Inorganic Chemistry
Article

Inorg. Chem. **2009**, *48*, 8515–8522 **8515**
DOI: 10.1021/ic901284t

Luminescence Study of Eu(III) Analogues of Esterase-Activated Magnetic Resonance Contrast Agents

Marco Giardiello and Mark P. Lowe*

Department of Chemistry, University Road, University of Leicester, Leicester, LE1 7RH, U.K.

Received July 3, 2009

A model for an accumulation and enzyme-activation strategy of a magnetic resonance contrast agent was investigated via the luminescence of Eu(III) analogues. Neutral $q = 2$ Eu(III) ethyl and acetoxymethyl ester LnaDO3A-based complexes showed increased emission intensity in the presence of serum concentrations of carbonate because of inner-sphere water molecule displacement by the anion. The affinity for carbonate is suppressed by the introduction of negative charge to the complex following enzymatic hydrolysis of the ester groups, resulting in quenching of Eu(III) luminescence and changes in spectral form. The conversion of neutral, carboxylic ester-containing complexes into free acid forms by enzymatic hydrolysis using pig liver esterase was demonstrated by luminescence (Eu) and ^{1}H NMR spectroscopic investigations (Y). These studies demonstrated that the concept of inhibition of anion binding as a result of enzyme activation is feasible.

Introduction

The use of gadolinium-containing contrast agents (CAs) for Magnetic Resonance Imaging (MRI) has increased dramatically in recent years. The introduction of chelates containing the paramagnetic Gd(III) ion enhances image contrast by increasing the longitudinal relaxation rate, R_1 ($1/T_1$), of surrounding water protons.[1] To minimize the inherent toxicity of the Gd(III) ion, first generation CAs were developed which encase the Gd(III) ion in multidentate chelating ligands, for example, ligands based on diethylenetriaminepentaacetic acid (DTPA) or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). The first generation CAs give good but non-specific image enhancement. Recent CA design has focused on the development of Gd(III) complexes where R_1 is varied as a result of changes in the physiological environment, such as pH,[2] the presence of biological molecules[3] or metal ions.[4] Magnetic Resonance (MR) CA development is entering the molecular and cellular imaging phase of its evolution. Current MR CAs are administered at a dose of ~0.1 mmol/kg. Relatively high concentrations are required to overcome the inherent low sensitivity of the NMR phenomenon. Concentrations

*To whom correspondence should be addressed. E-mail: mplowe@le.ac.uk.

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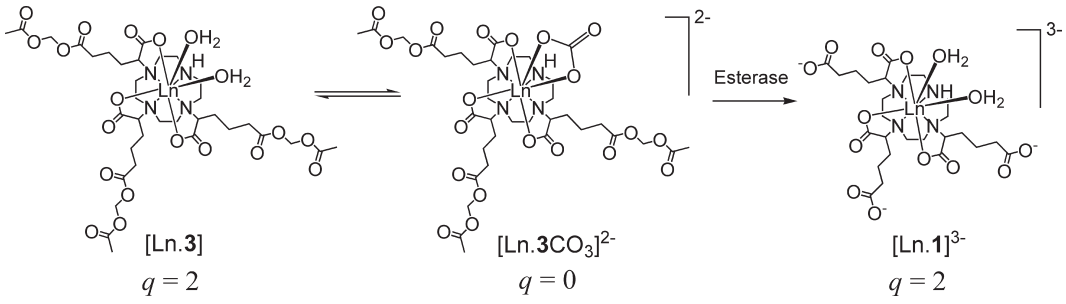
Published on Web 08/07/2009

Scheme 1



of >0.1 mM CA are typically needed to observe image enhancement. It is suggested that the absolute detection limit of such CAs is 30 μM.[5] Such high concentrations make imaging of, for example, cell surface receptors, problematic as concentrations are significantly lower than the administered CA dose. The generation of high relaxivity CAs, which are designed to target and accumulate in a specific location are required to overcome such sensitivity issues. To this end there have been essentially two approaches: to increase the "payload", that is, deliver more Gd(III) per molecule, or to "activate", that is, switch on the contrast in response to some in vivo event. With respect to the later, there has been great interest in the development of CAs that respond to the presence of an enzyme, which may be associated with a particular disease state. Several CAs have been designed which contain the substrate for a given enzyme.[3] Such substrates are incorporated into the molecule and mask the relaxometric properties of the CA, causing low MR image intensity. Enzymatic cleavage of the masking groups results in increased relaxivity of the CA and an increase in the MR image intensity, effectively "switching on" the CA. While an absolute "high" relaxivity value is desirable, it is a large percentage change in signal intensity that is important in these activated systems.

The efficiency of a CA is defined by its relaxivity, r_1; the total paramagnetic relaxation rate enhancement of the water protons per unit concentration of the CA ($mM^{-1}\ s^{-1}$). In the design of ligands for Gd(III) to be used as CAs, one or two coordination sites of the usually nine coordinate Gd(III) ion are reserved for binding of water molecules; these rapidly exchange with the bulk water. The relaxation contribution from the inner-sphere is often the most significant with regards to CA design. The longitudinal inner-sphere paramagnetic relaxation rate is expressed by eq 1.

