Supporting Information for

Protein labeling approach to improve lysosomal targeting and efficacy of antibody-drug conjugates

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1. General methods

The concentration of proteins was determined by absorption spectroscopy measurements at 280 nm using the extinction coefficient of the respective protein on NanoDrop^{TM} 2000 microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) and/or using a BCA protein assay (Thermo Fisher Scientific, USA). Coomassie brilliant blue R250 (Sangon Biotech, China) stained, fluorescently stained bands and chemiluminescent bands were imaged using a Tanon 5200 Multi imaging system (Tanon Science & Technology Co., Ltd., China). High-resolution mass spectra (HRMS) were measured on SYNAPT G2-Si quantitive time-of-flight mass spectrometer (Waters, USA) with a resolution higher than 60000 FWHM. The UltrafleXtreme Matrix-Assisted Laser Desorption/ Ionization Time of Flight (MALDI-TOF) mass spectrometer (Bruker, Germany) and Agilent 6120 Quadrupole LC/MS spectrometer with electrospray ionization were used to analyze the mass of proteins. Deconvolution was performed using ProMass.

2. Materials

Chemicals for peptide synthesis including Rink Amide-MBHA resin (100-200 mesh, 0.341mmol/g), Fmoc-protected amino acids, *N, N*′-diisopropylethylamine (DIPEA), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-hydroxybenzotriazole (HOBt) were purchased from GL Biochem (Shanghai, China). Maleimide-PEG_3-TAMRA was purchased from Biocone (Chengdu, China). Ethylisopropylamiloride (EIPA), nystatin and chlorpromazine were from Medchemexpress (New Jersey, USA), Yesen (Shanghai, China) and Solarbio (Beijing, China), respectively. The cell lines A431，MCF-7 and 293T were obtained from American Type Culture Collection

(Manassas, USA). For cell staining, LysoTracker® Green DND-26 and Hoechst 33342 was purchased from Yeasen (Shanghai, China) and Beyotime (Shanghai, China), respectively.

3. Molecular Docking

All of the molecular dockings were carried out based on docking ligands into a protein target using AutoDock VINA in YASARA.[1] In brief, in key steps in the dockings, a protein or a receptor was maintained rigid, while ligands were fully flexible. Protein was prepared as follows: The X-ray crystal structure of the phosphotyrosine binding domain (PTB) of mouse Disabled 2(Dab2) (PDB ID: 1M7E)[2] was downloaded from the Protein Data Bank and kept with chain A and then the polar hydrogen atoms were added in it. To remove bumps and correct the covalent geometry of the ligand, the structure was energy-minimized with the NOVA force field,[3] using the Particle Mesh Ewald algorithm[4] to treat longrange electrostatic interactions. After removal of conformational stress by a short steepest descent minimization, the procedure continued by simulated annealing (timestep 2 fs, atom velocities scaled down by 0.9 every 10th step) until convergence was reached.

The dockings were carried out in two steps. First, a blind docking was undertaken using boxes of size 35.81×35.81×35.81 Å for 1M7E, covering the key residues of the protein. In addition, with the setup done in YASARA molecular docking program, the first docks against the single receptor provided, while the latter docks against an ensemble of receptors, each with alternative high-scoring side-chain rotamers (which takes about five times as long) with 25 poses. Moreover, after cluster analysis, the results are divided into 9 clusters with free

energy of binding and interaction of hydrogen bonds shown in Fig. S1, Table S1 and Table S2.

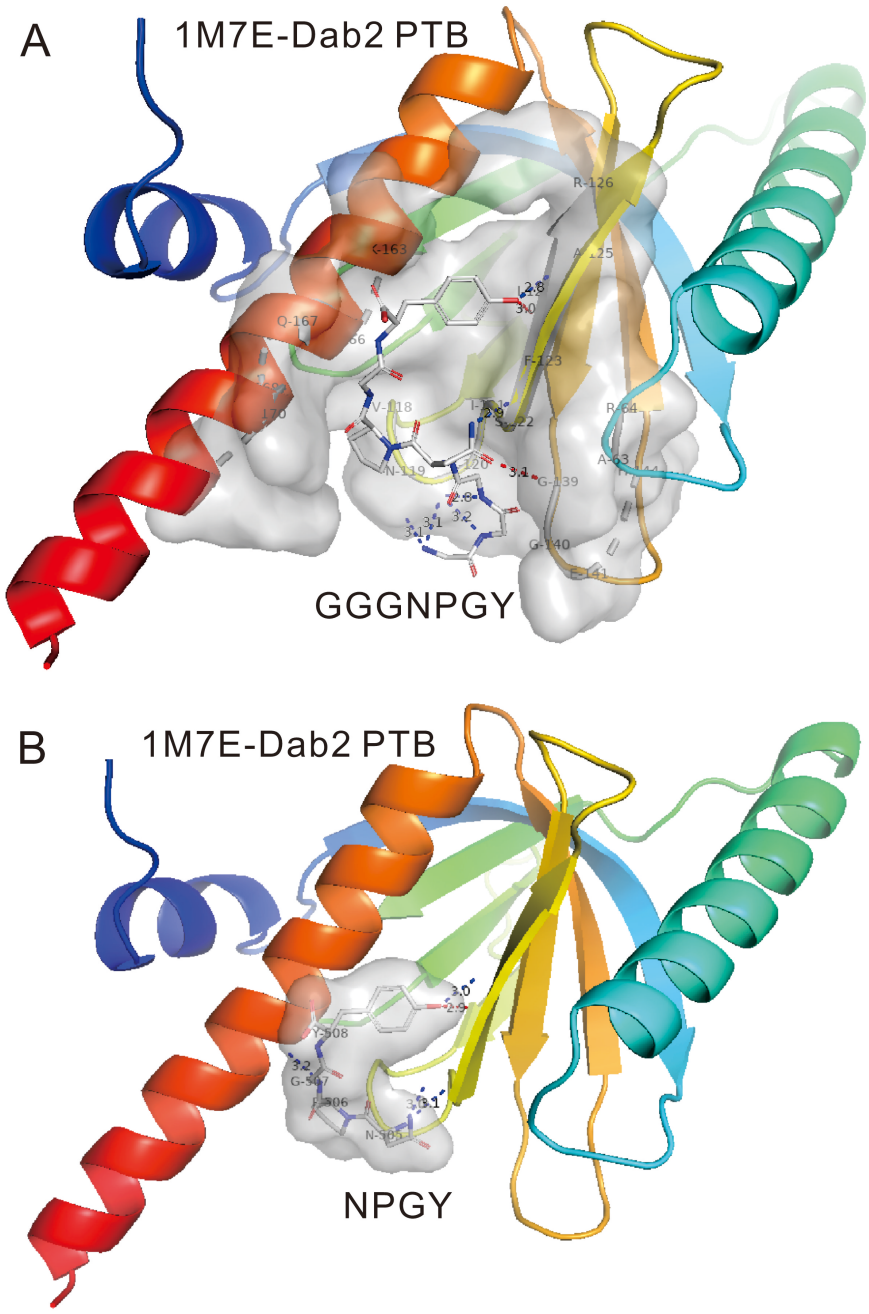


Fig. S1 Computationally docked models using using AutoDock VINA in YASARA. **A.** The docking complex of GGGNPGY motif and the PTB domain of mouse Dab2 (PDB ID: 1M7E). **B.** The docking complex of NPGY motif and the PTB domain of mouse Dab2 (PDB ID: 1M7E). The bones of oligopeptides are shown as sticks in silver with oxygen in red and nitrogen in blue and the interaction of hydrogen bonds among residues are shown in blue and red dashed lines.

Table S1. The free energy of binding and interaction of hydrogen bonds between PTB domain of mouse Dab2 (PDB ID: 1M7E)- 1M7E-Dab2 PTB and GGGNPGY motif (The numbers of residues are named from 502 to 508).

Free energy of binding (kcal/mol)		-7.19
Atoms in the Residues of 1M7E-Dab2 PTB	Atoms in the Residues of GGGNPGY	Hydrogen bond (Å)
ASN 119/O	GLY 502/N	3.06
ASN 119/O	GLY 504/N	2.80
ASN 119/O	GLY 503/N	3.17
SER 122/O	ASN 505/ND2	2.89
ILE 124/O	TYR 508/OH	2.76
ARG 64/NH1	ASN 505/OD1	3.14
ILE 124/N	TYR 508/OH	2.98

Table S2. The free energy of binding and interaction of hydrogen bonds between PTB domain of mouse Dab2 (PDB ID: 1M7E)- 1M7E-Dab2 PTB and NPGY motif (The numbers of residues are named from 505 to 508).

Free energy of binding (kcal/mol)		-6.95
Atoms in the Residues of 1M7E-Dab2 PTB	Atoms in the Residues of NPGY	Hydrogen bond (Å)
ILE 121/O	ASN 505/N	2.95
SER 122/O	ASN 505/ND2	3.12
ILE 124/O	TYR 508/OH	2.98
ILE 124/N	TYR 508/OH	2.87

4. Solid-phase peptide synthesis

The synthesis of peptides (Table S3) was performed based on Fmoc solid-phase peptide synthesis as previously reported.[5]

Table S3. The sequences of peptides synthesized by solid-phase peptide synthesis.

Peptides	Sequence
GGG	NH_2-GGG-$CONH_2$
P_1	NH_2-GGGNPGY-$CONH_2$
P_2	NH_2-GGGRRRRRRRRRNPGY-$CONH_2$
P_3	NH_2-GGGRRRRRRRRRR-$CONH_2$

Peptide was synthesized on a Rink Amide-MBHA resin (882 mg, 0.34 mmol/g, 1 equiv). After Fmoc deprotection (20% piperidine in DMF), Fmoc-protected amino acid (3 equiv) was coupled using 3 equiv of HBTU, 3 equiv of HOBt and 6 equiv of DIPEA in 5 mL of DMF for 2 h. The resin was washed for 30 sec each with three 5-mL portions of DCM, and then three 5-mL portions of DMF. After the coupling of each amino acid, the resulting peptide was cleaved by incubating the resin with 3 mL of cleavage cocktail (95% trifluoroacetic acid, 2.5% H_2O, 2.5% triisopropyl silane) for 2 h at room temperature. TFA was evaporated via N_2-stream, and the peptide was precipitated in 30 mL of diethyl ether. The crude product was purified by a semi-preparative HPLC column (column: Hypersil GOLD^{TM} 5 μm C18, 21.2 × 250 mm; solvent system: A = 0.1% TFA/H_2O, B = MeCN, flow rate 10 mL/min, 1%−65% B 0−45 min) to yield the desired peptide as a white trifluoroacetate.

