Multivalent, High-Relaxivity MRI Contrast Agents Using Rigid Cysteine-Reactive Gadolinium Complexes

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General Procedures and Materials

Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F_{254} plates with visualization by ultraviolet (UV) irradiation at 254 nm and/or staining with potassium permanganate. Purifications by flash chromatography were performed using EM silica gel 60 (230-400 mesh). The eluting system for each chromatography was determined by TLC analysis. Chromatography solvents were used without distillation. The solvents resulting from extraction procedures were dried using anhydrous Na_2SO_4 and removed under reduced pressure with a rotary evaporator. Water (ddH_2O) used in biological procedures or as a reaction solvent was deionized using a NANOpure™ purification system (Aldrich, Barnstead, USA).

Instrumentation and Sample Analysis Preparations

***NMR*:** ^1H and ^{13}C spectra were measured with a Bruker AVQ-400 (400 MHz), Bruker AVB-400 (400 MHz) or a Bruker AV-600 (600 MHz) spectrometer, as noted. ^1H NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 7.26, singlet), dimethyl sulfoxide-d_6 (δ 2.50, pentet) or methanol-d_4 (δ 3.31, pentet). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), br (broadened), or app (apparent). Coupling constants are reported as a *J* value in Hertz (Hz). The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. ^{13}C NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 77.2, triplet), or methanol-d_4 (δ 49.00, septet).

***Mass Spectrometry*:** High resolution Electrospray (ESI) and Fast Atom Bombardment (FAB^+) mass spectra were obtained at the UC Berkeley Mass Spectrometry Facility. Electrospray LC/MS analysis was performed using an API 150EX system (Applied Biosystems, USA) equipped with a Turbospray source and an Agilent 1100 series LC pump. Protein chromatography was performed using a Phenomenex Jupiter™ 300 5μ C18 300 Å reversed-phase column (2.0 mm x 150 mm) with a MeCN:ddH_2O gradient mobile phase containing 0.1% formic acid (250 μL/min). Protein mass reconstruction was performed on the charge ladder with Analyst software (version 1.3.1, Applied Biosystems).

***High Perfomance Liquid Chromatography*:** HPLC was performed on an Agilent 1100 Series HPLC System (Agilent Technologies, USA). Size exclusion chromatography was accomplished on an Agilent Zorbax® GF-250 with isocratic (0.5 mL/min) flow using an aqueous mobile phase (10 mM Na_2HPO_4, pH 7.2). Reversed-phase liquid chromatography on protein samples was accomplished on a Agilent Poroshell 300 SB-C18 column (2.1 x 75 mm) using a MeCN:ddH_2O gradient mobile phase containing 0.1% trifluoroacetic acid. Sample analysis for all HPLC experiments was achieved with an in-line diode array detector (DAD) and an in-line fluorescence detector (FLD).

***Protein Purification*:** General desalting and removal of other small molecules from biological samples were achieved using NAP-5 or NAP-10 gel filtration columns (GE Healthcare). Protein samples were concentrated by way of centrifugal ultrafiltration using Amicon Ultra-4 or Ultra-15 100 kDa molecular weight cut off (MWCO) centrifugal filter units (Millipore), or Amicon Microcon 100 kDa MWCO (Millipore) centrifugal filter units. Dialysis was achieved with 10,000 molecular weight cutoff Slide-A-Lyzer® Dialysis Cassettes (Pierce Biotechnology, Inc., USA) as indicated below.

***Gel Analyses*:** Sodium dodecyl sulfate-poly(acrylamide) gel electrophoresis (SDS-PAGE) was accomplished on a Mini-Protean apparatus from Bio-Rad (Hercules, CA) with 10-20% gradient polyacrylamide gels (BioRad, CA), following the protocol of Laemmli.[1] All electrophoresis protein samples were mixed with SDS loading buffer in the presence of dithiothreitol (DTT) and heated to 100 °C for 10 min to ensure reduction of disulfide bonds and complete denaturation unless otherwise noted. Commercially available molecular mass markers (Bio-Rad) were applied to at least one lane of each gel for calculation of the apparent molecular masses. Visualization of protein bands was accomplished by staining with Coomassie® Brilliant Blue R-250 (Bio-Rad, USA). Gel imaging was performed on an EpiChem3 Darkroom system (UVP, USA).

***Dynamic Light Scattering*:** DLS measurements were performed on a Zetasizer Nano ZS (Malvern Instruments, UK). Samples were taken in 12.5 mM HEPES buffer pH 7 at 24 ºC.

***Transmission Electron Microscopy (TEM)*:** TEM images were obtained at the UC-Berkeley Electron Microscope Lab (www.em-lab.berkeley.edu) using a FEI Tecnai 12 transmission electron microscope with 100 kV accelerating voltage.

***Inductively coupled plasma optical emission spectroscopy (ICP-OES)*:** Gd-content measurements were done on a Perkin Elmer Optima 7000DV ICP-OES in the Berkeley Department of Chemistry or a Perkin Elmer Optima 5300 DV ICP-OES instrument in the Berkeley College of Natural Resources.

Centrifugations were conducted with an Allegra 64R Tabletop Centrifuge (Beckman Coulter, Inc., USA). Relaxation time measurements were done on a 60 MHz Bruker mq60 NMR analyzer (Courtesy of the C. Chang Lab at UC Berkeley).

Experimental Procedures

N87C MS2 production

The pBAD-MS2 plasmid and protein expression has been previously reported.[2] The pBAD-MS2-N87C mutant was made by site-directed mutagenesis of the pBAD-MS2 plasmid. Position 87 was converted to a cysteine using the following forward and reverse primers:

Forward: 5'–AGCCGCATGGCGTTCGTACTTATGTATGGAACTAACCATTC–3'

Reverse: 5'–GAATGGTTAGTTCCATACATAAGTACGAACGCCATGCGGCT–3'

Growth and purification of MS2-N87C was identical to that of wtMS2. Yields are slightly less than the 100 mg/L reported for wtMS2.

*Synthesis and Characterization of Protein Reactive Compounds (**1**) to (**6a-c**)*

TAM-$(OBn)_2$-$(OPFP)_2$ bis-PFP ester of 2,3-bis(benzyloxy)terephthalic acid (2)

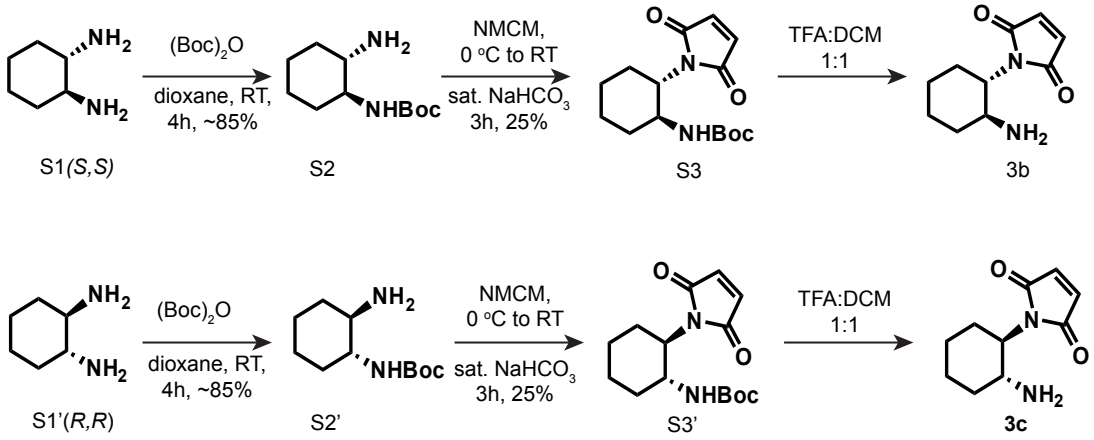
To a solution of 2,3-bis(benzyloxy)-terephthalic acid[3] (**1**, 100 mg, 0.264 mmol) in dry THF (20 mL) under a nitrogen atmosphere were added DIPEA (0.2 mL, 1.2 mmol, excess) and pentafluorophenyl acetate (0.114 mL, 0.661 mmol) slowly over 20 min, resulting in dense white fumes. The reaction was stirred for 1.5 h at RT, after which the solvent was removed using a rotary evaporator. The resulting material was diluted

