Supplementary Information

A short PEG linker alters the *in vivo* pharmacokinetics of trastuzumab to yield high-contrast immuno-PET images

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Contents

Materials, methods, and instrumentations

Methyltetrazine-PEG_4-NH_2 was procured through Click Chemistry Tools (Scottsdale, USA), p-SCN-Bn-NOTA was obtained from Macrocyclics (Texas, USA) and Trastuzumab was acquired from Roche (Basel, Switzerland). The reaming all the reagents were purchased from Sigma-Aldrich (St. Louis, MO), used as such without any further purification. Milli-Q water purification system (Millipore, MA, USA) was used to produce deionized water (>18.2 mΩ cm^{-1} at 25 °C), which then used for the preparation of buffer solutions and aqueous solutions for the study. The $^{64}CuCl_2$ was procured from Korea Institute of Radiological and Medical Sciences (KIRAMS) in Seoul, Korea and was synthesized by $^{64}Ni(p,n)^{64}Cu$ nuclear reaction in a MC50 cyclotron (Scanditronix, Sweden). ^1H and ^{13}C NMR was recorded by Varian Unity Inova400 and 500 MHz instrument (Varian, Midland, ON). Splitting patterns are indicated as follows: s, singlet; d, doublet; and m, multiplet. High-resolution mass spectral (HRMS) analyses were carried out at JEOL JMS700 (Jeol, Tokyo, Japan). HPLC traces were obtained using Waters 600 series HPLC system (Waters Corporation, Milford, MA) and Bioscan 2000 imaging scanner (Bioscan, Washington, DC, USA) was used to analyze radio-TLC data.

Preparation of NOTA-trastuzumab (1) conjugates

Trastuzumab (5 mg, 0.034 μmol) was added to a solution of NOTA-Bn-NCS (102 μg, 0.18 μmol) in 0.1 M sodium carbonate buffer (pH 8.5, 200 μL). The resulting solution was gently shaken at room temperature for 14 h. The solution was then transferred to a Centricon YM-50 centrifugal filter unit (Millipore, MA, USA), centrifuged, and washed with Milli-Q water three times at 4°C. The NOTA-trastuzumab conjugate was collected in 1 mL of Milli-Q water, lyophilized to obtain a white solid (3.55 mg), and stored at −20°C prior to use.

Radiolabeling of NOTA-trastuzumab

$^{64}CuCl_2$ (25.9 MBq) in 0.01 M HCl was added to a solution of NOTA-trastuzumab (1) (30 μg) in 100 μL of 0.1 M NH_4OAc buffer (pH 6.8) and incubated on a thermomixer (750 rpm) at 25°C for 30 min. The reaction was monitored using radio-TLC [iTLC-SG, 50 mM EDTA; R_f = 0.0], with a radiolabeling yield of 93.7%. The radiolabeled antibody (25.5 MBq) was then loaded onto the centrifugal filter unit with a molecular weight cutoff of 50,000 (Sartorius Vivacon-500 μl,

Regenerated Cellulose, Millipore Corp., Bedford, MA, USA) and centrifuged at 12000 rpm for 7 min. It was washed three times with 200 µL of PBS and isolated 17.98 MBq of ^{64}Cu-NOTA-trastuzumab conjugate in saline or PBS. The radiochemical purity of the final antibody conjugate was analyzed using radio-TLC [iTLC-SG, 50 mM EDTA; R_f = 0.0], and the radiolabeling purity was found to be 100%. In the radio-TLC experiments, ^{64}Cu-NOTA-trastuzumab (**6**) remained at the baseline, whereas free $^{64}CuCl_2$ was eluted to the solvent front.

Synthesis of NOTA-PEG$_4$-Tz (3)

