Supporting Information

A Triazine-Based Tool Box for Developing Peptidic PET Imaging Probes: Syntheses, Microfluidic Radio-labeling on Chip and Structure-Activity Evaluation

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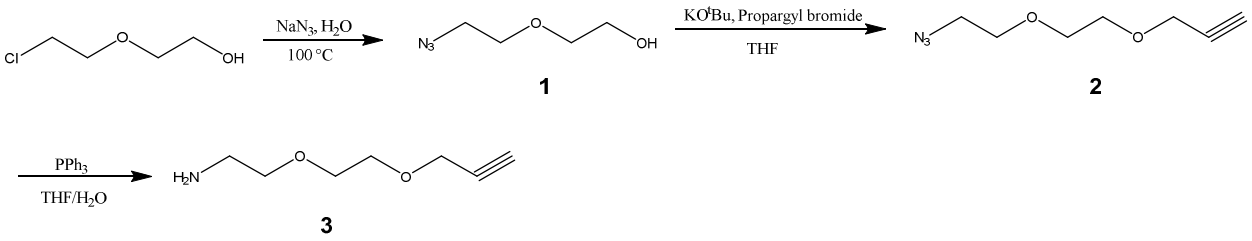
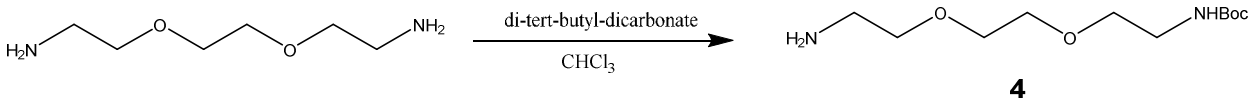
1. Synthesis.

Here we briefly reported the synthesis of PEG linker **3**[1] and linker **4**[2]**:**



Scheme S1. PEG Linker synthesis.



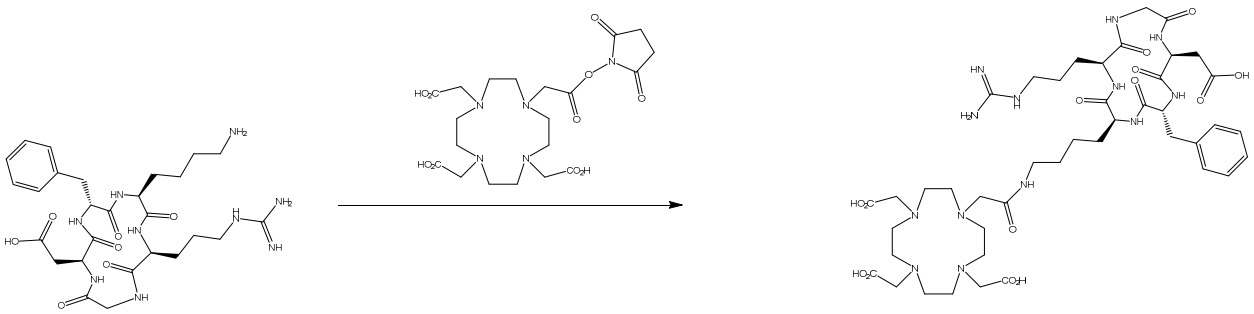
Scheme S2. PEG Linker synthesis.

2-(2-Azidoethoxy)ethan-1-ol (**1**). 2-(2-Chloroethoxy)ethanol (1 g, 8 mmol) and sodium azide (1.8 g, 24 mmol) were dissolved in DI H_2O (10 mL), and the reaction was heated to reflux for 48 h. After the reaction, the solution was cooled down to room temperature and concentrated. The product was extracted with dichloromethane (20 mL×5), and dried over anhydrous sodium sulfate. The solution was filtered and concentrated under vacuum. The product was dried under vacuum to afford viscous light yellow liquid (1g, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.69 (t, J = 4 Hz, 2H), 3.63 (t, J = 4 Hz, 2H), 3.55 (t, J = 4 Hz, 2H), 3.36 (t, J = 6 Hz, 2H), 2.67 (s, 1H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 72.45, 69.93, 61.63, 50.67. HRMS (ESI): calcd for $C_4H_9N_3O_2Na$ $[M+Na]^+$: 154.0587; found: 154.0587.

3-(2-(2-Azidoethoxy)ethoxy)prop-1-yne (**2**). 2-(2-Azidoethoxy)ethan-1-ol **1** (1g, 7.6 mmol) was dissolved in anhydrous THF (40 mL). Then potassium tert-butoxide (1 g, 9.1 mmol) was added to the solution and the solution was stirred at room temperature under argon flow. After 30 min, propagyl bromide in toluene (1 mL, 9.1 mmol) was added dropwise to the reaction solution, and the solution was stirred at room temperature overnight. After the reaction, the solution was filtered through a celite cake. The residue was purified by silica chromatography column (methanol/dichloromethane 2:98) to afford viscous yellow liquid (1.2 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.18 (d, *J* = 4 Hz, 2H), 3.70-3.64 (m, 6H), 3.37 (t, *J* = 4 Hz, 2H), 2.42 (t, *J* = 4 Hz, 1H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 79.46, 74.49, 70.36, 69.93, 69.05, 58.35, 50.56. HRMS (ESI): calcd for $C_7H_{11}N_3O_2Na$ $[M+Na]^+$: 192.0744; found: 192.0743.

2-(2-(Prop-2-ynyloxy)ethoxy)ethan-1-amine (**3**). 3-(2-(2-Azidoethoxy)ethoxy)prop-1-yne **2** (1.2 g, 7.1 mmol) was dissolved in mixed solvents of THF/H_2O (25 mL/150 μL). Triphenylphosphine (2.2 g, 8.2 mmol) was added to the reaction solution. The reaction was stirred at room temperature overnight. After the reaction, the solution was concentrated to dry and purified with silica chromatography column (methanol/dichloromethane 5:95 to triethylamine/methanol/dichloromethane 5:5:90) to afford viscous yellow liquid (0.9 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.17 (s, 2H), 3.68-3.46 (m, 4H), 3.47 (t, *J* = 6.0 Hz, 2H), 2.83 (t, *J* = 6.0 Hz, 2H), 2.41 (s, 1H), 1.53 (br, 2H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 79.43, 74.59, 72.38, 69.95, 68.96, 58.29, 41.26. HRMS (ESI): calcd for $C_7H_{14}NO_2$ $[M+H]^+$: 144.1019; found: 144.1023.

