Supporting Information

Structure Relaxivity Relationships of Serum Albumin Targeted MRI Probes Based on a Single Amino Acid Gd complex.

Eszter Boros and Peter Caravan*

The Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, 149 Thirteenth Street, Suite 2301, Charlestown, MA 02129.

Table of contents

General Methods and Materials:

^{1}H and ^{13}C NMR spectra were recorded on a Varian 500 NMR system. Chemicals were supplied by Aldrich Chemical Co., Inc., and were used without further purification. Solvents (HPLC grade) were purchased from various commercial suppliers and used as received**.** Longitudinal relaxation times, T_1, were measured by using the inversion recovery method on Bruker Minispecs. mq20 (20 MHz) and mq60 (60 MHz). Purification via HPLC of intermediates toward Fmoc-DOTAla was performed using method A: Injection of crude mixture onto preparative HPLC on a Rainin, Dynamax (column: 250 mm Kromasil C18) by using A: 0.1% TFA in water, B: 0.1% TFA in MeCN, flow-rate 15 mL/min, 0 – 1 min: 5 % B, 1 – 3 min: 5 – 40 % B, 3 – 15 min: 40 – 80 % B, 15 – 16 min: 80 – 95 % B, 16 – 20 min: 95 % B, 20 – 21 min: 95 – 5 % B, 21 – 23 min: 5 % B. HPLC purity analysis (both UV and MS detection) was carried out on an Agilent 1100 system (column: Phenomenex Luna, C18(2) 100/2 mm) with UV detection at 220, 254 and 280 nm by using method B: A gradient of A (0.1 % formic acid in water) to 95% B(0.1% formic acid in MeCN), flow-rate 0.8 mL/min. 0 – 1 min: 5 % B, 1 – 10 min: 5 – 95 % B, 10 – 12 min: 95 % B, 12 – 12.5 min: 95 – 5 % B, 12.5 – 15 min: 5 % B.

In order to measure HSA binding of the complexes, a 0.1 mM solution (determined by ICP-MS) of the Gd complex in 4.5% w/v HSA was prepared and pipetted into a Ultrafree-MC Microcentrifuge Filter (NMWL 5,000 Da, PLCC, Millipore). The mixture was incubated at 37 °C for 10 min and subsequently centrifuged at 12,000 rpm for 15 min. Binding is determined by measurement of Gd content in the filtrate by ICP-MS. % bound = ([initial] – [filtrate])/[initial].