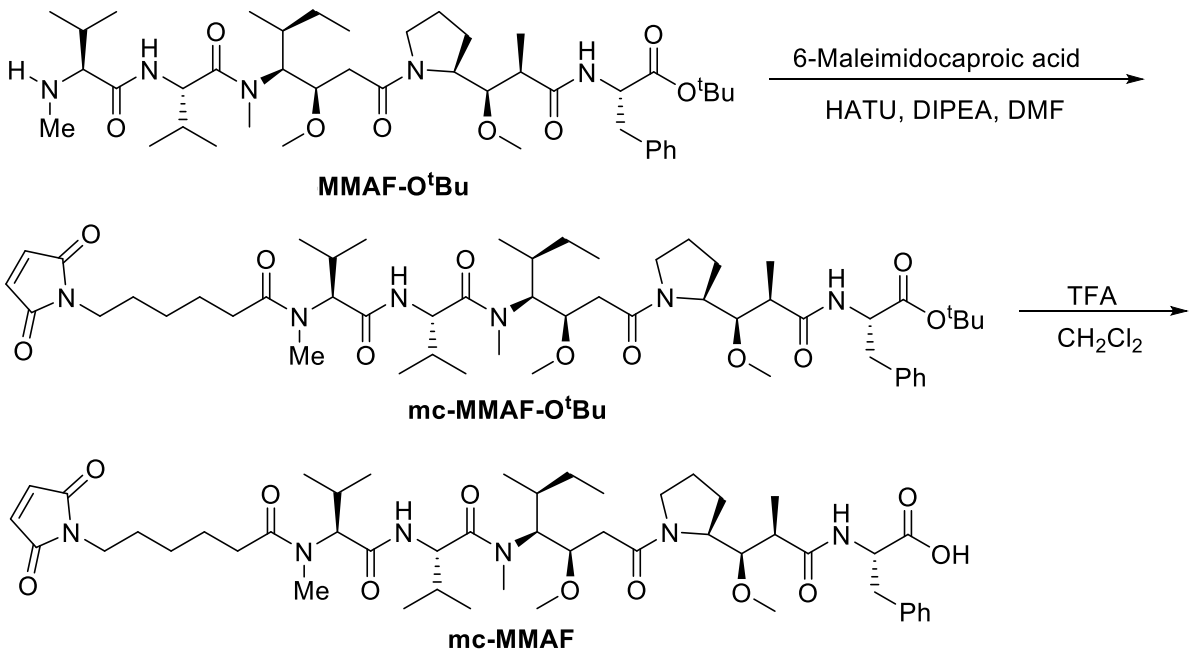
GGG ($C_6H_{12}N_4O_3$): HPLC retention time: 17.2 min; HRMS (ESI; m/z) 211.0805 $[M+Na]^+$ (calcd. m/z: 211.0802).

P_1 ($C_{26}H_{37}N_9O_9$): HPLC retention time: 14.1 min; HRMS (ESI; m/z) 620.2775 $[M+H]^+$ (calcd. m/z: 620.2787).

P_2 ($C_{80}H_{144}N_{44}O_{19}$): HPLC retention time: 18.8 min; HRMS (ESI; m/z) 507.2915 $[M+4H]^{4+}$ (calcd. m/z: 507.2986).

P_3 ($C_{60}H_{119}N_{39}O_{13}$): HPLC retention time: 18.9 min; HRMS (ESI; m/z) 532.3248 $[M+3H]^{3+}$ (calcd. m/z: 532.3356).

5. Synthesis of mc-MMAF



Scheme S1. Synthesis of mc-MMAF.

Synthesis of mc-MMAF-O^{t}Bu. Synthesis of MMAF-O^{t}Bu was described previously.[6] To an ice-cooled solution of mc-MMAF-O^{t}Bu (78.7 mg, 0.1 mmol) and 6-Maleimidocaproic acid (42.2 mg, 0.2 mmol) in DMF (2.5 mL) was added DIPEA (38.7 mg, 0.3 mmol). The solution was stirred at 0 °C for 5 min, then HATU (57 mg, 0.15 mmol) was added. The stirring was maintained at 0 °C for 30 min, then warmed to room temperature and stirred for 5 h. Water (5 mL) and ethyl acetate (15 mL) were added to the reaction mixture. The phases were separated,

and the aqueous layer was extracted with ethyl acetate (10 mL). The combined organic fractions were combined, washed successively with saturated aqueous solution of sodium bicarbonate (10 mL) and saturated brine (10 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (with 1/25 MeOH/CH_2Cl_2 as eluent) to give MMAF-O^{t}Bu (40.2 mg, 41%) as a white solid.

Synthesis of mc-MMAF. To a solution of mcMMAF-O^{t}Bu (19.6 mg, 0.02 mmol) in dichloromethane (2.5 mL) was added trifluoroacetic acid (0.5 mL). The reaction mixture was stirred at room temperature for about 6 h and then concentrated under reduced pressure. The crude residue was purified by a semi-preparative HPLC (column: Hypersil GOLD^{TM} 5 μm C18, 21.2 × 250 mm; solvent system: A = 0.1% TFA/H_2O, B = MeCN, flow rate 10 mL/min, 8%−38% B 0−30 min) to yield mc-MMAF (15.9 mg, 86%) as a white solid. HRMS (ESI; m/z) 925.5607 $[M+H]^+$ ($C_{49}H_{78}N_6O_{11}$ calcd. m/z: 925.5606).

6. Expression of recombinant 7D12 with peptides

Construction of 7D12-S85C-P_1, 7D12-S85C-P_2 and 7D12-S85C-P_3 expression plasmids. The plasmid pET28a-7D12-S85C-LPETG-His_6 was constructed as demonstrated in section 6. GAAAACCTGTACTTCCAGGGA sequence for TEV protease cleavage was added at the N-terminus after His_6 tag and the P_1 sequence was added in place of LPETG-His_6 at the C-terminus by PCR reaction with the sense primer (5'-GGAATTCCATATGGAAAACCTGT

ACTTCCAGGGACAGGTGAAACTGGAGGAAAGCG-3') and the anti-sense primer (5'-CCGCTCGAGTCAATAGCCCGGGTTGCCGCTGCTAACGGTCACTTGGGTACC-3') to yield the construct 7D12-S85C-P_1. 7D12-S85C-P_2 and 7D12-S85C-P_3 constructs were generated by similar procedure.

Expression and purification of 7D12-S85C-peptides. The 7D12-S85C-peptides constructs were transformed into *E. coli* BL21 (DE3) (Novagen, Germany), which was then grown in Luria broth media at 37 °C until the OD_{600} reached 0.6−0.8. The cells were then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Invitrogen, USA) and grown for another 16 h at 25 °C. Prior to purification, the cells were harvested and lysed. The lysate was centrifuged, and the resultant supernatant was applied to Ni-NTA agarose beads (Qiagen, Germany). The protein was eluted with a solution containing 400 mM NaCl, 50 mM Tris·HCl (pH 8.0) and 200 mM imidazole. The elution was then concentrated and buffer-exchanged to PBS (pH 7.4) for future use. 5 mM β-mercaptoethanol (β-ME) was added through the purification process.

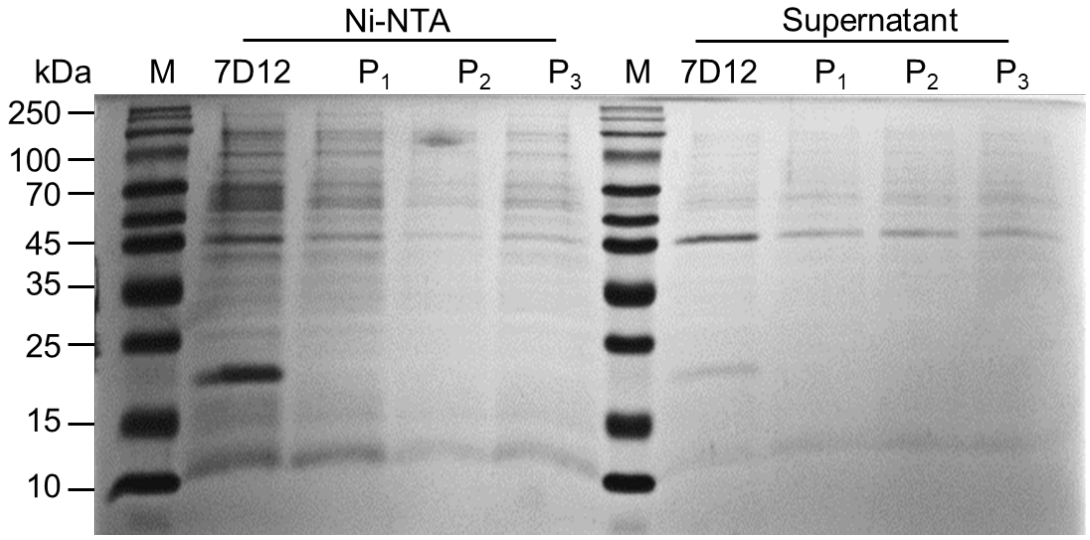


Fig. S2 SDS-PAGE analysis of recombinant 7D12 fusions. The bacteria colony were inoculated to 10 mL of LB medium and were induced when the OD_{600} reached about 0.7. M: marker; 7D12: 7D12-S85C-GGG; P_1: 7D12-S85C-P_1; P_2: 7D12-S85C-P_2; P_3: 7D12-S85C-P_3; Ni: samples pulled down from Ni-NTA beads; Supernatant: 20 μL of supernatant of cell lysate.

7. Preparation and characterization of 7D12 conjugates generated by sortagging

Construction of 7D12 expression plasmids. The plasmid containing 7D12 DNA fragment with the LPETG tag for sortagging at the C-terminus was synthesized by GenScript (Nanjing, China). The cloning plasmid pUC57-7D12-LPETG-His_6 was digested by *Nco*I and *Xho*I (Thermo Fisher, USA), and then recloned into pET28a expression vector (Novagen, Germany) to generate the construct pET28a-7D12-LPETG-His_6. Residue S85 was chosen for cysteine mutation to prepare site-specific labeling nanobodies. The mutant pET28a-7D12-S85C-LPETG-His_6 was obtained by PCR-based site-directed mutagenesis with the sense primer (5'-AAATGAACTGCCTGAAGCCGGAGGACACCG-3'), the anti-sense primer (5'-GGCTTCAGGCAGTTCATTTGCAGATCAACGGTGTTTT-3') and pET28a-7D12 as a template.

