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Supporting Information for

Two bifunctional desferrioxamine chelators for bioorthogonal labeling of biovectors with zirconium-89

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1) Materials

Unless otherwise noted, reagents and solvents were purchased from Sigma-Aldrich (Oakville, Canada) or VWR (Mississauga, Canada) and used without further purification. Fmoc-amino acids and HATU were purchased from ChemPep (Washington, USA). 2-Chlorotritylchloride resin was obtained from Advanced ChemTech (Kentucky, USA). Silica gel (40-63 µm) for flash chromatography was obtained from Silicycle (Quebec City, Canada). C18 SepPak cartridges were purchased from Waters (Mississauga, Canada). TLC was performed on pre-coated silica gel $60F_{254}$ aluminum sheets from Merck (Darmstadt, Germany). The compounds were visualized under ultra-violet light at 254 nm or 365 nm, or by brief immersion in an appropriate stain.
^{89}Zr was produced via the ^{89}Y(p,n)^{89}Zr reaction on the ACSI TR-19 cyclotron (Richmond, Canada) at the BC Cancer Agency Department of Functional Imaging as described previously.[1] Briefly, yttrium-89 foils (99.9%, 0.254 mm thickness) purchased from American Elements (Los Angeles, USA) were irradiated at 13 MeV. [^{89}Zr]Zr-oxalate (0.05 M) was obtained by dissolution of the foil and purification using a hydroxamate-based ZR resin generously supplied by Triskem (Bruz, France).[2] The resulting [^{89}Zr]Zr-oxalate was produced at 511 ± 40 MBq/mL (13.8 ± 0.1 mCi/mL).

2) Instrumentation and methods

Reverse phase HPLC was performed on an Agilent 1460 Series (Santa Clara, USA). For purification and analysis of the compounds, the following solvent were used: solvent A = 0.1% trifluoroacetic acid (TFA) in water (v/v) and solvent B = 0.1% TFA in acetonitrile (v/v). *System A =* Phenomenex Luna 10 µ C18 300 Å (250 × 10 mm, 10 µm) column (Torrance, USA) operated at a flow rate of 3 mL/min with a gradient of solvent A and B: t = 0 min, 95% A; t = 30 min, 10% A; t = 35 min, 10% A. *System B =* Phenomenex Luna 5 µ C18 90 Å (250 × 4.6 mm, 5 µm) column operated at a flow rate of 1 mL/min with a gradient of solvent A and B: t = 0 min, 95% A; t = 30 min, 10% A; t = 33 min, 10% A. During semi-preparative HPLC (system A), the mobile phase was monitored at 220, 250 and 270 nm, whereas only the UV absorbance at 250 nm was recorded during radio-analytical runs (system B). The HPLC was also equipped with a radioactivity detector (Raytest; Straubenhardt, Germany). Both UV and radioactivity detectors were connected in series, making retention times slightly different for radioactive peak and the corresponding UV signal.
Automated solid-phase peptide synthesis was performed on an Aapptec Focus Xi synthesizer (Louisville, USA).
NMR spectra were recorded in deuterated solvents on an Avance 400 MHz NMR Spectrometer (Bruker; Bremen, Germany) at room temperature. Chemical shifts (δ) are reported in ppm and coupling constant J in Hz. Residual solvent peaks were used as an internal reference. Abbreviations for the peak multiplicities are s (singulet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet) and br (broad).
Low-resolution MS were recorded on a Waters LC-MS (Milford, USA), while high resolution MS spectra were obtained on a Waters/Micromass LCT TOF-MS (Milford, USA).

Radioactivity measurements were made by using a CRC@-55TPET Dose Calibrator (Capintec; Florham Park, USA). Quantitative γ-counting of experimental samples was performed on a Wizard^2 2470 gamma counter (PerkinElmer; Waltham, USA). A system 200 imaging scanner (Bioscan; Washington, USA) with WinScan software was used for radioactive detection on TLC plates.

3) Synthesis and characterization of DFO-Cys (4) and DFO-CBT (10)

2,5-Dioxopyrrolidin-1-yl *N*-(*tert*-butoxycarbonyl)-*S*-tritylcysteinate (2). Boc-Cys(Trt)-OH (463 mg, 1 mmol), *N*-hydroxysuccinimide (253 mg, 2.2 mmol) and EDC (422 mg, 2.2 mmol) were dissolved into 7.5 mL of DMF. The reaction mixture was stirred at room temperature for 24 h. Then, the solvent was removed under vacuum. The resulting yellow oil was dissolved into EtOAc, washed with sat. $NaHCO_3$ and brine, and dried over Na_2SO_4. After evaporation of the solvent, the residue was purified by silica gel flash chromatography (95:5 DCM/EtOAc) to afford **2** (378 mg, 63%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.20-7.46 (m, 15H), 4.88 (d, 1H, J = 7.4 Hz) 4.33 (app. q, 1H, J = 5.8 Hz), 2.79 (s, 4H), 2.65-2.78 (m, 2H), 1.43 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.4, 166.8, 154.5, 144.1, 129.7, 128.4, 126.9, 80.6, 67.4, 51.1, 33.6, 28.3, 25.6. ESI-MS: *m/z* 564.3 $[M+H]^+$.

***tert*-Butyl (12,23,34-trihydroxy-5,13,16,24,27,35-hexaoxo-1,1,1-triphenyl-2-thia-6,12,17,23,28,34-hexaazahexatriacontan-4-yl)carbamate (3).** DFO mesylate (40 mg, 0.061 mmol) and Et_3N (34 µL, 0.244 mmol) were dissolved in 15 mL anhydrous DMF. Gentle heating at 45 °C was provided to obtain a homogeneous solution. **2** (69 mg, 0.123 mmol) in DMF (1 mL) was added and the reaction mixture was stirred for 18 h at room temperature. After evaporation of the solvent under vacuum, the residue was taken up into H_2O/CH_3CN (2:1) and loaded onto a large C18 SepPak (preconditioned with 6 mL MeOH and 12 mL H_2O). The C18 cartridge was eluted with a gradient of H_2O/CH_3CN (from 3:1 to 1:3) and fractions of 2 mL were collected. The fractions containing **3** were freeze-dried and the white product was used without further purification (47 mg, 77%). ESI-MS: *m/z* 1028.7 $[M+Na]^+$.

***N*^{1}-(5-(2-Amino-3-mercaptopropanamido)pentyl)-*N*^{1}-hydroxy-*N*^{4}-(5-(*N*-hydroxy-4-((5-(*N*-hydroxyacetamido)pentyl)amino)-4-oxobutanamido)pentyl)succinamide (4, DFO-Cys). 3** (50 mg, 0.05 mmol) was added to a solution containing TFA, H_2O and TIPS (10 mL, 95/2.5/2.5). The mixture was stirred for 2 h at room temperature. TFA was removed by using a gentle flow of N_2 gas. The remaining oil mixture was triturated with two portions of cold ether (10 mL) and a white precipitate was collected. The product was purified by HPLC (system A) with degassed mobile phase. Yield, 21 mg, 63%. ^{1}H NMR (400 MHz, D_2O): δ 4.13 (t, 1H, J = 6.0 Hz), 3.52-3.66 (m, 8H), 3.13-3.41 (m, 4H), 3.05 (dq, 2H, J = 5.6 and 14.8 Hz), 2.80 (dd, 4H, J = 6.8 and 7.2 Hz), 2.50 (dd, 4H, J = 6.8 and 7.2 Hz), 2.13 (s, 3H), 1.48-1.70 (m, 12H), 1.30-1.34 (m, 6H). ^{13}C NMR

(100 MHz, D_2O): δ 175.03, 175.00, 174.10, 174.05, 173.80, 167.83, 54.74, 48.06, 47.91, 47.88, 39.66, 39.38, 39.32, 30.66, 30.63, 28.12, 28.08, 27.98, 27.84, 26.47, 26.41, 26.33, 25.63, 25.10, 23.29, 23.24, 23.20, 19.45. ESI-MS: *m/z* 664.3 $[M+H]^+$, 686.4 $[M+Na]^+$. Purity: over 99%.

