An esterase-activated magnetic resonance contrast agent†

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A Gd(III) complex bearing pendant acetoxymethyl esters is activated on exposure to porcine liver esterase; the 84% increase in relaxivity is a result of suppression of HCO_3^-/CO_3^{2-} binding by the resulting negative charge.

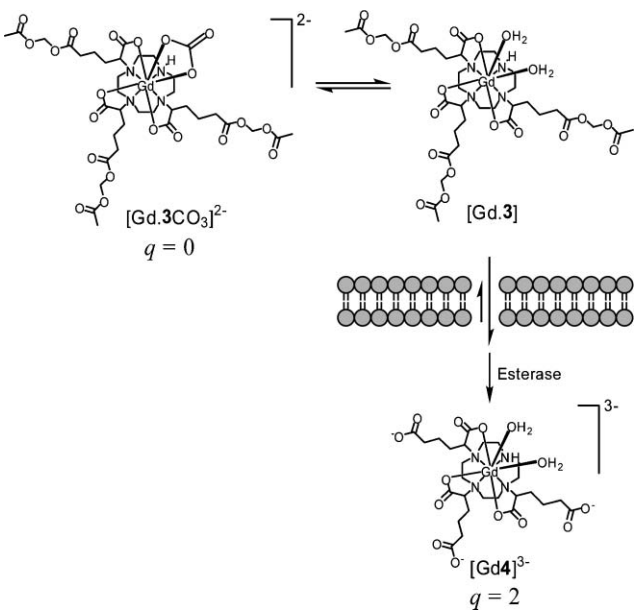
In recent years the focus of research into Gd(III)-based contrast agents has shifted to the development of 'smarter', responsive or activated contrast agents,[1] *i.e.* complexes whose relaxivities are modulated by a particular *in vivo* stimulus such as pH,[2] metal ion concentration[3] or enzyme-activity.[4] In order to image at the molecular level, smarter contrast agents are required that display significant changes in relaxivity, *i.e.* signal intensity on activation.

This communication describes an activation and accumulation approach to attempt to address the inherent insensitivity of the magnetic resonance imaging technique. Cyclen-based complexes of Gd(III) that are 7-coordinate with respect to ligand tend to possess two inner-sphere water molecules, *i.e.* their hydration state $q = 2$. It is well-established that such complexes generally make poor contrast agents; the expected high relaxivity engendered by two inner-sphere waters is not manifested *in vivo*. This is due to the affinity of this type of often cationic or neutral complex for endogenous serum anions such as hydrogencarbonate, phosphate, lactate and citrate, as well as carboxylate residues on proteins.[5] To some extent, such anions displace the inner-sphere waters, rendering the complex a poor contrast agent. Of these coordinating anions, HCO_3^- (bound as carbonate, CO_3^{2-}) is the most abundant in serum (20–30 mM), possesses a relatively high affinity for this type of $q = 2$ complex and is the focus of this communication.

It has been demonstrated that this affinity for HCO_3^- can be suppressed by introduction of negative charge into complexes of this type.[6] The work reported herein takes advantage of this difference in affinity for HCO_3^- between neutral and negatively charged complexes, by using enzyme-activation to switch from a neutral contrast agent with an affinity for carbonate-binding to a negatively charged one where the suppression of carbonate-binding is exploited. This enzyme-activation is manifested as a concomitant change in hydration state and hence relaxivity.

The rationale behind the design of these complexes is that the neutral species (in equilibrium with the carbonate-bound species) will be capable of crossing the cell membrane, *e.g. via* pinocytosis. Once internalised, the neutral complex is designed to be converted into the negatively charged species, *i.e. activated*, resulting in a change in hydration state (HCO_3^- no longer binding to Gd(III) at physiological pH). The outflow from the cell of the now negatively charged complex is expected to be significantly reduced, hence the agent is *accumulated* in the cell.[7] This hypothesis is illustrated in Scheme 1. The switch from neutral to negatively charged species is achieved by masking the negative charge with appended carboxylic esters. Acetoxymethyl esters have been used for a number of years to mask negative charge, *e.g.* to 'smuggle' charged complexes into cells.[7] Such esters are excellent substrates for non-specific intracellular esterases. A recent example of the use of acetoxymethyl esters is the trapping of fluorescent lipophilic ferrichrome analogues inside cells (for Fe^{3+} chelation) by Shanzer and co-workers.[8] Lippard and co-workers have recently utilised intracellular esterase activity to activate fluorescent sensors for Zn^{2+}.[9] The more hydrolytically robust ethyl ester-containing **2** was prepared as a model for the acetoxymethyl-containing **3** used in this study. The interaction of their Gd(III) complexes with carbonate in the presence and absence of porcine liver esterase is reported.

