Electronic Supplementary Material (ESI) for ChemComm.

Supplementary material

A Host−Guest ATP Responsive Strategy for Intracellular Delivery of Phosphopeptides

1. **Materials and methods**
2. **Supplementary figures**
3. **Peptide synthesis and characterization**

1、 Materials and methods

1.1 Peptide synthesis

All peptides were synthesized through the standard Fmoc-based solid phase peptide synthesis (SPPS). Rink Amide MBHA resins (GL Biochem, loading: 0.337 mmol/g) were swelled in DCM for 30 mins. Fmoc protecting group was removed by 20% piperidine in DMF. The resins were treated with Fmoc-amino acid (4 eq.) in the presence of HATU (4 eq.), HOAt (4 eq.) and DIEA(8 eq.) in DMF to couple with next residue. Triglycerol linker and 5(6)-carboxyfluorescein of fluorescent phosphopeptides were coupled as same as all building blocks. The N-terminal of non-fluorescent phosphopeptides was amidated by capping reagent (acetic anhydride). The Pep1 serial peptides as well as its side chain protecting groups were cleaved by a mixture of TFA/phenol/water/thioanisole/Me_2S/NH_4I (82.5/3/5/5/2/2.5) and the Pep2 serial peptides were treated by TFA/TIS/water (95/2.5/2.5). The released peptides were precipitated from the solution by cold diethyl ether and purified by preparative HPLC to afford pure products.

1.2 Synthesis of GC5A-12C

5,11,17,23,29-pentaguanidinium-31,32,33,34,35-penta-n-dodecyloxy calix[5]arene (GC5A-12C) was synthesized according to the previous literature.[1]

1.3 Preparation of 12C-NC

GC5A-12C dissolved in solution of methanol and PEG-12C dissolved in chloroform were mixed at a molar ratio of 1:1. Solvent was removed under vacuum pressure for 7-10 h and the residue was hydrated (10 mM HEPES buffer, pH = 7.4) by sonication at 80 °C for 4 h.

1.4 Characterization of the 12C-NC.

The dynamic light scattering (DLS) measurements were examined on a laser light scattering spectrometer (NanoBrook 173 plus) equipped with a digital correlator at 659 nm at a scattering angle of 90°. Zeta potential was measured by Zeta PALS + BI-90 instrument (Brookhaven Co, USA). Transmission electron microscopy (TEM) was measured by H-7650 Transmission Electron Microscope (Hitachi-Science &

Technology, Japan).

1.5 Binding affinities of 12C-NC with peptides

Fluorescence titrations were performed in a conventional quartz cell (light path 10 mm) on a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA) at ambient temperature. The complexation of 12C-NC with fluorescein (Fl) was measured by the direct fluorescence titration. A mixed solution containing Fl and 12C-NC was sequentially injected into 2.5 mL Fl solution in a quartz cuvette. The fluorescence intensity was measured (λ_{ex} = 500 nm) before the first addition and after every addition until a plateau was reached. By fitting the fluorescence intensity at 513 nm according to a 1:1 host-guest binding stoichiometry, the association constant was obtained. [2]

The complexation of 12C-NC with peptides were measured by the competitive fluorescence titrations. A solution containing Fl, 12C-NC and a certain peptide was sequentially injected into 2.5 mL Fl and 12C-NC mixed solution in a quartz cuvette. The fluorescence intensity was measured (λ_{ex} = 500 nm) before the first addition and after every addition until a plateau was reached. The association constant was obtained by fitting fluorescence intensity at 513 nm according to the n:1 competitive binding model.[3]

1.6 ATP-dependent phosphopeptides release

To study the ATP-dependent release of phophopeptides, the FAM modified peptides were employed. The fluorescence intensity of 20 μM peptide was measured (λ_{ex} = 495 nm) before and after addition of 20 μM 12C-NC, 5 μM (extracellular maximum concentration of ATP) ATP, and 1 mM (intracellular minimum concentration of ATP) ATP.

1.7 Cell culture and intracellular phosphopeptides delivery

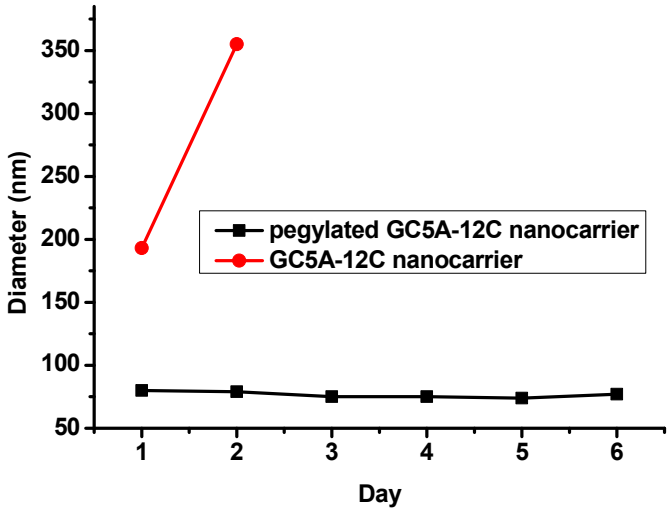
HeLa cells were maintained in DMEM (Corning) medium, which was supplemented with 10% FBS at 37 °C in 5% CO_2. N2a cells were maintained in 45% DMEM and 45% α-MEM (Corning) 10% FBS and A549 cells were maintained in 90% DMEM and 10% FBS. Before intracellular delivery of Phosphopeptides, 1.5×10^5 cells were seeded into 24-well plates (Corning) with 500 μL cell culture medium, 8×10^4 cells

were seeded into 8-well chambered cover glass (Cellvis) with 400 μL cell culture medium overnight. For formulation of phosphopeptides-nanocarriers complexes, different concentrations of 12C-NC were mixed with different concentration of phosphopeptides (molar ratio 1:1) in reduced serum-free Opti-MEM (Gibco) medium with a final volume of 100 μL at room temperature for 15 mins. Different concentration of phosphopeptides-nanocarriers complexes were added to plates with a final volume of 300 μL in each well of 24-well plates, 200 μL in each well of 8-well plates, respectively. After incubation for 4 h at 37 °C, the cell culture medium was removed. The redundant phosphopeptides-nanocarriers complexes were washed away with PBS buffer for 2 times. Cells in 24-well plates were collected after trypsinization (0.25% trypsin-EDTA， Corning) and centrifugation and resuspended with 500 μL PBS (Corning) buffer for flow cytometry. The fluorescence of each sample (10000 cells) was represented as the mean fluorescence intensity (fluorescence intensity of each cell) which was given by BD FACS Calibur. The results were expressed as the mean fluorescence intensity ± SEM for three replicates, 10000 cells for each replicate. The 8-hole plate was observed directly under confocal laser scanning microscopy (Zeiss LSM780).

