

Elucidating the role site-specific nitration of α -synuclein in the pathogenesis of Parkinson's disease using protein semisynthesis and mutagenesis

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Materials

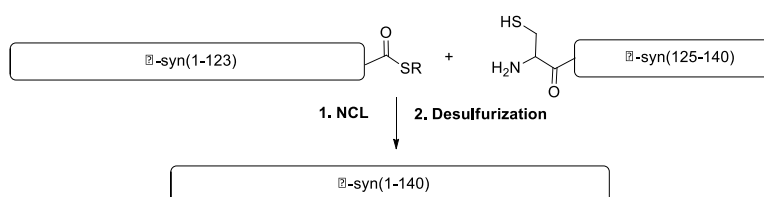
9-Fluorenylmethoxycarbonyl (Fmoc)-amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), were purchased from Novabiochem or AnaSpec. The side-chain functionalities of the Fmoc-amino acids were chemically protected as follows: Asp(OtBu), Glu(OtBu), Lys(Boc), Thr(tBu), Ser(tBu), Tyr(tBu), Cys(Trt), Gln(Trt), and Asn(Trt). Fmoc-3-nitro-Tyr-OH was obtained from Bachem AG (Bubendorf, Switzerland). Boc-Cys(Trt)-OH was purchased from Novabiochem. All other amino acids were not sidechain-protected. *N,N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), sodium 2-mercaptoethanesulfonate (MESNA), ammonium iodide, 1,2-ethanedithiol (EDT), thioanisole, 2-methyl-2-propanethiol (*tert*-butyl mercaptan, *t*BuSH), dimethylsulfide, guanidine hydrochloride (GuHCl) and tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. Piperidine and diethyl ether were purchased from Acros. 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) was purchased from Wako. The solvents *N*-methylpyrrolidone (NMP), dichloromethane, and HPLC-grade acetonitrile were purchased from VWR.

Methods

Solid Phase Peptide Synthesis and Purification

Solid phase peptide synthesis was performed on an automated CS 336X peptide synthesizer from CS Bio (California, USA) using standard Fmoc protocols including the *in situ* neutralization protocol described by Alewood and colleagues.¹ A preloaded Fmoc-Ala Wang resin was used. Syntheses were carried out on a 0.2 mmol scale, and coupling steps were performed with 5 eq. of Fmoc-amino acid, 5 eq. of HBTU/HOBt, and 10 eq. of DIPEA in NMP. For 3NT: the 5.0 eq. of Fmoc-3-Nitro-tyrosine was coupled in the presence of 5 eq. of HBTU/HOBt, and 10 eq. of DIPEA in NMP. N-terminal Cys residues were attached with Boc protecting groups on their N-terminus. Fmoc-group deprotections were performed with 20% piperidine in DMF. Peptide side chain deprotections, removal of N-terminal Boc group, and cleavage from the resin were performed by treatment with 81.5/5/3/5/2.5/2/1.5 TFA/water/phenol/thioanisole/EDT/Me2S/NH4I for 4 h on a rotating wheel to generate peptides with a C-terminal acid functionality. The crude product was subsequently precipitated three times with a 10-fold excess volume of cold diethyl ether, dissolved in water and lyophilized. Peptides were then purified by reversed-phase HPLC (Waters 600 system) using a Cosmosil C18 preparative column (20 mm × 250 mm, 5 μm particles, 300 Å pores) with a linear gradient of 20-70% B over 30 min (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). Peptide elution was monitored by UV absorbance at 214 and 280 nm. Peptide masses were confirmed by MALDI-TOF-MS and ESI-MS analysis. Peptide purity was confirmed by reinjection on a Water Acquity UPLC BEH 300 C4 column (2.1 mm × 100 mm, 1.7 μm) with a linear gradient of 10-90% B over 4 min (solvent A was water/0.1% TFA, and solvent B was acetonitrile/0.1% TFA).

A. Semisynthesis and purification of WT α-syn



Scheme S1. Scheme of the synthesis of WT α-syn by ligation at 123Glu: 124Cys

A.1. Synthesis and characterization of the α -syn C-terminal peptide

Peptide: α -syn(A124C-140)

Sequence: CYEMPSEEGYQDYEP EA

Synthetic specification: Coupling using HBTU, HOBt, double coupling performed at E-139, E-137, Q-134, M-127 and C-124.

Yield: 0.1 mmol scale, 40%.

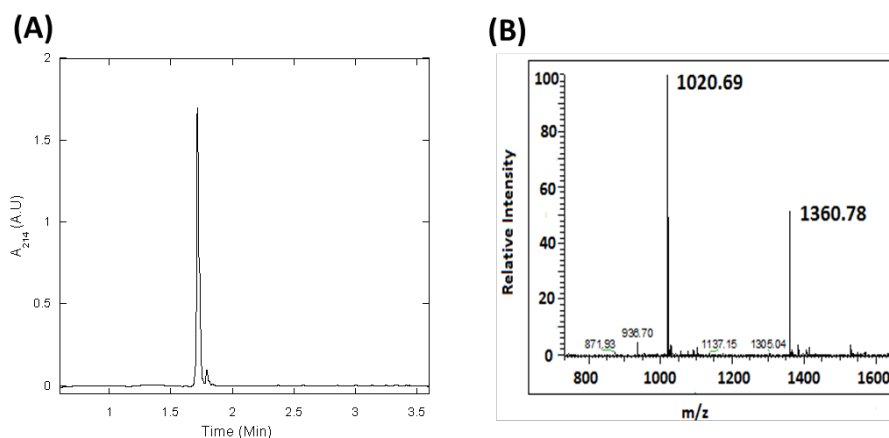


Figure S1. Analysis of the peptide used for α -Syn(124-140) synthesis

(A) RP-UPLC analysis of purified α -Syn(A124C-140) peptide on a Water Acquity UPLC BEH 300 C18 column (2.1 mm \times 100 mm, 1.7 μ m) with a linear gradient of 10–90% B over 4 min (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). **(B)** ESI-MS analysis (observed mass = 1020.69 Da, for $[M+2H]/2$, and 1360.78 Da, $[2M+3H]/3$, Exp mass 1020.87 $[M+2H]/2$ and 1360.83 Da $[2M+3H]/3$).

A.2. Expression and Purification of α -syn(1-123)SR

The α -syn(1-123) fragment was cloned into the pT7-7 vector as a fusion protein with the *Mxe* GyrA intein containing a C-terminal hexa-histidine tag, using standard molecular biology techniques. The plasmid was then transformed into BL21(DE3) *E. coli* cells which were used for protein expression. Typically, 4L of LB medium + 100 μ g/mL ampicillin was inoculated with an overnight pre-culture, and grown at 37°C on an orbital shaker to an OD600 of 0.6-0.8. Protein production was then induced by the addition of 1 mM isopropyl β -D-1-thiogalacto- pyranoside (IPTG). Cells were further incubated for 4 h at 37°C and then harvested by centrifugation (5000g, 4°C, 20 min). Bacterial pellets from 4L of culture were resuspended in 40 mL of lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, 15 mM imidazole, 5% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.2) and lysed by ultrasonication for 5 min. Then, 25 mM $MgCl_2$ was added to the lysates, which were centrifuged at high speed (48 000g, 20 min, 4°C). Supernatants were filtered (0.45 μ m) and injected (1 mL/min) onto an IMAC column (HisPrep FF 16/10, GE Healthcare) equilibrated with IMAC binding buffer (50 mM sodium phosphate, 300 mM of NaCl, 15 mM of imidazole, 5 % of glycerol, pH 7.2). The column was washed (3 mL/min) with 4 CV of binding buffer and followed by 5 CV of 7% IMAC elution buffer (50 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole, 5 % of glycerol, pH 7.4). α -syn(1-123)-Mxe-His₆ was then eluted with 100% IMAC elution buffer. To obtain the α -syn(1-123)SR thioester, 0.25 M MESNA were added to the pooled IMAC fractions, and the protein was incubated for 12 h at pH 7.4 at RT. After splicing with MESNA, TFA was added to lower the pH to 1, whereby most of the cleaved intein precipitated and was removed by centrifugation. The protein thioester remained in the supernatant. Acidification also inhibited the hydrolysis of the thioester. The protein 1-123SR was purified by reversed-phase HPLC on a Proto C4 column (20 x 250 mm, 10 μ m particles) using a linear gradient of 20% to 70% of solvent B in solvent A over 40 min at 10 mL/min. Solvents A and B were 0.1% TFA in water and 0.1% TFA in acetonitrile, respectively. Fractions containing pure α -syn(1-123)-SR were pooled and lyophilized. Final yield: 2 mg/L of bacterial culture.

