Supporting Information

Synthesis and Evaluation of Diaza-Crown Ether-Backboned Chelator Containing Hydroxamate Groups for Zr-89 Chelation Chemistry

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I. Materials and Methods

^{1}H and ^{13}C NMR spectra were obtained using a Bruker 300 instrument, and chemical shifts are reported in ppm on the δ scale relative to solvent. Electrospray iodization (ESI) high resolution mass spectra (HRMS) were obtained on JEOL double sector JMS-AX505HA mass spectrometer (University of Notre Dame, IN). All reagents were purchased from Sigma-Aldrich or Acros Organics and used as received unless otherwise noted.

2-Bromo-N-((4-methoxybenzyl)oxy)-N-methylacetamide (2).[14] To a solution of **1**[15] (400 mg, 2.39 mmol) in CH_2Cl_2 (8 mL) was added K_2CO_3 (363.5 mg, 2.63 mmol) and bromo acetyl bromide (531.1 mg, 2.63 mmol) at 0 °C. The resulting mixture was stirred for 1 h. The reaction mixture was filtered, concentrated to dryness, and purified via column chromatography on silica gel eluting with 15%-20% ethyl acetate in hexane to provide product **2** (530 mg, 77%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.25 (s, 3H), 3.82 (s, 3H), 3.89 (s, 2H), 4.87 (s, 2H), 6.92 (d, *J* = 8.7 Hz, 2H), 7.32 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.8 (t), 33.9 (q), 55.3 (q), 76.1 (t), 114.3 (d), 126.0 (s), 131.2 (d), 160.4 (s), 168.2 (s).

2,2'-(1,4,10,13-Tetraoxa-7,16-diazacyclooctadecane-7,16-diyl)bis(N-((4-methoxybenzyl)oxy)-N-methylacetamide) (4). To a solution of **3** (100 mg, 0.38 mmol) in CH_3CN (5 mL) was added K_2CO_3 (420.8 mg, 3.05 mmol) and **2** (241.6 mg, 0.83 mmol). The resulting mixture was stirred for 14 h at room temperature. The reaction mixture was filtered, concentrated to dryness, and purified via column chromatography on silica gel eluting with 3% CH_3OH in CH_2Cl_2 to provide product **4** (107.6 mg, 41%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.53 (s, 6H), 3.10-3.24 (m, 10H), 3.44-3.57 (m, 18H), 3.80 (s, 6H), 4.75 (s, 4H), 6.88 (d, *J* = 8.0 Hz, 4H), 7.28 (d, *J* = 9.0 Hz, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 33.3 (q), 54.9 (t), 55.4 (q). 57.9 (t), 67.1 (t), 68.8 (t), 75.6 (t), 114.1 (d), 126.1 (s), 131.5 (d), 160.4 (s), 173.5 (s). HRMS (positive ion ESI) Calcd for $C_{34}H_{52}N_4O_{10}$ $[M + H]^+$ m/z 677.3756. Found: [M + H]+ m/z 677.3770.

2,2'-(1,4,10,13-Tetraoxa-7,16-diazacyclooctadecane-7,16-diyl)bis(N-hydroxy-N-methylacetamide) (5). To compound **4** (107 mg, 0.16 mmol) was sequentially added TFA (2 mL) and triethyl silane (36.8

mg, 0.32 mmol) at 0 °C. The resulting mixture was stirred for 14 h at room temperature. The reaction mixture was evaporated to dryness and washed with CH_2Cl_2 to afford compound **5** (69 mg, 99%). ^{1}H NMR (D_2O, 300 MHz) δ 3.15 (s, 6H), 3.48-3.78 (m, 24H), 4.33 (s, 4H). ^{13}C NMR (D_2O, 75 MHz) δ 36.2 (q), 55.5 (2C, t), 64.2 (t), 69.8 (t), 164.9 (s). HRMS (positive ion ESI) Calcd for $C_{18}H_{36}N_4O_8$ $[M + H]^+$ m/z 437.2619. Found: [M + H]+ m/z 437.2606.

Radiolabeling of chelators with ^{89}Zr. All HCl solutions were prepared from the commercially available ultrapure HCl solution (JT baker, #6900-05). For metal-free radiolabeling, plasticware including pipette tips, tubes, and caps was soaked in 0.1 M HCl overnight and washed thoroughly with Milli-Q (18.2 MΩ) water and air-dried overnight. Ultrapure ammonium acetate (Sigma-Aldrich, #431311) was used to prepare 0.25 M NH_4OAc buffer solution (0.25 M, pH 7.0). After adjusting pH using 0.1 M HCl or 1 M HCl or 1 M NaOH solution, 0.25 M NH_4OAc buffer solution was treated with Chelex-100 resin (Biorad, #142-2842, 1 g/100 mL buffer solution), shaken overnight at RT, and filtered through a 0.22 μM filter (Corning, #430320) prior to use. Etheylenediaminetetraacetic Acid (EDTA, Sigma-Aldrich, #03609) was used to prepare 100 mM EDTA solution (pH 7.0 and pH 5.0). ^{89}Zr-Oxalate was purchased from Washington University (St. Louis, MO). Stock solution of ^{89}Zr-Oxalate was adjusted to pH 7.5~8.0 by adding 1 M Na_2CO_3 solution that was treated with Chelex-100 resin. ITLC-SA plates (1 × 9 cm, ITLC-silica acid, Agilent Technologies, #A120B12) with the origin line drawn at 0.5 cm from the bottom were prepared.

To a buffer solution (0.25M NH_4OAc, pH 7.0) in a capped microcentrifuge tube (1.5 mL) was sequentially added a solution of DA-18C6-BHA (0.25 mM, 2.2 μg, 0.5 μL/0.25 M NH_4OAc, pH 7.0) or DFO (0.25 mM, 3.3 μg, 0.5 μL/ 0.25 M NH_4OAc, pH 7.0) and a solution of ^{89}Zr (0.37 MBq, 10 μCi), and the total volume of the resulting solution was brought up to 20 μL by adding 0.25 M NH_4OAc solution (pH 7.0). The reaction mixture was agitated on the thermomixer (Eppendorf, #022670549) set at 1000 rpm at room temperature for 1 h. Radiolabeling efficiency was determined by ITLC-SA (eluent: 100mM EDTA, pH 5.0). A solution of reaction mixture (2 μL) was withdrawn at the designated time

points, spotted on an ITLC plate and eluted with the mobile phase. After completion of elution, the ITLC plate was warmed and dried on the surface of a hotplate maintained at 35 °C and scanned using a TLC scanner (Bioscan, #FC-1000). The radiolabeled complex ^{89}Zr-DA-18C6-BHA or ^{89}Zr-DFO was detected at ~30 mm from the bottom of the ITLC plate, while the unbound ^{89}Zr moved faster (~65 mm).