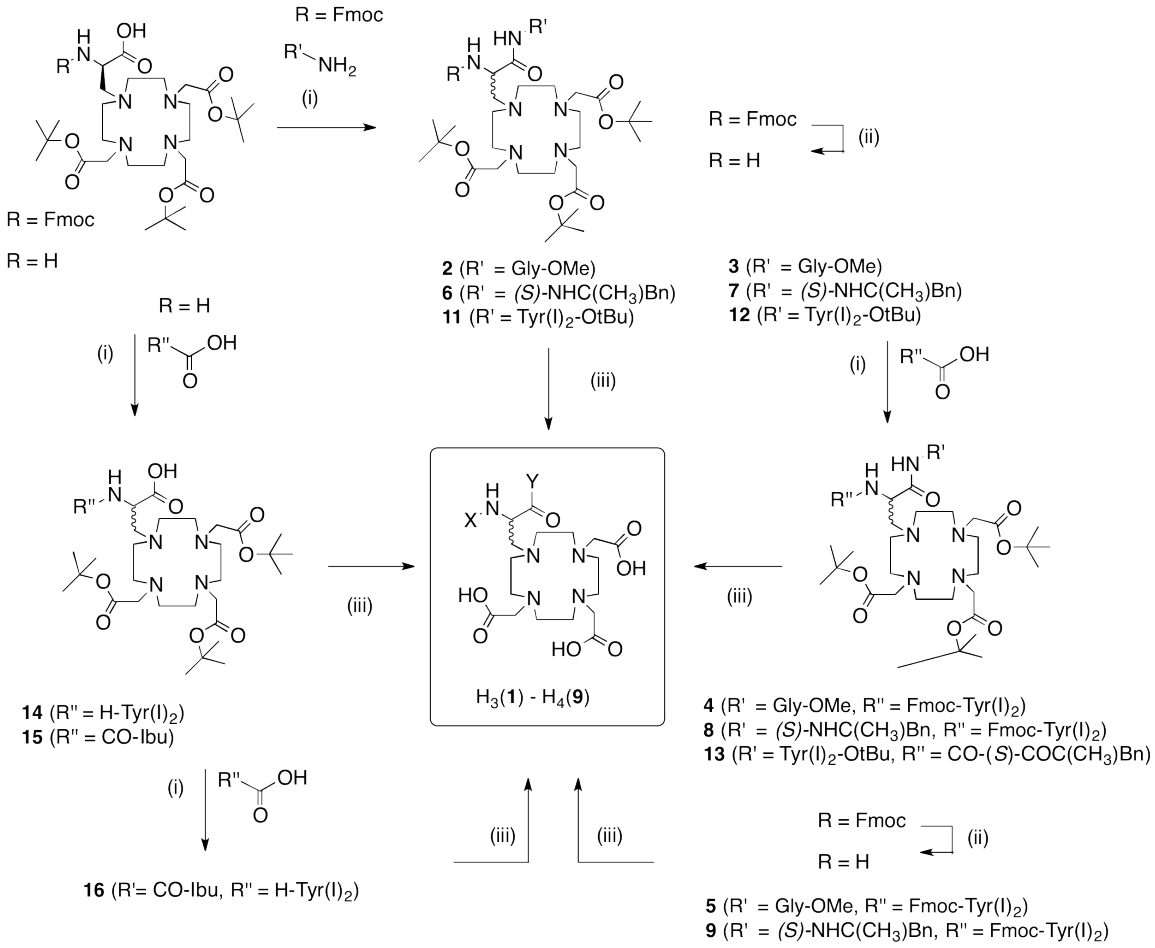
The synthesis of ligands was carried out as shown in Schemes 1,2 and 3. Chemicals were supplied by Aldrich Chemical Co., Inc., and were used without further purification. Solvents (HPLC grade) were purchased from various commercial suppliers and used as received. Fmoc-DOTAla (**1**) was synthesized according to reference. Glycine methyl ester hydrochloride salt (Sigma), Fmoc-diiodo-tyrosine (Bachem), R-phenylethylamine (Sigma) and R-2-phenylpropionic acid (Sigma) were used as received.

General procedure 1 (Coupling of Fmoc-protected amino acid with free amino synthon): The free carboxylate (0.05 mmol) was dissolved in DMF (5 mL). HATU (1.5 eq.), HOAt (1.5 eq.) and NMM (2.5 eq) were added and the mixture was agitated for 5 minutes in order to activate the carboxylate. Subsequently, the corresponding free amine (1.5 eq.) was added to the mixture and the solution was stirred at room temperature overnight. Reaction control via LCMS (method B, see above) was used in order to evaluate if product was present. The reaction mixture was purified using preparative HPLC (method A). Fractions containing the pure product were pooled, lyophilized and used for the subsequent reaction step after analysis.

General procedure 2 (Fmoc deprotection of intermediates): The Fmoc-protected intermediate synthon (0.01 - 0.05 mmol) was dissolved in DMF (1 mL) and added to a solution of solid-support-piperazine (SSP-pip, 10 eq.) suspended in DMF (5 mL). The mixture was stirred at room temperature over night. Reaction control via LCMS (method B, see above) was used in order to evaluate any residual starting material was present. Another batch of SSP-pip (10 eq.) was added if significant amounts of starting material were found to be present. Once the reaction was complete, the reaction mixture was filtered in order to remove the SSP-pip. The filtrate was used without further purification for the next reaction step if procedure 1 followed, or purified using preparative HPLC (method B), if no additional peptide couplings followed. For compounds 3, 6, 9, the solvent is removed and the intermediate used for the deprotection step without further purification. The only contaminant observed (1-((9*H*-fluoren-9-yl)methyl)piperazine) is removed via filtration of the subsequent reaction mixture.

General procedure 3 (^{t}Bu Deprotection): The starting material (0.01 – 0.02 mmol) was dissolved in a mixture of dichloromethane and trifluoroacetic acid (1:1, 2 mL) and stirred over night. LCMS provided reaction control. The solvent is removed in vacuo and the product is isolated as the trifluoroacetate salt.

General procedure 4 (Gd complex formation): The ligand (0.01 mmol) was redissolved in H_2O. The pH was adjusted to 3 and $GdCl_3 \bullet 6H_2O$ (0.07 mmol) was added and the pH was adjusted to 6.5 using NaOH (0.1 M). Complexation was found to be complete once less than 5 % free ligand was detected, as determined by LCMS (method A).



