Supporting information for:

Pretargeted Positron Emission Tomography Imaging that Employs Supramolecular Nanoparticles with *in Vivo* Bioorthogonal Chemistry

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1.1. Synthesis of (*E*)-2,5-dioxopyrrolidin-1-yl 6-(cyclooct-4-enylamino)-6-oxohexanoate (TCO-NHS)

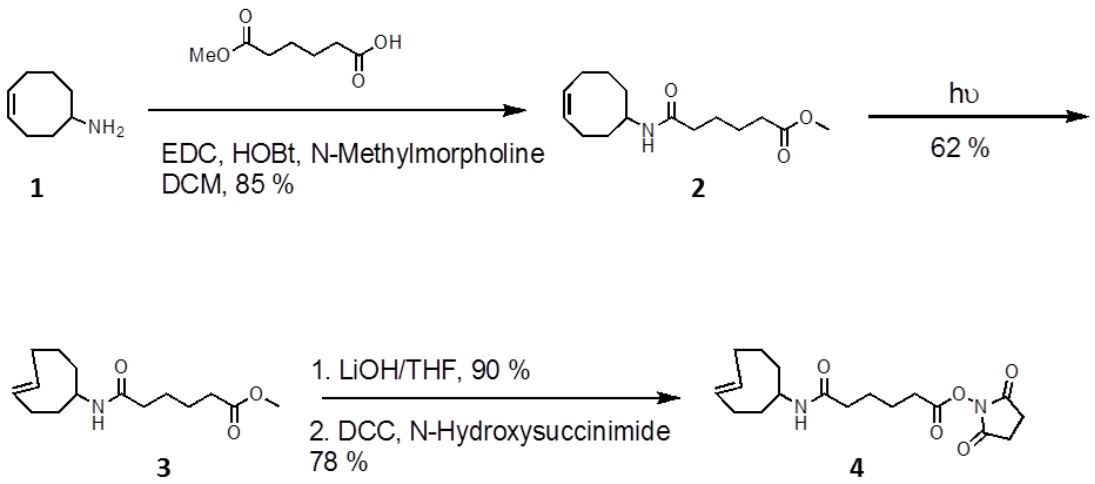
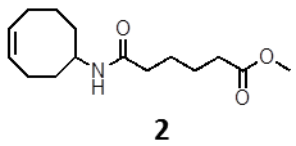


Figure S1. Schematic illustration of synthesis of (*E*)-2,5-dioxopyrrolidin-1-yl 6-(cyclooct-4-enylamino)-6-oxohexanoate (TCO-NHS, **4**)

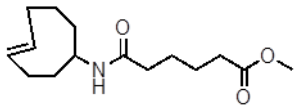


2

Methyl 5-((*Z*)-cyclooct-4-enylcarbamoyl)pentanoate (2)

A solution of **1** (12.5 g, 0.1 mol) in dried DCM (50 mL) was mixed with 6-methoxy-6-oxohexanoic acid (19.2 g, 0.12 mol), EDC (21 g, 0. 12 mol), HOBT (6.75 g, 0.5 mol) and DIPEA (1 mL). The mixture was stirred at room temperature overnight. Water (100 mL) and ethyl acetate (100 mL) were added to the mixture. The organic layer was washed with 2N HCl (100 mL) and water (100 mL x 2) sequentially, then concentrated *in vacuo* and purified by column chromatography on silica to afford **2** as a white solid (21 g, 78%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.63 (m, 2 H), 5.41 (m, 1 H), 3.92 (m, 1 H), 3.61 (s, 3 H), 2.07-2.34 (m, 8 H), 1.3-1.8 (m, 9 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.9, 170.1, 130.2, 129.6, 51.5, 49.4, 36.4, 35.1, 34.5, 33.6, 25.9, 25.7, 25.1, 24.3, 23.4; HRMS (ESI, *m/z*): Calcd for $C_{15}H_{25}NO_3$ ([*M*+H]$^+$): 268.1834, Found: 268.1891.

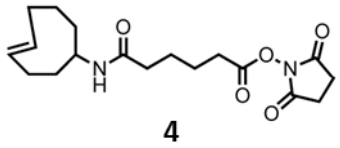


3

Methyl 5-((*E*)-cyclooct-4-enylcarbamoyl)pentanoate (3)

Compound **3** was photosynthesized by photolysis of compound **2,** following the previously reported method in the literature[1]. Compound **2** (1.00 g, 3.74 mmol) was added to 250 mL-mixture solution of ether and ethyl acetate (24:1). After 16 h of photolysis, **3** was collected (0.62 g, 62%). Major isomer contains approximately 20% of the *Z* isomer.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.6 (m, 3 H), 3.68 (s, 3 H), 3.41 (m, 1 H), 1.2-2.3 (m, 17 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.1, 171.2, 134.3, 133.7, 53.9, 45.2, 42.3, 41.3, 39.7, 36.4, 35.1, 34.5, 32.3, 30.1, 29.7, 26.9, 25.9, 25.1, 24.3, 23.6; HRMS (ESI, *m/z*): Calcd for $C_{15}H_{25}NO_3$ ([*M*+H]$^+$): 268.1834, Found: 268.1891.



