Tuning the properties of cyclen based lanthanide complexes for phosphodiester hydrolysis; the role of basic cofactors†

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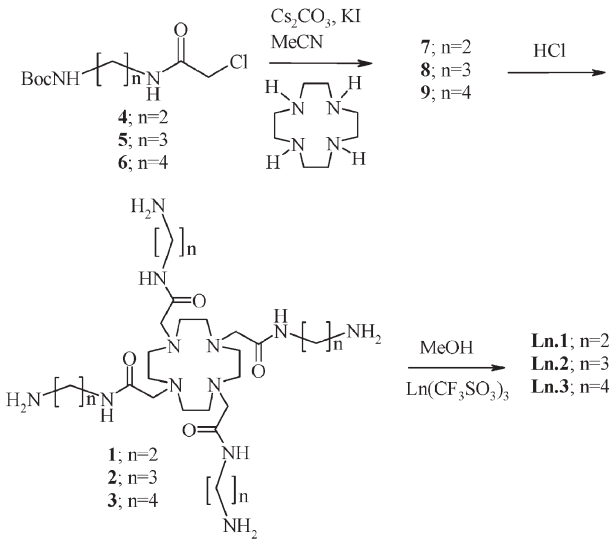
The synthesis of several cyclen based lanthanide [Eu(III) and La(III)] complexes is described; these metallo ribonuclease mimics are based on the use of alkyl amines as pendent arms, which give rise to fast hydrolysis within the physiological pH range of HPNP (an RNA model compound) that is highly dependent on the length of the alkyl spacer.

Phosphoesters play major structural and mechanistic roles in nature. They are the basis of energy formation and consumption, the centre of phosphorylation processes and crucially, form the backbone of both RNA and DNA. As protein synthesis is the consequence of the translation of the genetic code, being able to control the synthesis of non desirable proteins by directly targeting nucleic acids through phosphodiester hydrolysis of RNA or DNA is of great scientific and therapeutic value.[1,2] In nature, nucleases or ribonucleases achieve such hydrolysis in a fast and selective manner, through the synergetic action of one or several cofactors such as metal ion centres, basic amino acids and metal coordinated water molecules.[1,4] Hence, mimicking these features in a single molecule, is of great current interest.[1–7] We and others have developed such ribonuclease mimics using macrocyclic lanthanide cyclen (1,4,7,10-tetraazacyclododecane) complexes.[7–9] By functionalising these with hydrophobic amino acids and heterocycles as cofactors we have demonstrated fast hydrolysis of phosphodiesters such as **HPNP** (2-hydroxypropyl *p*-nitrophenyl phosphate)[10] and RNA oligomers. Even though this work has been extremely successful maximum activity has only been achieved in a narrow pH range; usually centred on pH ~ 8–10. This has been attributed to: (1) the binding of the phosphodiester to the coordinated lanthanide ion; and (2) the deprotonation of metal bound water molecules (and hence, become nucleophilic) which had pK_a's within this region.[9] In separate studies we have also demonstrated that the pK_a's of the aforementioned metal bound water molecules could be significantly tuned by the structure of the pendent arms; which can directly influence the hydrophobic environment around the metal ion, which effects the pK_a.[11]

With the aim of achieving fast hydrolysis within the physiological pH range, we set out to develop the new amino alkyl based lanthanide complexes **Ln1–Ln4**. The design rationale behind **Ln1–Ln4** was that the alkyl spacers would give rise to a hydrophobic environment around the lanthanide ions and at the same time the primary amines would be able to partake in general acid base catalysis (*i.e.* function as cofactors) and potentially lower the pK_a's of the metal bound water molecules. To the best of our knowledge these are the first examples of such lanthanide complexes to achieve such fast hydrolysis.

The synthesis of **1–3** was achieved in three steps from the mono Boc protected ethylene, propyl and butane diamine, which were reacted with chloroacetyl chloride to give the appropriate α-chloroamide derivatives **4–6**, Scheme 1. The tetra-substituted ligands **7–9** were formed by the reaction of cyclen with **4–6**, respectively, in MeCN, in the presence of Cs_2CO_3 and KI. Deprotection of the four amines was achieved using a 15% (v/v) aqueous HCl solution, giving ligands **1–3** as hygroscopic solids in *ca.* 90%. The La(III) and Eu(III) complexes of **1–3** were prepared by refluxing each of the ligands with 1.1 molar equivalents of the relevant lanthanide triflate in dry MeOH for 16 h, followed by precipitation using diethyl ether, filtration and washing. This gave the desired lanthanide complexes **La.1**, **Eu.1**, **La.2**, **Eu.2**, **La3** and **Eu.3** as pale yellow precipitates in 45–90% yields. All these complexes were fully characterised by elemental analysis, HRMS, IR and NMR spectroscopy (see ESI for full characterisation).† The ^{1}H NMR spectra (400 MHz, D_2O) of the Eu(III) complexes showed several resonances appearing between +25 and −13 ppm, for the shifted axial and equatorial protons of the cyclen ring and the acetamide protons of pendent arms, which is characteristic of square antiprism geometry found in similar cyclen complexes in solution.[9,12] The HRMS (ESMS) also indicated the formation of



