Supporting Information

Copper Binding and Subsequent Aggregation of α-Synuclein Are Modulated by N-Terminal Acetylation and Ablated by the H50Q Missense Mutation

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Contents

1. Methods

1.1 Protein expression and purification

All reagents and globular proteins were purchased from Sigma-Aldrich, UK. Human wild type α-syn was expressed in *E.coli* and purified as described previously[1]. N-terminally acetylated α-syn was produced through co-expression of the α-syn plasmid with the pNatB (pACYCduet-naa20-naa25) plasmid encoding the N-acetyltransferase B complex, a gift from Dan Mulvihill (Addgene plasmid # 53613)[2]. Successful modification was demonstrated by a +42 Da increase in mass over the unmodified protein, giving an observed mass of 14501.63 Da ±0.48. The H50Q mutant was created based on the pET23a plasmid encoding WT human α-syn with site directed mutagenesis using the QuikChange® II XL Site-Directed Mutagenesis Kit by Stratagene following manufacturer's instructions. Primers used were:

F: 5' GCGTTGTCCAAGGGGTTGCG 3' R: 5' CGCAACCCCTTGGACAACGC 3'

Plasmids were isolated using a QIAprep Spin Miniprep Kit, following manufacturer's instructions. Mutations were confirmed by DNA sequencing. The concentration of pure monomeric α-syn was determined using the molar extinction constant 5960 M^{-1} cm^{-1}.

1.2 Sample preparation

Samples were prepared by dissolving α-syn and $CuCl_2$ to a final concentration of 10 µM in 50 mM ammonium acetate pH 7. Protein to metal ratios of 1:0 and 1:1 were prepared and mixed immediately prior to analysis.

1.3 ESI-IMS-MS analysis

All experimentation was performed on a Synapt G2 HDMS instrument (Waters, Manchester, UK) equipped with a Triversa (Advion Biosciences) automated nano-ESI interface in positive mode. Positive ESI was used with a capillary voltage of 1.7 kV and nitrogen nebulizing gas pressure of 0.7 psi. The following instrument parameters were used for data acquisition: cone voltage 45 V, source temperature 60 °C, backing pressure 3.5 mbar, trap collision energy 15 V, transfer 20 V, IMS wave velocity 600 m/s, IMS wave height 40V, IMS gas flow 90 mL/min. Mass calibration was carried out by an infusion of CsI cluster ions. For attaining denatured spectra, all calibrants and mass standards were prepared before injection at 10-20 µM dissolved in 50% acetonitrile, 10% formic acid and 40% ultrapure water (v/v/v). A calibration curve for collision cross-sectional areas was obtained based on of multiple charge states of cytochrome c from horse heart, myoglobin from equine heart and ubiquitin from bovine erythrocytes as described previously[3]. Drift times were corrected for both

mass-dependent and independent time of flight. Arrival time distributions were determined by using the Mass Lynx v4.1 software (Waters, Manchester, UK). ATD for each charge state at its respective m/z value were extracted from the Driftscope plots available within the MassLynx suite of software. Plots were then fitted using least square regression to a minimum number of Gaussian distributions using multiples of the following equation:

$$y = \frac{A}{w\sqrt{\pi/2}}\, e^{\frac{-(x-x_0)^2}{2.w^2}}$$

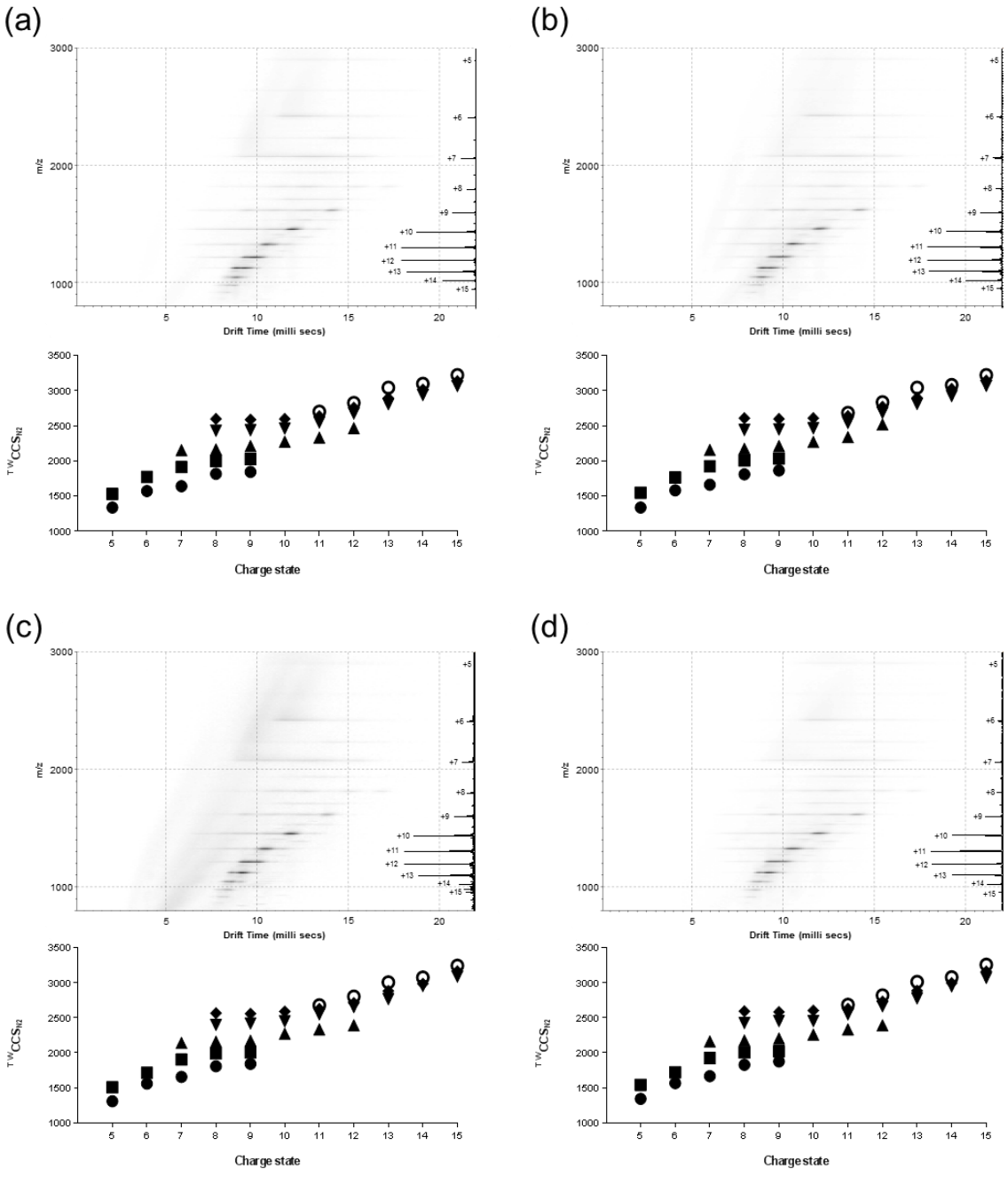
Where A = total area under the curve from the baseline, x_o = centre of the peak, and w = width of the peak at half height. This model describes a bell-shaped curve akin to the normal (Gaussian) probability distribution function.

