Supporting Information

Synthesis and Characterization of a Smart Contrast Agent Sensitive to Calcium

Kirti Dhingra,[a*] Martin E. Maier,[b] Michael Beyerlein,[a] Goran Angelovski,[a] Nikos K. Logothetis,[a,c*]

[a] Max Planck Institute for Biological Cybernetics, Department of Physiology of Cognitive Processes, Tübingen, Germany
[b] Institut für Organische Chemie, University of Tübingen, Tübingen, Germany

[c] Imaging Science and Biomedical Engineering, University of Manchester, Manchester, U.K.

Correspondence to be addressed to:

1. Kirti Dhingra, Max Planck Institute for Biological Cybernetics, Tuebingen, Germany

   Phone : 00 49 7071 601 917 ; fax : 00 49 7071 601 919.

   Kirti.Dhingra@tuebingen.mpg.de.

2. Nikos K. Logothetis, Max Planck Institute for Biological Cybernetics, Tübingen, Germany.

   Nikos.Logothetis@tuebingen.mpg.de

Materials and Method: All chemicals were purchased at the purest grade from commercial available sources. *o*-Nitroresorcinol, benzyl bromide, dibromopropane, lanthanide (III) chloride were purchased from Sigma-Aldrich, Germany. Cyclen was purchased from Strem, France. Acetonitrile (analytical and HPLC grade), dichloromethane and methanol were purchased from Acros Organics, Germany. Tris-*tert*-Bu-DO3A was synthesized according to a reported procedure[1] Column chromatography was performed on silica (60-200 mesh), Merck. Analytical thin layer chromatography was performed on aluminium sheet silica gel 60. HPLC was performed at room temperature on a Varian PrepStar Instrument, Australia, equipped with a PrepStar 335 photodiode array detector at 254 nm. Reversed phase analytical HPLC was performed in a stainless steel Chromsep C18 column (length 25 cm, internal diameter 4.6 mm, outside diameter 3/8 in. and particle size 8 μm, Varian advanced chromatographic solutions). The final ligand was purified using following method : 95% solvent A (H_2O, 0.1% HCOOH) and 5% solvent B (acetonitrile, 0.1% HCOOH) to 70% solvent B in 10 minutes and then to 100% in next 8 minutes running isocratic for 12 minutes after that and then back to 5% solvent B in next 2 minutes. The flow rate used for analytical HPLC was 1 ml/min and for preparative 50 ml/min. All solvents used were HPLC grade filtered through 0.45 μm nylon-66 Millipore filter prior to use. ESI-LRMS spectra were performed on a SL 1100 system (Agilent, Germany) with ion-trap detection in positive and negative ion mode. ESI-HRMS was performed on a Bruker Daltonics Apex II FT-ICR-MS (Bruker, Germany). Inductively coupled plasma optical emission spectrometry (ICP-OES) was performed on a Jobin Yvon Ultima 2 ICP-OES at the Department of Chemistry, Durham University, UK.

3-(Benzyloxy)-2-nitrophenol (1) A suspension of 2-Nitroresorcinol (5.0 g, 32.2 mmol) and K_2CO_3 (0.45 g, 3.22 mmol) in dry MeCN (25 ml) was heated at 60°C under nitrogen atmosphere for 1 h. To the resulting solution, benzyl bromide (0.39 ml, 3.22 mmol) dissolved in 10 ml of MeCN (dry) was added slowly within 1 h. The mixture was heated at the same temperature overnight under nitrogen conditions. It was then cooled down to room temperature, filtered and excess MeCN was evaporated. The resulting residue was purified by column chromatography using ethylacetate/hexane. Unreacted 2-nitroresorcinol was recovered in 75% yield as bright orange solid using 2% ethylacetate/hexane as eluent while the benzyl ether **1** was obtained in 0.66 g yield (85%) as bright yellow solid using 5-10% ethylacetate/hexane. ^{1}H NMR (400 MHz, $CDCl_3$, 25°C): δ ppm = 5.21 (s, 2 H), 6.61 (d, *J*=8.4 Hz, 1 H), 6.72 (d, *J*=8.4 Hz, 1 H), 7.32–7.38 (m, 2 H), 7.38–7.45 (m, 2 H), 7.47–7.52 (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$, 25°C): 71.3, 105.0, 110.9, 126.8, 128.1, 128.6, 135.4, 135.5, 154.6, 155.6. ESI-HRMS calculated for $[C_{13}H_{11}NO_4 - H]^-$ 244.06153, found 244.06156.

1-(Benzyloxy)-3-(3-bromopropoxy)-2-nitrobenzene (2). A mixture of phenol **1** (0.98 g, 4 mmol) and K_2CO_3 (1.4 g, 8.0 mmol) in dry DMF was heated to 70°C under nitrogen atmosphere for 1 h. After cooling to room temperature, dibromopropane (0.9 ml, 12 mmol) was dded. The resulting mixture was heated to 85°C for 2 hours. The reaction mixture was cooled down to room temperature and water was added to it. After extraction with chloroform, the organic layer was dried with anhydrous Na_2SO_4, filtered, and evaporated. The residue was purified by column chromatography using 7-10% ethylacetate/hexane to obtain the ether **2** as light yellow oil. Yield 1.20 g (88%). ^{1}H NMR (400 MHz, $CDCl_3$, 25°C): δ ppm = 2.13–2.24 (m, 2 H), 3.45 (t, *J*=6.4

Hz, 2 H), 4.10 (t, *J*=5.8 Hz, 2 H), 5.06 (s, 2 H), 6.55 (t, *J*=6.9 Hz, 2 H), 7.15–7.19 (m, 1 H), 7.19–7.25 (m, 1 H), 7.25–7.30 (m, 4 H). ^{13}C NMR (100 MHz, $CDCl_3$, 25°C): 29.6, 31.9, 66.7, 70.9, 105.6, 106.2, 126.9, 128.2, 128.7, 131.0, 135.5, 150.8, 150.9. ESI-HRMS calculated for $[C_{16}H_{16}BrNO_4 + Na]^+$ 365.01549, found 388.01569.