Scheme 1 Synthesis of the cyclen ligands **1–3** and the corresponding lanthanide complexes.

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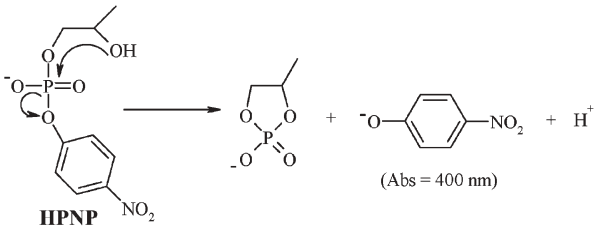

Scheme 2 Hydrolysis of **HPNP** by transesterification.[10]

the desired complexes with peaks found corresponding to the $[M + CF_3SO_3]^+$ ion and correct isotopic distribution patterns. The hydration state (q), the number of metal bound water molecules, was determined for the **Eu.1**, **Eu.2** and **Eu.3** complexes by measuring their Eu(III) excited state lifetimes in H_2O and D_2O, respectively.[12] These indicated that each Eu(III) complex possessed a single metal bound water molecule, giving an overall nine-coordinate environment for these complexes. In comparison, the La(III) complexes are usually found to have two metal bound water molecules, due to their higher coordination requirement.[9]

The ability of the above complexes to promote phosphodiester hydrolysis was evaluated using the RNA mimic substrate HPNP, Scheme 2. The rate constant k for the hydrolysis of **HPNP** can be obtained by monitoring spectrophotometrically the hydrolysis of HPNP and the formation of *p*-nitrophenolate at 400 nm.‡ We first evaluated the ability of the La(III) and Eu(III) complexes to hydrolyse **HPNP** at pH 7.4. The results from these measurements clearly indicated that the length of the alkyl spacer had an effect on the rate of hydrolysis. This can be seen in Table 1. For **La.1**, the hydrolysis is, to the best of our knowledge, the fastest for such stable coordination La(III) complexes, with a half-lifetime of 0.74 h. It is almost four times faster than **La.2** and twenty five times faster than **La.3**. The same trend is also seen for the Eu(III) analogues. Here, **Eu.1** gives rise to extraordinary fast hydrolysis, which is unprecedented for such Eu(III) coordination complexes, as in the past, such Eu(III)-cyclen based complexes have been reported to be either inactive, or very inefficient in promoting hydrolysis of **HPNP** at this pH. Furthermore, these results clearly demonstrate that the incorporation of 'cofactors' into our design, *e.g.* the terminus amino functionalities and the length of the alkyl spacers, play a crucial role in the promotion of hydrolysis.

With the aim of quantifying the role of the primary amines, we also carried out hydrolysis of **HPNP** using analogues of these molecules lacking the amino functions; and indeed these complexes did not give rise to any measurable hydrolysis, clearly demonstrating their importance in our current design. It is also clear that the length of the spacer is crucial to its activity. This can be deduced for two reasons; firstly, the alkyl spacers can give rise to a hydrophobic environment around the metal ion;[8] while secondly, positioning the amines at an optimal distance from the metal bound substrate. This enables the primary amines to either support the binding of the substrate through hydrogen bonding or activate the 2′ hydroxyl group, improving its nucleophilicity. Moreover, we predict that for the La(III) complexes, one of the metal bound water molecules may possibly be deprotonated by these amines.

Table 1 Results from the hydrolysis of **HPNP** at pH 7.4[a]

Complex	k/h^{-1}	$\tau_{1/2}$/h	k_{rel}[b]
La.1	0.933 (±0.005)	0.74	7775 (±42)
Eu.1	0.365 (±0.012)	1.89	3000 (±100)
La.2	0.170 (±0.011)	4.08	1417 (±92)
Eu.2	0.076 (±0.014)	9.02	633 (±116)
La.3	0.032 (±0.006)	21.73	266 (±50)
Eu.3	0.021 (±0.004)	33.01	175 (±33)

[a] The rate constant (k) values were determined by fitting the data to first order rate kinetics. [b] k_{rel}, is the ratio between k and the rate constant of the 'uncatalysed' hydrolysis of **HPNP**, $k_{un} = 0.00012$ h^{-1}, $\tau_{1/2} = 5.87 \times 10^3$ h, at pH 7.4. Every rate constant that is quoted is an average of 2–3 measurements, agreeing to within 15%.