Expression and purification of 7D12-LPETG-His_6 and 7D12-S85C-LPETG-His_6. The constructs pET28a-7D12-LPETG-His_6 and pET28a-7D12-S85C-LPETG-His_6 were transformed into *E. coli* BL21 (DE3) (Novagen, Germany), which was then grown in Luria broth media at 37 °C until the OD_{600} reached 0.6−0.8. The cells were then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Invitrogen, USA) and grown for another 16 h at 25 °C. Prior to purification, the cells were harvested and lysed. The lysate was centrifuged, and the resultant supernatant was applied to Ni-NTA agarose beads (Qiagen, Germany). The protein was eluted with a solution containing 400 mM NaCl, 50 mM Tris·HCl (pH 8.0) and 200 mM imidazole. The elution was then concentrated and buffer-exchanged to PBS (pH 7.4) for future use. For 7D12-S85C-LPETG-His_6, 5 mM β-mercaptoethanol (β-ME) was added through the purification process.

Site-specific labeling of 7D12-S85C-LPETG-His_6 with maleimide-PEG_3-TAMRA. 7D12-S85C-LPETG-His_6 was fully reduced in PBS buffer containing 5 mM tris(2-carboxyethyl)phosphine (TCEP, Thermo Fisher) for 30 min at room temperature. The remained TCEP was removed by dialysis with PBS buffer (pH 7.4) containing 1 mM EDTA at 4 °C. For a standard labeling reaction, 5 equiv of maleimide-PEG3-TAMRA in DMSO was mixed with 1 mg/mL protein solution. After incubation for 2 h at 4 °C, the free dye was removed by buffer exchange into PBS (pH 7.4) using Amicon® Ultra 10K centrifugal filter devices (Merck Millipore, USA). The obtained protein 7D12-TAMRA-LPETG-His_6 was stored at 4 °C for future use.

7D12-S85C-LPETG-His_6 conjugation with mc-MMAF. 7D12-S85C-LPETG-His6 was fully reduced in PBS buffer containing 5 mM TCEP for 30 min at room temperature. The remained TCEP was removed by dialysis with PBS buffer (pH 7.4) containing 1 mM EDTA at 4 °C. For a standard labeling reaction, 5 equiv of mc-MMAF in DMSO was mixed with 1 mg/mL protein solution. After incubation for 2 h at 4 °C, the excess amount of mc-MMAF was removed by buffer exchange into PBS (pH 7.4) using Amicon® Ultra 10K centrifugal filter devices (Merck Millipore, USA). The obtained protein 7D12-MMAF-LPETG-His_6 was stored at 4 °C for future use.

Sortase-mediated ligation of 7D12 conjugates. Expression and purification of $SrtA_{\Delta N59}$ was performed as previously described.[5a] 7D12-TAMRA-LPETG-His_6 and 7D12-MMAF-LPETG-His_6 were labeled by incubating with 1 equiv of 7D12 conjugates with 10 equiv of the synthesized peptides in the presence of 2 equiv of $SrtA_{\Delta N59}$ in a solution containing 150 mM NaCl, 50 mM Tris·HCl pH 7.5 and 5 mM $CaCl_2$ for 15 h at room temperature. The mixture was purified by Ni-NTA agarose beads with gradient elution and the obtained elute was analyzed by SDS-PAGE. The purified 7D12-TAMRA-peptide and 7D12-MMAF-peptide were buffer exchanged into PBS (pH 7.4) for further study.

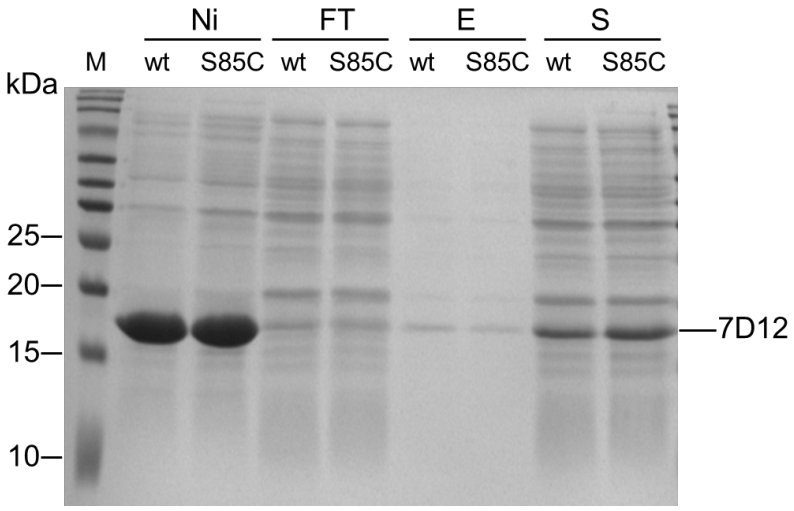


Fig. S3 SDS-PAGE analysis of wild-type 7D12-LPETG-His_6 and 7D12-S85C-LPETG-His_6. M: marker; wt: wild-type 7D12-LPETG-His_6; S85C: 7D12-S85C-LPETG-His_6; Ni: samples pulled down from Ni-NTA beads; FT: flow-through; E: elution buffer containing 20 mM imidazole; S: 20 μL of supernatant of cell lysate.

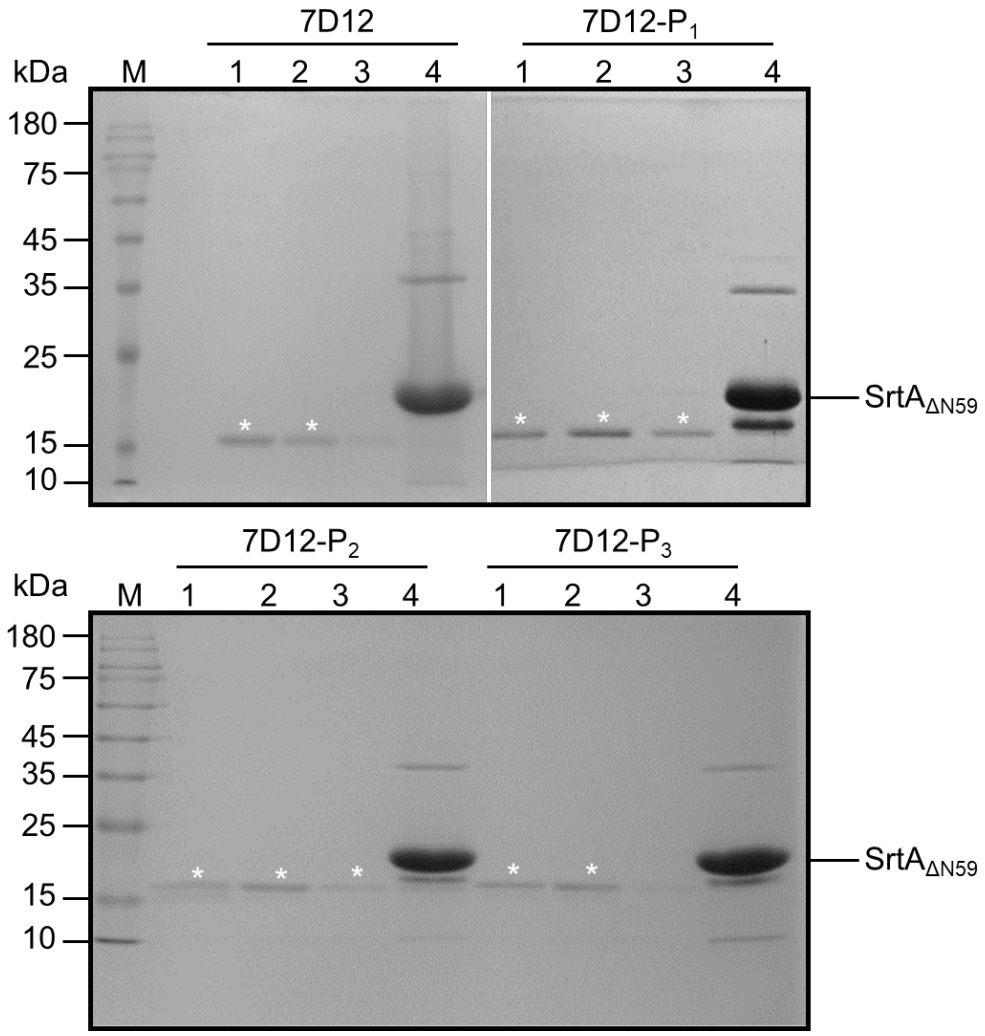


Fig. S4 Purification of 7D12-peptide conjugates by Immobilized Metal Ion Affinity Chromatography (IMAC). Lane M: marker; lane 1: flow through; lane 2−4: samples of elution with buffer containing 10, 20 and 800 mM imidazole, respectively. Flow-through and eluate containing 10 mM imidazole were concentrated and buffer-exchanged with PBS. Bands indicated with asterisks were from 7D12-peptide conjugates.

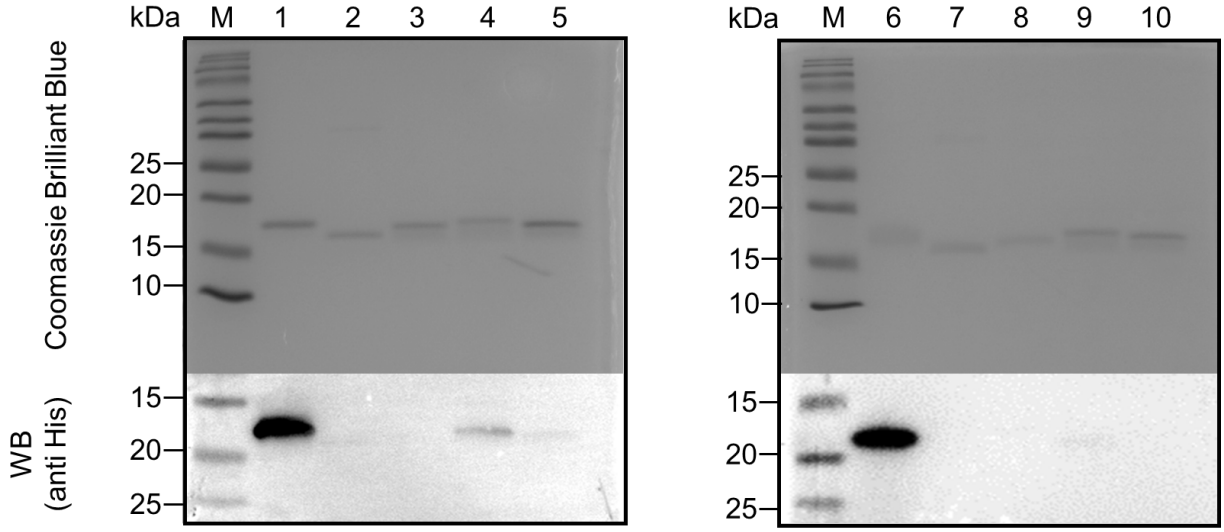


Fig. S5 SDS-PAGE and anti-His western blot characterization of 7D12-peptide conjugates. M: marker; lane 1: 7D12-TAMRA-LPETG-His_6; lane 2: 7D12-TAMRA; lane 3: 7D12-TAMRA-P_1; lane 4: 7D12-TAMRA-P_2; lane 5: 7D12-TAMRA-P_3; lane 6: 7D12-MMAF-LPETG-His_6; lane 7: 7D12-MMAF; lane 8: 7D12-MMAF-P_1; lane 9: 7D12-MMAF- P_2; lane 10: 7D12-MMAF- P_3.