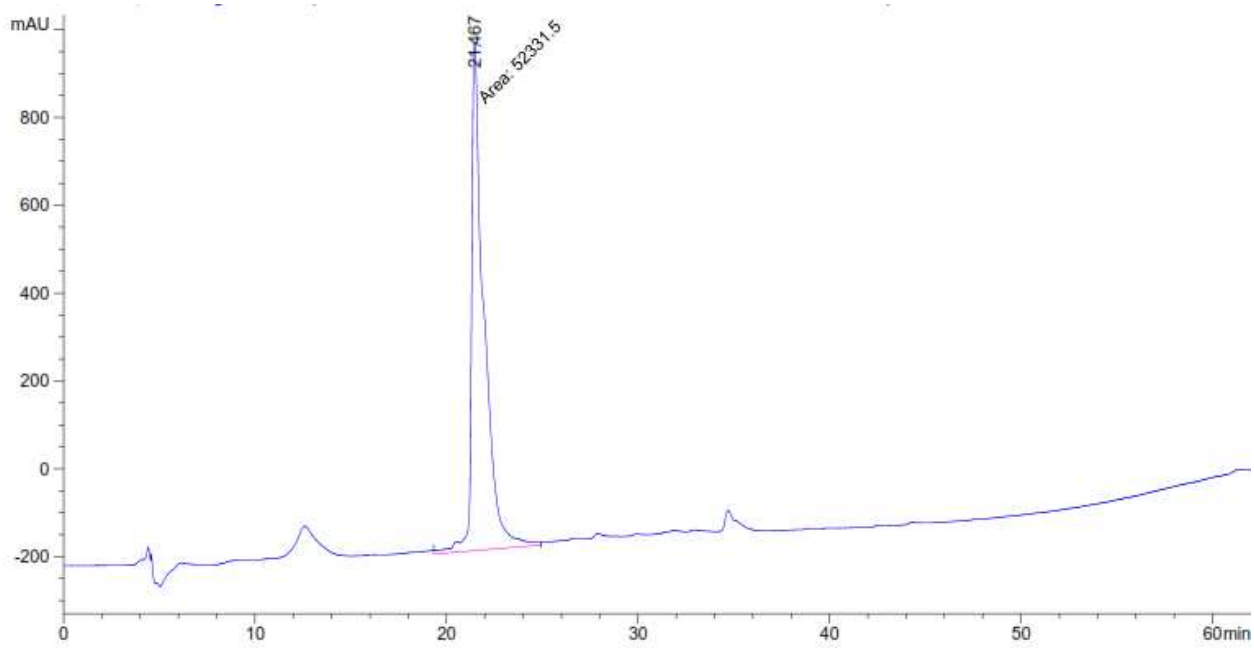
Tert-butyl-(2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (**4**). 2,2′-(Ethylenedioxy)bis(ethylamine) (2 g, 13.5 mmol) was dissolved in anhydrous chloroform (20 mL) under inert argon atmosphere. Di-tert-butyl-dicarbonate (0.3 g, 1.4 mmol) in chloroform (20 mL) solution was added dropwise to the reaction solution on an ice bath. After the addition, the ice bath was removed and the reaction solution was stirred overnight. The reaction solution was evaporated to dryness under vacuum. The residue was dissolved in H_2O (20 mL), and the product was extracted with dichloromethane (20 mL×3). The organic layers were combined and dried over anhydrous sodium sulfate. The product was further purified with silica chromatography (methanol/dichloromethane 2:98 to 15:85) to afford the viscous light yellow liquid (0.3 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.36 (br, 1H), 3.57 (s, 4H), 3.51-3.47 (m, 4H), 3.26 (d, *J* = 8.0 Hz, 2H), 2.84 (br, 2H), 1.39 (s, 9H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 155.82, 78.87, 73.30, 70.24, 70.09, 70.01, 41.58, 40.14, 28.23. HRMS (ESI): calcd for $C_{11}H_{24}N_2O_4$ $[M+H]^+$: 249.1809; found: 249.1809.



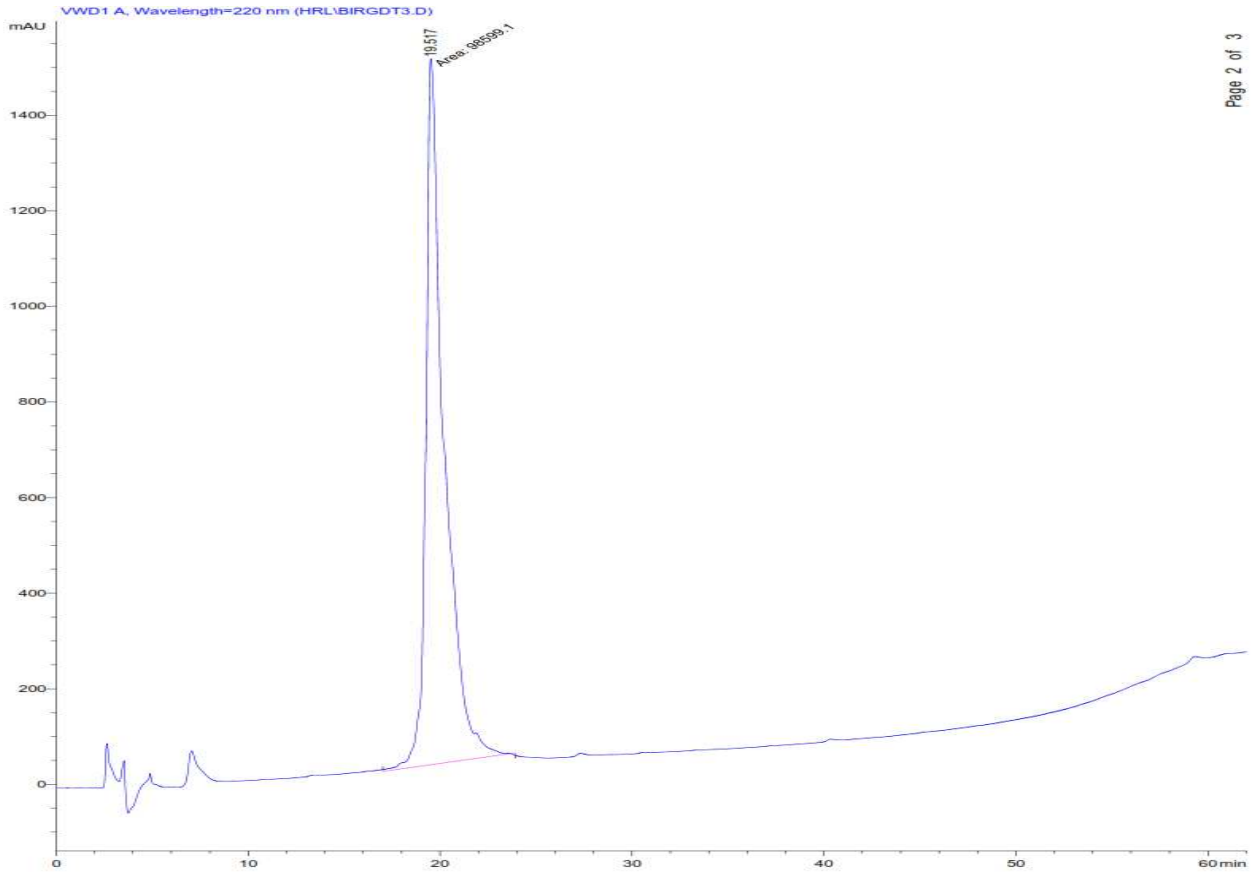
Scheme S3. Synthesis of DOTA-cyclo(RGDfK) peptide