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

where C is the concentration of paramagnetic ion, q is the number of coordinated water molecules, T_{1M} is the longitudinal relaxation time of the inner-sphere waters, and τ_M is the water exchange lifetime. It is clear from eq 1 that increasing the hydration state (q) of the complex will increase the overall relaxivity. The hydration state is normally restricted to $q \leq 2$ because of the necessity for the toxic (at the doses administered) Gd(III) ion to be encapsulated in a stable chelate. Several seven-coordinate (with respect to ligand) cyclen-based Gd(III) complexes have been prepared, which leave two vacant coordination sites for water molecules to exchange; however, such complexes display a much lower relaxivity than is to be expected when used in vivo.[6] The affinity of neutral or cationic $q = 2$ Gd(III) complexes for endogenous serum anions, such as carbonate, lactate, and phosphate, is the cause of such poor observed relaxivities as the coordinated inner-sphere waters are displaced by serum anions. Of these coordinating anions, HCO_3^- (bound as carbonate, CO_3^{2-}) is the most abundant in serum (20−30 mM) and possesses a relatively high affinity for this type of $q = 2$ complex (e.g., $K > 50{,}000\ M^{-1}$ for cationic, $K \sim 500\ M^{-1}$ for neutral, and $K \sim 25\ M^{-1}$ for anionic complexes at pH 8.9 and significantly lower at pH 7.4).[6c] It has been demonstrated that bidentate anion binding can be suppressed (at physiological pH) by the incorporation of negative charge into the complex: electrostatic repulsion disfavors anion binding, allowing waters to exchange at the Gd(III) center.[7] We have embraced the differing affinities for neutral and negatively charged complexes for anion binding and exploited it to prepare CAs where anion binding is disfavored on enzyme activation.[8] Scheme 1 shows the model which was hypothesized: the change from a neutral to a negatively charged species is achieved following enzymatic hydrolysis of acetoxymethyl esters, unmasking a negatively charged species resulting in the suppression of carbonate binding. This enzyme-activation is manifested as a concomitant change in hydration state and hence relaxivity.

The neutral $q = 2$ complex bearing acetoxymethyl esters is expected to be in equilibrium with the anion bound $q = 0$ complex in serum; thus the CA will have a lower than expected "average" hydration state and, consequently, lower relaxivity. As the CA is neutral in the $q = 2$ form, passage into the cell should possible (e.g., by endocytosis, pinocytosis, or pressure transport).[9] Once inside the cell, while carbonate is still present, albeit in lower concentration, intracellular esterases are expected to hydrolyze the acetoxymethyl ester groups, unmasking a negatively charged complex with $q = 2$.

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It is also expected that the outflow from the cell of the now negatively charged complex will be slowed.[10,11] Such acetoxymethyl ester hydrolysis strategies have been used in many aspects of drug development for cellular drug delivery and compartmentalization.[12,13] The now negatively charged species would disfavor binding of anions and allow waters to exchange with the metal center, crucially this $q = 2$ complex has been shown to have fast water exchange.[7] The hydration state is increased upon enzyme activation, and the relaxivity of the CA is therefore enhanced, leading to higher MR image intensity. The CA is both accumulated *and* activated inside the cell by enzymatic hydrolysis.

Herein the hypothesis has been explored in vitro by monitoring luminescent behavior of Eu(III) and ^{1}H NMR changes in Y(III) complexes, both of which are analogues of the previously reported Gd(III) complexes.[8] Luminescence is an effective tool for examining the hydration states and anion binding of these complexes. Conclusions drawn from studying Eu(III) complexes can be inferred for analogous Gd(III) complexes. Eu(III) emission is quenched by O−H oscillators; hence binding and displacement of water molecules by anions can be investigated in this manner; the form of the emission is also very sensitive to the coordination of anions. Preliminary data showing the promise of the acetoxymethyl ester model system **Gd.3** was reported in an earlier communication.[8] This contribution details studies carried out on the Eu(III) and Y(III) analogues of acetoxymethyl system and a more chemically robust ethyl ester model.

Experimental Section

Reagents. Porcine Liver Esterase, product number E3019 (lyophilized powder, 20 units per mg solid) was purchased from Sigma Aldrich.

Chromatography. Cation exchange chromatography was carried out using Dowex MAC-3 weak acid-cation exchange resin, prepared with 1% HCl.

Spectroscopy. ^{1}H NMR spectra were recorded using a Bruker 300 MHz spectrometer using deuterium oxide (D_2O) referenced to residual solvent peaks. All chemical shifts are recorded in ppm. Accurate mass ESMS were recorded on a Finnigan MAT 900 XLT high resolution double focusing mass spectrometer with tandem ion trap (polyethylenimine reference compound) at the EPSRC National MS Service at Swansea, U.K. IR measurements were carried out using a Perkin-Elmer Spectrum One FT-IR spectrometer. Concentrations of the complexes used in the studies were determined by ICP-MS using an Agilent Technologies 7500ce inductively coupled plasma mass spectrometer.

Luminescence Spectroscopy. Luminescence emission spectra were recorded using a Jobin Yvon Horiba FluoroMax-P spectrometer (using DataMax for Windows v2.2). Samples were held in a 10 × 10 nm or 10 × 2 nm quartz Hellma cuvette and a cutoff filter (550 nm) was used to avoid second-order diffraction effects. Eu(III) excitation was direct ($\lambda_{ex} = 395$ nm). Time-gated emission spectra obtained in phosphorimeter mode (for esterase hydrolysis experiments) employed a 0.1 ms delay. Flash time was 50 ms with 150 flashes per point. The sample window was 2 ms with an increment of 1 nm.

Hydration State, *q*, Determination. Lifetimes were measured by excitation (395 nm) of the sample with a short 40 ms pulse of light (500 pulses per point) followed by monitoring the integrated intensity of light ($\Delta J = 2$) emitted during a fixed gate time of 0.1 ms, at a delay time later. Delay times were set at 0.1 ms intervals, covering 4 or more lifetimes. Excitation and emission slits were set to 5:5 nm. The data is applied to the standard first order decay (eq 2), minimized in terms of k by iterative least-squares fitting operation using Microsoft Excel, where I_{obs} is the observed intensity, I_0 is the initial intensity, and t is the time (ms). The calculated k values are then applied to eq 3 to calculate the hydration state, q.[14,15]

$$I_{obs} = I_0 e^{-kt} + \text{offset} \quad (2)$$

$$q = 1.2[(k_{H_2O} - k_{D_2O}) - 0.25] \quad (3)$$

Equation 4 was applied to the lifetime data, recorded at pH 7.4 in the presence of carbonate, to estimate the mole fraction of carbonate bound complex present in solution, x, where $I_o^{CO_3^{2-}}$ and $k^{CO_3^{2-}}$ are the initial Eu(III) emission intensity and the calculated rate constant of the Eu(III) excited state decay of the carbonate bound species (i.e., measured at pH 10 where maximum carbonate binding occurs), $I_0^{H_2O}$ and $k_0^{H_2O}$ are the initial emission intensity and the calculated rate constant of the water bound species (i.e., measured at pH 5 where no carbonate binding occurs).

$$I_{obs} = xI_0^{CO_3^{2-}} e^{-k^{CO_3^{2-}}t} + (1-x)I_0^{H_2O} e^{-k^{H_2O}t} + \text{offset} \quad (4)$$

pH Titrations. pH measurements were recorded using a Jenway 3510 pH meter with a BDH probe, model 309−1025−02 calibrated at pH 4, 7, and 10. The luminescence versus pH titration was carried out in a background of constant ionic strength ($I = 0.1$ NaCl, 298 K). Aqueous solutions were made basic by addition of 1 or 0.1 M NaOH and titrated to acid pH by addition of small aliquots of 1 or 0.1 M HCl.

