Semi-Synthetic, Site-Specific Ubiquitin Modification of α-Synuclein Reveals Differential Effects on Aggregation

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Supporting Information

Chemicals and Reagents. Solvents and reagents were obtained from commercial sources (EMD, Novagen, Invitrogen, Fluka, Sigma Aldrich) and used without further purification. Ultrapure laboratory grade water (filtered, deionized, sterilized) was obtained from in-house MilliQ® water purification systems. All solutions were prepared in ultrapure laboratory water and filter sterilized through a 0.4 μM filter (Genesee Scientific). Media (LB broth, Miller, Novagen and S.O.C. broth, Sigma) were prepared, sterilized and stored according to the manufacturer and standard published protocols (Sambrook and Russel 2001). Media were handled in sterile conditions under an open flame. Antibiotic stock: stocks of ampicillin (Na salt, EMD) at a working concentration of 1000x (100 mg mL^{-1}) were stored at -20 °C. The anti-α-synuclein (Syn 211, AHB0261) and anti-oligomer (A11, AHB0052) were purchased from Invitrogen.

Plasmid Construction. All constructs were prepared using standard molecular cloning techniques. A pRK172 construct containing wild-type human α-synuclein inserted into Nde I and Hind III restriction sites has been described previously (Der-Sarkissian, Jao et al. 2003). Lysine to cysteine point mutations (K6C, K10C, K12C, K21C, K23C, K32C, K34C, K46C, and K96C) were generated using Quikchange mutagenesis (Stratagene). The full-length human ubiquitin gene was obtained by PCR with the previously reported pHub(1-75) plasmid (Chatterjee C *et al.* 2007). It was then ligated into pTXB1 vector into NdeI and SapI restriction sites. All constructs were characterized by DNA sequencing.

Ubiquitin-thiol Expression and Purification. *E. coli* BL21*(DE3)* cells, transformed with plasmid pUb, were grown in 6 L LB-amp (100 μg mL^{-1}) at 37 °C with shaking at 250 rpm to mid-log phase ($OD_{600\ nm}$ = 0.6-0.8). Overexpression was induced by the addition of 0.5 mM IPTG and cells were grown for an additional 23 h at 37 °C. The cells were then harvested by centrifugation at (10,000 x *g*, 30 min, 4 °C).

The cell pellet was resuspended in buffer A (200 mM NaCl, 50 mM Tris, 1 mM EDTA, pH 7.2). The cells were lysed by passage through a French Press and the soluble fraction separated from insoluble cellular debris by centrifugation at (18,500-20,000 x *g*, 20 min, 4 °C). After filtration through a 0.45 μm filter, supernatants were bound to a 50 mL chitin column, pre-equilibrated with 10 CV of buffer A, for 2 h at 4 °C. The resin was washed with 20 CV of buffer A, followed by 3 CV of buffer B (50 mM Tris, 200 mM NaCl, and 1 mM EDTA, pH 7.5). Ubiquitin was cleaved from the intein-CBD fusions by incubation with 1.5 CV of buffer B containing 50 mM of cystamine dihydrochloride, and 50 mM of Tris(2-carboxyethyl) phosphine, pH 7.75 for 48 h. The eluted protein, bearing the desired C-terminal aminoethanethiol linker, was subsequently purified by C18

process RP-HPLC employing a gradient of 25-60% B, over 60 min, followed by a second purification by C18 semi-preparative HPLC with a gradient of 25-60% B over 45 min. Typical yields of 4-6 mg were obtained for each of the proteins. Purity and identity of the final product was confirmed by SDS-PAGE, C18 analytical RP-HPLC and ESI-MS (M + H^+) **Ub-SH:** t_R = 21.48 min, observed = 8,627.3 Da ± 0.6 Da, expected = 8,624.9 Da.

Synthesis of disulfide activated ubiquitin. In a typical reaction, DTNP (10.0 mg, 32.266 µmol) dissolved in 3.697 mL of a 3:1 (v/v) acetic acid:water mixture was added to Ub-SH (16.0 mg, 1.855 µmol). The reaction was allowed to proceed for 48 h at 25 °C, prior to purification by C4 semi-preparative RP-HPLC with a 30-60% B gradient over 60 min. This yielded 9.2 mg (57%) of the pure Ub-S-nitro-2-pyridinesulfenyl (pNpys) disulfide adduct. The identity of the disulfide adduct was verified by C4 analytical HPLC, and ESI-MS (M + H^+) **Ub-S-pNpys** t_R = 23.2 min, observed: 8,781.0 Da ± 2.2 Da, expected: 8779.0 Da.

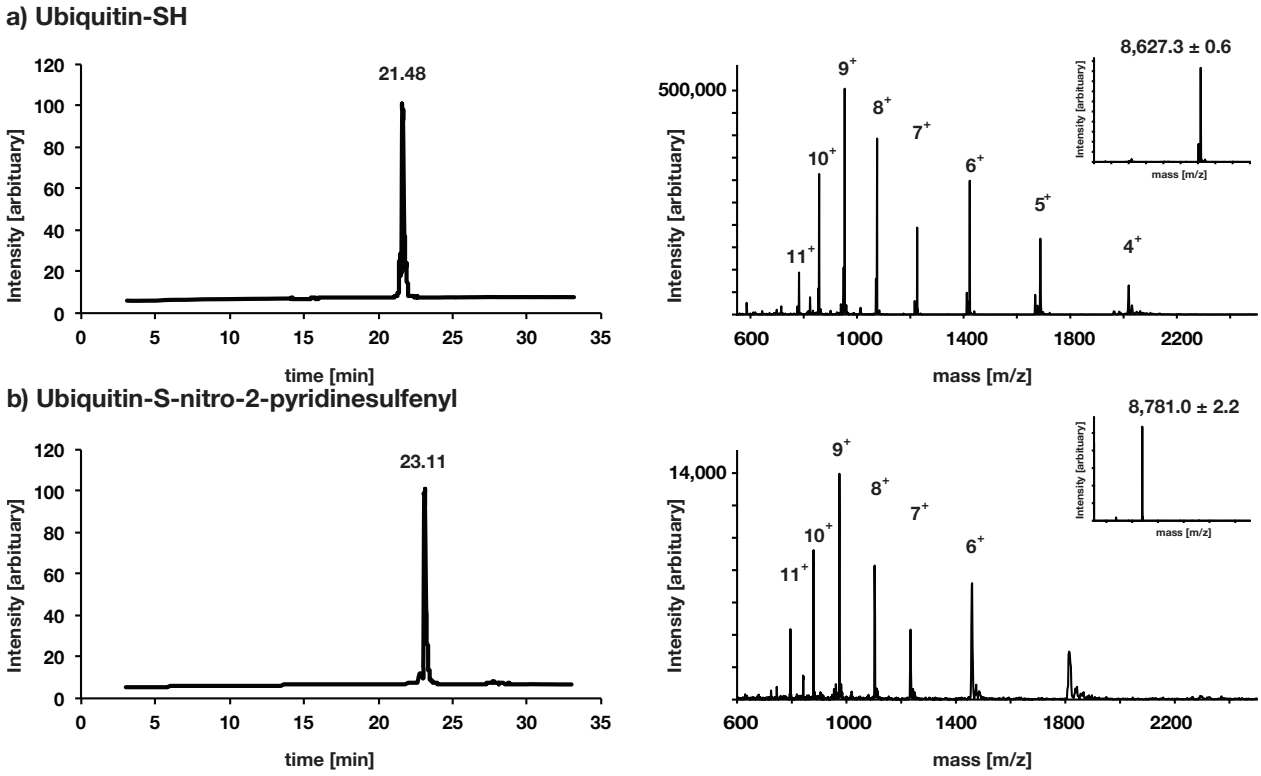


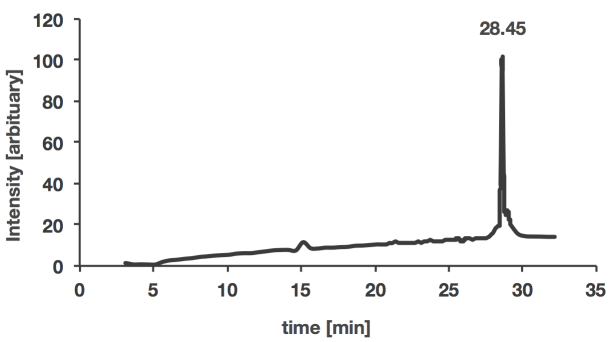
Figure S1. Characterization of ubiquitin. HPLC traces (Vydac® C4 analytical RP-HPLC column, 5-60% B gradient over 30 min) and mass spectra (API 3000 LC/MS-MS Systems, Applied Biosystems MDS SCIEX Electrospray, +ve, 800-3000 amu, deconvolution with Analyst Software) of ubiquitin C-terminal thiol and disulfide activated ubiquitin.