DOTA-NHS ester (9.4 mg, 12.3 μmol) was added to a 1 mL vessel. The vessel was charged with nitrogen. Cyclo(RGDfK) peptide (2.9 mg, 4.2 μmol) dissolved in anhydrous DMF (150 μL) was added to the solution. DIPEA (7.6 μL, 43.6 μmol) was added to the solution, and the reaction solution was stirred at room time for overnight. After the reaction, DI-water (50 μL) was added to the reaction solution, and the solution was stirred for 30 min. The product was purified by HPLC (method B, t_R = 15.19 min) and dried by lyophilization to afford white fluffy solid (3.6 mg, 76%). MALDI-TOF: cald for $C_{43}H_{67}N_{13}O_{14}$ $[M]^+$: 989.49; found: 989.13. HRMS (ESI): calcd for $C_{43}H_{66}N_{13}O_{14}KNa$ $[M-H+K+Na]^+$: 1050.4381; found: 1050.4297.

(A)



(B)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mAU
19.517
Area: 985599.1
Page 2 of 3
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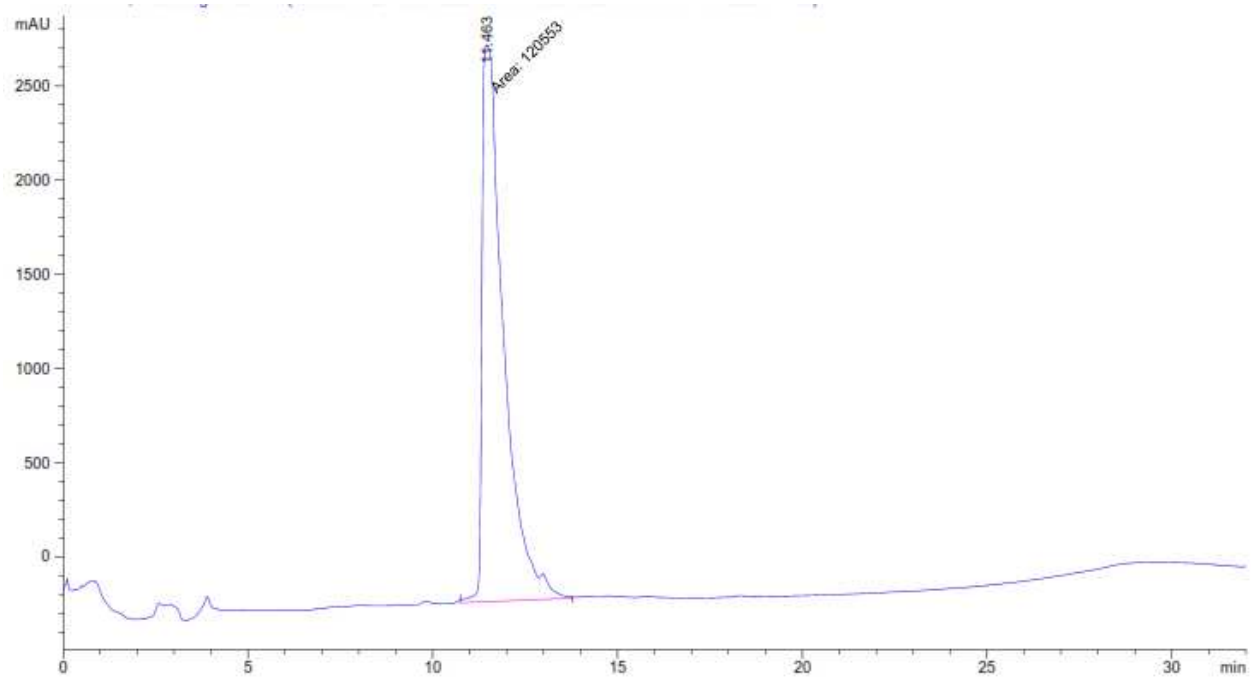
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60 min

