Supporting Information

Tetrazine-TCO Ligation: A Simple Approach to Improve Tumor Uptake through Enhanced Blood Circulation

Mengzhe Wang[†], Hui Wang[†], Crystal Q. Niu,[†] Tao Zhang,[†] Zhanhong Wu[†], and Zibo Li[†*]

[†] Department of Radiology and Biomedical Research Imaging Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, 27599

Correspondence should be sent to:

Zibo Li, PhD

Department of Radiology, Biomedical Research Imaging Center, and Lineberger Comprehensive Cancer Center
University of North Carolina at Chapel Hill, 125 Mason Farm Rd, Chapel Hill, NC 27599

Email: zibo_li@med.unc.edu

Running Title: Enhanced blood circulation of PET tracer

Contents

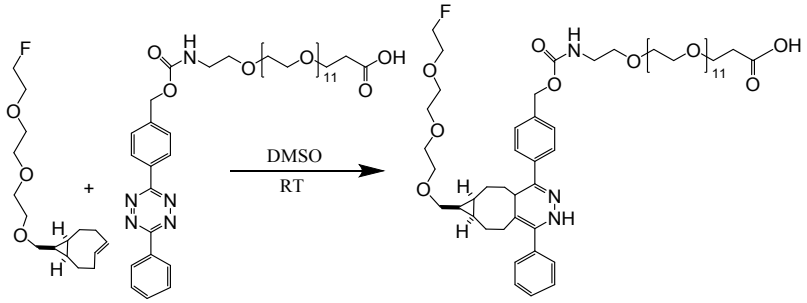
S1. General Considerations

All commercially available chemicals were purchased and used without further purification. Kinetex 5μ C18 column (250 x 4.6mm) was used as reversed-phase HPLC column. Mobile phase A was water with 0.1% TFA and mobile phase B was acetonitrile with 0.1% TFA. For HPLC program 1, 95% A and 5% B was maintained at 0 to 2min and ramped to 5% A and 95% B at 22min. For HPLC program 2, 75% A and 25% B was maintained at 0 to 2min and ramped to 35% A and 65% B at 22min.

High-resolution mass spectrums were obtained on a ThermoScientific Q Exactive HF-X system. MALDI-TOF data was taken on an AB Sciex 5800 MALDI-TOF/TOF system.

The *trans*-cyclooctenes TCO-F, sTCO-F, dTCO-F, their ^{18}F versions, DiPhTz-RGDyk, ^{19}F-sTCO-DiPhTz-RGDyK, DiPhTz-NT and ^{19}F-DiPhTz-NT were synthesized according to methods previously published.[1-4]

S2. Synthesis



^{19}F-sTCO-DiPhTz

DiPhTz (1 μL from a 10 mM solution in DMSO) was mixed with ^{19}F-sTCO (1 μL from a 24.5 mM solution in acetonitrile) at room temperature for 1 min. The crude reaction was analyzed and purified by HPLC using program 1. The product was eluted off at 18.5 min with program 1 and the yield is 53.6%. HRMS (ESI) *m/z*: $[M+H]^+$ $C_{59}H_{92}FN_3O_{19}$ is 1164.6334; found 1164.6230

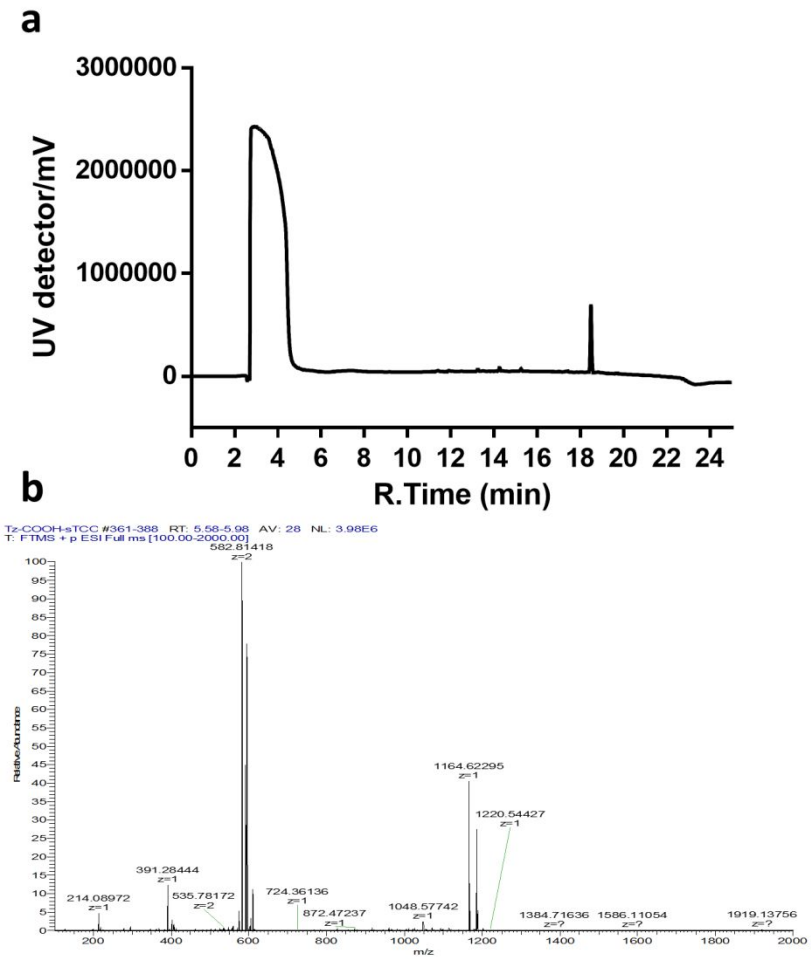
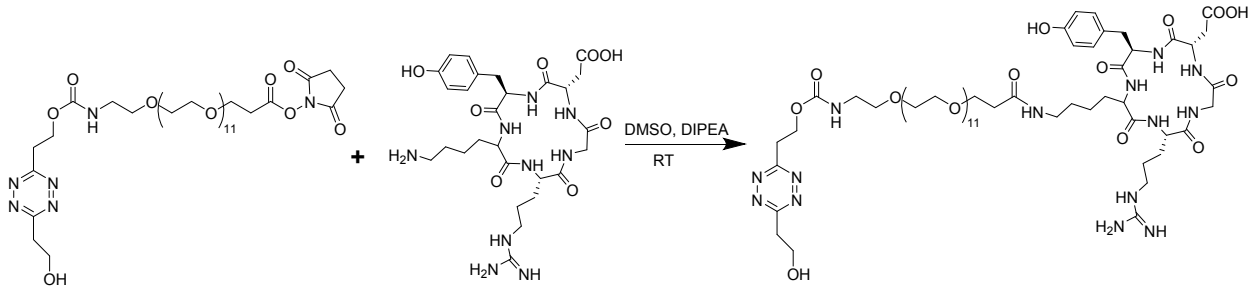
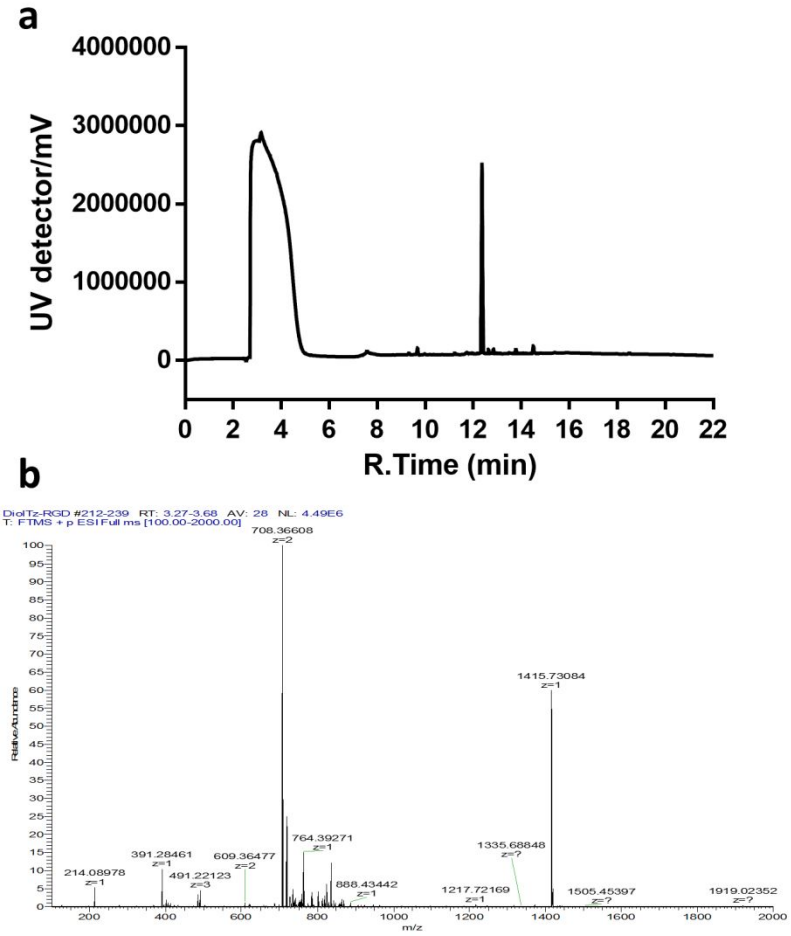