1.4 ThT fluorescence

Thioflavin T (ThT) fluorescence was monitored for 70 µM unmodified and acetylated α-syn, with and without equimolar $CuCl_{2.}$. Readings were taken every 60 minutes in 50 mM Tris pH 8 with 10 µM Thioflavin-T with a glass beads in each well of a black 96 well plate under agitation at 300 rpm at 37 °C using a BMG Labtech CLARIOstar. Lag time was determined by calculating the intercept between the maximum derivative and the pre-transitional base line.

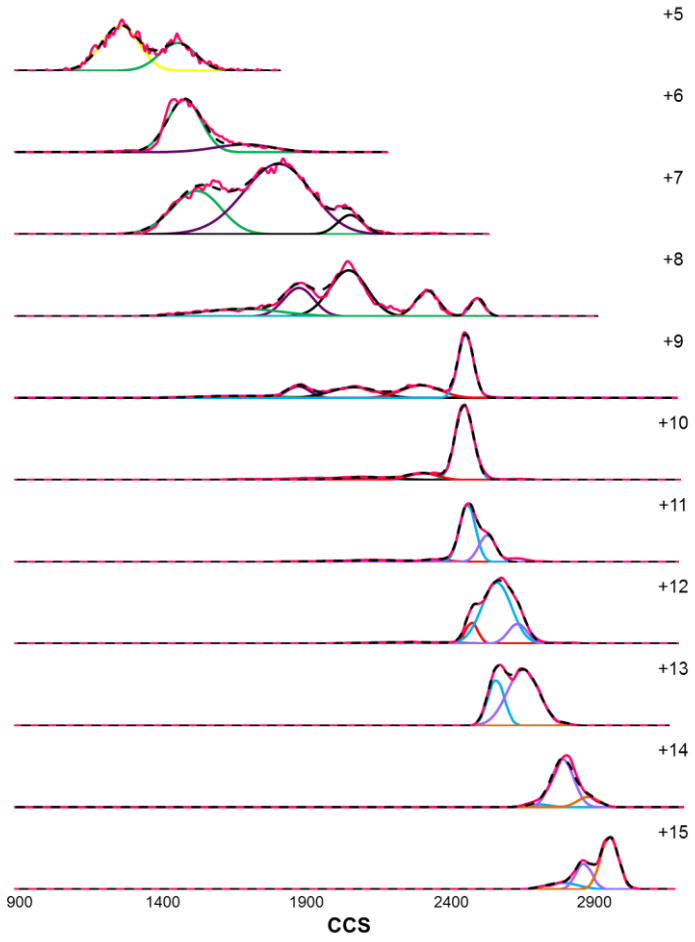
2. Figures

2.1 **Figure S1: Driftscope plot and overlaid mass spectrum of each of the four proteins.** Driftscope plot (square root display)/spectra and resolvable conformational families (determined by fitting ATD of each charge state ion to the minimum number of Gaussian distributions) obtained from 50 mM aqueous ammonium acetate solution of 10 µM (a) unmodified (b) acetylated (c) H50Q and (d) acetylated H50Q α-syn.



2.2 Figure S2: Arrival time distribution for each charge state ion of unmodified WT α-syn. (a) The peak area of each alternate population of differing CCS was calculated from Gaussian distributions using a least square fitting method. Each conformational family is assigned a colour starting with the most compact (1) yellow, (2) green, (3) purple, (4) black, (5) red, (6) blue, (7) lilac and the most expanded (8) orange. The raw ATD is displayed in pink and the sum of the fitted Gaussians is shown as a black broken line. (b) Resolvable conformational families as presented in (a), determined through fitting the ATD of each charge state ion to the minimum number of Gaussian distributions.

(a)