[4-[3-(3-Benzyloxy-2-nitro-phenoxy)-propyl]-7,10-bis-*tert*-butoxycarbonylmethyl-1,4,7,10tetraaza-cyclododec-1-yl]-acetic acid *tert*-butyl ester (4). A mixture of macrocycle **3** (1.84 g, 3.7 mmol) and K_2CO_3 (1.3 g, 9.25 mmol) in dry DMF (15 ml) was heated for 1 h under nitrogen atmosphere at 60°C. To this KI (0.083 g, 0.05 mmol) was added followed by slow addition of bromide **2** (1.75 g, 4.8 mmol) dissolved in dry DMF (5 ml). After complete addition the reaction mixture was heated overnight at the same temperature. The reaction mixture was cooled down and excess of DMF was evaporated. Water was added to it and the resulting mixture was extracted with dichloromethane The organic layer was dried with anhydrous Na_2SO_4, filtered, and evaporated to obtain a yellow oil. Crude product was purified by column chromatography using 2% MeOH/CH_2Cl_2 to obtain product **4** as light yellow fluffy powder. Yield 1.80 g (66%). ^{1}H NMR (250 MHz, $CDCl_3$): δ ppm = 1.72–1.88 (m, 2 H), 2.26 (s, 7 H), 2.49 (s, 4 H), 2.68 (br s, 4 H), 2.74 (s, 3 H), 2.83 (s, 2 H), 2.96 (d, *J*=6.9 Hz, 2 H), 3.05 (br s, 4 H), 3.94 (t, *J*=5.9 Hz, 2 H), 5.04 (s, 3 H), 6.57 (t, *J*=8.3 Hz, 2 H), 7.14–7.21 (m, 2 H), 7.25 (s, 4 H). ^{13}CNMR (100 MHz, $CDCl_3$, 25°C): 25.2, 27.8, 27.9, 50.1, 50.2, 50.7, 52.8, 55.8, 56.6, 68.1, 71.0, 82.6, 82.9, 106.0, 106.4, 127.1, 128.2, 128.6, 131.5, 135.7, 150.7, 151.0, 172.6, 173.6. ESI-HRMS calculated for $[C_{42}H_{65}N_5O_{10} + H]^+$ 800.48042, found 800.48049.

[4-[3-(2-Amino-3-hydroxy-phenoxy)-propyl]-7,10-bis-*tert*-butoxycarbonylmethyl-1,4,7,10tetraaza-cyclododec-1-yl]-acetic acid *tert*-butyl ester (5). A solution of nitro compound **4** (1.5 g, 1.87 mmol) in methanol (10 ml) was hydrogenated under 2 psi of hydrogen over Pd-C (10%, 0.15 mg) for 4 h in a Parr apparatus Thereafter, the mixture was filtered and the filtrate was concentrated in vacuo. The crude product was used as such for the next reaction without further purification. Yield 1.0 g (85%). ^{1}H NMR (400 MHz, $CDCl_3$, 25°C): δ ppm = 1.45 (s, 9 H), 1.46 (s, 18 H), 1.82 (s, 2 H), 2.50 (br. s., 7 H), 2.76 (s, 4 H), 2.83 (s, 4 H), 3.00 (br. s., 3 H), 3.05 (s, 3 H), 3.17 (s, 8 H), 4.21 (br. s., 2 H), 6.91 (d, *J*=8.1 Hz, 1 H), 6.97 (d, *J*=8.1 Hz, 1 H), 7.37 (t, *J*=8.4 Hz, 1 H). ^{13}C NMR (100 MHz, $CDCl_3$): 26.4, 27.8, 27.9, 50.4, 51.4, 52.7, 55.7, 56.5, 66.9, 82.5, 82.8, 103.8, 109.5, 117.3, 124.3, 145.3, 147.0, 172.6, 173.6. ESI-HRMS calculated for $[C_{35}H_{65}N_5O_8 + H]^+$ 680.45201, found 680.45891.