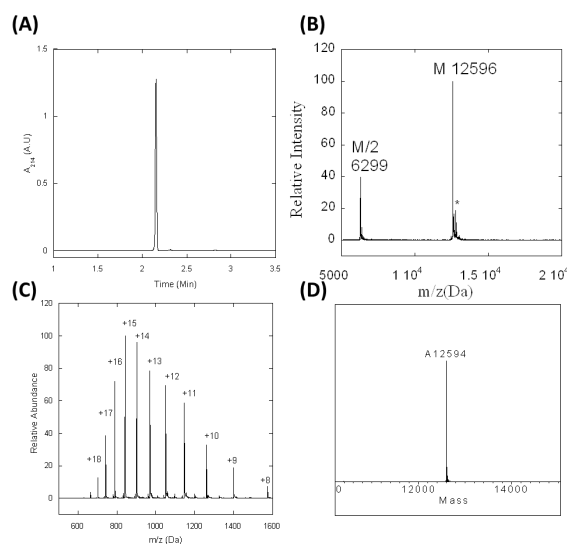


Figure S2. Analysis of α -syn(1-123)SR

(A) Water Acquity UPLC BEH 300 C4 column (2.1 mm \times 100 mm, 1.7 μ m) with a linear gradient of 10–90% B over 4 min (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). (B) MALDI-TOF-MS analysis (observed mass = 12596 Da, expected 12595 Da, M/2 and 6249 Da) (C) ESI-MS and (D) ESI-MS spectra corresponding to the mass calculated by deconvolution with MagTran.

A.3. Native Chemical Ligation and Desulfurization to produce α -syn WT

The semisynthesis of α -syn WT involved a NCL reaction between recombinant α -syn(1-123)SR and synthetic α -syn WT peptides (A124C-140). The NCL reactions were performed in degassed 6 M Gdn.HCl, 200 mM sodium phosphate buffer, pH 7.0, using 1 mM of α -Syn(1-123)SR and 3-fold-molar excess of C-terminal α -syn(A124C–140) peptide, in the presence of 50 mM TCEP and 30 mM MPAA at 37 °C with shaking at 1000 rpm. To monitor the NCL reactions, 2 μ L aliquots of the reaction mixture were removed and quenched with 58 μ L of water containing 0.1% TFA and subsequently analyzed by SDS-PAGE (15% polyacrylamide) and MALDI-TOF-MS. RP-HPLC could not be used due to the co-elution of α -syn(1-123)SR with the ligated product under all conditions tested. SDS-PAGE analysis indicated tha the desired ligated product was produced within 2 h. The MPAA was removed using a PD-10 column and the protein-containing fractions were pooled and lyophilized. The free-radical-based desulfurization of the semisynthetic α -syn was performed as previously described by Wan et al.² In our case, desulfurization was conducted at a protein concentration of 1 mM protein concentration by dissolving the lyophilized ligated product in NCL buffer (6 M Gdn.HCl, 200 mM sodium phosphate, 200 mM TCEP at pH 7.0). A solution of 2-methyl-2-propanethiol and 2-2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) free radical initiator was then added under argon to a final concentration of 10 mM and 1 M tBuSH. The reaction was incubated at 37°C with agitation at 1000 rpm in a ventilated fume hood for 3 h. The progress of desulfurization was followed by ESI-LC/MS.

A.4 Purification of the Semisynthetic WT α -syn

Prior to purification, the ligated desulfurized product of the desulfurization reaction was desalted through a 5 mL HiTrap desalting column (Amersham) with 20 mM sodium dihydrogen citrate, pH 4.0 as the mobile phase. The fractions containing the protein were pooled and injected on a 1 mL cation-exchange column HiTrap SP HP (Amersham). We exploited the charge difference between the full-length protein, the fragment α -syn(1-123)OH (hydrolyzed thioester), and the C-terminal A124C-140 peptide to perform a single-step purification. At pH 4.0, the peptide is negatively charged and does not bind to the column, whereas both the full-length protein and the fragment (1-123) are positively charged and hence bind to the column. At pH 5.0, the full-length protein and the fragment (1-123) have different charges and can be subsequently separated using a salt gradient. We initially allowed the peptide to pass in the flowthrough by washing the column after injection with 3 column volumes (CV) of buffer A (where A is 20 mM sodium citrate pH 4) and then with a linear gradient of NaCl from 0 to 15% buffer B (20 mM sodium citrate and 0.5 M NaCl, pH 4) over 5 CV with a flow rate 1 mL/min on an Akta Purifier system. Then, to elute bound proteins, we applied a linear gradient of NaCl from 0 to 70% B (where A is 20 mM sodium citrate pH 5 and B is 20 mM sodium citrate, 0.25 M NaCl) over 50 CV at a flow of 1 mL/min. The full-length protein eluted at approximately 22% B and the fragment (1-123) eluted at approximately 24% B. The fractions containing the protein were extensively dialyzed against deionized water and lyophilized. The final yield of pure lyophilized protein was approximately 46%, i.e., 3.69 mg from 7 mg of thioester.

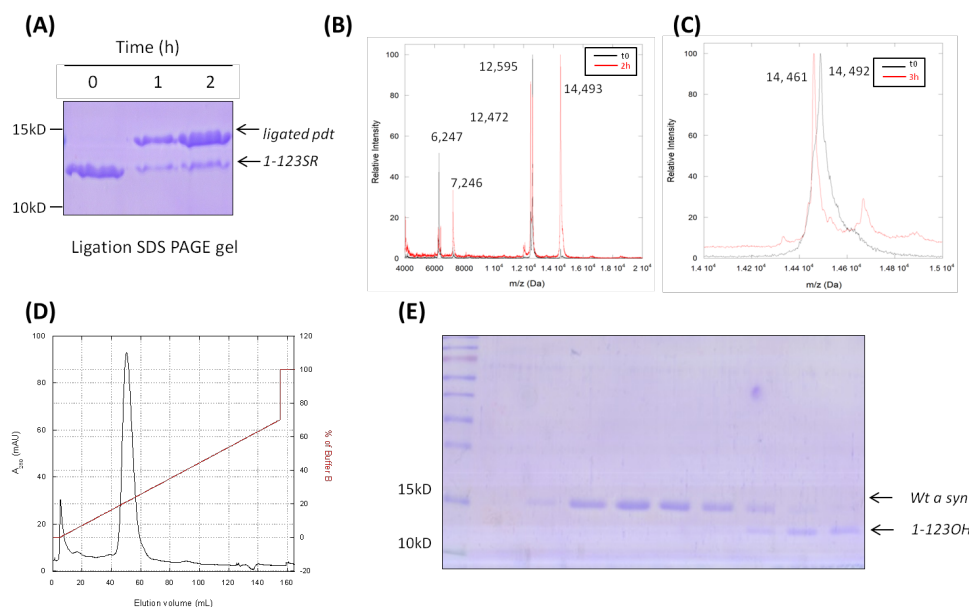


Figure S3. Semisynthesis, ligation, desulfurization and purification of WT α -Syn

(A) SDS-PAGE analysis of the NCL reaction between α -Syn(1-123)-SR and α -syn(A124C-140), (B) MALDI-TOF-MS analysis of the ligation reaction at t = 0, observed mass = 12596 Da, M/2 and 6247 Da, t = 2 h, observed mass = 14493 Da, M/2 7246 Da and hydrolyzed thioester mass observed = 12472 Da and M/2 6247 Da, and (C) MALDI-TOF-MS analysis of desulfurization, at t = 0, observed mass 14492 Da, t = 3 h observed mass 14461 Da. (D) Cation-

exchange chromatogram (pH 5). **(E)** SDS-PAGE analysis of the fractions containing WT α -syn (eluted from the cation-exchange column at pH 5).