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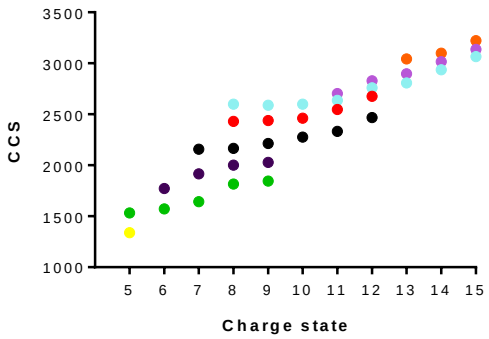
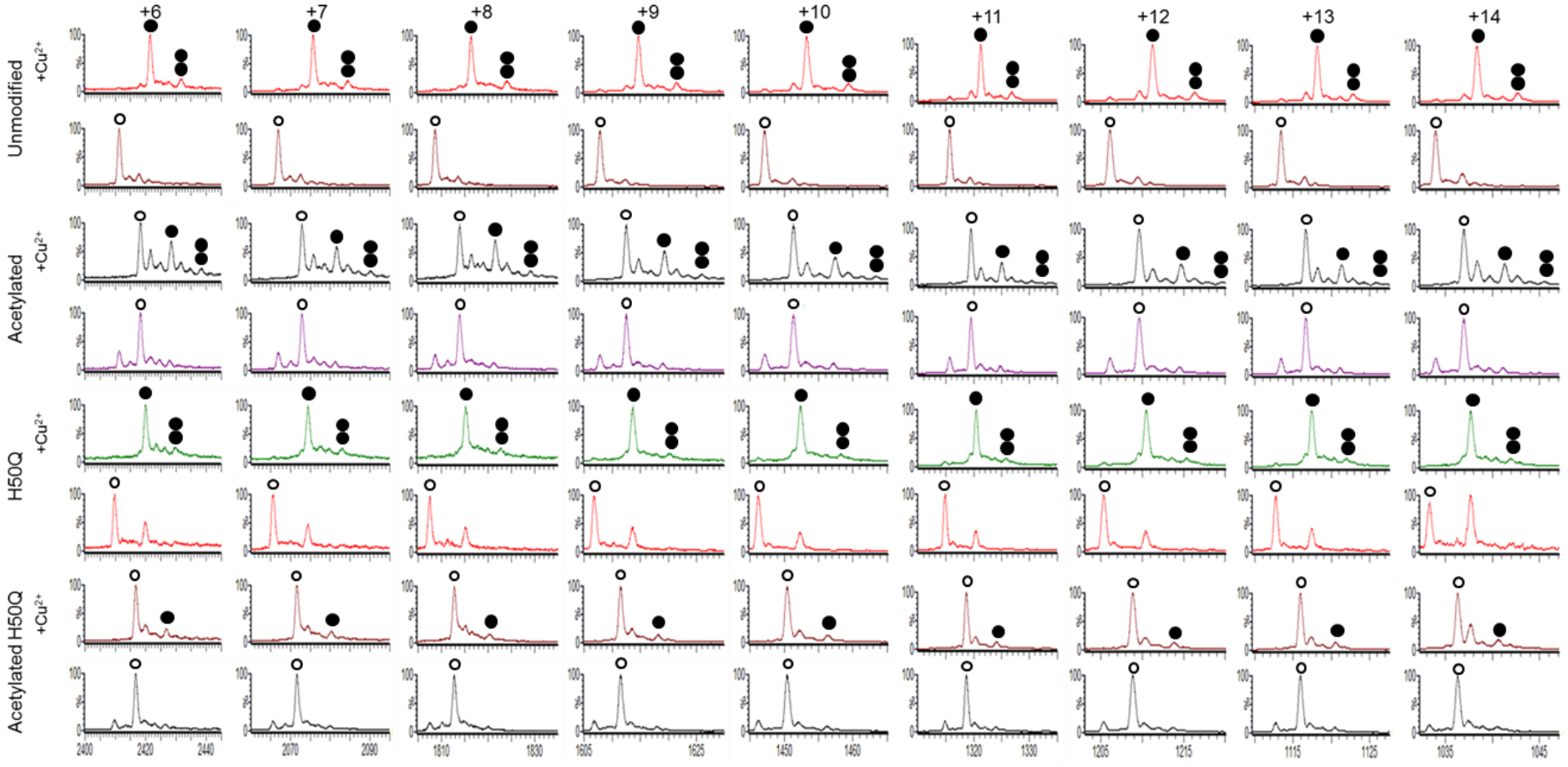
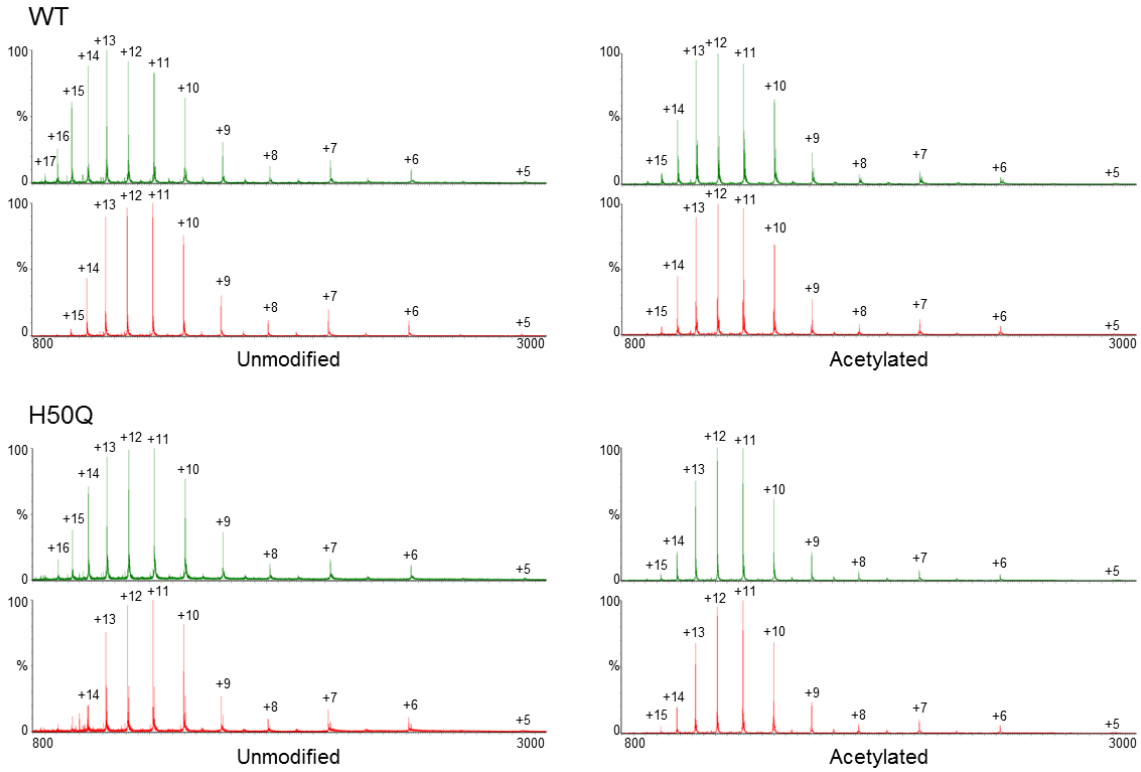


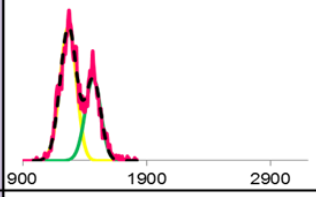
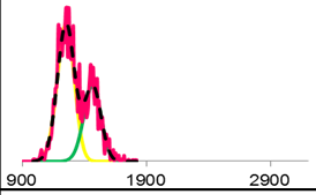
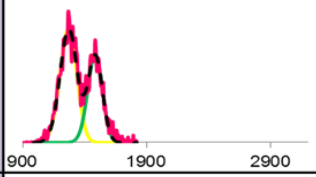
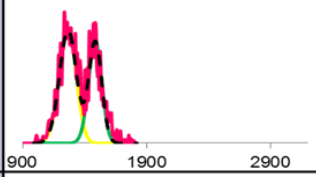
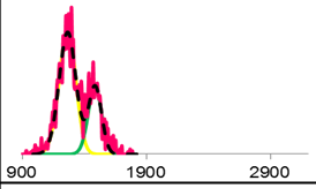
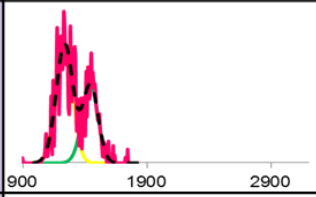
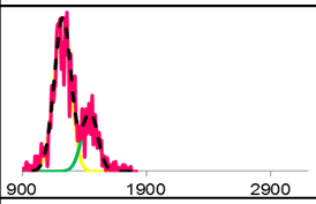
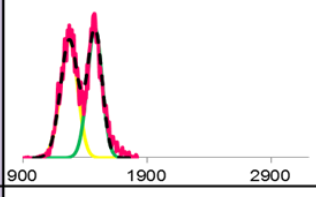
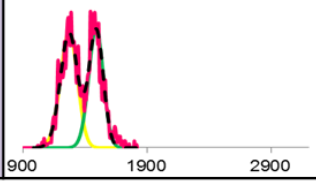
2.3 Figure S3: α-Syn spectra in the absence and presence of equimolar Cu^{2+}. Binding of Cu^{2+} to unmodified and acetylated WT and H50Q α-syn at the +6 to +14 charge state ions. Spectra were acquired at protein to $CuCl_2$ ratios of 1:0 and 1:1 in 50 mM ammonium acetate. The x axis represents *m/z* values and the y axis represents relative abundance.



