A Multimeric Near IR-MR Contrast Agent for Multimodal *In Vivo* Imaging

Victoria S. R. Harrison,[1] Christiane E. Carney,[1] Keith W. MacRenaris,[1] Emily A. Waters,[2] Thomas J. Meade[1,2*]

[1] Department of Chemistry, Molecular Biosciences, Neurobiology, Biomedical Engineering, and Radiology, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3113, United States

[2] Center for Advanced Molecular Imaging, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3113, United States

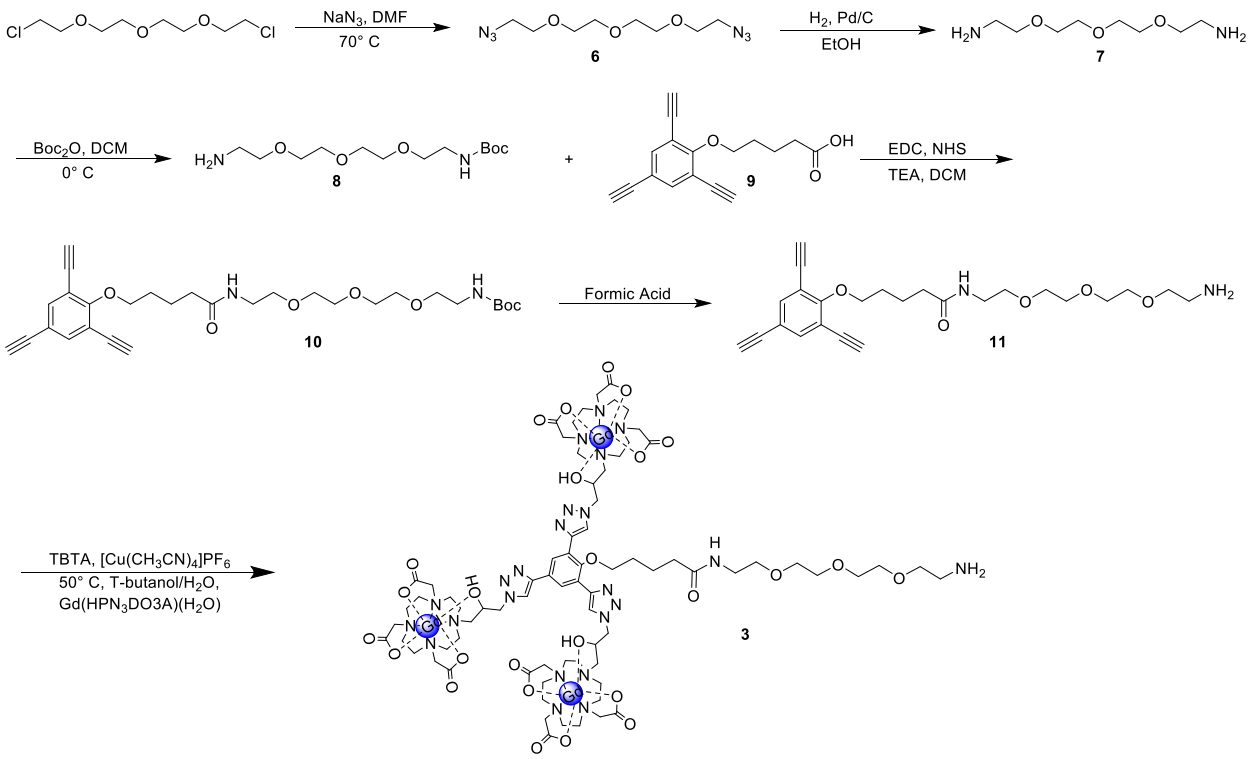
Email: tmeade@northwestern.edu

Author Contributions: C.E.C. and V.S.R.H. contributed equally to this work.

Supplemental Information

Table of Contents **Page**

Synthesis of **3** (**Scheme S1**)



Scheme S1. Synthesis of **3** from 1,11-dichloro-3,6,9-trioxa-undecane

1,11-diazido-3,6-9-trioxaundecane (6): To a solution of 1,11-dichloro-3,6,9-trioxa-undecane (1.0 mL, 6.3 mmol) in 15 mL DMF was added sodium azide (1.3 g, 20 mmol). The reaction was heated to 70°C and allowed to stir overnight under nitrogen. After 16 h, the reaction mixture was diluted with 150 mL water and extracted three times with diethyl ether (3x 50 mL). The combined organic layers were washed three times with water (3x 10 mL), and dried over Mg_2SO_4. Filtration and concentration gave 1.1 g of the product as a yellow oil (72% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.66 (m, 12H), 3.39 (t, J=5.1, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 50.67, 70.01, 70.68. MS (ESI positive): m/z observed= 267.10 [M + Na^+], 283.07 [M + K^+] m/z calculated= 267.12 [M + Na^+], 283.09 [M + K^+]