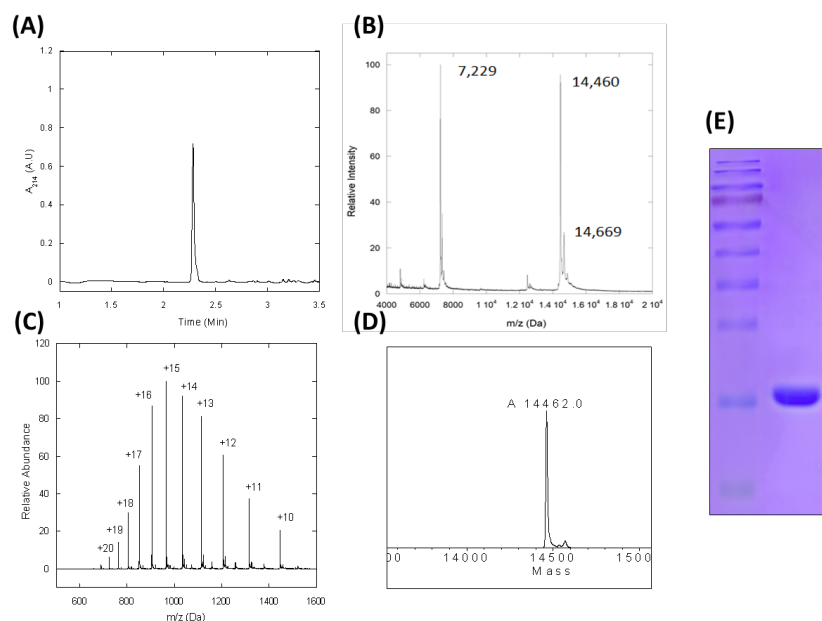
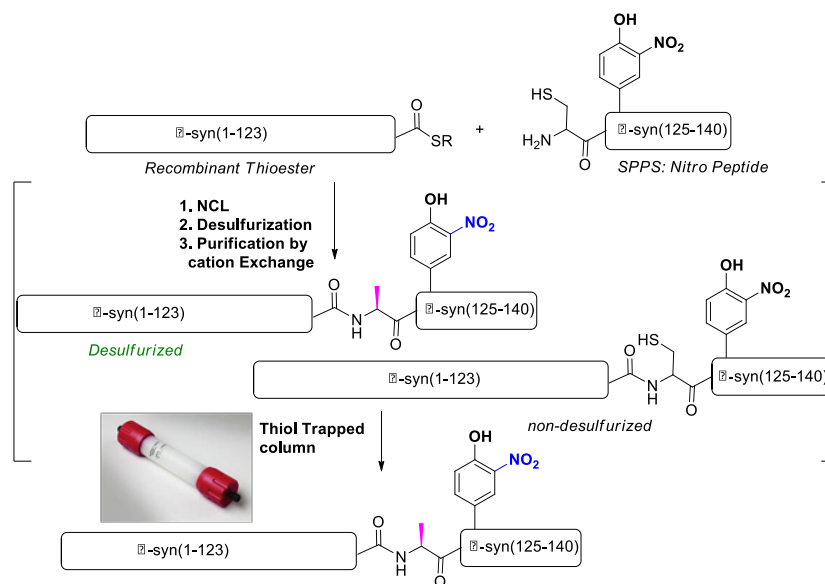


Figure S4. Analysis of semisynthetic WT α -syn

(A) RP-UPLC analysis of purified α -syn(1-140) on aWater Acquity UPLC BEH 300 C18 column (2.1 mm \times 100 mm, 1.7 μ m) with a linear gradient of 10-90% B over 4 min (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). **(B)** MALDI-TOF-MS analysis: observed mass = 14460 Da, expected mass = 14459 Da. The mass at 14669 corresponds to the sinapinic adduct. **(C)** ESI-MS analysis and **(D)** ESI-MS spectra corresponding to the mass calculated by deconvolution with MagTran. **(E)** SDS-PAGE analysis of purified α -syn(1-140).

B. Semisynthesis of nY125 α -synuclein



Scheme S2. Synthetic route for nY125 α -synuclein

B.1 Synthesis and characterization of the nY125 α -syn C-terminal peptide

Peptide: nY125 α -syn(A124C-140)

Sequence: Cn**Y**EMPSEEGYQDY**E**PEA

Synthetic specification : coupling used HBTU, HOBt, double coupling performed at E-139, E-137, Q-134, M-127 and nY-125, C-124. Yield: 0.1 mmol scale, 40%. Expected mass: 2084.75 Da

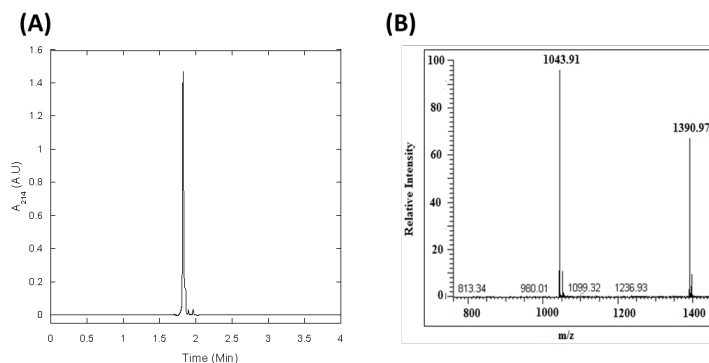


Figure S5. Analysis of nY125 α -syn(A124C-140)

(A) RP-UPLC analysis of purified α -syn(A124C-140), nY125 peptide on Water Acquity UPLC BEH 300 C18 column (2.1 mm \times 100 mm, 1.7 μ m) with a linear gradient of 10-90% B over 4 min (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). (B) ESI-MS analysis (observed mass = 1043.91 Da for $[M+2H]/2$, and 1390.97 Da for $[2M+3H]/3$, expected mass: 1043.37 Da $[M+2H]/2$ and 1390.83 Da $[2M+3H]/3$).

B.2. Native chemical ligation and desulfurization to produce of nY125 α -syn

The semisynthesis of nY125 α -syn involved an NCL reaction between recombinant α -syn (1-123)SR and the synthetic nY125 α -syn (A124C-140) peptide. NCL reactions were performed in 6 M Gdn.HCl, 200 mM sodium phosphate buffer, pH 7.0, using 1.0 mM of α -syn (1-123)-SR and 3-fold- molar excess of C-terminal nitro peptide in the presence of 50 mM TCEP and 30 mM MPAA at 37 °C under argon with shaking at 1000 rpm. To monitor the NCL reaction, 2 μ L aliquots of the reaction mixture were removed and quenched with 58 μ L of water containing 0.1% TFA and analyzed by SDS-PAGE (15% polyacrylamide) and ESI-MS. RP-HPLC could not be used due to the co-elution of α -syn(1-123)-SR with the ligated product under all conditions tested. According to SDS-PAGE analysis revealed that the desired ligated product was produced within 2 h.