Figure S1 (a) HPLC profile and (b) HRMS of purified ^{19}F-sTCO-DiPhTz



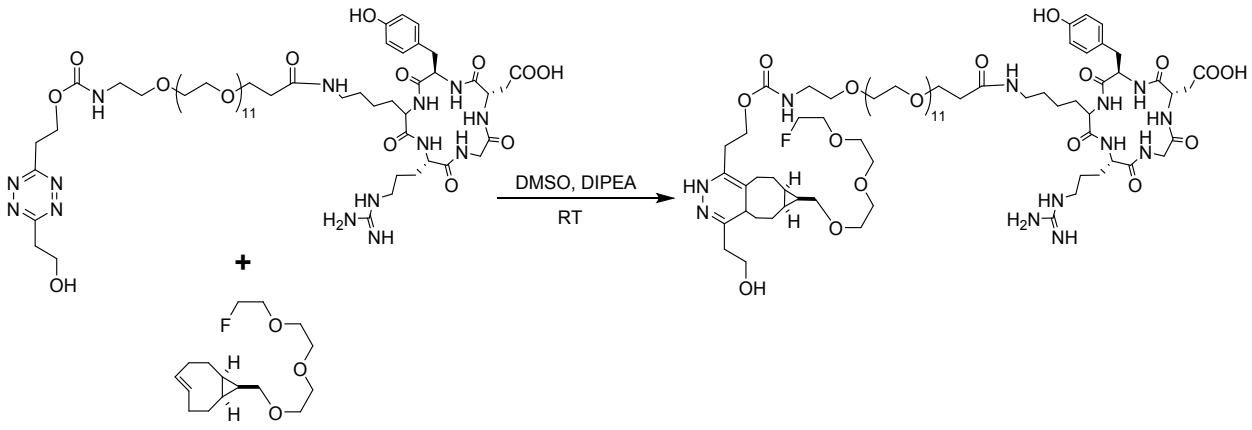
DiolTz-RGDyK

RGDyK (1mg, 1.6 μmol) was dissolved in anhydrous DMSO (30 μL), DiolTz-NHS (1.5 eq) was dissolved in 20 μL anhydrous DMSO before adding to the RGDyK solution. 5 μL of



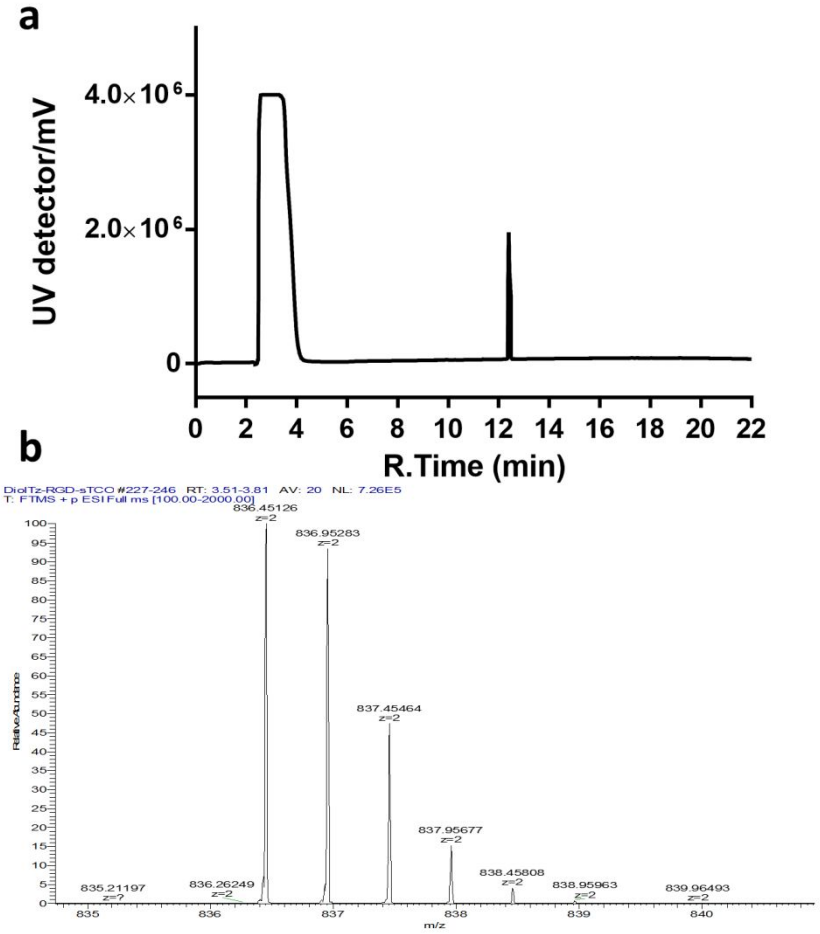
diisopropylethylamine was added to the mixture and incubated at room temperature for 2h. The reaction crude was purified by HPLC using program 1. The product was eluted off at 12.2 min with program 1 and the yield is 84.0%. HRMS (ESI) *m/z*: $[M+2H]^+$ $C_{61}H_{104}N_{14}O_{24}$ is 708.3674; found 708.3661

Figure S2 (a) HPLC profile and (b) HRMS of purified DiolTz-RGDyK



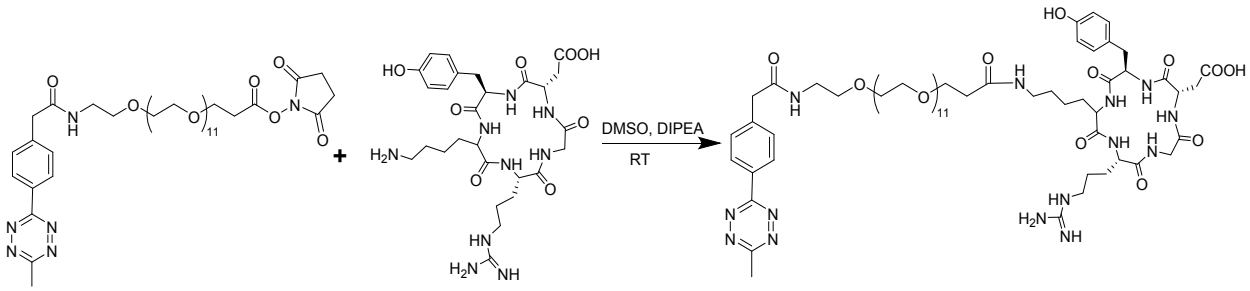
^{19}F-sTCO-DiolTz-RGDyK

DiolTz-RGDyK (1 μL from a 10 mM solution in DMSO) and ^{19}F-sTCO (1 μL from a 24.5 mM solution in acetonitrile) was mixed and incubated at room temperature for 1 min. The crude reaction was analyzed and purified by HPLC using program 1. The product was eluted off at



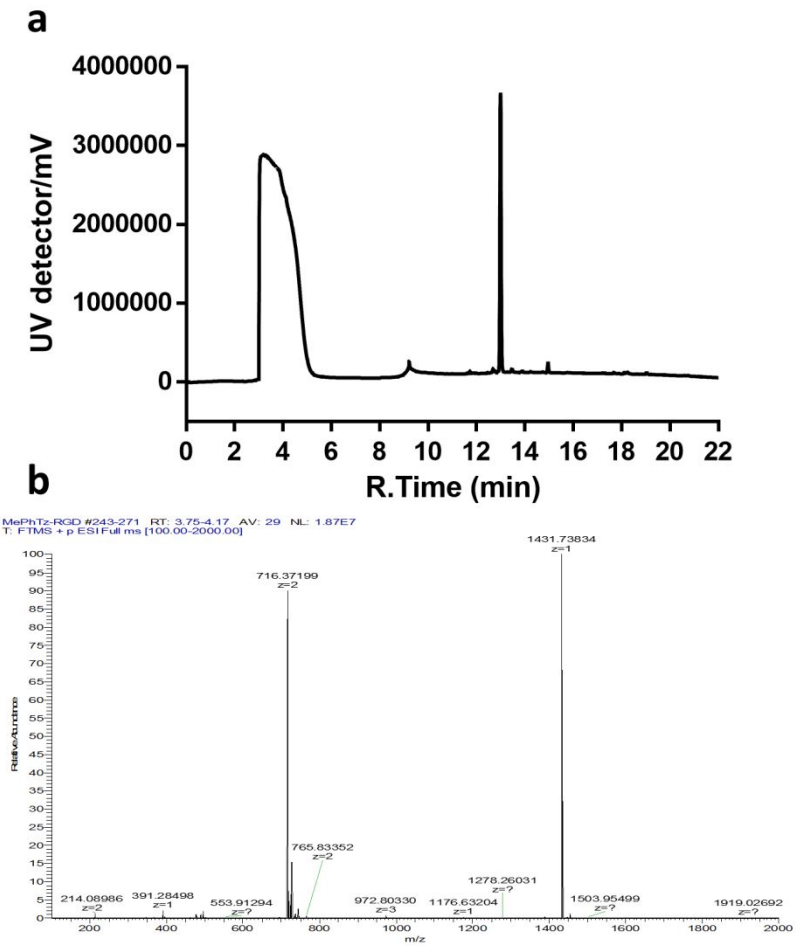
12.4 min with program 1 and the yield is 54.6%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ $C_{77}H_{131}FN_{12}O_{27}$ is 837.4616; found 837.4546