1,11-diamino-3,6,9-trioxaundecane (7): Ethanol (100 mL) was used to dissolve **6** (1.1 g, 4.4 mmol) and the solution was transferred to a hydrogenator flask. A catalytical amount of 10 wt % Pd/C was added and the vessel shaken on a hydrogenator reactor overnight under 3 atm of H_2. Complete reduction of the azide functional group was confirmed with ^{1}H NMR. The reaction mixture was filtered through Celite, rinsing with 100 mL of methanol. The solvent was removed from the filtrate to yield the product as 505 mg of a yellow oil (62% yield). ^{1}H NMR (500 MHz,

$CDCl_3$) δ 2.83 (t, J=5.2, 4H), 3.49 (m, 4H), 3.62 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 41.62, 70.33, 70.62, 73.51. MS (ESI positive): m/z observed= 193.09 [M + H^+], 215.06 [M + Na^+] m/z calculated= 193.16 [M + H^+], 215.14 [M + Na^+]

1-Boc-amine-11-amino-3,6,9-trioxaundecane (8): Dichloromethane (50 mL) was used to dissolve **7** (1.09, 5.7 mmol). After chilling the reaction on ice for 30 minutes, a solution of di-tert-butyl dicarbonate (0.53 g, 2.43 mmol) in 25 mL DCM was added. The reaction was left to stir for 1 hr at 0° C and 18 hr at room temperature. The organic phase was washed four times with 0.5 M NaOH (50 mL) and the organic layer was dried with Mg_2SO_4. Filtration and concentration gave 365 mg of the product as a yellow oil (23% yield). ^1H NMR (500 MHz, $CDCl_3$) δ 1.47 (s, 9H), 2.90 (t, J=5.2, 2H), 3.28 (m, 2H), 3.57 (m, 4H), 3.66 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 28.8, 40.5, 42.2, 70.4, 70.5, 70.7, 70.9, 79.4, 156.4 MS (ESI positive): m/z observed= 293.20 [M + H^+] m/z calculated= 293.38 [M + H^+]

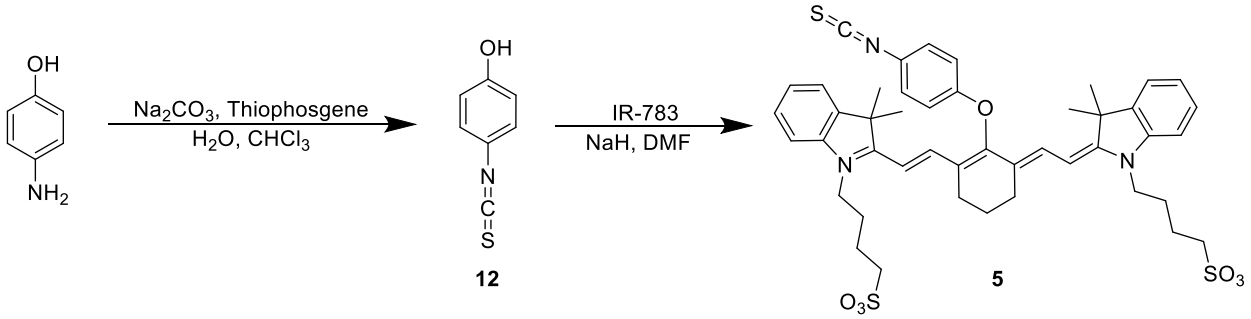
tert-butyl(13-oxo-17-(2,4,6-triethynylphenoxy)-3,6,9-trioxa-12-azaheptadecyl)carbamate (10): Under nitrogen, **9** (71.8 mg, 0.27 mmol), N-hydroxysuccinimide (NHS) (47 mg, 0.40 mmol), and 1-ethyl-3-(3-dimethylaminopropoyl)carbodiimide (EDC) (77 mg, 0.40 mmol) were dissolved in DCM (50 mL). After stirring for 2 hrs, 10 mL of triethylamine and **8** (197 mg, 0.67 mmol) were added and the reaction was left to stir overnight. The solvent was evaporated and the product was purified by column chromatography (95:5 dichloromethane: methanol) to give 123.0 mg of the product as an orange oil (85% yield). ^1H NMR (500 MHz, $CDCl_3$) δ 1.39 (m, 9H), 2.03 (m, 4H), 2.26 (m, 2H), 3.02 (s, 1H), 3.29 (m, 4H), 3.42 (m, 2H), 3.52 (m, 4H), 3.61 (m, 8H), 4.22 (m, 2H), 7.53 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.3, 29.24, 29.74, 26.23, 39.14, 40.36, 69.98, 70.18, 70.24, 74.04, 77.68, 78.59, 81.33, 82.70, 117.2, 117.66, 138.12, 156.00, 162.33, 172.92. MS (ESI positive): m/z observed= 563.36 [M + Na^+] m/z calculated= 563.27 [M + Na^+]