***tert*-Butyl 2-((2-cyanobenzo[*d*]thiazol-6-yl)oxy)acetate (7).** To a stirred solution of 2-cyano-6-hydroxybenzothiazole (311 mg, 1.77 mmol) in acetone (30 mL) was added *tert*-butyl bromoacetate (335 µL, 2.27 mmol), K_2CO_3 (386 mg, 2.79 mmol), and NaI (34 mg, 0.23 mmol). The mixture was refluxed overnight and then allowed to cool down to room temperature. Upon cooling, the insoluble material was removed by filtration and the filtrate was concentrated. After portioning over EtOAc (100 mL), the organic phase was washed with 5% aq. sodium thiosulphate (75 mL), H_2O (75 mL), brine (75 mL) and dried over Na_2SO_4. Solvent was removed under vacuum and the residue was purified by silica gel flash chromatography (100% DCM) to give compound **7** as a white powder (400 mg, 78% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (d, 1H, *J* = 9.1 Hz), 7.33 (d, 1H, *J* = 2.4 Hz), 7.28 (dd, 1H, *J* = 9.1, 2.4 Hz), 4.63 (s, 2H), 1.50 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.0, 158.6, 147.3, 137.1, 133.9, 125.9, 118.4, 113.1, 104.5, 82.9, 66.1, 28.0. ESI-MS: *m/z* 313.1 $[M+Na]^+$.

2-((2-Cyanobenzo[*d*]thiazol-6-yl)oxy)acetic acid (8). A solution of **7** (400 mg, 1.38 mmol) in $CHCl_3$ (12 mL) and TIPS (1 mL) was cooled in an ice bath and treated portion-wise with TFA (2 mL). The reaction mixture was stirred overnight at room temperature. After evaporation of the solvent, purification by flash chromatography (95:5 DCM/EtOAc to 100% EtOAc) gave the title compound as a white powder (225 mg, 70%). ^{1}H NMR (400 MHz CD_3OD): δ 8.02 (d, 1H, *J* = 9.1 Hz), 7.58 (d, 1H, *J* = 2.6 Hz), 7.31 (dd, 1H, *J* = 9.1, 2.6 Hz), 4.79 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.7, 160.1, 148.4, 138.7, 135.5, 126.5, 119.9, 114.1, 105.8, 66.2. ESI-MS: *m/z* 233.1 $[M-H]^-$.

2,5-Dioxopyrrolidin-1-yl 2-((2-cyanobenzo[*d*]thiazol-6-yl)oxy)acetate (9). A solution of 2-((2-cyanobenzo[*d*]thiazol-6-yl)oxy)acetic acid (100 mg, 0.43 mmol) in anhydrous DMF (5 mL) containing EDC (166 mg, 0.86 mmol) and NHS (100 mg, 0.86 mmol) was stirred under N_2 at room temperature for 48 h. After removal of the solvent *in vacuo*, the mixture was portioned over DCM (100 mL), then washed with 0.5 M HCl (2 x 100 mL), H_2O (100 mL), brine (100 mL), dried over Na_2SO_4, filtered and then concentrated to yield a colorless oil. Purification by flash chromatography (95:5 DCM/EtOAc) provided compound **9** as a white powder (60 mg, 42%). ^{1}H NMR (400 MHz, CD_3CN): δ 8.15 (d, 1H, *J* = 9.1 Hz), 7.68 (d, 1H, *J* = 2.6 Hz), 7.37 (dd, 1H, *J* = 9.1, 2.6 Hz), 5.21 (s, 2H), 2.79 (s, 4H). ESI-MS: *m/z* 332.2 $[M+H]^+$.

***N*^{1}-(5-(2-((2-Cyanobenzo[*d*]thiazol-6-yl)oxy)acetamido)pentyl)-*N*^{1}-hydroxy-*N*^{4}-(5-(*N*-hydroxy-4-((5-(*N*-hydroxyacetamido)pentyl)amino)-4-oxobutanamido)pentyl)succinamide (10, DFO-CBT).** DFO mesylate (60 mg, 0.09 mmol) and Et_3N (63.7 µL, 0.45 mmol) were dissolved in 5 mL

anhydrous DMF. Gentle heating at 45 °C was provided to obtain a homogeneous solution, after which **9** (30 mg, 0.09 mmol) was added. The resulting mixture was stirred at 45 °C for 4 h. Upon removal of solvent *in vacuo*, the product was purified by RP-HPLC (system A). Yield, 23 mg, 33%. ^{1}H NMR (400 MHz, CD_3CN): δ 9.56-9.61 (m, 3H), 8.13-8.14 (m, 1H), 8.13 (d, 1H, *J* = 9.2 Hz), 7.80 (d, 1H, *J* = 2.4 Hz), 7.72 (m, 2H), 7.34 (dd, 1H, *J* = 2.8 and 9.2 Hz), 4.56 (s, 2H), 3.36-3.41 (m, 6H), 3.06 (q, 2H, *J* = 6.8 Hz), 2.92-2.96 (m, 4H), 2.52 (t, 4H, *J* = 7.2 Hz), 2.21 (t, 4H, *J* = 7.2 Hz), 1.91 (s, 3H), 1.30-1.44 (m, 12 H), 1.51-1.17 (m, 6H). ^{13}C NMR (100 MHz, DMSO-*d6*): δ 172.43, 171.76, 170.59, 167.19, 158.78, 147.00, 137.82, 134.61, 125.74, 119.37, 114.04, 106.13, 67.86, 47.53, 47.24, 38.88, 30.35, 29.27, 28.02, 26.49, 23.95, 20.82. ESI-MS: *m/z* 777.5 $[M+H]^+$, 799.5 $[M+Na]^+$. Purity: over 99%.