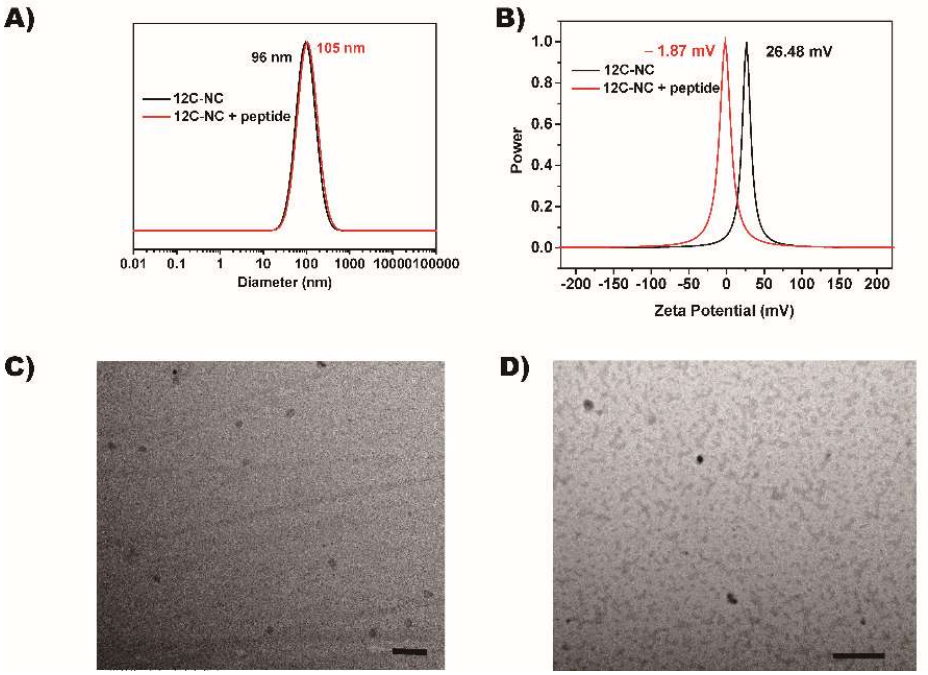
1.8 Experiments with Endocytosis Inhibitors

For the low temperature at 4 °C, the cells were pretreated at 4°C in Opti-MEM for 30 mins and then incubated with phosphopeptides/ 12C-NC complexes at 4 °C for 4 h. For the studies with endocytosis inhibitors, the cells were first incubated with chlorpromazine (CPZ, 100 μM), 5-(N-ethyl-N-isopropyl) amiloride (EIPA, 100 μM), methyl-β-cyclodextrin (MβCD, Solarbio,10 mM) or genistein (GST, MACKLIN， 200 μM) in Opti-MEM for 30 mins at 37°C and were further incubated with phosphopeptides/12C-NC complexes in Opti-MEM for 4 h before analyzed by confocal live cell imaging and flow cytometry analysis.

2. Supplementary figures



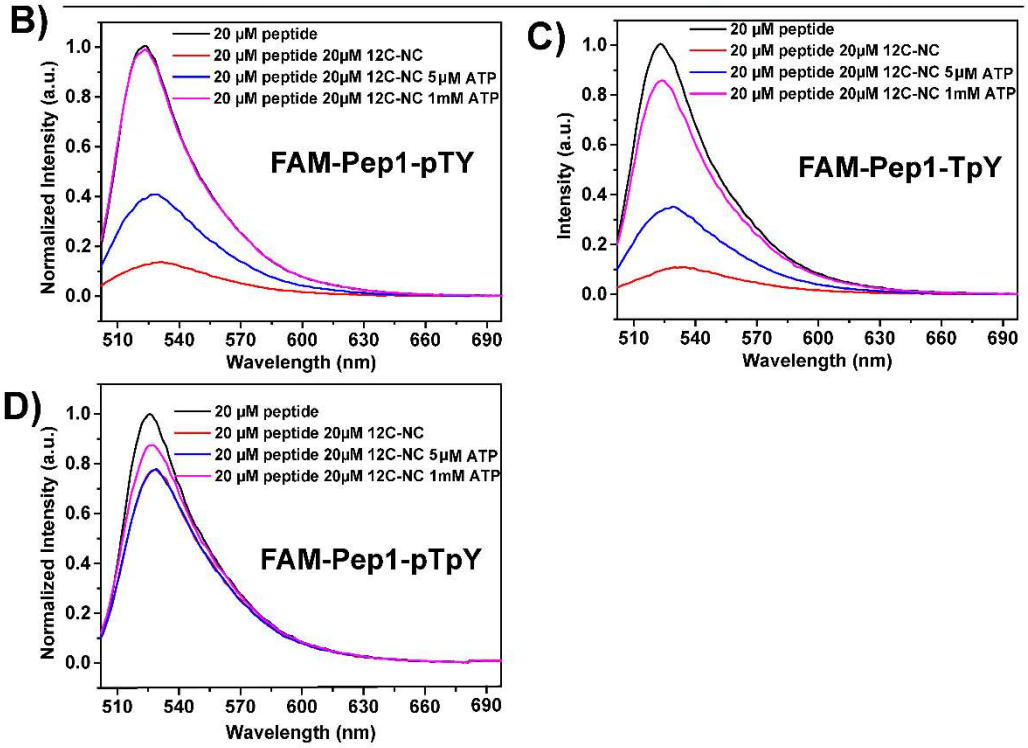
Supplementary Figure S1. The diameters of pegylated GC5A-12C nanocarrier and GC5A-12C nanocarrier within a few days after their preparation monitored by DLS.



