Supplementary Information (SI) for Organic & Biomolecular Chemistry.
This journal is © The Royal Society of Chemistry 2026

Supplementary Information

Facile Synthesis of Redox-Responsive Peptide Coacervates for Cytosolic Protein Delivery

Xiaona Han[a], Ruihui Han[a], Jiaxing Yang[a], Xiao Hua[b*], Yi-Ming Li [a*]

[a] School of Food and Biological Engineering, Engineering Research Center of Bio-process, Ministry of Education, Hefei University of Technology, Hefei 230009, China

[b] Tsinghua-Peking Center for Life Sciences, Department of Chemistry, Tsinghua University, Beijing 100084, China.

* To whom correspondence should be addressed: Yi-Ming Li (ymli@hfut.edu.cn)

Table of contents

1. Materials and general methods

1.1 Materials

Fmoc-protected amino acids, ethyl cyanoglyoxylate-2-oxime (Oxyma), 1-hydroxy-7-aza-benzotriazole (HOAT), 1-hydroxybenzotriazole (HOBT), and 2-(7-azabenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HATU) were purchased from GL Biochem (Shanghai, China). 2-Chlorotrityl resin (0.44 mmol/g), and Wang resin (0.44 mmol/g) were purchased from Tianjin Nankai HECHENG S&T Co., Ltd (Tianjin, China). 2-(Tritylthio) acetic acid, N, N'-diisopropylcarbodiimide (DIC), N, N-diisopropylethylamine (DIEA), hexafluoroisopropanol (HFIP), 1,2-dichloroethane, trifluoroacetic acid (TFA), 1,2-ethanedithiol (EDT), triisopropylsilane (TIPS), acetonitrile (HPLC grade), tris(2-carboxyethyl) phosphine (TCEP), 4-dimethylaminopyridine (DMAP), acetylacetone, and cysteamine were purchased from Energy Chemical and Bide Pharmatech (Shanghai, China). N, N-Dimethylformamide (DMF) and dichloromethane (DCM) were purchased from Chengdu Kelong Chemical. Piperidine, phenol, and sodium nitrite ($NaNO_2$) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Guanidine hydrochloride, urea, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris-base), sodium chloride (NaCl), and tryptone were purchased from Sangon Biotech (Shanghai, China). Isopropyl-β-D-thiogalactopyranoside (IPTG) and dithiothreitol (DTT) were purchased from Aladdin. Ampicillin, imidazole, and Ni-NTA resin were obtained from Lablead (Beijing, China). *Escherichia coli* (*E. coli*) BL21(DE3) competent cells were purchased from TransGen Biotech (Beijing, China). XB-C4 and XB-C18 column packing materials were purchased from Yuexu Technology. Yeast extract was purchased from Thermo Fisher, while 4-mercaptophenylacetic acid (MPAA) was purchased from Thermo Scientific. Glutathione (reduced form, GSH) was purchased from Macklin Reagent (Shanghai, China). Bovine serum albumin (BSA), Immunoglobulin G (IgG), FITC-NHS ester, and TAMRA-NHS ester were purchased from Sigma-Aldrich (USA). For cell culture and biological assays, Dulbecco's Modified Eagle Medium (DMEM), Opti-MEM, fetal bovine serum (FBS), 0.25% Trypsin-EDTA, and penicillin-streptomycin were purchased from Gibco (USA). The Cell Counting Kit-8 (CCK-8) was purchased from Biosharp (China).

1.2 RP-HPLC and ESI-MS

Analysis and purification of peptides were performed by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Shimadzu Prominence LC-20AT system. For analysis, an analytical Welch XB-C18 column (4.6 × 250 mm, 5 μm, 120 Å) was used at a flow rate of 1.0 mL/min. For purification, a semi-preparative Welch XB-C18 column (10 × 250 mm, 5 μm, 120 Å) was used at a flow rate of 4.0 mL/min. The mobile phase consisted of solvent A (0.1% TFA in water) and solvent B (0.08% TFA in acetonitrile). A linear gradient of solvent B was applied, and the elution was monitored by UV absorbance at 214 nm. Electrospray ionization mass spectrometry (ESI-MS) data were collected on a Shimadzu LCMS-2020 single quadrupole mass spectrometer.

