Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Efficient ^{125}I-radiolabeling of biomolecules using strain-promoted oxidation-controlled cyclooctyne −1, 2-quinone cycloaddition reaction

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Materials and Equipment

All chemicals, reagents, solvents, and materials including Human serum albumin protein (HSA) and human transferrin protein (hTf) were purchased from Sigma Aldrich and used without further purification. Peptide c[RGDyK] (FC-2202) was purchased from FUTURECHEM Co. Republic of Korea. All newly synthesized intermediate precursors were purified by silica gel chromatography, using high purity silica gel with average particle size 60μm. The reaction progress was monitored using glass or aluminium based silica plates (60 F_{254} pure silica gel). The compound spotted on TLC was visualized by using UV light wave length 254nm or using staining agent and heating. The reactions were performed at low temperature (4°C), or room temperature (25°C). The NMR (^{1}H and ^{13}C) analysis was performed using JEOL NMR spectrometer (500 MHz, KAERI, Jeongeup, Republic of Korea). The NMR spectra were obtained using chloroform-d ($CDCl_3$-d_1), dimethyl sulfoxide-d_6 (DMSO-d_6), or Methanol-d_4 (CD_3OD) as solvent. The high resolution mass spectroscopy of low molecular weight precursors was performed at Korea Basic Science Institute (Seoul, Republic of Korea) using Bruker Q-TOF high resolution mass spectroscopy system. For the MALDI-TOF analysis of proteins and peptides, AB SCIEX TOF/TOF 5800 MALDI-TOF system (Korea Institute of Radiological & Medical Science, Seoul, Republic of Korea) was used. 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic Acid) or 2,5-dihydroxybenzoic acid (DHB) was used as matrix for MALDI-TOF analysis. For biodistribution study accumulated radioactivity in each organ was measured using automatic gamma counter (PerkinElmer, USA). All SPECT/CT images were acquired using Inveon (Siemens) small-animal multimodality SPECT/CT system.

The cold and radioactive compounds were analyzed on analytical HPLC system supplied by Agilent Technologies. The analytical HPLC system (1290 infinite) was equipped with Eclipse XDB-C18 column (4.6 x 250 mm, 5μm) and gamma detector. For HPLC purification Agilent Technologies preparative HPLC system (1260 infinite) equipped with gamma detector and Eclipse XDB-C18 column (7μm, 21.2 x 150 mm) was used. All HPLC analysis were performed using solvent A (0.1% formic acid containing water) and solvent B (0.1% formic acid containing acetonitrile). For HPLC purification a preparative HPLC method A (flow rate: 10 mL/min, eluents gradient: 0–3 min: 95%A/5%B; 3–15 min: a linear gradient to 35%A/65%B from 95%A/5%B; 15–35 min: a linear gradient to 5%A/95%B from 35%A/65%B; 35–45 min: 5%A/95%B) or method B (flow rate: 10 mL/min, eluents gradient: 0–5 min: 95%A/5%B; 5–20 min: a linear gradient to 50%A/50%B from 95%A/5%B; 20–35 min: a linear gradient to 5%A/95%B from 50%A/50%B; 35–45 min: a linear gradient to 3%A/97%B from 5%A/95%B).The radioactive products were trapped inSep Pak C18 cartridge preconditioned with 5mL ethanol and 10mL water. The aqueous solution of [^{125}I]NaI (in 0.1M NaOH) with a 1GBq activity was purchased from PerkinElmer, Republic of Korea.

All animal experimental procedures were approved by the institutional animal committee for animal care at Korea Atomic Energy Research Institute, Jeongeup,

Republic of Korea and conducted in accordance with the Korean law of animal protection and welfare.

Synthesis of intermediate compound N-(3,4-dihydroxyphenethyl)-4-iodobenzamide (1)

4-iodobenzoic acid (1g, 4mmol) and HBTU coupling agent (1.5g, 3.9mmol) were dissolved in dimethylformamide DMF (10mL). 4-(2-aminoethyl)benzene-1,2-diol hydrochloride (0.76g, 4mmol) and N,N-diisopropylethylamine (DIPEA) base (1g, 7.7 mmol) were added under nitrogen gas atmosphere. The reaction mixture was stirred for 2.5h and quenched by the addition of 1N HCl. The crude product was dissolved in ethyl-acetate and washed with saturated NaCl solution and dried using anhydrous Na_2SO_4. Crude product was purified by silica gel chromatography and Ethyl-acetate/ n-Hexane (1:1) to give intermediate compound N-(3,4-dihydroxyphenethyl)-4-iodobenzamide (**1**) (0.9g, 2.3mmol, 58%). The HPLC retention time of product 1 was 22.0 min using method B (figure S9).^{1}H NMR (500 MHz, DMSO-d_6) δ 8.72 (s, 1H), 8.60 (s, 1H),8.55 (t, J=6.5 Hz, 1H),7.79 (d, J=8.5 Hz,2H), 7.55 (d, J=8.5 Hz, 2H), 6.40-6.59 (m, 3H, Ph), 3.31 (q, J=6.5Hz,2H), 2.59 (t, J=8.5, 2H);^{13}C NMR (125 MHz, DMSO-d_6) 165.8, 145.5, 144.0, 137.6, 133.8, 130.7, 129.6, 119.7, 116.5, 116.0, 99.1, 41.8, 35.0; HRMS ([M+H]$^+$) calculated: 384.1854; found: 384.1855.

Synthesis of intermediate compound N-(3,4-dihydroxyphenethyl)-4-(tributylstannyl)benzamide (2)

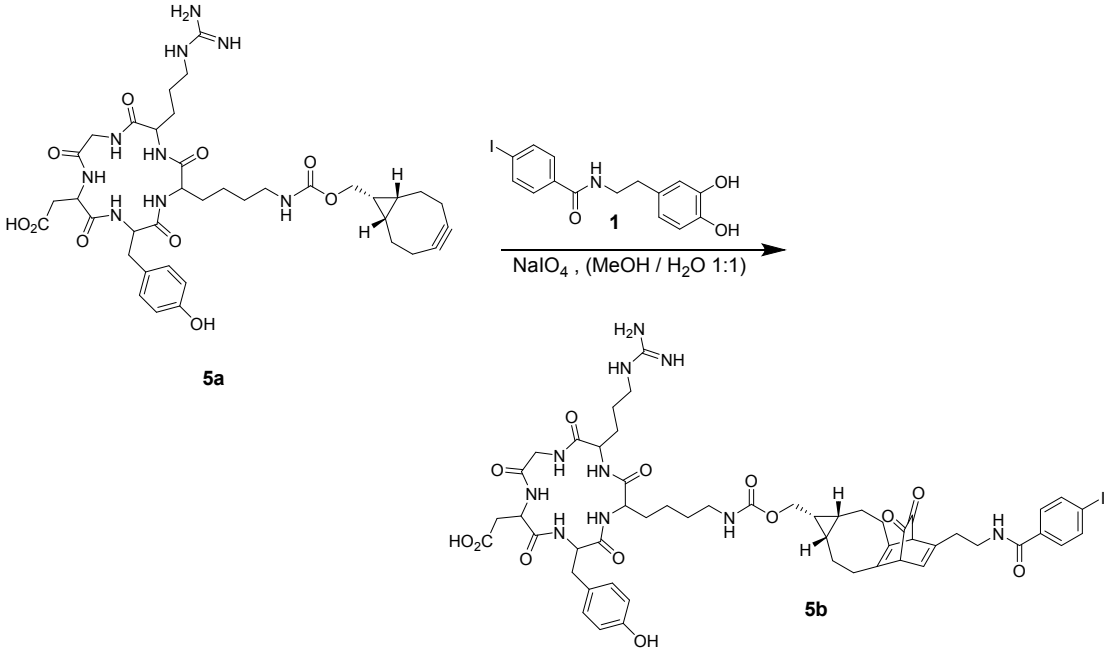
The reagent bis(tributyltin) (1.5g, 2.6mmol), tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$) (0.15g, 0.13), and intermediate compound **1** (0.5g, 1.3mmol) were dissolved in 25mL of 1,4 dioxane. The reaction mixture was stirred under reflux for 16h. The crude product was cooled down at 25°C and filtered through Whatman cellulose filter paper to remove undissolved impurities. The crude product was dissolved in ethyl-acetate and washed with saturated NaCl solution and dried using anhydrous Na_2SO_4. Crude product was purified by silica gel chromatography and Ethyl-acetate/ n-Hexane (3:7) to give intermediate compound N-(3,4-dihydroxyphenethyl)-4-(tributylstannyl)benzamide (**2**) (0.9g, 2.3mmol, 70%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.732 (s, 1H), 8.61 (s, 1H),8.44 (t, J=5.5 Hz, 1H),7.69 (d, J=7.5 Hz,2H), 7.47 (d, J=7.5 Hz, 2H), 6.41-6.60 (m, 3H, Ph), 3.34 (q, J=7.5 Hz, 2H), 2.59 (t, J=7.5 Hz, 2H), 1.40–1.55 (m, 6H), 1.21–1.28 (m, 6H), 0.95–1.13 (m, 6H), 0.80 (t, J=8.5 Hz, 9H);^{13}C NMR (125 MHz, DMSO-d_6) 166.8, 146.0, 145.5, 144.0, 136.5, 134.9, 130.7, 126.8, 119.7, 116.5, 116.0, 41.8, 35.2, 29.1, 27.2, 14.0, 9.7; HRMS ([M+H]$^+$) calculated : 548.3390; found: 548.3395.