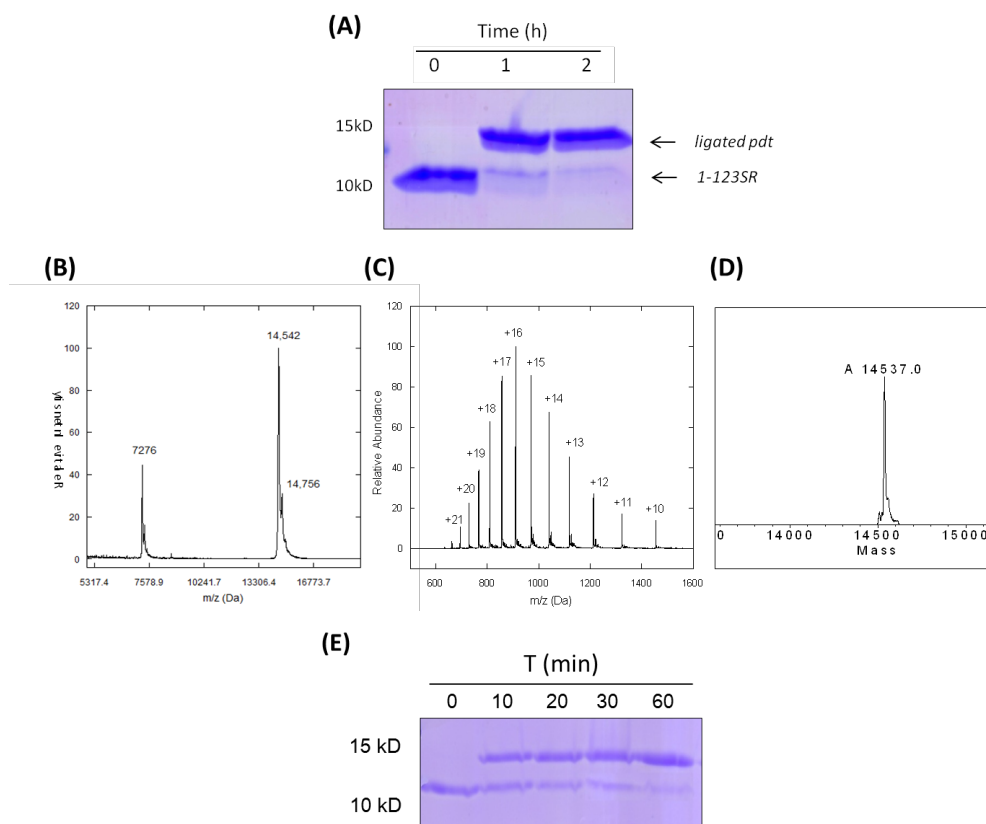


Figure S6. Ligation and characterization of 125nY α -syn A124C

(A) SDS-PAGE analysis of the NCL reaction between α -syn(1-123)SR and the nY125 α -syn(A124C-140) peptides. (B) MALDI-TOF-MS analysis of nY125 α -syn A124C, observed mass = 14542 Da, expected mass = 14537 Da, the mass of 14756 corresponds to the sinapinic adduct. (C) The ESI-MS analysis and (D) ESI-MS spectra correspond to the mass calculated by deconvolution with MagTran. (E) SDS-PAGE analysis of the NCL reaction between α -syn(1-123)SR and the nY125 α -syn(A124U-140) peptides.

B3 Desulfurization/deselenization conditions tested

Desulfurization conditions				
Protein	TCEP	VA-044	Ni ₂ B	Result
124 Cys, 125-nY α syn	100 eq	2 eq	-	NO ₂ reduction and desulfurization in 10 h
124 Cys, 125-nY α syn	50 eq	1 eq	-	NO ₂ reduction and desulfurization in 16h *
124 Cys, 125-nY α syn	-	-	10 eq	Complex mixtures in 10 min

In all cases, with 8% v/v tBuSH and reaction done pH 7.1 in NCL buffer at 37 °C *even at 25 °C with 250 mM TCEP and 6 mM Va-044 reduces both

Deselenization conditions				
Protein	TCEP	DTT	Ni ₂ B	Result
124 Sec, 125-nY α syn	50 eq	10 eq	-	NO ₂ reduction and desulfurization in 4 h
124 Sec, 125-nY α syn	50 eq	-	-	NO ₂ reduction and desulfurization in 20h
124 Sec, 125-nY α syn	10 eq	10 eq	-	NO ₂ reduction

In all cases, reaction done pH 6.8 in NCL buffer at 37 °C

Table S1. Desulfurization/Deselenization conditions

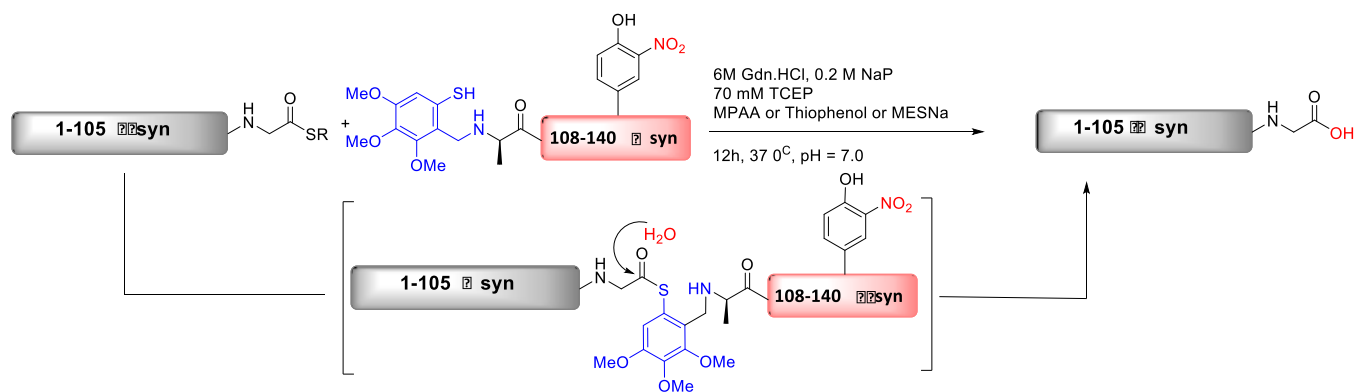


Figure S7. Auxiliary mediated ligation approach

(A) Cystein-based auxiliary mediated ligation is sluggish and leads to hydrolyzed thioester formation.

B.4. Desulfurization of nY125 α -syn A124C

Because MPAA quenches radical-mediated desulfurization of cysteine residues, it was removed using a PD-10 column in water. The protein containing fractions were pooled and lyophilized. For the free-radical-based desulfurization reaction, 7 mg of crude lyophilized nY125 α -syn A124C ligated protein was dissolved in 0.5 mL of degassed NCL buffer (6 M Gdn.HCl, 200 mM sodium phosphate, 150 mM TCEP at pH 7.0). Then, a solution of *t*-BuSH (8% (v/v)), 2-nitrobenzyl amine (10 mM) and free radical initiator VA-044 (10 mM) was added under argon. The reaction was incubated at 37°C with agitation at 1000 rpm in a ventilated fume hood for 20 h. To monitor the NCL reaction, 1 μ L aliquots of the reaction mixture were removed and quenched with 70 μ L of water containing 0.1% TFA, and the progress of the desulfurization was monitored by ESI-LCMS. After 16 h, no further reaction was observed, and thus an additional 50 mM TCEP, 4 mM of 2-nitrobenzyl amine and 0.2 M *t*BuSH. The reaction was stopped after 20 h, when trace amounts of nitro reduced product started to appear. The reaction was immediately passed through a PD-10 column to remove *t*BuSH and Gdn.HCl salt with 20 mM sodium dihydrogen citrate, pH 4.0 as the mobile phase. The protein-containing fractions were subsequently pooled and purified by FPLC purification.