Esterase Hydrolysis Studies. For luminescence (Eu) studies, four aqueous solutions were prepared, each containing 0.1 M NaCl and adjusted to pH 7.4 with small aliquots of HCl and NaOH; solutions contained 1.0 mM **Eu.L**; 1.0 mM **Eu.L**+ 30 mM $NaHCO_3$; 1.0 mM **Eu.L** + 100 units pig liver esterase; 1.0 mM **Eu.L** + 100 units pig liver esterase + 30 mM $NaHCO_3$. Luminescence and studies were conducted prior to and following incubation for ∼2 h at 37 °C.

Synthesis. The synthesis of [**Eu.1**]$^{3-}$, **Eu.2**, and **Eu.3** were published in an earlier communication.[8] The aliphatic side chains are present in six stereoisomeric forms defined by the absolute configuration at the α-carbon: *RRR*, *SSS*, *RRS*, *SSR*, *RSR*, and *SRS*. The precise equilibrium composition of the complexes is not known. It is assumed that each isomeric form is present in solution and contributes to the luminescent observations for each complex.

General synthesis of Y(III) complexes. The appropriate proligand was dissolved in water (10 mL) and $YCl_3 \cdot 6H_2O$ was added in slight excess to ensure all ligand was used in complexation. The solution was adjusted to pH 5.5 using NaOH and was heated at 90 °C for 24 h. The solution was then added to a suspension of Dowex MAC-3 weak acid-cation exchange resin and stirred for 10 min to ensure complete removal of unreacted

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YCl_3. This was monitored by addition of 200 μL aliquots of reaction solution to 800 μL of 0.1 mM xylenol orange solution (pH 5); if the solution remained orange there was no free Y(III) present; if the solution was pink, uncomplexed Y(III) ion was still in solution. The solution was then decanted from the resin and lyophilized to give the Y(III) complex.

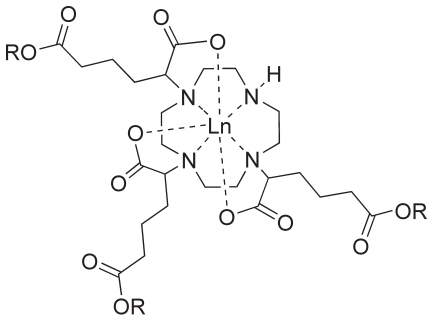
For the preparation of **Y.3** the reaction was carried out in MeOH and heated at 55 °C. On removal of the solvent the residue was dissolved in water at pH 5.5 at which point the previous procedure was followed.

Yttrium(III) 1,4,7-Tris[(4′-(ethoxycarbonyl)-1′-carboxybutyl]-1,4,7,10-tetraazacyclododecane, Y.2. 1,4,7-tris[(4′-(ethoxycarbonyl)-1′-carboxybutyl]-1,4,7,10-tetraazacyclododecane (43.6 mg, 63.0 μmol) and $YCl_3 \cdot 6H_2O$ (19.2 mg, 63.0 μmol). **Y.2** (45.2 mg, 93%) was obtained as a white hygroscopic powder. ν_{max} cm^{-1} 1681, (C═O), 1624, 1378, 1204, 1175, 1127; δ_H (300 MHz; D_2O) 1.1 (9 H, t, $^3J_{HH}$ 7.2 Hz, ~$CO_2CH_2CH_3$), 1.3−1.8 (12 H, br, ~$CHNCH_2CH_2$~ and ~$CH_2CH_2CH_2$~), 2.3 (6H, br, ~$CH_2CH_2CO_2$~), 2.4−3.5 (19H, br, ring CH_2 (16 H) and ~CNH (3 H)), 4.0 (6 H, br q, $^3J_{HH}$ 7.2 Hz, ~$CO_2CH_2CH_3$); *m*/*z* (HR-ESMS+) $[M+H]^+$ calcd for $C_{32}H_{54}N_4O_{12}Y$ 775.2791, found 775.2792.

Yttrium(III) 1,4,7-Tris[(4′-(acetoxymethoxycarbonyl)-1′-carboxybutyl]-1,4,7,10-tetraazacyclododecane, Y.3. 1,4,7-Tris[(4′-(acetoxymethoxycarbonyl)-1′-carboxybutyl]-1,4,7,10-tetraazacyclododecane (28.2 mg, 34.4 μmol) and $YCl_3 \cdot 6H_2O$ (10.4 mg, 34.4 μmol) **Y.3** (28.2 mg, 90%) was obtained as a white hygroscopic powder. ν_{max} cm^{-1} 2978, 1738 (C═O), 1415, 1370, 1198, 1139; δ_H (300 MHz; D_2O) 1.4−1.8 (12H, br, ~$CHNCH_2CH_2$~ and ~$CH_2CH_2CH_2$~), 1.9 (9H, s, ~CO_2CCH_3), 2.2 (6H, br, ~$CH_2CH_2CO_2$~), 2.4−3.4 (16 H, br, ring H), 3.6 (3H, s, ~CNH), 5.7 (6H, s, ~OCH_2O~); *m*/*z* (HR-ESMS+) $[M+H]^+$ calcd for $C_{35}H_{54}N_4O_{18}Y$ 907.2486, found 907.2476.