The Eu(III) and Gd(III) complexes of **2** and **3** were synthesised as shown in Scheme 2. [Eu.**4**]$^{3-}$ and [Gd.**4**]$^{3-}$ were prepared for comparison as the post-enzyme-activity complexes.[6] The ethyl ester-containing [Eu.**2**] and [Gd.**2**] were prepared as models for the corresponding acetoxymethyl-containing complexes [Eu.**3**] and [Gd.**3**]. The ethyl esters are more resistant to base-catalysed hydrolysis, thus enabling the pH-dependency of carbonate binding



Scheme 1 Proposed activation and accumulation strategy.

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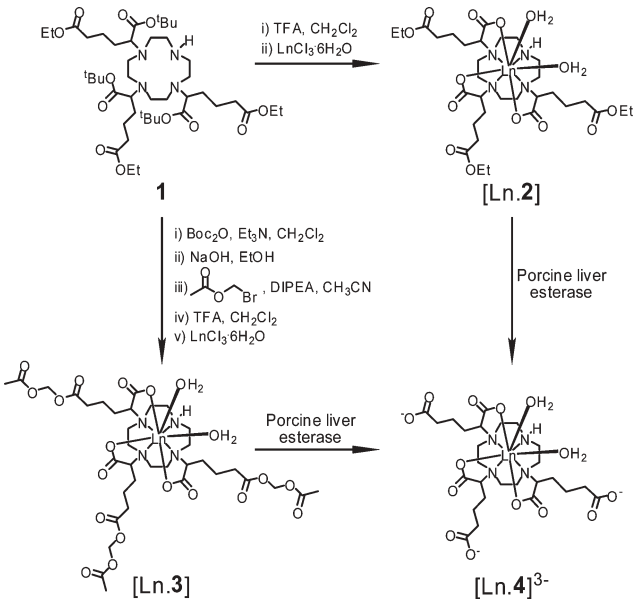

Scheme 2

to be probed (acetoxymethyl esters are very susceptible to hydrolysis under even mildly basic conditions). The mixed ethyl/^{t}butyl ester of racemic 2-bromoadipic acid was reacted with cyclen in the presence of K_2CO_3 in CH_3CN to give **1** as a statistical mix of six stereoisomeric forms due to the absolute configuration at the α-carbon (*RRR*/*SSS*, *RRS*/*SSR*, *RSR*/*SRS*). Acid hydrolysis of the ^{t}butyl esters in TFA yielded the pro-ligand, which was reacted with $EuCl_3{\cdot}6H_2O$ or $GdCl_3{\cdot}6H_2O$ in H_2O to give [Eu.**2**] and [Gd.**2**] respectively. Synthesis of [Eu.**3**] and [Gd.**3**] was slightly less straightforward: following base hydrolysis of the ethyl esters of **1** and Boc-protection, the acetoxymethyl esters were introduced by reaction with bromomethyl acetate and DIPEA in CH_2Cl_2. Removal of the Boc and ^{t}butyl groups in TFA yielded the pro-ligand which was reacted with $EuCl_3{\cdot}6H_2O$ or $GdCl_3{\cdot}6H_2O$ in MeOH to give [Eu.**3**] and [Gd.**3**] respectively.

