Supplemental Information

Materials:

Reagents used for chemical protein synthesis: Analytical grade dimethylformamide (DMF) was from Biotech. Resins were from Creosalus and all 9-fluorenylmethoxycarbonyl (Fmoc) and *tert*-butyloxycarbonyl (Boc) protected amino acids were from GL Biochem. The activating reagents, N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uroniumhexafluorophosphate (HBTU), 1-Hydroxybenzotriazolemonohydrate (HOBt), O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), 1-[Bis(dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), were from Luxembourg Bio Technologies. MPAA (4-Mercaptophenylacetic acid), MPA (3-Mercaptopropionic acid), MMP (Methyl 3-mercaptopropionate) were from Alfer Aeser. Fmoc-N-methyl-Cys(2-nitrobenzyl)-OH, Fmoc-3-Amino-4-methylamino-benzoic acid (Fmoc-MeDbz), and the thiazolidine (Thz) δ-mercaptolysine, were prepared using reported procedures [(1–3), respectively]. 2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) was from WAKO. Dichloromethane (DCM) was from Sigma-Aldrich. *tert*-Butyl mercaptan (t-BuSH) was from Sigma-Aldrich. *N*,*N*-Diisopropylethylamine (DIEA) was from Mercury. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was from Sigma-Aldrich.

Instruments used for chemical protein synthesis: Solid phase peptide synthesis (SPPS) was carried out in syringes equipped with Teflon filters (Torviq), or by using CS336X automated peptide synthesizer (CSBIO). Analytical, preparative, and semi preparative HPLC analysis were performed on Dionex Ultimate 3000 (Thermo) using the following columns: analytical HPLC column: Xbridge, BEH300 C4, 3.5μm, 4.6 × 150 mm (Waters); preparative HPLC columns: Jupiter, 10μm, C4 300 Å, 250 × 22.4 mm (Phenomenex), and XSelect, C4, 10 μm, 250 × 19 mm (Waters); and semi preparative HPLC column: Jupiter, C4 10 μm, 300 Å, 250 × 10 mm (Phenomenex). Following HPLC, the chemically synthesized proteins were further purified using the AKTA FPLC System (GE). Mass

spectrometry analysis was carried out using LCQ Fleet Ion Trap (Thermo). Circular dichroism was determined using Chirascan (Applied Photophysics) instrument.

Methods

Chemical synthesis of differentially ubiquitinated HA-α-globin:

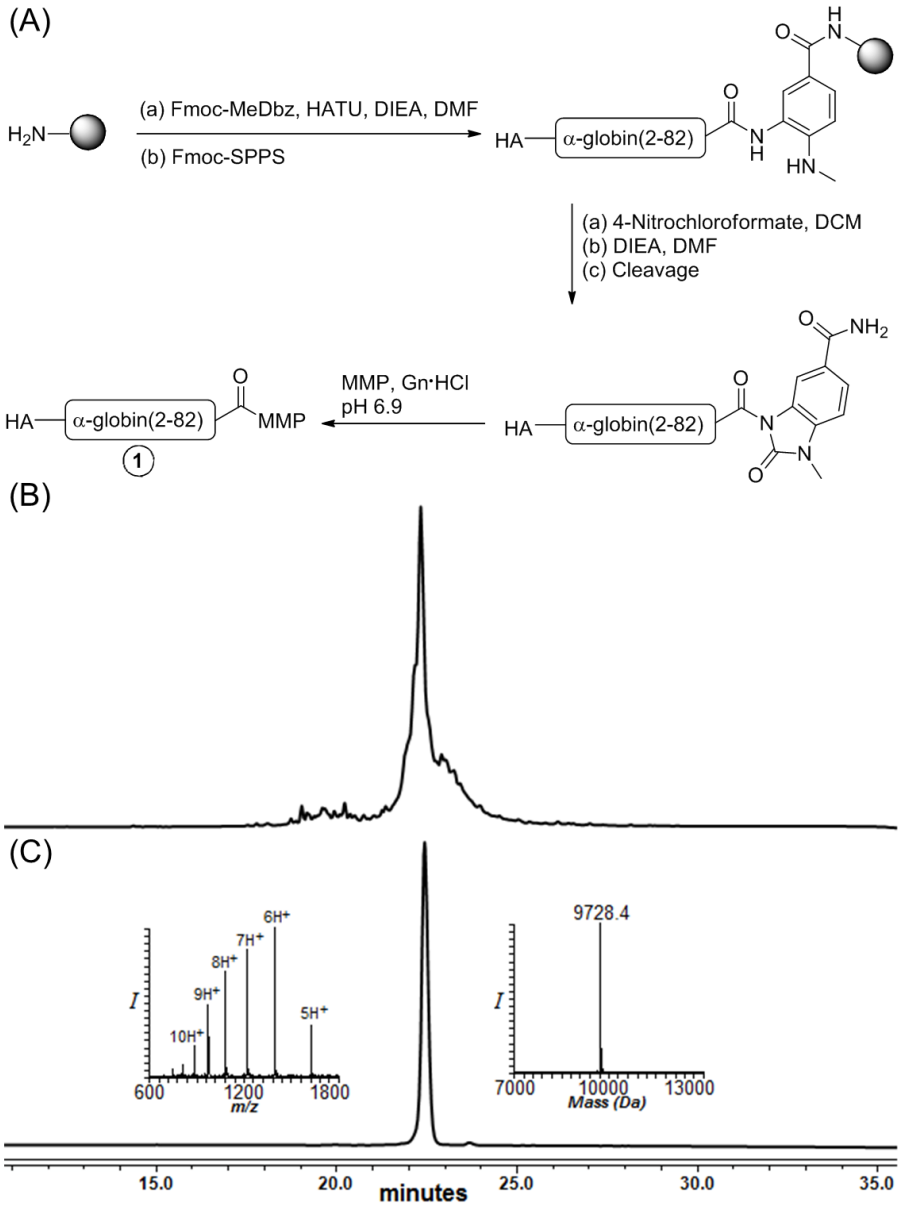

Fig. S1. Synthesis of HA-α-globin(2-82), Fragment **1**: (*A*) Synthesis was carried out on Knorr Resin (0.26 mmol/g, 0.2 mmol scale). Fmoc-MeDbz (4 equiv.) was coupled to the resin using HATU (4 equiv.), DIEA (8 equiv.) for 90 min. The Fmoc

was then deprotected by treatment of the resin with 20% piperidine for three cycles of 3, 5, and 3 min each. The C-terminal residue of Fragment **1** (Ser82) was coupled using HCTU (4 equiv.) and DIEA (8 equiv.) for 45 min (2 cycles). The remaining amino acids were coupled by standard Fmoc-SPPS on the synthesizer. After completion of SPPS, the peptide on the resin (0.1 mmol) was treated with p-nitrophenyl chloroformate (100 mg, 5 equiv.) in 4 ml dry DCM for 30 min (3 cycles), and was washed with DCM and DMF, followed by treatment in 4 ml of 0.5 M DIEA in DMF for 10 min (3 cycles). After cleaving the peptide from the resin, 25 mg of crude peptide were dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 6.9; 1.5 ml) containing MMP (200 equiv., 56 µl), and incubated at 37ºC for 1 h. Progress was monitored by analytical HPLC with a gradient of 0-60% buffer B (buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile) for 30 min. The reaction mixture was purified using a preparative column with the same gradient of the buffer system to obtain Fragment **1** (245 mg, 12.6% yield from 0.2 mmol). (*B*) HPLC of crude HA-α-globin(2-82)-MeDbz. (*C*) HPLC and mass analysis of purified Fragment **1** with observed mass of 9728.4 ± 0.7 Da (calculated 9729.8 Da).

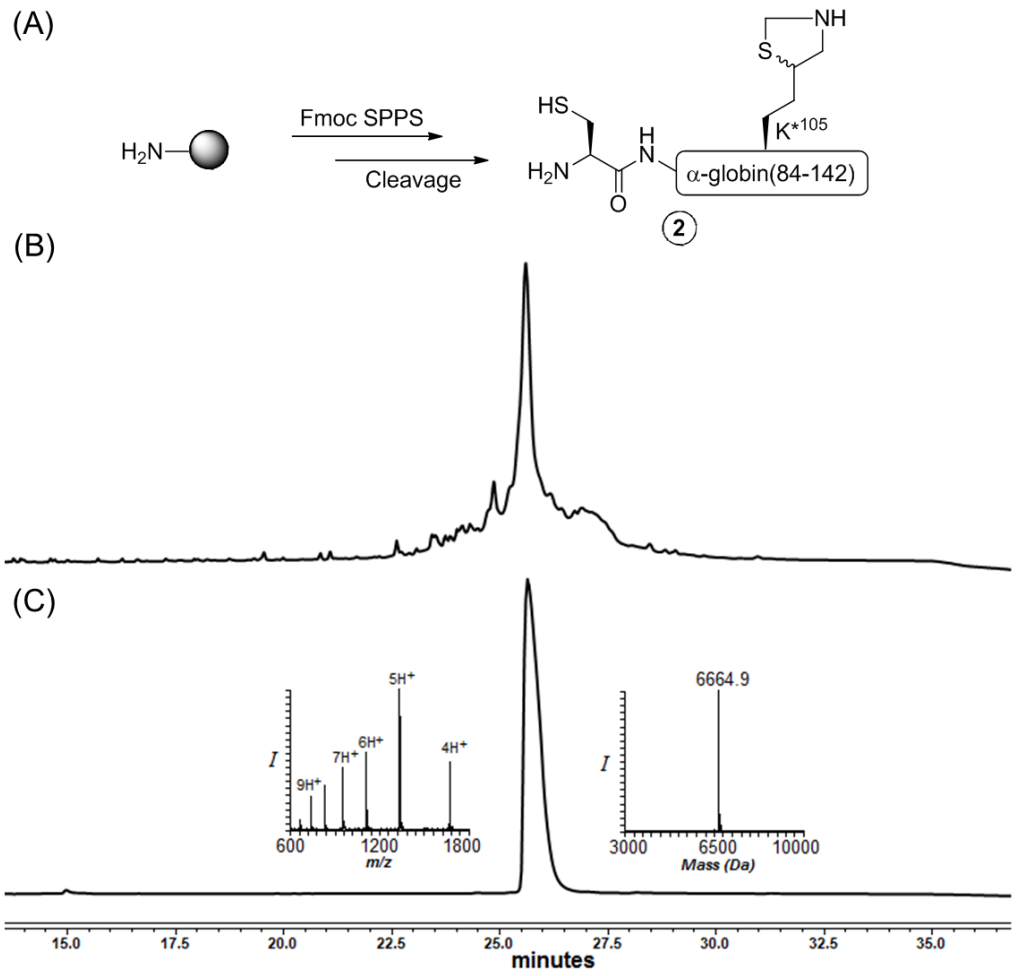

Fig. S2. Synthesis of Cys-α-globin(K*[105])(84-142), Fragment **2**: (*A*) Synthesis was carried out on Wang Resin (0.44 mmol/g, 0.2 mmol scale). The C-terminal residue (Arg142) was coupled using Fmoc-Arg(Pbf; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-OH (10 equiv.), HCTU (10 equiv.) and DIEA (20 equiv.) for 50 min (2 cycles). The Thz-protected δ-mercaptolysine (1.5 equiv.) was coupled at position 105 of α-globin (instead of Cys) using HBTU (1.5 equiv.), HOBT (1.5 equiv.) and DIEA (3 equiv.) for 90 min. The remaining amino acids were coupled by standard Fmoc-SPPS on the synthesizer to complete the peptide synthesis. The reaction was monitored and product purified as described in Fig. S1 to obtain Fragment **2** (205 mg, 15.4 % yield from 0.2 mmol). (*B*) HPLC of crude Fragment **2**. (*C*) HPLC and mass analysis of purified Fragment **2** with observed mass of 6664.9 ± 0.6 Da (calculated 6665.8 Da).

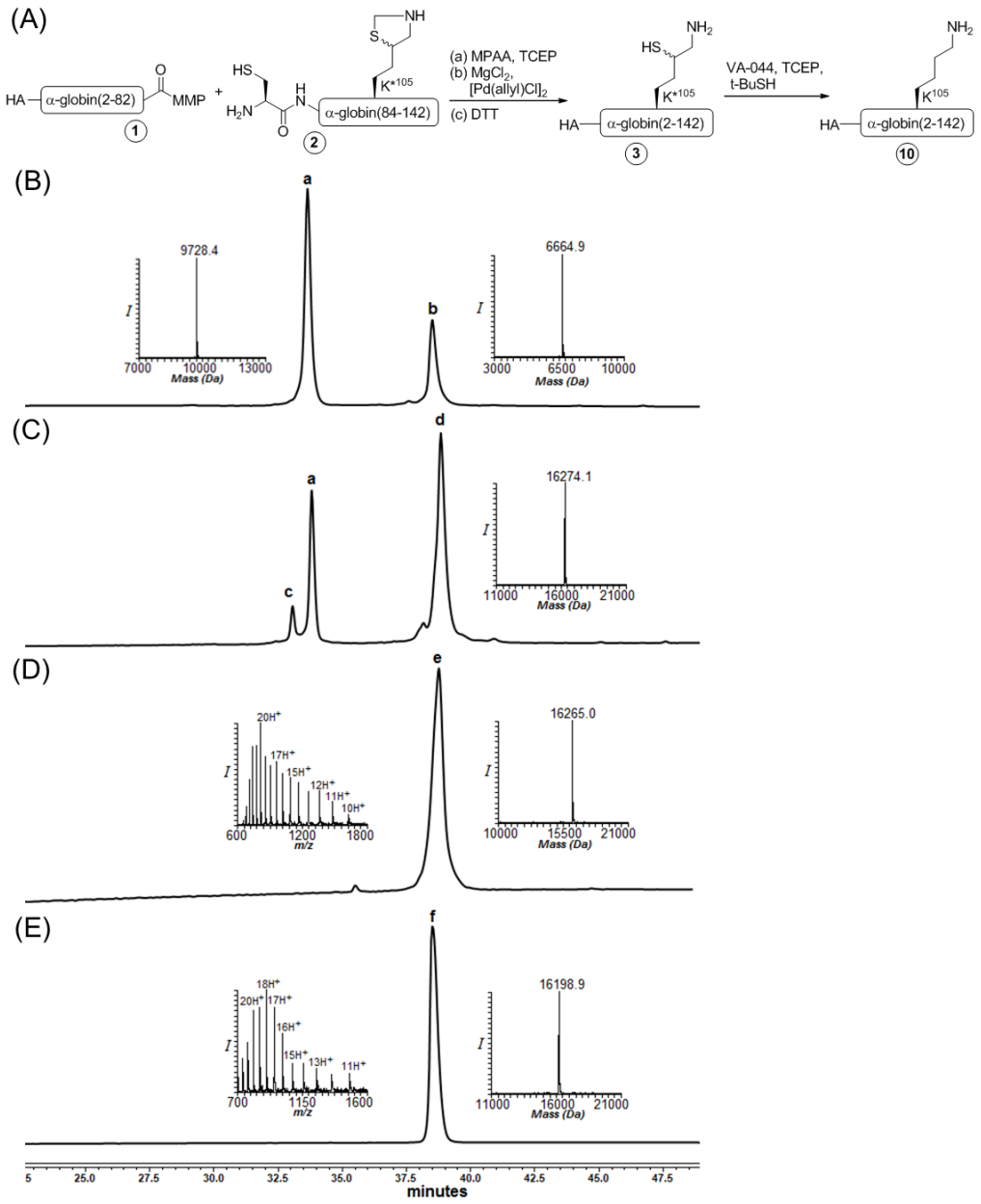

Fig. S3. Synthesis of HA-α-globin(K*^{105})(2-142), intermediate **3**, and HA-α-globin(K^{105})(2-142), construct **10**: (*A*) Synthesis of intermediate **3** was carried out in a similar manner as described for intermediate **5** in Fig. S12. The reaction was monitored and product purified using a gradient of 20-55% buffer B for 33 min to obtain intermediate **3** with 38% yield. HA-α-globin(K*^{105})(2-142), intermediate **3**, (4.9 mg, 3×10^{-4} mmol) was dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 7.3; 300 µl) and subjected to desulfurization by treatment with TCEP (23.6 mg, 0.25 mM), VA-044 (11.6 mg, 120 equiv.) and t-BuSH (33 µl) at 37ºC for 4 h. The