4) Coupling of DFO-Cys and DFO-CBT with their complementary bioorthogonal click handle and complexation reaction with ^{nat}Zr(IV)

***N*^{1}-Hydroxy-*N*^{1}-(5-(4-(hydroxy(5-(2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-5-carboxamido)pentyl)amino)-4-oxobutanamido)pentyl)-*N*^{4}-(5-(*N*-hydroxyacetamido)pentyl)succinamide (5a, DFO-Luc-OH).** To a mixture of DFO-Cys (5 mg, 7.5 µmol) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl, 2.5 mg, 8.5 µmol) in 1.5 mL of PBS buffer (0.1 M, pH 7.4) was added 2-cyano-6-hydroxybenzothiazole (1.5 mg, 8.5 µmol) in 0.5 mL of DMF. The resulting solution was stirred at room temperature for 4 h. Then the mixture was purified by semi-preparative HPLC (system A). Yield: 5 mg, 81%. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 10.23 (s, 1H), 9.16-9.66 (m, 2H), 8.11 (t, 1H, *J* = 5.2 Hz), 7.95 (d, 1H, *J* = 8.8 Hz), 7.78 (t, 2H, *J* = 5.2 Hz), 7.44 (d, 1H, *J* = 2.4 Hz), 7.05 (dd, 1H, *J* = 2.4 and 8.8 Hz), 5.23 (t, 1H, *J* = 9.6 Hz), 3.59-3.71 (m, 2H), 3.42-4.48 (m, 6H), 3.06-3.17 (m, 2H), 2.96-3.01 (m, 4H), 2.54-2.58 (m, 5H), 2.24-2.32 (t, 4H, *J* =7.2 Hz), 1.96 (s, 3H), 1.44-1.51 (m, 6H), 1.33-1.40 (m, 6H), 1.91-1.26 (m, 6H). ESI-MS: *m/z* 845.6 $[M+Na]^+$. Purity: over 99%.

^{nat}Zr-DFO-Cys (Zr-4). To a solution of DFO-Cys (10 mg, 15 µmol) and $ZrCl_4$ (5.3 mg, 23 µmol) in 1 mL of water was added a solution of 0.1 M Na_2CO_3 to adjust the pH to 7-7.5. The resulting solution was stirred at room temperature for 2 h. Then, the mixture was purified by semi-preparative HPLC (system A). Yield: 3 mg, 27%. ESI-MS: *m/z* 750.4 $[M+H]^+$.

2-(6-((9,20,31-trihydroxy-2,10,13,21,24,32-hexaoxo-3,9,14,20,25,31-hexaazatritriacontyl)oxy)benzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (11a, DFO-Luc-COOH). DFO-CBT (5 mg, 6 µmol) in 1.0 mL of PBS buffer (0.1 M, pH 7.4) was added to a freshly prepared solution of L-cysteine (1.5 mg, 12 µmol) in PBS buffer (0.5 mL). The resulting mixture was stirred at room temperature for 4 h and **11a** was purified by semi-preparative HPLC (system A). Yield, 5 mg, 95%. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 9.62-9.66 (m, 3H), 8.15-8.19 (m, 1H), 8.08 (d, 1H, *J* = 9.2 Hz), 7.72-7.80 (m, 3H), 7.27 (dd, 1H, *J* = 1.6 and 9.2 Hz), 5.42 (t, 1H, *J*

= 9.6 Hz), 4.58 (s, 2H), 3.65-3.80 (m, 2H), 3.43-3.46 (m, 6H), 3.09-3.16 (m, 2 H), 2.96-3.01 (m, 4H), 2.54-2.58 (m, 5H), 2.24-2.27 (t, 4H, *J* =7.2 Hz), 1.96 (s, 3H), 1.20-1.49 (m, 18H). ESI-MS: *m/z* 903.4 $[M+Na]^+$, 452.4 $[M+Na+H]^{2+}$. Purity: over 99%.

^{nat}Zr-DFO-CBT (Zr-10). Synthesis of Zr-**10** was performed according to the method described above for Zr-**4**. 10 mg (12 µmol) of DFO-CBT and 4.2 mg (18 µmol) of $ZrCl_4$ were used to prepared Zr-**10**. Yield, 4 mg, 39%. ESI-MS: *m/z* 863.4 $[M+H]^+$.

5) Labeling of DFO-Cys and DFO-CBT with ^{89}Zr(IV)

Labeling of DFO-Cys with ^{89}Zr(IV) ([^{89}Zr]-4, [^{89}Zr]Zr-DFO-Cys). Approximately 5 MBq (10 µL) of [^{89}Zr]Zr-oxalate (in 0.05 M oxalic acid) were transferred into a low-protein binding Eppendorf tube, followed by addition of 5 µL of 0.1 M sodium carbonate and 85 µL of water. The reaction solution was incubated for 3 min at room temperature. Thereafter, 197 µL of HEPES buffer (0.2 M, pH 7.0) and 3 µL of an aqueous solution of DFO-Cys (6 µg, 9 nmol) were added to the reaction vial. The pH of the reaction mixture was between 6.8 and 7.2. The solution was allowed to react at room temperature for 90 min. The identity and radiochemical yields of [^{89}Zr]-**4** were determined by analytical radio-HPLC (system B).

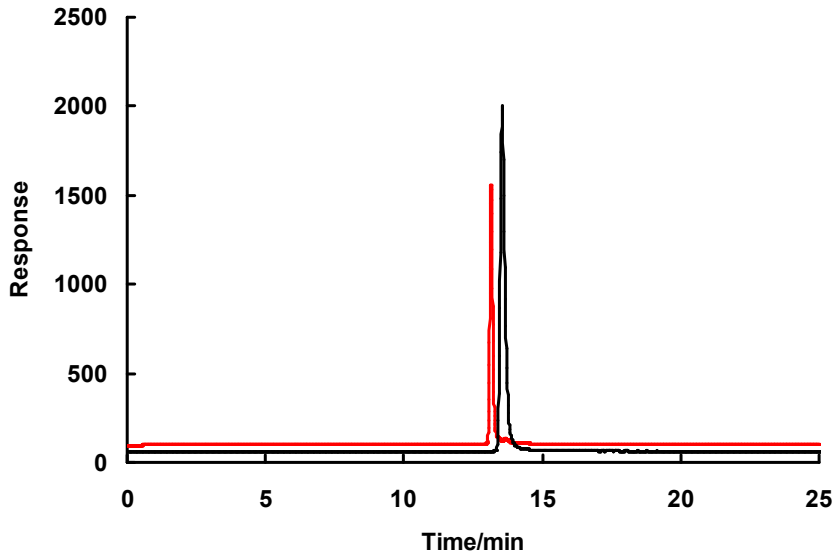

Figure S1. HPLC identification of [^{89}Zr]Zr-DFO-Cys. Radio-chromatogram of [^{89}Zr]Zr-DFO-Cys (black curve) and UV-chromatogram of its corresponding non-radioactive analog at 254 nm (red curve). The retention time of [^{89}Zr]Zr-DFO-Cys is 13.5 min and the retention time of non-radioactive ^{nat}Zr-DFO-Cys is 13.2 min.

Labeling of DFO-CBT with ^{89}Zr(IV) ([^{89}Zr]-10, [^{89}Zr]Zr-DFO-CBT). Approximately 5 MBq (10 µL) of [^{89}Zr]Zr-oxalate (in 0.05 M oxalic acid), 5 µL of 0.1 M sodium carbonate and 85 µL of water were placed into a low-protein binding Eppendorf tube. The mixture was incubated for 3 min at rt

and then 197 µL of HEPES buffer (0.2 M, pH 7.0) and 3 µL of an aqueous solution of **10** (7 µg, 9 nmol) were added to the reaction vial. The pH of the reaction mixture was between 6.8 and 7.2. The solution was allowed to react at rt for 90 min. The identity and radiochemical yields of [^{89}Zr]-**10** were evaluated by radio-HPLC (system B).