4

(*E*)-2,5-dioxopyrrolidin-1-yl 6-(cyclooct-4-enylamino)-6-oxohexanoate (TCO-NHS) (4):

A solution of **3** (200 mg, 0.75 mmol) in THF (2 mL) was cooled to 0-5^{o}C in an ice-bath. Lithium hydroxide (1M, 2 mL) was added slowly and the reaction mixture was stirred overnight at room temperature. Water was added and the aqueous layer was extracted with ethyl acetate (10 mL x 3). The collected organic layer was dried over $MgSO_4$ and the solvent was removed *in vacuo* to afford acid residue with quantitative yield. The acid residue was used for the next step without further purification.

The acid residue was then dissolved in DCM (10 mL) and NHS (88 mg, 0.75 mmol) was added to the solution. The reaction mixture was cooled in an ice-bath followed by addition of DCC (165 mg, 0.8 mmol). The reaction mixture was stirred at RT overnight and the solvent was removed *in vacuo*. The residue was purified by column chromatography to afford **4** as white powder (188 mg, 72%). Major isomer contains approximately 20% of the *Z* isomer.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.79 (s, 1 H), 5.8 (m, 1 H), 5.6 (m, 2 H), 4.1 (m, 1 H), 3.8 (m, 4 H), 1.5-2.4 (m, 18 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.1, 170.9, 169.5, 168.6,134.4, 133.7, 130.3, 129.8, 54.0, 49.6, 45.2, 42.5, 41.3, 39.7, 36.7, 36.0, 35.9, 35.1, 34.3, 29.8, 29.3, 26.1, 25.9, 25.7, 24.9, 24.3, 24.2, 23.6; HRMS (ESI, *m/z*): Calcd for $C_{18}H_{26}N_2O_5$ ($[M+H]^+$): 351.1842, Found: 351.1901.

1.2. Synthesis of CD-PEI.

To a solution of branched PEI (100 mg, 10.0 μmol) in 100 mL DMSO, 6-OTs-β-CD (1.29 g, 1.0 mmol) was added. After reaction at 70 ^{o}C for 3 days, the mixture was transferred to a Slide-A-Lyzer® dialysis cassette (MWCO, 10 kD) and dialyzed against deionized (DI) water for 6 days. After dialysis, the reaction mixture was filtrated to remove the unreacted 6-OTs-β-CD as white precipitate, and the filtrate was lyophilized overnight to afford CD-PEI (150 mg, 8.3 μmol) as a white floppy solid in an 83% yield. 1H NMR (400 MHz, D_2O) δ 4.92 (br, C_1H of CD), 3.27-3.66 (m, $C_{2\text{-}6}H$ of CD), 2.3-3.0 (br, OCH_2 of PEI). The CD/PEI ratio in a CD-PEI molecule was calculated based on the proton integration of C_1H of CD versus CH_2 of PEI. According to the 1H NMR spectrum, in CD-PEI, there are about 7 to 8 CD recognition units grafted on a branched PEI backbone.

1.3. Synthesis of TCO/CD-PEI

To a solution of CD-PEI (1.6 mg, 0.1 μmol, 1.0 equiv) in $NaHCO_3$ buffer solution (pH 8.4), TCO-NHS **4** (0.35 mg, 1.0 μmol, 10.0 equiv) in DMF (100 μL) was added, and the reaction mixture was stirred at RT for 6 h. After the reaction completed, the mixture was purified by dialysis (Slide-A-Lyzer® dialysis cassette, MWCO 10 kD) overnight and lyophilized to yield TCO/CD-PEI.

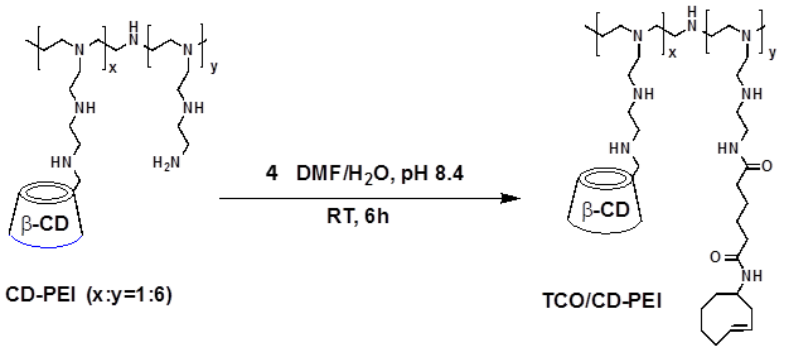


Figure S2. Schematic illustrated of synthesis of TCO/CD-PEI

2. Synthesis of 2,2',2''-(10-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7,18-trioxo-11,14-dioxa-2,8,17-triazanonadecan-19-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (Tz-DOTA)

