CHARACTERIZATION OF SEMISYNTHETIC AND NATURALLY N^{α}-ACETYLATED ALPHA-SYNUCLEIN *IN VITRO* AND IN INTACT CELLS: IMPLICATIONS FOR AGGREGATION AND CELLULAR PROPERTIES OF ALPHA-SYNUCLEIN *

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SUPPLEMENTAL FIGURE S1. Semisynthesis of N-Ac α-syn. **A:** SDS-PAGE/Coomassie staining analysis of the progression of the NCL reaction showing the migration shift upon addition of the 10-residue N-terminal peptide. **B**: MALDI-TOF-MS analyses of the progression of the NCL reaction. The ligation product (Ac-α-syn(1-140) A11C) is observed with a mass of 14531.5 Da (theoretical: 14535 Da). **C**: MALDI-TOF-MS analysis after the desulfurization reaction. The desulfurized N-Ac α-syn has an observed mass of 14505.2 Da (theoretical: 14503 Da). Stars indicate desulfurized, unreacted α-syn(11-140) starting material that is removed upon purification. **D:** Analytical RP-UHPLC was performed on a Waters Acquity H-Class UPLC system, with a Waters Acquity BEH300 C4 column (2.1x100 mm, 1.7µm particles) run at 0.6 mL/min. Samples were eluted with a linear gradient of 10% to 90% of solvent B (acetonitrile + 0.1% TFA) against solvent A (water + 0.1% TFA) over 4 min. Absorbance at 214 nm was monitored. **E:** Analytical reversed-phase UHPLC analysis of recombinant N^{α}-acetylated α-syn. Analysis was carried out as described panel **D**.

SUPPLEMENTAL FIGURE S2. Purification of recombinant N-Ac α-syn. **A:** Workflow scheme describing the protein production steps performed before purification. **B-D:** Chromatograms and representative SDS-PAGE /Coomassie staining analyses of each purification step. Left panels show UV absorption chromatograms and elution buffer gradients when applicable. Peaks framed in green correspond to the elution of N-Ac α-syn. Right panels show the corresponding SDS-PAGE analysis, and bands framed in green correspond to the fractions that were pooled before performing the next step. After the final purification by hydrophobic interaction chromatography, pure fractions were pooled and dialyzed overnight at 4°C against 20 mM sodium phosphate pH 7.4.

SUPPLEMENTAL FIGURE S3. Fragmentation mass spectra of Glu-C digested α-syn isolated from HEK cells overexpressing either scrambled shRNA or NatB shRNA-4. **A**: Annotated MS/MS spectrum of acetylated and oxidized α-syn N-terminal peptide ($_{Ac\text{-}Ox}$-MDVF$_{Ox}$-MKGLSKAKE, parent ion mass: 1556.76 Da) obtained by Glu-C digestion of immunoprecipitated α-syn from HEK cells transfected with a scrambled shRNA. **B**: Annotated MS/MS spectrum of non-acetylated and oxidized α-syn N-terminal peptide ($_{Ox}$-MDVF$_{Ox}$-MKGLSKAKE, parent ion mass: 1514.74 Da) obtained by Glu-C digestion of immunoprecipitated α-syn from NatB-silenced HEK cells (transfected with NatB-shRNA4), confirming that unacetylated α-syn is indeed present

in these cells. These plots were obtained using Scaffold version 3.4.5 (Proteome Software Inc.). The non-oxidized peptides were not detected.