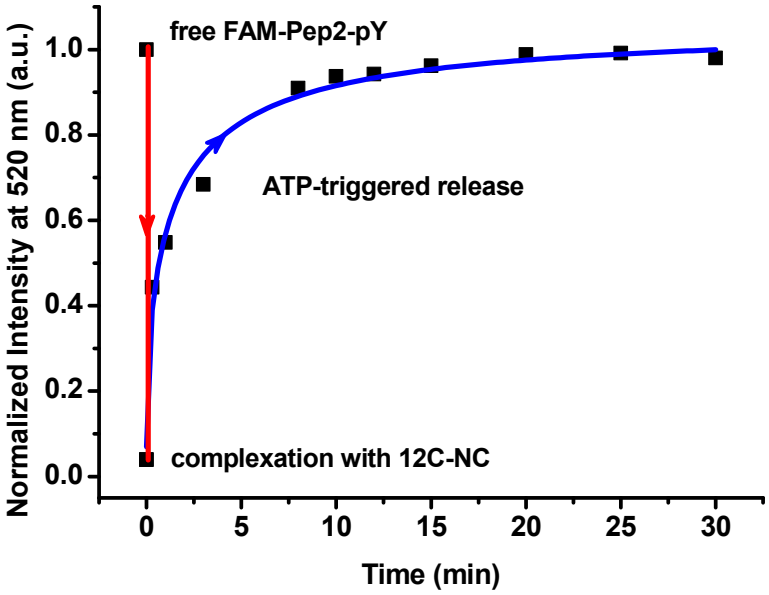
Supplementary Figure S2. (A) DLS, (B) Zeta potential and TEM images of 12C-NC in the (C) absence and (D) presence of FAM-Pep1-pTpY in HEPES buffer (10 mM, pH = 7.4), (scale bar 200 nm).

A)

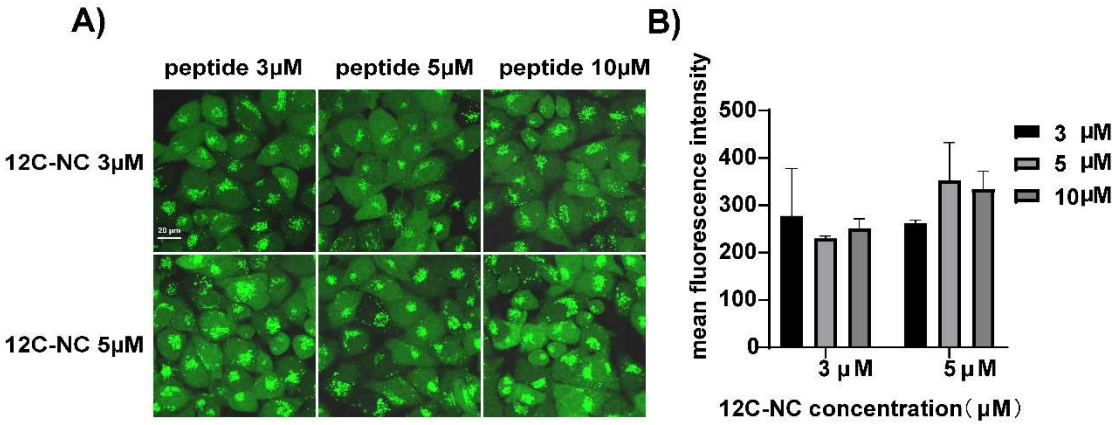
Non-fluorescent peptide				Fluorescent peptide	
Name	Sequence	K_a	n	Name	Sequence
Pep1-TY	Ac-QYFMTEYVA-NH_2	$(3.2\pm0.1)\times10^5$	1	FAM-Pep1-TY	FAM-PEG-QYFMTEYVA-NH_2
Pep1-pTY	Ac-QYFMpTEYVA-NH_2	$(2.5\pm0.0)\times10^7$	2	FAM-Pep1-pTY	FAM-PEG-QYFMpTEYVA-NH_2
Pep1-TpY	Ac-QYFMTEpYVA-NH_2	$(6.5\pm0.4)\times10^6$	2	FAM-Pep1-TpY	FAM-PEG-QYFMTEpYVA-NH_2
Pep1-pTpY	Ac-QYFMpTEpYVA-NH_2	$(2.0\pm1.0)\times10^8$	2	FAM-Pep1-pTpY	FAM-PEG-QYFMpTEpYVA-NH_2
Pep2-Y	Ac-YEEI-NH_2	$(2.1\pm1.3)\times10^5$	1	FAM-Pep2-Y	FAM-PEG-YEEI-NH_2
Pep2-pY	Ac-pYEEI-NH_2	$(1.4\pm0.1)\times10^8$	1	FAM-Pep2-pY	FAM-PEG-pYEEI-NH_2



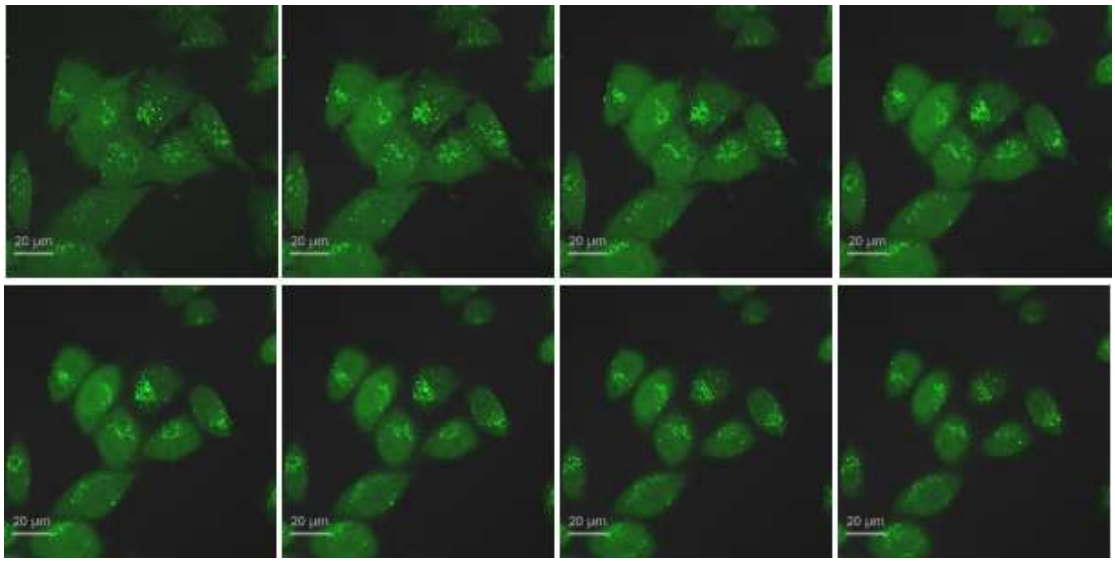
Supplementary Figure S3. The sequences of tested peptides and the binding affinities of 12C-NC system with non-fluorescent phosphopeptides and the control ones and ATP responsive release. (A) The sequences of tested peptides and binding affinities of 12C-NC with non-fluorescent peptides, Fluorescence spectra of (B) FAM-Pep1-pTY, (C) FAM-Pep1-TpY, (D) FAM-Pep1-pTpY, in the absence and presence of the 12C-NC (20 μM), and after the addition of ATP (5 μM and 1 mM) respectively.