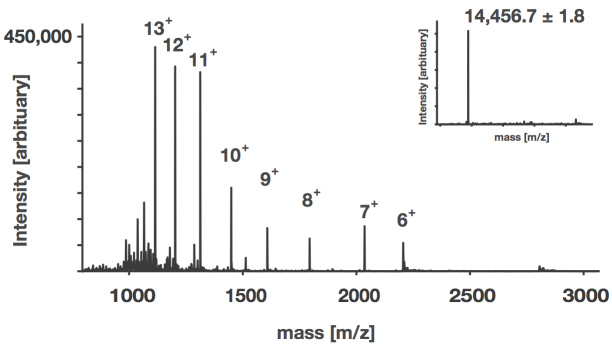
α-Synuclein Expression and Purification. The expression and purification of wild-type and mutant proteins was performed as described (Der-Sarkissian, A. et al. (2003) J. Biol. Chem. 278, 37530–37535) with minor modifications. *E. coli* BL21*(DE3)* cells were transformed with a single α-synuclein pRK172 construct by heat shock followed by

streaking on an LB agar plate containing ampicillin (LB-amp, 100 µg mL^{-1}) and incubation at 37 °C for 16 h. A single colony of transformed cells was used to inoculate 50 mL LB-amp. After incubation a shaker (250 rpm, 16 h, 37 °C), the cells from the cloudy starter culture were harvested by centrifugation (4,000 x *g*, 20 min, 4 °C). The supernatant was discarded whereas the pellet was resuspended in 1 L fresh LB-amp in a 3 L flask, followed by incubation in a shaker (250 rpm) at 37 °C to mid-log phase ($OD_{600\ nm}$ = 0.6-0.7). Protein expression was induced by addition of IPTG (Novagen) at a final concentration of 0.5 mM, and cultures were incubated in a shaker overnight at 25 °C. Cells were harvested by centrifugation (8,000 x *g*, 30 min, 4 °C) and washed with PBS.

To the resulting cell pellet was added 10 mL (per 1 L cell culture) of lysis buffer (500 mM NaCl, 100 mM Tris, 10 mM β-mercaptoethanol, 1 mM EDTA, pH 8.0). The cell lysate was then heated for 10 min at 80 °C and allowed to cool down to RT for 30 min. Protease inhibitor cocktail (c*o*mplete, Roche) was added and incubated for 30 min on ice, followed by centrifugation (43,000 x *g*, 30 min, 4 °C). The pH of the resulting supernatant was adjusted to 3.5 by addition of 10 M HCl to precipitate bacterial proteins, which were cleared by centrifugation (43,000 x *g*, 30 min, 4 °C). The final supernatant was dialyzed against 3 x 1 L of purification buffer (20 mM Tris, 1 mM dithiothreitol, 1 mM EDTA, pH 8.0,) and loaded onto a HiTrap Q XL column (5 mL, GE Healthcare) equilibrated in the same buffer. Proteins were eluted in a 0 – 1 M NaCl gradient (15-30% in 20 CV, 30-100% in 5 CV, 100% for 5 CV), and α-synuclein-containing fractions were identified by SDS-PAGE. α-Synuclein-containing fractions were pooled, dialyzed against 3 x 1 L of 1% acetic acid in H_2O and lyophilized. The resulting protein was purified by C4 semi-preparative RP-HPLC using a 40-50% B gradient over 60 min [A: 0.1% trifluoroacetic acid (TFA) in H_2O, B: 90% acetonitrile, 0.1% TFA in H_2O]. Typical yields of 5-12 mg L^{-1} were obtained for each of the proteins. All proteins were characterized by SDS-PAGE, C4 analytical RP-HPLC, and ESI-MS ($M + H^+$) **wild-type:** 5-12 mg L^{-1} yield, t_R = 28.45 min, observed = 14,457 Da ± XXX Da, expected = 14,460.1 Da; **K6C:** 11 mg L^{-1} yield, t_R = 28.82 min, observed = 14,437.5 Da ± 3.6 Da, expected = 14,435.1 Da; **K10C:** 7 mg L^{-1} yield, t_R = 28.87 min, observed = 14,438.8 Da ± 2.4 Da, expected = 14,435.1 Da; **K12C:** 7 mg L^{-1} yield, t_R = 28.82 min, observed = 14,440.2 Da ± 1.7 Da, expected = 14,435.1 Da; **K21C:** 4 mg L^{-1} yield, t_R = 51.63 min, observed = 14,438.4 Da ± 4.1 Da, expected = 14,435.1 Da; **K23C:** 3 mg L^{-1} yield, t_R = 55.44 min, observed = 14,438.9 Da ± 3.6 Da, expected = 14,435.1 Da; **K32C:** 4 mg L^{-1} yield, t_R = 51.44 min, observed = 14,438.3 Da ± 4.0 Da, expected = 14,435.1 Da; **K34C:** 3 mg L^{-1} yield, t_R = 31.11 min, observed = 14,440.5 Da ± 1.5 Da, expected = 14,435.1 Da; **K43C:** 7 mg L^{-1} yield, t_R = 28.89 min, observed = 14,440.7 Da ± 1.8 Da, expected = 14,435.1 Da; **K96C:** 8 mg L^{-1} yield, t_R = 28.78 min, observed = 14,438.5 Da ± 1.2 Da, expected = 14,435.1 Da.

a) alpha-synuclein Y136Y





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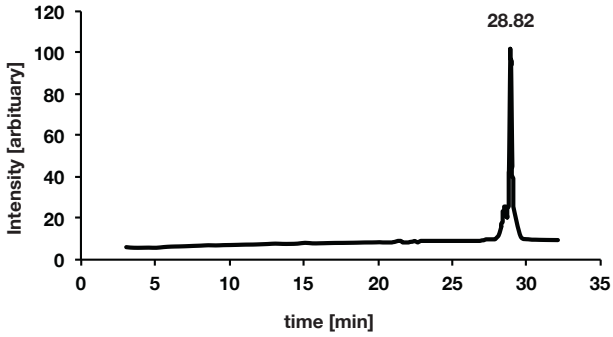

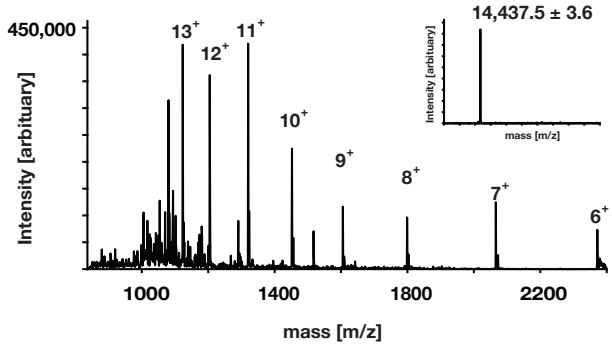

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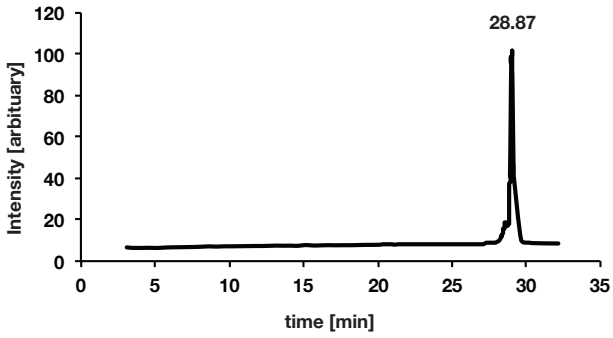

