Synthesis and characterization of a smart contrast agent sensitive to calcium†

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A novel first-generation Ca^{2+} sensitive contrast agent, Gd-DOPTRA has been synthesized and characterized. The agent shows ~100% relaxivity enhancement upon addition of Ca^{2+}. The agent is selective and sensitive to Ca^{2+} also in the presence of Mg^{2+} and Zn^{2+}. The relaxivity studies carried out in physiological fluids prove the prospects of the agent for *in vivo* measurements.

After the first report of Ca^{2+} as a signaling molecule in muscles, its role as a carrier of information has been recognized and this fact has invaded all corners of biology from biochemistry to cell biology and biophysics.[1] Ca^{2+} plays an important dual role as a carrier of electrical current and as a second messenger in the brain. Since its actions are mediated by a large array of proteins including protein kinases, the effects are much more diverse than that of the other second messengers such as cAMP (3′,5′-cyclic adenosine monophosphate) and DAG (diacylglycerol).[2] The concentration of Ca^{2+} outside the cell ($[Ca^{2+}]_o$) is 1.5–2 mM while it is only 50–100 nM inside the cells resulting in an extreme Ca^{2+} gradient of 15 000–40 000:1, outside to inside.[3] Studies done using ion selective micropipettes have shown that during normal brain activity, the $[Ca^{2+}]_o$ decreases ~15%. However, during maximal stimulation, $[Ca^{2+}]_o$ drops 30% while under traumatic events such as epileptic seizures and terminal anoxia it can decrease up to 90%.[4] The modulation in Ca^{2+} concentrations, both inside and outside the cell is a significant factor in determining nervous system function in both the normal as well as in pathological conditions.[5] Development of fluorescent dyes has greatly added to our understanding of this critical role played by Ca^{2+}. However the depth penetration limit of optical imaging techniques and production of toxic photobleaching byproducts of fluorescent dyes stimulated the development of 'smart contrast agents' for MRI which can provide information about physiological signals and biochemical events noninvasively and with high spatial resolution.[6] Along these lines, Li *et al.* have proposed a potential MRI contrast agent, Gd-DOPTA which is sensitive to Ca^{2+} concentration in the 0.1–10 μM range with an apparent dissociation constant of 0.96 μM.[6] We recently reported a modification of Gd-DOPTA which is suitable for $[Ca^{2+}]_o$ measurement, however the relaxivity response of this probe to Ca^{2+} is too low for *in vivo* measurements.[7]

With the objective of tracking the modulation in Ca^{2+} with a high relaxivity response, we synthesized a novel calcium sensitive MRI contrast agent, Gd-DOPTRA. Gd-DOPTRA has proven to be both selective and sensitive to Ca^{2+} over its competitor cation Mg^{2+}, with a relaxivity response of ~100% on addition of Ca^{2+}. Gd-DOPTRA has been designed by exploiting the Ca^{2+} chelating properties of APTRA (*o*-aminophenol-*N*,*N*,*O*-triacetate) linked to a Gd^{3+} loaded DO3A unit. The choice of a low affinity pentadentate Ca^{2+} chelator, APTRA (K_d = 20–25 μM)[8] was made because high affinity chelators (such as BAPTA, K_d = 0.1–0.4 μM) would most likely result in saturation of the indicator when the calcium concentration ($[Ca^{2+}]$) increases above 1 μM. Further, due to their slow kinetics, they have limited ability to follow rapid changes in $[Ca^{2+}]$ and significantly contribute to Ca^{2+} buffering capacity.[9] Low affinity chelators have been reported to show fewer problems with local saturation, Ca^{2+} binding kinetics, and Ca^{2+} buffering.[9] Another advantage of choosing APTRA as chelator is to simplify the overall synthesis of the contrast agent (CA) with just a simple seven-step synthesis (Scheme 1).

The synthesis started with 2-nitroresorcinol which was monobenzylated using benzyl bromide giving **1** in 85% yield. Alkylation of phenol **1** was done with 1,3-dibromopropane to give the alkyl bromide **2** in 88% yield. This was then used for alkylation of tris-*tert*-Bu-DO3A to give the macrocycle **4** in 66% yield. Thereafter, the NO_2 group was reduced with simultaneous removal of the benzyl group by hydrogenation using Pd–C as the catalyst to obtain **5** in 85% yield. The tris-*tert*-butylester **5** was then hydrolysed in neat TFA to give triacid **6** in 68% yield. As the alkylation of aniline **5** with *tert*-butylbromoacetate or with methylbromoacetate was not successful, yielding a mixture of two products which were difficult to separate, **7** was finally obtained in moderate yield by alkylation of **6** with bromoacetic acid and NaOH. The ligand **7** was purified by RP-HPLC and loaded with Ln^{3+} (Gd^{3+} or Eu^{3+}) using $LnCl_3{\cdot}6H_2O$ in water at pH 7. The final concentration of Gd^{3+} was determined by ICP-OES. Obtained complexes once formed were stable, however very slow hydrolysis of one acetate arm was observed similarly to a previously

