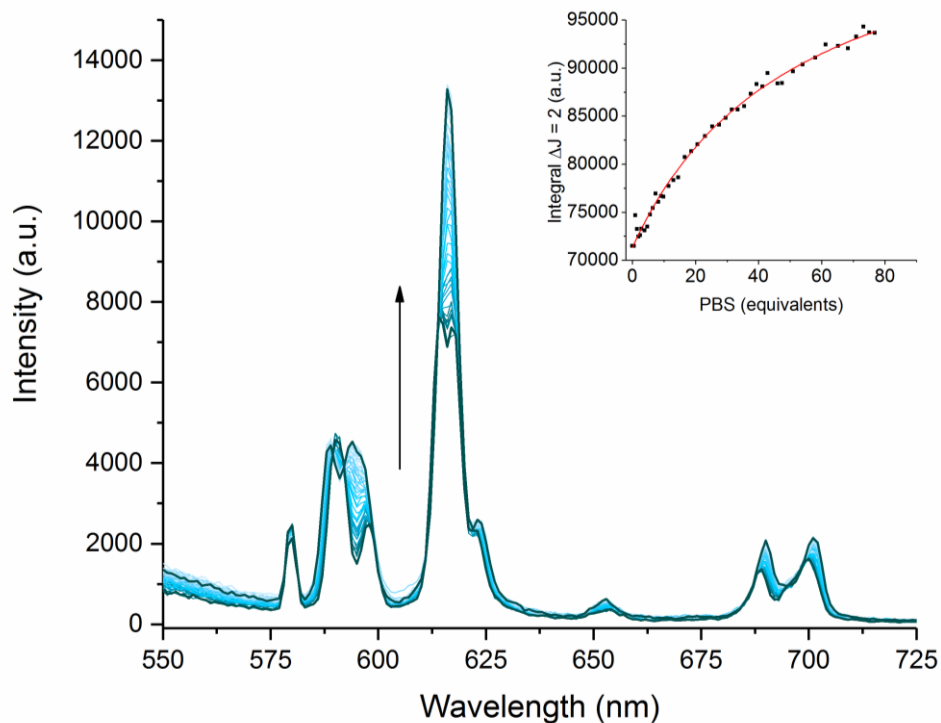


Figure 2.5: Europium fluorescence of 0.1 mM Eu.pDO3A in 10 mM HEPES with increasing [PBS].

Insert shows integral of $\Delta J = 2$ transition with increasing equivalents of PBS, $\lambda_{\text{ex}} = 394 \text{ nm}$, pH 7.4.

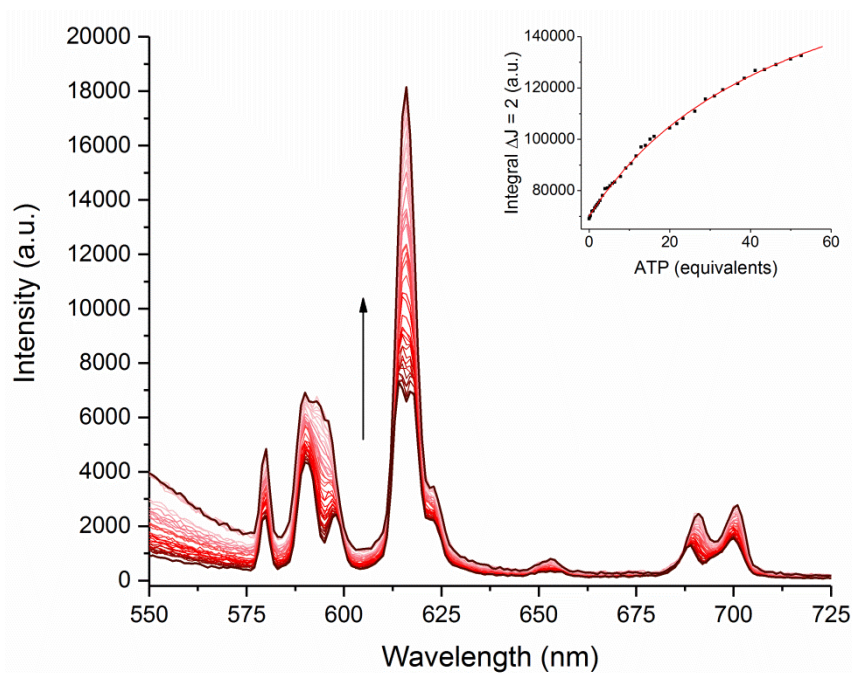


Figure 2.6: Europium fluorescence of 0.1 mM Eu.pDO3A in 10 mM HEPES with increasing [ATP].

Insert shows integral of $\Delta J = 2$ transition with increasing equivalents of ATP, $\lambda_{\text{ex}} = 394$ nm, pH 7.4.

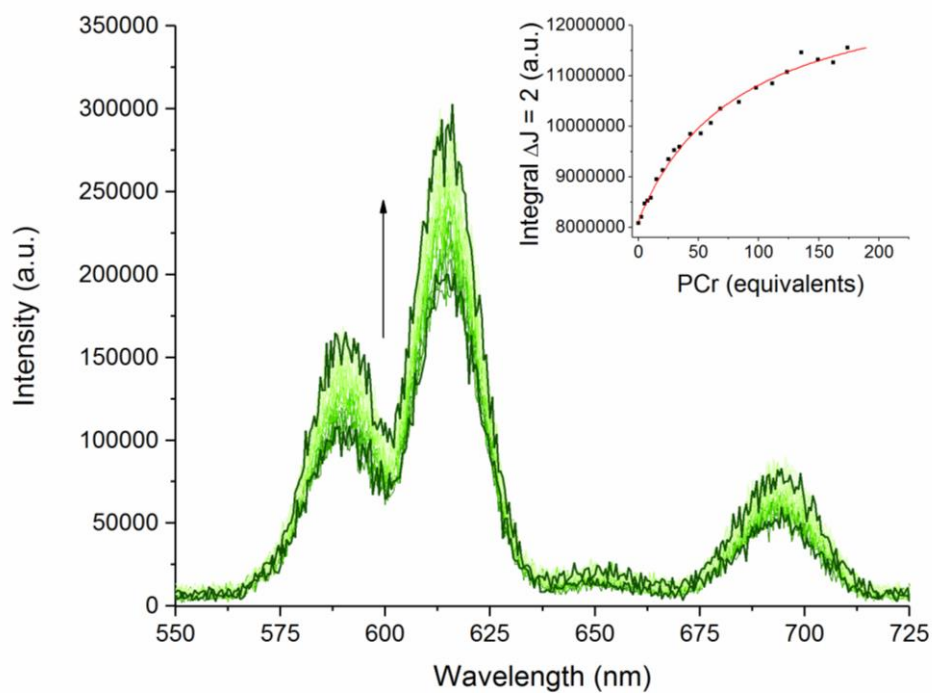


Figure 2.7: Phosphorescence of 0.1 mM Eu.pDO3A in 10 mM HEPES with increasing [PCr]. Insert

shows integral of $\Delta J = 2$ transition with increasing equivalents of PCr, $\lambda_{\text{ex}} = 394$ nm, pH 7.4.

The $\Delta J = 1$ transition is sensitive to changes in the crystal field and the magnetic anisotropy and so observation of changes in the shape of this transition can indicate whether the co-ordination is occurring in the same manner for each anion. The fluorescence spectrum of the endpoint of the PCr titration is shown in Figure 2.8, with the initial Eu.pDO3A for comparison of the fine structure. PCr and P_i show similar changes, where $\Delta J = 1$ maintains two peaks for PCr and for P_i . The first peak shifts only by 1 nm from 590 to 589 nm, while the second shifts 3 nm from 598 to 595 nm and displays a more significant intensity increase. ATP shows more distinctive coalescence of the two peaks maintaining the main peak at 590 nm. These changes are relatively small, but show that ATP may be binding in a subtly different way to P_i and PCr. This may be possible due to the multiple phosphate groups of ATP, which could allow three independent binding modes in fast exchange (Figure 2.9) and therefore all three species could be present in the luminescence spectrum

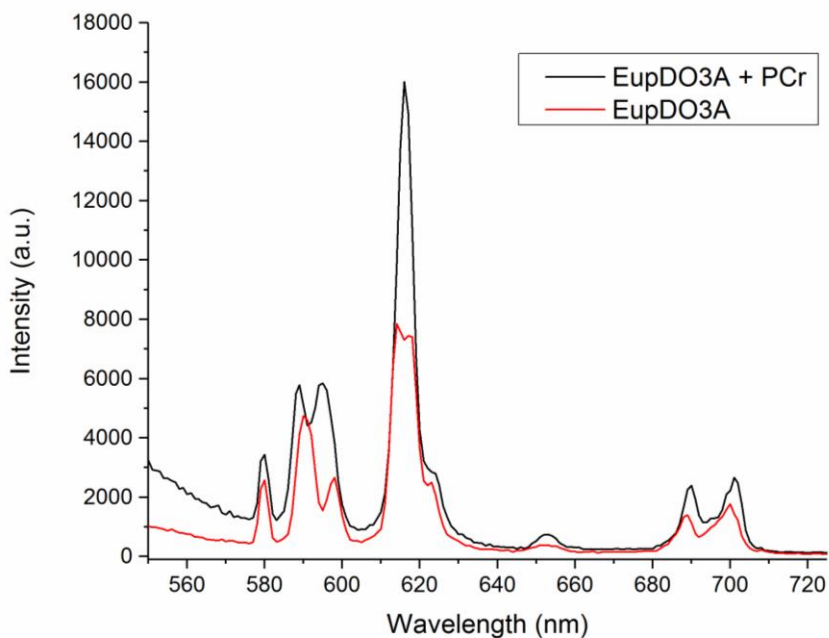


Figure 2.8: Europium fluorescence of 0.1 mM Eu.pDO3A in 10 mM HEPES (Red) and after the titration with 175 equivalents of PCr (black), $\lambda_{\text{ex}} = 394 \text{ nm}$, pH 7.4.

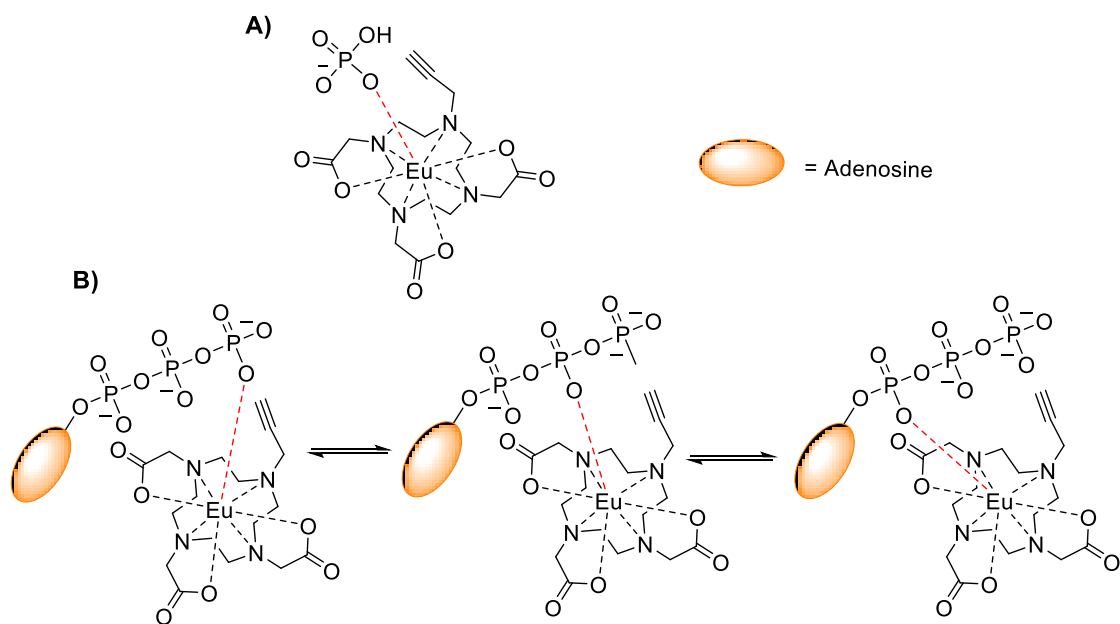


Figure 2.9: Suggested binding models of phosphate species with Eu.pDO3A: A) P_i and B) ATP.

The integral of the $\Delta J = 2$ transition was plotted against equivalents of phosphate species to determine the strength of the binding interaction. These were fitted using Dynafit[®] and modelled to the equilibrium in equation (2.3) to determine an association constant where K_a is defined as shown and X = phosphate.

$$\text{Eu} + \text{X} \leftrightarrow [\text{Eu} \cdot \text{X}] \quad (2.3)$$

$$K_a = \frac{[\text{Eu} \cdot \text{X}]}{[\text{Eu}][\text{X}]}$$

The values of K_a determined for P_i , ATP and PCr are shown in Table 2.4.

Table 2.4: Association constants (K_a) for Eu.pDO3A with each phosphate species, pH 7.4, $\lambda_{\text{ex}} = 394 \text{ nm}$.

Phosphate Species ^a	$K_a \text{ (M}^{-1}\text{)}^b$	95% Confidence Interval
P_i^c	194 ± 12	[172 – 218]
ATP	200 ± 8	[185 – 216]
PCr	120 ± 8	[104 – 138]

^aAll samples in 10 mM HEPES, with 0.1 mM Eu.pDO3A.

^b $\pm$ Standard deviation.

^cIn the absence of HEPES an apparent K_a of 261 ± 15 [232 – 292] was determined.

The interaction HEPES as a buffer was also investigated. Emission spectra of Eu.pDO3A in 10 mM HEPES and in distilled water were compared under the same conditions and found to show no apparent differences. The apparent association constant of Eu.pDO3A with PBS in water without HEPES was measured and found to

be 261 M^{-1} . Without the presence of a consistent buffer, this value is only an apparent association constant as pH is not controlled. However, as the values in the presence and absence of HEPES are relatively comparable, HEPES is thought to only show a weak interaction with Eu.pDO3A.

2.4.2. Europium emission lifetimes

The modified Horrock's equation (2.4) can be used to determine the number of bound solvent molecules (q) for the lanthanide complex in solution.²⁴

$$q = A(k_{\text{H}_2\text{O}} - k_{\text{D}_2\text{O}} - B) \quad (2.4)$$

The Eu(III) emission lifetimes of the complex (τ) are measured in H_2O and D_2O to determine the rate of depopulation of the lanthanide excited state. ($k = 1/\tau$). As explained in Chapter 1 (1.4.1), O-H oscillators are more efficient at quenching the lanthanide excited state than O-D oscillators, which allows the difference in lifetimes to be used to determine the hydration state. A is a proportionality constant, which is lanthanide specific and describes the affinity of the lanthanide for O-H molecules, while B accounts for the quenching contribution from outer-sphere solvent molecules. Equation 2.5 is relevant to europium and includes a contribution from exchangeable N-H oscillators (n_{NH}) in close proximity to the lanthanide. For the complex Eu.pDO3A, $n_{\text{NH}} = 0$

$$q_{\text{Eu}} = 1.2(k_{\text{H}_2\text{O}} - k_{\text{D}_2\text{O}} - 0.25 - 0.075n_{\text{NH}}) \quad (2.5)$$

The europium emission lifetimes of the excited state were recorded for Eu.pDO3A in H_2O and D_2O in order to determine the hydration number (q) in solution. Emission lifetimes were recorded for the complex in both water and in PBS; these and the calculated q values are displayed in Table 2.5. The calculated q value for the complex in water is close to 2, which is typical of heptadentate lanthanide complexes. The emission lifetimes of the complex with phosphate were recorded with 4000 equivalents of PBS to the complex, which ensures sufficient excess that all complex should be bound to one phosphate. The calculated q value in PBS is approximately 1, which is consistent with phosphate binding in a monodentate manner, as previously discussed (Section 2.3.2).

Table 2.5: Europium emission lifetimes of Eu.pDO3A in water and PBS, pH 7, $\lambda_{\text{ex}} = 394$ nm.

Complex	$\tau_{\text{D}_2\text{O}}$ (ms)	$\tau_{\text{H}_2\text{O}}$ (ms)	q
Water ^a	1.66	0.39	2.1
PBS ^b	1.73	0.64	0.9

^a[Eu] = 0.1 mM.

^b[Eu] = 10 μM , [PBS] = 40 mM.

2.5. ^{31}P –NMR Chemical Shifts

The ^{31}P relaxivity of ATP showed that γ and β experienced comparable relaxivity values, while α -ATP was only marginally lower. As Gd.DO3A has previously shown significantly higher relaxivity for the terminal γ -phosphate, presumably due to easier accessibility of the phosphate group furthest from the adenosine moiety, there is an

interest in the binding interaction of ATP with Gd.pDO3A. To study this, Tb.pDO3A was used, as terbium has a large magnetic anisotropy (Chapter 1) and therefore gives rise to a larger pseudo-contact shift. The effect is distance dependent and so the further the phosphate is from the Tb centre, the smaller chemical shift effect it is likely to experience. Tb.pDO3A was added to HEPES buffered ATP solution at increasing concentrations and the chemical shift monitored. A capillary insert containing NaH_2PO_4 was used as a reference. Figure 2.10 shows the effect of adding 0.5 equivalents of Tb.pDO3A to ATP on the ^{31}P chemical shift of the phosphate peaks.

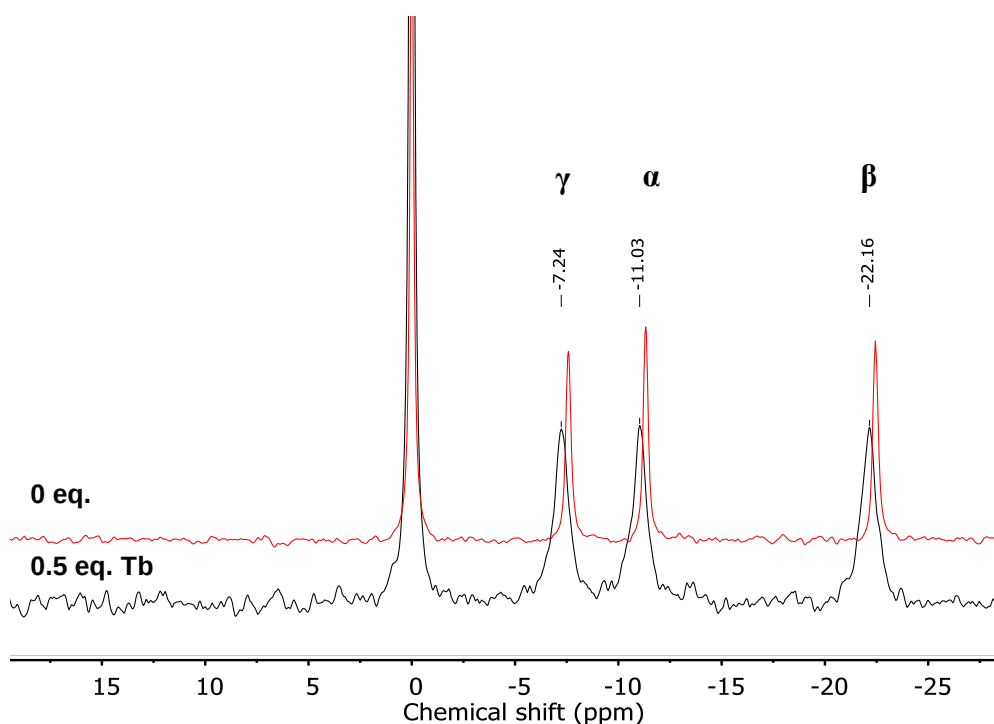


Figure 2.10: ^{31}P -NMR ATP peaks, 202 MHz, $[\text{ATP}] = 4.2 \text{ mM}$ in 20 mM HEPES buffer (black) and with 0.5 equivalents Tb.pDO3A (red), with 50 MHz line broadening. Referenced to PBS insert (0 ppm).

The effect is fairly small; there is a slight shift but mainly broadening of the peaks as a result of increasing T_2 due to the presence of the paramagnetic Tb ion. Figure 2.11

shows the effect of increasing Tb equivalents on the change in ^{31}P chemical shift of each phosphate of ATP.

The change in chemical shift is very small with less than 1 ppm difference and therefore as the errors due to line broadening are quite significant it is not possible to draw conclusions about the distance of each phosphate group from the Tb centre. The reason these shifts are so small is most likely to be due to the angle of the phosphate binding to Tb. Phosphate binds strongly in the equatorial position and therefore it is likely that the angle is approaching the magic angle where the effect of the pseudo-contact shift vanishes ($\theta = 54.7^\circ$), as shown in Figure 2.12.

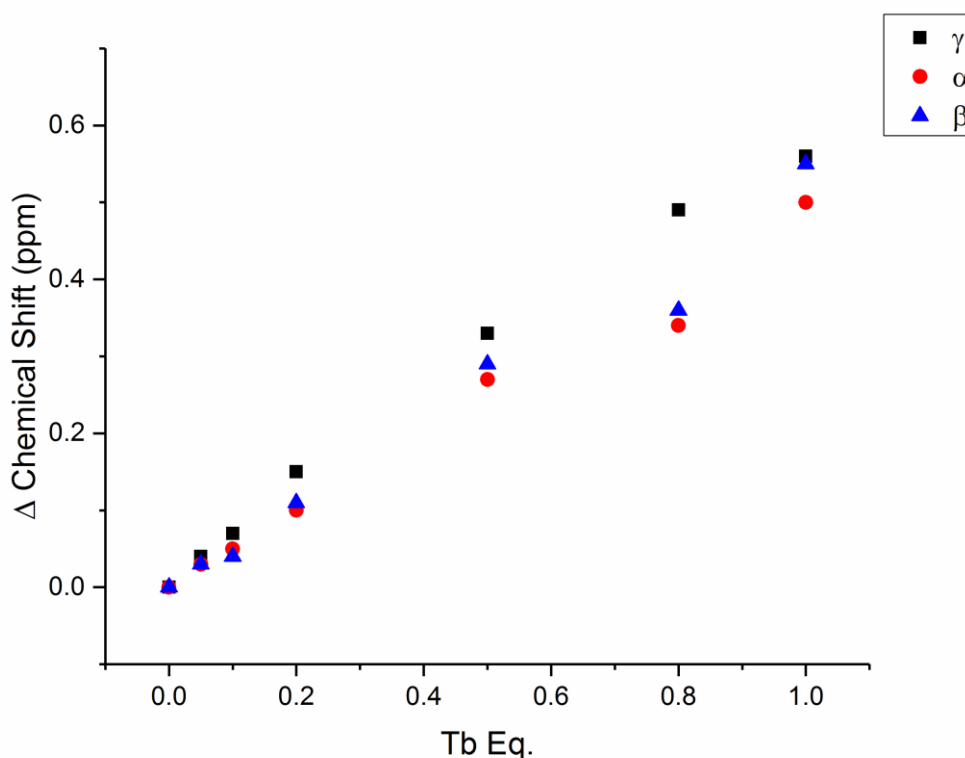


Figure 2.11: Change in ^{31}P -NMR chemical shifts of α - (●), β - (▲) and γ -ATP (■) with increasing equivalents of Tb.pDO3A, $[\text{ATP}] = 4.2 \text{ mM}$, in 20 mM HEPES, 202 MHz, pH 7.4, 298 K.

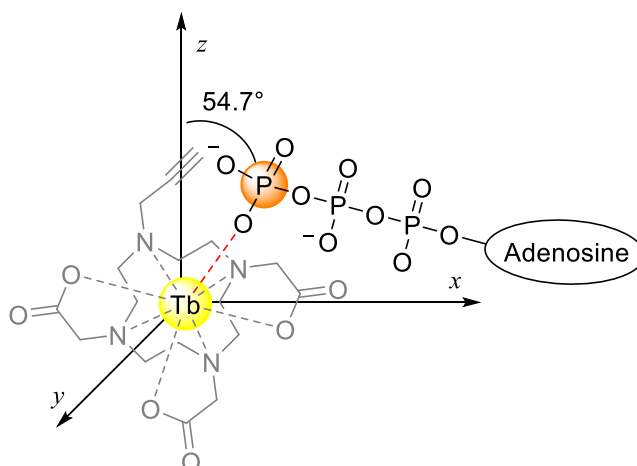


Figure 2.12: Suggested binding of ATP to Tb.pDO3A where the ^{31}P nucleus of γ -ATP is at the magic angle (54.7°).

2.6. Conclusions

Gd.pDO3A is an effective ^{31}P contrast agent in solution. It binds in fast exchange with P_i , ATP and PCr and exhibits significant relaxation of all the phosphate species. There is no particular specificity for any of the phosphate species with Gd.pDO3A as shown by the relaxivity and this is supported by the similarity in binding constants with Eu.pDO3A. ATP does not appear to show a specific preference for the terminal γ -phosphate as the relaxivity is comparable across the three phosphates. The ^{31}P -NMR chemical shifts with Tb.pDO3A do not give any indication of preferential phosphate group binding, as the chemical shifts are so small.

For Gd.pDO3A to be a useful contrast agent for ^{31}P -MRS it is necessary for it to come into contact with the species of interest *in vivo*. Therefore, cellular uptake experiments are required to determine the degree of uptake as well as the localisation intracellularly. Adaptations of the complex are likely to be required as Gd.pDO3A has

no particular functional groups that are known to facilitate transport across membranes.

2.7. References

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Chapter 3

Using ^{31}P -MRS to Establish the Stability of Commercial Contrast Agents

3.1. Introduction

In Chapter 2 it was demonstrated that seven co-ordinate gadolinium complexes like Gd.pDO3A have the potential to be used as ^{31}P contrast agents in solution. Most commercial complexes are octadentate and therefore phosphate species would have to act as a competitive ligand for gadolinium. In light of recent findings regarding the release of Gd^{3+} in the body from kinetically labile structures, studies of the kinetic stability of Gd complexes in the presence of biologically relevant compounds are of particular importance.¹

This chapter explores and compares the interaction of ATP, PCr and P_i with both linear and macrocyclic commercial contrast agents. The macrocyclic contrast agent Gd.DOTA (Dotarem[®]) and the linear contrast agents Gd.DTPA (Magnevist[®]) and Gd.DTPA-BMA (Omniscan[®]), were compared to the seven-coordinate complex previously discussed in Chapter 2—Gd.pDO3A. ^{31}P T_1 relaxation times can be a good indicator of the type of interaction of the phosphate species with Gd in solution.² In addition the changes in ^1H T_1 relaxation times of water can also indicate changes in the competition for the Gd binding site.³

3.2. ^{31}P Relaxivity

The interaction of commercial contrast agents with phosphate species ATP, PCr and P_i were assessed using ^{31}P and ^1H -MRS in an analogous method to that used for Gd.pDO3A. A model solution of ATP, PCr and P_i was used to allow competitive binding between the different phosphate species and to compare how the Gd complexes interact with each phosphate species. The concentration of each of the phosphate species in solution was confirmed using ^{31}P -MRS, through use of the metabolite hydrates. The stability of the phosphate solution was established over a 20 h period, by repeated single pulse-acquire spectra in which the ATP and PCr peaks were integrated and normalised to the peaks in the initial ^{31}P spectrum. This showed no apparent degradation of any of the phosphate species over the time course of the experiments (Figure A.1, Appendix).

The concentration range of contrast agents was chosen to try and reflect appropriate concentrations *in vivo*. The concentration of clinically administered (commercial) Gd-based contrast agents is below 2 mM in plasma *in vivo* one minute after injection according to manufacturer's directions.^{4,5} ^{31}P Relaxation rates ($R_1 = 1/T_1$) were measured using inversion recovery for ATP, PCr and P_i with increasing concentrations of each contrast agent. The effect of increasing the concentration of Gd contrast agents on the ^{31}P relaxation rate (R_1) is shown in Figure 3.1.

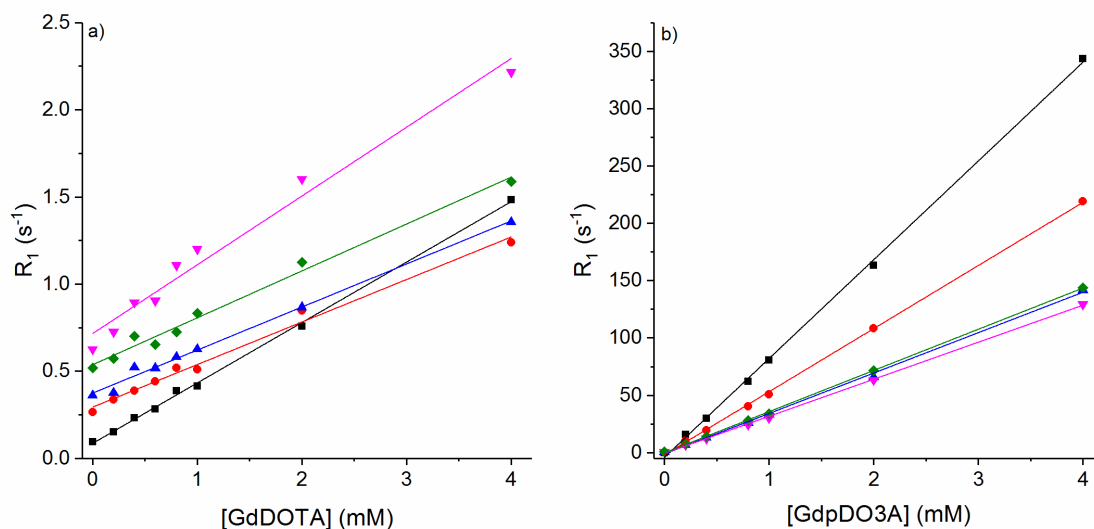


Figure 3.1: ^{31}P Relaxation rates (R_1) of P_i (■), PCr (●) and α - (▼), β - (◆), γ -ATP (▲) (8.7, 5.5 and 7.9 mM) versus concentration of contrast agent at pH 7.2, 9.4 T.

Increasing concentrations of the macrocyclic contrast agent Gd.DOTA resulted in a small linear relaxation enhancement across all five phosphate peaks (P_i , PCr, γ -ATP, α -ATP, β -ATP) (Figure 3.1a). The ^{31}P relaxivities ($r_1 = 1/([\text{Gd}]T_1)$) of all phosphate species were below $0.5 \text{ mM}^{-1}\text{s}^{-1}$. These relaxivity values for Gd.DOTA are more than 60 times smaller than the relaxivity values measured for Gd.pDO3A (Figure 3.1b). This demonstrates the significant advantage that Gd.pDO3A displays over currently available macrocyclic alternatives for ^{31}P relaxation rate enhancement. As Gd.DOTA is an octadentate complex and phosphate species do not have sufficient space to bind, this low level relaxivity observed with Gd.DOTA for all phosphate species can be attributed to an outer-sphere relaxation mechanism only. Outer-sphere relaxation is defined by diffusion, the distance of closest approach and the electronic relaxation time of the metal ion.⁶ For complexes of a similar size and molecular weight, the outer-sphere contribution is considered to be comparable and independent of

differences in functional groups on the ligand. On this basis, we can use the relaxivity measurements obtained using Gd.DOTA to estimate the inner sphere contribution to ^{31}P relaxivity with Gd.pDO3A, using equation 3.1:

$$r_1 = r_1^{\text{IS}} + r_1^{\text{OS}} \quad (3.1)$$

where the relaxivity (r_1) has both an inner-sphere (r_1^{IS}) and an outer-sphere (r_1^{OS}) contribution. The inner-sphere and outer-sphere estimated values for Gd.pDO3A are listed in Table 3.1.

Table 3.1: Inner-sphere (IS) and outer-sphere (OS) contribution to ^{31}P relaxivity in mM Gd $^{-1}\text{s}^{-1}$ of phosphate species with Gd.pDO3A, 9.4 T, pH7.2, 20°C.

Phosphate ^a	r_1 (mMGd $^{-1}\text{s}^{-1}$) ^b		% IS contribution
	IS	OS ^c	
P _i	85.78 ± 1.07	0.35 ± 0.01	99.6 ± 1.76
PCr	54.79 ± 0.50	0.24 ± 0.01	99.6 ± 1.29
α-ATP	31.91 ± 0.33	0.39 ± 0.03	98.8 ± 1.45
β-ATP	35.66 ± 0.29	0.27 ± 0.01	99.2 ± 1.15
γ-ATP	34.99 ± 0.60	0.25 ± 0.01	99.3 ± 2.42

^aPhosphate in solution: [PBS] = 8.7 mM, [PCr] = 5.5 mM, [ATP] = 7.9 mM.

^bR² > 0.96 for all linear fits, values ± standard error.

^cGd.DOTA r_1 values, assumed to be outer-sphere only.

Table 3.1 shows that the inner-sphere contribution to the overall observed ^{31}P relaxivity is very large and is clearly the dominant effect. For ^1H relaxation the outer-sphere relaxivity has been found to have a significant contribution to the observed relaxivity of around 40% (at imaging field strengths).^{7,8}

In Chapter 2, FBS was shown to reduce the proton relaxivity of Gd.pDO3A down to $2.09 \pm 0.08 \text{ mMg}^{-1}\text{s}^{-1}$ from $5.24 \pm 0.13 \text{ mMg}^{-1}\text{s}^{-1}$ in water. This was attributed to outer-sphere and second-sphere only contributions, due to the formation of a $q = 0$ complex, where the increase in molecular weight has negligible enhancement in relaxation enhancement at these field strengths. This would correlate with about a 40% outer-sphere and second-sphere contribution to the observed ^1H relaxivity of Gd.pDO3A. The higher contribution to outer-sphere relaxivity in ^1H relaxivity is due to the fact that there is a significantly higher concentration of water molecules, which can contribute to multiple second-sphere interactions. On the grounds that the total concentration of phosphate species will be less than 0.025% that of water (25 mM total phosphate species versus ~100 M water) the contribution of second-sphere phosphate molecules is likely to be relatively small. This is consistent with the small ^{31}P relaxivity values observed for Gd.DOTA. It is possible that Gd.pDO3A may exhibit more second-sphere interactions due to the differences in structure. However, this will still remain a relatively small proportion of the inner-sphere contribution observed.

The SBM equations outlined in previous chapters and shown below, describes the factors that affect the inner-sphere contribution that is observed here.

$$\frac{1}{T_1^{\text{IS}}} = \frac{qP_m}{T_{1m} + \tau_m} \quad (3.2)$$

The variation in inner-sphere ^{31}P relaxivity values for each phosphate species can be attributed to the differences in the binding constant and subsequently phosphate residency time (τ_m). The other parameters (q and T_{1m}) will remain reasonably constant as only one phosphate species can bind at one time ($q = 1$) and the relaxation time of the molecule T_{1m} is the inherent relaxivity of the molecule.

The ^{31}P relaxivity values of linear contrast agents Gd.DTPA and Gd.DTPA-BMA were measured using the same method. The results are shown in Figure 3.2. With increasing concentration of the acyclic contrast agent Gd.DTPA there is a small linear enhancement of the relaxation rate of P_i and PCr, which is of a similar magnitude to that observed for Gd.DOTA (Table 3.2)—and can therefore be attributed to outer-sphere interactions.

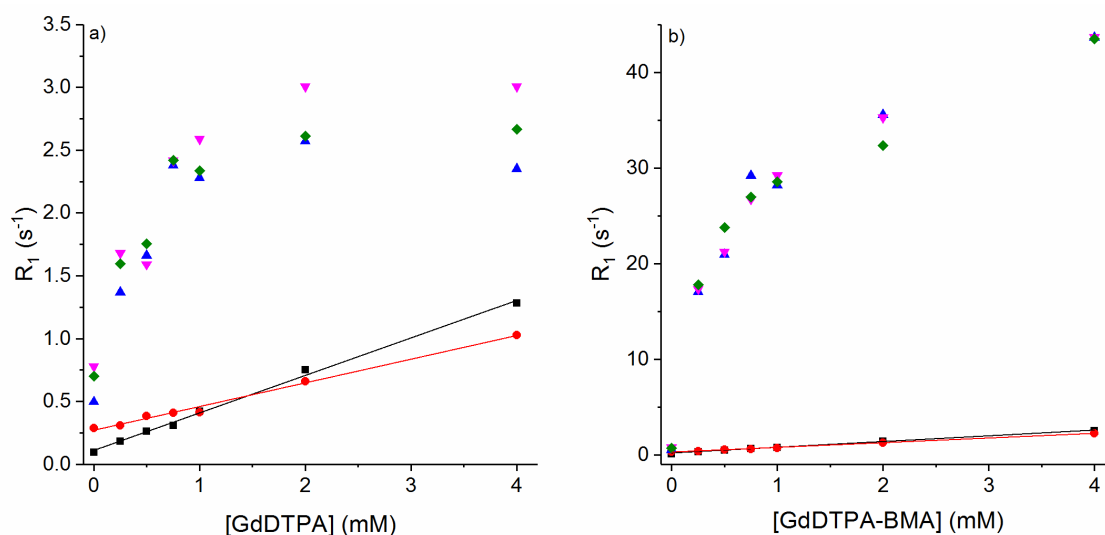


Figure 3.2: ^{31}P Relaxation rates (R_1) of P_i (■), PCr (●) and α - (▼), β - (◆), γ -ATP (▲) (8.7, 5.5 and 7.9 mM) versus concentration of contrast agent at pH 7.2, 9.4 T.