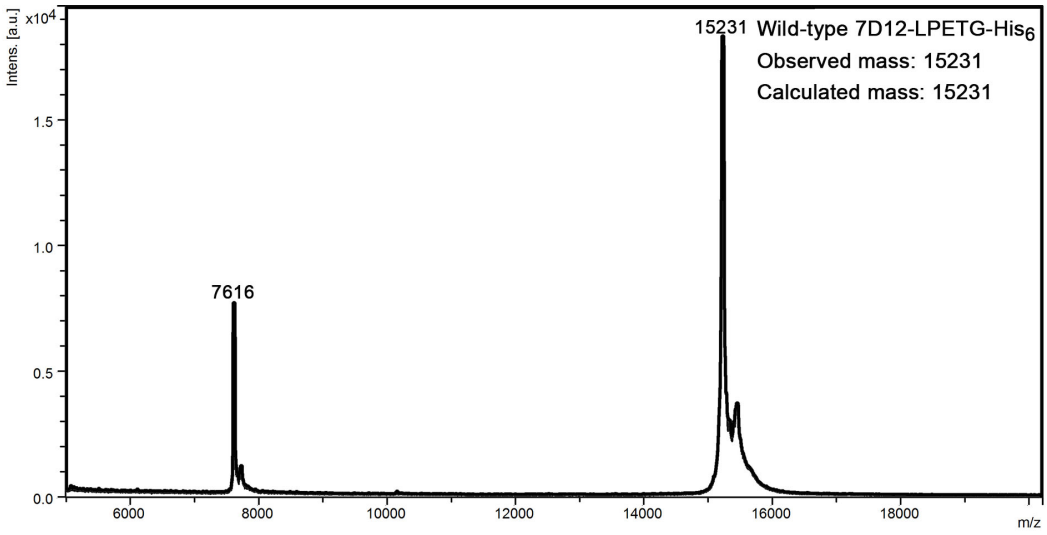


Fig. S6 MALDI-TOF mass spectra of wild-type 7D12-LPETG-His_6.

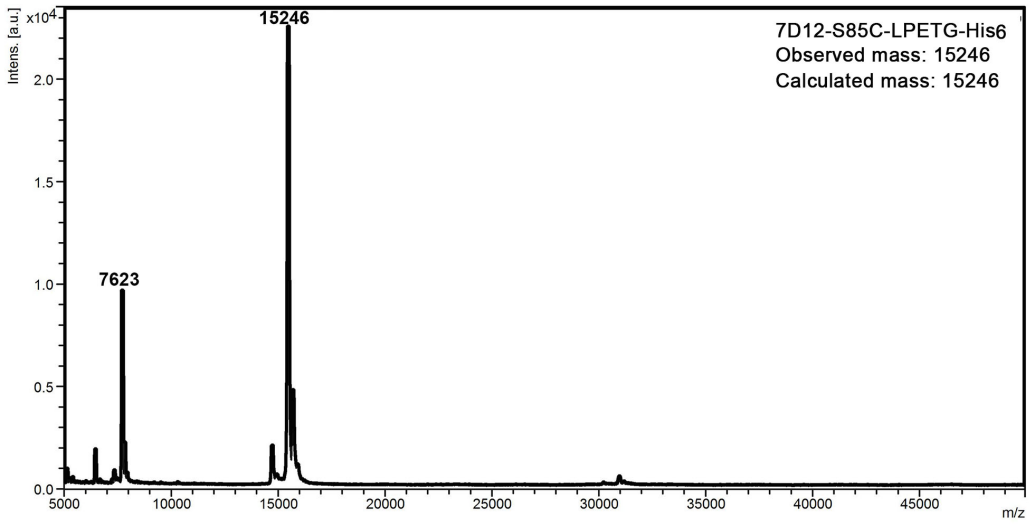


Fig. S7 MALDI-TOF mass spectra of 7D12-S85C-LPETG-His_6.

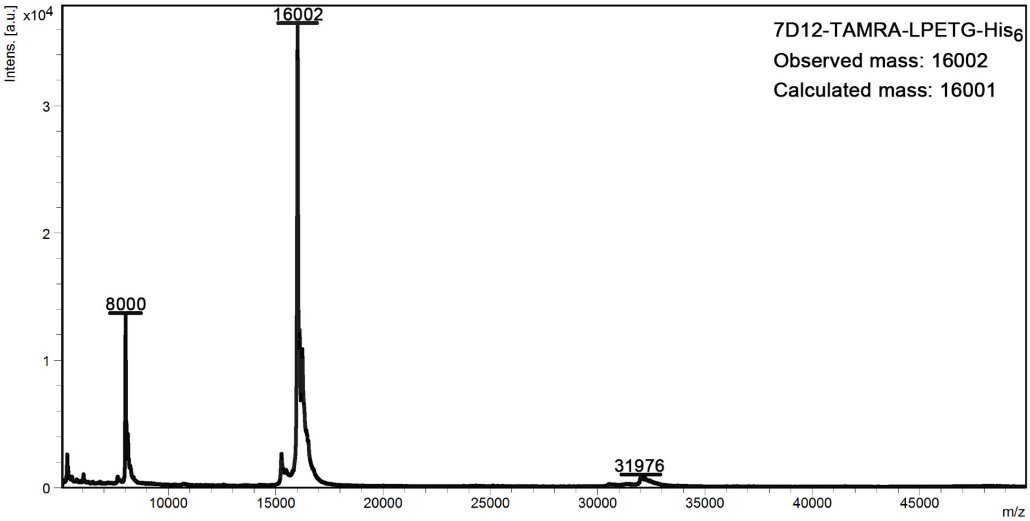


Fig. S8 MALDI-TOF mass spectra of 7D12-TAMRA-LPETG-His_6.

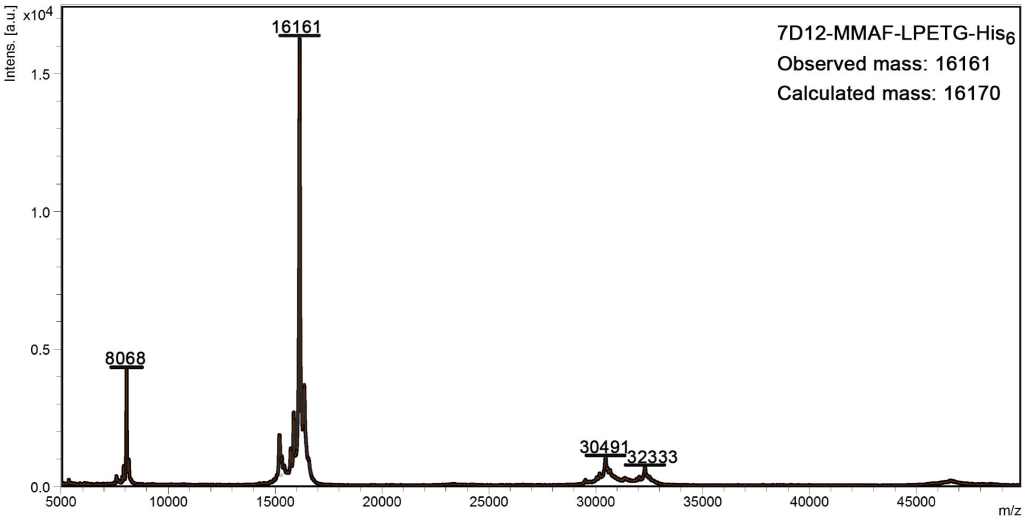


Fig. S9 MALDI-TOF mass spectra of 7D12-MMAF-LPETG-His_6.

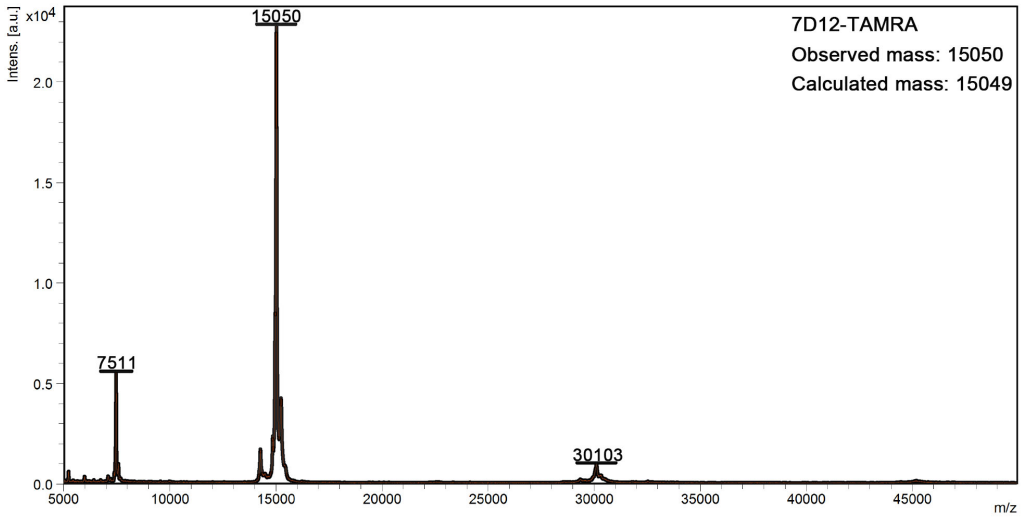


Fig. S10 MALDI-TOF mass spectra of 7D12-TAMRA.