Results and Discussion

The complexes reported are based on the aDO3A structure (1,4,7-tris[4′-carboxyl-1′-carboxybutyl]-1,4,7,10-tetraazacyclododecane).[7] Acetoxymethyl esters are readily hydrolyzed by esterase enzymes and are frequently used as masking groups for negative charge.[16] These were the substrates of choice for enzyme activation in this work. Two model compounds were prepared and used in initial luminescent (Eu) and relaxometric (Gd) studies to probe the feasibility of the enzyme activation hypothesis.[8] The previously reported negatively charged, post-hydrolysis, free acid complex, $[\mathbf{Ln.1}]^{3-}$ (at pH 7.4 the pendant non-coordinating carboxylic acids are deprotonated in **Ln.1** and so this complex will be referred to in its anionic form) was prepared as well as a neutral pre-hydrolysis complexes, **Ln.2** and **Ln.3**. The post-hydrolysis, carboxylic acid-containing complex $[\mathbf{Ln.1}]^{3-}$ was prepared as a control to monitor the anion binding affinity of both the pre- and post-enzyme activated complexes prior to esterase hydrolysis, that is, this is the hydrolysis product of **Ln.2** and **Ln.3**. Ethyl esters (**Ln.2**) were chosen for the neutral model compound because of their relative ease of synthesis and stability in comparison with the acetoxymethyl ester derivative (**Ln.3**). Furthermore, the ethyl esters are more resistant to base-catalyzed hydrolysis, thus enabling the pH-dependency of carbonate binding to be probed, a study precluded for acetoxymethyl esters. Such experiments cannot be carried out with the acetoxymethyl ester derivative because of its susceptibility to hydrolysis, even in mildly basic conditions.



Ln.1 R = H
Ln.2 R = CH_3CH_2
Ln.3 R = $CH_3CO_2CH_2$

Luminescence Studies. The ligands in $[\mathbf{Eu.1}]^{3-}$, **Eu.2**, and **Eu.3** are seven-coordinate, leaving two coordination sites vacant for coordination of water molecules to Eu(III). The presence of these two vacant sites is essential if bidentate anions are to bind. To confirm the hydration number, *q*, and therefore the number of readily available coordination sites, luminescent lifetime studies were carried out. The hydration state values in the absence of carbonate ($[\mathbf{Eu.1}]^{3-}$, **Eu.2**, and **Eu.3** all are $q = 2.1$)[8] indicate that two inner-sphere waters bind to Eu(III). It is therefore feasible that these waters can be displaced by bidentate anions, particularly carbonate, the most abundant bidentate serum anion with concentrations around 20−30 mM; it is present inside cells at around 10 mM.[17] There is negligible change in *q* for $[\mathbf{Eu.1}]^{3-}$ in the presence and absence of carbonate at pH 7.4 ($[\mathbf{Eu.1}]^{3-}$ + 30 mM $NaHCO_3$ $q = 2.0$); however for **Eu.2** and **Eu.3** in the presence of carbonate (**Eu.2**/**Eu.3** + 30 mM $NaHCO_3$ both are $q = 1.2$),[8] the apparent *q* indicates the presence of a mixture of both $q = 0$ (carbonate-bound) and $q = 2$ (water-bound) species (when the data is fit to two species an approximate 40:60 mixture of the two is indicated). Figure 1 shows the Eu(III) emission spectra for $[\mathbf{Eu.1}]^{3-}$, **Eu.2** and **Eu.3** in the presence and absence of carbonate at pH 7.4. The form of the spectra are typical, the $\Delta J = 0$ to $\Delta J = 2$ transitions ($^5D_0 \rightarrow {}^7F_J$) are shown. The three components in the $\Delta J = 1$ band are as expected for non-C4 symmetric complexes of this type. It is clear that there is a lower affinity for carbonate resulting from a much higher degree of anionic repulsion for the negatively charged, free acid compound $[\mathbf{Eu.1}]^{3-}$ in comparison to its neutral analogues **Eu.2** and **Eu.3** at physiological pH; there is little change in the form of the spectrum of $[\mathbf{Eu.1}]^{3-}$ in the presence of carbonate, but a significant change for **Eu.2** and **Eu.3**. As the O−H oscillators of water quench the Eu(III) excited state it is clear to see from the spectra in Figure 1 (left) that little or no water displacement occurs upon addition of carbonate to $[\mathbf{Eu.1}]^{3-}$; there is little change in emission intensity and spectral form. Addition of carbonate to the neutral complexes **Eu.2** and **Eu.3** results in an increase in emission

(16) (a) Tsien, R. Y. *Nature* **1981**, *290*, 527. (b) Meijler, M. M.; Arad-Yellin, R.; Cabantchik, Z. I.; Shanzer, A. *J. Am. Chem. Soc.* **2002**, *124*, 12666. (c) Woodroofe, C. C.; Lippard, S. J. *J. Am. Chem. Soc.* **2003**, *125*, 11458. (d) Woodroofe, C. C.; Won, A. C.; Lippard, S. J. *Inorg. Chem.* **2005**, *44*, 3112.

(17) Cooper, G. M.; Hausman, E. E. *The Cell; A Molecular Approach*; 4th ed.; Sinauer Associates, Inc.: Sunderland, MA, 2006.

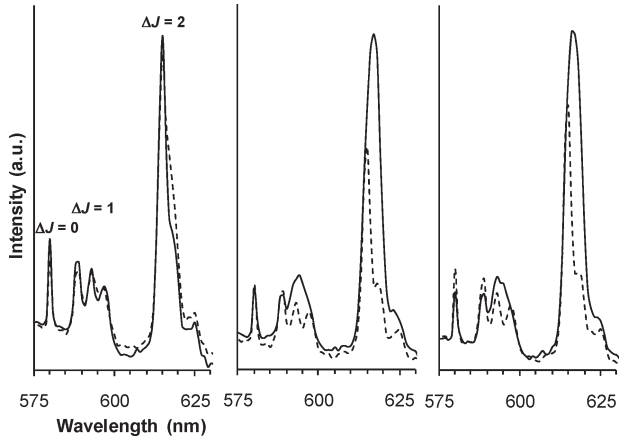


Figure 1. Eu(III) luminescent emission spectra in the presence (solid line) and absence (dotted line) of carbonate. 1.0 mM complex, 0.1 M NaCl, 30 mM $NaHCO_3$, λ_{ex} = 395 nm, pH 7.4, 25 °C: Left $[\mathbf{Eu.1}]^{3-}$, Middle **Eu.2**, Right **Eu.3**.