reaction was monitored and product purified as described for intermediate **3** to obtain HA-α-globin(K^{105})(2-142), construct **10** (3.0 mg, 61 % yield). (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to HA-α-globin(2-82)-MMP, Fragment **1**, peak b corresponds to Cys-α-globin(84-142), Fragment **2**. (*C*) HPLC of the ligation mixture after 1 h: Peak c corresponds to hydrolysis of HA-α-globin(2-82)-MPAA, peak d corresponds to the ligation product with observed mass of 16274.1 ± 0.3 Da (calculated 16275.5 Da). (*D*) HPLC of purified intermediate **3**: Peak e with observed mass of 16265.0 ± 0.8 Da (calculated 16263.5 Da). (*E*) HPLC and mass analysis of purified construct **10**: Peak f with observed mass of 16198.9 ± 0.6 Da (calculated 16199.5 Da).

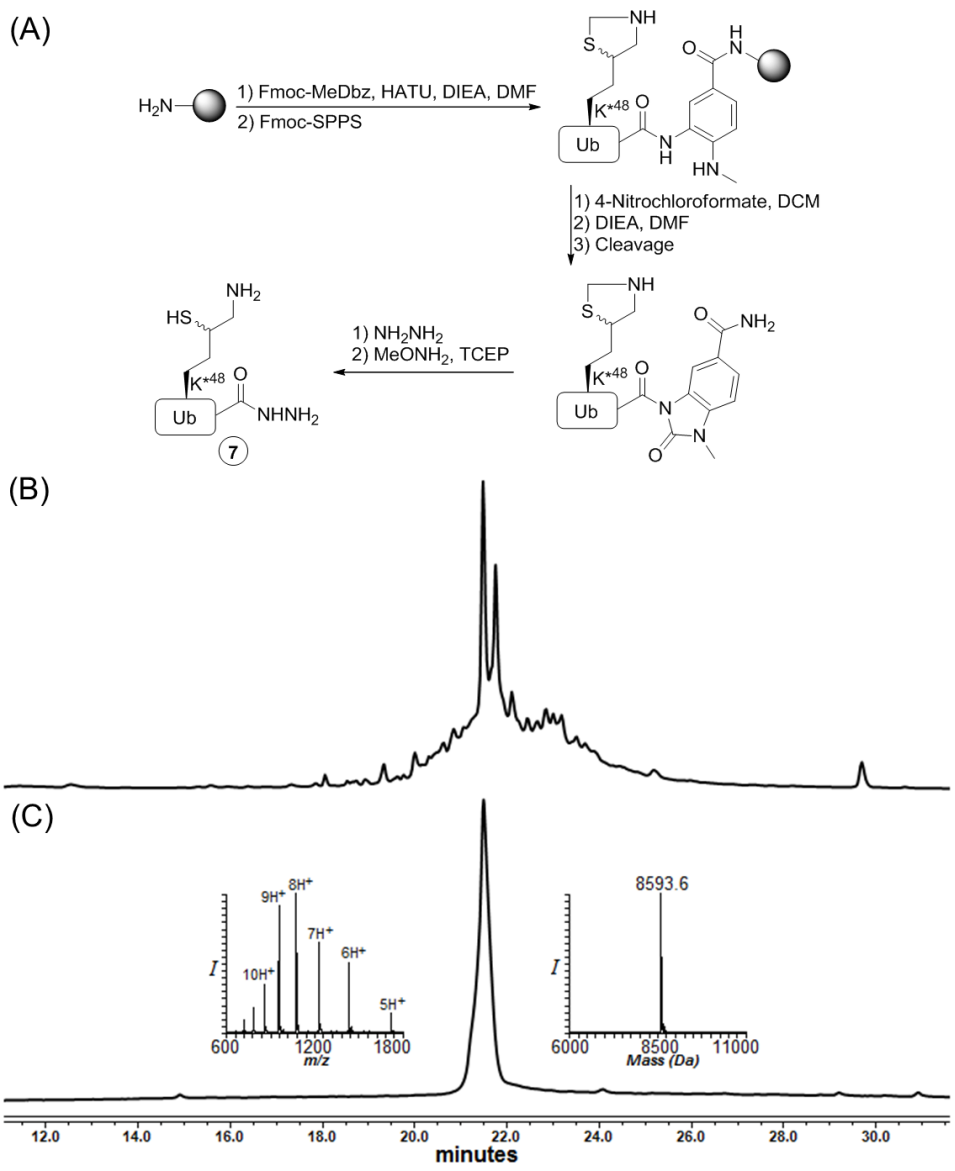

Fig. S4. Synthesis of Ub(K*[48])-$NHNH_2$, intermediate **7**: (*A*) Synthesis was carried out on Knorr Resin (0.26 mmol/g, 0.2 mmol scale). Fmoc-MeDbz (4 equiv.) was coupled to the resin using HATU (4 equiv.) and DIEA (8 equiv.) for 90 min. Thz-protected δ-mercaptolysine (1.5 equiv.) at position K^{48} of Ub was coupled using HBTU (1.5 equiv.), HOBT (1.5 equiv.) and DIEA (3 equiv.) for 90 min. The remaining amino acids were coupled by standard Fmoc-SPPS on the synthesizer. After completion of SPPS, the peptide on the resin (0.1 mmol) was treated with p-nitrophenyl chloroformate (100 mg, 5 equiv.) in 4 ml dry DCM for 30 min (3 cycles), and was washed with DCM and DMF, followed by treatment in 4 ml of 0.5 M DIEA in DMF for 10 min (3 cycles). After cleaving the peptide from the resin, 25 mg of

crude peptide was dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 7.2; 1.5 ml) containing hydrazine monohydrate (150 equiv., 21 µl). The reaction mixture was kept at room temperature for 1 h, followed by the addition of TCEP (10 mg) and $MeONH_2$·HCl (12 mg, 50 equiv.), and incubation at 37ºC for 3-4 h to unmask the Thz. The reaction was monitored and product purified as described under Fig. S1 to obtain Ub(K^{*48})-$NHNH_2$, intermediate **7** (280 mg, 16.3 % yield from 0.2 mmol). (*B*) HPLC of crude Ub(K^{*48})-MeDbz. (*C*) HPLC and mass analysis of purified intermediate **7** with observed mass of 8593.6 ± 0.3 Da (calculated 8592.8 Da).

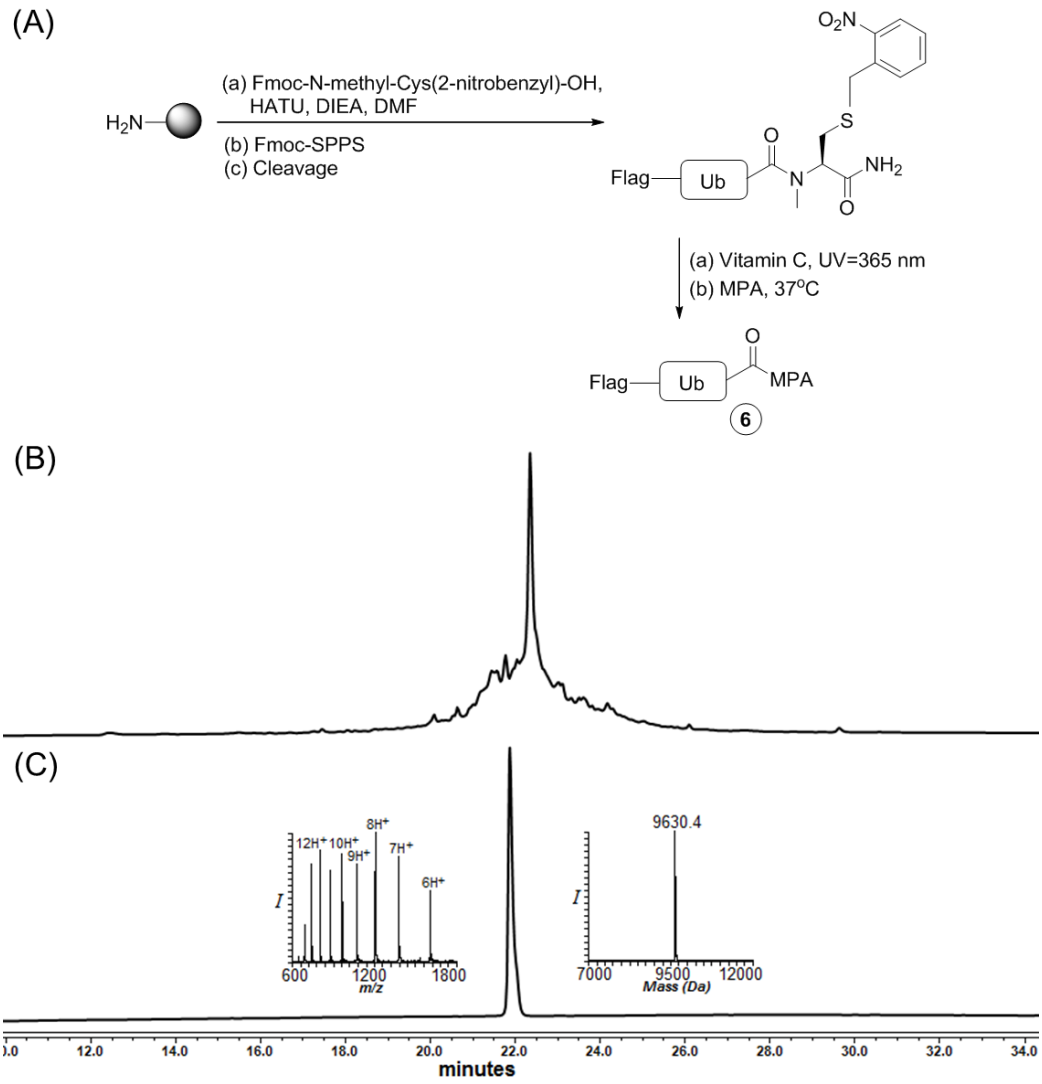

Fig. S5. Synthesis of Flag-Ub-MPA, intermediate **6**: (*A*) Synthesis was carried out as described for Ub-MPA (4), and purified to obtain intermediate **6** (272 mg, 14.1 % yield from 0.2 mmol). The reaction was monitored and product purified as described under Fig. S1. (*B*) HPLC of crude Flag-Ub-NmeCys. (*C*) HPLC and mass analysis of purified intermediate **6** with observed mass of 9630.4 ± 0.3 Da (calculated 9629.8 Da).