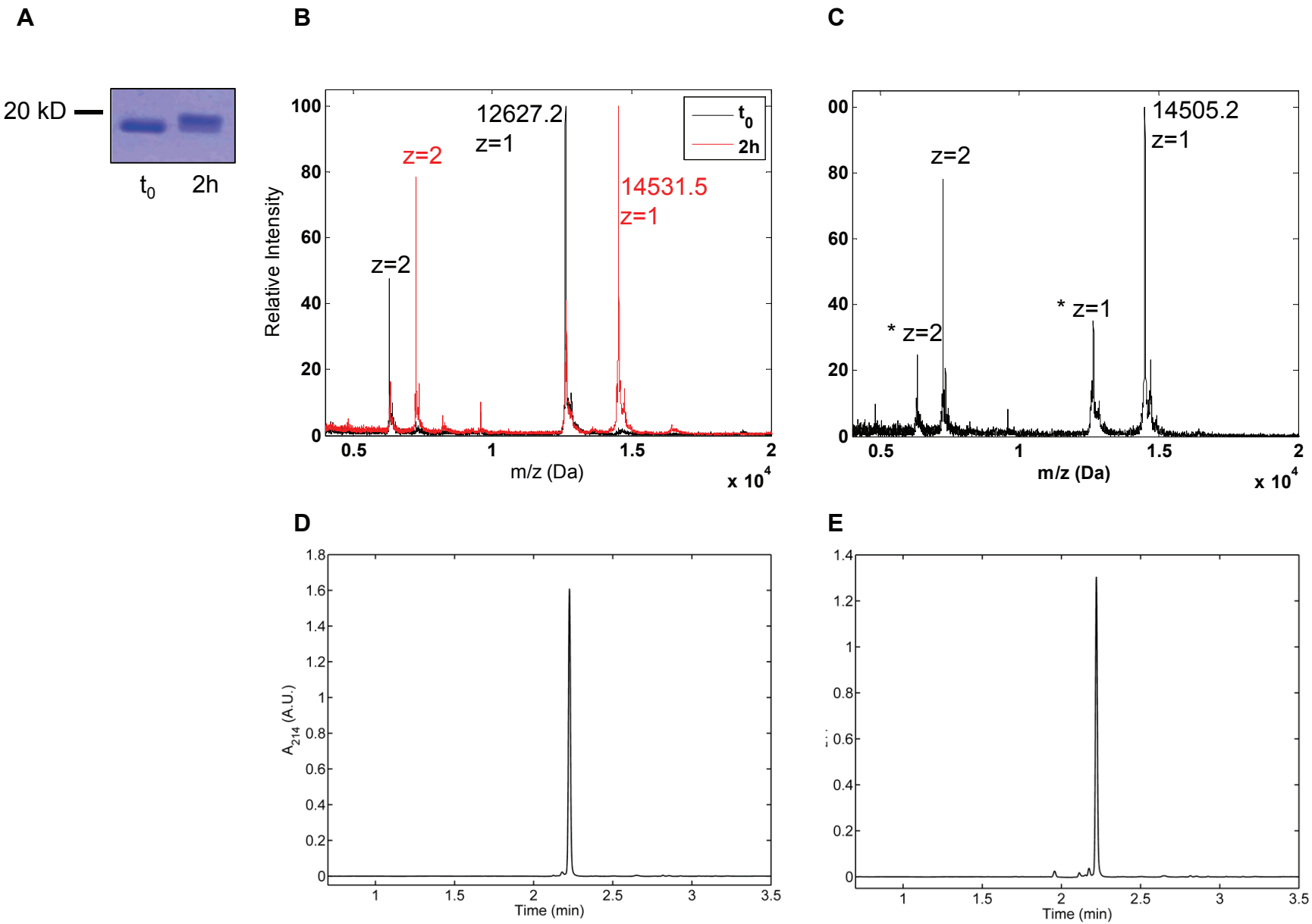
SUPPLEMENTAL FIGURE S4. Purification of recombinant N^{α}-acetylated α-syn in presence of glycerol/BOG. We attempted to replicate the findings reported by Trexler *et al.* (Trexler, A. J., and Rhoades, E. (2012) *Protein science*) by expressing and purifying recombinant N^{α}-acetylated α-syn under the same conditions they described. However, we found that the protein could not be purified to homogeneity just by anion-exchange and gel-filtration chromatography, because of the presence of a major contaminating protein at ~40 kDa. We thus performed an additional hydrophobic interaction chromatography step which allowed us to obtain sufficient purity for biophysical analyses. This method also has the advantage of removing most of the BOG detergent, which elutes just before α-syn. **A:** Preparative gel-filtration chromatogram (left panel) and SDS-PAGE/Coomassie blue analysis (right panel). 0.5 mL of sample obtained from ion-exchange chromatography were injected at 0.5 mL/min in a HiLoad 16/60 Superdex 200 column. SDS-PAGE analysis shows a major contaminant at ~40 kDa. **B:** Preparative hydrophobic interaction chromatogram (left panel) and SDS-PAGE/Coomassie blue analysis (right panel) of the purified protein. Pooled fractions from gel-filtration chromatography were injected in two HiTrap Phenyl HP 5 mL columns in series. The first peak contains the BOG detergent, and N^{α}-acetylated α-syn elutes in the second peak. **C:** circular dichroism spectra of denatured recombinant wt (non-acetylated) α-syn as a control (black line) and recombinant N^{α}-acetylated α-syn (5μM, red line) after dialysis of the pure HIC fractions. 10 spectra acquired using a 1 mm quartz cell were averaged. **D:** Analytical gel-filtration / light scattering analysis of denatured recombinant wt (non-acetylated) α-syn as a control (black lines) and recombinant N^{α}-acetylated α-syn (red lines). This analysis was done by equilibrating the analytical SEC column with a buffer free of glycerol, BOG, and ammonium sulfate, therefore the purified N^{α}-acetylated α-syn could be injected directly after HIC without the need for a prior dialysis step. 100 μL of samples were injected at 0.4 mL/min. Full lines represent normalized UV absorbance (left ordinate axis) while dashed lines correspond to calculated molecular weights(within the elution peak of the major species (right ordinate axis). Note that the results shown above may not be directly compared to those obtained by Trexler *et al.* (Trexler, A. J., and Rhoades, E. (2012) *Protein science*) because of the additional hydrophobic interaction chromatography step that we employed here.