2. Peptide synthesis methods

2.1 Preparation of Functionalized Solid Supports

For the synthesis of peptide hydrazides, 2-chlorotrityl chloride (2-CTC) resin (typically at 0.1 mmol scale) was initially hydrated in N, N-dimethylformamide (DMF) for 15 min. The resin was then functionalized into a hydrazine-loaded support by incubating with a 5% (*v/v*) hydrazine hydrate/DMF mixture at 37 °C (two 30-min cycles). To prevent non-specific elongation, unreacted active chloride sites were blocked utilizing 5% (*v/v*) methanol in DMF (two 10-min cycles) at ambient temperature, followed by extensive washing with DMF.

For peptides requiring a C-terminal carboxylic acid, Wang resin (0.1 mmol scale) was pre-swollen in a 1:1 (*v/v*) mixture of dichloromethane (DCM) and DMF. The initial Fmoc-protected amino acid (8.0 equivalents) was anchored to the resin using N, N'-diisopropylcarbodiimide (DIC, 8.0 equivalents) Oxyma (10.0 equivalents), and a catalytic amount of 4-dimethylaminopyridine (DMAP, 1 equivalents) under gentle agitation overnight at room temperature. Subsequent capping of the residual free hydroxyl groups was accomplished by treating the resin with an acetic anhydride/N, N-diisopropylethylamine (DIEA)/DMF cocktail (1:1:8 by volume) for 30 min. The loaded resin was then sequentially washed with DMF and DCM prior to chain elongation.

2.2 Automated Microwave-Assisted Peptide Elongation and Cleavage

General automated elongation: Following the manual preparation of the functionalized resins described above, general peptide chain elongation was carried out using a Liberty Blue™ automated microwave peptide synthesizer (CEM Corp., NC, USA). Standard coupling cycles utilized 4.0 equivalents of Fmoc-amino acids, 4.0 equivalents of Oxyma Pure, and 8.0 equivalents of DIC. The coupling reactions were accelerated under microwave irradiation at 90 °C for 2 min. Notably, to minimize racemization, histidine (His) residues were coupled at a milder temperature of 50 °C for 10 min, and this specific coupling step was repeated twice. Fmoc removal was achieved using a 20% (*v/v*) piperidine solution in DMF (supplemented with 0.1 M Oxyma) heated to 90 °C for 1 min in the microwave reactor. Intermittent washings between coupling and deprotection steps were performed thrice with DMF.

Specific modifications for peptides 5, 6, and C-terminal fragments: For the synthesis of the hydrazide-terminated N-terminal fragments of peptide derivatives 5 and 6, an orthogonal protecting group strategy was employed. First, Fmoc-Lys(ivDde)-OH was manually coupled to the hydrazine-loaded resin. The remaining sequence was then assembled via the automated microwave synthesizer. Importantly, the final N-terminal residue was coupled as Boc-Gly-OH instead of Fmoc-Gly-OH to prevent premature deprotection during the subsequent side-chain modification. After the automated assembly, the ivDde protecting group on the lysine side chain was orthogonally removed by treating the resin with 5% (*v/v*) hydrazine hydrate in DMF (5-6 cycles of 10 min each). The exposed lysine side chain was then sequentially coupled with Fmoc-AEEA-OH (as a linker) and the specific capping groups (benzoic acid for peptide 5 or indole-5-carboxylic acid for peptide 6) under standard conditions. Conversely, for the synthesis of all C-terminal fragments, the final building block coupled at the N-terminus via the microwave synthesizer was 2-(tritylthio)acetic acid.