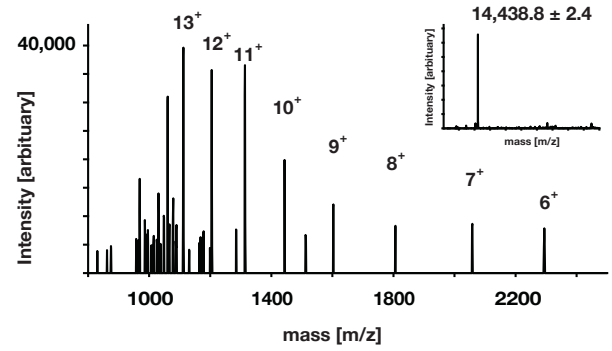

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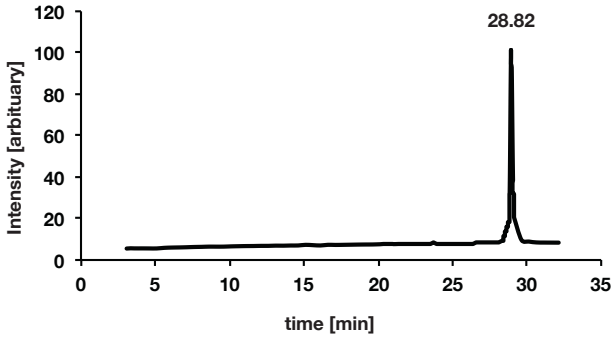

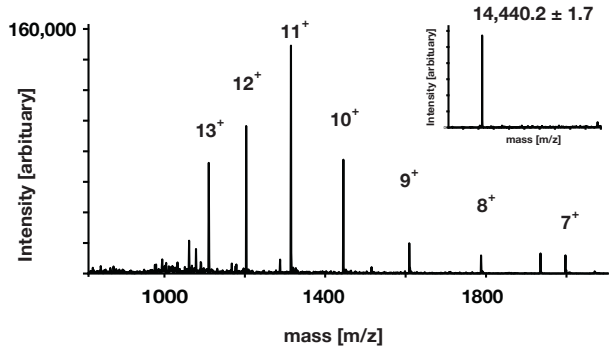

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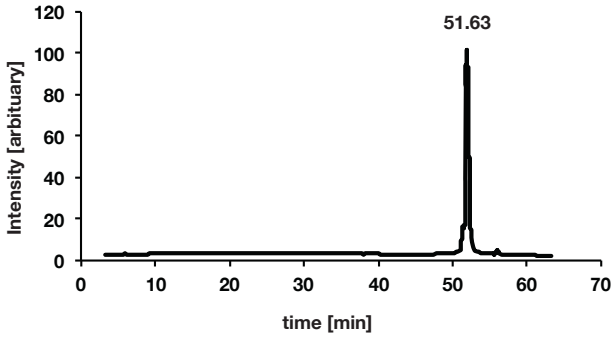

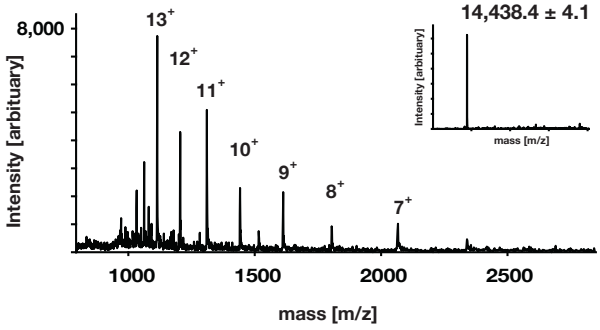

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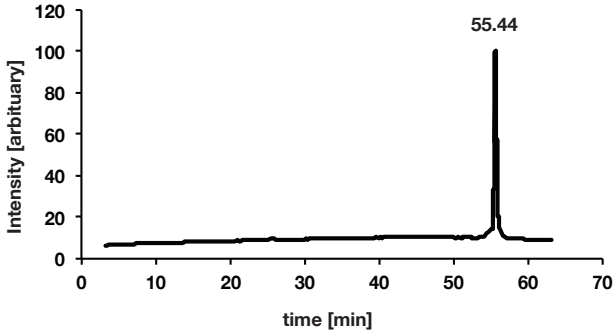

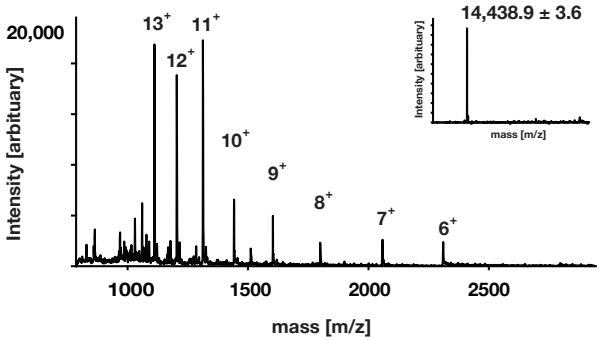

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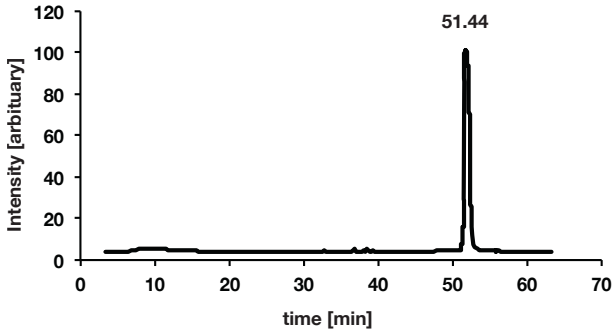

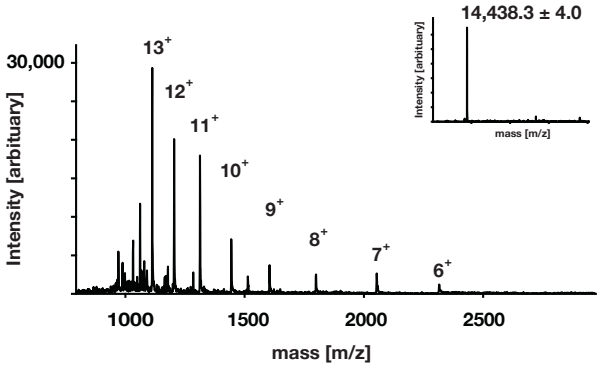

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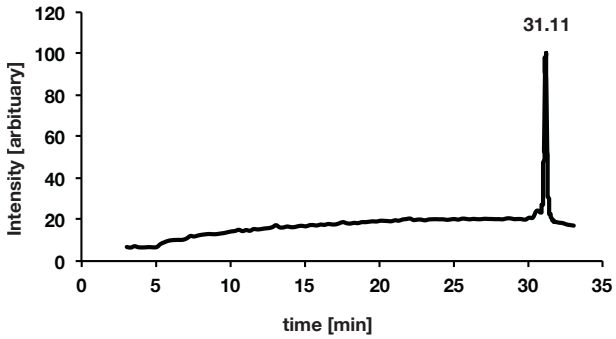
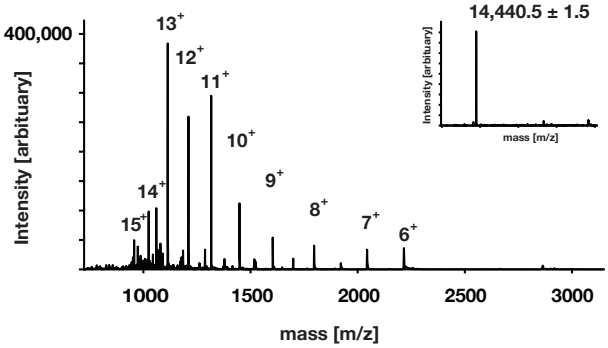