(C)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0
5
10
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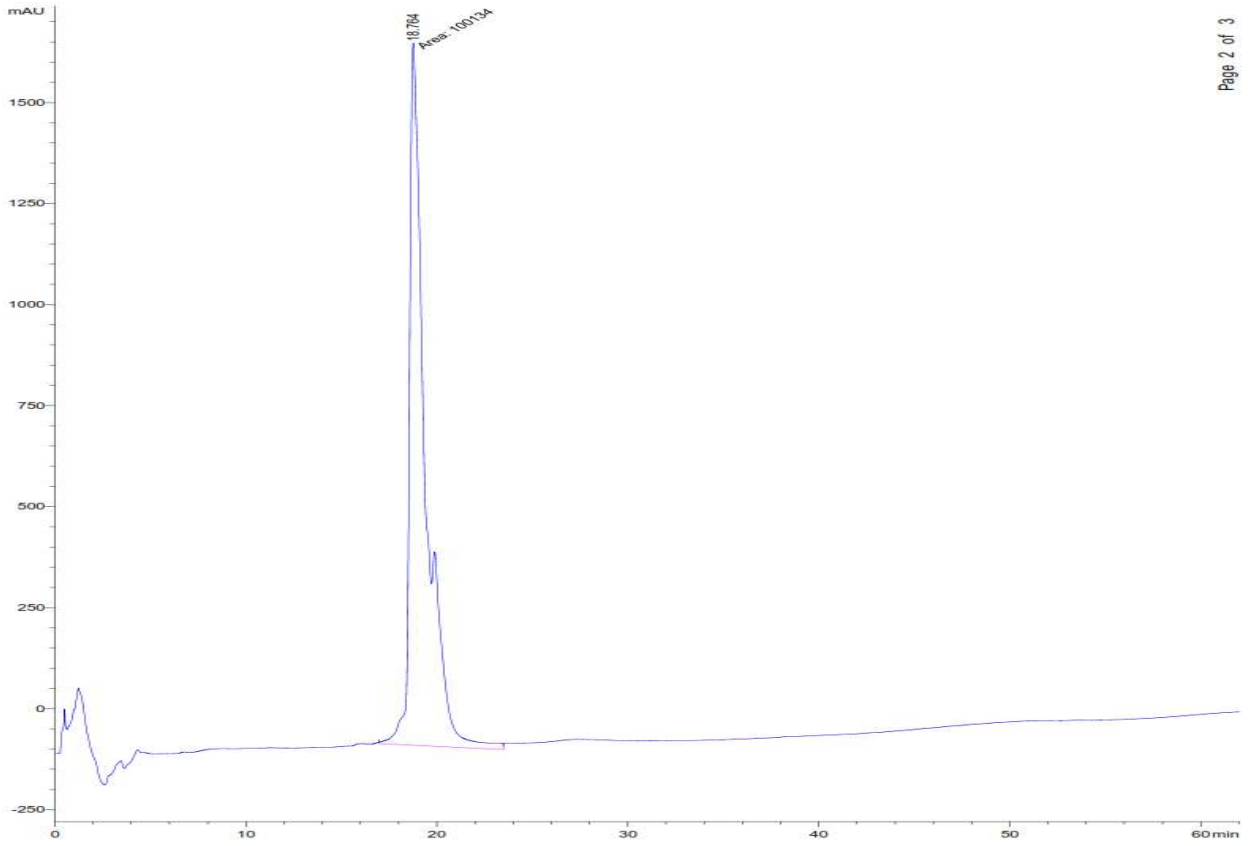
30
min

(D)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Page 2 of 3

(E)

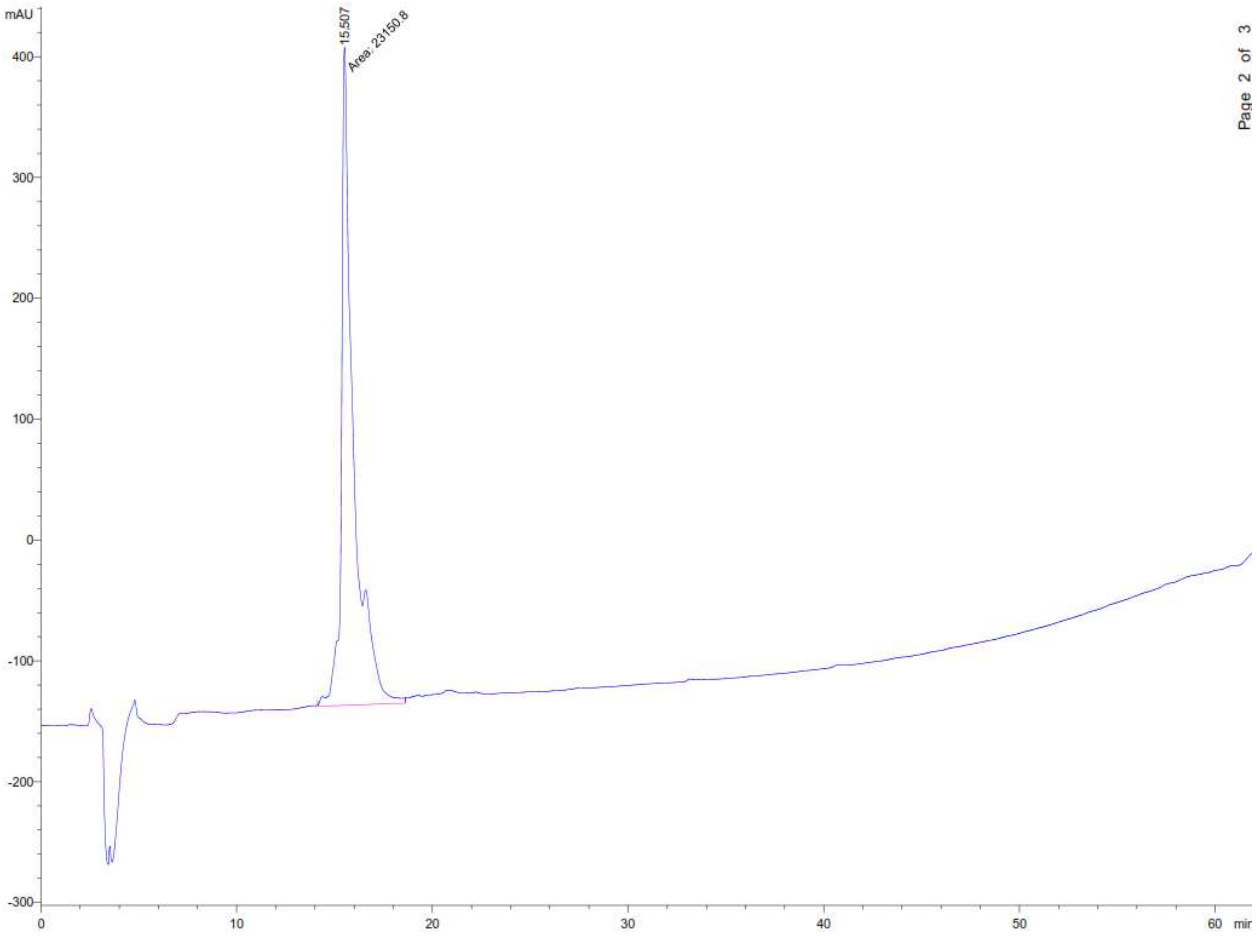

Figure S1. HPLC characterization spectra for (A) NH_2-TZ-Bis-cyclo(RGDfK) **10**; (B) DOTA-TZ-Bis-cyclo(RGDfK) **13**; (C) NH_2-TZ-cyclo(RGDfK) **11**; (D) DOTA-TZ-cyclo(RGDfK) **14**; (E) DOTA-"Click"-cyclo(RGDfK) **15**.