Figure S3 (a) HPLC profile and (b) HRMS of purified ^{19}F-sTCO-DiolTz-RGDyK



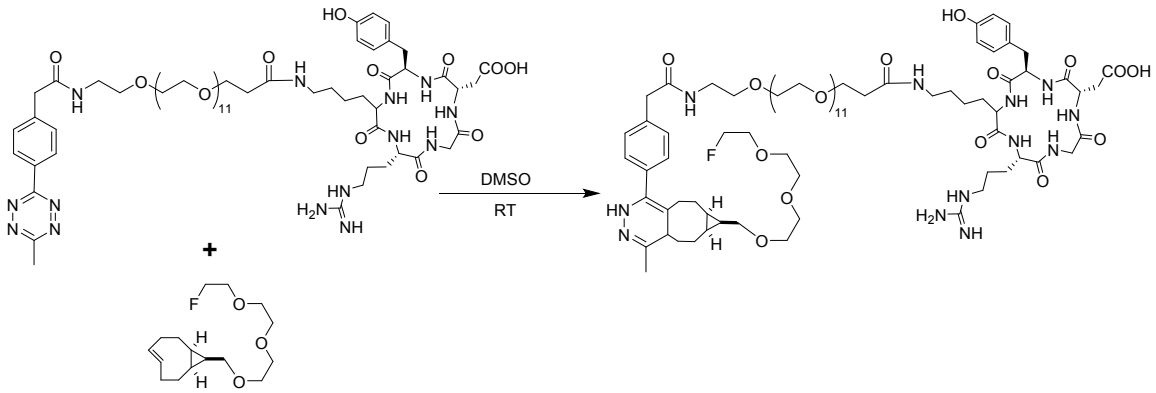
MePhTz-RGDyK

RGDyK (1mg, 1.6 μmol) was dissolved in anhydrous DMSO (30 μL), MePhTz-NHS (1.5 eq) was dissolved in 20 μL anhydrous DMSO before adding to the RGDyK solution. 5 μL of



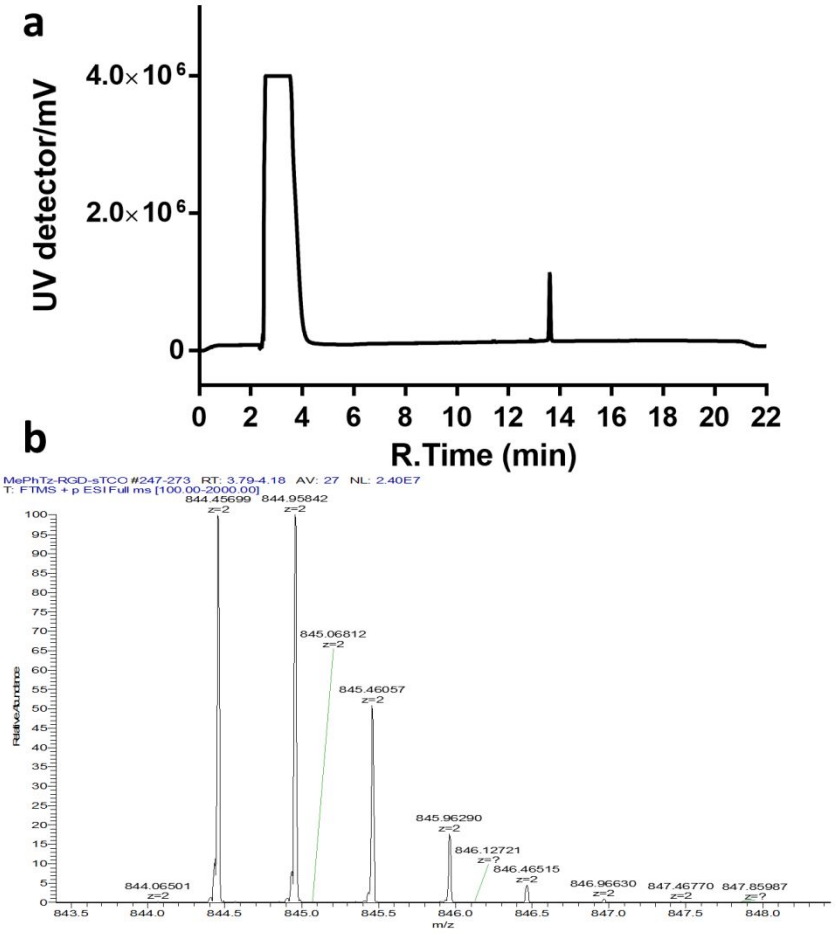
diisopropylethylamine was added to the mixture and incubated at room temperature for 2h. The reaction crude was purified by HPLC using program 1. The product was eluted off at 13.0 min with program 1 and the yield is 78.7%. HRMS (ESI) *m/z*: $[M+H]^+$ $C_{65}H_{104}N_{14}O_{22}$ is 716.3725; found 716.3720

Figure S4 (a) HPLC profile and (b) HRMS of purified MePhTz-RGDyK



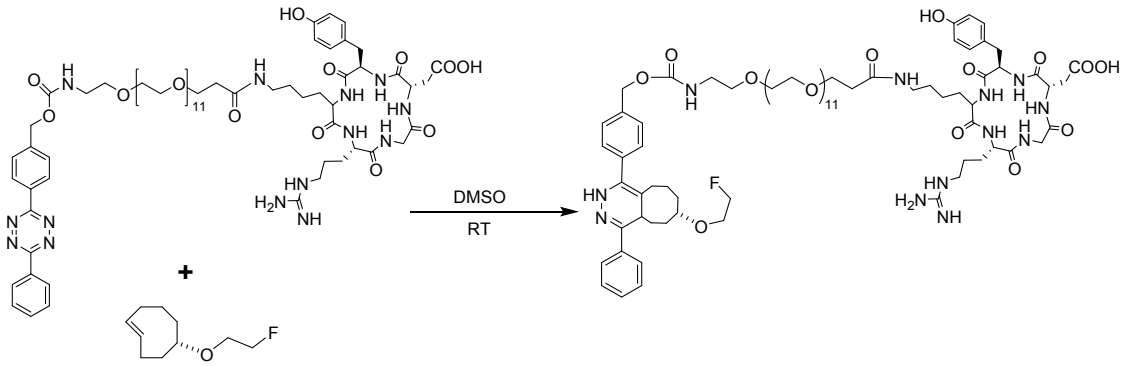
^{19}F-sTCO-MePhTz-RGDyK

MePhTz-RGDyK (1 μL from a 10 mM solution in DMSO) and ^{19}F-sTCO (1 μL from a 24.5 mM solution in acetonitrile) was mixed and incubated at room temperature for 1 min. The crude



reaction was analyzed and purified by HPLC using program 1. The product was eluted off at 13.6 min with program 1 and the yield is 71.0%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ $C_{131}H_{131}FN_{12}O_{25}$ is 845.4666; found 845.4606

Figure S5 (a) HPLC profile and (b) HRMS of purified ^{19}F-sTCO-MePhTz-RGDyK



^{19}F-TCO-DiPhTz-RGDyK

DiPhTz-RGDyK (1 μL from a 10 mM solution in DMSO) and ^{19}F-TCO (1 μL from a 58.1 mM solution in acetonitrile) was mixed at room temperature for 1 min. The crude reaction was analyzed and purified by HPLC using program 1. The product was eluted off at 14.9 min with program 1 and the yield is 55.6%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ $C_{80}H_{123}FN_{12}O_{24}$ is 827.4378; found 827.4314