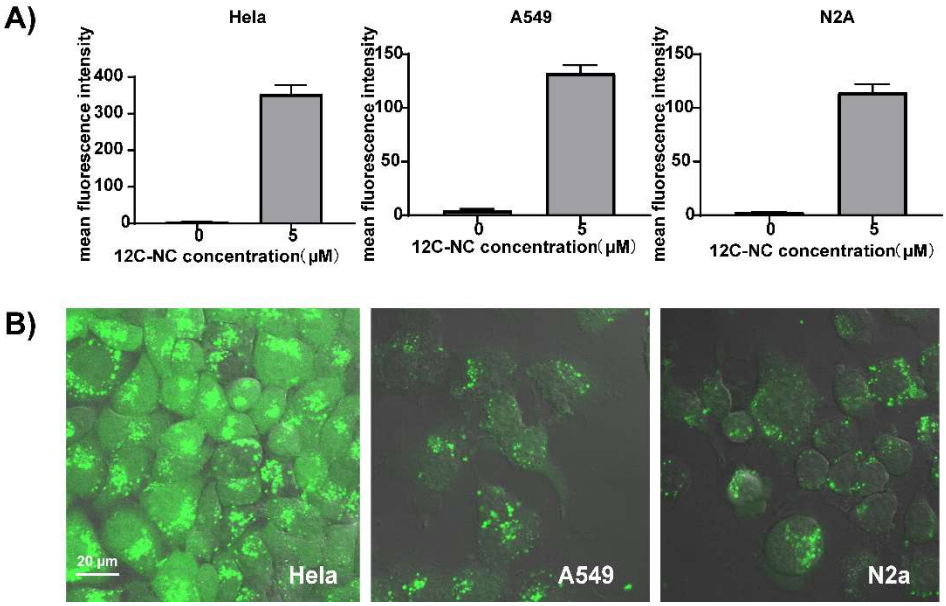
Supplementary Figure S4. Time-dependent ATP-triggered FAM-Pep2-pY release monitored by measuring the fluorescence intensity of peptide. The fluorescence intensity at 520 nm of 3 μM FAM-Pep2-pY in the presence of 3 μM 12C-NC was measured before and after the addition of 1 mM ATP. The fluorescence intensity was normalized by the fluorescence intensity of 3 μM free FAM-Pep2-pY.



Supplementary Figure S5. (A) CLSM images and (B) FACS of HeLa cells treated with different concentrations of FAM-Pep1-pTpY assembled with different concentrations of 12C-NC. Data are presented as mean ± SD (n=3). ***$p < 0.001$. (scale bar 20 μm)



Supplementary Figure S6. Confocal z-stacking fluorescence images of HeLa cells treated with 5 μM FAM-Pep1-pTpY/12C-NC complexes for 4 h. (scale bar 20 μm). Images were taken from every 0.64 micron apart.

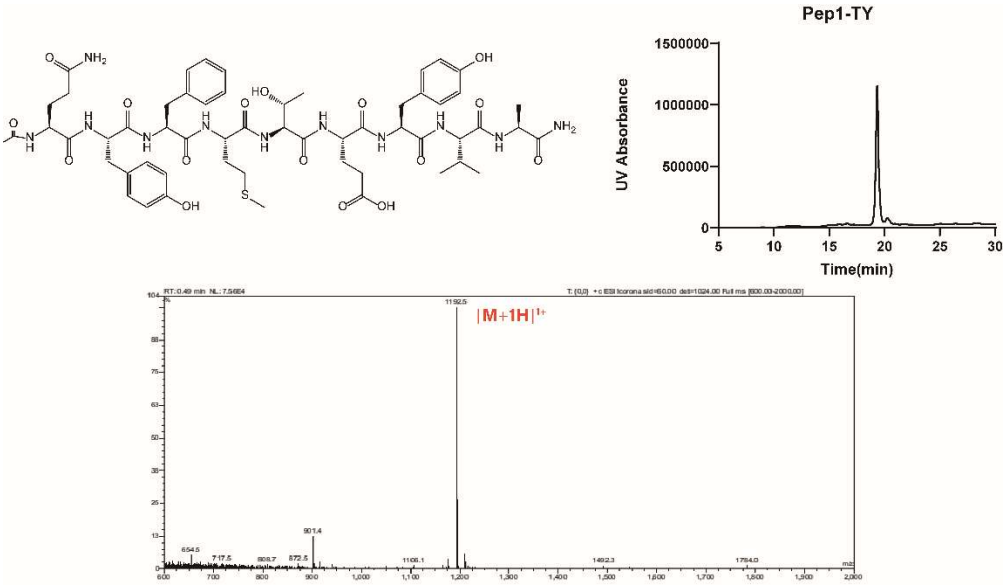


Supplementary Figure S7. (A) FACS and (B) CLSM images of HeLa, A549 and N2a cells treated with the FAM-Pep1-pTpY/12C-NC complexes for 4 h. The peptide and 12C-NC concentration were respectively 5 μM. Data are presented as mean ± SD (n=3). ***$p < 0.001$. (scale bar 20 μm)

3. Supplementary data of peptides

All peptides were synthesized through the standard Fmoc-protected solid phase peptide synthesis (SPPS) The analytical HPLC spectra of peptide was monitored at 215 nm on a C18 reversed-phase column at a flow rate of 0.8 mL/min. Conditions was 20% solution B to 80% solution B in 30 mins (Solution A: 0.06% TFA in water; B: 80% acetonitrile and 0.06% TFA in water)

