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Supporting Information

Synthesis and Characterization of a Hypoxia-Sensitive MRI Probe

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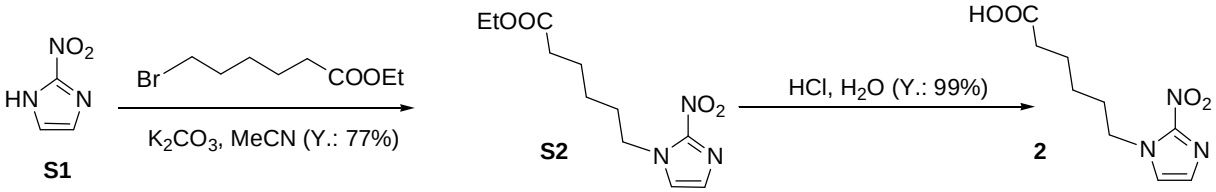
General

All reagents and solvents were purchased from commercial sources and used as received. ^{1}H and ^{13}C spectra were recorded using a Varian 400 spectrometer operating at 400 and 100 MHz, respectively. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). MALDI-TOF spectra were recorded on an Applied Biosystems Voyager 6115 mass spectrometer. ESI-mass spectra were obtained from HT Labs (San Diego, CA). Preparative HPLC was performed on a Waters DeltaPrep Preparative Chromatorgraphy System equipped with a Phenomenex Gemini C18 reverse phase column.

Synthetic procedures

The synthesis of 6-(2-nitro-1H-imidazoyl)hexanoic acid (**2**) was accomplished according to a published procedure with minor modifications.[S1] 1,4,7,10-Tetraazacyclododecane-1,4,7-tris(acetic acid tert-butyl ester) (DO3A tert-butyl ester) was prepared according to published procedures.[S2, S3] 1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid tert-butyl ester)-10-acetic acid methyl ester (**S4**) was converted to 1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid tert-butyl ester)-10-acetic acid diaminobutane amide (**1**) using a modification of a published procedure.[S4] DOTA-monobutylamide (**6**) was prepared as described in the literature.[S5] The synthesis of compounds 2 and 1 are outlined in Scheme S1 and S2.



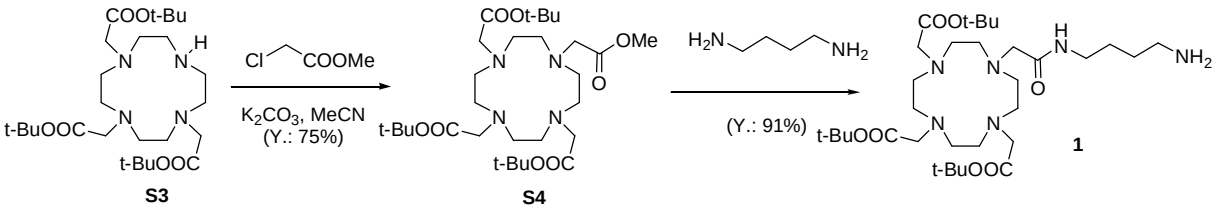
Scheme S1. Synthesis of 6-(2-nitro-1H-imidazoyl)hexanoic acid (**2**) (Y.: 76%).