2. TBTA and DOTA competitive binding study.

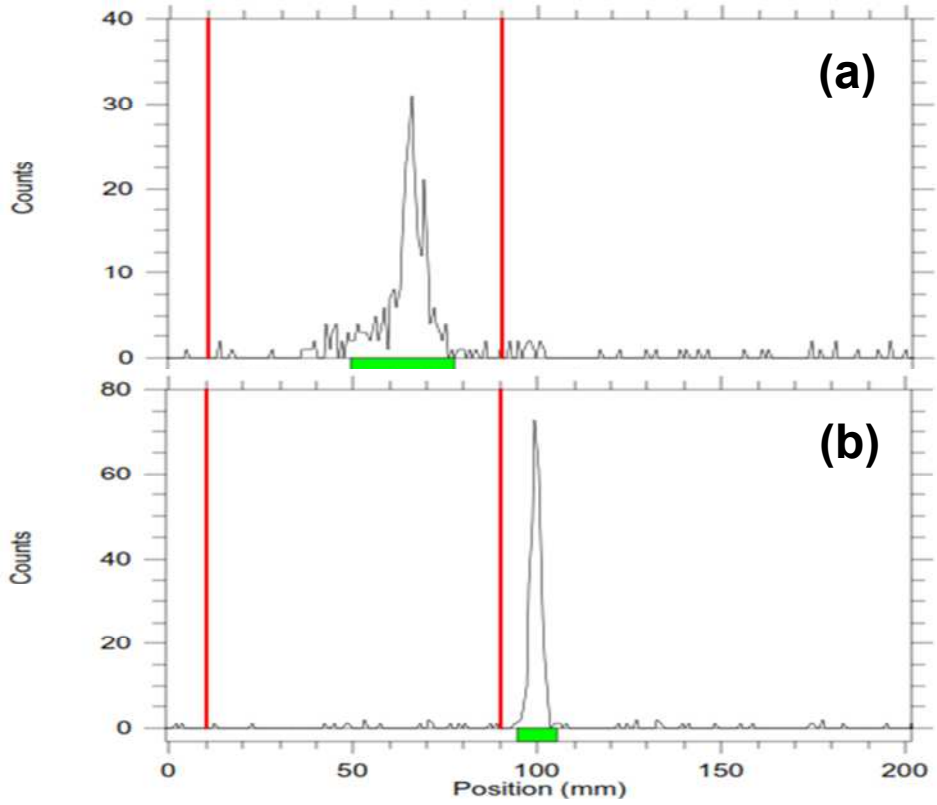

Figure S2. (a) TBTA+Cu (I) after purification; (b) DOTA+TBTA-Cu (I) after 15 min.

3. Reactions of "clickable" DOTA-triazine spacer.

Synthesis and HPLC condition:

DOTA-Cu-TZ-Et-Alkyne:

DOTA-TZ-Et-Alkyne (8 mg, 0.01 mmol) was dissolved in ammonia acetate (100 µL, pH=6.8, 0.1 M). Copper sulfate (100 µL, 100 mM) was added to the buffer solution, and the whole solution was stirred for 45 min at 80 °C. The product was purified on Sep-Pak C18 cartridge (Waters, Milford, MA) by removing the excessive salt with water, and the product was eluted by ethanol. MALDI-TOF calcd for $C_{36}H_{62}CuN_{11}O_{12}$ $[M+H]^+$: 903.39; found: 903.34.

DOTA-Gd-TZ-Et-Alkyne:

DOTA-TZ-Et-Alkyne (0.5 mg, 0.6 µmol) was dissolved in citrate buffer (500 µL, pH=6.8, 0.1 M). Gadolinium trichloride hexahydrate (4.4 mg, 11.8 mmol) was added to the buffer solution, and the whole solution was stirred for 16 h at 80 °C. The product was purified on Sep-Pak C18 cartridge by removing the excessive salt with water, and the product was eluted by ethanol. MALDI-TOF calcd for $C_{36}H_{62}GdN_{11}O_{12}$ $[M]^+$: 996.36; found: 996.05.

DOTA-Gd-TZ-Et-RGD:

Click reaction-conventional method: DOTA-TZ-Et-Alkyne (0.45 mg, 0.5 μmol) was dissolved in 100 μL methanol. Copper sulfate (5 μL, 100 mM) was reduced by sodium ascorbate (50 μL, 10 mg/1 mL) at room temperature, and the solution was vortexed for 5 min. TBTA (37.5 μL, 20 mM) was added to the copper catalyst solution and the solution turns colorless. Peptide cyclo(RGDfK)-N_3 (0.4 mg, 0.65 μmol) in methanol/H_2O (1:1, 200 μL) was added to the solution with the catalyst solution. The reaction solution was heated at 80 °C for 18 h. Then the reaction solution was analyzed by HPLC and MALDI-TOF mass spectrometry.

Click reaction-microwave method: DOTA-TZ-Et-Alkyne (0.45 mg, 0.5 μmol) was dissolved in 100 μL methanol. Copper sulfate (5 μL, 100 mM) was reduced by sodium ascorbate (50 μL, 10 mg/1 mL), and the solution was vortexed for 5 min. TBTA (37.5 μL, 20 mM) was added to the copper catalyst solution and mixed well. Peptide cyclo(RGDfK)-N_3 (0.4 mg, 0.65 μmol) in methanol/H2O (1:1, 200 μL) was added to the solution with the catalyst solution. The reaction solution was heated at 100 °C for 1 h under microwave condition. The reaction solution was purified by HPLC. The product was collected at 20.35 min, and analyzed by mass spectrometry. MALDI-TOF calcd for $C_{63}H_{100}CuN_{22}O_{19}$ $[M]^+$: 1531.68; found: 1531.80.

HPLC condition:

A: 0.1% TFA in H_2O; B: 0.1% TFA in acetonitrile. Flow rate: 1 mL/min. Method a: B 10% (0 min), 10% (2 min), 90% (24 min), 90% (28min), 10% (30 min), 10% (32 min). Method b: B 5% (0 min), 5% (2 min), 100% (60 min), 5% (62min).

Conversion yield (%)	Microwave 100 °C for 1 h	80 °C for 18 h
DOTA-TZ-Et-Alkyne	No reaction	Trace
Cu-DOTA-TZ-Et-Alkyne	100%	50%
Gd-DOTA-TZ-Et-Alkyne	30%	No reaction

Table S1. Reactions of "clickable" DOTA-triazine spacer with peptide cyclo(RGDfK)-N_3.

4. Determination of partition coefficient.

Peptide	Mean of Log *D*	SD
DOTA-TZ-Bis-cyclo(RGDfK)	-4.1	0.6
DOTA-TZ-cyclo(RGDfK)	-3.6	0.3
DOTA-"Click"-cyclo(RGDfK)	-3.3	0.5
DOTA-cyclo(RGDfK)	-4.3	0.7

Table S2. Determination of Log *D* values for peptide conjugates.