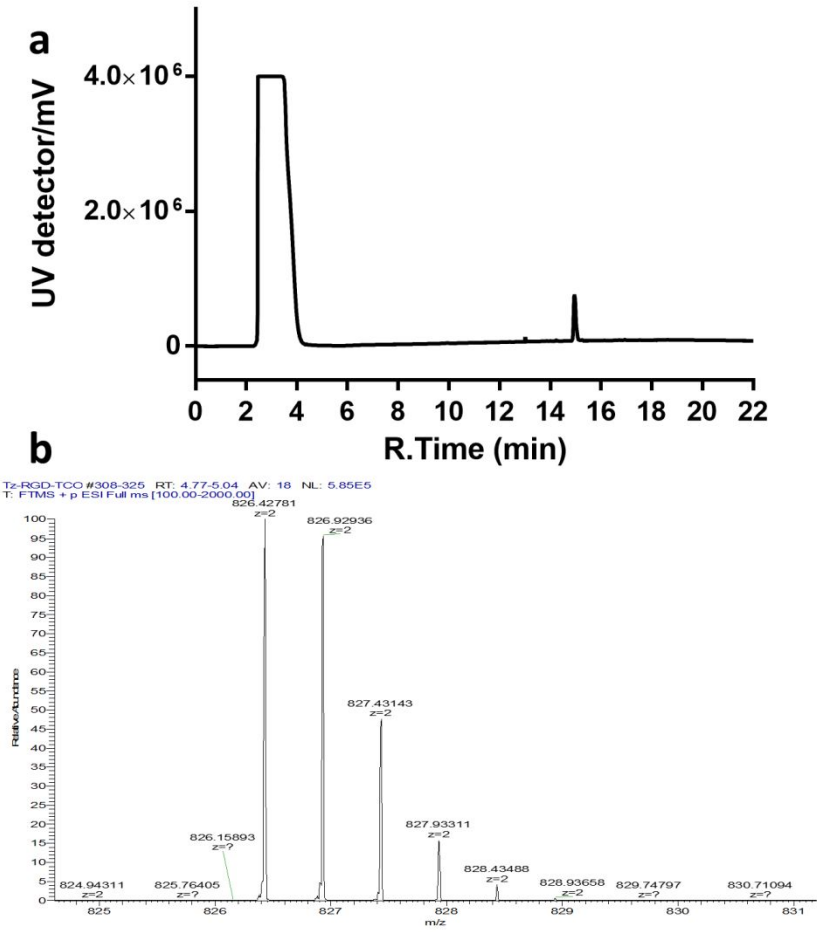
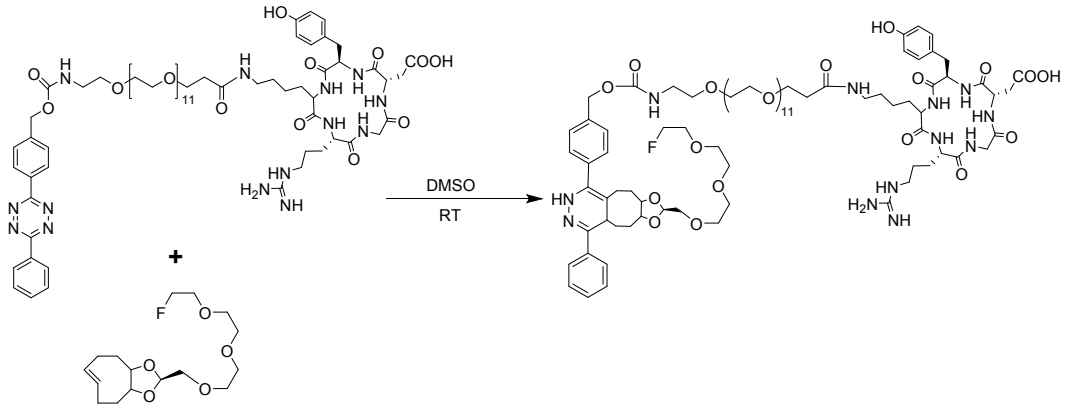


Figure S6 (a) HPLC profile and (b) HRMS of purified ^{19}F-TCO-DiPhTz-RGDyK



^{19}F-dTCO-DiPhTz-RGDyK

DiPhTz-RGDyK (1 μL from a 10 mM solution in DMSO) was added into ^{19}F-dTCO (1 μL from a 31.4 mM solution in acetonitrile) and incubated at room temperature for 1 min. The crude reaction was analyzed and purified by HPLC using program 1. The product was eluted off at 14.9 min with program 1 and the yield is 63.8%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ $C_{86}H_{133}FN_{12}O_{28}$ is 900.4668; found 900.4616

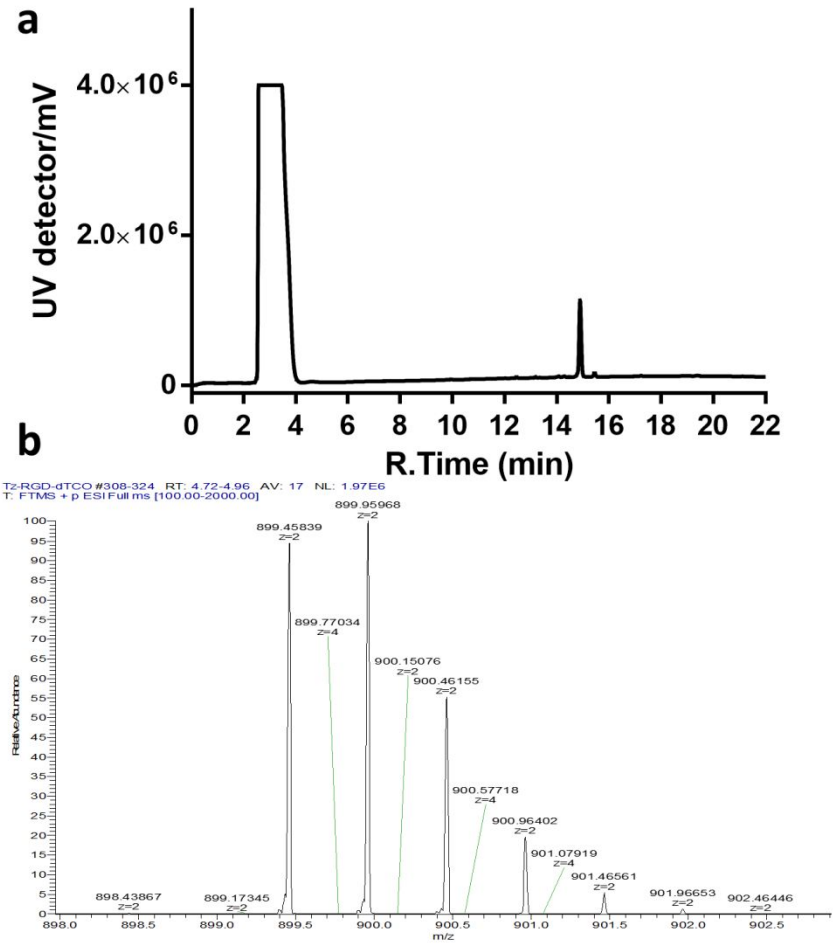
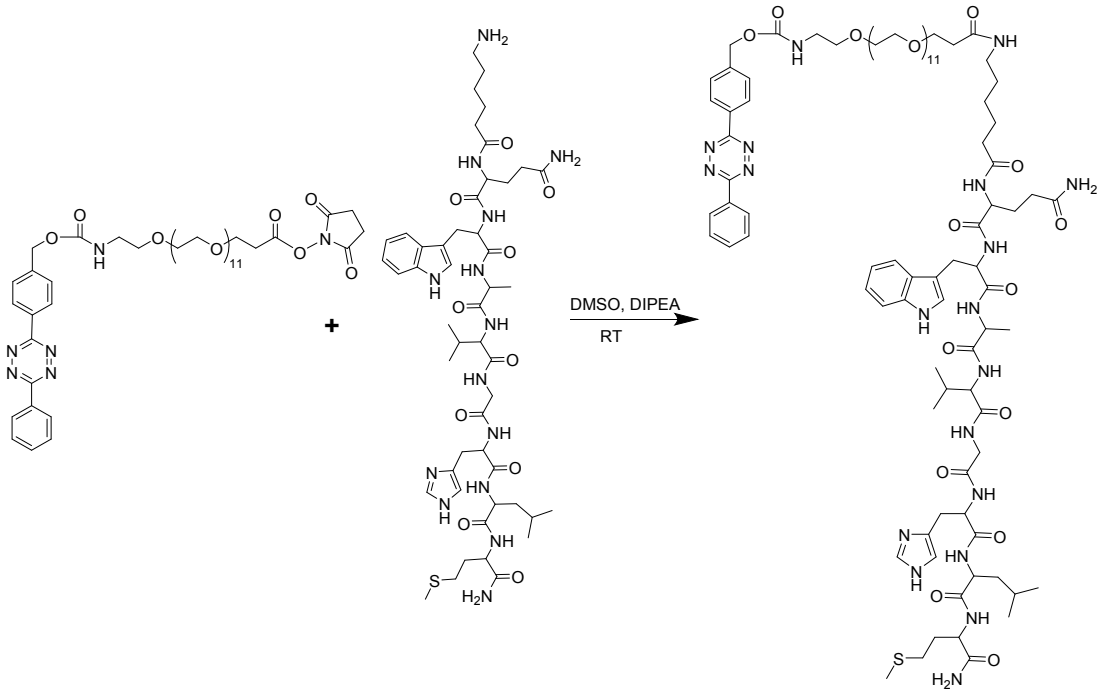


Figure S7 (a) HPLC profile and (b) HRMS of purified ^{19}F-dTCO-DiPhTz-RGDyK



DiPhTz-BBN

Bombesin (1mg, 0.9 μmol) was dissolved in 30 μL anhydrous DMSO and DiPhTz-NHS (1.5 eq) was dissolved in another 20 μL of anhydrous DMSO solution. The two solutions were mixed and added into 5 μL of diisopropylethylamine. The mixture was incubated at room temperature for 2h. The reaction crude was purified by HPLC using program 1. The product was eluted off at 15.5 min with program 1 and the yield is 85.8%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ $C_{92}H_{141}N_{19}O_{25}S$ is 972.0033; found 972.0039