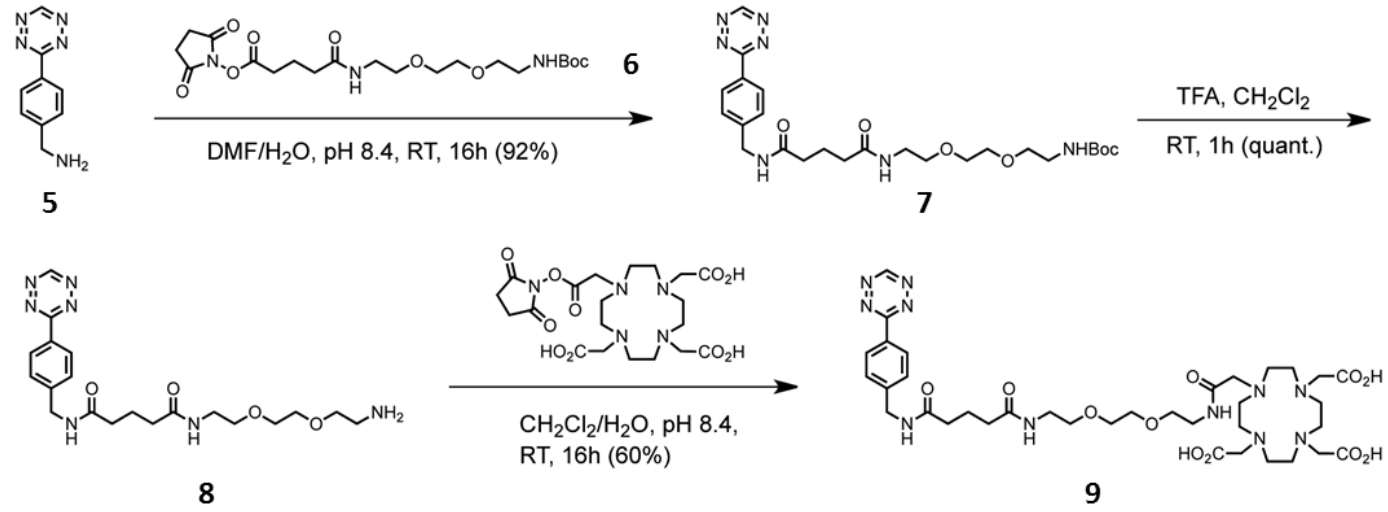
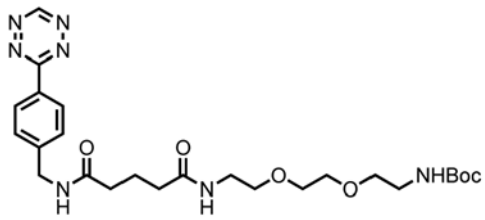


Figure S3. Schematic illustration of the synthesis of 2,2',2''-(10-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7,18-trioxo-11,14-dioxa-2,8,17-triazanonadecan-19-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (Tetrazine-DOTA, 9)

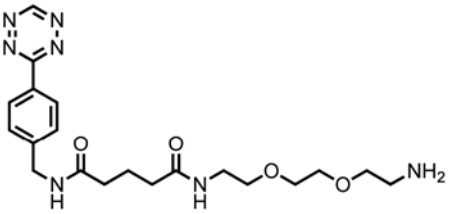


7

Tert-butyl1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14-dioxa-2,8-diazahexadecan-16-yl-carbamate (7)

Compound **5** was synthesized according to literature.[2] A solution of **6** (103 mg, 0.22 mmol) in DMF (1 mL) was added drop-wise to a solution of **5** (56 mg, 0.19 mmol) in $NaHCO_3$ buffer (pH= 8.4, 1 mL). The reaction mixture was stirred at room temperature for 16 h until the TLC indicated the completion of reaction. The aqueous layer was extracted with ether (10 mL x 3). The combined organic layer was dried over $MgSO_4$ and removed *in vacuo* to afford compound **7** as pink solid (91 mg, 92%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.18 (s, 1 H), 8.51(d, J = 11.2 Hz, 2 H), 7.52 (d, J = 10.8 Hz, 2 H), 6.68 (s, 1H), 6.19 (s, 1H), 5.02 (s, 1 H), 4.52 (m, 2 H), 3.25-3.57 (m, 12 H), 2.20-2.35 (m, 4 H), 1.95-2.03 (m, 2H), 1.25 (s, 9 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.7, 166.2, 157.8, 156.0, 144.2, 130.6, 129.9, 128.6, 128.5, 79.4, 70.2, 70.1, 69.7, 43.1, 40.3, 39.2, 35.3, 35.2, 28.4, 22.0; HRMS (ESI, *m/z*): Cacld for $C_{25}H_{37}N_7O_6$ ($[M+H]^+$): 532.2805, Found: 532.2884.

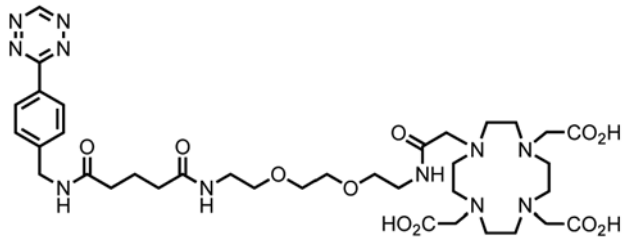


8

N1-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-N5-(2-(2-(2-aminoethoxy)ethoxy)ethyl)glutaramide (8)

A stirred solution of **7** (86 mg, 0.16 mmol) in dried CH_2Cl_2 (4 mL) was mixed with trifluoroacetic acid (0.8 mL). The mixture was stirred at room temperature for 1 h and then dried *in vacuo* to afford red powder of **8** in quantitative yield.