DIPEA (72 µL, 0.413 mmol) was added dropwise to a solution of NOTA-Bn-NCS (23.1 mg, 0.041 mmol) and methyltetrazine-PEG$_4$-NH$_2$ (15 mg, 0.041 mmol) in 2 mL of DMF. The resulting reaction mixture was stirred at room temperature for 14 h and then diluted with 1 mL of Milli-Q water and lyophilized, and the resulting crude solid was redissolved in MeOH and purified using Waters HPLC with a preparative X-terra C18 column (10 µm, 10 × 250 mm) by eluting with a gradient of 0.1% TFA for H_2O:CH_3CN (0–10 min, 95%:5%; 10–20 min, 80%:20%, 20–30 min, 70%:30%; and 30–40 min, 95%:5% at a flow rate of 1.8 mL/min). The collected fractions were lyophilized to yield NOTA-PEG$_4$-Tz (**3**) (10.6 mg, 31.5%) as a pink-colored solid. ^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.42 (dd, *J* = 7.0 Hz, 2H), 7.76 (brs, 1H), 7.43 (d, *J* = 8.5 Hz, 2H), 7.22 (d, *J* = 9.5 Hz, 4H), 4.23 (q, 2H), 3.97–381 (m, 2H), 3.80–3.79 (m, 4H), 3.62–3.61 (m, 4H), 3.57–3.55 (m, 8H), 3.42–3.39 (m, 3H), 3.31–3.21 (m, 5H), 3.06–3.00 (m, 8H), 2.82–2.76 (m, 2H), 2.64–2.61 (m, 1H); ^{13}C NMR (125 MHz, DMSO-d$_6$): δ 180.92, 173.80, 171.93, 169.75, 166.98, 163.41, 162.44, 158.68 (CH_3COOH), 129.67, 124.54, 115.85, 70.42, 69.27, 68.00, 59.14, 54.71, 53.71, 52.97, 51.92, 51.33, 44.00, 33.94, and 21.18. HR-MS (FAB) *m/z*: calculated for $C_{37}H_{51}N_9O_{10}S$ $[M + H]^+$: 813.3480; found: 814.3554.

Radiolabeling of NOTA-PEG$_4$-Tz

A solution of NOTA-PEG$_4$-Tz (**3**) (20 µg) in 100 µL of 0.1 M NH_4OAc buffer (pH 6.8) was prepared; then, $^{64}CuCl_2$ (316 MBq) in 0.01 M HCl was added to the reaction mixture and incubated at 37°C on a thermomixer at 750 rpm for 30 min. The reaction was monitored using radio-TLC [Merck, C–18 TLC plates developed with CH_3CN:H_2O (1:1); R_f = 0.55], and the radiolabeling yield was 96.3%. The radiolabeled chelate was further purified using reverse-phase HPLC [Waters HPLC using a X-bridge C18 column (5 µm, 4.6 × 150 mm); mobile phase A: 0.1% TFA/H_2O; B: 0.1% TFA/acetonitrile] by eluting with a gradient of 0%–20% B in 0–5 min, 20% B in 20 min, 20%–90%

B in 25 min, and 0% B in 30 min and continued up to 40 min at a flow rate of 1 mL/min. The collected fractions were concentrated under reduced pressure to yield ^{64}Cu-NOTA-PEG$_4$-Tz (**4**) (104 MBq) and collected in PBS buffer for further conjugation.

Synthesis of trastuzumab-PEG$_4$-TCO (5) conjugate

Trastuzumab (3.2 mg, 0.022 μmol) was added to a solution of TCO-PEG$_4$-NHS (55 μg, 0.11 μmol) in 200 μL of 0.1 M sodium borate buffer (pH 9.0). The resulting solution was incubated on a thermomixer at 25°C for 12.5 h. Then, the mixture was transferred to a Centricon YM-50 centrifugal filter unit (Millipore, MA, USA), centrifuged, and washed with Milli-Q water three times. The concentrated conjugate was collected in 1 mL of Milli-Q water and lyophilized to yield trastuzumab-PEG$_4$-TCO (**5**) (1.7 mg) as a white solid, and the conjugates were stored at −20°C.

Click reaction between ^{64}Cu-NOTA-PEG$_4$-Tz (4) and trastuzumab-PEG$_4$-TCO (5)

Trastuzumab-PEG$_4$-TCO (50 μg in 10 μL of Milli-Q water) was added to purified, radiolabeled ^{64}Cu-NOTA-PEG$_4$-Tz in 100 μL PBS (pH 7.4, 29.2 MBq). The reaction mixture was incubated at 25°C for 10 min, and the conjugation progress was monitored using radio-TLC (C-18, CH_3CN:H_2O [1:1]; R_f = 0), with a conjugation yield of 87.0%. The radiolabeled antibody (26.8 MBq) was then loaded onto the centrifugal filter unit with a molecular weight cutoff of 50,000 (Sartorius Vivacon-500 μl, Regenerated Cellulose, Millipore Corp., Bedford, MA) and centrifuged at 12000 rpm for 7 min. Then, it was washed three times with 200 μL of PBS, and 19.3 MBq of radiolabeled immunoconjugate ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) was collected in saline or PBS. The radiochemical purity of the final antibody conjugate was confirmed using radio-TLC (C-18, CH_3CN:H_2O [1:1]; R_f = 0), and the radiolabeling purity was found to be 100%. In the radio-TLC experiments, ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) remained at the baseline, whereas ^{64}Cu-NOTA-PEG$_4$-Tz (**4**) showed an R_f value of 0.55.