Global deprotection and cleavage: Upon completion of the sequence assembly and specific modifications, the peptidyl-resin was thoroughly washed with DCM and dried. Global deprotection and cleavage from the solid support were executed using a cleavage cocktail composed of trifluoroacetic acid (TFA)/thioanisole/H_2O/1,2-ethanedithiol (EDT) at a volumetric ratio of 87.5:5:5:2.5. The mixture was shaken at room temperature for 2 h. Subsequently, the resin was filtered off, and the filtrate was

concentrated under a gentle stream of nitrogen. The crude peptide was precipitated using ice-cold diethyl ether, collected via centrifugation, and ultimately subjected to semi-preparative RP-HPLC for purification.

2.3 Synthesis of Redox-Responsive Peptide Derivatives (1-6)

Preparation of the N-terminal thiol-containing fragments: The hydrazide-terminated peptides synthesized via microwave-assisted SPPS were first purified by semi-preparative RP-HPLC and lyophilized to yield pure peptide powders. To synthesize the N-terminal thiol-containing fragments (e.g., 1^{N}), the purified hydrazide peptide was dissolved in an acidic denaturing buffer (6 M guanidine hydrochloride, 0.2 M Na_2HPO_4, pH 2.3). Subsequently, acetylacetone (10.0 equiv.) and 4-mercaptophenylacetic acid (MPAA, 50.0 equiv.) were added to the solution. The reaction mixture was incubated at room temperature for 2 h to afford the MPAA-thioester intermediate. Cysteamine (7.0 equiv.) was then added to the mixture, and the pH of the solution was carefully adjusted to 7.0. The reaction was allowed to proceed for another 2 h at room temperature. Finally, the desired thiol-containing N-terminal fragment was isolated via RP-HPLC purification and lyophilized.

Preparation of the activated C-terminal fragments: The crude C-terminal peptides (e.g., 1^{C}) obtained directly from SPPS cleavage were used without prior chromatographic purification. The crude C-terminal peptide powder was dissolved in a neutral denaturing buffer (6 M guanidine hydrochloride, 0.2 M Na_2HPO_4, pH 7.0) and reacted with 2,2'-dipyridyldisulfide (DPDS, 1.5 equiv.). After 1 h of reaction at room temperature, the DPDS-activated C-terminal fragment (e.g., DPDS-1^{C}) was purified by RP-HPLC and lyophilized.

Ligation of the peptide derivatives: To assemble the complete redox-responsive peptide derivatives 1-6, the purified N-terminal fragment and the corresponding DPDS-activated C-terminal fragment were co-dissolved in a neutral denaturing buffer (6 M guanidine hydrochloride, 0.2 M Na_2HPO_4, pH 7.0). The disulfide exchange ligation was allowed to proceed for 1 h at room temperature. The final ligated products were purified by RP-HPLC, and their molecular weights were confirmed by ESI-MS.

3. Protein expression and purification

Expression of recombinant proteins: The plasmids encoding His-tagged eGFP, His-tagged RFP, and Ub were individually transformed into *Escherichia coli (E. coli)* BL21(DE3) competent cells. The transformed cells were cultured in Luria-Bertani (LB) medium supplemented with appropriate antibiotics (e.g., ampicillin) at 37 °C with shaking. Once the optical density at 600 nm (OD_{600}) of the culture reached 0.6–0.8, protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM. The cultures were further incubated overnight at 18 °C. Subsequently, the cells were harvested by centrifugation and lysed via sonication in an ice-cold lysis buffer.

Purification of His-tagged eGFP and RFP: For the His-tagged proteins (eGFP and RFP), the cell lysates were clarified by high-speed centrifugation. The resulting supernatants were loaded onto Ni-NTA affinity resin columns (Lablead). To remove non-specifically bound host proteins, the columns were extensively washed with a buffer containing 40 mM imidazole. Target proteins were then eluted using a buffer containing 250 mM imidazole. The collected fractions were further polished by Size Exclusion Chromatography (SEC) on an AKTA FPLC system equipped with a Superdex 75 or 200 size-exclusion column to obtain the final highly pure proteins.

