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Gd–XO: a colourimetric probe for the complexation of Gd^{3+} with DO3A-type ligands†

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Small molecule Gd-based MRI contrast agents possess both thermodynamic stability and kinetic inertness, and have been used safely in the clinic. Since the kinetics of Gd^{3+}-complex formation with macrocyclic ligands is slow, convenient methods to investigate their rates of formation are desirable. To this end, heptadentate macrocyclic ligands **1** and **2** were synthesized, and their binding with Gd^{3+} was investigated using the Gd^{3+}–Xylenol Orange (Gd–XO) complex as a colourimetric probe. A significantly faster rate of complex formation was observed with **1** in comparison with **2**, demonstrating that the hydrophobic functionality of **2** plays a role in reducing the rate of complex formation of the ligand with Gd^{3+}. Other macrocyclic ligands can be conveniently assessed for their binding and kinetic properties using the methods described.

FDA-approved small molecule gadolinium (Gd)-based Magnetic Resonance Imaging Contrast Agents (MRI CAs) employ a variety of polyaminocarboxylate (PAC) ligand architectures, generally either acyclic (such as DTPA) or macrocyclic (such as DO3A or DOTA) ligands, possessing different functionalities and hence a range of hydrophobicities.[1] However, MRI CAs based on acyclic ligands have been implicated in *in vivo* toxicity leading to nephrogenic systemic fibrosis in renally compromised patients.[2] Transcomplexation experiments have also demonstrated that the solution stability of MRI CAs based on macrocyclic ligands cannot be generalized,[3] thereby highlighting the need to evaluate these and analogous ligands on a case-to-case basis. Gd-complex stability has been thoroughly investigated by an NMR method involving the measurement of any decrease in the solution relaxivity of a Gd-complex in phosphate buffer upon challenge with Zn^{2+}.[4,5] However, not all research institutions may have access to such NMR capability.

Metal complexes of Arsenazo III (AAIII) are used as colourimetric probes to determine complex formation with PAC ligands *via* transcomplexation, particularly in the context of radiolabeling.[6] However, analysis of AAIII solution speciation may not be straightforward since metal-AAIII stoichiometries occur over a wide range, such as 1 : 1, 1 : 2, 2 : 1, and 2 : 2 metal-to-ligand ratios.[7]

Alternatively, Xylenol Orange (XO) (Fig. 1) has been shown to be a suitable colourimetric indicator for the presence of Gd^{3+} ions in solution, able to detect down to μM concentrations using standard UV-Vis spectrophotometry.[8] A solution of XO undergoes a dramatic colour change upon complexation with Gd^{3+} (from yellow to purple), and Gd–XO stoichiometry is well-defined as occurring in a 1 : 1 fashion.[9]

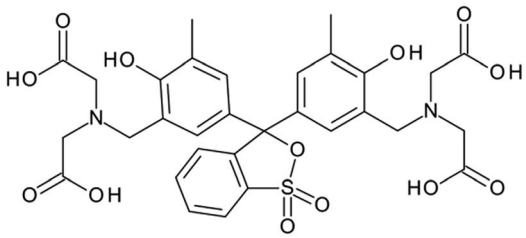

Fig. 1 Xylenol Orange.

A dilute solution of XO is yellow in colour and has an absorbance maximum (λ_{max}) at 434 nm, while a solution containing equimolar concentrations of Gd^{3+} and XO (*i.e.*, Gd–XO) is purple with a λ_{max} at 573 nm. Hence, the absorbance at 573 nm is an indicator for the presence of the Gd–XO complex. When a solution of Gd–XO is titrated with increasing concentrations of EDTA, the colour of the solution reverts back to yellow, indicating that EDTA sequesters Gd^{3+} from the Gd–XO complex.

$$Gd^{3+} + XO\ (\text{yellow}) \leftrightharpoons Gd\text{–}XO\ (\text{purple})$$

$$Gd\text{–}XO\ (\text{purple}) + L \leftrightharpoons Gd\text{–}L + XO\ (\text{yellow})$$

$$(L = \text{EDTA or test ligand})$$

This ligand exchange process can be monitored using UV-Vis spectrophotometry by noting any decrease in the absorbance at 573 nm, or a concurrent increase at 434 nm, with increasing

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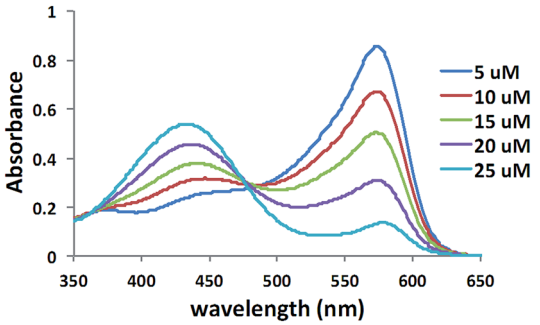

Fig. 2 Overlay of absorbance spectra of 25 μM Gd–XO with increasing concentrations of EDTA (pH 5.8).

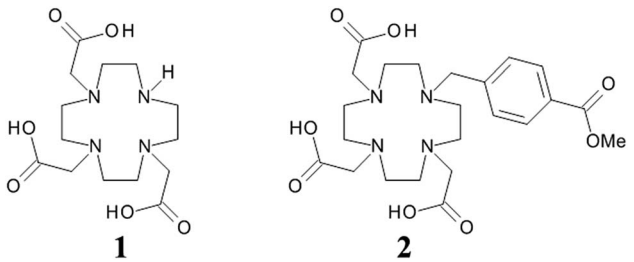

Fig. 3 Macrocyclic PAC ligands used in this study.

concentrations of EDTA, as previously described (Fig. 2).[8] Upon addition of EDTA, the change in solution colour was instantaneous. The relationship between the solution absorbance at 573 nm and ligand concentration is of excellent linearity (ESI Fig. S10†).

Heptadentate ligands **1** and **2** (Fig. 3) were synthesized as previously described, with slight modification.[10] When these were used in place of EDTA, no immediate change in solution colour was observed; indeed, a colour change was observed to occur slowly over a period of three days. This is in agreement with previous observations that complexes of Gd^{3+} with macrocyclic PAC ligands form relatively slowly and are kinetically inert.[11,12]

In order to determine the relative rate constants for the complexation of Gd^{3+} with these ligands, a 20-fold excess of the test ligand (*i.e.*, such that reaction conditions are pseudo-1st order with respect to [Gd^{3+}])[13,14] was added to a solution of initial concentration of 25 μM Gd–XO, and the absorbance at 573 nm was monitored at 30 s intervals. Observed rate constants (k_{obs}) were determined through non-linear regression of time-course data covering at least three half-lives of Gd–XO complex dissociation according to the equation,

$$A_t = A_f + (A_0 - A_f)\exp(-k_{obs}t)$$

Rate constants were determined at 25, 30, 35, and 40 °C, and activation energies (E_a) for complex formation were calculated from the Arrhenius equation $k_{obs} = A\exp(-E_a/RT)$ (Table 1).

Though both ligands are heptadentate, reaction with **1** was observed to have higher rate constants than **2** at all temperatures measured. The data suggest that the differences in rate constants for complexation between **1** and **2** can be accounted for by the presence of the hydrophobic functional group in the structure of **2**, which may impart some degree of steric hindrance for the approach of Gd^{3+} ions in order for complexation to occur. Hence, it may also be said that the difference in E_a between these two ligands can serve as a measure of the steric hindrance imparted by the hydrophobic functional group.

Some PAC ligands employed in MRI CAs possess a hydrophobic functional group by design, in order to increase the plasma circulation half-life of the agent[15] or to induce selective uptake in the liver,[16] for instance. Our results suggest that, in the preparation of small molecule Gd-based CAs in aqueous solution, it is insufficient to assume that pH-control alone ensures complete complexation; rather, the particulars of the macrocyclic ligand structure (*e.g.* functionalisation) can have a significant effect on the complex formation rate, and hence longer reaction/complexation times may be required to achieve complete synthesis of stable analogous Gd-based CAs, relative to those without a hydrophobic functionality.

The previously described NMR method for evaluating Gd-complex stability[4,5] demonstrated that complexes based on macrocyclic ligands are robust against Zn^{2+} challenge, whereas those based on acyclic ligands eventually undergo transmetallation over time. Combined with our observations, it can therefore be initially inferred that Gd-complexes based on a macrocyclic ligand are slow to form but are stable once formed, whereas complexes based on an acyclic ligand are quick to form but are less stable. It is not possible, however, to use our method to directly probe complex stability upon Zn^{2+} challenge since Zn–XO and Gd–XO exhibit the same absorbance profiles,[9] and therefore no change in solution colour will be observed if transmetallation occurs.

Conclusions

The kinetic assay described above permits the discrimination between macrocyclic PAC ligands of the same denticity but different hydrophobicities in terms of observed rates of complex formation with Gd^{3+}. The method described above may also be

Table 1 Summary of kinetic data

	k_{obs} ($\times 10^{-3}$ s^{-1})				
Ligand	25 °C	30 °C	35 °C	40 °C	E_a (kJ mol^{-1})
1	7.49 ± 0.63	11.04 ± 0.30	15.67 ± 1.93	20.57 ± 2.55	53 ± 3
2	1.01 ± 0.13	1.44 ± 0.14	2.24 ± 0.12	3.58 ± 0.43	66 ± 4

used to survey a wide range of related macrocyclic ligands of different denticities (for instance, hepta- in comparison with octadentate) and hydrophobicities for their kinetic properties in complexing Gd^{3+}, in the context of MRI CA development. Due to the excellent linear relationship between the solution absorbance at 573 nm and concentration of **1** (ESI Fig. S10 and S11†), we propose that Gd–XO may also be used in a manner similar to that previously described with Y-AAIII[6] in quantifying the number of ligands (analogous to DO3A) conjugated to nanoparticle-type or macromolecule-based MRI CAs towards the development of high-relaxivity agents.[17–19] Also, the methods described may also be extended or applied to monitor complex formation between macrocyclic PAC ligands and lanthanides other than Gd^{3+}, since the same absorbance profiles are observed with other cations of similar ion charge density.[9]

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