In vitro serum stability

The radiolabeled Immunoconjugates ^{64}Cu-NOTA-trastuzumab **(2)** and ^{64}Cu-NOTA-PEG$_8$-trastuzumab **(6), (**10 μL, 18.5-20.5 MBq) were incubated at 37 °C in a ThermoMixer (500 rpm) by diluting with 500 μL of fetal bovine serum and phosphate buffered saline (PBS, pH = 7.4)

individually. Demetalation was monitored by radio-TLC (iTLC-SG, 50 mM EDTA) at respective time point up to 48 h and each study was carried out in triplicate (n=3 each).

Cell uptake studies

The human fibroblast NIH3T3 cell line NIH3T6.7 and ovarian cancer cell line SKOV3 were used as HER2-positive cells, generally used in HER2 targeting studies.[1,2,3] Human embryonic kidney cell line (HEK293) and murine colon cancer cell line (CT26) were used as HER2-negative cells. All cell lines were obtained from Korean Cell Line Bank (Seoul, Korea) or American Type Culture Collection (Manassas, VA, USA). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium or Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics (Hyclone, Logan, UT, USA). Cells were incubated in an incubator at 37°C in an atmosphere containing 5% CO_2. All cells were seeded in a 6-well plate at a density of 1×10^5 cells per well and incubated overnight. ^{64}Cu-NOTA-trastuzumab (**2**) and ^{64}Cu-NOTA-PEG_8-trastuzumab (**6**) samples were prepared in relevant cell culture media with 37 kBq/mL. After adding the samples (1 mL) to each well (n = 3 for each cell line), cells were incubated for 1 h. Next, cells were washed three times with PBS and harvested with 0.25% trypsin-EDTA solution (Hyclone, Logan, UT, USA) for measurement with a gamma counter (Wallac Wizard 1480, PerkinElmer, France).

Animals

All animal experiments were conducted in accordance with relevant guidelines and regulations and were approved by the Animal Ethics Committee of Kyungpook National University (approval nos. 2019-0100 and 2019-0101). Animal experiments were conducted under anesthesia using 1%–2% isoflurane in O_2. Animals were euthanized by cervical dislocation after isoflurane gas overdose. Animals were purchased from Hyochang Science (Daegu, Korea).

***In vivo* clearance studies**

The half-lives of ^{64}Cu-NOTA-trastuzumab (**2**) and ^{64}Cu-NOTA-PEG_8-trastuzumab (**6**) in blood were determined with serial retro-orbital bleeds after tail vein injection of 8.5 MBq into 6-week-old male BALB/c mice (n = 3 per group). After injection, blood samples (80 μL) were collected through puncture of the retro-orbital vein plexus into heparin-coated glass tubes at several time points (10

min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h). Immediately after puncture, tube weights were measured, and then the tubes were counted in a gamma counter. The half-lives of ^{64}Cu-NOTA-trastuzumab (**2**) and ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) in blood were analyzed using a two-phase exponential decay model by GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Metabolism studies

To analyze urinary and fecal excretion of radioactive metabolites, 7.8 MBq of ^{64}Cu-NOTA-trastuzumab (**2**) or ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) was intravenously injected into 6-week-old male BALB/c mice (n = 3 per group). These mice were kept in metabolic cages (3 per cage) and urine and feces were collected separately 1, 2, 4, 8, 12, 18, and 24 h after injection. The radioactivity of the collected urine and feces was measured with a dose calibrator (CRC-Ultra; Capintec, Florham Park, NJ, USA). To profile the metabolites, urine samples 12 h after injection, which exhibited the highest radioactivity of the collected samples, was analyzed with radio-TLC (iTLC-SG, 50 mM EDTA).

Biodistribution studies

First, the biodistribution of ^{64}Cu-NOTA-trastuzumab (**2**) and ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) was evaluated in healthy mice. 12-week-old male BALB/c mice were randomly assigned to four groups (n = 4 per group). Then, 0.5 MBq of ^{64}Cu-NOTA-trastuzumab (**2**) or ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) was intravenously injected into the mice (specific activity: 15.2 - 18.2 μCi/μg, injected mass: 0.82 - 0.98 μg). Mice were sacrificed at 24 and 48 h after injection. Various tissues and organs (blood, heart, lung, skin, muscle, fat, bone, spleen, kidneys, intestine, and liver) were harvested and weighed. The radioactivity of the harvested tissues was counted using a gamma counter. Next, the biodistribution of ^{64}Cu-NOTA-trastuzumab (**2**) and ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) was verified in xenografts in nude mice. To establish xenografts, 5×10^6 NIH3T6.7 cells were subcutaneously inoculated into 6-week-old male BALB/c nude mice shoulders. At 10 days after inoculation, 0.5 MBq of ^{64}Cu-NOTA-trastuzumab (**2**) or ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) was intravenously injected to mice bearing NIH3T6.7 xenografts. As described above, the mice were sacrificed at 24 and 48 h after injection, and the relevant organs were harvested for gamma counter measurement. The accumulated radioactivity was calculated as the percentage of injected

dose per gram (%ID/g). Additionally, the radioactivity remaining in the mice was measured with a dose calibrator (CRC-Ultra, Capintec, NJ, USA) before euthanasia.