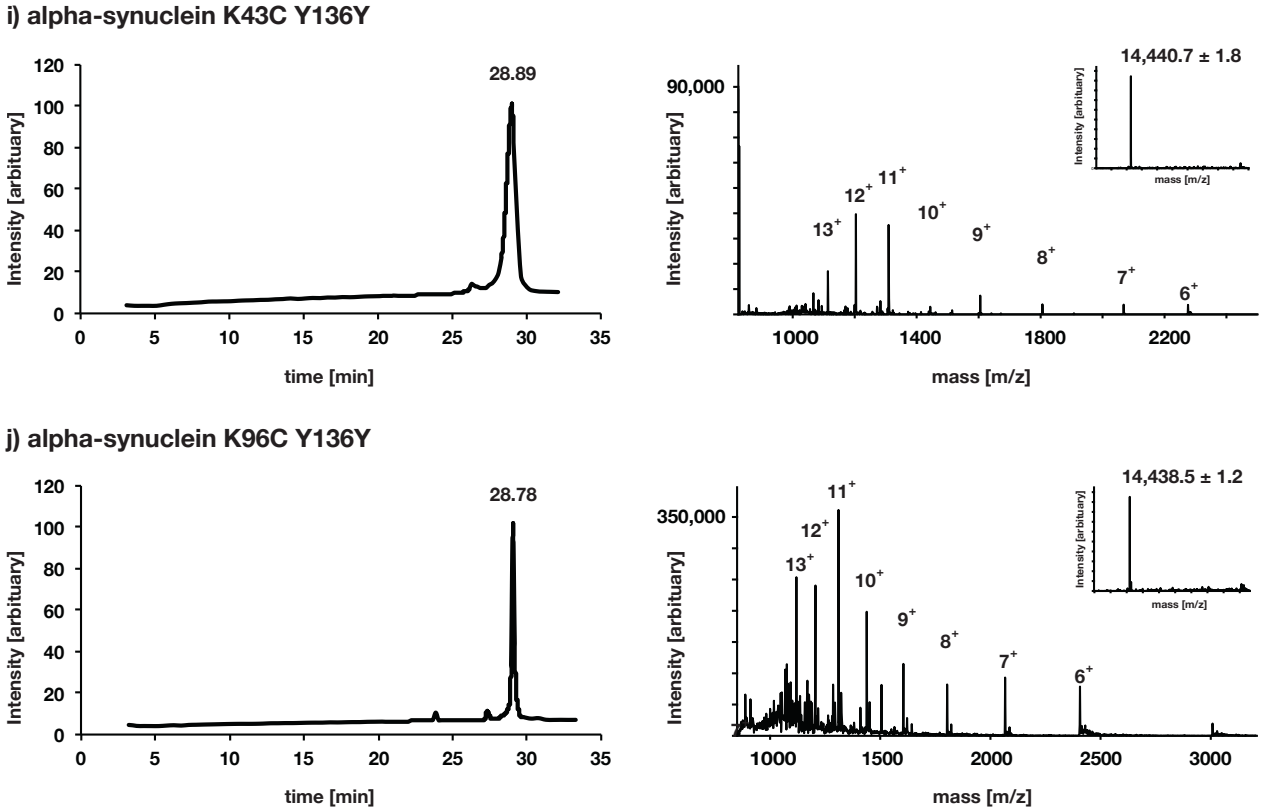


Figure S2. Characterization of α-synuclein and lysine to cysteine mutants. RP-HPLC traces (Vydac® C4 analytical RP-HPLC column, 5-60% B gradient over 30 min) and mass spectra (API 3000 LC/MS-MS Systems, Applied Biosystems MDS SCIEX Electrospray, +ve, 800-3000 amu, deconvolution with Analyst Software) of wild-type and K6C, K10C, K12C, K21C, K23C, K32C, K34C, K43C, and K96C mutants of α-synuclein.

Synthesis of disulfide-linked ubiquitin conjugated α-synuclein. In a typical reaction, 1.0 equivalent of α-synuclein cysteine mutant (3.0 mg, 0.208 μmol) and 2.0 equivalents of Ub-S-pNpys (3.5 mg, 0.406 μmol) were dissolved in 1024 μL of reaction buffer consisting of 1M HEPES, 6 M Gn-HCl, pH 6.93. The reaction was allowed for 1 h at 25 °C with continuous shaking. The reaction products were purified by C4 semi-preparative HPLC with a 30-60% B gradient to yield typically 1-3 mg α-synuclein K#C-Ub. Purity and identity of the final products were confirmed by SDS-PAGE, C4 analytical RP-HPLC and ESI-MS (M + H^+) **K6C-Ub:** 1.7 mg, 36% yield, t_R = 28.11 min, observed = 23,067.0 Da ± 2.4 Da, expected = 23,058.0 Da; **K10C-Ub:** 3.0 mg, 63% yield, t_R = 27.99 min, observed = 23,066.8 Da ± 2.4 Da, expected = 23,058.0 Da; **K12C-Ub:** 2.7 mg, 56% yield, t_R = 27.80 min, observed = 23,066.5 Da ± 2.1 Da, expected = 23,058.0 Da; **K21C-Ub:** 2.2 mg, 46% yield, t_R = 27.55 min, observed = 23,067.2 Da ± 6.3 Da, expected = 23,058.0 Da; **K23C-Ub:** 3.0 mg, 63% yield, t_R = 28.09 min, observed = 23,065.4 Da ± 5.8 Da, expected = 23,058.0 Da; **K32C-Ub:** 2.4 mg, 50% yield, t_R = 27.62 min, observed = 23,065.1 Da ± 4.7 Da, expected = 23,058.0 Da; **K34C-Ub:** 2.8 mg, 59% yield, t_R = 27.84 min, observed = 23,064.7 Da ± 5.3 Da, expected = 23,058.0 Da; **K43C-Ub:** 3.1 mg, 65% yield, t_R = 28.05 min, observed = 23,066.4 Da ± 2.2 Da, expected = 23,058.0 Da; **K96C-Ub:** 1.2 mg, 25% yield, t_R = 28.04 min, observed = 23,066.8 Da ± 2.2 Da, expected =

23,058.0 Da.