Luminescent lifetime measurements on the Eu(III) complexes in H_2O and D_2O enable the determination of the hydration state (q) of the complexes; the same values can be inferred for the corresponding Gd(III) complexes.[10] In the absence of HCO_3^- at pH 7.4, all three complexes possess two inner-sphere water molecules. For [Eu.**2**], [Eu.**3**] and [Eu.**4**]$^{3-}$ calculated values of q = 2.1 were obtained (Table 1). In the presence of 30 mM $NaHCO_3$ a significant change in excited state lifetime in H_2O ($1/k$) is noted for the two neutral complexes [Eu.**2**] and [Eu.**3**] (displacement of quenching inner-sphere waters giving longer lifetimes). Whilst the hydration state does not fall to zero at physiological pH at this concentration of HCO_3^-, it is lowered significantly, to give an apparent q = 1.2.‡ This indicates the presence of ~40% carbonate-bound species in solution. This carbonate-binding equilibrium has been shown previously to be pH-dependent for related q = 2 complexes, irrespective of the charge on the complex.[5] It is noteworthy that the affinity for HCO_3^- of the negatively charged complex [Eu.**4**]$^{3-}$ is essentially negligible at pH 7.4. In the presence of HCO_3^-, there is a ~70% increase in q moving from [Eu.**2**]/[Eu.**3**] to [Eu.**4**]$^{3-}$.

Table 1 Radiative rate constants (k) and calculated hydration states (q) for Eu(III) complexes (1 mM, λ_{ex} = 395 nm, pH 7.4, 298 K)

	k_{H_2O}/ms^{-1}	k_{D_2O}/ms^{-1}	q (±0.2)
[Eu.**2**]	3.32	1.36	2.1
[Eu.**3**]	3.26	1.23	2.1
[Eu.**4**]$^{3-}$	3.30	1.33	2.1
[Eu.**2**] + 30 mM $NaHCO_3$	2.83	1.61	1.2
[Eu.**3**] + 30 mM $NaHCO_3$	2.75	1.46	1.2
[Eu.**4**]$^{3-}$ + 30 mM $NaHCO_3$	3.26	1.38	2.0

The effect of carbonate-binding to the neutral and negatively charged Gd(III) complexes can be seen in Fig. 1. The plot shows the change in relaxivity of [Gd.**2**] and [Gd.**4**]$^{3-}$ *vs.* pH in the presence of 30 mM $NaHCO_3$ (the acetoxymethyl esters of [Gd.**3**] are too sensitive to base hydrolysis to allow titration to basic pH). The plot clearly shows that both neutral ([Gd.**2**]) and negatively charged ([Gd.**4**]$^{3-}$) complexes will bind carbonate at high enough pH. At pH 10, both complexes have a similar relaxivity of ~3.0 mM^{-1} s^{-1}, characteristic of a q = 0 complex; however, the major difference between these two species is the pH at the onset of carbonate binding. At pH < 5.0 both complexes are q = 2 as no carbonate is bound, but as the pH increases from 5.0 the neutral [Gd.**2**] starts to bind carbonate, indicated by a gradual decrease in relaxivity as inner-sphere water molecules are displaced. A similar change is seen for negatively charged [Gd.**4**]$^{3-}$, but the binding of carbonate and the subsequent decrease in relaxivity do not occur until the pH of the solution is > 7.0. The complicated form of the plotted data is due to the presence of various interlinked equilibrium processes: the pH-dependent speciation of carbonate, the pH-dependent deprotonation of bound water molecules and the carbonate-binding equilibrium. What Fig. 1 clearly demonstrates is the difference in relaxivity of [Gd.**2**] and [Gd.**4**]$^{3-}$ at physiological pH. There is a pronounced difference between the pre- and post-enzyme activated complexes ([Gd.**2**] and [Gd.**4**]$^{3-}$ respectively). The negatively charged post-ester hydrolysis species [Gd.**4**]$^{3-}$ has less affinity for HCO_3^-, suggesting a significant potential increase in relaxivity can be obtained upon activation of the contrast agent by esterase.

In the absence of HCO_3^-, all three of the Gd(III) complexes have a high relaxivity (Table 2). These values are a result of their

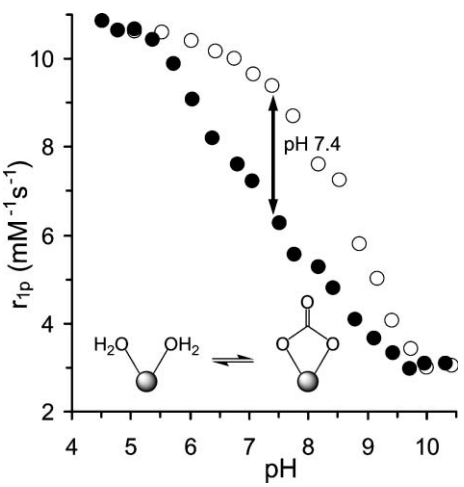

Fig. 1 Relaxivity (r_{1p}) *vs.* pH for 1 mM [Gd.**2**] (closed circles) and [Gd.**4**]$^{3-}$ (open circles) in 30 mM $NaHCO_3$ (298 K, 20 MHz).