^{1}H NMR (400 MHz, CD_3OD): δ = 10.29 (s, 1 H), 8.56 (d, J = 11.2 Hz, 2 H), 7.48 (d, J = 11.2 Hz, 2 H), 4.47 (s, 2 H), 3.67 (m, 6 H), 3.55 (m, 2 H), 3.36 (m, 2 H), 3.28 (m, 2 H), 3.07 (m, 2 H), 2.26 (m, 4 H), 1.89 (m, 2 H); ^{13}C NMR (100 MHz, CD_3OD): δ = 178.6, 178.5, 168.7, 165.8, 165.3, 159.9, 146.4, 132.6, 72.3, 72.2, 71.6, 69.1, 45.4, 41.8, 41.5, 37.65, 37.60, 24.5. HRMS (ESI, *m/z*): Cacld for $C_{20}H_{29}N_7O_4$ ([*M*+H]$^+$): 432.2281, Found: 432.2344.



9

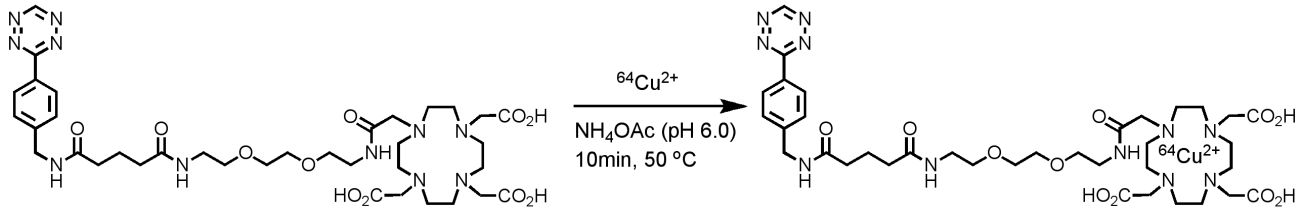
2,2',2''-(10-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7,18-trioxo-11,14-dioxa-2,8,17-triazanonadecan-19-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (9)

A solution of **8** (8.6 mg, 0.02 mmol) in $NaHCO_3$ buffer (pH=8.4, 0.5 mL) was mixed with DOTA-NHS (49 mg, 0.1 mmol) in DCM (2 mL). The reaction mixture was stirred at room temperature overnight and purified by HPLC to afford **9** as viscous pink oil (9.8 mg, 60%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.79 (d, J = 8.2 Hz, 2 H), 7.36 (d, J = 8.2 Hz, 2 H), 7.00 (s, 1 H), 5.59 (m, 1 H), 5.33 (m, 1 H), 4.49 (d, J = 12.5 Hz, 1 H), 4.42 (d, J = 12.5 Hz, 1H), 3.85 (t, J = 6.5 Hz, 2 H), 3.8-3.5 (m, 48 H), 3.09 (m, 1 H), 2.90 (t, J = 6.5 Hz, 2 H), 2.85 (s, 4 H), 2.43-1.40 (m, 10 H); ^{13}C NMR (100 MHz, D_2O): δ = 179.3, 179.2, 178.6, 178.5, 168.7, 165.8, 165.3, 159.9, 146.4, 132.6, 72.3, 72.2, 71.6, 69.1, 55.6, 55.5, 55.5, 53.2, 45.4, 41.8, 41.5, 37.65, 37.60, 24.5; HRMS (ESI, *m/z*): Calcd for $C_{36}H_{55}N_{11}O_{11}$ ([*M*+H]$^+$): 818.4083, Found: 818.4085.

3. Radiolabeling

3.1. Preparation of radiolabeled reporter (^{64}Cu-Tz) for pretargeted studies



All liquid was pretreated with Chelex-100 (Bio-Rad, Herchules, CA) to remove trace metal contaminants. The positron emitting isotope ^{64}Cu (copper chloride in 0.1 mol/L HCl; radionuclide purity, >99%) was provided by Mallinckrodt Institute of Radiology (Washington University School of Medicine, St. Louis, WA). The DOTA-conjugated tetrazine (**9**) was dissolved (1 mg/mL) in 0.2M ammonium acetate pH 6.0 and stored at -80°C before use. An aliquot of **9** was combined with a suitable amount of [^{64}Cu]copper chloride and the mixture was incubated for 10 min at 50^{o}C. Then, 5 μL-DTPA (10 mM) was added and the solution was incubated for additional 5 min. High radiochemical purity (> 98%) was achieved as determined by radio-HPLC (Figure S4).

3.2. Formation of ^{64}Cu-DHP/CD-PEI

An aliquot of ^{64}Cu-Tz (100 μCi, 100 μL in PBS) was added of to the solution of CD-PEI (10 μL, 1mg/mL). The resulting solution was incubated for an additional 10 min, a complete conversion of ^{64}Cu-Tz by radio-HPLC suggest the formation of dihydropyrazine (DHP) conjugation adduct ^{64}Cu-DHP/CD-PEI (Figure S4).