Pep1-TY



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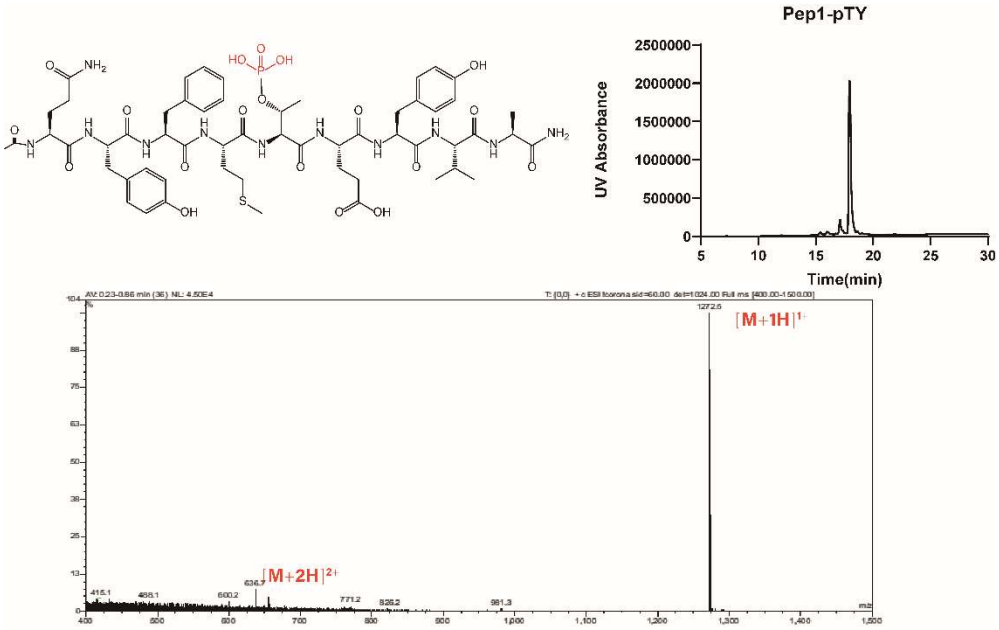

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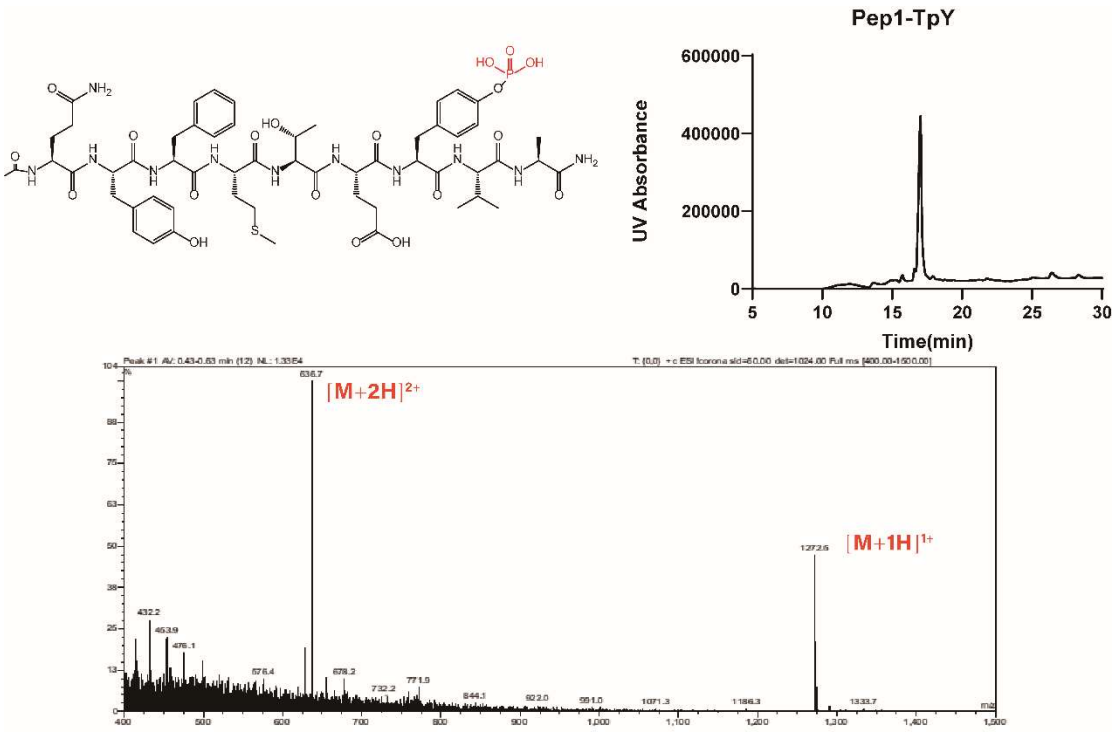
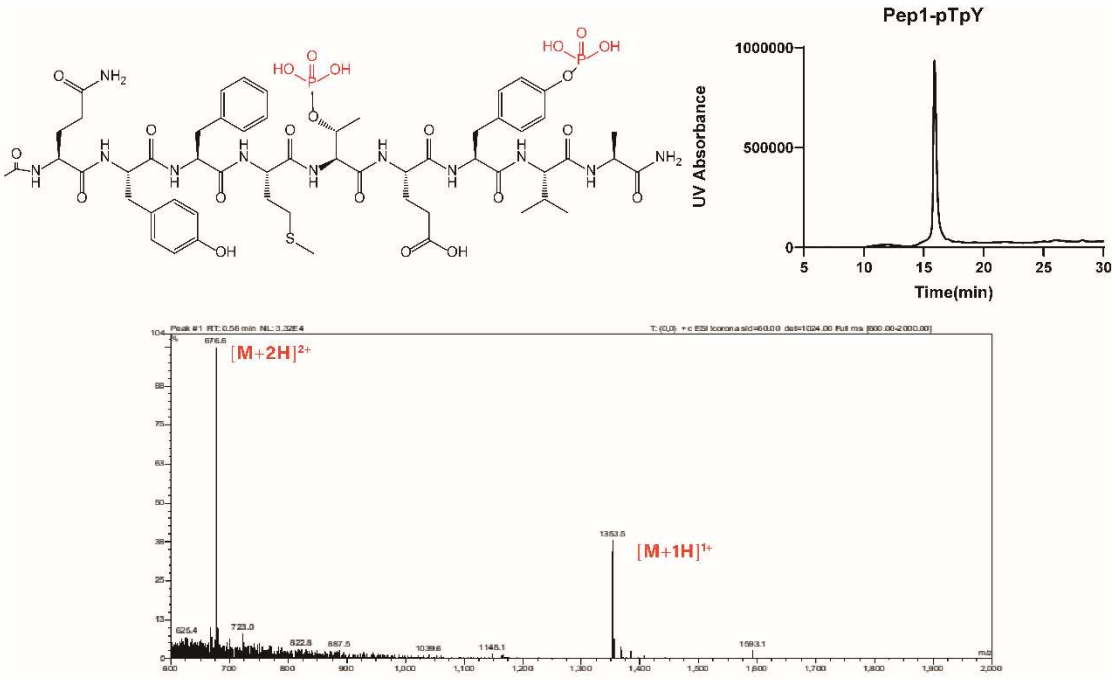
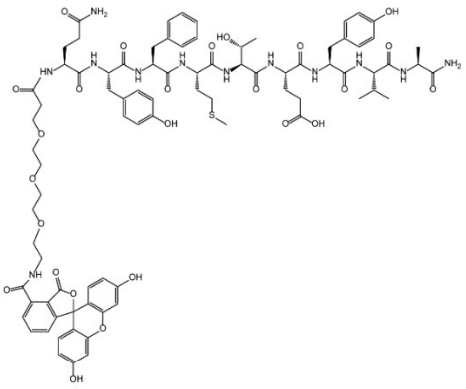
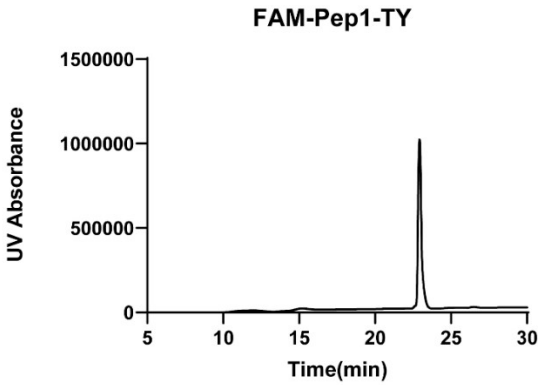