Table 3.2: ^{31}P relaxivity values for PCr and P_i with Gd.pDO3A (Gd.2.4), Gd.DOTA, Gd.DTPA and Gd.DTPA-BMA at pH 7.2, 9.4 T, 20°C.

Phosphate ^a	$r_1 (\text{mMGd}^{-1}\text{s}^{-1})^b$			
	Gd.pDO3A	Gd.DOTA	Gd.DTPA	Gd.DTPA-BMA
P_i	85.96 ± 1.00	0.35 ± 0.01	0.30 ± 0.01	0.60 ± 0.01
PCr	54.97 ± 0.48	0.24 ± 0.01	0.19 ± 0.01	0.49 ± 0.01

^aPhosphate in solution: [PBS] = 8.7 mM, [PCr] = 5.5 mM, [ATP] = 7.9 mM.

^b $R^2 > 0.96$ for all linear fits, values $\pm$ standard error.

However, the three phosphates of ATP demonstrate a non-linear relationship between R_1 and Gd.DTPA concentration (Figure 3.2a). As the gadolinium concentration is increased, there is an initial steep rise in R_1 of ATP. However, R_1 appears to reach a plateau above 1 mM Gd.DTPA. In comparison to Gd.DOTA, the R_1 values of α -, β - and γ -ATP at 1 mM Gd are about 2.0, 2.5 and 3.5 times larger respectively, while the PCr and P_i values remain comparable. Gd.DTPA-BMA exhibits a significantly more pronounced effect on the three phosphate peaks from ATP compared to that of Gd.DTPA. The relaxation rate enhancement of ATP is non-linear with respect to the concentration of Gd.DTPA-BMA and the R_1 of α -, β - and γ -ATP at 1 mM Gd are 22, 33 and 45 times larger respectively than with the same concentration of Gd.DOTA. Above 1 mM Gd, the relationship between R_1 of ATP and Gd concentration appears to continue to increase linearly rather than reaching a plateau. The relaxivity of P_i and PCr remain linear with increasing Gd.DTPA-BMA concentration and the values are comparable to that of Gd.DOTA, although slightly higher (Figure 3.2b). The higher relaxivities may be a result of second-sphere contributions.

The non-linear enhancement of R_1 of ATP with increasing concentrations of the linear Gd complexes (Gd.DTPA and Gd.DTPA-BMA) suggests there is a contribution from inner-sphere binding of ATP to the metal centre. This would require at least partial displacement of donor groups on octadentate ligands already bound to the lanthanide. Gd.pDO3A maintains a linear relationship of R_1 with Gd concentration due to fast exchange of all phosphate species with bulk phosphate. In comparison, the non-linearity observed may be due to a slow exchange regime, where strongly bound ternary complexes are formed, or complete Gd dissociation occurs. ATP is a chelating ligand and $\text{Ln}(\text{ATP})_n$ complexes have been previously characterised,⁹ so it is classed as a competing ligand.

The ^{31}P relaxivity values measured for these macrocyclic versus linear contrast agents, are consistent with earlier studies by Vander Elst *et al.*¹⁰ The non-linear enhancement of R_1 of ATP with increasing concentrations of the linear Gd complexes (Gd.DTPA and Gd.DTPA-BMA) was previously observed for these complexes at a lower magnetic field (81 MHz instead of 162 MHz). The relaxivity values observed were higher; this is most likely a combination of the difference in field strength and the use of the clinical formulations in our study. These clinical formulations contain low concentrations of excess ligand: Gd.DTPA-BMA contains 5% excess ligand (Ca.DTPA-BMA.Na, caldiamide sodium) and Gd.DTPA contains 0.2% excess ligand (diethylenetriaminepentaacetic acid) to prevent dissociation of Gd. This significantly reduces the amount of free Gd^{3+} formed, due to a shift in the equilibrium between the complex and the ligand with free Gd^{3+} .¹⁰

Vander Elst *et al.* reported the R_1 β -ATP/PCr ratio at 1 mM Gd as 20 for Gd.DTPA and 65 for Gd.DTPA-BMA using the pure complexes without excess

ligand; these values are much greater than the 5.7 and 39 respectively reported here. These results suggest that the interpretation by Vander Elst *et al.*—that the saturation plots observed are a result of competitive binding to Gd^{3+} —is the most likely explanation as it would be consistent with lower relaxation values, corresponding to a shift in the equilibrium as a result of excess ligand. From these results it is clear that, while excess ligand mitigates the problem of competitive binding, excess ligand does not completely prevent the release of Gd. Both Gd.DTPA and Gd.DTPA-BMA show evidence of some $\text{Gd}(\text{ATP})_n$ formation due to non-linearity in the R_1 of ATP versus Gd concentration plots. The significantly higher R_1 values for ATP with Gd.DTPA-BMA than with Gd.DTPA would suggest that the Gd.DTPA-BMA ligand is less kinetically stable and therefore allows more $\text{Gd}(\text{ATP})_n$ to be formed. These findings are also consistent with those of Frenzel *et al.* which demonstrated that Gd is still released in the presence of excess ligand, albeit more slowly than with a pure complex.¹¹ There has also been some evidence of phosphate playing a role in this dissociation.^{11,12}

3.3. ^1H T_1 and T_2 Measurements

^1H T_1 and T_2 relaxivities were recorded for Gd.DOTA, Gd.DTPA, and Gd.DTPA-BMA in phosphate solution and are displayed in Table 3.3. The values vary from most reported values due to their measurement at 9.4 T and room temperature (20°C), as opposed to 0.47–4.7 T and 37°C.¹³

Table 3.3: ^1H relaxivity of contrast agents in phosphate solution at pH 7.2, 9.4 T, 20°C^a

Contrast Agent	r (mMGd $^{-1}$ s $^{-1}$) ^b	
	r_1	r_2
Gd.DOTA ^c	3.89 ± 0.05	4.89 ± 0.07
Gd.DTPA	4.22 ± 0.07	5.33 ± 0.05
Gd.DTPA-BMA ^d	3.87 ± 0.04	4.78 ± 0.06

^a Phosphate solution contains 8.7, 5.5 and 7.9 mM P_i , PCr and ATP at pH 7.2.

^b $\pm$ Standard Error of fitted results. R^2 values > 0.99 for all fits.

^c In water Gd.DOTA $r_1 = 3.59 \pm 0.07$. ^d In water Gd.DTPA-BMA $r_1 = 3.83 \pm 0.01$

The relaxation rates increased linearly as the concentration of contrast agent increased. The ^1H r_1 of each contrast agent in the phosphate solution is comparable to that of Gd.pDO3A (4.36 ± 0.01 mM $^{-1}$ s $^{-1}$), albeit slightly lower. This was explained in Chapter one; that, in the presence of phosphate species, Gd.pDO3A has a high second-sphere relaxation contribution, which results in a relaxivity slightly higher than expected for a $q = 1$ complex. The ^1H r_1 of Gd.DOTA and Gd.DTPA-BMA were also measured in pure water without any phosphate species. The values are comparable to those in the phosphate solution: 3.59 ± 0.07 mM $^{-1}$ s $^{-1}$ for Gd.DOTA and 3.83 ± 0.01 mMGd $^{-1}$ s $^{-1}$ for Gd.DTPA-BMA. Gd.pDO3A demonstrated a significantly higher relaxivity in pure

water ($5.21 \pm 0.11 \text{ mM}\text{Gd}^{-1}\text{s}^{-1}$), which is representative of the ternary phosphate complexes reducing the hydration number of the complex from 2 to 1.

The octadentate commercial complexes all have a q value of 1 and as the value does not change significantly in the phosphate solution versus in water, this would suggest that the water co-ordination site remains consistent regardless of the presence of other anions. For Gd.DOTA this consistent relaxivity supports the almost negligible ^{31}P relaxivity enhancement in indicating that there is no significant phosphate interaction with the Gd complex. For Gd.DTPA-BMA, it would imply that a ternary complex that obscures the water co-ordination site is not forming. It would also suggest however, that any dissociation and formation of $\text{Gd}(\text{ATP})_n$ is either very low or the ^1H relaxivity of $\text{Gd}(\text{ATP})_n$ is comparable to Gd.DTPA-BMA. Muller *et al.* found that at a very high excess of ATP (4000-fold) with Gd.DTPA-BMA (the complex only and not the clinical formulation) an increase in the ^1H relaxivity was observed which corresponded with the higher relaxivity measured for $\text{Gd}(\text{ATP})_n$.¹⁰ Therefore, as no change was observed in the ^1H relaxivity of Gd.DTPA-BMA in our experiments, it is most likely that the quantity of $\text{Gd}(\text{ATP})_n$ formed is a very small proportion of the Gd in solution.

The reported ^{31}P relaxivity of $\text{Gd}(\text{ATP})_n$ at 81 MHz is approximately $550 \text{ mM}^{-1}\text{s}^{-1}$ as opposed to around $0.3 \text{ mM}^{-1}\text{s}^{-1}$ for outer-sphere relaxation.¹⁰ Therefore, a very low level of Gd dissociation may not noticeably affect the ^1H relaxivity, while the ^{31}P ATP relaxivity shows a more distinctive change because of the significantly larger ^{31}P relaxivity of $\text{Gd}(\text{ATP})_n$ compared to outer-sphere relaxation.

3.4. pH Dependence of ^{31}P R_1 Measurements

The pH will determine the extent of ligand protonation as well as the extent to which ATP is protonated. Protonation of the ligand aids dissociation of the complex and therefore a reduction in pH would be expected to enhance dissociation and make it easier for ATP to extract Gd. The ^{31}P relaxation rates were measured at two different pHs for each Gd.DTPA (pH 7.2 and 7.4) and Gd.DTPA-BMA (pH 7.1 and 7.2) to see if the results support extraction of Gd by ATP (Figure 3.3).

The non-linear trend of ATP relaxation with increasing Gd concentration was consistent at all pH values. However, the R_1 values of all ATP phosphates increased at lower pH for both contrast agents, while PCr and P_i values remained comparable at differing pH. Gd.pDO3A ^{31}P relaxivity values remain very similar within these small pH changes, due to the ternary complex formation, which does not depend strongly on pDO3A ligand protonation or dissociation. This indicates that the interaction of Gd.DTPA and Gd.DTPA-BMA with ATP is highly dependent on the extent to which the ligand and ATP is protonated and therefore full or partial dissociation of the ligand is likely to be occurring.

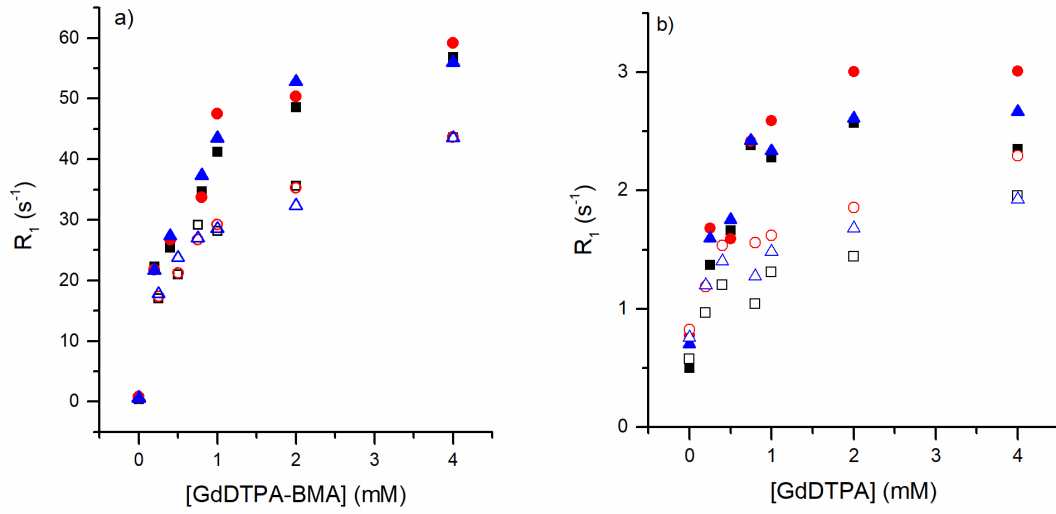


Figure 3.3: Effect of pH on ^{31}P R_1 of α - (●), β - (▲), γ - (■) ATP vs. $[\text{Gd}]$, where Gd is a) Gd.DTPA-BMA at pH 7.1 (closed symbols) and pH 7.2 (open symbols); b) Gd.DTPA at pH 7.2 (closed symbols) and pH 7.4 (open symbols).

3.5. Conditional Stability Constants

Using literature values for the protonation and stability constants we can estimate the conditional stability constants for each of the complexes at pH 7.2 in the presence of ATP. The equations to calculate these are shown below (equation 3.3, 3.4 and 3.5)¹⁴ and the values are summarised in Table 3.4.

$$K_{\text{Cond}} = \text{Log}K_f - \text{Log} \alpha_{\text{HL}} - \text{Log} \alpha_{\text{M.ATP}} \quad (3.3)$$

$$\alpha_{\text{HL}} = 1 + [\text{H}^+]K_1 + [\text{H}^+]^2K_1K_2 \dots \quad (3.4)$$

$$\alpha_{\text{M.ATP}} = 1 + [\text{ATP}]K_1 + [\text{ATP}]^2K_1K_2 \quad (3.5)$$

K_f is the formation constant of the relevant contrast agent, α_{HL} and $\alpha_{\text{M.ATP}}$ are the coefficients of the side reactions which are ligand protonation and ATP competition

respectively. The constants used for these calculations are summarised in Section A2 of the Appendix (Table A.1 and A.2) and based on previously measured values in the literature.^{15–18}

Table 3.4: Conditional stability constants for contrast agents at pH 7.2 with 7.9 mM ATP.

Contrast Agent	Conditional stability constant at pH
	7.2 with ATP (Log K_{cond})
Gd.DOTA	11.1
Gd.DTPA	12.3
Gd.DTPA-BMA	8.9

According to Eads *et al.*⁹ $\text{Gd}(\text{ATP})_2$ is likely to be the dominant species when the Gd/ATP ratio is below 0.5. However, if dissociation is occurring the ratio of Gd/ATP is likely to be very low and it is unknown if other species such as $\text{Gd}(\text{ATP})_3$ may form. Mixed ATP/ligand complexes have been previously observed for EDTA and BOPTA.^{16,19} BOPTA contains an aromatic moiety, which was thought to interact with the adenine group in ATP to form a ternary complex. While with EDTA, partial dissociation was thought to occur, which allowed simultaneous binding of EDTA and ATP. However, the absence of crystallographic data of these mixed ligand complexes means that the structures of these binding models are still unknown.

It is now well established that, in general, linear contrast agents have a lower kinetic stability than macrocyclic contrast agents and that kinetic stability is a better assessment of Gd dissociation *in vivo* than thermodynamic stability.^{11,20,21} Due to the high kinetic stability of Gd.DOTA, it is highly unlikely that any $\text{Gd}(\text{ATP})_n$ would be

formed in the timescale of these experiments, despite the conditional stability constant being lower than Gd.DTPA.²² This is consistent with the outer-sphere only relaxation mechanism for Gd.DOTA with ATP seen here. The size of the conditional stability constants suggests that at pH 7.2, if dissociation occurs, the quantity of $\text{Gd}(\text{ATP})_n$ formed must be very small in proportion to the quantity of complex in total even for Gd.DTPA-BMA. It is possible that the inorganic phosphate in the solution may aid the dissociation of the complexes as this has been previously observed,^{11,12} however this wasn't included in the calculation of the conditional stability constants.

3.6. Kinetic Stability Assessment

^{31}P relaxation rates were measured over time after mixing the contrast agent with the ATP, PCr, P_i solution, to assess the kinetics of the reaction. The stability of Gd.DTPA ^{31}P T_1 relaxation rates (R_1) of ATP were assessed over a 2 h period and found to increase significantly while the concentration of ATP remained constant (Figure 3.4). It was found that the R_1 of γ -ATP with 0.4 mM Gd.DTPA, for example, more than doubled between 20 mins and 2 h after addition of the contrast agent. This notable increase in ATP R_1 values for Gd.DTPA over time may be due to slow formation of $\text{Gd}(\text{ATP})_n$. This means that the time between mixing the solution and the measurement of R_1 , as well as the time taken for data acquisition, will affect the relaxation enhancement seen for ATP with Gd.DTPA.

The ^{31}P R_1 values of the other contrast agents with phosphate species were assessed over 12 h (Figure 3.5); the R_1 values of all ^{31}P -species remained constant over time following addition of Gd.DOTA and Gd.DTPA-BMA, despite significantly

higher R_1 values for ATP with Gd.DTPA-BMA than with Gd.DOTA. This is consistent with Gd.DOTA demonstrating no significant release of chelated Gd at all, while Gd.DTPA-BMA shows relatively fast release of chelated Gd reaching an equilibrium where no further Gd release occurs over time. It is therefore assumed that Gd.DTPA-BMA has already reached an equilibrium between Gd complex and $\text{Gd}(\text{ATP})_n$ prior to acquisition of the first data point ($t = 13$ min) and that no further dissociation occurs. Gd.pDO3A showed a slight increase in the R_1 values for ATP over 12 h, while for PCr and P_i , R_1 remained fairly constant. As for Gd.DTPA, this gradual increase in R_1 over time is consistent with slow release of Gd^{3+} .

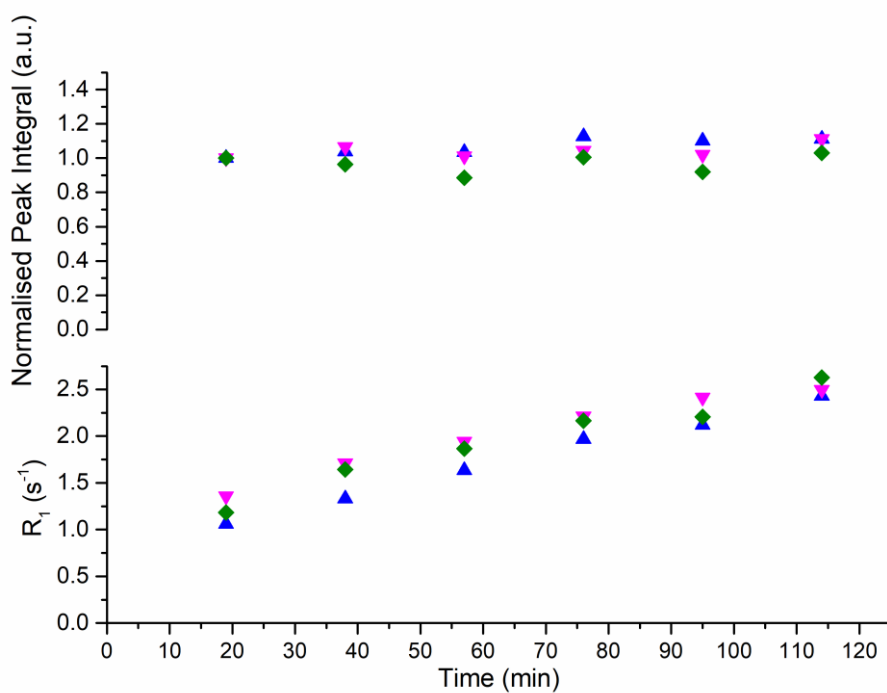


Figure 3.4: ^{31}P R_1 values of α - (▼), β - (◆), γ -ATP (▲) with 0.4 mM Gd.DTPA repeated over 2 h time period and the corresponding relative change in the ATP integrals over time.

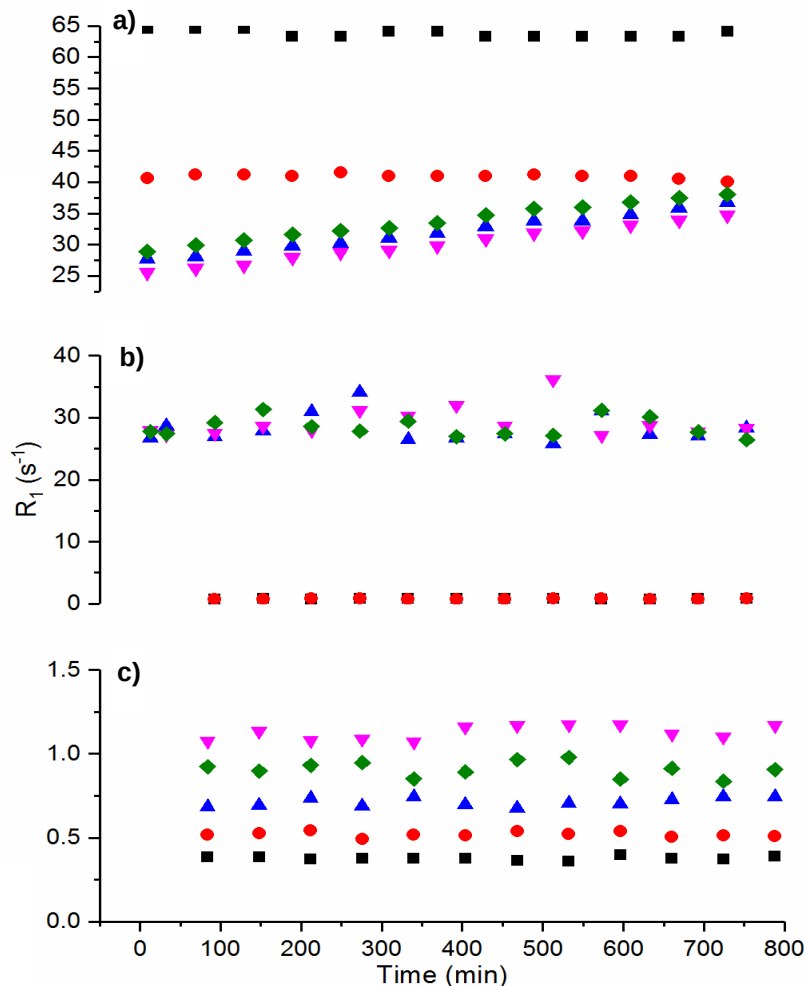


Figure 3.5: ^{31}P R_1 values of P_i (■), PCr (●) and α - (▼), β - (◆), γ -ATP (▲) repeated over a 12 h time period in the presence of 1 mM Gd contrast a) Gd.pDO3A, b) Gd.DTPA-BMA and c) Gd.DOTA.

The ^{31}P r_1 results presented here for Gd.pDO3A are similar to those previously reported for Gd.DO3A by Vander Elst *et al.*¹⁰ Gd.DO3A is structurally similar to Gd.pDO3A, but without a propargyl arm. Gd.DO3A demonstrates reduced kinetic stability to Gd.DOTA stemming from the loss of rigidity, as has been shown by Kumar *et al.*²³ The kinetic studies in Figure 3.5 demonstrate that Gd.pDO3A is also less

kinetically stable than Gd.DOTA, however the presence of the fourth propargyl arm is expected to increase the stability in comparison to Gd.DO3A.^{24,25}

3.7. HPLC Identification of Gd(ATP)_n

Reverse-phase HPLC was used to try and detect the formation of Gd(ATP)_n. 4 mM Gd(OAc)₃ was stirred with 10 mM ATP, which would ensure formation of Gd(ATP)_n—assumed to be Gd(ATP)₂ at these concentrations. The solution was checked with Xylenol Orange (XO), which showed no colour change by visual observation indicating that all Gd is complexed by ATP and that the Gd(ATP)₂ complex is more stable than the Gd-XO complex. However, using HPLC with both mass spectrometry and UV/Vis detection there was no peak that could be assigned to any form of Gd(ATP)_n complex, despite testing multiple conditions. It is possible that the complexes are too weak to remain intact on the HPLC C₁₈ column. HPLC of Gd.DTPA-BMA was conducted anyway in the presence of ATP and without to see if there were any significant changes apart from the additional metabolite. However, no conclusive results were obtained.

3.8. Conclusions

In light of recent reports regarding the safety and toxicity profiles of approved Gd contrast agents, measuring ^{31}P relaxivity of ATP species is a quick and effective way to identify issues with the stability of current or new T_1 agents used for routine (^1H) MR imaging. Non-linear relaxation enhancement of ATP with increasing contrast agent concentration is observed for the linear chelates DTPA and DTPA-BMA. This is

most likely explained by the chelation of free Gd^{3+} , especially in the knowledge of their kinetic lability. However, the heptadentate complex Gd.pDO3A demonstrates significant linear relaxation enhancement with increasing concentration of contrast agent resulting from transient binding of phosphate species to Gd, without the release of chelated Gd. This allows effective relaxation of bulk phosphate, which therefore shows excellent potential for development as a ^{31}P contrast agent. Native ^{31}P T_1 times are significantly reduced by Gd.pDO3A , which has a relaxivity 100-fold higher per mM of Gd than any of the commercial contrast agents tested.

As previously discussed in Chapter 2, in order for Gd.pDO3A to be considered as a possible ^{31}P contrast agent *in vivo*, studies of the intracellular uptake and toxicity will be conducted in Chapter 5.

3.9. References

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Chapter 4

Structural Adaptations for ^{31}P Contrast Agents

4.1. Introduction

The previous chapters have shown how Gd.pDO3A produces effective ^{31}P T_1 relaxation of relevant phosphate species in solution. However, key phosphate species involved in metabolism reside intracellularly, with a high proportion located in the cytosol and mitochondria.¹ While some lanthanide complexes show good cellular uptake,² internalisation of Gd.pDO3A is not anticipated. This is because it is structurally similar to Gd.DOTA for which cellular uptake is considered negligible.³ Further work on this will be conducted in Chapter 5. However, on this basis, structural adaptation of the Gd.pDO3A complex to enhance cellular uptake is desirable. In addition to this, a study of ^{31}P relaxivity with these structurally adapted complexes will help to understand structure-relaxivity relationships for ^{31}P and the potential for phosphate specificity.

The cell membrane is constructed of phospholipids, which makes it very lipophilic. Therefore, a key way to enhance cellular uptake is to increase the lipophilicity of the complex. Alternatively, utilising charge carriers and membrane transport systems can facilitate crossing the cellular membrane. In particular, lipophilic cations are known to be taken into cells and localise in the mitochondria;⁴ in part due

to the cationic charge on these compounds which allows them to cross the mitochondrial membrane potential.

Incorporation of a chromophore would also enhance visualisation of cellular uptake as fluorescent microscopy can be used to determine cellular localisation. A variety of structural adaptations of Gd.pDO3A were investigated as ways of enhancing cellular uptake. The complexes were required to be lipophilic, while maintaining high water solubility, and remain in fast exchange with phosphate species for effective relaxation enhancement.

4.2.Synthesis of Complexes

The complexes proposed as structural adaptations of Gd.pDO3A, which may enhance cellular uptake, are displayed in Figure 4.1.

As discussed briefly in Section 4.1, one simple method of enhancing cellular uptake primarily involves enhancing the lipophilicity of the complex in order to cross the cellular membrane. However, this needs to be balanced with maintaining sufficient water solubility in order for the complex to be applicable in cellular environments. The benzyl and acridone moieties in Ln.BenzylDO3A and Ln.AcridoDO3A are both relatively hydrophobic, which should help to enhance lipophilicity. The acridone moiety is also highly fluorescent, which will enable fluorescent microscopy studies to determine sub-cellular localisation of the complex.

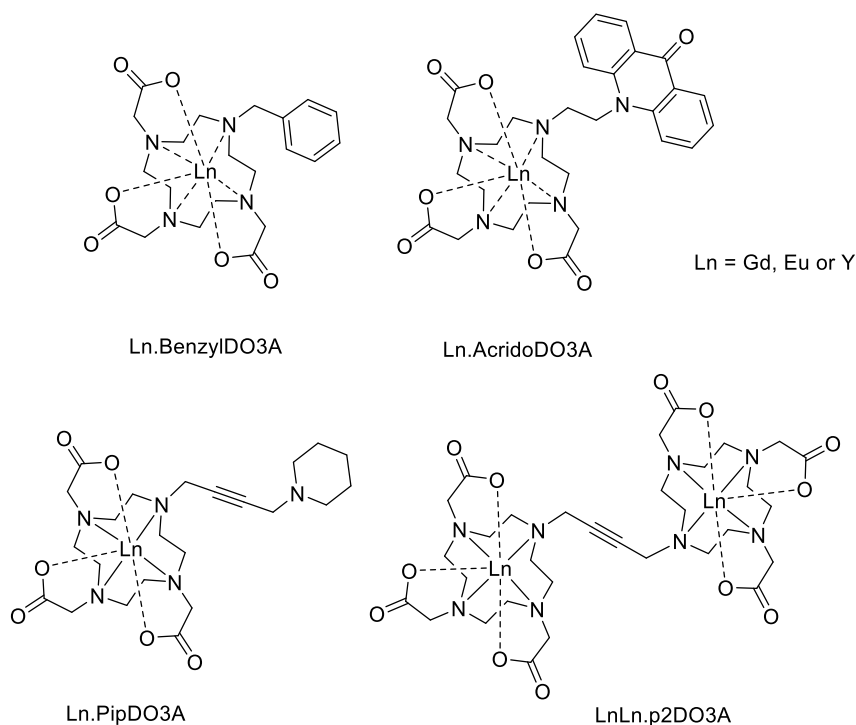


Figure 4.1: Synthesised complexes discussed in this chapter, which are structural adaptations of *Ln.pDO3A*.

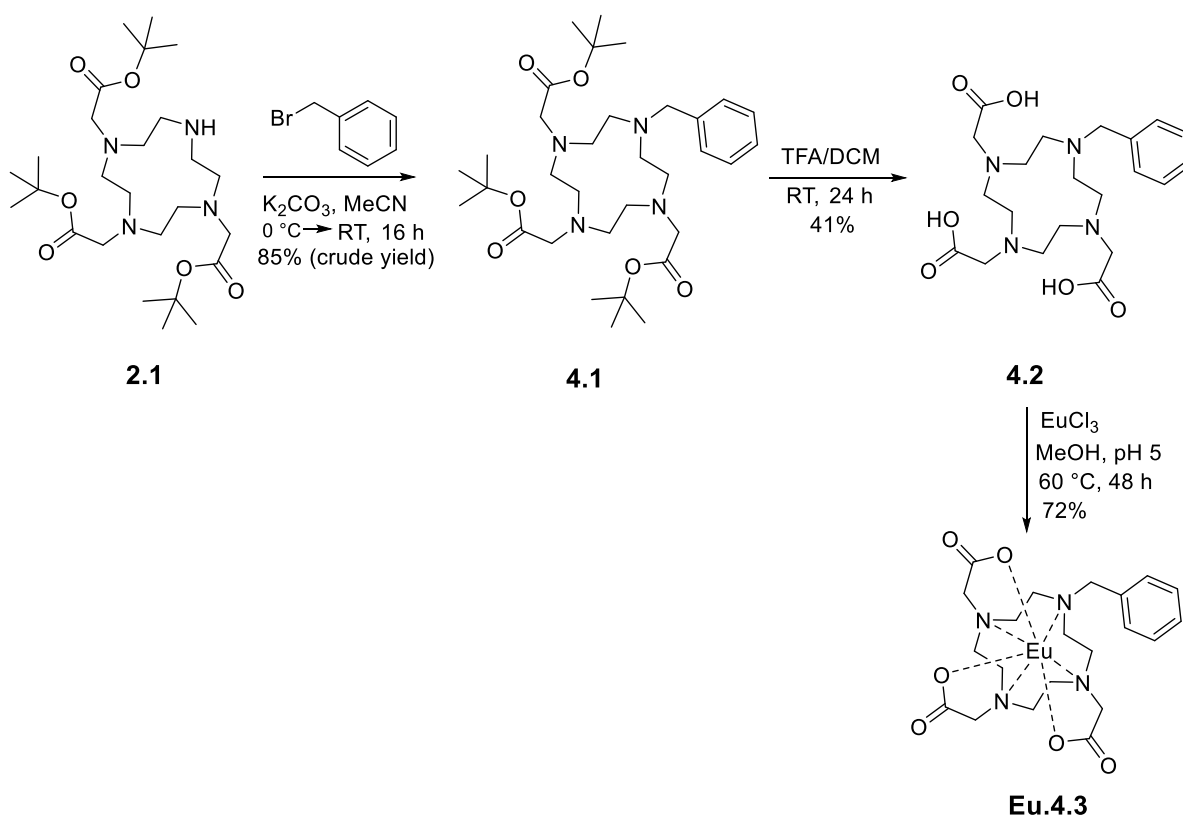
Ln.PipDO3A will be charged at physiological pH (pH 7.4), due to protonation of the nitrogen in the piperidine group. If the complex is sufficiently lipophilic, this may facilitate cellular uptake to the mitochondria as observed for many lipophilic cations.⁵

In addition to enhanced cellular uptake, the addition of hydrophobic and charged moieties may impact on the approach of phosphate anions and therefore alter the ^{31}P relaxivity of the complexes. There is the possibility these moieties may also introduce a level of selectivity by masking the approach of larger anions or forming electrostatic interactions. To enhance understanding of how these complexes interact with phosphate species and the mechanisms of ^{31}P relaxivity, *LnLn.p2DO3A* will also be investigated. The dimer would not be expected to display any enhanced cellular

uptake over Ln.pDO3A , however, the impact of two Ln centres may enhance relaxivity or facilitate bridging of phosphate species.

4.2.1. Benzyl complex

As previously mentioned, the benzyl complex was synthesised with the intention of increasing the lipophilicity of the complex without introducing any large substituents which would block access of phosphate to the lanthanide centre. The complex was synthesised following the scheme outlined in Scheme 4.1



Scheme 4.1: Synthesis of benzyl lanthanide complex.

To alkylate the fourth ring nitrogen of $^t\text{Bu}_3\text{DO3AH}$ (2.1), benzyl bromide was added dropwise in a 1:1 ratio to form compound 4.1, which was taken straight to the next step without purification. TFA deprotection of the compound yielded the ligand 4.2 as the TFA salt, which could then be purified by recrystallization from methanol. Complexation was achieved using an excess of the europium chloride salt (3:1) by heating at 60°C in methanol at pH 5 for two days. The europium complex was made initially for characterisation and to assess the luminescent properties. The complex was desalted using an Amberlite[®] ion exchange column. The paramagnetic ^1H -NMR of Eu.4.3 at pD 10 is shown in Figure 4.2. At pD 7 significant line broadening is observed due to the fast exchange of SAP and TSAP isomers typical in heptadentate DO3A complexes.

The resulting complex Eu.4.3 displayed significant precipitation from solutions upon standing. The resulting precipitate, when separated from the solution, proved highly insoluble. Upon further monitoring of the complexes in solution, precipitation appears to reduce over time. The possibility of formation of insoluble carboxylate complexes was tested by the addition of dry ice to a solution of complex Eu.4.3. However, the addition of dry ice did not catalyse the formation of the precipitate, which suggests that the precipitate is not a result of carboxylate ternary complexes. The europium emission lifetimes of the excited state were also measured in water and in D_2O in order to determine the hydration number (q) of the complex.

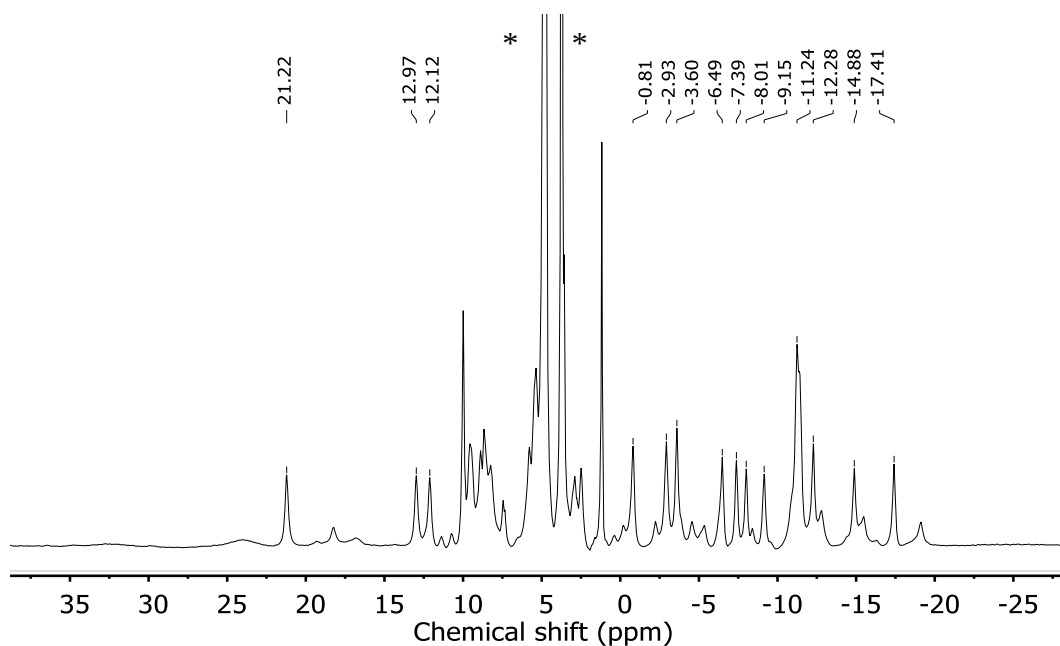


Figure 4.2: ^1H -NMR of Eu.4.3 in D_2O , pD10, 400 MHz, 298 K *Residual solvent.