5. *In vivo* study

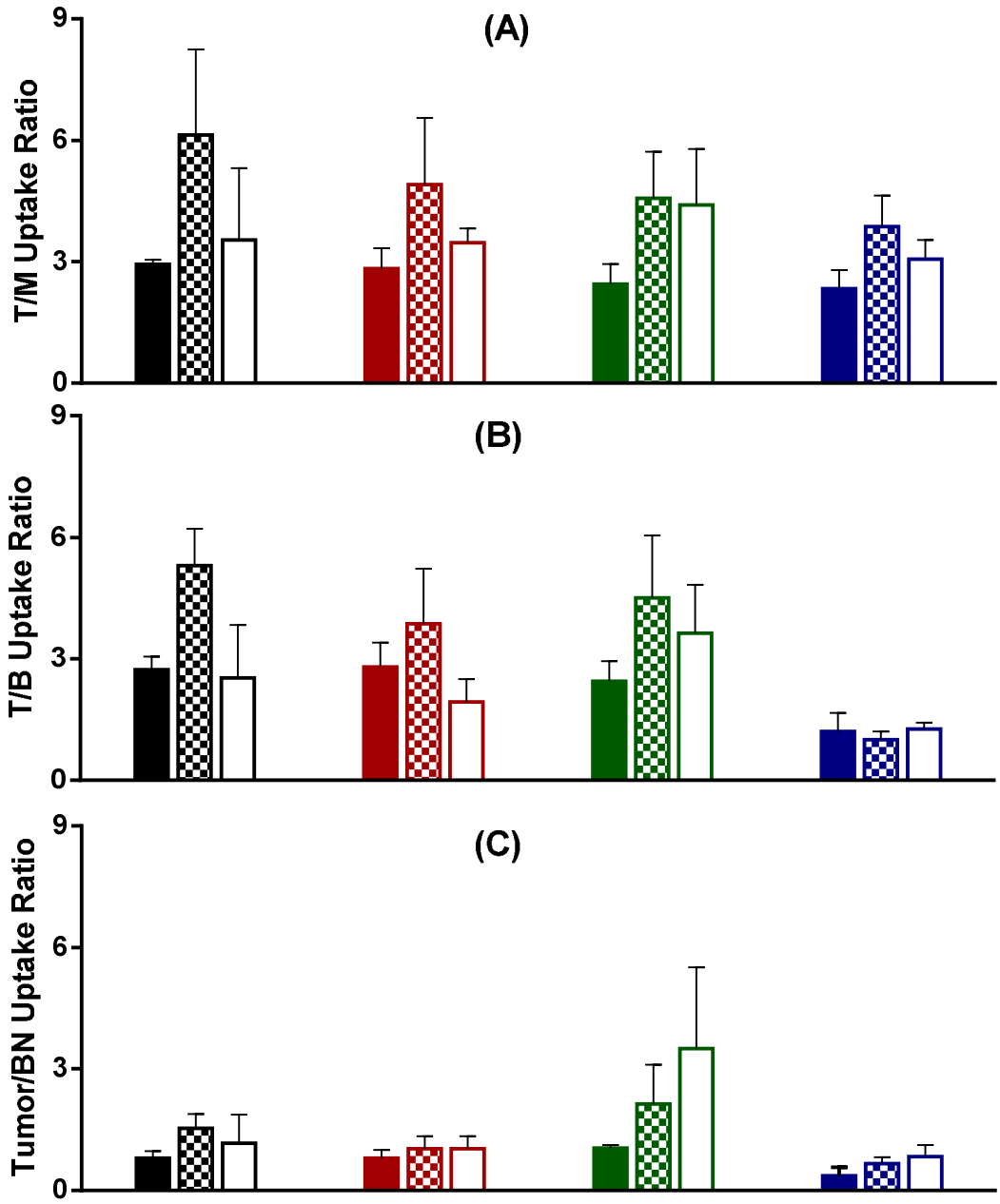

Figure S3. (A) Tumor to muscle uptake ratio; (B) Tumor to blood uptake ratio; (C) Tumor to bone uptake ratio. Black: DOTA-cyclo(RGDfK); red: DOTA-"Click"-cyclo(RGDfK) **15**; green: DOTA-TZ-cyclo(RGDfK) **14**; blue: DOTA-TZ-Bis-cyclo(RGDfK) **13**. Filled box: 1 h p.i.; dotted box: 4 h p.i.; open box: 24 h p.i.

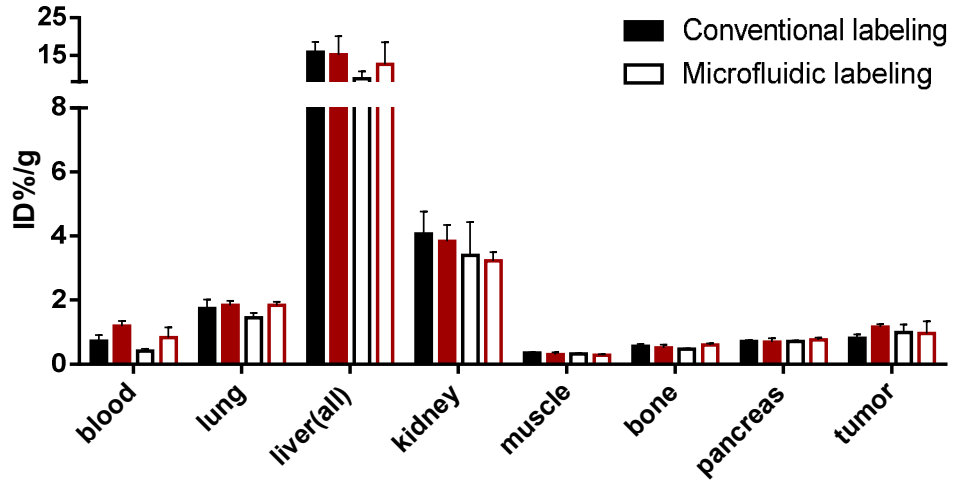

Figure S4. Comparison of biodistribution of ^{64}Cu-DOTA-TZ-Bis-cyclo(RGDfK) labeled by conventional method and microfluidic method. Black: 1 h p.i.; red: 4 h p.i.