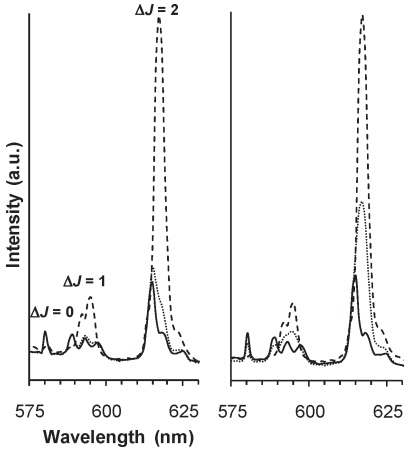


Figure 2. Eu(III) luminescent emission spectra in the presence of carbonate at pH 5.0 (solid line), pH 7.4 (dotted line), and pH 10.0 (dashed line). 1.0 mM complex, 0.1 M NaCl, 30 mM $NaHCO_3$, λ_{ex} = 395 nm, pH 7.4, 25 °C: Left $[\mathbf{Eu.1}]^{3-}$, Right **Eu.2**.

intensity and a change in spectral form (Figure 1, middle and right). Quenching water molecules are displaced as carbonate binding competes with water for ligation to the Eu(III) center. There is a shift in the emission maxima and intensity of the hypersensitive $\Delta J = 2$ band from λ_{em} = 615 nm to λ_{em} = 617 nm upon addition of carbonate. The increase in intensity of the $\Delta J = 2$ band is typical for carbonate coordination as the polarizability of the axial donor is changed.[18] Furthermore, the form of the $\Delta J = 1$ band at ~ 590 nm changes as three distinct sharp peaks are observed in the absence of carbonate, which become two broad peaks upon addition. This is indicative of the presence of a mixture of the carbonate-bound and water-bound species at this pH. The spectra shown in Figure 2 of $[\mathbf{Eu.1}]^{3-}$ and **Eu.2** in the presence of carbonate at pH 5.0, 7.4, and 10.0 clearly indicate that the spectra shown in Figure 1 (recorded at pH 7.4) are a superposition of the spectra recorded at pH 5.0 and pH 10.0 (for $[\mathbf{Eu.1}]^{3-}$ 95% pH 5.0 spectrum and **Eu.2** 58% pH 5.0 spectrum).

These observations were exploited further when studying the effects of esterase addition. Little spectral change is observed at pH 7.4 with the negatively charged $[\mathbf{Eu.1}]^{3-}$ complex as minimal carbonate binding occurs. The calculated hydration state values for both the pre- and post-enzyme activated species in the presence of 30 mM sodium hydrogen carbonate at pH 7.4 is reflected in the form of the spectra in Figure 1, the minor change in hydration state of the free acid complex $[\mathbf{Eu.1}]^{3-}$ suggests there may be a small quantity of the carbonate bound species present at pH 7.4.

The change in hydration state is much greater for the neutral complexes **Eu.2** and **Eu.3**; however, water molecules are not completely displaced. The hydration state of q = 1.2 suggests that at pH 7.4 there is a mixture of the q = 0 carbonate-bound and q = 2 water-bound species. This can be clearly seen from the titration monitoring Eu(III) emission intensity versus pH in the presence of carbonate (Figure 3). Figure 2 shows the representative spectra recorded at pH 5.0, 7.4, and 10.0 for $[\mathbf{Eu.1}]^{3-}$ (left) and **Eu.2** (right). pH titrations monitoring the Eu(III) emission intensity in the presence of carbonate were carried out to determine the extent of, and the optimum pH for binding to these complexes. Because of the base sensitivity to hydrolysis of acetoxymethyl esters, neither relaxivity (Gd) or emission intensity (Eu) versus pH titrations could be carried out as acetoxymethyl esters readily hydrolyze above pH 8. Because of the similarity of **Eu.3** to **Eu.2** similar conclusions can be inferred from the pH versus emission intensity titration of **Eu.2**.

The emission intensity versus pH profile incorporates the pH-dependent speciation of carbonate in aqueous solution, the coordination equilibrium established with the Eu(III) complex, and the deprotonation of coordinated water molecules. The solution pH determines the concentrations of HCO_3^-/CO_3^{2-} present. $[HCO_3^-]$ falls from ~ 90% at pH 8.9 to less < 2% at pH 4.9, and at pH > 9 there are significant quantities of CO_3^{2-} present (the pK_a for the H_2CO_3/HCO_3^- equilibria is 6.4 while the HCO_3^-/CO_3^{2-} equilibria is 10.2). In strongly basic media, carbonate is clearly coordinated to both the neutral, **Eu.2**, and the negatively charged, $[\mathbf{Eu.1}]^{3-}$, species as evidenced by both the form and the intensity of the emission spectra (Figure 2). Quenching water molecules are displaced, leading to more intense emission and a change in spectral form, particularly of the $\Delta J = 2$ band. Hydration states are calculated as q = 0.4 for both $[\mathbf{Eu.1}]^{3-}$ and **Eu.2** at pH 10.0. As the pH is lowered, water molecules begin to compete with carbonate for coordination to the Eu(III) centers. Because of the electrostatic repulsive effects of the negatively charged, free acid species $[\mathbf{Eu.1}]^{3-}$, carbonate binding does not occur to the same extent in the neutral analogue, **Eu.2**. Crucially, at physiological pH (7.4) Figures 2 and 3 clearly show carbonate binds to a much smaller extent for the post-enzyme $[\mathbf{Eu.1}]^{3-}$, than for the pre-enzyme, **Eu.2**, with the calculated hydration state values q = 2.0 and q = 1.2 for $[\mathbf{Eu.1}]^{3-}$ and **Eu.2** respectively. In acidic media hydration states were calculated as q = 2.1 for both $[\mathbf{Eu.1}]^{3-}$ and **Eu.2**. Eu(III) emission spectra recorded at pH 7.4 in the presence of carbonate are composed of the equilibrium contributions from both the carbonate bound and the water bound species. Table 1 shows the apparent q value of $[\mathbf{Eu.1}]^{3-}$ and **Eu.2** obtained at pH 7.4 when fit to