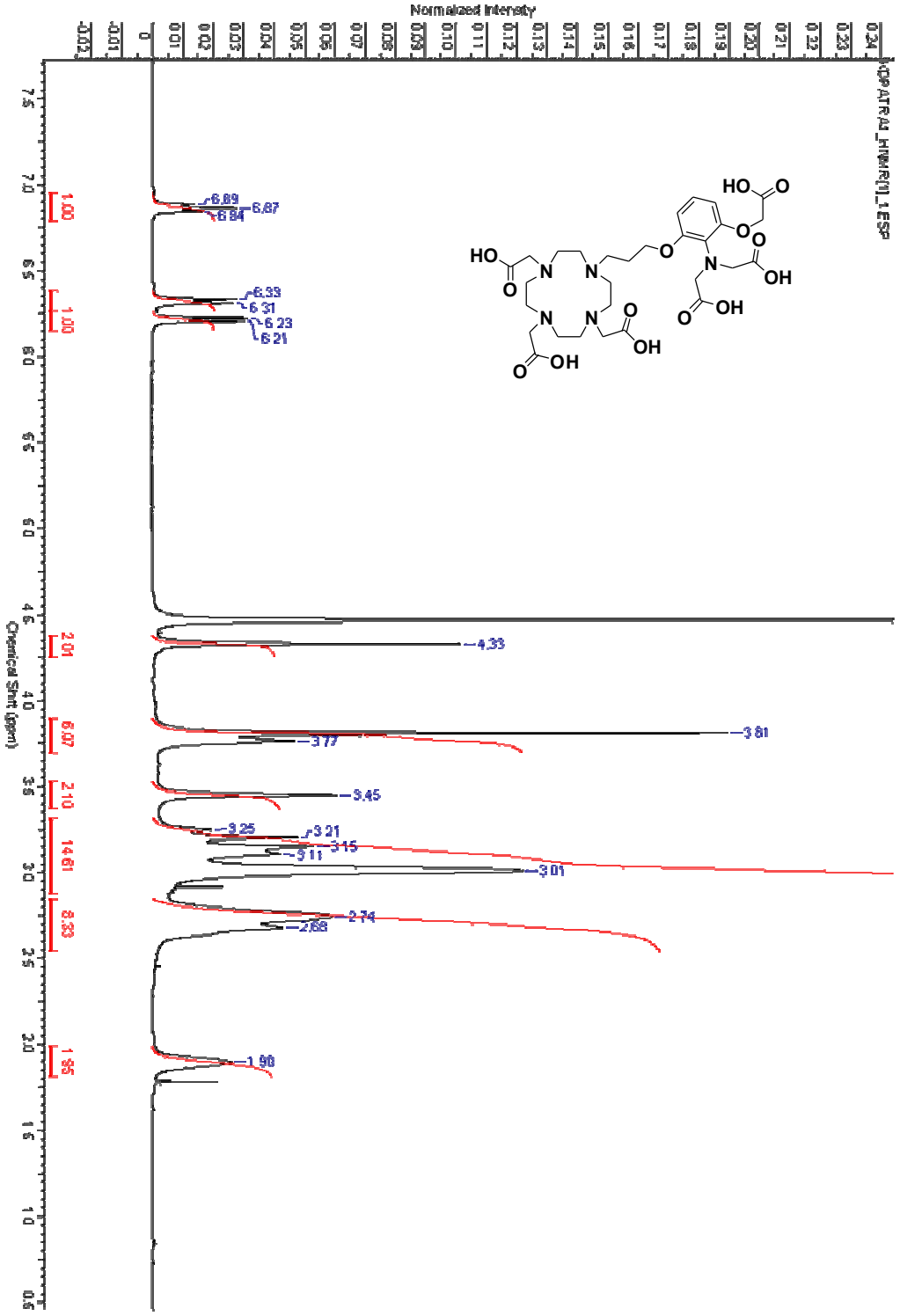
[4-{3-(2-Amino-3-hydroxy-phenoxy)-propyl}-7,10-bis-carboxymethyl-1,4,7,10tetraaza-cyclododec-1-yl]-acetic acid (6). The triester **5** (0.5 g, 0.7 mmol) was hydrolyzed in neat TFA (50 ml) for 24 h at room temperature. The TFA was then evaporated and the residue was dried on vacuum. The residue was then dissolved in water, pH was adjusted to 7 with 1 N NaOH and the this solution was purified by RP-HPLC using methanol as solvent B in method described before. Yield 0.25 g (68%). ^{1}H NMR (400 MHz, D_2O): δ ppm = 1.96 (br s, 2 H), 2.91–3.25 (m, 20 H), 3.42–3.65 (m, 6 H), 4.00 (t, *J*=5.3 Hz, 2 H), 6.48–6.57 (m, 2 H), 7.06–7.16 (m, 1 H). ^{13}CNMR (100 MHz, D_2O): 49.2, 50.0, 50.7, 54.6, 55.9, 66.6, 104.4, 107.4, 108.9, 130.2, 150.9, 152.9, 167.5. ESI-HRMS calculated for $[C_{23}H_{37}N_5O_8 + H]^+$ 512.27149, found 512.27093.

[4-{3-[2-(Bis-carboxymethyl-amino)-3-carboxymethoxy-phenoxy]-propyl}-7,10-bis-carboxymethyl-1,4,7,10tetraaza-cyclododec-1-yl]-acetic acid (7). A solution of aniline **6** (0.10 g, 0.2 mmol) in water (8 ml) was taken in a three neck round bottom flask equipped with a pH meter and water condenser. The pH was adjusted to 10 using solid NaOH followed by addition of bromoacetic acid (0.22 g, 1.6 mmol). The reaction mixture was heated to 90°C. The pH was maintained at 10 by occasional addition of solid NaOH. After the pH remained constant, the reaction mixture was heated for additional 2 h at pH 11. The reaction mixture was then cooled down to room temperature and pH was adjusted to 7 with 1 N HCl. The water was evaporated under vacuum. The residue was dissolved in water (1 ml) and loaded onto a sephadex LH-20 column to remove excess of salts. The elution was done with pure water. The ligand was finally purified by RP-HPLC using method described before. Yield 0.07 g (55%). ^{1}H NMR (400 MHz, D_2O): δ ppm = 1.90 (br s, 2 H), 2.60–2.85 (m, 8 H), 3.01 (br s, 8 H), 3.13 (s, 4 H), 3.21 (br. s., 1 H), 3.25 (br. s., 1 H), 3.45 (s, 2 H), 3.77 (br. s., 2 H), 3.81 (s, 4 H), 4.33 (s, 2 H), 6.22 (d, *J*=8.7 Hz, 1 H), 6.32 (d, *J*=8.39 Hz, 1 H), 6.81–6.91 (m, 1 H). ^{13}C NMR (100 MHz, D_2O): 21.3, 46.9, 47.0, 48.2, 49.7, 50.1, 50.9, 54.5, 57.5, 64.5, 64.8, 104.6, 104.8, 118.2, 128.9, 150.7, 150.8, 168.5, 170.4, 171.7, 172.6. ESI-HRMS calculated for $[C_{29}H_{43}N_5O_{14} + H]^+$ 686.28793, found 686.28920.