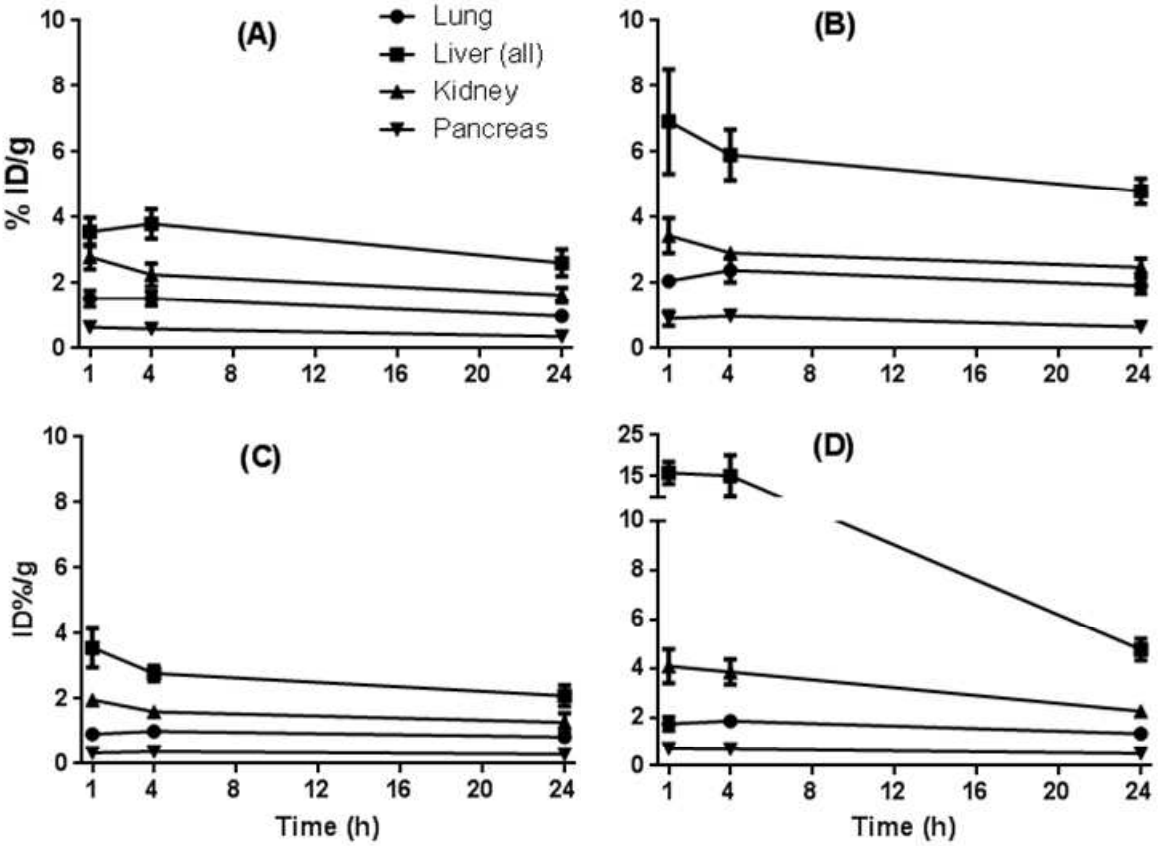

Figure S5. Time activity curves for different organs: lung (circle), liver (square), kidney (triangle) and pancreas (inverted pyramid) from biodistribution studies. (A) DOTA-cyclo(RGDfK); (B) DOTA-"Click"-cyclo(RGDfK) **15**; (C) DOTA-TZ-cyclo(RGDfK) **14**; (D) DOTA-TZ-Bis-cyclo(RGDfK) **13**.

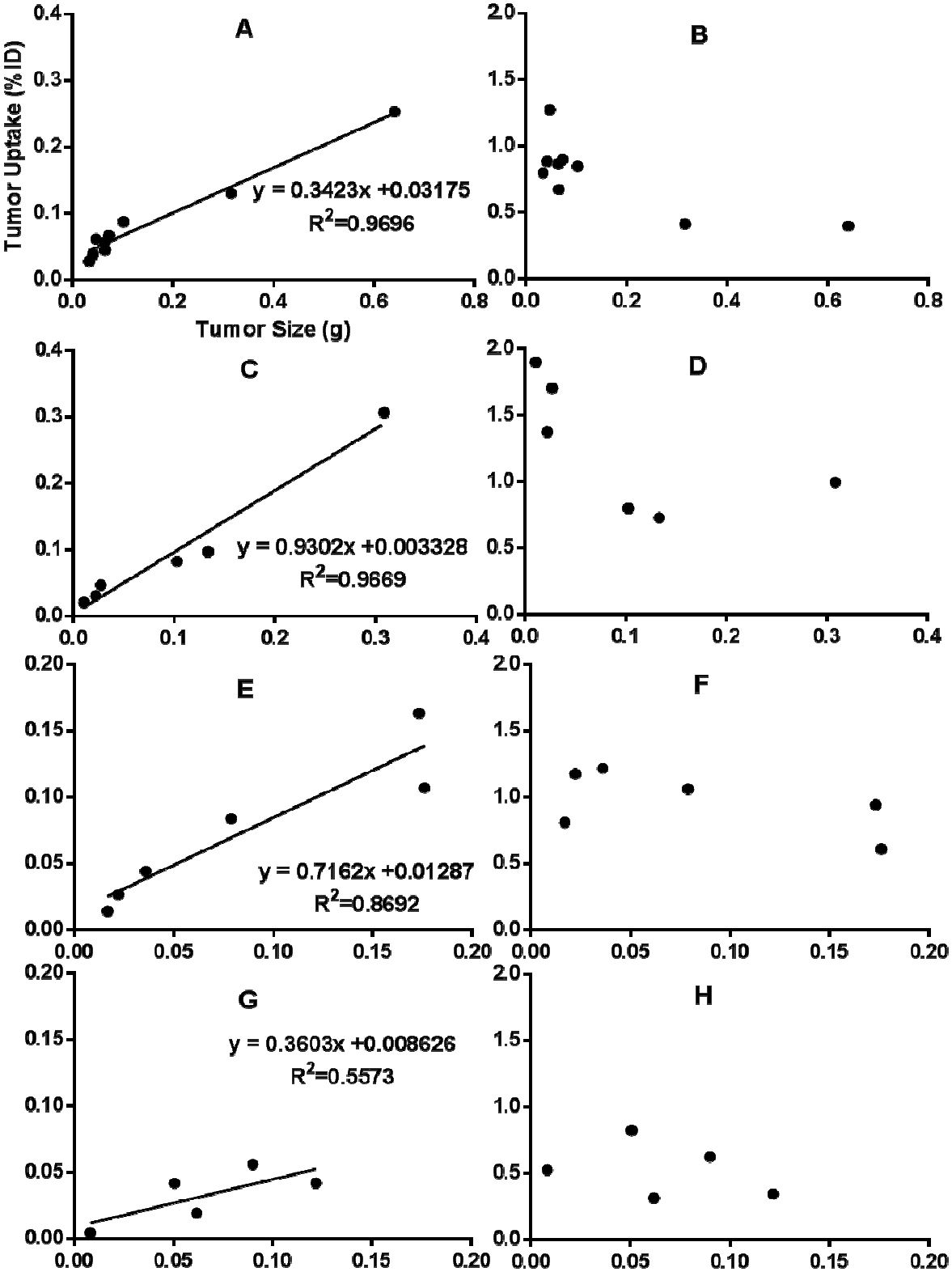
A
B
C
D
E
F
G
H
Tumor Uptake (% ID)
Tumor Size (g)
y = 0.3423x +0.03175
R2=0.9696
y = 0.9302x +0.003328
R2=0.9669
y = 0.7162x +0.01287
R2=0.8692
y = 0.3603x +0.008626
R2=0.5573