Table S1. Labeling conditions of DFO-Cys (4) and DFO-CBT (10)

Ligand	**4**	**10**
Amount (nmol)	9	9
T (˚C)	rt	rt
Reaction time (min)	90	90
pH	6.8-7.2	6.8-7.2
Radiochemical yield (%)	100	100
Molar activity (MBq/µmol)	556	556

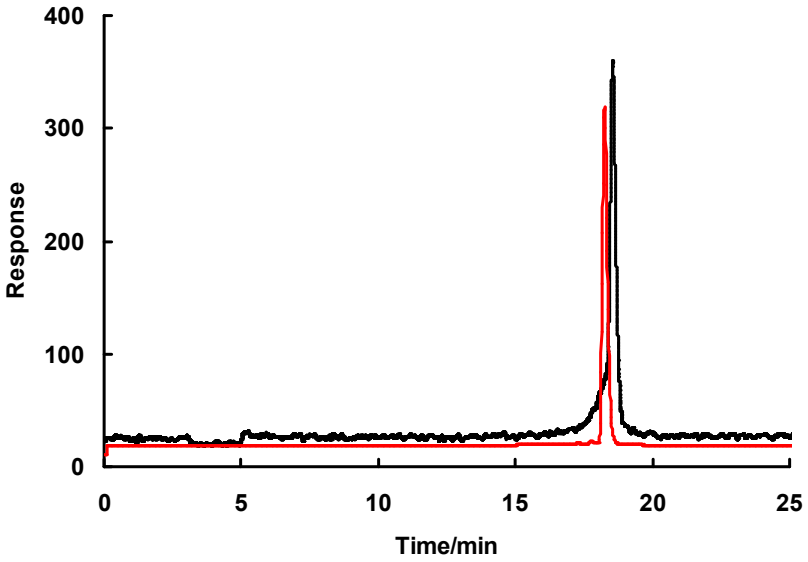

Figure S2. HPLC identification of [^{89}Zr]Zr-DFO-CBT. Radio-chromatogram of [^{89}Zr]Zr-DFO-CBT (black curve) and UV-chromatogram (310 nm) of its corresponding non-radioactive substance (red curve). The retention time of [^{89}Zr]Zr-DFO-CBT is 18.6 min and the retention time of non-radioactive ^{nat}Zr-DFO-CBT is 18.3 min.

6) Preparation of [^{89}Zr]Zr-DFO-Luc-OH ([^{89}Zr]-5a) and [^{89}Zr]Zr-DFO-Luc-COOH ([^{89}Zr]-11a)

Labeling of DFO-Luc-OH (5a) with ^{89}Zr. To a low-protein binding Eppendorf tube were added 5 MBq (10 µL) of [^{89}Zr]Zr-oxalate (in 0.05 M oxalic acid), 5 µL of 0.1 M sodium carbonate and 85 µL of water. After 3 min at room temperature, 200 µL of HEPES buffer (0.2 M, pH 7.0) and 4 µL of DFO-Luc-OH (8 µg, 10 nmol) were added. The pH of the reaction mixture was between 6.8 and 7.2. The solution was allowed to react at rt for 90 min. The product [^{89}Zr]-**5a** was analyzed by radio-HPLC (system B).

Coupling of [^{89}Zr]Zr-DFO-Cys with 6-OH-CBT ([^{89}Zr]-5a, [^{89}Zr]Zr-DFO-Luc-OH). 150 µL of [^{89}Zr]Zr-DFO-Cys (2.5 MBq, 4.5 nmol of DFO-Cys) were mixed with 200 µL of PBS buffer (0.4 M, pH 7.4). The mixture was stirred at room temperature for 1 min. Then, 1.5 µL of an aqueous solution of TCEP·HCl (1.5 µg, 5 nmol) and 1.8 µL of a methanolic solution of 6-OH-CBT (1.8 µg, 10 nmol) were sequentially added. The final pH was around 7.4. The reaction mixture was shaken at 37 °C for 1 h. The identity and radiochemical yields of [^{89}Zr]Zr-DFO-Luc-OH were determined by analytical radio-HPLC (system B).

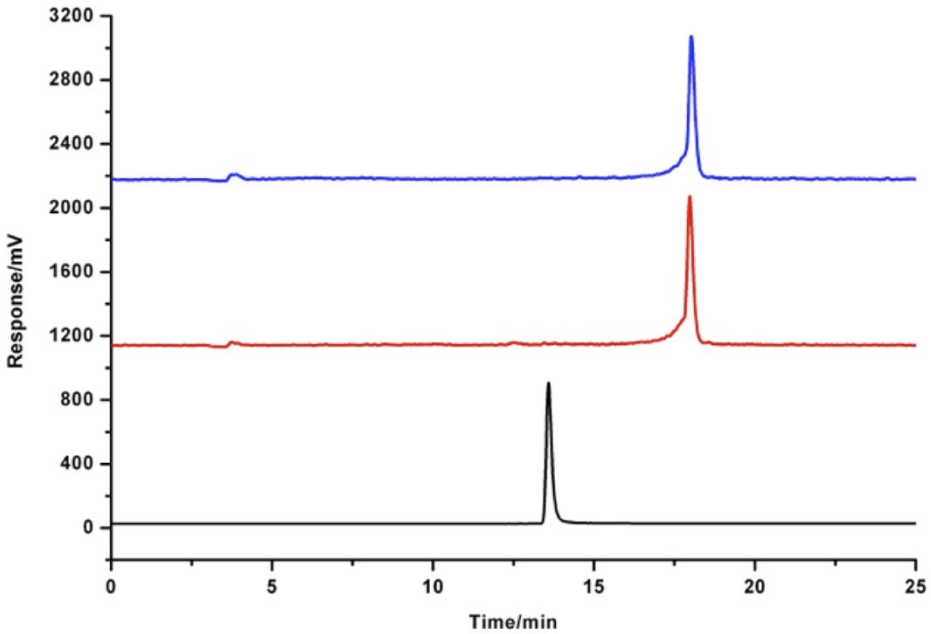

Figure S3. Radio-chromatograms of [^{89}Zr]Zr-DFO-Cys and [^{89}Zr]Zr-DFO-Luc-OH. [^{89}Zr]Zr-DFO-Luc-OH ([^{89}Zr]-**5a**) was either directly obtained from the labeling of DFO-Luc-OH (**5a**) with ^{89}Zr (blue curve) or by condensation of [^{89}Zr]Zr-DFO-Cys ([^{89}Zr]-**4**, black curve) with 6-OH-CBT (**6**) (red curve). Both labeling reactions resulted in a radio-complex with a retention time of 18 min.

Labeling of DFO-Luc-COOH (11a) with ^{89}Zr. [^{89}Zr]-**11a** was prepared according to the method described above for [^{89}Zr]-**5a**. The radiochemical yields of [^{89}Zr]-**11a** were evaluated by radio-HPLC (system B).