(18) Dickins, R.; Parker, D.; Bruce, J.; Tozer, D. *Dalton Trans.* **2003**, 1264.

a single exponential decay (k_{H_2O} = 3.26 ms^{-1}, k_{D_2O} = 1.38 ms^{-1} for $[\mathbf{Eu.1}]^{3-}$ and k_{H_2O} = 2.83 ms^{-1}, k_{D_2O} = 1.61 ms^{-1} for **Eu.2**). Estimates of the percentage of carbonate-bound species obtained from luminescent lifetime data using the calculated rate constant of the Eu(III) excited state decay of the carbonate-bound species (i.e., measured at pH 10) and the rate constant of the water-bound species (i.e., measured at pH 5) obtained in H_2O is fit to two decaying species, one carbonate-bound and one water-bound.

The calculated values for both $[\mathbf{Eu.1}]^{3-}$ and **Eu.2** were q = 2.1 and q = 0.4 in acidic and basic media, respectively. The apparent q value at pH 7.4 in the presence of carbonate for $[\mathbf{Eu.1}]^{3-}$ comprises 95% of the q = 2.1 species and 5% of the q = 0.4 species, which is consistent with the observed value of q = 2.0. Likewise, the apparent q value for **Eu.2** comprises 58% of the q = 2.1 species and 42% of the q = 0.4 species, which is once again consistent with the observed value of q = 1.2 in the presence of carbonate at pH 7.4 (obtained if the data is fit to a single exponential decay). This suggests an 8-fold difference in the proportion of bound carbonate to the neutral, ester bearing complex than for the negatively charged complex at pH 7.4, highlighting the reduced affinity for carbonate of the unmasked post-enzyme-activated complex. It can therefore be hypothesized that the carbonate binding equilibrium established at pH 7.4 for **Eu.3** would be similar to that of **Eu.2**, where it was estimated that ~42% of the carbonate bound species was present in solution (the sensitivity to base hydrolysis precludes this experiment for **Eu.3**).

The clear difference in hydration states between the pre- and post-enzyme activated Eu(III) complexes in the presence of carbonate, mirror the observations for the relaxometric studies of the Gd(III) complexes. The relaxivity versus pH titrations in the presence of carbonate were carried out for the Gd(III) analogues.[8] These complexes behave in a similar manner to the Eu(III) complexes; however, displacement of inner-sphere water molecules has an opposite effect: Gd(III) relaxivity is enhanced by the presence of inner-sphere water molecules, whereas Eu(III) emission is quenched and the form of the emission spectrum is altered. As carbonate displaces water molecules in basic media, relaxivity decreases (Gd) whereas emission intensity and excited state lifetime increases (Eu), as the hydration state q decreases. At physiological pH there is a clear difference in observed relaxivity for the pre- and post-enzyme complexes as carbonate has less affinity for the free acid species, $[\mathbf{Gd.1}]^{3-}$. This behavior mirrors that described here for the Eu(III) analogues.

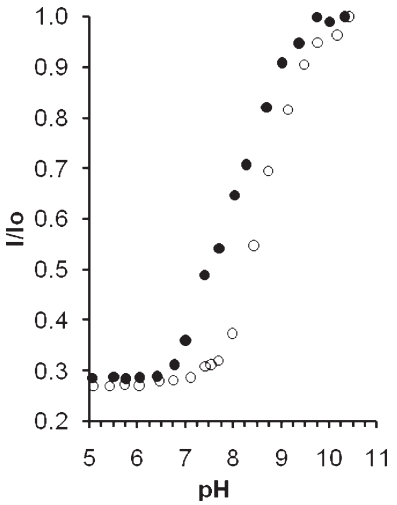


Figure 3. Eu(III) luminescent emission intensity (I/I_0 ΔJ = 2) vs pH for **Eu.2** (filled circles) and $[\mathbf{Eu.1}]^{3-}$ (open circles) in the presence of carbonate. 1.0 mM Eu, 0.1 M NaCl, 30 mM $NaHCO_3$, λ_{ex} = 395 nm, 25 °C.

Luminescence Studies in the Presence of Esterase. Enzyme activation was monitored via the luminescent properties of the Eu(III) analogues. Figure 1 showed that upon addition of carbonate to the neutral complexes **Eu.2** and **Eu.3** at pH 7.4 the emission intensity of the ΔJ = 2 band increased, while the emission maxima shifted from λ_{max} = 615 nm to λ_{max} = 617 nm. No such spectral change was observed at pH 7.4 for the negatively charged $[\mathbf{Eu.1}]^{3-}$ model as little carbonate was seen to be binding to the Eu(III) center. Mirroring the enzyme-activation experiments we reported for the relaxometric investigation of $[\mathbf{Gd.1}]^{3-}$, **Gd.2**, and **Gd.3**,[8] solutions were prepared containing the Eu(III) complex alone; complex + 30 mM $NaHCO_3$; complex + esterase; and complex + 30 mM $NaHCO_3$ + esterase. Eu(III) emission spectra and excited state lifetimes of the solutions were measured (pH 7.4, 25 °C) 2 h after incubation at 37 °C. These experiments were carried out to determine any change in hydration state upon addition of esterase and were recorded both before and after incubation in the presence and absence of esterase. In order eliminate the complication of any background fluorescence from esterase, a 0.1 ms delay was incorporated prior to emission being recorded. The four samples were incubated at 37 °C at pH 7.4 (Figure 4).

