# Supporting Information

## Generation of antibody-drug conjugation by proximity-driven acyl transfer and sortase-mediated ligation

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**General information**



**Experimental Section**

## General Information

*Materials*

Fmoc-protected amino acids, Rink amide MBHA resin, and 2-chlorotrityl chloride resin were purchased from GL Biochem Ltd (Shanghai). *N, N'*-dimethylformamide (DMF), dichloromethane (DCM), methanol, trifluoroacetic acid (TFA, HPLC grade or analytic pure), *N, N'*-diisopropyl-carbodiimide (DIC), cyano(hydroxyamino)acetate (oxyma pure), m-cresol, *N, N'*-diisopropylethylamine (DIPEA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), Fmoc-Dap(Boc)-OH, disuccinimidyl suberate, biotin, 6-Fmoc-amino hexanoic acid, bis(N, N-diethylthiocarbamoyl)disulfide, Fmoc-2-(2-(2-Aminoethoxy)ethoxy) acetic acid (AEEA), methyl thioglycolate (MTG), thioglycolic acid (MPA), 2-(tritylthio)acetic acid, m-cresol and trimethylsilane (TIS) were purchased from Energy-Chemical Ltd. Acetonitrile (HPLC grade) was purchased from J. T. Baker (Phillipsburg, NJ, USA). Formic acid (LC-MS grade) for mass spectrometry was purchased from Fisher Scientific.

*HPLC, Mass Spectrometry (MS)*

HPLC experiments were performed with a high-performance liquid chromatography system (LC-20AT, Shimadzu). ReproSil-Pur Basic C18 (4.6 × 250mm, 5μm) column (Serial No. r15.b9.s2546, column A) or ChromCore 120 C18 (4.6 × 250mm, 5μm) column (Serial No. A001-050012-04625s, column B) was used for the purity analysis of the mentioned peptides and the reaction progress analysis, and the flow rate for the HPLC machine was set to 1.0 mL/min. The purification of the peptides was conducted on a Vydac C18 (10 × 250 mm) column, and the flow rate for HPLC machine was set to 3.0 mL/min. There are two mobile phases for HPLC running. **Solvent A** is water phase that contains 1% acetonitrile and 0.1% TFA. **Solvent B** is acetonitrile that contains 1% water and 0.1% TFA. The molecular weight of the mentioned peptides was determined by an LC-MS mass spectrometer (Thermo LTQ-Orbitrap). The water phase for LC-MS is water contains 0.1% formic acid, and the organic phase is acetonitrile containing 0.1% formic acid.

*General procedures for Solid-Phase Peptide Synthesis*

The resins we used were Rink-amide MBHA resin (0.68 mmol/g) or 2-chlorotrityl chloride resin (0.56 mmol/g). The choice of the resin type was based on the C-terminal moiety of the peptide.

*Preparation of 2-Chlorotrityl Hydrazine Resin (300 μmol)*

0.54 g of 2-Chlorotrityl Chloride resin (0.56 mmol/g) was transferred to a 10-mL syringe. Wash the resin with 6.0 mL of DCM. The resin was swelled in DCM at room temperature for 20 min. After DCM discarding, 5 mL of freshly prepared 5% (v/v) hydrazine hydrate mixture (DMF 4.75 mL/hydrazine hydrate 0.25 mL) was added to the resin. Keep the resin at room temperature for 30 min. Discard the hydrazine hydrate mixture and repeat the step of hydrazine treatment once again. Then, 5.0 mL of freshly prepared 20% methanol in DMF (DMF/methanol, 4.0 mL/1.0 mL) was added to the resin. Keep the resin at room temperature for 20 min. The resin

was washed with DMF (~4 times), then can be directly used for peptide synthesis.

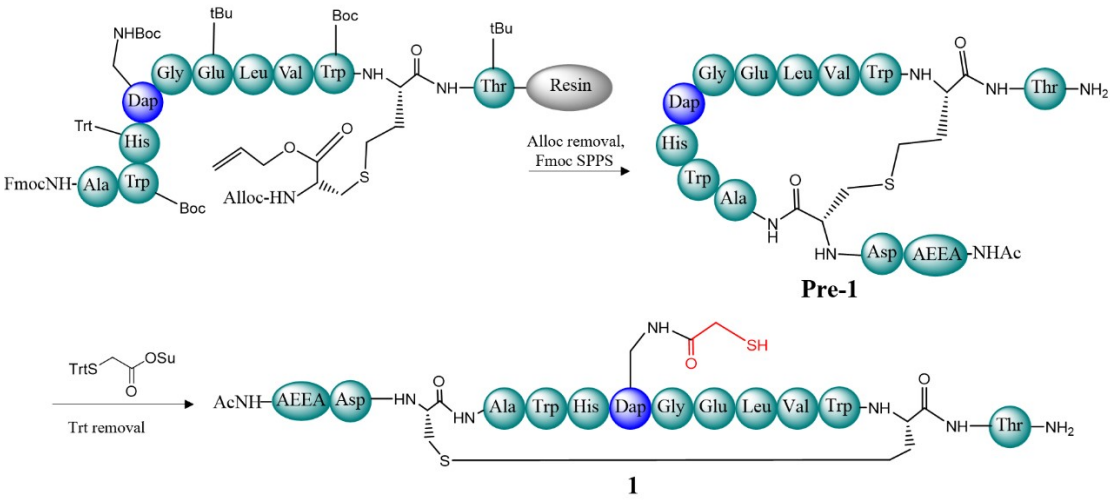
*Solid-Phase Peptide Synthesis (300 μmol)*

All peptides were assembled manually by the procedure of Fmoc SPPS. Fmoc group deprotection was performed by 20% piperidine in DMF (v/v), rt, 6 min×2. The amino acid coupling was carried out under DIC/Oxyma-based conditions (4.5 equiv DIC, 4.5 equiv oxyma, and 4.5 equiv Fmoc-protected amino acids, 55°C, 40 min). After DMF washing, the remaining amino group was capped with a capping reagent (DMF/$Ac_2O$/2, 6-lutidine, 89/5/6, rt, 2 min). After DMF washing, the resin was treated with 20% piperidine in DMF (rt, 6 min×2). After the assembly of the peptide, the resin was thoroughly washed by DMF and DCM. To the dried resin was added a freshly prepared TFA solution (TFA/m-cresol/water/TIS, 88/5/5/2, v/v). After 2-hour incubation, the TFA mixture was collected. To the TFA mixture was added a pre-chilled $Et_2O$ (8~10 volume of TFA mixture). After centrifugation and ether washing, the crude peptide was obtained as a powder. The crude peptide was dissolved into **Solvent A**, and analysed by HPLC and ESI-MS.

**Experimental Section**

*Synthesis of peptides mentioned in this study*

Synthesis of **1**




Synthesis of **Pre-1**

**Pre-1** was synthesized from Rink amide MBHA resin (150 μmol) using Fmoc-based SPPS. Diaminodiacid was coupled to the resin as follows. S-C bridged diaminodiacid (127.8 mg, 1.5 eq), PyAOP (391.1 mg, 5.0 eq), HOAT (102.0 mg, 5.0 eq) and NMM (134.1 μL, 8.0 eq) were added to 6.0 mL DMF, and then transferred to the resin. Keep the reaction at 30°C for 3 h. After Ala coupling, the alloc and the allyl groups were removed by the use of $Pd[PPh_3]_4$. $Pd[PPh_3]_4$ (180.0 mg, 1.0 eq) and phenylsilane ( 93.0 μL, 5.0 eq) were dissolved in 6.0 mL of DMF/DCM (1:1). Then, the mixture was then transferred to the resin. Keep the reaction at room temperature for 3 h (in dark). The resin was thoroughly washed with sodium diethyldithiocarbamate (100.0 mg sodium diethyldithiocarbamate in 20.0 mL of DMF) until the solution became clear. Then,

Fmoc group of Ala residue was removed by 20% piperidine in DMF, followed by the intramolecular cyclization. PyAOP (0.78 g, 5.0 eq), HOAT (0.21 g, 5.0 eq), and NMM (0.34 mL, 10.0 eq) were dissolved in 5.0 mL DMF. The mixture was transferred to the resin. Keep the reaction at room temperature for 4 h. Wash the resin five times with DMF. The remaining amino acids were assembled according to standard Fmoc-based SPPS. Note that, the N-terminal AEEA residue is 2-(2-(2-Aminoethoxy)ethoxy) acetic acid. After TFA cleavage and ether precipitation, **Pre-1** was obtained as a white powder. After preparative HPLC and lyophilization, we obtained the purified **Pre-1** as a white powder (27.5 mg, 16.5 μmol, 27.8%). HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 23.3 min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{75}H_{106}N_{20}O_{22}S$ 1671.75; Found 1671.80.

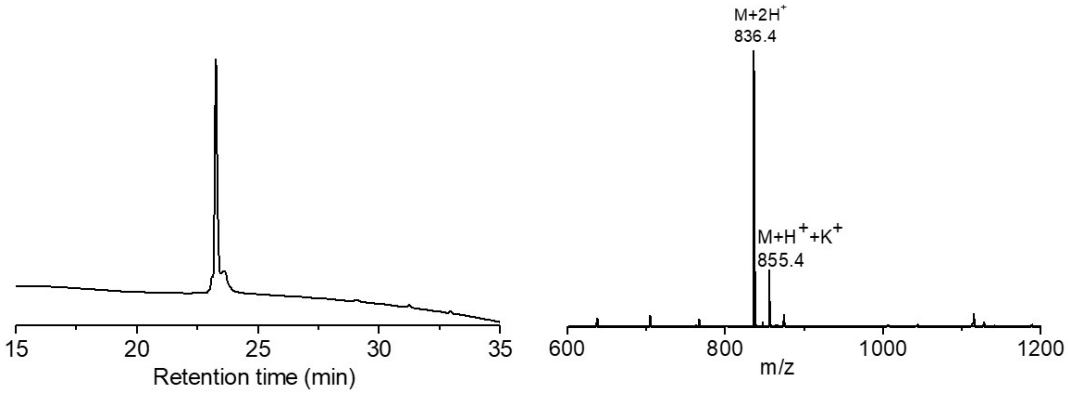



Figure S1: (a) HPLC trace (210nm) and (b) MS spectrum of the purified **Pre-1**.

Synthesis of **1**

2-(tritylthio)acetic acid (2.9 mg, 2.0 eq) was dissolved in 100.0 μL DMF. 4.4 mg HOSu (8.0 eq) in 50.0 μL of DMF was added to the solution of 2-(tritylthio)acetic acid, followed by the addition of DIC (5.3 μL, 8.0 eq). Keep the reaction at room temperature for 1h. Then, the purified **Pre-1** (7.4 mg, 1.0 eq) in 100 μL of DMF was added to the mixture. Then, DIEA (17.6 μL, 24.0 eq) was added. Keep the reaction at room temperature for 1h. Quench the reaction mixture with **Solvent A**. The reaction was analysed by HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm). The desired product **Pre-1'** was purified by HPLC and lyophilized as a white powder (4.8 mg).

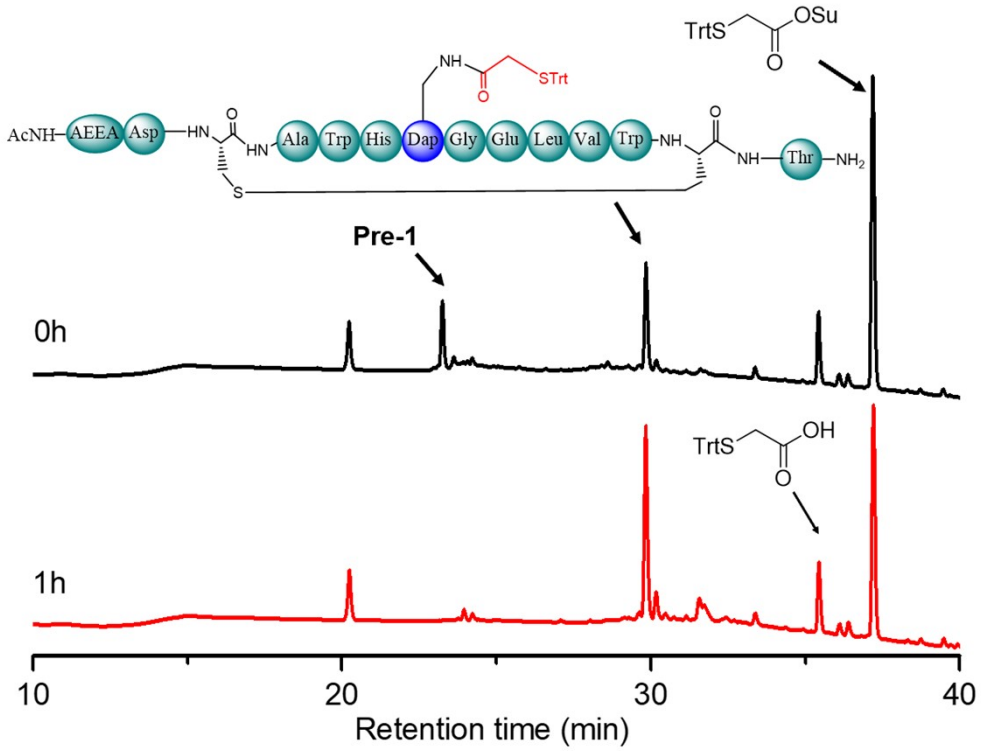



Figure S2: HPLC trace (210nm) the coupling of **Pre-1** to 2-(tritylthio)acetic acid.

**Pre-1'** (8.0 mg, 4.0 μmol) was dissolved in a TFA mixture containing 1% DTT and 9% $H_2O$ (0.9 mL TFA, 0.1 mL $H_2O$, 10 mg DTT). Keep the reaction at room temperature for 90 min. After ether precipitation, HPLC purification and lyophilization, the desired **1** was obtained as a white powder (4.0 mg).

HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 24.1 min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{77}H_{108}N_{20}O_{23}S_2$ 1744.73; Found 1745.60.

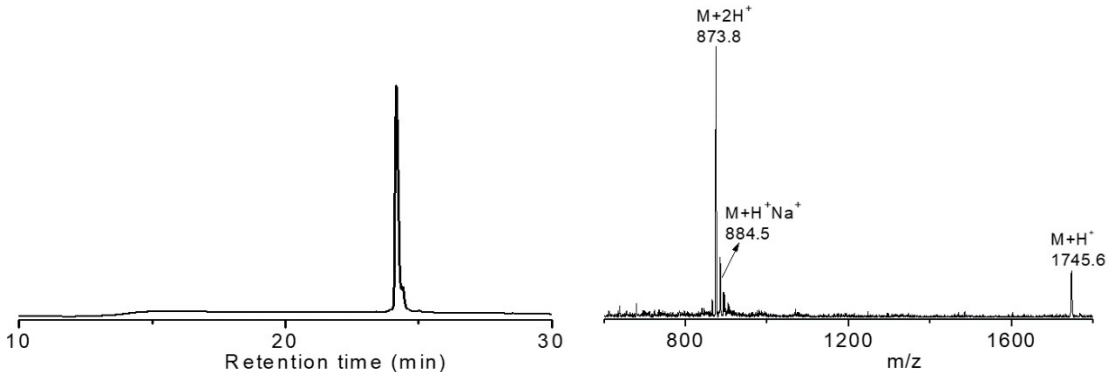
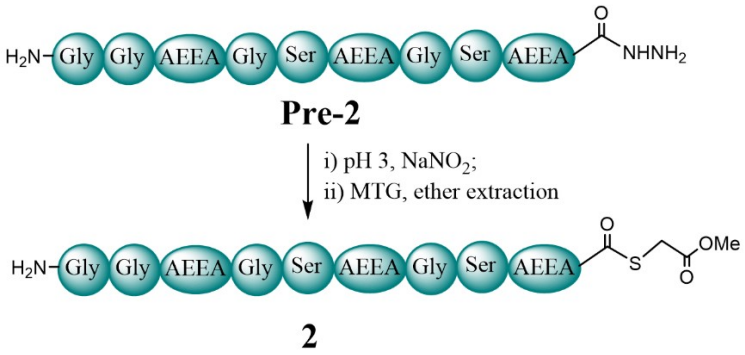



Figure S3: (a) HPLC trace (210nm) and (b) MS spectrum of the purified **1**.

Synthesis of **2**




**Pre-2** was assembled by Fmoc-based SPPS using 2-Chlorotrityl Hydrazine resin (200 µmol). After TFA cleavage and ether precipitation, **Pre-2** was obtained as a white powder. The crude **Pre-2** (9.0 mg, 9.8 µmol) was dissolved in 5.0 mL of pH 3 buffer (6.0 M Gn·HCl, 0.2 M $NaH_2PO_4$). Keep the reaction mixture in an ice-salt bath (-15°C) for 5 min. Then, $NaNO_2$ (8.0 eq, 5.4 mg) in water was added to the mixture. 20 min later, methyl thioglycolate (MTG, 80.0 eq, 70 µL) was added. Keep the reaction mixture at room temperature for 20 min. Then, the excess MTG was extracted by ether, followed by analysing the reaction by HPLC. **Pre-2** was scaled up to 69.0 mg. After preparative HPLC and lyophilization, we obtained the purified **2** as a white powder (22 mg, 23.3 µmol, 27.1%).

HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 19.4 min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{35}H_{61}N_9O_{19}S$ 944.38; Found 944.30.

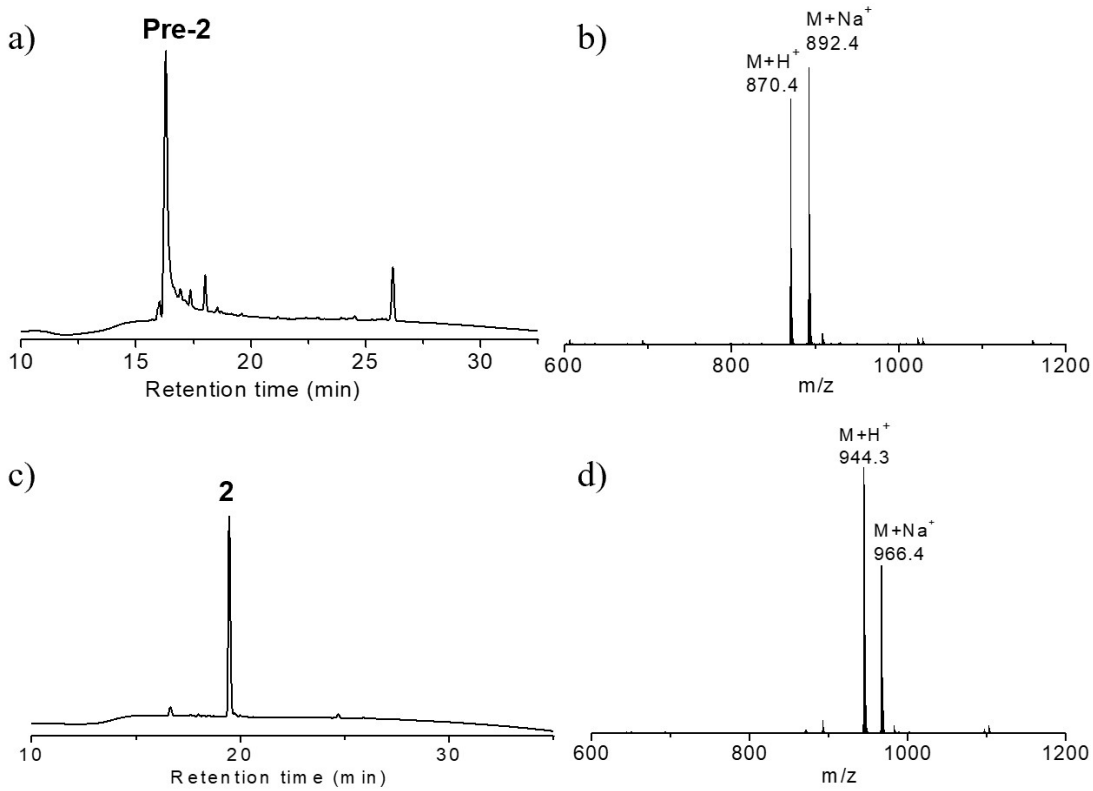
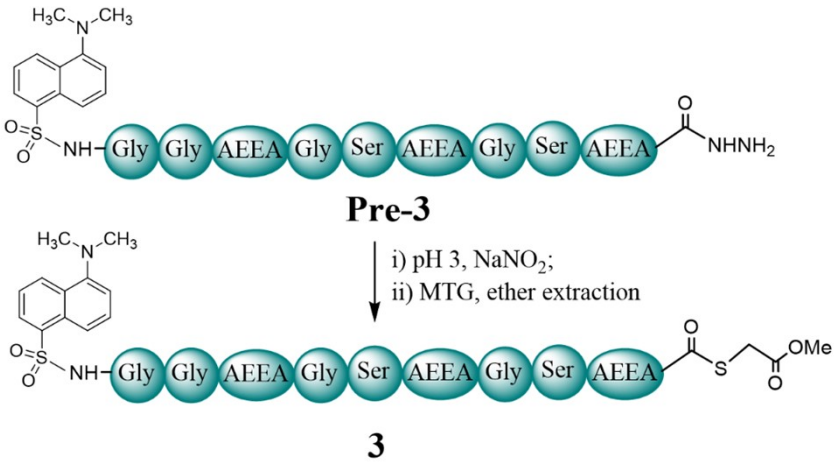



Figure S4: (a) HPLC trace (210nm) and (b) MS spectrum of the crude **Pre-2**; (c) HPLC trace (210nm) and (d) MS spectrum of the purified **2.**

Synthesis of **3**




**Pre-3** was assembled by Fmoc-based SPPS using 2-Chlorotrityl Hydrazine resin (100 μmol). The dansyl chloride coupling conditions: dansyl chloride (82 mg, 3.0 eq) in 4 mL DMF, 100 μL of DIEA (6.0 eq), 25℃, 6~10h. After TFA cleavage and ether precipitation, **Pre-3** was obtained as a yellow powder. The crude **Pre-3** (11.0 mg, 9.8 μmol) was dissolved in 5.0 mL of pH 3.0 buffer (6.0 M Gn·HCl, 0.2 M $NaH_2PO_4$). Keep the mixture in an ice-salt bath (-15℃) for 5 min. Then, $NaNO_2$ (8.0 equiv, 5.4 mg) in water was added. 20 min later, methyl thioglycolate (MTG, 80.0 equiv, 70.0 μL) was added. Keep the reaction at room temperature for 20 min. Then, the excess MTG was extracted by ether, followed by analysing the reaction by HPLC. **Pre-3** was scaled up 33.2 mg. After preparative HPLC and lyophilization, we obtained the purified **3** as a yellow powder (11 mg, 9.4 μmol, 25.8%).