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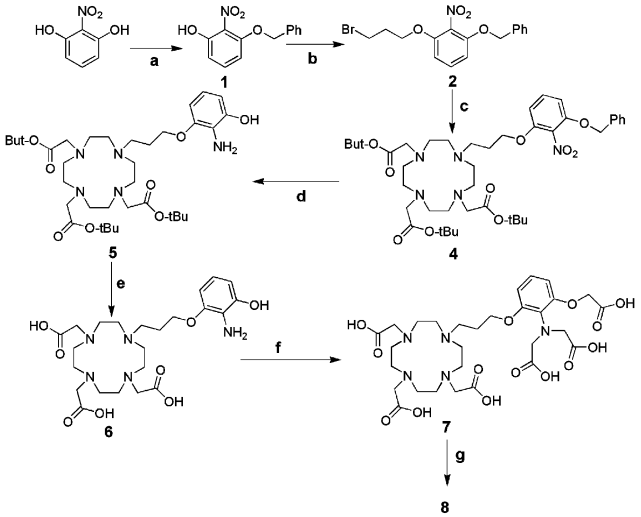


Scheme 1 Synthesis of Gd-DOPTRA. *Reagents and conditions*: (a) benzyl bromide, K_2CO_3, MeCN; (b) 1,3-dibromopropane, K_2CO_3, DMF; (c) tris-*tert*-Bu-DO3A, K_2CO_3, DMF; (d) H_2, Pd–C; (e) TFA (neat); (f) bromoacetic acid, NaOH, H_2O; (g) $GdCl_3{\cdot}6H_2O$.

reported molecule.[10] The mechanism of hydrolysis has yet to be explored and is under investigation.

The relaxivity response of the synthesized CA was checked at 400 MHz in KMOPS buffer at pH 7.4. In the absence of Ca^{2+} the relaxivity observed was 3.5 mM^{-1} s^{-1} which increased by 97% to 6.9 mM^{-1} s^{-1} upon addition of 1 equiv. of Ca^{2+} and leveled off with further addition (Fig. 1(a)), demonstrating the high sensitivity of the CA toward Ca^{2+}. Furthermore, the binding of CA to Ca^{2+} is reversible; addition of EDTA to a solution of CA saturated with Ca^{2+} brings back the increased relaxivity to the initial value (Fig. 1(a)).

The Mg^{2+} concentration inside the brain during neural activity remains nearly constant. Thus we checked the selectivity of Gd-DOPTRA by measuring its relaxivity response toward Ca^{2+} in a Mg^{2+} containing buffer. At resting state the cerebro spinal fluid (CSF) within the brain has 0.7 mM of Mg^{2+} as compared to 1 mM of Ca^{2+}.[11] (CSF is the fluid that occupies the subarachnoid space and ventricular system around and inside the brain. The extracellular space of the brain freely communicates with the CSF compartment and therefore the compositions of the two fluids are similar).[11,12] When the CA was dissolved in a buffer containing more than half an eq. of Mg^{2+} (0.62 equiv.), the relaxivity observed was 4.4 mM^{-1} s^{-1} which is only 25% higher than the relaxivity of CA in Mg^{2+} free buffer, whereas the increase in relaxivity was ~60% with the same amount of Ca^{2+} added. When Ca^{2+} was added to the above Mg^{2+} containing buffer, 70% relaxivity enhancement was observed. This shows that the agent is selective to [Ca^{2+}] changes even in presence of a constant [Mg^{2+}]. We also checked the effect on relaxivity of CA due to Zn^{2+} binding, as a similar ligand has been found to show a Zn^{2+} binding effect.[13] The Ca^{2+} titration was performed with Zn^{2+} containing (0.5 equiv.) buffer. The initial relaxivity observed was 4.2 mM^{-1} s^{-1}, which is 17% higher than the relaxivity observed in Zn^{2+} free buffer, whereas the relaxivity enhancement was ~49% with the same amount of Ca^{2+} added. Further addition of Ca^{2+} to Zn^{2+} containing buffer