with ethyl acetate, washed twice with 30 mL portions of dilute aqueous $NaHCO_3$ solution and one portion of brine, and then dried over anhydrous Na_2SO_4. After purification by gradient flash chromatography (5% EtOAc:Hexanes), **2** was obtained as a pale-yellow solid (146 mg, 78 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.21 (s, 4H), 7.31-7.52 (m, 10H), 7.89 (s, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 160.2, 154.5, 136.9, 128.7, 128.5, 128.1, 126.5. HRMS (ESI) calculated for $C_{34}H_{16}F_{10}O_6$ ([M+Na]) 733.0679, found 733.0666.

***tert*-butyl (1*S*,2*S*)-2-aminocyclohexylcarbamate (S2)[4].**

(1*S*,2*S*)-cyclohexane-1,2-diamine (**S1**) (10.7 g, 93 mmol) was dissolved in 200 mL of dioxane in a clean dry flask. A solution of di-*tert*-butylpyrocarbonate (Boc-anhydride, 2.04g, 9.34 mmol) in 60 mL of dioxane was added dropwise over 1 h at RT. After the completion of reaction (5 h), the solvent was removed under reduced pressure. The reaction mixture was then extracted with four 50 mL portions of CH_2Cl_2, dried over

Scheme SI-1



Na_2SO_4, and concentrated under reduced pressure to afford **S2** (~85% pure), which was directly taken to the next step without further purification.

***N*-Boc-(1*S*,2*S*)-cyclohexyldiamine mono maleimide (S3).**

The crude product from the previous step (**S2**, ~500 mg, 2.33 mmol) was dissolved in 45 mL of saturated aqueous $NaHCO_3$ solution by sonication for 1 h. The solution was then cooled 0 ºC. Freshly powdered *N*-(methoxycarbonyl)maleimide (452 mg, 2.91 mmol) was added to the rapidly stirring solution, and stirring was continued at 0 ºC for 1 h, followed by RT for an additional 1.5 h. The reaction mixture was extracted with four 30 mL portions of EtOAc, washed with brine, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure. Gradient flash chromatography with 1:1 EtoAc: Hexanes afforded **S3** (172 mg, 25%) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.62 (s, 2H), 4.3 (br d, 1H, J = 9.2 Hz), 3.97 (br m, 1H), 3.64 (m, 1H, J = 4 Hz), 2.36 (q, 1H, J = 10 Hz), 2.04 (br d, 1H, J = 10.5 Hz), 1.82-1.71 (m, 4H), 1.44-1.22 (m, 11H).^{13}C NMR (100 MHz, $CDCl_3$): δ, 24.67, 25.30, 28.22, 28.56, 33.07, 50.21, 55.66, 79.13, 133.8, 155.17, 171.04. HRMS (ESI) calculated for $C_{15}H_{22}N_2O_4$ ([M+Na]) 317.1472, found 317.1470.

1-((1*S*,2*S*)-2-aminocyclohexyl)-1*H*-pyrrole-2,5-dione (3b)
To a solution of **S3** (50 mg, 0.17 mmol) in 1 mL of CH_2Cl_2 was added 1 mL of TFA. The resulting solution was stirred at RT for 15 min. The volatile components were removed under a stream of N_2, and the material was further concentrated under reduced pressure. After drying under high vacuum, the deprotected amine **(3b)** was used directly for reaction with **2**.

All of the reactions corresponding to the *R,R*-enantiomer were done following the same procedures as for the *S*,*S*-enantiomer.
S3' data: 1.39-1.19 (m, 12H), 1.77-1.66 (m, 3H), 1.99 (m, 1H), 2.31(q, 1H, *J* = 12 Hz), 3.61 (d of t, 1H, *J* = 12,3.2 Hz), 3.93 (m, 1H), 4.34 (d, 1H, *J* = 8 Hz), 6.58 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.64, 25.26, 28.19, 28.52, 32.96, 50.16, 55.57, 79.03, 133.84, 155.14, 171.03. HRMS (ESI) calculated for $C_{15}H_{22}N_2O_4$ ([M+Na]) 317.1472, found 317.1472.

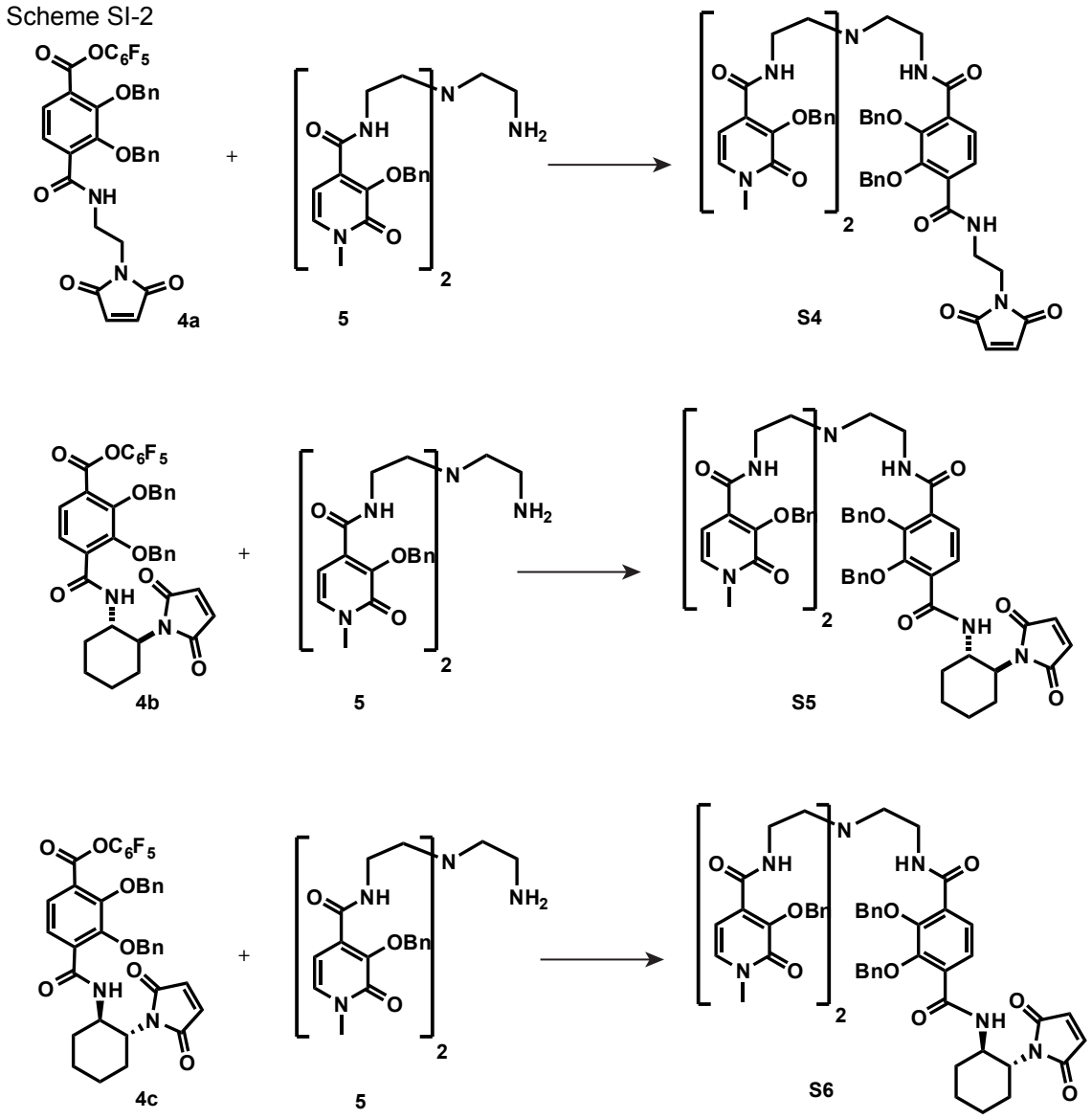
TAM-(OBn)$_2$-(OPFP)-ethyl-maleimide (4a)
A solution of **2** (450 mg, 0.63 mmol) was dissolved in 270 mL of DCM, after which DIPEA (0.5 mL, excess) was added. A solution of 1,2-amino-ethyl-maleimide (**3a**, 14.4 mg, 0.103 mmol) in 130 mL of dichloromethane was added slowly over 24 h using a slow-addition set-up. After completion of the reaction, the solvents were concentrated under reduced pressure and the residue was purified by flash chromatography (35% EtOAc in Hexanes) to give **4a** (146 mg, 69%) as a foamy white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.47 (app q, 2H, *J* = 6 Hz), 3.62 (t, 2H, *J* = 5 Hz), 5.16 (s, 2H), 5.19 (s, 2H), 6.62 (s, 2H), 7.26-7.48 (m, 10H), 7.88 (d, 1H, *J* = 8 Hz), 7.94 (d, 1H, *J* = 8 Hz), 7.97 (t, 1H, *J* = 5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 37.3, 38.67, 125.55, 126.25, 127.01, 128.48, 128.52, 128.75, 128.87, 128.99, 132.57, 134.01, 134.14, 135.63, 136.10, 136.66, 151.69, 153.57, 160.3, 164.15, 170.64. HRMS (ESI) calculated for $C_{34}H_{23}N_2O_7F_5$ ([M+Na]) 689.1318, found 689.1310.