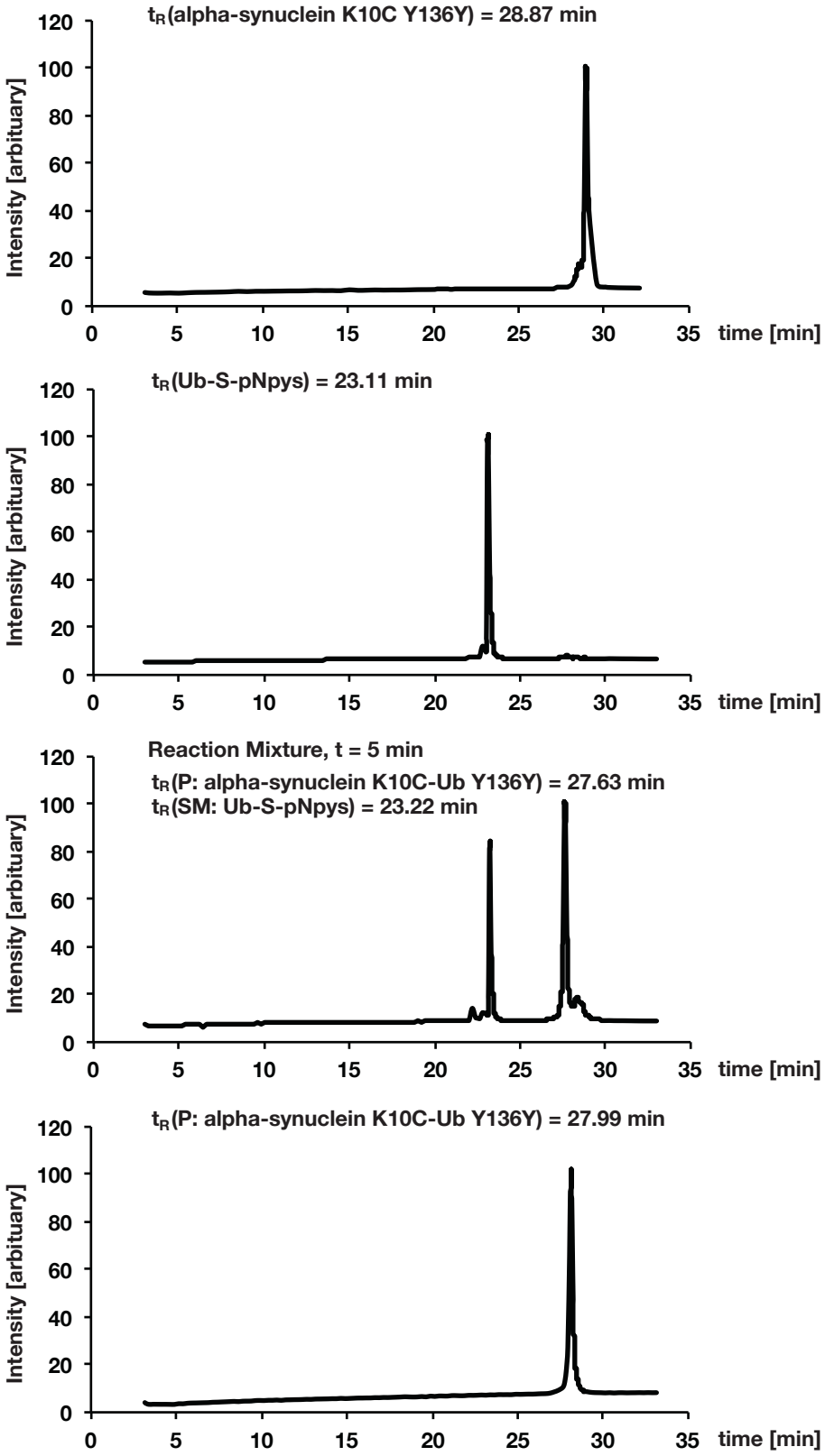
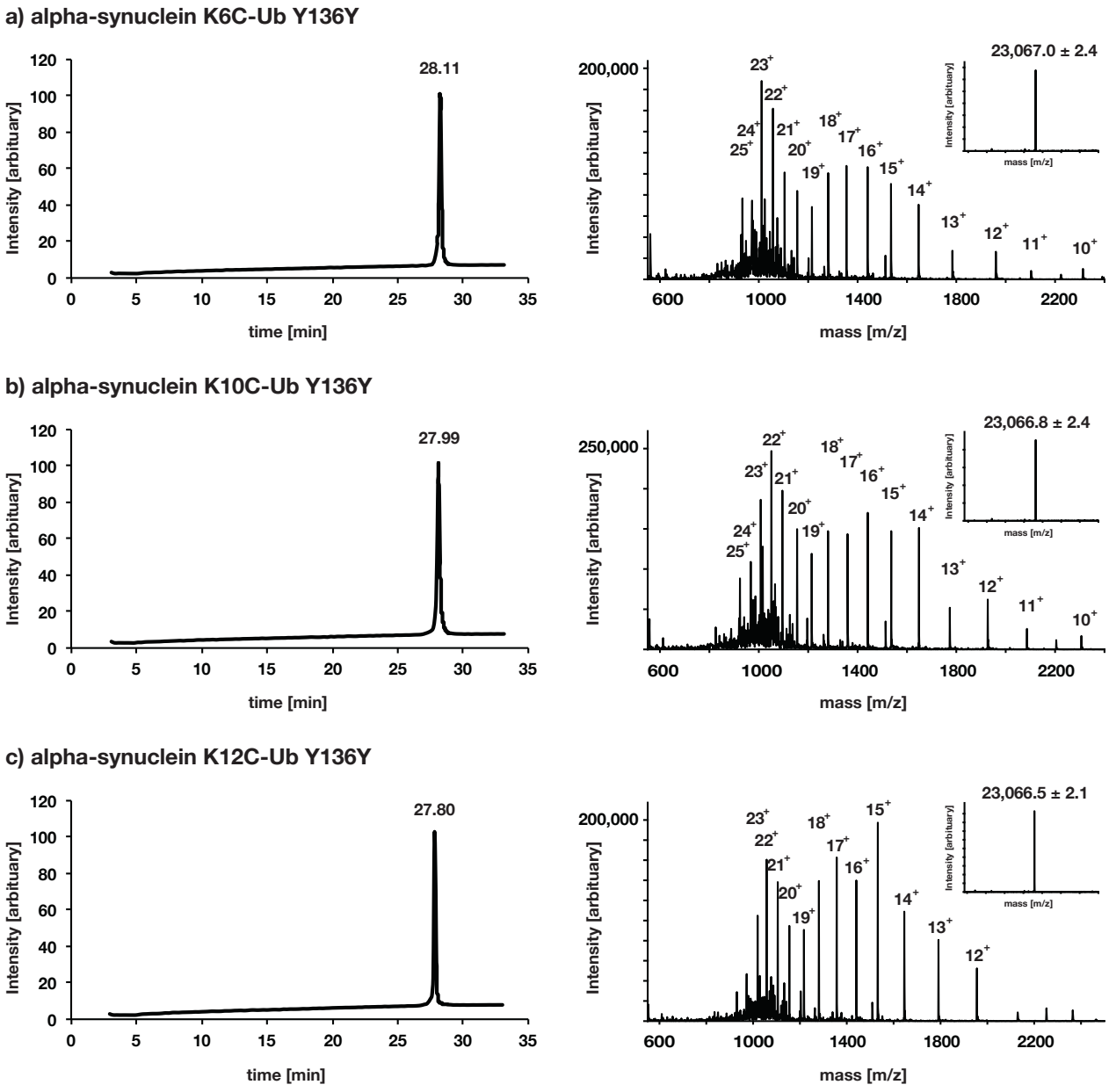
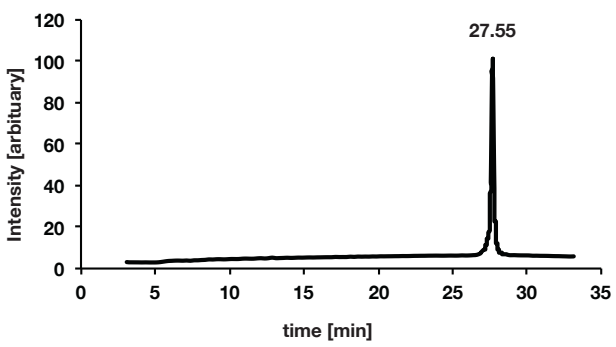
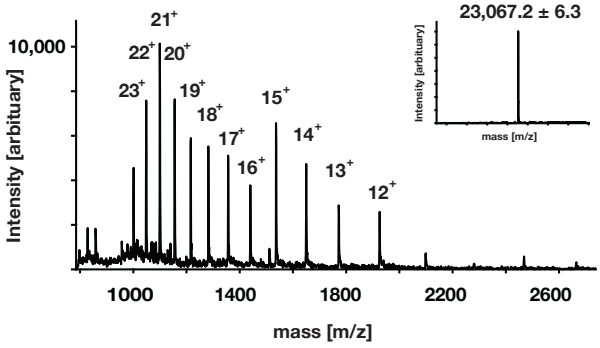

t_R(alpha-synuclein K10C Y136Y) = 28.87 min
Intensity [arbituary]
time [min]
t_R(Ub-S-pNpys) = 23.11 min
Intensity [arbituary]
time [min]
Reaction Mixture, t = 5 min
t_R(P: alpha-synuclein K10C-Ub Y136Y) = 27.63 min
t_R(SM: Ub-S-pNpys) = 23.22 min
Intensity [arbituary]
time [min]
t_R(P: alpha-synuclein K10C-Ub Y136Y) = 27.99 min
Intensity [arbituary]
time [min]

Figure S3. Disulfide-directed ubiquitination of α-synuclein. The reaction of ubiquitination of K10C was followed by HPLC (Vydac® C4 analytical RP-HPLC column, 5-60% B gradient over 30 min).