2.4 Figure S4: Charge state distributions of α-syn in the absence and presence of equimolar Cu^{2+}. Spectra were acquired at protein to $CuCl_2$ ratios of 1:0 (red spectra) and 1:1 (green spectra) in 50 mM ammonium acetate. The x axis represents *m/z* values and the y axis represents relative abundance.



2.5 Figure S5: Arrival time distributions of each charge state ion of all alpha-synuclein species studied. Experimental collisional cross sections of all charge state of unmodified and acetylated WT and H50Q α-syn. Apo and Cu^{2+} bound forms resulting from mixing a 50 mM aqueous ammonium acetate solution of 10 μM α-syn with 10μM $CuCl_2$. The percentage of alternate populations of differing CCS were calculated using Gaussian fitting. The raw ATD is displayed in pink and the sum of the fitted Gaussians is shown as a black broken line.

+5 ion	Unbound	1x Cu^{2+}	2x Cu^{2+}
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

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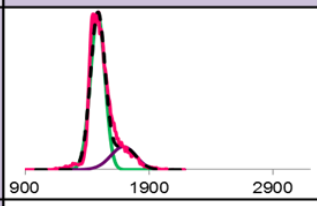
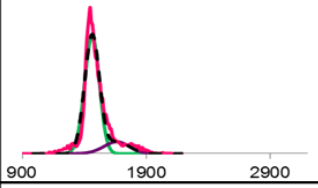
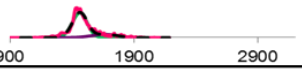
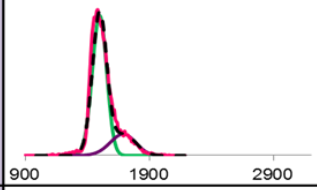
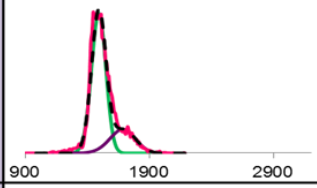
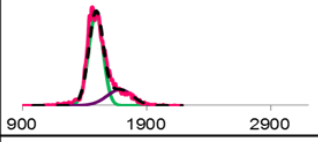
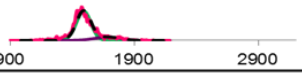
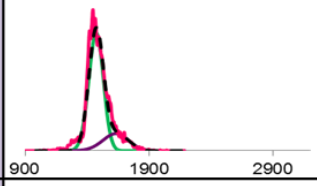
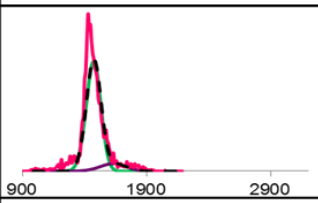
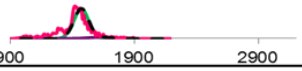
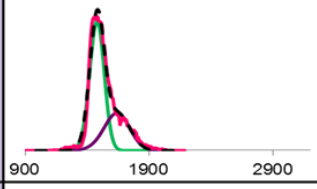
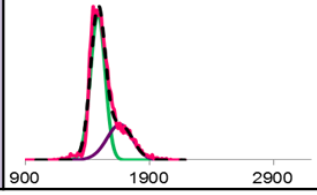
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

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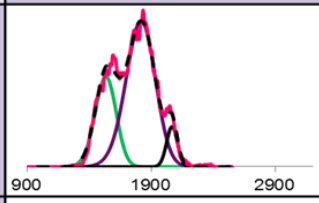
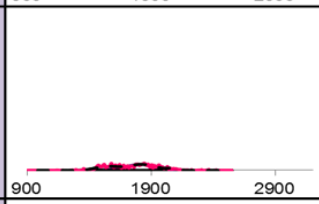
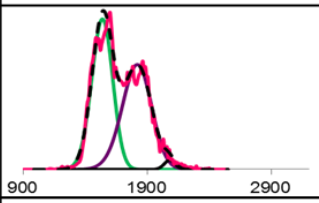
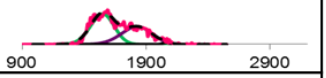
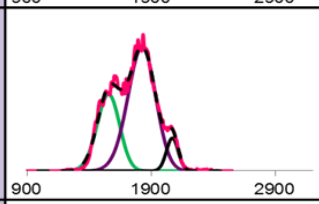
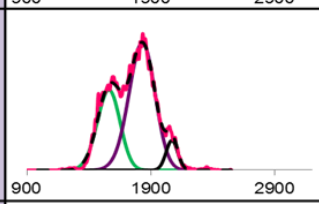
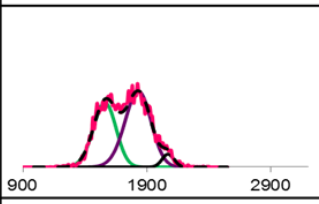
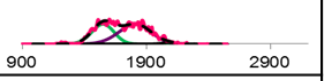
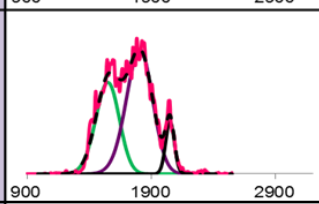
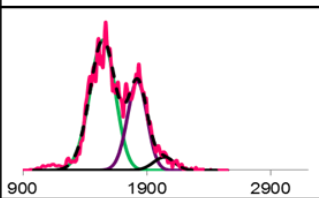
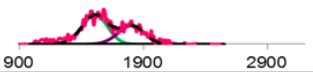
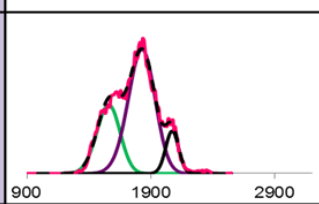
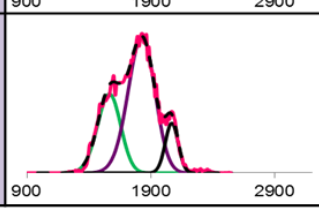
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