Ethyl-(2-nitroimidazoyl)hexanoate (S2). 2-nitroimidazole (S**1**) (0.40 g, 3.54 mmol) and ethyl bromohexanoate (0.83 g, 3.72 mmol) were suspended in acetonitrile (6 mL) in the presence of potassium carbonate (0.98 g, 7.08 mmol) and heated at 60°C for six days. The solvent was then evaporated under vacuum, the residue was partitioned between ethyl acetate and water and the organic layer was washed with water three times. The organic layer was dried over sodium sulfate, filtered and the solvent evaporated under vacuum to yield 0.69 g (77%) of a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) d (ppm): 7.05 (d, 1H, Ar), 6.93 (d, 1H, Ar), 4.26 (t, 2H, $N_{ring}C\underline{H}_2$), 3.92 (q, 2H, $CO_2C\underline{H}_2CH_3$), 2.11 (t, 2H, $C\underline{H}_2CO_2$), 1.69 (m, 2H, $N_{ring}CH_2C\underline{H}_2CH_2CH_2CH_2CO_2$), 1.48 (m, 2H, $N_{ring}CH_2CH_2CH_2C\underline{H}_2CH_2CO_2$),1.21 (m, 2H, $N_{ring}CH_2CH_2C\underline{H}_2CH_2CH_2CO_2$) 1.04 (t, 2H, $CO_2CH_2C\underline{H}_3$); ^{13}C NMR (100 MHz, $CDCl_3$) d (ppm): 172.78 ($CH_2\underline{C}O_2$), 144.32 ($\underline{C}_{Ar}$-NO_2), 127.93 ($\underline{C}_{Ar}$), 126.10 ($\underline{C}_{Ar}$), 59.82 ($CO_2\underline{C}H_2CH_3$), 49.57 ($N_{ring}\underline{C}H_2$), 33.38 ($\underline{C}H_2CO_2$), 29.70 ($N_{ring}CH_2\underline{C}H_2CH_2CH_2CH_2CO_2$), 25.29 ($N_{ring}CH_2CH_2\underline{C}H_2CH_2CH_2CO_2$), 23.73 ($N_{ring}CH_2CH_2CH_2\underline{C}H_2CH_2CO_2$), 13.84 ($CO_2CH_2\underline{C}H_3$).

6-(2-Nitro-1H-imidazoyl)hexanoic acid (2). A solution of ethyl-(2-nitroimidazoyl)hexanoate (**S2**) (0.69 g, 2.71 mmol) in concentrated hydrochloric acid (2 mL) was stirred overnight at room temperature. Solvent was then evaporated under vacuum to yield 0.71 g (99%) of a yellow oil that slowly crystallized.

^{1}H NMR (400 MHz, CD_3OD) d (ppm): 7.50 (d, 1H, Ar), 7.12 (d, 1H, Ar), 4.46 ($N_{ring}C\underline{H}_2$), 2.30 ($C\underline{H}_2CO_2$), 1.87 ($N_{ring}CH_2C\underline{H}_2CH_2CH_2CH_2CO_2$), 1.61 ($N_{ring}CH_2CH_2CH_2C\underline{H}_2CH_2CO_2$), 1.39 ($N_{ring}CH_2CH_2C\underline{H}_2CH_2CH_2CO_2$); ^{13}C NMR (100 MHz, CD_3OD) d (ppm): 175.95 ($CH_2\underline{C}O_2$), 144.61 ($\underline{C}_{Ar}$-NO_2), 127.33 (Ar), 127.16 (Ar), 49.76 ($N_{ring}\underline{C}H_2$), 33.31 ($\underline{C}H_2CO_2$), 29.82 ($N_{ring}CH_2\underline{C}H_2CH_2CH_2CH_2CO_2$), 25.59 ($N_{ring}CH_2CH_2\underline{C}H_2CH_2CH_2CO_2$), 24.10 ($N_{ring}CH_2CH_2CH_2\underline{C}H_2CH_2CO_2$).



Scheme S2. Synthesis of 1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid tert-butyl ester)-10-acetic acid diaminobutane amide (**1**) (Y.: 53%).

1,4,7,10-Tetraazacyclododecane-1,4,7-tris(acetic acid tert-butyl ester)-10-acetic acid methyl ester (S4). A suspension of DO3A t-butyl ester (**S3**) (5.26 g, 10.2 mmol), methyl chloroacetate (1.14 g, 10.5 mmol) and potassium

carbonate (3.37 g, 24.4 mmol) in acetonitrile (60 mL) was heated to 55°C for two days. The base was then filtered and the solvent evaporated under vacuum. The brown residue was dissolved in ether and washed four times with water. The organic layer was dried over sodium sulfate, filtered and the solvent evaporated under vacuum to yield 4.52 g (75%) of an amber oil.