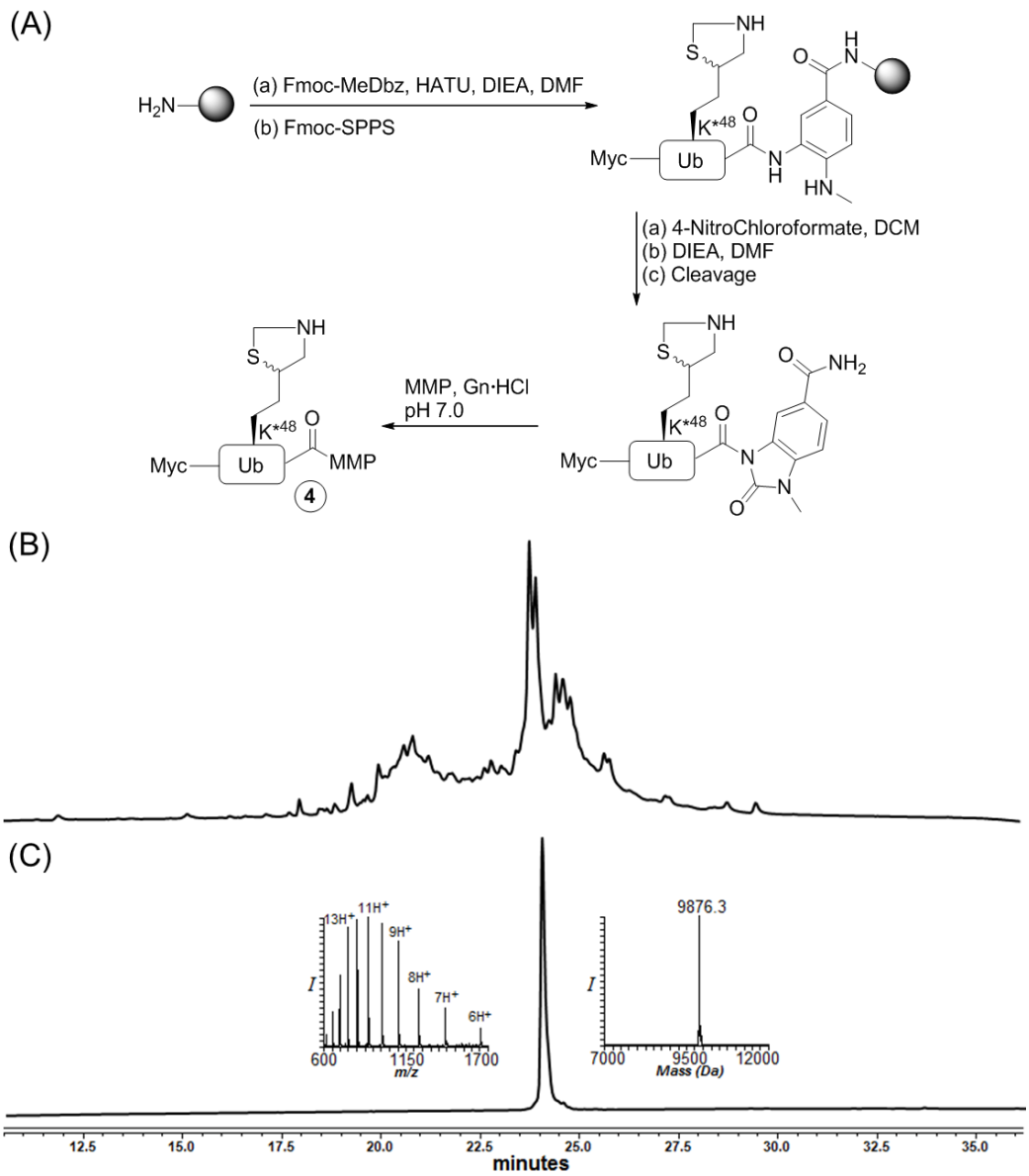

Fig. S6. Synthesis of Myc-Ub(K*[48])-MMP, intermediate **4**: (*A*) Synthesis was carried out as described under Fig. S4. Crude peptide (25 mg) was dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 7.0; 1.5 ml) containing MMP (200 equiv., 56 µl) and incubated at 37ºC for 1 h. The reaction was monitored and product purified as described under Fig. S1 to obtain Myc-Ub(K*[48])-MMP, intermediate **4** (253 mg, 12.8 % yield from 0.2 mmol). (*B*) HPLC of crude Myc-Ub(K*[48])-MeDbz. (*C*) HPLC and mass analysis of purified intermediate **4** with observed mass of 9876.3 ± 0.3 Da (calculated 9878.1 Da).

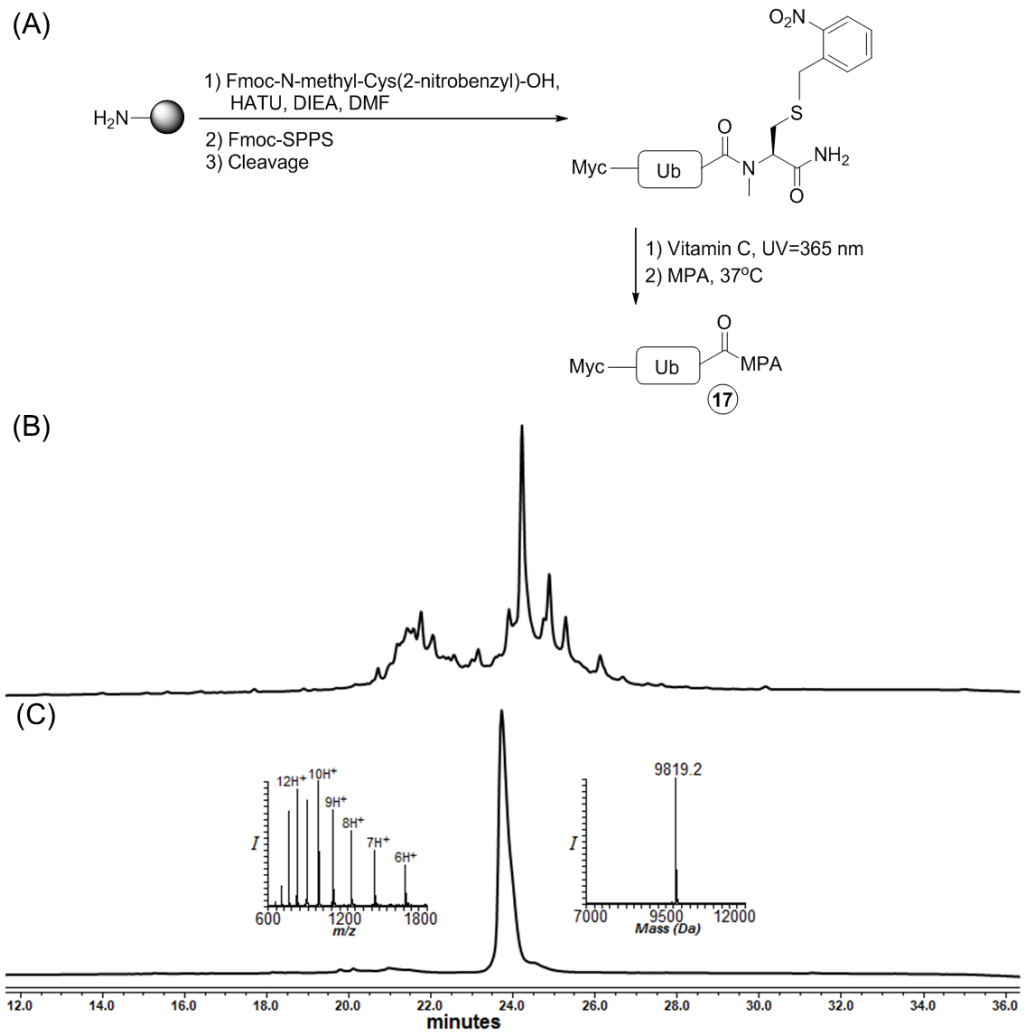

Fig. S7. Synthesis of Myc-Ub-MPA, intermediate **17**: (*A*) Synthesis, analysis, and purification of Myc-Ub-MPA were carried out as described for intermediate **6** under Fig. S5 to obtain intermediate **17** (271 mg,13.8 % yield from 0.2 mmol). (*B*) HPLC of crude Myc-Ub-NmeCys. (*C*) HPLC and mass analysis of purified intermediate **17** with observed mass of 9819.2 ± 0.3 Da (calculated d 9820.1 Da).

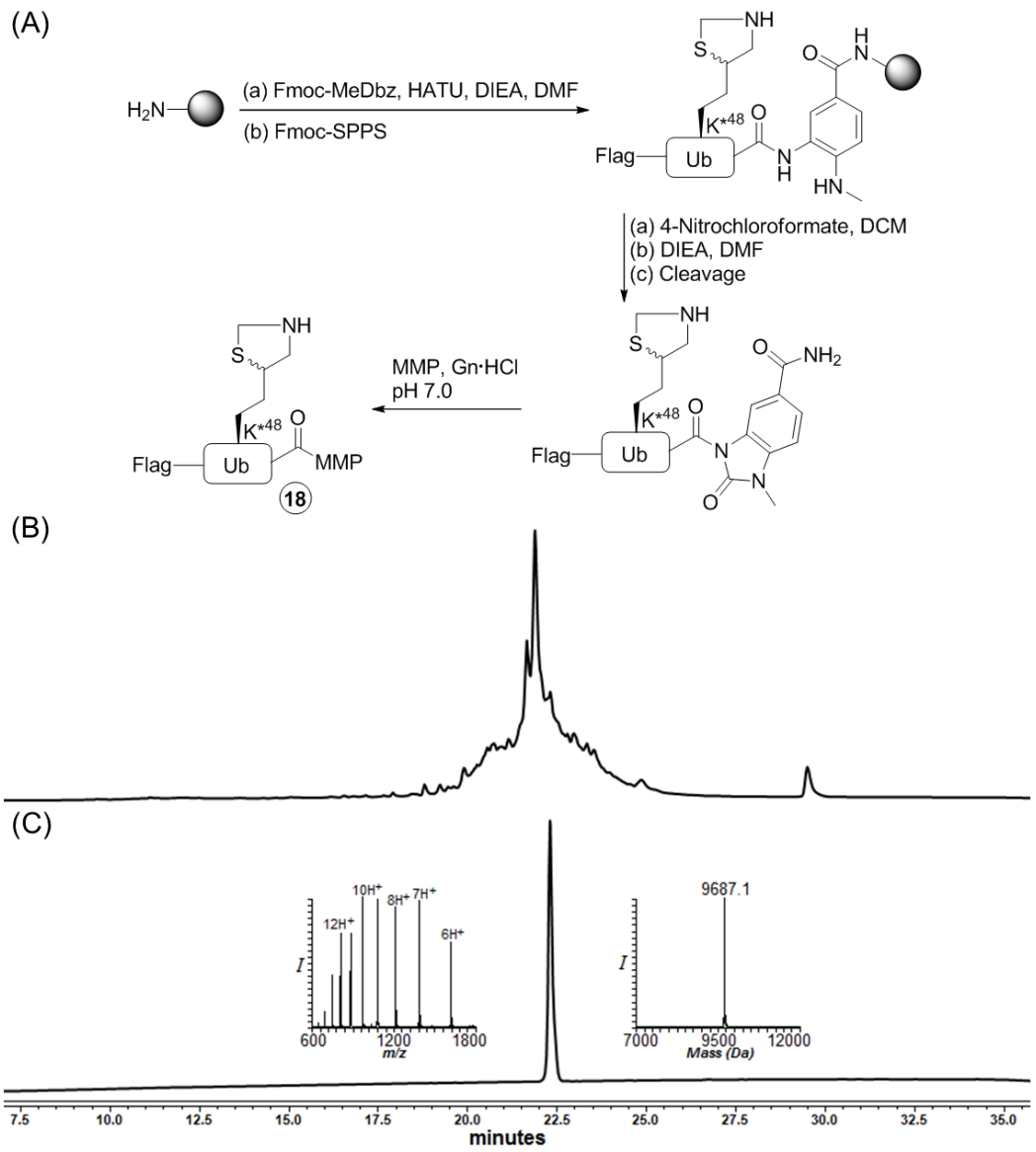

Fig. S8. Synthesis of Flag-Ub(K*[48])-MMP, intermediate **18**: (*A*) Synthesis, analysis, and purification of Flag-Ub(K*[48])-MMP were carried out as described for intermediate **4** under Fig. S6 to obtain intermediate **18** (262 mg, 13.5% yield from 0.2 mmol). (*B*) HPLC of crude Flag-Ub(K*[48])-MeDbz. (*C*) HPLC and mass analysis of purified intermediate **18** with observed mass of 9687.1 ± 0.3 Da (calculated 9687.8 Da).

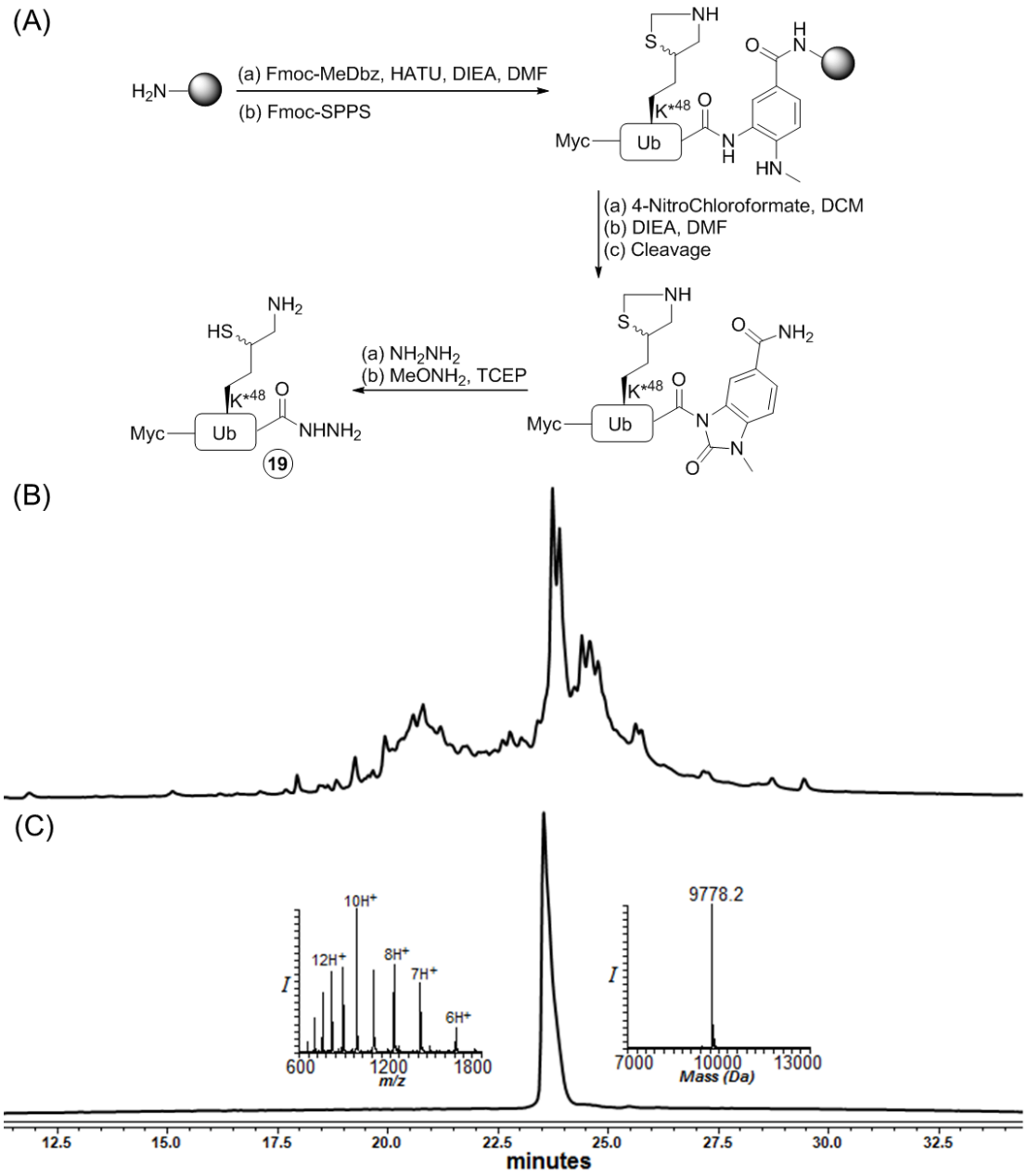

Fig. S9. Synthesis of Myc-Ub(K*[48])-$NHNH_2$, **19**: (*A*) Synthesis, analysis, and purification of Myc-Ub(K*[48])-$NHNH_2$ were carried out as described for intermediate **7** under Fig. S4 to obtain intermediate **19** (237 mg,12.1 % yield from 0.2 mmol). (*B*) HPLC of crude Myc-Ub(K*[48])-MeDbz. (*C*) HPLC and mass analysis of purified intermediate **19** with observed mass of 9778.2 ± 0.6 Da (calculated 9778.1 Da).