Higher intensity Eu(III) emission of the ΔJ = 2 band is observed in the presence of carbonate (dashed line) than in its absence (solid line) of carbonate for **Eu.2** and **Eu.3** as inner-sphere waters are displaced to an extent at pH 7.4 (Figure 4, middle and right). Furthermore, the spectral form of the ΔJ = 1 band is consistent with the increase of carbonate bound complex present in solution. The spectral form in the presence and absence of carbonate remains the same as that observed before incubation, which confirms the ethyl and acetoxymethyl ester groups of **Eu.2** and **Eu.3** remain intact and, in the absence of esterase, do not undergo hydrolysis under these experimental conditions (pH 7.4, 37 °C). The similarity in intensity and spectral form of the two esterase-containing solutions of **Eu.2** and **Eu.3** suggests that complete hydrolysis of the ester groups has occurred; moreover, the

Table 1. Radiative Rate Constants (k) at pH 5 and 10, Measured Hydration State, q, Values at pH 7.4 for $[\mathbf{Eu.1}]^{3-}$ and **Eu.2**, and the Percentage of Carbonate Bound Species Present in Solution at pH 7.4. 1.0 mM Eu, 0.1 M NaCl, 30 mM $NaHCO_3$, pH 7.4, 25 °C

	k_{H_2O} (ms^{-1}) pH 10	k_{H_2O} (ms^{-1}) pH 5	q pH 7.4	% CO_3^{2-} bound pH 7.4
$[\mathbf{Eu.1}]^{3-}$	2.14	3.36	2.0	5.0
Eu.2	2.20	3.67	1.2	42.0

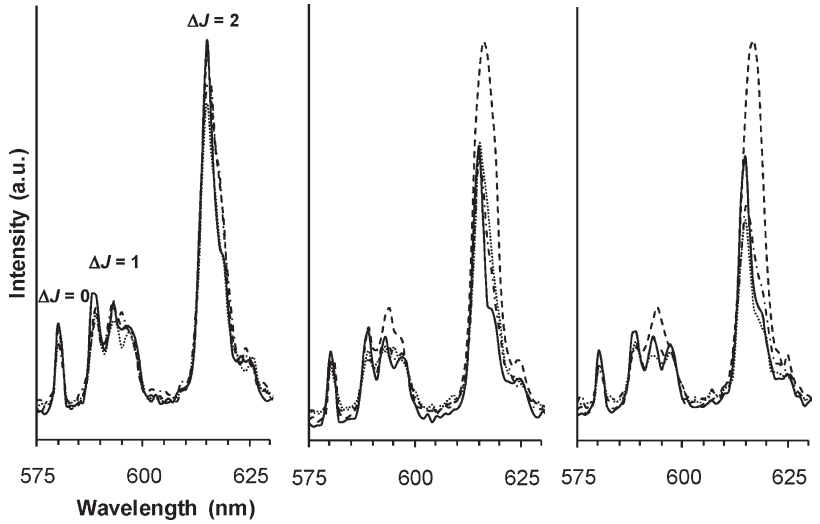


Figure 4. Eu(III) luminescent emission spectra for **Eu.L** (solid line), **Eu.L** + $NaHCO_3$ (dashed line), **Eu.L** + esterase (dotted line), and **Eu.L** + $NaHCO_3$ + esterase (dot-dash line) following 2 h incubation at 37 °C. λ_{ex} = 395 nm, 0.1 ms delay, pH 7.4, 25 °C: Left $[\mathbf{Eu.1}]^{3-}$, Middle **Eu.2**, Right **Eu.3**.

spectra are now identical to those obtained for the control $[\mathbf{Eu.1}]^{3-}$ (this can be seen more clearly for **Eu.3** in Figure 5).

The reduction in emission intensity of the $\Delta J = 2$ band and the presence of three components in the $\Delta J = 1$ band again suggests that carbonate binding is disfavored as the negatively charged species has been generated. As a control, the same solution compositions of the post-enzyme activated model, $[\mathbf{Eu.1}]^{3-}$, were also prepared, and their Eu(III) emissive properties were investigated following incubation at 37 °C (Figure 4, left). As there is very little difference between the spectral forms and intensities of each of the four solutions of $[\mathbf{Eu.1}]^{3-}$ it can be concluded that the presence of esterase has no effect on the nature of the Eu(III) emission observed. Likewise, as the emission spectra of the esterase hydrolyzed **Eu.3** are very similar to those seen for the post-enzyme hydrolyzed model complex $[\mathbf{Eu.1}]^{3-}$, it can be concluded that the species is indeed being converted from **Eu.3** to $[\mathbf{Eu.1}]^{3-}$ upon exposure to the enzyme. Figure 5 shows more clearly $[\mathbf{Eu.1}]^{3-}$ and **Eu.3** under the same experimental conditions. The two pairs of spectra of the esterase-containing solutions post-incubation (Figure 5 (c) and (d)) clearly indicate that hydrolysis has occurred as both solutions now contain $[\mathbf{Eu.1}]^{3-}$, hence have essentially identical spectra. The difference between the two compounds in the absence of esterase (Figure 5 (a) and (b)) is marked, particularly in the presence of carbonate.

Luminescent lifetime studies were carried out for each of the four solutions of $[\mathbf{Eu.1}]^{3-}$, **Eu.2**, and **Eu.3** to determine the hydration states of the complexes for each of the experimental solutions. The change in emission spectra as a consequence of hydration state variations for both the neutral complexes **Eu.2** and **Eu.3** are consistent with these data (Supporting Information). A decrease in the calculated hydration state, q, is observed upon the addition of carbonate as an equilibrium is established between the $[\mathbf{Eu.L(H_2O)_2}]$ and $[\mathbf{Eu.LCO_3}]^{2-}$ (**L** = **2** or **3**) complexes at pH 7.4, resulting in the apparent hydration state value of q = 1.2. Upon addition of esterase there is an increase in observed hydration state as **Eu.L** is converted to $[\mathbf{Eu.1}]^{3-}$. The calculated q value of