Animal PET imaging studies

MicroPET imaging studies were performed in xenografts using nanoscan PET/CT (PET 82S, Mediso, Budapest, Hungary). To prepare xenografts, NIH3T6.7 cells were subcutaneously inoculated into the bilateral shoulders (left: 5×10^6 cells; right: 1×10^7 cells, respectively) of 6-week-old male BALB/c nude mice. After 10 days of inoculation, the tumor attained a size of approximately 100 mm^3. At this point, 7–10 MBq of ^{64}Cu-NOTA-trastuzumab (**2**) or ^{64}Cu-NOTA-PEG_8-trastuzumab (**6**) was intravenously injected to NIH3T6.7 xenografts for animal PET imaging scans (specific activity: 15.9 - 18.2 μCi/μg, injected mass: 13.2 - 14.8 μg). The scanning was conducted with 1:3 coincidence mode for 45 min at both 24 and 48 h. The microPET images were reconstructed with a Tera-Tomo three-dimensional OSEM reconstruction algorithm using Nucline software. CT images were acquired consecutively immediately after microPET imaging scans without a contrast agent. MicroPET/CT images were automatically co-registered and then analyzed using Inter View Fusion software. The color bar of the microPET images was calibrated in standardized uptake values.

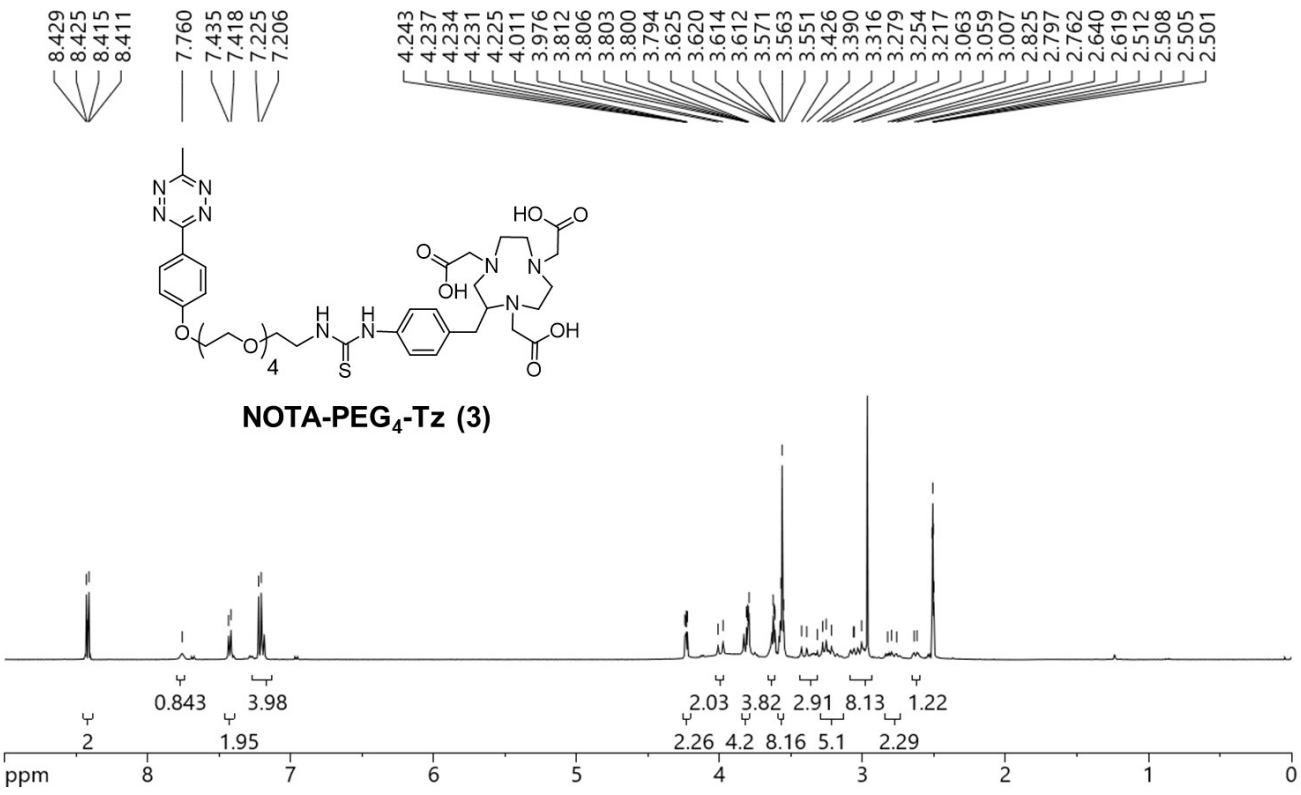

Figure S1. ^{1}H-NMR of NOTA-PEG$_4$-Tz **(3)** in DMSO-d$_6$.

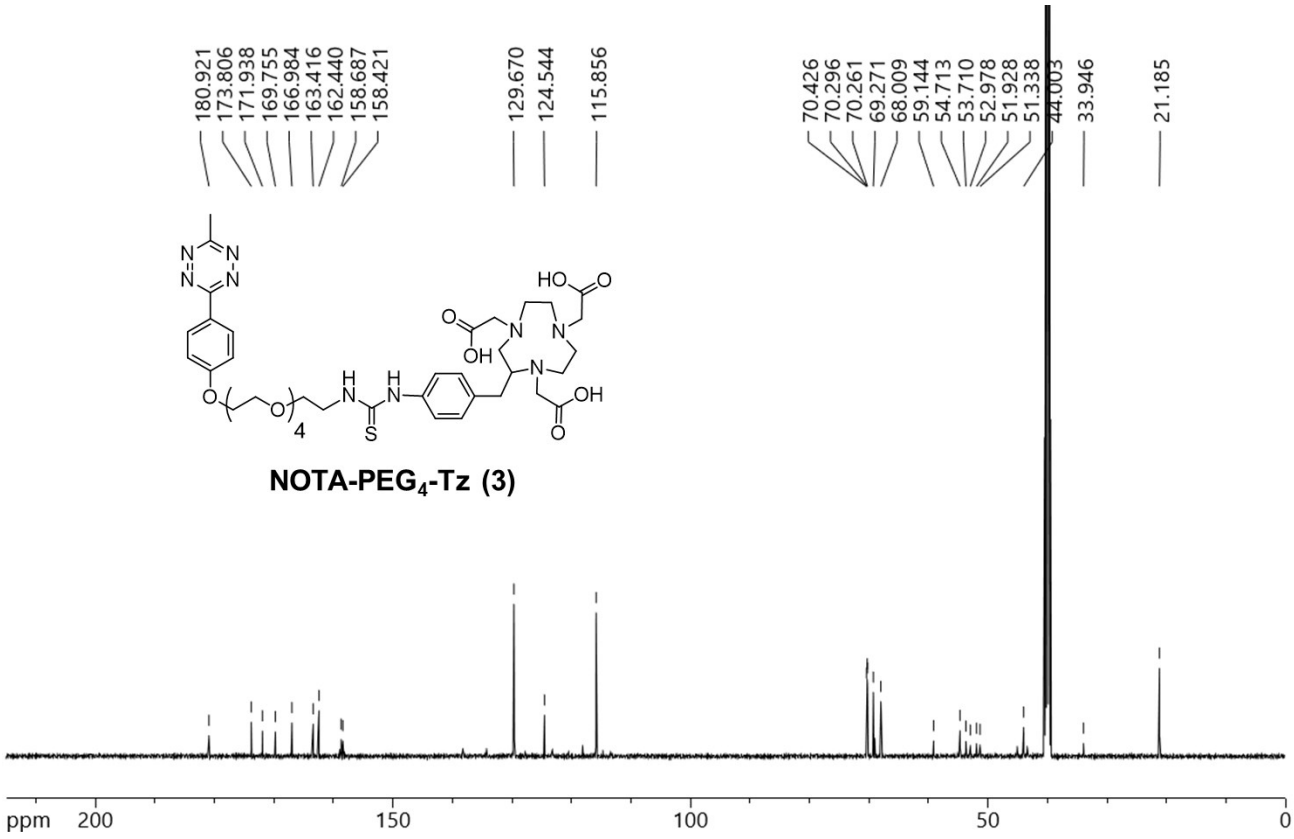

Figure S2. ^{13}C-NMR of NOTA-PEG$_4$-Tz **(3)** in DMSO-d$_6$.