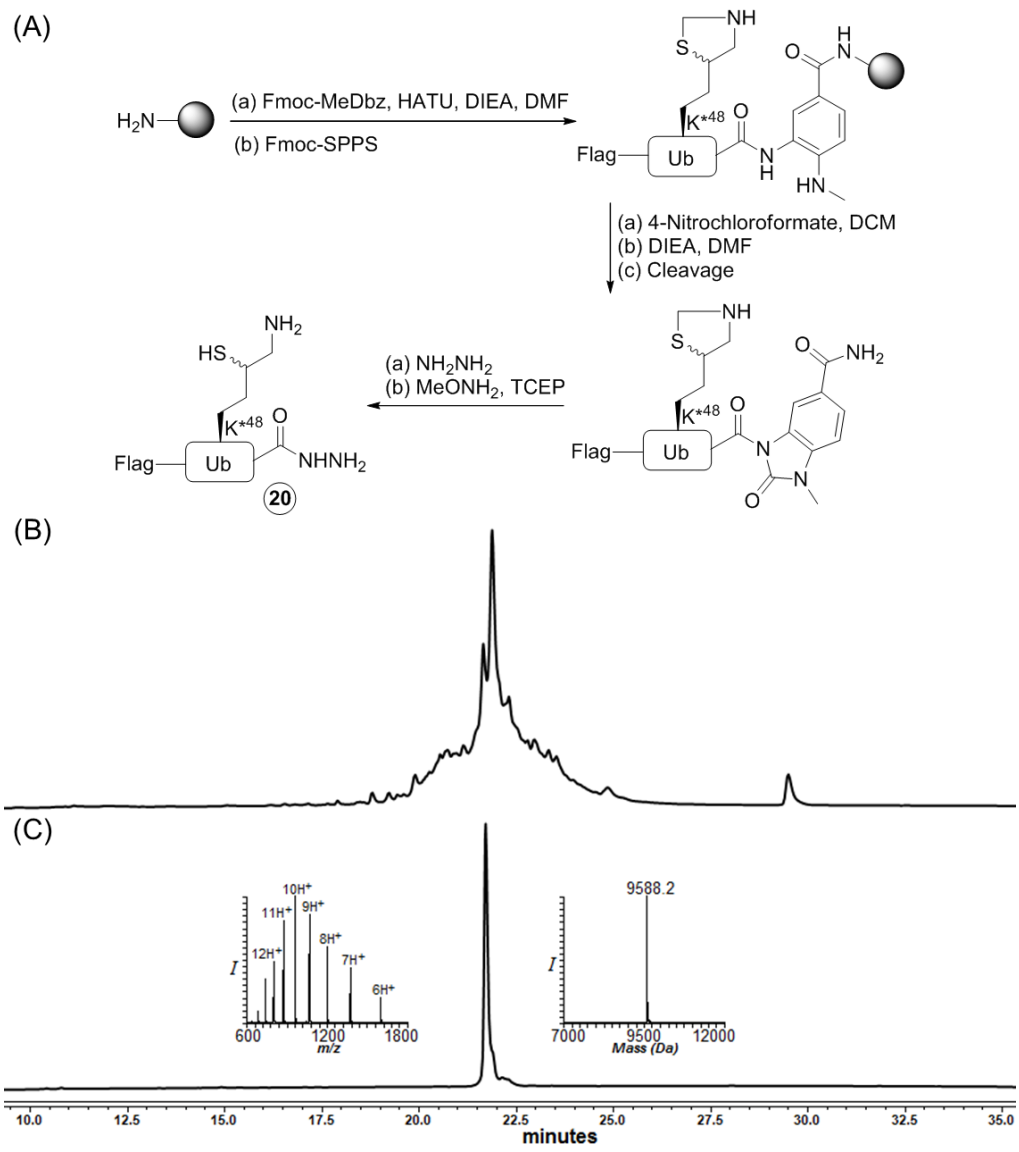

Fig. S10. Synthesis of Flag-Ub(K*[48])-$NHNH_2$, intermediate **20**: (*A*) Synthesis, analysis, and purification of Synthesis of Flag-Ub(K*[48])-$NHNH_2$ were carried out as described for intermediate **7** under Fig. S4 to obtain intermediate **20** (249 mg, 13.0% yield from 0.2 mmol). (*B*) HPLC of crude Flag-Ub(K*[48])-MeDbz. (*C*) HPLC and mass analysis of purified intermediate **20** with observed mass of 9588.2 ± 0.4 Da (calculated 9587.8 Da).

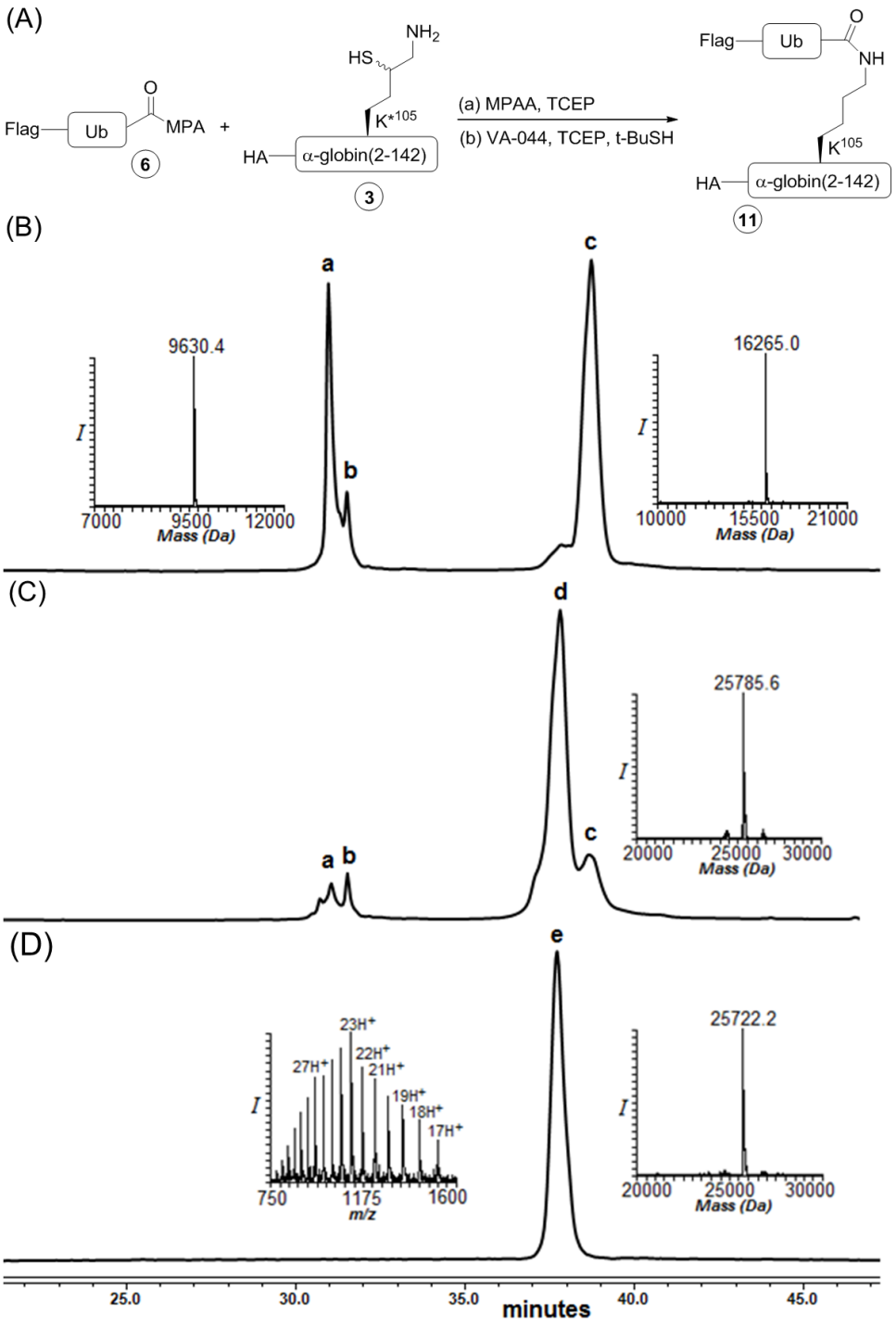

Fig. S11. Synthesis of Flag-Ub-HA-α-globin(K^{105})(2-142), construct **11**: (*A*) Flag-Ub-MPA, intermediate **6**, (3.5 mg, 3.6×10^{-4} mmol) and HA-α-globin(K^{*105})(2-142), intermediate **3**, (4.9 mg, 3.0×10^{-4} mmol) were dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 7.0; 150µl) containing MPAA (100 mM) and TCEP (50 mM). After incubation at 37ºC for 4 h, the reaction mixture was dialyzed using a Slide-A-

Lyzer 3.5K dialysis cassette (Thermo, 0.1-0.5 ml) against 6 M Gn·HCl/200 mM phosphate buffer (pH 7.3; 500 ml) for 12 h. The mixture (300 µl) was subjected to desulfurization by treatment with TCEP (23.5 mg, 0.25 mM), VA-044 (11.6 mg, 120 equiv.), and t-BuSH (33 µl) at 37ºC for 4 h. The reactions were monitored and product purified by HPLC with a gradient of 20-55% buffer B for 33 min to obtain Flag-Ub-HA-α-globin(K^{105})(2-142), construct **11**, (2.8 mg, 37 % yield). (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Flag-Ub-MPA, intermediate **6**, peak b corresponds to Flag-Ub-MPAA, and peak c corresponds to HA-α-globin(K^{*105})(2-142), intermediate **3**. (*C*) HPLC of the ligation mixture after 2 h: Peak d corresponds to the ligation product with observed mass of 25785.6 ± 1.1 Da (calculated 25787.2 Da). (*D*) HPLC and mass analysis of purified construct **11**: Peak e with observed mass of 25722.2 ± 0.7 Da (calculated 25723.2 Da).

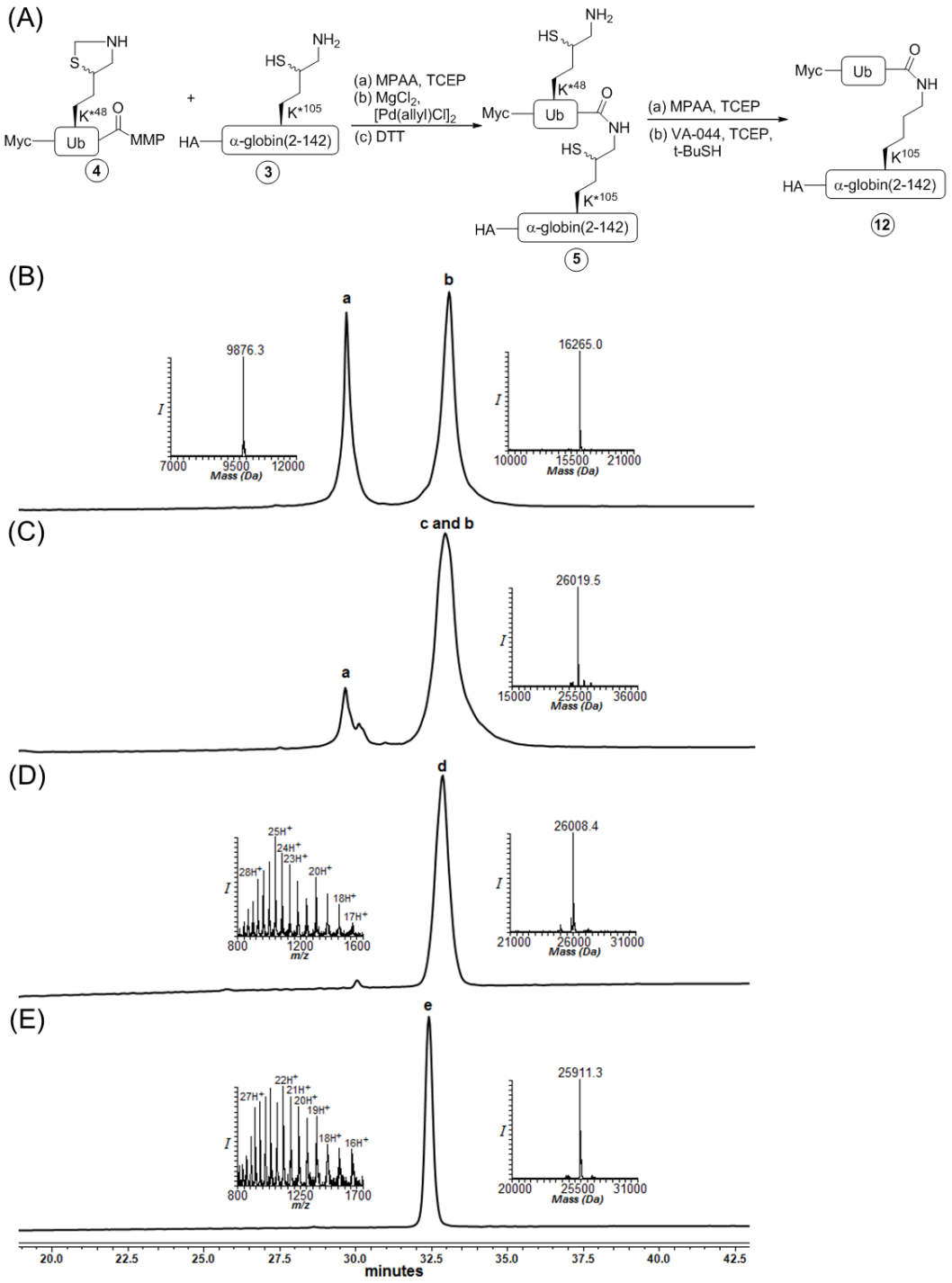

Fig. S12. Synthesis of Myc-Ub(K*^{48})-HA-α-globin(K*^{105})(2-142), intermediate **5**, and Myc-Ub-HA-α-globin(K^{105})(2-142), construct **12**: (*A*) Myc-Ub(K*^{48})-MMP, intermediate **4**, (11.9 mg, 1.2×10^{-3} mmol) and HA-α-globin(K*^{105})(2-142), intermediate **3**, (16.3 mg, 1×10^{-3} mmol) were dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 7.0; 500 µl) containing MPAA (60 mM) and TCEP (30 mM).