***In vitro* serum stability of ^{89}Zr-radiolabeled complexes.** Human serum was purchased from Gemini Bio-products (#100110). ^{89}Zr-DA-18C6-BHA and ^{89}Zr-DFO were prepared by reaction of DA-18C6-BHA (1 mM, 8.7 μg, 2 μL/ 0.25 M NH_4OAc, pH 7.0) or DFO (1 mM, 13 μg, 2 μL/ 0.25 M NH_4OAc, pH 7.0) with ^{89}Zr (1.05 μL, 1.11 MBq, 30 μCi) in 0.25 M NH_4OAc buffer (pH 7.0), respectively. The total volume of the resulting solution was brought up to 20 μL by adding 0.25 M NH_4OAc solution (pH 7.0). Completion of radiolabeling was determined by ITLC-SA (eluent: 100mM EDTA, pH 5.0), and the resulting complexes ^{89}Zr-DA-18C6-BHA and ^{89}Zr-DFO were directly used for serum stability studies without further purification. ^{89}Zr-DA-18C6-BHA (1.11 MBq, 30 μCi, 20 μL) or ^{89}Zr-DFO (1.11 MBq, 30 μCi, 20 μL) was added to human serum (200 μL) in a microcentrifuge tube. The stability of the radiolabeled complexes in human serum was evaluated at 37 °C for 7 days. The serum stability of the radiolabeled complexes was assessed by measuring the transfer of the radionuclide from each complex to serum proteins using ITLC-SA (eluent: 100 mM EDTA, pH 5.0). A portion of the radiolabeled complex in serum was withdrawn at the designated time point and evaluated by ITLC-SA. The radiolabeled complex ^{89}Zr-DA-18C6-BHA or ^{89}Zr-DFO was detected at ~30 mm from the bottom of the TLC plate, while the unbound ^{89}Zr moved faster (~65 mm).

EDTA challenge of ^{89}Zr-radiolabeled complexes. ^{89}Zr-DA-18C6-BHA and ^{89}Zr-DFO were prepared by reaction of DA-18C6-BHA (1 mM, 8.7 μg, 2 μL/ 0.25 M NH_4OAc, pH 7.0) and DFO (1 mM, 13 μg, 2 μL/ 0.25 M NH_4OAc, pH 7.0) with ^{89}Zr (1.05 μL, 1.11 MBq, 30 μCi) in 0.25 M NH_4OAc buffer (pH 7.0), respectively. The total volume of the resulting solution was brought up to 20 μL by adding 0.25 M NH_4OAc solution (pH 7.0). Completion of radiolabeling was determined by ITLC-SA (eluent: 100mM

EDTA, pH 5.0), and the resulting complexes ^{89}Zr-DA-18C6-BHA and ^{89}Zr-DFO were directly used for EDTA challenge studies without further purification. A solution of EDTA (18 μl, 100 mM/ H_2O, pH 7.0) at a 100-fold molar excess was added to a solution of ^{89}Zr-DA-18C6-BHA (0.99 MBq, 27 μCi, 18 μl/ 0.25 M NH_4OAc, pH 7.0) or a solution of ^{89}Zr-DFO (0.99 MBq, 27 μCi, 18 μl/ 0.25 M NH_4OAc, pH 7.0). The resulting mixture was incubated at 37 ^oC for 7 days. At each of the time points, the stability of ^{89}Zr-DA-18C6-BHA and ^{89}Zr-DFO complex in the presence of EDTA at a 100-fold molar excess was determined using ITLC-SA (eluent: 100mM EDTA, pH 5.0). The radiolabeled complex ^{89}Zr-DA-18C6-BHA or ^{89}Zr-DFO was detected at ~30 mm from the bottom of the TLC plate, while unbound ^{89}Zr moved faster (~65 mm).

Apotransferrin challenge of ^{89}Zr-radiolabeled complexes. Apotransferrin was purchased from Sigma-Aldrich (#T1147). Phosphate buffered saline (1×, pH 7.4, Corning, #21-040-CV) was treated with Chelex-100 resin (Biorad, #142-2842, 1 g/100 mL buffer solution), shaken overnight at room temperature, and filtered through a 0.22 μM filter (Corning, #430320). The chelexed PBS was used to prepare a solution of apo-transferrin (500 μM). ^{89}Zr-DA-18C6-BHA and ^{89}Zr-DFO was prepared by reaction of DA-18C6-BHA (1mM, 8.7 μg, 2 μL in 0.25M NH_4OAc, pH 7.0) or DFO (1mM, 13 μg, 2 μL in 0.25M NH_4OAc, pH 7.0) with ^{89}Zr (0.88 μL, 1.11 MBq, 30 μCi) in 0.25 M NH_4OAc buffer (pH 7.0), respectively. The total volume of the resulting solution was brought up to 20 μL by adding 0.25 M NH_4OAc solution (pH 7.0). Completion of radiolabeling was determined by ITLC-SA (eluent: 100mM EDTA, pH 5.0), and the resulting complexes ^{89}Zr-DA-18C6-BHA and ^{89}Zr-DFO were directly used for apotransferrin challenge assays without further purification. A solution of apotransferrin (200 μl, 500 μM in PBS) at a 5-fold molar excess was added to a solution of ^{89}Zr-DA-18C6-BHA (1.11 MBq, 30 μCi/20 μl 0.25 M NH_4OAc buffer). A solution of apotransferrin (200 μl, 500 μM in PBS) at a 5-fold molar excess was added to a solution of ^{89}Zr-DFO (1.11 MBq, 30 μCi/20 μl 0.25 M NH_4OAc buffer). The resulting mixture was incubated at 37°C for 7 days. The stability of ^{89}Zr-DA-18C6-BHA and ^{89}Zr-

DFO complex in the presence of apotransferrin at a 5-fold molar excess was determined using ITLC-SA (eluent: 100mM EDTA, pH 5.0). The radiolabeled complex ^{89}Zr-DA-18C6-BHA or ^{89}Zr-DFO was detected at 20 - 45 mm from the bottom of the TLC plate, while unbound radionuclide ^{89}Zr moved faster (50 - 75 mm).

***In vivo* biodistribution studies.** All animal experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of the University of Iowa. Six to eight weeks old CD-1 mice were obtained from Charles River Laboratories and housed one week prior to the studies. ^{89}Zr-labeled chelator (DA-18C6-BHA) was freshly prepared prior to *in vivo* biodistribution studies. ^{89}Zr-labeled DFO was also evaluated for comparison. Stock solution of the radioisotopes (75 μL, 3 mCi) was adjusted to pH 7.0 by adding 1 M Na_2CO_3 solution. Each chelator (50 μg) in buffer solution (0.25 M NH_4OAc, pH 7.0) and ^{89}Zr (100 μCi) were sequentially added to a microcentrifuge tube (1.5 mL). The total volume of the reaction mixture was 30 μL. The resulting mixture was reacted in a thermomixer (Eppendorf, 5355) set at 300 rpm at 37 ºC for 1 h.

Completion of Zr-89 labeling was determined by TLC followed by phosphor imaging (Typhoon, FLA 7000). An aliquot of ^{89}Zr-radiolabeled complex (3 μCi) was intravenously injected via the tail vein in a volume of 100 μL of sterilized saline. At 1 h, 4 h and 24 h post-injection, mice were sacrificed, and blood, liver, kidney, muscle, and bone were collected, weighed, and counted in a Cobra II auto gamma counter. The radioactivity from each tissue/organ was decay-corrected by a known aliquot of the injected dose, and the percent-injected dose per gram of tissue (% ID/g) was calculated. Values were presented as mean ± SD for each group of 4 mice.

II. NMR Spectra of compounds 2, 4, and 5.

Figure S1. ^{1}H NMR spectrum of compound 2

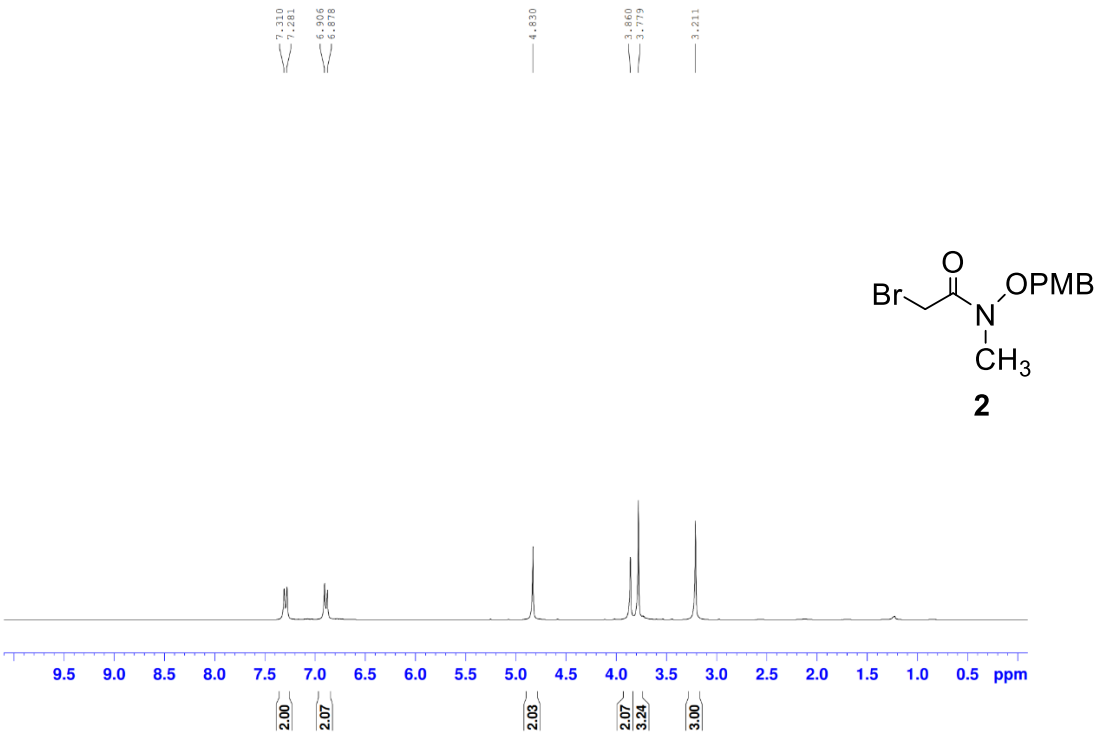


Figure S2. ^{13}C NMR spectrum of compound 2

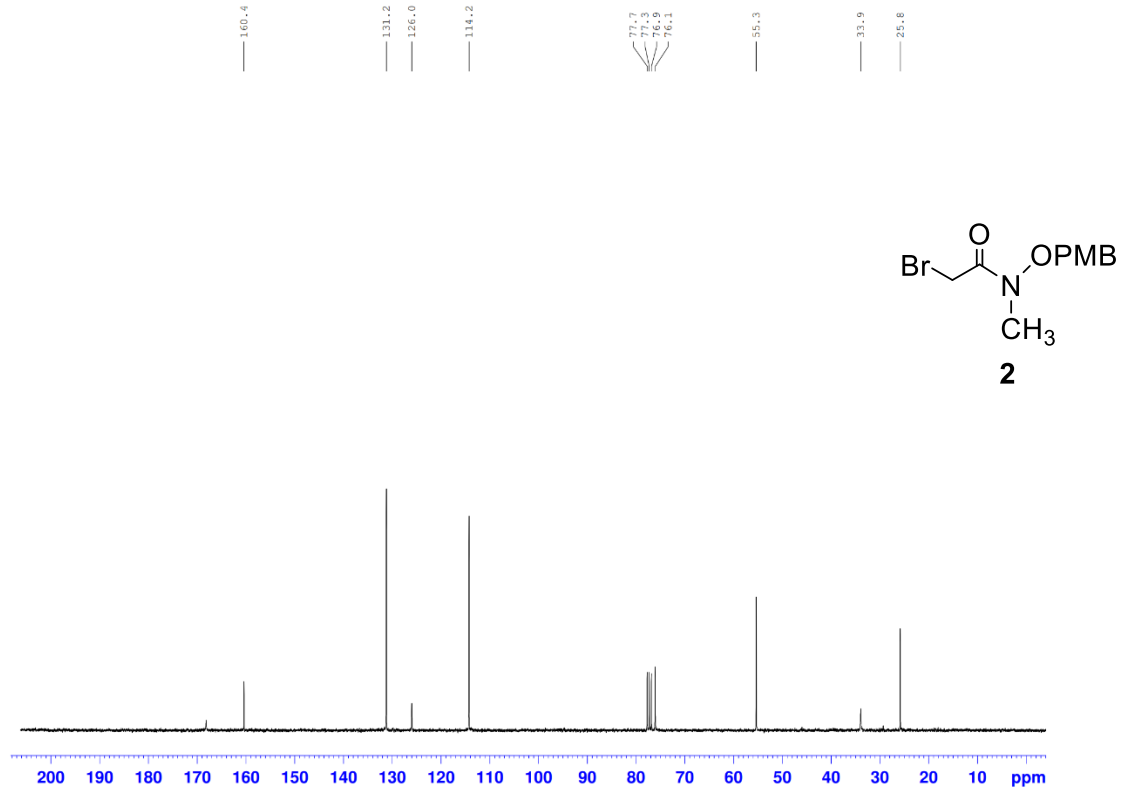


Figure S3. ^{1}H NMR spectrum of compound 4

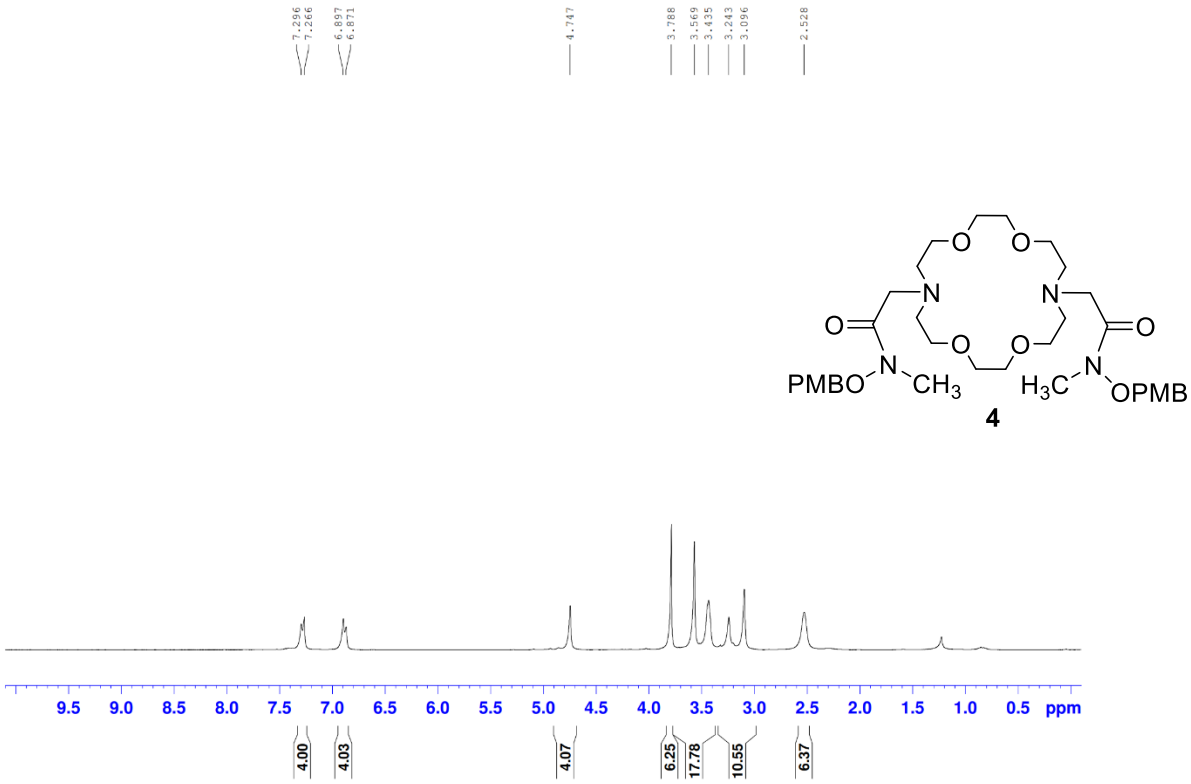


Figure S4. ^{13}C NMR spectrum of compound 4

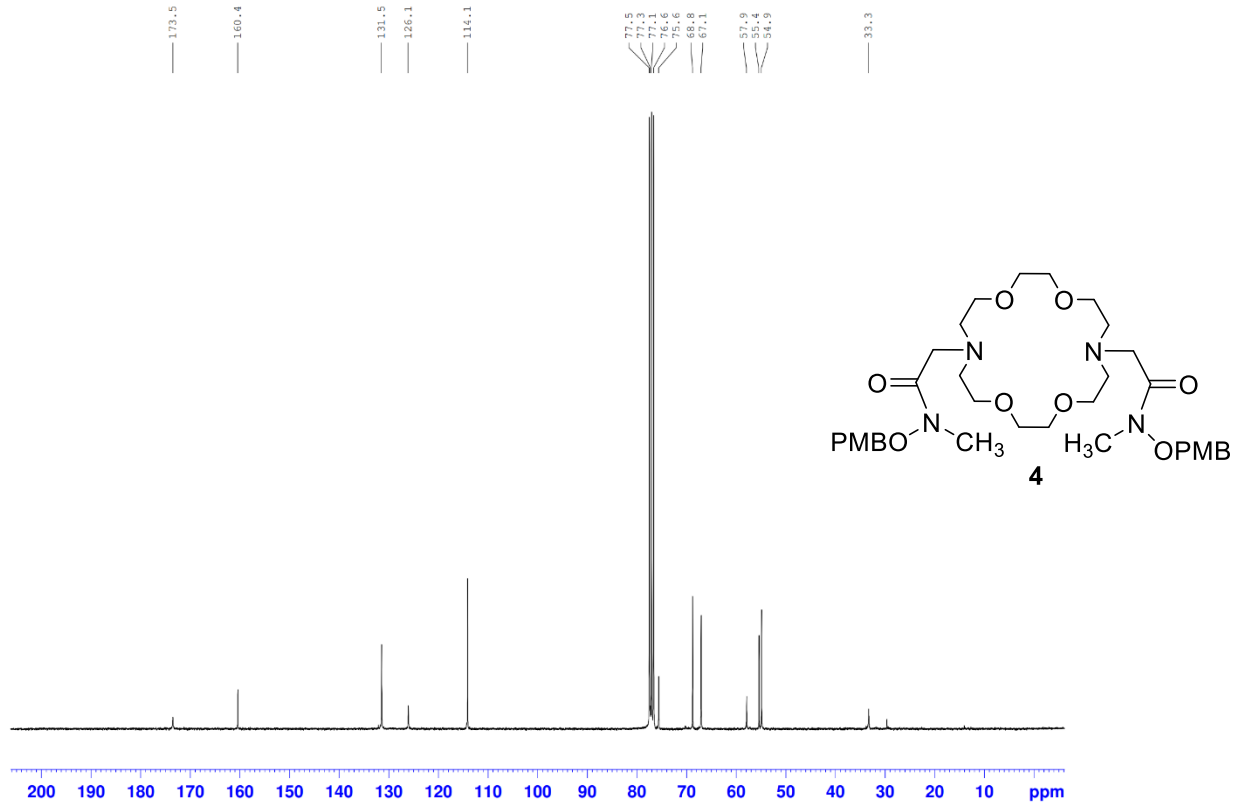


Figure S5. ^{1}H NMR spectrum of compound 5

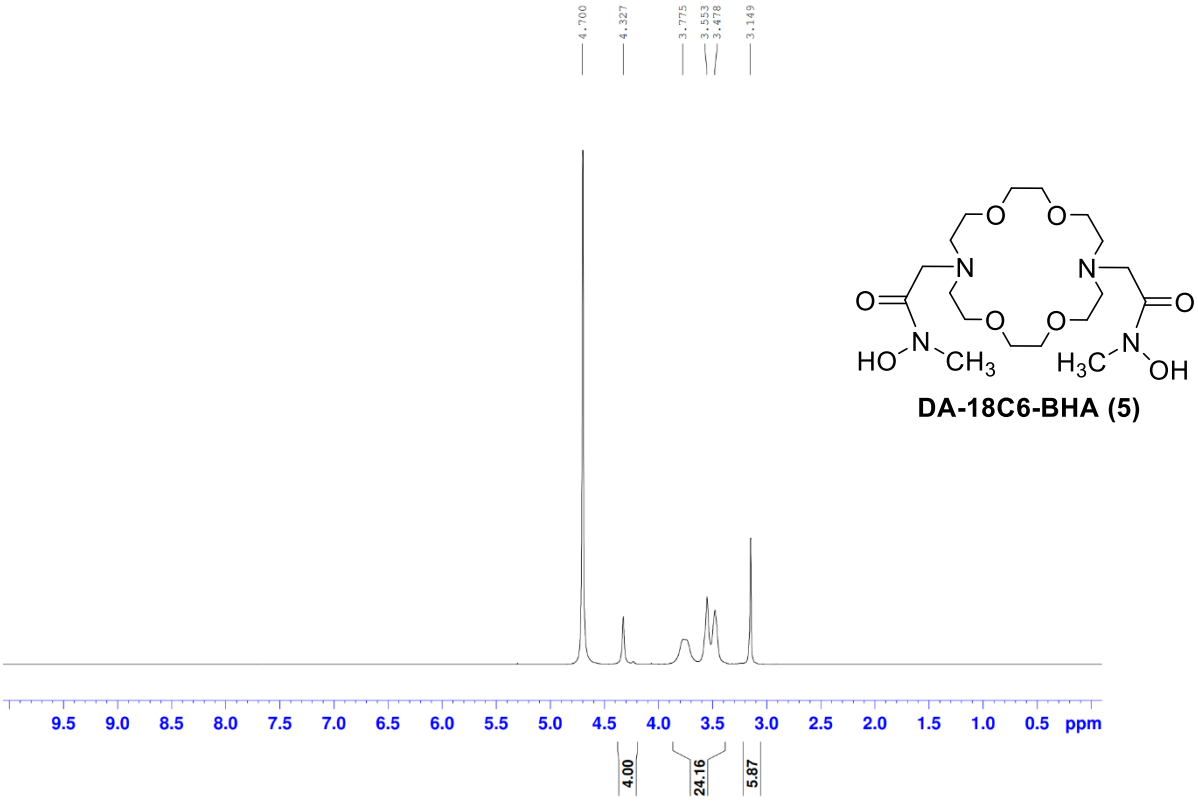
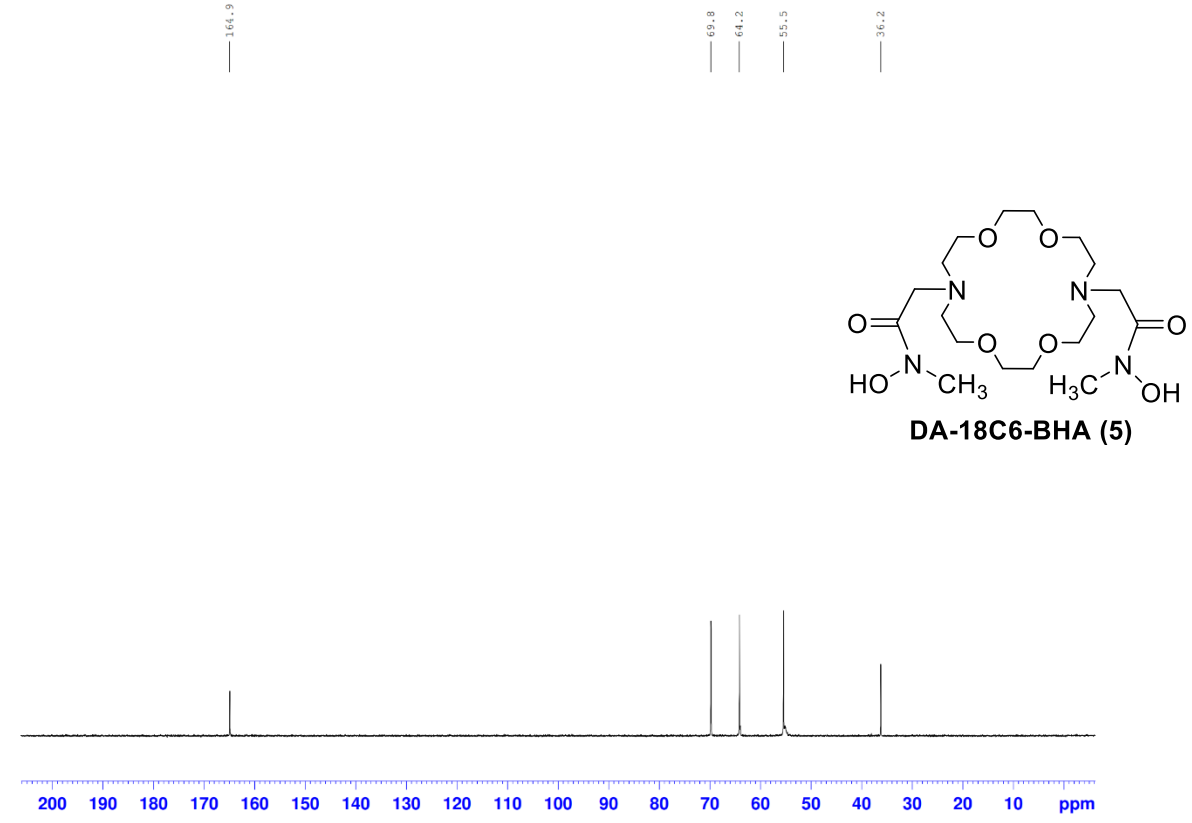