Gd-DOPTRA, Eu-DOPTRA: To a solution of ligand **7** (70 mg, 0.1 mmol) in water (3 ml) in a two neck flask fitted with pH meter, $GdCl_3$ or $EuCl_3$ solution (0.12 mmol) was added and the mixture heated in a water bath at 60°C. The pH was maintained at 7 by adding 1N NaOH. After the pH remained constant at 7, the content was heated at the same temperature for 3 to 4 h. Chelex 100 was added and the mixture was stirred at room temperature for 1 h. It was filtered

and water was evaporated under vaccum. The content was loaded onto a sephadex LH-20 column (13 * 2.5 cm) to ensure complete removal of free Gd^{3+} or Eu^{3+}. The elution was done with water. The fractions were checked with ESI-LRMS and evaporated to obtain the solid product. Yield 43 mg (50%). ESI-MS calculated for $[C_{39}H_{38}GdN_5O_{14} - H]^-$ 837.2, found 837.2 $[M - H]^-$, 861.1 $[M – H + Na]^-$ with appropriate isotopic distribution for Gd^{3+}. ESI-MS calculated for $[C_{39}H_{40}EuN_5O_{14} - H]^-$ 834.2, found 834.3 with appropriate isotopic distribution for Eu^{3+}.

^{1}H NMR of the ligand 7



^{13}C NMR of the ligand 7

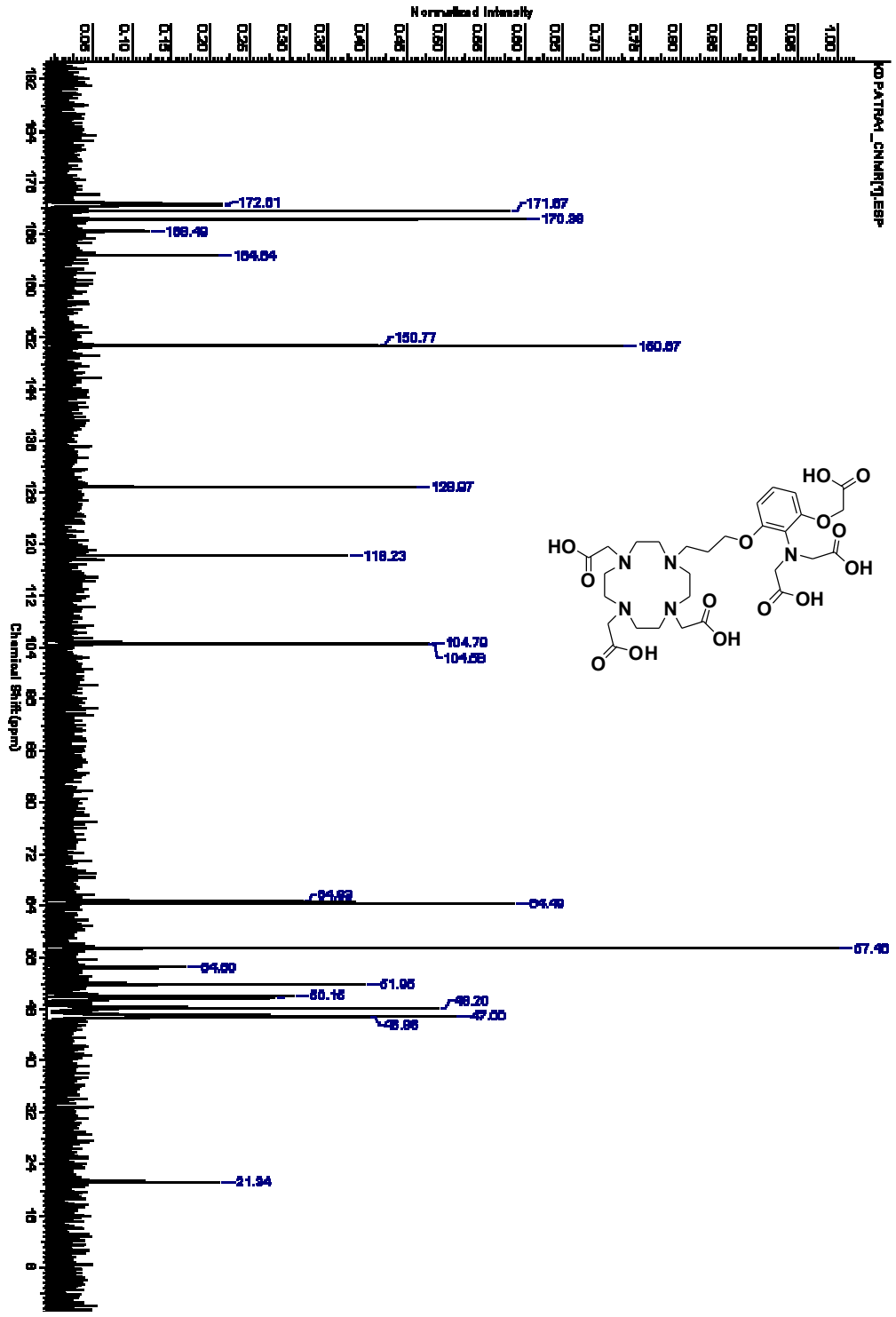


Figure S1: ESI-MS of Gd-APTRA

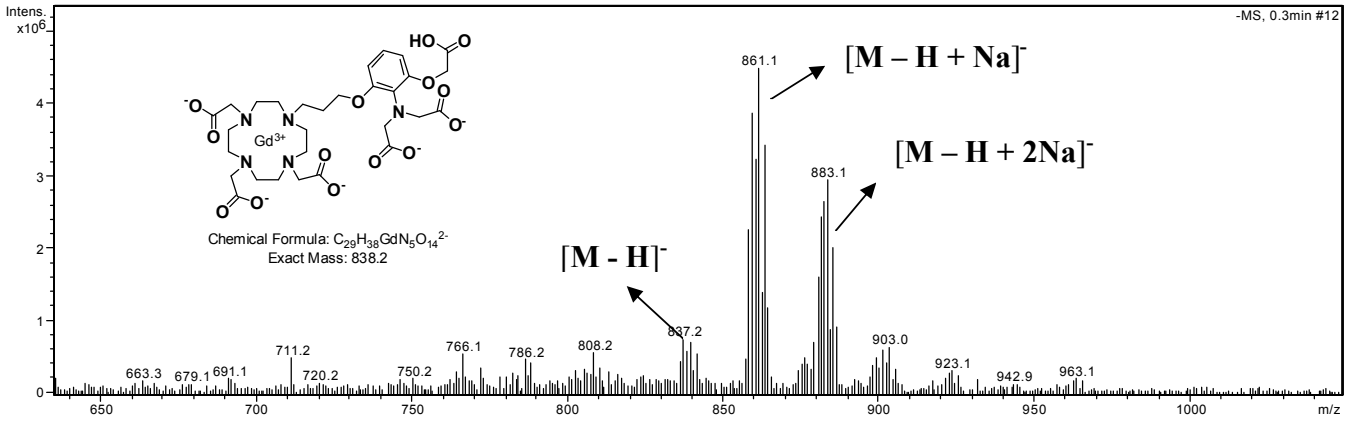
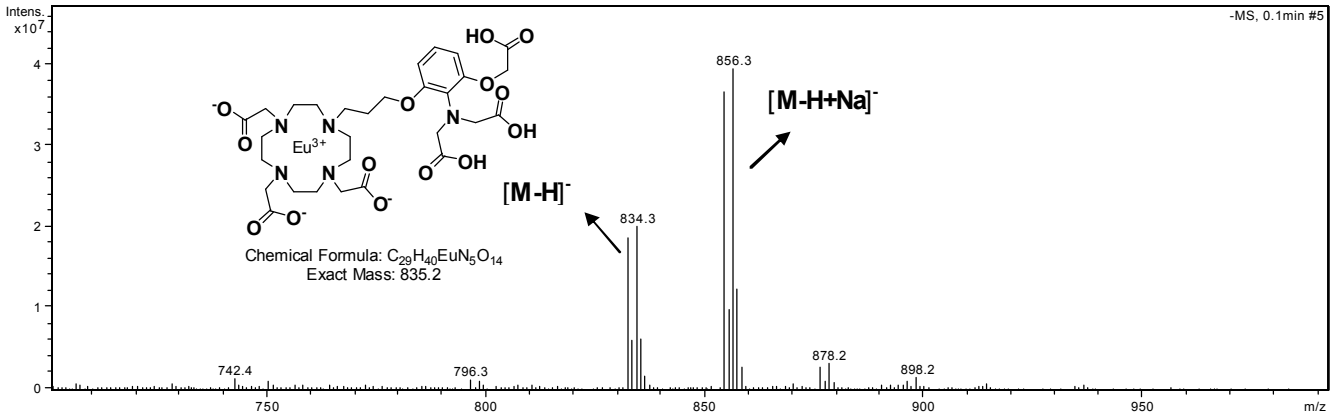


Figure S2: ESI-MS of Eu-APTRA



MR experiments

The T_1 measurements were done on a 400 MHz Bruker advance spectrometer using the system software Topspin® for data acquisition and T_1 evaluation. An inversion-recovery measurement was used with 32 logarithmic inversion time steps between 50 μs and 3 s. The inversion delay was 6 s and the power for the 90° reference pulse was adjusted for every sample individually.

T_1 was calculated by fitting the intensities (I) of the spectrum proton peaks into equation [1].

$$I_{(t_i)} = I_0 \cdot \left(1 - 2 \cdot A \cdot \exp\left(-t_i / T_1\right)\right) \qquad [1]$$

Where $I_{(t_i)}$ is the measured proton peak intensity at inversion time t_i, I_0 is the proton peak intensity without inversion, T_1 is longitudinal relaxation time and factor A takes the finite inversion delay in to account.