TAM-(OBn)$_2$-(OPFP)-*S,S*-cyclohexyl-maleimide (4b)
A solution of **2** (242 mg, 0.34 mmol) was dissolved in 200 mL of DCM, after which DIPEA (0.3 mL, excess) was added. A solution of 1,2-amino-ethyl-maleimide (**3b**, 33 mg, 0.17 mmol) in 150 mL of dichloromethane was added slowly over 24 h using a slow-addition set-up. After completion of the reaction, the solvents were concentrated under reduced pressure and the residue was purified by flash chromatography (35% EtOAc in Hexanes) to give **4b** (80.9 mg, 66%) as a foamy white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.63 (d of q, 1H, *J* = 3.2, 9.2 Hz), 1.12 (m, 1H), 1.36 (m, 1H), 1.65 (br d, 1H, *J* = 13 Hz), 1.73 (br d, 1H, *J* = 12 Hz), 1.81 (br d, 2H, *J* = 13 Hz), 2.39 (d of q, 1H, *J* = 10,3Hz), 3.64 (d of t, 1H, *J* = 12,4 Hz), 4.47 (d of t, 1H, *J* = 15.6, 4.4 Hz), 5.18 (m, 4H), 6.57 (s, 2H), 7.26-7.48 (m, 10H), 7.76 (d, 1H, *J* = 8 Hz), 7.83 (d, 1H, *J* = 8 Hz), 7.89 (d, 1H, *J* = 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.33, 25.21, 28.5, 32.06, 49.56, 55.03, 125.49, 126.42, 126.77, 128.47, 128.51, 128.54, 128.78, 128.82, 128.86, 131.93, 133.72, 135.77, 136.01, 151.84, 153.41, 160.27, 162.91, 171.04. HRMS (ESI) calculated for $C_{38}H_{29}N_2O_7F_5$ ([M+Na]) 743.1787, found 743.1781.

TAM-(OBn)$_2$-(OPFP)-*R,R*-cyclohexyl-maleimide (4c)
A solution of **2** (362 mg, 0.51 mmol) was dissolved in 250 mL of DCM, after which DIPEA (0.3 mL, excess) was added. A solution of 1,2-amino-ethyl-maleimide (**3c**, 49.5 mg, 0.26 mmol) in 170 mL of dichloromethane was added slowly over 24 h using a slow-addition set-up. After completion of the reaction, the solvents were concentrated under reduced pressure and the residue was purified by flash chromatography (35% EtOAc in Hexanes) to give **4c** (123 mg, 67%) as a foamy white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.64 (d of q, 1H, *J* = 12, 3.2Hz), 1.01-1.41 (m, 2H), 1.65 (d, 1H, *J* = 12 Hz), 1.73(d, 1H, *J* = 12 Hz), 1.81 (d, 2H, *J* = 12 Hz), 2.39 (d of q, 1H, *J* = 12,3.2Hz), 3.64 (m, 1H), 4.47 (m, 1H, *J* = 12,4Hz), 5.2-5.15 (m, 4H), 6.58 (s, 2H), 7.34-7.48 (m, 10H), 7.77 (d, 1H, *J* = 8 Hz), 7.83 (d, 1H, *J* = 8 Hz), 7.89 (d, 1H, *J* = 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.33, 25.21, 28.5, 32.06, 49.59, 55.02, 60.41,

125.52, 126.40, 126.77, 128.47, 128.51, 128.54, 128.78, 128.83, 128.87, 131.90, 133.73, 135.77, 136.01, 151.85, 153.41, 160.27, 162.98, 171.04. HRMS (ESI) calculated for $C_{38}H_{29}N_2O_7F_5$ ([M+Na]) 743.1787, found 743.1782.

Scheme SI-2



TREN-Bis(*N*-methyl-3, 2-HOPO-OBn)-TAM-(OBn)$_2$-ethyl-maleimide (S4)

TREN-bis(*N*-methyl-3,2-HOPO-OBn) (**5**, 55 mg, 0.09 mmol) and TAM-(OBn)$_2$-(OPFP)-ethyl-maleimide (**4a**, 50 mg, 0.075 mmol) were dissolved in 10 mL of dichloromethane. To this solution was added 0.2 mL of DIPEA. The reaction mixture was stirred for 15 h at RT, after which the solvent was removed under reduced pressure. The residue was further concentrated under high vacuum. The crude product was purified by gradient flash chromatography using 5% MeOH in CH_2Cl_2 to give the product as a pale-white solid (67.5 mg, 81%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.25 (t, 4H, J = 6 Hz), 2.32 (t, 2H, J = 6 Hz), 3.09 (br d, 4H, J = 7 Hz), 3.16 (br d, 2H, J = 5.6 Hz), 3.49 (apparent q, 2H, J = 6 Hz), 3.55 (s, 6H), 3.61 (t, 2H, J = 6 Hz), 5.07 (s, 2H), 5.11 (s, 2H), 5.27 (s, 4H), 6.57 (s, 2H), 6.61 (d, 2H, J = 7 Hz), 7.07 (d, 2H, J = 7 Hz), 7.26-7.36 (m, 20H), 7.56 (t, 1H, J = 5 Hz), 7.64 (d, 1H, J = 8 Hz), 7.72 (d, 1H, J = 8 Hz), 7.82 (t, 2H, J = 4 Hz), 7.99 (t, 1H, J = 6 Hz).^{13}C-NMR (100 MHz, $CDCl_3$): δ 37.2, 37.3, 37.4, 37.7, 38.5, 41.9, 52.0, 52.2, 53.5, 74.7, 104.7, 125.8, 126.0, 128.4, 128.5, 128.7, 128.72, 128.8, 128.82, 128.9, 130.3, 130.7, 131.7, 132.3, 134.0, 136.0, 136.1, 136.4, 146.3, 150.2, 150.4, 159.5, 163.3, 164.7, 165.1, 170.7. HRMS (ESI) calculated for $C_{62}H_{62}N_8O_{12}$([M+H]) 1111.4560, found 1111.4544.