Scheme S1. Synthetic scheme for compounds **2**-**16**. (i) HATU (1.2 eq), HOAt (1.2 eq), NMM (1.2 eq), DMF, rt, 14h. (ii) SSP-piperazine (10 eq), DMF, rt, 14h. (iii) TFA/DCM (1:1), rt, 14h.

tri-*tert*-butyl 2,2',2''-(10-(2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-((2-methoxy-2-oxoethyl)amino)-3-oxopropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, (**2**). ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm): 8.75(s (br), NH), 7.75 (d, 2H), 7.61 (m, 2H), 7.41 – 7.28 (m, 4H), 4.77 (s(br), 1H), 4.41 – 3.05 (m, 31H), 1.43 – 1.39 (m, 27H). ^{13}C-NMR ($CDCl_3$, 125 MHz, ppm): 169.9, 169.3, 161.3, 161.0, 143.7, 143.5, 141.3, 141.2, 127.8, 127.1, 125.2, 119.9, 117.4, 115.0, 113.1, 82.6, 67.5, 54.8, 52.2, 50.9, 46.9, 41.1, 29.7, 28.0. LC-ESI-MS: calcd. for $C_{47}H_{71}N_6O_{11}$: 895.5. Found: 895.5 $[M+H]^+$, R_t= 6.36 min.

tri-*tert*-butyl 2,2',2''-(10-(2-amino-3-((2-methoxy-2-oxoethyl)amino)-3-oxopropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, (**3**). ^{1}H-NMR (D_2O, 500 MHz, ppm): 3.95 (d, 2H), 3.63 (s, 3H), 3.48 – 2.2 (m, 24H), 1.42 – 1.34 (m, 27H). LC-ESI-MS: calcd. for $C_{32}H_{61}N_6O_9$: 673.5 Found: 673.5 $[M+H]^+$, R_t= 5.02 min.

tri-*tert*-butyl 2,2',2''-(10-(2-(2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-hydroxy-3,5-diiodophenyl)propanamido)-3-(((*S*)-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate,(**4**). ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm): 7.73 (d, 2H), 7.61 – 7.54 (m, 4H), 7.41 – 7.28 (m, 4H), 4.95 (s(br), 1H), 4.39 – 2.94 (m, 31H), 1.47 – 1.41 (m, 27H). ^{13}C-NMR ($CDCl_3$, 125 MHz, ppm): 169.9, 161.5, 161.2, 156.6, 152.7, 143.7, 141.2, 141.1, 140.0, 139.9, 127.7, 127.2, 125.4, 125.3, 119.8, 117.4, 115.0, 82.8, 82.4, 67.5, 58.4, 56.9, 55.1, 52.2, 49.3, 48.6, 46.9 41.3, 28.0, 27.9. LC-ESI-MS: calcd. for $C_{56}H_{78}I_2N_7O_{13}$: 1310.4. Found: 1310.3 $[M+H]^+$, R_t= 7.2 min.

tri-*tert*-butyl 2,2',2''-(10-(2-(2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-hydroxy-3,5-diiodophenyl)propanamido)-3-(((*S*)-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate,(**5**). ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm): 7.59 (s, 2H), 7.41 – 7.28 (m, 4H), 4.95 (m, 2H), 3.9 – 2.94 (m, 32H), 1.50 – 1.44 (m, 27H). LC-ESI-MS: calcd. for $C_{41}H_{68}I_2N_7O_{11}$: 1088.3 Found: 1088.3 $[M+H]^+$, R_t= 5.2 min.

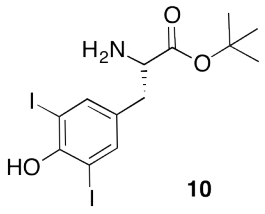
tri-*tert*-butyl 2,2',2''-(10-(2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-oxo-3-(((*R*)-1-phenylethyl)amino)propyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, (**6**). ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm): 8.33 (s, 1H), 8.25 (s, 1H), 7.74 (m, 2H), 7.63 – 7.59 (m, 2H), 7.41 – 7.16 (m, 9H), 5.01 (m, 1H), 4.69(s, 1H), 4.39 – 2.21 (m, 3H), 3.72 – 2.92 (m, 25H) 1.47 – 1.42 (m, 31H). ^{13}C-NMR ($CDCl_3$, 125 MHz, ppm): 169.4, 161.3, 161.0, 143.5, 141.3, 128.6, 128.5, 127.7, 127.2, 127.1, 126.2, 126.1, 125.2, 119.9, 117.4, 82.6, 55.1, 53.4, 51.0, 49.9, 49.8, 46.9, 28.9, 22.0. LC-ESI-MS: calcd. for $C_{52}H_{75}N_6O_9$: 927.5 Found: 927.4 $[M+H]^+$, R_t= 6.76 min.