Table 2 Relaxivities r_{1p} (mM^{-1} s^{-1}) ±5% of Gd(III) (0.2 mM) complexes in the presence and absence of $NaHCO_3$ (10 mM) and porcine liver esterase (100 units) (pH 7.4, 298 K, 20 MHz)

	Complex only	Complex + esterase	Complex + $NaHCO_3$	Complex + $NaHCO_3$ + esterase
[Gd.**2**]	10.2	10.8	5.7	10.8
[Gd.**3**]	9.9	10.5	5.7	10.5
$[Gd.\mathbf{4}]^{3-}$	11.3	10.8	10.8	10.8

hydration state $q = 2$ and size (longer rotational correlation times τ_R *cf.* $[GdDOTA]^-$). These values are similar to those of GdHOPO-based $q = 2$ complexes.[11]

To demonstrate the ability of an enzyme to activate the contrast agents, the relaxivities of the three Gd(III) complexes were measured in the presence and absence of HCO_3^- and porcine liver esterase. The results of these studies are shown in Table 2. Solutions were prepared containing complex alone; complex + 10 mM $NaHCO_3$; complex + esterase; and complex + 10 mM $NaHCO_3$ + esterase (intracellular concentration of HCO_3^- is ~10 mM). Relaxivities of the solutions were measured (at 298 K, 20 MHz) 2 h after incubation at 310 K. The results clearly demonstrate ester hydrolysis is occurring; an increased relaxivity is noted for the neutral complexes [Gd.**2**] and [Gd.**3**] in the presence of the enzyme as they are converted to $[Gd.\mathbf{4}]^{3-}$. This is entirely expected as $[Gd.\mathbf{4}]^{3-}$ possesses a slightly higher relaxivity than [Gd.**2**] or [Gd.**3**]. Both [Gd.**2**] and [Gd.**3**] exhibit a fall in relaxivity in the presence of 10 mM $NaHCO_3$ ($r_{1p} = 5.7$ mM^{-1} s^{-1} for both); this correlates with the pH-dependency of carbonate-binding depicted in Fig. 1. The slight lowering of relaxivity of $[Gd.\mathbf{4}]^{3-}$ from 11.3 to 10.8 mM^{-1} s^{-1} in the presence of 10 mM $NaHCO_3$ is again expected due to the low affinity for carbonate of this complex at pH 7.4. The most important observation is the relaxivity enhancement of both [Gd.**2**] or [Gd.**3**] in the presence of 10 mM $NaHCO_3$ when exposed to porcine liver esterase. The increase in relaxivity from 5.7 mM^{-1} s^{-1} to 10.8 and 10.5 mM^{-1} s^{-1} respectively for [Gd.**2**] or [Gd.**3**] is due to their conversion to the negatively charged complex $[Gd.\mathbf{4}]^{3-}$ with its much reduced affinity for carbonate at pH 7.4, *i.e.* complete conversion occurs. The effect of enzyme-activation produces an 89% and 84% increase in relaxivity for complexes [Gd.**2**] and [Gd.**3**] respectively. Such a large percentage increase is a significant change with respect to magnetic resonance imaging.

In conclusion, neutral Gd(III) complexes have been developed and their propensity to bind endogenous HCO_3^- has been exploited. On activation by esterase, the relaxivities of these complexes increased by ~85% at physiological pH and $NaHCO_3$ concentration, as anion binding is inhibited by the unmasked negative charge. This augurs well for developing the proposed accumulation and activation strategy for cellular MR imaging. Indeed, one of the few enzyme-activated agents to be used in 'molecular imaging', Meade's 'benchmark' EgadMe (β-galactosidase substrate), shows a 57% increase in q and signal intensity on enzyme activation; sufficient for *in vivo* imaging of gene expression.[4] Studies are underway to incorporate targeting vectors to render the complexes more site-specific, as are studies to fine-tune the carbonate-binding affinity to maximise the percentage change in relaxivity on esterase activation.

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Notes and references

‡ More details on the fitting of the decay curves are contained in the supplementary information.

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