N-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-5-(2,4,6-triethynylphenoxy)pentanamide (11): 10 (123.0 mg, 0.23 mmol) was dissolved in 20 mL of formic acid and stirred for 4 hours or until completion was determined by TLC (90:10 dichloromethane: methanol). The solvent was evaporated and the residue was purified by column chromatography (88:10:2 chloroform: methanol : ammonium hydroxide) to give 100 mg of the product as a yellow oil (85 % yield). ^1H NMR (500 MHz, $CDCl_3$) δ 1.87 (m, 4H), 2.29 (m, 2H), 2.92 (m, 2H), 3.02 (s, 1H), 3.34 (s, 2H), 3.46 (m, 2H), 3.52 (m, 4H), 3.61 (m, 8H), 4.23 (m, 2H), 7.52 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.35, 29.65, 36.20, 39.17, 41.19, 70.03, 70.41, 71.63, 74.15, 77.80, 78.55, 81.32, 82.81, 117.05, 117.61, 138.10, 162.33, 173.58. MS (ESI positive): m/z observed= 441.34 [M + H^+] m/z calculated= 441.54 [M + H^+]

N-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-5-(3-(2,4,6)-Tris(1-2(hydroxyl-3-(1H-1,2,3-triazol-1-yl(propyl)-3,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecylgadolinium (III)phenoxy)-pentanamide (3): To a solution of Gd(HPN_3DO3A)(H_2O) (242 mg, 0.404 mmol) in a 40 mL mixture of 2:1 T-butanol:H_2O was added **11** (51 mg, 0.12 mmoles). Nitrogen was bubbled through the solution for 15 minutes, followed by the addition of $[Cu(MeCN)_4]PF_6$ (4.3 mg, 0.01 mmole) and tris[(1-benzyl-1-H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (6.15 mg, 0.01 mmole). The reaction was heated to 50°C and stirred under a nitrogen atmosphere for 48 hours. The solvent was removed by lyophilization and the product was purified by reverse phase HPLC

using a C18 column, held at 5% for 5 minutes and eluting with a gradient of 5% - 15% acetonitrile in pH 10.38 buffered water over 11 min, t_r= 15.1 min. This gave 120 mg of the product as a white solid (45% yield). The purity and identity of the product was confirmed using analytical HPLC-MS on a C18 column, held at 5% for 5 minutes and eluting with a gradient of 5%-30% acetonitrile in water, over 30 min, t_r = 22.82 min. MS (MALDI-TOF): m/z observed= 2240.57 $[M + H^+]$, 2262.57 $[M + Na^+]$ m/z calculated= 2240.65 $[M + H^+]$, 2262.31$[M + Na^+]$

Synthesis of **5** (**Scheme S2**)



Scheme S2. Synthesis of **5** from 4-aminophenol

4-isothiocyanatophenol (12): To 4-aminophenol (0.60 g, 5.5 mmol) dissolved in 30 mL $CHCl_3$ and 40 mL aqueous Na_2CO_3 (1.0 g in 40 mL H_2O) was added thiophosgene (0.85 mL, 1.18 mmol). After stirring for 3 hours, the organic layer was washed with brine, and dried over Mg_2SO_4. The solvent was removed via evaporation, and the product was purified by column chromatography (9:1 hexanes : ethyl acetate). This yielded 435 mg of the product as a yellow solid (52% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.24 (s, 1H), 6.99 (d, J=8.8, 2H), 7.26 (d, J=8.8, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 116.49, 123.78, 17.34, 133.62, 154.42

4-(2-{4-[(4-isothiocyanatophenyl)oxy]-7-[3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl}-3,3-dimethyl-3H-indolio)butanesulfonate (5): To a slurry of 33 mg of NaH (60% in mineral oil, 0.85 mmol) and 20 mL anhydrous DMF, under N_2, was added **12** (60 mg, 0.39 mmol) over ice. After 30 minutes, the reaction was allowed to warm to room temperature and IR-783 (102 mg, 0.136 mg) was added. The reaction was left overnight under N_2. The solvent was removed in vacuo, keeping the water bath temperature below 40°C, and the product was purified by column chromatography using a gradient of 10-30% methanol: dichloromethane. This afforded 100 mg of the product as a dark purple solid (87% yield). ^{1}H NMR (500 MHz, Methanol-d_4) δ 1.40 (s, 12H), 1.94 (s, 8H), 2.07 (m, 2H), 2.79 (t, J = 6.1 Hz, 4H), 2.90 (t, J = 7.0 Hz, 4H), 4.16 (s, 4H), 6.24 (d, J = 14.1 Hz, 2H), 7.21 (s, 4H), 7.38 (m, 9H), 7.94 (d, J = 14.1 Hz, 2H). ^{13}C NMR (126 MHz, MeOD) δ 20.97, 22.18, 23.81, 25.83, 26.78, 43.49, 48.91, 50.38, 100.02, 110.78, 115.91, 121.76, 121.90, 125.39, 127.46, 128.42, 135.29, 140.95, 141.64, 142.07, 158.64, 163.01, 172.60. MS (ESI positive): m/z observed= 842.34 $[M + H^+]$ m/z calculated= 842.09 $[M + H^+]$