TREN-Bis(*N*-methyl-3, 2-HOPO-OBn)-TAM-(OBn)$_2$-*S,S*-cyclo-hexyl-maleimide (S5)

A solution of **4b** (60 mg, 0.083 mmol) in 5 mL of dichloromethane was added to a solution of **5** (68.1 mg, 0.11 mmol) in 5 mL of dichloromethane. To this solution was added 0.3 mL of DIPEA. The reaction mixture was then stirred for 15 h at RT, after which the solvent was removed under reduced pressure. The crude product was purified by flash chromatography, eluting with a gradient of 100% CH_2Cl_2 to 7% MeOH: 93% CH_2Cl_2. The protected ligand was obtained as a pale white solid (88 mg, 91%) after drying under high vacuum. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.71 (m, 1H), 1.1-1.4 (m, 2H), 1.65 (br d, 1H, *J* =12 Hz), 1.72 (br d, 1H, *J* = 12 Hz), 1.81 (br t, 3H), 2.2-2.4 (m, 6H), 2.9-3.14 (m, 6H), 3.57 (s, 6H), 3.63 (m, 1H), 4.47 (m, 1H), 5.07 (m, 2H), 5.15 (m, 2H), 5.28 (s, 4H), 6.54 (s, 2H), 6.61 (d, 2H, *J* = 7.2 Hz), 7.06 (d, 2H, *J* = 7.2 Hz), 7.25-7.34 (m, 12H), 7.35-7.45 (m, 6H), 7.5 (m, 2H), 7.57 (t, 1H), 7.61 (d, 1H, *J* = 8 Hz), 7.66 (d, 1H, *J* = 8 Hz), 7.70 (d, 1H, *J* = 8 Hz), 7.81 (t, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 24.38, 25.23, 28.56, 32.14, 37.21, 37.66, 49.42, 52.02, 52.27, 55.02, 74.70, 104.6, 125.54, 126.16, 128.39, 128.51, 128.66, 128.72, 128.76, 128.91, 129.68, 130.58, 131.47, 132.21, 133.87, 135.89, 136.18, 136.30, 146.26, 150.07, 150.46, 159.43, 163.28, 163.69, 164.59, 171.04. HRMS (ESI) calculated for $C_{66}H_{68}N_8O_{12}$([M+H]) 1165.5029, found 1165.5009.

TREN-Bis(*N*-methyl-3, 2-HOPO-OBn)-TAM-(OBn)$_2$-*R,R*-cyclo-hexyl-maleimide (S6)

A solution of **5** (91 mg, 0.143 mmol) and **4c** (80.3 mg, 0.11 mmol) in 10 mL of DCM along with 0.2 mL DIPEA were stirred for 15 h at RT. After completion of reaction, the solvent was removed under reduced pressure and the product was obtained after gradient flash chromatography using 7% MeOH-DCM as a pale-white solid (113 mg, 87%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.7 (d of q, 1H, *J* = 12,4Hz), 1.05-1.39 (m, 2H), 1.62 (d, 1H, *J* =12 Hz), 1.69 (d, 1H, *J* = 12 Hz), 1.79 (t, 2H, *J* = 12 Hz), 2.2-2.4 (m, 7H), 3.01-3.17 (m, 6H), 3.53 (s, 6H), 3.58-3.65 (d of t, 1H, *J* = 12,4 Hz), 4.46 (m, 1H, *J* = 12,4 Hz), 5.04 (s, 2H), 5.15 (s, 2H), 5.25 (s, 4H), 6.52 (s, 2H), 6.57 (d, 2H, *J* = 8 Hz), 6.99 (d, 2H, *J* = 8 Hz), 7.25 (m, 12H), 7.32-7.43 (m, 6H), 7.48 (br d, 2H, *J* = 8 Hz), 7.52-7.58 (m, 2H), 7.62 (d, 1H, *J* = 8 Hz), 7.69 (d, 1H, *J* = 8 Hz), 7.93 (t, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 24.4, 25.2, 28.6, 32.1, 37.21, 37.66, 49.4, 52.0, 52.2, 55, 74.7, 104.6, 125.5, 126.1, 128.4, 128.5, 128.6, 128.7, 128.8, 128.9, 129.7, 130.6, 131.5, 132.2, 133.9, 135.9, 136.2, 136.3, 146.3, 150.1, 150.5, 159.4, 163.3, 163.7,164.6,171. HRMS (ESI) calculated for $C_{66}H_{68}N_8O_{12}$([M+H]) 1165.5029, found 1165.4998.

TREN-Bis(*N*-methyl-3, 2-HOPO)-TAM-ethyl-maleimide (6a)

TREN-Bis(*N*-methyl-3,2-HOPO-OBn)-TAM-(OBn)$_2$-ethyl-maleimide **S4** (33 mg, 0.03 mmol) was dissolved in 2 mL DCM, and to this was added a pre-mixed solution of 200 µL thio-anisole and 2 mL trifluoroacetic acid. The reaction mixture was stirred for 3 h at RT and the solvents were evaporated by drying under high vacuum for 2 h. The residue left was dissolved in minimum amount of methanol and precipitated by adding to 10 mL cold ethyl ether. The mixture was centrifuged, the ether layer decanted and the precipitate was resuspended in fresh cold ether to repeat the last step 3-4 times (to remove thio-anisole). The product was obtained as a white solid after drying the precipitate under high vacuum (20.6 mg, 61 %). ^{1}H-NMR (400 MHz, MeOD): δ 3.34-3.49 (overlapped m+s, 16H), 3.64-3.71 (m, 8H), 6.09 (d, 2H, *J* = 8 Hz), 6.63-6.74 (overlapped d+s, 4H), 6.91 (d, 1H, *J* = 8 Hz), 7.00 (d, 1H, *J* = 8 Hz). ^{13}C NMR (150 MHz, MeOD): δ 29.35, 34.98, 36.51, 36.98, 37.99, 42.13, 54.46, (60.36, 77.64), 99.98, 103.88, 108.41, 115.99, 116.8, 117.76, 123.43, 126.84, 129.26, 134.14, 149.11, 158.98, 161.56, 165.19, 171.23. HRMS (ESI) calculated for $C_{34}H_{38}N_8O_{12}$([M+H]) 751.2682, found 751.2696.

TREN-Bis(*N*-methyl-3, 2-HOPO)-TAM-*S,S*-cyclohexyl-maleimide (6b)

To a solution of **S5** (50 mg, 0.043 mmol) in 3 mL of CH_2Cl_2 was added a pre-mixed solution of thio-anisole (300 µL) and 3 mL of trifluoroacetic acid. The reaction mixture was stirred for 3 h at RT, after which the solvents were evaporated by drying under high vacuum for 2 h. The residue was dissolved in a minimal amount of methanol and **6b** was precipitated by adding this solution to 10 mL of cold ethyl ether.