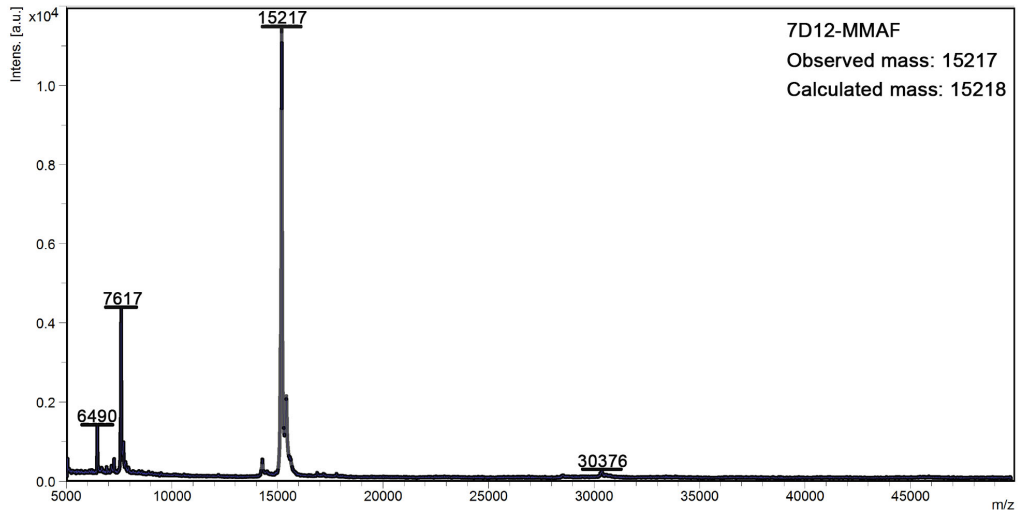


Fig. S11 MALDI-TOF mass spectra of 7D12-MMAF.

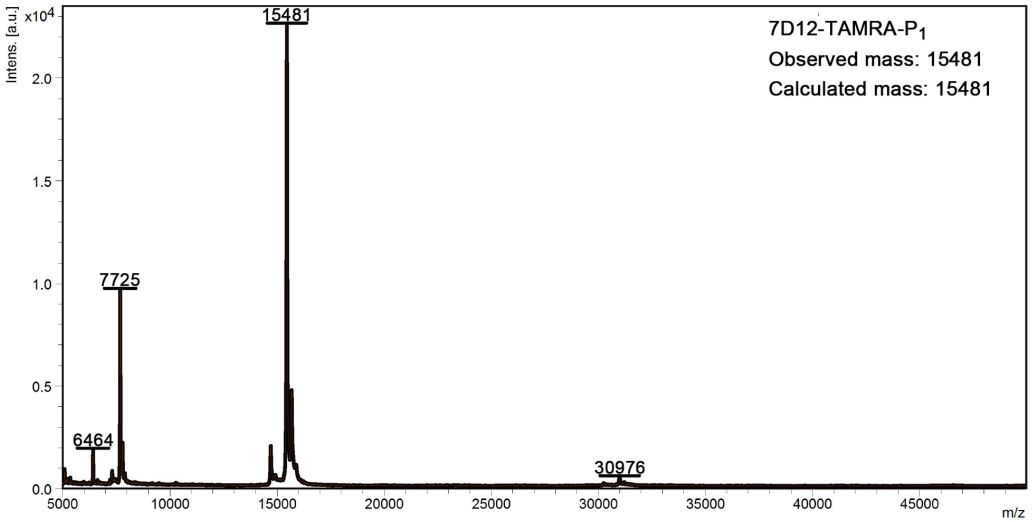


Fig. S12 MALDI-TOF mass spectra of 7D12-TAMRA-P_1.

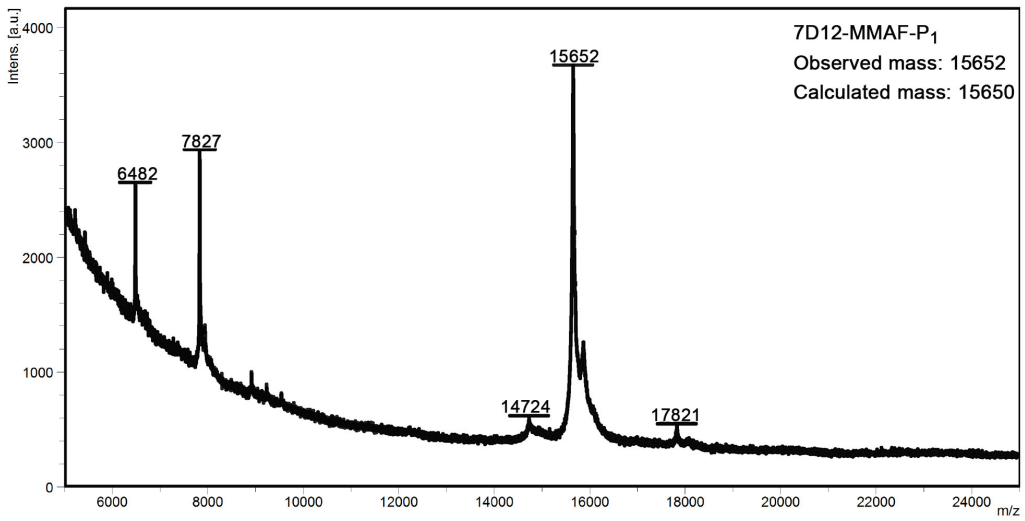


Fig. S13 MALDI-TOF mass spectra of 7D12-MMAF-P_1.

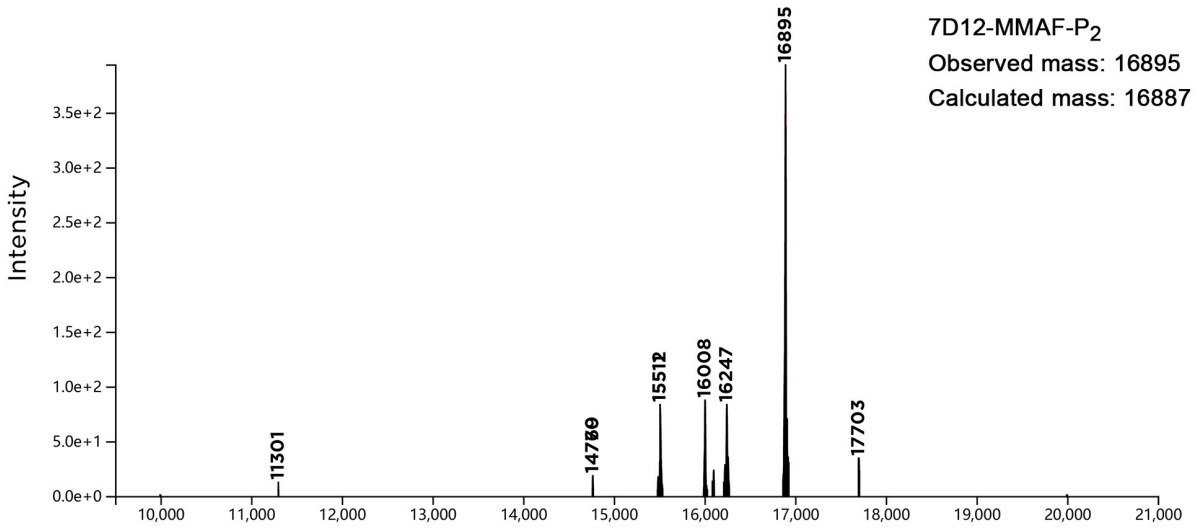


Fig. S14 Deconvoluted ESI–MS spectrum of 7D12-TAMRA-P_2.

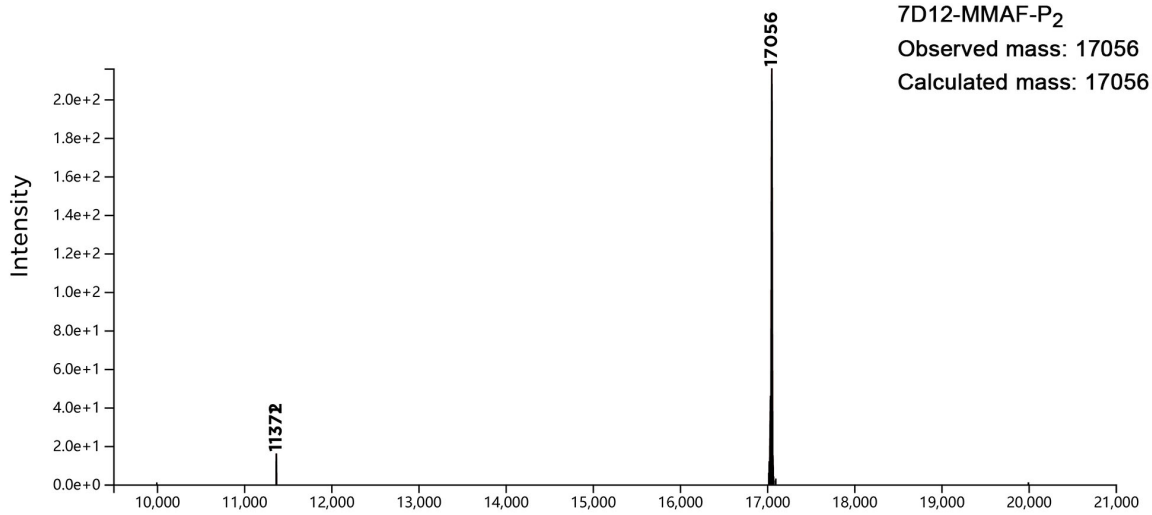


Fig. S15 Deconvoluted ESI–MS spectrum of 7D12-MMAF-P_2.