After incubation at 37ºC for 4 h, $MgCl_2$ (50 equiv., 4.8 mg) was added to the reaction mixture, that was further incubated for 10 min at 37ºC. After that, the reaction mixture was treated with 15 equiv. of $[Pd(allyl)Cl]_2$ (5.5 mg) and incubated at 37ºC for 1 h to unmask the Thz. The reaction was quenched by adding 60 equiv. of DTT, followed by centrifugation to pellet the solid material, and the supernatant was further analyzed. The progress of the reaction was monitored and the product purified using HPLC with a gradient of 15-55% buffer B for 30 min to obtain intermediate **5** (10.7 mg, 41 % yield). Synthesis of construct **12** was carried out as described for construct **10** under Fig. S3. The reactions were monitored and products purified by HPLC with a gradient of 15-55% buffer B for 30 min. Construct **12** was folded in 50 mM Tris/150 mM NaCl (pH 7.4), and further purified by FPLC. (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Myc-Ub(K^{*48})-MMP, intermediate **4**, and peak b corresponds to HA-α-globin(K^{*105})(2-142), intermediate **3**. (*C*) HPLC of the ligation mixture after 2 h: Peak c corresponds to the ligation product with observed mass of 26019.5 ± 1.0 Da (calculated 26021.6 Da). (*D*) HPLC of purified intermediate **5**: Peak d with observed mass of 26008.4 ± 0.8 Da (calculated 26009.6 Da). (*E*) HPLC and mass analysis of purified construct **12**: Peak e with observed mass of 25911.3 ± 0.9 Da (calculated 25913.6 Da).

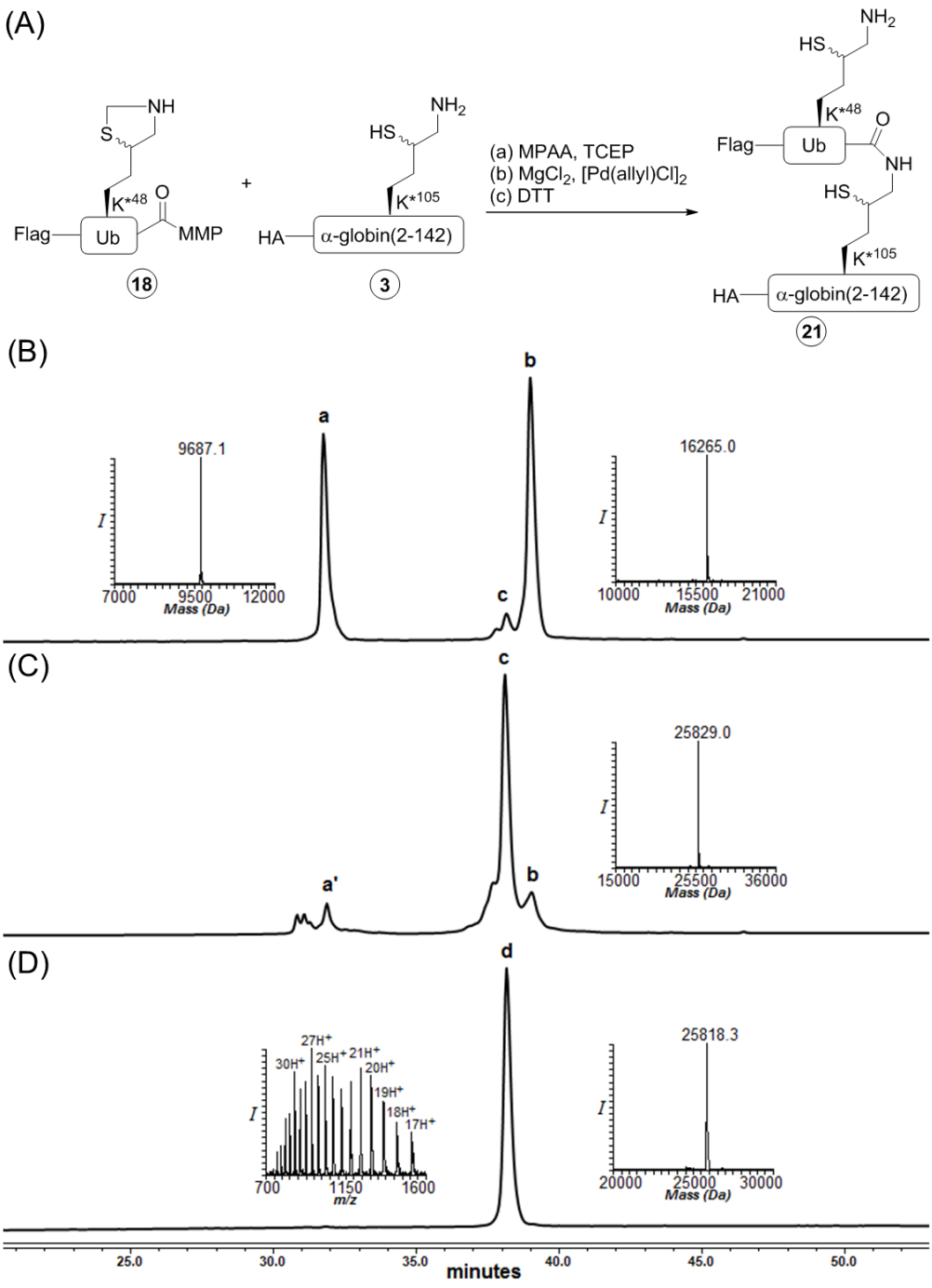

Fig. S13. Synthesis of Flag-Ub(K*[48])-HA-α-globin(K*[105])(2-142), intermediate **21**: (*A*) Synthesis of Flag-Ub(K*[48])-HA-α-globin(K*[105])(2-142) was carried out by ligating intermediates **18** and **3** using a similar procedure as described for intermediate **5** under Fig. S12. The reaction was monitored and product purified by HPLC using a gradient of 20-55% buffer B for 33 min to obtain intermediate **21** with 42% yield. (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak

a corresponds to Flag-Ub(K*^{48})-MMP, intermediate **18**, peak b corresponds to HA-α-globin(K*^{105})(2-142), intermediate **3**. (*C*) HPLC of the ligation mixture after 3 h: Peak c corresponds to the ligation product with observed mass of 25829.0 ± 0.8 Da (calculated 25831.2 Da). (*D*) HPLC and mass analysis of purified intermediate **21**: Peak d with observed mass of 25818.3 ± 0.6 Da (calculated 25819.2 Da).

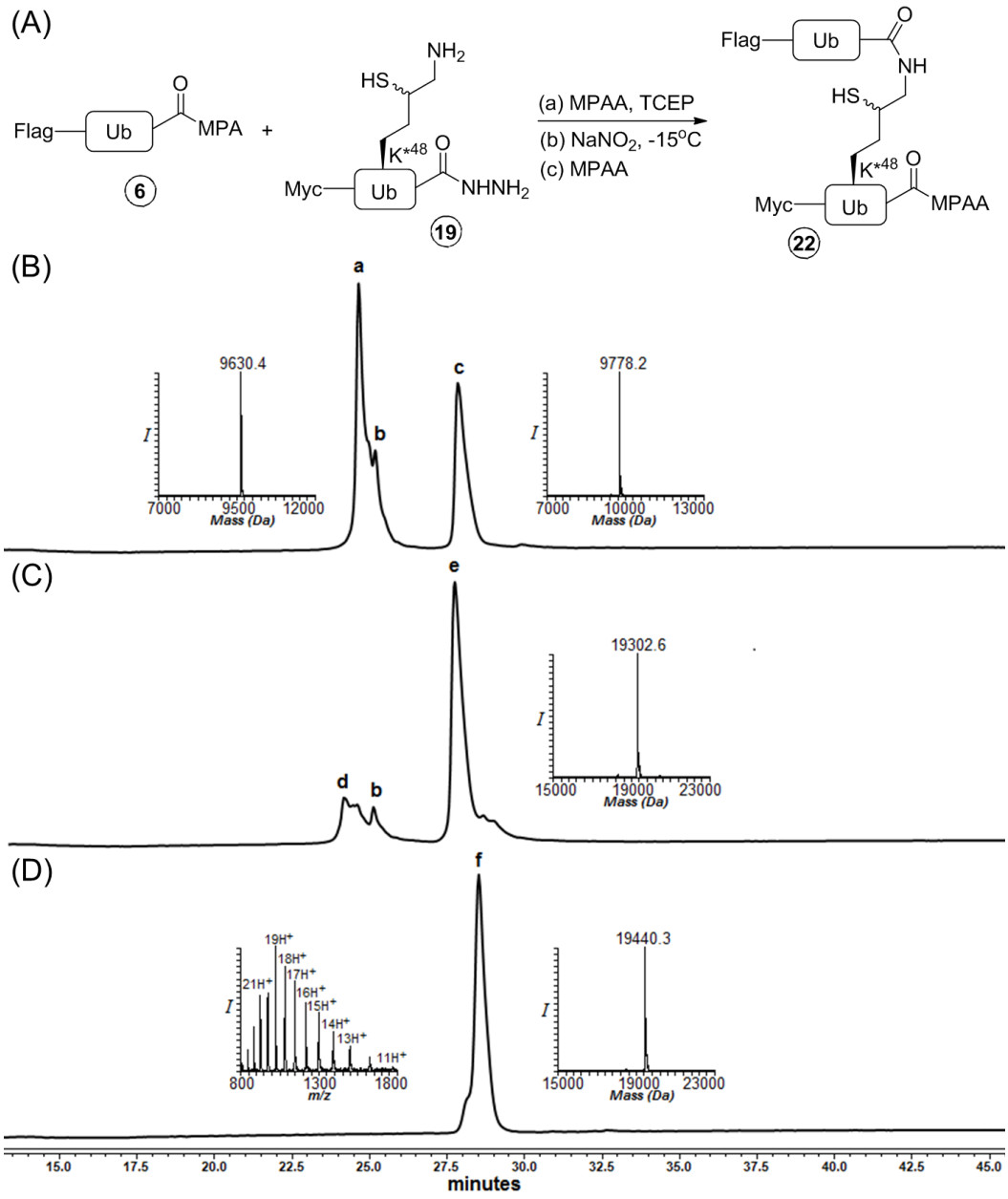

Fig. S14. Synthesis of Flag-Ub-Myc-Ub-MPAA, intermediate **22**: (*A*) Synthesis of Flag-Ub-Myc-Ub-MPAA was carried out by ligating intermediates **6** and **19** in a similar procedure as described for intermediate **8** under Fig. S18. The reaction was monitored and product purified by HPLC using a gradient of 25-65% buffer B for 40 min to obtain intermediate **22** with 38% yield. (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Flag-Ub-MPA, intermediate **6**, peak b corresponds to Flag-Ub-MPAA, and peak c corresponds to Myc-Ub-$NHNH_2$, intermediate **19**. (*C*) HPLC of the ligation mixture after 2 h: Peak d corresponds to

Flag-Ub-MPA hydrolysis product Flag-Ub-COOH, and peak e corresponds to the ligation product Flag-Ub-Myc-Ub-$NHNH_2$ (the precursor of intermediate **22**) with observed mass of 19302.6 ± 0.5 Da (calculated 19301.9 Da). (*D*) HPLC and mass analysis of purified intermediate **22**: Peak f with observed mass of 19440.3 ± 0.6 Da (calculated 19437.9 Da).