Coupling of [^{89}Zr]Zr-DFO-CBT with L-cysteine ([^{89}Zr]-11a, [^{89}Zr]Zr-DFO-Luc-COOH). 150 µL of [^{89}Zr]Zr-DFO-CBT (2.5 MBq, 4.5 nmol of DFO-CBT) and 200 µL PBS buffer (0.4 M, pH 7.4) were stirred at rt for 1 min. Then, 1.2 µL of a freshly prepared aqueous solution of L-cysteine (1.2 µg, 10 nmol) were added. The final pH was around 7.4. The reaction mixture was shaken at 37 °C for 10 min and the final product [^{89}Zr]-**11a** was analyzed by radio-HPLC (system B).

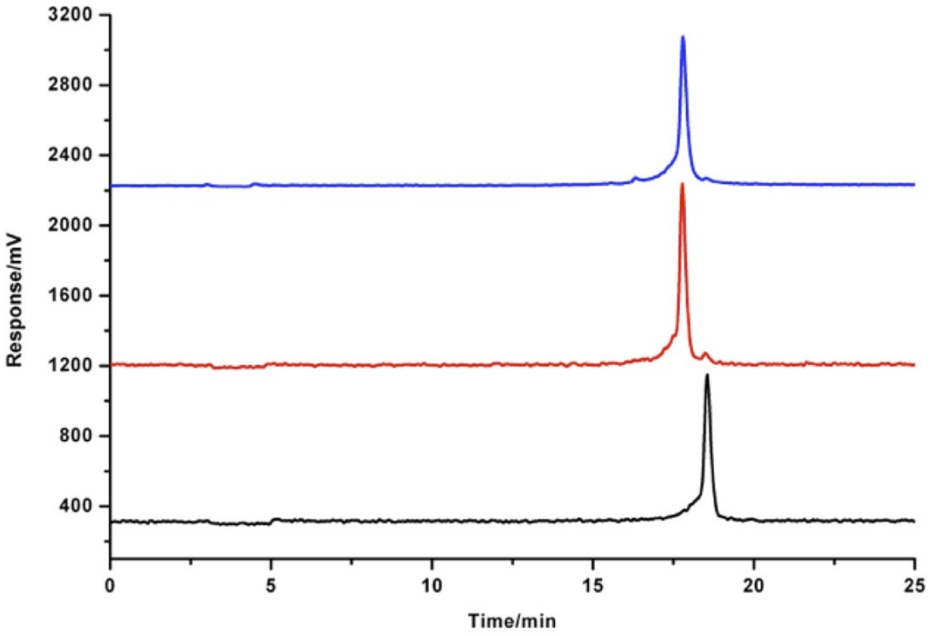

Figure S4. Radio-chromatograms of [^{89}Zr]Zr-DFO-CBT and [^{89}Zr]Zr-DFO-Luc-COOH. [^{89}Zr]Zr-DFO-Luc-COOH ([^{89}Zr]-**11a**) was either directly obtained from the labeling of DFO-Luc-COOH (**11a**) with ^{89}Zr (blue curve) or by condensation of [^{89}Zr]Zr-DFO-CBT ([^{89}Zr]-**10**, black curve) with L-cysteine (red curve). Both labeling reactions resulted in a radio-complex with identical retention time.

7) Optimization and kinetic studies of the condensation reaction

Influence of pH and temperature on the coupling between [^{89}Zr]Zr-DFO-Cys ([^{89}Zr]-4) and 6-OH-CBT (6). 75 µL of an aqueous solution of [^{89}Zr]Zr-DFO-Cys were added to 100 µL of PBS buffer (0.4 M, pH 7.4), followed by the addition of TCEP·HCl (1 µg, 3.5 nmol) and an excess of 6-OH-CBT (5.3 µg, 30 nmol). *pH*: pH was adjusted with a solution of HCl (0.1 N) for pH 4 and 6. The reaction mixtures were incubated at 37 °C for 10 min. *Temperature:* The reaction mixture were incubated at room temperature or 37 °C for 10 min. The coupling yields of [^{89}Zr]Zr-DFO-Cys with **6** were monitored by HPLC (system B).

Temperature dependence of the coupling between [^{89}Zr]Zr-DFO-CBT ([^{89}Zr]-10) and L-cysteine. To [^{89}Zr]Zr-DFO-CBT (75 µL) in PBS buffer (100 µL, 0.4 M, pH 7.4) was added L-cysteine (3.6 µg, 30 nmol) in excess. The mixture was incubated at room temperature or 37 °C for 10 min. Radiochemical yields of [^{89}Zr]Zr-DFO-Luc-COOH ([^{89}Zr]-**11a**) were evaluated by radio-HPLC (system B).

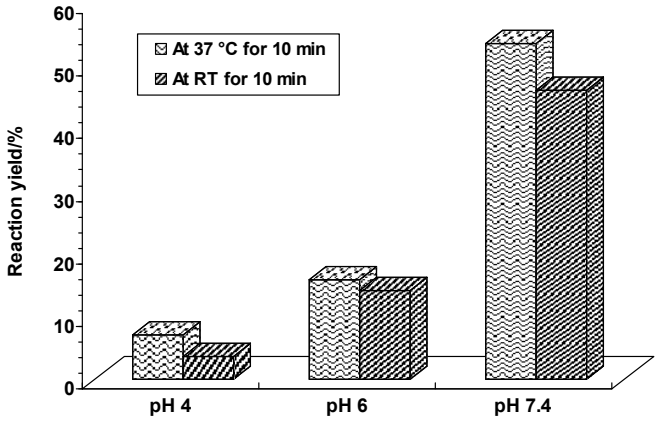

Figure S5. Influence of pH and temperature on the condensation reaction between [^{89}Zr]Zr-DFO-Cys and 6-OH-CBT.

Kinetic study of the formation of [^{89}Zr]Zr-DFO-Luc-OH ([^{89}Zr]-5a). [^{89}Zr]Zr-DFO-Cys (5 MBq, 300 µL) was added into 400 µL of PBS buffer (0.4 M, pH 7.4) containing 3 µg of TCEP·HCl (10.5 nmol) and an excess of 6-OH-CBT (16 µg, 90 nmol). The resulting mixture was stirred at 37 °C and at different time points a sample was taken out. Acetic acid (10% solution) was added to the sample to quench the reaction. The coupling efficiency was monitored by measuring the radiochemical yield of [^{89}Zr]Zr-DFO-Luc-OH by radio-HPLC (system B).

Kinetic study of the formation of [^{89}Zr]Zr-DFO-Luc-COOH ([^{89}Zr]-11a). 300 µL of an aqueous solution of [^{89}Zr]Zr-DFO-CBT (5 MBq, 9 nmol DFO-CBT) were added into 400 µL of PBS buffer (0.4 M, pH 7.4) containing an excess of L-cysteine (11 µg, 90 nmol). The resulting mixture was stirred at 37 °C. At different time points a sample was withdrawn and quenched with a 10% solution of acetic acid. The coupling efficiency was determined by monitoring the radiochemical yield of [^{89}Zr]-**11a** by radio-HPLC (system B).