Purification of untagged ubiquitin via acid precipitation: For the untagged Ub protein, an acid precipitation strategy was employed based on its unique high structural stability. Briefly, perchloric acid was carefully added to the clarified cell lysate to a final concentration of 0.07% (*v/v*). The acidified mixture was stirred at 4 °C for 5 min, which induced the precipitation of most endogenous E. coli host proteins while Ub remained soluble in the supernatant. Following centrifugation to remove the precipitates, the supernatant containing the target Ub was neutralized, dialyzed against a physiological buffer, and finally polished by SEC using a Superdex 75 column to ensure high purity.

Preparation of Fluorescently Labeled Proteins Bovine serum albumin (BSA) and Immunoglobulin G (IgG) were purchased from commercial suppliers and utilized without further purification. Since eGFP and RFP are intrinsically fluorescent proteins, they were directly used for intracellular delivery imaging. To visually track the cytosolic delivery of the other non-fluorescent cargo proteins, Ub, BSA, and IgG were

covalently conjugated with amine-reactive fluorescent dyes (TAMRA-NHS or FITC-NHS).

To maintain their native structural integrity, the target proteins were prepared in standard phosphate-buffered saline (PBS). FITC-NHS or TAMRA-NHS (pre-dissolved in anhydrous DMSO) was added dropwise to the protein solutions at a strict molar ratio of 3:1 (dye to protein). The reaction mixtures were incubated in the dark at room temperature for 2 h under gentle agitation. Subsequently, the unreacted free dyes were completely removed via repeated centrifugal ultrafiltration using Amicon Ultra centrifugal filter units with appropriate molecular weight cutoffs. During this ultrafiltration process, the protein retentate was continuously diluted and washed with fresh PBS until the flow-through filtrate became visually colorless, indicating the complete removal of free dye molecules. The resulting fluorescently labeled proteins (TAMRA-Ub, TAMRA-BSA, and FITC-IgG) were quantified using a NanoDrop spectrophotometer and stored appropriately for subsequent delivery assays.

4. *In Vitro* Characterization and Cellular Assays

4.1 Turbidity Measurements and pH-Dependency Assay

The LLPS behavior of the peptide derivatives was evaluated by monitoring the solution turbidity, measured as the optical density at 600 nm (OD_{600}) using a microplate reader. To ensure complete dissolution and prevent premature assembly, peptide derivatives were first dissolved in 10 mM acetic acid to prepare a 3 mM monomeric stock solution. For the assay, the stock solution was mixed with buffers of varying pH (ranging from 3.0 to 9.0) at a volume ratio of 1:9 in a transparent 384-well plate, yielding a final peptide concentration of 300 μM. The plate was subjected to gentle orbital shaking for 10 s to ensure homogeneous mixing. After an incubation period of 5 min at room temperature to reach phase equilibrium, the OD_{600} values were recorded. The relative turbidity was calculated by normalizing the measured OD_{600} values, setting the minimum value (typically observed at acidic pH < 6.0, where peptides are fully soluble) as 0% and the maximum plateau value (indicating maximal coacervation) as 100%.

4.2 *In Vitro* Redox-Responsive Disassembly of Coacervates

To demonstrate the redox-responsiveness of the assemblies, the pre-formed bare peptide coacervates were treated with a reducing agent. Specifically, dithiothreitol (DTT, final concentration 5 mM) was added to the coacervate suspension. The morphological changes and the disassembly process were directly monitored using bright-field microscopy until the microdroplets completely dissolved into a clear solution.

4.3 Cell Culture

Human cervical carcinoma (HeLa), human hepatocellular carcinoma (HepG2), human osteosarcoma (U2OS), human lung adenocarcinoma (A549), and murine macrophage (RAW 264.7) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. Human normal bronchial epithelial (BEAS-2B) cells were cultured in a specialized BEAS-2B cell culture medium. All cells were maintained in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. Upon reaching 80–90% confluence, the cells were routinely subcultured. Notably, to maintain cell viability and adherence properties, the RAW 264.7 macrophages were passaged mechanically using a cell scraper rather than via standard enzymatic digestion.