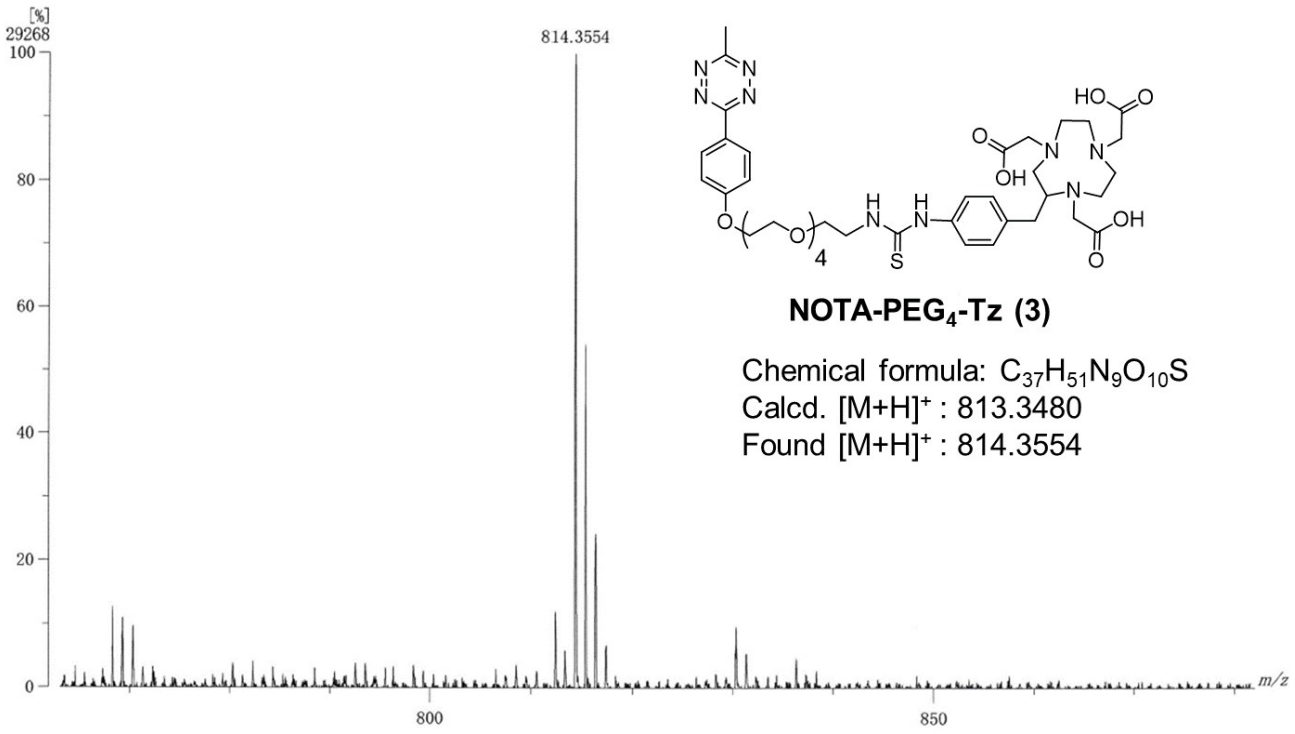

Figure S3. HRMS of NOTA-PEG$_4$-Tz **(3).**

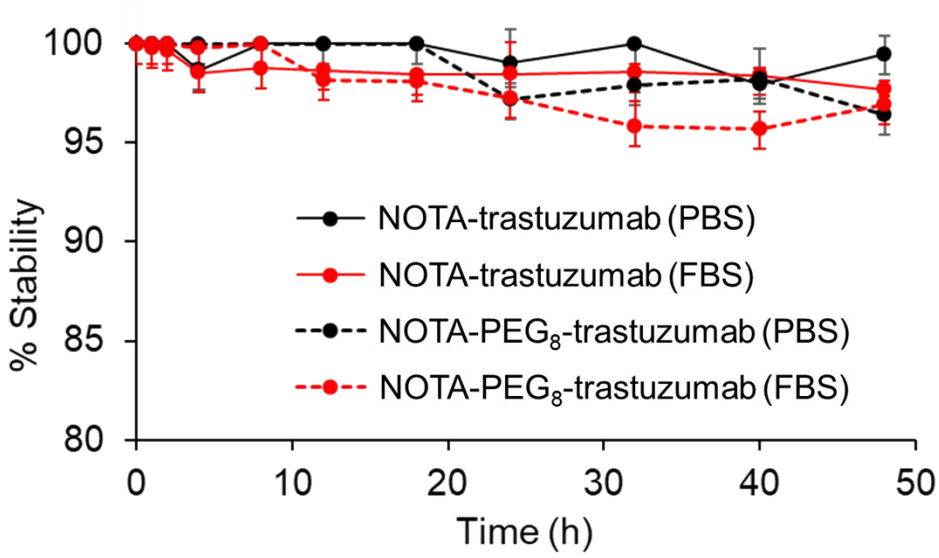

Figure S4. Stability of radio-Immunoconjugates NOTA-trastuzumab (**2**) and NOTA-PEG_8-trastuzumab (**6**) in both phosphate buffer and fetal bovine serum (FBS) for 48 h at 37 °C (n=3 each).