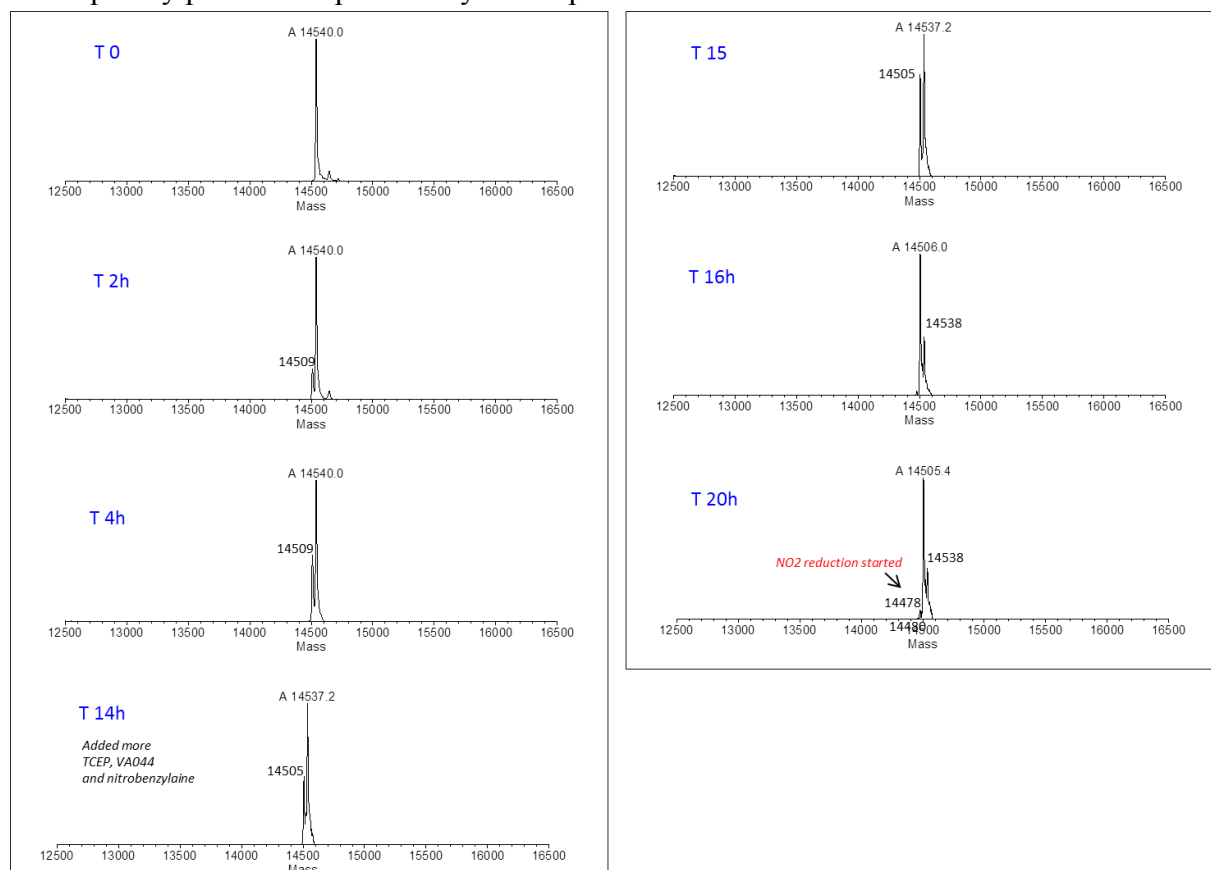


Figure S8. Desulfurization kinetic of nY125 α -syn A124C

The kinetics of the desulfurization were monitored by ESI-MS. The ESI-MS spectra correspond to the mass calculated by deconvolution with MagTran. At $t = 16$ h, 50 mM TCEP, 4 mM of 2-nitrobenzyl amine and 0.2 M *t*BuSH were added. The reaction was stopped at $t = 20$ h, when trace amounts of nitro reduced product were first detected.

B.5. Purification of semisynthetic nY125 α -syn following desulfurization

Desulfurized protein fractions of the PD-10 column were manually injected on a 1mL cation-exchange column HiTrap SP HP (Amersham) at 0.5 mL/min. We exploited the charge difference between the full-length protein, the fragment α -syn(1-123)OH (hydrolyzed thioester), and the C-terminal 124A-140, nY125 peptide to perform a single-step purification. The protein was purified following the same protocol used to purify WT semisynthetic α -syn. At pH 4.0, the peptide was removed by washing the column after injection with 3 column volumes (CV) of buffer A (20 mM sodium citrate pH 4) followed by a linear gradient of NaCl from 0 to 15 % buffer B (20 mM sodium citrate and 0.5 M NaCl, pH 4) over 5 CV with a flow rate of 1 mL/min on an Akta Purifier system. To elute bound proteins, we applied 3 CV of buffer C (20 mM sodium citrate pH 5) and then a linear gradient of NaCl from 0 to 70% buffer D (20 mM sodium citrate and 0.25 M NaCl) over 50 CV at a flow of 1 mL/min. The full-length protein eluted approximately 34% D and the fragment α -syn-(1-123)SR eluted at approximately 36% D. The fractions containing the protein were subsequently passed through a thiol capture column.

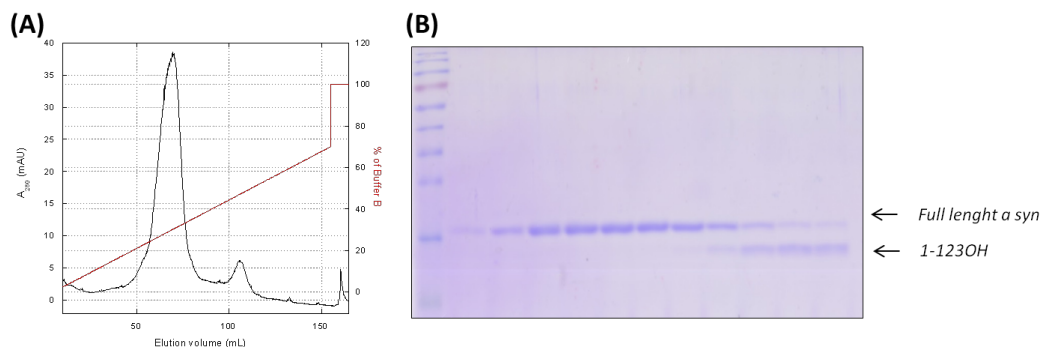


Figure S9. FPLC purification to remove 1-123OH and peptide

(A) Elution from the cation exchange column at pH 5 and (B) SDS-PAGE analysis of the fractions containing α -syn, 125nY eluting from the cation exchange column at pH 5.

B.6. Purification by thiol exchange column

The FPLC-purified fractions were lyophilized and re-dissolved in degassed binding buffer (50 mM Tris and 20 mM NaCl, pH 7.5). The protein was injected into a 5 mL thiol-exchange column packed with Activated Thiol Sepharose 4B beads from (GE Healthcare) at a flow rate of 0.06 mL/min. Then, the column was left at RT for 1 h for thiol exchange (binding). The desulfurized product, which lacks free SH-group did not bind to the column and was eluted when the column was washed with binding buffer (50 mM Tris and 20 mM NaCl, pH 7.5) at a flow rate of 0.06 mL/mL. The flow through fractions containing the desired protein were collected and extensively dialyzed against deionized water and lyophilized. The final yield was approximately 24%, i.e., 2.0 mg from 7 mg of thioester.