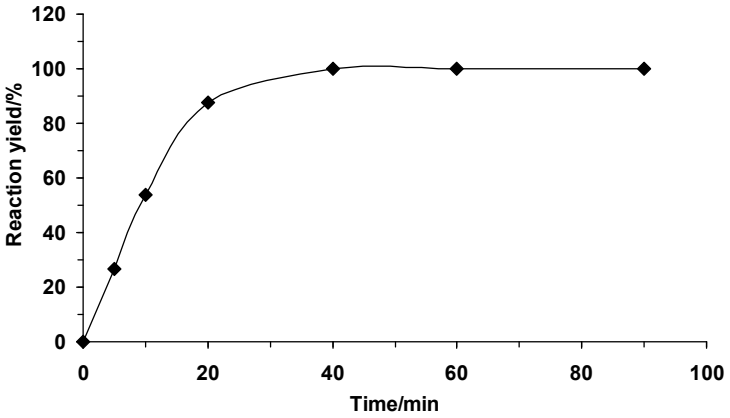

Figure S6. Time course of the condensation reaction between [^{89}Zr]Zr-DFO-Cys and 6-OH-CBT.

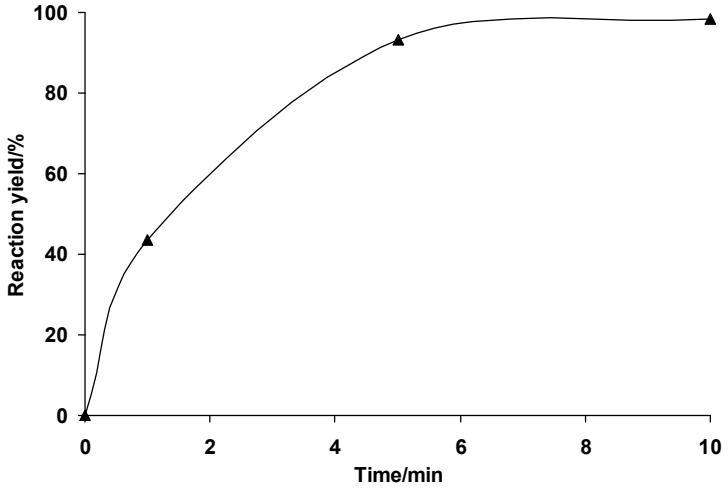

Figure S7. Time course of the condensation reaction between [^{89}Zr]Zr-DFO-CBT and L-cysteine.

Table S3. Evaluation of the click reaction efficiency at different time points

Labeled ligand	[^{89}Zr]-**4**	[^{89}Zr]-**10**
Reaction time (min)	**Click efficiency (%)** [a]	
0	0	0
1	-	43.7
5	26.8	93.2
10	53.7	98.4
20	87.4	-
40	99.9	-
60	99.9	-
90	99.9	-

[a] Click reaction was carried out at 37 °C and pH 7.4. 6-OH-CBT was used for the click reaction with [^{89}Zr]-**4** and L-cysteine with [^{89}Zr]-**10**.

8) Stability studies of [^{89}Zr]Zr-DFO-Luc-OH and [^{89}Zr]Zr-DFO-Luc-COOH complexes

Challenge study in cysteine solution. 100 µL of [^{89}Zr]-**5a** or [^{89}Zr]-**11a** were added to 400 µL of a cysteine solution (final concentrations 2 nM and 0.2 nM). The mixtures were vortexed and incubated at 37 °C for 1 h and 24 h. Aliquots of the reaction mixtures were taken and analyzed by radio-HPLC (system B).

Stability in PBS and human serum. 100 µL of [^{89}Zr]-**5a** or [^{89}Zr]-**11a** were mixed with 300 µL of 0.1 M PBS (pH 7.4) or 300 µL of human serum. After 1 h or 12 h of incubation at 37 °C, samples were analyzed by radio-HPLC. Serum samples were first treated with ethanol (twice the volume of the mixture) and centrifuged at 4 °C for 10 min (13,200 rpm). Supernatant was filtrated (VWR sterile syringe filter, 0.2 µm PES) and the filtrate was injected onto HPLC to determine the percentage of intact radioligand at each time point.

Protein binding assay. 100 µL [^{89}Zr]-**5a** or [^{89}Zr]-**11a** were incubated in 300 µL of fresh human serum for 4 h at 37 °C. Subsequently, ethanol (800 µL) was added and the mixture was centrifuged at 4 °C for 10 min (13,200 rpm). Protein binding was determined by measuring in triplicates the activity in the precipitate and the activity in the liquid phase in a gamma counter.

9) Synthesis of c(RGDfK)-CBT (12) and c[RGDyK(C)] (13)

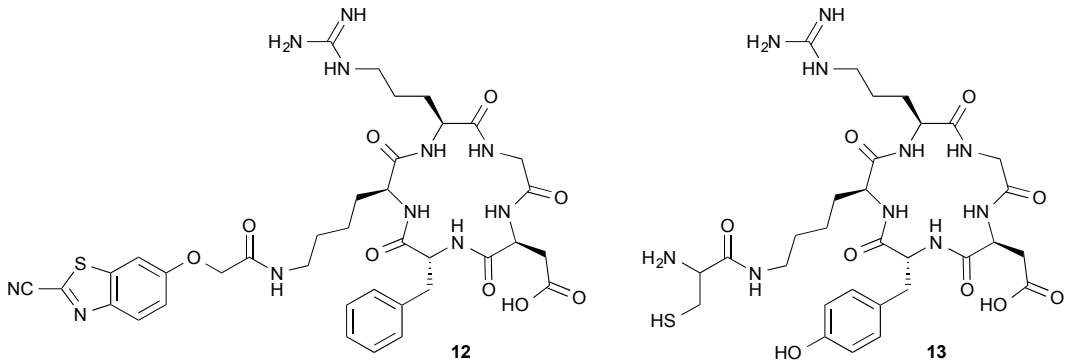

Figure S8. c(RGDfK)-CBT (**12**) and c[RGDyK(C)] (**13**).

Synthesis of c(RGDfk)-CBT (12). Monomeric cyclic RGD peptide c(RGDfK) was synthesized as previously reported with slight modification.[3] The fully protected linear pentapeptide H-Asp(OtBu)-*D*-Phe-Lys(Boc)-Arg(Pbf)-Gly-OH was synthesized by Fmoc-based solid phase peptide synthesis according to established methods on a 2-chlorotritylchloride resin (1.60 mmol/g). Coupling with *Nα*-protected amino acid (5 equiv.) were performed at room temperature for 2 h upon pre-activation with 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU; 4.9 equiv.) and DIEA (7.5 equiv.) or 2,3,5-collidine (15 equiv. for *D*-Phe coupling). The *N*-Fmoc groups were removed with 20% piperidine in DMF (2 × 10 min) at room temperature. The cycle of deprotection and coupling was checked by Kaiser test and repeated until the whole sequence was assembled. The side-chain protecting groups were chosen as *tert*-butyl for aspartic acid, *tert*-butoxycarbonyl (Boc) for lysine and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for arginine. The fully protected linear peptide was cleaved from the resin by treatment with a solution of AcOH/TFE/DCM (1:1:10) at room temperature for 2 h. Head-to-tail cyclization was carried out in DMF solution (6 mg/mL) with DPPA (3.2 equiv.) and $NaHCO_3$ (6.0 equiv.). All side-chain protecting groups were then removed by TFA/TIPS/H_2O (95:2.5:2.5) at room temperature for 2 h. The material was triturated with iced-cold diethyl ether and dried to afford a powder. The unprotected cyclic peptide was treated in anhydrous DMF with **9** (1 equiv.) in presence of DIPEA (5 equiv.) for 12 h at room temperature. Upon removal of solvent *in vacuo*, the product was purified by RP-HPLC (system A). ESI-MS: *m/z* 820.3 $[M+H]^+$.