The samples were measured in 40 µl capillary tubes inserted in 5 mm NMR tubes. The relaxation rate of the solvent in absence of a paramagnetic solute ($R_{1d} = 1/T_{1d}$) was determined from the plot of different concentrations of Gd-DOPTRA versus $R_{1,obs}$ (observed relaxation rate, $1/T_{1,obs}$) via linear regression. Gd concentrations ([Gd]) were determined by ICP-OES. Relaxivity with different [Ca^{2+}] was then calculated using equation [2].[2]

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

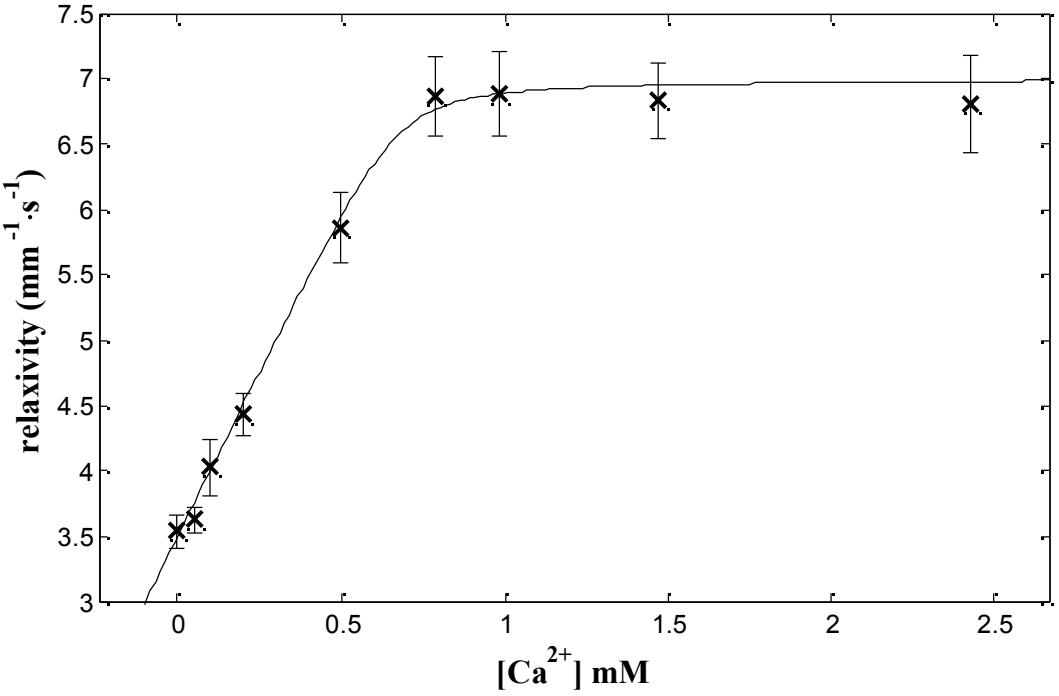
where $r_{1,obs}$ is the observed relaxivity. For determining the dissociation constant, the relaxivity observed in buffer with different [Ca^{2+}] was fitted in to the equation [3].[3]

$$r_{obs} = \left(r_f \cdot [CA]_t + (r_b - r_f) \cdot \left(\frac{n \cdot [CA]_t + [Ca^{2+}] + K_d - \sqrt{\left(n[CA]_t + [Ca^{2+}] + K_d\right)^2 - 4 \cdot n \cdot [CA]_t \cdot [Ca^{2+}]}}{2n} \right) \right) \Big/ [CA]_t$$

[3]

where K_d is equilibrium dissociation constant, r_f is the relaxivity of unbound or free CA, r_b is the relaxivity of the Ca-CA complex or bound CA and n is the number of Ca^{2+} binding sites on CA. The fitting was done using K_d, r_f, r_b, and n as variable parameters. Using 3.5 $mM^{-1}s^{-1}$ for r_f and 7.0 $mM^{-1}s^{-1}$ for r_b from the measured relaxivities and setting n to one, fitting for Kd provides a value of 11±11µM. The plotted curve using K_d = 11µM is shown in Figure **S1**.

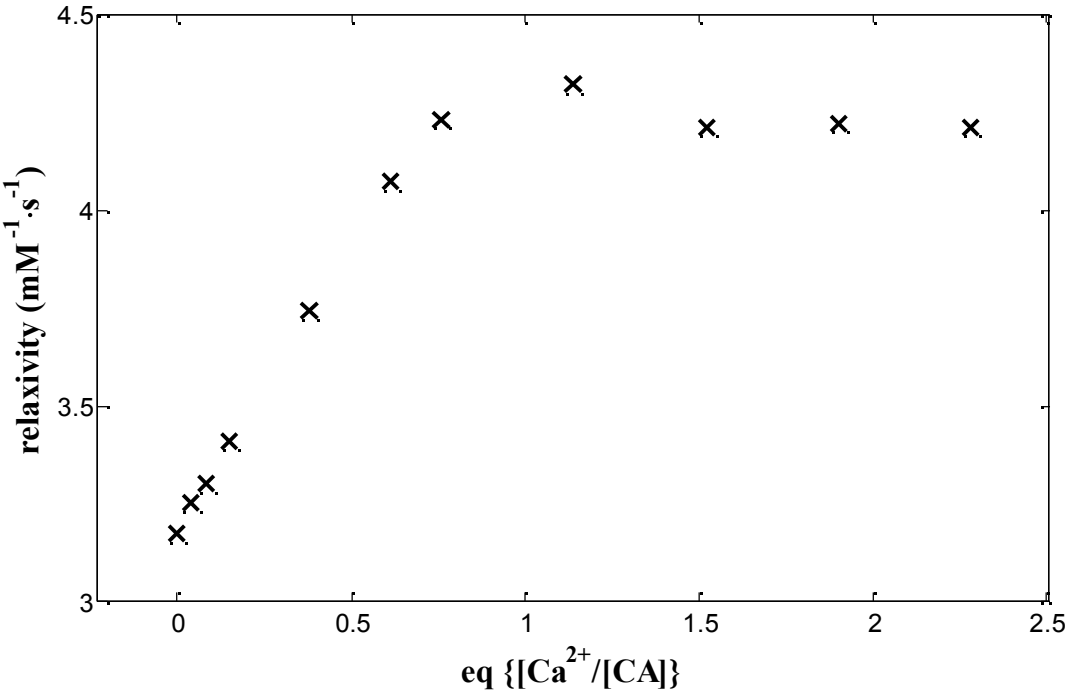
Figure S3: Ca^{2+} dependent relaxivity enhancement of Gd-DOPTRA at 27°C in KMOPS buffer. The plotted graph was done for r_f = 3.5$mM^{-1}s^{-1}$, r_b = 7 $mM^{-1}s^{-1}$, n = 1.0, $[CA]_t$ = 0.7mM, K_d = 11μM.