The modified Horrock's equation (4.1) was previously used in Chapter 2 to determine q and will also be applied here.

$$q_{\text{Eu}} = 1.2(k_{\text{H}_2\text{O}} - k_{\text{D}_2\text{O}} - 0.25 - 0.075n_{\text{NH}}) \quad (4.1)$$

The number of N-H oscillators (n_{NH}) is zero for Eu.4.3. The emission lifetimes and the calculated q value are displayed in Table 4.1.

Table 4.1: Excited state Eu(III) emission lifetimes for complex Eu.4.3 in D_2O and H_2O and calculated hydration number (q), λ_{em} 616 nm, λ_{ex} 394 nm, pH 7, 298 K.

Complex	$\tau_{\text{D}_2\text{O}}$ (ms) ^a	$\tau_{\text{H}_2\text{O}}$ (ms) ^a	q
Eu.BenzylDO3A ^b	1.49	0.34	2.4

^a Typical errors on measured values are 10%

^b [Eu] = 0.25 mM in distilled water or D_2O .

The q value calculated for Eu.4.3 is approximately 2, which indicates two bound water molecules. The slightly higher value of q indicates there could be a slightly larger contribution of outer-sphere quenching oscillators than is accounted for by the B value (0.25) in the Horrock's equation. A q value of 2 suggests that no ternary molecules are present in solution, although this does not eliminate the fact that the precipitate may still be a ternary complex. Another possibility is that the precipitate is the result of the formation of π - π bonds between the complexes. This may cause dimers or extended structures that are less soluble than the monomeric complex. The emission lifetimes demonstrated monoexponential decay and did not show an improved fit to a biexponential model, which indicates there is only one emissive Eu species present in solution. Therefore, if dimeric species are forming, the q value indicates that either there is no obstruction of water binding sites or that the dimers are completely insoluble in solution.

Due to the unexplained precipitation of the complex, no further work on Eu.4.3 was conducted as the properties are inadequate for relaxivity and for cellular studies.

4.2.2. Acridone complex

Acridone is a highly fluorescent chromophore which has been previously applied as a fluorescent tag for antibody catalysis.⁶ The chromophore is relatively lipophilic and has an excitation wavelength around 405 nm, which is compatible with the excitation wavelengths typically applied in fluorescent microscopy. The triplet state energy of acridone in polar solvents is $21\,050\text{ cm}^{-1}$,⁷ which is high enough to be able to excite the $^5\text{D}_1$ level of Eu^{3+} calculated to be around $18\,700\text{ cm}^{-1}$.⁸ The quantum yield of acridone fluorescence in polar solvents is 0.75 compared to that of intersystem crossing at 0.014 which shows that intersystem crossing is significantly less favourable than fluorescence.⁹ However, close proximity to lanthanide ions can induce heavy atom and paramagnetic effects, which have been shown to enhance intersystem crossing.^{10,11} For this reason, europium acridone complexes have been previously investigated, with effective sensitization of europium by the acridone moiety. The structures of these complexes, which have been previously reported in the literature, are shown in Figure 4.3.

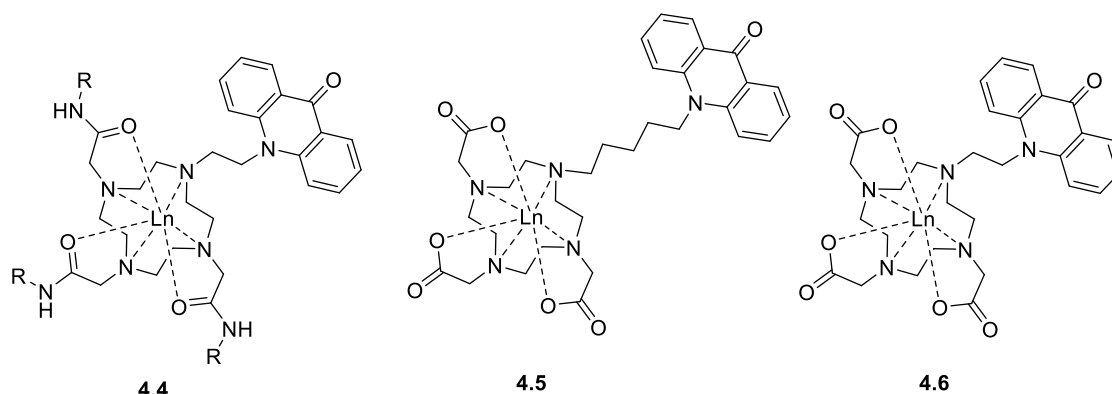
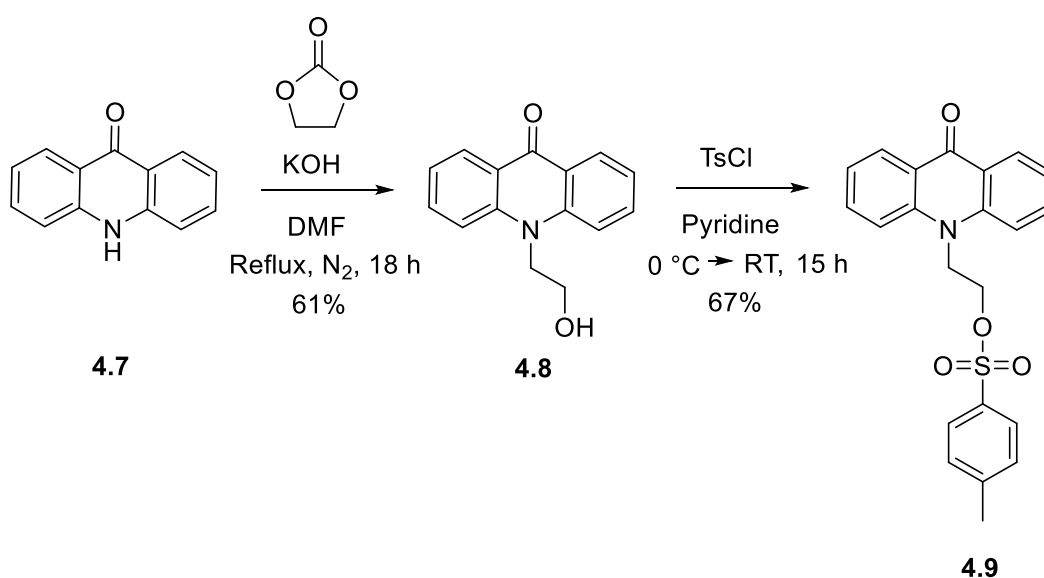


Figure 4.3: Structures of synthesised lanthanide acridone complexes previously reported in the literature.^{9,12}

The series of triamide complexes (4.4) made by Bretonniere *et al.*¹² are highly charged (+3), which would lower the exchange rate of anions, such as phosphate species, with the lanthanide centre. In order to maintain fast exchange of phosphate for effective ^{31}P relaxation, complexes with a neutral charge located on the lanthanide are preferred. The two DO3A scaffold complexes synthesised by Dadabhoy *et al.*⁹ are both neutral and therefore should remain in fast exchange with the phosphate species. Complex 4.5 has a five carbon chain linker between the lanthanide and the acridone, which is too long for effective sensitisation of the lanthanide by Förster energy transfer.⁹ However, the two carbon chain linker (4.6) is short enough to allow energy transfer from acridone to europium and therefore europium emission can be used for luminescent cellular imaging.

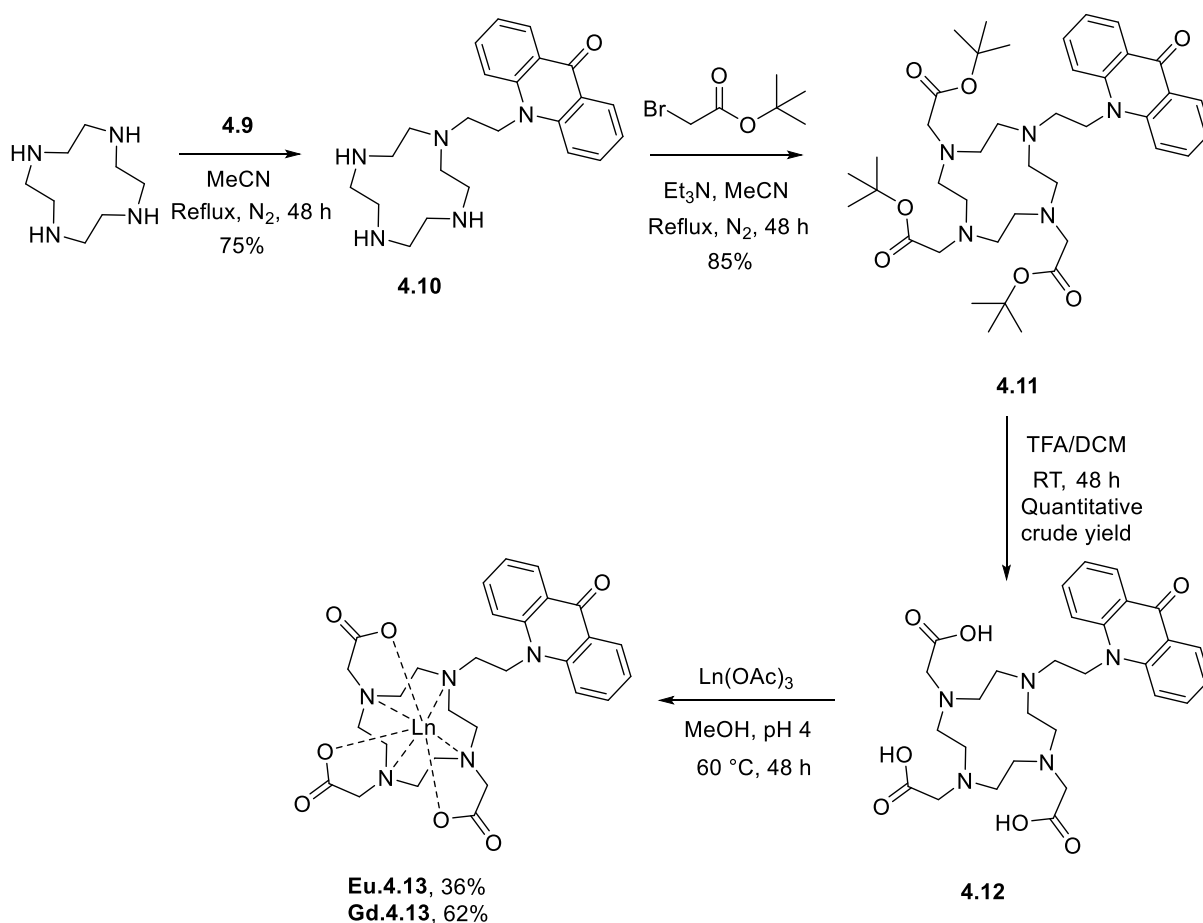
The synthesis of the lanthanide acridone complexes was adapted from literature procedures. The synthesis of the acridone arm is outlined in Scheme 4.2 and the subsequent lanthanide complex in Scheme 4.3.



Scheme 4.2: Synthesis of acridone arm (adapted from literature procedures).^{12,13}

Firstly, acridone (4.7) was reacted with ethylene carbonate to add an ethyl hydroxyl chain forming product 4.8, which was purified by recrystallisation.¹² The hydroxyl group was substituted for a tosyl group, by reaction with tosyl chloride and recrystallisation of the product yielded 4.9.

Previous work by Dadabahoy *et al.* found that addition of the carboxylate arms to cyclen, prior to the acridone moiety, caused an elimination product to be formed instead.⁹ Therefore, 4.9 was added directly to cyclen by refluxing in acetonitrile with an excess of cyclen and no base.



Scheme 4.3: Synthesis of lanthanide acridone complex adapted from literature procedures.⁹

Alkylation of the remaining three ring nitrogens of 4.10 was achieved by reaction with tert-butylbromoacetate using trimethylamine as a base to give 4.11. Full conversion of 4.10 to 4.11 could not be achieved, resulting in a small percentage of the DO2A side product. The product was deprotected by reaction with TFA in DCM to give ligand 4.12. Recrystallisation of the product from methanol yielded co-crystallisation of the DO3A and DO2A products due to their similarity in structure. Separation of the two compounds was achieved using preparative reverse-phase HPLC. Details of the HPLC method can be found in the experimental methods section of this thesis. Briefly, basic eluent conditions were employed using a shallow $\text{H}_2\text{O}/\text{MeCN}$ gradient. The product was collected by mass detection at $568\ m/z$ ($[\text{M}+\text{H}]^+$). Re-injection of the product determined the purity of the ligand by analytical HPLC (Figure A6, Appendix). ^{19}F -NMR showed the product was the free ligand, with no TFA salt present.

Once the acridone ligand (4.12) was HPLC purified, the ^1H -NMR spectrum shows concentration dependence as seen in Figure 4.4. At higher concentrations, the spectrum shows the appearance of additional peaks in the aromatic region and doubling of the peaks at 4.43 ppm and 2.96 ppm. These additional peaks, and the associated line broadening, would suggest that there is aggregation between the acridone moieties at higher concentrations; the peaks which remain unaffected are those of the cyclen ring and carboxylate arms.

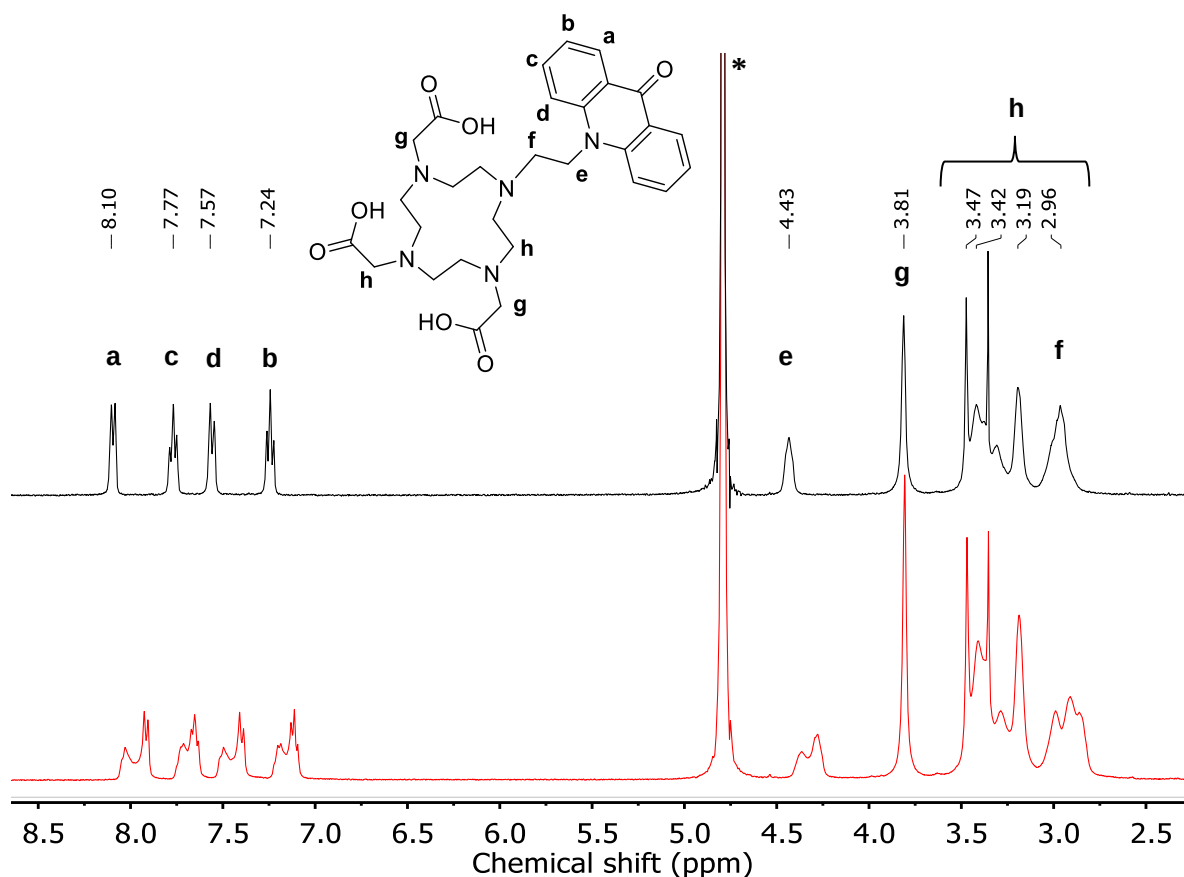


Figure 4.4: ^1H -NMR of acridone ligand at low (~ 5 mg/ml, black) versus high (~ 20 mg/ml, red) concentration, D_2O , pD 7. * Residual solvent.

Lanthanide complexation of 4.12 was found to be highly dependent on solubility and the counter-ion in the lanthanide salt. The ligand is only partially soluble in methanol and use of the co-ordinating counter-ion acetate allowed complete complexation of the ligand at pH 4, while triflate and chloride did not. The lanthanide complexes were de-salted using ion-exchange columns and the absence of free lanthanide ions confirmed with xylenol orange. Complexes were assessed for purity and the absence of free ligand by analytical RP-HPLC (Figure A.7, Appendix). Complexes were found to be highly soluble in ethanol, but due to aggregation effects had mixed solubility in aqueous solutions that was highly dependent on salt concentration. The Eu.4.13 and

Gd.4.13 complexes were made for luminescent and relaxivity studies respectively. Figure 4.5 shows the ^1H -NMR of Eu.4.13 in D_2O at pD 10, which is typical of heptadentate europium complexes, exhibiting line broadening as a consequence of exchange between diastereoisomers of the complexes.

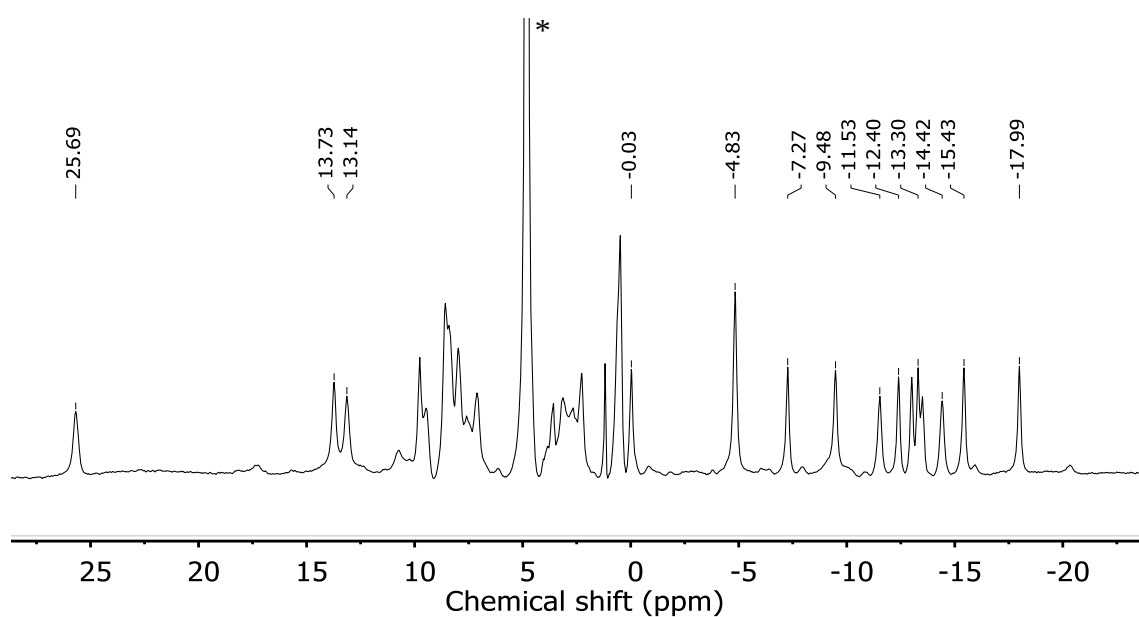


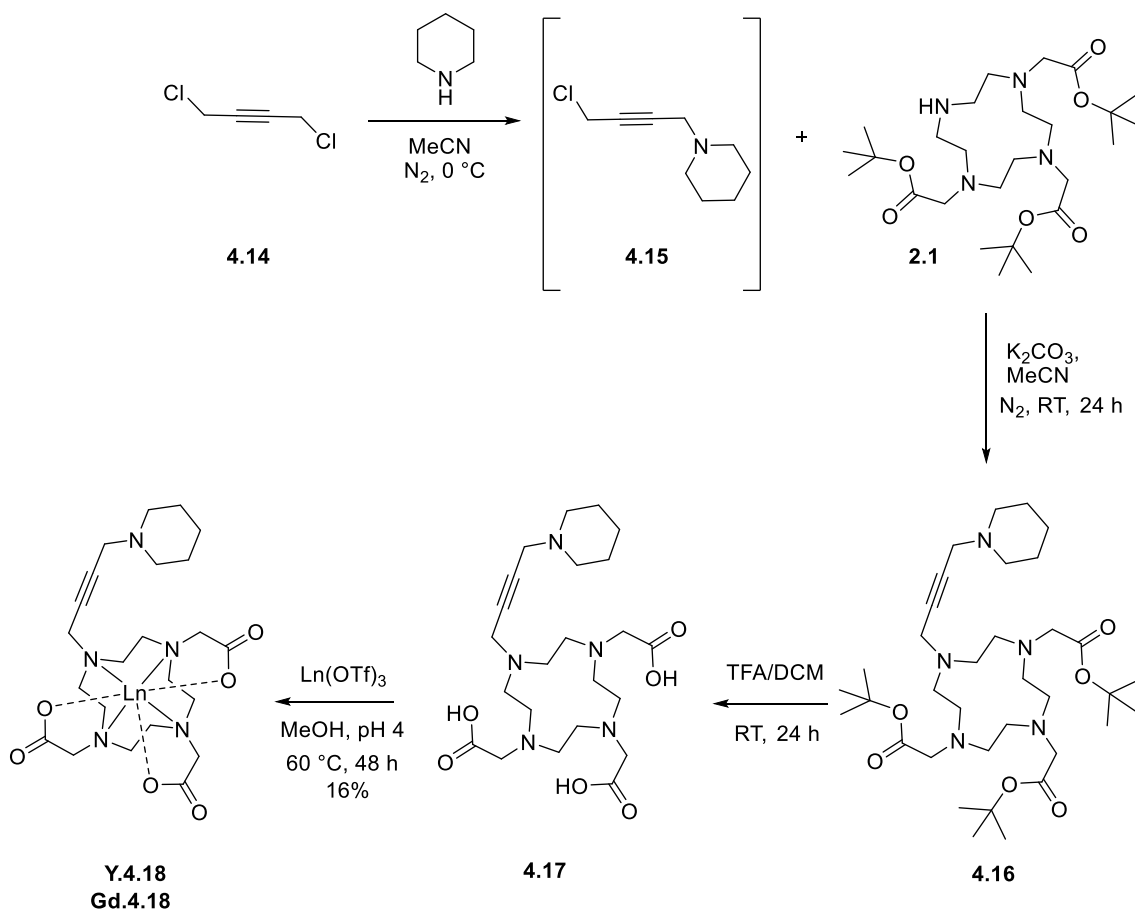
Figure 4.5: ^1H -NMR spectra of europium complex 5.10 in D_2O at pD 10. * Residual solvent

4.2.3. Piperidine complex

It has been widely reported that lipophilic cations such as Rhodamine B and triphenylphosphine groups localise in the mitochondria due to their lipophilicity and ability to cross the mitochondrial membrane potential.¹⁴⁻¹⁶ We reasoned that a piperidine functional group, attached to the propargyl moiety, will be protonated on the nitrogen at pH 7 and therefore charged *in vivo*. As the charge is not located on the lanthanide centre, it will not necessarily affect the rate of exchange of phosphate species.

The synthesis of the piperidine complex is outlined in Scheme 4.4. The ligand was synthesised in a one-pot synthesis where one molar equivalent of piperidine was added dropwise to 1,2-dichloropropylene (4.14). The reaction was monitored by mass spectrometry and TLC. The reaction was judged to have reached completion by the disappearance of the starting material 4.14 in the mass spectrum ($m/z = 123$ $[\text{M}+\text{H}]^+$) and in TLC. $^t\text{Bu}_3\text{DO3AH}$ (2.1) was then added to the reaction mixture with potassium carbonate as a base. The product was purified by column chromatography on alumina to remove any di-substituted 1,2-dipiperidine propylene and any unreacted $^t\text{Bu}_3\text{DO3AH}$.

The product 4.16 was also found to contain a small quantity of the dimeric DO3A ligand (p2DO3A). The compound was deprotected first using TFA to form 4.17 and then the ligand was purified by preparative reverse-phase HPLC to remove the dimeric DO3A ligand. A shallow $\text{H}_2\text{O}/\text{MeCN}$ gradient was employed in basic conditions—further details of the HPLC conditions can be found in the experimental methods section. The product was collected on mass detection at 482 m/z ($[\text{M}+\text{H}]^+$). The ligand 4.17 was isolated as the TFA salt, as confirmed by ^{19}F -NMR spectroscopy.



Scheme 4.4: Synthesis of lanthanide piperidine complex.

Complexation of the ligand was carried out in methanol, adding the appropriate lanthanide triflate salt at a 1:1 ratio. The lanthanide complexes were de-salted using ion-exchange columns and the absence of free lanthanide ions confirmed with xylenol orange. The yttrium complex Y.4.18 was made for characterisation because it is diamagnetic, while the gadolinium complex Gd.4.18 was made for relaxivity studies. ^1H -NMR and peak assignments of the piperidine ligand are shown in Figure 4.6. The yttrium complex ^1H -NMR showed changes in chemical shifts and broadening consistent with complexation in a fast exchanging system (Figure A.2, Appendix).

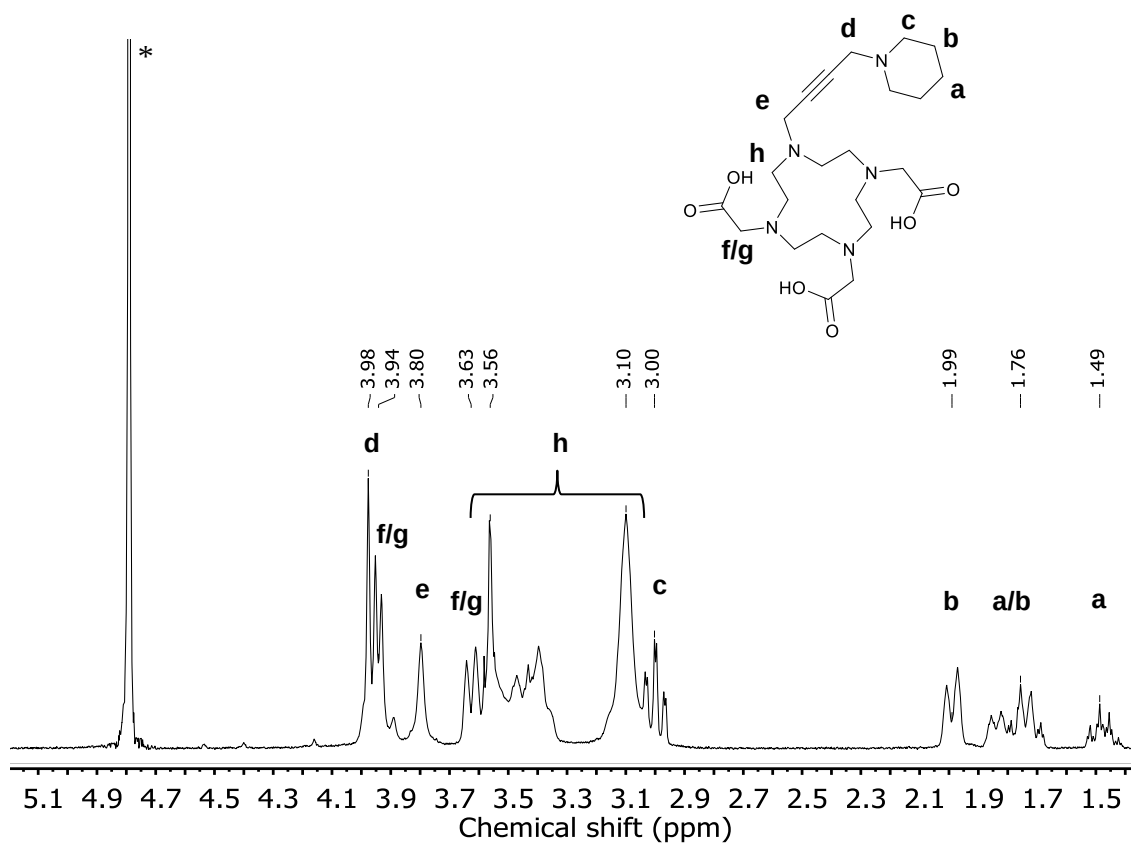
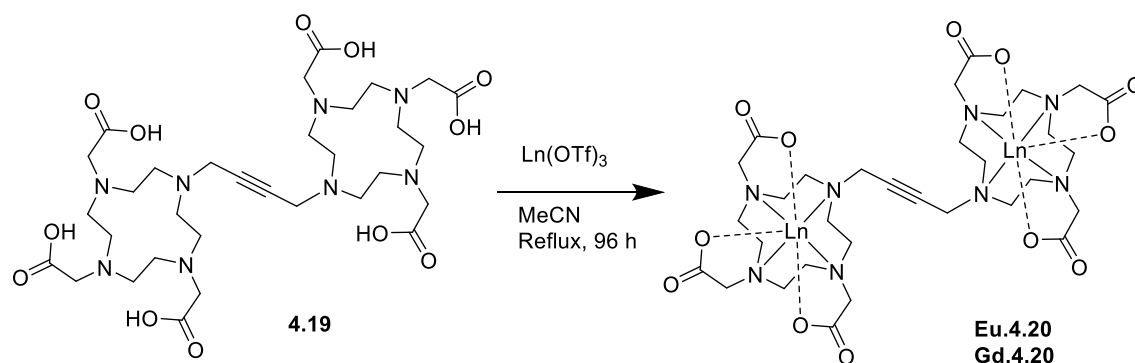


Figure 4.6: ^1H -NMR spectra of 4.17 in D_2O , 298 K, 400 MHz. * residual solvent.

4.2.4. Dimer complex

A side product of the PipDO3A (4.17) synthesis, was the p2DO3A dimer in Scheme 4.5 (4.19) which was removed by HPLC. The Gd.4.20 and Eu.4.20 complexes were made from this ligand to see how phosphate species interact with a dimeric Gd complex and whether there is an element of selectivity for specific phosphate species, due to bridging effects. The ligand has been previously made in the group and was donated by Matthew Jamshed.¹⁷ Purity was confirmed by analytical reverse-phase HPLC (Figure A.9, Appendix), mass spec. and NMR (experimental methods). Scheme 4.5 details the complexation of the ligand. Complexation was achieved over 96 h under reflux in acetonitrile and the product was formed as a precipitate. The precipitate was washed with acetonitrile and de-salted using an Amberlite[®] ion-exchange column. The absence of free lanthanide was confirmed using Xylenol Orange.



Scheme 4.5: Synthesis of LnLn.p2DO3A complexes.

4.3. Relaxivity

4.3.1. ^{31}P relaxivity

^{31}P relaxivities of all complexes with the phosphate species P_i , ATP and PCr were determined following the same procedure as that used for Gd.pDO3A in Chapter 2. Figure 4.7 shows the ^{31}P relaxation rates recorded with increasing concentration of each Gd complex and the corresponding linear fits, with Gd.pDO3A for comparison. The ^{31}P relaxivity values resulting from the fitted data are outlined below in Table 4.2. Figure 4.7 demonstrates that all complexes maintain a linear ^{31}P relaxation rate enhancement with increasing concentration of Gd. As demonstrated in Chapter 3, this suggests that ATP is unable to compete significantly with any of the DO3A ligands to extract Gd.

Table 4.2: ^{31}P Relaxivity of Gd(III) Complexes Gd.pDO3A (Gd.2.4), Gd.4.13, Gd4.20 and Gd.4.18 in phosphate solution,^a pH 7.2, 298 K, 162 MHz.

Gd Complex	^{31}P r_1 (mMGd $^{-1}\text{s}^{-1}$) ^b				
	P_i	PCr	γ -ATP	α -ATP	β -ATP
a) Gd.2.4	85.96 $\pm$ 1.00	54.97 $\pm$ 0.48	35.30 $\pm$ 0.64	32.35 $\pm$ 0.46	35.97 $\pm$ 0.35
b) Gd.4.13	13.41 $\pm$ 0.25	17.87 $\pm$ 0.49	33.93 $\pm$ 1.04	40.55 $\pm$ 0.39	42.46 $\pm$ 1.40
c) Gd.4.20	50.36 $\pm$ 0.87	29.11 $\pm$ 0.49	21.73 $\pm$ 0.26	20.82 $\pm$ 0.05	23.11 $\pm$ 0.45
d) Gd.4.18	44.84 $\pm$ 0.51	29.55 $\pm$ 1.11	29.68 $\pm$ 0.49	27.04 $\pm$ 0.81	30.01 $\pm$ 0.79

^aPhosphate solution = 8.7 mM PBS, 5.5 mM PCr and 7.9 mM ATP at pH 7.2.

^b $\pm$ Standard error of fitted results. R^2 values > 0.99 for all fits.

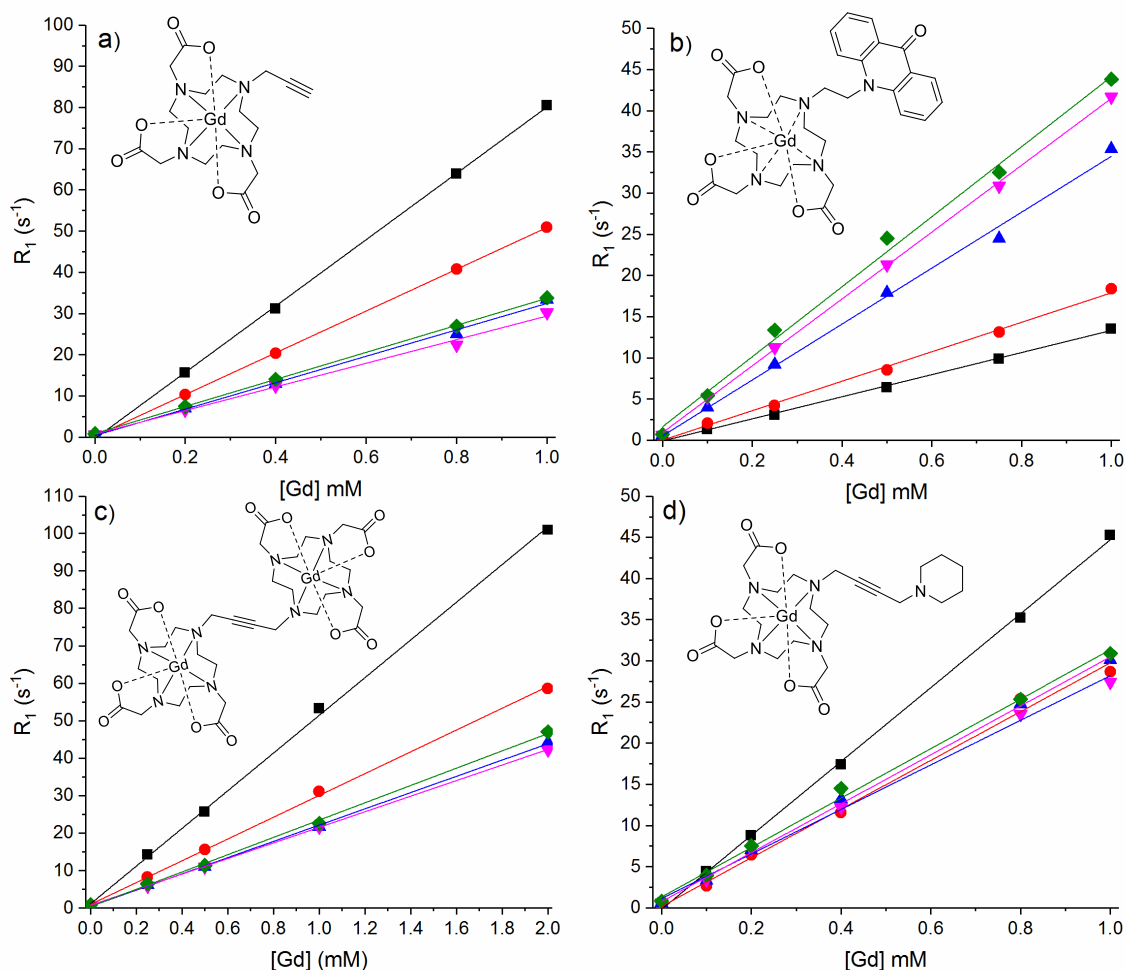


Figure 4.7: ^{31}P Relaxation rates (R_1) of P_i (■), PCr (●) and α - (▼), β - (◆), γ - (▲) ATP (8.7, 5.5 and 7.9 mM) versus concentration of Gd for contrast agents a) Gd.pDO3A Gd.2.4, b) Gd.AcridoDO3A Gd.4.13, c) GdGd.p2DO3A Gd.4.20, d) Gd.PipDO3A Gd.4.18. Recorded at pH 7.2, 162 MHz, 298 K.