The mixture was centrifuged, the ether layer decanted, and the precipitate was resuspended in fresh cold ether. This rinsing process was repeated 3-4 times to remove the thioanisole. The product was obtained as a white solid after drying the precipitate under high vacuum (22 mg, 63 %). ^{1}H-NMR (400 MHz, MeOD): δ 1.29-1.59 (m, 3H), 1.84 (q, 3H, *J* = 12 Hz), 2.05 (br d, 1H, *J* = 12 Hz), 2.34-2.45 (m, 1H), 3.3 (m, 6H), 3.63 (br m, 6H), 3.85 (br s, 6H), 3.91-3.98 (m, 1H), 4.57 (m, 1H), 6.23 (d, 2H, *J* = 8 Hz), 6.7 (s, 2H), 6.82 (d, 2H, *J* = 8 Hz), 7.01 (d, 1H, *J* = 8 Hz), 7.08 (d, 1H, *J* = 8 Hz). ^{13}C NMR (150 MHz, MeOD): δ 24.32, 25.59, 26.00, 26.51, 30.06, 36.70, 38.13, 58.48, 103.42, 105.49, 117.32, 117.89, 118.29, 118.32, 118.41, 119.26, 126.87, 128.61, 129.11, 132.59, 135.17, 148.94, 152.04, 152.15, 159.89, 160.24, 162.9, 169.14, 172.86. HRMS (ESI) calculated for $C_{38}H_{44}N_8O_{12}$ ([M+H]) 805.3151, found 805.3155.

TREN-Bis(*N*-methyl-3, 2-HOPO)-TAM-*R,R*-cyclohexyl-maleimide (6c)

To a solution of **S6** (50 mg, 0.043 mmol) in 3 mL of CH_2Cl_2 was added a pre-mixed solution of thioanisole (300 μL) and 3 mL of trifluoroacetic acid. The reaction mixture was stirred for 3 h at RT, after which the solvents were evaporated by drying under high vacuum for 2 h. The residue was dissolved in a minimal amount of methanol and **6c** was precipitated by adding this solution to 10 mL cold ethyl ether. The mixture was centrifuged, the ether layer decanted, and the precipitate was resuspended in fresh cold ether. This rinsing process was repeated 3-4 times to remove the thioanisole. The product was obtained as a white solid after drying the precipitate under high vacuum (21.4 mg, 62 %). ^{1}H-NMR (400 MHz, MeOD): δ 1.38-1.58 (m, 3H), 1.78-1.89 (q, 3H), 2.05 (br d, 1H, *J* = 12 Hz), 2.36-2.39 (m, 1H), 3.4-3.6 (overlapped m+s, 12H), 3.8 (br s, 6H), 3.86-3.98 (m, 1H), 4.53-4.59 (m, 1H), 6.25 (d, 2H, *J* = 8 Hz), 6.69 (s, 2H), 6.83 (d, 2H, *J* = 8 Hz), 7.01 (d, 1H, *J* = 8 Hz), 7.07 (d, 1H, *J* = 8 Hz). ^{13}C NMR (150 MHz, MeOD): 25.99, 26.51, 30.06, 33.29, 36.06, 36.52, 38.12, 43.65, 51.10, 55.5, 55.99, 58.48, 65.37, 67.02, 105.39, 117.89, 118.19, 118.38, 118.88, 124.99, 128.13, 128.40, 128.61, 129.48, 129.53, 129.99, 130.82, 132.78, 149.03, 150.27, 160.22, 169.24, 169.74, 171.58, 172.86. HRMS (ESI) calculated for $C_{38}H_{44}N_8O_{12}$ ([M+H]) 805.3151, found 805.3157.

Bioconjugation Reactions, Purifications, and Analyses

Typical MS2 modification with maleimides 6a-c

To a solution of N87C MS2 (800 μL, 160 μM based on monomer in 10 mM Chelex-treated phosphate buffer at pH 7.5) was added 100 μL of 100 mM TRIS (pH 8) and the corresponding maleimide (**6a**, **6b** or **6c**, 1.8-1.9 mg, delivered in 100 μL of a 50 mM stock solution in DMF, 15 equiv). The reaction mixture was incubated at RT for 3 h. Size-exclusion chromatography purification was used to remove excess small-molecule (NAP-10, 10 mM NaOAc, pH 4.5 eluent), followed by multiple rounds of centrifugation to reach 200-250 μM using Amicon® Ultra-4 (100,000 MWCO) spin concentrators. Labeling efficiency was determined to be virtually quantitative by ESI-MS for all three samples (Figure S1 and Figure 2 of the main paper).

Initially, the LC-ESI spectrum/protein reconstruct of the modified capsids showed a peak corresponding to [bioconjugated pdt +55], probably because adventitious Fe was complexed by the HOPO-ligands (the HOPO-ligands have a very strong affinity for iron[5]). This problem was circumvented by treatment of the capsids with Chelex-treated buffers in the final protein purification steps, and by repeated EDTA washes of the LC-MS lines using MeCN-H_2O-ammonium-formate as the solvent system before sample injection.

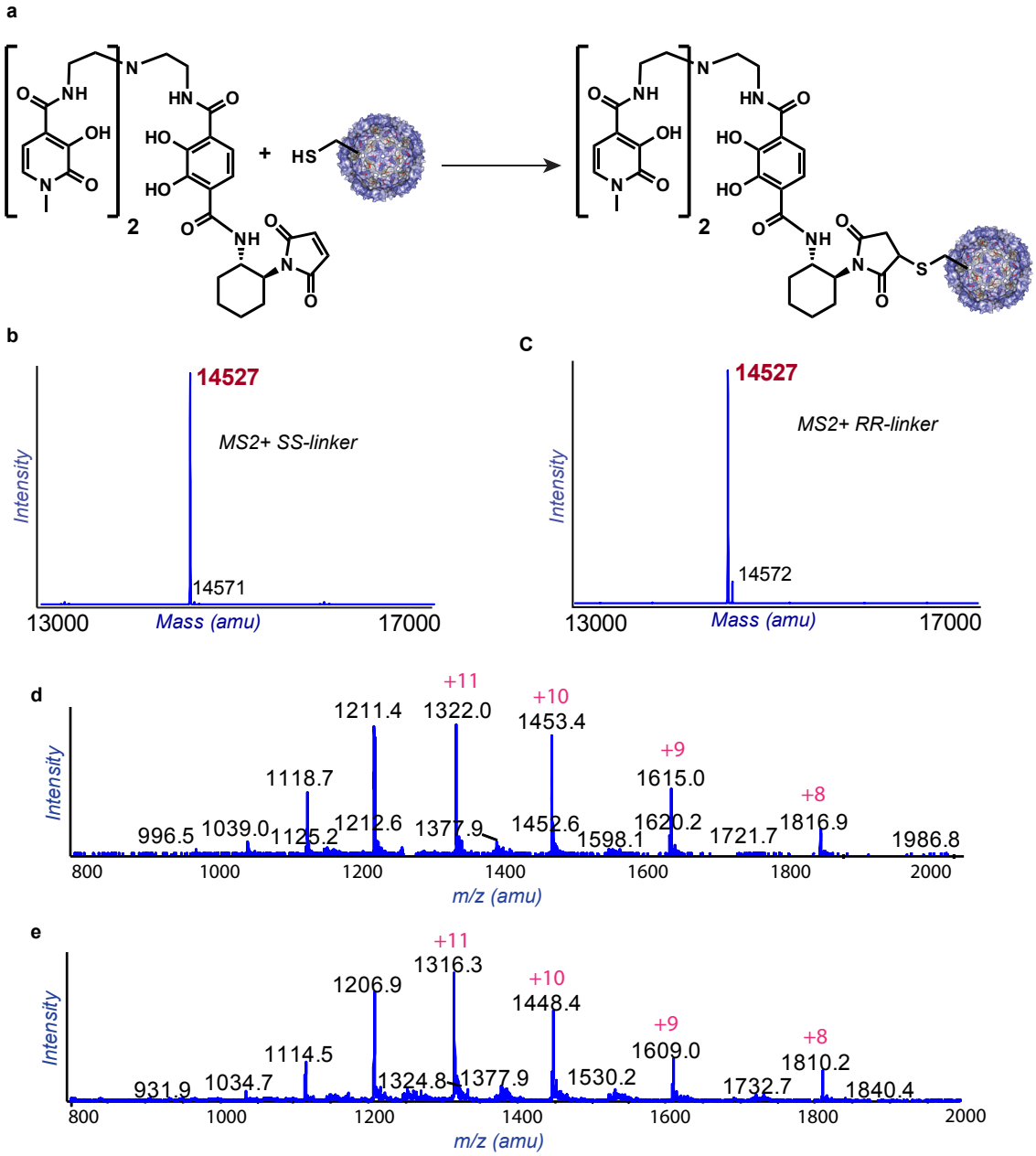


Figure S1: Mass spectral analysis of MS2-conjugates. (a) Bioconjugation of MS2 with the S,S- rigid linker (b) ESI- reconstruct of bioconjugated rigid *S,S*- cyclohexyl linker. (c) ESI- reconstruct of the MS2-*R,R*-cyclohexyl conjugate. (d) Protein charge distribution ladder of MS2-*S,S*- cyclohexyl conjugate and (e) Protein charge distribution ladder of MS2-Lin-conjugate. Almost quantitative conversion was seen in the conjugation reactions with maleimides leading to 150-180 copies of the contrast agent per viral capsid.

Gd-chelation reactions

Gd-complexation of the internally modified capsids was performed by a slight modification to the previously reported protocol.[6,7] A typical procedure involved the addition of 0.95-1 equiv. of aqueous $GdCl_3$ solution (1.5 equiv. was added in the earlier report) to the modified capsids in NaOAc buffer at pH 4.5, and then adjusting to pH 7.

To a solution of the MS2-conjugates in 10 mM NaOAc, pH 4.5 (500 µL, ~250 µM in capsid monomer), was added a previously standardized aqueous solution of $GdCl_3.6H_2O$ solution (0.95 equivalents, 2.64 µL of 48.5 mM solution in 0.1 M HCl) in 0.5 µL aliquots. The solution was then incubated at RT for 15 min, after which it was adjusted to pH 7 by the slow addition of HEPES buffer (100 mM, pH 7.5). The resulting

sample was then purified using a NAP-10 size exclusion column (25 mM HEPES, pH 7), followed by dialysis using 0.5-3 mL cassettes against 25 mM HEPES buffer, pH 7, containing 0.5 mM ammonium citrate for 2 h at RT. The buffer solution was next changed to a fresh solution of 12.5 mM HEPES, pH 7 for overnight dialysis. Following this, the samples were concentrated using Amicon® Ultra-4 (100,000 MWCO) spin-concentrators.

Metallation Control Experiments

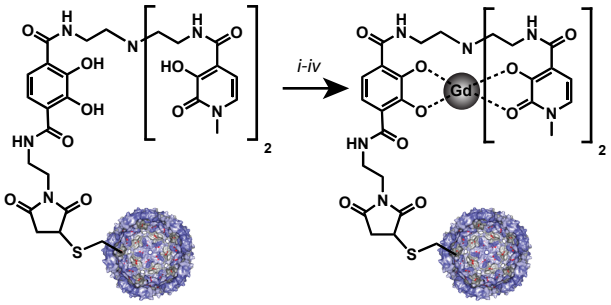


Figure S2. Formation of Gd- Contrast agents. Conditions: (i) 0.95 eq. $GdCl_3$, 10 mM NaOAc, pH 4.5 (ii) pH raised to 7.0 with 100 mM HEPES pH 7.5 (iii) NAP-10 with 25 mM HEPES pH 7 (iv) Dialysis with 25 mM HEPES pH 7 containing citrate (0.5 mM) for 2h, over-night dialysis with 12.5 mM HEPES pH 7 at RT.

Control 1: A solution of MS2 N87C was treated with excess (15 equiv) of *N*-ethyl maleimide for 2 h. The conjugate was purified using NAP-10 size-exclusion chromatography columns eluting with 10 mM NaOAc at pH 4.5 and concentrated to ~200-250 µM using Amicon Ultra-4 spin concentrators. This sample was then treated with 0.95-1 equiv.of $GdCl_3$ stock solution (48.5 mM in 0.1 M HCl). After 15 min, the solution was adjusted to pH 7 with 100 mM HEPES (pH 7.5). After purification using a NAP-10 gel filtration column (eluting with 25 mM HEPES at pH 7), the sample was dialyzed against 25 mM HEPES buffer, pH 7, containing 0.5 mM ammonium citrate for 2 h at RT. This step was followed by overnight dialysis with fresh 12.5 mM HEPES buffer, pH 7, and the sample was concentrated using an Amicon® Ultra-4 (100,000 MWCO) spin-concentrator. Gd-content analysis by ICP-AES indicated that 49% of the original Gd had remained attached to the protein (Figure 3a, Entry 1 in main text).

Control 2: MS2 N87C was treated with an excess of a non-maleimide HOPO complex (HOPO-Ethyl amine) and then purified using an analogous procedure as that described for compounds **6a-c** and Control 1. The resulting conjugate was incubated with an additional equiv. of HOPO-ethyl amine and 0.95-1 equiv. of $GdCl_3$, and then subjected to the same purification steps listed above. Gd-content analysis by ICP-AES indicated that < 5% of the original Gd remained after all the steps. This indicated that the non-specific binding to MS2 N87C observed in the earlier case was prevented when a strong HOPO-ligand was present in solution (Figure 3a, Entry 2 in main text).

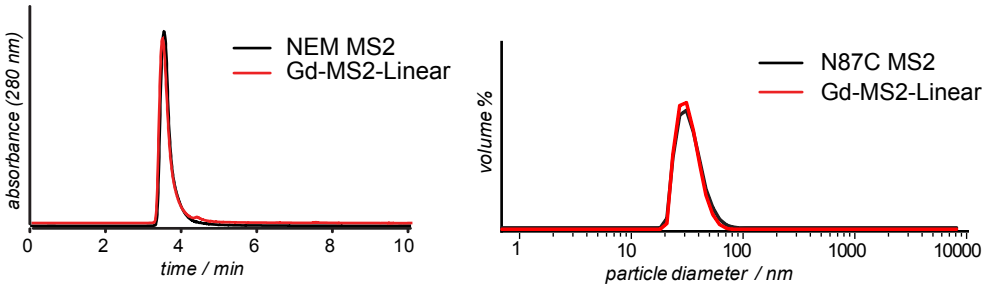


Figure S3. Analysis of Gd-MS2 complexes **(a)** Size exclusion chromatography of control sample (NEM- MS2) and Gd-MS2-Linear sample **(b)** dynamic light scattering analysis of N87C MS2 and Gd-MS2-Linear confirm that the capsids remained assembled after modification.