tri-*tert*-butyl 2,2',2''-(10-(2-amino-3-oxo-3-(((*R*)-1-phenylethyl)amino)propyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, (**7**). ^{1}H-NMR (D_2O, 500 MHz, ppm): 7.26 – 7.21 (m, 5H), 4.62 (d, 1H), 4.81 (s, 1H), 3.41 -2.80 (m, 26H), 1.35-1.25 (m,31 H LC-ESI-MS: calcd. for $C_{37}H_{65}N_6O_7$: 705.5 Found: 705.4 $[M+H]^+$, R_t= 5.5/5.7 min.

tri-*tert*-butyl 2,2',2''-(10-(2-(2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-hydroxy-3,5-diiodophenyl)propanamido)-3-oxo-3-(((*R*)-1-phenylethyl)amino) propyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, (**8**). ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm): 7.71 – 7.18 (m, 15H), 4.41 - 3.95 (s(br), 1H), 4.81 (m, 1h) 4.39 – 2.74 (m, 29H), 1.51 – 1.25 (m, 31H). ^{13}C-NMR ($CDCl_3$, 125 MHz, ppm): 172.0, 171.0, 169.9, 161.4, 155.8, 152.9, 143.8, 141.1, 140.0, 139.8, 128.5, 127.7, 127.6, 127.2, 125.9, 125.2, 125.1, 120.0, 119.9, 119.8, 83.2, 82.0, 67.3, 56.9 – 46.9 (9 broad peaks), 28.0, 22.3. LC-ESI-MS: calcd. for $C_{61}H_{82}I_2N_7O_{11}$: 1342.4. Found: 1342.3 $[M+H]^+$, R_t= 7.44 min.

tri-*tert*-butyl2,2',2''-(10-(2-(2-amino-3-(4-hydroxy-3,5-diiodophenyl) propanamido)-3-oxo-3-(((*R*)-1-phenylethyl)amino)propyl)-1,4,7,10-tetraaza-cyclododecane-1,4,7-triyl)triacetate, (**9**). ^{1}H-NMR (CD_3OD, 500 MHz, ppm): 7.64 (d, 2H), 7.27 – 7.15 (m, 5H), 5.02 (d, 1H), 4.10 – 2.85 (m, 28H), 1.51 – 1.25 (m, 30H). LC-ESI-MS: calcd. for $C_{46}H_{72}I_2N_7O_9$: 1120.3 Found: 1120.2 $[M+H]^+$, R_t= 5.6/ 5.8 min.

2,2',2''-(10-(2-acetamido-3-oxo-3-(((*R*)-1-phenylethyl)amino)propyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid H_3(**3**). tri-*tert*-butyl 2,2',2''-(10-(2-amino-3-oxo-3-(((*R*)-1-phenylethyl)amino)propyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (**9**, 11 mg, 0.015 mmol), was dissolved in dichloromethane (2 mL). Triethylamine (2 eq., 5 uL), followed by acetic anhydride (5 eq.) was added and the reaction was stirred over night at room temperature. LCMS analysis indicated complete conversion of starting material. The product was purified using method A. The fractions containing product were pooled and lyophilized to afford the protected precursor (1 mg, 0.0015 mmol, 10% yield after purification). Subsequent deprotection of the tert-butyl acetate moieties yielded the final product H_3(**3**). (for spectral data see article).