^{1}H NMR (400 MHz, $CDCl_3$) d (ppm): 3.55 (s, 3H, $CO_2C\underline{H}_3$), 3.29 (s, 2H, $N_{ring}C\underline{H}_2$), 3.16 (s, 2H, $N_{ring}C\underline{H}_2$), 3.15 (s, 4H, $N_{ring}C\underline{H}_2$), 2.69 (b, 16H, $C_{ring}\underline{H}_2$), 1.32 (s, 27H, $CO_2C(C\underline{H}_3)_3$); ^{13}C NMR (100 MHz, $CDCl_3$) d (ppm): 172.25 ($\underline{C}O_2CH_3$), 171.21 ($\underline{C}O_2C(CH_3)_3$), 171.18 ($\underline{C}O_2C(CH_3)_3$), 80.73 ($CO_2\underline{C}(CH_3)_3$), 80.72 ($CO_2\underline{C}(CH_3)_3$), 56.72 ($N_{ring}\underline{C}H_2CO_2C(CH_3)_3$), 56.69 ($N_{ring}\underline{C}H_2CO_2C(CH_3)_3$), 55.60 ($N_{ring}\underline{C}H_2CO_2CH_3$), 52.18 ($\underline{C}_{ring}H_2$), 52.13 ($\underline{C}_{ring}H_2$), 52.06 ($\underline{C}_{ring}H_2$), 51.24 ($CO_2\underline{C}H_3$), 28.34 ($CO_2C(\underline{C}H_3)_3$). MALDI-TOF *m/z*: 587.4 $[M+H]^+$ (50%), 609.4 $[M+Na]^+$ (100%).

1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid tert-butyl ester)-10-acetic acid diaminobutane amide (1). A solution of 1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid tert-butyl ester)-10-acetic acid methyl ester (**S4**) (2.00 g, 3.41 mmol) in diaminobutane (6.01 g, 68.17 mmol) was stirred at room temperature for three days. The excess diaminobutane was then distilled off under vacuum (740 mtorr) at 60 °C to yield 2 g (91 %) of an amber oil.

^{1}H NMR (400 MHz, $CDCl_3$) d (ppm): 8.56 (b, 1H, amide), 3.19 (s, 2H, $N_{ring}C\underline{H}_2CO_2C(CH_3)_3$), 3.14 (s, 4H, $N_{ring}C\underline{H}_2CO_2C(CH_3)_3$), 2.94 (s, 2H, $N_{ring}C\underline{H}_2CO_2C(CH_3)_3$), 2.80 (b, 4H, $N_{ring}C\underline{H}_2$), 2.75 (b, 4H, $N_{ring}C\underline{H}_2$), 2.63 (b, 8H, $N_{ring}C\underline{H}_2$), 2.42 (b, 4H, $CONC\underline{H}_2CH_2CH_2C\underline{H}_2NH_2$), 1.44 (b, 4H, $CONCH_2C\underline{H}_2C\underline{H}_2CH_2NH_2$), 1.35 (s, 27H, $CO_2C(C\underline{H}_3)_3$); ^{13}C NMR (100 MHz, $CDCl_3$) d (ppm): 172.36 ($\underline{C}O$), 170.74 ($\underline{C}O$), 81.09 ($CO_2\underline{C}(CH_3)_3$), 80.99 ($CO_2\underline{C}(CH_3)_3$), 58.31 ($N\underline{C}H_2$), 56.77 ($N\underline{C}H_2$), 56.31 ($N\underline{C}H_2$), 54.78 ($N\underline{C}H_2$), 53.35 ($N\underline{C}H_2$), 52.50 ($N\underline{C}H_2$), 52.09 ($N\underline{C}H_2$), 49.91 ($N\underline{C}H_2$), 41.51 (alkyl chain methylene), 39.14 (alkyl chain methylene), 28.28 ($CO_2C(\underline{C}H_3)_3$). MALDI-TOF *m/z*: 643.8 $[M+H]^+$ (100%), 665.8 $[M+Na]^+$ (70%).