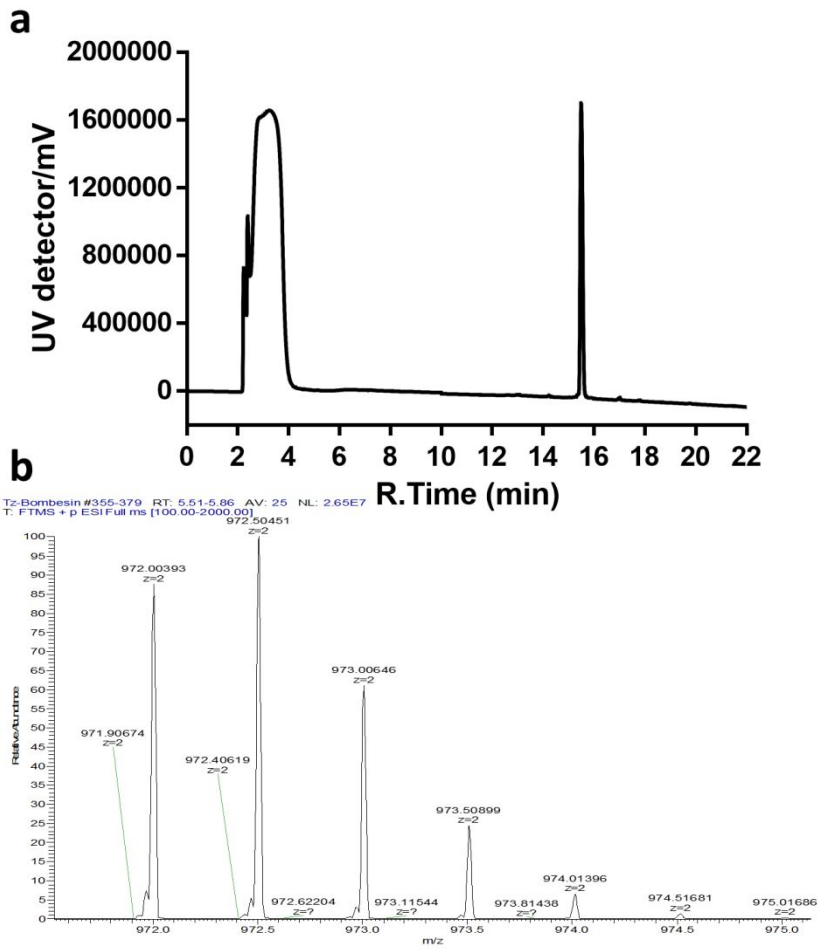
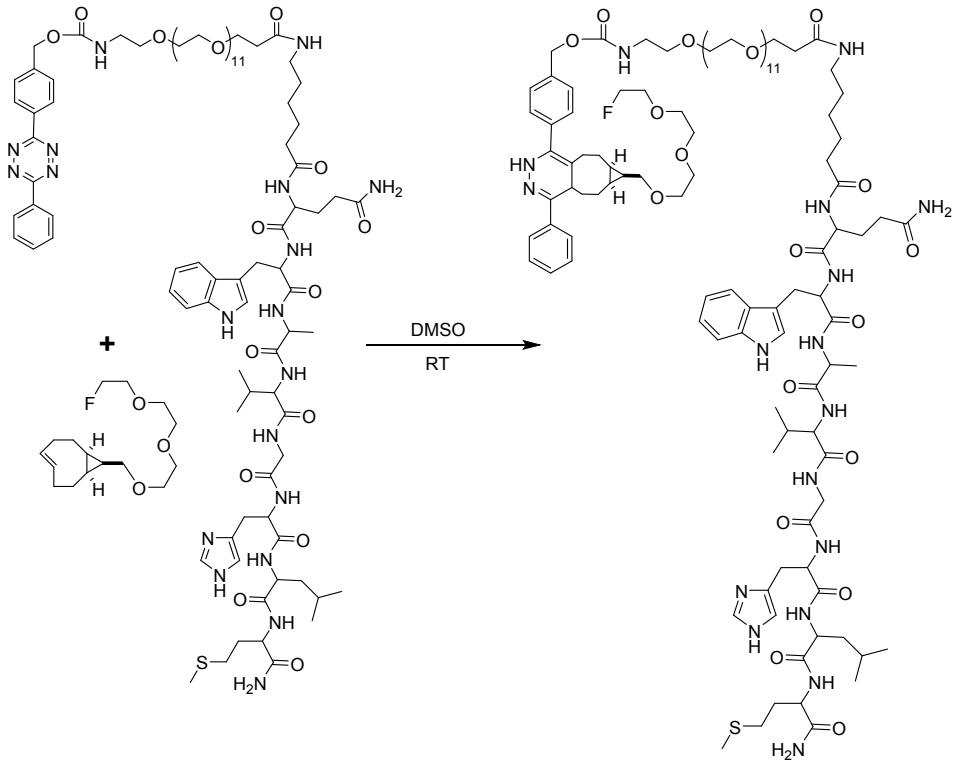


Figure S8 (a) HPLC profile and (b) HRMS of purified DiPhTz-BBN



^{19}F-sTCO-DiPhTz-BBN

DiPhTz-BBN (1 μL from a 10 mM solution in DMSO) and ^{19}F-sTCO (1 μL from a 24.5 mM solution in acetonitrile) was mixed and incubated at room temperature for 1 min. The crude reaction was analyzed and purified by HPLC using program 1. The product was eluted off at 16.1 min with program 1 and the yield is 51.1%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ $C_{108}H_{168}FN_{17}O_{28}S$ is 1109.0950; found 1109.0888

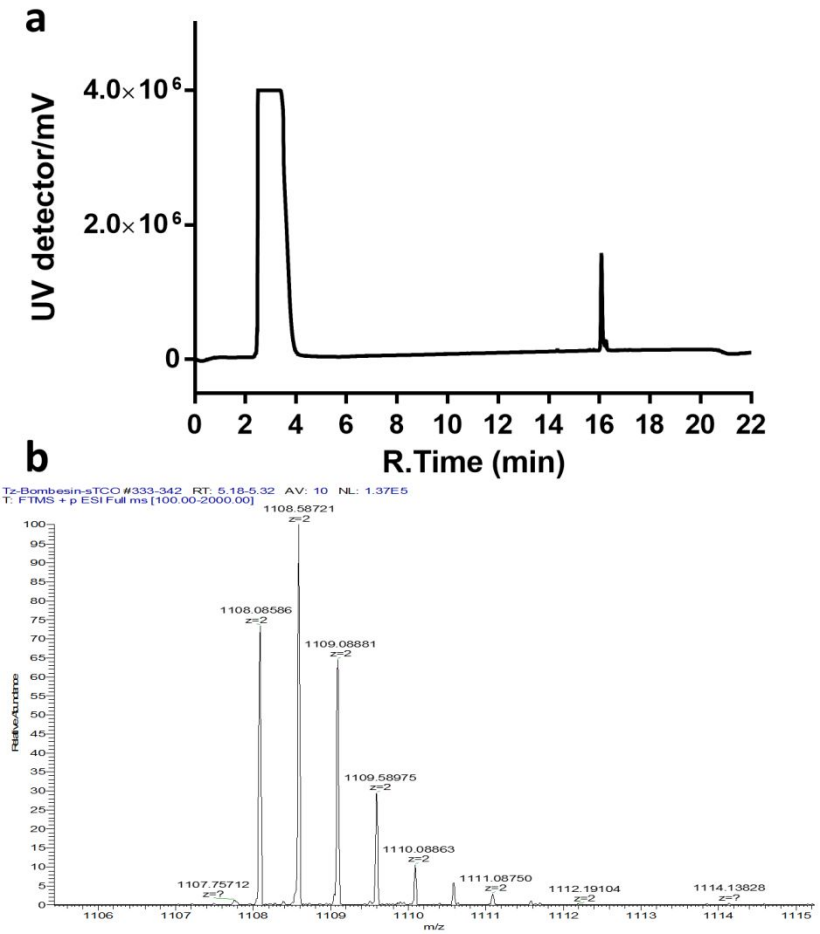
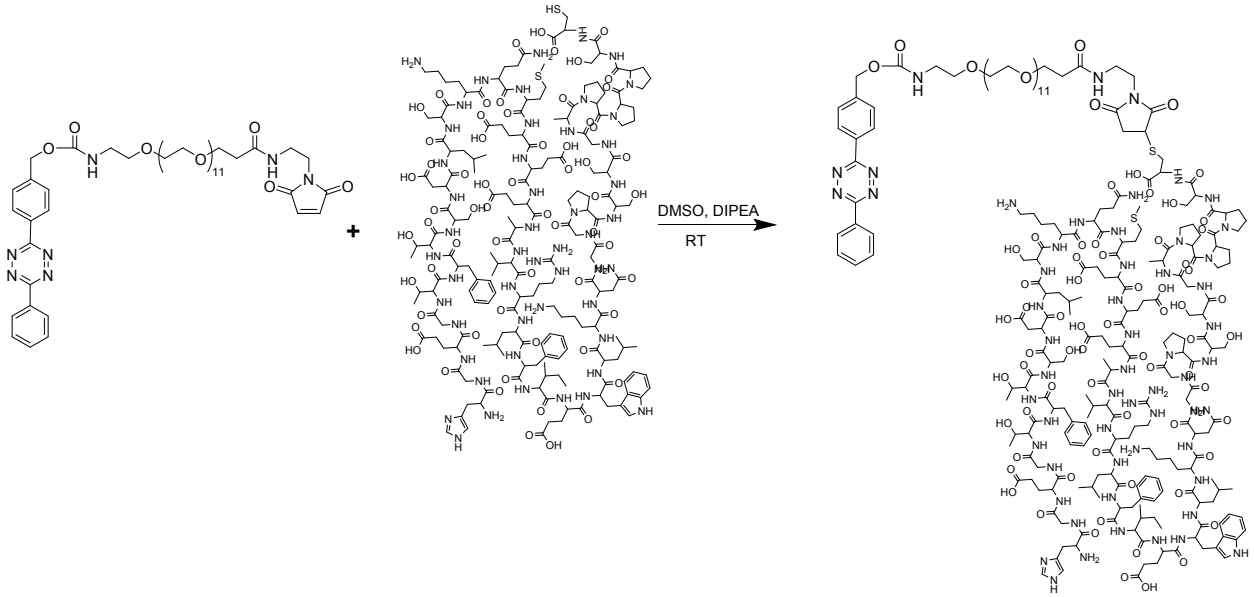