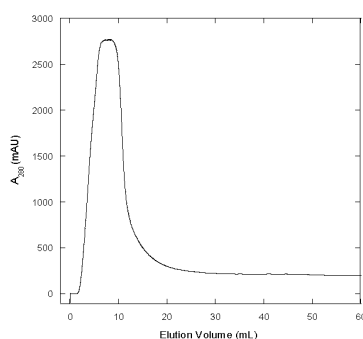


Figure S10. Thiol-exchange purification to remove non-desulfurized product

Chromatogram of the thiol-exchange column washed with binding buffer. The flow through fractions contain pure nY125 α -syn

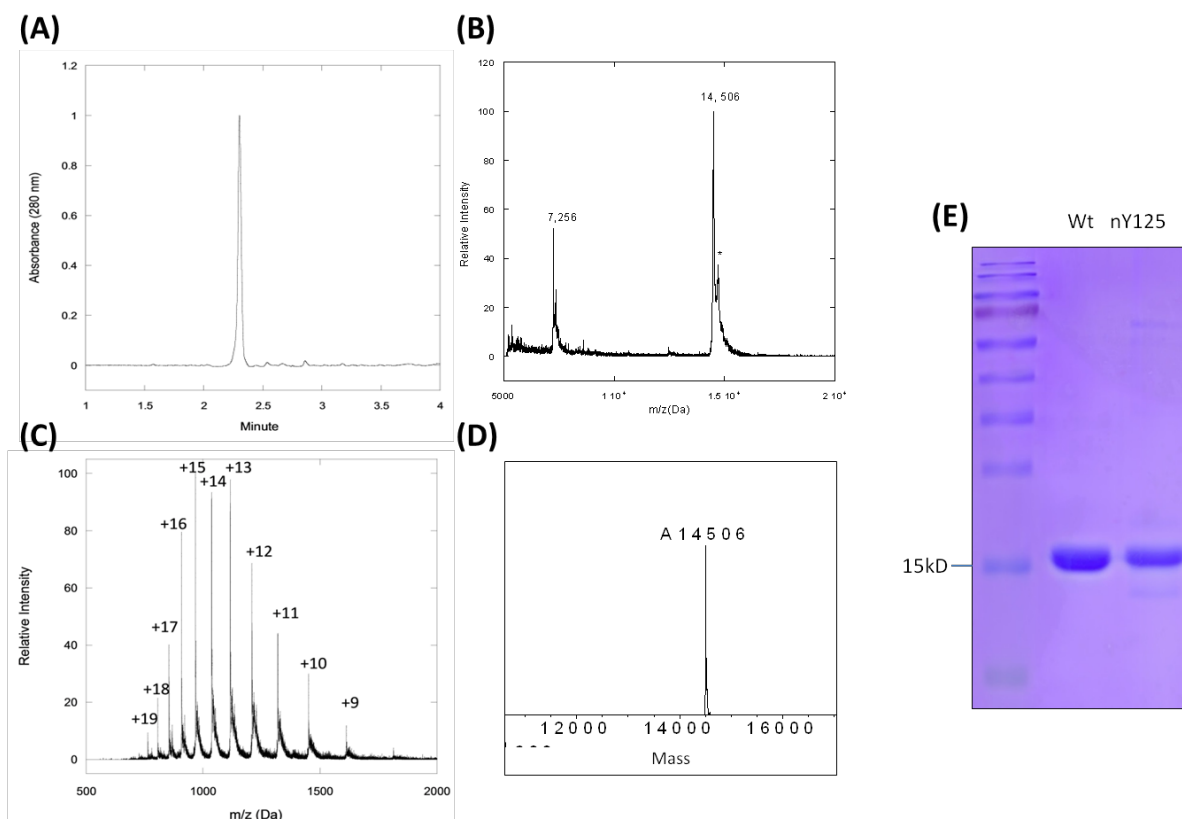
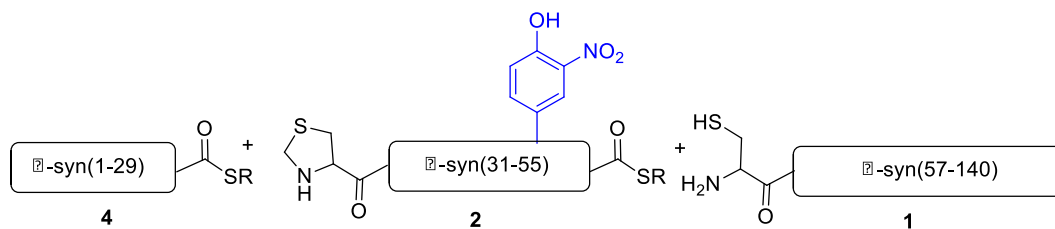


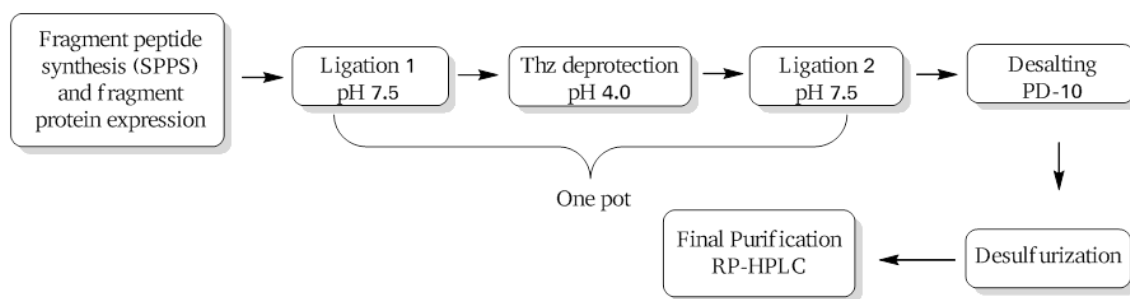
Figure S11. Analysis of semisynthetic nY125 α -syn

(A) RP-UPLC analysis of purified nY125 α -syn(1-140) on Water Acquity UPLC BEH 300 C18 column (2.1 mm \times 100 mm, 1.7 μ m) with a linear gradient of 10–90% B over 4 min (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). (B) MALDI-TOF-MS analysis, observed mass = 14506 Da, expected mass= 14505 Da, * corresponds to the sinapinic adduct. (C) ESI-MS analysis and (D) ESI-MS spectra correspond to the mass calculated by deconvolution with MagTran. (E) SDS-PAGE analysis of the purified nY125 α -syn(1-140).