10

(*S*)-*tert*-butyl 2-amino-3-(4-hydroxy-3,5-diiodophenyl)propanoate, (**10**). (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-hydroxy-3,5diiodophenyl) propanoic acid (0.67g, 1 mmol) was

dissolved in tert-butyl acetate (7 mL). Perchloric acid (1.5 eq, 90 uL) was added and the solution was stirred at room temperature over night. The acid was neutralized with a saturated aqueous solution of $NaHCO_3$ and the product was extracted three times with dichloromethane. The organic layer was collected, dried with Na_2SO_4 and concentrated. The crude product was subsequently purified with a 0-20% gradient of EtOAc in hexanes on a silica combiflash column. The intermediate product is isolated as yellow oil, which solidifies upon standing. This intermediate was taken up in DMF and the Fmoc protective group was removed according to general procedure 2. The deprotected synthon (**10**) was separated by filtration of the solid support beads and added to the subsequent reaction without further purification steps.

tri-*tert*-butyl 2,2',2''-(10-(2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((*S*)-1-(*tert*-butoxy)-3-(4-hydroxy-3,5-diiodophenyl)-1-oxopropan-2-yl)amino)-3-oxopropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, (**11**). ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm): 7.71 – 7.26 (m, 10H), 4.47 - 2.90 (m, 29H), 1.48 – 1.25 (m, 27H). ^{13}C-NMR ($CDCl_3$, 125 MHz, ppm): 169.9, 160.7, 160.5, 152.6, 152.6, 143.4, 141.3, 141.2, 139.9, 129.7, 128.3, 127.8, 127.1, 127.1, 125.2, 124.9, 120.1, 116.5, 114.2, 83.2, 82.0, 67.5, 67.3, 55.1, 54.5, 54.1, 46.9, 46.9, 46.7, 27.8. LC-ESI-MS: calcd. for $C_{57}H_{81}I_2N_6O_{12}$: 1295.4. Found: 1295.4 $[M+H]^+$, R_t= 7.9 min

tri-*tert*-butyl 2,2',2''-(10-(3-(((*S*)-1-(*tert*-butoxy)-3-(4-hydroxy-3,5-diiodophenyl)-1-oxopropan-2-yl)amino)-3-oxo-2-((*R*)-2-phenylpropanamido)propyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, (**13**). ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm): 8.07 (d, 1H), 7.53 – 7.26 (m, 7H), 4.86 (m, 1H), 4.78 (m, 1H) 4.47 - 2.90 (m, 26H), 1.48 – 1.25 (m, 30H). LC-ESI-MS: calcd. for $C_{51}H_{79}I_2N_6O_{11}$: 1205.4. Found: 1205.3 $[M+H]^+$, R_t= 7.3 min.

2-((*S*)-2-amino-3-(4-hydroxy-3,5-diiodophenyl)propanamido)-3-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)propanoic acid, (**14**).
^{1}H-NMR (CD_3OD, 500 MHz, ppm): 7.69 (d, 2H), 5.08 (m, 1H), 4.59 (m, 1H), 4.2 – 2.98 (m, 26H), 1.55 – 1.47 (m, 27H). LC-ESI-MS: calcd. for $C_{38}H_{63}I_2N_6O_{10}$: 1017.3. Found: 1017.3 $[M+H]^+$, R_t= 5.3 min.

2-(2-(4-isobutylphenyl)propanamido)-3-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)propanoic acid, (**15**). ^{1}H-NMR (CD_3OD, 500 MHz, ppm): 7.29 – 7.11 (m, 4H), 5.05 (m, 1H), 3.97 – 2.98 (m, 24H), 2.45 (m, 2H), 2.15 (s, 3H), 1.83 (m, 1H), 1.52 – 1.43 (m, 30H), 1.30 (s, 3H), 0.87 (m, 6H). ^{13}C-NMR (CD_3OD, 125 MHz, ppm): 175.9, 175.4, 160.9, 160.6, 140.5, 138.1, 129.2, 117.5, 115.2, 84.2, 82.8, 55.4, 55.0, 54.5, 53.8, 45.6, 44.5, 30.0, 29.3, 27.1, 22.8, 21.4. LC-ESI-MS: calcd. for $C_{42}H_{72}N_5O_9$: 790.5. Found: 790.5 $[M+H]^+$, R_t= 7.0 min.

tri-*tert*-butyl 2,2',2''-(10-(3-(((*S*)-1-(*tert*-butoxy)-3-(4-hydroxy-3,5-diiodophenyl)-1-oxopropan-2-yl)amino)-2-(2-(4-isobutylphenyl)propanamido)-3-oxopropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, (**16**). ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm): 7.73 – 7.04 (m, 6H), 6.63 (s, 1H), 6.08 (s, 1H), 5.90 (s, 1H), 4.85 (m, 1H), 4.50 (m, 1H), 4.39 (m, 1H), 3.68 – 2.40 (m, 28H), 1.83 (m, 1H), 1.55 – 0.87 (m, 45 H). LC-ESI-MS: calcd. for $C_{55}H_{88}I_2N_6O_{11}$: 1261.4. Found: 1261.3 $[M+H]^+$, R_t= 8.0 min.