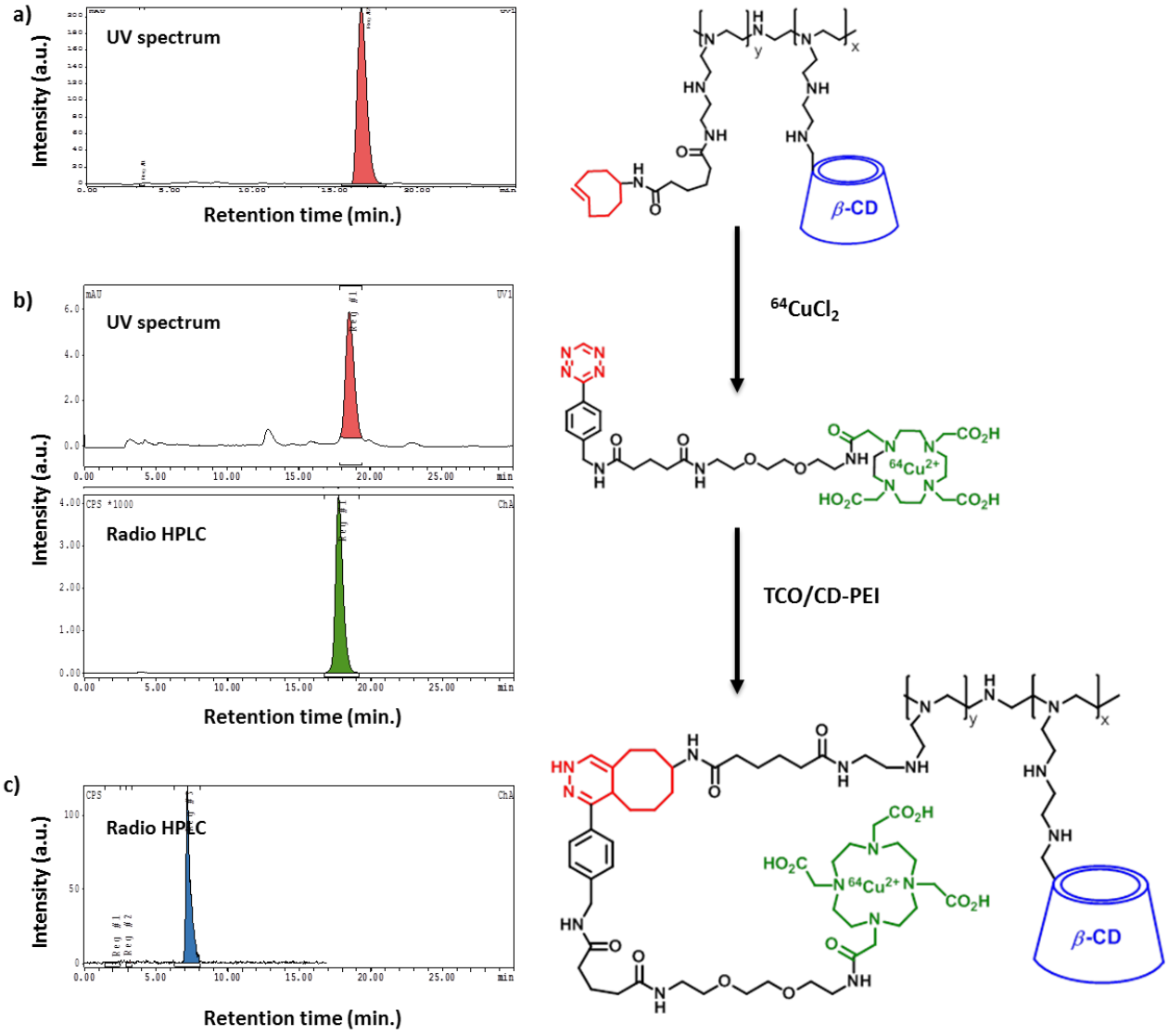


Figure S4. Analytical HPLC profile of (a) cold tetrazine (R_t = 17.1 min) in the UV profile, (b) the labeled ^{64}Cu-Tz (R_t = 17.8 min) in the UV and radioactive profile, and (c) ^{64}Cu-DHP/CD-PEI in the radioactive profile. (15% acetonitrile, 85% water, 0.2% trifluoroacetic acid, 1 ml/min, 290 nm, Phenomenex Luna C18(2), 4.6 x 250 mm).

3.3. ^{64}Cu-Tz stability in PBS and serum

The ^{64}Cu-Tz solution (30 μCi of ^{64}Cu, 1 nmol of Tz) stability was assessed at different time points by radioHPLC (Table S1; 15% acetonitrile, 85% water, 0.2% trifluoroacetic acid, 1 ml/min, 290 nm, Phenomenex Luna C18(2), 4.6 x 250 mm). The purity of ^{64}Cu-Tz, which possesses retention time of 17.8

min, was calculated based on the peak area which was divided by the total radioactivity of ^{64}Cu. The solution volume was 10 μL in either PBS or serum. The mixture was allowed to sit for 30 min, 4 h, 8 h, 24 h, and 48 h at room temperature before analysis.