C. One-pot three-fragment ligation and desulfurization to produce nY39 α -syn



Scheme S3. Fragment design for the three-fragment ligation scheme to obtain nY39 α -syn



Scheme S4. Workflow diagram illustrating the synthesis and purification procedures

C.1. Synthesis of peptide thioesters

Peptide thioesters were generated based on the Nbz method developed by Blanco-Canosa et al.³ 3-Fmoc-3,4-diaminobenzoic acid (Fmoc-Dbz-OH) was prepared by reacting 500 mg (3.3 mmol) of 3,4-diaminobenzoic acid with 1 eq of Fmoc-OSu (1.1 g, 3.3 mmol) in 10 mL of 1:1 acetonitrile:0.1 M NaHCO₃, pH 7.9 at RT with stirring for 12-16 h. Then, the mixture was acidified until the pH decreased to approximately 1 and filtered through a sintered glass funnel. The filtrate was washed with diethyl ether, hexanes, and MeOH (at least 3x each), and finally transferred into a glass flask and vacuum-dried. Synthesis of peptide thioesters started with the loading of 0.2 mmol of Rink amide MBHA resin with Fmoc-Dbz-OH. The resin was treated with 5 eq. of Fmoc-Dbz-OH, 5 eq. of HATU and 10 eq. of DIPEA twice for 2 h each at RT. Then, the 4-amino position on the loaded Dbz linker was protected as described by Mahto and colleagues⁴ by treating the resin with 350 mM allyl chloroformate and 1 eq. of DIPEA in dry DCM for 24 h at RT, followed by washing with DCM and DMF. After deprotection of the 3-Fmoc group on the Alloc-protected Dbz linker, SPPS was continued as described earlier. After completion of the peptide elongation, the resin was swelled in argon-sparged DCM and the Alloc group was removed by adding 20 eq. of phenylsilane, and 0.35 eq. of tetrakis(triphenylphosphine)palladium(0). The deprotection was conducted for 30 min and repeated as needed. After deprotection of the 4-amino position, the Nbz group was activated by sequential treatments with 5 eq. of 4-nitrophenylchloroformate in DCM, followed by 0.5 M DIPEA in DMF. Nbz activation cycles were also repeated if needed. Peptides were then cleaved from the solid support and deprotected using Reagent H, as described earlier, precipitated in cold diethyl ether and lyophilized. Treatment with 50 mM MESNA in 6.0 M Gdn.HCl, 200 mM sodium phosphate pH 7.5, displaced the C-terminal Nbz group to obtain a thioester. Peptides were purified by RP-HPLC on a C18 preparative column (Vydac 218MS, 20 mm ID x 250 mm length, 5 μ m), lyophilized and stored at -20°C until use.

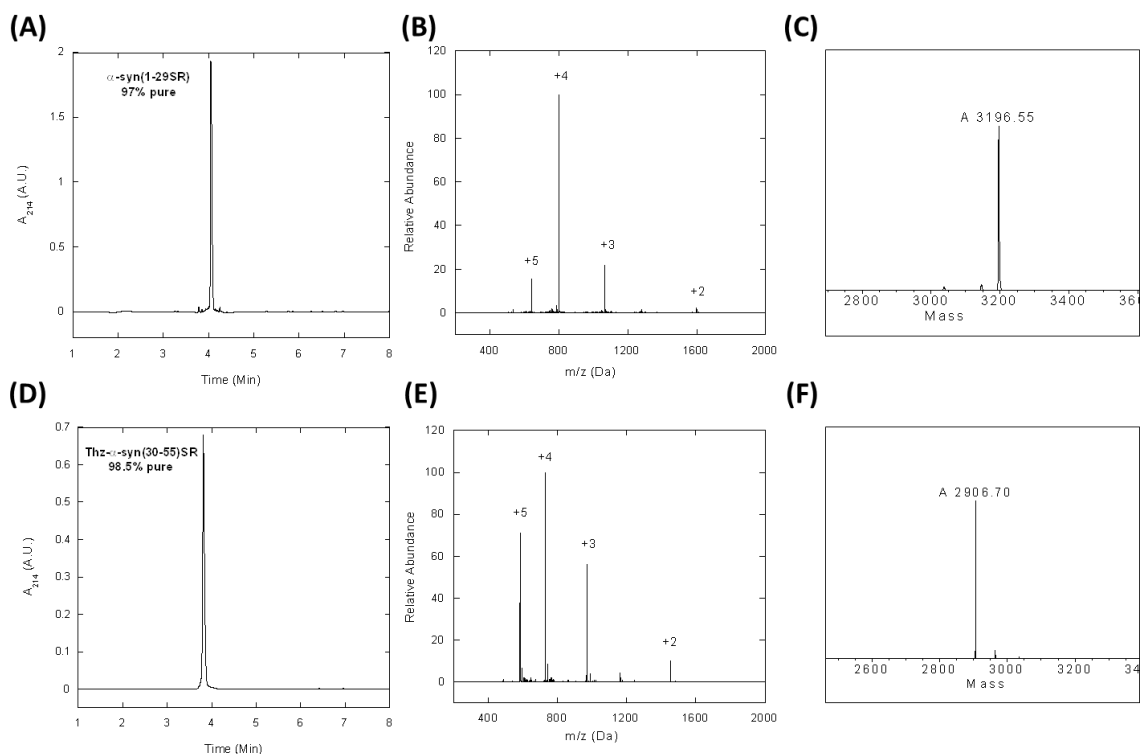


Figure S12. Analysis of the peptides α -syn(1-29)SR and Thz- α -syn(30-55)SR, nY39 used for nY39 α -syn synthesis

(A) Analytical RP-UPLC of the purified α -syn(1-29)SR, (B) the ESI-MS and (C) ESI-MS spectra corresponding to the mass calculated by deconvolution with MagTran. (D) Analytical RP-UPLC of the purified Thz- α -syn(30-55)SR, (E) the ESI-MS and (F) ESI-MS spectra corresponding to the mass calculated by deconvolution with MagTran.

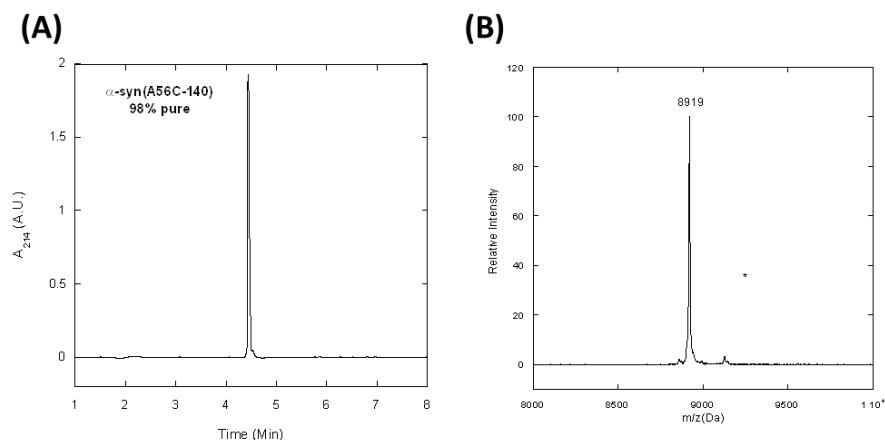


Figure S13. Analysis of α -syn(A56C-140) used for nY39 α -syn synthesis

(A) Analytical RP-UHPLC and (B) MALDI-TOF MS analysis of purified α -Syn(A56C-140).