To confirm this, we investigated the pH dependence on the rate of hydrolysis for **La.1**. Fig. 1 shows that the complex is very active over a wide pH range. A 'pseudo' bell-shaped curve is observed with a maximum rate constant centred on pH 7.4, and a shoulder at *ca.* pH 8.3. These striking results indicate that the maximum hydrolysis of **HPNP** does not occur within the pH range associated with the pK_a values expected for the two metal bound water molecules of **La.1** (see below). Having established this fast hydrolysis for **La.1**, we measured the second order rate constant for the hydrolysis of **HPNP** by **La.1** at the rate maxima, pH 7.4. Here the plot of k *vs.* [**La.1**] increased linearly (see ESI),† indicating that the reaction was first order in the complex concentration, with a second order rate constant of 1.41 M^{-1} s^{-1}. This is significantly faster than those previously obtained for both tetra-amide cyclen based La(III) complexes (0.016 M^{-1} s^{-1}), and a dinuclear Zn(II) complex (0.25 M^{-1} s^{-1}) reported for the cleavage of **HPNP**.[7,13] Furthermore, the latter of these was reported to be one of the fastest second order rate constants determined for the hydrolysis of **HPNP** by metal based ribonuclease mimics; clearly showing the advantage of **La.1** complex in promoting such transesterification.§

With the aim of gaining further insight into the role of the amino moiety of the pendent arms, in the mechanism of hydrolysis, potentiometric pH titrations were carried out on the above La(III) and Eu(III) complexes of **1**. For **1**, five pK_a's were determined (*cf.* Speciation diagram in Fig. 2) using the non-linear least squares regression programme HYPERQUAD; pK_{a1} 10.59 (±0.01), pK_{a2} 9.70 (±0.04), pK_{a3} 8.88 (±0.01), pK_{a4} 8.35 (±0.05), and pK_{a5} 6.10 (±0.09). The last three possible protonations of the ligand occurred at very acidic pH and therefore could not be accurately measured using a glass electrode. The first and the second deprotonations, pK_{a1} and pK_{a2}, respectively, may be attributed to the protonation of the alkyl amines of the pendent donor arms. The protonations pK_{a3}, pK_{a4} and pK_{a5} are attributed to the protonation of two of the cyclen amines and one further alkyl amine in the pendent donor arms. For **La.1**, there are six

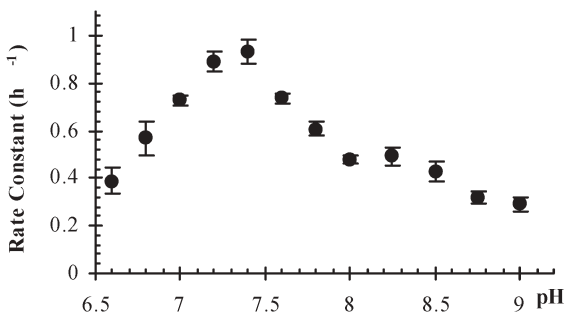

Fig. 1 The pH–rate profile for the hydrolysis of 0.14 mM **HPNP** at 37 °C by **La.1** (0.18 mM). The error bars are determined from the average of 2–3 measurements.

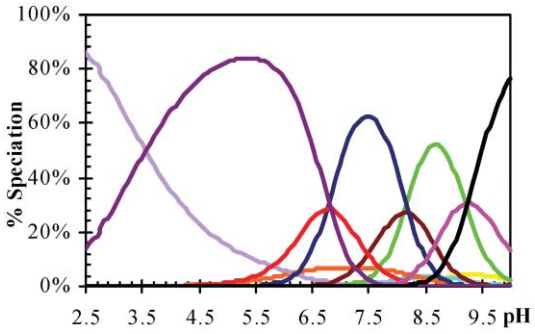

Fig. 2 Speciation variation of ligand **1**, showing the species present in H_2O at various pH in which $[\mathbf{1}]_{total} = 7.2 \times 10^{-3}$ M, $[La(III)]_{total} = 7.0 \times 10^{-3}$ M, I = 0.10 M (NEt_4ClO_4) at 25 °C. Speciation is shown relative to the total concentration of ligand **1**. yellow: $\mathbf{1H_2}$; blue: $\mathbf{1H_3}$; orange: $\mathbf{1H_4}$; light pink: $\mathbf{1H_5}$; green: **La.1**; brown: **La.1H**; navy blue: $\mathbf{La.1H_2}$; red: $\mathbf{La.1H_3}$; purple: $\mathbf{La.1H_4}$; pink: $\mathbf{La.1OH_2}$; black: $\mathbf{La.1(OH_2)_2}$.