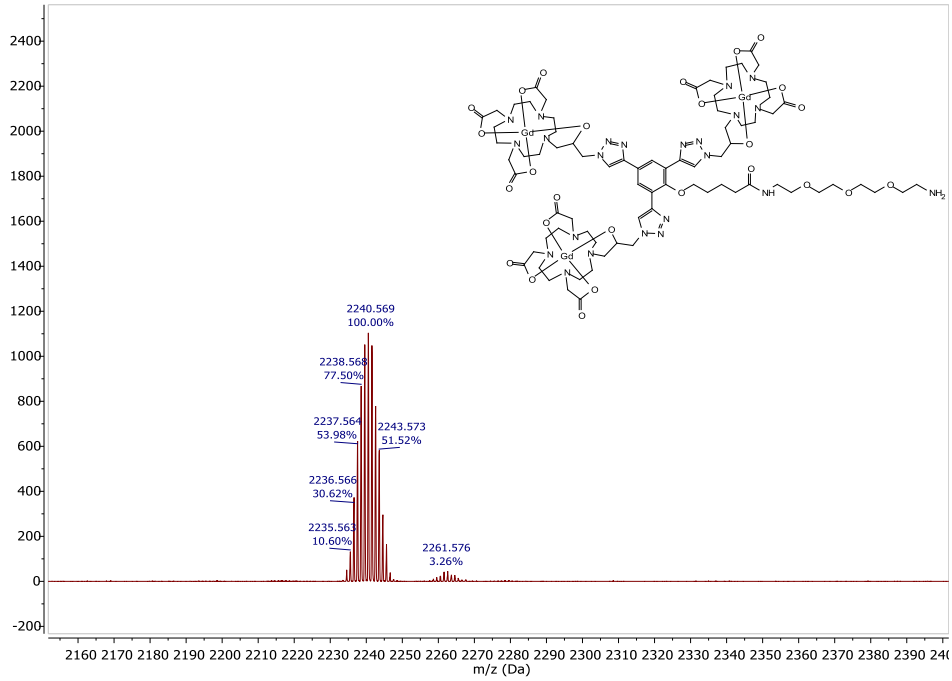


Figure S1. MS (MALDI-TOF) of **3**

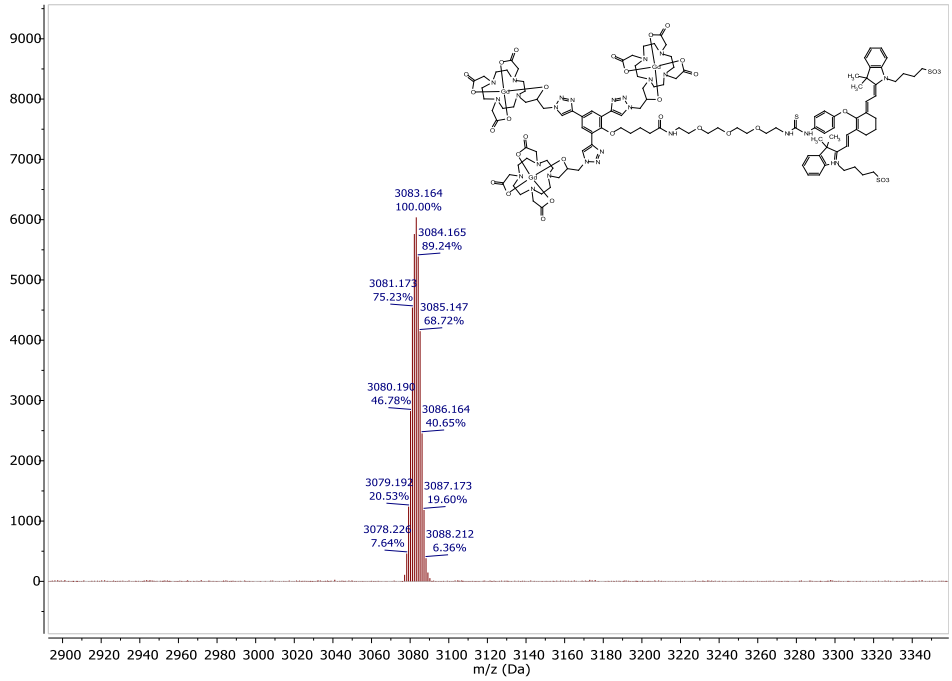


Figure S2. MS (MALDI-TOF) of **1**

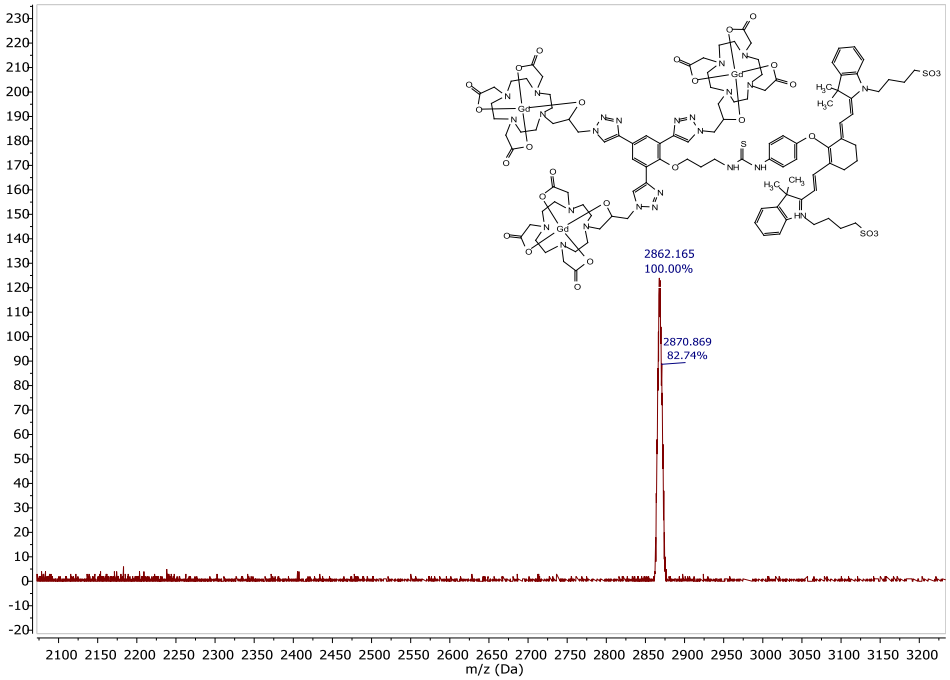


Figure S3. MS (MALDI-TOF) of **2**

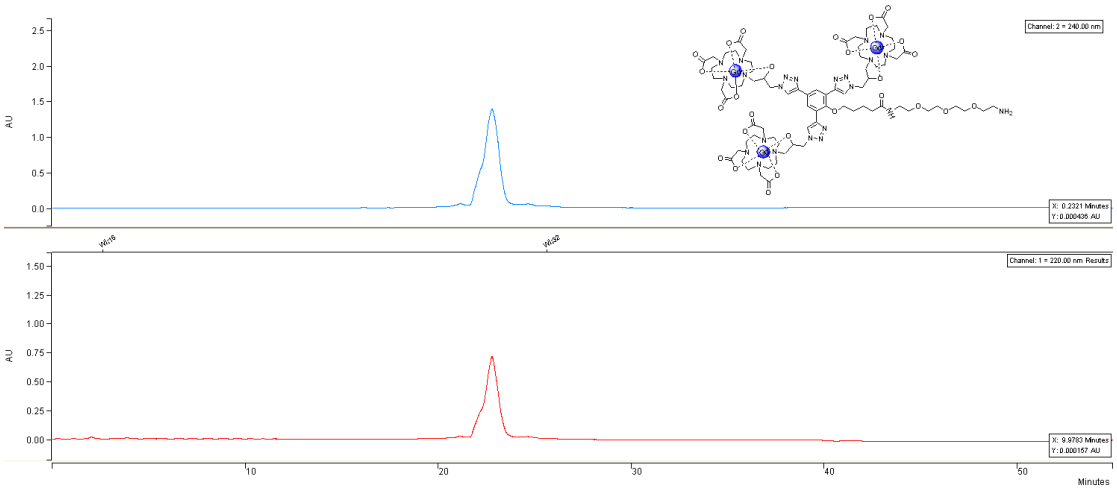


Figure S4. Analytical HPLC trace, monitored at 220 and 240 nm, of **3**

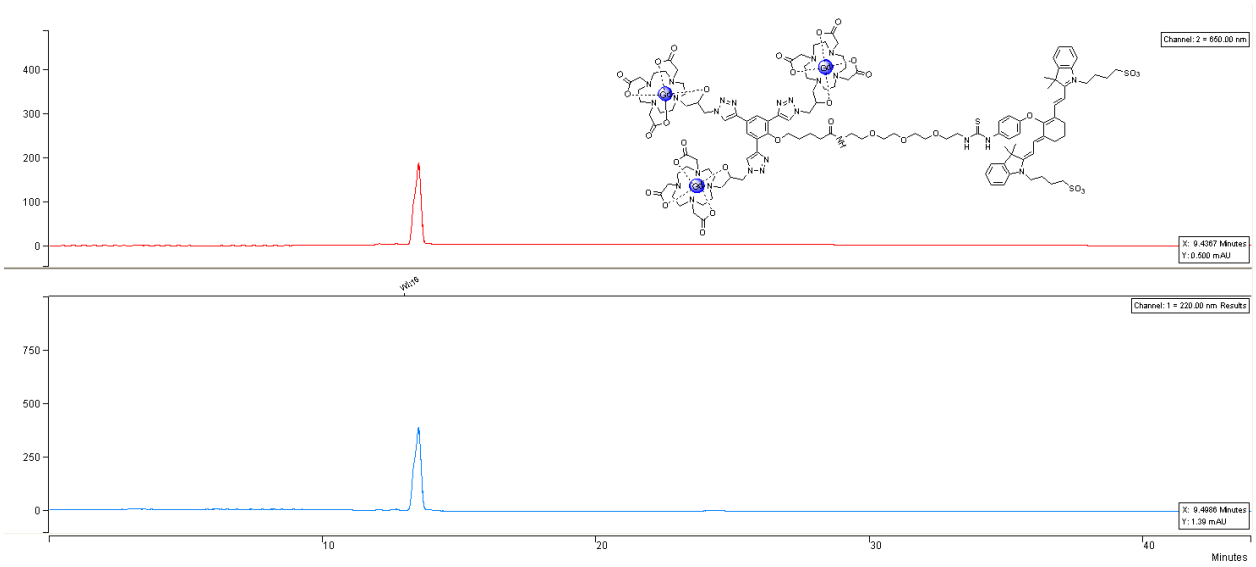


Figure S5. Analytical HPLC trace, monitored at 220 and 650 nm, of **1**

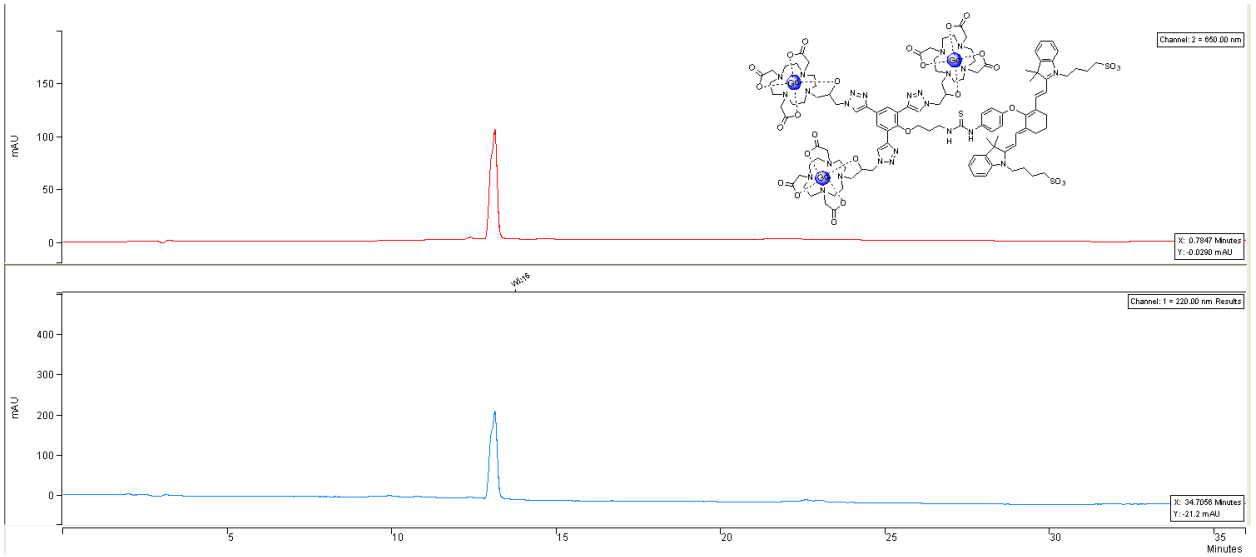