Synthesis of BCN installed cRGD peptide 5a

Peptide (cRGDyK) (20 mg, 0.032 mmol) was added to the solution (1R, 8S, 9s)-Bicyclo[6.1.0]non-4-yn- 9-ylmethylN-succinimidyl carbonate (BCN-succinimidyl ester) (9.3 mg, 0.31 mmol) of in DMSO (100 µL). The pH of solution was adjusted to 8.2-8.5 by using (DIPEA) (17µL).The reaction was carried out for 2.5h and the crude mixture was purified by preparative HPLC (method A) to give **5a** (19 mg, 0.23mmol, 74%, retention time :15.9 min). MALDI-TOF ([M+H]$^+$) found: 796.4450.

Synthesis of iodinated cRGD peptide 5b

Intermediate compound **1** (5 mg, 0.013 mmol) dissolved in 1mL MeOH was treated with sodium periodate ($NaIO_4$) (1eq, 1mL H_2O) for 10min. The intermediate compound **5a** (10mg, 0.012 mmol) was added to the reaction mixture. After 30 min crude product was purified by preparative HPLC (method A) to give iodinated cRGD peptide (**5b**) (12.5 mg, 0.010 mmol, retention time: 19.0 min).MALDI-TOF ([M+H]$^+$) found: 1177.3569.



Scheme S1 Synthesis of iodinated cRGD peptide **5b**

Synthesis of BCN installed Human transferrin protein 6a

Human transferrin protein (hTf) (500 µmol, pH 8.2, 100µL, PBS) was incubated with BCN-succinimidyl ester (20 mM, 10µL in DMSO) for 4h at 4°C. For the purification of **6a**, crude product was diluted with saline (2.5mL) and passed through PD-10 desalting column. The extracted product was further subjected to centrifugation (4500 rpm, 12 min, 4°C) using Amicon filter (30 kDa). BCN modified protein **6a** was found in residue and analyzed using MALDI-TOF mass spectroscopy system and concentration (mg/mL) was determined using protein quantification system.

Synthesis of BCN installed human serum albumin 7a

BCN installed human serum albumin protein was synthesized using same protocol as adopted for hTf.

Radiochemistry

Radiosynthesis of intermediate precursor [^{125}I]1

The synthesis of **[^{125}I]1** was performed according to published procedure after slight modifications[1]. Briefly, intermediate precursor **2** (1 mg) was dissolved in 1mL of DMSO. The 0.5 M phosphoric acid (250 µL) and 0.5 M peracetic acid (500 µL) were added. To start reaction [^{125}I]NaI (74MBq, 7µL) in 0.1M NaOH was added and reaction was carried out at room temperature for 10 min. The reaction was quenched by adding excess amount of aqueous sodium metabisulfite solution (0.5M). The crude product was diluted with distilled water to make final volume 5mL and purified by preparative HPLC system (method B).The radioiodinated product **[^{125}I]1** trapped in preconditioned Sep Pak C18 cartridge and eluted with methanol (100µL) to give **[^{125}I]1** (analytical HPLC retention time 22.5 min, figure S10) in high isolated radiochemical yield (85%±3, n=5) and radiochemical purity (>99).

Radiosynthesis of ^{125}I-cRGD peptide ([^{125}I]5) (SPOCQ)

Purified intermediate precursor **[^{125}I]1** (7.4 MBq, 10µL in methanol) was treated with sodium periodate ($NaIO_4$) (80 µg, 50µL in water) for 10min. To this solution intermediate compound **5a** (40 µL in methanol) was added. Total reaction volume was 100µL and final concentration of BCN installed cRGD peptide was 25, 10 or 5 µM. The reaction progress in terms of radiochemical yield was monitored at different time points using radio-HPLC system (method A) and observed radiolabeling results were summarized in table 1.

Radiosynthesis of ^{125}I-TF ([^{125}I]6) and ^{125}I-HSA ([^{125}I]7)

Purified intermediate precursor **[^{125}I]1** (7.4 MBq, 10µL in methanol) was treated with sodium periodate ($NaIO_4$) (80µg, 40µL in water) for 10min. The reaction mixture was diluted with 40mL of distilled water and radioactivity was trapped in preconditioned Sep Pak C18 cartridge to remove unreacted $NaIO_4$. Oxidized radiotracer was eluted with 100 µL of methanol and solvent volume was reduced to 10 µL under reduced pressure. The radioactive product was then incubated with **6a** or **7a** (100uL, 1xPBS) so that the final concentration of **6a** or **7a** was 25, 10 or 5 µmol. Reaction progress in terms radiolabeling yield was monitored using radio-TLC system at different time points and important results were summarized in table 1.

Radiosynthesis of ^{125}I-HSA ([^{125}I]8) (direct radioiodination)

HSA (25µmol, 100µL in saline) was treated with [^{125}I]NaI (37MBq, 3.5µL) and aqueous solution of chloramine-T (100 µg, 10µL) for 10 min at 4°C.The reaction was quenched by 10µL of aqueous sodium metabisulfite solution (1mg, 20 µl). Crude product was purified by PD-10 desalting column to give **[^{125}I]8** with 93% radiochemical yield and more than 99% radiochemical purity as determined by radio-TLC system.

Radiosynthesis of ^{125}I-HSA ([^{125}I] 9) (Bolton-Hunter reagent precursor)

Reagent 3-(4-Hydroxyphenyl) propionic acid *N*-hydroxysuccinimide ester was purchased from Sigma Aldrich, Korea. The radioiodination for HSA protein was performed following procedure published elsewhere[2]. Crude product was purified by PD-10 desalting column to give **[^{125}I]9** with 85% radiochemical yield and more than 99% radiochemical purity .