Figure S9 (a) HPLC profile and (b) HRMS of purified ^{19}F-sTCO-DiPhTz-BBN.



DiPhTz-Exendin-4

Exendin-4 (4mg, 0.9 μmol) was dissolved in 100 μL anhydrous DMSO and DiPhTz-Mal (1.5 eq) was dissolved in 20 μL of anhydrous DMSO solution. The mixture was incubated at room temperature for 2h. The reaction crude was purified by HPLC using program 1. The product was eluted off at 15.2 min with program 1 and the yield is 83.4%. MALDI-TOF/TOF *m/z*: $[M+H]^+$ $C_{242}H_{366}N_{59}O_{81}S_2$ is 5462.0; found 5462.1

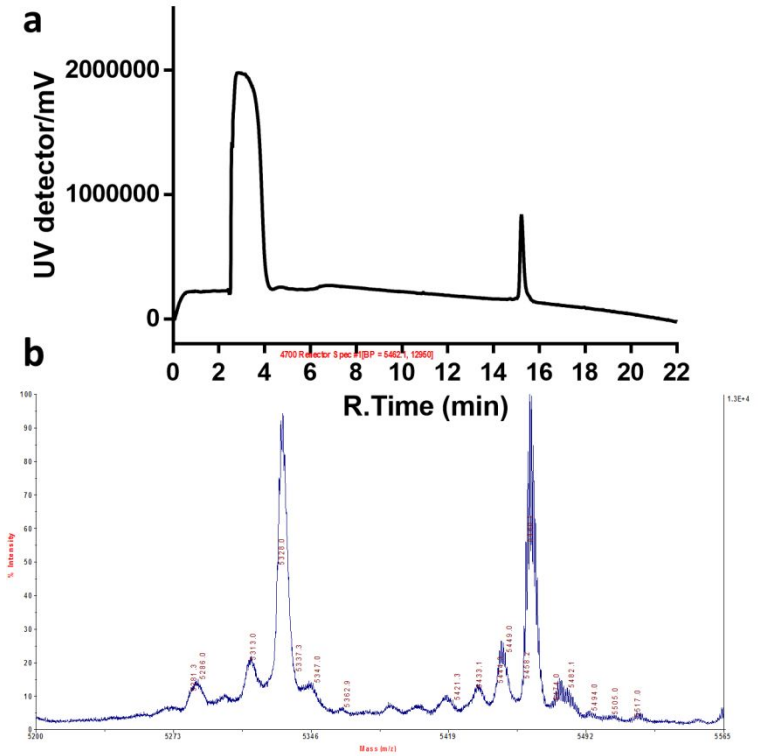
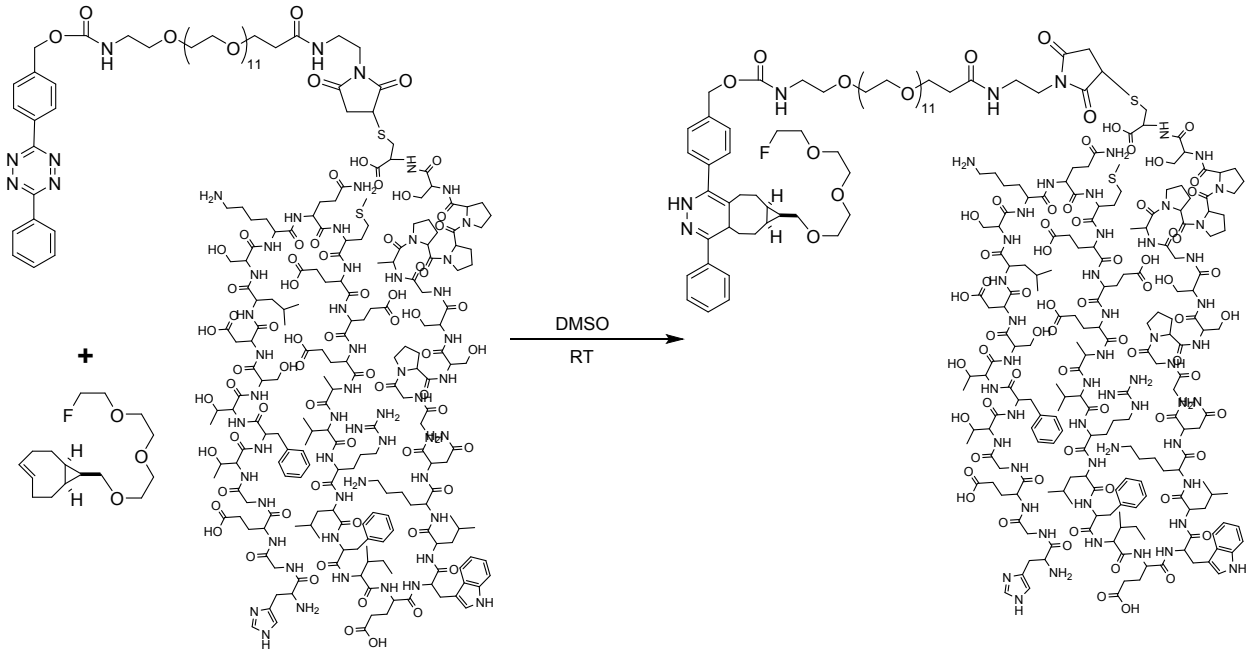
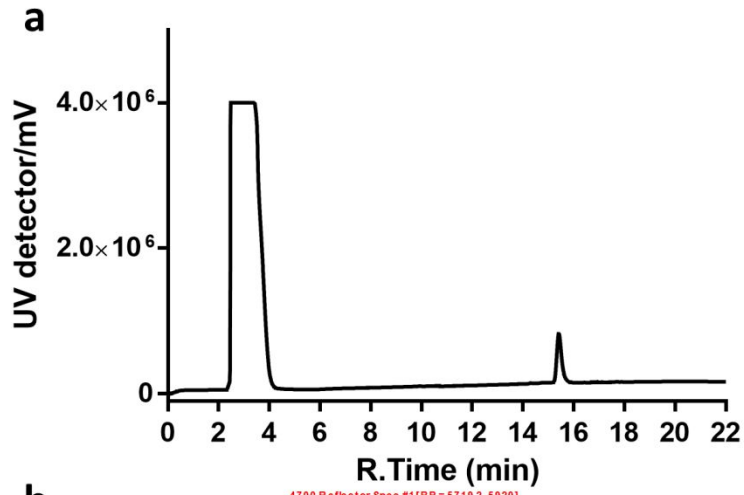


Figure S10 (a) HPLC profile and (b) MALDI-TOF/TOF result of purified DiPhTz-Exendin4



^{19}F-sTCO-DiPhTz-Exendin-4

DiPhTz-Exendin-4 (1 μL from a 10 mM solution in DMSO) and ^{19}F-sTCO (1 μL from a 24.5 mM solution in acetonitrile) was mixed and incubated at room temperature for 1 min. The crude reaction was analyzed and purified by HPLC using program 2. The product was eluted off at 15.5 min with program 1 and the yield is 60.9%. HRMS (ESI) *m/z*: $[M+H]^+$ $C_{258}H_{393}FN_{57}O_{84}S_2$ is 5719.4; found 5719.3