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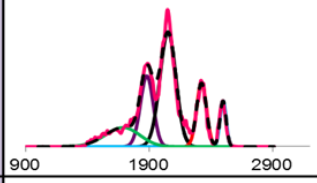
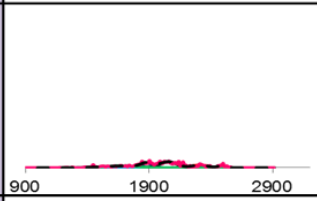
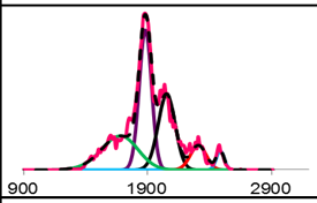
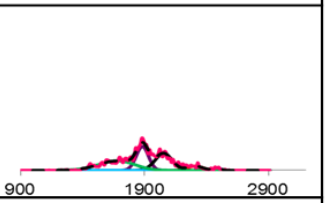
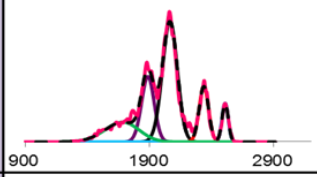
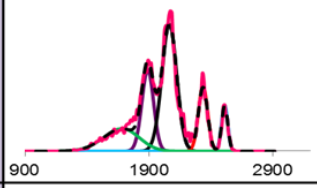
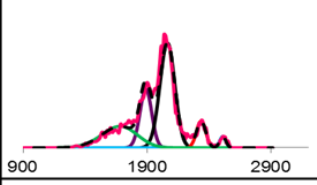
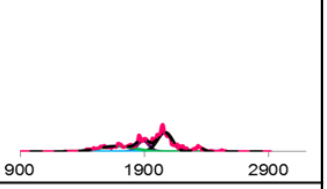
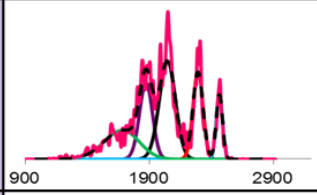
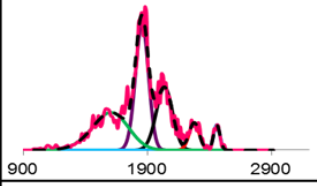
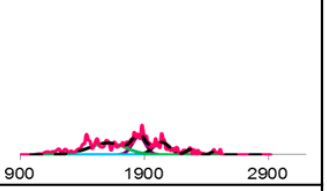
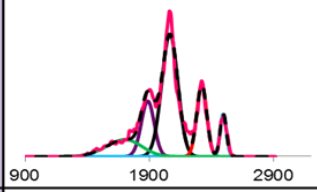
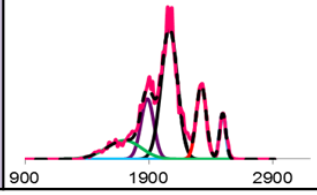
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

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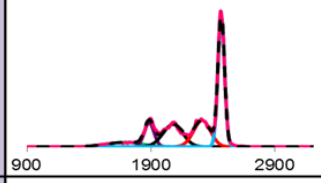
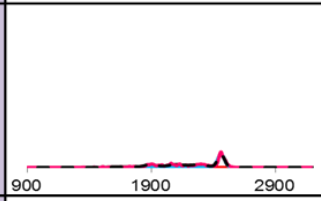
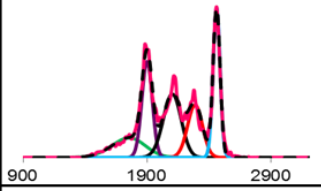
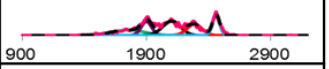
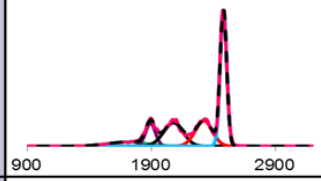
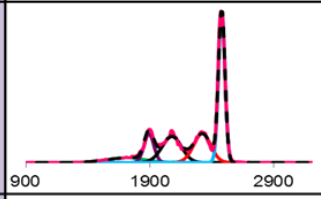
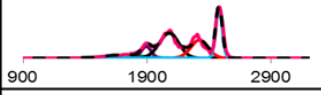
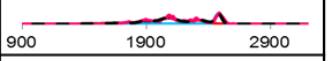
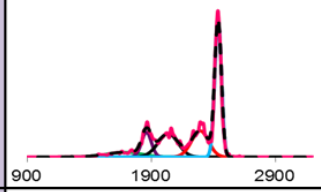
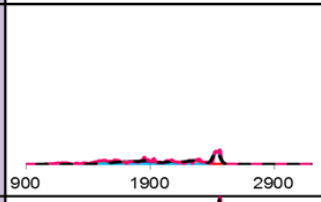
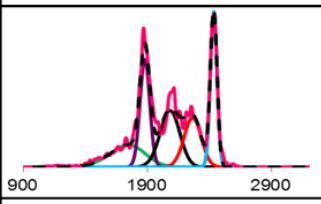
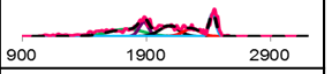
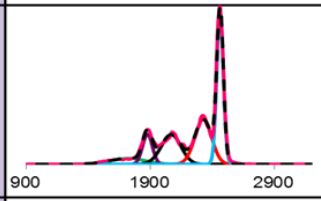
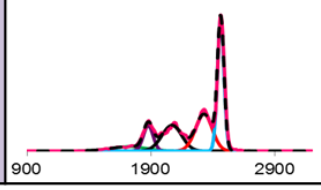
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