4.4 Cytotoxicity Assay

The *in vitro* cytotoxicity of the peptide derivatives 1-6 was evaluated on HeLa cells using the Cell Counting Kit-8 (CCK-8, Biosharp) assay under conditions identical to those used in the intracellular delivery experiments. Briefly, HeLa cells were seeded into 96-well plates at a density of 5,000 cells per well in 100 μL of complete DMEM and allowed to adhere overnight. To exactly mimic the delivery formulation, the peptide stock solution (3 mM in 10 mM acetic acid) was mixed with 9 volumes of a neutral buffer (10 mM phosphate, 79 mM NaCl, pH 7.0) to induce coacervation. This turbid suspension was subsequently diluted with 9 volumes of Opti-MEM. The cell culture medium in the 96-well plate was then removed and replaced with 100 μL of this prepared Opti-MEM/coacervate mixture. After 4 h of co-incubation at 37 °C, the

coacervate-containing medium was carefully removed and replaced with 100 μL of fresh complete DMEM. Subsequently, 10 μL of CCK-8 reagent was added to each well. The plates were further incubated for exactly 1 h at 37 °C in the dark. The absorbance at 450 nm was measured using a microplate reader, and cell viability was calculated as a percentage relative to the untreated control cells.

4.5 Intracellular Protein Delivery and Confocal Imaging

For intracellular protein delivery, cells (e.g., HeLa, HepG2, A549, U2OS, RAW 264.7, and BEAS-2B) were seeded in confocal dishes and allowed to adhere overnight. To prepare the protein-loaded coacervates, the peptide stock solution (3 mM in 10 mM acetic acid) was mixed at a 1:9 volume ratio with a neutral buffer (10 mM phosphate, 79 mM NaCl, pH 7.0) containing the desired cargo proteins (eGFP, RFP, TAMRA-Ub, TAMRA-BSA, or FITC-IgG at 0.1 mg/mL. typically, 10 μL of peptide stock solution was mixed with 90 μL of protein solution). The culture medium was then removed and replaced with a mixture containing 1 volume of the prepared coacervate suspension and 9 volumes of Opti-MEM(i.e., 100 μL of coacervate suspension diluted with 900 μL of Opti-MEM to give a total volume of 1 mL per dish). After exactly 4 h of co-incubation at 37 °C, the medium was removed, and the cells were washed twice with standard PBS to remove uninternalized coacervates. Subsequently, phenol red-free DMEM was added to the dishes. Intracellular fluorescence distributions were acquired using a Confocal Laser Scanning Microscope (CLSM, LSM 980, Carl Zeiss).

4.6 Flow Cytometry Analysis

To quantitatively evaluate the cellular uptake efficiency, flow cytometry analysis was specifically performed using HeLa cells. HeLa cells were seeded in multi-well plates and treated with eGFP-loaded coacervates exactly as described for the confocal imaging experiments. Following the 4 h incubation at 37 °C, the cells were washed twice with standard PBS to remove uninternalized coacervates. The cells were subsequently detached using 0.25% Trypsin-EDTA, harvested by centrifugation, and resuspended in cold PBS. The fluorescence intensity of the cells was analyzed using a flow cytometer. A minimum of 10,000 events were recorded for each sample, and the percentage of fluorescently positive cells was determined using FlowJo software.