Table S1: Stability of **^{64}Cu-Tz** at different time points in PBS and serum.

Entry	30 min	4 h	8 h	24 h	48 h
PBS	>95%	>95%	>95%	90%	85%
Serum	>95%	>95%	>95%	78%	60%

4. Synthesis of Ad-PAMAM and Ad-PEG

4.1. Synthesis of Ad-PAMAM

A methanol solution containing PAMAM (20% wt, 100 mg, 0.07 mmol) was added into a round-bottom flask. Methanol was evaporated *in vacuo* and the sticky solid was redissolved in 10 mL dry THF. 1-adamantane isocyanates (244.6 mg, 1.4 mmol) in 10 mL dry THF was directly added the PAMAM solution. After the reaction mixture was stirred at rt for 2 h, the solvent was removed *in vacuo*. Ether (100 mL) was added to the reaction residue to generate a white precipitate, which was collected through filtration. The white precipitate was washed with ether (100 mL x 3), and dried to give 8-Ad-PAMAM (169 mg, 0.06 mmol) as a white solid in 85% yield. 1H NMR (400 MHz, $CDCl_3$): δ 7.82-7.98 (m, 12 H, CONH), 6.10 (s, 8 H, NHCONH), 5.37 (s, 8 H, NHCONH), 3.24 (br, 32 H, $COCH_2$), 2.34-2.76 (m, 64 H, NCH2), 1.64-2.03 (m, 120 H, protons on Ad). ESI-MS: calcd. for $C1_{52}H_{252}N_{34}O_{20}$ [M + H]+: m/z = 2875.98; found: 2875.78 (100%).

4.2. Synthesis of Ad-PEG

To a solution of 1-adamantanamine hydrochloride (187.7 mg, 1.0 mmol, 5.0 equiv.) in 10 mL CH_2Cl_2, triethylamine (105 mg, 1.0 mmol, 5.1 equiv.) and mPEG-NHS (1 g, 0.2 mmol, 1.0 equiv.) was added in sequence. The reaction mixture was stirred at rt for 2 h. After the reaction, the solvent was subsequently

removed *in vacuo*, and water was added to the reaction residue. The solution was transferred into a centrifuge tube and centrifuged at 10,000 rpm for 10 min to remove the unreacted 1-adamantanamine. The solution was dialyzed with Slide-A-Lyzer®dialysis cassette (MWCO, 2 kD) against water overnight and lyophilized to yield Ad-PEG (0.92 g, 0.18 mmol) a white powder in 91% yield. 1H NMR (400 MHz, DMSO-d6): δ 3.42-3.54 (br, 440H, OCH_2), 1.13-1.18 (br, 15H, protons on Ad).

5. Characterization of TCO⊂SNPs

5.1. Preparation of TCO⊂SNPs

To a solution of Ad-PEG (150 μg) in 150-μL of PBS buffer, Ad-PAMAM (5 μg) in 10-μL DMSO was slowly injected *via* a Hamilton under vigorous stirring. A 100-μL of PBS buffer containing CD-PEI (50 μg) and TCO-CD-PEI (50 μg) was sequentially added into the mixture to obtain TCO⊂SNPs. The morphology and sizes of TCO⊂SNPs were examined using a transmission electron microscope (Figure S5). TCO⊂SNPs possess spherical shape with the size of 90.9 ±8.6 nm. Sizes were determined by averaging the measured value from 100 single TCO⊂SNPs in TEM images.

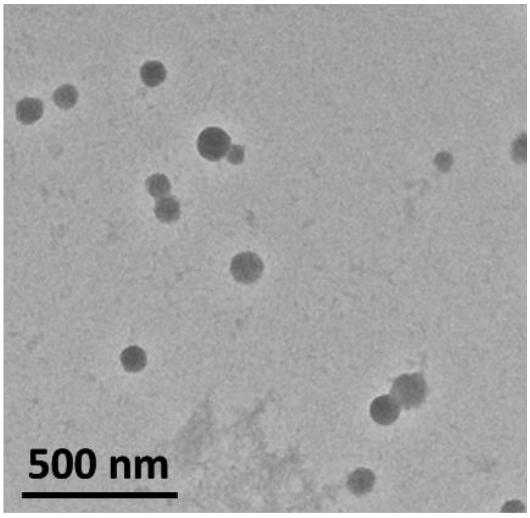


Figure S5. TEM image of TCO⊂SNPs in low magnification

5.2. Toxicity test of TCO⊂SNPs

Viability of cells with TCO⊂SNPs was also examined with Cell Counting Kit-8 (CCK-8) test (Dojindo Molecular Techonology Inc.; Code #: CK04-05) together with control materials (*i.e.*, PEI, CD-PEI, and TCO/CD-PEI). U87MG cells (1×10^4 cells/well) were seeded into a 96-well plate and cultured for 24 h before TCO⊂SNPs' and control materials' treatment. Next, the cells were incubated with various concentrations of PEI, CD-PEI, TCO/CD-PEI, and TCO⊂SNPs, which were calculated based on PEI concentration of each materials. After 24 h-incubation, CCK-8 solution was added into each well and incubated for 1 h, and then the absorption at 450 nm of each well was measured with microplate reader (Infinite M200Pro, TECAN, Switzerland). Cell viability was defined as the absorption intensity of cells with the four materials' treatment divided by that of U87MG cells without any treatment at a corresponding time point. As shown in Figure S6, cells treated with CD-PEI, TCO/CD-PEI, TCO⊂SNPs shows no toxicity with the PEI concentration upto 50 μg/mL while PEI shows high toxicity with the concentration over 30 μg/mL.