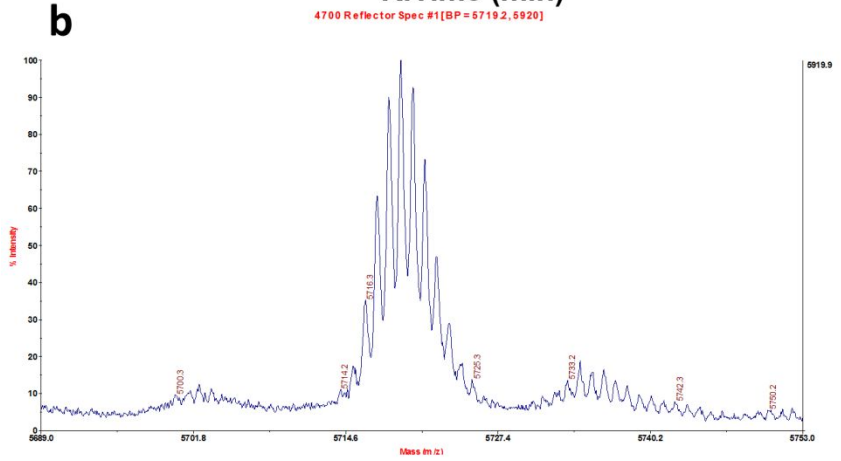


Figure S11 (a) HPLC profile and (b) MALDI-TOF/TOF result of purified ^{19}F-sTCO-DiPhTz-Exendin-4

S3. Radiochemistry

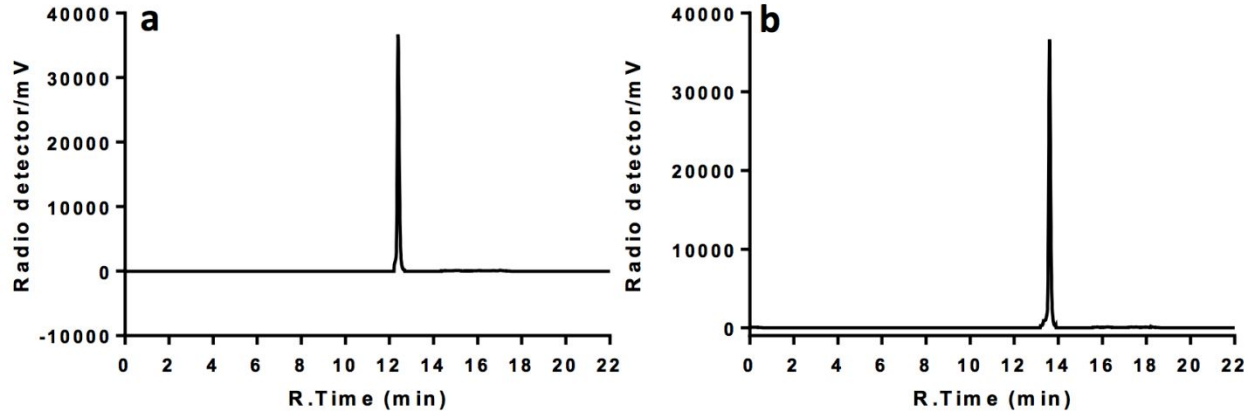


Figure S12 Radio HPLC profile of purified (a) ^{18}F-sTCO-DiolTz-RGDyK and (b) ^{18}F-sTCO-MePhTz-RGDyK

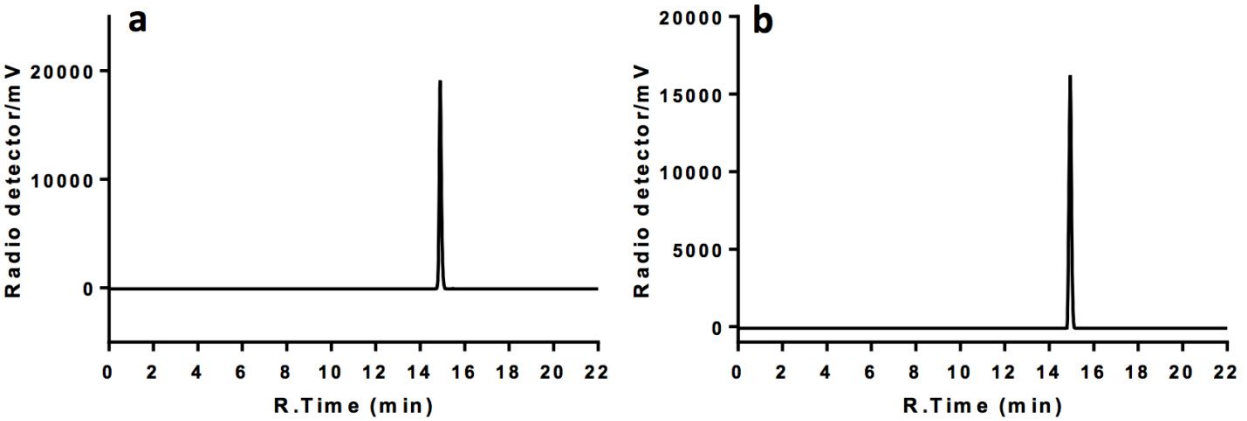


Figure S13 Radio HPLC profile of purified (a) ^{18}F-dTCO-DiPhTz-RGDyK and (b) ^{18}F-TCO-DiPhTz-RGDyK

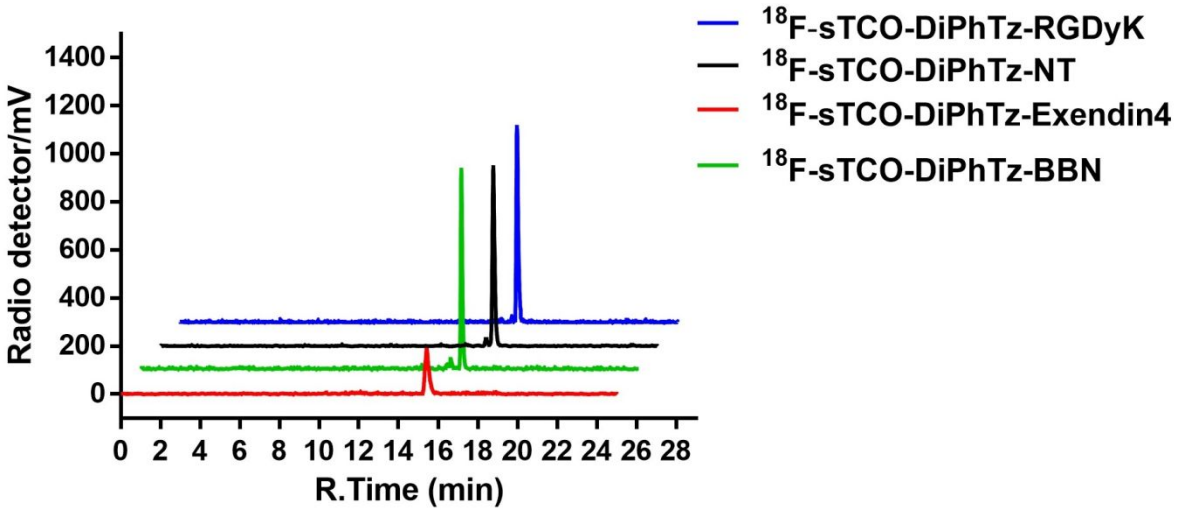


Figure S14 Radio HPLC profile of purified ^{18}F-sTCO-DiPhTz-RGDyK, ^{18}F-sTCO-DiPhTz-NT, ^{18}F-sTCO-DiPhTz-Exendin4 and ^{18}F-sTCO-DiPhTz-BBN