Characterization data for Gd complexes

Compound Gd(**1**). LC-ESI-MS calcd. for $C_{20}H_{34}GdN_6O_9$: 660.2. Found: 660.15 $[M+H]^+$. R_t: 1.0 min (Method C).

Compound Gd(**2**). LC-ESI-MS calcd. for $C_{25}H_{38}GdN_6O_7$: 692.2. Found: 692.25 $[M+H]^+$. R_t: 8.7/9.1 min (Method C).

Compound Gd(**3**). LC-ESI-MS calcd. for $C_{27}H_{40}GdN_6O_8$: 734.21. Found: 734.2 $[M+H]^+$. R_t: 7.2 min (Method C).

Compound Gd(**4**). LC-ESI-MS calcd. for $C_{26}H_{36}GdI_2N_6O_{10}$: 1004.0 Found: 1004.0 $[M^-+2H]^+$. R_t: 8.6/9.0 min (Method C).

Compound Gd(**5**). LC-ESI-MS calcd. for $C_{29}H_{41}GdI_2N_7O_{11}$: 1075.0 Found: 1075.0 $[M+H]^+$. R_t: 8.0/9.0 min (Method C).

Compound Gd(**6a**). LC-ESI-MS calcd. for $C_{34}H_{45}GdI_2N_7O_9$: 1105.0 Found: 1105.1 $[M+H]^+$. R_t: 10.6 min (Method C).

Compound Gd(**6b**). LC-ESI-MS calcd. for $C_{34}H_{45}GdI_2N_7O_9$: 1105.0 Found: 1105.1 $[M+H]^+$. R_t: 12.2 min (Method C).

Compound Gd(**7**). LC-ESI-MS calcd. for $C_{35}H_{44}GdI_2N_6O_{11}$: 1136.0 Found: 1136.1 $[M^-+2H]^+$. R_t: 12.4 min (Method C).

Compound Gd(**8**). LC-ESI-MS calcd. for $C_{39}H_{52}GdI_2N_6O_{11}$: 1192.1 Found: 1192.1 $[M^-+2H]^+$. R_t: 14.1 min (Method C).

Compound Gd(**9**). LC-ESI-MS calcd. for $C_{30}H_{45}GdN_5O_9$: 777.2 Found: 777.3 $[M^-+2H]^+$. R_t: 13.4/13.7 min (Method C).