Biodistribution study of ^{125}I-HSA

For detail tissue distribution study 25 ICR male mice were purchased from Orientbio Co., Ltd (Republic of Korea). The animals were randomly divided into into 5 groups so that each group contains 5 mice. Each mouse was injected with^{125}I-HSA (**[^{125}I]7**), (37 KBq, 100μL in saline). At given time point animals were sacrificed and important organs and blood were harvested. Collected organs and blood were weighted and accumulated radioactivity was measured using gamma counter. The final tissue distribution data were represented as % injected dose per gram of blood or organ of mouse (%ID/g). All animal experiments were performed under the guidelines of Institutional Animal Ethical Committee. Same procedure was adopted for tissue distribution study of radioiodinated HSA protein **[^{125}I]8** and **[^{125}I]9**

Competitive reaction study

To determine the reaction preference ofoxidized **[^{125}I]1** toward BCN group two sets of experiment were conducted. In experiment (I) oxidized **[^{125}I]1** (7.4 MBq, 20μL in methanol) was incubated with a mixture containing BCN installed cRGD peptide (25μmol) and non modified HSA protein (25μmol) in 200μL of 1xPBS at rt (pH 7.5). The reaction was carried out for 30 min and subjected to centrifugation (4500 rpm, 12min, 4°C) using 50 kDa Amicon filter. The radioactivity associated to cRGD peptide (filtrate) or HSA protein (residue) was determine using gamma counter system. In experiment (II) oxidized **[^{125}I]1** (7.4 MBq, 20μL in methanol) was incubated with BCN installed HSA protein (25μmol) and non modified cRGD peptide (25μmol) under similar conditions used for experiment (I). Each experiment was repeated 3 times and products identity was confirmed by radio-HPLC or radio-TLC system. The final radiolabeling results were summarized in table S1.

Table S1 Radiolabeling results for competitive reaction studies

Experiment	Molecule	%RCY	^{125}I-molecule
I	c(RGDyK) 25μmol + HSA-BCN 25μmol	93-97	^{125}I-HSA
II	c(RGDyK)-BCN 25μmol + HAS 25μmol	90-94	^{125}I-c(RGDyK)

SPECT/ CT imaging of ^{125}I-HSA

^{125}I-HSA (**[^{125}I]7**) (5.5 MBq, 100 μL in saline), was intravenously injected to ICR mouse. At given time point (0.25, 1, 4, 16, 24 h) SPECT/CT images were acquired. For comparison SPECT/CT images were also acquired for **[^{125}I]8** using same procedure**.**