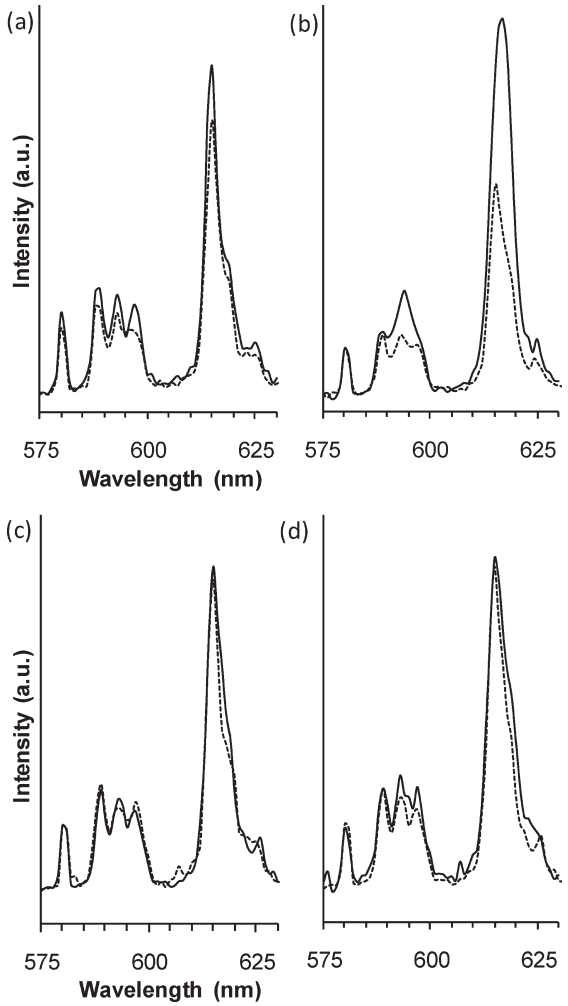


Figure 5. Eu(III) luminescent emission spectra for (a) **Eu.3** (solid line) and $[\mathbf{Eu.1}]^{3-}$ (dotted line) in H_2O, (b) + 30 mM $NaHCO_3$, (c) + esterase, (d) + 30 mM $NaHCO_3$ + esterase following 2 h incubation at 37 °C λ_{ex} = 395 nm, 0.1 ms delay, pH 7.4, 25 °C.

the carbonate containing solution (post-esterase) is lower than that containing esterase but no carbonate. This is consistent with the slight carbonate affinity of the ester

hydrolyzed complex $[\mathbf{Eu.1}]^{3-}$, which is now present in both solutions. These data are consistent with the observed changes in spectral form.

^{1}H NMR Investigation of Esterase Hydrolysis of Y.3. To study further the effect of esterase on the acetoxymethyl ester complex, the yttrium analogue **Y.3** was prepared. Yttrium is non-paramagnetic, and therefore amenable to ^{1}H NMR study as chemical shift values and line widths are not affected as they are for the paramagnetic Eu(III) analogues (the ionic radius of Y(III) is similar to Ho(III) at $r = 102$ pm cf. Gd(III) $r = 105$ pm). Two solutions were prepared in D_2O, each containing **Y.3** (13 mM), and 100 units of pig liver esterase was added to one solution. After incubation at 37 °C for 24 h, hydrolysis is observed by the disappearance of the $\sim OCH_2O\sim$ resonances of the acetoxymethyl ester groups at $\delta = 5.7$ ppm and the $\sim CO_2CCH_3$ resonances at $\delta = 1.9$ ppm upon the addition of esterase. The resulting ^{1}H NMR spectra (Supporting Information) consist of the typically broad resonances of this type of seven-coordinate complex, the resonances spanning $\delta = 2.4$ to 3.4 ppm are those of the 16 methylenic ring hydrogens and $NCH(CO_2)CH_2$. The remaining resonances from $\delta = 1.4$ to 2.3 ppm correspond to the aliphatic methylenes. The spectral line broadening occurs as a result of the fluxionality of the complex; however, the more remote and hence more mobile acetoxymethyl ester resonances $\delta = 5.7$ ppm and $\delta = 1.9$ ppm could clearly be observed in the room temperature ^{1}H NMR spectrum. The spectrum recorded in the absence of esterase shows the acetoxymethyl ester groups remain intact following incubation. This proves that the compound is stable in solution under these conditions (37 °C at pD 7.4). Following incubation of the sample in the presence of esterase, a marked decrease in intensity of these resonances is noted, indicating loss of the acetoxymethyl group, that is, ester hydrolysis has occurred. The equivalent ^{1}H NMR study was carried out with the Y(III) ethyl ester analogue, **Y.2** monitoring the production of ethanol following the hydrolysis of the ethyl esters as resonances appear at $\delta = 1.0$ and 3.5 ppm, corresponding to the CH_3 and CH_2 protons of ethanol, respectively. At the same time, ethyl ester resonances at $\delta = 1.1$ and 4.0 ppm disappear upon hydrolysis, once again corresponding to the methyl and CH_2 protons. The data from these experiments using Y(III) is consistent with the observations for the Eu (luminescence) and Gd (relaxivity) analogues.

Summary and Conclusions

In a prior communication we demonstrated that neutral $q = 2$ Gd(III) complexes (**Gd.2** and **Gd.3**) show decreased relaxivity in the presence of serum concentrations of carbonate because of the inner-sphere water molecule displacement by bidentate anionic binding. This decrease in relaxivity is not as great for the negatively charged analogues of such complexes, ascribed to the increased electrostatic repulsion and hence a lower affinity for anionic binding at pH 7.4.[6−8] The conversion of neutral, carboxylic ester containing complexes into free acid forms by enzymatic hydrolysis using pig liver esterase has been further demonstrated by luminescence (Eu) and ^{1}H NMR spectroscopic (Y) investigations. The studies have confirmed that the concept of inhibition of anion binding as a result of enzyme activation works. Changes in spectral form and the lifetime of the excited state of the Eu(III) complexes as a result of perturbations of the hydration state complement the changes in relaxivity seen for the corresponding Gd(III) complexes. The ^{1}H NMR spectra of the Y(III) analogues demonstrate ester hydrolysis in the presence of pig liver esterase, evidenced by the disappearance of the ester resonances post-incubation. The model shows great scope for future development, first by modification of the aliphatic side chains, incorporating more enzyme-specific substrates for monitoring enzyme activities associated with specific disease sites. Second, the issues of delivery to the cell either by addition of a targeting vector to the nonsubstituted cyclen ring nitrogen or via viral encapsulation are being pursued.

Acknowledgment. The authors thank the EPSRC National Mass Spectrometry Service Centre, Swansea, U.K. for high resolution ESMS.

Supporting Information Available: Luminescent lifetime data and ^{1}H NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.