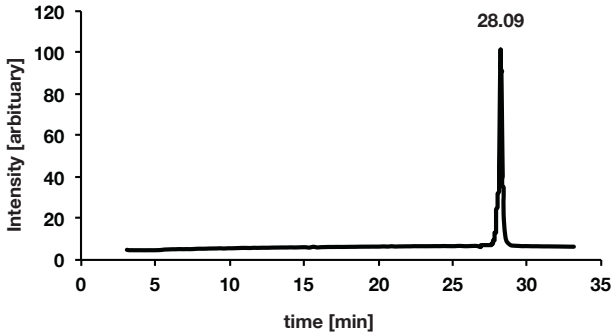


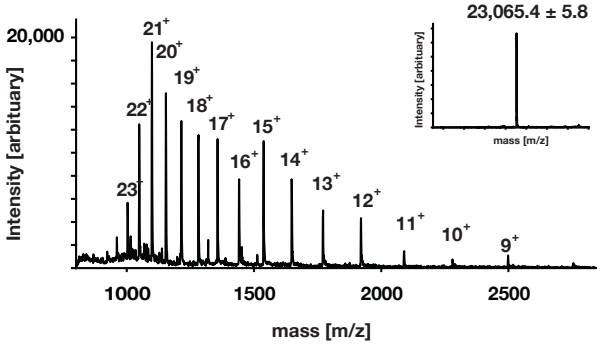
d) alpha-synuclein K21C-Ub Y136Y



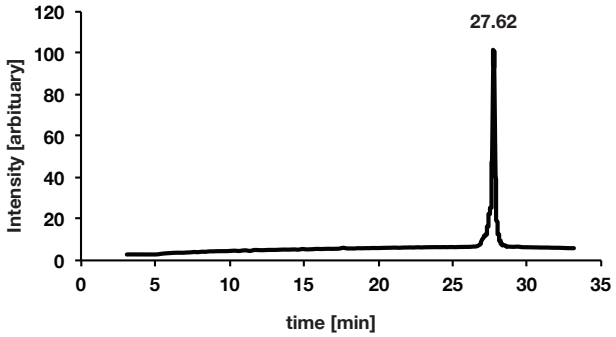


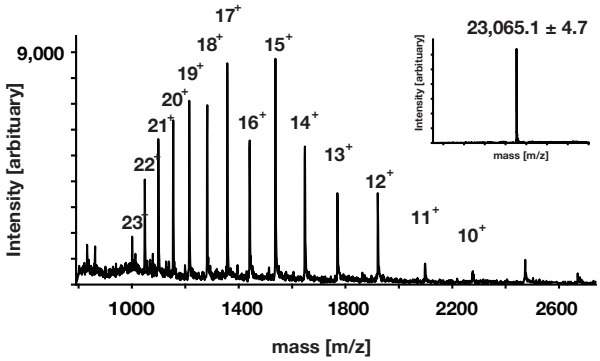
e) alpha-synuclein K23C-Ub Y136Y



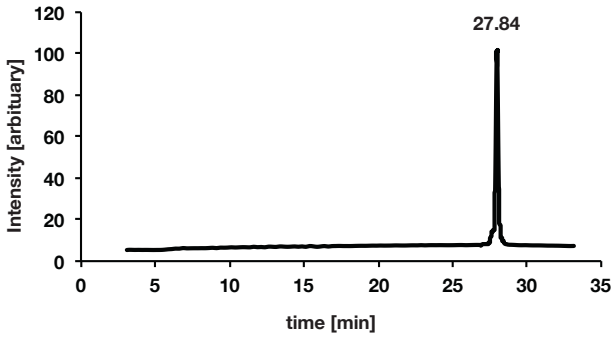


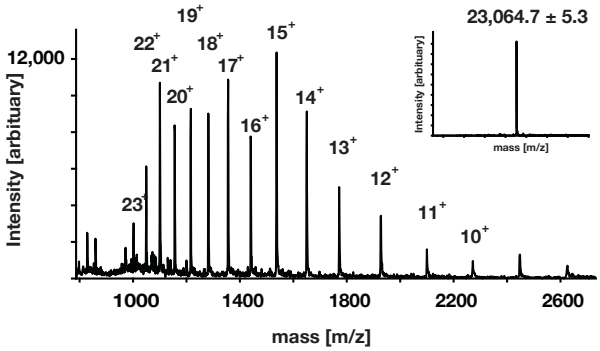
f) alpha-synuclein K32C-Ub Y136Y





g) alpha-synuclein K34C-Ub Y136Y





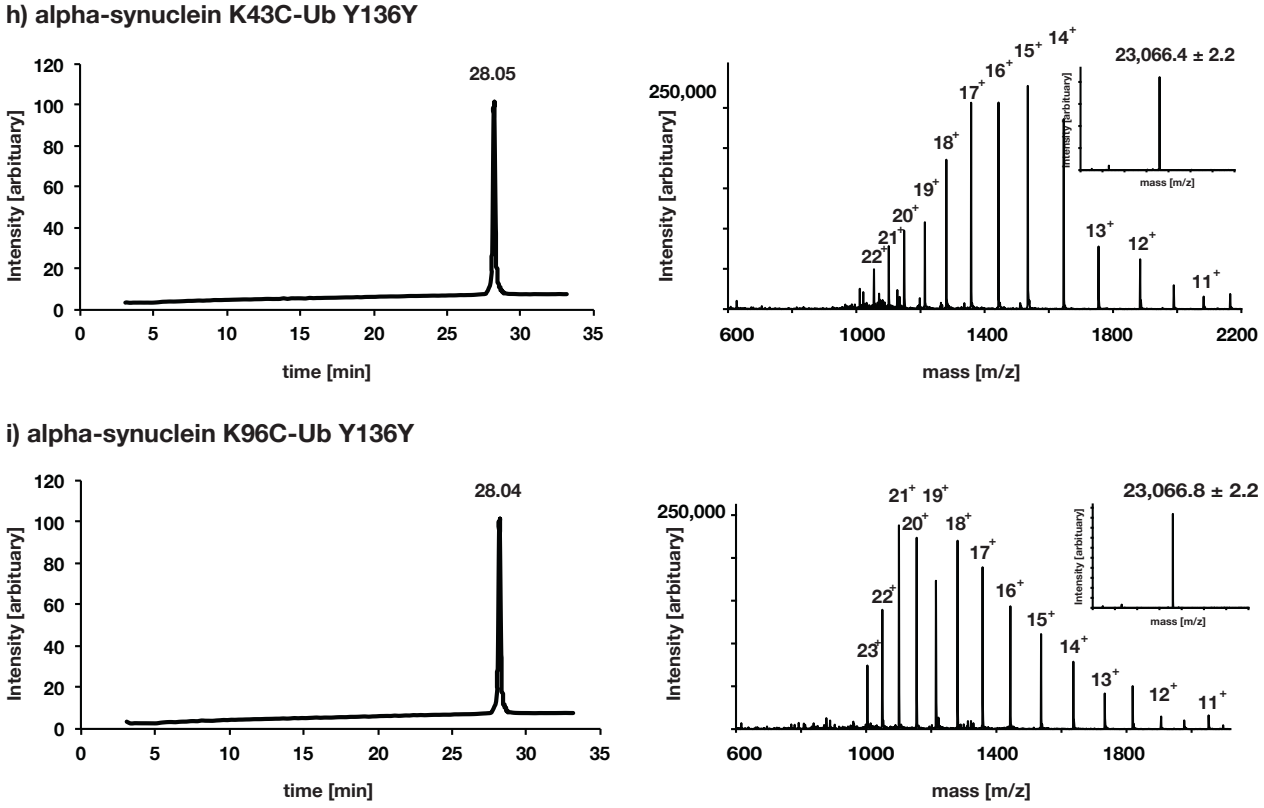


Figure S4. Characterization of disulfide-directed ubiquitinated α-synuclein. HPLC traces (Vydac® C4 analytical RP-HPLC column, 5-60% B gradient over 30 min) and mass spectra (API 3000 LC/MS-MS Systems, Applied Biosystems MDS SCIEX Electrospray, +ve, 800-3000 amu, deconvolution with Analyst Software) of disulfide-directed ubiquitinated α-synuclein.

Circular dichroism (CD) measurements. CD spectra were obtained on a Jasco J-815 CD spectrometer equipped with a constant temperature cell holder and analyzed with Spectra Analysis Version 2. The Far UV-CD spectra (190–250 nm) were collected at 25 °C in a 1 mm path length quartz cuvette containing 15 μM protein in 10 mM phosphate buffer, pH 7.4. For all spectra, an average of 4 scans was obtained with a step size of 0.1 nm, 1.0 nm bandwidth, 50 nm min^{-1} scanning speed, and DIT of 4 s. The backgrounds of buffers were subtracted for all samples, and the data were converted into mean residue ellipticity.