5. Supplementary Figures

5.1 Characterization of the Final Peptide Derivatives

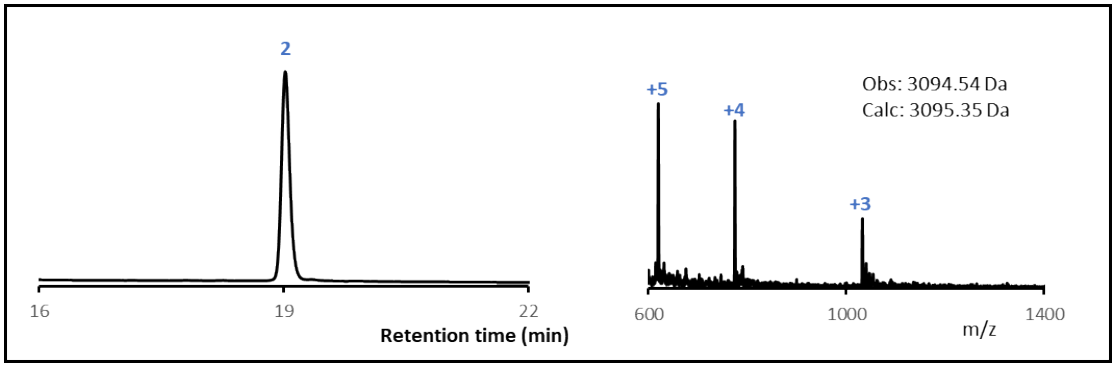

Figure S1. Characterization of the purified peptide derivative 2. Analytical RP-HPLC chromatogram (5 to 80% MeCN (with 0.08% TFA) in 30 min, λ = 214 nm) of peptide 2 on a C18 column. ESI-MS spectrum of the purified peptide 2. Calculated mass: 3095.35 Da, Observed mass: 3094.54 Da.

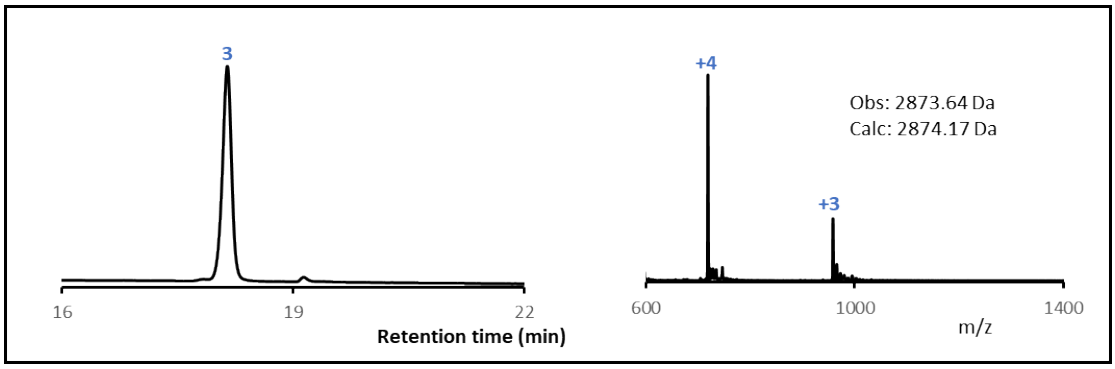

Figure S2. Characterization of the purified peptide derivative 3. Analytical RP-HPLC chromatogram (5 to 80% MeCN (with 0.08% TFA) in 30 min, λ = 214 nm) of peptide 3 on a C18 column. ESI-MS spectrum of the purified peptide 3. Calculated mass: 2874.17 Da, Observed mass: 2873.64 Da.

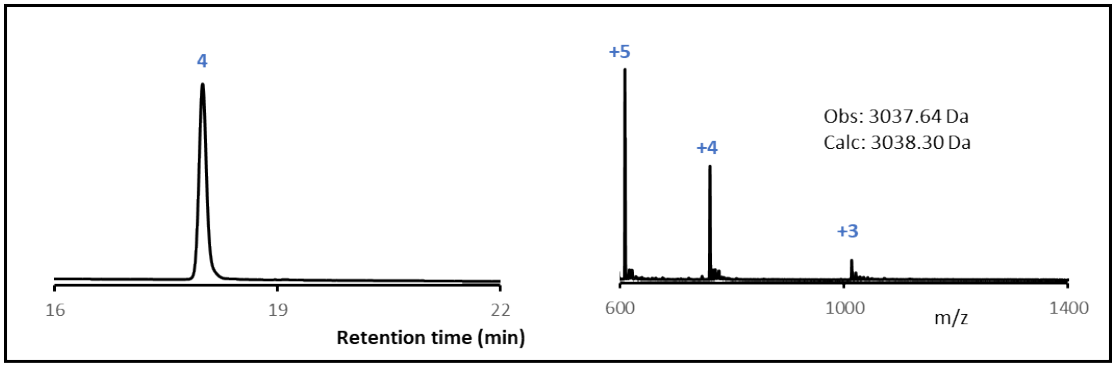

Figure S3. Characterization of the purified peptide derivative 4. Analytical RP-HPLC chromatogram (5 to 80% MeCN (with 0.08% TFA) in 30 min, λ = 214 nm) of peptide 4 on a C18 column. ESI-MS spectrum of the purified peptide 4. Calculated mass: 3038.30 Da, Observed mass: 3037.64 Da.