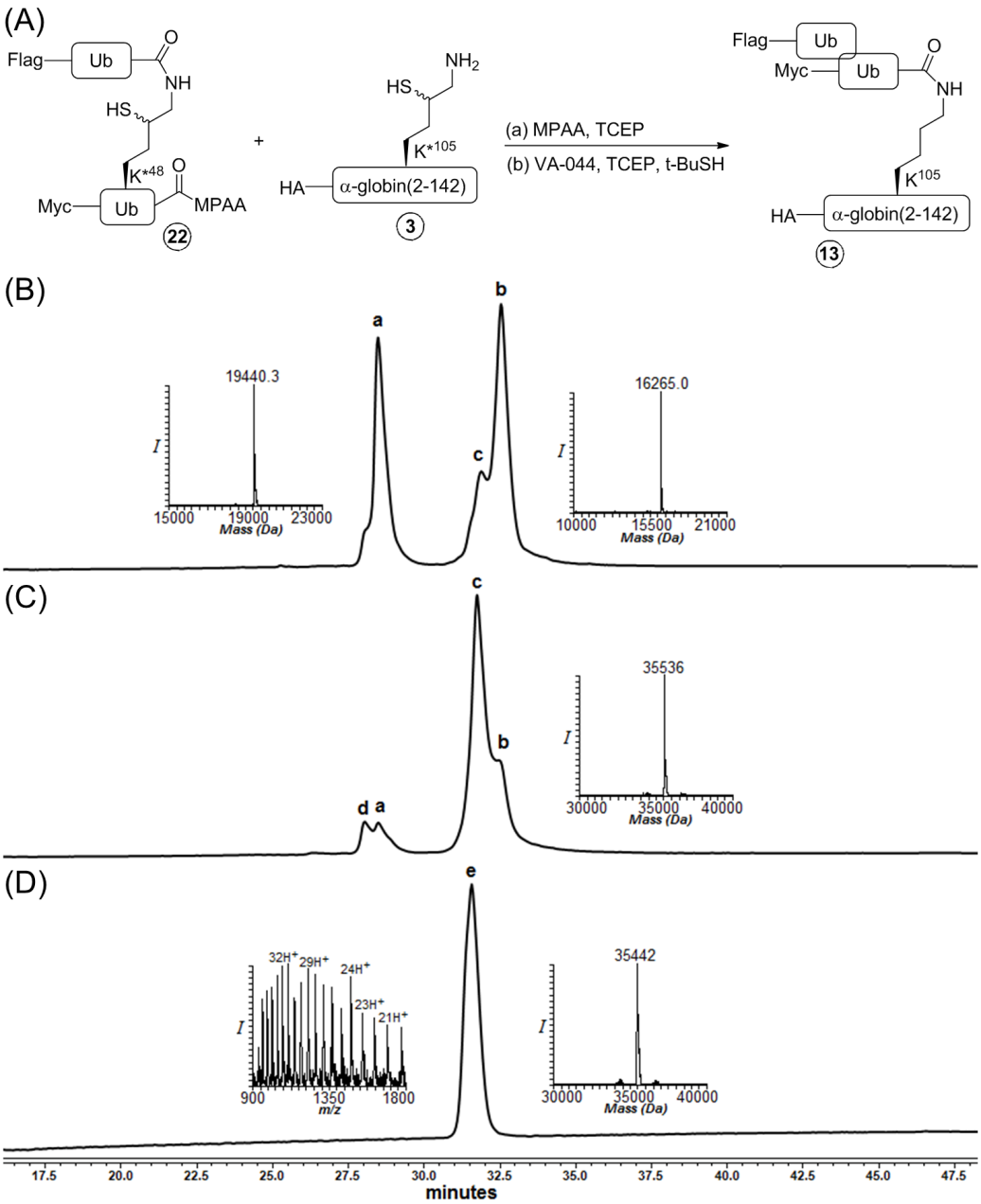

Fig. S15. Synthesis of Flag-Ub-Myc-Ub-HA-α-globin(K^{105})(2-142), construct **13**: (*A*) Synthesis of Flag-Ub-Myc-Ub-HA-α-globin(K^{105})(2-142) was carried out by ligating intermediates **22** and **3** in a similar procedure as described for construct **15** under Fig. S20. The reaction was monitored and product purified by HPLC using a gradient of 25-65% buffer B for 40 min to obtain construct **13** with 37% yield. (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Flag-Ub-Myc-Ub-MPAA, intermediate **22**, and peak b corresponds to HA-α-globin(K^{*105})(2-142), intermediate **3**. (*C*) HPLC of the ligation mixture after

1.5 h: Peak c corresponds to the ligation product with observed mass of 35536 ± 1.4 Da (calculated 35534 Da), and peak d corresponds to hydrolysis product of intermediate **22**. (*D*) HPLC and mass analysis of purified construct **13**: Peak e with observed mass of 35542 ± 1.5 Da (calculated 35438 Da).

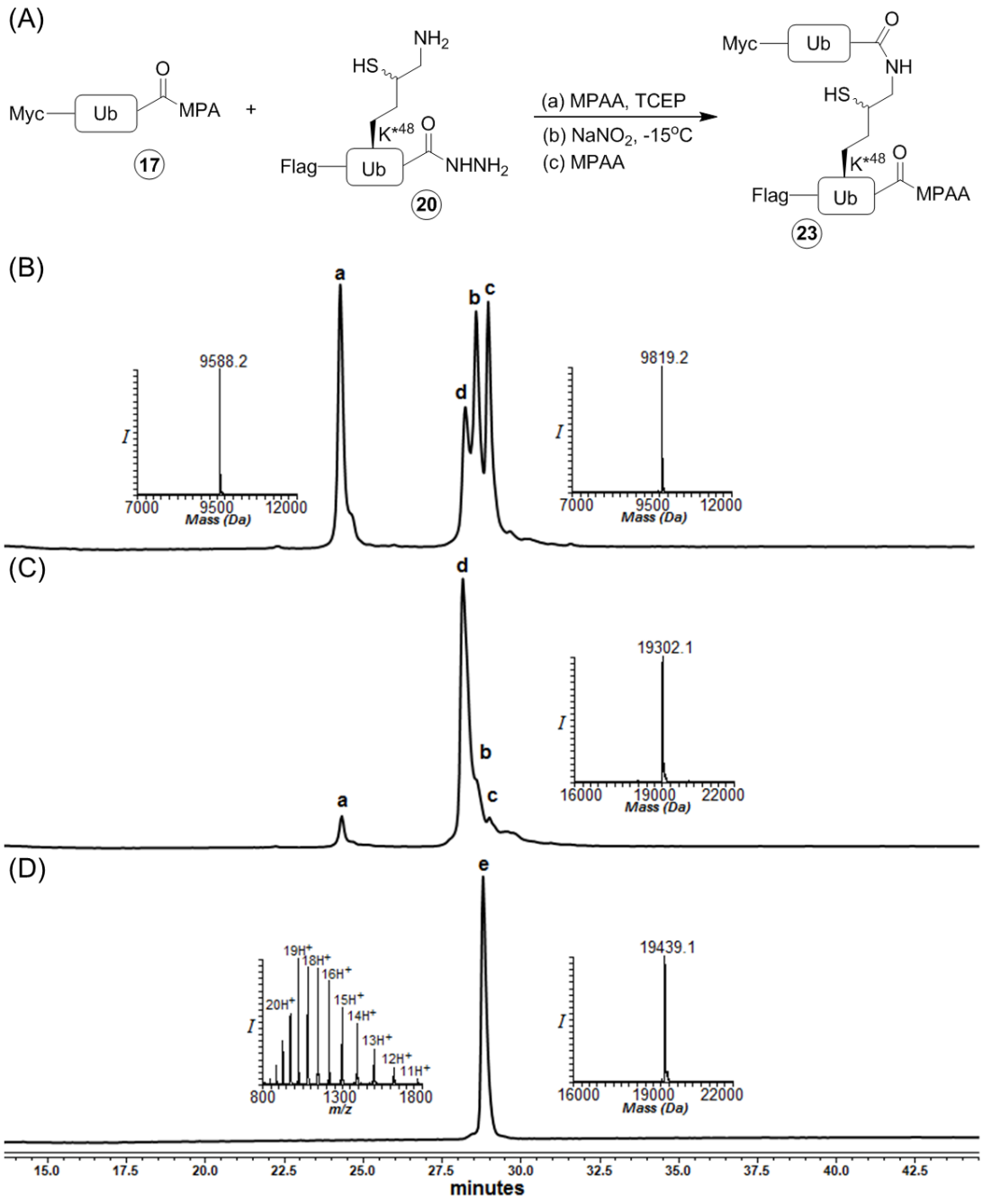

Fig. S16. Synthesis of Myc-Ub-Flag-Ub-MPAA, intermediate **23**: (*A*) Synthesis, analysis, and purification of Myc-Ub-Flag-Ub-MPAA were carried out by ligating intermediates **17** and **20** as described for intermediate **22** under Fig. S14 to obtain intermediate **23** with 38% yield. (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Flag-Ub(K*^{48})-$NHNH_2$, intermediate **20**, peak b corresponds to Myc-Ub-MPA, intermediate **17**, and peak c corresponds to Myc-Ub-MPAA. (*C*) HPLC of the ligation mixture after 80 min: Peak d corresponds to

the ligation product Myc-Ub-Flag-Ub-$NHNH_2$ (the precursor of intermediate **23**) with observed mass of 19302.1 ± 0.5 Da (calculated 19301.9 Da). (*D*) HPLC and mass analysis of purified intermediate **23**: Peak e with observed mass of 19439.1 ± 0.7 Da (calculated 19437.9 Da).

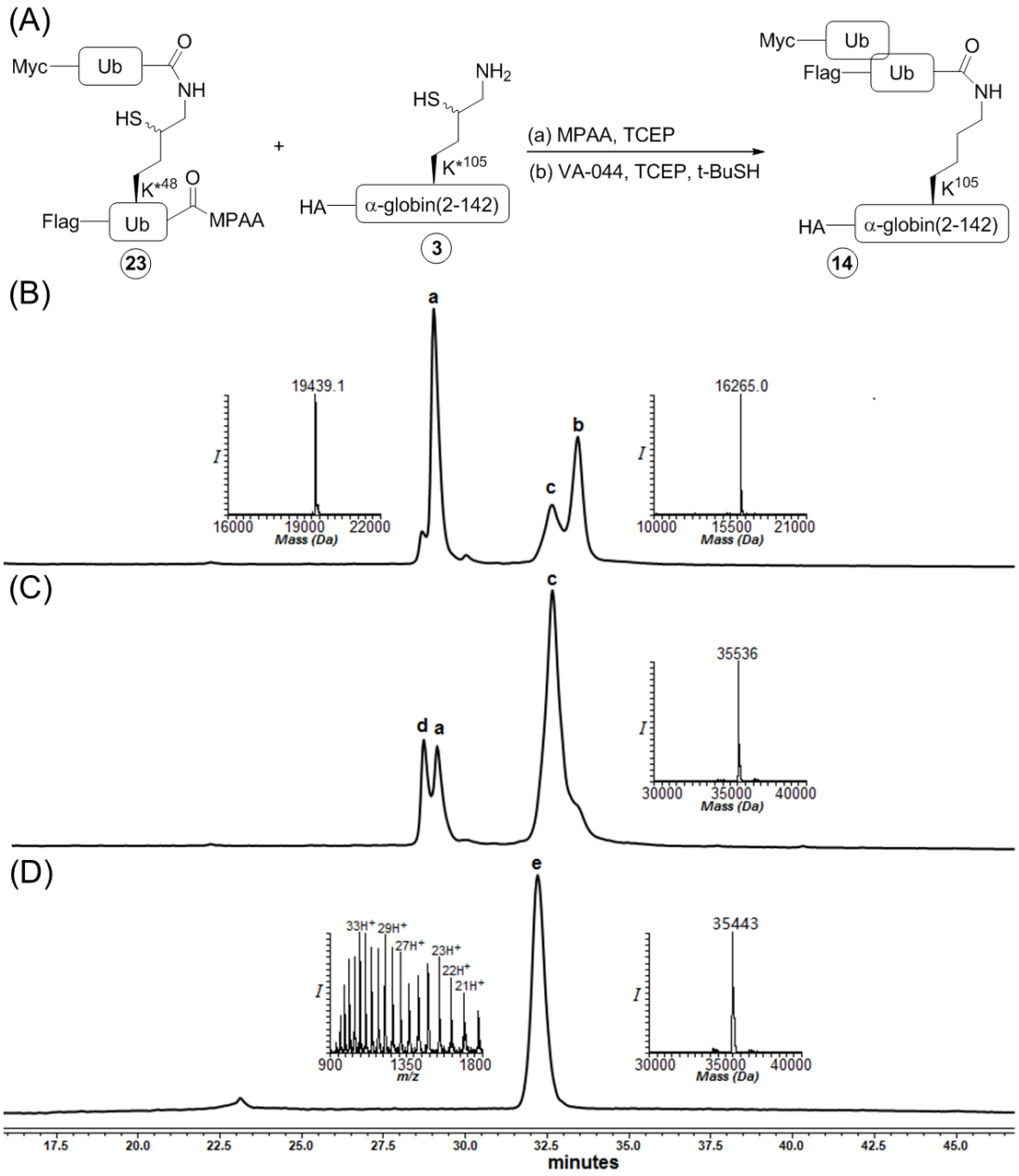

Fig. S17. Synthesis of Myc-Ub-Flag-Ub-HA-α-globin(K^{105})(2-142), construct **14**: (*A*) Synthesis, analysis and purification of Myc-Ub-Flag-Ub-HA-α-globin(K^{105})(2-142) were carried out by ligating intermediates **23** and **3** as described for construct **13** under Fig. S15 to obtain construct **14** with 37% yield. (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Myc-Ub-Flag-Ub-MPAA, intermediate **23**, and peak b corresponds to HA-α-globin(K^{*105})(2-142), intermediate **3**. (*C*) HPLC of the ligation mixture after 1.5 h: Peak c corresponds to the ligation product with observed mass of 35536 ± 1.6 Da (calculated 35534 Da),

and peak d corresponds to the hydrolysis product of the Myc-Ub-Flag-Ub-MPAA. (*D*) HPLC and mass analysis of purified construct **14**: Peak e with observed mass of 35543 ± 1.6 Da (calculated 35438 Da).