Figure S6. Tumor uptake with the increasing tumor size at 1 h p.i.: (A, B) DOTA-TZ-Bis-cyclo(RGDfK) **13**; (C,D) DOTA-"Click"-cyclo(RGDfK) **15**; (E, F) DOTA-cyclo(RGDfK); (G, H) DOTA-TZ-cyclo(RGDfK) **14**.

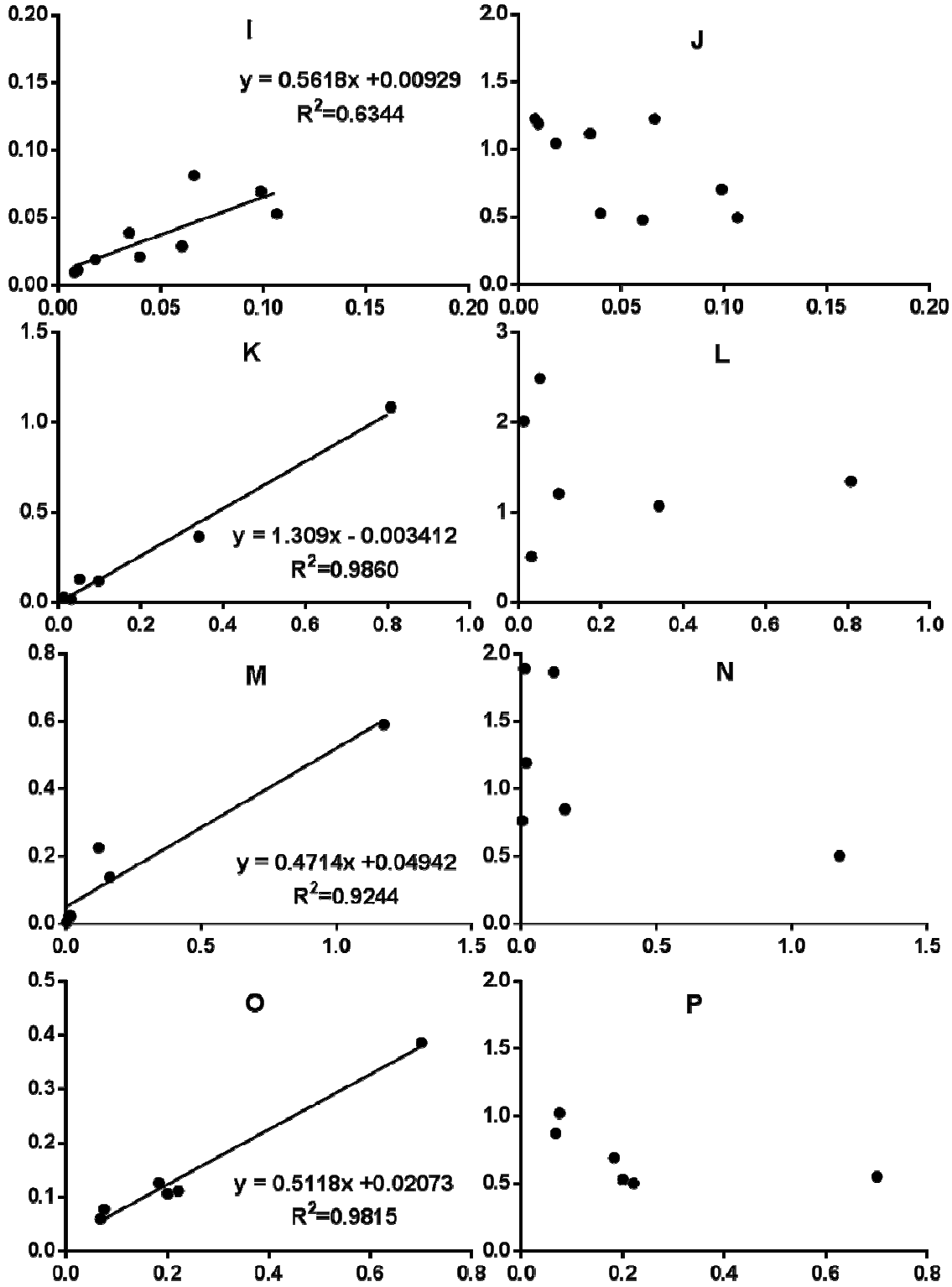

Figure S7. Tumor uptake with the increasing tumor size at 4 h p.i.: (I, J) DOTA-TZ-Bis-cyclo(RGDfK) **13**; (K, L) DOTA-"Click"-cyclo(RGDfK) **15**; (M, N) DOTA-cyclo(RGDfK); (O, P) DOTA-TZ-cyclo(RGDfK) **14**.

Reference

1. Zhang, A. X.; Murelli, R. P.; Barinka, C.; Michel, J.; Cocleaza, A.; Jorgensen, W. L.; Lubkowski, J.; Spiegel, D. A., A Remote Arene-Binding Site on Prostate Specific Membrane Antigen Revealed by Antibody-Recruiting Small Molecules. *Journal of the American Chemical Society* **2010,** *132* (36), 12711-12716.
2. van Dongen, S. F. M.; Clerx, J.; Norgaard, K.; Bloemberg, T. G.; Cornelissen, J. J. L. M.; Trakselis, M. A.; Nelson, S. W.; Benkovic, S. J.; Rowan, A. E.; Nolte, R. J. M., A clamp-like biohybrid catalyst for DNA oxidation. *Nature Chemistry* **2013,** *5* (11), 945-951.