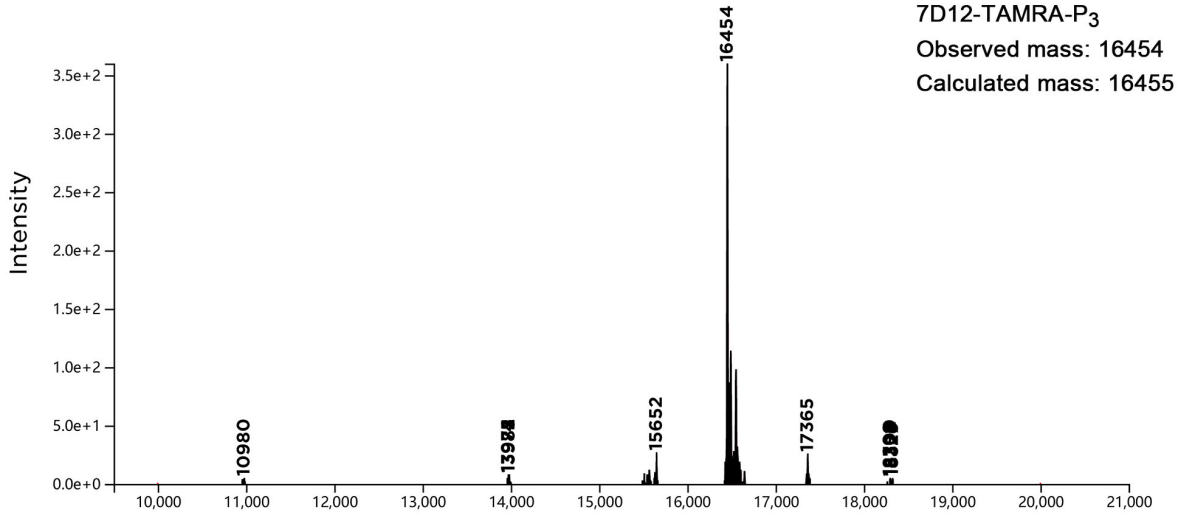


Fig. S16 Deconvoluted ESI–MS spectrum of 7D12-TAMRA-P_3.

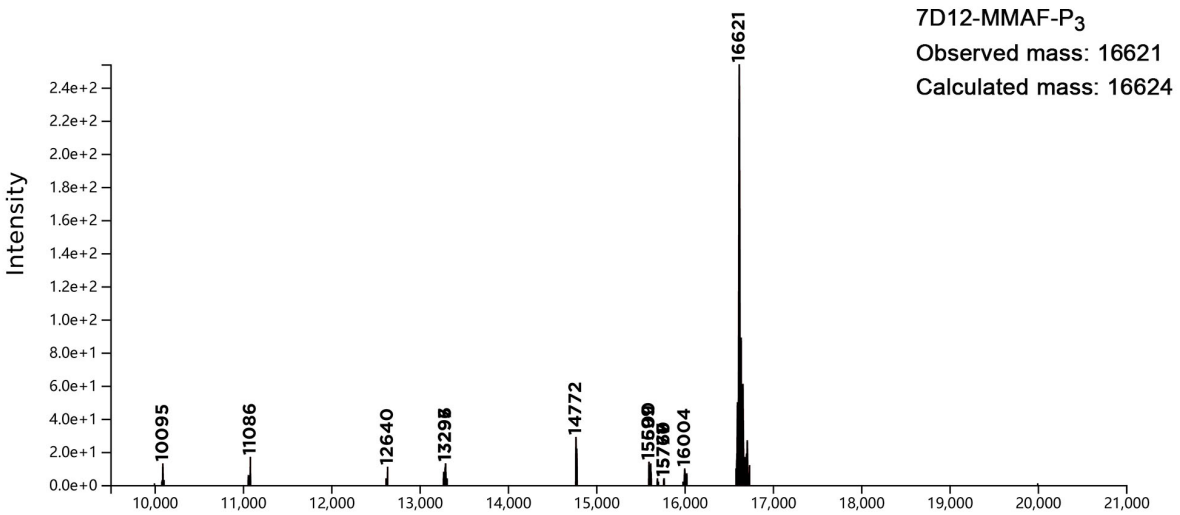


Fig. S18 Deconvoluted ESI–MS spectrum of 7D12-MMAF-P_3.

8. Cell culture

The A431, MCF-7 and 293T cells were grown and maintained in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)–streptomycin (100 g/mL). All cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

9. Confocal microscopy for conjugate internalization and colocalization with lysosome

A431 or 293T cells were seeded on a 35-mm glass-bottom dish (Cellvis, Sunnyvale, CA) (4×10^4 cells/well) at 60% confluence. Cells were then treated with a fresh serum-free medium containing TAMRA-labeled 7D12 conjugates (2 μM), and incubated for 4 h. Unbound conjugates were removed by washing with PBS, and the cells were treated with LysoTracker® Green (75 nM in PBS buffer) for 30 min. After washes three times with cold PBS, cells were stained with Hoechst 33342 (5 μg/mL in PBS buffer) at room temperature for 10 min. All images were acquired by laser scanning confocal microscope (Olympus FV3000, Olympus Corporation, Japan). Triplicate was required in this experiment. Fluorescence colocalization was calculated with Image J (https://imagej.nih.gov/ij/) and data were analyzed with Graphpad Prism6 (https://www.graphpad.com/). EGFR negative cell line 293T was used as a negative control.

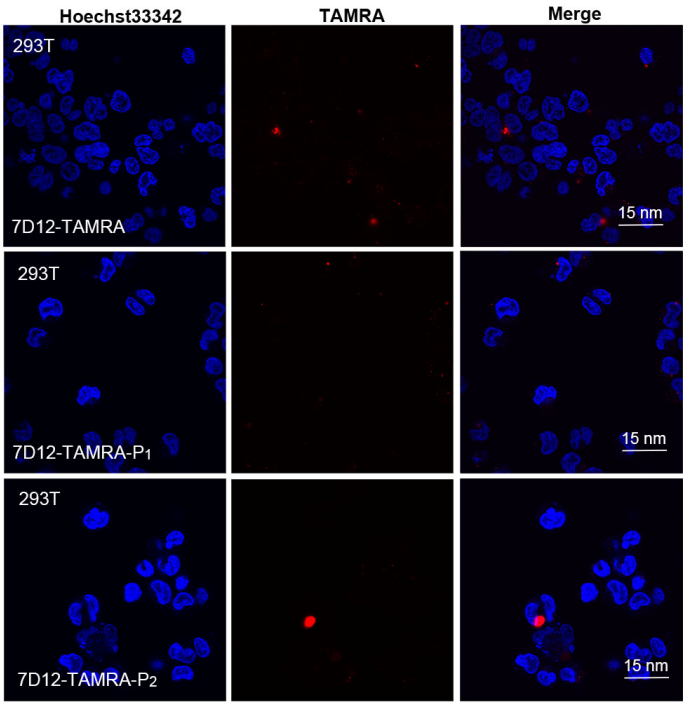


Fig. S19 The confocal images of 293T cells incubated with 7D12-TAMRA, 7D12-TAMRA-P_1 and 7D12-TAMRA-P_2, respectively. Cells were treated with a fresh serum-free medium containing TAMRA-labeled 7D12 conjugates (2 μM) and incubated for 2 h. Internalized TAMRA-labeled 7D12 conjugates were hardly visualized in 293T cells.

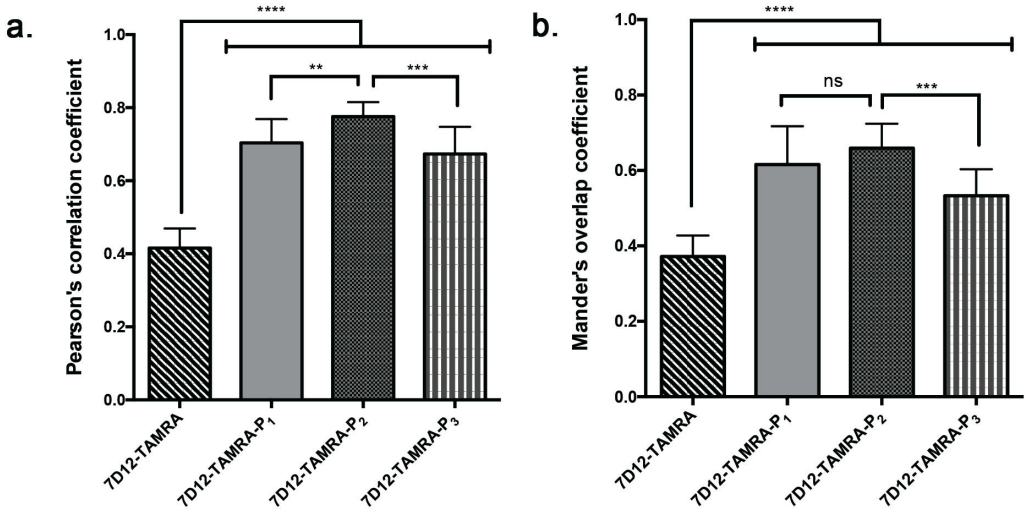


Fig. S20 The Pearson's correlation and Mander's correlation coefficients between 7D12-TAMRA-peptide and lysosomes (mean ± SEM). Error bars represent SEM (n=20). ns: not significant. *P<0.05, **P<0.01, ***P<0.001, ****P < 0.0001 (Student's *t* test).

Table S4. The Pearson and Mander's corelation coefficients between TAMRA-labeled 7D12 conjugates and lysosomes.

7D12-TAMRA conjugates	PCC	MOC
7D12-TAMRA	0.4157	0.3717
7D12-TAMRA-P_1	0.7236	0.6157
7D12-TAMRA-P_2	0.7504	0.6592
7D12-TAMRA-P_3	0.6580	0.5329

PCC: Pearson's correlation coefficient
MOC: Mander's overlap coefficient

10. Flow cytometry

A431 cells were plated on a 12-well plate until reaching at approximately 60% confluence. The culture medium was then replaced with a fresh serum-free medium containing 2 μM TAMRA-labeled 7D12 conjugates for variable time phases. Following this, cells were transferred on ice to stop internalization and washed twice with 0.2 M glycine buffer containing 0.15 M NaCl (pH 3.0) and then PBS to remove antibodies bound on the cell surface. Trypsinized cells were fixed in 4% PFA (paraformaldehyde) for 15 min, and analyzed by flow cytometry system (Beckman CytoFLEX S, USA). FlowJo software (https://www.flowjo.com/) was used to analyze the flow cytometry data. Each assay was performed in triplicate.

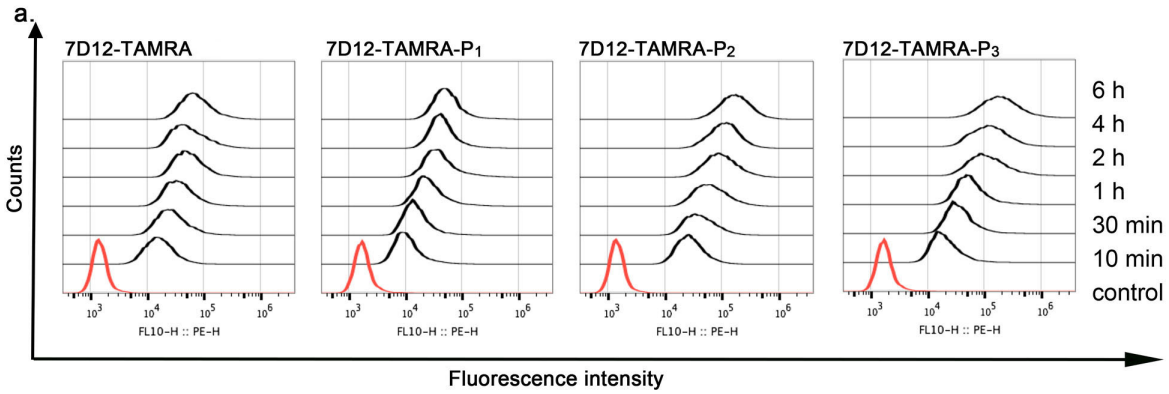


Fig. S21 Representative flow cytometric histograms showing the internalization of 7D12-TAMRA conjugates at different points of time. A431 cells were incubated with different 7D12-TAMRA conjugates for 10, 30 min, and 1, 2, 4 and 6 h, followed by analysis via flow cytometry.