S4. Small Animal PET Imaging

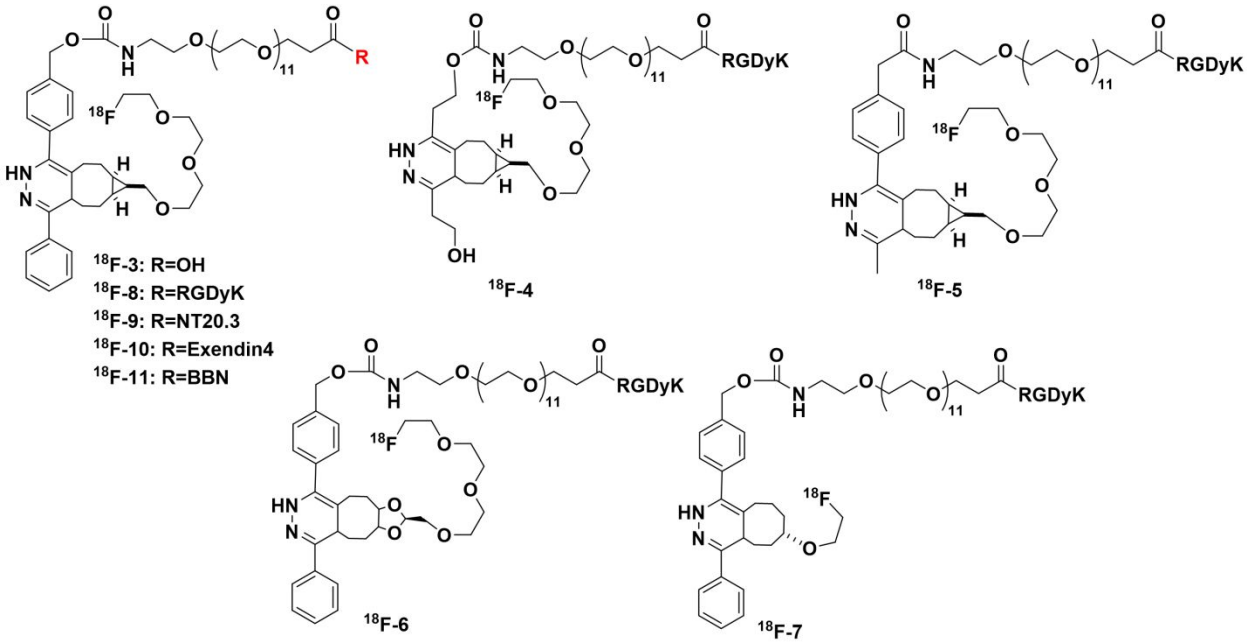


Figure S15 Chemical structures of all TCO/Tz system constructed PET probes

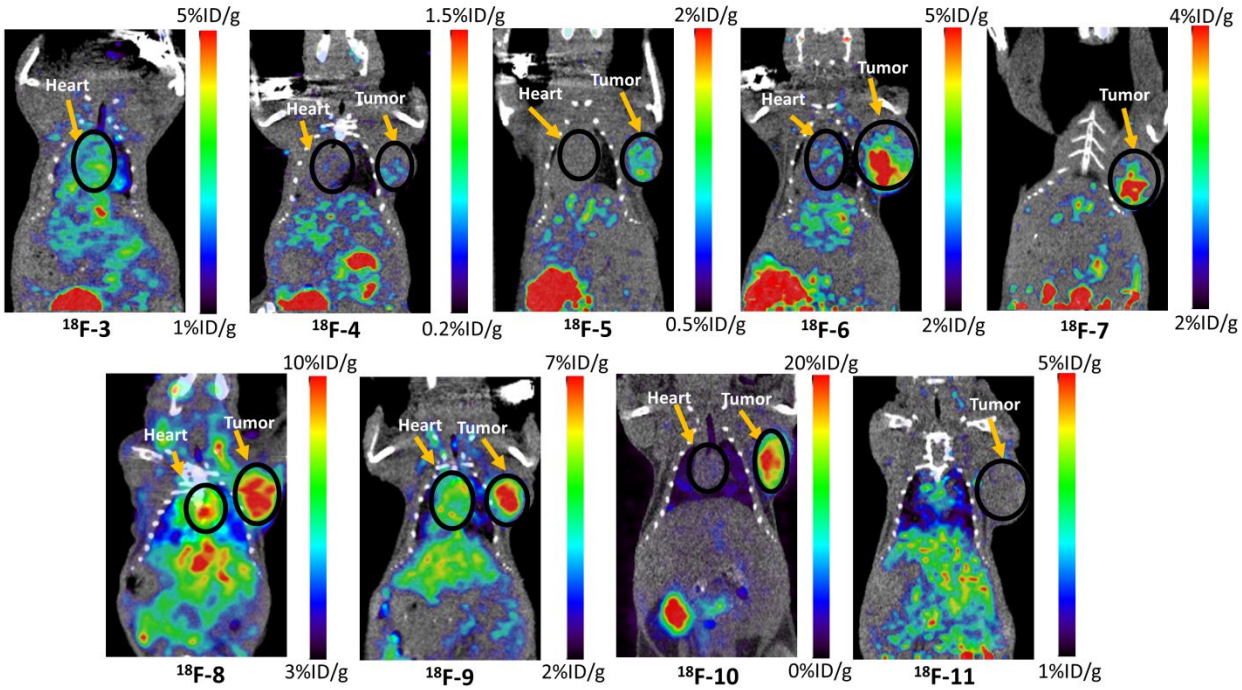


Figure S16 Representative PET/CT images of all TCO/Tz system constructed PET probes at 4 h post injection.

Table S1 Comparison between ^{18}F-sTCO-DiPhTz labeled tracer with other commonly used strategies labeled tracer using same targeting ligands

		1h post injection		Late time point	
Tracer	Yield%	Tumor uptake (%ID/g)	Tumor to muscle ratio	Tumor uptake (%ID/g)	Tumor to muscle ratio
^{18}F-sTCO-DiPhTz-RGD	49.6	5.3	3.9	8.9 at 4h	12.8 at 4h
^{18}F-SFB-RGD[5]	35.0~45.0	2.6	5.1	1.5 at 2h	8.2 at 2h
^{18}F-sTCO-DiPhTz-NT	42.7	4.3	7.8	4.6 at 4h	9.3 at 4h
^{18}F-AlF-NOTA-NT[6]	34.0	3.1	7.8	1.0 at 4h	5.1 at 4h
^{18}F-sTCO-DiPhTz-Ex4	40.1	8.2	6.9	11.4 at 4h	23.7 at 4h
^{18}F-FBEM-Ex4[7]	30.0	25.3	~25	20.0 at 2h	~40 at 2h
^{18}F-sTCO-DiPhTz-BBN	46.2	3.2	4.7	2.2 at 4h	5.4 at 4h
^{18}F-SFB-BBN[8]	24.0~30.0	2.5	11.1	N/A	N/A

Table S2 Quantitative uptake of ^{18}F-sTCO-DiPhTz-NT in major organs derived from PET images of AsPC-1 tumor bearing mice

	1h post injection (%ID/g)	4h post injection (%ID/g)
Liver	4.8 ± 0.3	4.2 ± 0.4
Tumor	4.3 ± 0.1	4.6 ± 0.1
Kidneys	4.9 ± 0.2	4.6 ± 0.2
Muscle	0.6 ± 0.1	0.5 ± 0.1

Table S3 Quantitative uptake of ^{18}F-sTCO-DiPhTz-Exendin-4 in major organs derived from PET images of INS-1 tumor bearing mice

	1h post injection (%ID/g)	4h post injection (%ID/g)
Liver	3.7 ± 0.6	2.4 ± 0.3
Tumor	8.2 ± 2.9	11.4 ± 4.7
Kidneys	24.8 ± 3.5	22.4 ± 2.4
Muscle	1.2 ± 0.1	0.5 ± 0.2

Table S4 Quantitative uptake of ^{18}F-sTCO-DiPhTz-BBN in major organs derived from PET images of PC-3 tumor bearing mice

	1h post injection (%ID/g)	4h post injection (%ID/g)
Liver	5.0 ± 0.7	2.6 ± 0.1
Tumor	3.2 ± 0.9	2.2 ± 0.3
Kidneys	4.7 ± 0.9	2.5 ± 0.1
Muscle	0.8 ± 0.5	0.4 ± 0.1

References

(1) Li, Z., Cai, H., Hassink, M., Blackman, M. L., Brown, R. C. D., Conti, P. S., and Fox, J. M. (2010) Tetrazine–trans-cyclooctene ligation for the rapid construction of ^{18}F labeled probes. *Chemical Communications 46*, 8043-8045.

(2) Wang, M., Svatunek, D., Rohlfing, K., Liu, Y., Wang, H., Giglio, B., Yuan, H., Wu, Z., Li, Z., and Fox, J. (2016) Conformationally Strained trans-Cyclooctene (sTCO) Enables the Rapid Construction of ^{18}F-PET Probes via Tetrazine Ligation. *Theranostics 6*, 887-95.

(3) Wang, M., Vannam, R., Lambert, W. D., Xie, Y., Wang, H., Giglio, B., Ma, X., Wu, Z., Fox, J., and Li, Z. (2019) Hydrophilic ^{18}F-labeled trans-5-oxocene (oxoTCO) for efficient construction of PET agents with improved tumor-to-background ratios in neurotensin receptor (NTR) imaging. *Chem Commun 55*, 2485-2488.

(4) Feng, H., Zhang, H., Wang, M., Vannam, R., Wang, H., Yan, X., Ouyang, W., Jia, X., Fox, J., and Li, Z. (2020) Improving Tumor-to-Background Contrast Through Hydrophilic Tetrazines: The Construction of ^{18}F Labeled PET Agents Targeting Non-Small Cell Lung Carcinoma. *Chemistry Accepted Author Manuscript*.

(5) Chen, X., Park, R., Shahinian, A. H., Tohme, M., Khankaldyyan, V., Bozorgzadeh, M. H., Bading, J. R., Moats, R., Laug, W. E., and Conti, P. S. (2004) ^{18}F-labeled RGD peptide: initial evaluation for imaging brain tumor angiogenesis. *Nucl Med Biol 31*, 179-89.

(6) Wang, M., Zhang, H., Wang, H., Feng, H., Deng, H., Wu, Z., Lu, H., and Li, Z. (2018) Development of [^{18}F]AlF-NOTA-NT as PET Agents of Neurotensin Receptor-1 Positive Pancreatic Cancer. *Mol Pharm 15*, 3093-3100.

(7) Kiesewetter, D. O., Gao, H., Ma, Y., Niu, G., Quan, Q., Guo, N., and Chen, X. (2012) ^{18}F-radiolabeled analogs of exendin-4 for PET imaging of GLP-1 in insulinoma. *Eur J Nucl Med Mol Imaging 39*, 463-73.

(8) Richter, S., Wuest, M., Krieger, S. S., Rogers, B. E., Friebe, M., Bergmann, R., and Wuest, F. (2013) Synthesis and radiopharmacological evaluation of a high-affinity and metabolically stabilized 18F-labeled bombesin analogue for molecular imaging of gastrin-releasing peptide receptor-expressing prostate cancer. *Nucl Med Biol 40*, 1025-34.