GdGd.p2DO3A (Figure 4.7(c), Gd.4.20) demonstrates similar behaviour in ^{31}P relaxivity to Gd.pDO3A (Figure 4.7(a), Gd.2.4). P_i experiences the highest relaxation rate enhancement, followed by PCr and then all three phosphates of ATP with comparable relaxivity values. The relaxivity per mmolGd is lower for all phosphate species with GdGd.p2DO3A than with Gd.pDO3A. This would suggest there are no significant concurrent relaxation effects on the same phosphate molecule from both Gd

centres. Previous studies have shown that dimeric Gd complexes tend to display higher water relaxivity than monomeric complexes due to the increase in rotational correlation time.¹⁸ However, as discussed in Chapter 2, at 9.4 T the effect of increasing the rotational correlation time will be negligible on the observed relaxivity.¹⁹ The lower relaxivity observed for the dimeric complex 4.20 compared to the monomeric Gd.pDO3A is therefore most likely due to the close proximity of the two Gd centres. Each phosphate molecule is relatively large and negatively charged, so, when one phosphate species is bound, steric hindrance or charge repulsion may restrict the approach of phosphate species to the second Gd centre.

Gd.PipDO3A demonstrates slightly lower relaxivity values than Gd.pDO3A (Figure 4.7(d), Gd.4.18 vs Figure 4.7(a), Gd.2.4) most likely due to the addition of a bulky arm that limits access to the Gd centre. The values remain relatively high and P_i still maintains the highest relaxivity. However, PCr is reduced to values comparable to ATP, which is again most likely due to limited access to the Gd centre.

Gd.AcridoDO3A (Figure 4.7(b), Gd.4.13) demonstrates the most significant difference in relaxivity trends in comparison to the other complexes. All three ATP phosphate groups have the highest relaxivity, with values similar to those of Gd.pDO3A. PCr demonstrates significantly lower relaxivity, followed by P_i . This is a reversal of the trend observed for all the previous complexes. In addition, a notable difference in the three phosphates of ATP, is that γ (the terminal phosphate group) has a relaxivity lower than both α - and β -ATP. Previously, the relaxivity values of all three phosphate groups within ATP have been comparable, with α -ATP being slightly lower. ATP contains adenine, which may be able to form hydrogen bonds with the carbonyl group of acridone or π - π interactions. This interaction may mean that the phosphate

groups closest to the Gd centre are in fact α - and β -ATP, rather than the less inhibited γ -ATP. It is also possible that these ligand-centred interactions between acridone and ATP increase the second sphere relaxation effects on ATP, by increasing the number of co-ordinated ATP molecules.

4.3.2. ^1H relaxivity

^1H relaxivity was also measured in deionised water and in the phosphate solution; the results are shown in Table 4.3. GdPipDO3A (Gd.4.18) demonstrates very similar relaxivity to Gd.pDO3A in both deionised water and the phosphate solution. The dimer GdGd.p2DO3A (Gd.4.20) also has a comparable relaxivity. In water, the relaxivity per Gd with GdGd.p2DO3A is very similar to Gd.pDO3A, whereas in the phosphate solution, it is slightly lower than all the other complexes at $4.04 \text{ mM Gd}^{-1}\text{s}^{-1}$. It is possible that due to having two Gd centres, ATP and PCr may be able to bridge, thereby forming a stronger bond and lowering the exchange rate of water with the Gd centre. The slightly lower relaxivity of PCr and ATP, would also support this theory.

Gd.AcridoDO3A (Gd.4.13) exhibits comparable relaxation to Gd.pDO3A in the phosphate solution, but in deionised water the ^1H relaxivity is much lower—approximately half the relaxivity of Gd.pDO3A. The results are also more variable at higher concentrations ($> 0.5 \text{ mM}$), and above 1 mM the complex has significant solubility issues in water. This would suggest that the lower ^1H relaxivity in deionised water is due to aggregation of the complexes in solution, most likely π - π stacking of the acridone moieties. The presence of salt in the phosphate solution and coordinating anions help to significantly reduce aggregation and improve solubility and therefore the reduction is not observed in the ^1H relaxivity of the phosphate solution.

Table 4.3: ^1H Relaxivity of Gd(III) Complexes Gd.pDO3A, Gd.4.13, Gd4.20 and Gd.4.18 in H_2O and phosphate solution, pH 7, 298 K, 400 MHz.

Gd Complex	$^1\text{H } r_1 \text{ (mMGd}^{-1}\text{s}^{-1})^{\text{a}}$	
	Phosphate Solution ^b	H_2O
Gd.pDO3A Gd.2.4	4.36 ± 0.02	5.22 ± 0.11
Gd.PipDO3A Gd.4.18	4.47 ± 0.03	5.31 ± 0.08
GdGd.p2DO3A Gd.4.20	4.04 ± 0.06	5.41 ± 0.15
Gd.AcridoDO3A Gd.4.13	4.45 ± 0.09	2.66 ± 0.07

^a $\pm$ Standard error of fitted results. R^2 values > 0.99 for all fits.

^b Phosphate solution = 8.7 mM PBS, 5.5 mM PCr and 7.9 mM ATP at pH 7.2.

4.4. Photophysical Characterisation

4.4.1. Luminescence and binding data

In Chapter 2, the binding of the phosphate species ATP, PCr and P_i was determined by using the europium analogue of pDO3A and measuring the change in luminescence intensity of the complex, as the concentration of phosphate is increased. The same method was used for the Eu.AcridoDO3A (Eu.4.13) and EuEu.p2DO3A (Eu.4.20) complexes to compare the strength of phosphate binding to that of Eu.pDO3A (Eu.2.4) and to try and rationalise the differences in the phosphate relaxivity of the Gd complexes.

Phosphorescence spectra of Eu.AcridoDO3A (Eu.4.13) were recorded, with excitation at 420 nm, to minimise residual acridone emission (~ 450 nm), which is observed in the fluorescence spectrum. The effect of adding P_i (PBS, Figure 4.8), ATP (Figure 4.9), or PCr (Figure 4.10) on Eu(III) emission can be seen in the figures below. The most notable difference between addition of each phosphate group is the effect on the $\Delta J = 1$ transition. P_i and ATP both result in coalescence of the two peaks of $\Delta J = 1$, although differing in shape. P_i results in a main peak at 595 nm with a shoulder remaining at 590 nm, while ATP results in the main peak at 590 nm into which the other peak merges. The biggest difference is observed with PCr, where the two peaks remain distinctive at 588 and 595 nm, the second of which shows the most significant increase. This shows a distinctive change in the crystal field of Eu(III) in the presence of PCr; the $\Delta J = 1$ transition is particularly sensitive to changes in magnetic anisotropy.²⁰ The intensity and splitting of the $\Delta J = 1$ is indicative of changes in the magnetic anisotropy factor (D), which is affected by the ligand and axial donor atoms.

Previous studies on axial symmetric C_4 complexes have shown that switching a neutral axial ligand for a polarizable ligand (fluoride), reduces $\Delta J = 1$ splitting.²¹ One of the other notable differences is that in the presence of ATP and PCr, the intensity of the $\Delta J = 0$ transition at 580 nm increases. For ATP and PCr, the intensity increases by a factor of 3 and 2 respectively, while for PBS it stays constant—increasing only marginally, by a factor of 1.2.

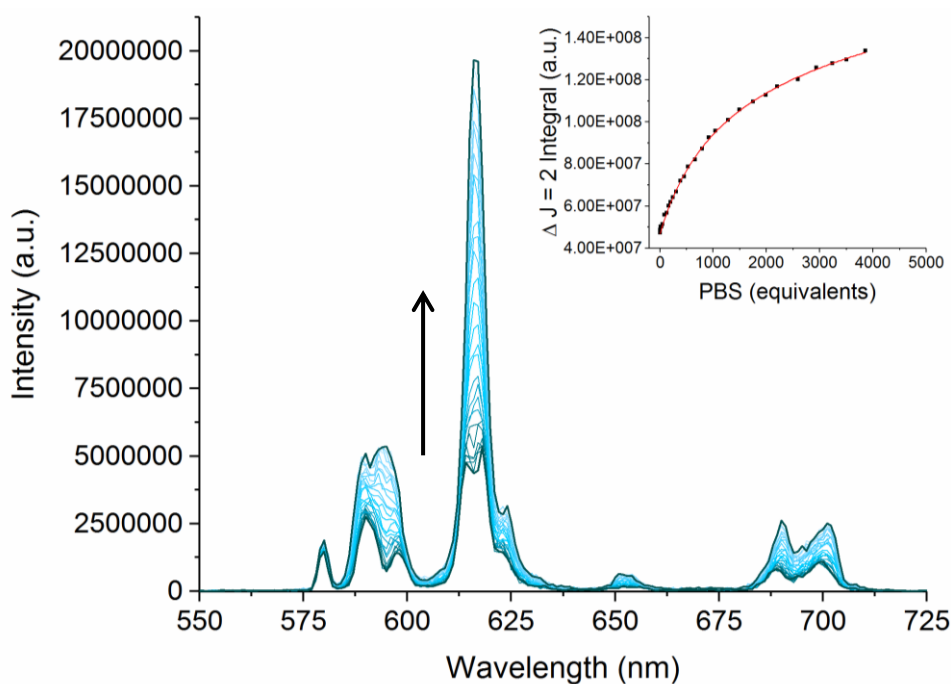


Figure 4.8: Eu(III) emission of Eu.AcridoDO3A (10 μM) in 10 mM HEPES buffer with increasing concentration of PBS. $\lambda_{\text{ex}} = 420$ nm. Insert: $\Delta J = 2$ integral at 616 nm with PBS; Eu(III) equivalents.

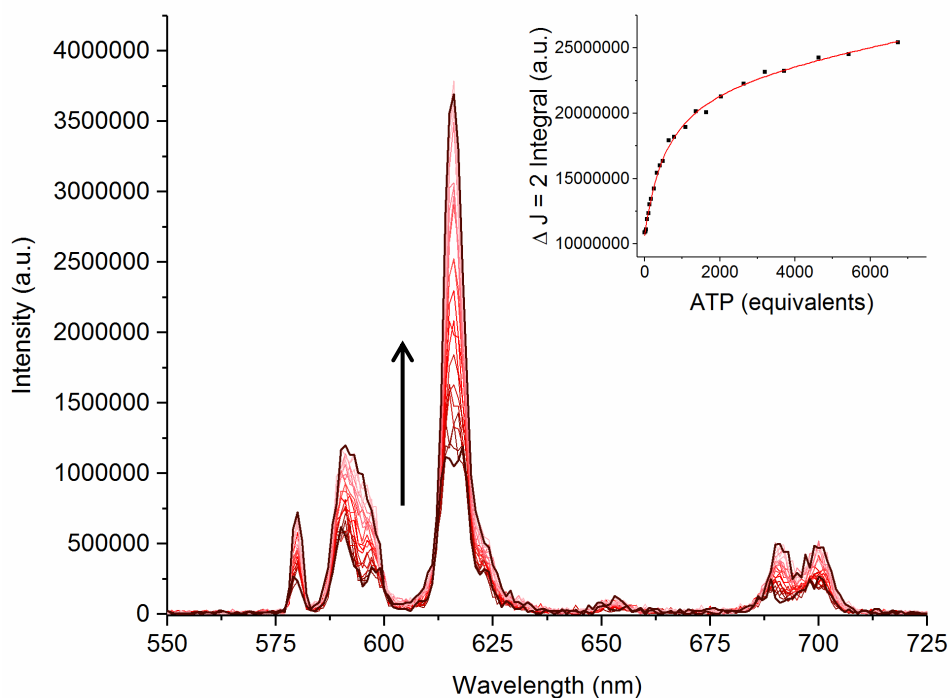


Figure 4.9: Eu(III) emission of Eu.AcridoDO3A (2 μM) in 10 mM HEPES buffer with increasing concentration of ATP. $\lambda_{\text{ex}} = 420$ nm. Insert: $\Delta J = 2$ integral at 616 nm with ATP: Eu(III) equivalents.

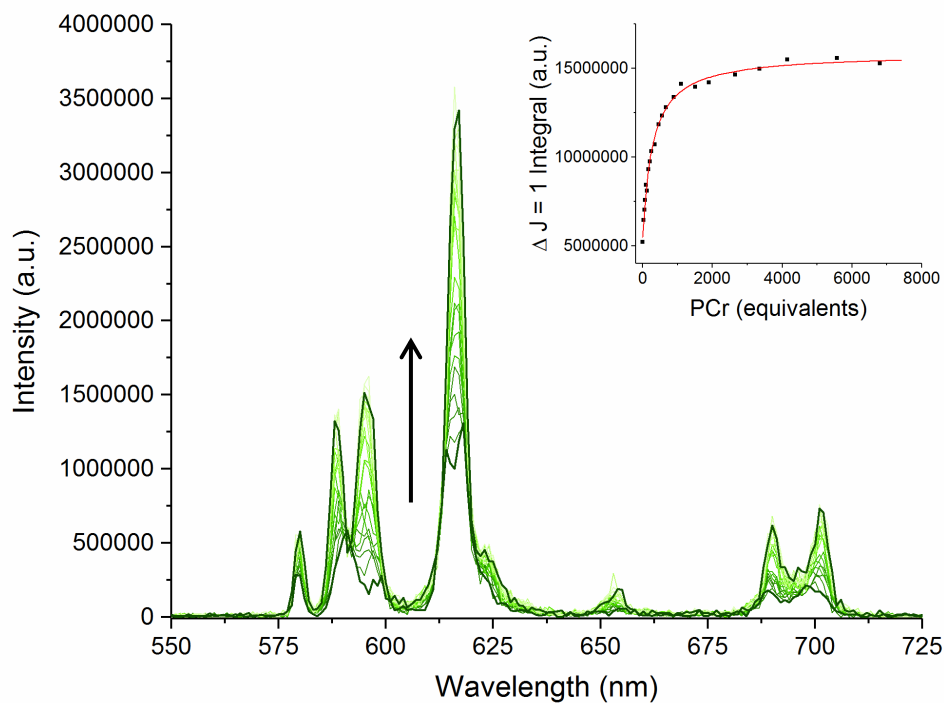


Figure 4.10: Eu(III) emission of Eu.AcridoDO3A (2 μM) in 10 mM HEPES buffer with increasing concentration of PCr. $\lambda_{\text{ex}} = 420$ nm. Insert: $\Delta J = 1$ integral at 616 nm with PCr: Eu(III) equivalent.,.

Phosphocreatine also has a carboxylic acid moiety, which is capable of binding to the lanthanide centre. The maintenance of the magnitude of the $\Delta J = 1$ splitting and the increase in $\Delta J = 0$ has been previously noted as a characteristic related to sodium acetate binding in some europium complexes.²² To establish whether phosphocreatine may bind to Eu.AcridoDO3A (Eu.4.13) via the carboxylate terminus, the emission spectrum of Eu.AcridoDO3A with an excess of creatine was also recorded. The result is shown in Figure 4.11, where it is clear that the spectrum shows distinct differences to that of phosphocreatine in Figure 4.10. The intensity of the $\Delta J = 0$ band is consistent with that of Eu.AcridoDO3A in buffer and the splitting of $\Delta J = 1$ is maintained. This would suggest that the binding mode of phosphocreatine involves direct binding of the phosphate group to Eu, as the effect on the Eu crystal field differs from that with creatine.

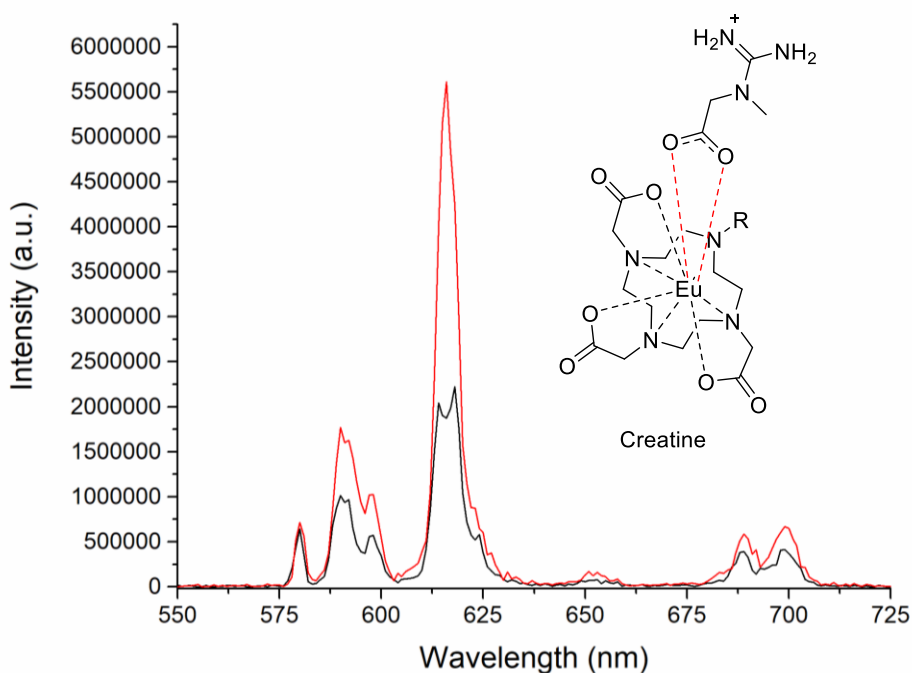


Figure 4.11: Eu(III) emission of 10 μM Eu.AcridoDO3A (Eu.4.13) in 10 mM HEPES buffer (Black) and with 40 mM Creatine (Red), with proposed creatine binding model.

EuEu.p2DO3A (Eu.4.20) was also tested for binding with ATP, PCr and P_i to determine whether multiple phosphate species can bind at once or whether larger species can bridge between the Eu centres. Figure 4.12 shows the modulation of Eu(III) emission of EuEu.p2DO3A with addition of PBS. Phosphorescence spectra were recorded using direct Eu(III) excitation at 394 nm. The lower intensity of emission limits the resolution of the spectra and therefore changes in the fine structure, which indicate changes in the crystal field, cannot be visualised on timescales compatible with the number of measurements required for titrations. Figure 4.13 shows the change in the $\Delta J = 2$ transition integral upon increasing equivalents of PCr and ATP.

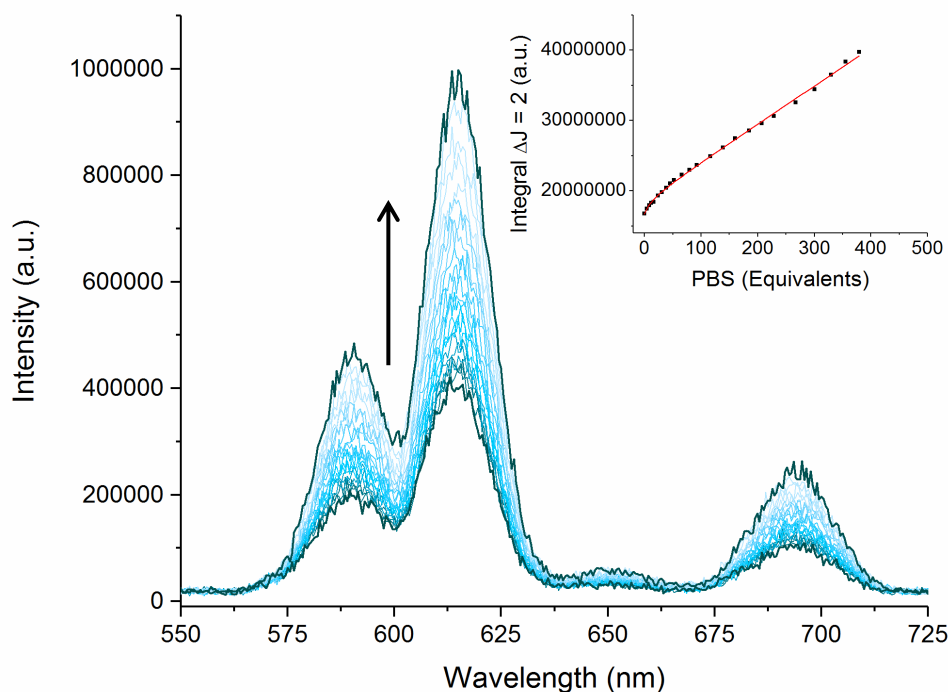


Figure 4.12: Eu(III) emission of EuEu.p2DO3A (50 μM) in 10 mM HEPES buffer with increasing concentration of PBS. $\lambda_{\text{ex}} = 394$ nm. Insert: $\Delta J = 2$ integral at 616 nm with PBS: Complex equivalents.

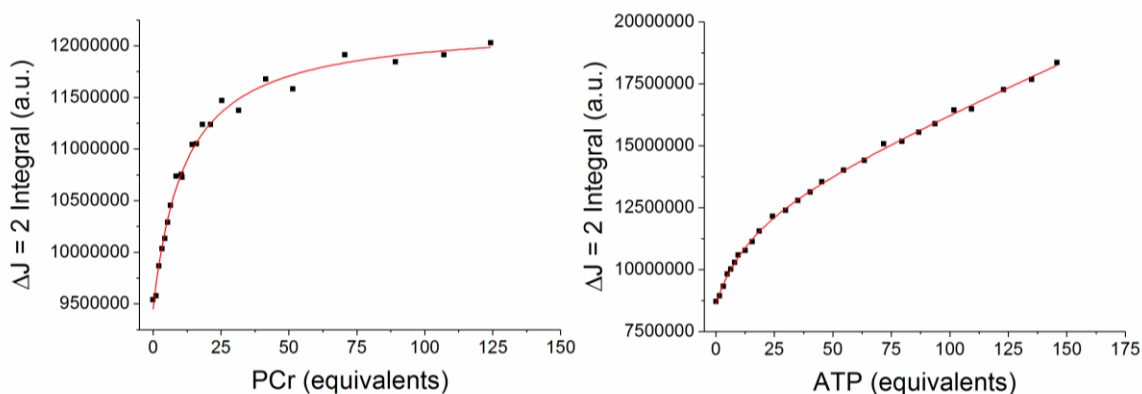


Figure 4.13: $\Delta J = 2$ integral at 616 nm Eu(III) emission of EuEu.p2DO3A (50 μM) in 10 mM HEPES buffer with increasing equivalents of PCr and ATP to Eu complex.. $\lambda_{\text{ex}} = 394$ nm.

4.4.2. Determination of binding constants

The integral of the intensity of the $\Delta J = 1$, $\Delta J = 2$ and $\Delta J = 4$ transitions were globally fitted using Dynafit[®] software to determine an apparent binding constant, K_a . All three phosphate species with both Eu complexes were initially fitted to a 1:1 binding model:

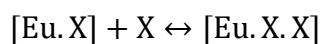


Where x is the relevant phosphate molecule; ATP, PCr or P_i . The binding constant, K_1 is defined as:

$$K_1 = \frac{[\text{Eu} \cdot \text{X}]}{[\text{Eu}][\text{X}]} \quad (4.3)$$

Only PCr demonstrated a reasonable fit for this model with both EuEu.p2DO3A (Eu.4.20) and Eu.AcridoDO3A (Eu.4.13). Therefore, model discrimination analysis in Dynafit[®] was applied to determine the best model for the data.

A 1:2 binding model was found to be the best fit for ATP and P_i data, modelled by the equation below:



K_1 is defined by the same equation (4.3), and a second binding constant K_2 is defined as follows:

$$K_2 = \frac{[\text{Eu} \cdot \text{X} \cdot \text{X}]}{[\text{Eu} \cdot \text{X}][\text{X}]} \quad (4.5)$$

The resulting fits for each data set are displayed in Figures 4.8 – 4.13 above. A comparison of the 1:1 and 1:2 binding models for ATP and Eu.AcridoDO3A is shown in Figure 4.14, which demonstrates the improved fit of the 1:2 model to the data.

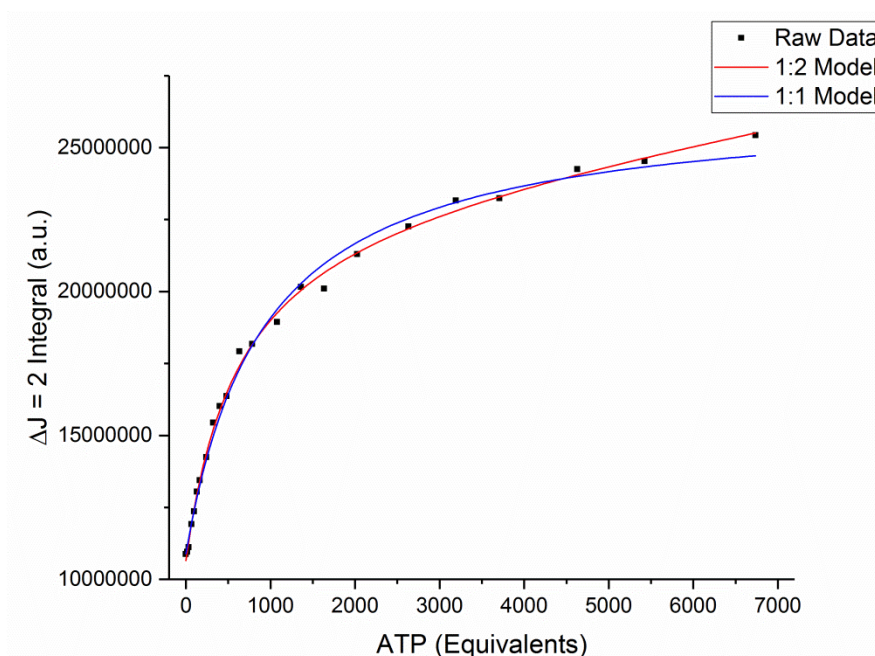


Figure 4.14: Comparison of exponential fits of Eu.AcridoDO3A (Eu.4.13) with ATP binding. The integral of the $\Delta J = 2$ transition is plotted against increasing ATP equivalents to Eu complex. The proposed models are a 1:1 or a 1:2 binding model.

The values of the association constants K_1 and, where relevant, K_2 are displayed in Table 4.4, with the values previously determined for Eu.pDO3A (Eu.2.4). Although a 1:2 binding model was determined to be the best fit for ATP and P_i with both complexes, Dynafit[®] was unable to determine an accurate value for K_2 in most cases. The K_2 values in Table 4.4 have very large confidence intervals and standard deviation, which indicates a significant uncertainty in these values. For Eu.AcridoDO3A with P_i , K_2 was fixed at different values (from 0.01–100) and found to have a minimal effect on the determined K_1 value. This indicates that the contribution of the second binding event (K_2) is very small and this could be the reason why Dynafit[®] struggles to determine a value for K_2 .

ATP and Eu.AcridoDO3A demonstrated a larger contribution from the second binding event as fixing the value of K_2 (0.001–100) varied the determined value of K_1 (834–1246). P_i and ATP binding with EuEu.p2DO3A illustrates a higher dependence on the value of K_2 , as fixing the value above a certain threshold (30 for ATP and 4 for P_i) causes the fitting to fail.

Two binding events for EuEu.p2DO3A are anticipated, due to the presence of two Eu centres. However, for Eu.AcridoDO3A, with one Eu centre, the explanation of two binding events is less obvious. Previous evidence in this chapter has indicated the aggregation of the ligand (4.12) and the Gd complex (Gd.4.20) at high concentrations. It is therefore not unreasonable to suggest there may be a small proportion of aggregated complexes in solution at low concentration. These may facilitate a second binding event, which would have a small overall contribution to the observed binding if the majority of molecules are not aggregated.

Table 4.4: Association constants of Eu(III) complexes Eu.2.4 Eu.4.13 and Eu.4.20 in 10 mM HEPES with PBS, ATP and PCr at pH 7.4, 298 K.

Eu Complex ^b	$K_I (\text{M}^{-1})^a$		
	PBS	PCr	ATP
Eu.pDO3A	194 ± 12	120 ± 8	200 ± 8
Eu.2.4	[172–218]	[104–138]	[185–216]
Eu.AcridoDO3A	74 ± 10 ^c	1759 ± 80	1006 ± 204
Eu.4.13	[68–83]	[1604–1927]	[752–1510]
	($K_2 = 3 \times 10^{-4} \pm 470$ [1.8×10^{-8} –117])		($K_2 = 45 \pm 46$ [1×10^{-4} –139])
EuEu.p2DO3A	811 ± 316 ^d	1819 ± 115 ^e	1250 ± 227
Eu.4.20	[488–1347]	[1600–2065]	[1039–1496]
	($K_2 = 8 \times 10^{-5} \pm 1$ [1×10^{-5} – 2×10^{-3}])		($K_2 = 2 \times 10^{-3} \pm 13$ [4×10^{-6} –16])

^a ± SD, [95% confidence intervals]^b All Samples in 10 mM HEPES^c In the absence of HEPES apparent binding constants are $K_I = 350 \pm 250$ [300–500] and $K_2 = 38 \pm 9$ [37–40] M^{-1} .^d In the absence of HEPES apparent binding constants are $K_I = 1400 \pm 210$ [997–1870] and $K_2 = 57 \pm 31$ [8–150] M^{-1} .^e For creatine $K_I = 29 \pm 23$ [10–251] and $K_2 = 29 \pm 18$ [5–50]

The interaction of HEPES buffer with both complexes was tested and found to show a significant interaction with EuEu.p2DO3A and a small interaction with Eu.AcridoDO3A. In the case of EuEu.p2DO3A, binding to HEPES appears stronger than to PBS. This has previously been observed for binuclear pyridine-bridged

complexes, where HEPES and TRIS buffers demonstrated more competitive binding than PBS.²⁴ Given the structure of HEPES, in comparison to PBS (Figure 4.15), and the fact that no significant buffer interaction was observed with Eu.pDO3A, it is possible that HEPES may have bridging capabilities. For Eu.Acridodo3A, the buffer may be able to hydrogen bond with the carbonyl of acridone and demonstrate some association. Apparent binding constants of PBS without buffer for both complexes were found to be significantly higher and K_2 could also be determined more accurately—for EuEu.p2DO3A $K_1 = 1400$ and $K_2 = 57 \text{ M}^{-1}$, while for Eu.Acridodo3A $K_1 = 350$ and $K_2 = 38 \text{ M}^{-1}$. Eu.Acridodo3A and EuEu.p2DO3A both demonstrate a decrease in Eu(III) emission intensity in the presence of HEPES, which is more pronounced for EuEu.p2DO3A. Without the presence of HEPES, EuEu.p2DO3A demonstrates an initial decrease in Eu(III) emission intensity with the first binding event of PBS that is not apparent when HEPES is present (Figure 4.15). In light of this, the binding constants in Table 4.4 are apparent binding constants in the presence of a competitive ligand and should be used as a qualitative guide only. The difference in the affinity of each complex for HEPES, in addition to the low ionic strength of HEPES, will result in inconsistencies between measurements.

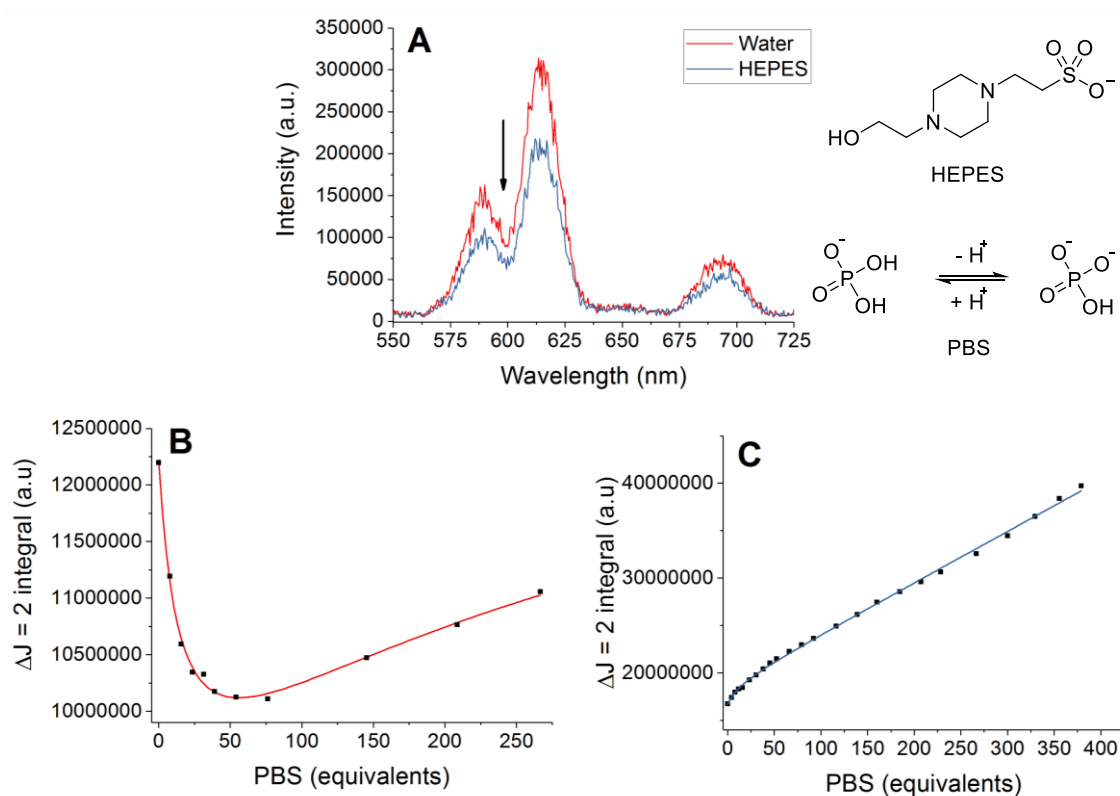


Figure 4.15: Interaction of HEPES (10 mM) with Eu.4.20. A) Modulation of Eu(III) emission intensity with addition of HEPES. B) $\Delta J = 2$ integral of Eu.4.20 (50 μM) emission intensity in water with increasing equivalents of PBS. C) Eu.4.20 (100 μM) in 10 mM HEPES with increasing equivalents of PBS.

The values of K_I for ATP with Eu.AcridoDO3A and EuEu.p2DO3A both indicate stronger binding of ATP with these complexes than to Eu.pDO3A. In the case of Eu.AcridoDO3A, this could be explained by π - π interactions between the acridone moiety and adenosine in ATP. This supports the hypothesis that was proposed in section 4.3.1 as the reasoning for the higher ^{31}P relaxivity of α - and β -ATP over terminal γ -ATP. For EuEu.p2DO3A, the stronger interaction with ATP could be explained by bridging of the phosphates between the two Eu centres. However, as the

data demonstrates an improved fit to a 1:2 model over a 1:1 model, this does not support bridging of a single ATP molecule.

PCr, however, does exhibit 1:1 binding with EuEu.p2DO3A in addition to a relatively high binding constant, both of which are consistent with PCr bridging. The structure of PCr would allow bridging via the phosphate and carboxylate group as demonstrated in Figure 4.16. The binding of creatine to EuEu.p2DO3A was also measured to rationalise the high affinity for PCr. Creatine demonstrated almost equivalent values for K_1 and K_2 both of which were relatively small ($K_1 = K_2 = 29 \text{ M}^{-1}$). This indicates carboxylate binding of creatine to Eu occurs and the binding of one molecule does not inhibit the binding of the second. This is not what is observed for the K_2 values of the phosphate molecules. This suggests that the very small values of K_2 recorded for EuEu.p2DO3A are more likely a result of anion-repulsion reducing the affinity for the second phosphate species, as opposed to steric hindrance. Steric hindrance would affect the binding of both creatine and phosphate to some extent.