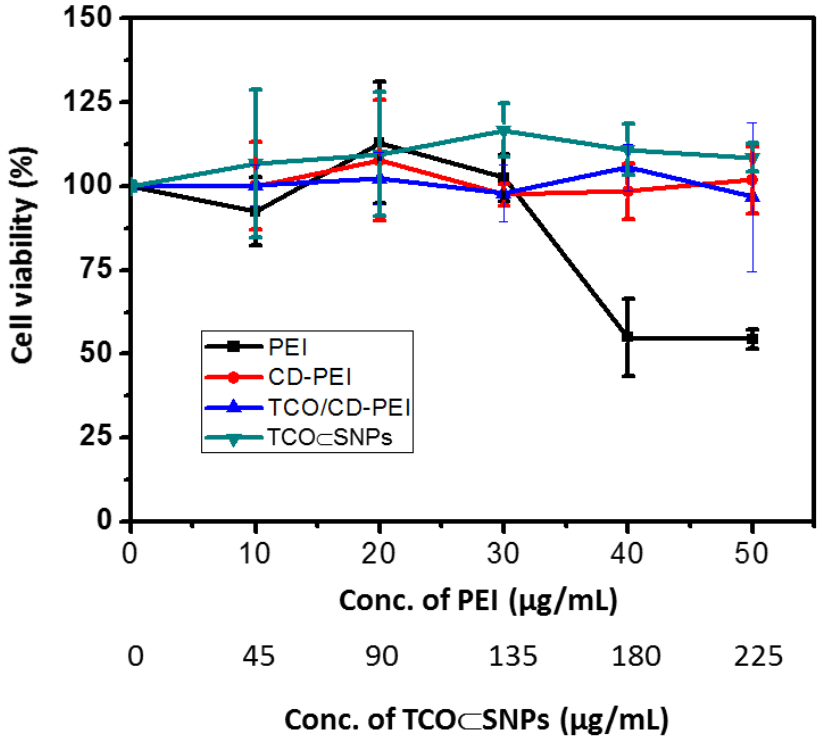


Figure S6. *In vitro* toxicity comparison of PEI, CD-PEI, TCO/CD-PEI, and TCO⊂SNPs with U87MG

6. Preparation of ^{64}Cu-DHP⊂SNPs

The ^{64}Cu-Tz solution (300 μCi of ^{64}Cu, equivalent 10 nmol of Tz) in 10 μL of ammonium acetate buffer (pH 6.0) was mixed with TCO⊂SNPs (100 μg, equivalent 1 nmol of TCO, 100 μL PBS). The mixture was incubated for 15 min at 60^{o}C, and the ^{64}Cu-labeled SNPs products was purified by a molecular weight cutoff filter (CentriconYM10, Billerica, MA) with a spin speed of 10000 rcf for 10 min. The radiochemical purity (>95%) was determined by measuring the radioactivity in the filter, the filtrate and the retaintate, respectively. The ^{64}Cu-DHP⊂SNPs were then re-suspended in saline for *in vivo* injections.

7. Probe (^{64}Cu-Tz) Characterization

7.1. Pharmacokinetics of the radiolabeled reporter (^{64}Cu-Tz)

Animals were purchased from Jackson Laboratory (Bar Harbor, ME). All animal manipulations were performed with sterile technique and were approved by the University of California at Los Angeles Animal Research Committee. MicroPET/microCT imaging was performed with a microPET FOCUS 220 PET scanner (Siemens Preclinical Solutions, Knoxville, TN, USA) and a MicroCAT II CT scanner (Siemens Preclinical Solutions, Knoxville, TN, USA). Fifteen minutes before imaging, mice were anesthetized by using 1.5–2% isoflurane in a heated (30°C) induction chamber, and then transferred to a heated isolation/imaging chamber. ^{64}Cu-Tz (300 μCi of ^{64}Cu, equivalent 10 nmol of Tz) was injected *via* tail vein; the volumes used for the tail vein injection were ~ 150 μL. At various time points the anesthetized animals were positioned on the PET scanner bed, and a static PET scan was acquired for 10 min. Mice were moved to the microCT in the same isolation/imaging chamber. PET images were reconstructed by filtered back projection. Immediately, after the microPET scan mice underwent a 10-min microCT scan, using routine image acquisition parameters. The microCT scan was used for anatomical localization of the tissue concentrations of the ^{64}Cu-Tz over time by microPET (Figure S7).