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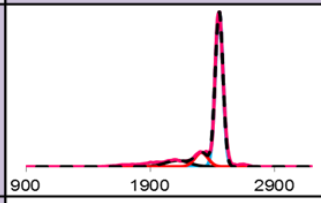
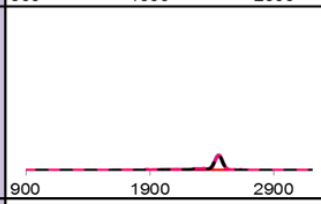
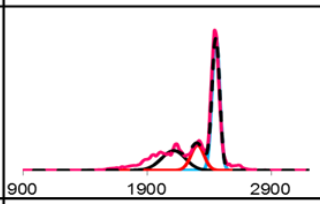
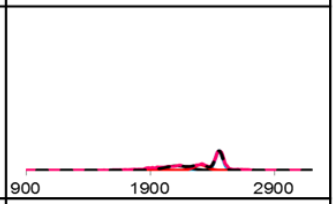
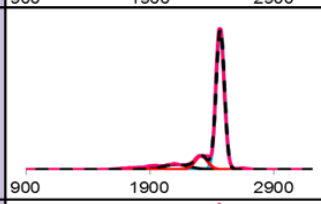
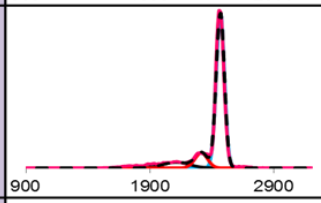
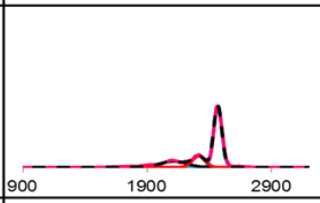
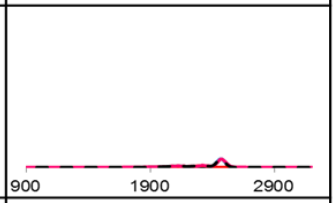
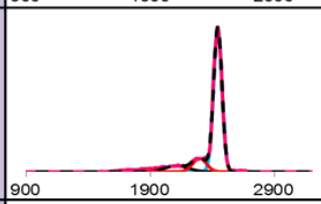
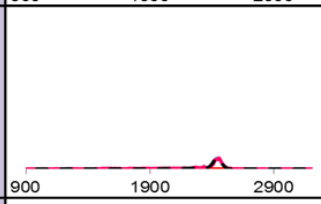
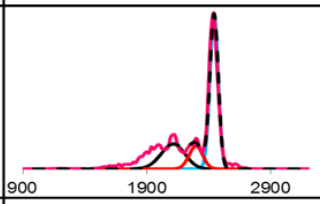
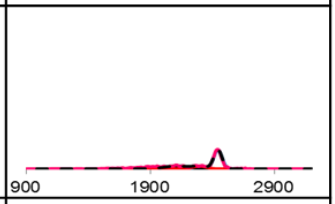
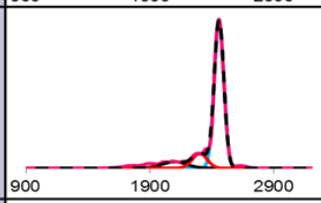
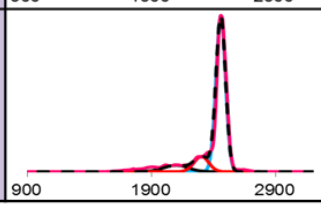
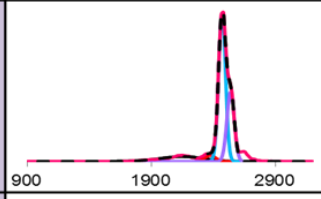
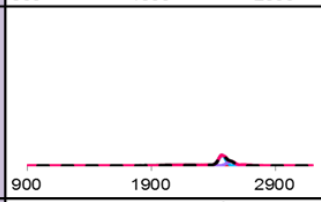
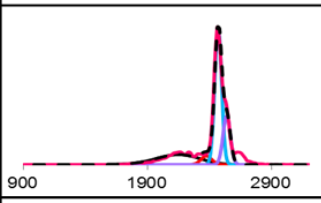
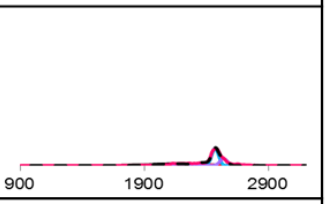
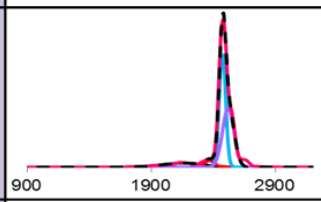
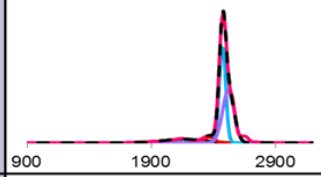
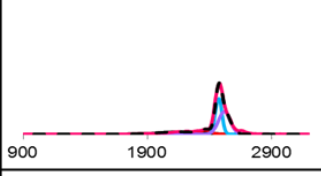
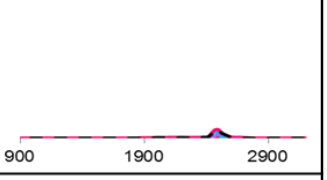
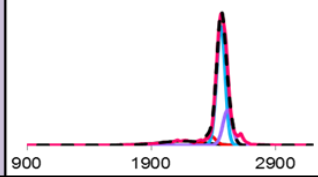
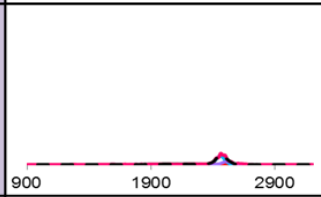
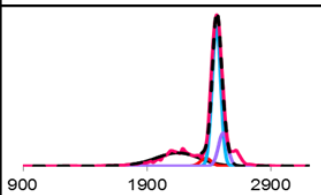
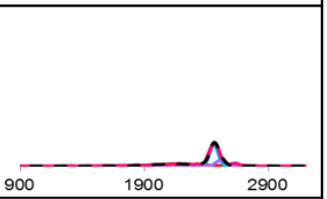
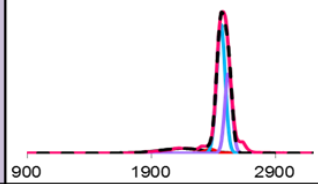
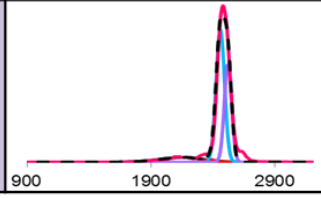
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

+11 ion	Unbound	1x Cu^{2+}	2x Cu^{2+}
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

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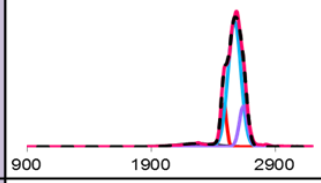
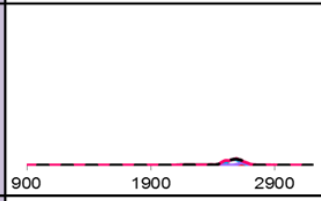
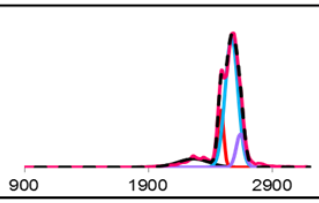
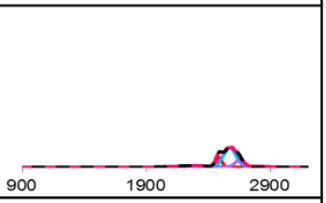
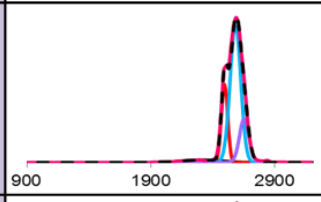
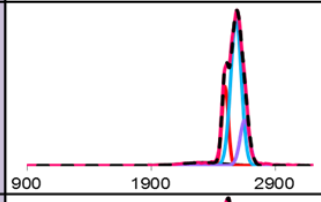
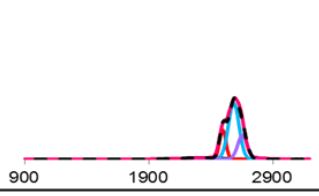
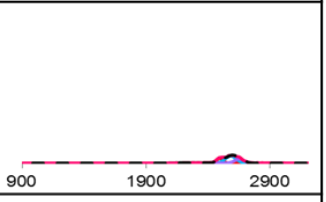
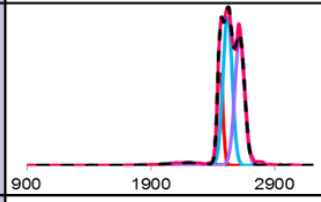
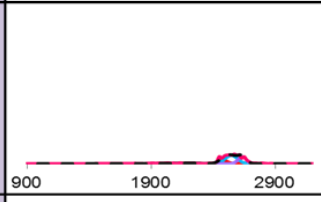
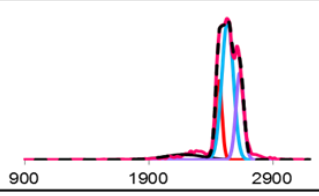
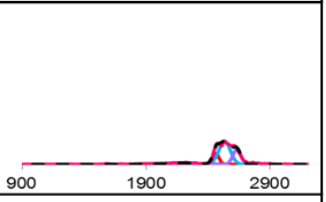
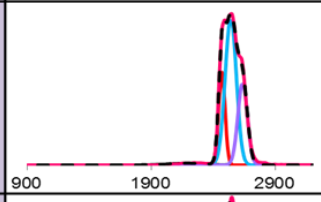
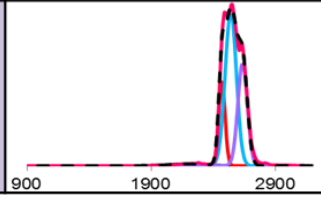
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