Gadolinium Content Analysis by ICP-OES

Standard Gd(III) solutions (100 - 1000 ppb, 10 mL each) in water or 2% HNO_3 in water were prepared from a standard aqueous Gd solution (1005 ppm in 1 wt. % HNO_3). The Gadolinium-content of these solutions was measured by ICP-OES. The gadolinium wavelengths monitored were 342.2, 336.2 and 335.1 nm. Calibration curves at each wavelength were generated from these measurements. The complex solutions and the complex-conjugated MS2 solutions were diluted with water so that their concentrations would range between 100-1000 ppb and then the intensity at each wavelength was measured. The Gd-contents were obtained from the calibration curves that had been generated from the standards.

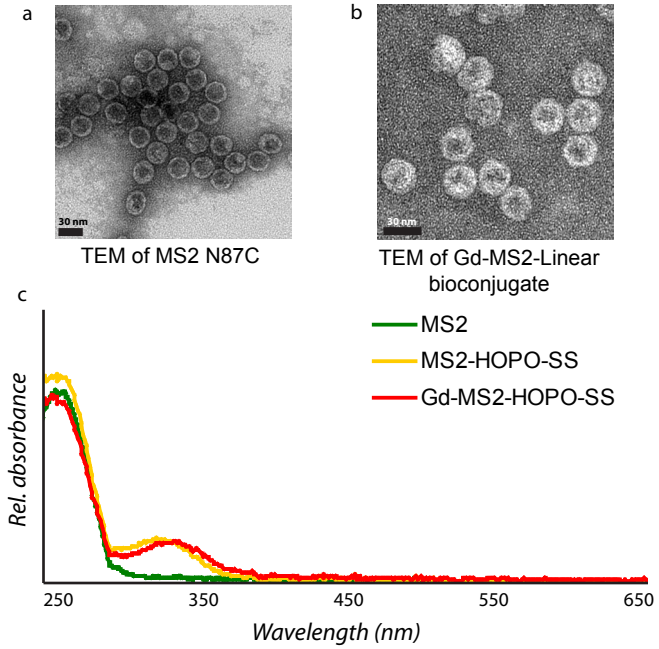


Figure S4. Structural characterization of MS2 contrast agents **(a, b)** Transmission electron microscopy (TEM) was used to verify the aggregate state after Gd-complexation.**(c)** Sample UV-Visible spectra of the protein, bioconjugated protein and Gd-complexed contrast agent.

Determination of MS2-HOPO Conjugate Concentration

A sample of linear HOPO maleimide **6a** was first analyzed using UV/vis to determine the extinction coefficients at 280 and 335 nm. The ratio of these values was calculated to be 1.18. The extinction coefficient at 280 nm was also determined for an unmodified MS2 capsid sample at known concentration. Following the dialysis steps, the concentration of each MS2-HOPO-Gd sample was determined by measuring its absorbance at 280 nm and applying the following correction:

$$A_{280}(\text{protein only}) = A_{280}(\text{conjugate}) - 1.18 * A_{335}$$

The comparison of the protein concentration to the Gd(III) concentration measured using ICP-OES provided an estimate of the number of complexes housed within each capsid shell. We take these estimates to be within 5-10% of the actual values.

Relaxivity at 60 MHz using T_1 measurements

A Bruker Minispec mq60 NMR analyzer (proton Larmor frequency 60 MHz) was used to acquire all the T_1 values using a standard inversion-recovery procedure. All the samples were prepared in 12.5 mM HEPES solution pH 7 and were measured in 7 mm NMR tubes. Gd concentrations were determined by ICP-OES and relaxivities were calculated using the equation $(1/T_1\text{-}1/T_{1d})/$ [Gd], where T_1 is the relaxation

time in the presence of Gd complex and T_{1d} is the inherent diamagentic relaxation time.

Transmission Electron Microscopy

TEM grids were prepared by charging carbon-coated, Formvar-supported copper mesh grids with argon plasma (40 mA at 0.1 mbar for 30 s) in a Cressington 108 Auto Sputter Coater. Protein samples were prepared for TEM analysis by pipetting 5 µL samples onto these grids and allowing them to equilibrate for 3 minutes. The samples were then wicked with filter paper and rinsed with ddH_2O. The grids were then exposed to 5 µL of a 1% (w/v) aqueous solution of uranyl acetate for 90 s as a negative stain. After excess stain was removed, the grid was allowed to dry in air.

Molecular Modeling Studies

Starting structures for the maleimide-substituted Gd-HOPO complexes (Gd-**7b** and Gd-**7c**) were generated based on crystallographically-characterized Gd-HOPO-Tren complex **S7**.[8] Three different coordination isomers were generated for each linker (either *S*,*S* or *R*,*R*) by attaching it to one of the sites indicated in Figure S5. The six resulting complexes were then minimized using MacroModel (version 9.9) with an OPLS 2005 force field and no specified solvent. Although there is an additional chiral center that is formed upon addition to the maleimide, it is assumed to have a negligible influence on the display of the ligands relative to (1) the chiral centers on the bulky-but-rigid cyclohexane ring and (2) the isomer of the Gd-HOPO complex that is actually preferred (see example in Figure S6). A segment of the MS2 capsid protein[9] consisting of 6 monomers was next generated using UCSF Chimera, such that two Cysteine 87 groups were located in the center, Figure S7. The maleimide groups of two copies of the desired Gd complex were then attached to the sulfur atoms of the cysteines using the Maestro 9.2 modeling environment. The resulting structures were then minimized using MacroModel with the OPLS 2005 force field and no specified solvent. The atoms of the Gd complexes were fully unconstrained, as were all amino acid residues within a 20 Å sphere. The remaining atoms of the protein were frozen during the calculation. In all cases, convergence was achieved after 3000-4500 cycles. The images shown in Figure 3 of the main text and Figures S5-S7 were generated using Mac PyMol 1.4.

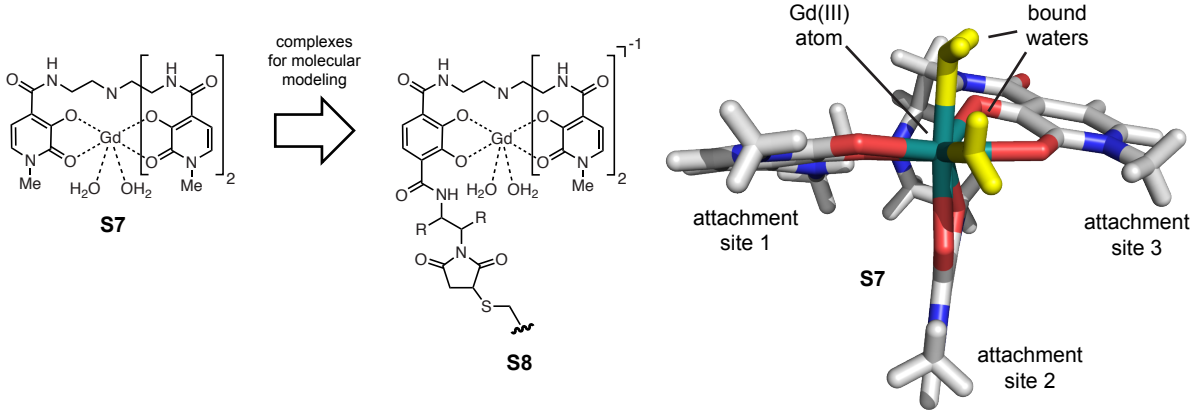


Figure S5. Attachment sites for the generation of the three coordination isomers. The crystal structure of **S7** has been reported previously.[8]

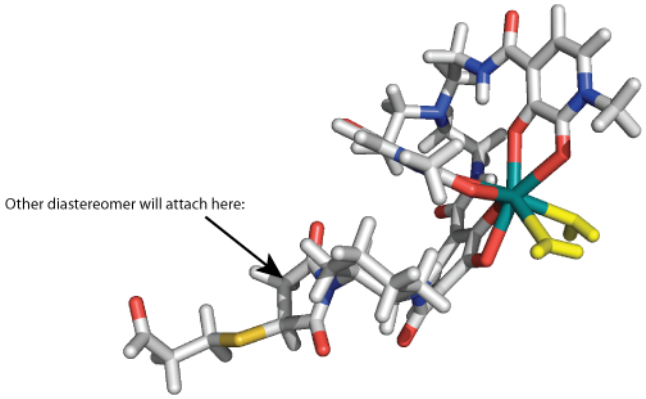


Figure S6. Comparison of diastereomeric attachment points for maleimides after attachment to MS2 capsids.