C.2. One-pot native chemical ligation to produce of nY39 α -syn A30C, A56C

The one-pot synthesis of α -syn was performed according to the methods described by Bang & Kent⁵. **Ligation 1:** Syntheses were typically performed at a 1 μ mol scale and peptide concentrations were kept at 1 mM at pH 7.5. An 8.92 mg quantity of peptide **1** (α -syn(A56C-140)) was dissolved in degassed NCL buffer (6.0 M Gdn.HCl, 0.2 M sodium phosphate, 30 mM TCEP, 50 mM MPAA, pH 7.5). Following dissolution of peptide **1**, 1.5 eq. (4.35 mg) of peptide **2** (Thz- α -syn(30-55)SR, nY39) was added, and the reaction was incubated at 37°C with orbital agitation at 950 rpm under argon and monitored regularly by withdrawing 2 μ L aliquots for RP-UHPLC, SDS-PAGE, and mass spectrometry analyses. Aliquots were immediately diluted 50-fold in 95:5 water:acetonitrile + 0.1 % TFA, centrifuged (20000 g, 4°C, 5 min) and kept on ice to quench the reaction. For RP-UHPLC analysis, 10 μ L of the diluted aliquots was loaded on a Waters Acquity UPLC BEH300 C4 column (2.1x100 mm, 1.7 μ m) and eluted with a 10% to 90% B gradient over 8 min at 0.6 mL/min (A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile). For SDS-PAGE analysis, diluted aliquots were mixed with an equal volume of 2X Laemmli sample buffer and 10 μ L was loaded on a 17% polyacrylamide SDS gel. For analysis by mass spectrometry, 10 μ L of diluted aliquots was analyzed by LC-ESI-MS (Thermo LTQ instrument) or by MALDI-TOF MS (AB Sciex 4700). The ligation **1** reaction was completed in 8 h, with a conversion yield of ~ 90% (measured by RP-UPLC). **Thz deprotection:** 30 mM fresh TCEP and 100 mM methoxyamine were added and the pH was adjusted to 4.0 under argon. The reaction was then placed back at 37°C under agitation and monitored by mass spectrometry to follow the -12 Da mass shift, indicative of the removal of the Thz protection of the intermediate **3a** (Thz- α -Syn(30-140) nY39, A56C) to yield the intermediate product **3** (α -syn(30-140) A30C, nY39, A56C). **Ligation 2:** Following completion of the Thz deprotection analyzed by mass spectrometry, the pH of the reaction mixture was adjusted back to 7.5. Then 30 mM fresh TCEP and 1.5 eq. (4.8 mg) of peptide **4** (α -syn(1-29)SR) were added and incubated at 37°C with orbital agitation at 950 rpm and monitorin as for ligation-1. Ligation 2 was completed in 3 h and yielded product **5** (nY39 α -syn(1-140) A30C, A69C), with a reaction yield of 50%, as determined by RP-UPLC analysis.

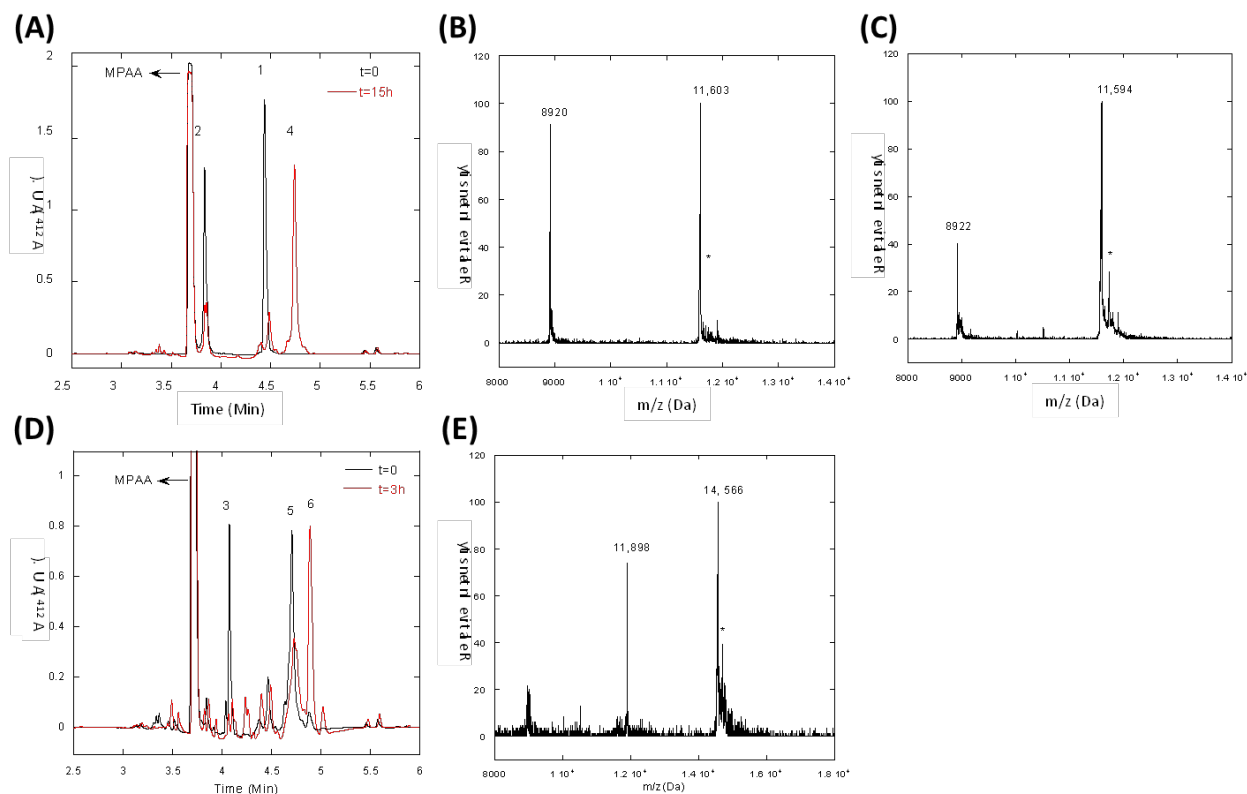


Figure S14. UPLC and MALDI-TOF MS ligations analyses

(A) RP-UHPLC and (B) MALDI-TOF MS analysis of the *Ligation 1* product **3a** (observed mass = 11603 Da, expected mass = 11606 Da). (C) MALDI-TOF MS analysis of the Thz deprotected intermediate product **3** (observed mass = 11594 Da, expected mass = 11594 Da). (D) RP-UHPLC and (E) MALDI-TOF MS analysis of the *Ligation 2* product **5** (observed mass = 14566 Da, expected mass = 14570 Da).

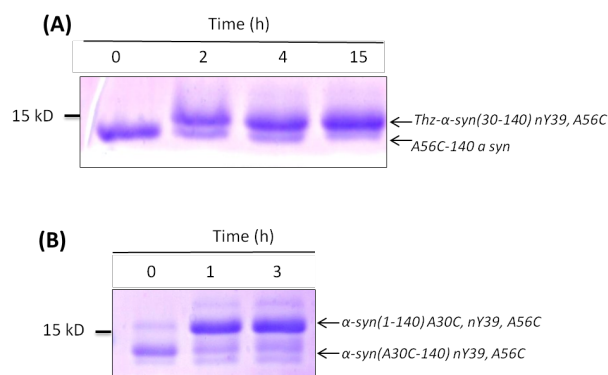


Figure S15. SDS-PAGE analysis of the ligations

(A) SDS-PAGE analysis of *Ligation-1* and (B) *Ligation -2*.

C.3. Desulfurization of nY39 α -syn A30C, A56C

The ligation reactions were performed in presence of an excess of MPAA, which inhibits the desulfurization reaction. Thus, a prior desalting step was performed using a PD-10 column (GE Healthcare). The desalted protein fractions were lyophilized, and then dissolved in argon-sparged 6.0 M Gdn.HCl, 0.2 M sodium phosphate, pH 7.0 (1 mL). Then, TCEP, 2-2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044), 2-methyl-2-propanethiol and 2-nitrobenzyl amine were added to final concentrations of 150 mM, 10 mM, 8% (v/v) and 10 mM, respectively. The reaction tube was sealed under argon and placed at 37°C under agitation. The loss of 64 Da (for two desulfurized cysteine residues desulfurized) was monitored by mass spectrometry. After 4 h, another 30 mM TCEP, 5 mM VA044 and 4 mM nitrobenzyl amine were added and incubated for another 2 h, at which point the reaction was stopped as NO₂ reduction started to appear. The mixture was desalted using a PD-10 column, in order to separate the protein from the thiol, and then purified by RP-HPLC on a semi-preparative C18 column (Cosmosil protein-R, 10x250 mm, 5 μ m) using a gradient of 30-45% B over 40 min at 5 mL/min. (A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile). