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Design and synthesis of calcium responsive magnetic resonance imaging agent: Its relaxation and luminescence studies

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ABSTRACT

Calcium concentration modulation both inside and outside cell is of considerable interest for nervous system function in normal and pathological conditions. MRI has potential for very high spatial resolution at molecular/cellular level. Design, synthesis and evaluation of Gd-DO3A-AME-NPHE, a calcium responsive MRI contrast agent is presented. The probe is comprised of a Gd^{3+}-DO3A core coupled to iminoacetate coordinating groups for calcium induced relaxivity switching. In the absence of Ca^{2+} ions, inner sphere water binding to the Gd-DO3A-AME-NPHE is restricted with longitudinal relaxivity, $r_1 = 4.37\ mM^{-1}\ s^{-1}$ at 4.7 T. However, addition of Ca^{2+} triggers a marked enhancement in $r_1 = 6.99\ mM^{-1}\ s^{-1}$ at 4.7 T (60% increase). The construct is highly selective for Ca^{2+} over competitive metal ions at extracellular concentration. The r_1 is modulated by changes in the hydration number (0.2 to 1.05), which was confirmed by luminescence emission lifetimes of the analogous Eu^{3+} complex. T_1 phantom images establish the capability of complex of visualizing changes in $[Ca^{2+}]$ by MRI.

1. Introduction

Calcium ion, Ca(II), is one of the most versatile and universal signalling agent in the biological system. Around 1% of calcium in the body is present in solution form in the intra and extracellular fluids at varying concentrations (100 nM–2 mM) [1,2]. Its essentiality can be well understood from the multitude of cellular process controlled by it right from the cell birth, throughout life and unto death [3]. A number of intracellular processes in the brain are dependent on Ca^{2+}, both pre synaptic and post synaptic [4] which include its essential presence for neurotransmitter release, as a mediator of synaptic plasticity and in cellular processes supporting learning and memory. The modulation in Ca^{2+} concentration both inside and outside the cell is a significant factor in determining the nervous system function in both the normal as well as diseased state. Therefore, tracking the fluctuations in the systematic concentration of Ca^{2+} can well serve as a Ca^{2+} signalling "tool kit" for understanding its participation in neuronal regulation and abnormalities in diseased states [5]. The multiple roles of calcium are being majorly understood in greater details with the help of selective fluorescent reporters [6]. However, intrinsic depth limitations associated with optical imaging and production of toxic photobleaching byproducts of fluorescent dyes restricts these applications to superficial regions [7]. With the development of metal selective MRI contrast agents, MRI has evolved as one of the most promising alternative for tracking $[Ca^{2+}]$ changes for biomedical research where depth penetration is not a restriction [8]. With the better understanding of Bloembergen–Solomon–Morgan theory [9–11], significant progress has been made in the past two decades, towards the development of contrast agents and their applications to provide insight into the relationship between the structure and efficacy of a contrast agent [12–14]. For the detection of functional activity by MRI, the main challenge lies in translating the activity at molecular level into changes in the MR image contrast [15,16]. In pursuit of this, conventional linear polyamine or polyaza macrocycle based gadolinium complexes of diethylenetriaminepentaacetic acid, [Gd(DTPA)(H_2O)] and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, [Gd(DOTA)(H_2O)], respectively with coordinating acetate arms, have been commercially employed as first generation approved contrast agents. These complexes are sufficiently stable with one coordination site left available for

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surrounding water molecules [17–19]. However, their non-specific nature has directed the focus in designing new contrast agents with improved magnetic properties as well as specificity. Thus the quest for novel complexes that are able to detect particular changes in their environment by altering relaxivity thereby generating a MRI signal has led to the development of various MR contrast agents that provide information about biochemical events and physiological signals [20].

Smart contrast agents responsive to the essential metal ions of life *viz-a-viz*. Ca^{2+} [21–23], Zn^{2+} [24,25], Cu^{2+} [26–29] and Mg^{2+} [30–32] are of paramount importance for biomedical research prompting development of many metal sensitive contrast probes. Towards this goal, Meade and co-workers have developed gadolinium (Gd) based smart MRI contrast agents responsive towards calcium leading to changes in the longitudinal relaxation time of water protons (T_1) in its presence [15,33,34]. Similarly, Logothetis, Toth and co-workers have developed agents for studying fluctuation in extracellular Ca^{2+} levels using BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid bisamide, EDTA (ethylenediamine tetraacetic acid), DTPA and EGTA (ethyleneglycoltetraacetic acid) linked DO3A cores which show 10–40% increase in relaxivity in response to excess Ca^{2+} [21,22,35]. DO3A system conjugated with metal-selective molecular recognition elements provides a promising new class of chemosensors for molecular imaging of biological systems. However, EDTA, EGTA and their derivatives exhibit strong chelation for Ca^{2+} and form complexes with log k_{CaL} ~10 [36]. Consequently, for the development of Ca^{2+} chelator for extracellular calcium concentration ranges, modifications in their structure is required without compromising the selectivity towards Ca^{2+} over other ions. Encouraged by the successful development of a Gd based bis-polyazamacrocyclic non ionic MRI contrast agent, DO3A-AME-DO3A, exhibiting enhanced r_1 [37], an attempt has been made to develop a metal ion specific T_1 agent derived from Gd^{3+}-DO3A linked to chelating iminoacetate groups. Towards this objective, we have synthesized Gd-DO3A-AME-NPHE which is selective towards calcium and is showing potential influence of variation in physiological Ca^{2+} concentration by reflecting changes in T_1 relaxivity.

As the MRI probes must be compatible with biological systems and their practical properties includes water-soluble, non-toxic, and able to image extracellular and/or intracellular spaces, thus the MRI probe discussed here has been synthesized from a biomolecule, an amino acid L-phenylalanine which was derivatized to introduce iminoacetate groups, the site of recognition for Ca^{2+}. It was finally linked to DO3A *via* a linker. The r_1 was modulated by the presence and absence of calcium ion thus inducing changes in hydration number due to the alteration in the structure of the agent. The selectivity and sensitivity of the system was studied in the presence of biologically relevant alkali, alkaline earth and d-block metal ions. Luminescence and UV–Vis absorbance measurements on the corresponding Eu-complex were carried out to assess the hydration state and its variation on Ca^{2+} addition.

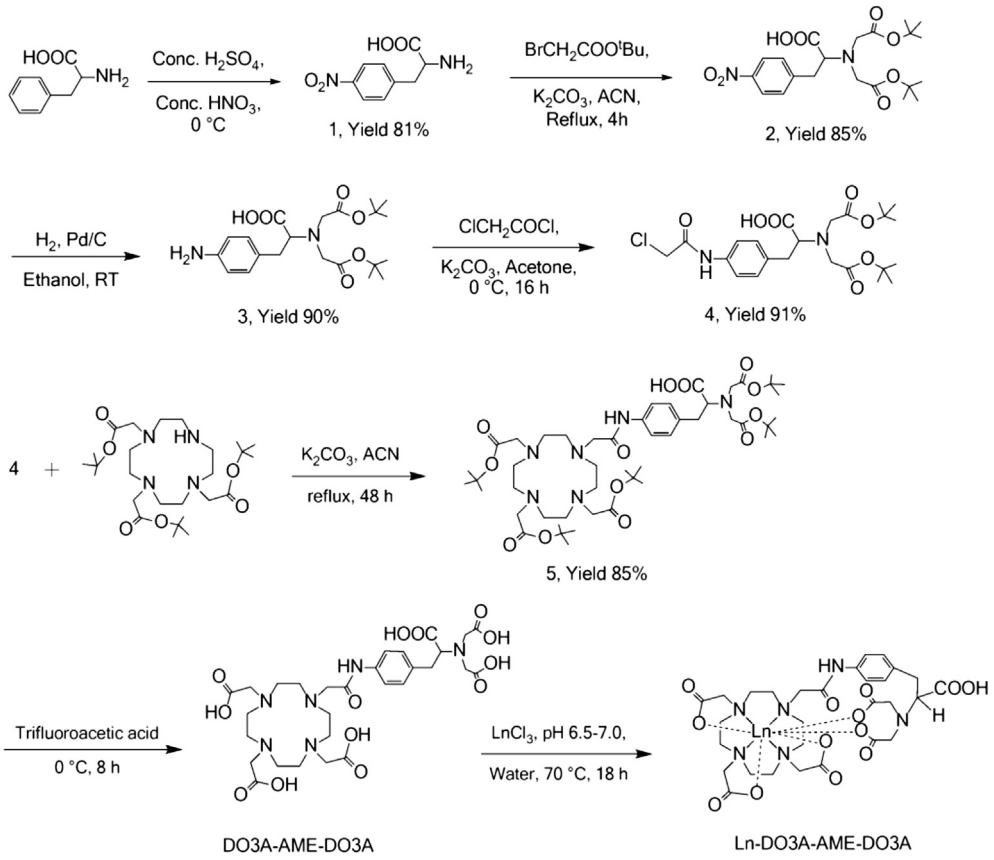
2. Results and discussion

Molecular imaging techniques are being utilized for the diagnosis of different diseases and identification of specific markers upregulation during various physiological processes [38–40]. With the aim to develop a calcium specific contrast agent for magnetic resonance imaging, a synthetic strategy was designed based on a framework derived from an optically active amino acid, L-phenylalanine and DO3A derivative. L-phenylalanine being an essential amino acid is expected to exhibit less toxicity *in-vivo* and was further derivatized to a reactive group to serve the role of bifunctional chelating agent. It possessed desirable rigidity and two nucleophilic reactive centres which were poised for introduction of carboxylates moieties to bind strongly with Ca^{2+} metal ion and another moiety for alkylation of secondary amine of DO3A.

2.1. Synthesis and characterization

A synthetic strategy for calcium specific magnetic resonance contrast agent was designed based on a framework derived from DO3A derivative and L-phenylalanine. The synthesis of DO3A-AME-NPHE, 2,2′,2″-(10-(2-(4-(2-(bis(carboxymethyl)amino)-2-carboxyethyl) phenylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetic acid, was achieved in multi-step reactions. Amino bis (methylene acetates) are known to exhibit efficient chelating properties for biologically relevant ions like Ca^{2+}, Mg^{2+} or Zn^{2+}. But the studies on these type of functionalities are more focussed for Ca^{2+} due to its selective binding with desirable formation constants in the concentration region suitable for tracking changes of Ca^{2+} [41–43]. Thus the properties of the probe were tuned for Ca^{2+} sensing by appending its functionalities with carboxylate groups. L-phenylalanine was nitrated at the para position by carrying out electrophilic nitration with concentrated H_2SO_4 and concentrated HNO_3 giving compound **1** in 81.0% yield. The amino group was derivatized to introduce bis (methylene acetate) coordinating groups which was followed by the reduction of nitro group to the amino group using standard procedure to give compound **3**. Finally, reaction of compound **3** with 2-chloroacetyl chloride gave compound **4**, (2-(bis-(2-*tert*-butoxy-2-oxoethyl) amino)-3-(4-(2-chloroacetamido)phenyl) propanoic acid). Among the variety of linkers available 2-chloroacetyl chloride was used as the source for linkage as its synthetic procedure is straightforward and simple [44]. DFT studies [45] on DO3A-AME-NPHE were performed to calculate the binding proximity on the basis of their chemical potentials and its chemical hardness based on HOMO and LUMO energy parameters with Ca^{2+}. It was found that with 2-chloroacetyl chloride as a linker there was an energy gap of 0.21 between these energy parameters along with a chemical potential of −5.423 eV which suggests that 2-chloroacetyl chloride provides suitable proximity consequently augmenting the binding properties of acetates. It also possessed desirable rigidity and two nucleophilic reactive centres which were poised for introduction of carboxylates moieties to bind strongly with Ca^{2+} metal ion and another moiety for alkylation of secondary amine of DO3A.

Compound **4** was then conjugated with *t*-Bu-DO3A yielding compound **5** which on treatment with trifluoroacetic acid gave the title compound **6**, DO3A-AME-NPHE with a yield of 82% (Scheme 1). Column chromatography was used for the purification of compounds. Structure elucidation was done using ^{1}H NMR, ^{13}C NMR, HETCOR, DEPT and ESI-MS spectrometric techniques. Finally, the complexation of the ligand DO3A-AME-NPHE with the lanthanide ions (Ln = Gd^{3+}, Eu^{3+}) was carried out at a pH 6.5–7.0 at a temperature of 70 °C. After the complexation reaction of DO3A-AME-NPHE with the lanthanide ion, the reaction mixture was passed through chelex-100 (widely used for binding of lanthanide metals) at room temperature to trap the free lanthanide ion and lanthanide complex of DO3A-AME-NPHE was collected as an eluent. Absence of free Ln^{3+} was checked by xylenol orange indicator [46,47]. The lanthanide complexes of DO3A-AME-NPHE were characterized by MS spectrometry. Characteristic isotopic pattern of peaks in the ESI-MS for the complexes confirmed the presence of respective lanthanide ions. The relaxometric, UV and luminescence studies and spectrophotometric studies were carried out with metal-ligand complex to check the potential of the developed compound as a calcium sensing agent.



Scheme 1. Synthetic scheme for 2,2′,2″-(10-(2-(4-(2-(bis(carboxymethyl)amino)-2-carboxyethyl) phenylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetic acid (DO3A-AME-NPHE) and Ln-DO3A-AME-NPHE.

2.2. Relaxometric Ca^{2+} titration of Gd-DO3A-AME-NPHE

Gd-DO3A-AME-NPHE was evaluated as a Ca^{2+} sensing MRI agent by studying its relaxation behaviour in the absence and presence of Ca^{2+}. The longitudinal and transverse relaxation rates of the Gd-DO3A-AME-NPHE protons were measured. Gd-DO3A-AME-NPHE displays a relaxivity (Fig. 1) of $r_1 = 4.37 \pm 0.12$ mM^{-1} s^{-1} and $r_2 = 3.13 \pm 0.25$ mM^{-1} s^{-1}. On addition of Ca^{2+} the relaxivity r_1 gradually enhanced reaching to a maximum of 60% to 6.99 ± .0.16 mM^{-1} s^{-1} at 1.0 equivalent of calcium metal ion and levelled off with further addition. This significant enhancement in investigations thus confirmed our presumption that in the absence of Ca^{2+}, the amino bis(methylene acetate) groups exhibit weak interaction ~3–4 Å with Gd^{3+} through ionic attraction leaving no space for water to interact. However, in the presence of Ca^{2+}, they rearranged to bind Ca^{2+} thus increasing the probability of water to bind directly to Gd^{3+} leading to enhancement of r_1. The sensitivity of the Gd^{3+} complex at different concentration levels was also investigated for which the titration curves were drawn by plotting the relaxivity as a function of the Ca^{2+}/Gd- molar ratio. Upon addition of equimolar amount of EDTA after titration of Gd-DO3A-AME-NPHE with Ca^{2+}, the relaxivity of the complex returned to the initial value observed in Ca^{2+}-free solution, indicating the reversible nature of r_1 and its exclusive dependence on Ca^{2+}. Further, to check the contribution of the α-COOH of L-phenylalanine on the relaxometric response, methyl ester derivative of phenylalanine was used for the preparation of the ligand and relaxation studies were performed on its gadolinium complex. The initial r_1 and r_2 values in the presence and absence of Ca^{2+} ion were found to be similar with the methyl ester derivatized ligand **7** suggestive of participation of α-COOH in the interaction/coordination with either Gd^{3+} or Ca^{2+} was negligible and did not contribute in relaxivity.

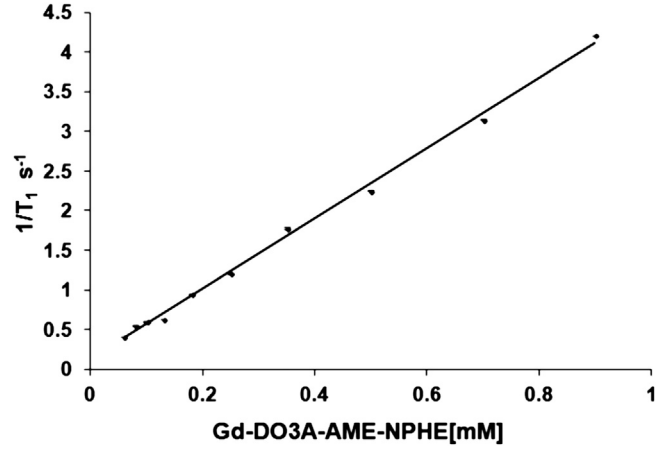


Fig. 1. Dependence of T_1 on the concentration of Gd-DO3A-AME-NPHE at pH 7.4, 4.7 T, 27 °C.

The relaxivity results of Gd-DO3A-AME-NPHE are found to be better than the relaxivities of MRI contrast agents prepared from Gd-DOPTA (500 MHz, 298 K, 3.26 to 5.76 mM^{-1} s^{-1}), bismacrocyclic Gd^{3+} chelate with a BAPTA-bisamide conjugated Gd-DO3A core (500 MHz, 298 K, 3.35 to 3.85 mM^{-1} s^{-1}), DTPA-Gd-derivative (500 MHz, 298 K, 4.7 to 5.4 mM^{-1} s^{-1}). EDTA based Gd-derivative

(500 MHz, 298 K, 3.44 to 6.29 mM^{-1} s^{-1}) exhibited 32% increase in relaxivity in response to Ca^{2+} [23,24,38]. Gd-DO3A-AME-NPHE displayed longitudinal relaxivity, r_1, higher than that of $[Gd(DOTA)(H_2O)]^-$ (3.38 mM^{-1} s^{-1}, 37 °C) which can be attributed to the introduction of rigidity due to the phenyl group in the molecule. However it was found to be quite close to the r_1 measured in the calcium sensing MRI probes (GdL^1-GdL^4) wherein variation in the r_1 was in response to the structure [23].

2.3. Selectivity studies

The selectivity of the MRI contrast agent is influenced by the architecture of the molecule including the type of donor sites used for recognition (hard/soft, neutral/charged, etc.), number of donors, cavity size, and geometry which can all be tailored to match a metal ion of choice. With no vector conjugated to the developed agent which could aid its internalization of the cell, so with the objective to track the changes in extracellular Ca^{2+} concentration, studies were performed to confirm that the amino bis(methylene acetates) would afford higher selectivity for Ca^{2+} over other abundant extracellular metal ions by measuring its relaxivity values in the presence of other competing, biologically relevant metal ions with added calcium at physiological condition. The selectivity experiments were conducted with alkali metal ions, Na^+ (10.0 mM) and K^+ (2.0 mM), alkaline, Mg^{2+}(2.0 mM) and d-orbital, Zn^{2+} (2.0 mM) metal ions were used to check the selectivity of the probe (Fig. 2). The addition the metal ions, Na^+ and K^+ led to a slight increase in the r_1 from the original (<3%), however interference of Zn^{2+} ion upon addition brought 6 and 8% variation in r_1 calculated in the absence and presence of the calcium. The variation was <2 and 6% in the response with Mg^{2+}. Selectivity experiments conducted proved that the ions triggered little variation in the relaxivity of Gd-DO3A-AME-NPHE and their presence led to slight relaxivity enhancements or interfered with calcium ion turn-on responses.

The potential of the title compound to selectively target Ca^{2+} was further demonstrated by the competitive co-binding experiment involving the addition of Ca^{2+} to the Gd^{3+} complex containing Zn^{2+}/Mg^{2+} metal ion solutions. Impressively, 55% increase in relaxivity was observed upon the addition of calcium to the solution. The selectivity studies imply that the developed MRI sensors respond strongly to calcium metal ion thus reducing the probability of false signals and contrast will not be altered by the high background of cations.

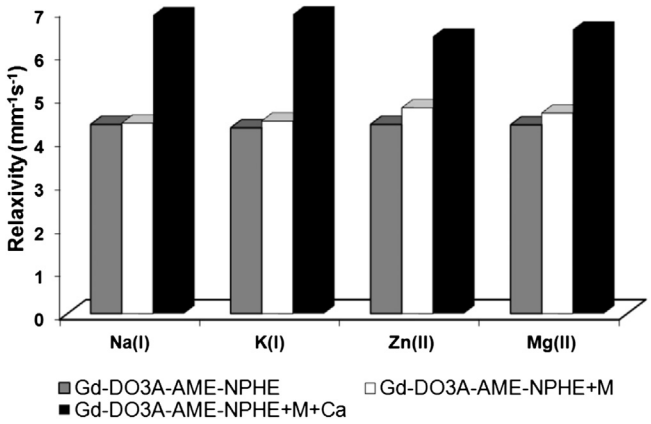


Fig. 2. Selectivity studies of Gd-DO3A-AME-NPHE (0.5 mM) towards calcium in presence of Na^+, K^+, Mg^{2+} and Zn^{2+} (10.0, 2.0, 2.0 and 2.0 mM respectively) before and after the addition of Ca^{2+} (pH 7.4, 4.7 T, 27 °C).

2.4. Luminescence studies

To assess the complex hydration, measurement of the radiative rate constants for the decay of Eu^{3+} or Tb^{3+} excited state in H_2O and D_2O are useful. Therefore for examining the behaviour of Gd-DO3A-AME-NPHE, it was both prudent and informative to study the photophysical properties of europium analogues. Eu^{3+} complex of DO3A-AME-NPHE was prepared and it was assumed that the number of inner sphere water molecules estimated for Eu^{3+} should be close to the corresponding Gd^{3+} complexes. Luminescence lifetime measurements on Eu-DO3A-AME-NPHE showed specific transition of Eu^{3+} complex ($^7F_0 \rightarrow {}^5D_0$) at 578–582 nm. Upon addition of calcium to the solution of Eu-DO3A-AME-NPHE (0.04 mM) the emission band did not produce any change in its pattern but the intensity of the band increased by 25% at pH 7.4 maintained with HEPES buffer.

The hydration number was determined in the first coordination sphere before and after Ca^{2+} is added by measuring the luminescence lifetimes of lanthanide complex. The hydration numbers were calculated according to the Equation (3) [48].

$$q = A'(1/\tau_{H_2O} - 1/\tau_{D_2O}) \quad (3)$$

where, A′ is 1.05 ms.

In the absence of Ca^{2+}, the luminescence τ values were found to be, $\tau_{H_2O} = 0.618$ ms and $\tau_{D_2O} = 0.713$ ms for 10^{-4} M solution of the complex (Table 1). Luminescence lifetime remained constant over the pH range 7.0–8.0, consistent with a single species in solution under these conditions. The experiment was carried out in a set of six. The time resolved luminescence experiment for the complex, Eu-DO3A-AME-NPHE recorded in H_2O and D_2O shows a hydration number (q) of 0.2. Luminescence decay constant was recorded in presence of calcium metal ion an increase in the hydration number (q) to 1.05 was observed with $\tau_{H_2O} = 0.495$ ms and $\tau_{D_2O} = 0.984$ ms. The pattern of the band did not show any variation in the presence of K^+ and Na^+ ions while the intensity decreased by ~3–4% (K^+; 3%, Na^+;4%) in their presence.

2.5. K_d measurements

The absorption spectrum of a solution of Eu-DO3A-AME-NPHE shows an absorbance band at 277 nm changing linearly with an increase in the concentration of Ca^{2+} up to molar ratio of 1:1 Ca^{2+}/Eu-DO3A-AME-NPHE and remained as a plateau upon adding more Ca^{2+} (Fig. 3). Binding analyses using the method of continuous variations obtained through UV–Vis absorption measurements, showed inflection points at a mole fraction of 0.5 for both the Gd-DO3A-AME-NPHE and calcium ion. Furthermore, plots of relaxivity versus [Ca^{2+}] revealed that the observed relaxivity reached a maximum at 1.0 equiv of Ca^{2+} and levelled off at higher concentrations of added Ca^{2+}. These data are consistent with a 1:1 Ca^{2+}/Gd-DO3A-AME-NPHE binding stoichiometry, which was then assumed in all of the K_d calculations. The dissociation constant (K_d) for 1:1 complex was estimated to be 0.027×10^{-3} M.

Table 1
Luminescence lifetime measurements of Eu-DO3A-AME-NPHE at pH 7 and 25 °C.

Ca^{2+} (mM)	τ_{H_2O} (ms)	τ_{D_2O} (ms)	τ_{H_2O}/τ_{D_2O}	q
0.0 mM	0.618	0.713	0.866	0.22
1.0 mM	0.495	0.984	0.503	1.05

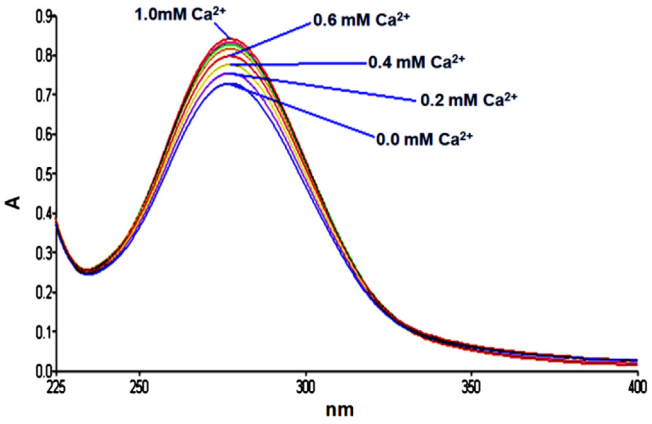


Fig. 3. UV–Vis titration of Eu-DO3A-AME-NPHE with at pH 7.2 in presence and absence of various concentrations of Ca^{2+} ions (0–1.0 equivalent). On addition of Ca^{2+} the relative intensity of the band increases but the spectrum hardly changed (A = Absorbance).

2.6. T_1 weighted imaging studies

With spectroscopic data exhibiting the turn on relaxivity responses, binding properties, metal ion selectivity and calcium induced change in q value for DO3A-AME-NPHE, the ability of probe was tested to detect the change in aqueous calcium using MRI. T_1 weighted images were acquired in the presence and absence of Ca^{2+} at different concentration. Images were taken in absence and presence of (1.0, 0.5, 0.2, 0.1 mM) solution of Ca^{2+} ion to the solution of complex (Fig. 4). T_1 weighted images acquired got brighter upon addition of Ca^{2+} ions and a linear expression was observed between the concentration of Ca^{2+} and brightness. The phantom MR depicts that the title compound can detect contrast between samples with and without added Ca^{2+} and readily visualizes difference in Ca^{2+} level.

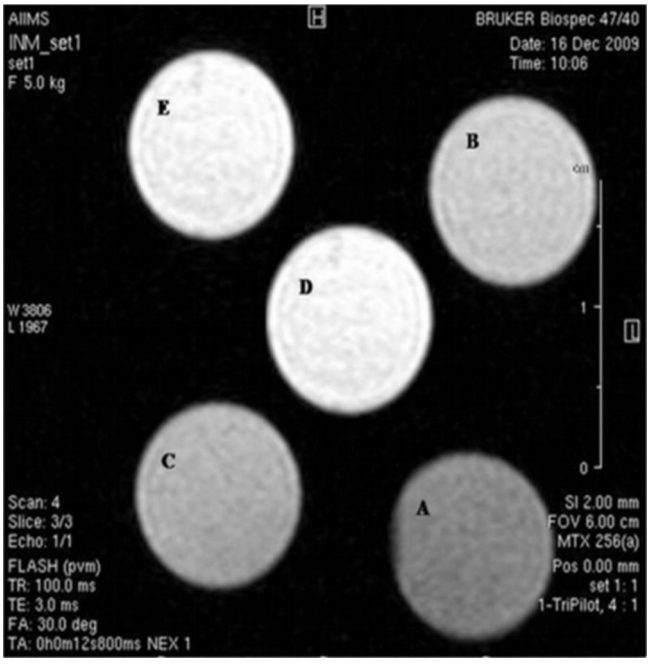


Fig. 4. T_1-weighted phantom MR images of a 0.5 mM solution of Gd-DO3A-AME-NPHE in phosphate buffered saline at various concentrations of Ca^{2+}. A: 0.0 mM Ca^{2+}, B: 0.10 mM Ca^{2+}, C: 0.20 mM Ca^{2+}, D: 0.50 mM Ca^{2+}, E: 1.0 mM Ca^{2+}.

3. Conclusion

A calcium specific magnetic resonance contrast agent was developed based on a framework derived from an optically active amino acid, L-phenylalanine and DO3A derivative. The preliminary results demonstrate the capability of the probe, Gd-DO3A-AME-NPHE as a new Ca^{2+} responsive MRI contrast agent. The probe exhibits a distinct Ca^{2+} triggered enhancement (ca. 60%) in relaxivity with specific selectivity towards Ca^{2+} over alkali (K^+ and Na^+), alkaline earth (Mg^{2+}) and d-block (Zn^{2+}) metal cations. The hydration number of analogous Eu-DO3A-AME-NPHE was determined from luminescence lifetime measurements in the absence ($q = 0.2$) and presence of Ca^{2+} ($q = 1.05$). Visual changes in T_1 phantom images with Ca^{2+} demonstrate the potential feasibility of the MR sensor for molecular imaging applications.

4. Experimental section

4.1. Chemistry

4.1.1. General

1,4,7,10-Tetraazacyclododecane was procured from Strem Chemicals, France. 2-Chloroacetyl chloride, *tert*-butyl-bromoacetate, trifluoroacetic acid, L-phenylalanine, nitric acid, sulphuric acid, hydrochloric acid, triethylamine, ammonium hydroxide, sodium hydroxide, chelex-100, xylenol orange, palladium over activated charcoal, celite, acetonitrile, chloroform, gadolinium trichloride hexahydrate, europium trichloride hexahydrate, zinc chloride, calcium chloride, potassium carbonate, sodium carbonate, were purchased from Aldrich, Germany. Column chromatography was carried out using silica MN60 (60–200 μm), TLC on aluminium plates coated with silica gel 1160, F254 (Merck, Germany). HEPES buffer (0.01 M) of pH 7.4 was used in relaxation and luminescence studies.

NMR spectra were recorded on a Bruker Avance II 400 MHz spectrometer. Electrospray ionization mass spectrometry (ESI-MS) was performed on 6310 system (Agilent, Germany) with ion trap detection in the positive and negative modes. HPLC analyses were performed on Agilent 1200 LC coupled to UV detector ($\lambda = 214$ nm). The C-18 RP Agilent column (5μ, 1 mm × 125 mm) were used applying elution system as described. The flow rate was 3 mL/min with mobile phase being isocratic with 20% 10 mM ammonium acetate and 80% acetonitrile.

For DFT methodology, all the calculations were performed with the Jaguar 7.9 quantum chemistry module of Schrödinger software. The calculated stationary points were characterized by calculating vibrational frequencies with the Hessian obtained during the geometry optimization. Real frequencies were obtained for optimized structures.

4.1.2. Synthesis of 4-nitrophenylalanine (**1**)

In a round bottom flask, conc. H_2SO_4 (0.183 mol, 10.0 mL) was taken and L-phenylalanine (0.030 mol, 5.0 g) was added slowly to dissolve completely. The reaction mixture was cooled down to 0 °C and conc. HNO_3 (0.019 mol, 1.70 mL) was added dropwise to the reaction mixture. The reaction was stirred for 1.0 h at 0 °C. The reaction mixture was then poured into ice-cooled water (50.0 mL) and stirred for 15 min, heated to boil and neutralized by the addition of ammonium hydroxide solution after bringing down to room temperature. The reaction mixture was concentrated under vacuum and kept overnight for crystallization. The crystals formed were filtered, washed with water and recrystallized in water-ethanol solvent system to give product **1** (5.10 g, 81.0%) as white crystalline solid. δ_H (400 MHz, D_2O, Me_4Si): 3.16–2.96 (2H, m, CH_2), 3.52 (1H, t, CH), 7.34 (2H, d, CH), 8.17 (2H, d, CH); δ_C (100 MHz, D_2O,

Me_4Si): 36.54 (CH_2), 55.61 (CH), 1230.42, 130.26 (CH aromatic), 143.87,147.36 (C aromatic), 171.70 (COOH); *m*/*z* (ESI) 211.2 (M^++H $C_9H_{10}N_2O_4$ requires 210.0). Elemental analysis calcd for $C_9H_{10}N_2O_4$: C, 51.43; H, 4.80; N, 13.33. Found: C, 51.38; H, 4.71; N, 13.40.

4.1.3. Synthesis of 2-(bis-tert-butoxycarbonylmethyl-amino)-3-(4-nitrophenyl)-propionic acid (**2**)

Under an atmosphere of nitrogen, 4-nitrophenylalanine (**1**) (0.007 mol, 1.50 g) was dissolved in acetonitrile (15.0 mL), potassium carbonate (0.014 mol, 1.95 g) was added to the solution and stirred for 30.0 min. To the stirred solution *tert*-butylbromoacetate (0.014 mol, 2.09 mL) in acetonitrile (10.0 mL) was added dropwise. After the complete addition, the reaction mixture was refluxed for 18.0 h. The reaction mixture was cooled down to room temperature, filtered and the filtrate was evaporated under reduced pressure. The compound was purified by column chromatography (silica gel, 30% ethyl acetate in petroleum ether, $R_f = 0.55$) to give the final compound **2** (2.66 g, 85.0%) as yellow solid. δ_H (400 MHz, $CDCl_3$, Me_4Si): 1.45 (18H, s, $C(CH_3)_3$), 3.05–3.20 (2H, dd, CH_2), 3.27–3.36 (2H, *q*, CH_2), 3.69 (1H, t, CH), 4.57 (2H, *q*, CH_2), 7.45 (2H, d, CH aromatic), 8.11 (2H, d, CH aromatic); δ_C (100 MHz, $CDCl_3$, Me_4Si): 27.30 (CH_3), 38.95, 49.46, 61.19 (CH_2), 81.37, 82.40 ($C(CH_3)_3$), 123.73, 130.55 (CH aromatic), 141.32, 146.93 (C aromatic), 166.63, 170.63, 172.57 (CO); *m*/*z* (ESI) 439.6 (M^++H $C_{21}H_{30}N_2O_8$ requires 438.2). Elemental analysis calcd for $C_{21}H_{30}N_2O_8$: C, 57.52; H, 6.90; N, 6.39. Found C, 57.48; H, 6.88; N, 6.43.

4.1.4. Synthesis of 3-(4-amino-phenyl)-2-(bis-tert-butoxycarbonylmethyl-amino)-propionic acid (**3**)

In a three necked round bottom flask under an inert atmosphere of nitrogen 2-(bis-*tert*-butoxycarbonylmethyl-amino)-3-(4-nitrophenyl)-propionic acid **2** (4.60 mol, 2.0 g), was taken and dissolved in absolute ethanol (20.0 mL) with stirring. Palladium over activated carbon (10.0%, 2.30 mol, 4.87 g) was added to the stirred solution. The hydrogen gas was purged into the solution till the completion of reaction. The progress of the reaction was monitored by TLC, ethyl acetate: petroleum ether; 1:1. The solution was filtered through celite, filtrate was collected and evaporated under reduced pressure. The compound was purified by column chromatography (silica gel, ethyl acetate/petroleum ether 1:1, $R_f = 0.50$) to give the final compound **3** (1.67 g, 90.0%) as a pale yellow solid. δ_H (400 MHz, $CDCl_3$, Me_4Si): 1.44 (18H, s, $C(CH_3)_3$), 2.87–3.10 (2H, dd, CH_2), 3.26–3.42 (2H, *q*, CH_2), 3.63 (1H, t, CH), 4.59 (2H, s, CH_2), 6.62 (2H, d, CH aromatic), 7.03 (2H, d, CH aromatic); δ_C (100 MHz, $CDCl_3$, Me_4Si): 28.69 (CH_3), 38.33, 49.71, 61.20, 62.40 (CH_2), 81.19, 82.15 ($C(CH_3)_3$), 126.09, 130.14 (CH aromatic), 145.13 (C aromatic), 166.54, 170.73, 173.21 (CO); *m*/*z* (ESI) 409.6 (M^++H $C_{21}H_{32}N_2O_6$ requires 408.2). Elemental analysis calcd for $C_{21}H_{32}N_2O_6$: C, 61.75; H, 7.90; N, 6.86. Found C, 61.72; H, 7.76; N, 6.92.

4.1.5. Synthesis of 2-(bis-(2-tert-butoxy-2-oxoethyl)amino)-3-(4-(2-chloroacetamido) phenyl) propanoic acid (**4**)

In a three necked round bottom flask, under inert atmosphere, compound **3** (0.0012 mol, 0.50 g) was taken and dissolved in dry acetone (15.0 mL). The reaction mixture was brought to 0 °C. Triethylamine (0.0015 mol, 205.0 μL) was added and the reaction mixture was stirred for 15.0 min. To the reaction flask 2-chloroacetyl chloride (0.0015 mol, 118.0 μL) dissolved in dry acetone (10.0 mL) was added dropwise at 0 °C and stirred overnight. The solvent was evaporated under reduced pressure and the residue obtained was extracted in dichloromethane/water mixture. The organic solvent was collected and evaporated under reduced pressure. The compound **4** was purified by column chromatography (silica gel, 50.0% ethyl acetate in petroleum ether, $R_f = 0.65$) and obtained (0.539 g, 91.0%) as a cream coloured solid. δ_H (400 MHz, $CDCl_3$, Me_4Si): 1.46 (18H, s, $C(CH_3)_3$), 2.96–3.14 (2H, dd, CH_2), 3.23–3.40 (2H, *q*, CH_2), 3.61 (1H, t, CH), 4.20 (2H, s, CH_2), 4.50 (2H, s, CH_2), 7.27 (2H, d, CH aromatic), 7.49 (2H, d, CH aromatic); δ_C (100 MHz, $CDCl_3$, Me_4Si): 27.93 (CH_3), 38.76, 42.86, 49.12, 61.32, 61.75 (CH_2), 81.30, 82.62 ($C(CH_3)_3$), 120.17, 129.43, 130.06, 135.43 (CH aromatic), 163.67, 166.47, 170.72, 172.96 (CO); *m*/*z* (ESI) 485.0 (M^++H $C_{21}H_{32}N_2O_6$ requires 484.2). Elemental analysis calcd for $C_{23}H_{33}ClN_2O_7$: C, 56.96; H, 6.86; N, 5.78. Found C, 57.06; H, 6.77; N, 5.88.

4.1.6. Synthesis of 2-(bis(2-tert-butoxy-2-oxoethyl)amino)-3-(4-(2-(4,7,10-tris(2-tert-butoxy-2-oxoethyl) 1,4,7,10-tetraazacyclododecane-1-yl)acetamido) phenyl) propanoic acid (**5**)

Under an inert atmosphere *t*-Bu-DO3A[22] (0.001 mol, 0.50 g) was taken in a round bottom flask and dissolved in acetonitrile (10.0 mL). Potassium carbonate (0.0019 mol, 0.27 g) was added and stirred for 30.0 min. To the stirred solution of reaction mixture compound **4** (0.0015 mol, 0.71 g) dissolved in acetonitrile was added dropwise. After the complete addition of compound **4**, the reaction was refluxed for 18.0 h. The progress of the reaction was checked by TLC (dichloromethane: methanol/9:1). The reaction was cooled down to room temperature and filtered. The filtrate was evaporated under reduced pressure and the obtained residue was purified by column chromatography (silica gel, 5.0% methanol in dichloromethane, $R_f = 0.51$) to give the white compound **5** (0.79 g, 85.0%). δ_H (400 MHz, $CDCl_3$, Me_4Si): 1.43 (54H, s, $C(CH_3)_3$), 2.77–1.73 (18H, br, CH_2), 3.10–2.90 (2H, dd, CH_2), 3.43–3.21 (2H, *q*, CH_2), 3.63 (1H, t, CH), 4.49 (2H, s, CH_2), 7.06 (2H, d, CH aromatic), 7.71 (2H, d, CH aromatic); δ_C (100 MHz, $CDCl_3$, Me_4Si): 27.95 (CH_3), 39.00, 49.04, 53.41, 55.66, 61.32, 61.75 (CH_2), 81.04, 82.12 ($C(CH_3)_3$), 120.40, 129.01, 131.26, 138.13 (CH aromatic), 166.58, 167.41, 170.97, 172.95 (CO); *m*/*z* (ESI) 963.7 (M^++H $C_{21}H_{32}N_2O_6$ requires 962.59). Elemental analysis calcd for $C_{49}H_{82}N_6O_{13}$: C, 61.10; H, 8.58; N, 8.73. Found C, 61.07; H, 8.47; N, 8.79.

4.1.7. Synthesis of 2,2′,2″-(10-(2-(4-(2-(bis(carboxymethyl)amino)-2-carboxyethyl) phenylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetic acid (DO3A-AME-NPHE, **6**)

The ligand **5** (0.0033 mol, 1.0 g) was dissolved in 3.0 mL of neat trifluoroacetic acid at 0 °C and stirred for 4.0 h. After that the reaction mixture was stirred at room temperature for additional 10.0 h. The solvent was evaporated under reduced pressure and the residue was dissolved in 1.0 mL of MeOH, followed by addition of 30.0 mL of diethyl ether dropwise at 0–5 °C. The reaction solution was stirred for 1.0 h at room temperature. The compound was dried, dissolved in water and neutralized to pH 7.0 by the addition of 1.0 M aqueous NaOH. The crude product was purified by preparative HPLC to give compound **6** (0.58 g, 82.0%). δ_H (400 MHz, D_2O, Me_4Si): 4.35–2.75 (18H, br, CH_2), 4.51 (1H, t, CH), 7.21 (2H, d, CH aromatic), 7.39 (2H, d, CH aromatic); δ_C (100 MHz, D_2O, Me_4Si): 34.49, 48.25, 60.34, 62.37, 65.95 (CH_2), 120.60, 121.09, 130.05 (CH aromatic), 162.31, 162.66, 163.37, 167.85, 168.53, 170.71 (CO); *m*/*z* (ESI) 681.2 (M^+−H $C_{29}H_{42}N_6O_{13}$ requires 682.2). Elemental analysis calcd for $C_{29}H_{42}N_6O_{13}$: C, 51.02; H, 6.20; N, 12.31. Found C, 51.01; H, 6.12; N, 12.35.

4.1.8. Synthesis of 2,2′,2″-(10-(2-(4-(2-(bis(carboxymethyl)amino)-3-methoxy-3-oxopropyl)phenylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (DO3A-AME-NPHME, **7**)

The synthetic procedures followed were similar to the procedures used in the synthesis of compound **1**–**6** wherein instead of L-phenylalanine, methyl ester of L-phenylalanine was taken as one of the precursors. The yield of the final purified compound **7** was 85.2%. δ_H (400 MHz, D_2O, Me_4Si): 4.32–2.81 (18H, br, CH_2), 3.74(3H,

s, $COOCH_3$) 4.52 (1H, t, CH), 7.21 (2H, d, CH aromatic), 7.39 (2H, d, CH aromatic); δ_C (100 MHz, D_2O, Me_4Si): 34.45, 48.25, 60.40, 62.41, 65.91 (CH_2),42.01(CH_3) 120.60, 121.12, 130.00 (CH aromatic), 162.41, 162.60, 163.29, 167.05, 168.33, 170.71 (CO); *m*/*z* (ESI) 696.2 (M^+–H $C_{30}H_{45}N_6O_{13}$ requires 697.2).

4.1.9. Synthesis of lanthanide (III) complexes of DO3A-AME-NPHE

The introduction of lanthanide ion into the macrocyclic framework was done at pH 7.0. To a stirred aqueous solution of ligand DO3A-AME-NPHE (10^{-4} mol), a solution of 1.0 M NaOH was added until pH 7.0 was attained. A solution of $LnCl_3$ was prepared in water and was added dropwise to the ligand solution in 1:1 M ratios. The reaction mixture was heated at 65–70 °C for 18.0 h and the pH of the solution was periodically adjusted to 7.0 by addition of aliquots of NaOH solution. After 18.0 h, the reaction mixture was cooled to room temperature and then passed through an ion-exchange column packed with chelex-100 resin at room temperature. The absence of any free lanthanide (III) ion was checked by xylenol orange indicator. The compound was lyophilized, and white solid was obtained. The formation of the metal complex Ln-DO3A-AME-NPHE (Ln = Gd^{3+}, Eu^{3+}) was confirmed by mass spectrometry. Calculated mass [M^+] 834, found *m*/*z* (ESI) 833.0 (M^+–H Gd-DO3A-AME-NPHE, $C_{29}H_{37}GdN_6O_{13}$ requires 834), Elemental analysis calcd for $C_{29}H_{37}GdN_6O_{13}$: C, 41.77; H, 4.35; N, 10.08. Found C, 41.90; H, 4.28; N, 10.21. *m*/*z* (ESI) 826.0 (M^+–H Eu-DO3A-AME-NPHE, $C_{29}H_{37}EuN_6O_{13}$ requires 827). Elemental analysis calcd for $C_{29}H_{37}EuN_6O_{13}$: C, 42.04; H, 4.38; N, 10.14. Found C, 41.95; H, 4.28; N, 10.27.

4.2. Relaxation studies

Experiments were carried out on a 4.7 T horizontal Bruker Biospec 47/50 (Bruker, Ettlingen, Germany) at 27 °C. The measurement of longitudinal and transverse relaxation rates (r_1 and r_2) of the complex Gd-DO3A-AME-NPHE were carried out at different concentrations. A set of Gd-complex solution of different concentration (0.06–6.0 mM) were prepared. The total volume of the solution was kept constant (5.0 mL) and the sampling was done by avoiding air gap in the tube. The solutions were prepared in HEPES buffered saline of physiological pH. A stock solution of calcium chloride of concentration (10.0 mM) was prepared and its appropriate concentration was added to the Gd-DO3A-AME-NPHE solutions. An incubation time of 30 min was given to the solutions prior to the experiments. Relaxation rate measurements were performed at 27 °C on a 4.7 T MRI machine.

Longitudinal (T_1) measurements were performed with an inversion-recovery pulse sequence (increment of inversion delay: 10 ms with 456 increments) followed by a RARE imaging sequence (RARE Factor: 16; TR/TE_{eff}: 5000/7.7 ms; FOV: 25 × 25 mm; Matrix: 128 × 128; slice thickness: 1 mm). T_1 values were measured from six data points generated by inversion recovery pulse sequence. Fitting of T_1 value was done voxel wise on selected ROIs using IGORpro (Wavemetric).

Transverse (T_2) measurements were performed with a Carr-Purcell-Meiboon-Gill imaging sequence (TR: 5000, inter-echo time: 5 ms, number of echoes: 128; FOV: 25 × 25 mm; Matrix: 128 × 128; slice thickness: 1 mm). The longitudinal relaxivity (r_1) and transverse relaxivity (r_2) was measured from concentration dependent relaxation times (T_1 and T_2) of the Gd-complex. The effective relaxivity was determined from the equation [9–11]:

$$1/T_{obs,(1,2)} = 1/T_{d,(1,2)} + r_{(1,2)}[Gd] \quad (1)$$

Where, $T_{obs,(1,2)}$ is the observed longitudinal relaxation time, $T_{d,(1,2)}$ is the diamagnetic contribution in the absence of the paramagnetic substance, and [Gd] is the concentration of Gd^{3+} complex. Relaxivities, r_1 and r_2 were calculated from the slope of linear regression fits of $1/T_1$ and $1/T_2$ against the gadolinium concentration.

For metal ion selectivity experiments, stock solution (1.0 M) of NaCl, KCl, $ZnCl_2$ and $MgCl_2$ were prepared and appropriate concentrations (NaCl: 10.0 mM, KCl: 2.0 mM, $MgCl_2$: 2.0 mM, $ZnCl_2$: 2.0 mM) were prepared by dilution method. Solutions of Gd-DO3A-AME-NPHE of concentration, 0.5 and 0.75 mM were used for the selectivity studies. The titrations were performed at 4.7 T, 25 °C and pH 7.0–7.4. Metal ion solutions of known concentration were added in stepwise portions to the complex solution and the longitudinal proton relaxation time (T_1) was measured after each addition of the analyte. The solution of Ca^{2+} was added in the 1:1 M ratio and the relaxation time, T_1 was again measured. The relaxivity (r_1) was calculated using the actual Gd^{3+} concentration each point of the titrations.

4.3. UV–Vis and luminescence studies

UV–vis spectrum of the Eu-DO3A-AME-NPHE complex was obtained on a Cary Varian double beam spectrophotometer (Cary BIO 100, Australia at 450–600 nm). A stock solution of Eu-DO3A-AME-NPHE of concentration 0.1 M was prepared and diluted as per requirement. The emission wavelength for ligand was found at 348 nm while for specific transition of Eu(III) complex ($^7F_0 \rightarrow {}^5D_0$) was found 578–582 nm.

The lifetime experiment was carried out in D_2O and H_2O solutions in the absence and presence of Ca^{2+} metal ions. Luminescence decay was recorded in the short phosphorescence lifetime mode and was repeated 6 times under each condition. Luminescence lifetime was calculated from the mono exponential fitting of the average decaying data. A stock solution (0.10 M) of Eu-DO3A-AME-NPHE complex was prepared and diluted as per requirement. The pH of the solution was adjusted to 7.0 and the solution was stirred overnight at 25 °C.

The pseudo-first-order kinetics were measured by putting the conditions M >> L. Thermodynamic parameters were calculated by using standard least-square procedures to fit the data to the expression.

Luminescence measurements were carried out on spectrofluorimeter FS920 (Edinburgh Instruments, UK) equipped with Xenon arc lamp. The temperature of the sample holder was regulated with a peltier cooled thermostat. Luminescence lifetime measurements were calculated by customized integrated steady state spectrofluorimeter and fluorescence lifetime instrument FL900CDT (Edinburgh Analytical Instruments, UK). The excitation source was hydrogen gas filled nanosecond flash lamp nF900 filled with low hydrogen gas pressure of 0.4 bar operating at frequency of 40 KHz. The intensity decay curves were obtained at emission maximum and fitted as sum of exponentials as:

$$I_t = I_0 \sum A_i \exp(-t/\tau_i) \quad (2)$$

where, τ_i and A_i represent the fluorescence lifetime and pre exponential factor for *i*th decay component. The luminescence decay was noted in a short phosphorescence lifetime mode and repeated at least five times under each condition. The luminescence lifetime was determined from the mono exponential fitting of the average decay data.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.05.046.

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