Figure S6. ^{13}C NMR spectrum of compound 5



III. Evaluation of chelators for radiolabeling with ^{89}Zr

Table S1. Radiolabeling efficiency (%) with ^{89}Zr (pH 7, RT, ITLC)

Time	Radiolabeling efficiency (%)	
	DA-18C6-BHA	DFO
1 min	90.2	99.1
10 min	98.2	99.1
30 min	99.5	99.4

*Radiolabeling efficiency (mean ± standard deviation%) was measured using ITLC (eluent: 100mM EDTA, pH 5.0).

Figure S7. Control ITLC of unbound radionuclide (^{89}Zr-oxalate)

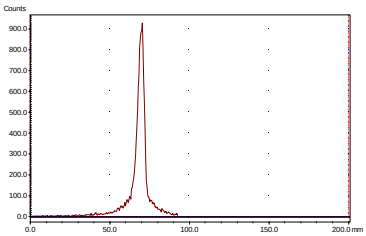


Figure S8. Radiolabeling of DA-18C6-BHA with ^{89}Zr

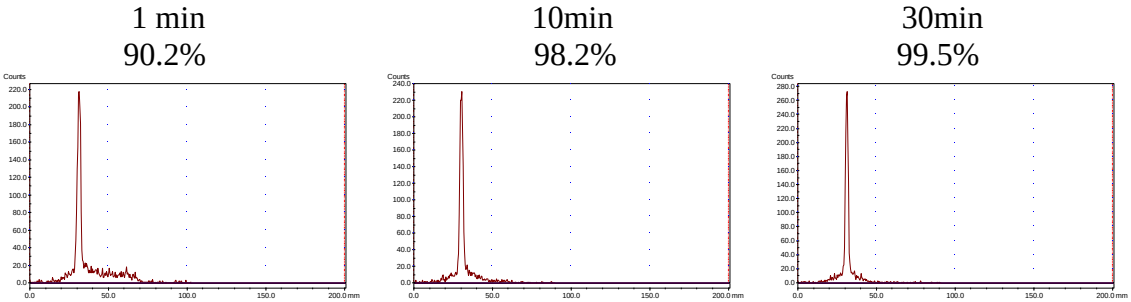
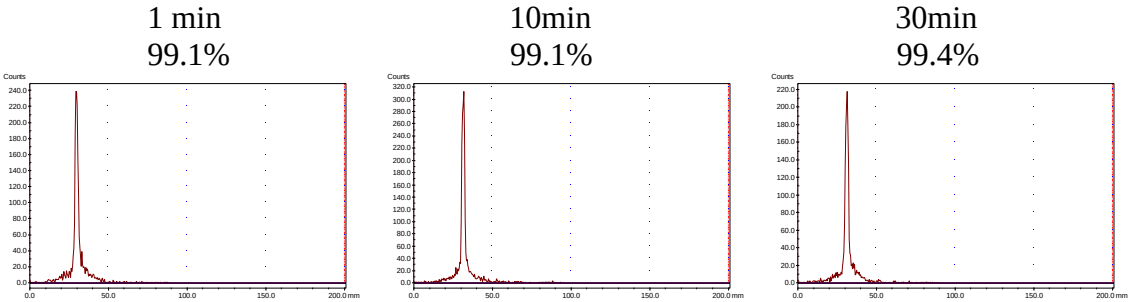


Figure S9. Radiolabeling of DFO with ^{89}Zr



IV. Evaluation of ^{89}Zr-labeled complexes for stability in human serum (pH 7.0, 37 ℃, ITLC, duplicate).

Figure S10. Complex Stability of ^{89}Zr-labeled DA-18C6-BHA in human serum

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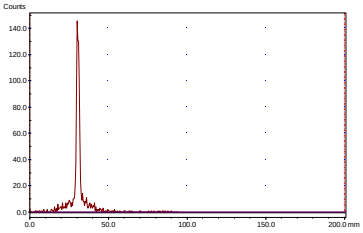

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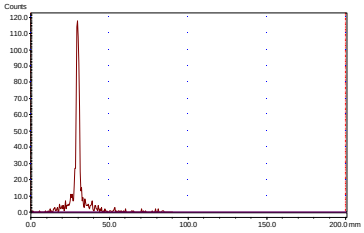

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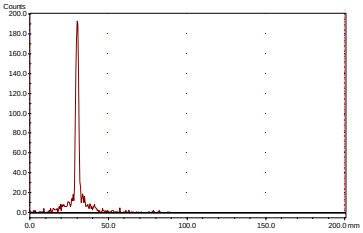

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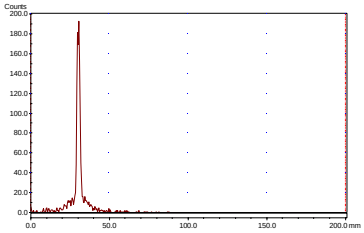

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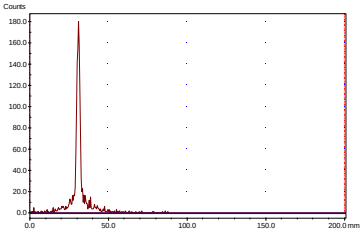

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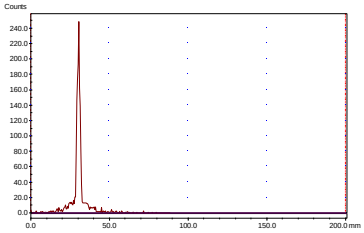

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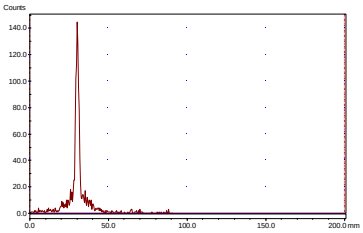

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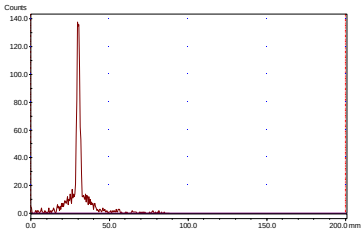

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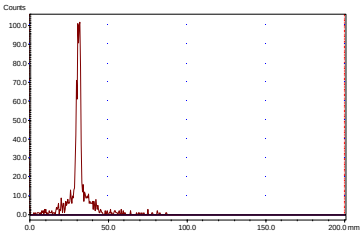

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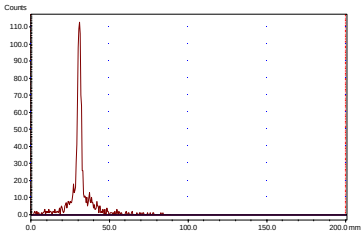

Figure S11. Complex Stability of ^{89}Zr-labeled DFO in human serum

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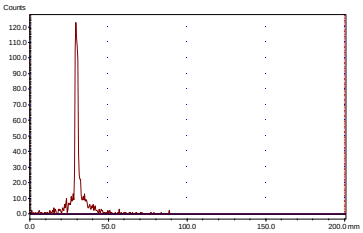

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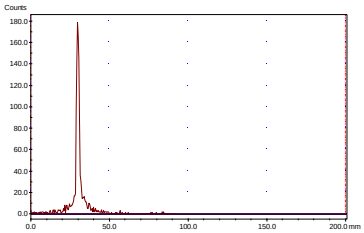

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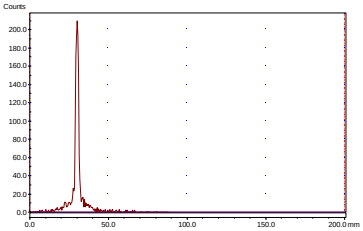

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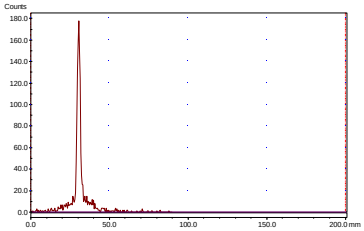

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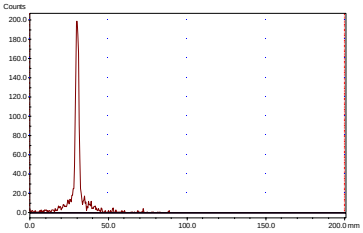

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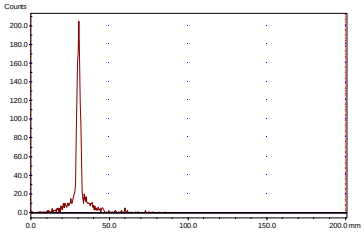

72 hr

1st run
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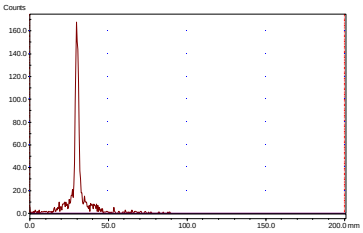
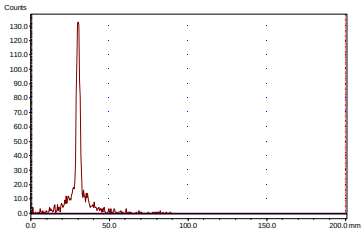
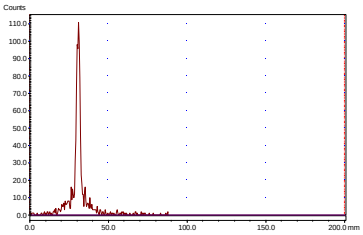


2nd run
99.2%

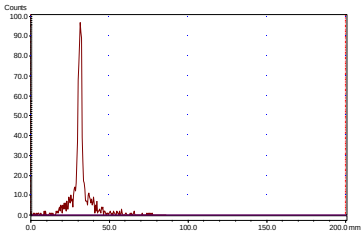


168 hr

1st run
98.6%



2nd run
99.3%

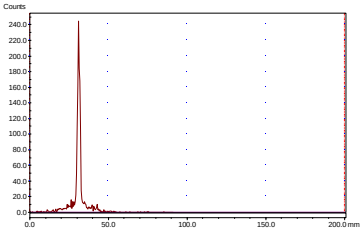


V. Evaluation of ^{89}Zr-labeled complexes for stability in EDTA solution (100-fold molar excess, pH 7.0, 37°C, ITLC, duplicate).

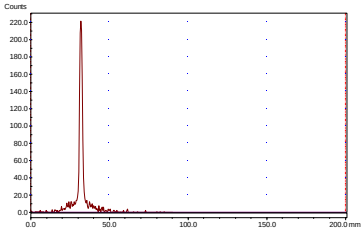
Figure S12. Complex Stability of ^{89}Zr-DA-18C6-BHA in EDTA solution

0hr

1st run
99.3%

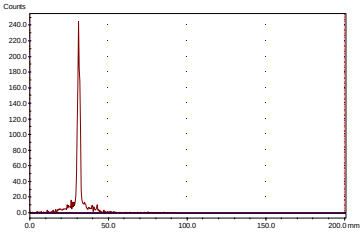


2nd run
99.2%

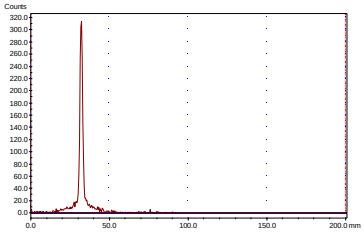


1hr

1st run
99.7%

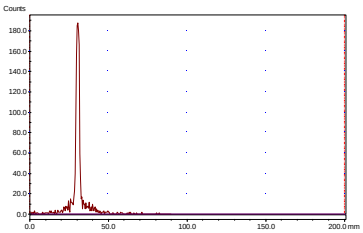


2nd run
99.5%

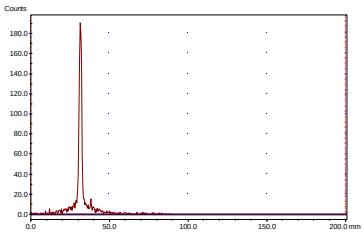


24hr

1st run
99.1%

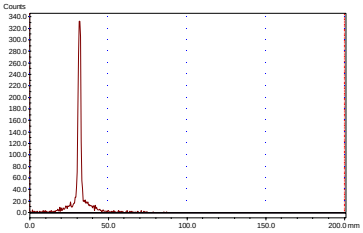


2nd run
98.8%

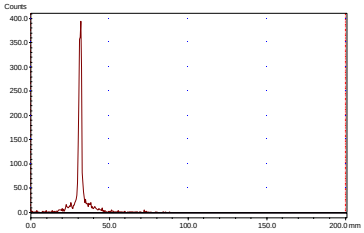


48hr

1st run
99.1%

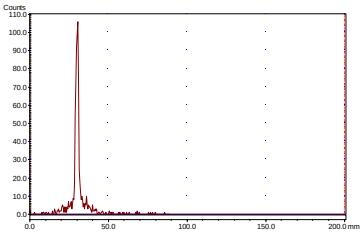


2nd run
98.8%



168hr

1st run
98.9%



2nd run
99.3%

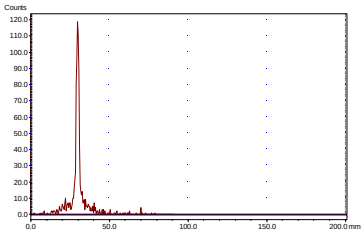
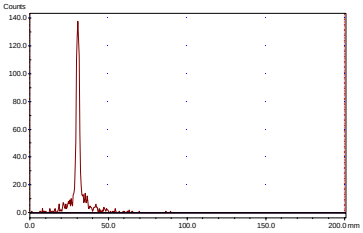


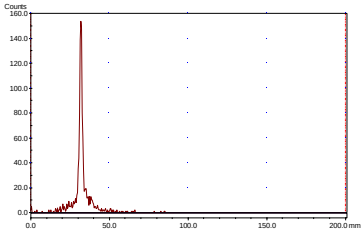
Figure S13. Complex Stability of ^{89}Zr-DFO in EDTA solution

0hr

1st run
99.3%

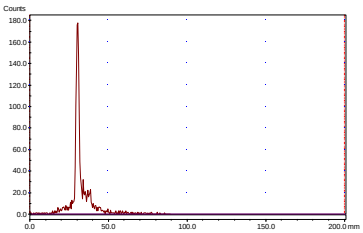


2nd run
99.4%

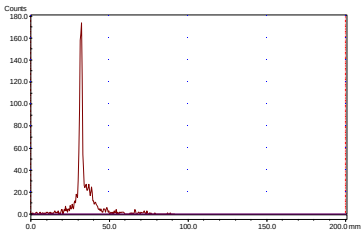


1hr

1st run
98.3%

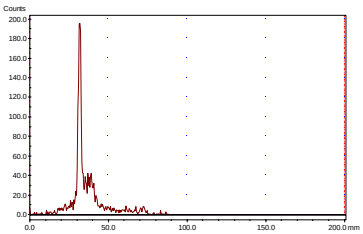


2nd run
97.9%

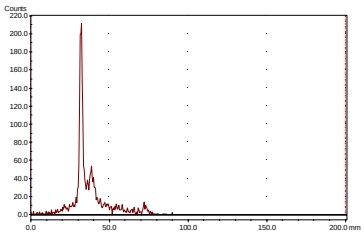


24hr

1st run
92.8%

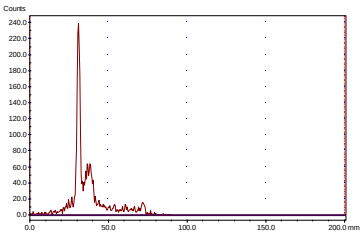


2nd run
92.2%

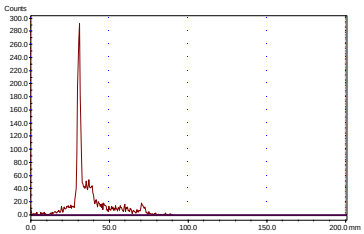


48hr

1st run
89.2%

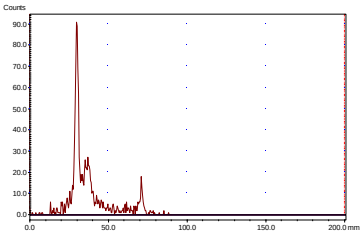


2nd run
88.9%

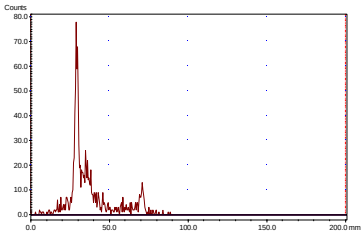


168hr

1st run
88.5%



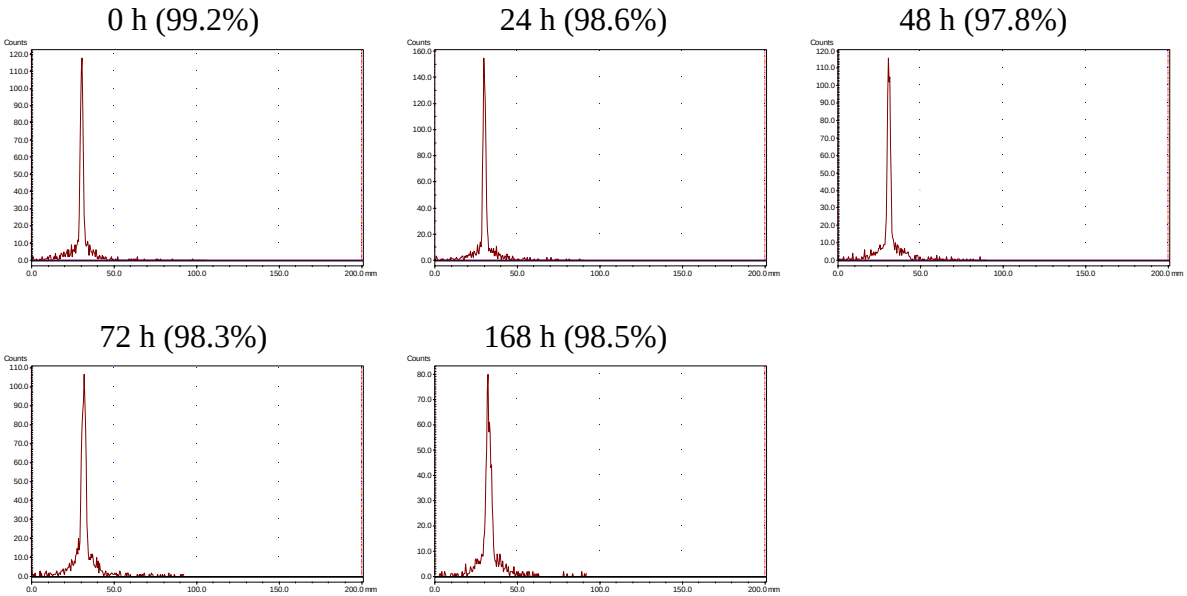
2nd run
89.0%



VI. Evaluation of ^{89}Zr-labeled complexes for stability in apotransferrin solution (5-fold molar excess, pH 7.0, 37°C, ITLC, duplicate).