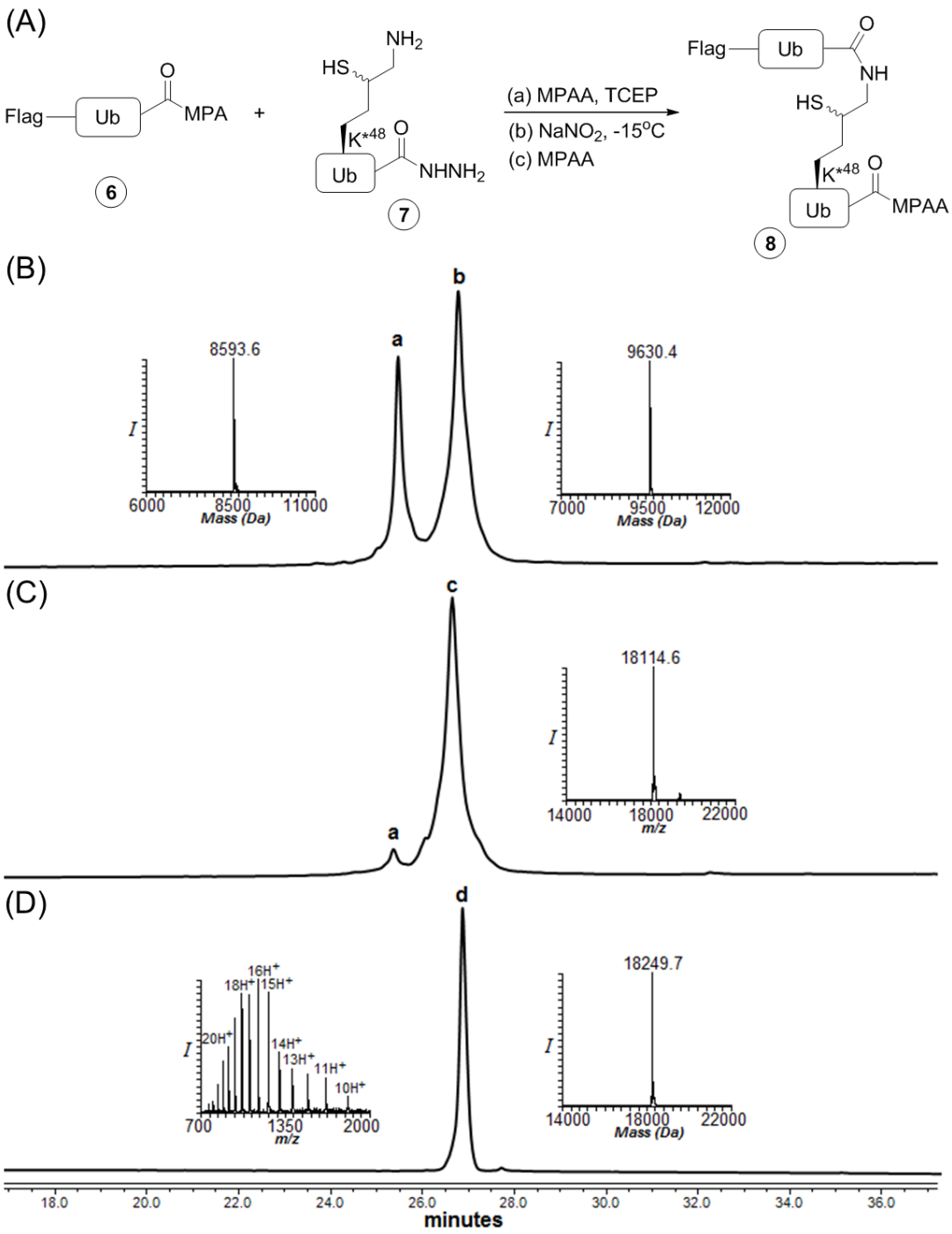

Fig. S18. Synthesis of Flag-Ub-Ub-MPAA, intermediate **8**: (*A*) Flag-Ub-MPA, intermediate **6**, (23.1 mg, 2.4×10^{-3} mmol) and Ub(K*[48])-$NHNH_2$, intermediate **7**, (17.2 mg, 2.0×10^{-3} mmol) were dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 7.0; 1000 µl) containing MPAA (100 mM) and TCEP (50 mM). After incubation at 37ºC for 4 h, the reaction mixture was dialyzed in a Slide-A-Lyzer 3.5K dialysis

cassette (Thermo, 3.0 ml) against 6 M Gn·HCl/200 mM phosphate buffer (pH 7.3; 500 ml) for 12 h. The mixture was adjusted to pH 3.0 and cooled to -15 ºC with salt ice bath for 20 min. $NaNO_2$ (15 equiv. dissolved in 10 µl H_2O) was added to the reaction mixture that was kept at -15 ºC for additional 20 min. Subsequently, MPAA (30 equiv.) dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 6.0; 200 µl) was added to the mixture. Following additional 5 min at room temperature and addition of TCEP (30 mg), the mixture was resolved using preparative HPLC. The reactions were monitored and product purified by HPLC with a gradient of 15-55% buffer B for 30 min to obtain Flag-Ub-Ub-MPAA, intermediate **8**, (13.9 mg, 38% yield). (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Ub(K^{*48})-$NHNH_2$, intermediate **7**, and peak b corresponds to Flag-Ub-MPA, intermediate **6**. (*C*) HPLC of the ligation mixture after 3 h: Peak c corresponds to the ligation product Flag-Ub-Ub-$NHNH_2$ (the precursor of intermediate **8**) with observed mass of 18114.6 ± 1.4 Da (calculated 18116.6 Da). (*D*) HPLC and mass analysis of purified intermediate **8**: Peak d with observed mass of 18249.7 ± 1.6 Da (calculated 18252.6 Da).

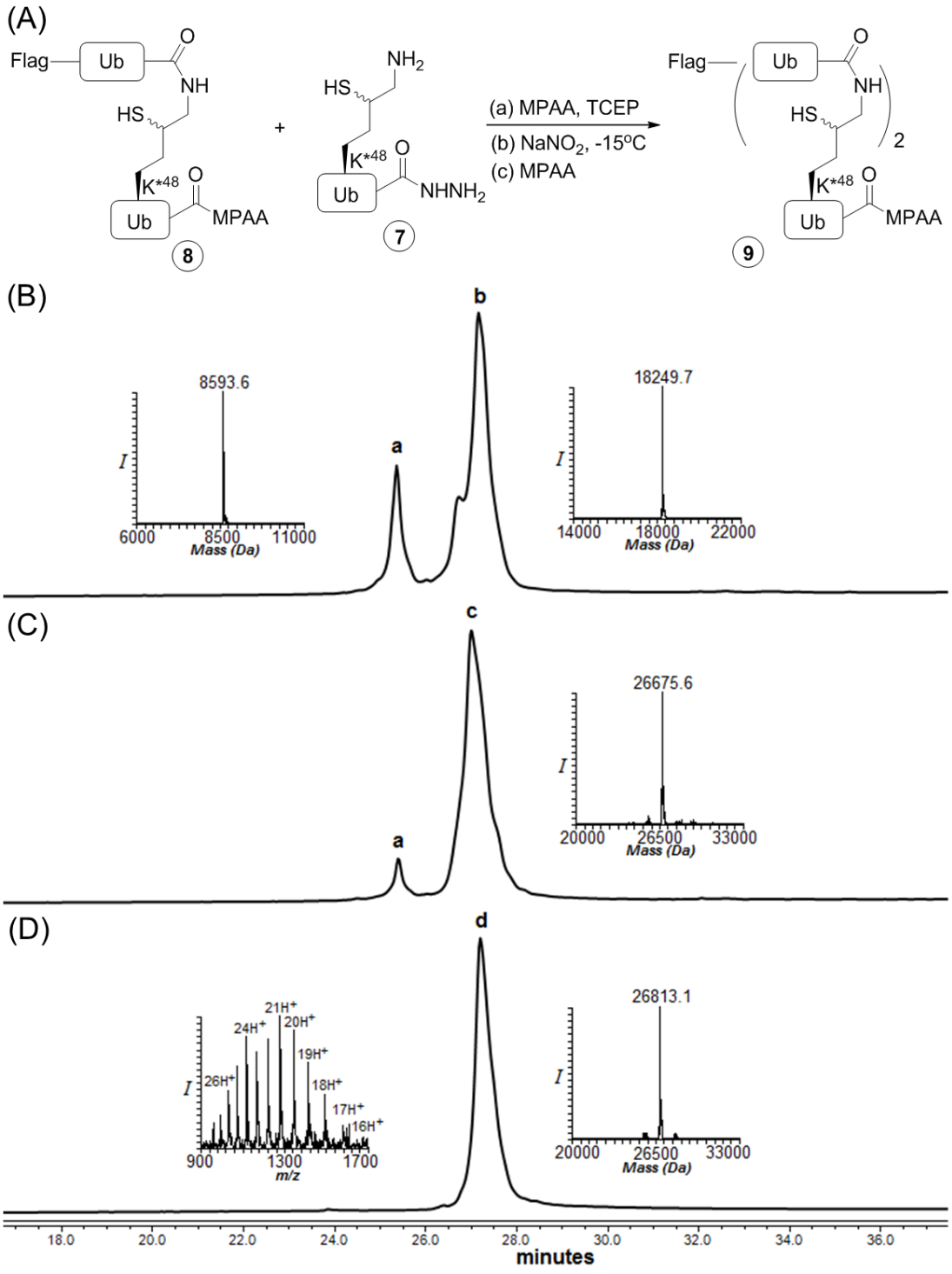

Fig. S19. Synthesis of Flag-Ub-Ub-Ub-MPAA, intermediate **9**: (*A*) Synthesis, analysis, and purification of Flag-Ub-Ub-Ub-MPAA were carried out by ligating intermediates **8** and **7** as described for intermediate **8** under Fig. S18 to obtain intermediate **9** with 35% yield. (*B*) HPLC and mass traces of the ligation mixture at

time 0: Peak a corresponds to Ub(K^{*48})-$NHNH_2$, intermediate **7**, and peak b corresponds to Flag-Ub-Ub-MPAA, intermediate **8**. (*C*) HPLC of the ligation mixture after 4 h: Peak c corresponds to the ligation product Flag-Ub-Ub-Ub-$NHNH_2$ (the precursor of intermediate **9**) with observed mass of 26675.6 ± 0.8 Da (calculated 26677.4 Da). (*D*) HPLC and mass analysis of purified Flag-Ub-Ub-Ub-MPAA, intermediate **9**: Peak d with observed mass of 26813.1 ± 0.8 Da (calculated 26813.4 Da).

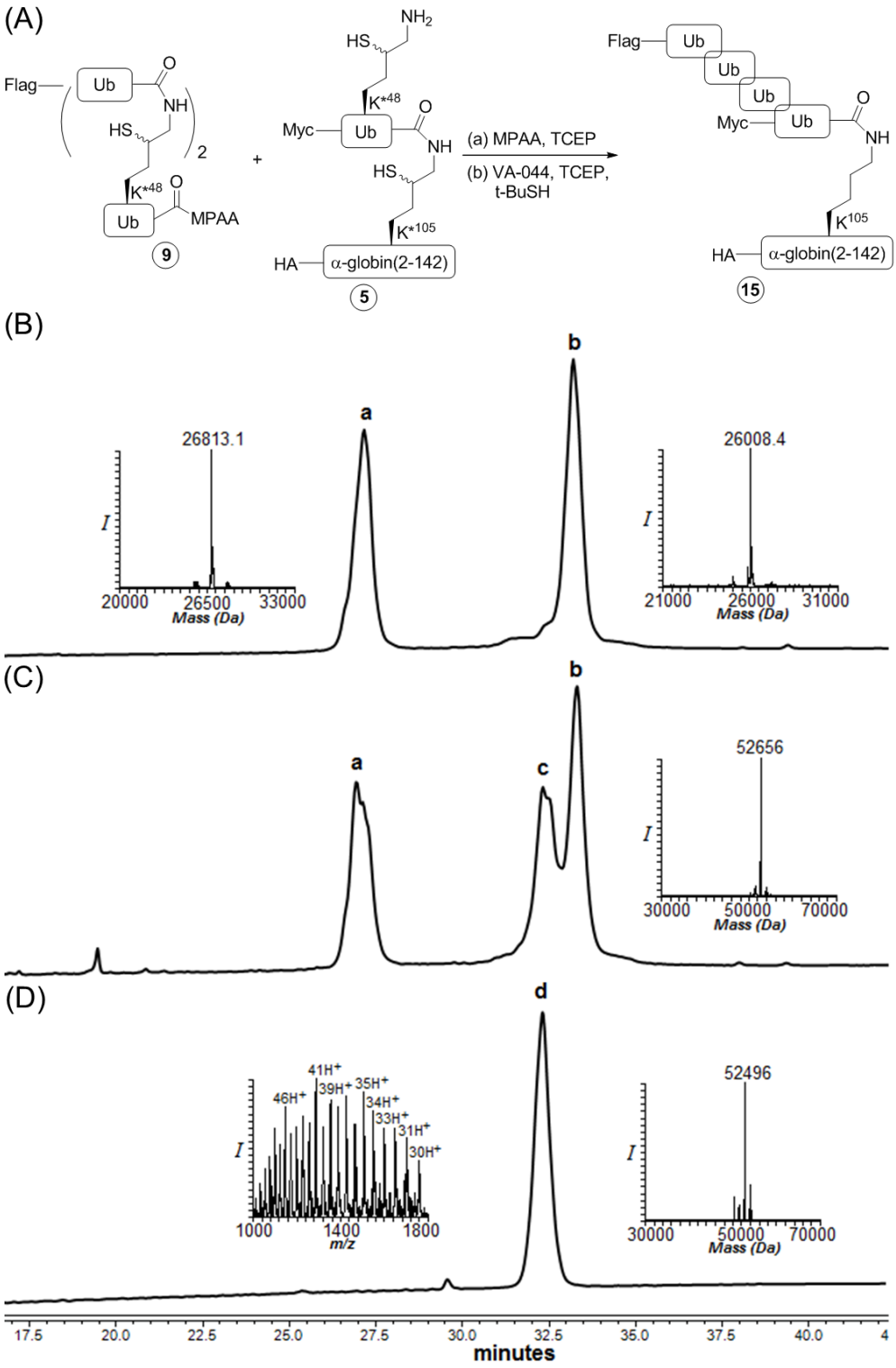

Fig. S20. Synthesis of Flag-Ub-Ub-Ub-Myc-Ub-HA-α-globin(K^{105})(2-142), construct **15**: (*A*) Flag-Ub-Ub-Ub-MPAA, intermediate **9** (7.0 mg, 2.6×10^{-4} mmol) and Myc-Ub(K^{*48})-HA-α-globin(K^{*105})(2-142), intermediate **5** (5.2 mg, 2.0×10^{-4}

mmol) were dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 6.5; 100 µl) containing MPAA (100 mM) and TCEP (50 mM). After an overnight incubation at 37ºC, the reaction mixture was dialyzed using a Slide-A-Lyzer 3.5K dialysis cassette (Thermo, 0.1-0.5 ml) against 6 M Gn·HCl/200 mM phosphate buffer (pH 7.3; 500 ml) for 12 h. Reaction mixture (200 µl) was desulfurized at 37ºC for 15 h using TCEP (15.7 mg, 0.25 mM), VA-044 (25.8 mg, 400 equiv) and t-BuSH (22 µl). The reactions were monitored and product purified by HPLC with a gradient of 15-55% buffer B for 30 min to obtain construct **15** (3.25 mg, 31 % yield). (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Flag-Ub-Ub-Ub-MPAA, intermediate **9**, and peak b corresponds to Myc-Ub(K^{*48})-HA-α-globin(K^{*105})(2-142), intermediate **5**. (*C*) HPLC of the ligation mixture after 4 h: Peak c corresponds to the ligation product with observed mass of 52656 ± 2.1 Da (calculated 52655 Da). (*D*) HPLC and mass analysis of purified construct **15**: Peak d with observed mass of 52496 ± 2.1 Da (calculated 52495 Da).