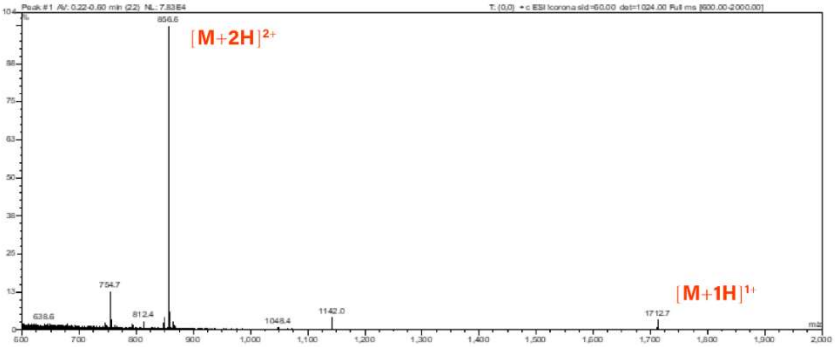
Pep1-pTpY


Pep1-pTpY
UV Absorbance
1000000
500000
0
5
10
15
20
25
30
Time(min)
[M+2H]2+
[M+1H]1+
676.6
1353.5
625.4
723.0
822.8
887.5
1039.6
1145.1
1593.1
m/z

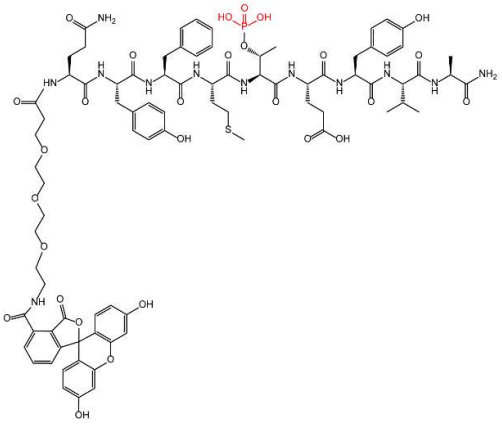
FAM-Pep1-TY

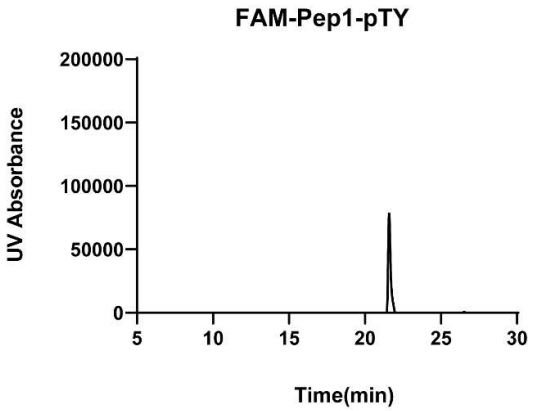


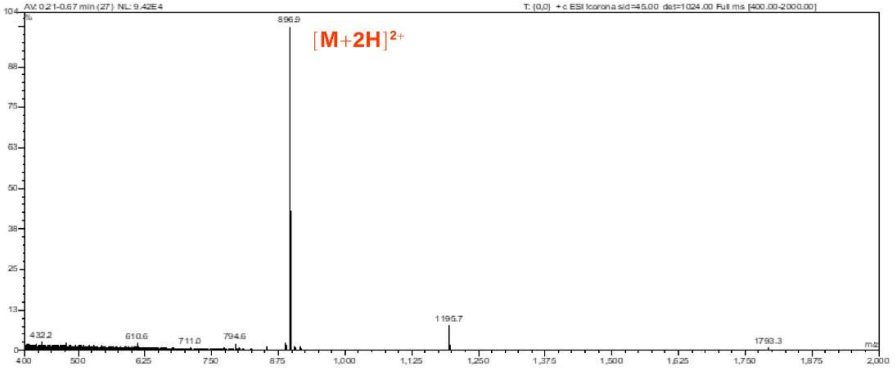




FAM-Pep1-pTY







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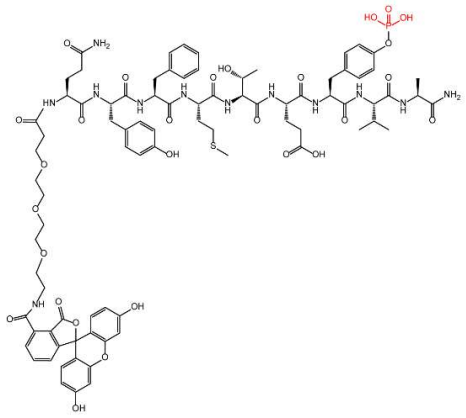