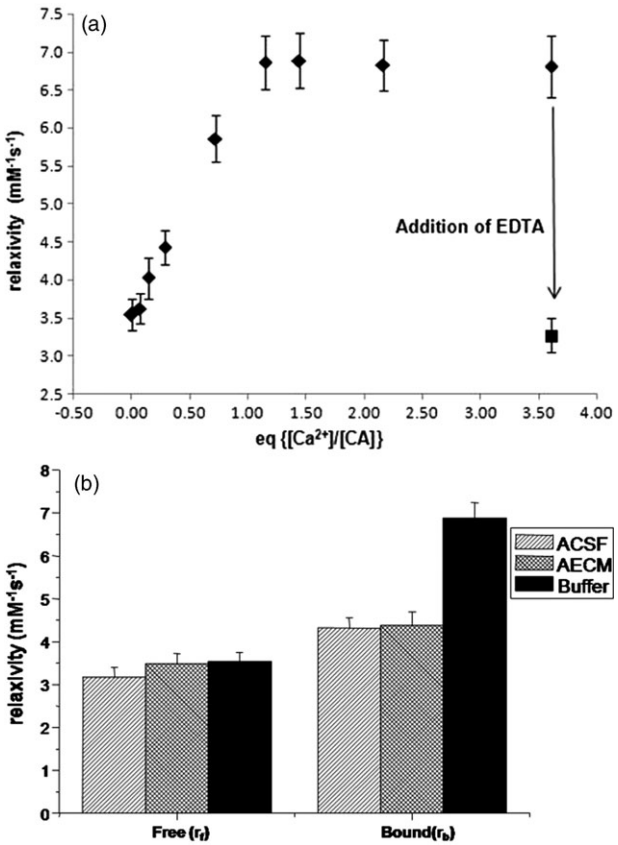
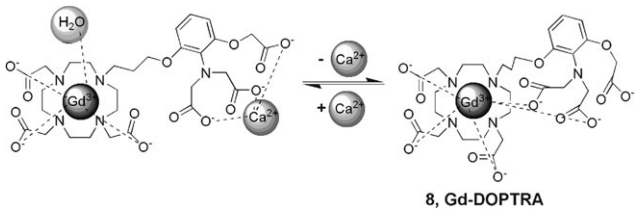


Fig. 1 (a) Relaxivity enhancement profile of Gd-DOPTRA with Ca^{2+} in KMOPS buffer at pH 7.4, 27 °C (◆), relaxivity observed on addition of EDTA after addition of 3.6 eq. of Ca^{2+} (■). (b) Comparative relaxivity enhancement profile of Gd-DOPTRA in KMOPS buffer, ACSF and AECM; r_f and r_b are the relaxivity value corresponding to free form and bound form of the agent to Ca^{2+}.

solution of CA resulted in 70% relaxivity enhancement. This shows the selectivity of CA for Ca^{2+} over Zn^{2+} as well. As the concentration of Zn^{2+} in the extracellular space of the brain is much lower[14] as compared to Ca^{2+} and Mg^{2+}, the observed weak Zn^{2+} binding to the CA should not interfere with its response to large Ca^{2+} modulation observed during synaptic transmission.

We further checked the relaxivity response in Ca^{2+} free artificial cerebro spinal fluid (ACSF) at 37 °C (see ESI†). The relaxivity enhancement was observed to be 36%. This signifies both the selectivity and sensitivity of the agent in a physiological environment toward Ca^{2+}. To further explore the efficacy of CA, we performed relaxivity measurement in the artificial extracellular matrix (AECM, for exact composition see ESI†). ECM is a lattice of proteins, polysaccharides and various compounds attached to the plasma membrane. ECM materials are mostly present in intercellular spaces between neurons and glia.[15] The maximum changes observed were 27% at 27 °C and 25% at 37 °C (Fig. 1(b)). The drop in relaxivity in biological media is likely due to anion binding to Gd in the presence of Ca^{2+}. Anion binding will also block water access and this problem is well established for the DO3A class of complexes.[16,17] However these changes could be sufficient to report dynamics of Ca^{2+} in the brain.



Scheme 2 Equilibrium reaction with Ca^{2+}.

The dissociation constant (K_d) was determined using paramagnetic relaxation enhancement (PRE) method. The relaxivity enhancement plot of CA *vs.* [Ca^{2+}] was fitted to the equation described in the ESI.† The K_d was found to be $\sim$11 μM.

In order to elucidate the main parameter responsible for relaxivity enhancement, we have performed luminescence lifetime measurements on Eu^{3+} loaded ligand (Eu-DOPTRA) in H_2O and D_2O solutions. The hydration number, q was calculated according to the revised equation of Beeby *et al.*[18] In the absence of Ca^{2+}, q was observed to be 0.17 while in the presence of Ca^{2+}, it increases to 0.88 (Scheme 2). This proves that the relaxivity enhancement of Gd-DOPTRA in the presence of Ca^{2+} is largely determined by the changes in hydration number of the complex.[7,10,16]

In conclusion, we have reported the synthesis of a novel first-generation calcium-sensitive MRI contrast agent, Gd-DOPTRA. The probe showed $\sim$100% increase in relaxivity upon addition of Ca^{2+}. Relaxivity studies carried out in physiological fluids such as ACSF and AECM prove the prospects of the agent for *in vivo* measurements. The structural design of the agent also offers the possibility for various modifications that could be made to synthesize a series of derivatives with a range of Ca^{2+} binding affinities. Further investigations aim at modifying the present molecule in order to achieve an even better selectivity toward Ca^{2+}, particularly in physiological fluids.

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