NMR spectra of intermediate precursors

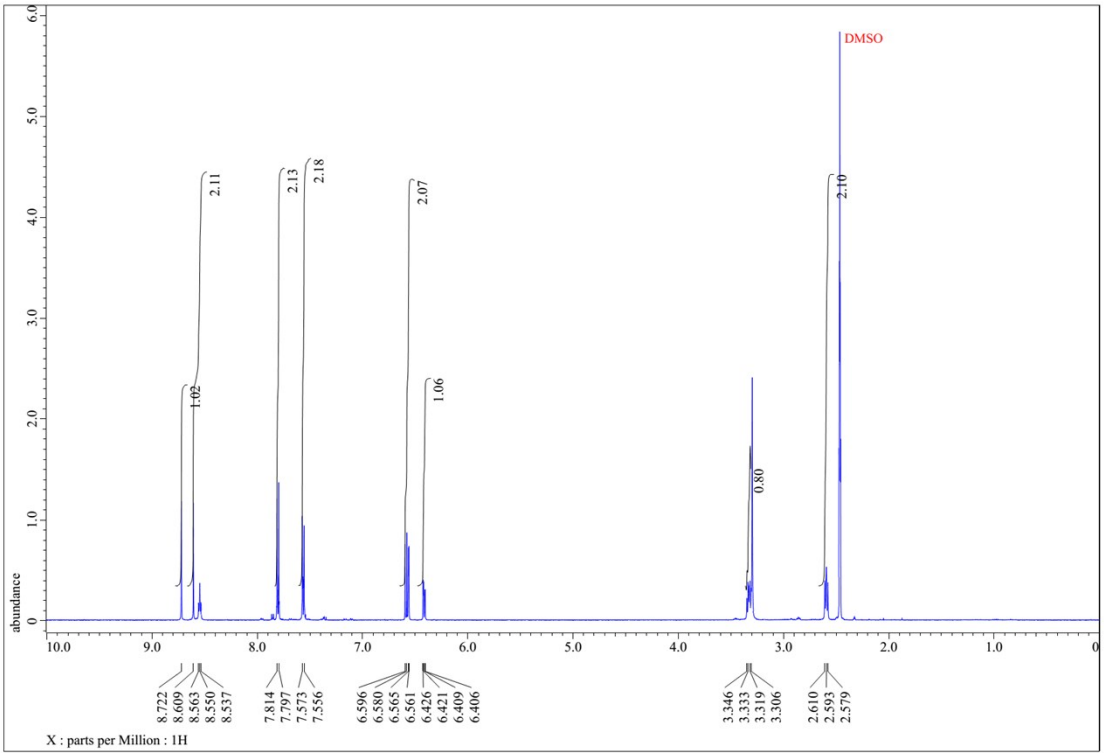


Figure S1 ^{1}H-NMR of the compound**1** in DMSO-d_6

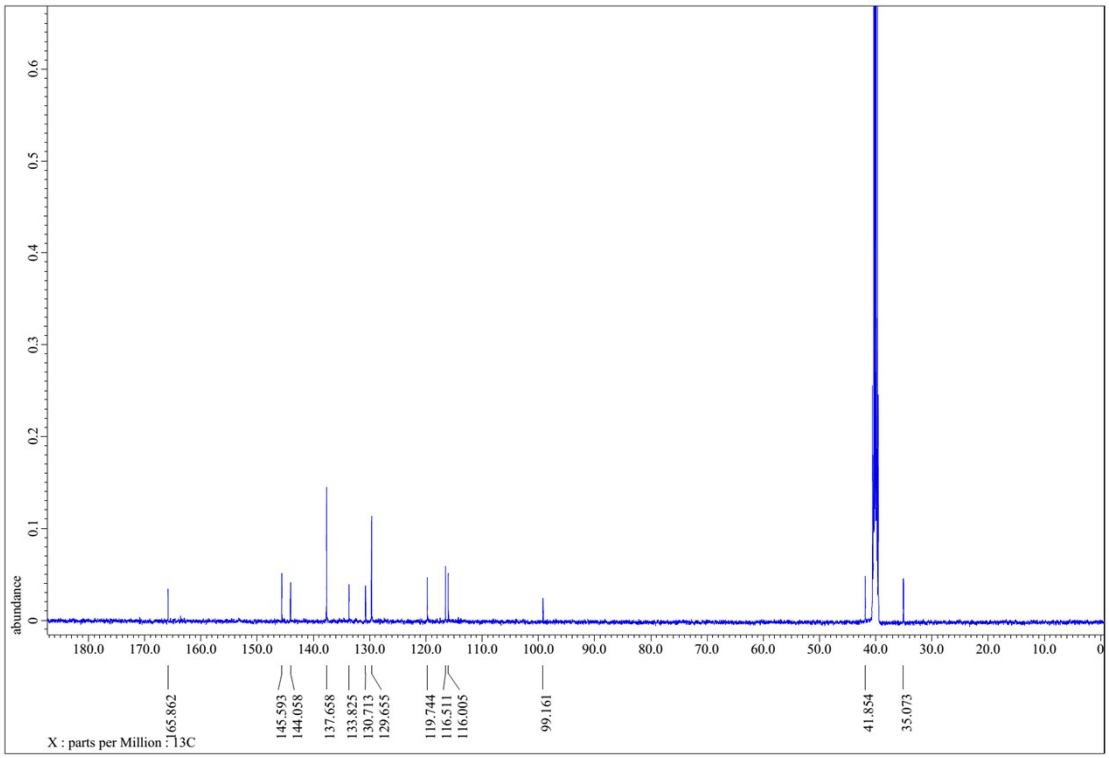


Figure S2 ^{13}C-NMR of the compound **1** in DMSO-d_6

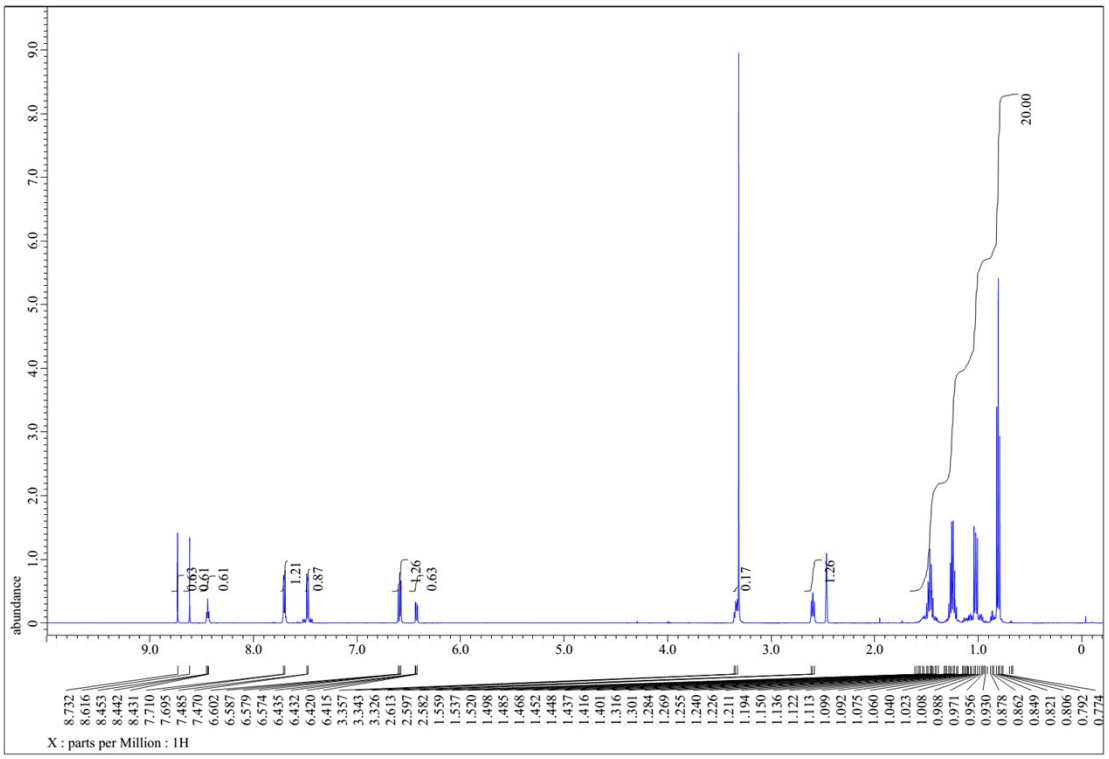


Figure S3 ^{1}H-NMR of the compound **2**in DMSO-d_6

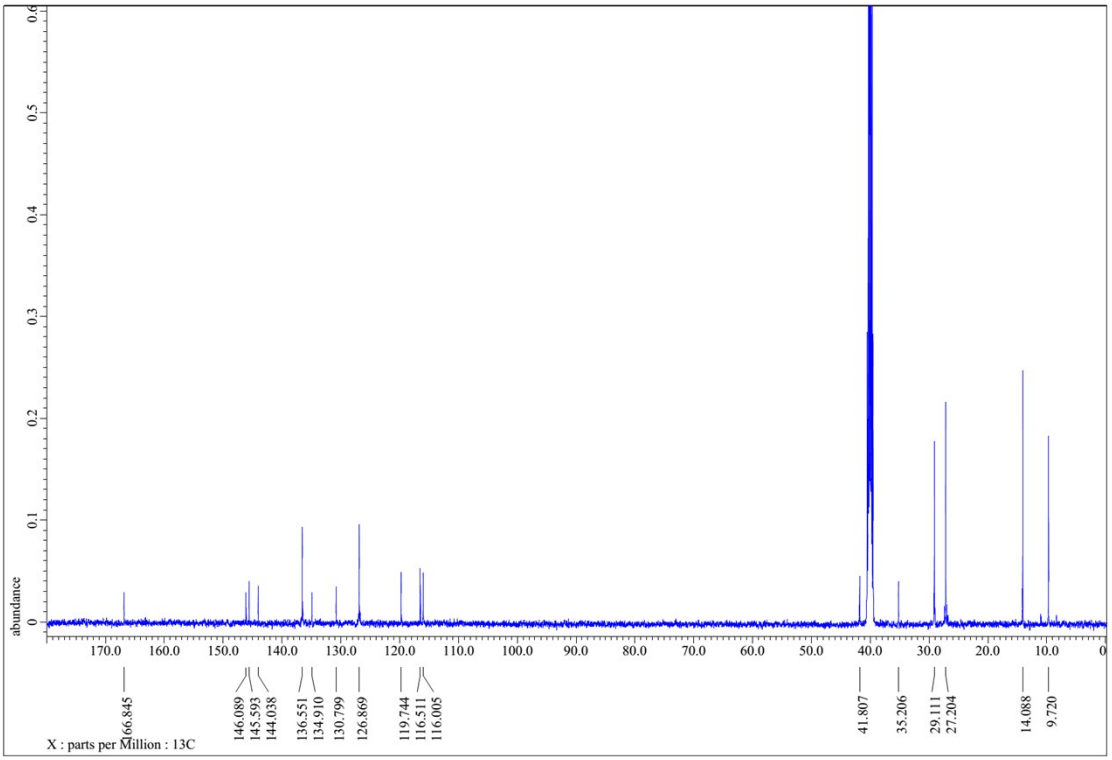


Figure S4 ^{13}C-NMR of the compound **2**in DMSO-d_6

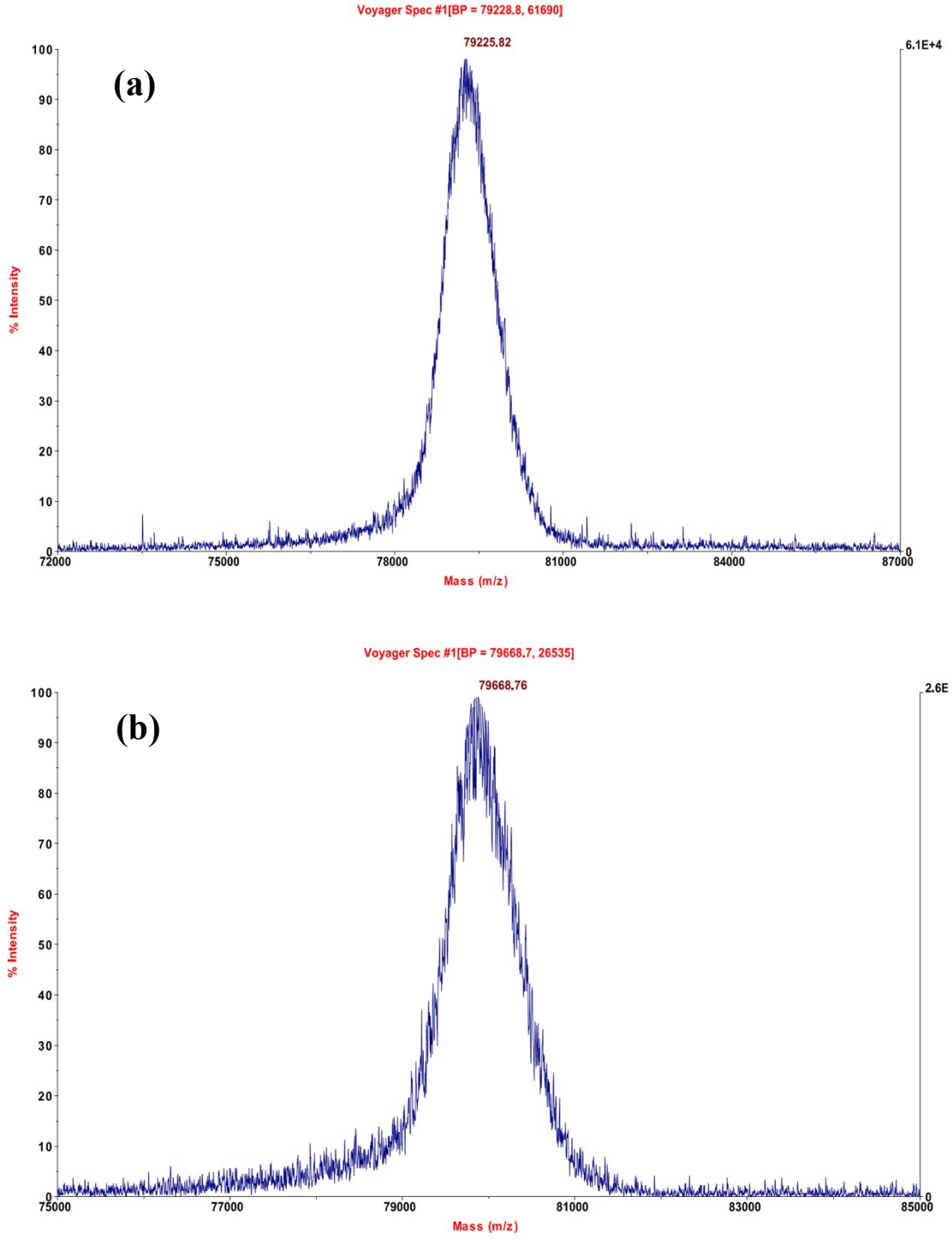


Figure S5 MALDI-TOF mass spectra: (a) non-modified hTf **6**, (b) BCN group installed hTf **6a**