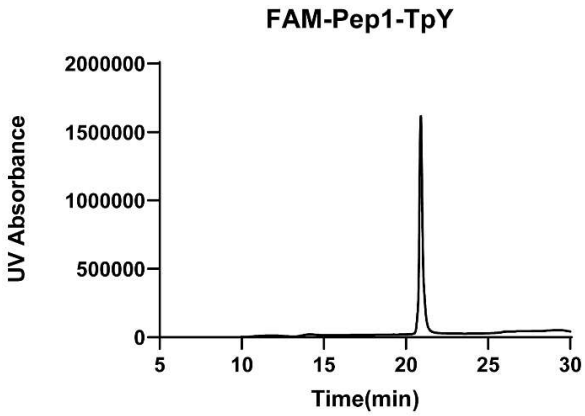

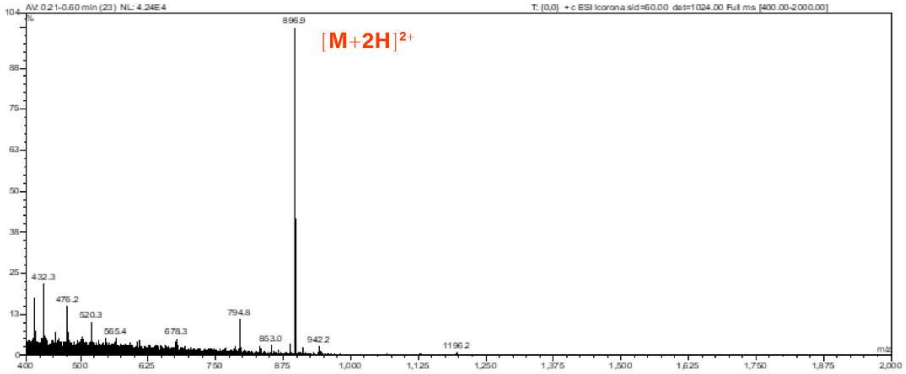

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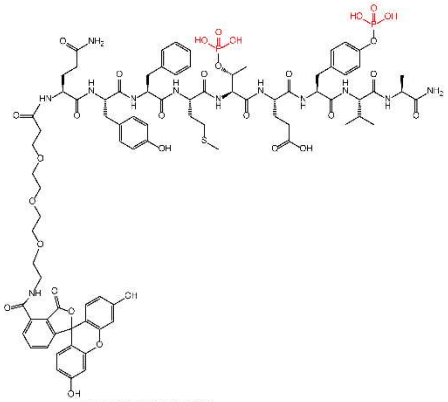
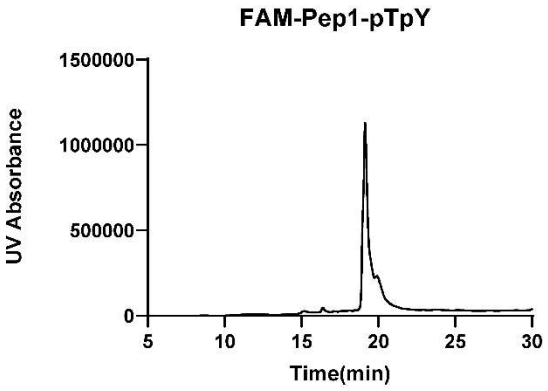
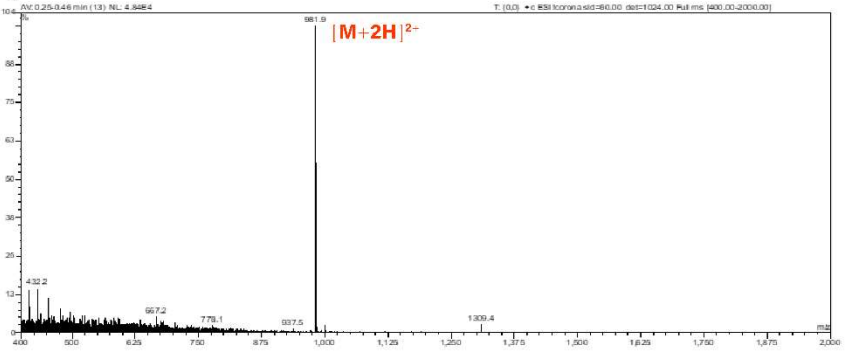
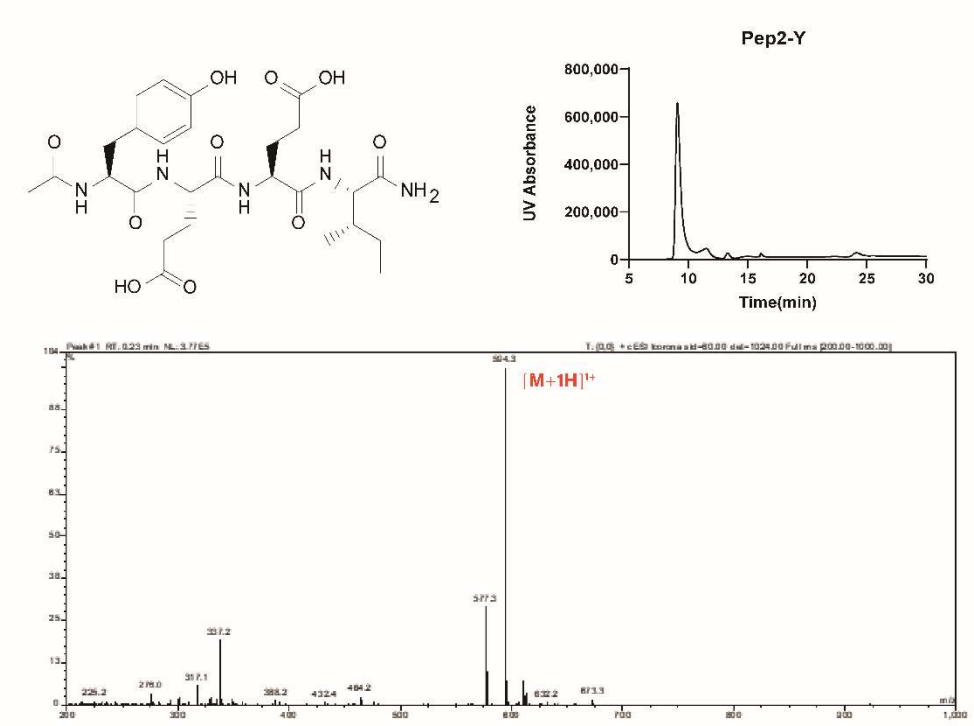






Pep2-Y



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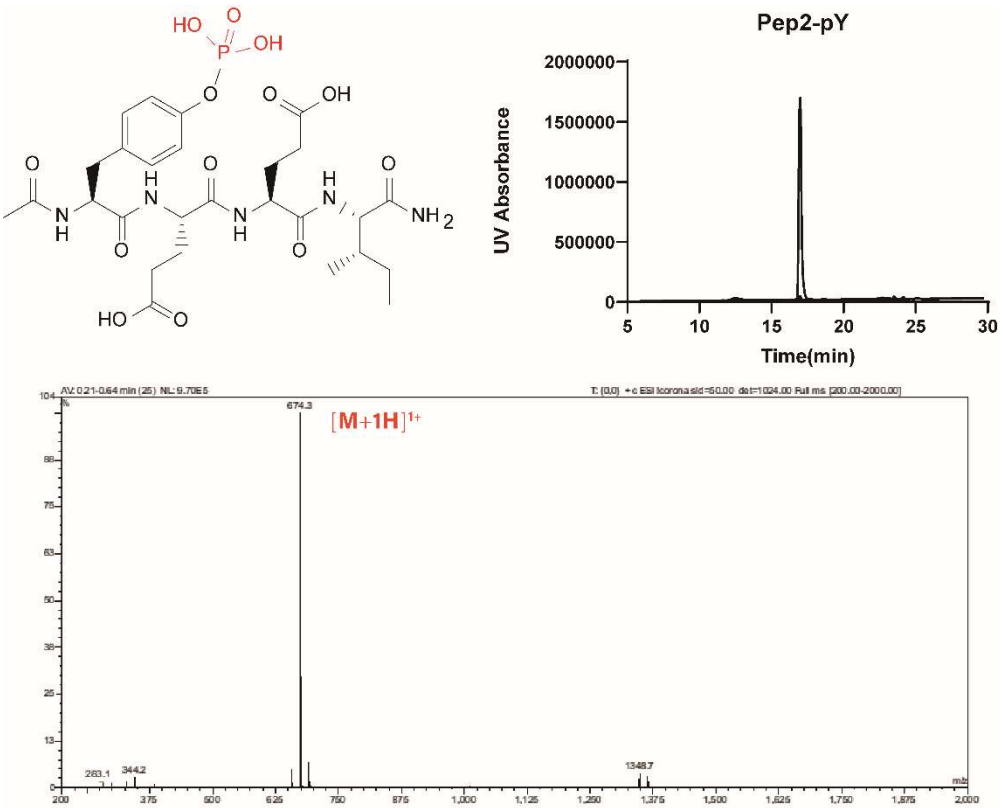

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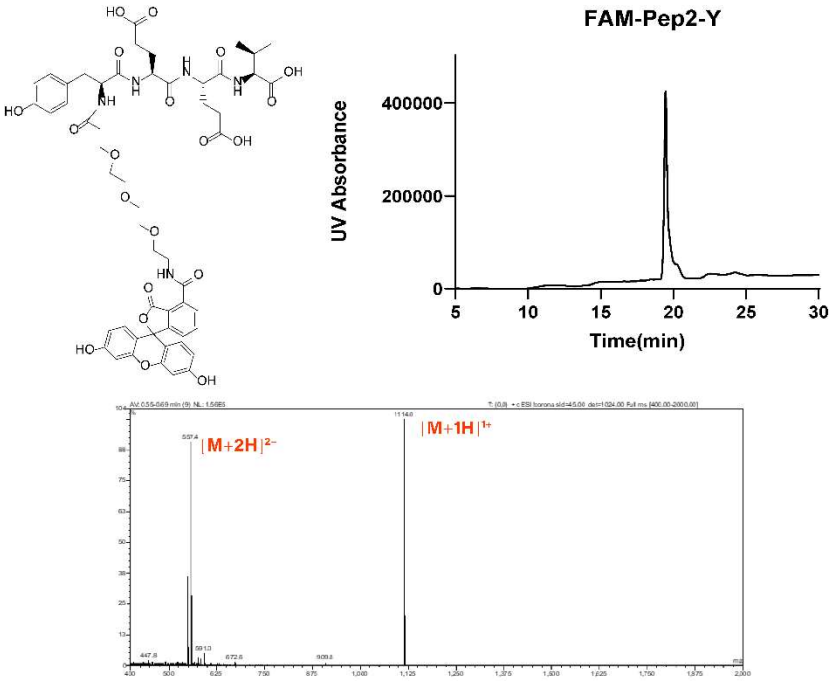

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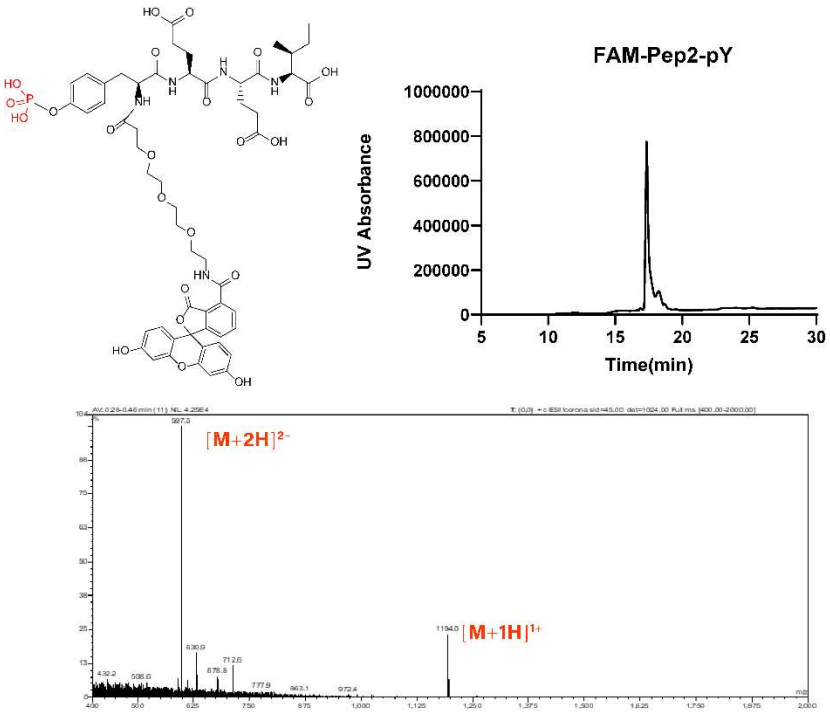

Reference:

1. J. Gao, J. Li, W.-C. Geng, F.-Y. Chen, X. Duan, Z. Zheng, D. Ding and D.-S. Guo, *J. Am. Chem. Soc.*, 2018, **140**, 4945-4953.
2. D.-S. Guo, V. D. Uzunova, X. Su, Y. Liu and W. M. Nau, *Chem. Sci.*, 2011, **2**, 1722.

3. G. Ghale, A. G. Lanctôt, H. T. Kreissl, M. H. Jacob, H. Weingart, M. Winterhalter and W. M. Nau, *Angew. Chem. Int. Ed.*, 2014, **53**, 2762-2765.