Figure S6. Analytical HPLC trace, monitored at 220 and 650 nm, of **2**

Table S1. r_2 of **1-4** at 1.41 and 7 T at 37° C in 10 mM MOPS buffer, pH 7.4

	Relaxivity 1.41 T (60 MHz)		Relaxivity 7 T (300 MHz)	
	Ionic ($mM^{-1}s^{-1}$)	Molecular ($mM^{-1}s^{-1}$)	Ionic ($mM^{-1}s^{-1}$)	Molecular ($mM^{-1}s^{-1}$)
1	23.2 ± 0.6	69.8 ± 1.8	25.0 ± 0.3	75.0 ± 0.9
2	24.6 ± 0.5	73.8 ± 1.5	28.5 ± 0.4	85.5 ± 1.2
3	15.4 + 0.4	46.2 ± 1.2	21.0 ± 0.4	63.0 ± 1.2
4	17.8 ± 0.4	53.4 ± 1.0	16.2 ± 0.3	48.6 ± 0.9

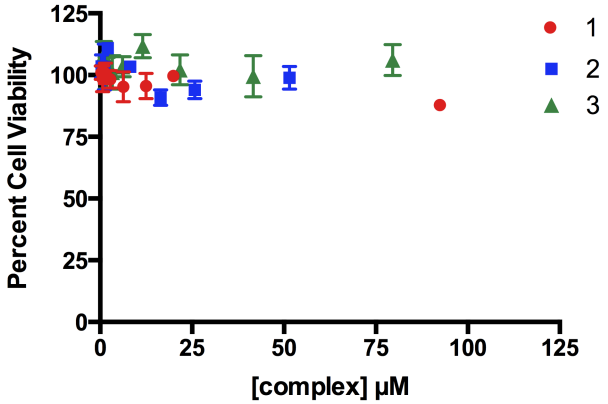


Figure S7. Toxicity of **1-3** was determined using a luminescent-based cell viability assay. MCF7 cells incubated with concentrations of **1-3** ranging from 0 – 100 µM showed ≥ 90% viability. Error bars represent the standard deviation of triplicate experiments.

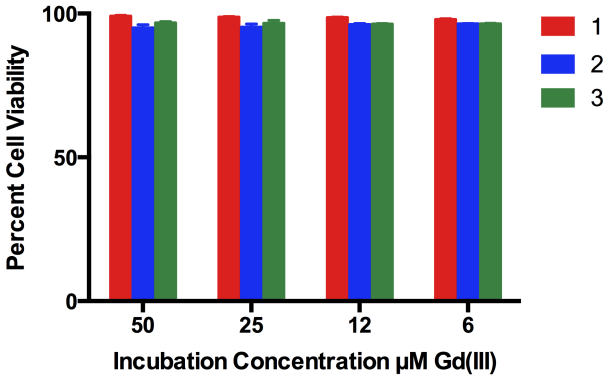


Figure S8. Cell viability was confirmed using microcapillary flow cytometry. MCF7 cells incubated with 6 – 50 µM **1-3** showed no significant decrease in viability. Error bars represent the standard deviation of triplicate experiments.