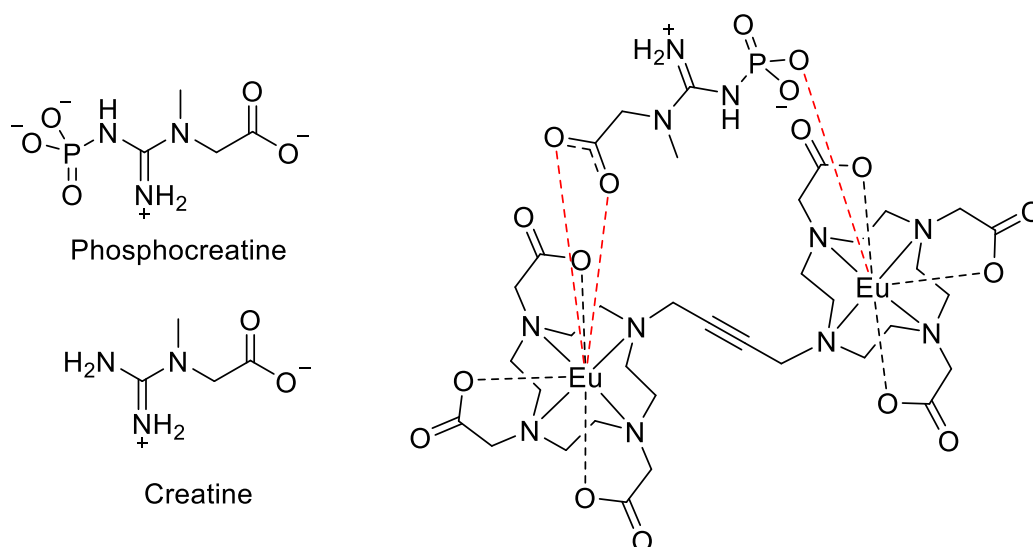


Figure 4.16: Possible bridging of PCr with EuEu.p2DO3A (Eu.4.20).

Based on the apparent K_2 of PBS with EuEu.p2DO3A (57 M^{-1}) the tiny K_2 values for phosphate species are likely to be artificially small as a result of the binding constants being masked by competitive binding with HEPES. However, the size difference between K_1 and K_2 for the phosphate species remains apparent.

With Eu.AcridoDO3A, PCr demonstrates a high affinity and a 1:1 binding mode. In the previous section it was noted that the Eu(III) emission spectrum displayed some distinct spectral differences to the other metabolites. Figure 4.17 demonstrates some possible binding modes of PCr to Eu.AcridoDO3A to account for these results.

Option A comprises of simple phosphate binding with a stabilising effect from hydrogen bonding of the NH_2 group in PCr with the acridone carbonyl. Alternatively, option B postulates that a six-membered ring could form between the phosphate group the NH_2 and the Eu centre.

In previous studies of heptadentate lanthanide complexes, chelating guests such as amino acids and peptides display bidentate binding through the OH and NH_2 groups.^{22,23} Four to six membered rings are favoured and therefore although this type of chelation has not previously been reported, it may occur. This conformation in option B could be encouraged by the presence of H-bonding as seen in option A, the absence of which in Eu.pDO3A may disfavour this binding mode. Option C proposes a 9-membered ring which is disfavoured and there are no reports of this type of interaction occurring.²³

The Eu(III) emission spectrum of Eu.AcridoDO3A with creatine did not display the same crystal field effects that PCr did. This indicates that options D and E are not possible binding modes as the same coordination via the carboxylate could also be observed for creatine.

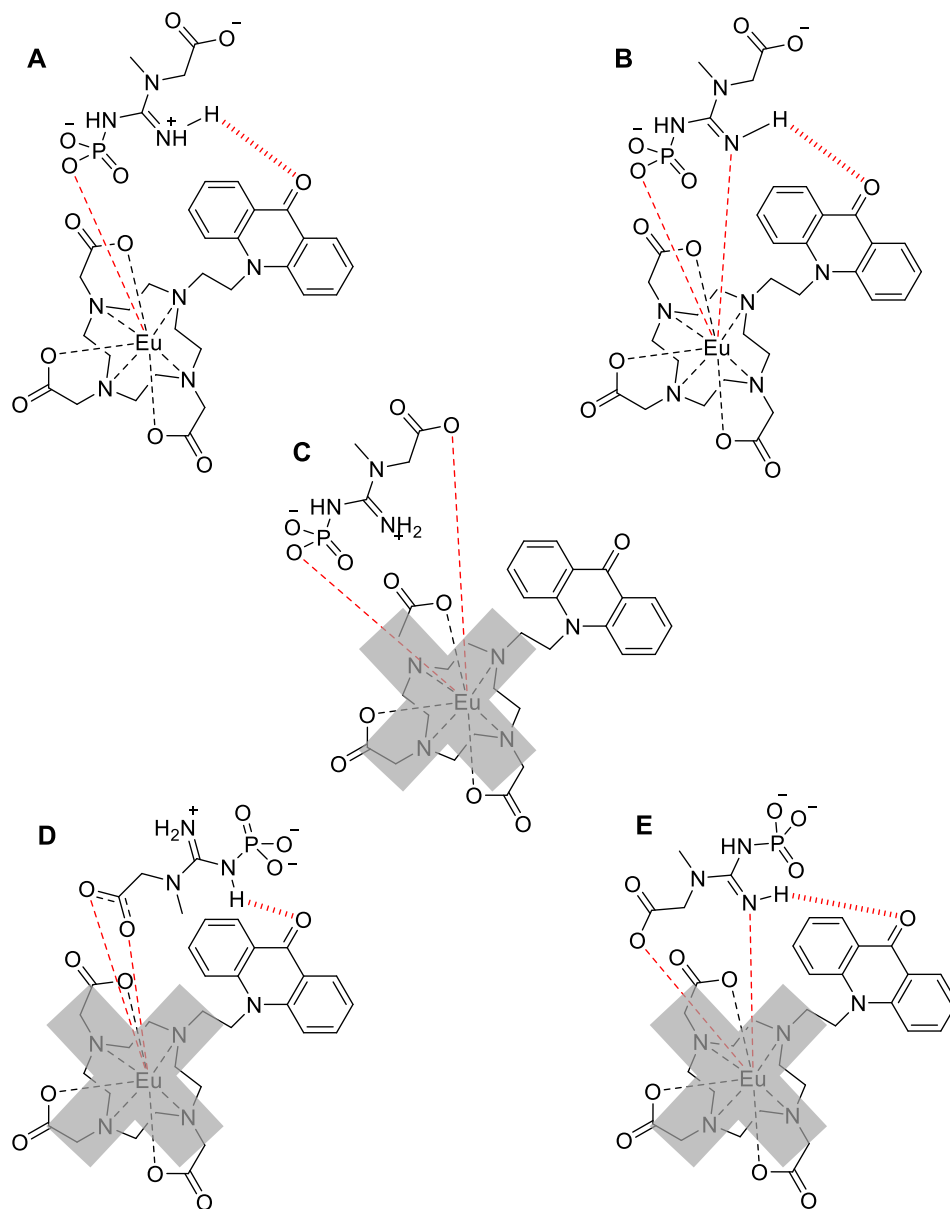


Figure 4.17: Examples of possible binding mechanisms for PCr with Eu.AcridoDO3A (Eu.4.13) with unlikely models crossed out.

Previous Eu.DO3AM complexes have been found to display an element of chemoselectivity for phosphate coordination of phosphorylated amino-acids over the peptide N terminal, while Tm/Yb.DO3AM bind via both mechanisms.^{25,26} More

information regarding the association of PCr with Eu.AcridoDO3A could be obtained by measuring the Eu(III) emission lifetimes.

4.4.3. Eu(III) emission lifetimes

Emission lifetimes of the excited state were recorded for each complex in D_2O and H_2O in order to determine the hydration number (q) in solution. q was calculated for each complex using the modified Horrocks' equation, outlined earlier in this chapter (section 4.2.1). n_{NH} , the number of N-H oscillators in close proximity to the lanthanide, remains as zero for all the complexes investigated in this section. The emission lifetimes and calculated q values are shown in Table 4.5.

Eu.pDO3A and EuEu.p2DO3A emission lifetimes fit well to a single exponential decay model, while Eu.AcridoDO3A exhibited an improved fit to a double exponential decay. This indicates there are two Eu.AcridoDO3A species present in solution with different hydration numbers (around 2 and 0). The single heptadentate Eu complex would be expected to have a q value of 2 and it is likely that the lower hydration number is a result of aggregation of the molecules, which prevents water binding to the europium centre. This is consistent with the evidence of aggregation already observed for the ligand in ^1H -NMR and for the Gd complex in the ^1H r_1 values.

The Eu(III) emission lifetime of Eu.AcridoDO3A was repeated at a much lower concentration of 2 μM and with 10 mM HEPES to try and minimise aggregation effects, however this had no significant effect on the measured lifetimes or calculated q values (2.4 and 0), as there were still two components present. This suggests aggregation is possible even at very low concentrations, which is not unprecedented

for co-ordinatively unsaturated complexes at physiological pH.²⁷ In the previous section (4.4.2) it was suggested that aggregation at low concentrations may explain the preference of a 1:2 model for Eu.AcridoDO3A with PBS and ATP. The biexponential decay of the excited state Eu(III) emission lifetimes present even at low concentrations in HEPES buffer also supports this.

Table 4.5: Excited state Eu(III) emission lifetimes for complexes Eu.2.4, Eu.4.13 and Eu.4.20 in D₂O and H₂O and calculated hydration number (q), λ_{em} 616, pH 7, 298 K.

Complex ^a	$\tau_{\text{D}_2\text{O}}$ (ms) ^b	$\tau_{\text{H}_2\text{O}}$ (ms) ^b	q
Eu.pDO3A	1.66	0.39	2.1
Eu.2.4^c			
Eu.AcridoDO3A	1.39	0.32, 1.01	2.6, 0.0
Eu.4.13^d			
EuEu.p2DO3A	1.54	0.43	1.7
Eu.4.20^c			

^a[Eu] = 0.1 mM unless otherwise stated. ^bTypical errors on measured values are 10%.

^c λ_{ex} = 394 nm. ^d λ_{ex} = 420 nm.

The calculated q value for EuEu.p2DO3A (Eu.4.20) is around 2, indicating that both Eu centres have two hydration sites. The value is slightly lower than 2, which may be a result of the close proximity of the two europium centres which introduces a small amount of steric hindrance in access to the hydration sites. The q value of Eu.AcridoDO3A is slightly higher than 2, which may be due to additional outer-sphere oscillators as suggested for Eu.BenzylDO3A (Eu.4.3).

The inner-sphere hydration of the complexes in the presence of phosphate was also calculated, by measurement of the Eu(III) emission lifetime in PBS. The hydration number in the presence of phosphate was calculated using equation 4.1, where H_2O and D_2O both contain 40 mM PBS. These values are displayed in Table 4.6.

Table 4.6: Excited state Eu(III) emission lifetimes for complexes Eu.2.4, Eu.4.13 and Eu.4.20 with PBS in D_2O and H_2O and calculated hydration number (q), λ_{em} 616, pH 7, 298 K.

Complex ^a	$\tau_{\text{PBS}_{\text{D}_2\text{O}}}$ ^b (ms) ^c	$\tau_{\text{PBS}_{\text{H}_2\text{O}}}$ ^b (ms) ^c	q
Eu.pDO3A			
Eu.2.4 ^d	1.73	0.64	0.9
Eu AcridoDO3A			
Eu.4.13 ^e	1.57	0.59	1.0
EuEu.p2DO3A			
Eu.4.20 ^d	1.90	0.62	1.0

^a[Eu] = 0.01 mM. ^b[PBS] = 40 mM. ^cTypical errors on measured values are 10%

^d λ_{ex} = 394 nm. ^e λ_{ex} = 420 nm.

Initial measurements of Eu.AcridoDO3A in PBS at 0.1 mM concentration were difficult to fit—possibly due to a second component. However, lowering the concentration to 10 μM and maintaining the same PBS concentration gave a good fit to a single component exponential decay. This suggests that the aggregation effects can be reduced by the presence of co-ordinating ions such as PBS, while non-co-ordinating compounds such as HEPES were unable to reduce aggregation. The q values for the complexes in the presence of PBS are all approximately 1, which is consistent with

monodentate binding of phosphate to lanthanides as previously reported in Chapter 1 and in the literature.²² This also suggests that for EuEu.p2DO3A, the europium centres are sufficiently spaced to allow one phosphate molecule to bind to each Eu centre, since the emission lifetimes suggest they are equivalent.

4.5. Conclusions

A series of structurally adapted pDO3A complexes were synthesised and their properties assessed in comparison to pDO3A. Successfully synthesised complexes, with appropriate water solubility, included Ln.AcridoDO3A (Ln.4.13), Ln.PipDO3A (Ln.4.18) and LnLn.p2DO3A (Ln.4.20). Ln.AcridoDO3A demonstrates significant aggregation effects in water, which appear to be reduced by the presence of co-ordinating ions, such as phosphate. This is demonstrated by ^1H relaxivity and Eu(III) emission lifetimes. Relaxivity measurements and luminescent binding studies show interesting differences in the interaction of phosphate species with these structurally adapted complexes, in comparison to Ln.pDO3A.

Phosphocreatine in particular demonstrates a much stronger affinity for both EuEu.p2DO3A and Eu.AcridoDO3A. With EuEu.p2DO3A, PCr most likely bridges between the two Eu centres, while with Eu.AcridoDO3A H-bonding interactions are likely to occur with the carbonyl of acridone. The enhanced bonding lowers the ^{31}P relaxivity of PCr with the Gd complexes Gd.4.13 and Gd.4.20 due to a reduced rate of exchange of PCr with the bulk metabolite in solution.

Inorganic phosphate displays similar interactions across all complexes. The ^{31}P relaxivity is similar with GdGd.p2DO3A and Gd.PipDO3A to Gd.pDO3A, although

slightly lowered by the increased bulk of the complexes. Gd.AcridoDO3A, however, shows significantly lower ^{31}P relaxivity of P_i which is the lowest ^{31}P relaxation enhancement of the phosphate species with Gd.AcridoDO3A. This is unusual due the small size of P_i , which generally increases the accessibility to the Gd centre and maintains a fast exchange of bulk phosphate. Binding studies showed a very weak interaction of Eu.AcridoDO3A with P_i , so the ^{31}P relaxivity may be reduced by stronger interactions with the other phosphate species present in the solution.

ATP shows a similar size ^{31}P relaxivity enhancement across all the complexes tested. Gd.AcridoDO3A demonstrates a slightly higher ^{31}P relaxivity for ATP, but the most notable difference is the lower relaxivity of γ -ATP in comparison to α and β . As the terminal phosphate this is unexpected due to the easier accessibility of the γ -ATP. Luminescence binding studies indicated a reasonably strong affinity of ATP for Eu.AcridoDO3A, which was attributed to π - π interactions between acridone and adenosine. These interactions may alter the positioning of ATP, when binding to Gd.AcridoDO3A, from that of the other complexes and may explain the different trend in the ATP relaxivities. EuEu.p2DO3A did not demonstrate clear evidence of ATP bridging between the Eu centres, due to the preference for a 1:2 binding model.

It is clear that by modifying the ligand-sphere the interactions with different phosphate species can be tailored. This can introduce an element of specificity for particular phosphate species primarily by altering the strength of association to the complex and lowering the rate of exchange of species. While this cannot be utilised in MRI, luminescence can allow quantification of species using this methodology. For MRI it is much harder to introduce an element of specificity, as increasing affinity for the molecule only slows down the rate of exchange. It is therefore noted with

particular interest that acridone has an element of selectivity for ATP in terms of ^{31}P relaxivity, which must be related to ligand-sphere interactions with ATP. It is possible that these interactions allow the distance and the angle of the phosphorus nuclei from Gd to be altered—which may explain the difference in α -, β - and γ -ATP ^{31}P relaxivity.

4.6. References

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Chapter 5

Cellular Uptake of ^{31}P Contrast Agents

5.1. Introduction

The compounds that were discussed in Chapters 2 and 4 show good potential as ^{31}P contrast agents with phosphate species in solution. However, as previously mentioned, in order for these complexes to be effective *in vivo* they must come into contact with the phosphate species of interest which reside in the cytosol. Most high energy phosphate species are located in the mitochondria or in the cytoplasm¹ and therefore the complexes would need to localise in these areas. The toxicity of these complexes also needs to be assessed on the time scale of exposure *in vivo*, especially in light of recent studies regarding Gd deposition. Cellular uptake of the complexes will slow clearance time from the body, and it is important the complexes remain intact and are excreted completely from the cells to be applied *in vivo*.

Initially, we studied the cellular uptake of Eu.AcridoDO3A by fluorescence microscopy, which is a preferred method for investigating the sub-cellular localisation profile. Following this, ^{31}P -NMR and ^1H -NMR methods were used to explore the uptake of Gd.pDO3A and Gd.PipDO3A into cells and the results correlated with ICP-MS. Acute toxicity of the complexes at long exposure times are tested for all the complexes by MTT assay.

5.2. Fluorescence Microscopy

The cellular uptake of Eu.AcridoDO3A (Eu.4.13) was assessed using fluorescence microscopy. Microscopy experiments were performed at the Rutherford Appleton Laboratory in collaboration with Prof. Sofia Pascu. Cell culture methods were conducted by Dr. Haobo Ge and assistance with imaging and equipment was provided by Prof. Stan Botchway. The cellular uptake experiments were conducted on prostate cancer PC3 cells, which were relevant to the collaborative project.

The cells were incubated with 0.2 mM Eu.AcridoDO3A in serum-free media for 20 minutes and cell uptake visualised using confocal fluorescence microscopy with an excitation wavelength of 405 nm. These images were compared to control cells to check for auto fluorescence. PC3 cells showed localised uptake in specific regions of the cells. Using two-photon excitation at 810 nm, emission spectra could be extracted from these regions of the cellular image, to identify the emission wavelengths. The emission spectrum is dominated by direct acridone emission at 450 nm, which is the main source of the fluorescence.

As previously mentioned in Section 4.2.3, acridone fluorescence is more favourable than intersystem crossing and therefore even though europium is sensitised by acridone, a significant amount of acridone emission remains. The emission spectrum of Eu.AcridoDO3A in solution under these conditions demonstrates that, although present, the low intensity of the europium emission at 616 nm is unlikely to be easily observed at the low concentrations inside the cell without applying a very different approach to time-gating that favours the long emission lifetime of europium. Given the restraints of available instrumentation, gadolinium complexes can be just as

effective as the europium complexes when used directly to assess cellular uptake using only acridone emission.

Cellular localisation experiments were conducted to determine the localisation of the Eu.AcridoDO3A complex intracellularly. The cells were initially incubated with Eu.AcridoDO3A followed by either LysoTracker[®] Red, MitoTracker[®] Red or ER-Tracker[™]. The commercial tracker probes are all excited in the region 543 nm, while Eu.AcridoDO3A is excited at 405 nm. Co-localisation of LysoTracker[®] Red and Eu.AcridoDO3A can be seen in Figure 5.1. MitoTracker[®] Red and ER-tracker[™] Red did not localise in the same regions as Eu.AcridoDO3A. Therefore, Eu.AcridoDO3A is clearly taken up by lysosomes, late stage endosomes, or similar acidic organelles which are targeted by LysoTracker[®] Red. In other systems, macropinocytosis has been found to be the most common method of uptake for lanthanide complexes.² This results in encapsulation of the complexes inside endosomes, but the complexes can then leak out inside the cell and localise in different subcellular compartments. In this case, EuAcridoDO3A either remains in late stage endosomes or localises in lysosomes. This has previously been observed for a number of lanthanide complexes.^{3,4} For MRI purposes this means that the complexes do not come into contact with any intracellular phosphate species for use in ^{31}P -MRS, and that they also do not “see” the bulk intracellular water. At high concentrations the ^1H relaxivity is therefore likely to experience a quenching effect as previously reported for Gd complexes.⁵

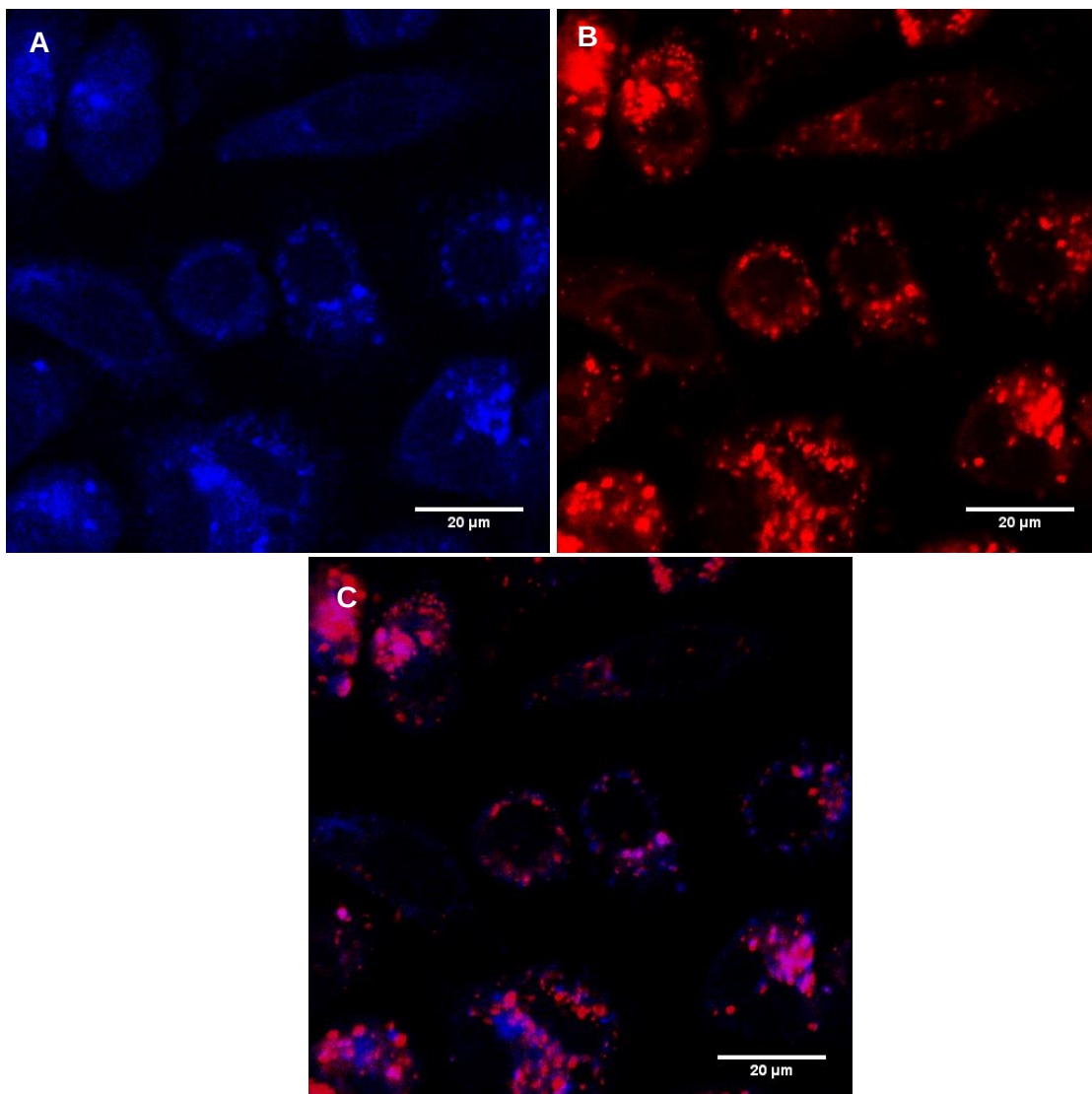


Figure 5.1: Confocal fluorescence microscopy of PC3 cells incubated with 200 μM Eu.AcridoDO3A and 100 nM LysoTracker[®] red A) Eu.AcridoDO3A emission ($\lambda_{\text{ex}} = 405 \text{ nm}$) and B) LysoTracker[®] red emission ($\lambda_{\text{ex}} = 543 \text{ nm}$). C) Overlay of images A and B.

Live cell samples were fixed after imaging with paraformaldehyde for further investigations. However, imaging of the cells post-fixation shows significant changes in the localisation of the complex (Figure 5.2). This has been previously reported for cell-penetrating peptides, where significant movement within the cell is observed upon fixation.⁶ In this case, the complex appears to remain bound within the outer cellular

membrane, but most notably is able to travel into the nucleus and cytoplasm. Due to the characteristically long lifetimes of the complex, phosphorescence lifetime imaging (PLIM) was also used to try and confirm the presence of the europium complex. Fluorescence lifetime imaging (FLIM) is a method by which the image contrast is created by differences in the fluorescence emission lifetime. It is therefore not dependent on concentration or the intensity of the fluorophore. The emission lifetimes are affected by binding of molecules or changes in pH, which means lifetime imaging can be used to detect functional changes in the cell.⁷ In the case of Eu.AcridoDO3A the millisecond-order lifetimes will be longer than anything else in the cellular environment, so PLIM imaging should allow for detection of the complex and also for any binding to endogenous anions. Unfortunately, no signal could be detected in the live cells—only in fixed cells where it is clear there was significant movement of the complex and chemical speciation is unknown.

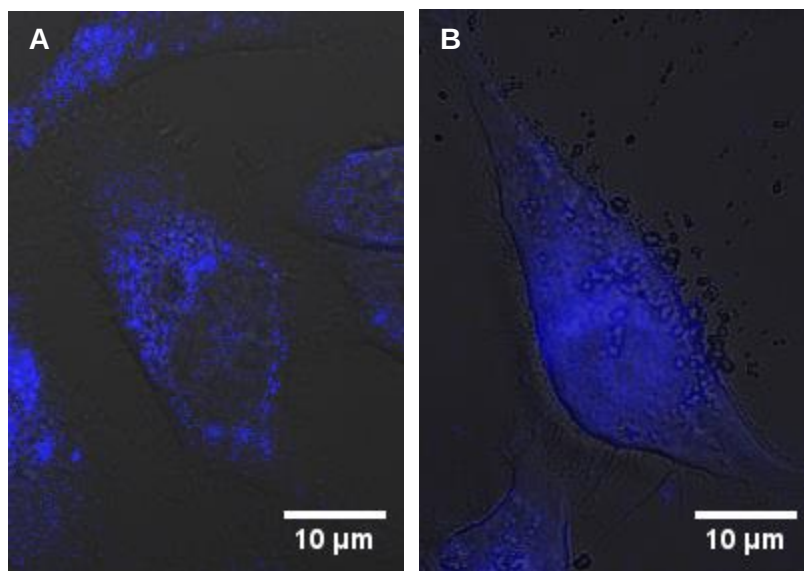


Figure 5.2: PC3 cells incubated with 200 μM Eu.AcridoDO3A, live cell imaging (A) and post-fixation

(B), $\lambda_{\text{ex}} = 405 \text{ nm}$.

One of the reasons for this may be the rapid pulse repetition used in this system, which means that most of the laser power goes to waste since the pulse cycles much faster than the lanthanide emission. Alternative approaches to observing lanthanide emission have used Nd:YAG pumped excitation sources, where the slower repetition rate (Hz instead of MHz) allows high energies to be used to excite the sample without incurring tissue damage.⁸ Since no such equipment was available, the microscopy was not pursued during this project.

5.3. ^{31}P Cell NMR

^{31}P -NMR spectra of live cells were acquired with and without Gd.pDO3A to determine whether the effects of the contrast agent in action on phosphate species could be observed. The cells were incubated for 30 minutes with 0.5 mM Gd.pDO3A and compared to control cells. Due to the insensitivity of the ^{31}P nucleus and the low concentration of metabolites, large cell numbers, of the order of 10^8 cells, were required. The samples were run in Shigemi tubes so that the cell sediment remained within the NMR coil throughout the NMR experiment. The phosphate species involved in metabolism are time-sensitive once the cells leave the incubator. This is because the production of ATP is reduced, while the cells continue to consume energy. To try and make the samples comparable and to maximise ATP in the sample, ice cold medium was used and the cells were kept on ice until the NMR experiment began, to slow cell metabolism as much as possible. The first scan was run close to 30 minutes after incubation time ended and this was kept as constant as possible between experiments. Each FID consisted of 1000 scans and took 17 minutes to acquire. During this time metabolism changes will be occurring but keeping all parameters constant means the

spectra are comparable. All spectra acquired were referenced externally to 80% H_3PO_4 . The spectra are not quantitative, due to the short repetition time and the flip angle used, which do not allow full relaxation of the spins. However, as the same parameters are used for each experiment, if the contrast agent reduces the relaxation time of the species, an increase in the signal of the peaks should be observed. The effect of Gd.pDO3A on the ^{31}P -NMR of 3T3 fibroblast cells is shown in Figure 5.3. The cells remained $\geq 95\%$ viable throughout the experiments as determined by trypan blue exclusion assay after completion of the experiments.

The Gd.pDO3A and control spectra have to be normalised to account for differences in the cell count. The spectra were normalised to the α -ATP peak, as an intracellular phosphate species, where the degradation is less time-sensitive than β -ATP and γ -ATP, due to the overlap of α -ATP and α -ADP.

Both the initial and the second spectra show a notable increase and broadening of the P_i peak. This is consistent with the interaction of extracellular phosphate with Gd.pDO3A, which is known to occur. There appears to be a small enhancement of the phosphomonoesters (PME) and a slight broadening in the second spectra. The PMEs consist mainly of phosphatidylcholine and phosphatidylethanolamine, which are phospholipids that are major constituents of the cellular membrane.⁹ This would suggest there may be a small amount of interaction of Gd.pDO3A with the membrane of the cell. None of the other ATP related peaks show any significant and consistent change over time in the presence of Gd.pDO3A.

PCr is not apparent in the spectra as it is hidden in the baseline noise at approximately -3 ppm. It is likely that in the anaerobic environment PCr is used up

and not replenished; previous studies have shown that PCr is observable in perfused cell lines when creatine is added to the perfusion medium.^{10,11}

^{31}P -NMR spectra of the media once the cells had been removed showed only P_i —most likely excreted from the cells. As expected, this was enhanced in the sample containing Gd.pDO3A (Section A.5, Appendix).

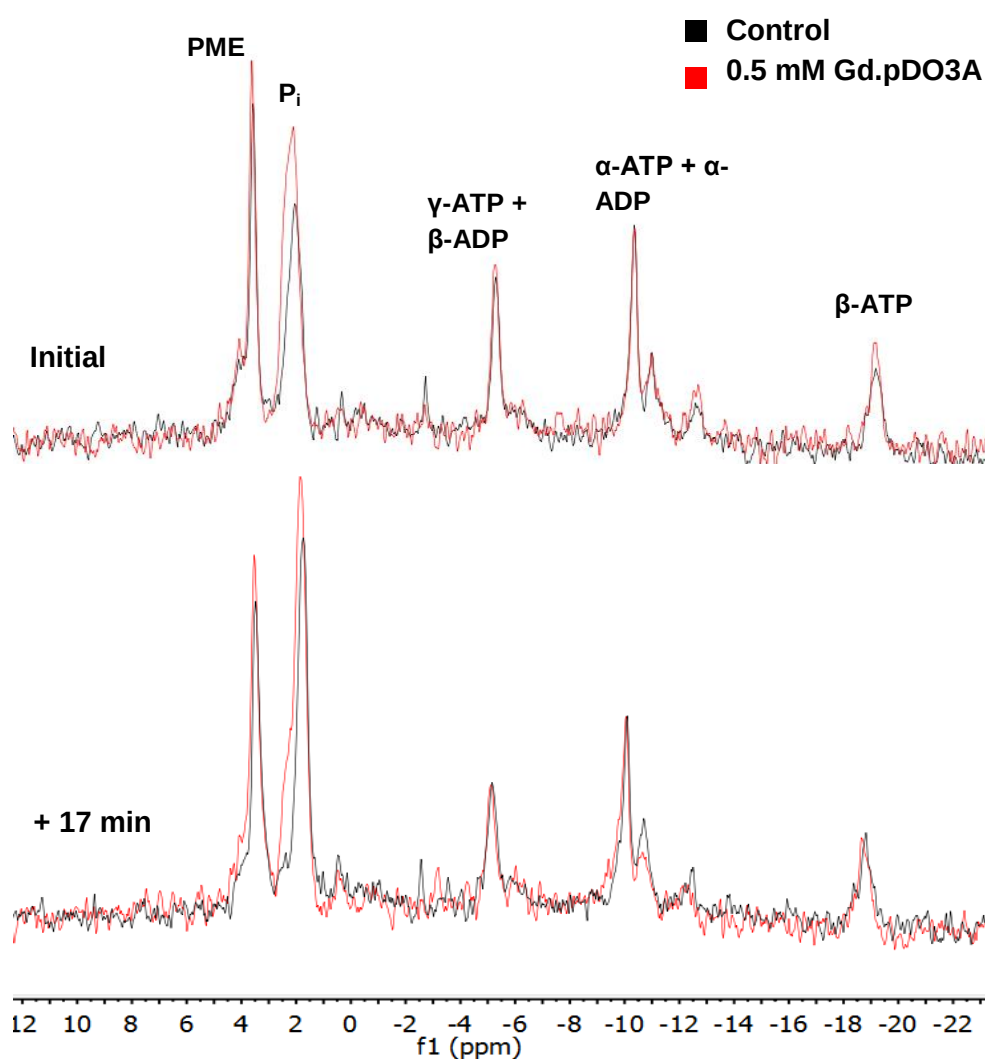


Figure 5.3: ^{31}P -NMR of 3T3 cells ($\sim 1 \times 10^8$ cells) in serum-free DMEM (+ 5% D_2O). Control (black) versus 0.5 mM Gd.pDO3A (red), two spectra show consecutive acquisitions on the same samples each of 1000 scans (17 mins) at 298 K, 202 MHz. First scan of initial spectrum is acquired ~ 30 mins after incubation endpoint. Each spectrum is the sum of $n = 2$. Externally referenced to H_3PO_4 .

Although the quantity of P_i expelled from the cells cannot be normalised to account for differences in cell number, the increase seen is significant and Gd.pDO3A is known from the previous experiments to enhance the relaxation rate of P_i in solution.

More detailed interpretation of the spectra proved difficult due to the time-sensitive nature of the phosphate species. This is demonstrated more clearly in Figure 5.4; the changes over a series of three consecutive 17 minute scans can be clearly seen. The PME peak shows a broadening over time, which may be an increase in AMP (also a phosphomonoester), which has a chemical shift in this region.¹² The inorganic phosphate (P_i) peak consists of both intra- and extra-cellular phosphate.

The P_i concentration clearly increases significantly over time. This is because as the ATP is used up and the cells begin to die they excrete phosphate out of the cell into the medium, increasing the concentration of extracellular phosphate. The P_i chemical shift is very sensitive to pH and a small shift upfield is also observed, which is a sign that the extracellular media is increasing in acidity.¹²

β -ADP as a terminal phosphate group, becomes similar to γ -ATP and the peak occurs to the right of the γ -ATP peak. γ -ATP shows significant decrease over time, in addition to an increase in the β -ADP peak to the right. α -ATP and α -ADP coincide – the increasing shoulder on left hand side of the peak over time may be a result of the increasing ADP content over time as ATP degrades.

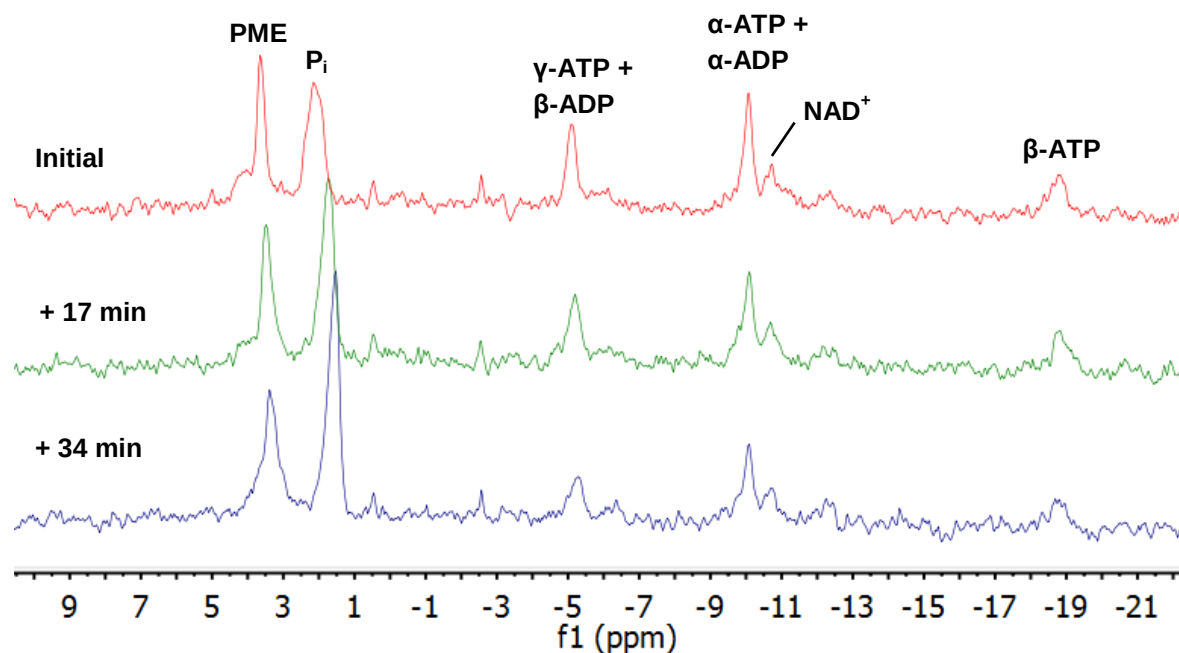


Figure 5.4: ^{31}P -NMR of NIH-3T3 fibroblast cells (1.6×10^8 cells/ml) in serum-free DMEM (+ 5% D_2O).