Dynamic light scattering. Dynamic light scattering measurements were collected on a Wyatt Technologies Dynastar. All samples represent above aggregation reactions at time = 0 h. For all spectra, an average of 10 scans was obtain with background subtraction of buffer.

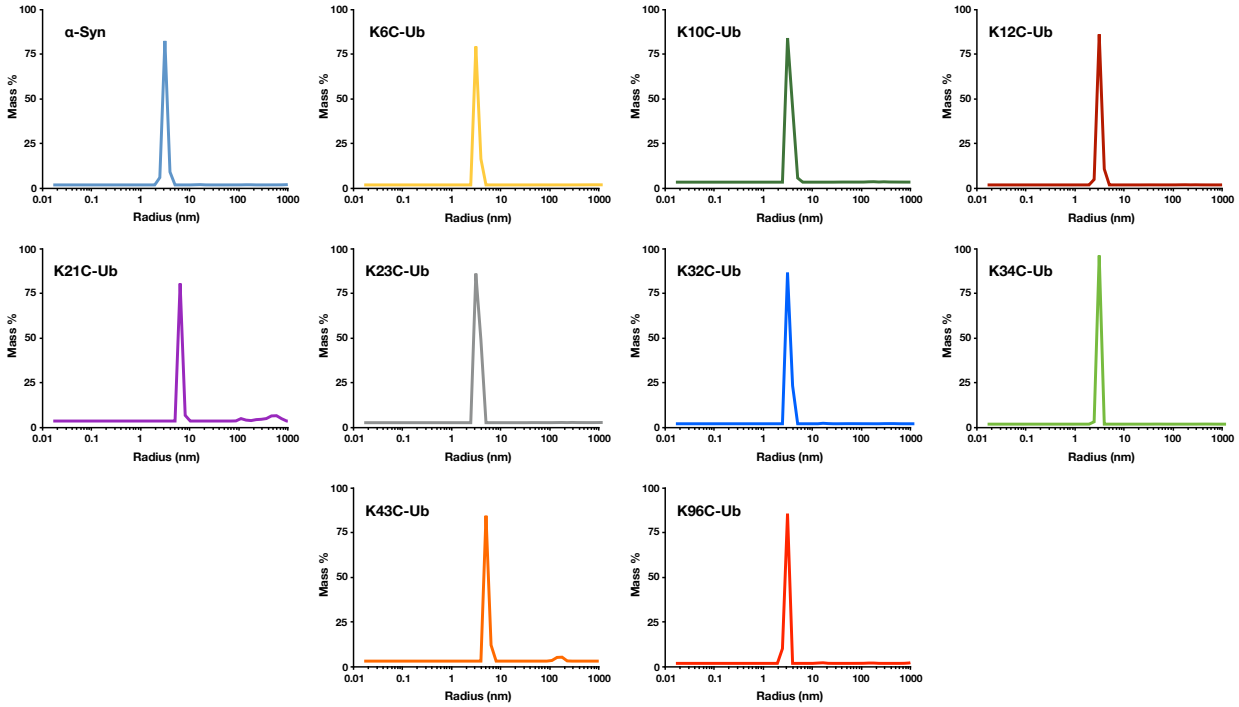


Figure S5. Dynamic light scattering. Unmodified α-synuclein and α-synuclein lysine to cysteine ubiquitinated derivatives (K#C-Ub) were analyzed by light scattering and plotted as mass percentage against Stokes radius.

Dot Blotting. Protein (~3 µg) was spotted on dry nitrocellulose membrane (0.45 µg, BioRad). Membranes were then blocked in TBST (0.1% Tween-20, 150 mM NaCl, 10 mM Tris, pH 8.0) containing 10% nonfat milk for 1 h at 4° C under constant rocking. After washing them three times with TBST, the membranes were incubated with the following primary antibodies at 4° C under constant rocking overnight: anti-α-synuclein (1:5,000 dilution, Syn 211), anti-oligomer (1:2,000 dilution, A11) Membranes were washed three times with TBS-T, followed by incubation with the HRP-conjugated secondary antibody (1:10,000 dilution, Jackson ImmunoRearch) in blocking buffer for 1 h at 4° C under constant rocking. After being washed three ore times with TBST, the blots were developed using ECL reagents (BioRad) and visualized on a ChemiDoc XRS+ molecular imager (BioRad).

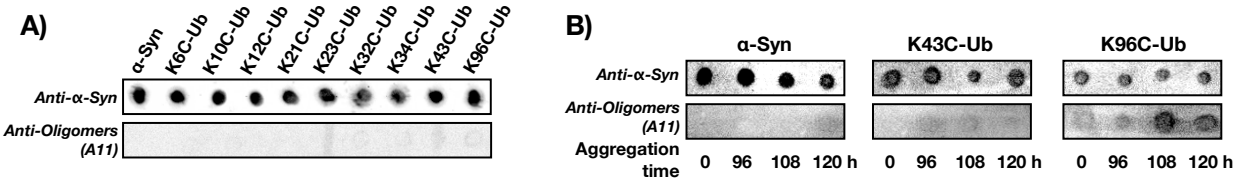


Figure S6. Dot blotting analysis. A) Equal amounts (~3 µg) of synuclein (α-Syn) and α-synuclein lysine to cysteine ubiquitinated derivatives (K#C-Ub) were analyzed by dot-blotting with an anti-α-synuclein antibody (Syn 211) followed by deactivation and incubation with an oligomer-specific antibody (A11).

α-Synuclein Aggregation. Proteins were dissolved to a concentration of 100 µM in phosphate buffer (10 mM phosphate buffer pH 7.4 with 0.05% NaN_3) at 0 °C. The resulting solution was cleared of any aggregates by centrifugation (14,000 x *g*, 15 min, 4 °C) and aliquoted in triplicate in microcentrifuge tubes. Samples were incubated at 37 °C with constant agitation using a small magnetic stirring bar for indicated times. Aliquots were removed every 12 h over the course of the experiment to analyze aggregation behavior.

Thioflavin T (ThT) fibrillation assay. Readings were carried out at a ThT concentration of 20 µM and a protein concentration of 2.5 µM in 10 mM phosphate buffer, pH 7.4. Measurements were recorded on a Tecan GENios spectrometer (Phenix Research Products). The protein was incubated for 1 min prior emission scans with a step size of 1 nm and integration of 1 s. An average of 4 scans was obtained. The fluorescence intensity was monitored using an excitation wavelength of 450 nm ± 10 nm and measuring the emission at 482 nm. All samples were analyzed in triplicate and corrected for the fluorescence level of ThT in buffer.

Transmission electron microscopy (TEM). Samples were negatively stained for transmission electron microscopy studies. Specifically, carbon-coated formvar films mounted on copper grids were floated on a 10 µL droplet of sample for 5 minutes and the excess liquid was removed from the grids with a filter paper. The grids were then stained with 1% uranyl acetate for 5 minutes, followed by two washes in 1% uranyl acetate. A JEM-1400 transmission electron microscope (JEOL) accelerated to 100 kV was used for specimen observation.