SUPPLEMENTAL TABLE S1. Tandem mass spectrometry analysis of the N-terminal peptide obtained by Glu-C digestion of wt α-syn immunoprecipitated from HEK cells overexpressing wt human α-syn. Parent ion m/z: Observed: 1525.71 Da, theoretical, 1525.78 Da

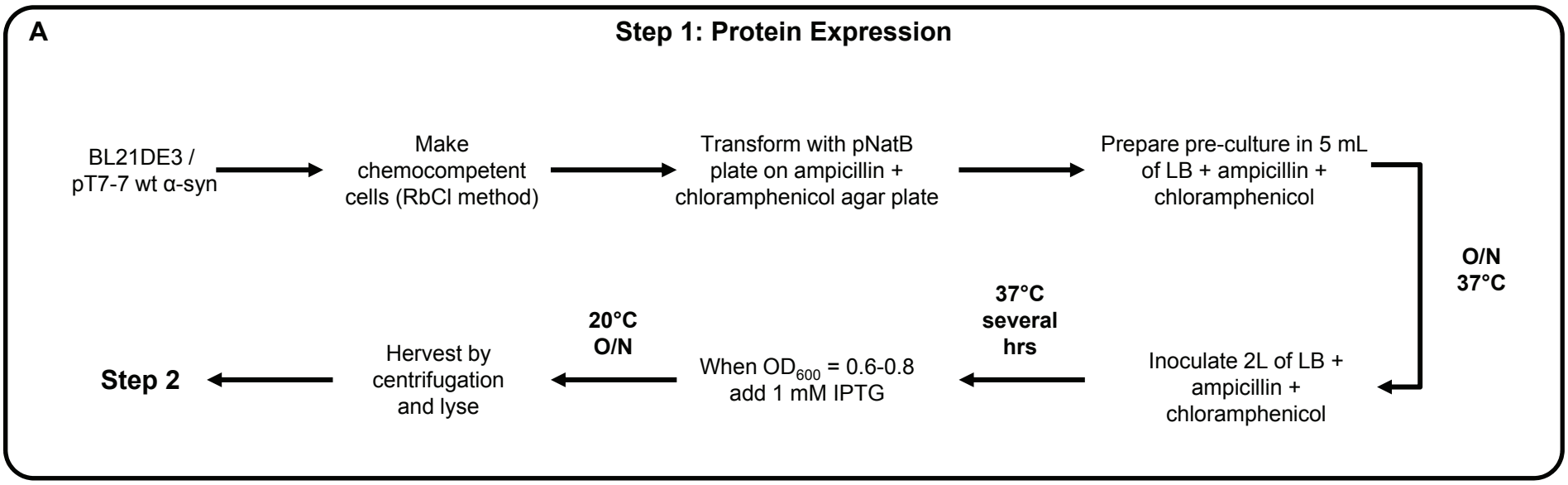
b ion series			y ion series		
Fragment Ion	Observed m/z (Da)	Theoretical m/z (Da)	Fragment Ion	Observed m/z (Da)	Theoretical m/z (Da)
b1	174.07	174.06	y1	N/A	148.06
b2	289.09	289.09	y2	276.14	276.16
b3	388.13	388.15	y3	347.15	347.19
b4	535.13	535.22	y4	475.21	475.29
b5	N/A	666.26	y5	562.25	562.32
b6	N/A	794.36	y6	675.34	675.40
b7	851.36	851.38	y7	732.37	732.42
b8	964.46	964.6	y8	860.48	860.52
b9	1051.47	1051.49	y9	991.52	991.56
b10	1179.63	1179.59	y10	1138.63	1138.63
b11	1250.68	1250.63	y11	1237.72	1237.70
b12	1378.85	1378.72	y12	1352.80	1352.72
b13	N/A	1507.76	y13	N/A	1525.78

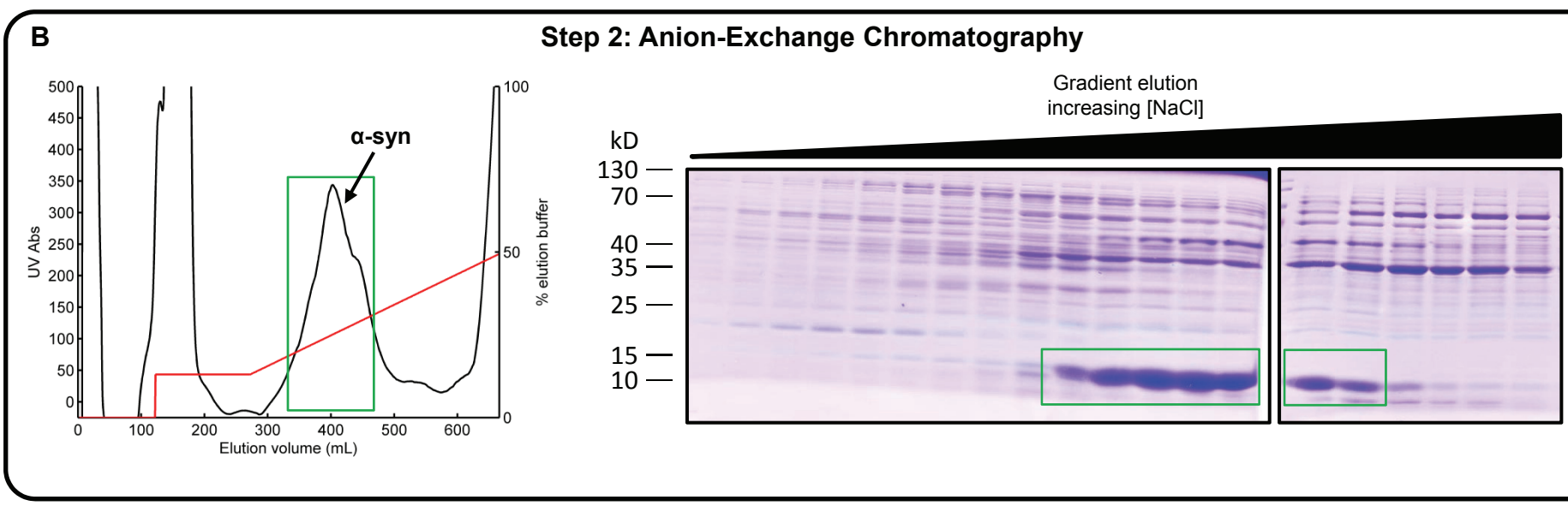
SUPPLEMENTAL TABLE S2. Tandem mass spectrometry analysis of the N-terminal peptide obtained by Glu-C digestion of D2P α-syn immunoprecipitated from HEK cells overexpressing D2P human α-syn. Parent ion m/z: Observed:1334.68 Da, theoretical, 1334.75 Da