Figure S14. Complex Stability of ^{89}Zr-DA-18C6-BHA in apotransferrin solution

1st run



2nd run

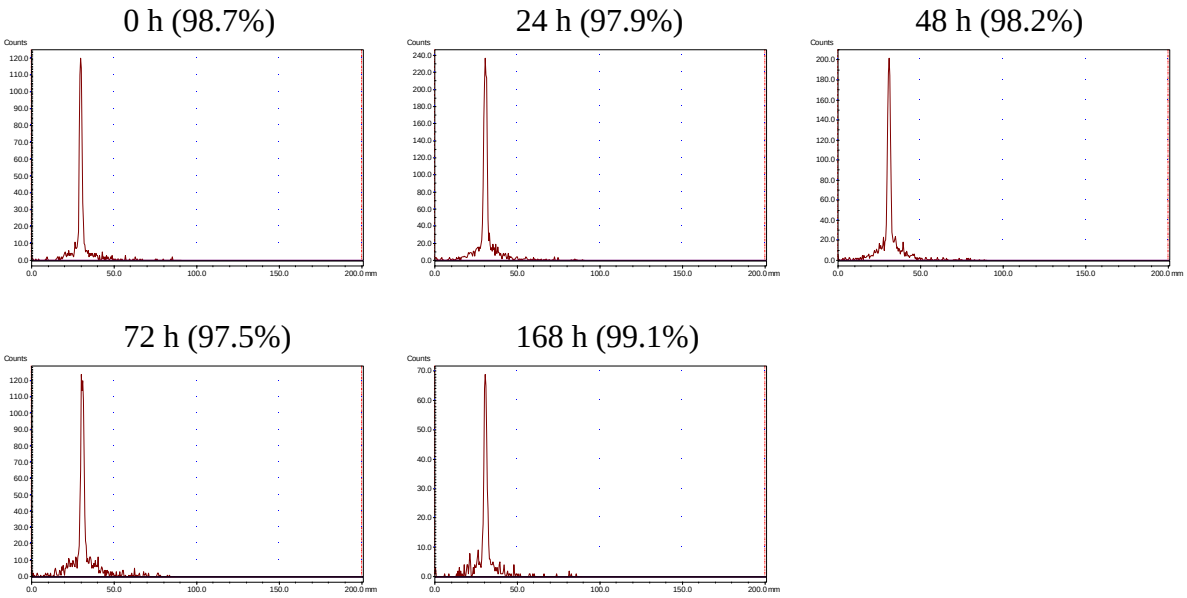
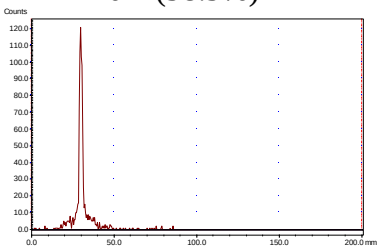


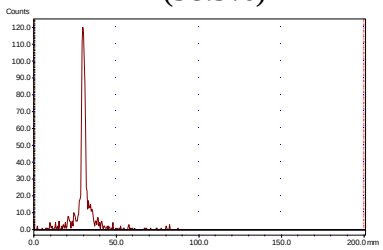
Figure S15. Complex Stability of ^{89}Zr-DFO in apotransferrin solution

1st run

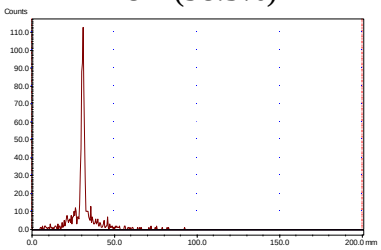
0 h (98.9%)



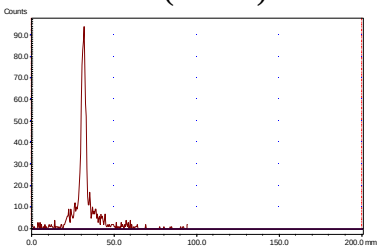
24 h (98.9%)



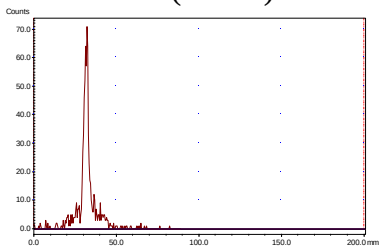
48 h (98.3%)



72 h (97.6%)

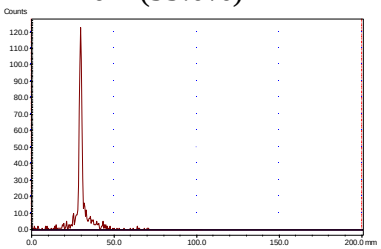


168 h (98.7%)

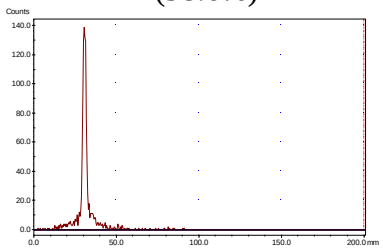


2nd run

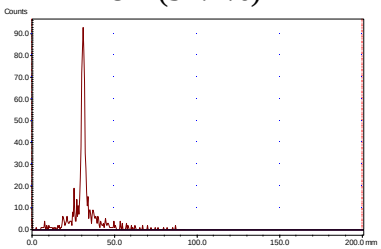
0 h (99.0%)



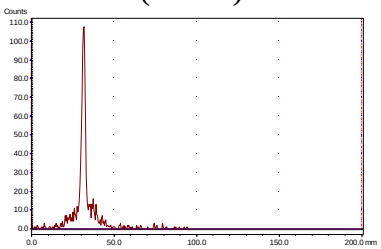
24 h (98.6%)



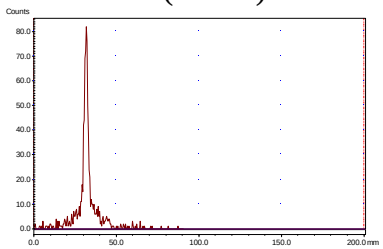
48 h (97.2%)



72 h (98.3%)



168 h (98.0%)



VII. *In Vivo* Biodistribution of ^{89}Zr-DFO and ^{89}Zr-DA-18C6-BHA in CD-1 Mice.

Table S2. Percentage of injected dose per gram of tissue (%ID/g)

Tissue	Time points (%ID/g)					
	1 h		4 h		24 h	
	DFO	DA-18C6-BHA	DFO	DA-18C6-BHA	DFO	DA-18C6-BHA
blood	0.02 ± 0.02	0.05 ± 0.04	0.04 ± 0.02	0.12 ± 0.14	0.02 ± 0.01	0.03 ± 0.01
liver	0.03 ± 0.01	0.43 ± 0.06	0.04 ± 0.01	0.44 ± 0.03	0.01 ± 0.00	0.30 ± 0.05
kidney	0.23 ± 0.03	0.21 ± 0.02	0.25 ± 0.03	0.15 ± 0.02	0.16 ± 0.02	0.12 ± 0.01
muscle	0.04 ± 0.02	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.05 ± 0.02
bone	0.06 ± 0.02	0.11 ± 0.01	0.09 ± 0.03	0.09 ± 0.02	0.04 ± 0.02	0.07 ± 0.01