First scan is acquired ~ 30 mins after incubation endpoint. 1000 scans were accumulated per spectrum

(17 minutes) at 202 MHz, 298 K. Externally referenced to H_3PO_4 .

β -ATP shows a time-dependent decrease, as ATP degrades. All peaks in the spectrum therefore show time-dependent changes, so a combination of variation in cell number and different stages of metabolism make determining the enhancement of the contrast agent intracellularly very challenging unless the enhancement is significant. In order to quantify enhancement in ^{31}P -NMR by contrast, perfusion or *in vivo* studies would be required to stabilise the system.

5.4. ^1H Diffusion NMR

As a result of the difficulties with measuring phosphorus metabolites in cell samples and the time restrictions, a more quantitative method was required. ^1H -NMR can be used to indicate whether the complex is intracellular, and also to clarify whether or not it is in contact with the phosphate species of interest. If not then this would lend weight to the hypothesis that the complex is encapsulated in lysosomes rather than in the cytosol. In collaboration with Professor Andrew Baldwin and Mr Gogulan Karunanithy, a method they developed to separately interrogate the intracellular and extracellular R_1 times of the water, was used. The intracellular and extracellular water can be separated based on diffusion times, as intracellular water is diffusion limited inside the cell (and the diffusion of the cell itself occurs very slowly due to its bulk).

In diffusion NMR, gradient pulses are applied to dephase the nuclear spins. The rephasing of the spins after diffusion time results in signal attenuation as a result of the Brownian motion of molecules during the diffusion time.¹³ Increasing the strength of the gradient pulse increases spatial selectivity, so as the pulse is increased the signal that remains will primarily be intracellular water. R_1 values for intracellular versus extracellular water can be determined by using a set of increasing diffusion times, with the same gradient pulses. The initial signal intensity for each water population can be determined and then as the diffusion time is increased, the intensity will decrease exponentially with increasing R_1 .

HeLa cells were used for this experiment due to the high durability of the cells, which minimises cell death during the time frame of the NMR experiments. The cells were suspended in Shigemi NMR tubes, which prevented sedimentation of the cells outside of the NMR coil. Settling of the cells during the experiments appeared to cause

a slight chemical shift in the water peak. To avoid this problem, 0.2% low melting point agarose was used to keep the cells suspended. Low melting point agarose has been previously applied in cell NMR experiments to reduce cell sedimentation¹⁴, and did not significantly affect the observed water diffusion rates. Cells were incubated in 0.5 mM of complex for 20 minutes prior to the experiment. After harvesting the cells, they were re-suspended in the cell NMR buffer, which contained 0.5 mM complex, PBS, glucose and low melting point agarose. The complex was added to the extracellular solution to prevent forming a negative concentration gradient of Gd complex out of the cell.

NMR measurements and data analysis were performed by Mr Gogulan Karunanithy, an outline procedure can be found in the experimental methods (Section 7.8.2, Chapter 7). In brief, the model fits the change in the intensity of the water peak by taking into account the following factors: diffusion of water molecules, water exchange across the cellular membrane, restriction on diffusion of intracellular water and the relaxation of water molecules. Using this model, the relaxation rate of intra- and extra-cellular water (R_1^{in} and R_1^{ex}) in the sample can be determined. In addition to R_1 values, the other fitting parameters of the model are: extra- and intra-cellular water diffusion coefficients (D_1 and D_2), the exchange parameter (K_{ex}), mole fraction of intracellular water (p_{α}), cell radius and overall intensity factor (I_0). The cell radius averages at about 10 μM , which is comparable with the values determined by the cell counter. Approximately 1×10^7 cells were used per sample. For this number of cells approximate values of the other parameters are: $K_{\text{ex}} = 6 \text{ s}^{-1}$, $D_1 = 1.5 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, $D_2 = 1.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ and $p_{\alpha} = 0.95$.

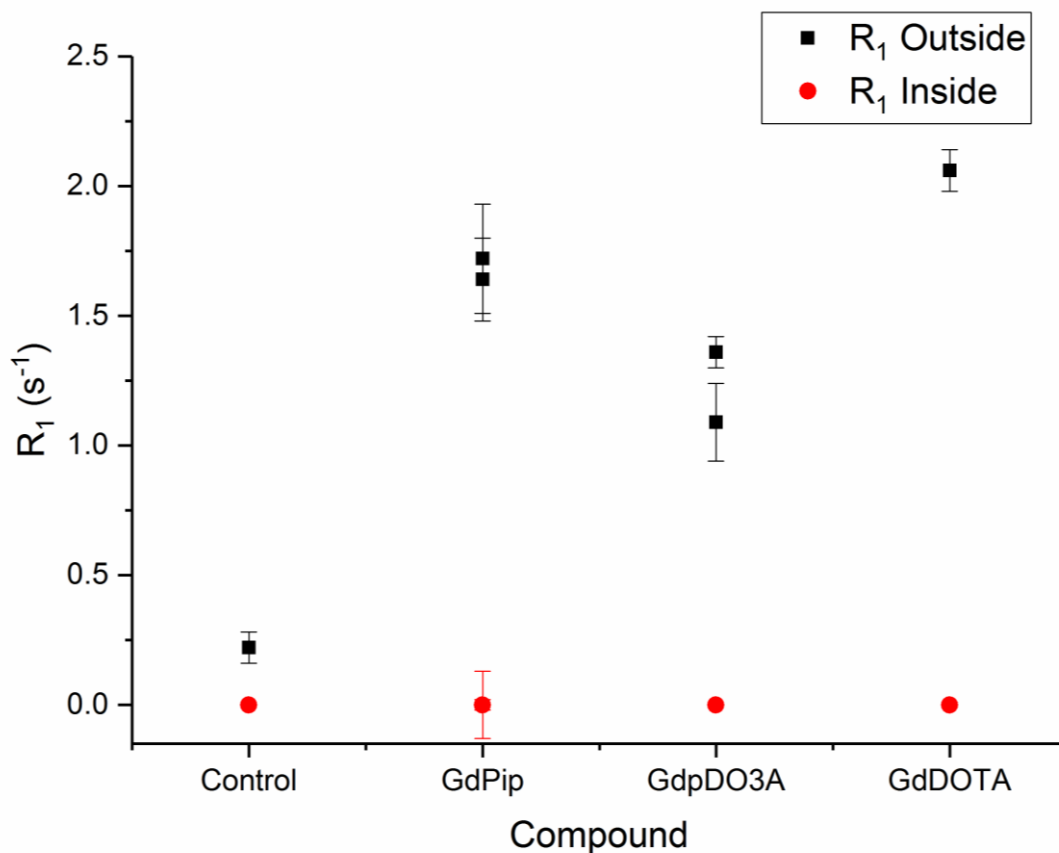


Figure 5.5: Relaxation rates (R_1) of intracellular water (●) and extracellular water (■) of HeLa cells in PBS with 0.5 mM Gd.PipDO3ADO3A, Gd.pDO3A and Gd.DOTA at 298 K, 600 MHz. Cells were pre-incubated with 0.5 mM of the complex for 20 min at 37°C in serum-free media. Error bars are $\pm$ one SD.

The results are displayed in Figure 5.5. The viability of the cells was $\geq 90\%$ for all experiments and remained above 90% for up to 3 h after initial harvesting as determined by Trypan Blue assay. The results show that there is very little difference in the intracellular relaxation rate of the cells in the presence of any of the complexes. A limitation of the model is that it fails to fit a specific value to R_1^{in} at low values, which would be expected in cases of low uptake. However, if the complex was entering the cell in significant amounts then R_1^{in} would be larger and the model could

fit a value. As discussed in Chapter 1, quenching of the observed R_1^{in} can occur when the complex is trapped in intra-vesicular compartments due to the water exchange rate across the membrane limiting the concentration of the complex in the cytosol.⁵ This creates a saturation effect of R_1^{in} at increasing concentrations of internalised Gd. However, it is unlikely that this is occurring here, as no notable relaxation enhancement of R_1^{in} is observed at all.

R_1^{ex} is enhanced in the presence of all Gd contrast agents, with Gd.DOTA having the most notable effect. In previous chapters we have demonstrated that Gd.pDO3A and Gd.PipDO3A both still demonstrate higher relaxivity in PBS than Gd.DOTA. Therefore, the lower R_1^{ex} for Gd.pDO3A compared to Gd.DOTA (1.23 s^{-1} vs 2.06 s^{-1}) and even Gd.PipDO3A (1.69 s^{-1}) would suggest that there may be a lower concentration of Gd.pDO3A and Gd.PipDO3A than Gd.DOTA in the extracellular solution. However, the NMR solution contains other components which also need to be tested for their interaction with the Gd complexes. The NMR solution consists of PBS supplemented with 90 mM Glucose and 0.2% low melting point agarose, both of which were tested for their interaction with the complexes at 400 MHz. Gd.pDO3A (1 mM) demonstrated an enhanced R_1 in glucose supplemented PBS as opposed to in PBS alone (5.28 s^{-1} vs 4.96 s^{-1}). It was found that with added glucose the relaxation rate of Gd.pDO3A becomes closer to the relaxation rate in water (5.31 s^{-1}). This may be due to the large glucose molecules at a high concentration reducing the interaction of PBS with Gd.pDO3A, because glucose itself does not interact with Gd.pDO3A. The effect of 0.2% low melting point agarose on the relaxation rate was also tested with both Gd.DOTA and Gd.pDO3A. The R_1 of Gd.pDO3A was significantly lowered in the presence of the agarose (2.12 s^{-1}), which becomes close to the relaxivity of a $q = 0$

complex, as recorded in the presence of FBS (2.09 s^{-1}). This suggests that Gd.pDO3A demonstrates significant interaction with the agarose even at low concentrations, which results in obstruction of the water binding sites. In comparison, Gd.DOTA demonstrated a minimal change in R_1 in the presence of the agar (4.12 s^{-1} vs 3.89 s^{-1}), which indicates there are no interactions with the Gd centre that prevent inner-sphere water binding.

Agarose is a polysaccharide that consists of alternate D-galactose and 3,6-anhydro-L-galactose units. Gelling occurs at low temperatures through crosslinking of the double-helical polymer units by H-bonds and electrostatic interactions.¹⁵ Glucose and galactose are structurally very similar, but, as demonstrated, glucose did not exhibit the same interaction with Gd.pDO3A. As Gd.DOTA does not exhibit the same reduction in relaxation rate that Gd.pDO3A does, the effect is most likely due to the ability of heptadentate complexes to bind to different anions and not an effect of the gelling properties of agarose on relaxation enhancement. Due to the ability of agarose to form strong crosslinked polymer chains based on electrostatic interactions, Gd.pDO3A may be able to partake in some of these interactions. The lower R_1^{ex} values obtained in these experiments for Gd.PipDO3A and Gd.pDO3A compared to Gd.DOTA, are therefore likely to be a result of interactions with the agarose and not an indication of the concentration of complex in solution. There is a possibility that some of the complex may still be interacting with the membrane phospholipids at a surface level in addition to the agarose.

5.5. Cell Viability

The experiments so far have involved relatively short exposure times and concentrations below 1 mM. Realistically, if these compounds are used *in vivo* the exposure time will be on the order of hours, depending on the excretion rate of the compound. In patients with renal impairment, longer exposure times to GdDTPA-BMA were the reason for the cause of NSF.¹⁶

To test the acute toxicity of these compounds in a more realistic biological time frame, NIH-3T3 fibroblast cells were exposed to the Gd complexes for 20 h at 37°C, at concentrations from 0.05–2 mM. The cell viability was assessed using an MTT assay, which assesses cell metabolic activity.¹⁷ Viability is reported relative to the control cells, which are taken to be 100%. Prior to the experiment the initial viability of the cells was assessed by trypan blue exclusion to ensure the viability was > 90%. The results are displayed in Figure 5.6. Gd.pDO3A clearly shows no evidence of toxicity. The percentage viability of >100% for some of the samples at higher concentrations may be due to increased proliferation of the cells, which results in higher cell numbers relative to the control. Gd.PipDO3A and Gd.AcridODO3A both show concentration dependent reduction in viability in comparison to the control which is more pronounced for Gd.AcridODO3A. At 1 mM the toxicity appears to converge and is almost the same as at 2 mM for both complexes. At 2 mM the viability of the cells in the presence of Gd.PipDO3A is 77%, while for Gd.AcridODO3A it is down to 62%.

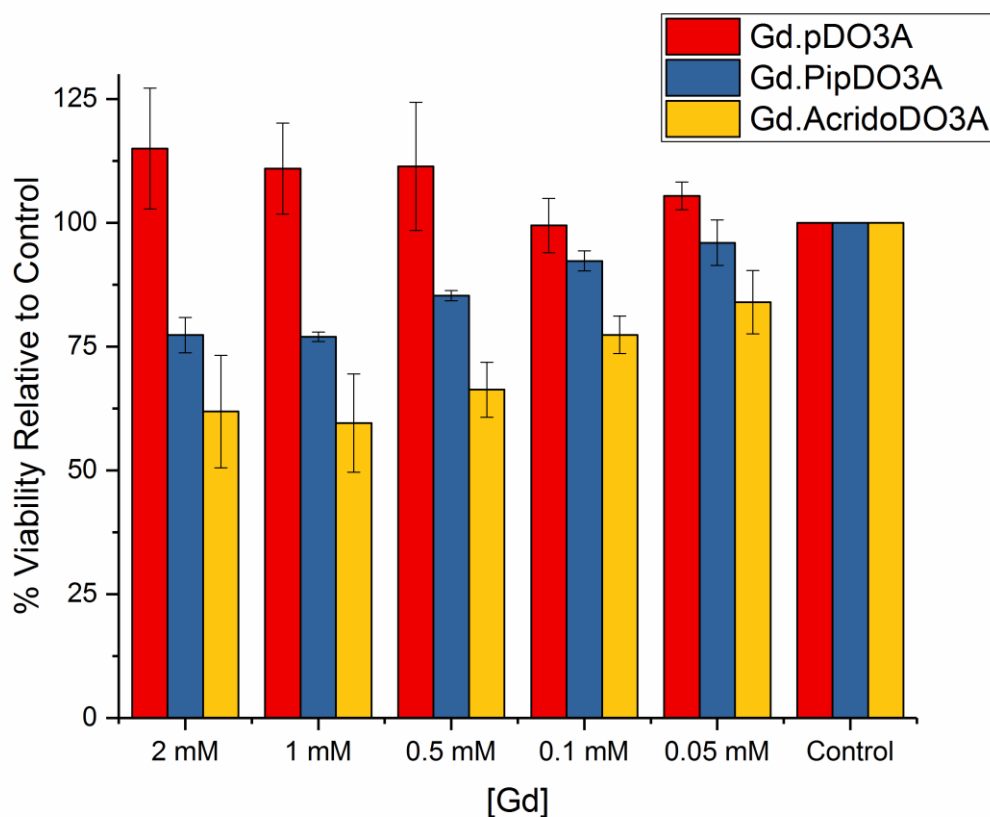


Figure 5.6: Percentage viability of 3T3 cells after 20 h exposure to each Gd compound. Viability was measured by MTT assay and viability is reported as the percentage of the control. $n = 3$, error bars correspond to $\pm$ SEM.

The results suggest that Gd.pDO3A shows no evidence of toxicity as it remains extracellular, as demonstrated by our previous experiments. Gd.AcridoDO3A demonstrates the most significant toxicity and we have also demonstrated that this enters cells and localises in lysosomes. The Gd.PipDO3A complex demonstrated no significant relaxation enhancement of the intracellular water in the diffusion experiments. But the toxicity may suggest there is some uptake or interaction of the complex which affects cell metabolism at long exposure times.

Some acridone derivatives are known to display significant cytotoxicity in cells for which they have attracted interest as chemotherapeutic agents.^{18,19} These compounds demonstrate IC_{50} (half-maximal inhibitory concentration) values around 10 μM , that are thought to be caused by DNA intercalation of acridone.^{20,21} Gd.AcridoDO3A has a relatively low toxicity in comparison, as the complex does not reach 50% cell viability in the concentration range studied. Fluorescent lanthanide complexes require much lower concentrations to be visualised intracellularly than MRI contrast agents. Therefore IC_{50} values around 200 μM for fluorescent complexes are not considered particularly problematic if the concentration used is 50 μM . For MRI contrast agents higher concentrations (millimolar range) are required and therefore, for intracellular agents, it is more challenging to maintain high cell viability.

The previous cell uptake experiments in this chapter used exposure times between 20–30 minutes and demonstrated no significant toxic effects. The viability tests in the previous experiments all made use of trypan blue exclusion assay. In this method, trypan blue stains cells which have increased membrane permeability, as a marker of cell death. In contrast, the MTT assay measures metabolism to determine viability. This is an important distinction to make, and in this case the MTT assay is thought to provide more useful information in regard to the use of these compounds.

These results show how important it is to run complete toxicity tests, which are relevant to the application of the compound. At long exposure times, Gd.AcridoDO3A and Gd.PipDO3A are clearly unsuitable to be used in a cellular environment. However, GdpDO3A does not demonstrate any evidence of toxicity from these preliminary experiments and therefore may be considered for use in cellular systems.

5.6. Conclusions

In this chapter we showed that Eu.AcridoDO3A is internalised by PC3 cells and localises in lysosomes or late stage endosomes, as demonstrated by fluorescent microscopy. It proved challenging to achieve quantitative results using ^{31}P -NMR in cells, which are not perfused or *in vivo*, due to the rapid degradation of phosphate metabolites. However, Gd.pDO3A demonstrates enhancement of extracellular phosphate as it is released from cells and therefore can act effectively as an extracellular ^{31}P contrast agent to separate intra- and extra-cellular phosphate species. ^1H diffusion NMR did not show any apparent uptake of Gd.pDO3A or Gd.PipDO3A in HeLa cells, which correlates with the Gd.pDO3A results of the ^{31}P -NMR study in 3T3 cells. The relatively low relaxation rate of Gd.pDO3A and Gd.PipDO3A in extracellular solution suggests that the concentration is lower than expected, which may be a result of interaction of the complexes with the phospholipids of cells. This would be consistent with the minor enhancement by ^{31}P -NMR of the PME peak, which shows only a small interaction, but possibly enough to lower the concentration of Gd in solution and reduce the relaxation rate. Therefore, the results suggest only extracellular enhancement, although there is the possibility of surface level interactions.

On the timescale of these studies none of the complexes showed any toxicity to the cells. However at longer exposure times, which are more applicable to the use of the complex *in vivo*, significant toxicity to 3T3 cells was observed with Gd.AcridoDO3A and to a lesser extent with Gd.PipDO3A. As it is known from our fluorescence microscopy studies that Gd.AcridoDO3A is likely to be entering cells, it is clear that interactions inside the cell are causing significant toxicity. It is reasonable

to suggest that the toxicity of Gd.PipDO3A may also suggest a small amount of internalisation of the complex. The lack of toxicity observed for Gd.pDO3A would appear consistent with the evidence presented that the complex remains extracellular. Considering the high concentrations of Gd often required for MRI, especially in visualising cells, it is clearly not viable to load even relatively low concentrations into cells. Due to the heptadentate nature of these complexes, it is likely they may interact with many different parts of the cell machinery, not just the phosphate species and in some cases may bind with much higher affinity. At this point it does not seem reasonable to suggest an intracellular phosphate probe of this design, unless this issue can be addressed, or another method can be utilised.

However, these probes do show the potential for simplifying ^{31}P MR in living systems by differentiating extra- and intra-cellular phosphate species. Gd.pDO3A is probably the best candidate for such an application, since it exhibits no apparent cytotoxicity.

5.7. References

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Chapter 6

Conclusions and Future Work

This work has investigated the development of ^{31}P contrast agents as a novel concept and their potential as molecular probes for high-energy phosphate species *in vivo*.

The prototype Gd.pDO3A compound was evaluated for the key physical properties required for a suitable ^{31}P contrast agent. The phosphate species, PCr, ATP and P_i were found to bind in fast exchange with Gd.pDO3A in solution and all exhibit pronounced ^{31}P relaxation. In cellular environments, however, Gd.pDO3A does not come into contact with any of the intracellular phosphate species of interest, which reside in the cytosol.

Structural adaptation of the Gd.pDO3A complex led to a number of further complexes, which all exhibited similar levels of ^{31}P relaxation enhancement. The ligand-sphere was found to have an impact on the selectivity of the complex for phosphate species in terms of both ^{31}P relaxivity and binding affinity with Eu analogues. Affinity of the complexes for different phosphate species were able to be tuned by introducing non-covalent interactions with different substituents on the ligand or by an additional lanthanide centre. With these types of complexes, luminescence methods could be applied to quantify phosphate species in biological environments.

Cellular uptake was observed for Eu.AcridoDO3A by fluorescence microscopy with sub-cellular localisation in lysosomes, most likely internalised by

macropinocytosis. Gd.PipDO3A did not display any cellular uptake by measurement of the intracellular ^1H relaxation rate using diffusion-NMR. However, both these complexes displayed significant toxicity in cellular environments at long exposure times. These are anticipated to be comparable with exposure times *in vivo*, which is therefore likely to render these complexes incompatible with biological application. Possible new approaches to ^{31}P -MRS contrast agents and their development are detailed below.

6.1. ^{31}P -MRS for Extracellular Contrast

It is clear from this work that Gd.pDO3A remains outside cells and does not exhibit any acute cell toxicity. Therefore, Gd.pDO3A can relax extracellular phosphate which has been excreted from the cell. Future work on Gd.pDO3A would involve investigating the potential for differentiation of intra- and extra- cellular phosphate in biological systems. The overlap of intra and extra-cellular P_i in the ^{31}P -MRS spectrum makes the separation of the signals in this way highly desirable. The Gd contrast agent could be used to effectively shorten the T_1 times of extra-cellular P_i (and potentially other phosphate species) and therefore separate fast-relaxing species in the ^{31}P -MRS spectrum, based on T_1 times.

Alternatively, extra-cellular signal-suppression could be achieved using T_2 relaxation. Signal suppression of water in ^1H -NMR can be achieved using water attenuation by transverse relaxation (WATR).¹ Dysprosium (Dy) complexes have previously been applied to enhance water-suppression by shortening the T_2 of water, as Dy exhibits more pronounced T_2 relaxation than Gd.² Therefore, it is possible that

Dy.pDO3A may be able to suppress the signal of any extracellular phosphate species, by shortening the T_2 times. This would allow only intra-cellular phosphate species to be observed in the ^{31}P -MRS spectrum.

Another approach to separating intra- and extra-cellular species is to use paramagnetic shift reagents. Pr.DO3A has previously been used to separate intra- and extra-cellular lactate by their chemical shift.³ This thesis has indicated that the scope of these DO3A complexes as ^{31}P paramagnetic shift reagents for phosphate is limited due to the lack of change in chemical shift observed with Tb.pDO3A—potentially due to binding of phosphate at the magic angle. In order to confirm this, and to further understand phosphate binding with this series of complexes, a wider investigation of different lanthanides may be informative. In particular, Ytterbium (Yb) has a smaller T_2 contribution and therefore will have lower line broadening than Tb, while erbium (Er) demonstrates a different orientation of the magnetic susceptibility tensor.^{4,5} It has recently been shown that the orientation of the magnetic susceptibility tensor can account for the differences in observed paramagnetic chemical shifts.^{6,7} Therefore, as these DO3A complexes are non-axially symmetric, lanthanides such as Er, with a different orientation of the magnetic susceptibility tensor, may result in more pronounced ^{31}P chemical shifts.

6.2. ^{31}P -MRS for Intracellular Contrast

The ^{31}P contrast agents studied in this work demonstrated low cellular uptake and those that were internalised became encapsulated inside vesicles. As a result, none of the complexes appeared to come into contact with the high-energy phosphate species of interest internally. In order to access intracellular ^{31}P contrast further work needs to focus on the delivery of complexes into the cytosol. The ability of the complexes to cross the cellular membrane could be improved by using polyarginine and TAT-based peptides,^{8–10} but access of phosphate species to the Gd centre would need to be maintained in the presence of bulky substituents. In addition to this, localisation in the cytosol or mitochondria is required upon uptake into the cell, which proves challenging to predict. Localisation in the mitochondria must also not perturb the mitochondrial membrane potential as this is likely to cause apoptotic cell death.¹¹

An alternative approach to cellular uptake is to use physical methods such as electroporation, hypotonic swelling or sonoporation. Electroporation involves the use of electrical pulses to permeabilise the cell membrane, while hypotonic swelling uses the osmotic potential.¹² This makes these methods incompatible with use *in vivo*, however they may allow proof of concept and a greater understanding of the potential of these contrast agents. Sonoporation, however, has more potential for *in vivo* application. This involves the application of ultrasound to cells, which temporarily permeabilises the membrane and enhances the uptake of molecules from the surrounding blood plasma.¹³ Microbubbles, which are used clinically as ultrasound contrast agents, significantly amplify the cell-permeabilisation effects of ultrasound

when co-administered with target molecules. These methodologies result in cytosolic uptake of any local species outside the cell which is therefore useful for testing intracellular ^{31}P relaxivity. The internalisation of high concentrations of complex is likely to reduce cell viability and therefore high ^{31}P relaxivity complexes will be required, as it is not desirable biologically to load high concentrations into cells.

In these studies, the complexes Gd.AcridoDO3A and Gd.PipDO3A were found to display acute toxicity despite localisation inside lysosomes with no apparent cytosolic uptake. As heptadentate lanthanide complexes, it is likely they may be able to interfere with cellular machinery or disrupt cellular membranes by interaction with phospholipids. It therefore presents a significant challenge to design complexes, which can interact only with phosphate species, localise in the cytosol and elicit no cytotoxic effects. Based on the evidence in this thesis, a new and innovative approach will be required in order to achieve this. However, the potential of these probes offers a novel technique to improve the SNR in ^{31}P -MRS, which may pave the way to future advances in molecular imaging.

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Chapter 7

Experimental Methods

7.1. General Procedures

Commercially available solvents and chemicals were purchased and used without further purification (unless otherwise stated). Anhydrous solvents, where used, were degassed with nitrogen and dried by passing through a MBraun MPSP-800 column. Deionised water was obtained using an Elix[®] Essential water purification system. Neutral alumina for column chromatography was purchased from Sigma-Aldrich (aluminium oxide activated, neutral Brockmann I, pore size 58 Å, 150 mesh) and soaked in ethyl acetate overnight prior to use. Silica gel for column chromatography was purchased from Merck (Geduran, pore size 60 Å, 230–400 mesh, 40–63 µm). Silica TLC plates were TLC gel 60 purchased from Merck.

NMR spectra were acquired at 298 K. 500 MHz ¹H-NMR, 202 MHz ³¹P-NMR and 126 MHz ¹³C-NMR spectra were recorded on a Bruker Avance III NMR equipped with an 11.75 T magnet ¹³C-NMR spectra at 126 MHz were also recorded on a Bruker Avance NMR equipped with a 11.75 T magnet and a ¹³C detect cryoprobe. 400 MHz ¹H-NMR, 101 MHz ¹³C-NMR and 162 MHz ³¹P-NMR spectra were obtained using a Bruker Avance III HD nanobay NMR equipped with a 9.4 T magnet. All spectra are referenced to the residual solvent peak, unless otherwise stated. The chemical shifts (δ) are reported in parts per million (ppm) and coupling constants in Hz. NMR

abbreviations used are as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broad (br).

Mass spectra were acquired using either an Agilent Technology 1260 Infinity or a Waters LCT premier XS. High resolution accurate mass spectra were recorded to 4 decimal places using Bruker μ TOF and Micromass GCT systems. MALDI mass spectra were obtained on a Waters MALDI MicroMX spectrometer.

Infrared spectra were recorded in solid-state using a Bruker Tensor 27 FT-IR spectrometer with an IR Diamond Attenuated Total Reflectance module. Data was processed using Bruker OPUS software.

pH values were measured using a Hanna Instruments pH210 Microprocessor pH Meter with a HI 1131B electrode.

Xylenol Orange test

Xylenol Orange was prepared and used in accordance with previous reports.¹ 50 mM acetate buffer at pH 5.8 was prepared by diluting acetic acid (2.87 mL, $d = 1.05$ g/mL, 50 mmol) in 800 mL water. The pH was adjusted to 5.8 with 1 M NaOH and the buffer made up to 1 L with water. Xylenol Orange (3 mg, 4.5 μ mol) was dissolved in 250 mL acetate buffer to give an 18 μ M solution. Aliquots of the solution were frozen immediately for storage and defrosted for use as required. Ln complexes were tested for free Ln^{3+} as follows: 10 μ L of 44 mM Ln complex was added to 100 μ L Xylenol Orange solution to give a final concentration of 4 mM Ln complex, and the results determined by eye.

Amberlite® XAD16N

Complexes were desalted on an Amberlite® XAD16N (Sigma Aldrich, 20–60 mesh) ion-exchange column. Amberlite® was soaked in water for 10 min, then in MeCN for 10 min. The column was loaded in MeCN and left to settle for a minimum of 30 min, before washing through with water (x 3 column volumes) and loading the complex in water (0.1 mg/ml). The column was then washed multiple times with water (minimum 1 L), before elution of the product with 10–20% MeCN.

Dowex®-50WX8 beads

Dowex®-50WX8 (Acros Organics, 100–200 mesh) was used to remove lanthanide ions still present after desalting with Amberlite®. The beads were washed with water, then MeOH and again with water. The beads were then stirred with 5% NH₄OH (aq.) three times, before stirring with a solution of the Ln complex in water for 1 h. The solution was filtered and the solvent removed under reduced pressure, before re-testing the complexes with Xylenol Orange.

Lewatit® –TP207

Lewatit®–TP207 beads (sodium form, Sigma Aldrich) were also tested for their ability to remove free lanthanide from the solution. The beads were washed firstly with MeOH, then 0.1 M NaOH and converted to the proton form by stirring in 1 M HCl. The beads were then washed repeatedly until water was at pH 7. The beads were then stirred with the lanthanide complex in solution, maintaining the pH around 7 with 0.1 M NaOH. The solution was then filtered and the solvent removed, before the complexes were retested with Xylenol Orange.

HPLC methods

HPLC was performed using a Waters Autopurification system, equipped with an ACQUITY QDa performance mass spectrometer and UV-Vis absorption spectrometer. UV-Vis absorption spectra were acquired on the HPLC. MilliQ[®] water (Milipore, Merck) and HPLC-grade MeCN were used as eluents A and B respectively, for all HPLC methods. Neutral conditions consisted of original solvents, basic conditions contain 0.05% NH₄OH (pH 8) in both solvents and acidic conditions contain 0.1% TFA in both solvents (pH 2), gradient and column conditions are described below. Crude samples were dissolved in 95:5 H₂O/MeCN and filtered (nylon, 0.2µM).

Preparative HPLC All preparative HPLC was performed in basic conditions with a Sunfire[®] OBD prep column (5 µm, 19 mm x 150 mm), an injection loop of 1 ml and a flow rate of 10 ml/min. Samples were injected in 500–750 µL aliquots with mass-directed purification.

Gradient A: At 0 min, solvent mixture A/B = 95:5 v/v, at 7 min, A/B = 70:30 v/v, at 8 min, A/B = 0:100 v/v and at 9 min A/B = 95:5 v/v.

Gradient B: At 0 min, solvent mixture A/B = 98:2 v/v, at 7 min, A/B = 80:20 v/v, at 8 min, A/B = 0:100 v/v and at 9 min A/B = 98:2 v/v.

Analytical HPLC Samples were injected in 5 µL aliquots using either column 1:

Sunfire[®] C₁₈ column (5 µm, 4.6 mm x 150 mm) with a flow rate of 1 ml/min, or column 2: Waters X-bridge[®] C₁₈ column (5 µm, 4.6 mm x 50 mm) with a flow rate of 1.3 ml/min.

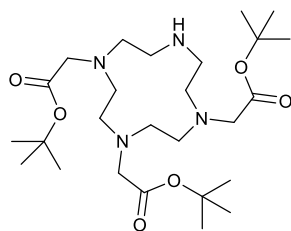
Gradient A: At 0 min, solvent mixture A/B = 98:2 v/v, at 7.9 min, A/B = 50:50 v/v, at 8 min, A/B = 5:95 v/v and at 9 min A/B = 98:2 v/v.

Gradient B: At 0 min, solvent mixture A/B = 95:5 v/v, at 5 min, A/B = 5:95 v/v, at 7 min, A/B = 5:95 v/v and at 7.1 min A/B = 95:5 v/v.

Gradient C: At 0 min, solvent mixture A/B = 95:5 v/v, at 7.9 min, A/B = 60:40 v/v, at 8 min, A/B = 0:100 v/v and at 8.5 min A/B = 95:5 v/v.

7.2. Synthetic Methods

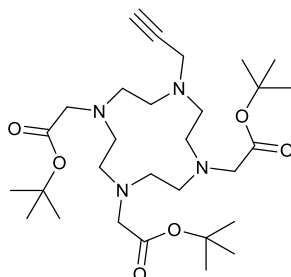
^tBuDO3AH: 1,4,7-Tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane, hydrobromide salt² (2.1)



Cyclen (7.00 g, 40 mmol) was added to dry MeCN (175 mL) with sodium hydrogen carbonate (11.30 g, 134 mmol) and stirred for 20 minutes in an ice bath. *Tert*-butyl bromoacetate (19.75 mL, 134 mmol) dissolved in dry MeCN (75 mL) was added dropwise over 30 minutes. The solution was then allowed to warm to room temperature and stirred under nitrogen for 48 h. The inorganic solids were removed by filtration and the solvent removed under reduced pressure to give a pale cream solid. The solid was dissolved in a minimum amount of DCM, hexane added and triturated to remove excess *tert*-butyl bromoacetate. The resulting cream solid was washed with toluene and then recrystallized from hot toluene to yield the product as a white solid (7.65 g, 37%).

¹H-NMR (400 MHz, CDCl₃): δ_H 9.99 (s, 1H, NH), 3.35 (s, 4H, 1,7-CH₂CO₂), 3.27 (s, 2H, 4-CH₂CO₂), 3.08 (s, 4H, 9,11-CH₂NH), 2.91–2.85 (s, 12H, 2,3,5,6-CH₂), 1.44 (s, 27H, ^tBu) ppm. ¹³C-NMR (126 MHz, CDCl₃): δ_C 170.65, 169.76, 81.99, 81.83, 58.38, 51.52, 49.36, 47.66, 28.38, 28.34 ppm. ES-MS (MeOH): *m/z* 515.4 [M+H]⁺, 537.4 [M+Na]⁺.

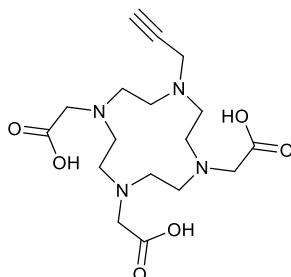
Propargyl-^tBu₃DO3A: 1,4,7-tris(tert-butoxycarbonylmethyl)-10-(prop-2-ynyl)-1,4,7,10-tetraazacyclododecane (Adapted from Jauregui *et al.* 2009.³) (2.2)



^tBu₃-DO3AH, **2.1** (3.00 g, 5.83 mmol) was added to dry MeCN (60 mL) with potassium carbonate (2.03 g, 11.66 mmol) and stirred for 20 minutes. The flask was cooled on ice, propargyl bromide (0.52 mL, 5.83 mmol) added dropwise and the reaction left stirring overnight at room temperature under nitrogen. Inorganic solids were removed by filtration and the solvent removed under reduced pressure. The resulting oil was dissolved in toluene and washed with water. The organic layer was dried with MgSO₄, filtered and the solvent removed to give an orange oil (1.87 g, 58%).

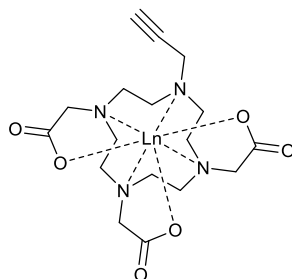
¹H-NMR (400 MHz, CDCl₃): δ_H 3.43 (d, ⁴J_{H-H} = 2.3 Hz, 2H, CH₂C≡C), 3.28 (s, 6H, CH₂CO₂), 2.82–2.69 (m, 16H, CH₂), 2.14 (t, ⁴J_{H-H} = 2.3 Hz, 1H, C≡CH), 1.45 (s, 27H, ^tBu) ppm. ¹³C-NMR (101 MHz, CDCl₃): δ_C 171.26, 80.85, 79.49, 72.62, 56.95, 52.30, 51.98, 51.78, 43.25, 28.39, 21.61 ppm. ES-MS (MeOH): *m/z* 553.4. [M + H]⁺, 575.4 [M + Na]⁺.

pDO3A: 1,4,7-tris(carboxymethyl)-10-(prop-2-ynyl)-1,4,7,10-tetraazacyclododecane³ (2.3)



Propargyl ^tBu₃DO3A, **2.2** (1.87 g, 3.38 mmol) was dissolved in DCM (10 mL) and TFA (10 mL) was added dropwise and the reaction stirred overnight at room temperature. Extra DCM was added and the solvent removed under reduced pressure, this was repeated with DCM and then twice with MeOH. The orange residue was dissolved in a minimum amount of MeOH and triturated with diethyl ether repeatedly to yield a pale brown solid. The product was precipitated from MeOH with a few drops of DCM, as the white trifluoroacetate salt in quantitative yield.