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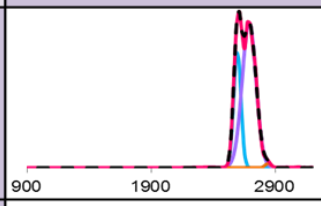
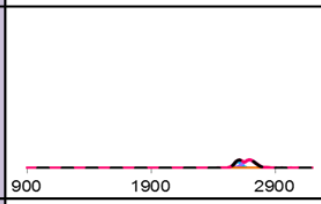
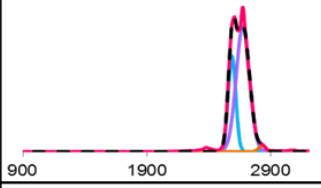
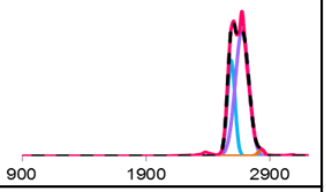
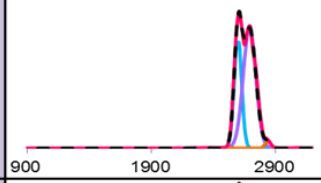
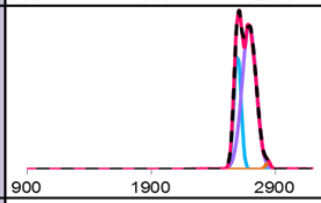
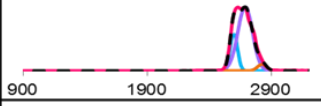
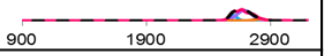
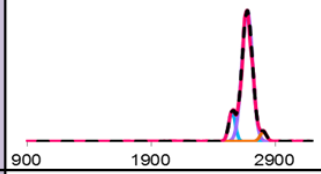
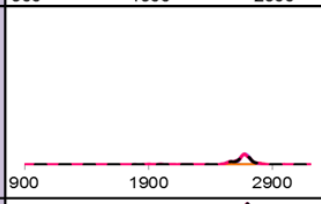
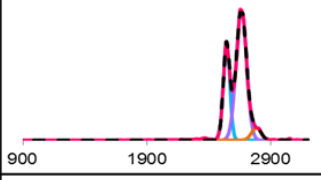
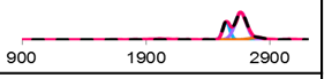
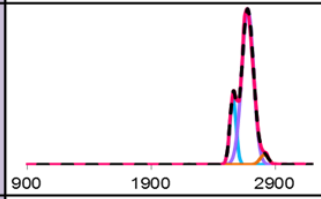
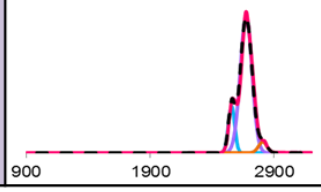
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

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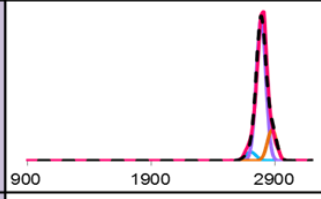
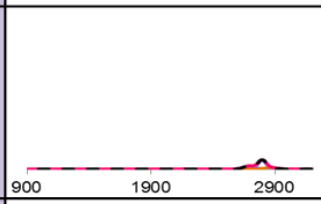
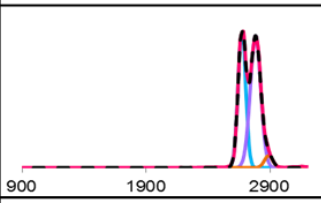
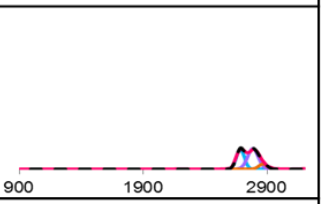
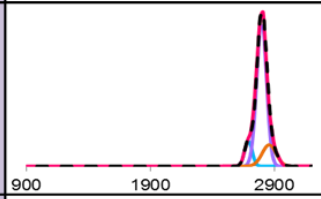
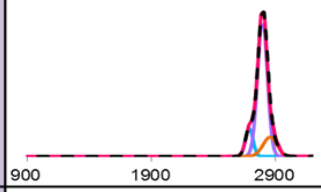
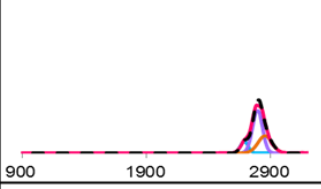
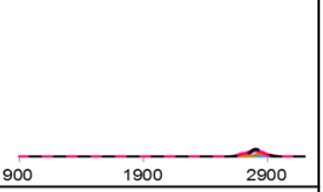
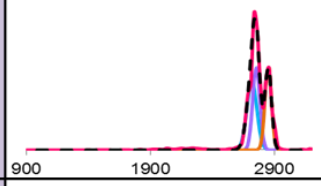
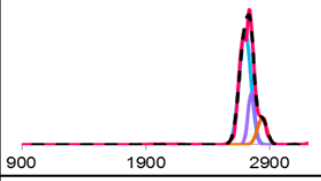
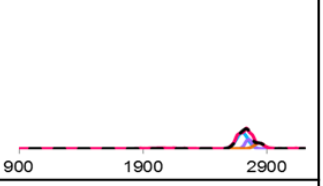
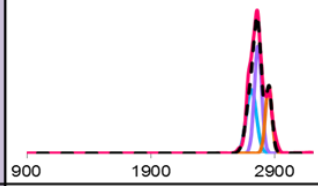
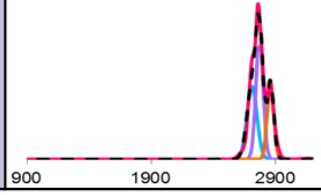
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