1,4,7,10-tetraazacyclododecane-1,4,7-tri(acetic acid)-10-acetic acid [N-(4-aminobutyl)-6-(2-nitro-1H-imidazol-1-yl)hexan amide] amide (3). A solution of 6-(2-nitro-1H-imidazoyl) hexanoic acid (2) (0.76 g, 3.36 mmol) and diisopropylethyleneamine (DIEA) (0.87 g, 6.73 mmol) in dimethylformamide (DMF) (6 mL) was placed in an ice bath. A solution of O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (1.34 g, 3.53 mmol) and N-hydroxybenzotriazole (HOBt) (0.023 g, 0.17 mmol) in DMF (2.5 mL) was added to the reaction vessel and the mixture was allowed to stir in the ice bath for 15 min. A solution of 1,4,7,10-tetraazacyclododecane-1,4,7-tri(acetic acid t-butyl ester)-10-acetic acid diaminobutane amide (1) (2 g, 3.11 mmol) in DMF (6 mL) was added and the mixture allowed to stir overnight at room temperature. The solvent was removed by rotary evaporation and the resulting residue was dissolved in dichloromethane and washed three times with water. The organic layer was dried over sodium sulfate, filtered, and the solvent evaporated under vacuum to yield a thick, brown oil. This was redissolved in dichloromethane, the crude product was precipitated with ether, and the resulting residue was washed twice with ether and dried under vacuum. A solution of this foam in dichloromethane (3 mL) was mixed with trifluoroacetic acid (TFA) (5 mL) and stirred at room temperature overnight. The solvent and excess TFA was then removed by rotary evaporation and the residue washed three times with ether. The resulting off-white white solid was dried under vacuum. The crude product was purified by high performance liquid chromatography on a C18 reverse phase column [water-acetonitrile-trifluoroacetic acid (0.1%)] to yield 0.96 g (28%) of an off-white solid. The trifluoroacetate salt was converted into the hydrochloride by repeated dissolution in dilute HCl followed by lyophilization.

^{1}H NMR (400 MHz, D_2O) δ (ppm): 7.28 (s, 1H, aromatic), 7.01 (s, 1H, aromatic), 4.21 (t, 2H, $C\underline{H}_2$ a to nitroimidazole), 3.93 (b, $NC\underline{H}_2$), 3.22 (br, $NC\underline{H}_2$), 2.94 (br, $NC\underline{H}_2$), 2.88 (br, $NC\underline{H}_2$), 1.97 (t, 2H, $C\underline{H}_2$ ε- to nitroimidazole), 1.59 (q, 2H, $C\underline{H}_2$ β to nitroimidazole), 1.33 (q, 2H, $C\underline{H}_2$ δ to nitroimidazole), 1.22 (br, 4H, diaminobutyl methylenes), 1.03 (q, 2H, $C\underline{H}_2$ γ to nitroimidazole); ^{13}C NMR (100 MHz, D_2O) δ (ppm): 176.75 (amide carbonyl), 174.17 (br, carbonyl), 168.77 (br, carbonyl), 165.65 (br, carbonyl), 143.33 ($\underline{C}$-NO_2), 127.98 (aromatic C), 126.51 (aromatic C), 55.11 (br, $N\underline{C}H_2$), 54.29 (br, $N\underline{C}H_2$), 53.24 (br, $N\underline{C}H_2$), 51.42 (br, $N\underline{C}H_2$), 50.42 ($N\underline{C}H_2$), 48.66 (br, $N\underline{C}H_2$), 39.17 (br, alkyl chain methylene), 35.40 (alkyl chain methylene), 28.97 (br, alkyl chain methylene), 25.81 (alkyl chain methylene), 25.77 (alkyl chain methylene), 24.92 (alkyl chain methylene), 24.82 (alkyl chain methylene). ESI-MS, *m/z*: 684.5 $[M+H]^+$ (100%), 706.5 $[M+Na]^+$ (60%). Anal. Calcd. for $C_{29}H_{49}N_9O_{10}$ 2HCl x $5H_2O$ (%): C 41.13; H 7.26; N 14.89. Found (%): C 41.26; H 7.37; N 14.67.

Preparation of gadolinium complexes. The ligand 1,4,7,10-tetraazacyclododecane-1,4,7-tri(acetic acid)-10-acetic acid [N-(4-aminobutyl)-6-(2-nitro-1H-imidazol-1-yl)hexanamide] amide (**3**) was dissolved in water and mixed with an aqueous solution of gadolinium chloride with the ligand in 2% excess. The pH was maintained between 5 and 6 by the addition of dilute sodium hydroxide. The mixture was stirred until the pH stabilized. The pH was then raised to 8 with sodium hydroxide and the solution was filtered through 0.45 µm syringe filter and lyophilized to give Gd(**3**). The Gd(III)-complex of DOTA monobutylamide, Gd(**6**), was prepared similarly. **Gd(3).** MALDI-TOF, *m/z*: 839 $[M+H]^+$. Gd-content, ICP-OES: 10.7%. **Gd(6)**. MALDI-TOF, *m/z*: 615 $[M+H]^+$. Gd-content, ICP-OES: 17.9%.

pH potentiometry. pH-potentiometric titrations were carried out using a Thermo Orion ion analyzer EA940 pH meter using a 6.0234.100 Metrohm combined electrode in a mixing vessel thermostated at 25.0°C. A Metrohm DOSIMATE 665 autoburette (5 mL capacity) was used for base additions and 1.0 M KCl was used to maintain the ionic strength. All equilibrium measurements (direct titrations) were carried out in 10.00 mL samples with magnetic stirring. During the titrations, argon gas was passed over the sample to maintain the cell free of CO_2. The electrodes were calibrated according to the standard two point calibration procedure with commercially available buffers (0.01 M borax, pH = 9.180 and 0.05 M KH-phthalate, pH = 4.005). The concentrations of H^+ ions were calculated from the measured pH values using the method proposed by Irving et al.[S6] Using this method, diluted stock solutions of HCl (approx. 0.027 M in 1.0 M KCl) are titrated with standardized KOH solutions and the difference between the measured and calculated pH was used to correct the pH values obtained in the titration experiments. The log K_w of water was also calculated from these titration data and was found to be 13.793.

Equilibrium studies. The $LnCl_3$ stock solutions were prepared by dissolving the hepta- or hexahydrate chloride salts in double-distilled water. The concentrations of the stock solutions were determined by complexometric titration with Na_2H_2EDTA stock solution using xylenol orange as indicator at pH = 5.5 – 5.8 (set by addition of 40 % hexamethylenetetraamine solution).[S7] Stock solutions of **3** and **6** were prepared by dissolving a weighed amount of solid ligand in double-distilled water. The exact concentration of the ligand in this stock solution and the amount of the dissociable protons (C_{H+}/C_L) were determined by pH-potentiometry on the basis of the titration curves obtained in the absence and presence of an excess of $CaCl_2$ (C_{Ca}/C_L ratio was approximately 50).

The protonation constants (log K_i^H) of **3** and **6** were obtained by titrating 1.7 and 3.7 mM samples (312 and 301 data points, respectively) with standardized KOH solution (0.1874 M), in the absence of Ca^{2+}, over the pH range of 2.05 – 12.01. Before each potentiometric titration, the pH of each sample was adjusted to 1.9 – 2.0 with a measured volume of strong acid (HCl) of known concentration. The titration data were fitted to Equation S1 using PSEQUAD.[S8]

$$K_i^H = \frac{[H_iL]}{[H_{i-1}L][H^+]} \qquad \text{(S1)}$$

where i = 1, 2, ..., 5 and $[H_{i-1}L]$ and $[H^+]$ are the concentrations of the ligand (i = 0) and its protonated forms, and of the hydrogen ions, respectively.

Complexation of a trivalent lanthanide (III) (Ln^{III}) ion by a macrocyclic ligand such as **3** or **6** is typically slow at low pH values; about four to five weeks are required to achieve equilibrium at room temperature. Hence, the "out-of-cell" titration method (also known as batch method) was used for the determination of the stability constants. Typically, a 25.0 mL mixture of Ln^{III} and ligand (2.5 mM adjusted with HCl or KOH to cover the pH range, 1.8 – 3.1 (the pH range where complexation takes place as predicted by model calculations). The samples were then sealed and placed in a 40 °C incubator for 21 days followed by an additional 42 days at room temperature to ensure that the samples had reached equilibrium. UV-vis experiments performed on the samples with Ce^{3+} indicated that this time period was sufficient for the samples to reach equilibrium. Literature data shows that of the rate of complex formation for LnDOTA–type complexes is slowest for Ce^{III} compared to the other Ln^{3+} ions.[S9-S11] Given this prior knowledge, we assumed that all other Ln(**3**) and Ln(**6**) complexes would reach equilibrium more quickly than Ce^{III} using identical experimental conditions. Once equilibrium had been reached, the samples were opened, the pH of each sample was measured, and the stability constants of the complexes were calculated from the titration data using PSEQUAD.[S8]

Table S1. Protonation constants[a] of some alkyl ethylenediamine-tris acetic acid (EDTA-R) derivative ligands (EDTA-[$C_1 – C_{12}$], T = 25 ^{0}C).[S12-S14]

Ligand	R	log K_1^H	log K_2^H	log K_3^H	log K_4^H
EDTA-C_1	$–CH_3$	10.31[a]	5.42	2.45	1.9
		10.19[b]	5.55	2.40	1.7
EDTA-C_4	$–(CH_2)_3–CH_3$	10.04[c]	6.64	1.8	–
EDTA-C_8	$–(CH_2)_7–CH_3$	9.76[c]	6.53	1.9	–
EDTA-C_{12}	$–(CH_2)_{11}–CH_3$	9.77[c]	6.56	2.0	–

[a] I = 0.1 M K^+ salt from ref. [S12]; [b] I = 0.5 M Na^+ salt from ref. [S13]; [c] I = 0.1 M K^+ salt from ref.[S14].

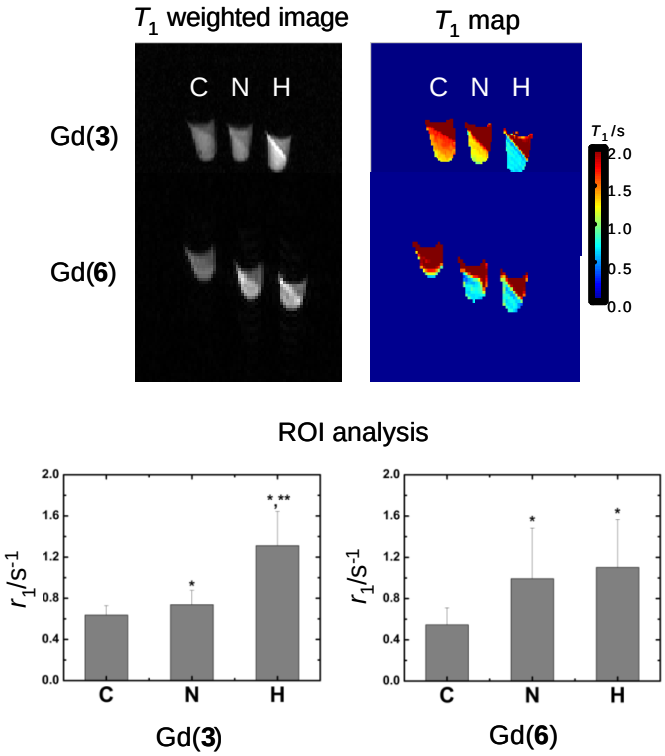


Figure S1 (color version of Fig 2 form the manuscript). *In vitro* MRI imaging of 9L rat glioma cells after exposure to either Gd(**3**) or Gd(**6**) (top). T_1-weighted images (T_R = 300 msec; T_E = 10 msec) and T_1 maps of negative control (C), normoxic (N) and hypoxic (H) cells. Relaxation rates (R_1) for the packed cell layers (bottom) (* = p <0.05 compared to negative control (C); ** = p<0.05 compared to normoxic sample (N)).

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