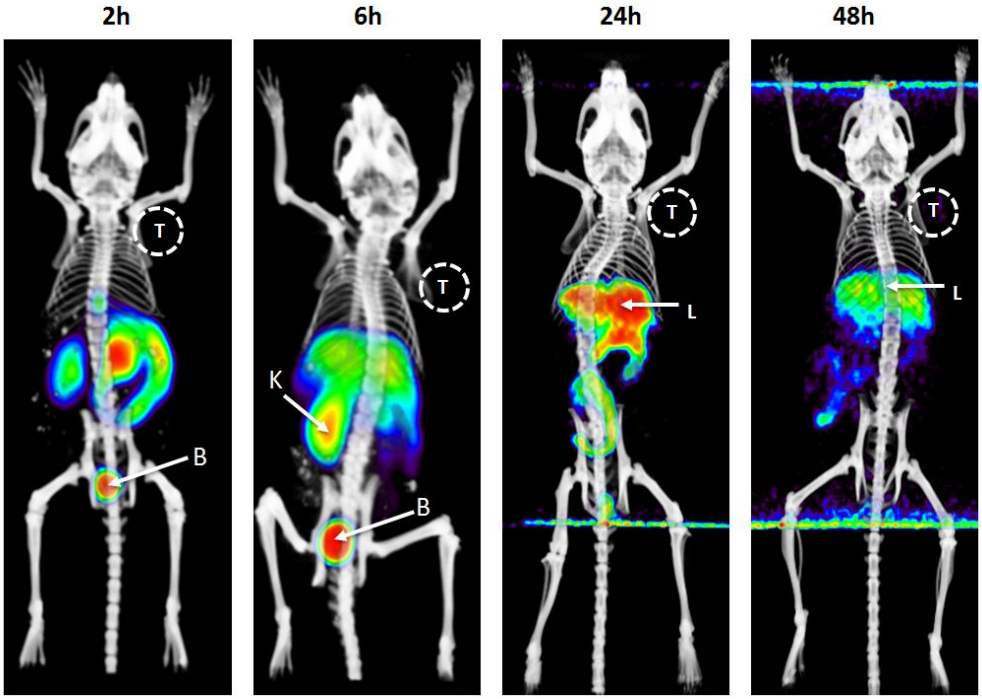


Figure S7. Maximum intensity microPET/CT projections of the radiolabeled reporter (^{64}Cu-Tz) at various time points post injection. Labels T, L, K, and B correspond to tumor, liver, kidney, and bladder respectively; dotted circle denotes the location of the tumor. Most unbound ^{64}Cu-Tz was quickly cleared out of the mouse body *via* the kidneys to the bladder as can be seen at the time points of 2h and 6h. The relatively high liver uptake at the 24-h time point is presumably due to the well-known demetalation (*i.e.*, dissociation of $^{64}Cu^{2+}$ from the DOTA ligand) of the $^{64}Cu^{2+}$-labeled reporter (^{64}Cu-Tz).

7.2. ^{64}Cu-Tz half-life in blood

Mice that were administered ^{64}Cu-Tz underwent repeated cheek bleeding at various time points to determine the ^{64}Cu-Tz concentration in blood as a function of time (Figure S8).

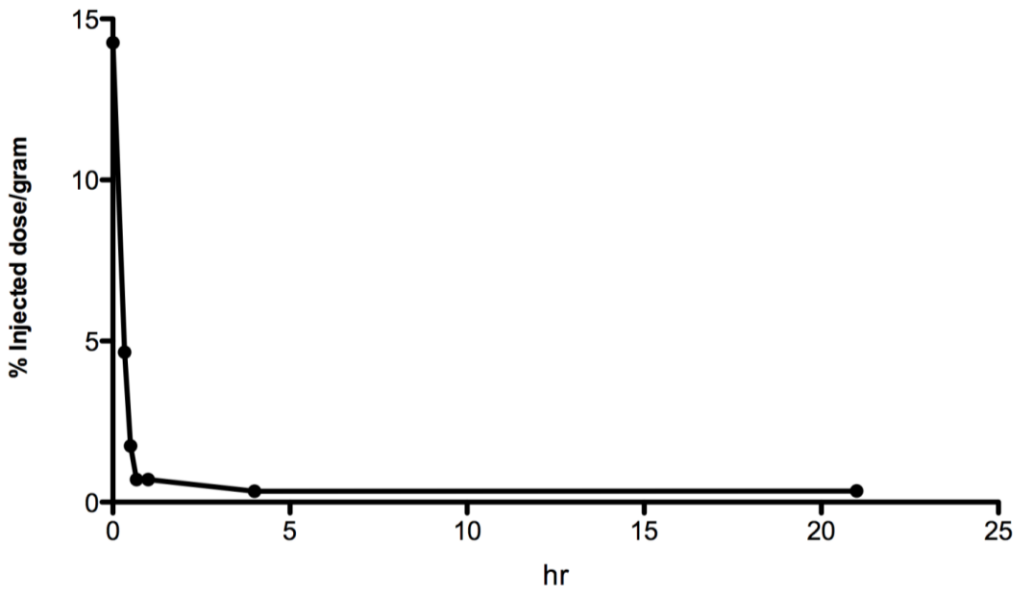


Figure S8. The time dependent concentration of radiolabeled imaging agent (^{64}Cu-Tz) in blood at various time points post injection. The data were obtained by quantifying blood samples collected *via* cheek bleeding.

References:

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