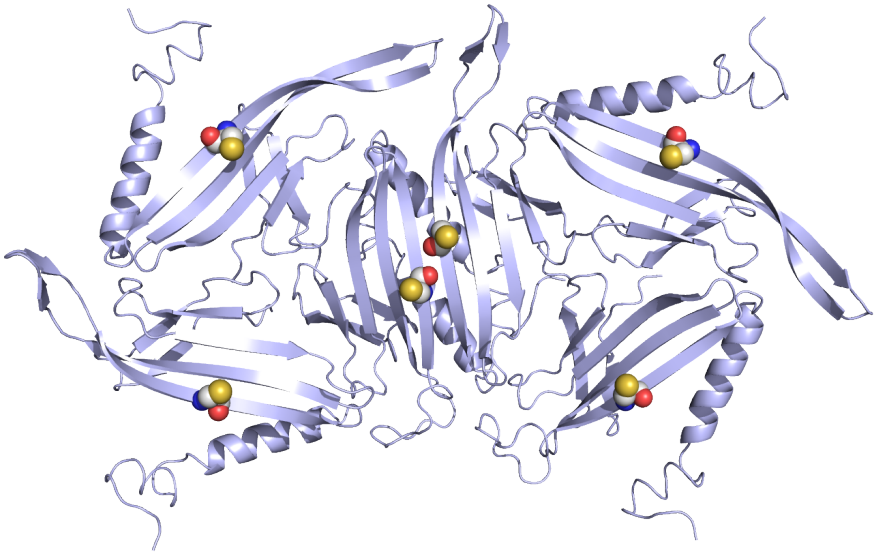
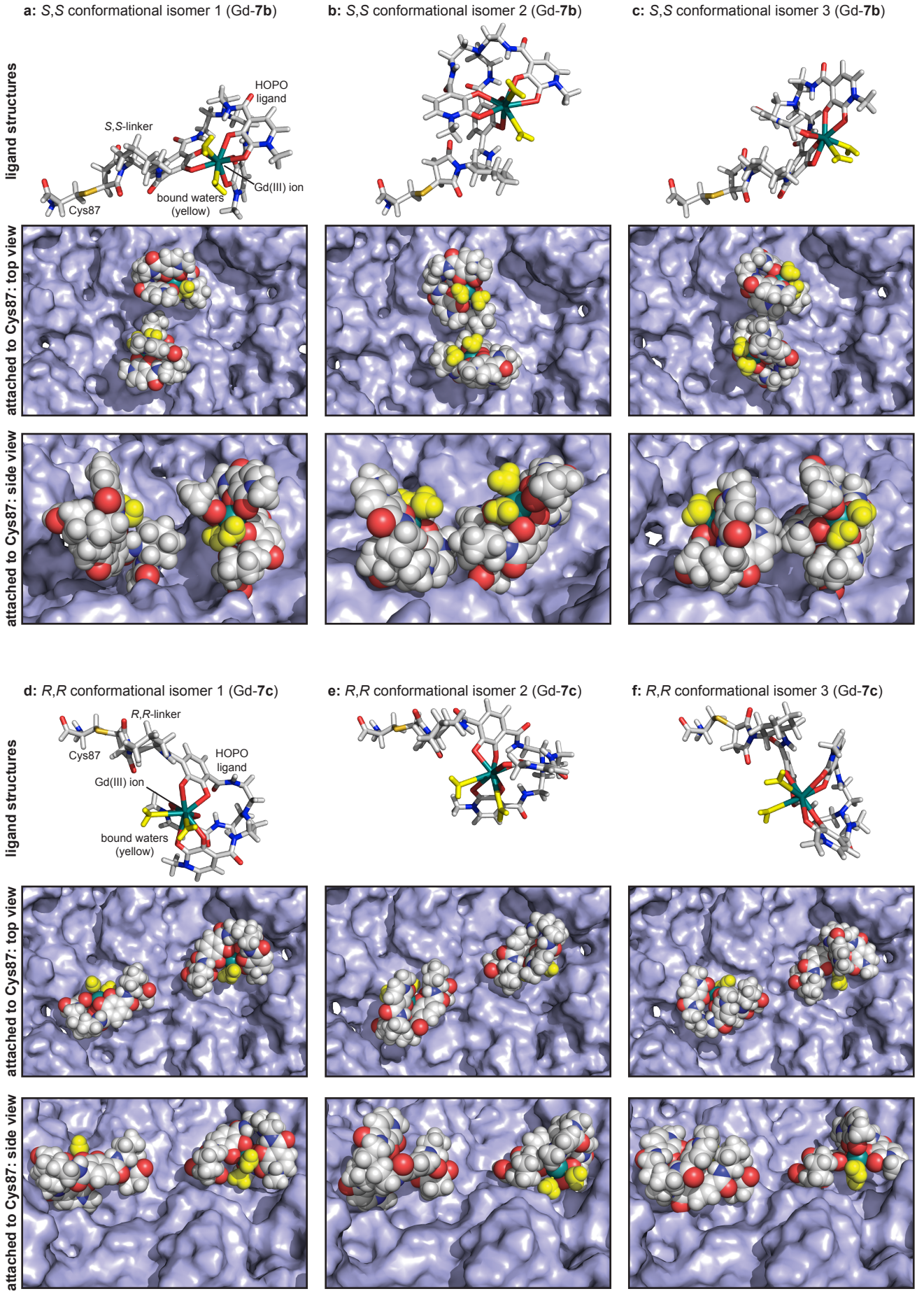


Figure S7. A C2-symmetric MS2 structure was generated for the modeling studies.[9] The Cys 87 groups are shown. Identical complexes were attached to the two central cysteine groups (the sulfur atoms of which are 9.6 Å apart). For these studies, the complexes attached to the more remote cysteine atoms (at a distance of 35.6 Å) were assumed to have minimal interactions with these sites.

Figure S8 (next page). Models of sterically hindered Gd-HOPO complexes attached to the interior MS2 surface. Structures attached to two adjacent Cys87 groups were minimized simultaneously using MacroModel, and three different coordination geometries were considered for each (see Figure S5 for differences). The Gd coordination spheres were based on the crystal structure reported in reference 8. The minimized structures of the three conformational isomers are shown, along with van der Waals renderings after attachment to the Cys87 residues.


a: S,S conformational isomer 1 (Gd-7b)
b: S,S conformational isomer 2 (Gd-7b)
c: S,S conformational isomer 3 (Gd-7b)
ligand structures
HOPO ligand
S,S-linker
Gd(III) ion
Cys87
bound waters (yellow)
attached to Cys87: top view
attached to Cys87: side view
d: R,R conformational isomer 1 (Gd-7c)
e: R,R conformational isomer 2 (Gd-7c)
f: R,R conformational isomer 3 (Gd-7c)
ligand structures
R,R-linker
Cys87
HOPO ligand
Gd(III) ion
bound waters (yellow)
attached to Cys87: top view
attached to Cys87: side view

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Full Reference 19 from Main Text:

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