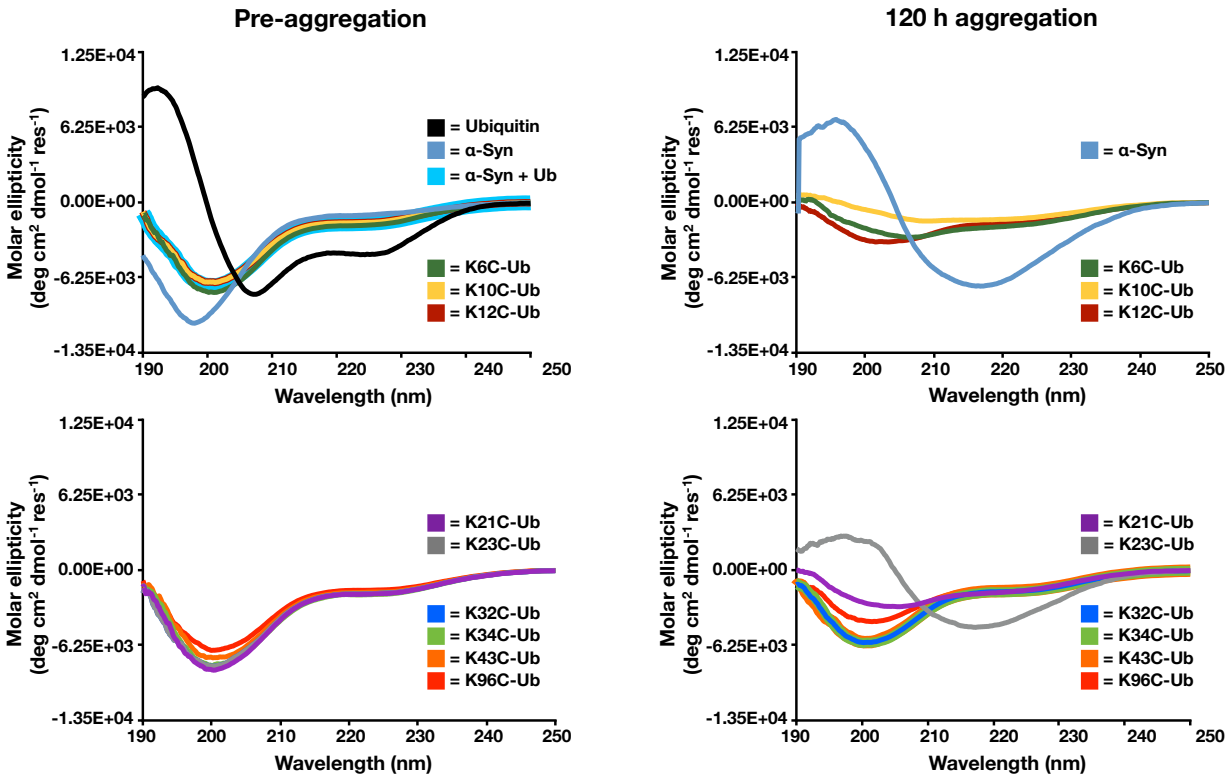


Figure S7. Aggregation of ubiquitinated α-synuclein by CD. CD spectra of purified proteins as in Figure 2B and purified α-synuclein (α-Syn) and the disulfide-directed ubiquitinated derivatives (K#C-Ub) at a concentration of 100 μM incubated at 37 °C for 5 days.

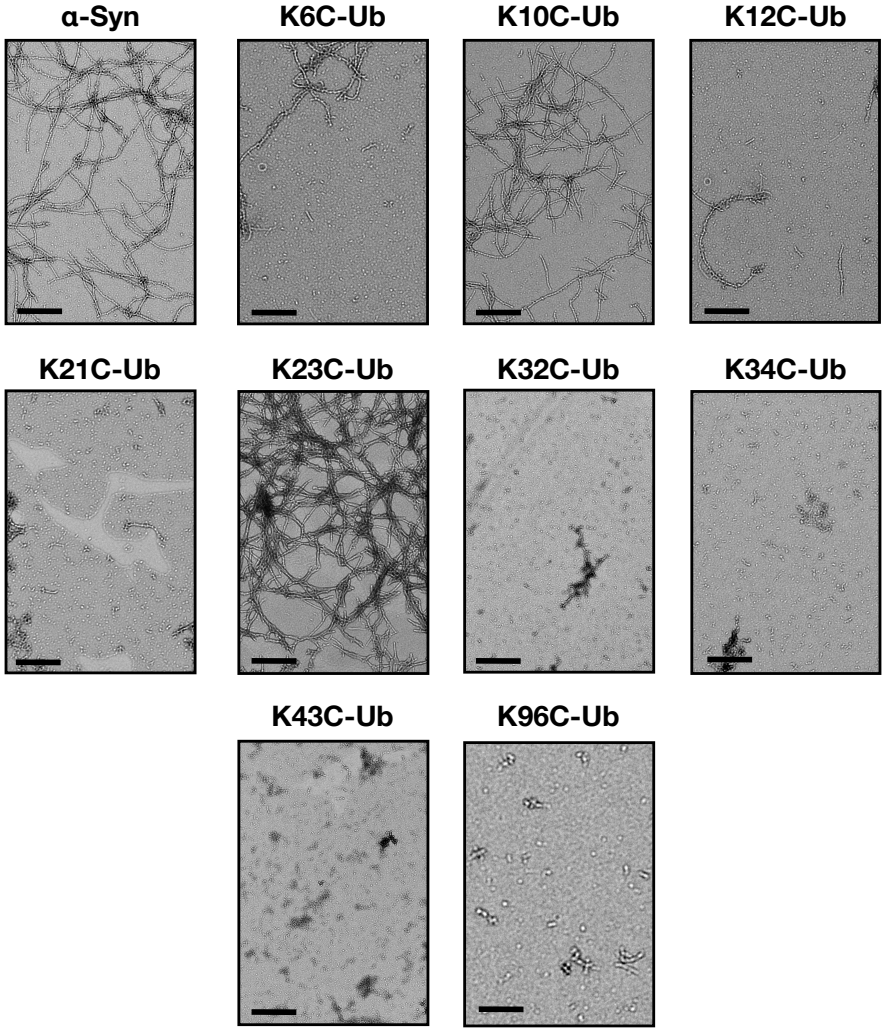


Figure S8. Aggregation of ubiquitinated α-synuclein by TEM. Purified α-synuclein (α-Syn) and the disulfide-directed ubiquitinated derivatives (K#C-Ub) at a concentration of 100 μM were incubated at 37 °C for 5 days and analyzed by TEM; scale bar: 500 nm.

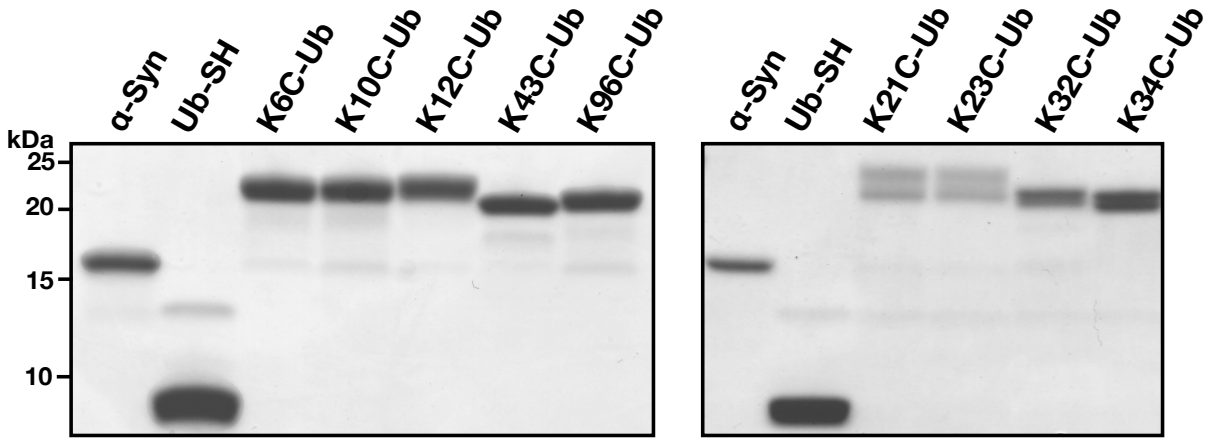


Figure S9. Characterization of ubiquitinated α-synuclein post aggregation. α-Synuclein (α-Syn), ubiquitin C-terminal thiol (Ub-SH, **2**), α-synuclein lysine to cysteine ubiquitinated derivatives (K#C-Ub) from the end of the aggregation assay were boiled to dissolved aggregates and separated by SDS-PAGE and analyzed by Coomassie staining.

SDS-PAGE. Samples (5 µg, 15 µL) were mixed with 4X loading buffer (5 µL, with or without β-mercaptoethanol as indicated). After thorough mixing and boiling at 98° C for 5 min, the samples were loaded on precast Tris HCl gels (any kD™ Criterion™ TGX™ Precast Gel, BioRad) with 5 µL protein marker (Precision Plus Protein™ Dual Color Standards, BioRad). The gel was run in TRIS/Glycine/SDS Running Buffer (BioRad) at 195 V. For visualization of proteins, gels were soaked in Coomassie Blue (Coomassie Brilliant Blue R-250 Staining Solution, BioRad) and destained with 10% acetic acid, 40% Methanol and 50% H_2O.

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