HPLC of **3** (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 22.6 min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{47}H_{72}N_{10}O_{21}S_2$ 1177.43; Found 1177.40.

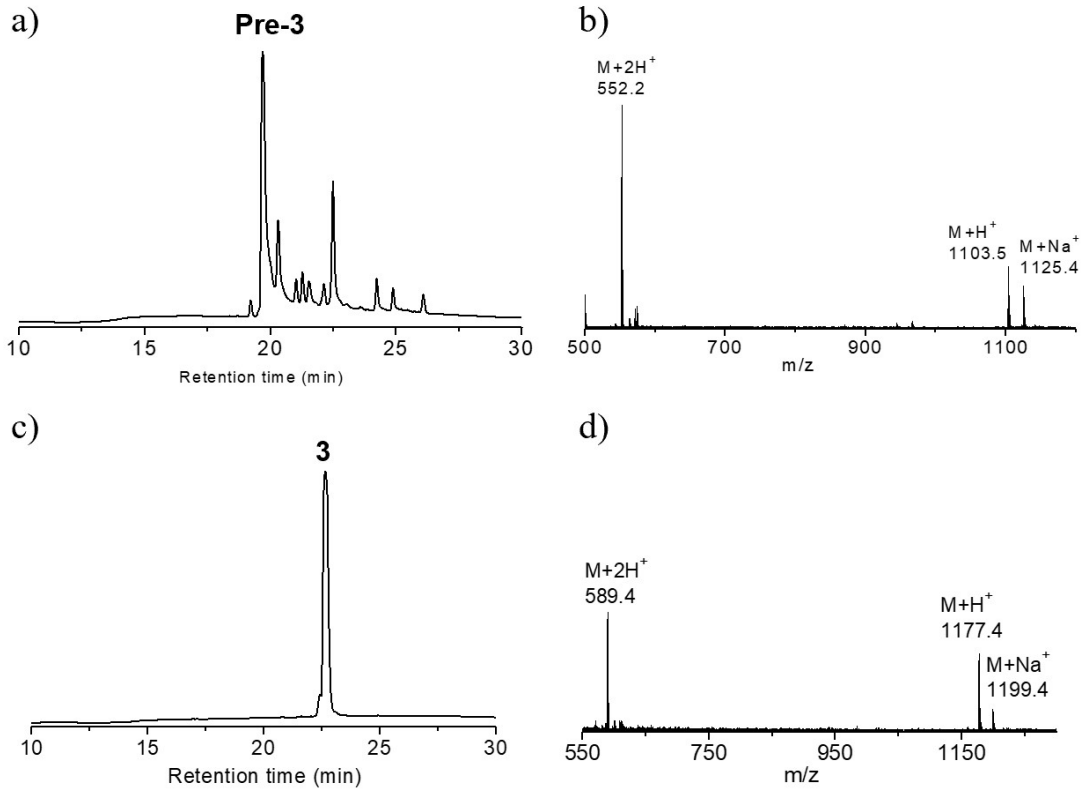
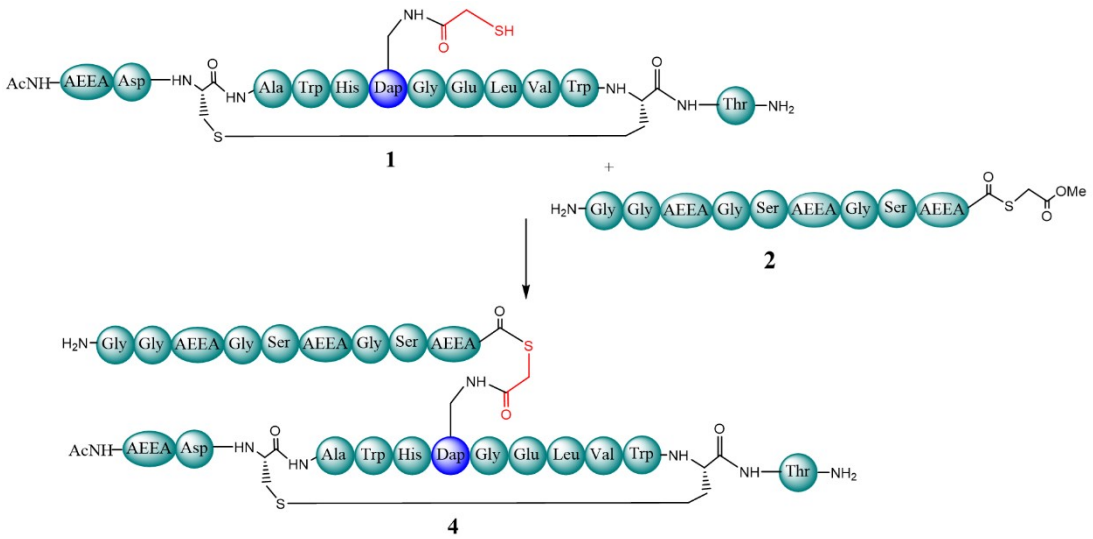



Figure S5: (a) HPLC trace (210nm) and (b) MS spectrum of the crude **Pre-3**; (c) HPLC trace (210nm) and (d) MS spectrum of the purified **3.**

Synthesis of **4**




**1** (0.7 mg, 1.0 eq) was dissolved in 0.2 mL of pH6.6 buffer (6.0 M Gn ·HCl, 0.2 M $Na_2HPO_4$, pH 6.6), and 3.6 mg of **2** (10.0 eq) was dissolved in 0.2 mL of pH6.6 buffer (6.0 M Gn ·HCl, 0.2 M $Na_2HPO_4$, pH 6.6). The solution of **2** was added to the solution of **1**. Keep the reaction mixture at room temperature. The reaction was analysed by HPLC. After HPLC purification and lyophilization, the desired **4** was obtained as a white powder (0.7 mg, 0.27 μmol, 67.5%). HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm), tR = 22.6 min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{109}H_{163}N_{29}O_{40}S_2$ 2584.11; Found 2584.00.

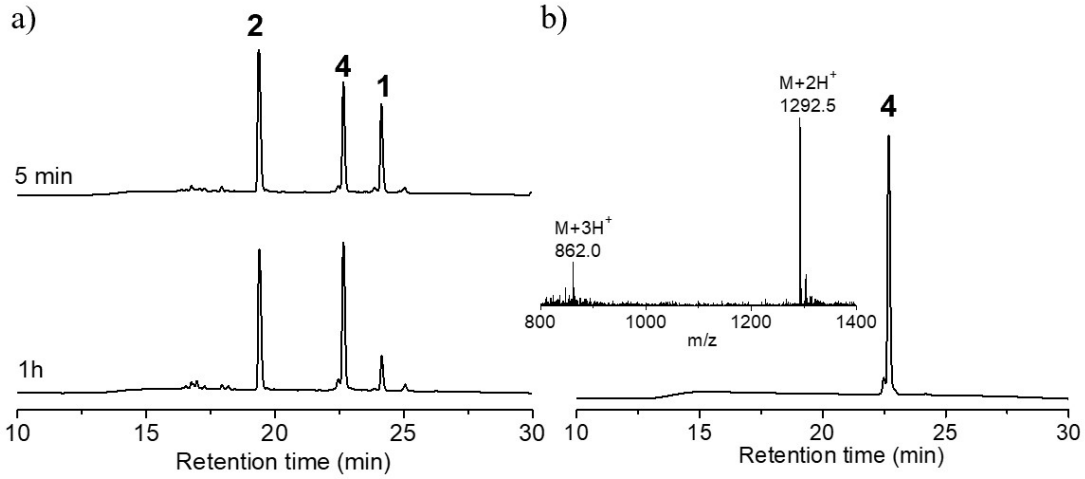
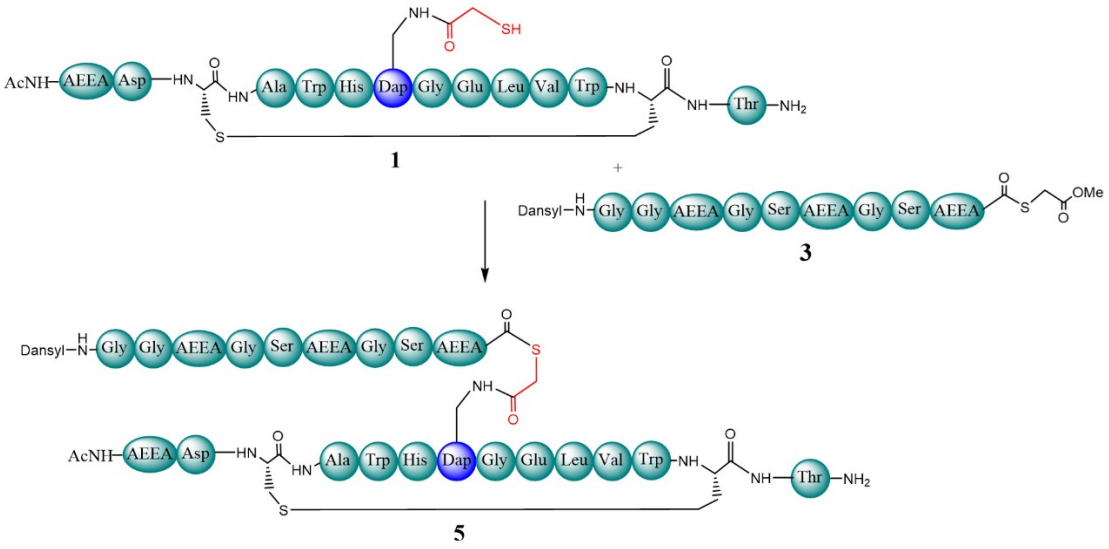



Figure S6: (a) HPLC trace (210nm) of the ligation between **1** and **2** at the time point of 5 min and 60 min; (b) HPLC trace (210nm) and MS spectrum of the purified **4**.

Synthesis of **5**




0.7 mg of **1** (1.0 eq) was dissolved in 0.2 mL of pH 6.6 buffer (6.0 M Gn ·HCl, 0.2 M $Na_2HPO_4$, pH 6.6), and 4.8 mg of **3** (10.0 eq) was dissolved in 0.2 mL of pH 6.6 buffer (6.0 M Gn ·HCl, 0.2 M $Na_2HPO_4$, pH 6.6). The solution of **3** was added to the solution of **1**. Keep the reaction mixture at room temperature. The reaction was analysed by HPLC. After HPLC purification and lyophilization, the desired **5** was obtained as a white powder (0.9 mg, 0.32 μmol, 80.0%). HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm), tR = 24.5 min (major). MS (ESI) m/z:$[M+H]^+$ Calcd for $C_{121}H_{174}N_{30}O_{42}S_3$ 2817.16; Found 2817.20.

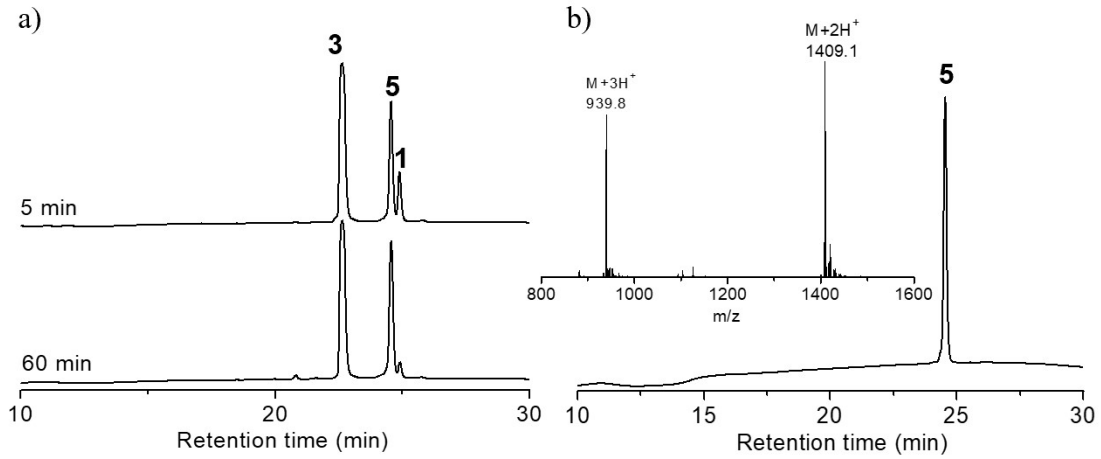
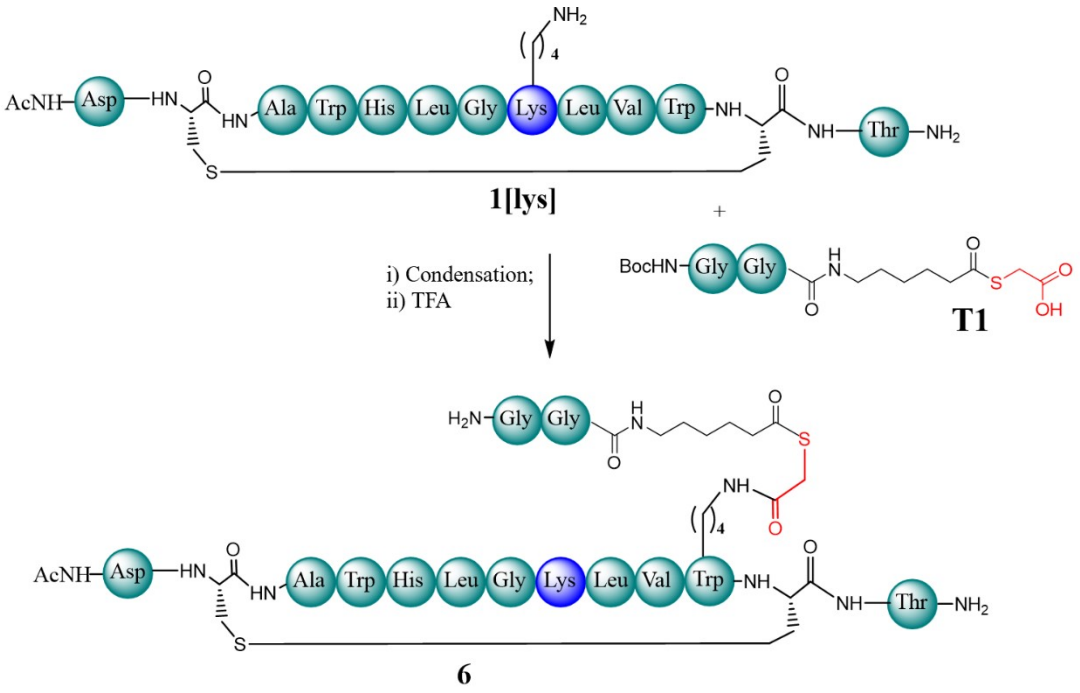



Figure S7: (a) HPLC trace (210nm) of the ligation between **1** and **3** at the time point of 5 min and 60 min; (b) HPLC trace (210nm) and MS spectrum of the purified **5**.

Synthesis of **6**




Synthesis of **1[lys]**

**1[lys]** was synthesized by diaminodiacid Fmoc-based SPPS using Rink amide MBHA resin (150 μmol). After TFA cleavage and ether precipitation, **1[lys]** was obtained as a powder. After preparative HPLC and lyophilization, we obtained the purified **1[lys]** as a white powder (13.6 mg, 8.8 μmol, 15.1%).

HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 24.2 min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{73}H_{105}N_{19}O_{17}S$ 1554.77; Found 1554.0.

Synthesis of **T1**

**T1** was assembled by Fmoc-based SPPS using 2-Chlorotrityl Hydrazine resin (150 μmol). After HFIP cleavage and ether precipitation, the peptide hydrazide **Pre-T1** was obtained as a white powder. The crude **Pre-T1** (4.0 mg, 11.1 μmol) was dissolved in 0.3 mL of pH 3 buffer (6.0 M Gn·HCl, 0.2 M $NaH_2PO_4$). Keep the reaction mixture in an ice-salt bath (-15℃) for 5 min. Then, $NaNO_2$ (7.8 mg, 10.0 eq) in water was added. 20 min later, thioglycolic acid (63.0 μL, 80.0 eq) was added. The pH value was adjusted to 4.0 by NaOH. Keep the reaction mixture at room temperature for 30 min. Then, the excess MTG was extracted by ether, followed by analysing the reaction by HPLC. The crude **Pre-T1** was scaled up to 28.0 mg. After preparative HPLC and lyophilization, we obtained the purified **T1** as a white powder (6.9 mg, 16.4 μmol, 21.1%).

HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 22.6 min (major). MS (ESI) m/z:$[M+H]^+$ Calcd for $C_{17}H_{29}N_3O_7S$ 420.2; Found 420.2.

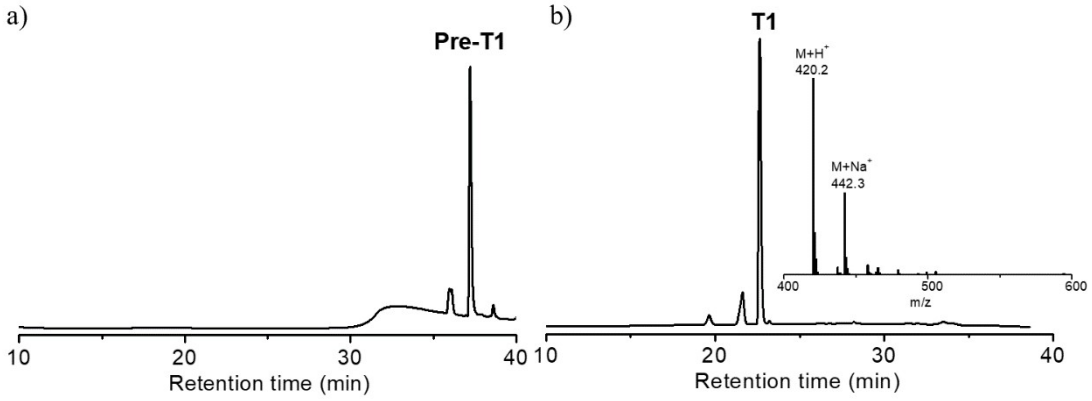



Figure S8: (a) HPLC trace (210nm) of the crude **Pre-T1** and (b) HPLC trace and MS spectrum of **T1.**

Synthesis of **6**

7.4 mg of **1[lys]** (1.0 eq) was dissolved in 0.25 mL DMF. 30 mg of **T1** (15.0 eq) was dissolved in 0.25 mL of DMF. The solution of **1[lys]** was added to the solution of **T1**, followed by the addition of WSC·HCl (14 mg, 15.0 eq). Keep the reaction mixture at room temperature. Overnight, the reaction was analysed by HPLC. After HPLC purification and lyophilization, the desired **Pre-6** was obtained as a white powder (1.9 mg, 0.97 μmol, 20.1%). 10.0 mg of **Pre-6** was dissolved in the TFA mixture (0.9 mL TFA, 0.1 mL $H_2O$). After 1h at room temperature, the desired **6** was precipitated by ether to afford a white powder. After preparative HPLC and lyophilization, we obtained the purified **6** as a white powder (5.0 mg, 2.7 μmol, 52.6%).

HPLC (gradient: 2-90% B in 40 min, column B, flow rate = 1.0 mL/min, I = 210 nm) tR = 23.9 min(major) MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{85}H_{124}N_{22}O_{21}S_2$ 1854.88; Found 1855.40.

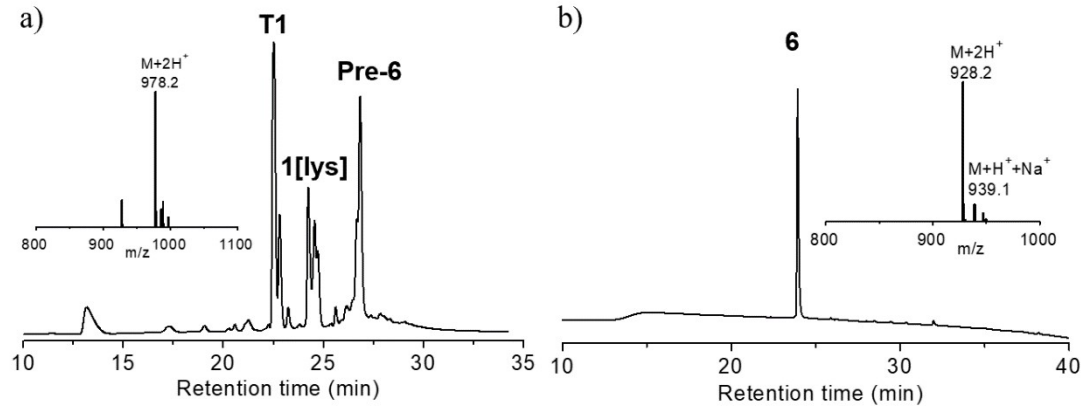
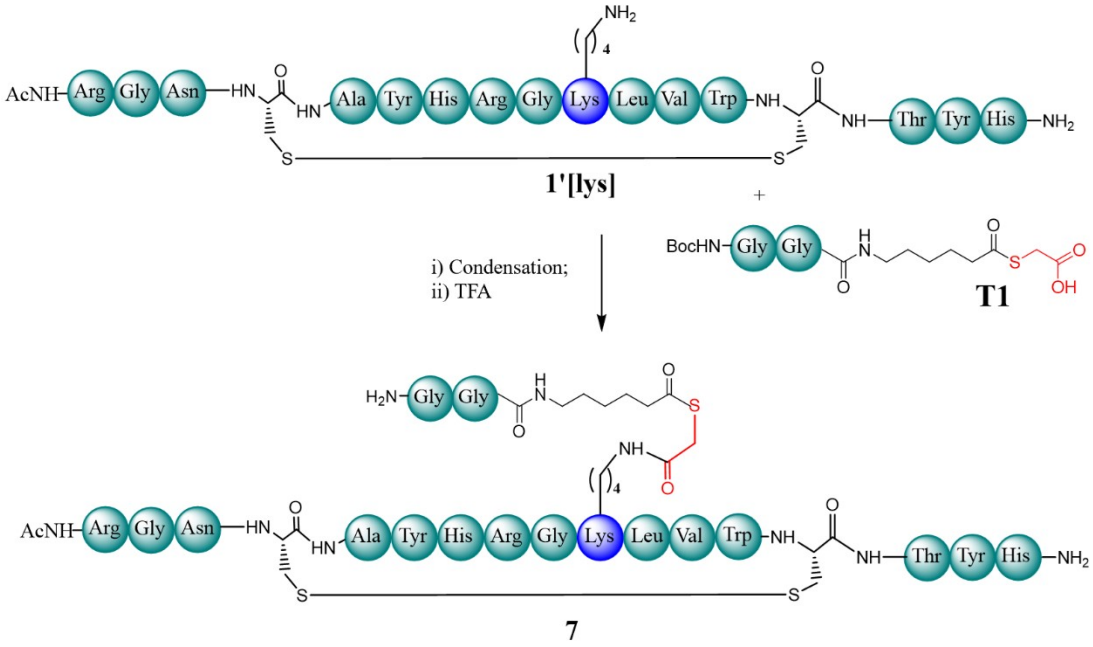



Figure S9: (a) HPLC trace (210 nm) of the reaction between $\mathbf{T_1}$ and **1[lys]** at the time point of ~8h; (b) HPLC trace (210 nm) and MS spectrum of **6**.

Synthesis of **7**




Synthesis of **1'[lys]**

**1'[lys]** was synthesized by Fmoc SPPS using Rink amide MBHA resin (150 μmol). After TFA cleavage and ether precipitation, the linear precursor of **1'[lys]** was obtained as a powder. After preparative HPLC and lyophilization, we obtained the purified linear precursor of **1'[lys]** as a white powder (23.8 mg, 11.3 μmol, 15.3%).

30 mg of reduced GSH and 6 mg of oxidized GSSH were dissolved in 100 mL water. Then, 10 mg of the linear precursor of **1'[lys]** was added to the solution. The pH value of the mixture was adjusted to 7.9-8.1. Keep the reaction mixture at room temperature for 8 h. After preparative HPLC and lyophilization, we obtained the purified **1'[lys]** as a white powder (2.1 mg, 1.0 μmol, 21.0%).

HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR =20.6 min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{93}H_{135}N_{31}O_{22}S_2$ 2104; Found 2105.6.

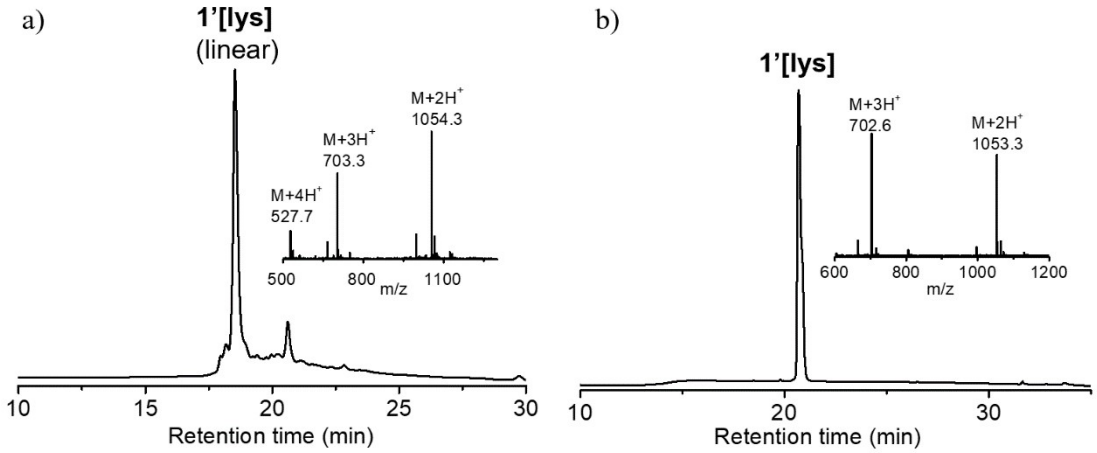



Figure S10: HPLC trace (210nm) and MS spectrum of (a) the linear precursor of **1'[lys]** and

(b) **1'[lys]**.

Synthesis of **7**

10.0 mg of **1'[lys]** (1.0 eq) was dissolved in 0.25 mL of DMF. 30 mg of **T1** (15.0 eq) was dissolved in 0.25 mL DMF. The solution of **1'[lys]** was added to the solution of **T1**, followed by the addition of WSC ·HCl (14 mg, 15.0 eq). Keep the reaction mixture at room temperature. Overnight, the reaction was analysed by HPLC. After HPLC purification and lyophilization, the desired **Pre-7** was obtained as a white powder (2.4 mg, 0.96 μmol, 19.8%). 10.0 mg of **Pre-7** was dissolved in the TFA mixture (0.9 mL TFA, 0.1 mL $H_2O$). After 1h at room temperature, the desired **7** was precipitated by ether to afford a white powder. After preparative HPLC and lyophilization, we obtained the purified **7** as a white powder (5.2 mg, 2.2 μmol, 54.1%).

HPLC (gradient: 2-90% B in 40 min, column B, flow rate = 1.0 mL/min, I = 210 nm) tR = 20.6 min(major) MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{105}H_{154}N_{34}O_{26}S_3$ 2405.80; Found 2406.70.

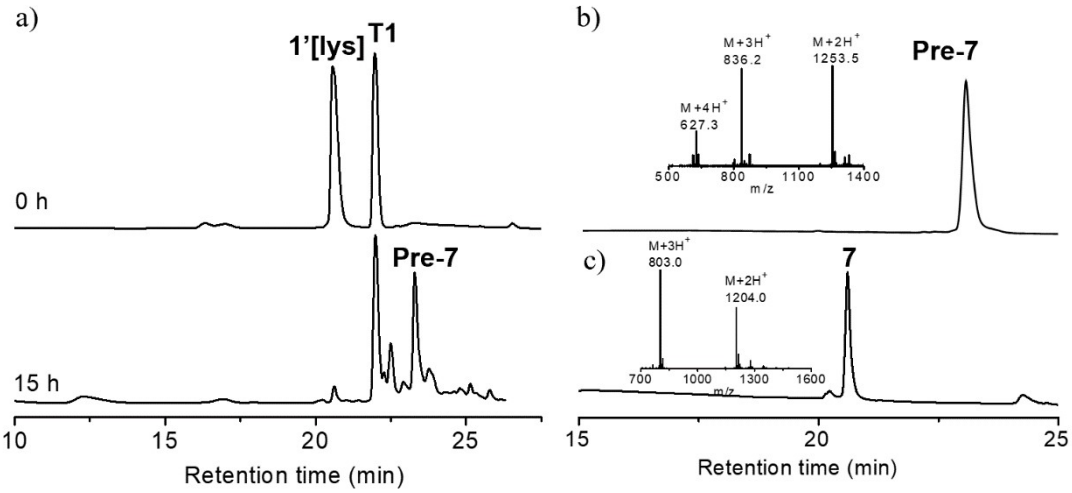
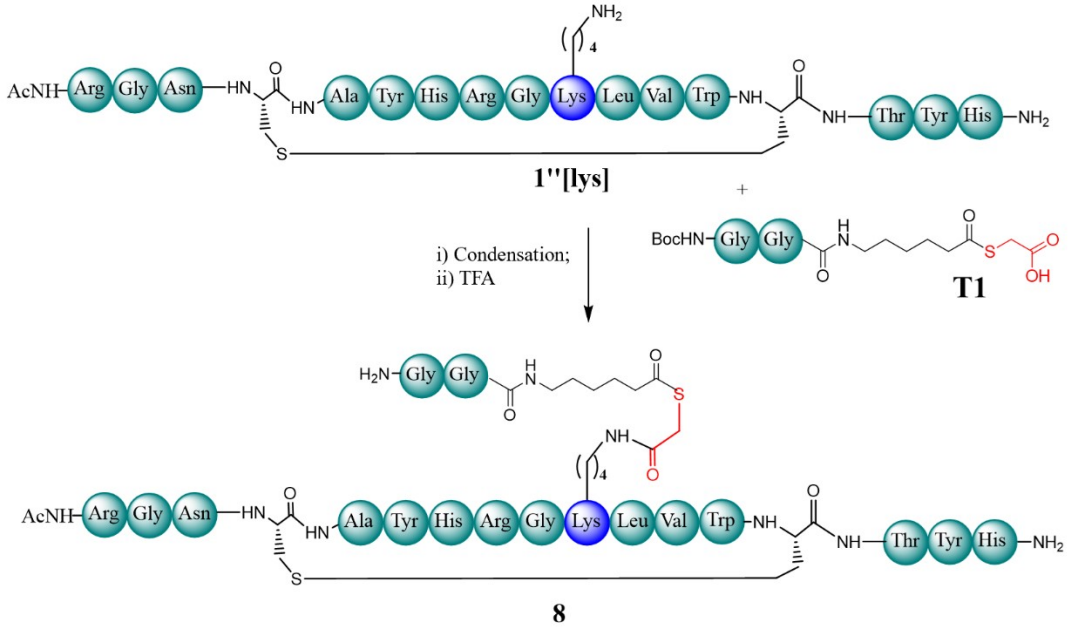



Figure S11: (a) HPLC trace (210nm) of the ligation between **1'[lys]** and **T1** at the time point of 0 h and 15 h; (b) HPLC trace (210nm) and MS spectrum of the purified **Pre-7**; (c) HPLC trace (210nm) and MS spectrum of the purified **7**.

Synthesis of **8**




Synthesis of **1''[lys]**

**1''[lys]** was synthesized by diaminodiacid-based Fmoc SPPS using Rink amide MBHA resin (150 μmol). After TFA cleavage and ether precipitation, **1''[lys]** was obtained as a powder. After preparative HPLC and lyophilization, we obtained the purified **1''[lys]** as a white powder (17.3 mg, 8.3 μmol, 15.4%).

HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR =18.2 min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{94}H_{137}N_{31}O_{22}S$ 2086.03; Found 2086.4.

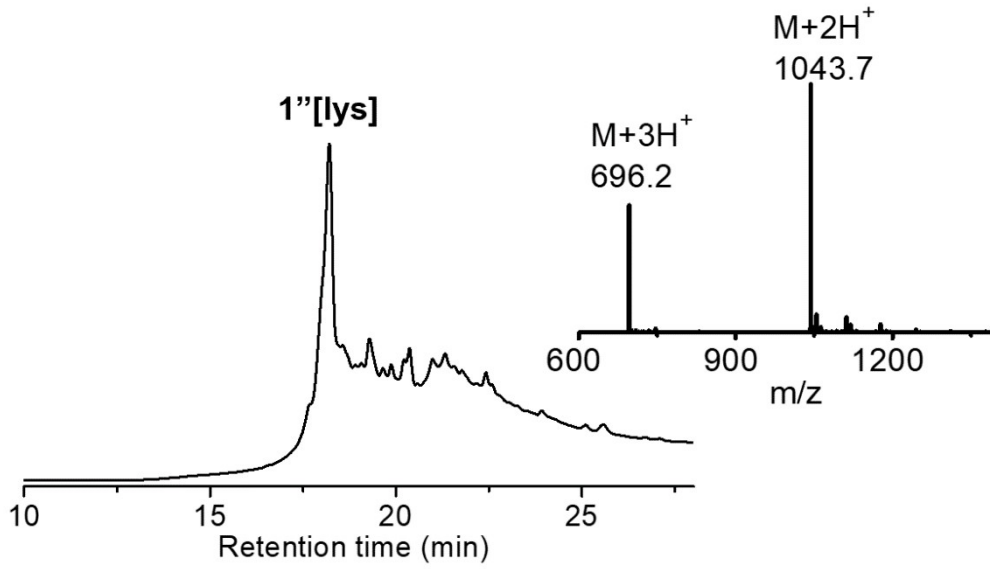



Figure S12: HPLC trace (210nm) and MS spectrum of **1''[lys]**.

Preparation of **8**

**1''[lys]** (10.0 mg, 1.0 eq) was dissolved in 0.25 mL of DMF. **T1** (30 mg, 15.0 eq) was dissolved in 0.25 mL of DMF. The solution of **1''[lys]** was added to the solution of **T1**, followed by the addition of WSC·HCl (14 mg, 15.0 eq). Keep the reaction mixture at room temperature. Overnight, the reaction was analysed by HPLC. After HPLC purification and lyophilization, the desired **Pre-8** was obtained as a white powder (2.4 mg, 0.96 μmol, 20.3%). 10.0 mg of **Pre-8** was dissolved in the TFA mixture (0.9 mL TFA, 0.1 mL $H_2O$). After 1h at room temperature, the desired **8** was precipitated by ether to afford a white powder. After preparative HPLC and lyophilization, we obtained the purified **8** as a white powder (4.7 mg, 1.97 μmol, 48.9%).

HPLC (gradient: 2-90% B in 40 min, column B, flow rate = 1.0 mL/min, I = 210 nm) tR = 18.2 min(major) MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{106}H_{156}N_{34}O_{26}S_2$ 2387.10; Found 2387.80.

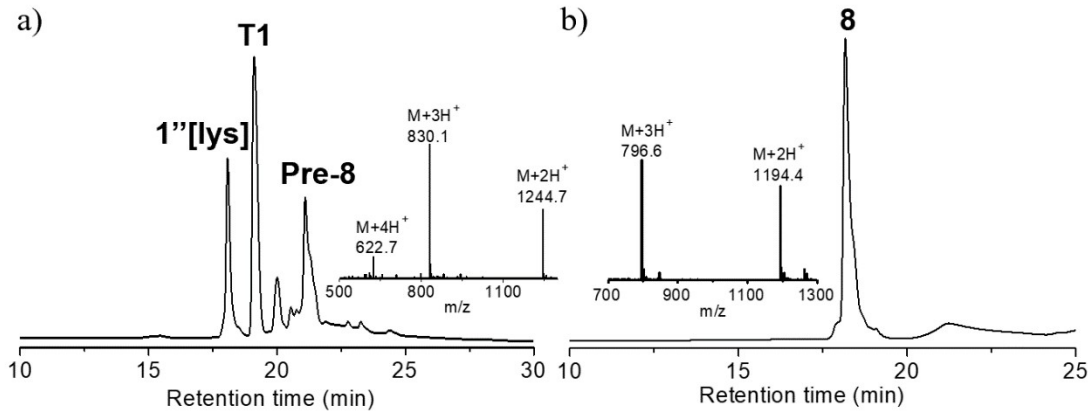
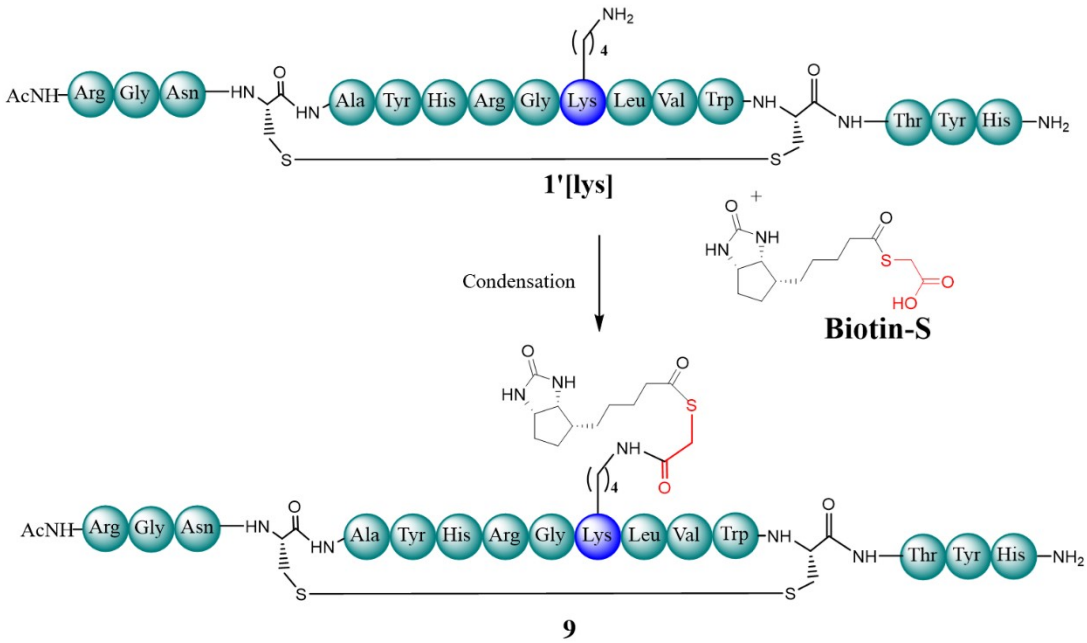



Figure S13: (a) HPLC trace (210nm) of the ligation between **1''[lys]** and **T1** at the time point of 4 h; (b) HPLC trace (210nm) and MS spectrum of the purified **8**.

Synthesis of **9**



Synthesis of **Biotin-S**

**Biotin-OSu** was prepared by the coupling of Biotin (1.0 eq) and HOSu (1.0 eq) in the presence of DIC (1.0 eq). **Biotin-OSu** (10.0 mg, 1.0 eq) was dissolved in 0.5 mL of DMF, followed by the addition of thioglycolic acid (10.0 eq) in 0.5 mL of PBS buffer (0.2 M $Na_2HPO_4$, pH 7.4). Keep the reaction mixture at room temperature. After 1h, the reaction was analysed by HPLC. After preparative HPLC and lyophilization, we obtained the desired **Biotin-S** as a white powder (6.2 mg, 19.5 μmol, 66.5%).

HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 22.5 min(major) MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{12}H_{18}N_2O_4S_2$ 319.07; Found 319.20.

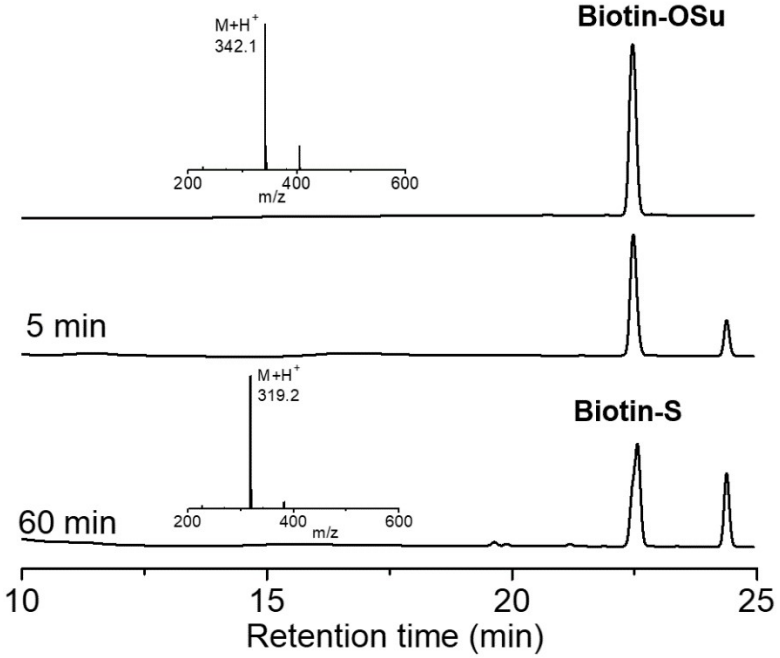



Figure S14: HPLC trace (210nm) of the reaction between **Biotin-OSu** and thioglycolic acid at the time point of 5 min and 60 min (retention time of **Biotin-OSu** is the same as **Biotin-S**).

Preparation of **9**

**Biotin-S** (1.0 mg, 1.0 eq), HOSu (6.0 eq), and DIC (6.0 eq) were dissolved in 75.0 μL of DMF. Keep the reaction mixture at room temperature for 1 h. To this mixture was added **1'[lys]** (3.0 mg) in 75.0 μL of DMF. DIEA (12.0 eq) was added to this mixture. After 15 min, the reaction mixture was analysed by HPLC. After purification and lyophilization, the desired **9** was obtained as a white powder (0.7 mg, 0.29 μmol, 19.5%).

HPLC of **9** (gradient: 2-90% B in 40 min, column B, flow rate = 1.0 mL/min, I = 210 nm) tR = 22.9 min(major) MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{105}H_{151}N_{33}O_{25}S_4$ 2404.05; Found 2404.60

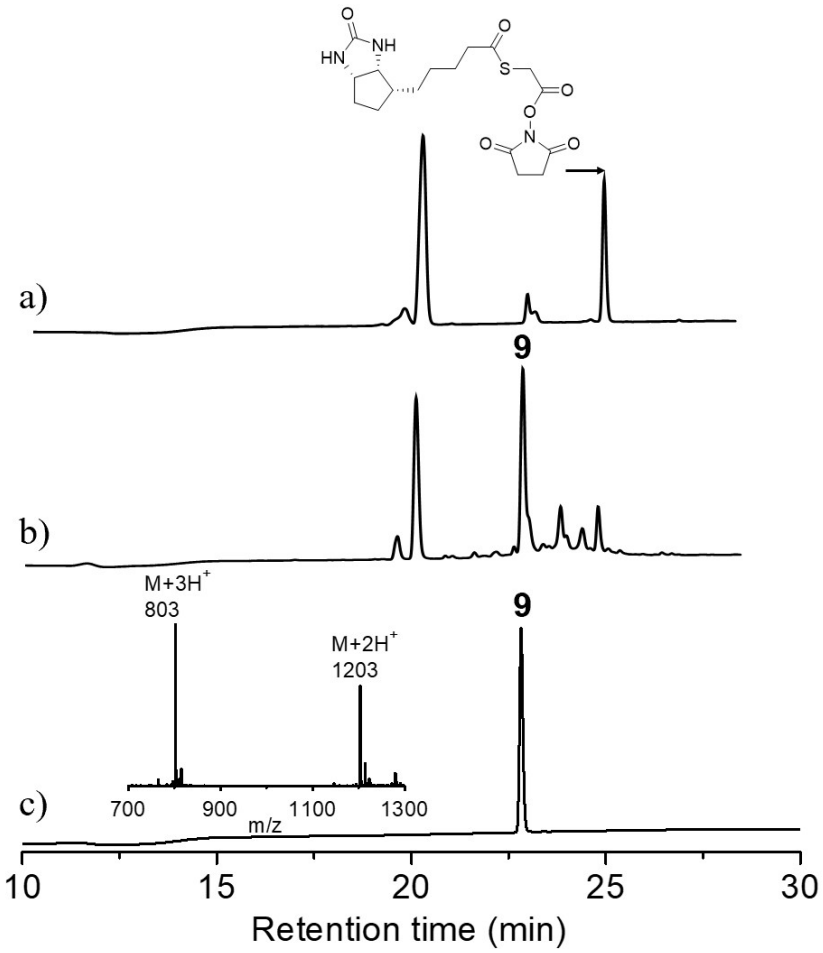
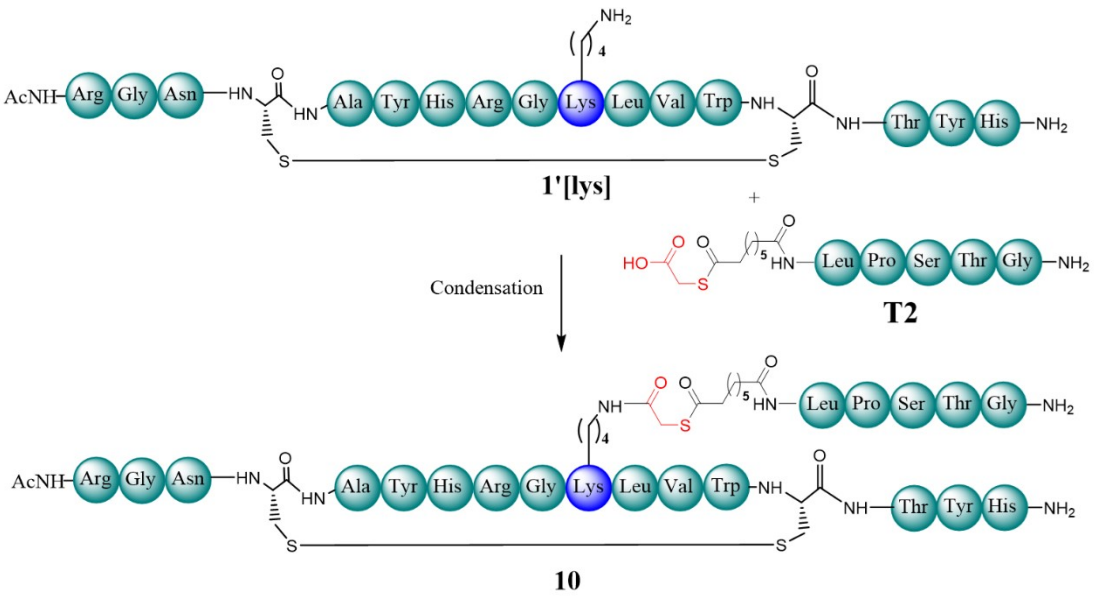
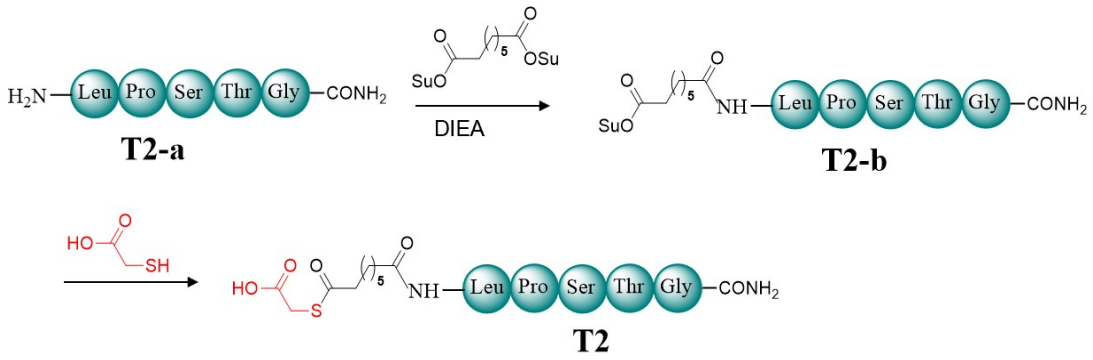



Figure S15: HPLC trace (210nm) of the reaction between **Biotin-S** and **1'[lys]**: (a) conversion of **Biotin-S** into its corresponding NHS-ester; (b) the reaction of **1'[lys]** and **Biotin-S** NHS-ester at the time point of 15 min; (c) the purified **9** and its MS spectrum.

Synthesis of **10**



Synthesis of **T2**




**T2-a** was synthesized by Fmoc-based SPPS with Rink amide MBHA resin (150 μmol). After TFA cleavage and ether precipitation, the crude **T2-a** was obtained as a white powder (62.0 mg). 10.0 mg of **T2-a** was dissolved in 0.2 mL of DMF. Disuccinimidyl suberate (DSS) (78.0 mg, 10.0 eq) was dissolved in 0.8 mL of DMF. The solution of **T2-a** was mixed with the solution of DSS, followed by the addition of DIEA (7.4 μL, 2.0eq). After 10 min, the reaction was completed as conformed by HPLC. **T2-b** (2.0 mg, 1.0 eq) was dissolved in 0.2 mL of DMF, followed by the addition of thioglycolic acid (2 μL, 10.0 eq) in 0.2 mL PBS solution (0.2 M $Na_2HPO_4$, pH 7.4). Keep the reaction mixture at room temperature. **T2-b** was scaled up to 6.4 mg. After purification and lyophilization, the desired **T2** was obtained as a white powder (1.9 mg, 2.7 μmol, 30.6%).

HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm), tR = 19.7 min(major) MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{30}H_{50}N_6O_{11}S$ 703.3; Found 703.7.

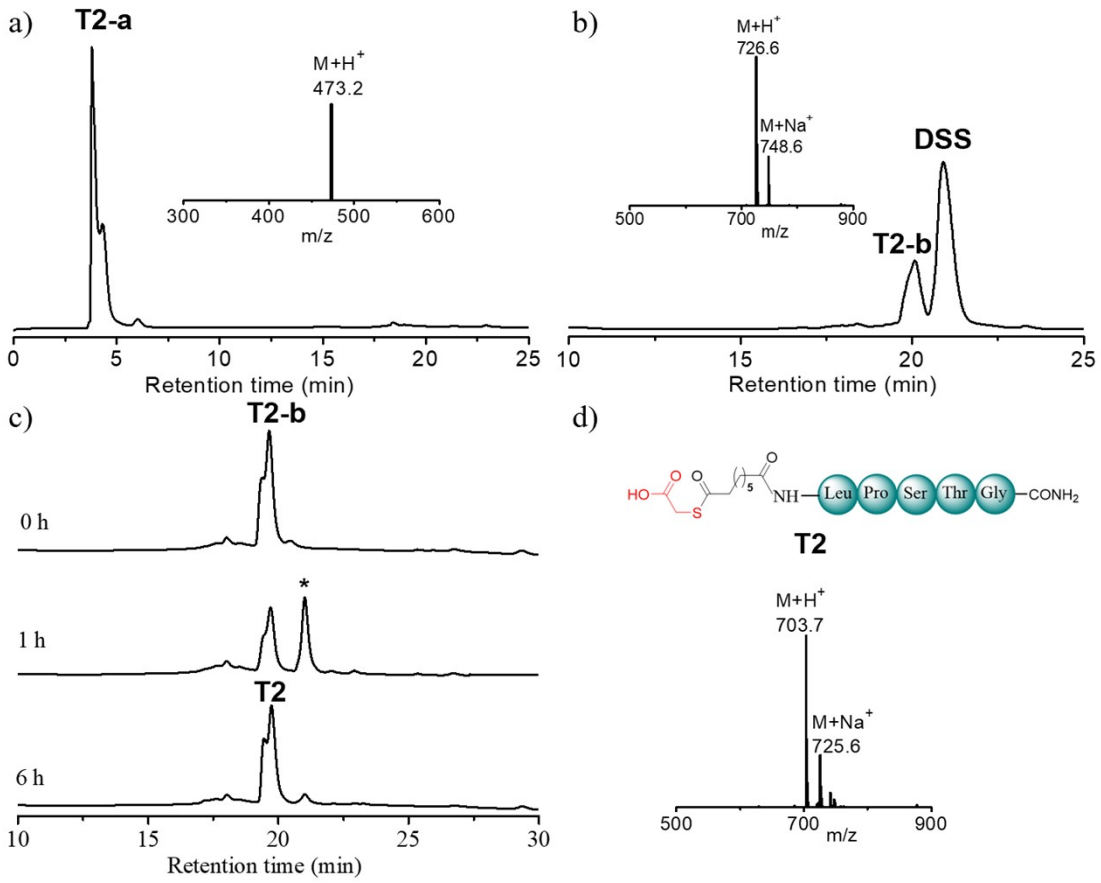



Figure S16: HPLC trace (210nm) for the preparation of **T2**: (a) the crude **T2-a**; (b) **T2-a** reacts with DSS to afford **T2-b**; (c) T2-b reacts with thioglycolic acid to afford **T2** (**T2** and **T2-b** almost overlaps, * disappears after 6-hour incubation); (d) MS spectrum of **T2**.

Preparation of **10**

3.0 mg of **T2** (1.0 eq), HOSu (6.0 eq), and DIC (6.0 eq) were dissolved in 112.0 µL of DMF. Keep the reaction mixture at room temperature for 1 h. To this mixture was added 4.5 mg of **1'[lys]** in 112.0 µL of DMF. DIEA (12.0 eq) was added to this mixture. After 15 min, the reaction mixture was analysed by HPLC. After purification and lyophilization, the desired **10** was obtained as a white powder (1.1 mg, 0.39 µmol, 19.6%).

HPLC of **10** (gradient: 2-90% B in 40 min, column B, flow rate = 1.0 mL/min, I = 210 nm) tR = 20.7 min(major) MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{123}H_{183}N_{37}O_{32}S_3$ 2789.3; Found 2789.4.

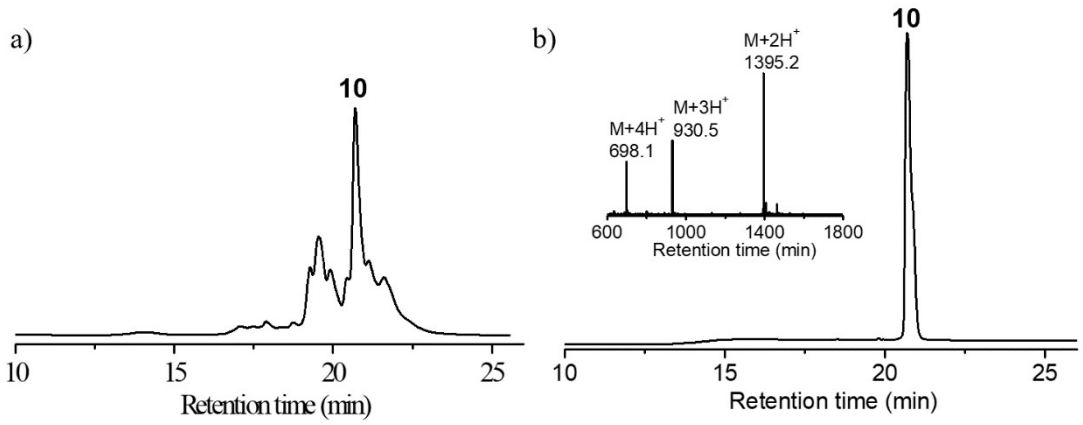



Figure S17: HPLC trace (210nm) of the reaction between **T2** and **1'[lys]**: (b) the reaction of **1'[lys]** and **T2** NHS-ester at the time point of 15 min; (c) the purified **10** and its MS spectrum.

Synthesis of **GLP-1**

**FITC**-AEEA-Tyr-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Glu-Thr-Gly-$NH_2$

**GLP-1** was assembled by Fmoc-based SPPS using Rink amide MBHA resin (150 μmol). Aib: 2-aminoisobutyric acid; AEEA: 2-(2-(2-aminoethoxy)ethoxy)acetic acid. The procedure for the coupling of FITC (Fluorescein 5-isothiocyanate) was shown as follows. 175.2 mg of FITC (3.0 eq) in 1.5 mL of DMF was added to the resin (150 μmol), followed by the addition of DIEA (78 μL, 3.0 eq). Keep the reaction mixture at 33℃ for 3 h. After TFA cleavage and ether precipitation, **GLP-1** was obtained as a yellow powder. After purification and lyophilization, the desired **GLP-1** was obtained as a white powder (53.0 mg, 10.5 μmol, 18.9%). HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 28.5min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{237}H_{340}N_{52}O_{70}S$ 5070.9; Found 5070.1.

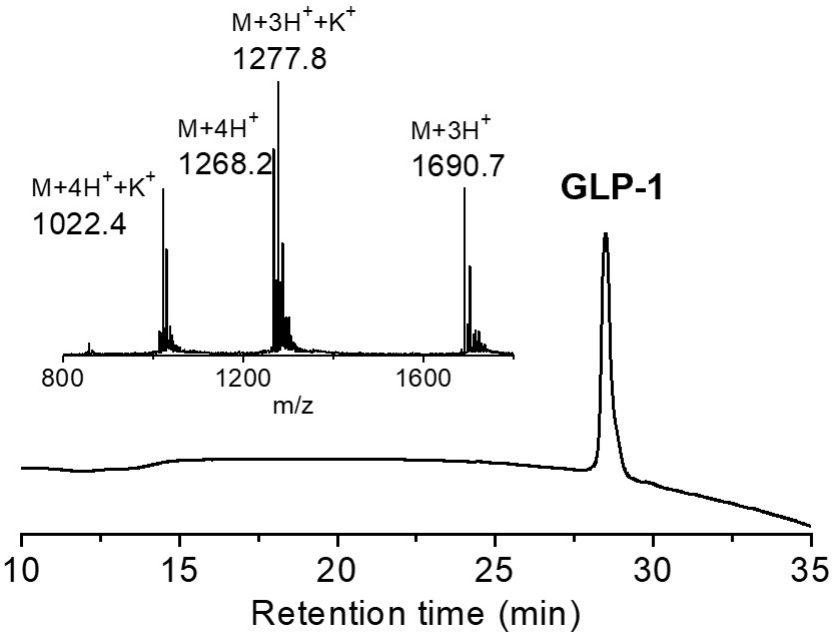



Figure S18: HPLC trace (210nm) and MS spectrum of the purified **GLP-1**.

Synthesis of **GLP-1'** containing a side-chain GG motif

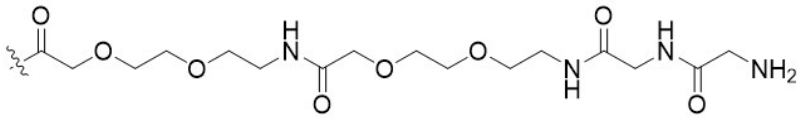
BocNH-Tyr-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-**Lys(ivDde)**-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser

Rink amide resin

i) 3% hydrazine, 15 min x 2;
ii) coupling of Fmoc-AEEA-OH;
iii) repeat step ii);
iv) coupling of Fmoc-Gly-OH;
v) coupling of Boc-Gly-OH;
vi) TFA cleavage

Tyr-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-**Lys(Z)**-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-$NH_2$

**Z**:



**GLP-1'** was assembled by Fmoc-based SPPS using Rink amide MBHA resin (150 μmol). Aib: 2-aminoisobutyric acid. After the coupling of N-terminal Tyr residue, the ivDde group of Lys was deprotected by 3% hydrazine in DMF for 15 min at room temperature. 3% hydrazine in DMF: 85% hydrazine solution was diluted by DMF 28 times to afford 3% hydrazine. Repeat the ivDde deprotection once again. After the coupling of AEEA and Gly residues, the resin was treated by TFA mixture. After purification and lyophilization, the desired **GLP-1'** was obtained as a white powder (34.0 mg, 7.6 μmol, 26.6%). HPLC (gradient: 2-90% B in 40 min, column

A, flow rate = 1.0 mL/min, I = 210 nm) tR = 27.5 min (major). MS (ESI) m/z:$[M+H]^+$ Calcd for $C_{204}H_{311}N_{49}O_{64}$ 4474.27; Found 4473.72.

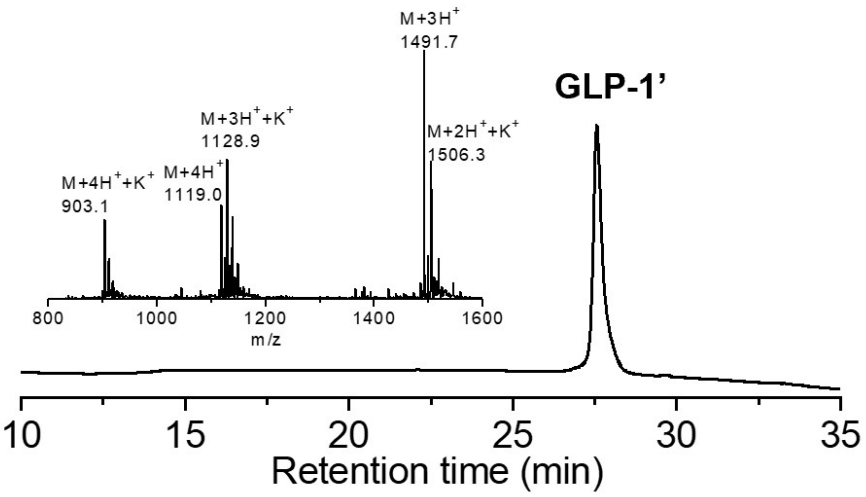
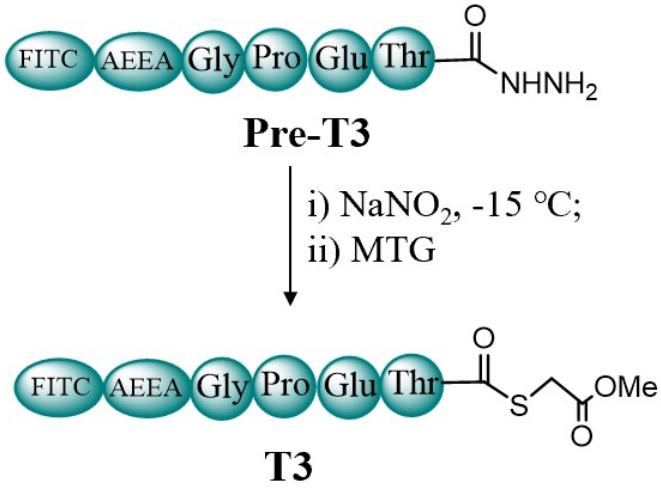



Figure S19: HPLC trace (210nm) and MS spectrum of the purified **GLP-1'**.

Synthesis of **T3**




**Pre-T3** was synthesized by Fmoc-based SPPS using 2-Chlorotrityl Hydrazine resin (150 µmol scale). After TFA cleavage and ether precipitation, **Pre-T3** was obtained as a yellow powder (144.0 mg). The crude **Pre-T3** (9.3 mg, 9.8 µmol) was dissolved in 2.0 mL of pH 3.0 buffer (6.0 M Gn·HCl, 0.2 M $NaH_2PO_4$). Under an ice-salt bath (-15°C), $NaNO_2$ (5.4 mg, 8.0 eq) in water was added to the solution of **Pre-T3**. 20 min later, methyl thioglycolate (MTG, 70 µL, 80.0 eq) was added. Keep the reaction mixture at room temperature for 20 min. Excess MTG was extracted by ether. **Pre-T3** was scaled up to 144 mg. After preparative HPLC and lyophilization, the desired **T3** was obtained as a yellow powder (46.8 mg, 45.6 µmol, 27.2%). HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 25.7 min (major). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{46}H_{52}N_6O_{17}S_2$ 1025.28; Found 1025.5.

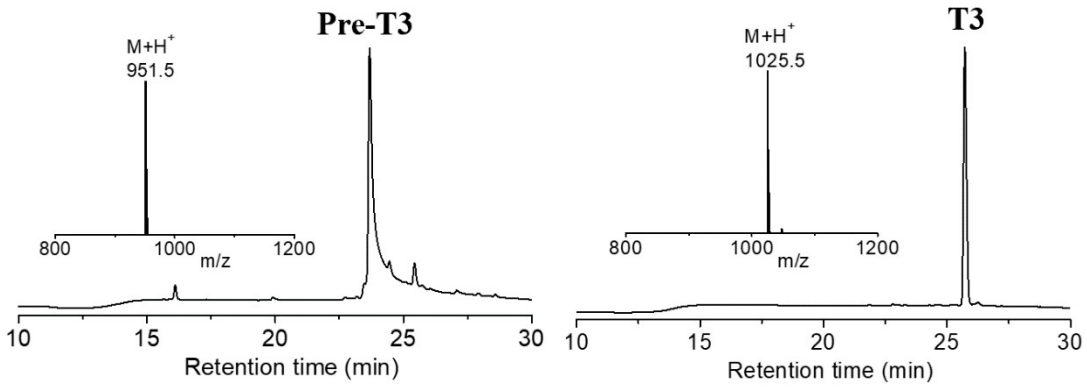
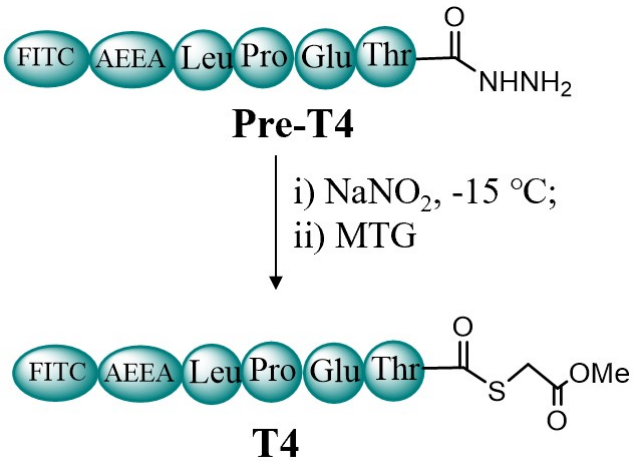



Figure S20: HPLC trace (210nm) and MS spectrum of the purified **Pre-T3** and **T3**.

Synthesis of **T4**




**Pre-T4** was synthesized by Fmoc-based SPPS with 2-Chlorotrityl Hydrazine resin (150 μmol scale). After TFA cleavage and ether precipitation, **Pre-T4** was obtained as a yellow powder (130.0 mg). 9.8 mg of the crude **Pre-T4** (9.8 μmol) was dissolved in 2.0 mL pH 3.0 buffer (6.0 M Gn·HCl, 0.2 M $NaH_2PO_4$). Under an ice-salt bath (-15°C), $NaNO_2$ (8.0 eq, 5.4 mg) in water was added to the solution of **Pre-T4**. 20 min later, methyl thioglycolate (MTG, 80.0 eq, 70 μL) was added. Keep the reaction mixture at room temperature for 20 min. Excess MTG was extracted by ether. **Pre-T4** was scaled up to 130 mg. After preparative HPLC and lyophilization, the desired **T4** was obtained as a yellow powder (41.6 mg, 38.5 μmol, 24.6%). HPLC (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 27.8 min (major). MS (ESI) m/z:[M+H]+ Calcd for $C_{50}H_{60}N_6O_{17}S_2$ 1081.35; Found 1081.6.

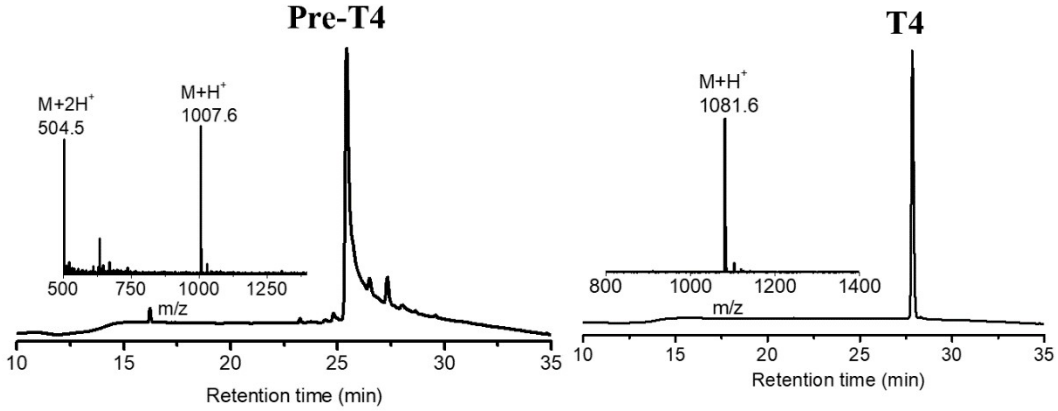



Figure S21: HPLC trace (210nm) and MS spectrum of the purified **Pre-T4** and **T4**.

### ***4, 5, 6, 7, 8, 9****-directed Fc conjugation*

Treatment of bevacizumab with **5**

To 35.0 µL of PIPES buffer (50.0 mM PIPES, pH 6.6) were added 10.0 µL of bevacizumab (1.0 eq, final concentration: 9.0 µM) and 5.0 µL of **5** or **3** (6.0 eq, final concentration: 54.0 µM) in PIPES buffer (50.0 mM PIPES, pH 6.6). After 6h at 25°C, the reaction mixture was analysed by SDS-PAGE.

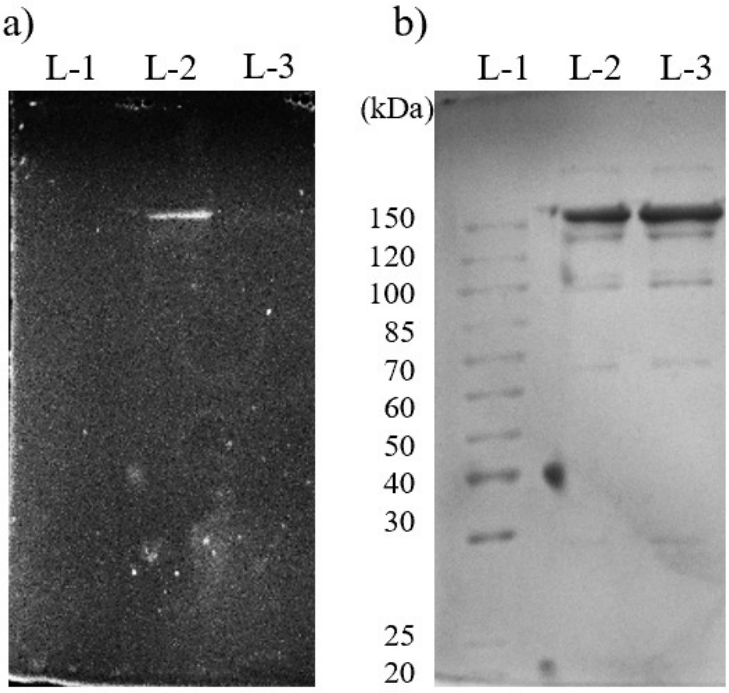



Figure S22: SDS-PAGE (without DTT) of bevacizumab samples treated by **5** or **3** (right gel: coomassie staining; left gel: fluorescence image). L-1: MW protein ladder; L-2: bevacizumab treated by **5**; L-3: bevacizumab treated by **3**.

Treatment of bevacizumab with **4**

6.70 mg of bevacizumab (1.0 eq) and 0.50 mg of **4** (6.0 eq) were dissolved in PIPES buffer (50.0 mM PIPES, pH 6.6). After 6 h at 25℃, the modified bevacizumab was purified by molecular sieve by running a Tris buffer (20.0 mM Tris-HCl, 0.5 M NaCl, pH7.5). The collected samples were analysed by SDS-PAGE (without DTT). The **4**-treated bevacizumab was analysed by LC-MS.

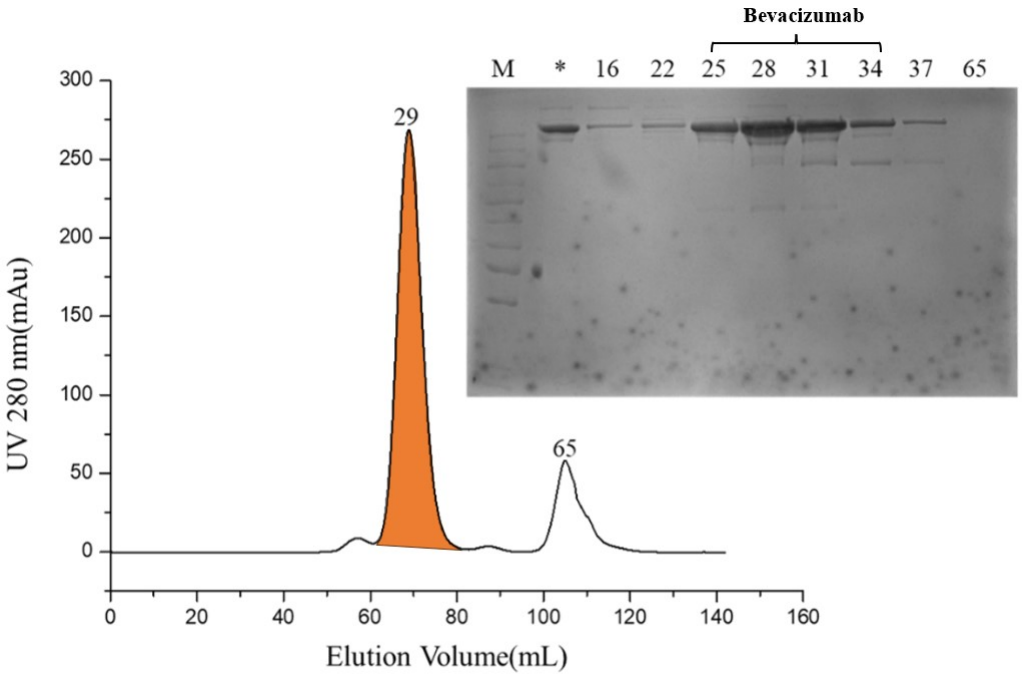



Figure S23: SEC purification of **4**-treated bevacizumab and the corresponding SDS-PAGE (without DTT). "*" represents the naïve bevacizumab.

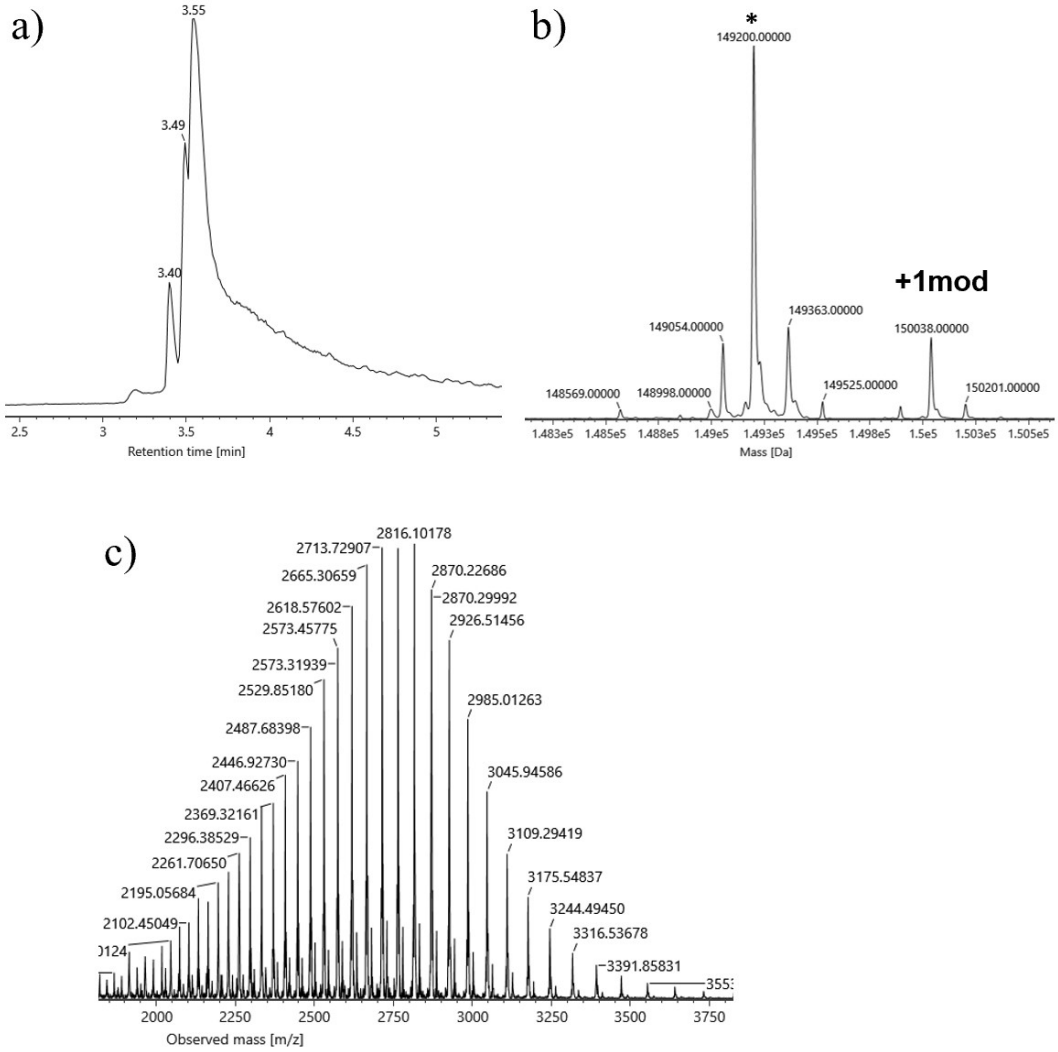



Figure S24: LC-MS spectra of bevacizumab treated with **4** for 6 h, "*" represents the naïve bevacizumab. (a) HPLC of **4**-treated bevacizumab; (b) deconvoluted MS of the sample, "**+1 mod**" is bevacizumab modified with one N-GlyGly peptide; (c) MS of the sample.

SrtA-mediated ligation of **4**-treated bevacizumab with **GLP-1**

12.0 μL of **4**-treated bevacizumab, 5.0 μL of **GLP-1**, and 5.0 μL of SrtA were added to 28.0 μL of HEPES buffer (50.0 mM HEPES, 100.0 mM NaCl, 2.0 mM $CaCl_2$, pH7.0). Note that, the final concentration of bevacizumab was set to be 9.0 μM; the final concentration of **GLP-1** was set to be 9.0 μM, or 36.0 μM, or 72.0 μM; the final concentration of SrtA was set to be 4.5 μM. Keep the reaction mixture at 25℃. After 3 min, 60 min, and 120 min, the reaction mixture was analyzed by SDS-PAGE (without DTT).

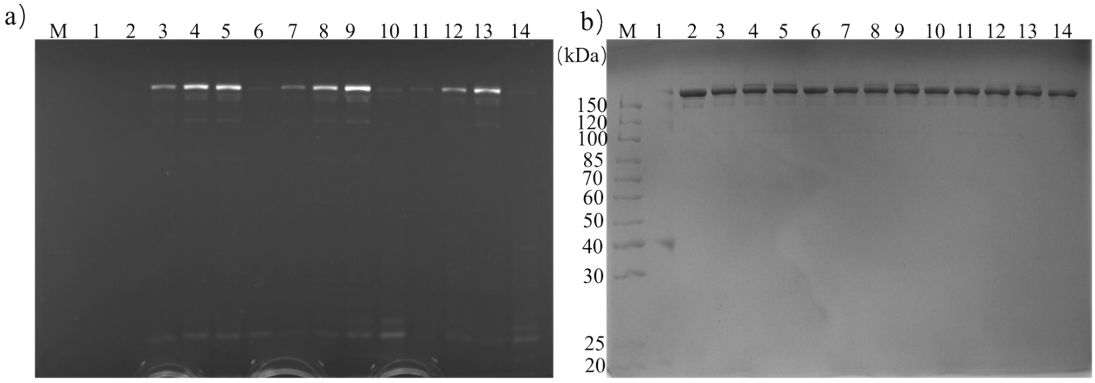



Figure S25: SDS-PAGE of SrtA-mediated ligation of **GLP-1** and **4**-treated bevacizumab. (a) Fluorescence image; (b) coomassie staining. lane 2: naïve Bevacizumab; lane 3: **4**-treated bevacizumab (9.0 μM), **GLP-1** (9.0 μM), 3-min incubation; lane 4: **4**-treated bevacizumab (9.0 μM), **GLP-1** (36.0 μM), 3-min incubation; lane 5: **4**-treated bevacizumab (9.0 μM), **GLP-1** (72.0 μM), 3-min incubation; lane 6: naïve bevacizumab (9.0 μM), **GLP-1** (72.0 μM), 3-min incubation; lane 7: **4**-treated bevacizumab (9.0 μM), **GLP-1** (9.0 μM), 60-min incubation; lane 8: **4**-treated bevacizumab (9.0 μM), **GLP-1** (36.0 μM), 60-min incubation; lane 9: **4**-treated bevacizumab (9.0 μM), **GLP-1** (72.0 μM), 3-min incubation; lane 10: naïve bevacizumab (9.0 μM), **GLP-1** (72.0 μM), 60-min incubation; lane 11: **4**-treated bevacizumab (9.0 μM), **GLP-1** (9.0 μM), 120-min incubation; lane 12: **4**-treated bevacizumab (9.0 μM), **GLP-1** (36.0 μM), 120-min incubation; lane 13: **4**-treated bevacizumab (9.0 μM), **GLP-1** (72.0 μM), 120-min incubation; lane 14: naïve bevacizumab (9.0 μM), **GLP-1** (72.0 μM), 120-min incubation.

Treatment of bevacizumab with **6**

73.0 μL of PIPES buffer (50.0 mM PIPES, pH 7.4) was mixed with 30.0 μL DMF. Then, 30.0 μL of **6** (0.2 mg, 10.0 eq) in DMF was added to the solution, followed by the addition of 167 μL PIPES buffer (50.0 mM PIPES, pH7.4) containing naïve bevacizumab (1.5 mg, 33.4 μM,1.0 eq) in. Keep the reaction mixture at 37°C. After 6-hour incubation, the **6**-treated bevacizumab was purified by protein A resin and analysed by LC-MS.

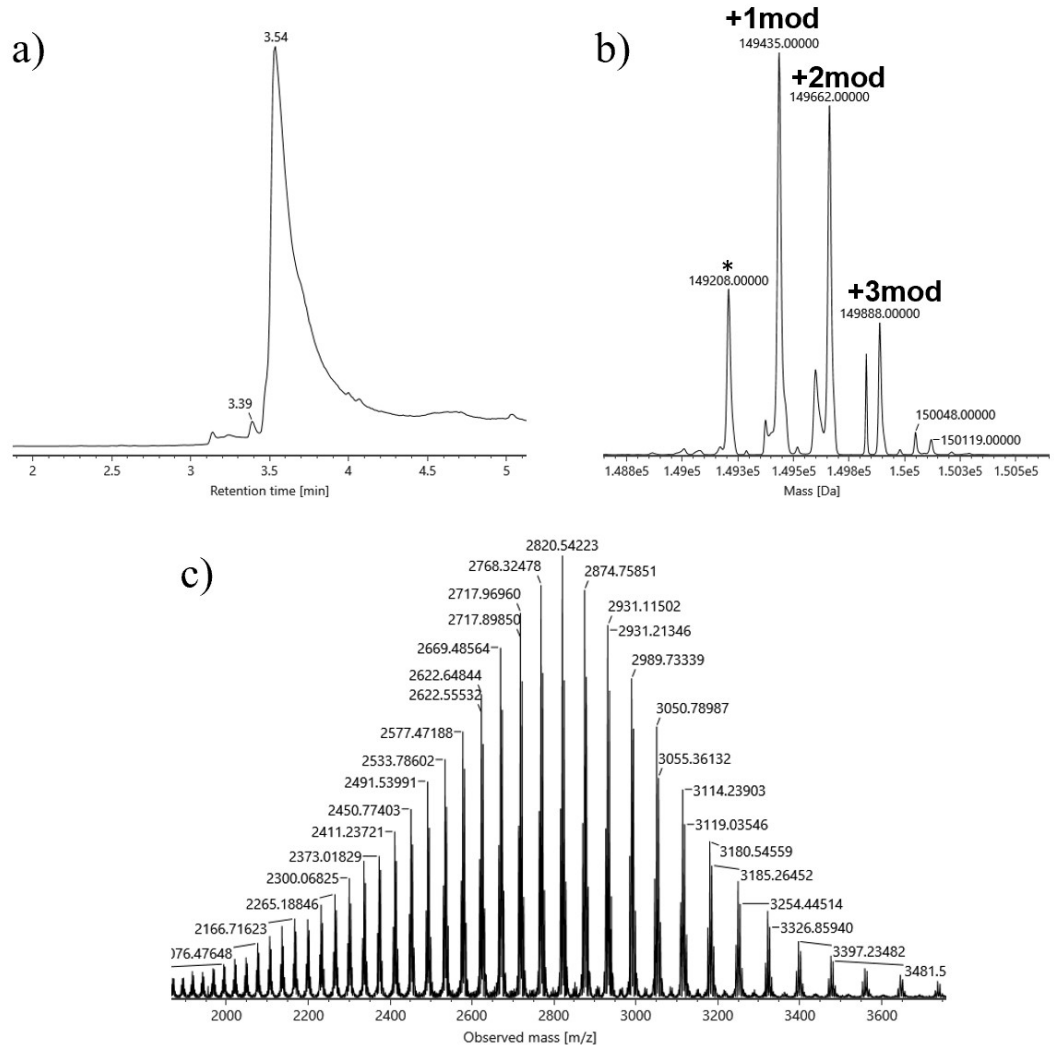



Figure S26: LC-MS spectra of bevacizumab treated with **6** for 6h, "*" represents the naïve bevacizumab. (a) HPLC of **6**-treated bevacizumab; (b) deconvoluted MS of the sample, "**+1 mod**", "**+2 mod**" and "**+3mod**" are the bevacizumabs modified with one, two, and three N-GlyGly peptides; (c) MS of the sample.

Treatment of bevacizumab with **7**

73.0 µL of PIPES buffer (50.0 mM PIPES, pH 7.4) was mixed with 30.0 µL DMF. Then, 30.0 µL of **7** (0.2 mg, 10.0 eq) in DMF was added to the solution, followed by the addition of 167 µL PIPES buffer (50.0 mM PIPES, pH7.4) containing naïve bevacizumab (1.5 mg, 33.4 µM,1.0 eq) in. Keep the reaction mixture at 37℃. After 1-hour or 6-hour incubation, the **7**-treated bevacizumab was purified by protein A resin and analysed by LC-MS.

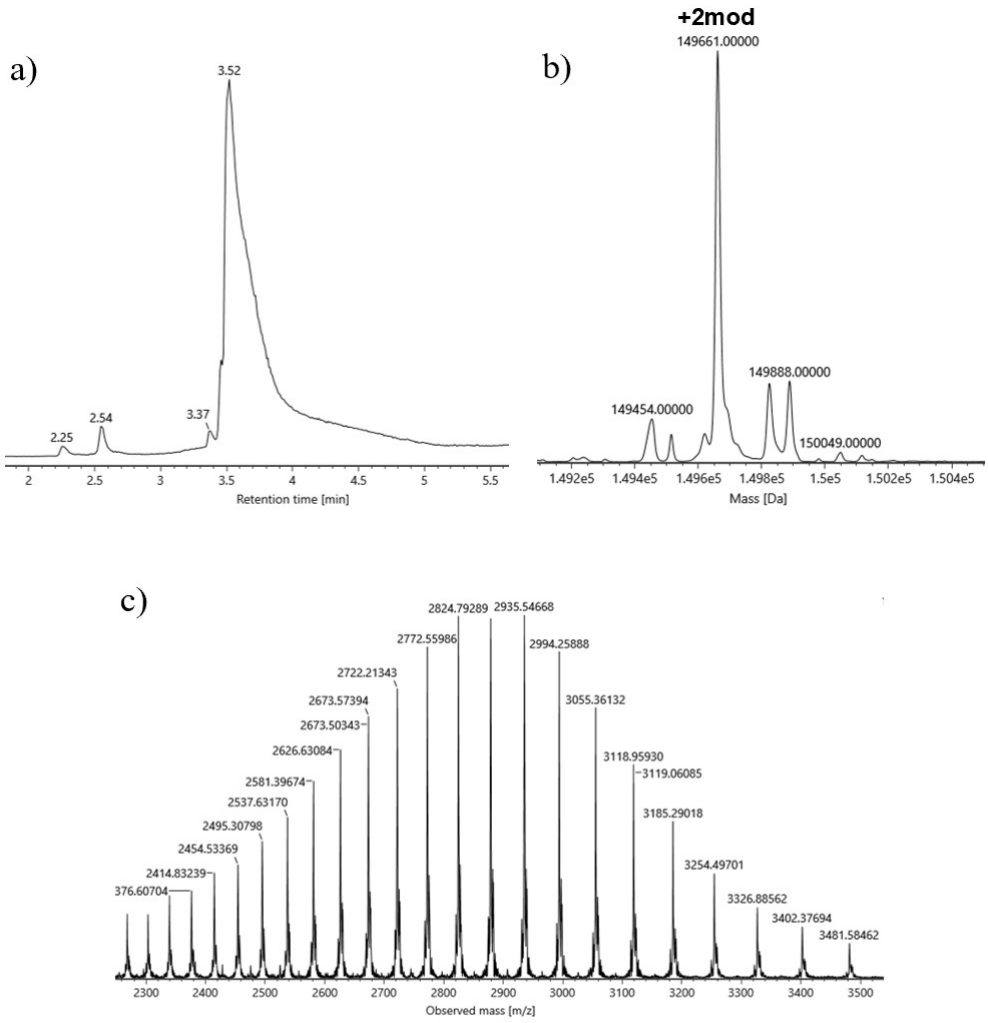



Figure S27: LC-MS spectra of bevacizumab treated with **7** for 1 h. (a) HPLC of **7**-treated bevacizumab; (b) deconvoluted MS of the sample, "**+2 mod**" is the bevacizumab modified with one, two, and three N-GlyGly peptides; (c) MS of the sample.

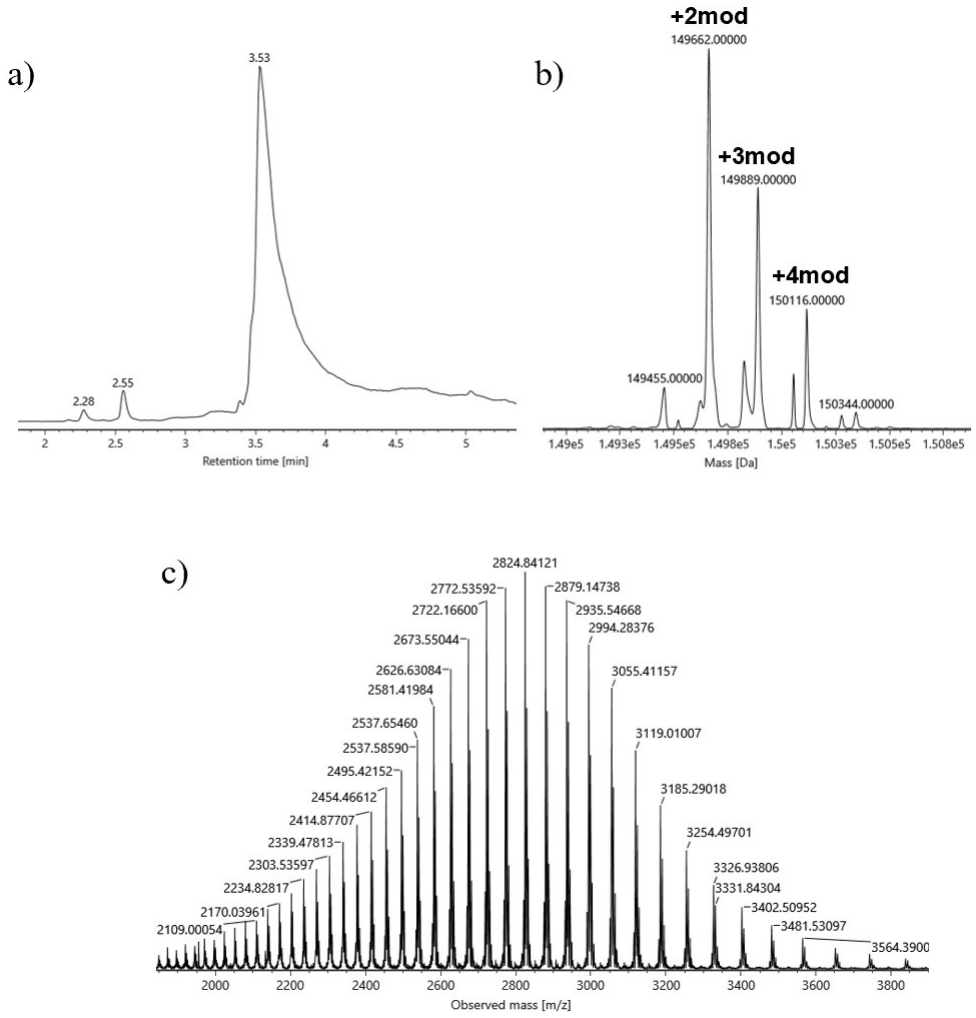



Figure S28: LC-MS spectra of bevacizumab treated with **7** for 6 h. (a) HPLC of **7**-treated bevacizumab; (b) deconvoluted MS of the sample, "**+2 mod**", "**+3mod**" and "**+4mod**" are the bevacizumabs modified with two, three, and four N-GlyGly peptides; (c) MS of the sample.

Treatment of bevacizumab with **8**

136.6 µL of PIPES buffer (50.0 mM PIPES, pH 7.4) was mixed with 30.0 µL DMF. Then, 30.0 µL of **8** (0.24 mg, 10.0 eq) in DMF was added to the solution, followed by the addition of 103.4 µL PIPES buffer (50.0 mM PIPES, pH7.4) containing naïve bevacizumab (1.5 mg, 33.4 µM,1.0 eq) in. Keep the reaction mixture at 37℃. After 1-hour or 6-hour incubation, the **8**-treated bevacizumab was purified by protein A resin and analysed by LC-MS.

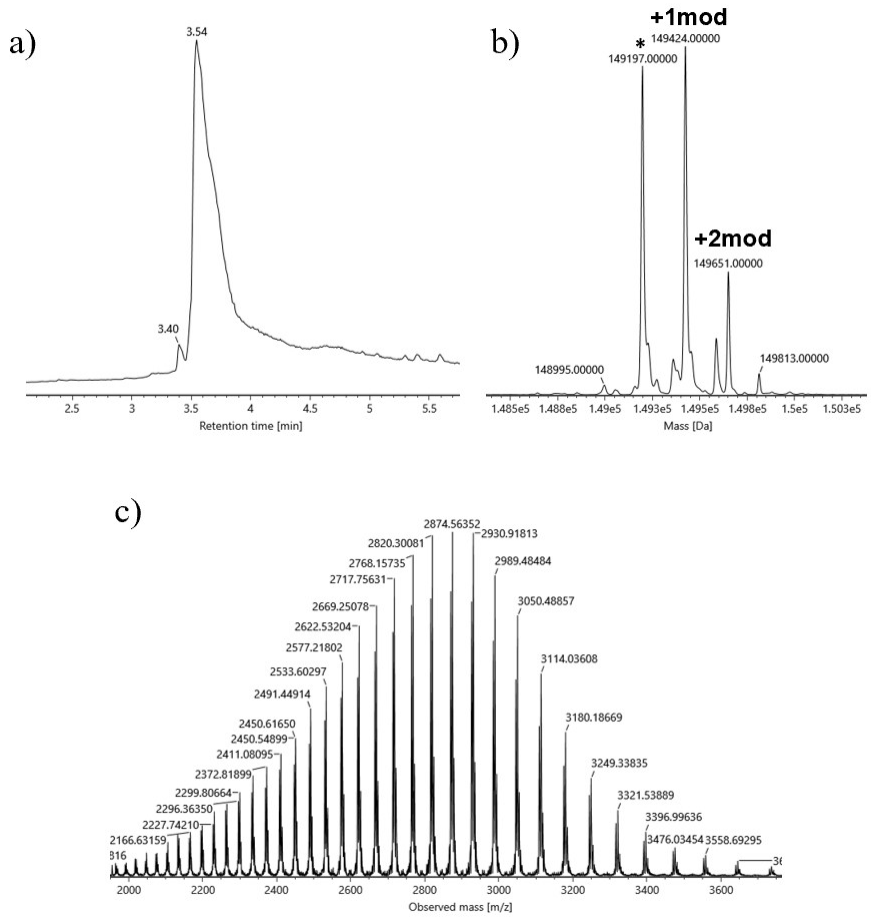



Figure S29: LC-MS spectra of bevacizumab treated with **8** for 1 h, "*" represents the naïve bevacizumab. (a) HPLC of **8**-treated bevacizumab; (b) deconvoluted MS of the sample, "**+1 mod**" and "**+2mod**" are the bevacizumabs modified with one and two N-GlyGly peptides; (c) MS of the sample.

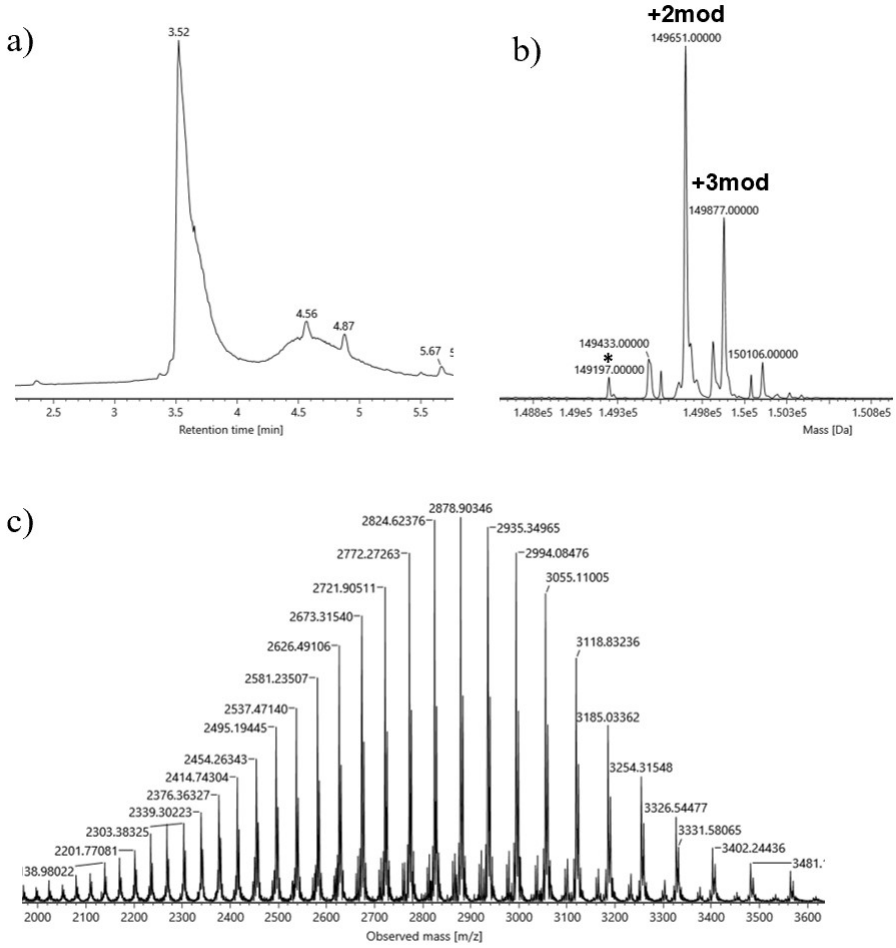



Figure S30: LC-MS spectra of bevacizumab treated with **8** for 6 h, "*" represents the naïve bevacizumab. (a) HPLC of **8**-treated bevacizumab; (b) deconvoluted MS of the sample, "**+2 mod**" and "**+3mod**" are the bevacizumabs modified with two and three N-GlyGly peptides; (c) MS of the sample.

Treatment of bevacizumab with **9**

123.0 µL of PIPES buffer (50.0 mM PIPES, pH 7.4) was mixed with 50.0 µL DMF. Then, 50.0 µL of **9** (0.4 mg, 10.0 eq) in DMF was added to the solution, followed by the addition of 277.0 µL PIPES buffer (50.0 mM PIPES, pH7.4) containing naïve bevacizumab (2.5 mg, 33.4 µM,1.0 eq) in. Keep the reaction mixture at 37°C. After 6-hour incubation, the **9**-treated bevacizumab was purified by protein A resin and analysed by LC-MS.

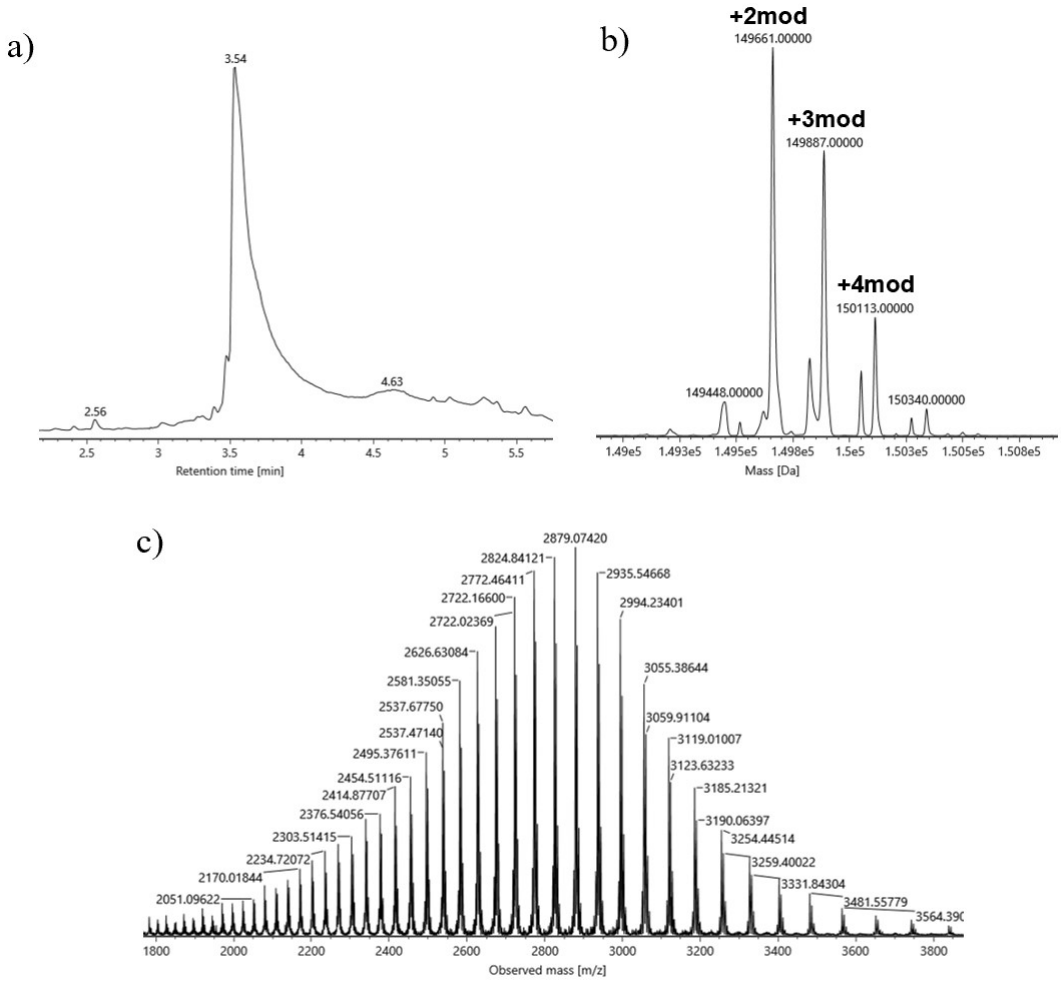



Figure S31: LC-MS spectra of bevacizumab treated with **9** for 6h. (a) HPLC of **9**-treated bevacizumab; (b) deconvoluted MS of the sample, "**+2 mod**", "**+3mod**" and "**+4mod**" are the bevacizumabs modified with two, three and four N-GlyGly peptides; (c) MS of the sample.

*SrtA-mediated GLP-1 attachment-*

SrtA-mediated ligation of **7**-treated bevacizumab with **GLP-1**

100.0 μL of **7**-treated Bevacizumab in HEPES buffer (50.0 mM HEPES, 100.0 mM NaCl, 2.0 mM $CaCl_2$, pH7.0) and 47.6 μL of **GLP-1** in HEPES buffer were added to 281.0 μL of HEPES buffer, followed by the addition of 47.6 μL of SrtA. Note that, the final concentration of **7**-treated bevacizumab was set to be 9.0 μM; the final concentration of **GLP-1** was set to be 72.0 μM; the final concentration of SrtA was set to be 4.5 μM. Keep the reaction mixture at 25℃. After 60 min, the bevacizumab was purified by protein A resin and analysed by LC-MS.

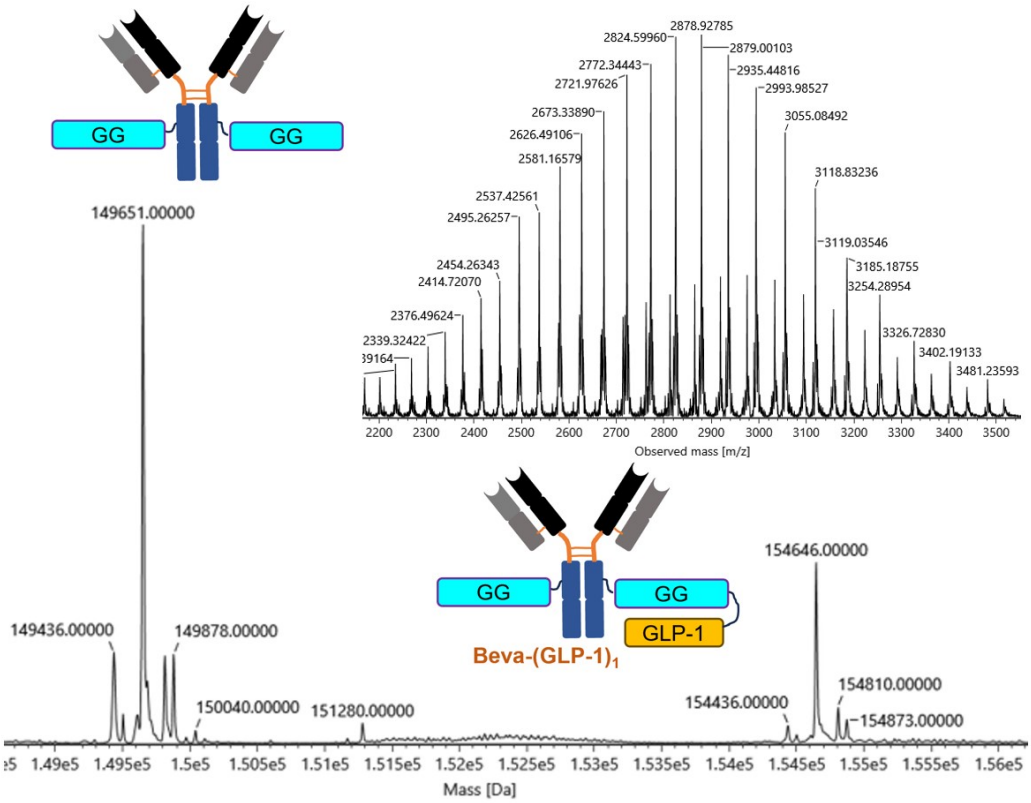



Figure S32: MS spectra of the product of SrtA-mediated ligation of **7**-treated bevacizumab with **GLP-1.**

SrtA-mediated ligation of **T3** and **GLP-1'**

1.0 mg of **T3** was dissolved into 32.3 µL of DMSO. 1.2 mg of **GLP-1'** was dissolved into 100.0 µL of DMSO. Then, 10.0 µL of the **T3** solution (final concentration: 100.0 µM) and 10.0 µL of the **GLP-1'** solution (final concentration: 9.0 µM) were added to 2972.4 µL of HEPES buffer (50.0 mM HEPES, 150.0 mM NaCl, 5.0 mM $CaCl_2$, pH 7.0), followed by the addition of 7.6 µL of SrtA (final concentration: 4.5 µM). Keep the reaction at 37℃. The reaction was monitored by HPLC. HPLC of the ligation product **GLP-2** (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 28.3 min (major). MS (ESI) m/z: [M+H]+ Calcd for $C_{247}H_{357}N_{55}O_{79}S$ 5393.54; Found 5393.8.

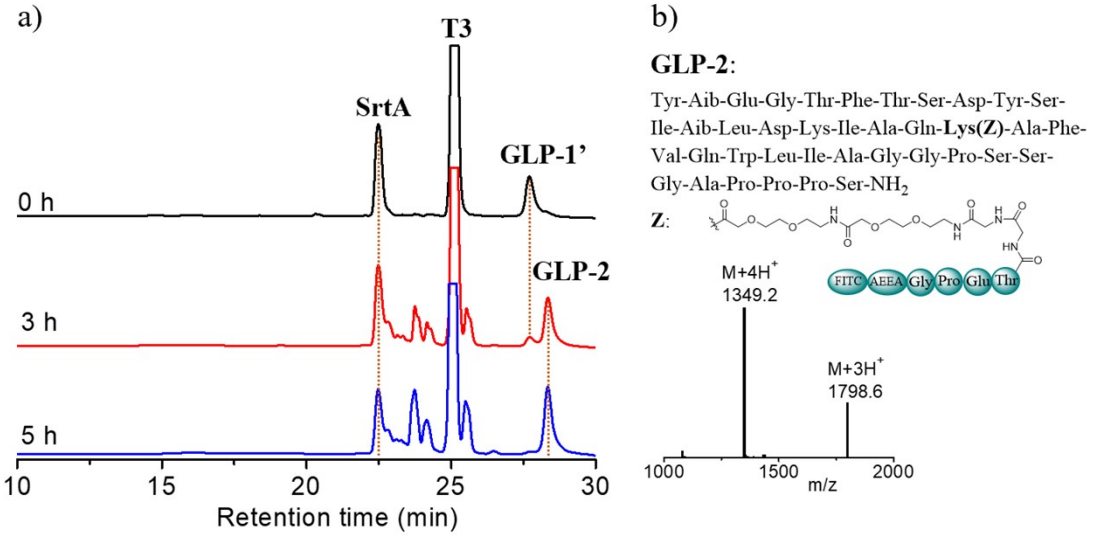



Figure S33: (a) HPLC trace (210nm) of the SrtA-mediated ligation of **T3** and **GLP-1'**; (b) the sequence of the ligation product **GLP-2** and its MS spectrum.

SrtA-mediated ligation of **T4** and **GLP-1'**

1.0 mg of **T4** was dissolved into 31.3 μL of DMSO. 1.2 mg of **GLP-1'** was dissolved into 100.0 μL of DMSO. Then, 10.0 μL of the **T4** solution (final concentration: 100.0 μM) and 10.0 μL of the **GLP-1'** solution (final concentration: 9.0 μM) were added to 2972.4 μL of HEPES buffer (50.0 mM HEPES, 150.0 mM NaCl, 5.0 mM $CaCl_2$, pH 7.0), followed by the addition of 7.6 μL of SrtA (final concentration: 4.5 μM). Keep the reaction at 37°C. The reaction was monitored by HPLC. HPLC of the ligation product **GLP-3** (gradient: 2-90% B in 40 min, column A, flow rate = 1.0 mL/min, I = 210 nm) tR = 28.5 min (major). MS (ESI) m/z: [M+H]+ Calcd for $C_{251}H_{365}N_{55}O_{79}$ S 5449.6; Found 5449.9.

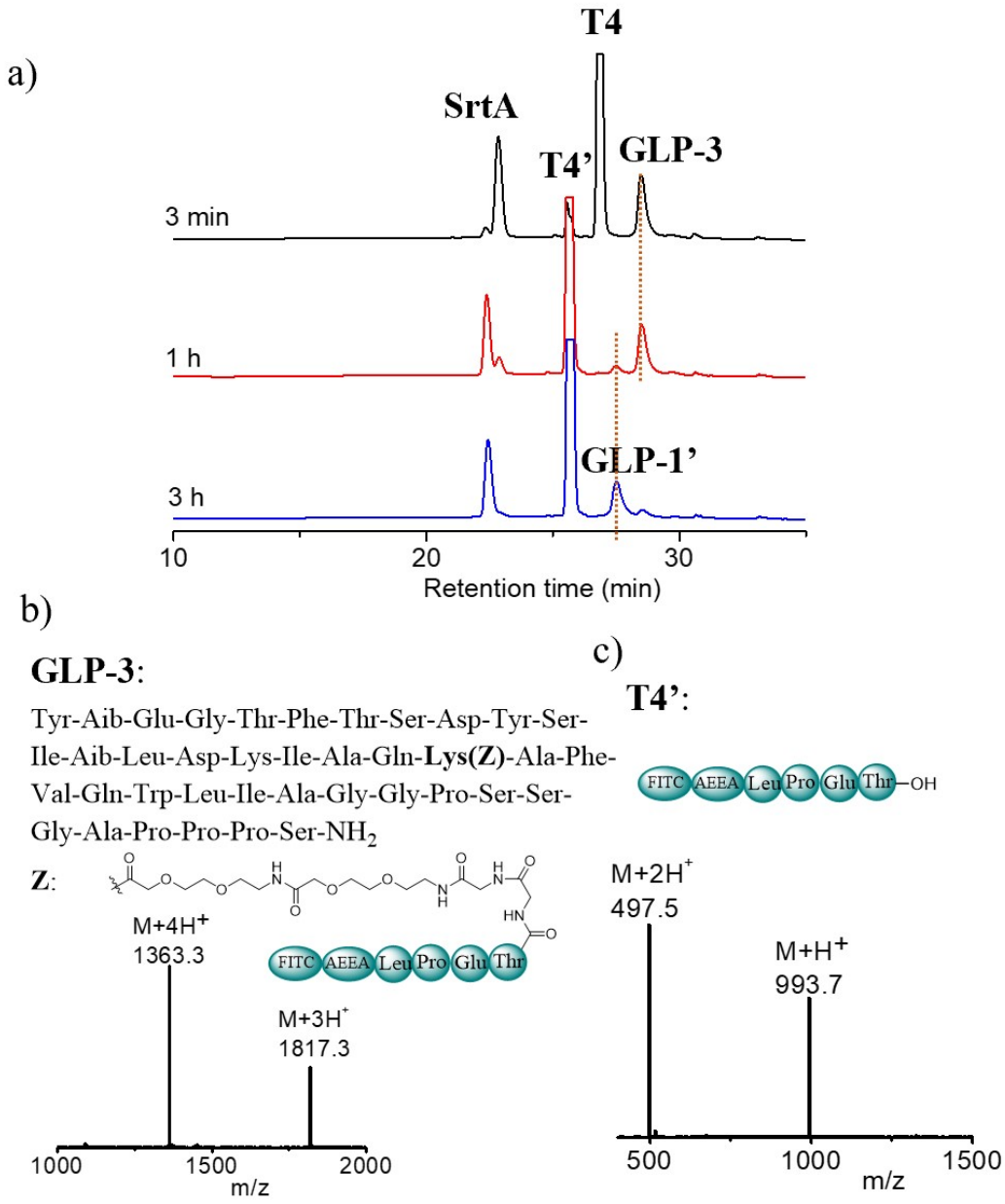



Figure S34: (a) HPLC trace (210nm) of the SrtA-mediated ligation of **T4** and **GLP-1'**; (b) the sequence of the ligation product **GLP-3** and its MS spectrum; (c) the sequence of hydrolysis product **T4'** and its MS spectrum. Note that, GLP-3 is completely hydrolysed by SrtA after 3 h.

SrtA-mediated ligation of **7**-treated bevacizumab with **T3**

34.1 μL of **7**-treated bevacizumab in HEPES buffer (50.0 mM HEPES, 100.0 mM NaCl, 2.0 mM $CaCl_2$, pH7.0) and 5.0 μL of **T3** in HEPES buffer were added to 5.9 μL of HEPES buffer, followed by the addition of 5.0 μL of SrtA. Note that, the final concentration of **7**-treated bevacizumab was set to be 9.0 μM; the final concentration of **T3** was set to be 100.0 μM, or 200.0 μM, or 400.0 μM. The final concentration of SrtA was set to be 4.5 μM. Keep the reaction

mixture at 37℃. After 3 min, 2 h, 4 h, 6 h, and 8 h, the reaction mixture was analyzed by SDS-PAGE (with DTT).

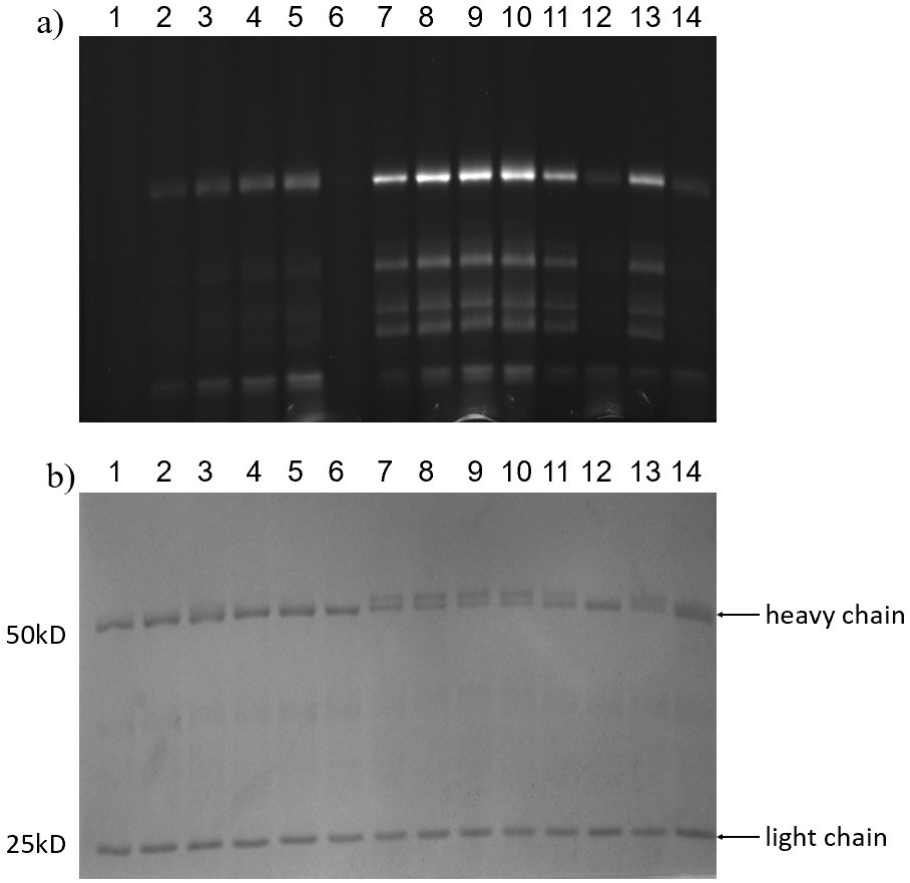



Figure S35: SDS-PAGE of SrtA-mediated ligation of **T3** and **7**-treated bevacizumab. (a) Fluorescence image; (b) coomassie staining. lane 1: naïve bevacizumab (9.0 μM), **T3** (400.0 μM), 3-min incubation; lane 2: naïve bevacizumab (9.0 μM), **T3** (400.0 μM), 2-hour incubation; lane 3: naïve bevacizumab (9.0 μM), **T3** (400.0 μM), 4-hour incubation; lane 4: naïve bevacizumab (9.0 μM), **T3** (400.0 μM), 6-hour incubation; lane 5: naïve bevacizumab (9.0 μM), **T3** (400.0 μM), 8-hour incubation; lane 6: **7**-treated bevacizumab (9.0 μM), **T3** (400.0 μM), 3-min incubation; lane 7: **7**-treated bevacizumab (9.0 μM), **T3** (400.0 μM), 2-hour incubation; lane 8: **7**-treated bevacizumab (9.0 μM), **T3** (400.0 μM), 4-hour incubation; lane 9: **7**-treated bevacizumab (9.0 μM), **T3** (400.0 μM), 6-hour incubation; lane 10: **7**-treated bevacizumab (9.0 μM), **T3** (400.0 μM), 8-hour incubation; lane 11: **7**-treated bevacizumab (9.0 μM), **T3** (100.0 μM), 6-hour incubation; lane 12: naïve bevacizumab (9.0 μM), **T3** (100.0 μM), 6-hour incubation; lane 13: **7**-treated bevacizumab (9.0 μM), **T3** (200.0 μM), 4-hour incubation; lane 14: naïve bevacizumab (9.0 μM), **T3** (200.0 μM), 4-hour incubation.

To 260.0 μL of **7**-treated bevacizumab in HEPES buffer (50.0 mM HEPES, 100.0 mM NaCl, 2.0 mM $CaCl_2$, pH7.0) were added 30.3 μL of **T3** and 30.3 μL of SrtA. Note that, **T3** and SrtA were dissolved in HEPES buffer. The final concentration of **7**-treated bevacizumab was set to be 9.0 μM; the final concentration of **T3** was set to be 400.0 μM; the final concentration of SrtA was set to be 4.5 μM. Keep the reaction mixture at 37℃. After 4 h, the bevacizumab was purified by protein A resin and analysed by LC-MS.

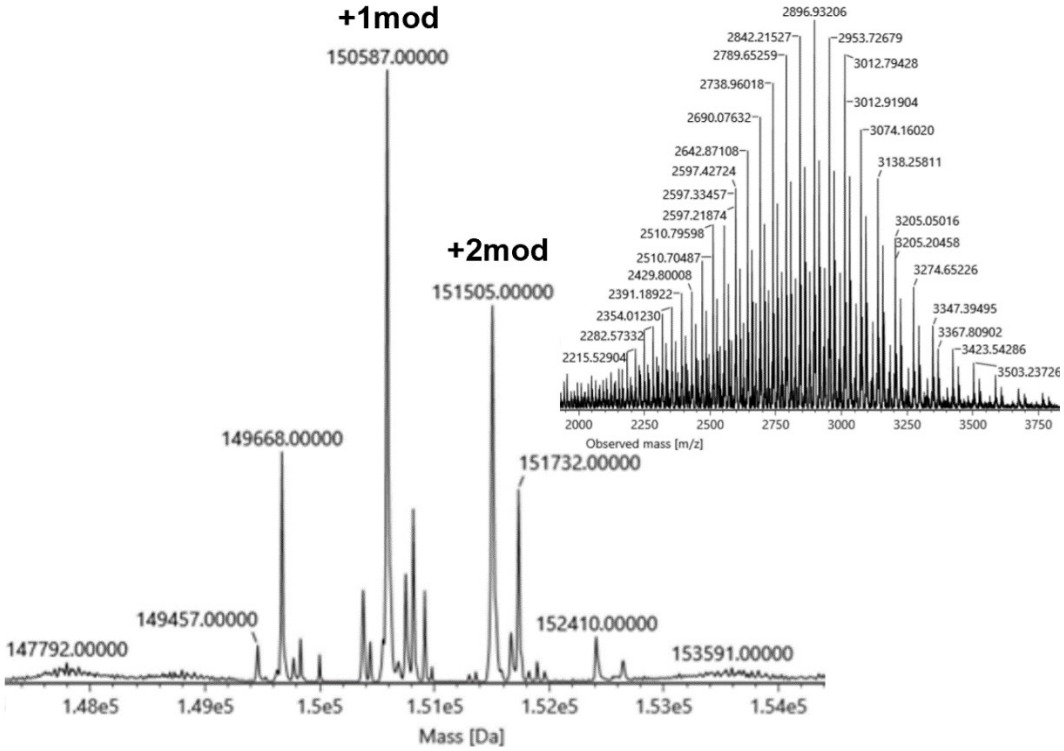



Figure S36: MS spectra of the product of SrtA-mediated ligation of **7**-treated Bevacizumab with **T3**. +**1mod**, +**2mod**: Bevacizumab modified with one, two **T3** peptides.

### *10-directed Fc conjugation and SrtA-mediated GLP-1 attachment*

Treatment of bevacizumab with **10**

170.7 μL of PIPES buffer (50.0 mM PIPES, pH 7.4) was mixed with 30.0 μL DMF. Then, 30.0 μL of **10** (0.28 mg, 10.0 eq) in DMF was added to the solution, followed by the addition of 69.3 μL PIPES buffer (50.0 mM PIPES, pH7.4) containing naïve bevacizumab (1.5 mg, 33.4 μM,1.0 eq) in. Keep the reaction mixture at 37℃. After 1-hour, 3-hour or 6-hour incubation, the **10**-treated bevacizumab was purified by protein A resin and analysed by LC-MS.

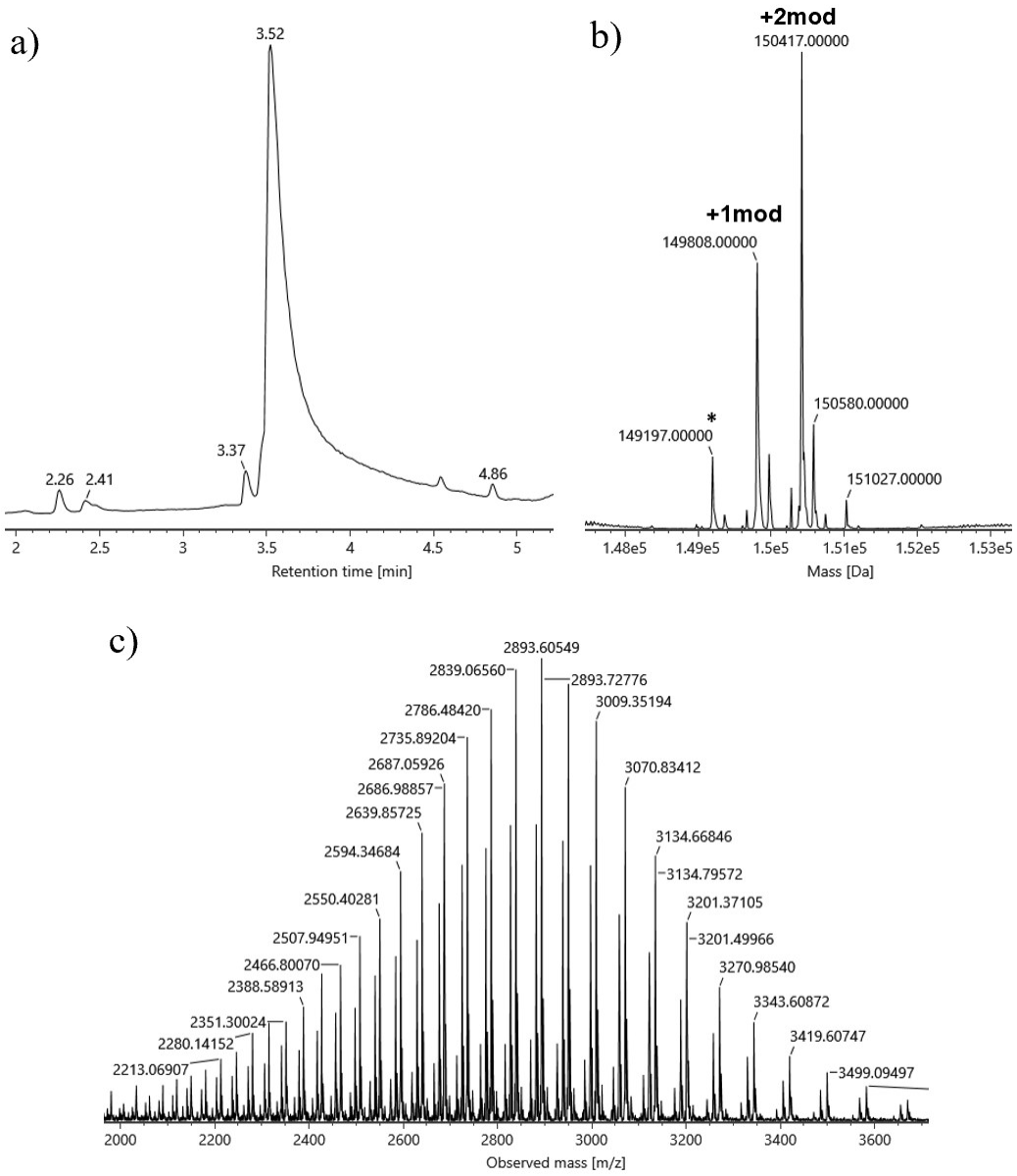



Figure S37: LC-MS spectra of bevacizumab treated with **10** for 1 h. (a) HPLC of **10**-treated bevacizumab, "*" represents the naïve bevacizumab; (b) deconvoluted MS of the sample, "**+1 mod**", and "**+2mod**" are the bevacizumabs modified with one, and two LPSTG peptides; (c) MS of the sample.

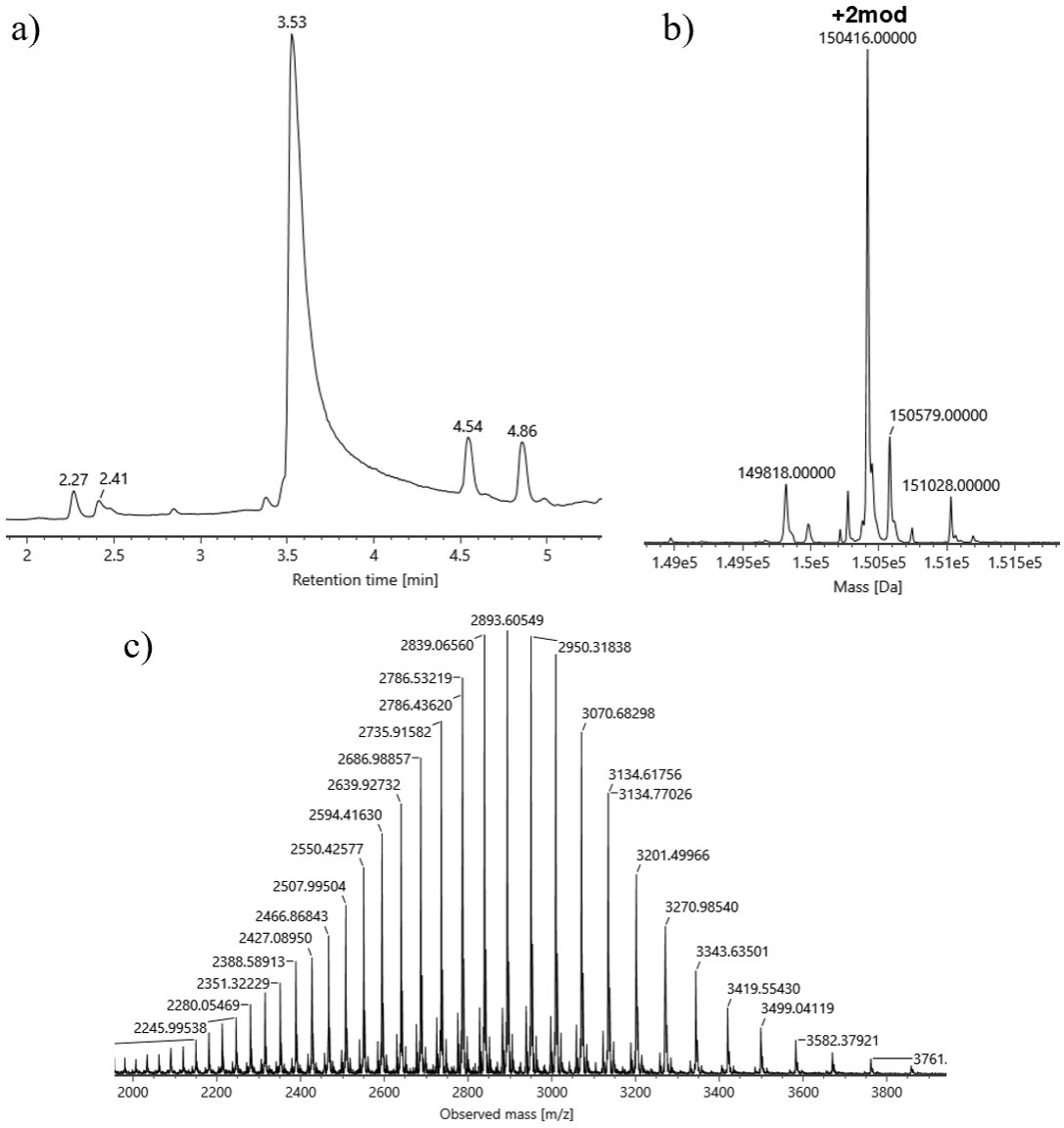



Figure S38: LC-MS spectra of bevacizumab treated with **10** for 3 h. (a) HPLC of **10**-treated bevacizumab; (b) deconvoluted MS of the sample, "+**2mod**" is the bevacizumab modified with two LPSTG peptides; (c) MS of the sample.

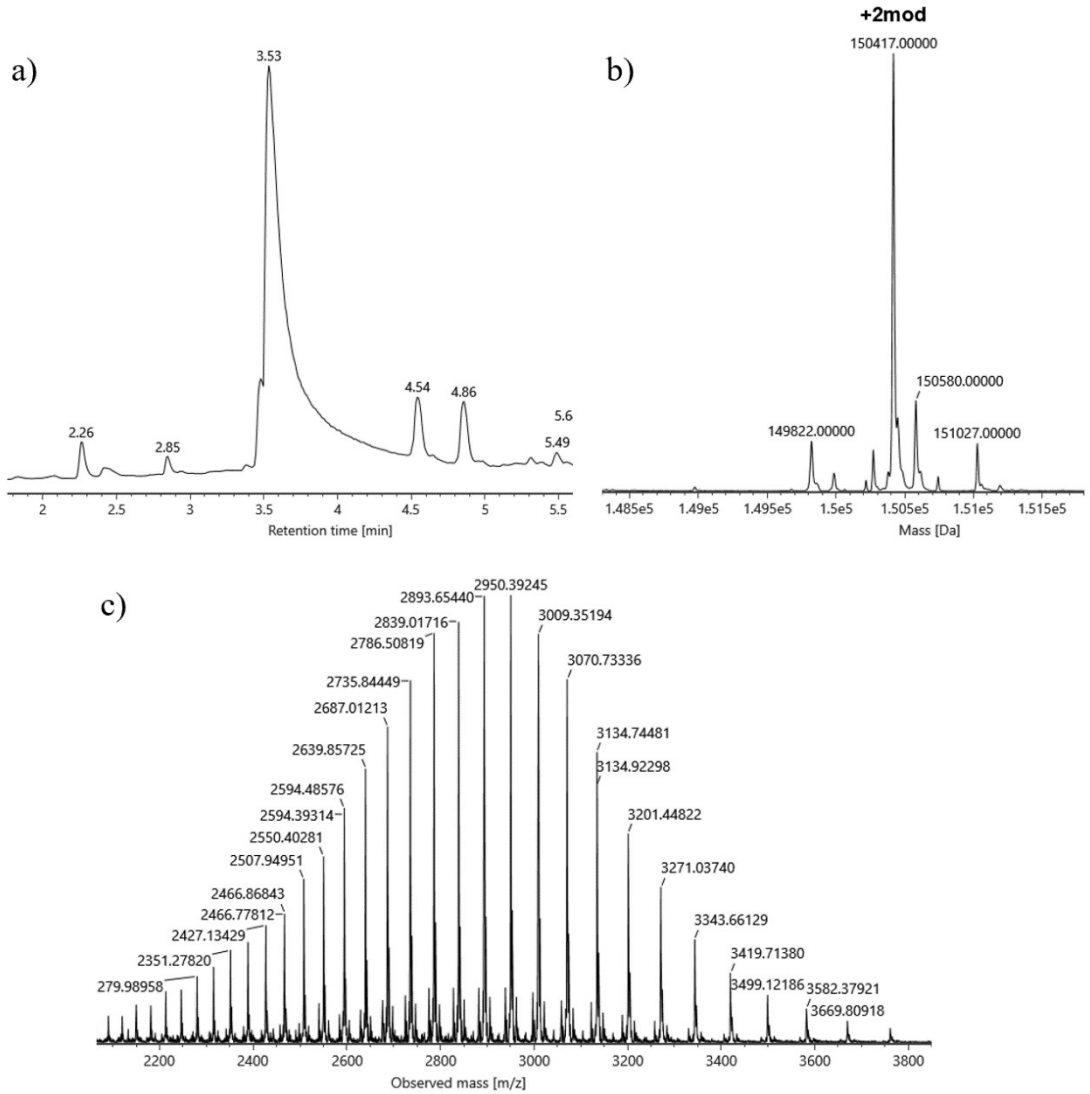



Figure S39: LC-MS spectra of bevacizumab treated with **10** for 6 h. (a) HPLC of **10**-treated bevacizumab; (b) deconvoluted MS of the sample, "**+2mod**" is the Bevacizumab modified with two LPSTG peptides; (c) MS of the sample.

SrtA-mediated ligation of **10**-treated bevacizumab with **GLP-1'**

125.0 μL of **10**-treated bevacizumab in HEPES buffer (50.0 mM HEPES, 100.0 mM NaCl, 2.0 mM $CaCl_2$, pH7.0) and 6.3 μL of **GLP-1'** in HEPES buffer were added to 79.7 μL of HEPES buffer, followed by the addition of 3.1 μL of SrtA. Note that, the final concentration of **10**-treated bevacizumab was set to be 10.0 μM; the final concentration of **GLP-1'** was set to be 500.0 μM. The final concentration of SrtA was set to be 5.0 μM. Keep the reaction mixture at 25℃. After 3 h, the reaction mixture was analyzed by SDS-PAGE (with DTT), and purified by Protein A resin.

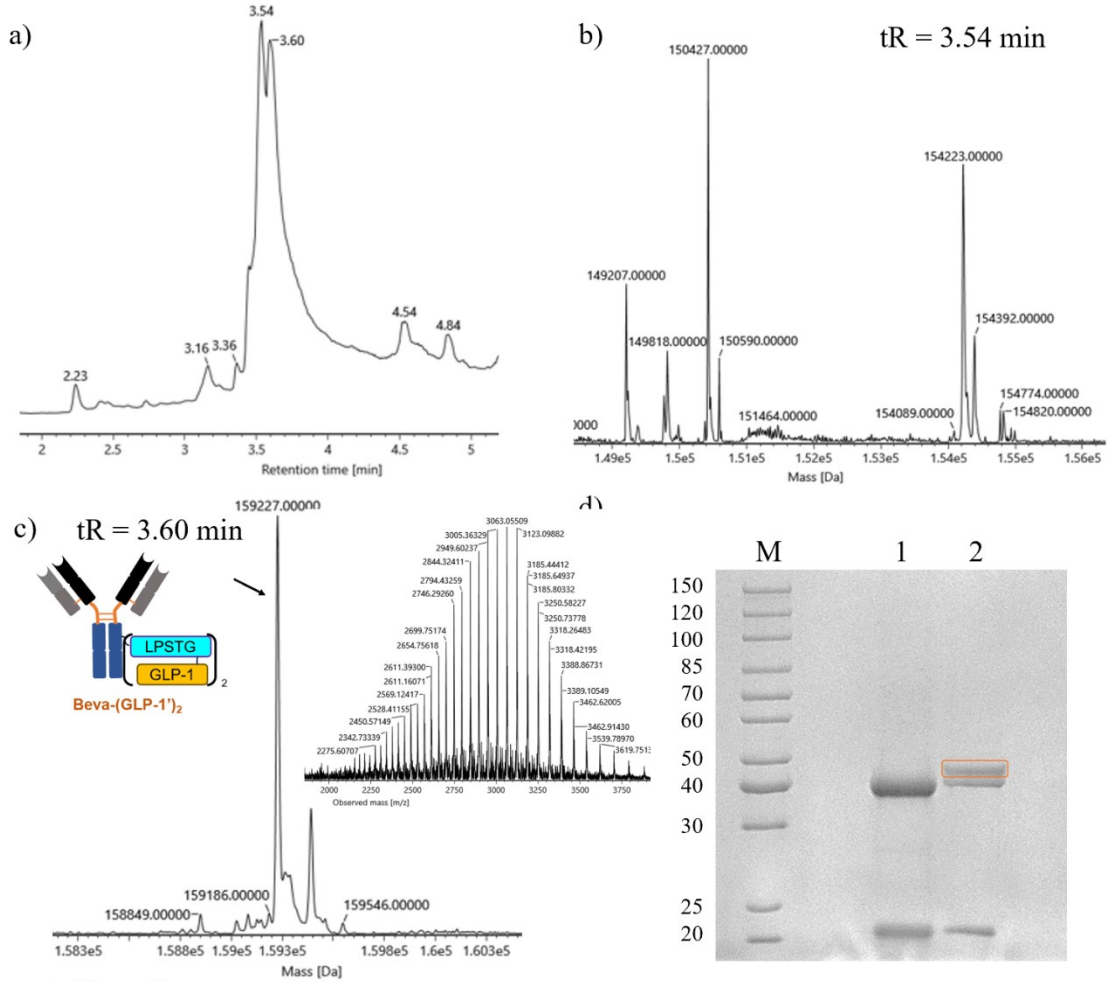



Figure S40: LC-MS spectra of SrtA-mediated ligation of **10**-treated bevacizumab with **GLP-1'**. (a) HPLC of the sample; (b) MS of the retention time of 3.54 min; (c) MS of the retention time of 3.60 min; (d) SDS-PAGE (with DTT) of the sample. M: MW protein ladder, lane 1: naïve bevacizumab, lane 2: the product after SrtA-mediated ligation, the red one is heavy chain label with **GLP-1'**.

Bevacizumab modification with **GLP-1'** conjugated **10**

[10.0 equiv of **GLP-1'** conjugated **10**]

To 100 µL of PIPES buffer (50.0 mM PIPES, 100.0 mM NaCl, 2.0 mM $CaCl_2$, pH6.9) were added 4 µL of **10** (0.02 µmol, final concentration: 170 µM) in DMSO, 4 µL of **GLP-1'** (0.1 µmol, final concentration: 850 µM) in DMSO, and 10 µL of sortase (0.001 µmol, final concentration: 8.5 µM) in HEPES buffer (pH7.0). The pH value of the mixture was around 6.9. The mixture was incubated at 37°C for 1 hour. Then, 5.6 µL of 200 mM EDTA in water (pH 8.0) was added to the mixture to afford the final EDTA concentration of 10.0 mM, followed by the addition of 25 µL of DMF. To the mixture was added 12.0 µL of bevacizumab in pH 6.0 PBS buffer (0.3 mg, final concentration: 17.0 mM) and 8.0 µL of 0.3 M NaOH. The pH of the mixture was around 7.4. Keep the mixture in a shaker at 37°C. At the time point of 5 min, 1 hour, 3 hour, and 6 hour, 4.0 µL of the reaction mixture was diluted with 16.0 µL water. 4.0 µL

of the diluted reaction mixture was mixed with 1.0 μL of SDS PAGE loading buffer containing DTT, heated 10 min at 90℃. The treated mixture was kept at 4℃ for SDS-PAGE analysis.

[20.0 equiv of **GLP-1'** conjugated **10**]

To 100 μL of PIPES buffer (50.0 mM PIPES, 100.0 mM NaCl, 2.0 mM $CaCl_2$, pH 6.9) were added 4 μL of **10** (0.02 μmol, final concentration: 170 μM) in DMSO, 4 μL of **GLP-1'** (0.1 μmol, final concentration: 850 μM) in DMSO, and 10 μL of sortase in HEPES buffer (pH7.0, 0.001 μmol, final concentration: 8.5 μM). The pH value of the mixture was around 6.9. The mixture was incubated at 37℃ for 1 hour. Then, 5.6 μL of 200 mM EDTA in water (pH 8.0) was added to the mixture to afford the final EDTA concentration of 10.0 mM, followed by the addition of 22 μL of DMF. To the mixture was added 6.0 μL of bevacizumab in pH 6.0 PBS buffer (0.15 mg, final concentration: 8.0 mM) and 5.0 μL of 0.3 M NaOH. The pH of the mixture was around 7.4. Keep the mixture in a shaker at 37℃. At the time point of 5 min, 1 hour, 3 hour, and 6 hour, 4.0 μL of the reaction mixture was diluted with 6.0 μL water. 4.0 μL of the diluted reaction mixture was mixed with 1.0 μL of SDS PAGE loading buffer containing DTT, heated 10 min at 90℃. The treated mixture was kept at 4℃ for SDS-PAGE analysis.