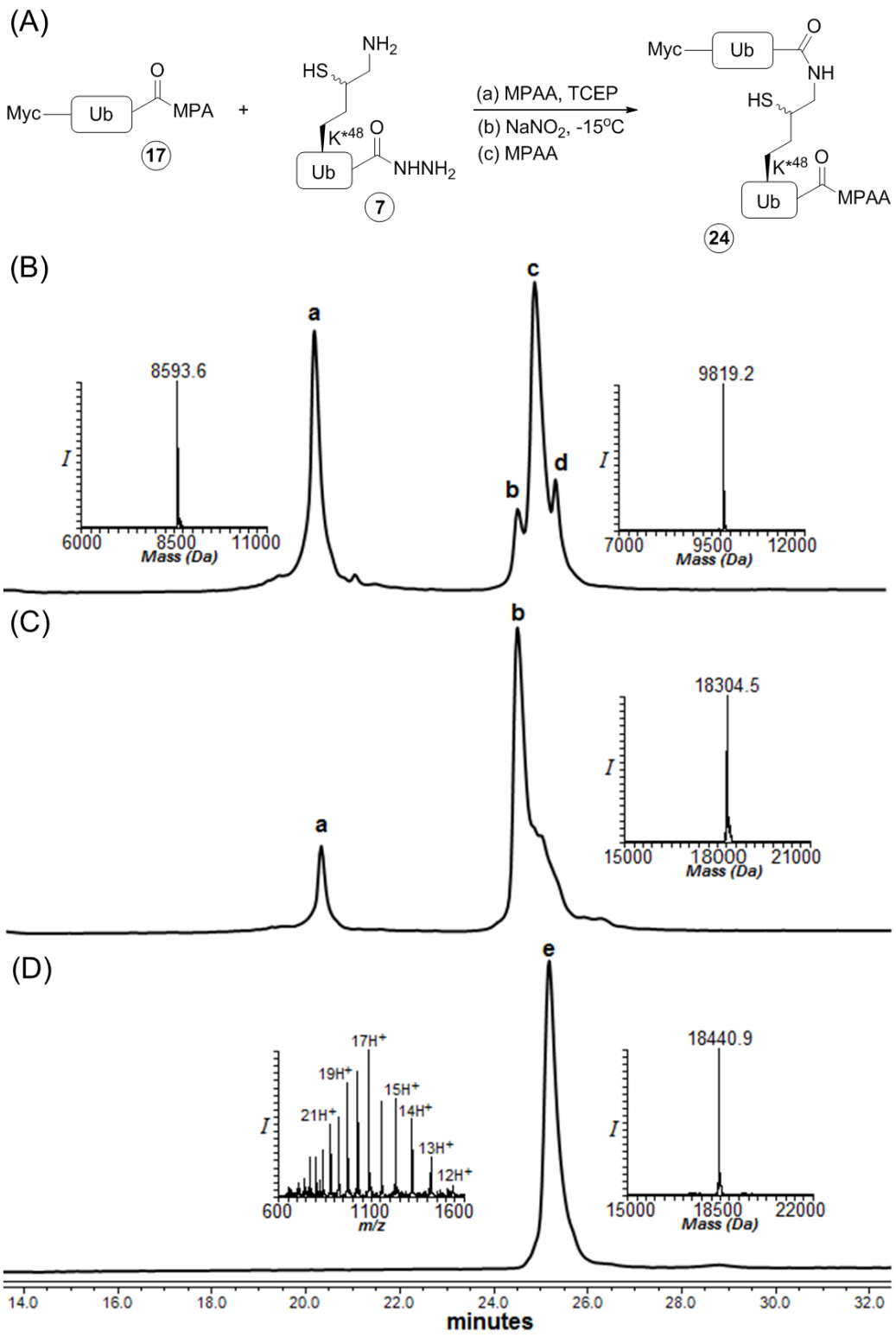

Fig. S21. Synthesis of Myc-Ub-Ub-MPAA, intermediate **24**: (*A*) Synthesis of Myc-Ub-Ub-MPAA was carried out by ligating intermediates **17** and **7** in a similar procedure as described for intermediate **8** under Fig. S18. The reaction was

monitored and product purified by HPLC using a gradient of 20-65% buffer B for 38 min to obtain intermediate **24** with 38% yield. (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Ub(K^{*48})-$NHNH_2$, intermediate **7**, peak c corresponds to Myc-Ub-MPA, intermediate **17**, and peak d corresponds to Myc-Ub-MPAA. (*C*) HPLC of the ligation mixture after 4 h: Peak b corresponds to the ligation product Myc-Ub-Ub-$NHNH_2$ (the precursor of intermediate **24**) with observed mass of 18304.5 ± 0.8 Da (calculated 18306.9 Da). (*D*) HPLC and mass analysis of purified intermediate **24**: Peak e with observed mass of 18440.9 ± 1.6 Da (calculated 18442.9 Da).

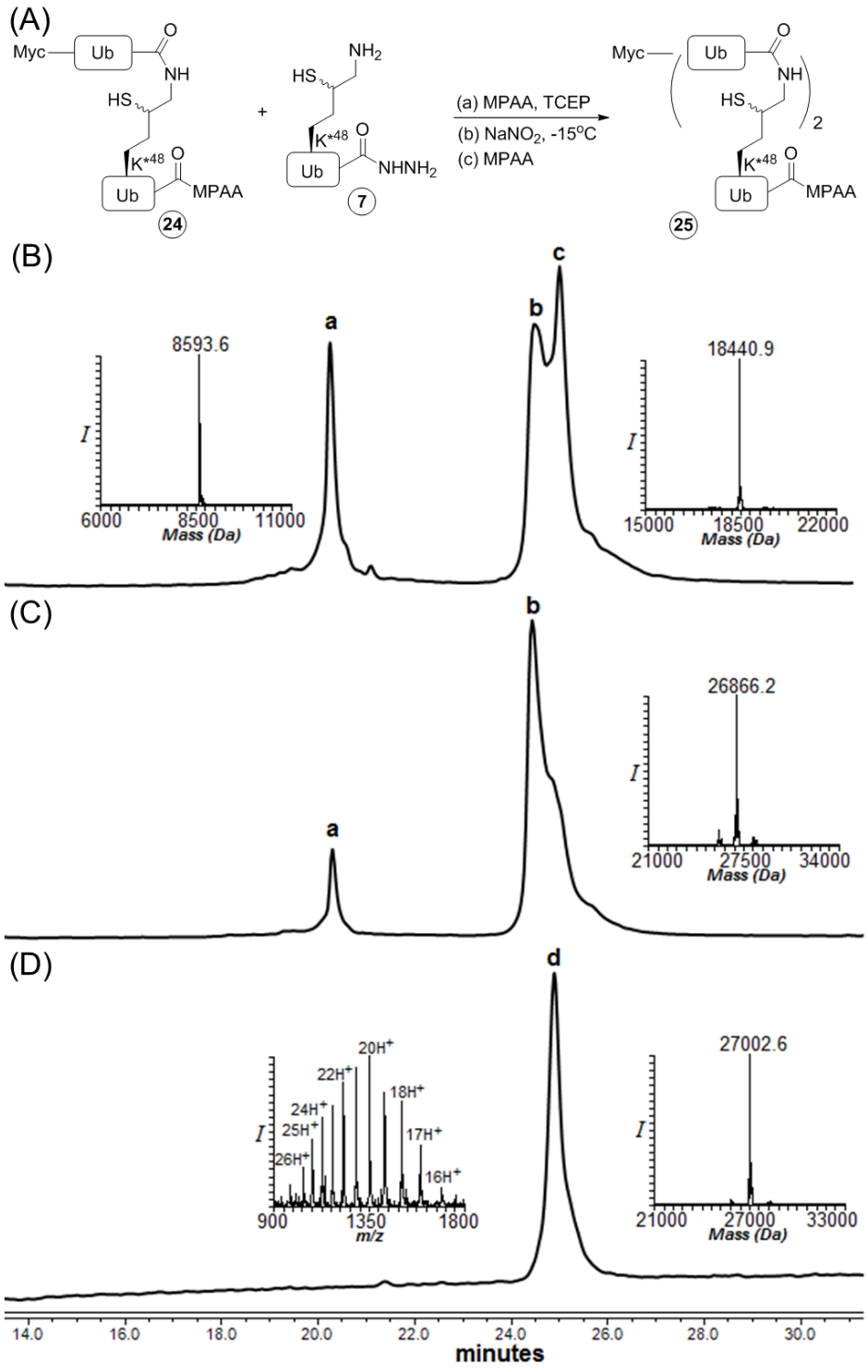

Fig. S22. Synthesis of Myc-Ub-Ub-Ub-MPAA, intermediate **25**: (*A*) Synthesis, analysis, and purification of Myc-Ub-Ub-Ub-MPAA were carried out by ligating intermediates **24** and **7** as described for intermediate **24** under Fig. S21 to obtain intermediate **25** with 35% yield. (*B*) HPLC and mass traces of the ligation mixture

at time 0: Peak a corresponds to Ub(K^{*48})-$NHNH_2$, intermediate **7**, peak c corresponds to Myc-Ub-Ub-MPAA, intermediate **24**, and peak b corresponds to the ligation product (the precursor of intermediate **25**). (*C*) HPLC of the ligation mixture after 4 h: Peak b (the precursor of intermediate **25**) with observed mass of 26866.2 ± 1.9 Da (calculated 26867.7 Da). (*D*) HPLC and mass analysis of purified intermediate **25**: peak d with observed mass of 27002.6 ± 1.6 Da (calculated 27003.7 Da).

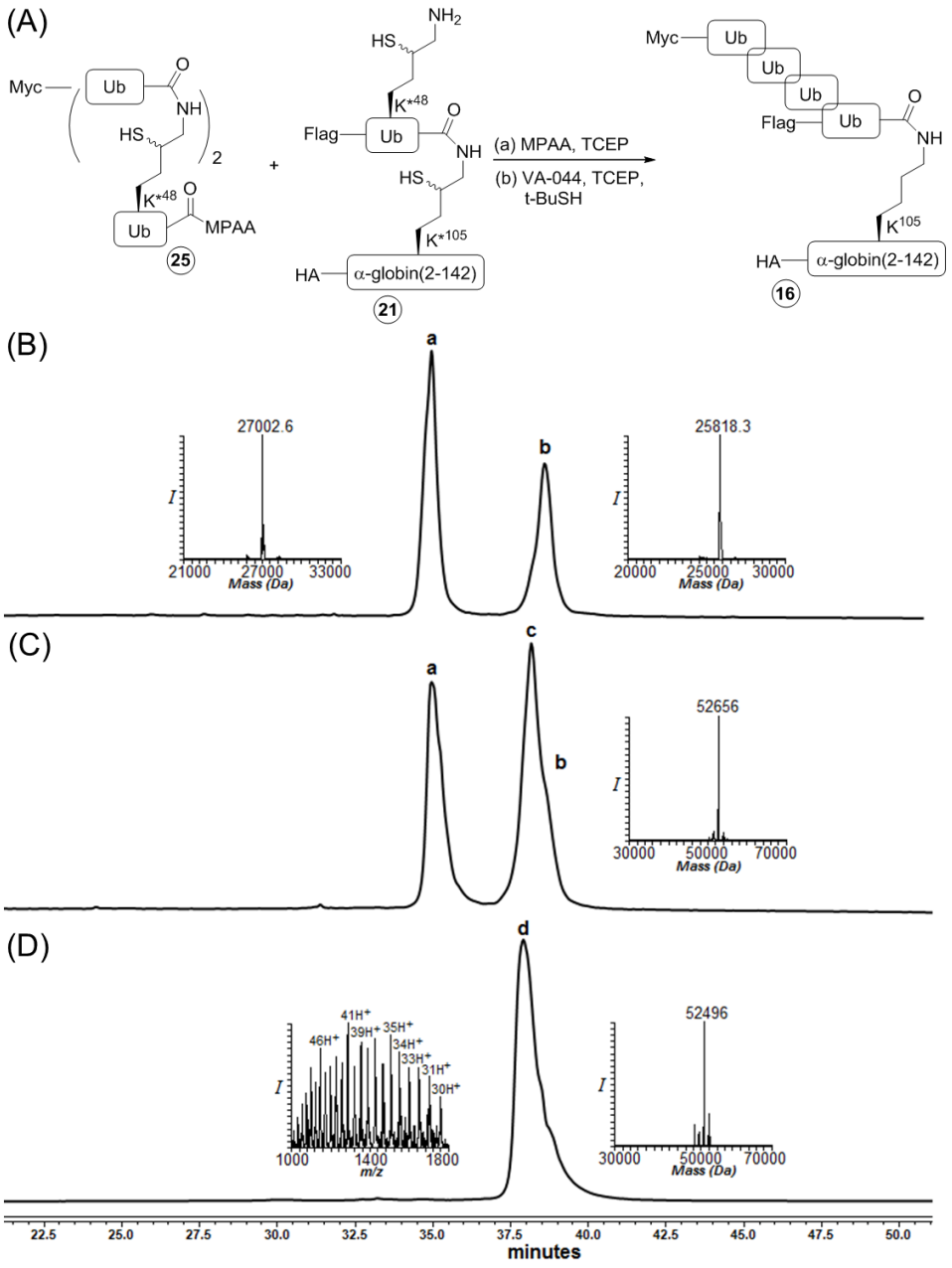

Fig. S23. Synthesis of Myc-Ub-Ub-Ub-Flag-Ub-HA-α-globin(K^{105})(2-142), construct **16**: (*A*) Synthesis of Myc-Ub-Ub-Ub-Flag-Ub-HA-α-globin(K^{105})(2-142) was carried out by ligating intermediates **25** and **21** in a similar procedure as described for construct **15** under Fig. S20. The reaction was monitored and product purified by HPLC using a gradient of 20-55% buffer B for 33 min to obtain construct **16** with 30% yield. Construct **16** was folded in 50 mM Tris/150 mM NaCl (pH 7.4), and further purified by FPLC. (*B*) HPLC and mass traces of the ligation mixture at time 0: Peak a corresponds to Myc-Ub-Ub-Ub-MPAA, intermediate **25**, and peak

b corresponds to Flag-Ub(K^{*48})-HA-α-globin(K^{*105})(2-142), intermediate **21**. (*C*) HPLC of the ligation mixture after 12 h: Peak c corresponds to the ligation product with observed mass of 52656 ± 2.1 Da (calculated 52655 Da). (*D*) HPLC and mass analysis of purified **16**: Peak d with observed mass of 52496 ± 2.1 Da (calculated 52495 Da).

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