Relaxation rate experiments in ACSF

ACSF was prepared according to the composition described elsewhere[4]:- NaCl (123.7mM), KCl (2.9mM), $MgCl_2$ (0.7mM), $NaHCO_3$ (23.3mM), HEPES buffer (70mM). T_1 experiments were performed in the similar manner as in KMOPS buffer. Results are shown in the Figure **S4**.

Figure S4: Ca^{2+} dependent relaxivity enhancement of Gd-DOPTRA in ACSF at 37°C. (Error for all r_1 values was less than ± 0.20 $mM^{-1}s^{-1}$ and is not displayed.)



Relaxation rate experiments in AECM

Stock solution of AECM was prepared afresh by mixing 5 ml of Dulbecco's Modified Eagle Medium (D-MEM) liquid (high glucose) (Invitrogen, Catalog Number: 21068028), 5 ml of F-12 Nutrient Mixture (Ham) (Invitrogen, Catalog Number: 21765029) and 100 µl of N-2 Supplement liquid (Invitrogen, Catalog Number: 17502048). D-MEM is well suited for supporting the growth of a broad spectrum of mammalian cell lines. F-12 Nutrient Mixture was originally formulated for single cell plating of near-diploid Chinese Hamster Ovary (CHO) cells.[5] N-2 supplement is a chemically defined, 100X concentrate of Bottenstein's N-2 formulation. This supplement is recommended for the growth and expression of neuroblastomas and for the survival and expression of post-mitotic neurons in primary cultures.[6] The exact compositions are listed in the Table **S1**. T_1 experiments in AECM were performed in the similar manner as in KMOPS buffer. Results are shown in the Figure **S5** and Table **S2**.

Table S1. Compositions of DMEM, F-12 and N2 (for further details see www.invitrogen.com)

D-MEM		**F-12**		**N2**	
components	Conc.(mM)	components	Conc.(mM)	components	Conc.(mM)
Amino Acids		Amino Acids		Proteins	
Glycine	0.4	Glycine	0.1	Human Transferrin (Holo)	1
L-Arginine hydrochloride	0.398	L-Alanine	0.1	Insulin Recombinant Full Chain	0.0861
L-Cystine 2HCl	0.201	L-Arginine hydrochloride	1	Other Components	
L-Histidine hydrochloride-H2O	0.2	L-Asparagine	0.0985	Progesterone	0.002
L-Isoleucine	0.802	L-Aspartic acid	0.1	Putrescine	10.01
L-Leucine	0.802	L-Cysteine hydrochloride	0.228	Selenite	0.003
L-Lysine hydrochloride	0.798	L-Glutamic Acid	0.1		
L-Methionine	0.201	L-Glutamine	1		
L-Phenylalanine	0.4	L-Histidine hydrochloride	0.1		
L-Serine	0.4	L-Isoleucine	0.305		
L-Threonine	0.798	L-Leucine	0.1		
L-Tryptophan	0.0784	L-Lysine hydrochloride	0.199		
L-Tyrosine disodium salt dihydrate	0.398	L-Methionine	0.0302		
L-Valine	0.803	L-Phenylalanine	0.0303		
Vitamins		L-Proline	0.3		
Choline chloride	0.0286	L-Serine	0.1		
D-Calcium pantothenate	0.00839	L-Threonine	0.1		
Folic Acid	0.00907	L-Tryptophan	0.01		
i-Inositol	0.04	L-Tyrosine	0.298		
Niacinamide	0.0328	L-Valine	0.1		
Pyridoxal hydrochloride	0.0196	Vitamins			
Riboflavin	0.00106	Biotin	0.0000299		
Thiamine hydrochloride	0.0119	Choline chloride	0.1		
Inorganic Salts		D-Calcium pantothenate	0.00105		
Ferric Nitrate ($Fe(NO_3)3"9H_2O$)	0.00248	Folic Acid	0.00295		
Magnesium Sulfate ($MgSO_4$) (anhyd.)	**0.814**	i-Inositol	0.1		
Potassium Chloride (KCl)	5.33	Niacinamide	0.00295		
Sodium Bicarbonate ($NaHCO_3$)	44.05	Pyridoxine hydrochloride	0.000291		
Sodium Chloride (NaCl)	110.34	Riboflavin	0.000985		

Sodium Phosphate monobasic (NaH_2PO_4-H_2O)	0.906	Thiamine hydrochloride	0.0089		
Other Components		Vitamin B12	0.00103		
D-Glucose (Dextrose)	25	Inorganic Salts			
Phenol Red	0.0399	**Calcium Chloride**	**0.299**		
		Cupric sulfate	0.00001		
		Ferric sulfate	0.003		
		Magnesium Chloride	**0.601**		
		Potassium Chloride	2.98		
		Sodium Bicarbonate	14		
		Sodium Chloride Sodium Phosphate dibasic	131.02 1		
		Zinc sulfate	0.003		
		Other Components			
		D-Glucose (Dextrose)	10.01		
		Hypoxanthine	0.0294		
		Linoleic Acid	0.003		
		Lipoic Acid	0.00102		
		Phenol Red	0.00319		
		Putrescine 2HCl	0.001		
		Sodium Pyruvate	1		
		Thymidine	0.0089		

Figure S5: Ca^{2+} dependent relaxivity enhancement of Gd-DOPTRA in AECM at 27°C. (Error for all r_1 values was less than ±0.35 $mM^{-1}s^{-1}$ and is not displayed). x axis represents the equiv of Ca^{2+} added to the solution of CA in AECM containing ~0.15mM of Ca^{2+} (see compositions in **Table S1**).