- Number of BCN group per transferrin molecule = (molecular weight of BCN conjugated hTf – molecular weight of hTf) / molecular weight of BCN linker = (79225.82 – 79668.76) / 177 = 2.5

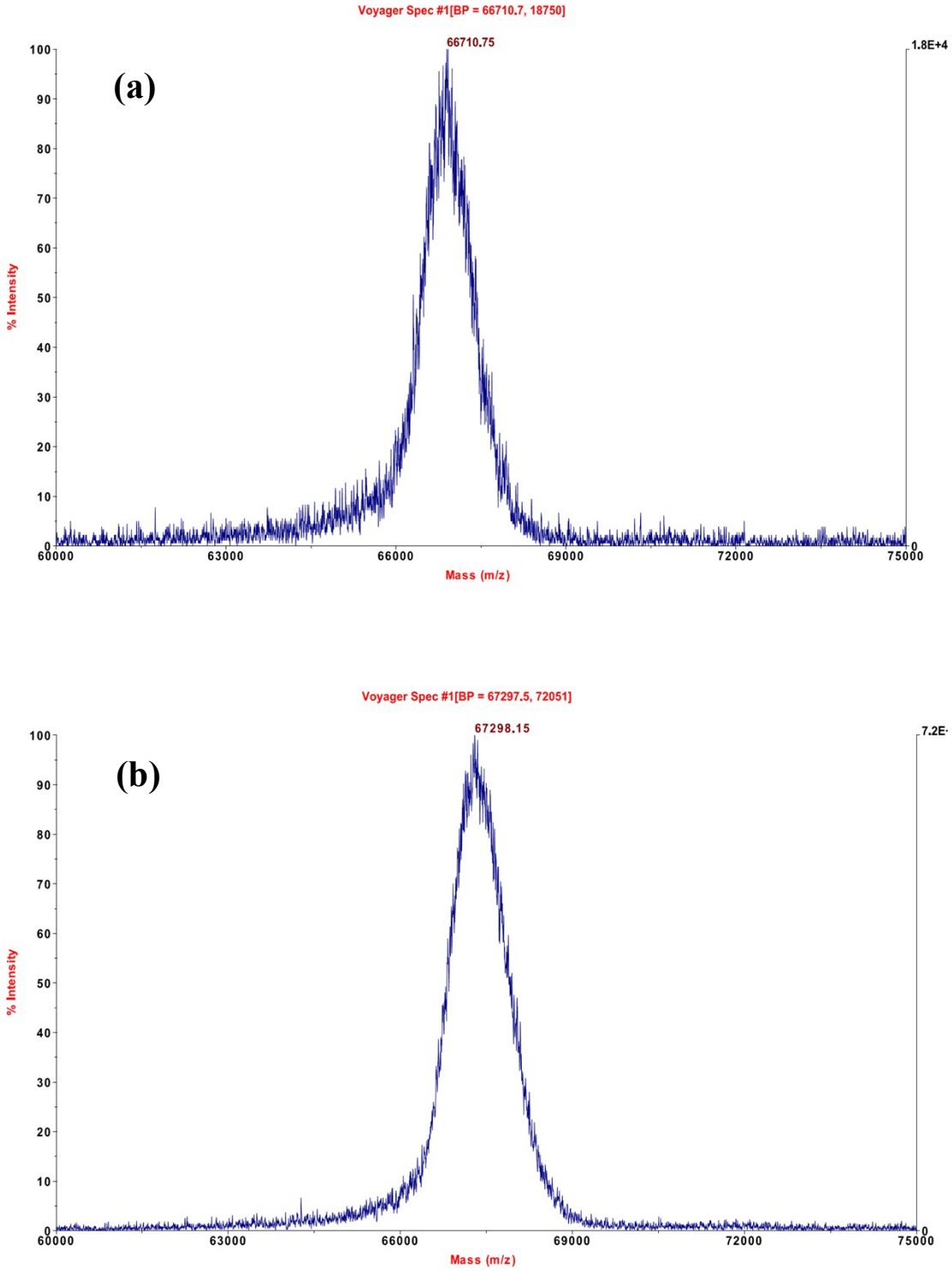


Figure S6 MALDI-TOF mass spectra: (a) non-modified HSA **7**, (b) BCN group installed HSA **7a**

- Number of BCN group attached per HSA molecule = (molecular weight of BCN conjugated HSA – molecular weight of HSA) / molecular weight of BCN linker = (67298.15 – 66710.75) / 177 = 3.3