Synthesis of c[RGDyK(C)] (13). Synthesis of the linear sequence H-Asp(OtBu)-*D*-Tyr(OtBu)-Lys(Boc-Cys(Trt))-Arg(Pbf)-Gly-OH, cleavage from the resin and head-to-tail cyclization were performed according to the same protocols described above. Fmoc-Lys(Boc-Cys(Trt))-OH was prepared as previously reported.[4] Upon precipitation of the product in iced-cold diethyl ether, the resulting peptide was purified by HPLC (System A). ESI-MS: *m/z* 723.4 $[M+H]^+$.

10) Synthesis of peptide conjugates DFO-Luc-c(RGDfK) (5b) and DFO-Luc-c(RGDyK) (11b)

DFO-Luc-c(RGDfk) (5b). DFO-Cys (7.0 mg, 10.6 µmol) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl, 3.0 mg, 10.6 µmol) in 1.5 mL of PBS (0.2 M, pH 7.4) were added to **12** (9.0 mg, 11.0 µmol) in DMF (3.5 mL). The resulting mixture was stirred at room temperature for 6 h. Then, the crude product was purified by semi-preparative HPLC (system A). Yield, 11 mg, 71%. ESI-MS: *m/z* 757.2 $[M+2Na]^{2+}$.

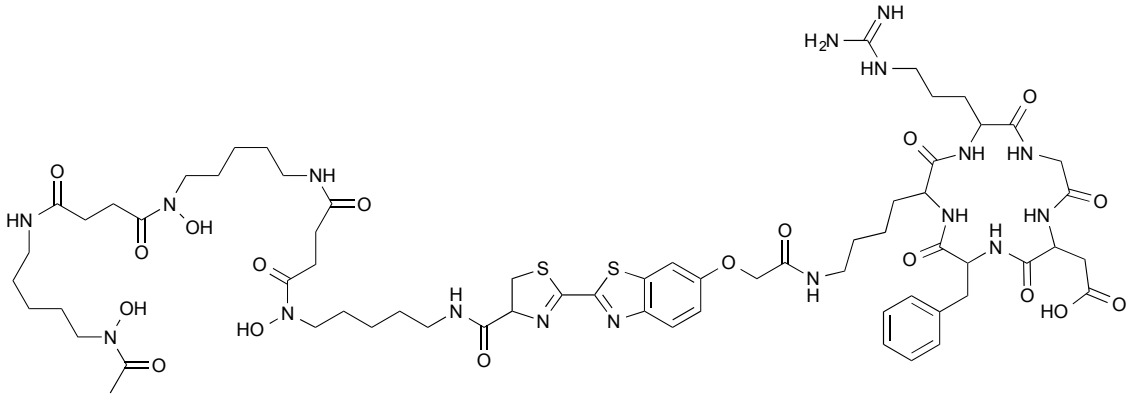

Figure S9. DFO-Luc-c(RGDfK) (**5b**)

DFO-Luc-c(RGDyk) (11b). DFO-CBT (8.0 mg, 10.3 µmol) in 1.5 mL of PBS (0.2 M, pH 7.4) was added to a DMF solution of **13** (8.0 mg, 10.6 µmol, 3.5 mL) pre-treated with TCEP.HCl (3.0 mg, 10.5 µmol). The reaction mixture was stirred at rt for 6 h. Then, **11b** was purified by semi-preparative HPLC (system A). Yield, 10 mg, 65%. ESI-MS: *m/z* 765.1 $[M+2Na]^{2+}$.

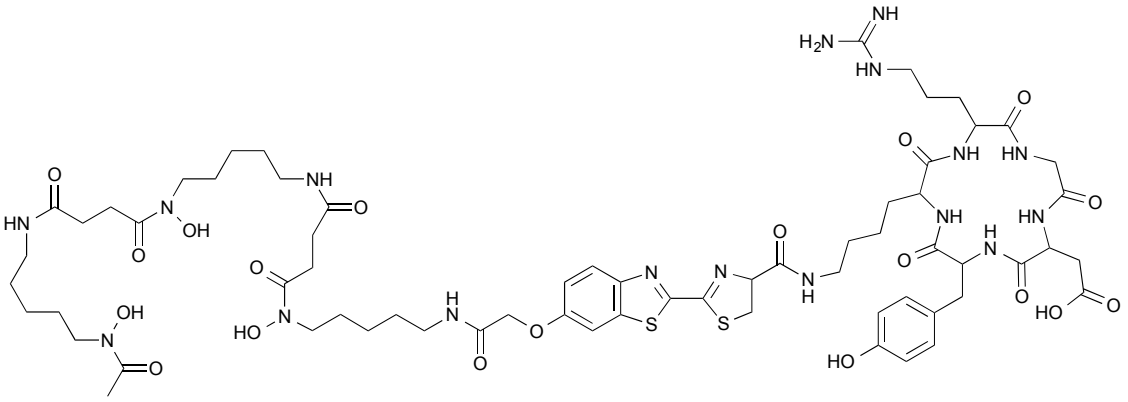

Figure S10. DFO-Luc-c(RGDyK) (**11b**)

11) Labeling of c(RGDfK)-CBT (12) with [^{89}Zr]Zr-DFO-Cys ([^{89}Zr]-4)

Coupling [^{89}Zr]Zr-DFO-Cys with c(RGDfK)-CBT ([^{89}Zr]-5b). In a low-protein binding Eppendorf tube, 300 µL of an aqueous solution of [^{89}Zr]Zr-DFO-Cys (5 MBq, 9 nmol DFO-Cys) were added to 200 µL of PBS buffer (0.4 M, pH 7.4). The mixture was stirred at room temperature for 1 min. 3 µL of an aqueous solution of TCEP·HCl (3 µg, 10 nmol) and 16.5 µL of a DMF solution of **12** (33 µg, 40 nmol) were sequentially added. The final pH was around 7.4. The reaction mixture was shaken at 37 °C for 90 min. Radiochemical yield of [^{89}Zr]-**5b** was determined by analytical radio-HPLC (system B).