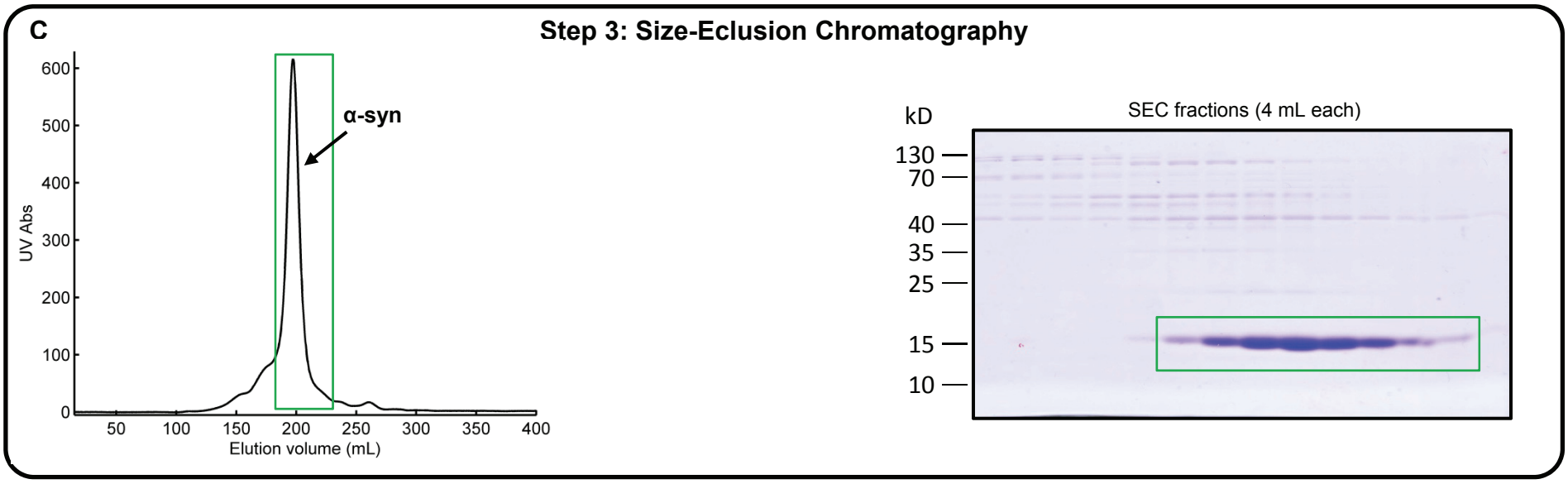
b ion series			y ion series		
Fragment Ion	Observed m/z (Da)	Theoretical m/z (Da)	Fragment Ion	Observed m/z (Da)	Theoretical m/z (Da)
b1	N/A	98.06	y1	N/A	148.06
b2	197.12	197.13	y2	276.16	276.16
b3	344.18	344.20	y3	347.16	347.19
b4	475.24	475.24	y4	475.24	475.29
b5	603.32	603.33	y5	562.29	562.32
b6	660.33	660.35	y6	675.33	675.40
b7	773.43	773.44	y7	732.39	732.43
b8	860.50	860.47	y8	860.50	860.52
b9	988.61	988.57	y9	991.63	991.56
b10	1059.68	1059.60	y10	1138.74	1138.63
b11	1187.84	1187.70	y11	N/A	1237.70
b12	N/A	1316.74	y12	N/A	1334.75