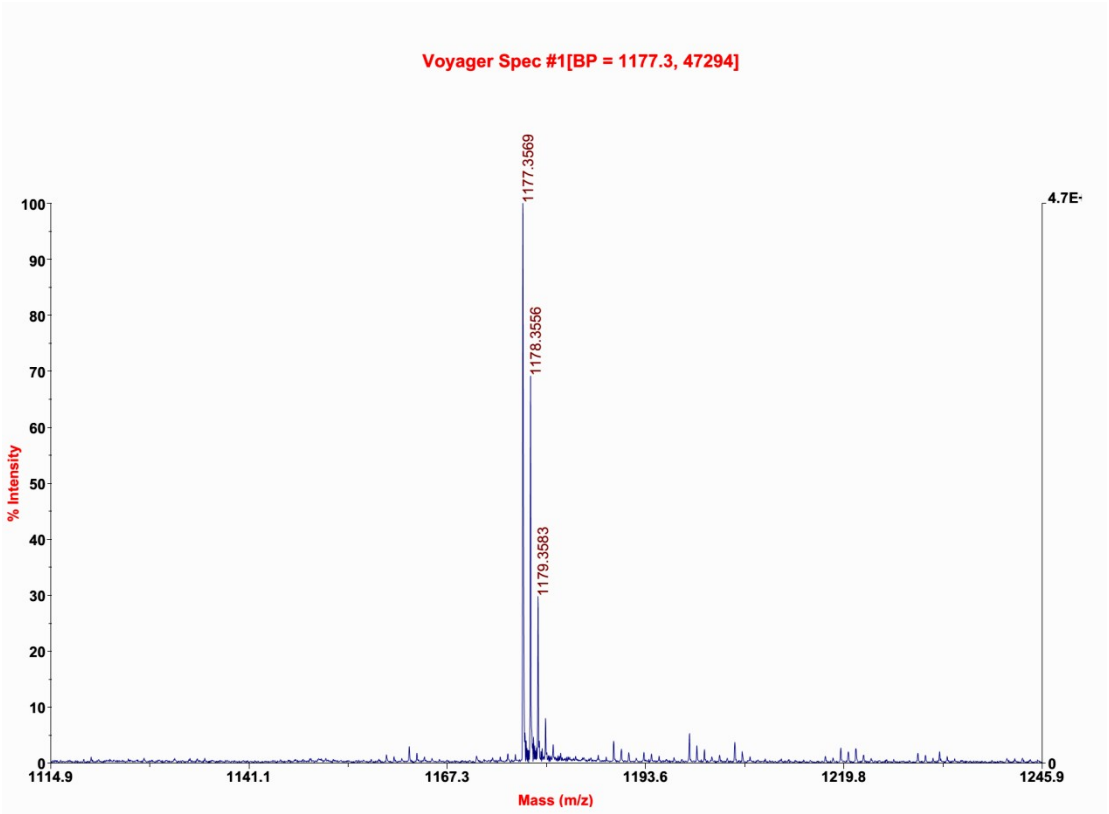


Figure S7 MALDI-TOF mass spectrum of iodinated cRGD peptide **5b**

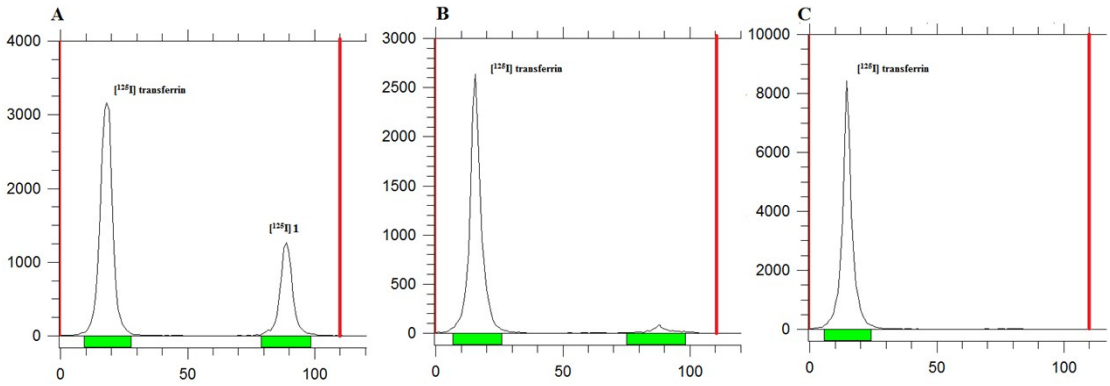


Figure S8 Radio-TLC analysis for reaction between BCN installed TF protein **6a** and **[^{125}I]1** at 25 ^{o}C in 10% methanol in 1xPBS; (A) **6a** (5 μmol) and oxidized **[^{125}I]1** 7.4 MBq for 15 min; (B) **6a** (10 μM) and oxidized **[^{125}I]1** 7.4 MBq for 15 min;(C) **6a** (25 μM) and oxidized **[^{125}I]1** 7.4 MBq for 15 min; (eluent: Ethyl acetate)

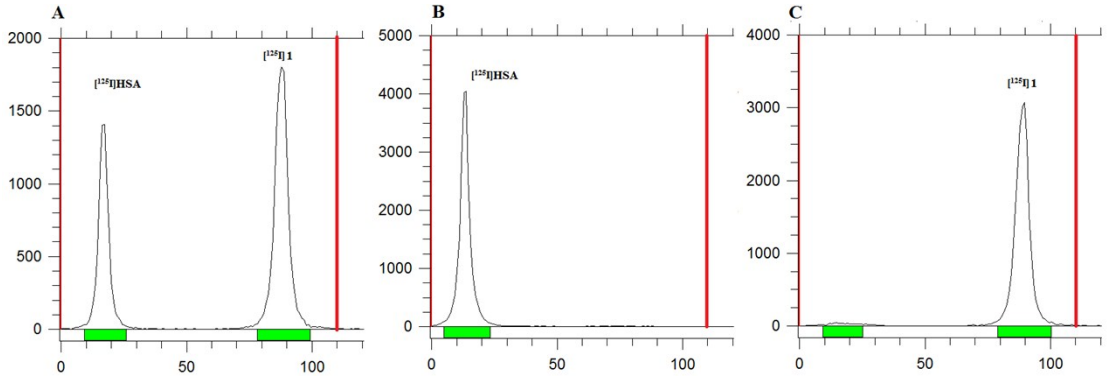


Figure S9 Radio-TLC analysis for reaction between BCN installed HSA protein **7a** and **[^{125}I]1** at 25 °C in 10% methanol in 1xPBS; (A) **7a** (25 µmol) and oxidized **[^{125}I]1** 7.4 MBq for 5 min; (B) **7a** (25 µmol) and oxidized **[^{125}I]1** 7.4 MBq for 15 min;(C) **7a** (25 µmol) and non-oxidized **[^{125}I]1** 7.4MBq for 15 min; (eluent: Ethyl acetate)

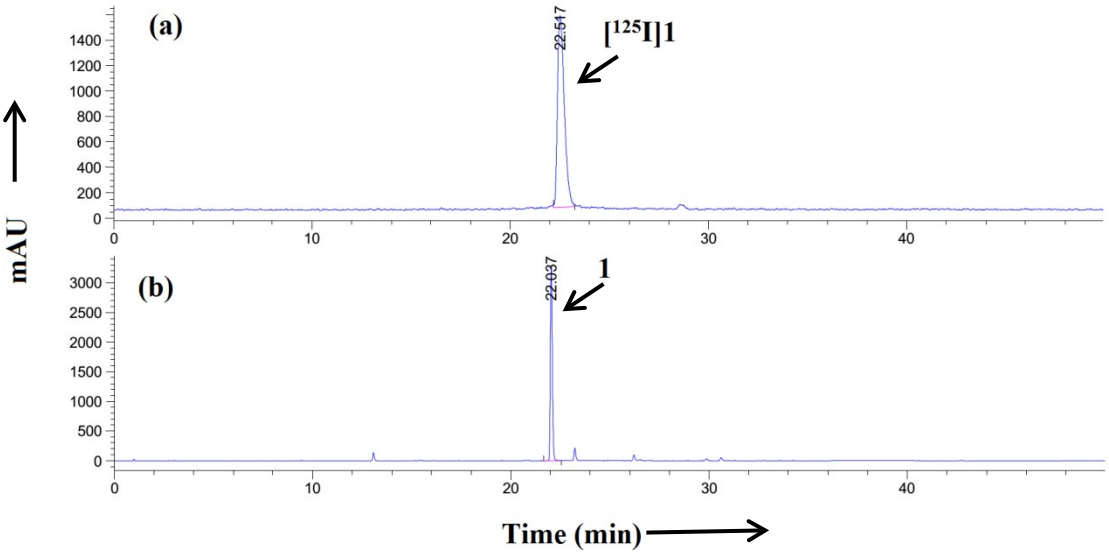


Figure S10 HPLC chromatogram (a) radio chromatogram of purified **[^{125}I]1** (b) UV chromatogram of standard compound **1**