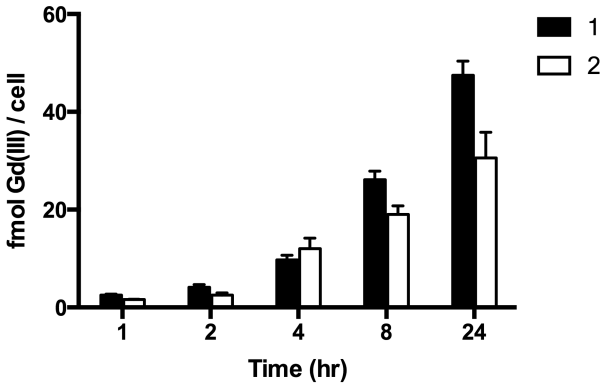


Figure S9. Time-dependent uptake of complexes **1** and **2** incubated at 20 μM with MCF7 cells. These data show a time-dependent increase in cell uptake. Based on these results, an incubation time of 24 hours was chosen for subsequent experiments to maximize labeling.

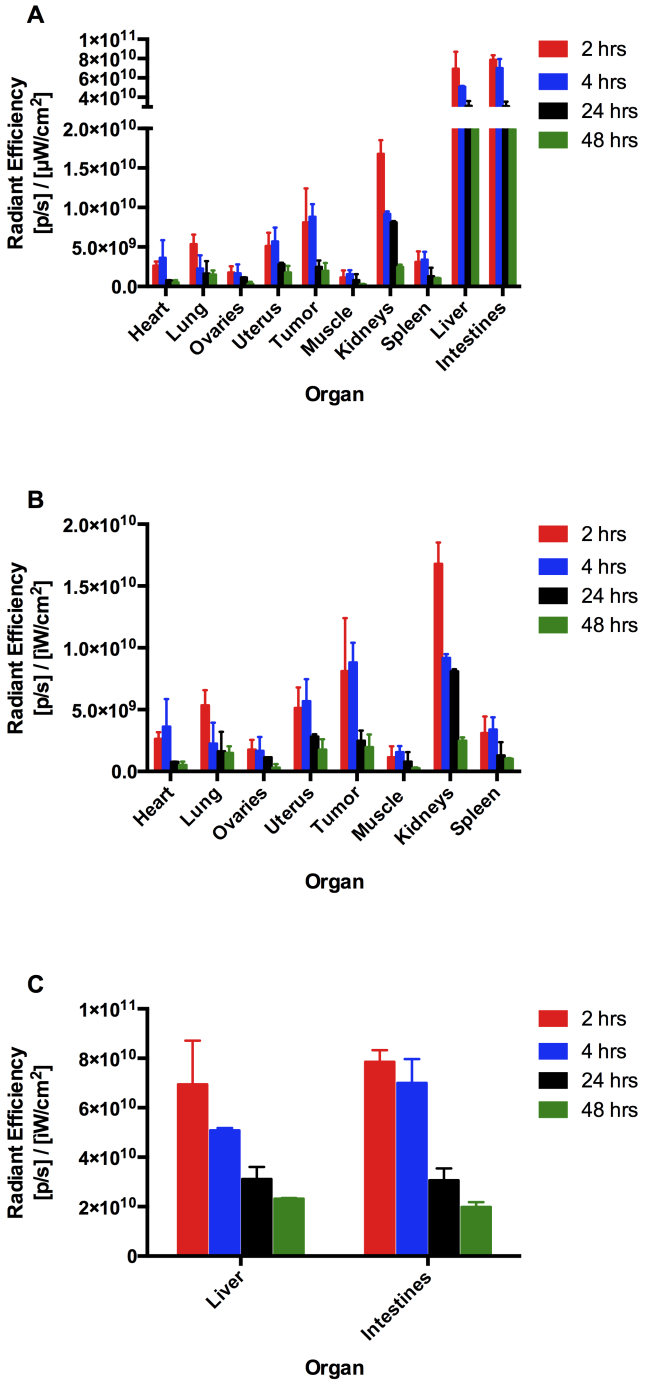


Figure S10. Biodistribution of **1** was determined 2, 4, 24, and 48 hours post-i.p. injection in tumor bearing nude mice. The heart, lungs, ovaries, uterus, tumor, muscle, kidneys, liver, and intestines were harvested and imaged *ex vivo* with near-IR fluorescence imaging. These data show that the most significant accumulation of **1** occurs in the liver and intestines followed by the kidneys, tumor, and uterus. Over time, fluorescence signal decreases in each organ suggesting clearance of the complex. **A**: all organs, **B:** organs with lower accumulation of **1**, **C:** organs with greatest accumulation of **1**.