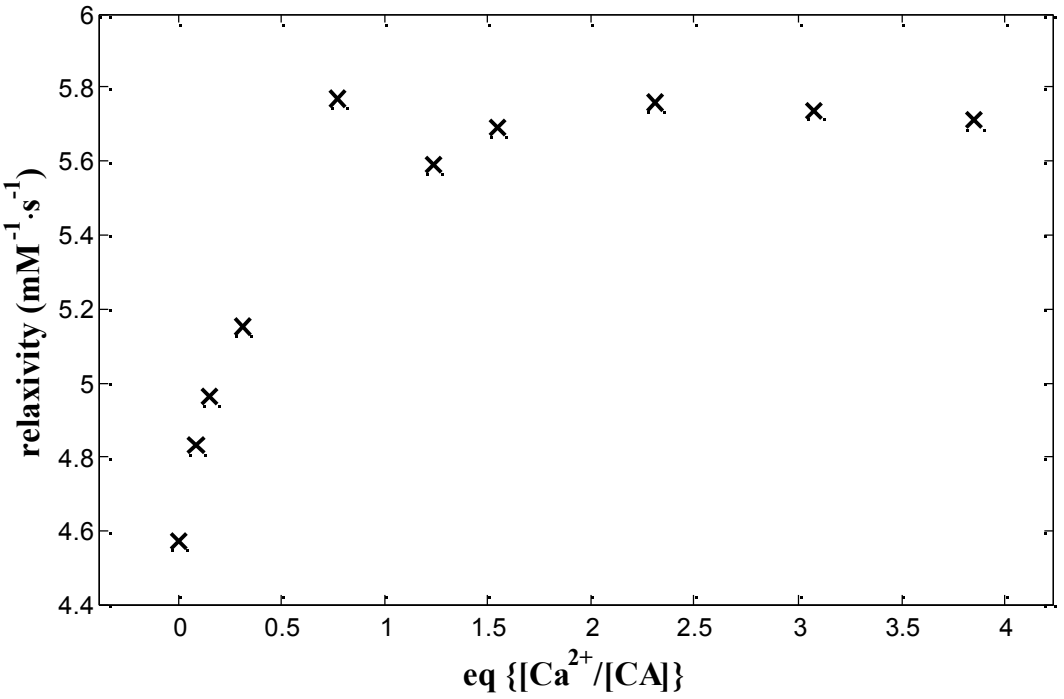


Table S2: Ca^{2+} dependent relaxivity enhancement of Gd-DOPTRA in AECM at 37°C. Eq{[Ca^{2+}]/[CA]} represents the eq of Ca^{2+} added to a solution of CA in AECM.

Eq{[Ca^{2+}]/[CA]}	r ($mM^{-1}s^{-1}$)
0	3.48±0.24
2	4.37±0.33

Luminescence measurement: The luminescence measurements were performed on a Varian eclipse spectrofluorimeter, equipped with a 450 W xenon arc lamp, a microsecond flash lamp and a red-sensitive photomultiplier (300–850 nm). The luminescence spectra were obtained after excitation at $^5L_6 \leftarrow ^7F_0$ band (394 nm) and emission at 615 nm. 3 mM solution of Eu-APTRA was

prepared in 30 mM KMOPS buffer at pH 7.4 in H_2O and in D_2O. Hydration number, q, is calculated according to the equation [4][2,7]:

$$q = 1.2(\tau_{H2O}^{-1} - \tau_{D2O}^{-1} - 0.25) \quad [4]$$

Table S3: Luminescence life times (ms) and calculated hydration number (q)

	τ_{H2O} (ms)	τ_{D2O} (ms)	q
Without Ca^{2+}	0.328	0.377	0.17
With Ca^{2+}	0.410	0.684	0.88

References

1 K. Dhingra, P. Fousková, G. Angelovski, M. E. Maier, N. K. Logothetis, E. Tóth. *J. Biol. Inorg. Chem.* 2008, **13,** 3-41.

2 A. E. Merbach, É. Tóth (eds) (2001) *The Chemistry of Contrast Agents in Medical MRI*. Wiley, Chichester.

3 J. E. Bottenstein, (1985) Cell Culture in the Neurosciences, Bottenstein, J.E. and Harvey, A.L., editors, p. 3, Plenum Press: New York and London.

4 V. Henrotte, L. V. Elst, S. Laurent, R. N. Muller, *J. Biol. Inorg. Chem.* **2007**, 12, 929-937**.**

5 G. G. Somjen, (2004). *Ions in the Brain*; *Normal function, Seizures and Strokes*. Oxford Press.

6 R. G. Ham, *Proc. Nat. Acad. Sci*., **1965**, 53, 288.

7 A. Beeby, I. M. Clarkson, R. S. Dickins, S. Faulkner, D. Parker, L. Royle, A. S. de Sousa, J. A. G. Williams, M. Woods, *J. Chem. Soc. Perkin. Trans.2,* 1999, **2**, 493.