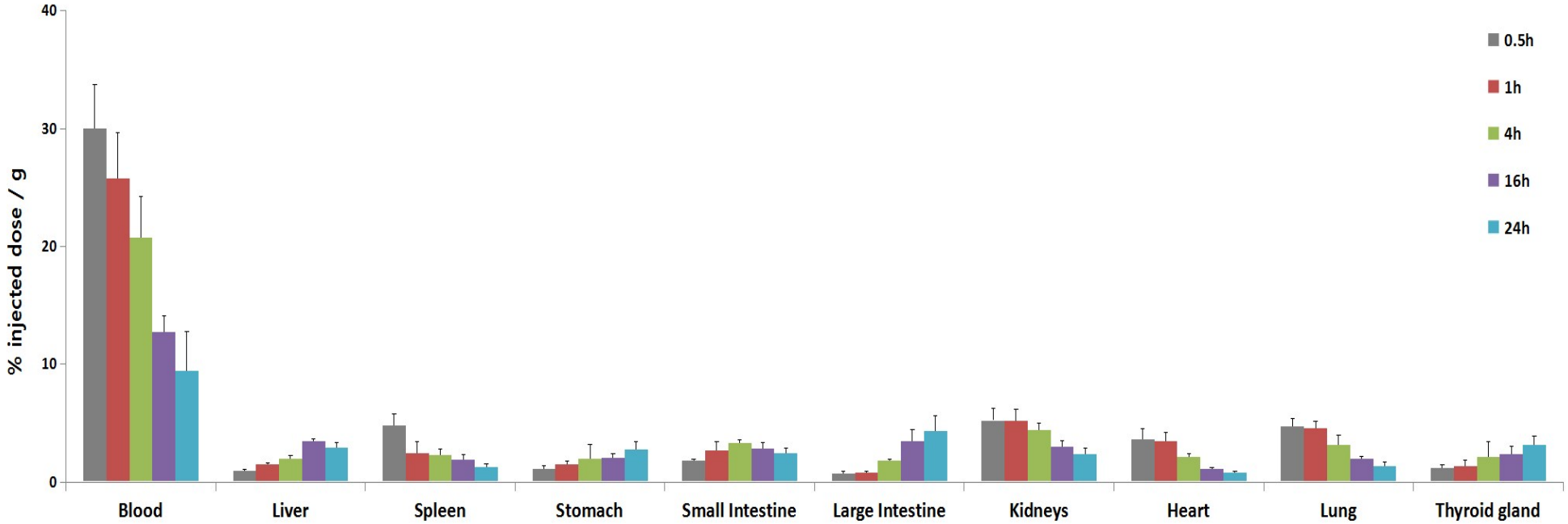


Figure S11 Biodistribution results of radioiodinated HSA **[^{125}I]7,** n=5 mice per group.

Table S2 Biodistribution results of radioiodinated HSA **[^{125}I]8**

Time (h)	%ID/g [a]									
	Blood	Liver	Spleen	Stomach	Small Int.	Large Int.	Kidneys	Heart	Lungs	Thyroid
0.5	31.0±2.02	4.98±0.55	4.32±1.46	1.55±2.02	1.77±0.16	0.96±0.21	7.52±0.96	6.35±1.32	9.33±1.20	3.10±1.11
1.0	30.1±8.47	3.10±1.08	3.06±1.59	2.02±0.99	1.78±0.98	0.88±0.44	5.10±2.22	4.55±2.22	7.21±2.55	4.93±3.20
4.0	12.3±3.04	2.72±1.47	2.10±0.56	10.3±1.08	1.66±0.42	1.05±0.29	3.55±1.25	3.67±1.68	5.25±1.44	59.21±18.50
16	11.2±1.07	2.16±0.30	1.79±0.47	18.4±1.55	1.70±0.21	1.44±0.26	3.10±0.32	3.22±1.52	6.49±3.21	177.65±53.2
24	9.48±1.23	1.99±0.33	1.67±0.32	25.2±4.22	1.75±0.40	1.47±0.29	2.93±0.43	2.86±1.10	5.21±1.33	320.10±78.3

[a] n=5 mice per group; % injected dose /gram of organ.

Table S3 Biodistribution results of radioiodinated HSA **[^{125}I]9**

Time (h)	%ID/g [a]									
	Blood	Liver	Spleen	Stomach	Small Int.	Large Int.	Kidneys	Heart	Lungs	Thyroid
0.5	24.0±1.82	4.17±0.56	3.55±0.66	1.61±0.33	1.92±0.32	0.74±0.12	5.88±0.22	6.12±1.22	7.66±1.22	2.44±0.35
1.0	22.9±1.25	3.22±0.49	3.21±0.81	2.00±0.90	2.65±0.26	1.31±0.17	6.22±0.57	5.44±0.77	6.66±0.87	3.38±1.61
4.0	15.8±0.95	3.53±0.62	2.88±0.44	8.21±3.11	2.75±0.32	2.01±0.27	6.31±0.76	4.28±0.86	6.25±2.31	50.21±9.30
16	7.41±0.78	2.42±0.28	1.58±0.20	10.3±2.41	2.85±0.30	2.74±0.23	3.88±0.33	2.32±0.47	4.22±0.47	130.65±45.3
24	5.51±0.48	1.85±0.45	1.16±0.17	19.1±3.95	2.80±0.29	1.99±0.10	4.84±0.18	2.01±1.11	3.85±2.32	298.10±95.0

[a] n=5 mice per group; % injected dose /gram of organ.