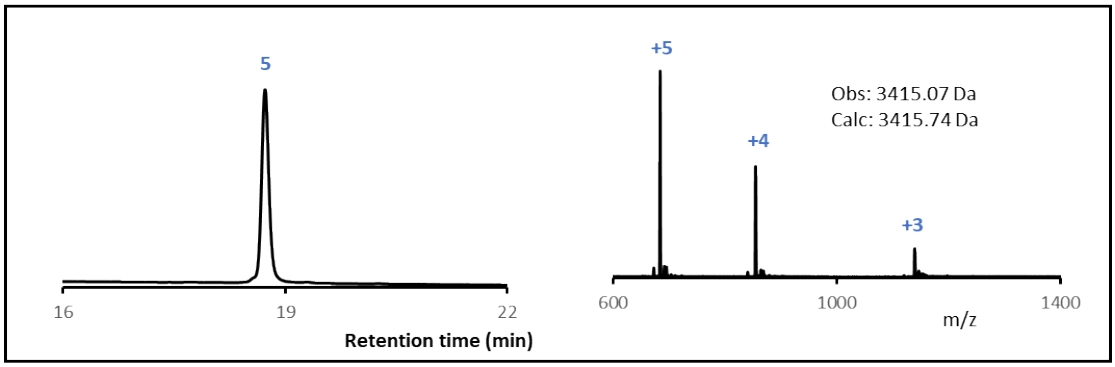

Figure S4. Characterization of the purified peptide derivative 5. Analytical RP-HPLC chromatogram (5 to 80% MeCN (with 0.08% TFA) in 30 min, λ = 214 nm) of peptide 5 on a C18 column. ESI-MS spectrum of the purified peptide 5. Calculated mass: 3415.74 Da, Observed mass: 3415.07 Da.

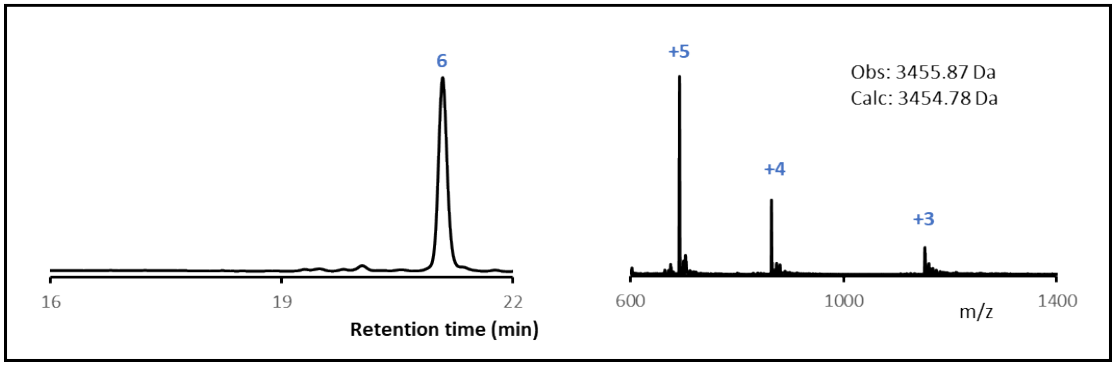

Figure S5. Characterization of the purified peptide derivative 6. Analytical RP-HPLC chromatogram (5 to 80% MeCN (with 0.08% TFA) in 30 min, λ = 214 nm) of peptide 6 on a C18 column. ESI-MS spectrum of the purified peptide 6. Calculated mass: 3454.78 Da, Observed mass: 3455.87 Da.