11. Fluorescence quantification assay

A431 cells were seeded on a 12-well plate (8×10^5 cells/well), and incubated for 24 h. The culture medium was then replaced with a culture medium containing 2 μM TAMRA-labeled 7D12 conjugates (7D12-TAMRA, 7D12-TAMRA-P_1, 7D12-TAMRA-P_2 and 7D12-TAMRA-P_3). After incubation for 6 h, the cell culture was washed twice with 0.2 M glycine buffer containing 0.15 M NaCl (pH 3.0) and then PBS. Cells were then trypsinized using tripsin-EDTA (0.25%), and the resulting cell suspension was counted and transferred to a 12-well plate (1×106 cells/well). Following this, 500 μL of RIPA Lysis Buffer (20 mM Tris·HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/mL leupeptin, 1 mM PMSF) was added. The resultant lysates were incubated on ice for 15 min. The suspension was then centrifuged at 12,000 × g at 4 °C for 15 min, and the resultant supernatant was collected. In the end, all the samples were analyzed by FlUOstar Omega Microplate Reader (BMG Labtech, Germany) with monitoring at 561-nm excitation and 595-nm emission. The background assay was conducted using the

same protocol without the addition of any 7D12 conjugate. Calibration curves were obtained at eight concentrations of 7D12-TAMRA (2.5 nM, 3.25 nM, 5 nM, 7.5 nM, 10 nM, 15 nM, 20 nM and 30 nM) in cell lysis buffer. All assays were performed in triplicate.

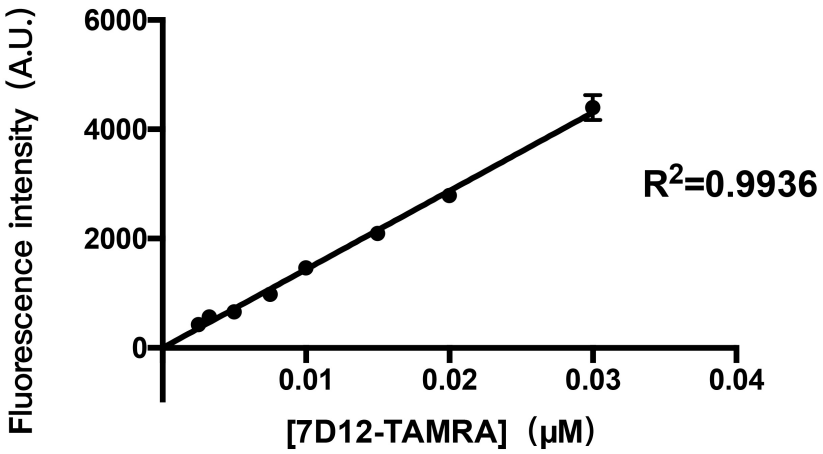


Fig. S22 Calibration curve for 7D12-TAMRA fluorescence. Fluorescence values were obtained for 7D12-TAMRA of varying concentrations. Error bars represent SEM (n=3). R^2 = 0.9936.

12. *In vitro* cytotoxicity assay

A431 or 293T cells were seeded on a 96-well plate in triplicate at a density of 5×10^3 cells/well and cultured for 24 h, followed by the treatment of 7D12 conjugates of varying concentrations for 48 h. Cell proliferation was determined using CCK8 reagent (Beyotime Biotechnology, China) according to the manufacturer's protocol. Absorbance was detected at 450 nm using a Model Flex Station 3 microtiter plate reader (Molecular Devices, USA). The percentage of the control sample was also calculated. Percentage viability was calculated using the following formula: %viability= (OD of treated cells/OD of control cells) ×100.

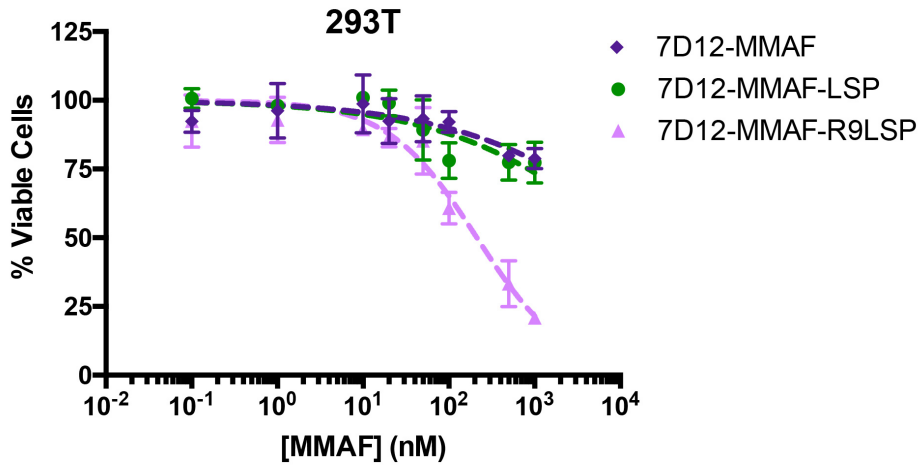


Fig. S23 *In vitro* cytotoxicity of the indicated 7D12 conjugates in EGFR-negative 293T cells. 293T cells were treated with the indicated 7D12-MMAF conjugates of varying concentrations for 96 h. Cell viability is plotted against the concentration of the tested conjugates. Data are expressed as mean ± SEM (n=3).

13. *In vitro* study via a 3D tumor spheroid model

Three-dimensional tumor spheroid model. Tumor spheroids were formed with A431, MCF-7 or 293T cells using the agarose method.[7] The wells of a 24-well plate or 96-well plate were coated with a heated (60 °C) 1.5% agarose solution in PBS buffer (200 μL/well or 50 μL/well) to provide a non-adhesive surface. A431, MCF-7 or 293T cells (5×10^4 cells/ml) were placed onto the solidified agarose-based plate. Soft agarose cultures were incubated at 37 ^{o}C for 48 h until the cellular aggregates reached around 100 μm or 200 μm. The multicellular spheroids were subsequently used to evaluate the lysosome-targeting or antitumor activity of the tested 7D12 conjugates.

Microscopy for conjugate trafficking via a 3D tumor spheroid model. Cell cultures were

harvested when majority of spheroids reached 100 μm. The tumor spheroids were then incubated with 2 μM TAMRA-labeled 7D12 conjugates (7D12-TAMRA, 7D12-TAMRA-P_2) at 37 ^{o}C for 6 h, and the unbound conjugates were removed by washing with PBS. The obtained spheroids were treated with LysoTracker® Green (75 nM in PBS buffer) for 30 min, and then rinsed three times with cold PBS followed by staining with Hoechst 33342 (5 μg/mL in PBS buffer). The tumor spheroids were then transferred to glass bottom dish and analyzed by confocal microscopy. The tumor spheroids were scanned by layer to obtain Z-stack images. The scanning began from the top of the spheroid to the equatorial plane. Each scanning layer was 10 μm in thickness, and the total scan was 200 μm in depth.

Cell viability assay via a 3D tumor spheroid model. Cell cultures were harvested when majority of spheroids reached 200 μm. The formed spheroids were then transferred into a culture medium containing the tested conjugates of varying concentrations (0.00 nM, 0.25 nM, 2.50 nM, 25.0 nM, 50.0 nM, 125 nM, 250 nM, 1.25 μM and 2.50 μM), and incubated at 37 ^{o}C for 10 days. The cell viability was evaluated every day by measuring the size of the tumor spheroids using an inverted Nikon TS100 microscope (Nikon, Japan).

Besides A431 cells, tumor spheroids were also formed with MCF-7 (low EGFR expression)[8] or 293T (EGFR-negative) cells using the agarose method.

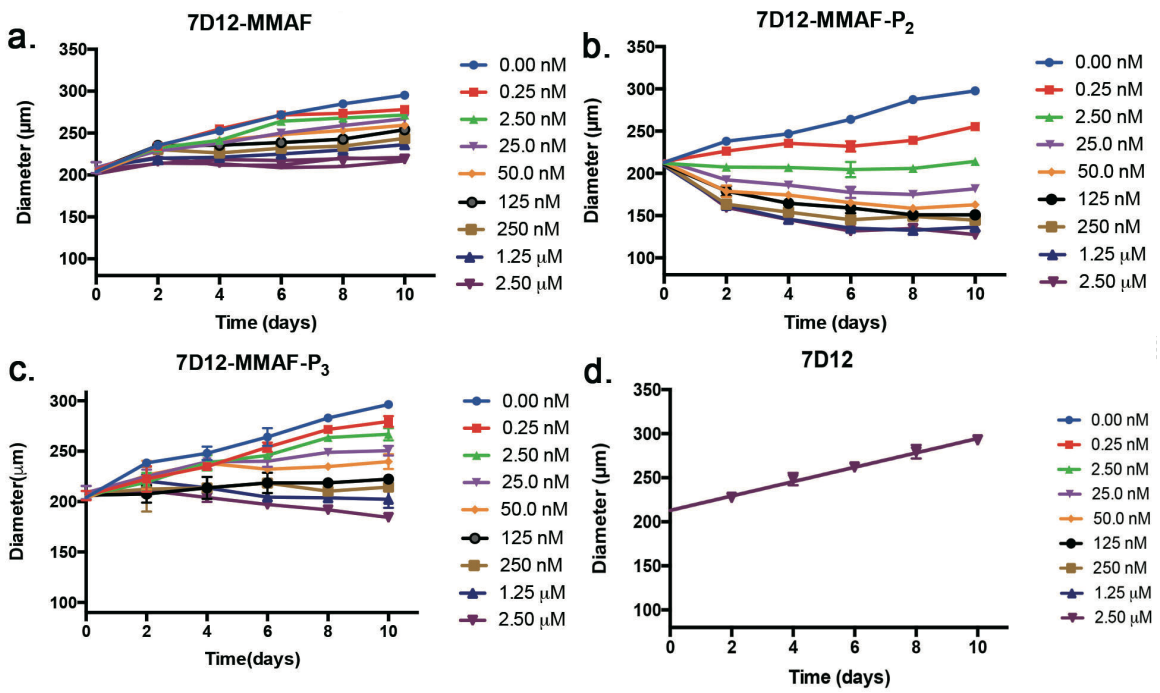


Fig. S24 Antitumor activity of lysosome-targeting 7D12 conjugates in A431 cells. a. The inhibitory effect on the growth of A431 spheroids at varying concentrations of 7D12-MMAF. b. The inhibitory effect of 7D12-MMAF-P_2. c. The inhibitory effect of 7D12-MMAF-P_3. d. 2.50 μM 7D12 was added as a control. Data are expressed as mean ± SEM (n=3).