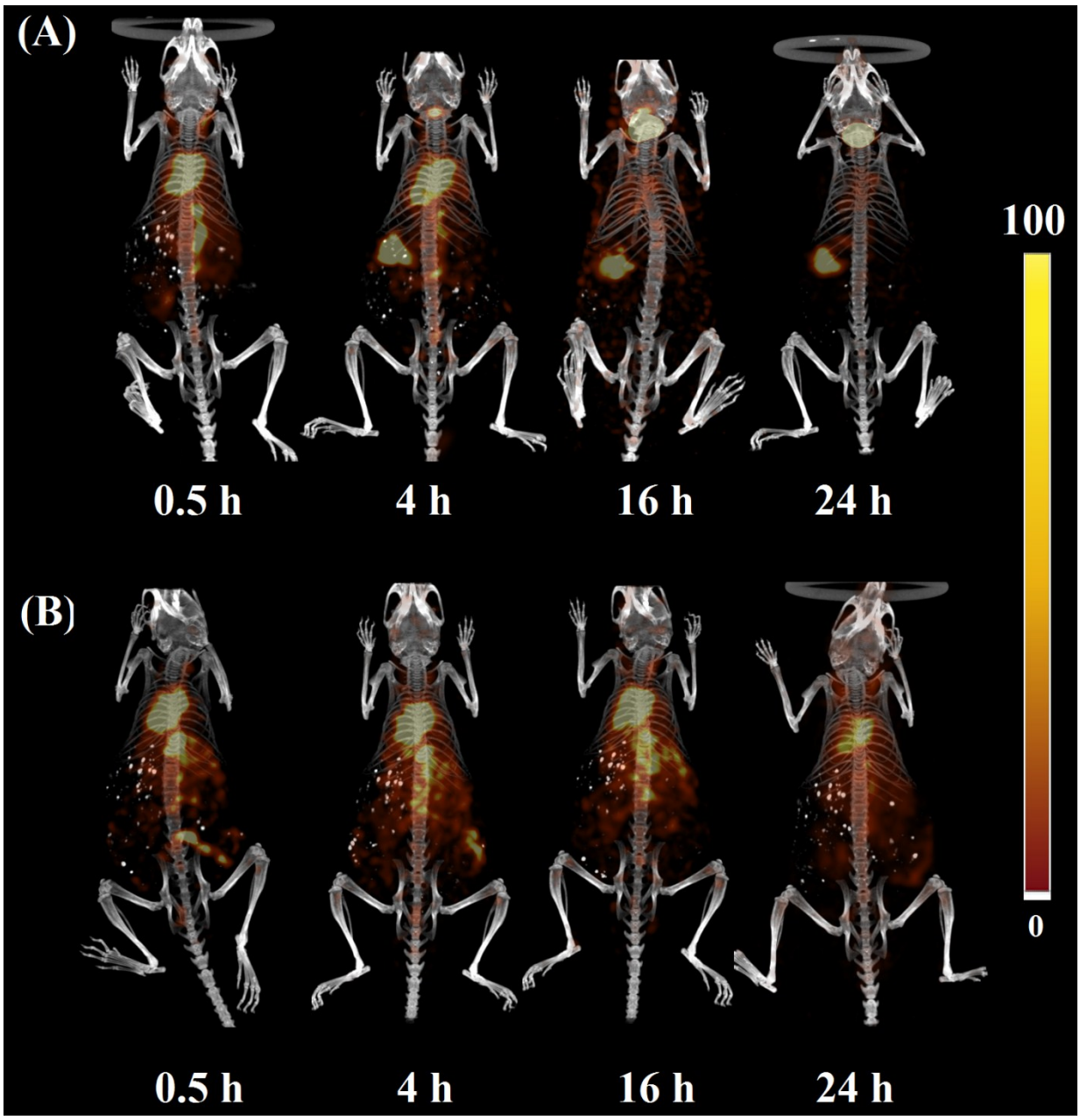


Figure S12 Representative SPECT/CT images of radioiodinated HSA (5.5 MBq) IV injection to ICR male mice (A) direct radioiodination **[^{125}I]8** (B) SPOCQ reaction **[^{125}I]7**

***In vitro* stability [^{125}I] 1**

The purified **[^{125}I] 1** (100µL, 3.7 MBq) was incubated with 900µL of mouse serum or PBS (pH 7.5) or saline at 37°C, pH 7.5. At each time point (0.5, 4, 16, 24, 48h) 10 µL of each mixture were withdrawn and analyzed using radio-HPLC or radio-TLC system.

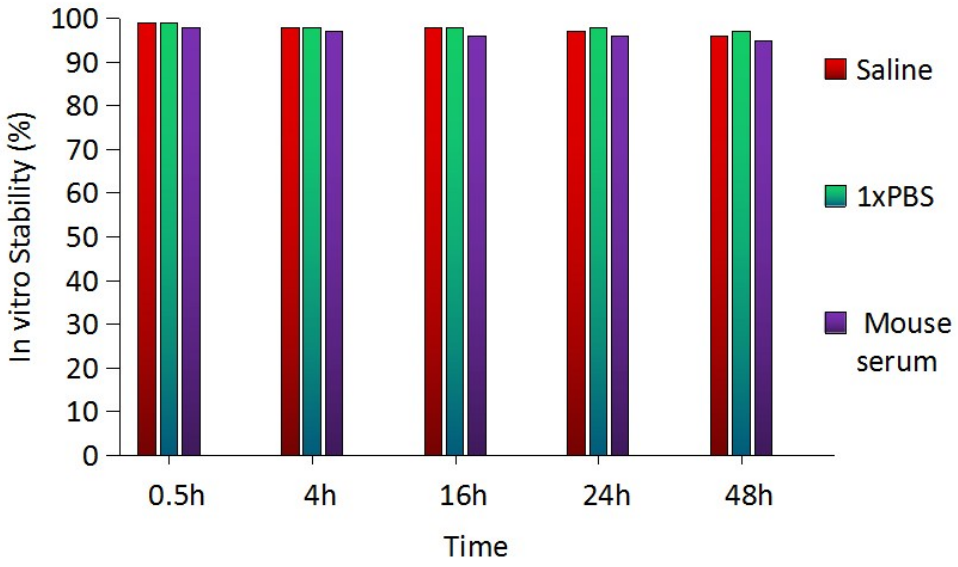


Figure S13 *In vitro* stability **[^{125}I] 1** in various media under physiological conditions

Comparison of thyroid uptake of free radioiodine from [^{125}I]7, [^{125}I]8 or [^{125}I]9

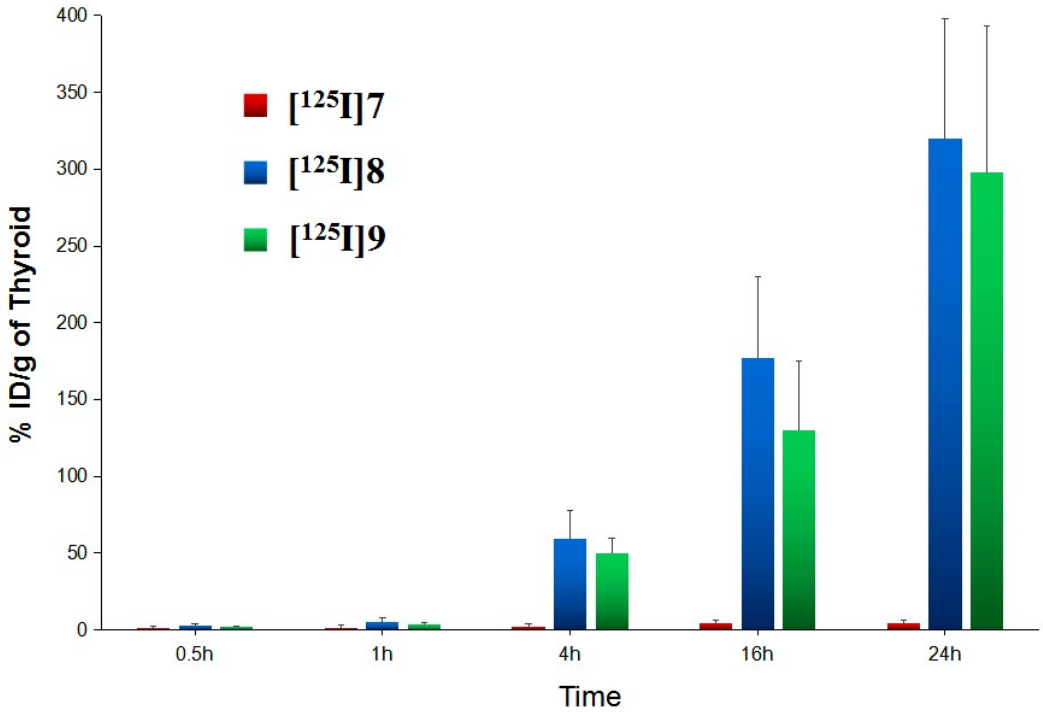


Figure S14 *In vivo* deiodination and uptake of free **^{125}I** in thyroid from radioiodinated HSA **[^{125}I]7, [^{125}I]8** or **[^{125}I]9.** The radioiodinated HSA **[^{125}I]7** (SPOCQ based radioiodination) found to be more stable as compared with other two radioiodination strategies.

Reference

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