¹H-NMR (400 MHz, D₂O): δ_H 3.85 (m, 6H, -CH₂CO₂), 3.61 (s, 2H, -CH₂C≡C), 3.44–3.33 (m, br, 8H), 3.18 (s, br, 8H), 2.77 (s, br, 1H, C≡CH) ppm. ¹³C-NMR (126 MHz, D₂O): δ_C 174.45, 169.85, 56.12, 53.19, 51.64, 49.00, 47.78, 42.29 ppm. ES-MS (MeOH): *m/z* 385.2 [M + H]⁺, 521.2 [M + NaCO₂CF₃]⁺.

General method for Ln.pDO3A complexes³ (Ln.2.4)

pDO3A, **2.3** (0.2 g, 0.4 mmol, 1 eq.), was dissolved in 3 mL MeOH and the appropriate lanthanide triflate salt added (0.4 mmol, 1 eq.). The reaction was stirred at 60 °C for 30 min and the pH adjusted to pH 5 by with 1 M NaOH (aq.) and then stirred for a further 48 h at 60 °C, maintaining the solution at pH 5. The solvent was removed under reduced pressure and the solid dissolved in 2 mL water. The complexes were purified using Amberlite[®] XAD16N, eluted with 10 % MeCN. Purified complexes were tested for the absence of free lanthanide with Xylenol Orange. If free lanthanide remained, the complex was stirred with Dowex-50 beads and then re-tested.

Gd.pDO3A (Gd.2.4)

Yield: 50 mg, 18%. HRMS (ES⁺), found (m/z): 540.10926 [M-H]⁺; C₁₇H₂₆N₄O₆¹⁵⁸Gd requires 540.10879. ($|\Delta m/z| = 0.87$ ppm).

Eu.pDO3A (Eu.2.4)

Complex was recrystallized from hot MeOH/Acetone, after desalting methods.

Yield: 45 mg, 16%. ¹H-NMR (400 MHz, D₂O, pD 10): δ_{H} 30.75, 22.98, 17.75, 13.78, 11.35, 10.18, -0.65, -1.13, -3.82, -6.00, -6.56, -9.28, -11.08, -11.42, -12.26, -12.61, -14.52, -17.50, -19.14 ppm. Only resolved peaks outside the region 0–10 ppm are

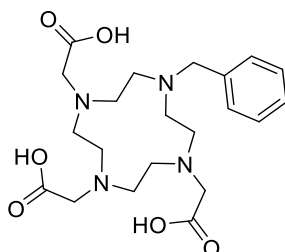
reported. ESMS (MeOH): m/z 535.1 $[M+H]^+$. Analytical RP-HPLC t_R = 4.17 min (column 1, neutral, gradient A).

Tb.pDO3A (Tb.2.4)

Complex was recrystallized from hot MeOH/Acetone, after desalting methods.

^1H -NMR (400 MHz, D_2O , pD 10): δ_{H} 278.20, 142.76, 111.77, 88.11, 81.24, 72.98, 57.49, 48.96, 28.57, 16.04, -31.80, -42.10, -50.69, -58.63, -64.05, -64.71, -70.93, -80.64, -85.78, -93.98, -114.78, -155.38, -177.62, -229.74, -252.24, -314.93 ppm. Only resolved peaks outside the region 0–10 ppm are reported. ES-MS (MeOH): m/z 541.1 $[M+H]^+$. Analytical RP-HPLC t_R = 4.21 min (column 1, neutral, gradient A).

BenzylDO3A: 1,4,7-tris(carboxymethyl)-10-benzyl-1,4,7,10-tetraazacyclododecane (4.2)



^tBu-DO3AH, **2.1** (2.00 g, 3.89 mmol) was dissolved in 60 ml dry MeCN with K₂CO₃ (1.60 g, 11.58 mmol) and stirred for 20 min under N₂. The solution was cooled to 0°C on ice then benzyl bromide (0.46 ml, 3.89 mmol) added dropwise and stirred for 16 h at room temperature. The solution was filtered and the solvent removed under reduced pressure to give a pale yellow oil. The oil was dissolved in 50 ml DCM and washed with saturated NaHCO₃ (25 ml) and 2 x water (20 ml). The product (**4.1**) was taken to the next step without purification (2.00 g, 85% crude yield).

ES-MS (MeOH): *m/z* 605.4 [M+H]⁺, 627.4 [M+Na]⁺

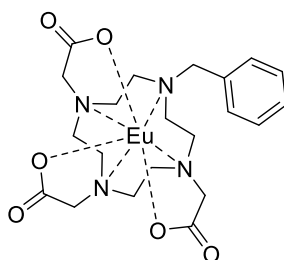
Benzyl-^tBuDO3A, **4.1** (2.00 g, 3.31 mmol) was dissolved in DCM (10 ml) and TFA (10 ml) was added dropwise. The solution was left to stir overnight at room temperature. The solvent was removed under reduced pressure before more DCM was added (20 ml) and removed again. This was repeated once more with DCM (20 ml) and then twice with MeOH (20 ml). The brown oil was then dissolved in a minimum amount of MeOH, triturated with diethyl ether and the excess ether pipetted off. This was repeated until a pale brown solid was collected.

Ion exchanger iii strongly basic anion exchanger –OH form (Merck) were used to removed excess TFA. The beads were washed twice with water, then added to the

product dissolved in water and stirred with the beads overnight. The beads were filtered and stirred with 20% acetic acid overnight again. On filtering, the filtrate was collected and the solvent evaporated. Recrystallisation from MeOH yielded the pure product (0.59 g, 41%).

^1H -NMR (400 MHz, D_2O): δ_{H} 7.48 (br, 2H, ArH), 7.39 (br, 3H, ArH), 3.95 (br, 2H, CH_2), 3.65–3.02 (m, 22H) ppm. ^{13}C -NMR (126 MHz, D_2O): δ_{C} 170.35, 130.32, 129.32, 128.53, 57.20, 55.51, 54.18, 50.13, 48.59, 47.82, 20.67 ppm. ES-MS (MeOH): m/z 437 $[\text{M}+\text{H}]^+$.

Eu.BenzylDO3A (Eu.4.3)

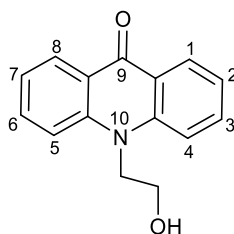


BenzylDO3A, **4.2** (100 mg, 0.23 mmol) and Eu(III) chloride hexahydrate (194 mg, 0.53 mmol) were stirred in MeOH (3 ml) at 60 °C. After 30 min the pH was adjusted to pH 5 with NaOH (1 M) and then stirred for 48 h. The pH was adjusted to pH 10 with NaOH (1 M), with precipitate forming at pH 7 and then at pH 10. The solution was centrifuged to remove the precipitate and then filtered through an inorganic membrane filter. The complex was purified by Amberlite[®] (XAD16N) column, eluting first with water to remove salts and then with 20–50% MeCN to elute the product (97 mg, 72%).

^1H NMR (400 MHz, D_2O , pD 10) δ 21.15, 18.18, 12.90, 12.04, -0.90, -3.02, -3.69, -6.58, -7.48, -8.10, -9.24, -11.33, -12.37, -12.88, -14.98, -15.58, -17.51, -19.23 ppm.

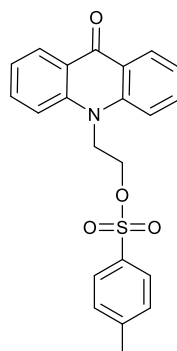
Only resolved peaks outside the region 0–10 ppm are reported. ES-MS (MeOH) m/z : 587 $[M+H]^+$. UV-Vis (H_2O): $\lambda_{max} = 255$ nm. Analytical RP-HPLC $t_R = 2.93$ min (column 2, neutral, gradient B).

N-(2-Hydroxyethyl)-acridone (Adapted from Bretonniere *et al.* 2004.⁴) (4.8)



9(10*H*)-Acridanone (2.0 g, 10.2 mmol) was dissolved in 20 ml dry DMF with ethylene carbonate (1.8 g, 20.5 mmol, 2 eq.) and a catalytic amount of KOH (0.2 g). The solution was refluxed overnight under N_2 (15 h at 150 °C). The solvent was removed under reduced pressure to give an orange oil and water added to precipitate the product. The solid was collected by filtration and recrystallized from MeOH to give yellow crystals (1.5 g, 61%).

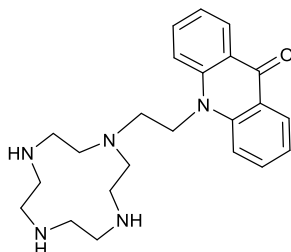
^1H -NMR (400 MHz, $DMSO-d_6$): δ_H 8.35 (dd, $^3J_{H-H} = 8.0$, $^4J_{H-H} = 1.8$ Hz, 2H, acridone-1H,8H), 7.90 (d, $^3J_{H-H} = 8.8$ Hz, 2H, acridone-4H,5H), 7.82 (ddd, $^3J_{H-H} = 8.8$, 6.8, $^4J_{H-H} = 1.8$ Hz, 2H, acridone-3H,6H), 7.33 (ddd, $^3J_{H-H} = 8.0$, 6.8, $^4J_{H-H} = 0.9$ Hz, 2H, acridone-2H,7H), 5.06 (m, 1H, OH), 4.60 (t, $^3J_{H-H} = 6.1$ Hz, 2H, CH_2), 3.89 (q, $^3J_{H-H} = 6.1$ Hz, 2H, CH_2) ppm. ^{13}C -NMR (101 MHz, $DMSO-d_6$): δ_C 176.48, 142.12, 133.95, 126.55, 121.56, 121.22, 116.42, 58.32, 47.58 ppm. ES-MS (MeOH): m/z 240.1 $[M+H]^+$, 501.1 $[2M + Na]^+$, 740.2 $[3M + Na]^+$

N-(2-(Tosylethyl)-acridone⁵ (4.9)

4.8 (1.10 g, 4.60 mmol) was dissolved in 2 ml pyridine and stirred on ice under N₂. 4-Toluenesulfonyl chloride (1.14 g, 5.98 mmol, 1.3 eq) was added to the solution and left to stir overnight at room temperature (16 h). DI water (20 ml) was added to the solution to precipitate the product. The solution turned dark red/purple on standing. The product was filtered and washed with water to yield the crude product as a pale pink solid. Recrystallisation from hot toluene and ethanol yields the product as yellow crystals (1.22 g, 67%).

¹H-NMR (400 MHz, CDCl₃): δ_H 8.51 (dd, ³J_{H-H} = 8.0, ⁴J_{H-H} = 1.8 Hz, 2H, acridone-1H,8H), 7.70 (ddd, ³J_{H-H} = 8.8, 7.0, ⁴J_{H-H} = 1.8 Hz, 2H, acridone-3H,6H), 7.40 (d, ³J_{H-H} = 8.8 Hz, 2H, acridone-4H,5H), 7.35–7.28 (m, 4H, ArH), 6.94 (d, ³J_{H-H} = 8.0 Hz, 2H, ArH), 4.67 (t, ³J_{H-H} = 6.2 Hz, 2H, CH₂), 4.55 (t, ³J_{H-H} = 6.2 Hz, 2H, CH₂), 2.30 (s, 3H, CH₃) ppm. ¹³C-NMR (101 MHz, DMSO-d₆): δ_C 176.39, 144.68, 141.46, 133.81, 130.88, 129.46, 126.62, 126.47, 121.60, 121.32, 115.96, 67.98, 43.15, 21.02 ppm. ES-MS (MeOH): *m/z* 394 [M+H]⁺, 416 [M+Na]⁺.

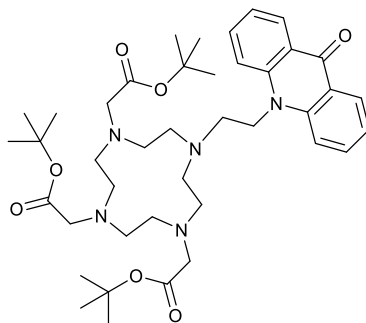
N-(2-Acridylethyl)-1,4,7,10-tetraazacyclododecane (Adapted from Dadabhoy *et al.* 2002.²) **(4.10)**



4.9 (0.58 g, 1.81 mmol) and cyclen (0.65 g, 3.78 mmol, 2 eq.) in dry MeCN (7 ml) and refluxed under N₂ (48 h at 85 °C). The solvent was removed under reduced pressure and the resulting oil suspended between 2 M HCl (25 ml) and DCM (25 ml). The aqueous layer was separated, basified with conc. NaOH solution to pH 10 and extracted with 3 x 15 ml DCM. The solution was dried with MgSO₄, filtered and evaporated to give the product as a waxy yellow solid (0.53 g, 75%).

¹H NMR (500 MHz, CDCl₃): δ_H 8.56 (dd, ³J_{H-H} = 8.0, ⁴J_{H-H} = 1.8 Hz, 2H, acridone-1H,8H), 7.74 (ddd, ³J_{H-H} = 8.7, 6.9, ⁴J_{H-H} = 1.8 Hz, 2H, acridone-3H,5H), 7.57 (d, ³J_{H-H} = 8.7 Hz, 2H, acridone-4H,5H), 7.29 (m, 2H, acridone-2H,7H), 4.55 (m, 2H, CH₂), 2.96 (m, 2H, CH₂), 2.86–1.95 (16 H, cyclen) ppm. ¹³C-NMR (126 MHz, CDCl₃): δ_C 178.12, 141.89, 134.34, 128.20, 122.69, 121.57, 114.66, 52.61, 51.63, 47.23, 45.71, 44.50 ppm. ES-MS (MeOH): *m/z* 394.2 [M+H]⁺.

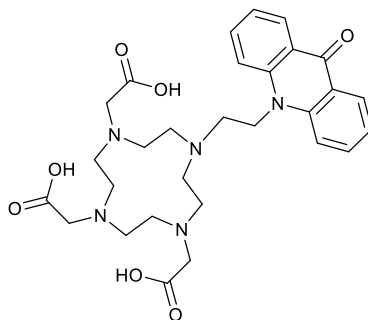
Acridone-^tBuDO3A: N-{2-[4,7,10-Tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecan-1-yl]ethyl}acridone² (4.11)



4.10 (0.53 g, 1.36 mmol) was dissolved in MeCN (40 ml) with trimethylamine (0.86 ml, 6.17 mmol, 4.5 eq.) and *tert*-butyl bromoacetate (0.71 ml, 4.80 mmol, 3.5 eq.). The solution was refluxed under N₂ for 48 h (80 °C). The solvent was removed under reduced pressure, the oil dissolved in DCM (25 ml) and washed with 3 x 15 ml water. The solution was dried with MgSO₄, filtered and the solvent evaporated to give the product as a pale orange foamy solid (0.85 g, 85%).

¹H-NMR (400 MHz, CDCl₃): δ_H 8.56 (dd, ³J_{H-H} = 8.1, ⁴J_{H-H} = 1.8 Hz, 2H, acridone-1H,8H), 7.77 (ddd, ³J_{H-H} = 8.6, 6.8, ⁴J_{H-H} = 1.8 Hz, 2H, acridone-3H,6H), 7.63 (d, ³J_{H-H} = 8.6 Hz, 2H, acridone-4H,5H), 7.30 (m, 2H, acridone-2H,7H), 4.77 (2H, CH₂), 3.62–2.87 (24H, m, CH₂), 1.45–1.32 (27H, m, ^tBu) ppm. ¹³C-NMR (126 MHz, CDCl₃): δ_C 177.93, 169.63, 169.25, 141.65, 134.55, 128.31, 126.03, 122.74, 121.80, 114.42, 82.61, 56.22, 55.99, 51.92, 51.32, 50.83, 28.18, 28.04. ES-MS (MeOH): *m/z* 736.4 [M+H]⁺, 758.5 [M+Na]⁺.

AcridoDO3A: N-{2-[4,7,10-Tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl]ethyl}acridone² (4.12)

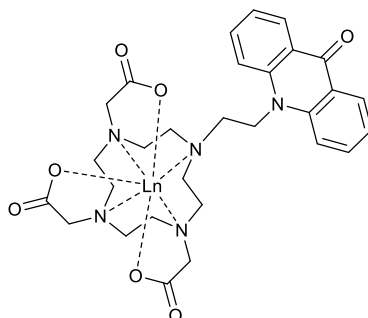


4.11 (0.85 g, 1.15 mmol) was stirred with TFA (8 ml) and DCM (8 ml) and stirred for 48 h. The solvent was removed under reduced pressure and the oil re-dissolved in DCM and the solvent evaporated (x2), and then repeated with MeOH (x2). The product was re-dissolved in a min. amount of MeOH and diethyl ether added to give a precipitate. The precipitate was collected by filtration and the process repeated to give a pale yellow solid in quantitative yield. The ligand was then HPLC purified using prep HPLC (basic, gradient A).

¹H-NMR (400 MHz, D₂O): δ_{H} 8.10 (d, ³J_{H-H} = 7.9 Hz, 2H, acridone-1H,8H), 7.77 (dd, ³J_{H-H} = 8.4, 7.3 Hz, 2H, acridone-3H,6H), 7.57 (d, ³J_{H-H} = 8.4 Hz, 2H, acridone-4H,5H), 7.24 (dd, ³J_{H-H} = 7.9, 7.3 Hz, 2H, acridone-2H,7H), 4.43 (m, br, 2H, CH₂), 3.81 (s, br, 4H), 3.47–3.19 (m, br, 16H), 2.96 (m, br, 4H). ¹³C-NMR (126 MHz, D₂O): δ_{C} 178.96, 170.00, 141.02, 135.39, 126.45, 122.22, 120.79, 115.39, 56.93, 55.07, 51.87, 50.88, 48.82, 48.50, 48.15, 41.83. ES-MS (MeOH): *m/z* 568.23 [M+H]⁺. HRMS (ES⁺), found (*m/z*): 566.26160 [M-H]⁺, C₂₉H₃₆N₅O₇ requires 566.26202. ($|\Delta m/z| = 0.75$ ppm). UV-Vis (H₂O): λ_{max} = 255, 390, 400 nm. Analytical RP-HPLC *t_R* = 4.87 min (column 1, acidic, gradient C).

General procedure for synthesis of Ln.AcridoDO3A Complexes² (Ln.4.13)

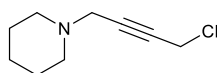
AcridoDO3A, **4.12** (50 mg, 0.4 mmol, 1 eq.), was dissolved in 3 mL MeOH and the appropriate lanthanide acetate salt added (0.4 mmol, 1 eq.). The reaction was stirred at 60 °C for 30 min and the pH adjusted to pH 3 with 1 M NaOH (aq.) and then stirred for a further 48 h at 60 °C, maintaining the solution at pH 3. The solvent was removed under reduced pressure and the solid dissolved in 1 mL water. The complexes were purified using Amberlite[®] XAD16N, eluted with 20 % MeCN. Purified complexes were tested for the absence of free lanthanide with Xylenol Orange. If free lanthanide remains, the complex was stirred with Dowex-50 beads and then re-tested.

**Eu.AcridoDO3A (Eu.4.13)**

Yield: 21 mg, 36%. ¹H NMR (400 MHz, D₂O, pD 10): δ 25.68, 13.72, 13.13, -0.03, -4.83, -7.27, -9.48, -11.53, -12.40, -13.01, -13.30, -13.50, -14.41, -15.42, -17.99 ppm. Only resolved peaks outside the region 0–10 ppm are reported. HRMS (ES⁺), found (*m/z*): 738.15479 [M+Na]⁺, C₂₉H₃₄N₅O₇¹⁵¹Eu²³Na requires 738.15489 (|Δ *m/z*| = 0.14 ppm). UV-Vis (H₂O): λ_{max} = 255, 390, 408 nm. Analytical RP-HPLC t_R = 3.22 min (column 1, basic, gradient C).

Gd.AcridoDO3A (Gd.4.13)

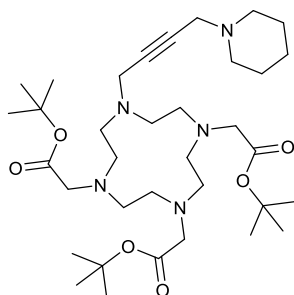
Yield: 45 mg, 62%. HRMS (ES⁺), found (*m/z*): 745.15970 [M+Na]⁺, C₂₉H₃₄N₅O₇¹⁵⁸Gd²³Na requires 745.15915 ($|\Delta m/z| = 0.74$ ppm). UV-Vis: $\lambda_{\text{max}} = 255, 390, 405$ nm.

1-chloro-,4-piperidyl-but-2-yne (Adapted from Zhang *et al.* 2007.⁶) (4.15)

1,4-Dichloro-2-butyne (0.80 ml, 8.2 mmol, 1.03 eq.) was stirred in dry MeCN (25 ml) under N₂ on ice. Piperidine (0.79 ml, 8.0 mmol) diluted in 25 ml MeCN was added dropwise over 30 min. Reaction was monitored by TLC 9:1 DCM/MeOH. Starting material was separated by extraction with 1 M HCl, basify aq. Layer with 2 M NaOH and extract back into DCM. Separate bis-substituted product by silica gel column chromatography (90:10 DCM/MeOH + 1% NH₄OH) (0.13 g, 9%).

¹H-NMR (400 MHz, CDCl₃): δ_{H} 4.18 (s, 2H, C-CH₂-Cl), 3.43 (s, 2H, R₂N-CH₂-C), 2.63 (br, 4H, N-CH₂) 1.71 (m, br, 4H, CH₂) 1.48 (m, br, 2H, CH₂) ppm. ES-MS (MeOH): *m/z* 172 [M+H]⁺.

Piperidine-butyne-^tBuDO3A: 1,4,7-tris(tert-butoxycarbonylmethyl)-10-(4-piperidyl-but-2-yne)-1,4,7,10-tetraazacyclododecane (4.16)

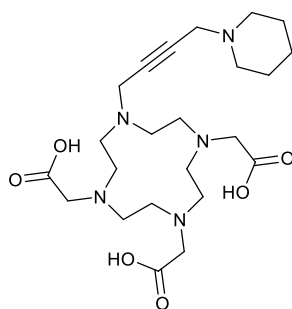


One-step synthesis:

Piperidine (0.30 mL, 3.0 mmol) in dry MeCN (30 mL) was added slowly dropwise to a stirred solution of 1,4-dichloro-2-butyne (0.29 mL, 3.0 mmol) dry MeCN (30 mL) on ice. The reaction was monitored by ESMS, after 2 h ^tBuDO3AH 2.1 (1.23 g, 2.4 mmol) was added with potassium carbonate (0.90g, 6.6 mmol) and the solution stirred for a further 24 h. The solution was filtered and the solvent removed under reduced pressure to yield a dark brown oil. The oil was dissolved in DCM (20 mL), washed with water (3 x 10 mL), dried with MgSO₄, filtered and the solvent removed under reduced pressure. The crude product was purified by column chromatography on alumina in 1:2 EtOAc/Hexane + 0.1 % Et₃N increased to 4:5:1 Hexane/EtOAc/MeOH, after first fraction was eluted. R_f (Al₂O₃, 1:2 EtOAc/Hexane): 0.28.

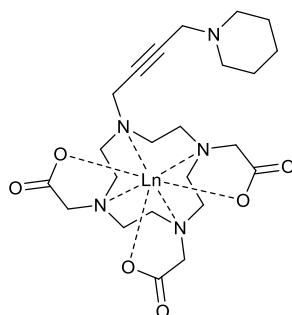
¹H-NMR (400 MHz, CDCl₃): δ_H 3.43 (s, 2H, CH₂-C≡C), 3.37–3.19 (m, 8H, N-CH₂), 2.82–2.69 (m, 16H, cyclen), 2.47 (m, 4H, N-CH₂, piperidine) 1.61 (m, 4H, CH₂ piperidine), 1.45 (27H + 2H, ^tBu + CH₂ piperidine) ppm. ES-MS (MeOH): *m/z* 650 [M+H]⁺, 325 [M+2H]²⁺.

PipDO3A: 1,4,7-tris(carboxymethyl)-10-(4-piperidyl-but-2-yne)-1,4,7,10-tetraazacyclododecane (4.17)



4.16 (0.23 g, 0.35 mmol) was dissolved in DCM (5 ml) and TFA added dropwise (5 ml). The solution was stirred at room temperature overnight. The solvent was removed, the residue redissolved in DCM and removed twice. This process was repeated with 2x MeOH. The oil was dissolved in a min. amount of MeOH and triturated with diethyl ether to yield a fine pale brown powder in quantitative yield. The product was then purified by HPLC using prep HPLC (basic, gradient B) to yield the product as the TFA salt.

$^1\text{H-NMR}$ (400 MHz, D_2O): δ_{H} 3.98–3.93 (m, 6H, $\text{N-CH}_2\text{-CO}_2\text{H}$), 3.80 (s, 2H, $\text{N-CH}_2\text{-C}\equiv\text{C}$), 3.64–3.40 (m, 12H, cyclen), 3.10–2.97 (m, 4H+6H, cyclen + N-CH_2), 1.98 (m, 2H, $\text{CH}_2\text{-piperidine}$), 1.86–1.68 (m, 3H, $\text{CH}_2\text{-piperidine}$), 1.53–1.42 (m, 1H, $\text{CH}_2\text{-piperidine}$) ppm. $^{13}\text{C-NMR}$ (101 MHz, D_2O): δ_{C} 163.03, 162.68, 120.66, 117.76, 114.86, 55.48, 53.31, 52.79, 51.09, 50.47, 48.52, 48.02, 46.00, 42.92, 22.83, 20.84 ppm. ES-MS (MeOH): m/z 482.3 $[\text{M}+\text{H}]^+$, 241.6 $[\text{M}+2\text{H}]^{2+}$. HRMS (ES^+), found (m/z): 482.29742 $[\text{M}+\text{H}]^+$, $\text{C}_{23}\text{H}_{40}\text{N}_5\text{O}_6$ requires 482.29731. ($|\Delta m/z| = 0.24$ ppm). Analytical RP-HPLC $t_{\text{R}} = 1.81$ min (column 1, neutral, gradient C).

General procedure for Ln.PipDO3A complexes (Ln.4.18)

PipDO3A, **4.17** (50 mg, 0.40 mmol, 1 eq.), was dissolved in 3 mL MeOH and the appropriate lanthanide triflate salt added (0.40 mmol, 1 eq.). The reaction was stirred at 60 °C for 30 min and the pH adjusted to pH 4 with 1 M NaOH (aq.) and then stirred for a further 48 h at 60 °C, maintaining the solution at pH 4. The solvent was removed under reduced pressure and the solid dissolved in 1 mL water. The complexes were purified using Amberlite[®] XAD16N, eluted with 20 % MeCN. Purified complexes were tested for the absence of free lanthanide with Xylenol Orange. If free lanthanide remains, the complex was stirred with Dowex-50 beads and then re-tested.

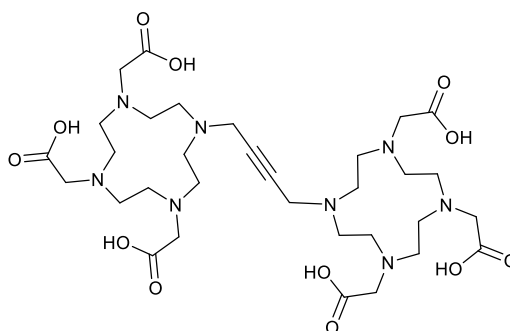
Y.PipDO3A (Y.4.18)

HRMS (ES⁺), found (*m/z*): 568.17957 [M+H]⁺; C₂₃H₃₇O₆N₅⁸⁹Y requires 568.17968. (|Δ *m/z*| = 0.21 ppm).

Gd.PipDO3A (Gd.4.18)

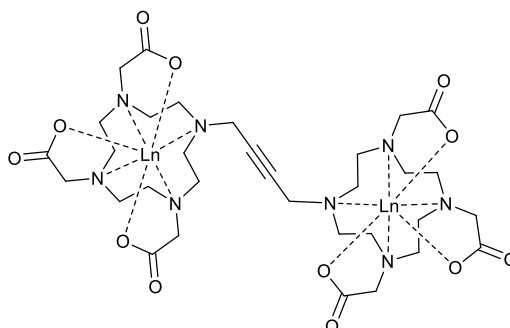
Yield: 20 mg, 16%. IR-ATR ν = 2160 (C≡C), 1584 (C=O). ES-MS (MeOH/H₂O): *m/z* 637 [M+H]⁺.

p2DO3A: 1,4-bis-((1,4,7,10-tetraazacyclododecane-1,4,7-triyl)(triacetic acid))-but-2-yne.⁷ (4.19)



¹H-NMR (400 MHz, D₂O): δ_{H} 3.91–3.78 (br, 12 H, N-CH₂-CO₂H), 3.67 (br, 4 H, N-CH₂-C $\equiv$ C), 3.36–3.22 (m, 32 H, cyclen) ppm.

¹³C-NMR (101 MHz, D₂O): δ_{C} 171.56, 162.79, 116.44, 55.40, 54.38, 53.04, 51.77, 49.84, 49.01, 43.68, 42.18, 27.21 ppm (TFA underlined). ES-MS (MeOH): m/z 743 [M+H]⁺, 372 [M+2H]²⁺ Analytical RP-HPLC t_{R} = 0.97 min (column 1, basic, gradient C).

General procedure for LnLn.p2DO3A complexes⁸ (Ln.4.20)

p2DO3A, **4.19** (50 mg, 0.07 mmol, 1 eq.) was dissolved in MeCN (3 ml) and Ln triflate (81 mg, 0.14 mmol, 2 eq.) was added. The pH was adjusted to pH 3 with 1 M NaOH and the solution refluxed for 96 h. A pale brown precipitate was formed. The precipitate was collected by centrifugation and washed twice with MeCN. The product was desalted on an Amberlite[®] column, eluted with 20% MeCN and tested for absence of free Ln³⁺ with Xylenol Orange.

GdGd.p2DO3A (Gd.4.20)

MALDI-MS (α -MeOH): m/z 1073 [M+Na]⁺, 1089 [M+K]⁺

EuEu.p2DO3A (Eu.4.20)

¹H NMR (400 MHz, D₂O, pD 10) δ 23.54, 16.88, 14.18, 11.73, -3.41, -5.48, -6.23, -8.66, -9.04, -10.70, -10.97, -11.97, -12.29, -14.04, -15.38, -17.21, -19.78 ppm. Only resolved peaks outside the region 0–10 ppm are reported.

7.3. Cell Culture Methods

Cells were grown in the tissue culture facility in the Chemistry Research Laboratory, University of Oxford. Cell culture was performed in a laminar flow hood using aseptic techniques. NIH-3T3 cells were acquired from Dr. Jianzhou Chen (Department of Oncology, University of Oxford), HeLa cells were acquired from Dr. Illaria Pettinati (Chemistry Research Laboratory, University of Oxford). The growth medium used for both cells lines was high glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich) supplemented with 2 mM L-Glutamine (Sigma Aldrich) and 10% fetal bovine serum (FBS, Sigma-Aldrich, non-USA origin). Cells were incubated at 37 °C and 5% (v/v) CO₂ (humidity check) and grown in tissue-culture treated flasks (T-Flasks) for adherent cell lines.

7.3.1. Subculture

The media in the flasks was discarded and the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS, Sigma Aldrich). 3–5 mL 0.25% Trypsin-EDTA (Sigma Aldrich) was added to the flasks and incubated at 37 °C for 5 minutes until the cells had detached. Trypsin was neutralised by the addition of 5 mL warm growth medium (containing 10% FBS) and suspensions were pelleted by centrifugation at (1200 x g) for 3–5 minutes. The supernatant was removed and the cells re-suspended in growth medium by trituration with a pipette. Cell suspensions were typically split in a ratio of 1:10 or 1:8 into fresh T-flasks, unless plated for subsequent experiments.

7.3.2. Trypan blue exclusion assay

Cell numbers were determined using an Invitrogen Countess™ II FL Automated Cell Counter. Cells were suspended in PBS and a sample mixed in a 1:1 ratio with Trypan Blue solution (0.4%, Invitrogen). 10 µl was inserted into Countess™ cell counting chamber slides (Invitrogen), yielding cell count and percentage viability. An average of two readings was used to determine cell count and viability for each cell suspension.

7.3.3. Freezing and thawing of cells

Cells were grown to a high density in a T-175 flask and harvested using trypsinisation (7.3.1). Freezing medium was made containing 10% DMSO, 20% FBS in DMEM and sterile filtered. The cells were re-suspended in 4 mL freezing medium and 1 mL transferred to 4 cryovials. The cryovials were placed in a CoolCell® freezing container (Corning®) at -80 °C for slow cooling and then after 24 h transferred into falcon tubes and stored at -80 °C.

Cells were thawed rapidly in a 37 °C water bath. Pre-warmed growth medium (10 mL) was added slowly to dilute the cells. Cells were centrifuged at (200 x g) for 5 minutes to remove DMSO and the supernatant discarded. The cells were re-suspended in growth medium and plated at a high density in a T-75 flask.

7.3.4. MTT viability assay

NIH-3T3 cells were plated in triplicate in 96-well plates at approximately 1×10^4 cells per well in growth medium and incubated overnight at 37 °C. 20 mM stock solutions of the test compounds were made in DI water (DPBS for Gd.AcridoDO3A due to solubility issues) and diluted to the appropriate concentration (2, 1, 0.5, 0.1, 0.05 mM)

in growth medium. The growth medium was removed from the cells and fresh growth medium (100 μ L) of each concentration was added to the cells in triplicate, in addition to control cells with no contrast agent and blank wells with no cells. The cells were incubated for 20 h with the compound, before MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reagent (5 mg/mL in PBS, sterile filtered, 10 μ L) was added to each well and the cells incubated for a further 3 h. The media was carefully removed from each well and 100 μ L DMSO added to dissolve the purple formazan crystals, with trituration using a pipette. Absorbance was recorded at 570 nm using a PHERAstar[®] FSX (BMG Labtech) plate reader and the absorbance of the blank wells subtracted. The mean absorbance at each concentration was calculated from the triplicate wells and the viability calculated relative to the control cells. Each compound was repeated three times with separate flasks of cells and the mean viability and standard error of the mean calculated from the three separate flasks.

7.4. Relaxometry

7.4.1. ATP, PCr and P_i sample preparation for ³¹P-MRS

Solutions of ATP disodium salt hydrate (Sigma-Aldrich) and PCr disodium salt hydrate (Sigma-Aldrich) in Dulbecco's Phosphate Buffered Saline (DPBS), Sigma-Aldrich were prepared and adjusted to either pH 7.1–7.2, or pH 7.3–7.4 with 1 M NaOH as measured by pH meter. Aliquots of the solution were frozen until use. The concentration of phosphate in solutions was checked using ³¹P-NMR spectroscopy due to the use of hydrates with unknown water content. Quantitative ³¹P-NMR spectra were acquired with no decoupling, delay time (TR) = 60s ($>5 \cdot T_1$), 64 averages. All solutions contained 10 mM PBS which was used to normalise the integrals of all other phosphate peaks and determine relative concentration of each species.

GdDTPA (Magnevist[®], Bayer), GdDTPA-BMA (Omniscan[®], GE Healthcare) and GdDOTA (Dotarem[®], Guerbet) clinical formulations of 0.5 M were used as received and diluted with distilled water to the specified concentration. The contrast agent dilutions were added to the ATP, PCr and DPBS aliquots to give final concentrations of 7.9 mM ATP, 5.5 mM PCr and 8.7 mM DPBS and the appropriate contrast agent concentration (0–4 mM).

The FBS solution was prepared by dissolving 1 x DPBS tablet in 100 mL FBS to a concentration of 10 mM. Contrast agent solutions were added to 100% FBS + 10 mM PBS samples, resulting in a final FBS concentration of 90% (v/v).