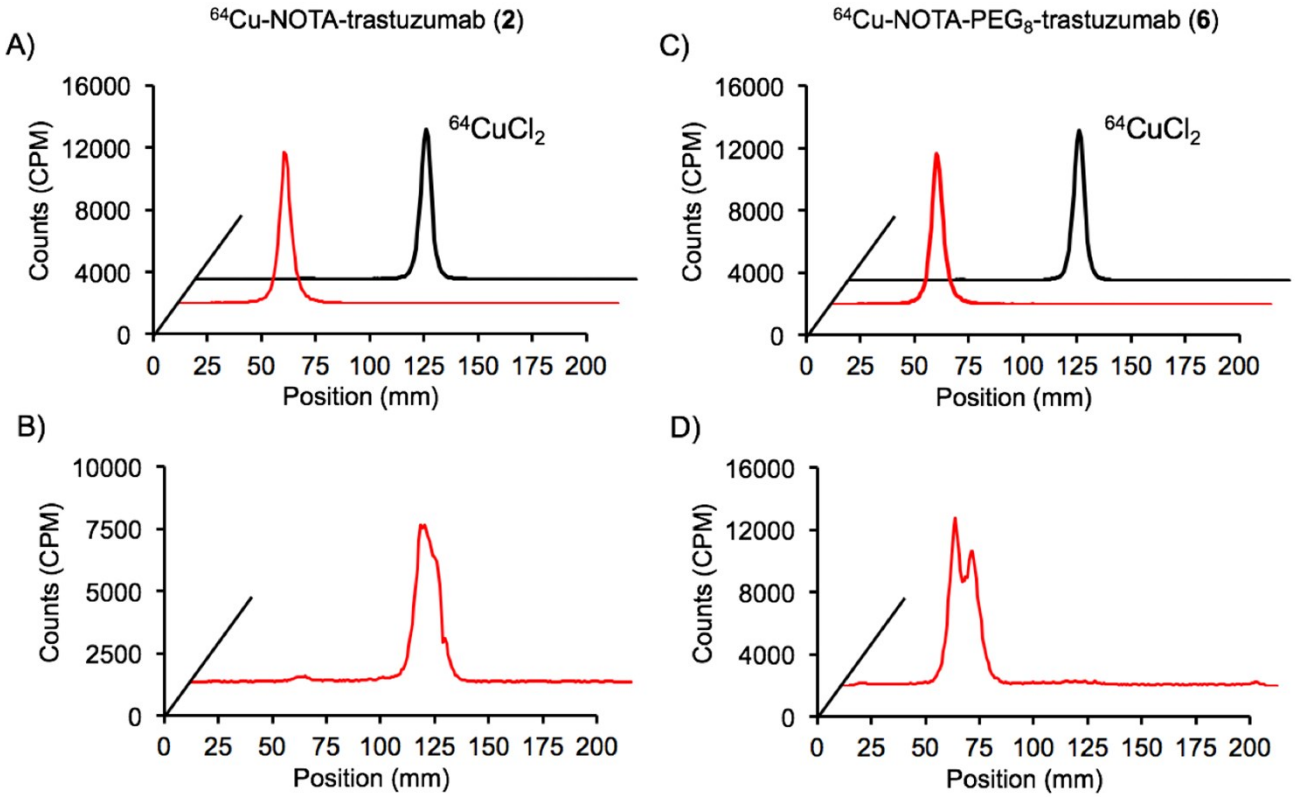

Figure S5. Radio-TLC profiles. A) Free $^{64}CuCl_2$ (black), intact ^{64}Cu-NOTA-trastuzumab (**2**) (red). B) Urine sample of ^{64}Cu-NOTA-trastuzumab (**2**) collected 12 h after injection. C) $^{64}CuCl_2$ (black); intact ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) (red). D) urine sample of ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) collected 12 h after injection. (iTLC-SG, 50 mM EDTA).

Table S1. Biodistributions of ^{64}Cu-NOTA-trastuzumab (**2**) and ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) in normal BALB/c mice 24 h and 48 h post-injection (n = 4). The radioactivity in the tissues was expressed as the % ID/g of tissue.