possible sites for protonation; two assigned to metal bound water molecules [$\mathbf{La.1OH_2}$ and $\mathbf{La.1(OH_2)_2}$] as well as four alkyl amines ($\mathbf{La.1H_4}$, $\mathbf{La.1H_3}$, $\mathbf{La.1H_2}$ and **La.1H**). As expected the pK_a values decreased on increasing protonation; 8.13 (±0.06), 8.17 (±0.01), 6.80 (±0.01) and 6.76 (±0.05), were assigned to the alkyl amines. The pK_a values 9.21 (±0.03) and 9.26 (±0.03) were assigned to the deprotonation of the two metal bound water molecules, which are very close. However, the titration curve shows that this process occurs from pH 8–10 (see ESI).† Moreover, from the speciation diagram in Fig. 2 it can be seen that the formation of $\mathbf{La.1OH_2}$ and $\mathbf{La.1(OH_2)_2}$ occurs where the activity of **La.1** is decreasing in Fig. 1. This clearly indicates that these two species do not partake directly in the hydrolysis, for instance through nuclephilic activation of the 2′ hydroxyl group of **HPNP**, as previously demonstrated by us for related complexes where maximum activity was achieved at alkaline pH. However, under physiological conditions, one of these water molecules is most likely replaced upon binding of the substrate to the La(III) centre (Lewis acid activation). Such an activation mode is also possible for **Eu.1**. However, here a single pK_a = 8.00 (±0.06) was determined for the deprotonation of the metal bound water. We demonstrated this by measuring the hydration state in the presence of a phosphodiester for **Eu.1**, and a q ~ 0 was determined. From Fig. 2, it can be seen that within the pH window 6.5–8.5, which most closely overlaps with that of the pH–rate profile in Fig. 1, the most active species is $\mathbf{La.1H_2}$ (*ca.* 60% at pH 7.5) while lesser contributions are observed from $\mathbf{La.1H_3}$ and $\mathbf{La.1H_4}$. This suggests that the amines can strongly influence the reaction through two possible modes of action, *e.g.* through hydrogen bonding and/or by a general acid–base catalysis. Furthermore, they may be acting as a general base by deprotonating the second sphere waters molecules, which themselves become nucleophilic.[14] For **Eu.1** a similar species distribution (*cf.* ESI)† was observed; clearly demonstrating the important role of the primary amines in pendent donor arms in the hydrolytic process. The amines may also participate directly in the transesterification by functioning as nucleophiles (*e.g.* pendent arm base nucleophilic activation). These results clearly show that the trend observed in Table 1, *e.g.* the decrease in activity as a function of the length of the alkyl spacer, is a measure of the role the spacers plays in locating the primary amines around HPNP.

In summary, we have demonstrated that by simply incorporating primary amines into lanthanide cyclen complexes, the rate of hydrolysis of phosphodiesters can be greatly increased and maximum activity shifted towards the physiological pH range. Moreover, such modifications greatly modulate the mechanism for the hydrolysis, which can be *switched* from being dominantly dependent on nucleophilic activation by metal bound hydroxide molecules, to ligand based activation. We are currently investigating these and related systems in greater detail.

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Notes and references

‡ All of the experiments discussed in this paper were performed using either an *Agilent 8453* or a *Cary 50 Scan* spectrophotometer, both fitted to a circulating temperature controlled water bath, and mechanically stirred. A 50 mM HEPES buffer solution was prepared in deionised water and the pH of the solution was adjusted to the desired pH using 2 M NaOH or 2 M HCl solutions. A 0.18 mM HPNP (Abs = 1.22 at 300 nm, ε = 6777 M^{-1} cm^{-1}) solution was subsequently prepared using this buffered solution and 2.4 mL of this HPNP solution was then incubated in a UV cell at 37 °C for 10 min. The addition of 0.18 mM EDTA had no effect on $\boldsymbol{k}$.
§ As anticipated, the lanthanide complexes of **1** were thermodynamically stable with log$K$ of 13.64 (±0.01) and 20.64 (±0.01) for **1.La**, and **1.Eu**, respectively, as established using HPERCHEM programme.

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