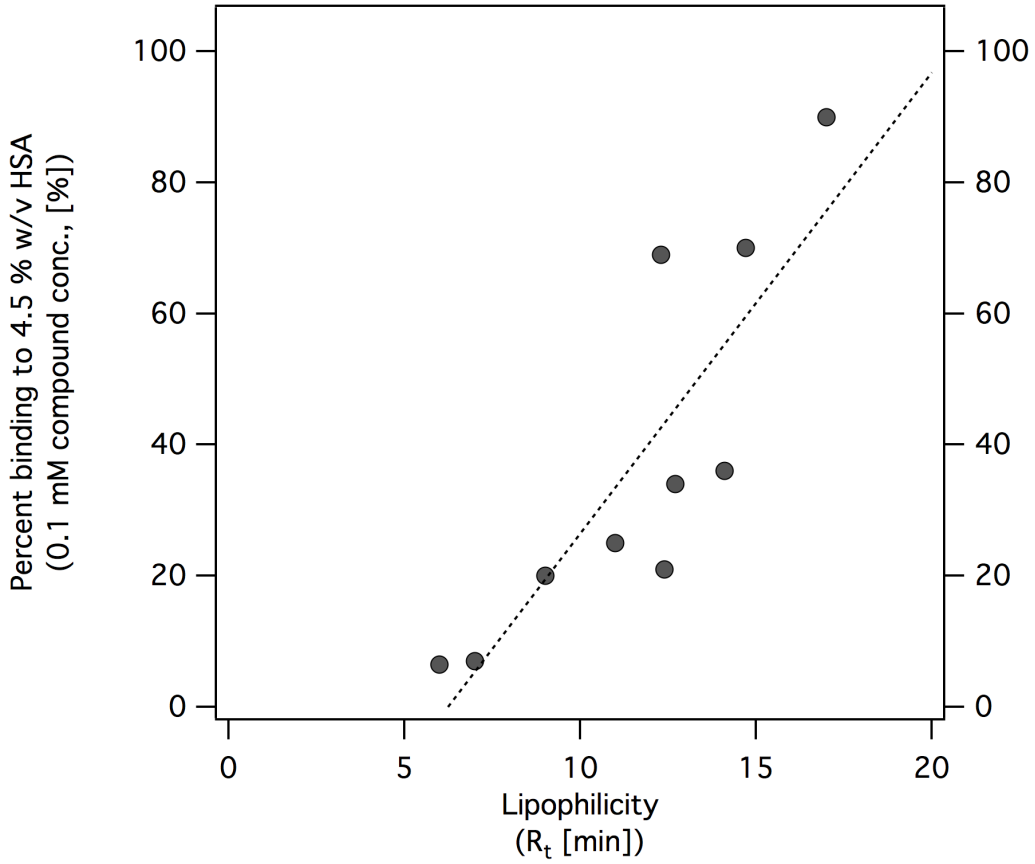


Figure S1. Correlation plot of lipophilicity versus percent binding to HSA. $R^2 = 0.72$.

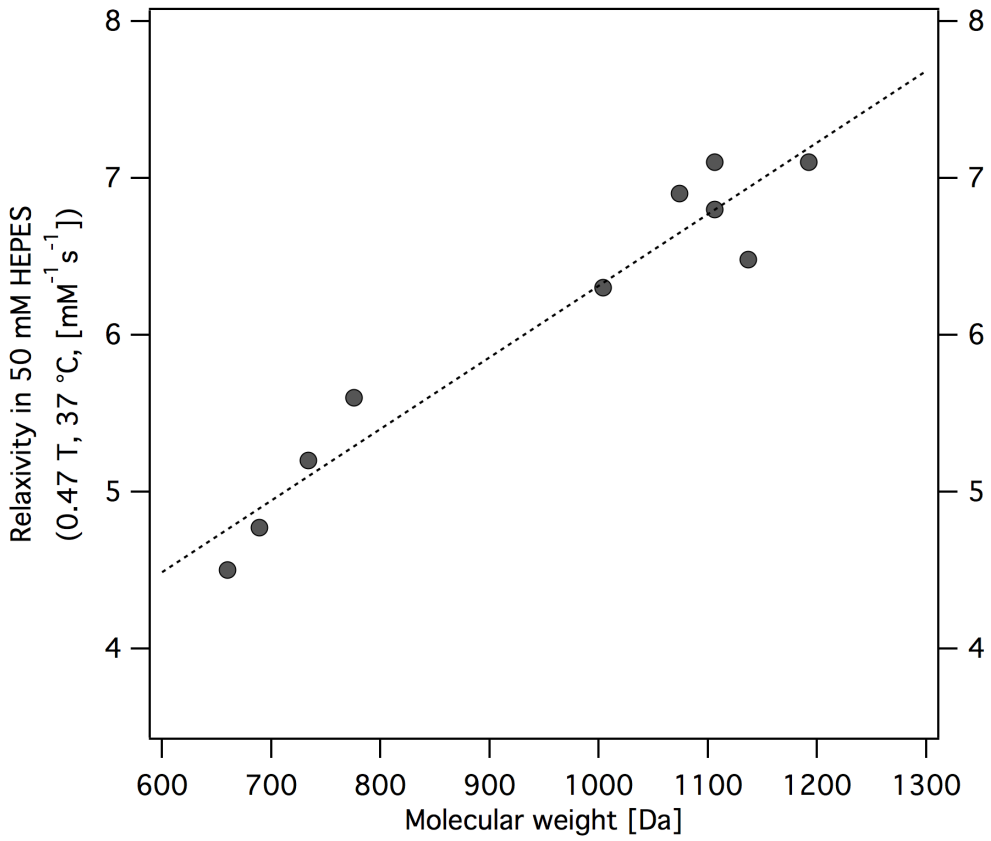


Figure S2. Correlation plot of molecular weight versus relaxivity in HEPES buffer at 0.47 T. $R^2 = 0.94$.

Abbreviations

DO3A	1,4,7,10-Tetraazacyclododecane-1,4,7-triacetate
HSA	Human serum albumin
DTPA	Diethylene triamine pentaacetic acid
HATU	(*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate)
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
ICP-MS	Inductively coupled plasma mass spectrometry
MeCN	Acetonitrile