Organs	Radioimmunoconjugates			
	^{64}Cu-NOTA-trastuzumab (**2**)		^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**)	
	24 h	48 h	24 h	48 h
Blood	20.90 ± 1.42	13.93 ± 1.82	3.07 ± 0.31	1.42 ± 0.94
Heart	3.32 ± 1.41	3.54 ± 0.20	1.57 ± 0.21	1.20 ± 0.13
Lung	5.30 ± 0.39	5.79 ± 0.52	2.58 ± 0.20	1.33 ± 0.40
Skin	2.88 ± 0.25	1.67 ± 0.11	1.66 ± 0.21	1.96 ± 0.60
Muscle	1.50 ± 0.62	1.18 ± 0.26	0.66 ± 0.06	1.50 ± 0.95
Fat	1.68 ± 0.03	2.06 ± 0.28	0.96 ± 0.07	1.41 ± 1.11
Bone	3.33 ± 0.20	2.31 ± 0.15	1.19 ± 0.11	1.11 ± 0.63
Spleen	11.77 ± 1.08	5.93 ± 0.73	7.31 ± 0.50	7.36 ± 0.83
Kidneys	5.12 ± 0.75	6.29 ± 0.26	2.47 ± 0.31	2.28 ± 0.39
Intestine	2.04 ± 0.25	1.65 ± 0.21	1.08 ± 0.18	0.86 ± 0.17
Liver	17.28 ± 0.81	13.69 ± 0.88	21.94 ± 1.16	21.46 ± 2.58

Table S2. Biodistributions of ^{64}Cu-NOTA-trastuzumab (**2**) and ^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**) in NIH3T6.7 tumor-bearing BALB/c nude mice 24 h and 48 h post-injection (n = 3). The radioactivity in the tissues was expressed as the % ID/g of tissue.

Organs	Radioimmunoconjugates			
	^{64}Cu-NOTA-trastuzumab (**2**)		^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**)	
	24 h	48 h	24 h	48 h
Blood	15.65 ± 0.17	12.92 ± 0.64	0.97 ± 0.06	0.71 ± 0.04
Heart	2.86 ± 0.27	3.14 ± 0.31	1.05 ± 0.07	1.09 ± 0.06
Lung	3.75 ± 0.94	4.74 ± 0.95	1.87 ± 0.07	1.66 ± 0.32
Skin	4.48 ± 1.24	4.79 ± 1.29	0.16 ± 0.03	0.27 ± 0.02
Muscle	0.89 ± 0.16	1.51 ± 0.19	0.33 ± 0.02	0.30 ± 0.03
Fat	0.77 ± 0.16	0.69 ± 0.04	0.07 ± 0.003	0.24 ± 0.02
Bone	1.71 ± 0.21	3.02 ± 0.26	0.42 ± 0.01	0.83 ± 0.10
Spleen	13.41 ± 1.75	9.42 ± 0.29	1.77 ± 0.09	2.32 ± 0.13
Kidneys	4.25 ± 0.56	4.25 ± 0.63	1.52 ± 0.17	1.62 ± 0.23
Intestine	2.95 ± 0.62	2.16 ± 0.14	0.97 ± 0.12	0.68 ± 0.04
Liver	11.65 ± 1.17	6.79 ± 0.58	4.10 ± 0.30	3.24 ± 0.42
Tumor	12.07 ± 0.53	13.84 ± 1.13	16.50 ± 2.51	16.25 ± 3.41

Table S3. Tumor-to-blood/liver/spleen/kidney/muscle uptake ratio of radioimmunoconjugates. Quantification was calculated on the basis of biodistribution studies depicted in Figure 5. Significantly higher than others at the same time point (* $p < 0.05$. ** $p < 0.005$. *** $p < 0.001$).

Ratio	Radioimmunoconjugates			
	^{64}Cu-NOTA-trastuzumab (**2**)		^{64}Cu-NOTA-PEG$_8$-trastuzumab (**6**)	
	24 h	48 h	24 h	48 h
Tumor-to-blood	0.77 ± 0.03	1.08 ± 0.14	17.13 ± 3.64**	22.79 ± 4.36***
Tumor-to-liver	1.04 ± 0.12	2.06 ± 0.34	4.06 ± 0.79**	4.98 ± 0.47***
Tumor-to-spleen	0.88 ± 0.13	0.72 ± 0.08	2.32 ± 0.20***	1.39 ± 0.12**
Tumor-to-kidney	2.86 ± 0.33	3.32 ± 0.69	10.92 ± 1.84**	10.34 ± 3.28*
Tumor-to-muscle	13.87 ± 2.38	9.32 ± 1.73	50.11 ± 6.01***	54.34 ± 8.69***

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