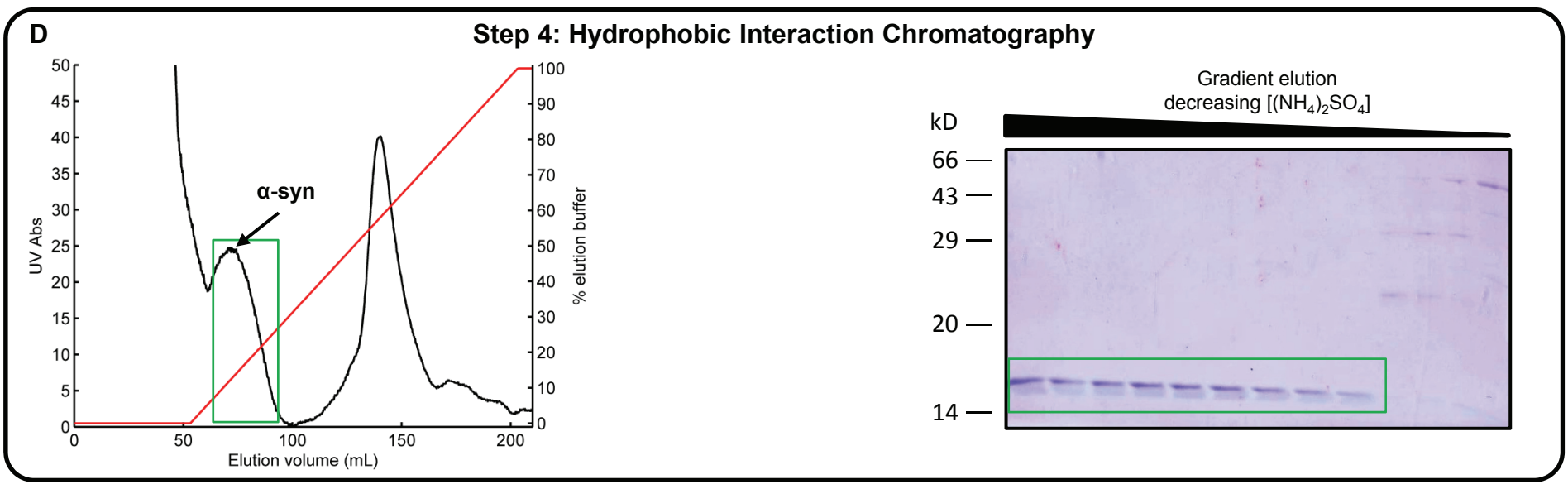


Supplemental Figure S1









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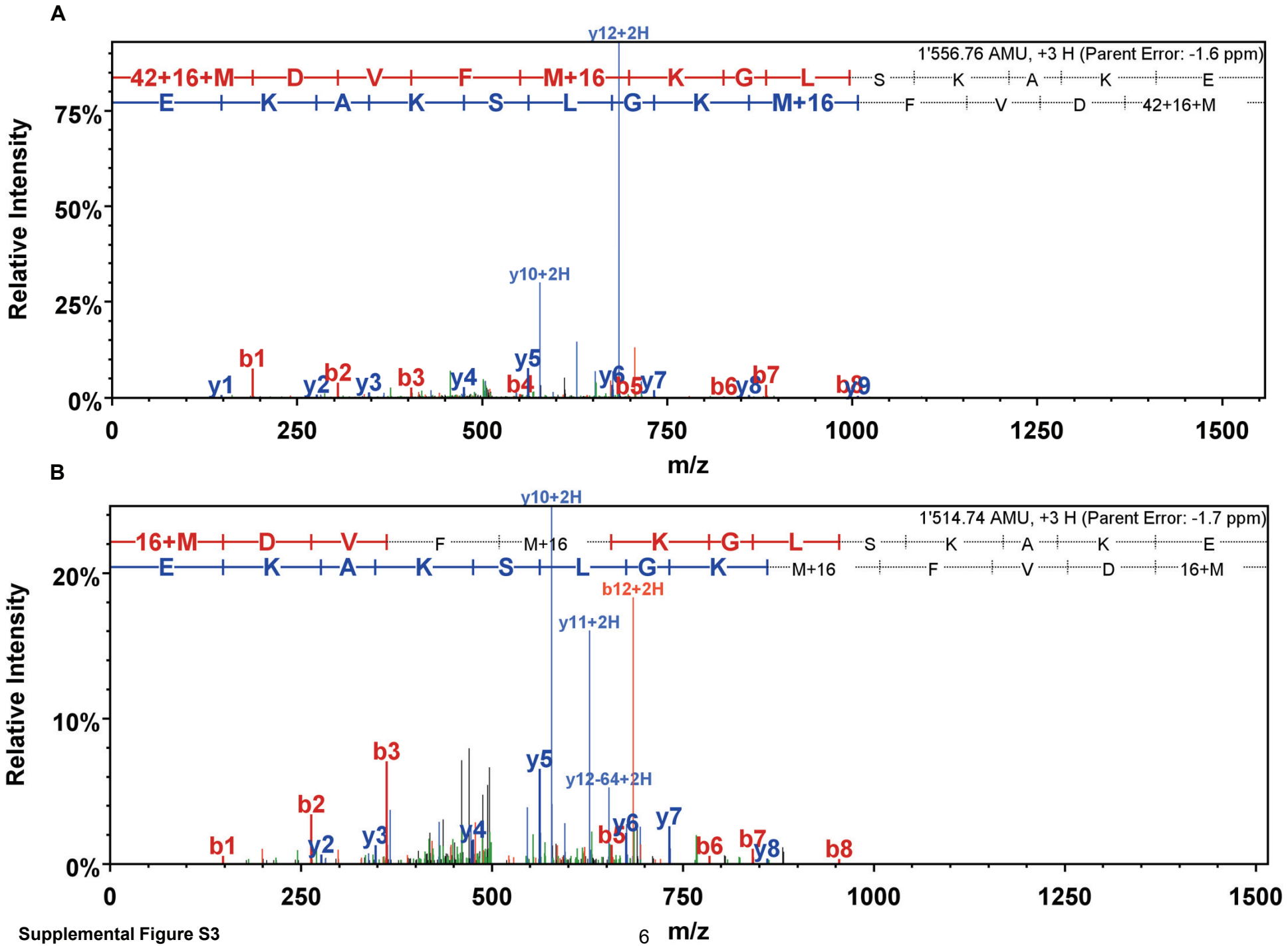

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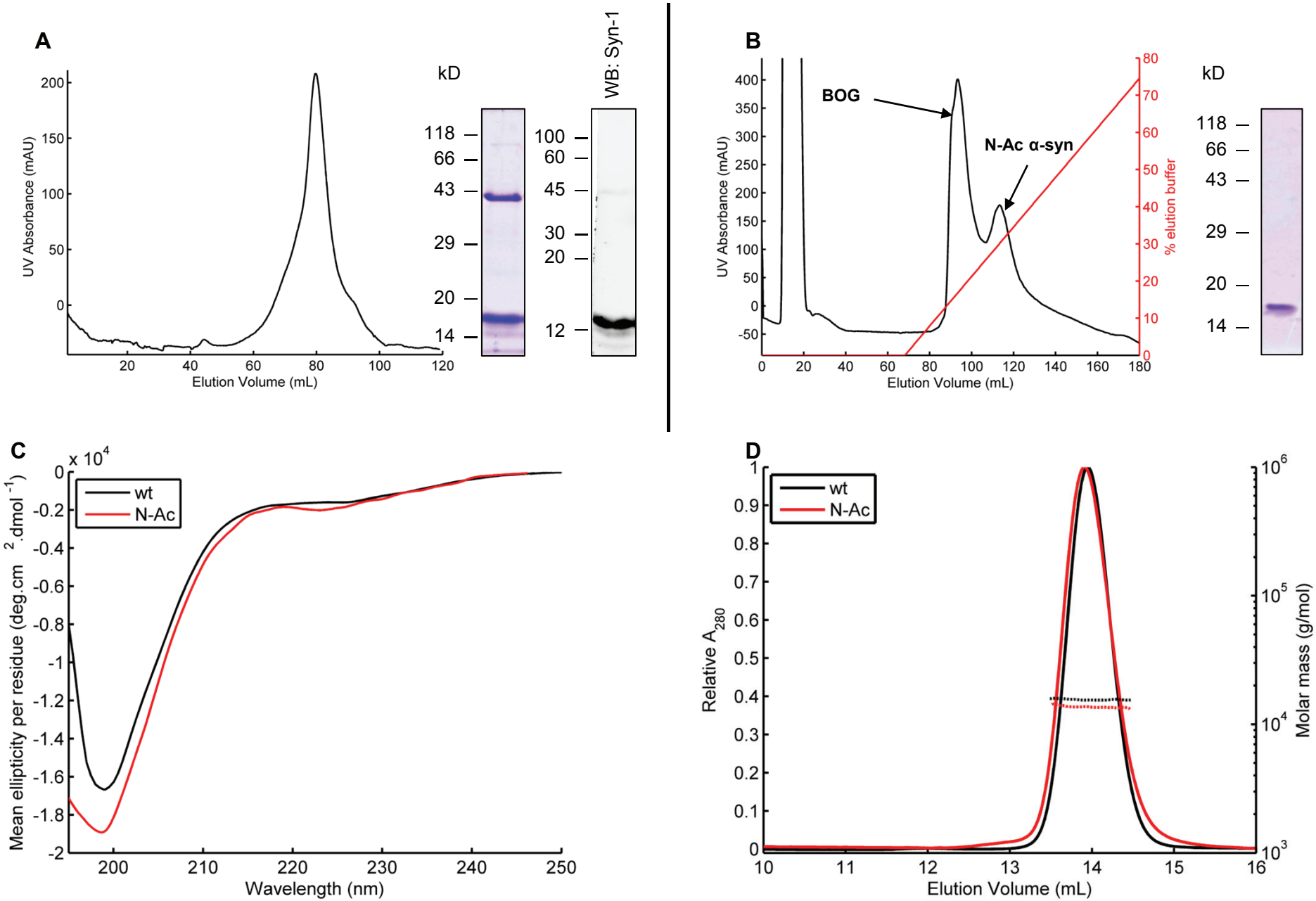

Supplemental Figure S4