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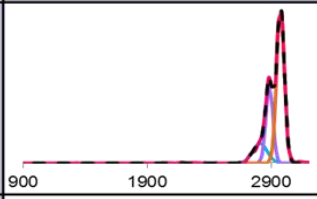
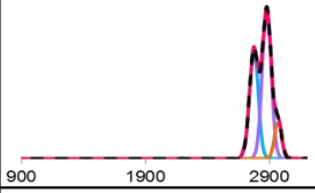
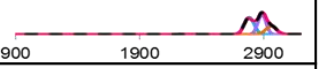
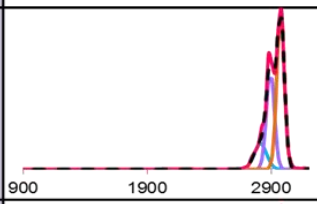
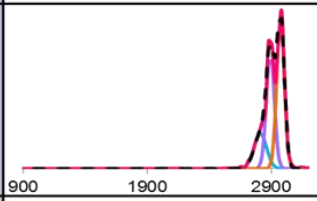
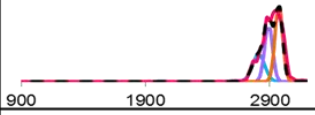
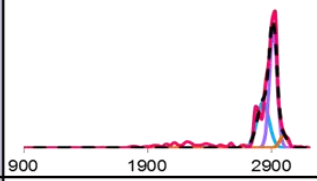
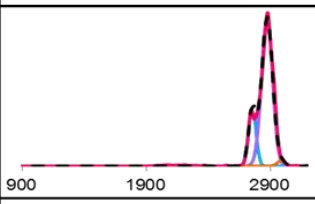
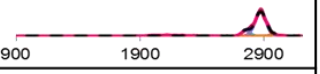
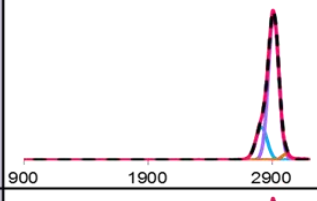
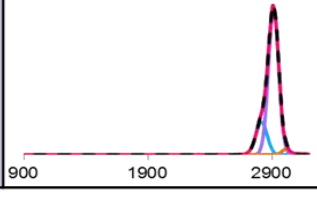
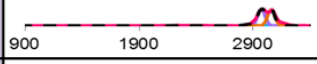
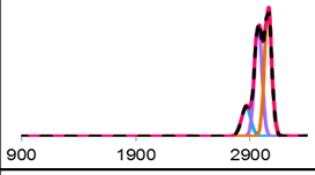
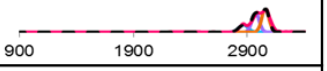
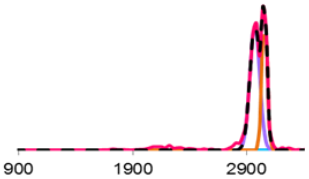
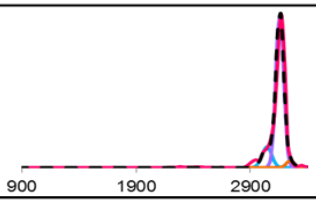
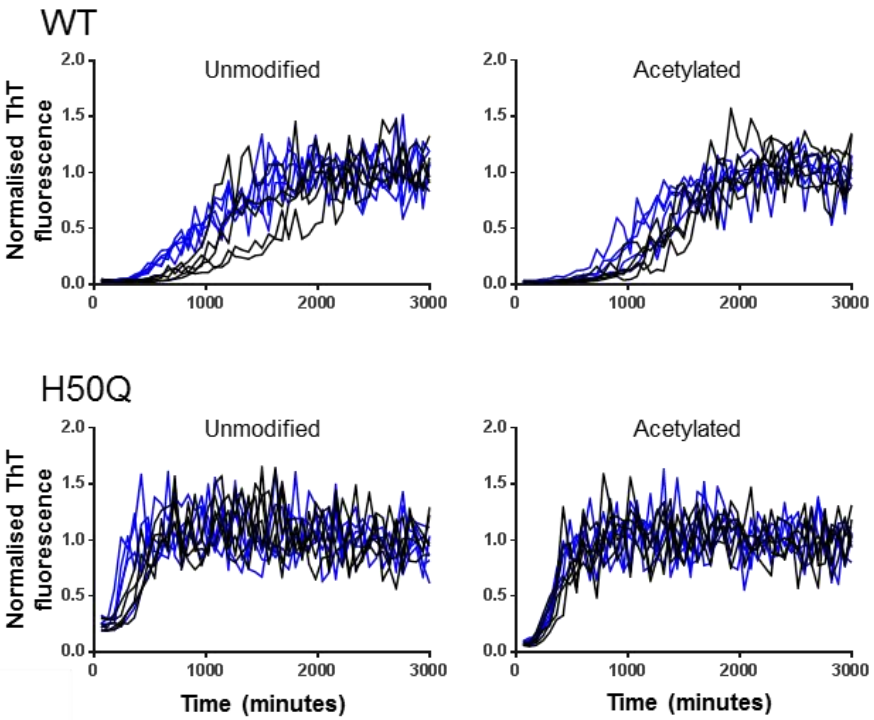
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

+ 16 ion	Unbound	1x Cu^{2+}	2x Cu^{2+}
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

+17 ion	Unbound	1x Cu^{2+}	2x Cu^{2+}
Unmodified			
+ copper			
Acetylated			
+ copper			
H50Q			
+ copper			
Acetylated H50Q			
+ copper			

2.6 Figure S6: Copper induced aggregation of α-syn as monitored by Thioflavin-T. ThT fluorescence of unmodifed (left) and acetylated (right) α-syn. Wild type α-syn (top) and α-syn H50Q (bottom). α-Syn incubated at a concentration of 70 μM alone is shown in black, or in the presence of equimolar $CuCl_2$, shown in blue. In agreement with previous studies[4], in the presence of Cu^{2+} unmodified α-syn shows an increase in the rate of aggregate formation over the apo form, whereas there is little effect on the aggregation rates of the acetylated version of the protein in the presence of this metal. Copper does not affect the aggregation of N-terminally acetylated α-syn H50Q.



4. References

(1) Smith, D. P., Tew, D. J., Hill, A. F., Bottomley, S. P., Masters, C. L., Barnham, K. J., and Cappai, R. (2008) Formation of a high affinity lipid-binding intermediate during the early aggregation phase of alpha-synuclein. *Biochemistry 47*, 1425–34.

(2) Johnson, M., Coulton, A. T., Geeves, M. A., and Mulvihill, D. P. (2010) Targeted amino-terminal acetylation of recombinant proteins in E. coli. *PLoS One 5*, e15801.

(3) Smith, D. P., Knapman, T. W., Campuzano, I., Malham, R. W., Berryman, J. T., Radford, S. E., and Ashcroft, A. E. (2009) Deciphering drift time measurements from travelling wave ion mobility spectrometry-mass spectrometry studies. *Eur. J. Mass Spectrom. (Chichester, Eng). 15*, 113–30.

(4) Moriarty, G. M., Minetti, C. A. S. A., Remeta, D. P., and Baum, J. (2014) A revised picture of the Cu(II)-α-synuclein complex: the role of N-terminal acetylation. *Biochemistry 53*, 2815–7.