7.4.2. Relaxivity measurements

Commercial contrast agent measurements were performed on a horizontal 9.4 T MR system equipped with a VnmrS Direct Drive console (Agilent Technologies, Santa Clara, US) and using a home-built ^{31}P solenoid (inner diameter 5 mm, tuned to 161.7 MHz). ^1H T_1 times were also measured using the same solenoid coil (without retuning) at 400 MHz. 110 μl samples were contained in 0.2 ml PCR tubes and measured at room temperature (thermostatically regulated around 20 °C). T_1 and T_2 data were analysed using the scanner software (VnmrJ4.2, Agilent Technologies)

Novel contrast agent measurements were performed using a Bruker Avance III HD nanobay NMR equipped with a 9.4 T magnet at 25°C. For ^{31}P T_1 measurements, 495 μl samples were contained in standard 5 mm NMR tubes and ran unlocked. For ^1H T_1 measurements, 50 μl samples were contained in a co-axial insert (Wilmad[®]) NMR tube and the outer tube filled with CDCl_3 for locking. T_1 data was analysed using Bruker TopSpin3.5 NMR software.

^{31}P T_1 times were measured by inversion recovery ($TR > 5* T_1$, 8–32 averages, 8 kHz bandwidth). ^1H T_1 times were also measured by inversion recovery ($TR > 5* T_1$, 4 kHz bandwidth, 4–16 averages), while ^1H T_2 times were measured by CPMG echo ($TR > 5* T_1$, $TE = 1\text{--}20$ ms, 4–16 averages, 4 kHz bandwidth).

7.4.3. ^{31}P -MRS phosphate stability measurements.

The stability of the phosphate solution was measured overnight by a series of twenty fully relaxed pulse-acquire spectra ($TR = 60\text{s}$, 90° flip angle, $7.5\ \mu\text{s}$ hard pulse, 60 averages, 512 complex points, 8 kHz bandwidth). Pulse-acquire spectra ($TR = 20\ \text{s}$, 90° flip angle, $6.5\ \mu\text{s}$ hard pulse, 16 averages, 8 kHz bandwidth, 512 complex points)

of the phosphate solutions with added contrast agent were acquired immediately before the IR experiments. To measure sample stability over the course of the IR measurements, the peak integrals from the pulse-acquire spectra were normalised to that of P_i and compared to the normalised integrals from the final spectrum on the IR curve.

7.5. Fluorescence Spectrometry

Emission and excitation spectra and emission lifetimes were all recorded using a HORIBA Jobin Yvon FluoroLog3 fluorimeter equipped with a double-grating emission monochromator. Lifetime decay curves were fitted using Origin® software.

7.5.1. Titration methodology

Concentrated stock solutions of the lanthanide complexes in deionised water were made prior to the titration experiments, and a separate concentrated stock solution of the anion (at pH 7.4). For the experiment, the same volume of the stock solution was used for both the initial solution and the titrant solution. Concentrated HEPES buffer was added to both solutions. To the titrant solution an aliquot of the anion solution was added to yield a concentration at least 100 times that of the complex. Both solutions were diluted to the final concentration with deionised water, to give equivalent concentrations of the complex and prevent dilution during the titration. The pH of the final solutions was checked to ensure it was at pH 7.4 (± 0.2). The initial solution (2 mL) was added to a quartz fluorescence cuvette and small increments of the titrant added with emission spectra recorded at each point.

7.5.2. Dynafit[®] scripting

The integrals of the peak intensity were calculated for $\Delta J = 1$ (~560–600 nm), $\Delta J = 2$ (~600–640 nm) and $\Delta J = 4$ (~670–720 nm), as a function of increasing anion concentration. These values were globally fitted using Dynafit[®] software⁹ and associated binding constants determined. Model discrimination methodology was employed to determine the best model for anion binding.

Example Dynafit[®] Script for model discrimination:

```
[task]

task = fit

data = equilibria

model = 1:1?

algorithm = LM

[mechanism]

Eu + PBS <==> complex1 : K1 assoc

[constants]

K1 = 100 ??

[concentrations]

Eu = 1E-5

[responses]

[data]

variable PBS

file ./605-630.txt | response Eu = 4.7E12 , complex1 =
1.4E13 ?
```

```
file ./680-710.txt | response Eu = 1.4E12 , complex1 =  
4.5E12 ?
```

```
file ./580-605.txt | response Eu = 2.6E12 , complex1 =  
8.2E12 ?
```

```
[output]
```

```
directory ./output/
```

```
[task]
```

```
task = fit
```

```
data = equilibria
```

```
model = 1:2?
```

```
[mechanism]
```

```
Eu + PBS <==> complex1 : K1 assoc
```

```
complex1 + PBS <==> complex2 : K2 assoc
```

```
[constants]
```

```
K1 = 100 ??
```

```
K2 = 1 ??
```

```
[data]
```

```
variable PBS
```

```
file ./605-630.txt | response Eu = 4.7E12 , complex1 =  
1.4E13 ?, complex2 = 2E13?
```

```
file ./680-710.txt | response Eu = 1.4E12 , complex1 =  
4.5E12 ?, complex2 = 5E12?
```

```
file ./580-605.txt | response Eu = 2.6E12 , complex1 =  
8.2E12 ?, complex2 = 9E12?  
[end]
```

7.6. Experiments at the Rutherford Appleton Laboratory, Harwell

Campus

7.6.1. Cell preparation for microscopy

Cell culture and incubation for microscopy was conducted by Dr. Haobo Ge (University of Bath). PC3 cells (1.5×10^5) were plated in 35 mm dishes (20 mm microwell) and incubated at 37 °C for over 48 h prior to the experiments. Cells were washed three times with PBS and 0.99 ml of serum-free RPMI media was added along with 10 uL of complex to a final concentration of 200 μ M and incubated for 20 minutes at 37 °C. Post-incubation cells were washed three times with PBS and fresh serum-free RPMI added.

For co-localisation studies: cells were initially incubated with the novel probe and then one of the commercially available tracker probes: LysoTracker[®] Red at 100 nM for 30 minutes, MitoTracker[®] Red at 200 nM for 30 minutes, ER-Tracker[®] Red at 1 μ M for 30 minutes.

For cell fixation: serum-free RPMI was removed and 1 mL 4% paraformaldehyde was added. The plate was left for 10 minutes at room temperature before the fixing agent was removed, the plate was washed three times with PBS and then inverted and left to dry at room temperature.

7.6.2. Microscopy

The experiments were performed at the Central Laser Facility, STFC Rutherford Appleton Laboratory, Harwell Campus, Didcot. Fluorescence confocal microscopy images were recorded using a Nikon TE2000-U microscope. The microscope was set up for two photon excitation FLIM using a 690-1000nm mode locked Mira titanium sapphire laser (Coherent Lasers Ltd) producing 180 fs pulses at 75 MHz, tuned to 810 nm excitation wavelength and focussed to a diffraction limited spot through a water immersion UV corrected objective. The system was equipped with an external fast microchannel plate photomultiplier tube detector (R3809-U, Hamamatsu, Japan). A Time-Correlated Single Photon Counting (TCSPC) PC module SPC830 was used for lifetime measurements.¹⁰

PLIM measurements were recorded on a confocal Nikon microscope connected to a Becker and Hickl DCS-120 confocal scanning FLIM system and a 405 nm diode laser (BDL-405-SMC). The diode laser was on/off controlled for PLIM imaging by the Becker and Hickl FLIM module.¹¹

7.7. Live Cell ^{31}P -NMR spectroscopy

7.7.1. Cell preparation for ^{31}P -NMR spectroscopy

NIH-3T3 cells were grown in T-175 flasks to a high cell density; two flasks were used per experiment yielding approximately 5×10^7 cells. The medium was removed from the cells, the cells washed with DPBS and 5 mL serum-free DMEM medium was added containing 0.5 mM Gd.pDO3A (Gd.2.4), or the same volume of water for the control, and incubated at 37°C for 30 minutes. The cells were harvested using trypsinisation (7.3.1) and the cells re-suspended in 390 μL ice-cold serum-free DMEM media containing 0.5 mM Gd.pDO3A (water in the control) and 5% D_2O . 5 μL of the cell suspension was set aside for trypan blue viability testing after the experiment. The cells were transferred into a Shigemi tube (Bruker, D_2O) and kept on ice until the experiment began (up to 15 minutes). Time between the incubation endpoint and the first scan was approximately 30 minutes for each cell sample.

7.7.2. Live cell ^{31}P -NMR acquisition

^{31}P -NMR spectra of live cells— $\sim 5 \times 10^7$ cells in 0.385 ml in Shigemi (Bruker, D_2O) tubes — were acquired at 202 MHz, 298 K and referenced externally to 80% H_3PO_4 . 1000 transients were accumulated per FID with 8 kHz bandwidth, 8192 complex points, $TR = 0.5$ s, 30° flip angle and the spectrum centred at -5 ppm. Total scan time: 17 minutes. Consecutive scans were acquired on the same sample to monitor changes in phosphate speciation. Experiments were repeated with separate flasks of cells and the sum of the two spectra was calculated in TopSpinTM (Bruker) and normalised to the α -ATP peak at -10 ppm.

7.8. Diffusion NMR Experiments

7.8.1. Cell preparation for diffusion NMR experiments

HeLa cells were grown in T-175 flasks, 1–2 flasks per experiment were used yielding $1\text{--}4 \times 10^7$ cells. 50.5 mM stock solutions of the contrast agent in water were made and added to serum free DMEM media to yield 0.5 mM concentrations. The media was removed from the flasks, the cells washed with DPBS and serum-free DMEM media added (5 mL) containing 0.5 mM contrast agent or no contrast for the control. The cells were then incubated for 20 minutes before the cells were harvested using trypsinisation (7.3.1). The cell pellet was re-suspended in DPBS and a sample taken for cell counting with Trypan Blue. The cell suspension was centrifuged again and DPBS removed. The cells were then kept on ice until the experiment (up to 30 min). The NMR solution was made by supplementing DPBS with 90 mM glucose, 0.2% low melting point agarose and 5% D₂O. 0.5 mM contrast agent was added to the relevant solutions and water added to the control. The cells were re-suspended in 0.22 ml NMR solution and triturated with a pipette to gain a single cell suspension. The suspension was transferred to a Shigemi (Wilma[®], Agilent, D₂O) tube and placed in the fridge for a few minutes to ensure complete gelling of the agarose.

7.8.2. ¹H-NMR acquisition and data analysis

All diffusion NMR data acquisition and analysis was carried out by Mr. Gogulan Karunanithy. Diffusion ¹H-NMR was carried out at 298 K and 14.1 T (600 MHz) on a narrow bore solution-state NMR spectrometer equipped with a room temperature probe. A stimulated echo pulse sequence is applied with rectangular pulsed field

gradients along the z-axis for a time period of 2 ms. Varying gradient strengths are employed (11 values at 4–60 Gcm⁻¹). Each experiment is repeated with a series of diffusion times (typically 0.05 s, 0.1 s, 0.2 s, 0.3 s 0.4 s and 0.5 s), in order to extract R_1 values from the diffusion analysis. All 1D spectra are recorded with 8 transients, an acquisition time of 2 s and recycle delay of 1.5 s. The spectra are Fourier transformed and phased using NMRPipe.¹²

1D spectra were analysed using in-house python scripts using NmrGlue, NumPy, SciPy and LmFit python modules. The water intensity was integrated in each spectra to give a series of intensities as a function of applied diffusion time and the gradient, which are then globally fitted. Parameter errors are calculated by taking the standard deviation of each parameter from 1000 bootstrap runs. Synthetic datasets are generated by randomly picking intensity sets with particular diffusion times with replacement until the synthetic dataset has the same number of points as the original dataset. Extensive Monte Carlo simulations determined that the intracellular R_1 is underestimated by this model (for values < 0.5 s⁻¹), which leads to an unrealistic value of R_1 . At greater values of R_1 the model is capable of fitting a more realistic value. This signifies this is an error in the given fits of the particular model, rather than an error in the model itself.

7.9. References

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Appendix

A.1. Phosphate Solution Stability

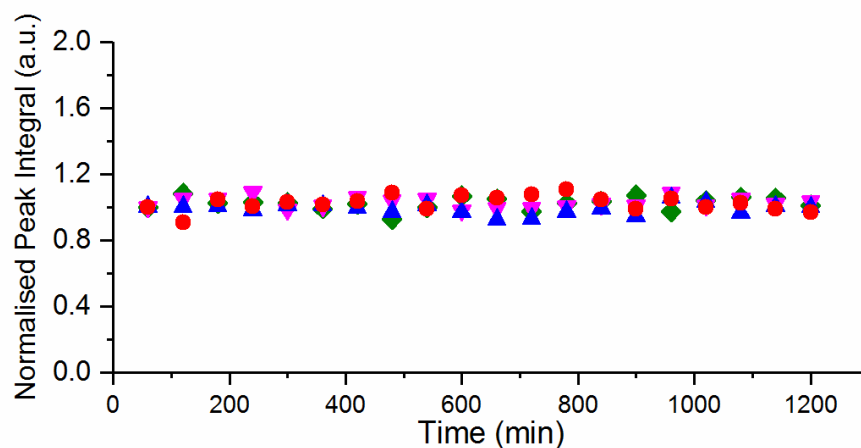


Figure A.1: Relative change in the integrals of α - (▼), β - (◆) γ -ATP (▲) and PCr (●) of phosphate solution containing P_i , PCr and ATP (8.7, 5.5 and 7.9 mM) over a 20 h period with no Gd contrast.

A.2. Values for Conditional Stability Constant Calculations

To calculate conditional thermodynamic equilibrium constants of commercial contrast agents with ATP at pH 7.2 (section 3.5) the following literature values in Table A.1 and A.2 were used.

Table A.1: Formation constants and ligand pK_a values.

Reaction	Log K_a
Gd.DOTA Formation	24.7 ^b
DOTA pK_a 1	12.6 ^b
DOTA pK_a 2	9.7 ^b
GdDTPA Formation	22.5 ^c
Gd.DTPA pK_a 1	10.2 ^c
DTPA pK_a 2	8.6 ^c
Gd.DTPA-BMA Formation	16.9 ^c
DTPA-BMA pK_a 1	9.4 ^c

Values from ^aSherry et al.¹, ^bCacheris et al.² and ^cCacheris et al.³ all values reported in 0.1 M NaCl, comparable to 0.12 M NaCl in this study.

Table A.2: Formation constants and pK_a values for ATP with Gd.

Reaction	Log K_a
Gd.ATP	6.34
ATP pK_a 1	6.24
Gd(ATP) ₂	3.64

^aValues from Bianchi et al.⁴ measured with 0.15 M NaCl, comparable to 0.12 M NaCl in this study.

The conditional stability constant of GdATP at pH 7.2 was calculated to be $\text{Log}K_{\text{cond}} = 6.30$ using equation 3.2 and 3.3. This value was used for subsequent calculations.

A.3. ^1H -NMR of Y.PipDO3A

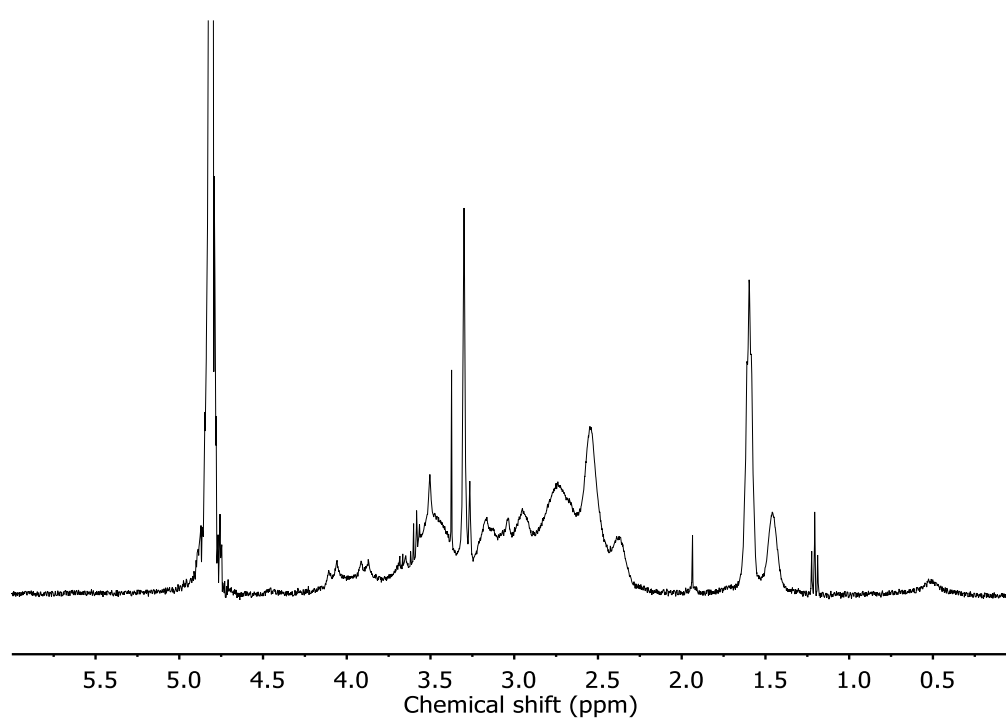


Figure A.2: ^1H -NMR of Y.PipDO3A (Y.2.18) in D_2O at pH 10

A.4. HPLC Data

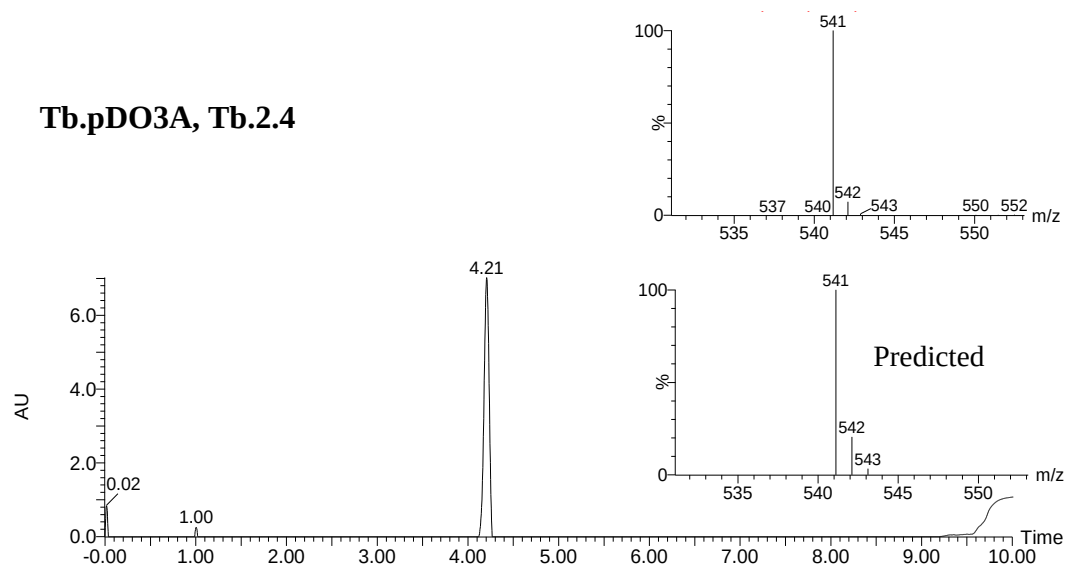


Figure A.3: HPLC-UV trace of Tb.pDO3A (Tb.2.4) with corresponding integrated mass spectrum at 4.21 minutes, versus predicted mass spectrum of compound. Trace obtained using a Sunfire[®] column (4.6 mm x 150 mm: flow rate 1 ml/min) with an eluent system comprising of H₂O/MeCN at a gradient of 98:2 v/v to 50:50 v/v.

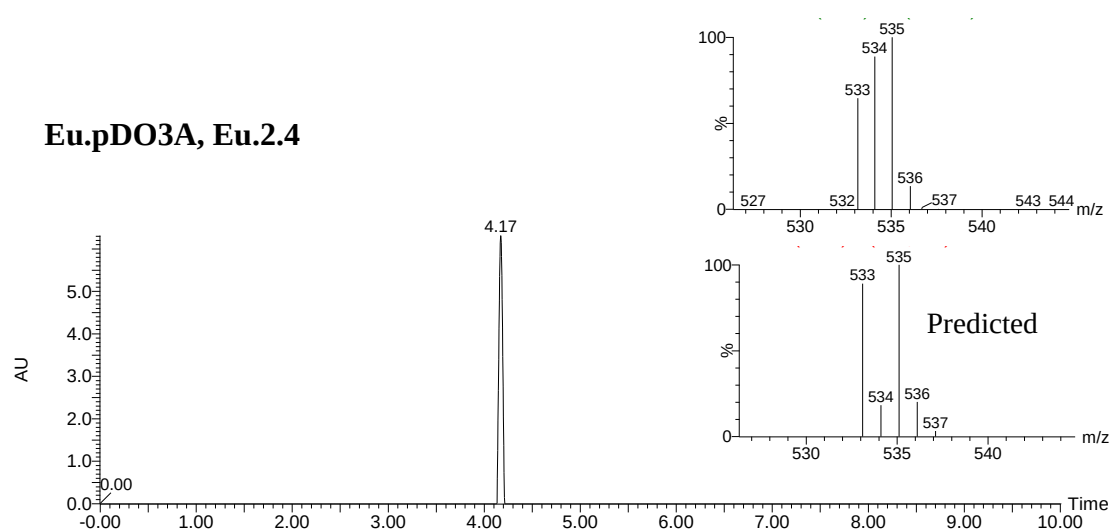


Figure A.4: HPLC-UV trace of Eu.pDO3A (Eu.2.4) with corresponding integrated mass spectrum at 4.17 minutes, versus predicted mass spectrum of compound. Trace obtained using a Sunfire[®] column (4.6 mm x 150 mm: flow rate 1 ml/min) with an eluent system comprising of H₂O/MeCN at a gradient of 98:2 v/v to 50:50 v/v.

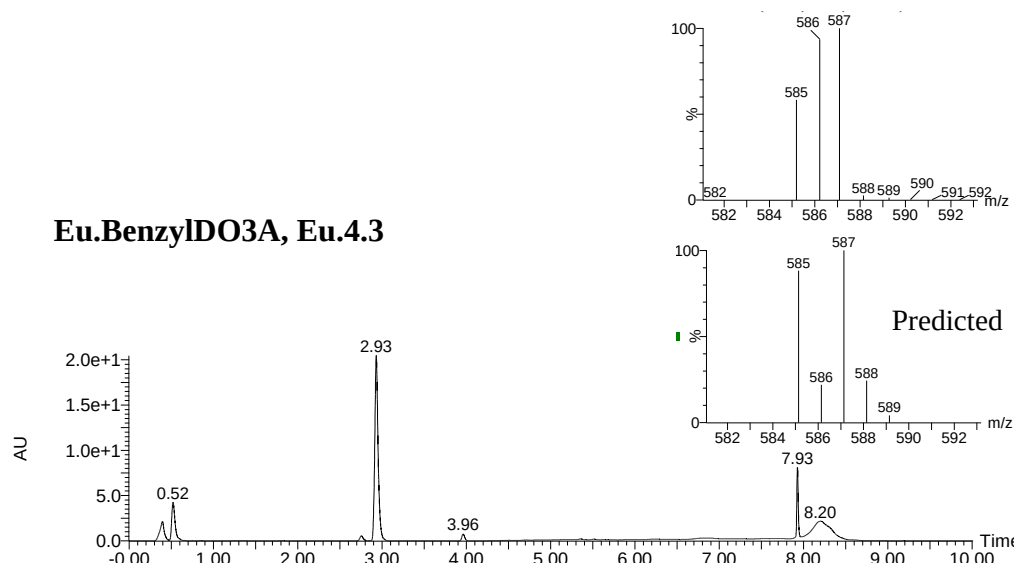


Figure A.5: HPLC-UV trace of Eu.BenzylDO3A (Eu.4.3) with corresponding integrated mass spectrum at 2.93 minutes, versus predicted mass spectrum of compound. Trace obtained using an X-bridge[®] column (4.6 mm x 50 mm: flow rate 1.3 ml/min) with an eluent system comprising of H₂O/MeCN at a gradient of 95:5 v/v to 5:95 v/v.

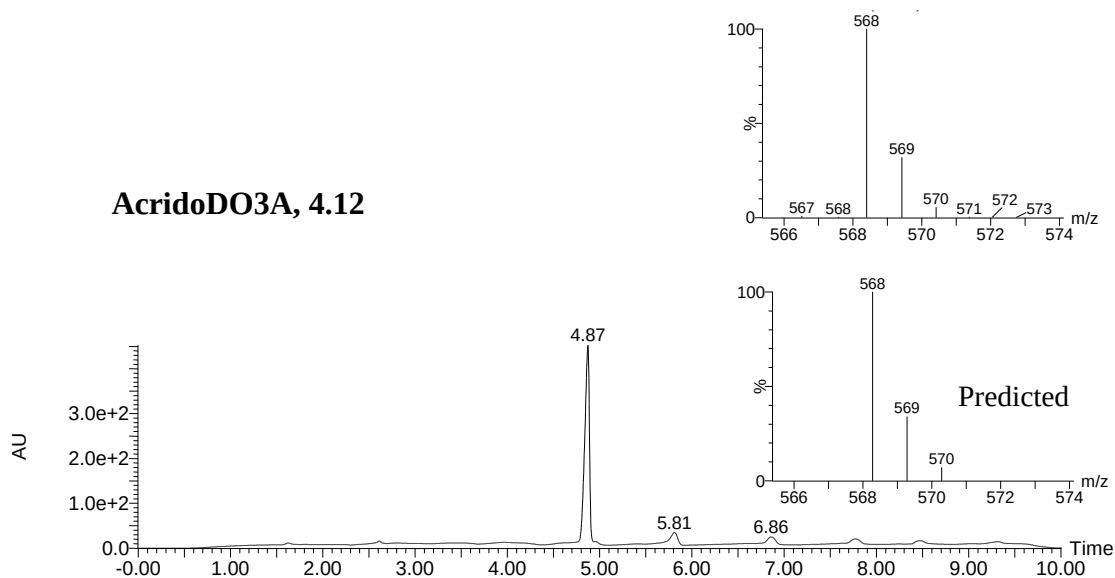


Figure A.6: HPLC-UV trace of AcridoDO3A (4.13) with corresponding integrated mass spectrum at 4.87 minutes, versus predicted mass spectrum of compound. Trace obtained using a Sunfire[®] column (4.6 mm x 150 mm: flow rate 1 ml/min) with an eluent system comprising of H₂O+0.1% TFA/MeCN+0.1% TFA at a gradient of 95:5 v/v to 60:40 v/v.

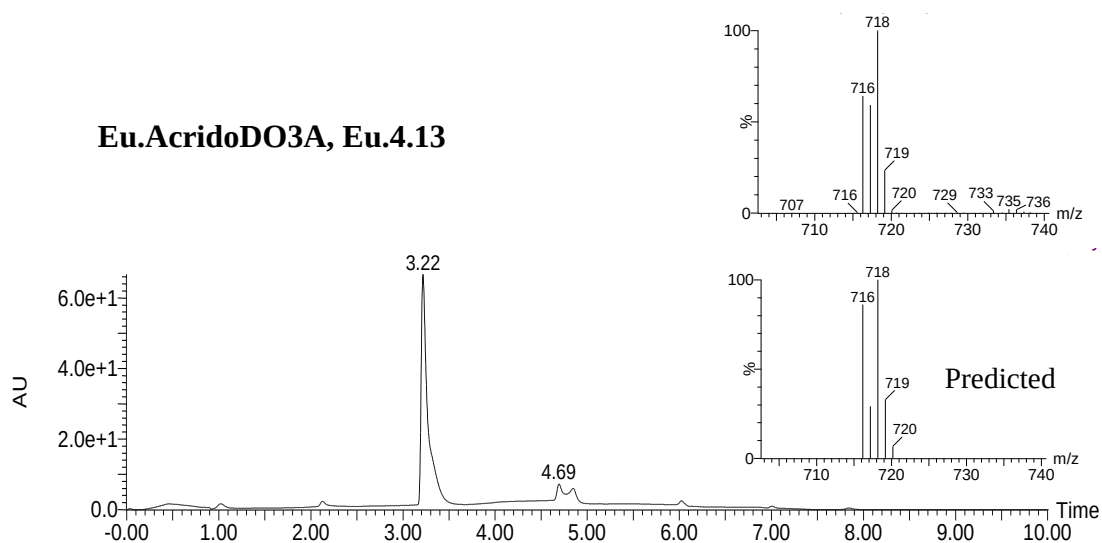


Figure A.7: HPLC-UV trace of Eu.AcridoDO3A (Eu.4.13) with corresponding integrated mass spectrum at 3.22 minutes, versus predicted mass spectrum of compound. Trace obtained using a Sunfire[®] column (4.6 mm x 150 mm; flow rate 1 ml/min) with an eluent system comprising of $H_2O + 0.05\% NH_4OH / MeCN + 0.05\% NH_4OH$ at a gradient of 95:5 v/v to 60:40 v/v.

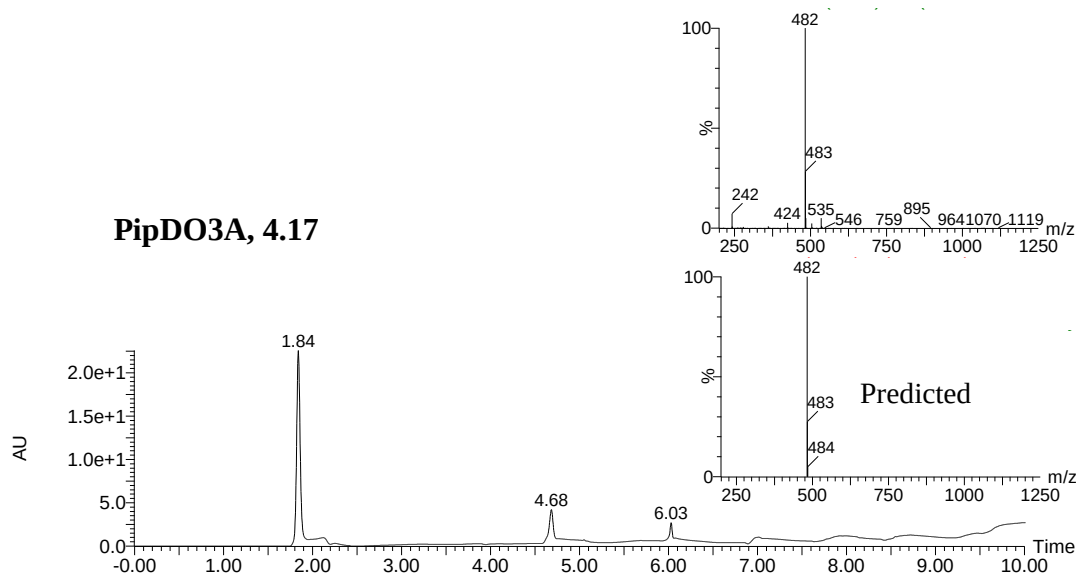


Figure A.8: HPLC-UV trace of PipDO3A (4.17) with corresponding mass spectrum at 1.87 minutes, versus predicted mass spectrum of compound. Trace obtained using a Sunfire[®] column (4.6 mm x 150 mm; flow rate 1 ml/min) with an eluent system comprising of $H_2O / MeCN$ at a gradient of 95:5 v/v to 60:40 v/v.

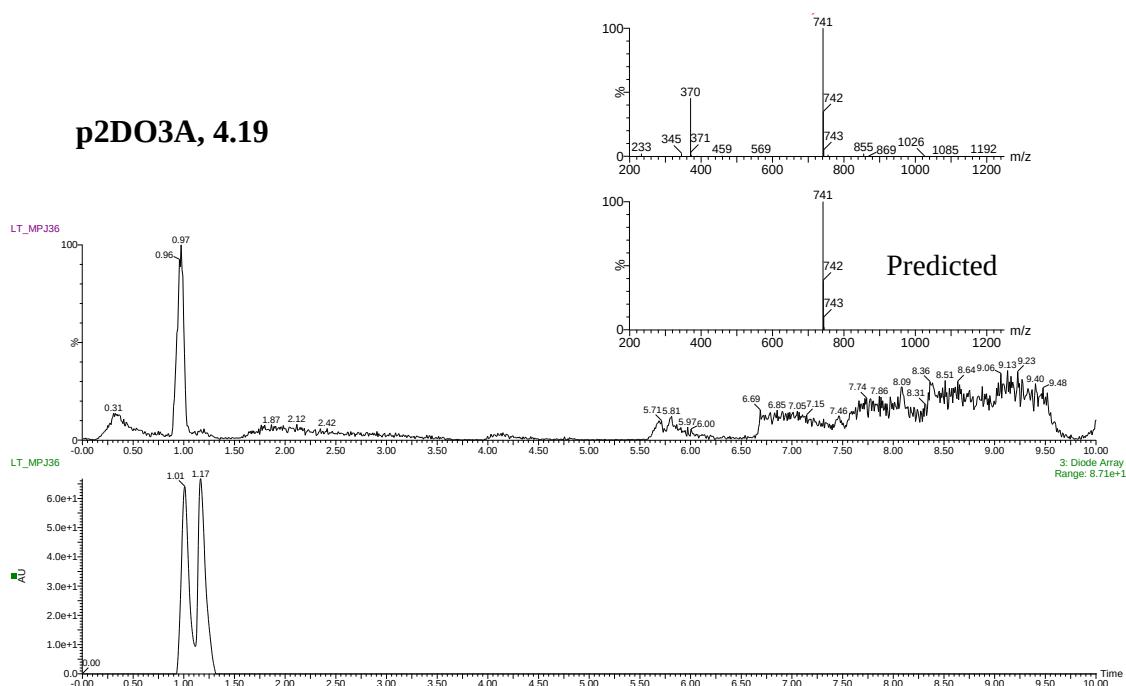


Figure A.9: HPLC-UV and ES⁻ trace of p2DO3A (4.19) with corresponding mass spectrum at 0.97 minutes, versus predicted mass spectrum of compound. Both peaks in UV trace correspond to the same mass (ES⁺ and ES⁻). Trace obtained using a Sunfire[®] column (4.6 mm x 150 mm; flow rate 1 ml/min) with an eluent system comprising of H₂O+0.05% NH₄OH/MeCN+0.05% NH₄OH at a gradient of 95:5 v/v to 60:40 v/v.

A.5. Live Cell ^{31}P -NMR Spectroscopy

Extracellular medium of 3T3 cells was tested after ^{31}P -NMR experiments. The cell suspension was centrifuged to remove the cells and the supernatant put into an NMR tube and analysed by ^{31}P -NMR. Samples were referenced externally to 80% H_3PO_4 , but not normalised. Cell numbers and timescale of the experiments were comparable, so the concentration of P_i in each sample should be similar. Therefore the increase in signal observed in the spectrum is a result of Gd.pDO3A shortening the T_1 times of P_i .

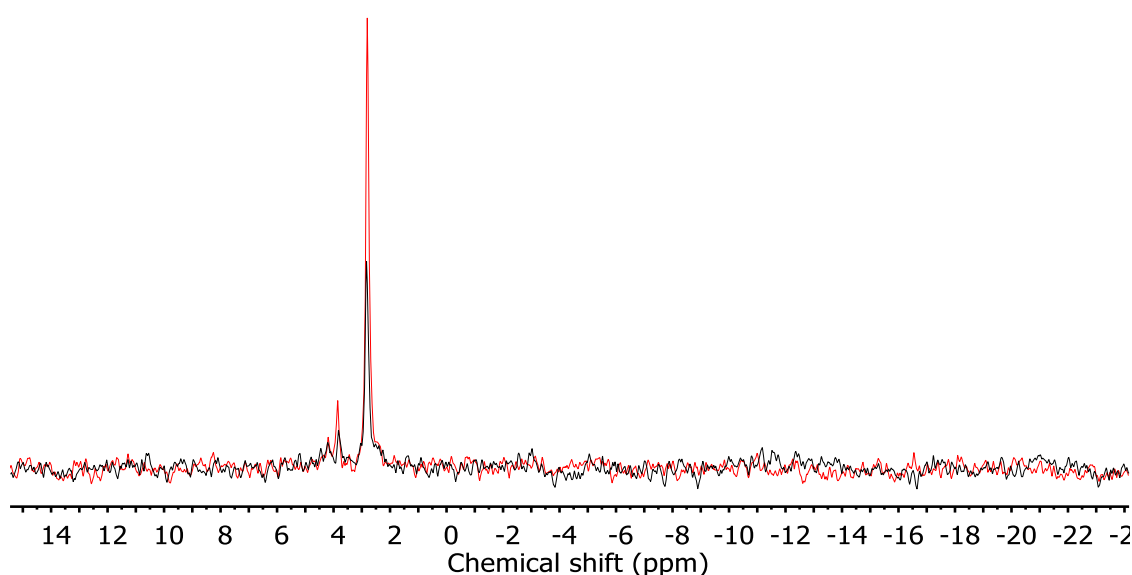


Figure A.10: ^{31}P -NMR spectra of extracellular DMEM medium after ^{31}P -NMR experiments on 3T3 cell; spectra are with Gd.pDO3A (red) and without (black). Spectra are not normalised, but cell numbers were comparable ($\sim 1 \times 10^8$ cells). Externally referenced to H_3PO_4 .

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