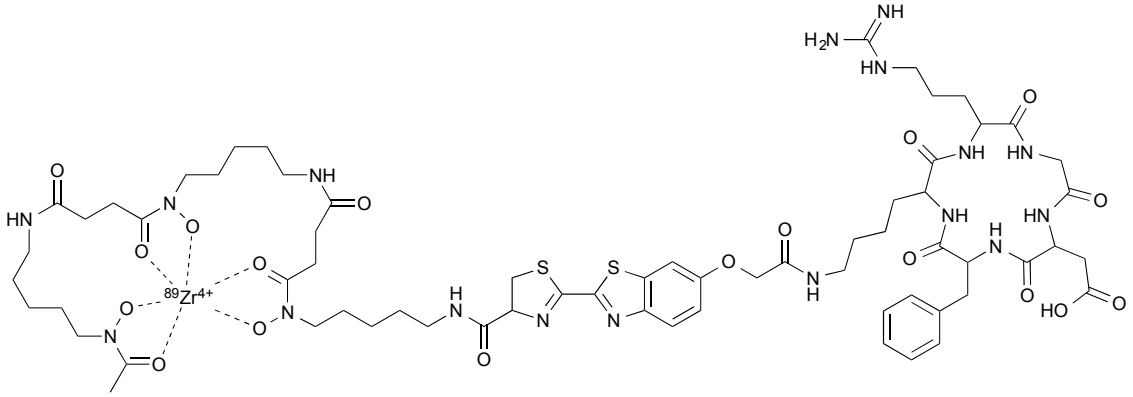

Figure S11. Structure of [^{89}Zr]Zr-DFO-Luc-c(RGDfK) ([^{89}Zr]-**5b**)

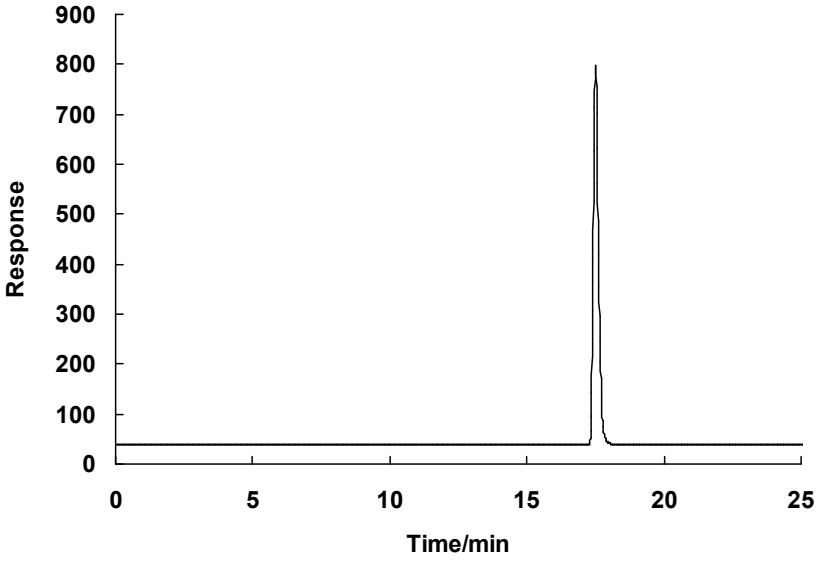

Figure S12. Radio-chromatogram of [^{89}Zr]Zr-DFO-Luc-c(RGDfK) ([^{89}Zr]-**5b**).

12) Labeling of c[RGDyK(C)] (13) with [^{89}Zr]Zr-DFO-CBT ([^{89}Zr]-10)

Coupling [^{89}Zr]Zr-DFO-CBT with c[RGDyK(C)] ([^{89}Zr]-11b). 300 µL of [^{89}Zr]Zr-DFO-CBT (5 MBq, 9 nmol DFO-CBT) were added to 200 µL of PBS buffer (0.4 M, pH 7.4). The mixture was stirred at

room temperature for 1 min. Then, 14.5 µL of a solution of **13** (14.5 µg, 20 nmol) in degassed H_2O was added. The final pH was around 7.4 and the reaction mixture was shaken at 37 °C for 90 min. Radiochemical yield of [^{89}Zr]-**11b** was evaluated by analytical radio-HPLC (system B).

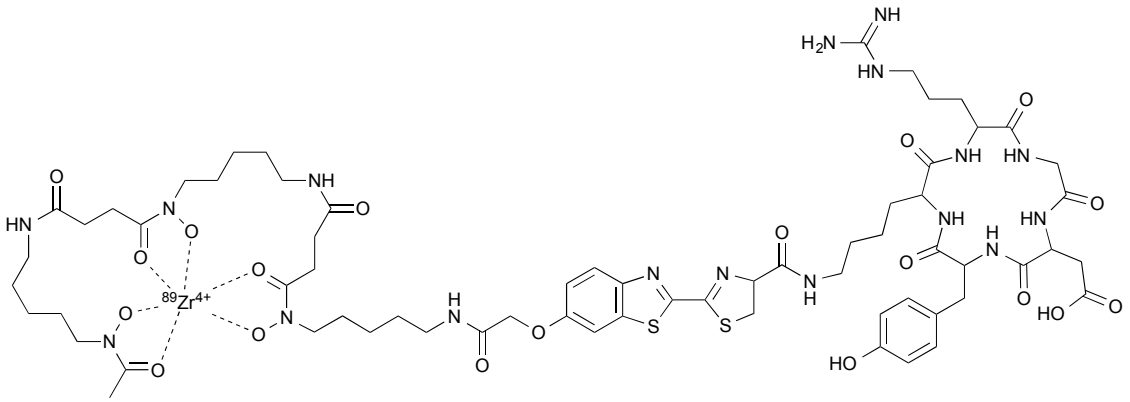

Figure S13. Structure of [^{89}Zr]Zr-DFO-Luc-c(RGDyK) ([^{89}Zr]-**11b**)

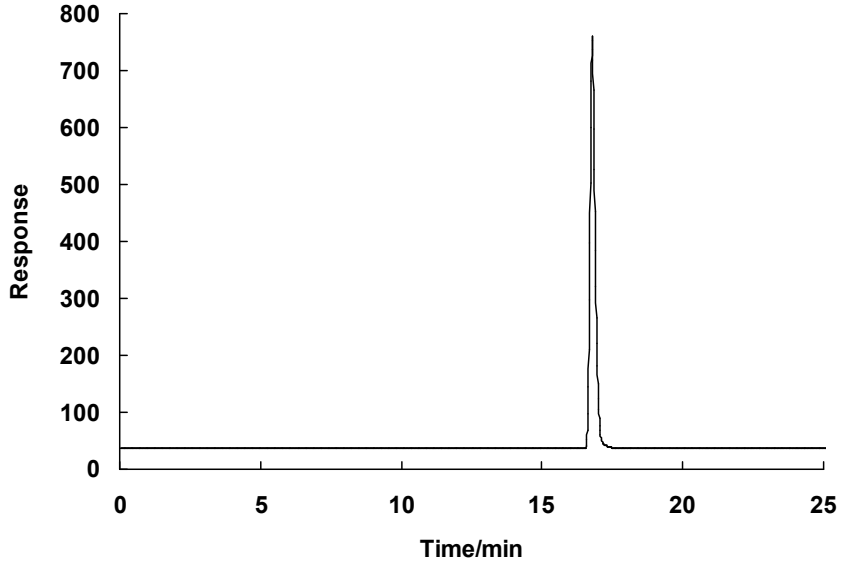

Figure S14. Radio-chromatogram of [^{89}Zr]Zr-DFO-Luc-c(RGDyK) ([^{89}Zr]-**11b**).

13) References

1. G. M. Dias, C. F. Ramogida, J. Rousseau, N. A. Zacchia, C. Hoehr, P. Schaffer, K. S. Lin and F. Benard, *Nuclear Medicine and Biology,* 2018, **58**, 1-7.
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3. X. Chen, R. Park, A. H. Shahinian, J. R. Bading, P. S. Conti, *Nuclear Medicine and Biology,* 2004, **31**, 11-19.
4. J. Kuil, M. J. E. Fisher, N. J. de Mol, R. M. J. Liskamp, *Organic & Biomolecular Chemistry*, 2011, **9**, 820-833.