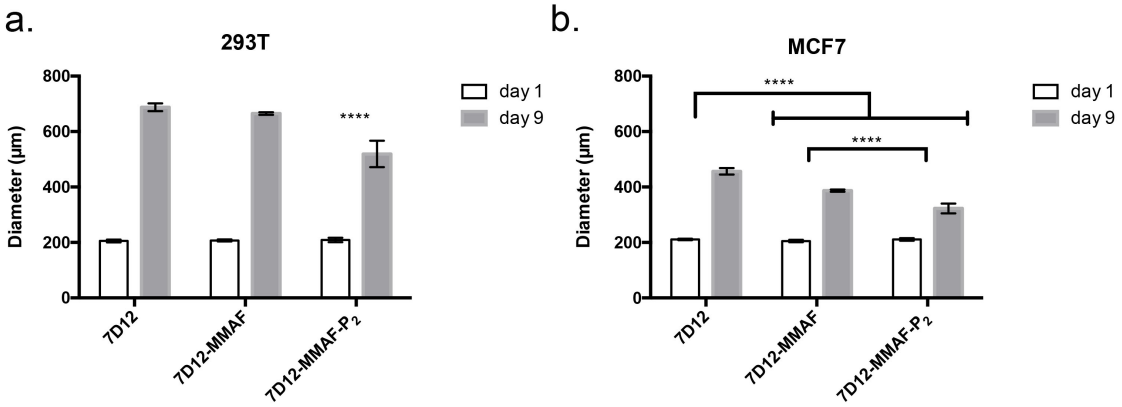


Fig. S25 Antitumor activity of lysosome-targeting 7D12 conjugates in 293T and MCF-7 cells. **a.** The inhibitory effect on the growth of 293T spheroids at varying concentrations of the tested conjugates (7D12, 7D12-MMAF, 7D12-MMAF-P_2). **b.** The inhibitory effect on the growth of MCF-7 tumor spheroids at varying concentrations of the tested conjugates (7D12, 7D12-MMAF, 7D12-MMAF-P_2). Data are expressed as mean ± SEM (n=3). Error bars represent SEM. **P<0.01, ****P < 0.0001.

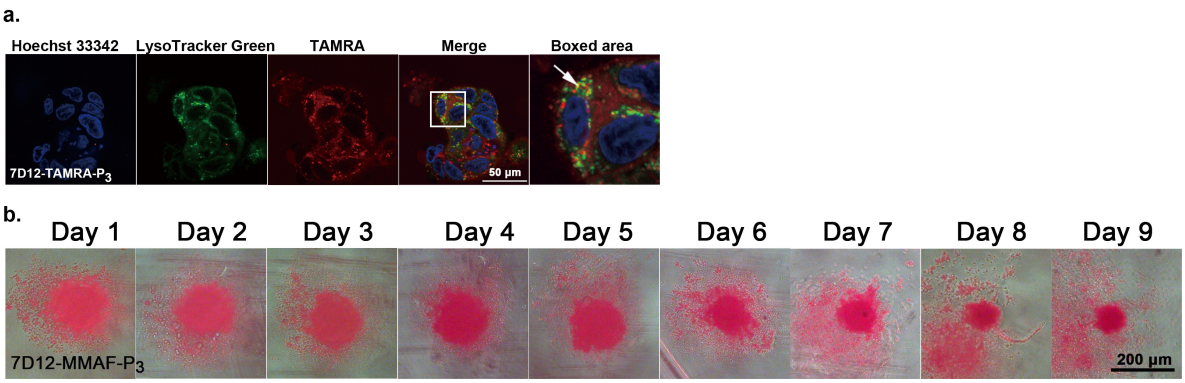


Fig. S26 a. Confocal images of A431 tumor spheroids incubated with 7D12-TAMRA-P_3 for 6 h followed by the treatment of LysoTracker® Green. Nuclei were stained with Hoechst 33342. **b.** Representative images of A431 tumor spheroids treated with 2.5 μM 7D12-MMAF-P_3 over nine days.

14. Endocytosis mechanism study

To probe the effect of temperature on the internalization of 7D12 conjugates, A431 cells were treated with 7D12-TAMRA and incubated either at 37 °C or 4 °C for 30 min. Cells were then washed with PBS three times, and images were acquired by confocal microscope. To further investigate the probable mechanistic pathway of cellular uptake of 7D12 conjugates, cells were pretreated with chlorpromazine (10 μg/mL for 1h), ethylisopropylamiloride (10 μg/mL for 45 min), or nystatin (20 μg/mL for 45 min) in PBS at 37 °C. After washes with cold PBS, cells were incubated with TAMRA-labeled 7D12 conjugates (2 μM) in fresh serum-free medium at 37 °C for 2 h. Confocal fluorescence imaging studies were performed with an Olympus confocal microscope.

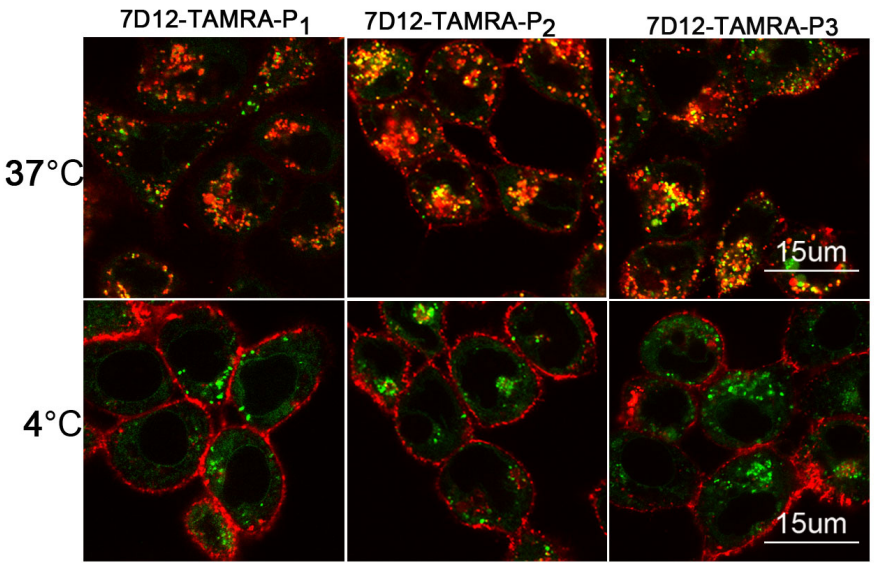


Fig. S27 Effect of temperature on the internalization of 7D12-TAMRA-peptide conjugates.

15. Colocalization study of 7D12-TAMRA-peptide with caveolin and clathrin

A431 cells were seeded on a 35-mm glass-bottom dish at 60% confluence. Following this, cells were treated with a fresh serum-free medium containing 2 μM TAMRA-labeled 7D12 conjugates (7D12-TAMRA-P_1, 7D12-TAMRA-P_2 and 7D12-TAMRA-P_3)), and then incubated for 4 h. Cells were fixed and permeabilized by paraformaldehyde/Triton X-100. Subcellular compartments were visualized with anti-clathrin heavy chain antibody (Bioss, China) or anti-cavolin-1 antibody (Bioss, China) at 37 °C for 1 h, followed by a FITC-labeled anti-rabbit IgG antibody (Bioss, China) at room temperature for 1 h. After washes with PBS, nuclei were stained by incubating cells in PBS containing 5 μg/mL Hoechst 33342 at room temperature for 10 min. All images were acquired by laser scanning confocal microscope (Olympus FV3000, Olympus Corporation, Japan). Triplicate was required in this experiment.

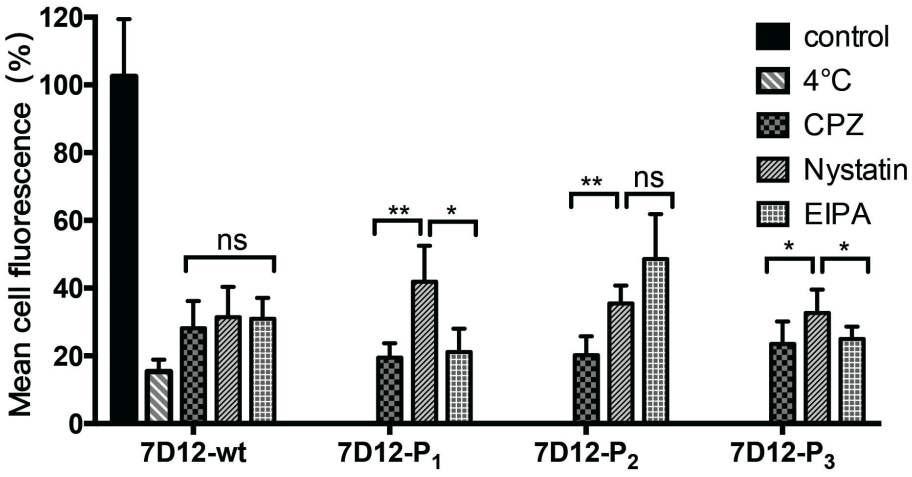


Fig. S28 Endocytosis mechanism studies of TAMRA-labeled 7D12 conjugates. The inhibitory effect was expressed as a percentage of fluorescence intensity relative to the control. Error bars represent SEM (n=20). ns: not significant. *P < 0.05. **P < 0.01 (Student's t test).

16. Statistical analysis.

All data are represented as the mean ± SD. The number of biological replicates for every experiment was represented as 'n' numbers. While the 'n' in imaging studies is the total number of cells which were analyzed, and the data are represented as the mean ± SEM. Student's *t* test or one-way analysis of variance (ANOVA) was performed in statistical evaluation. A *P*-value less than 0.05 is considered to be significant.

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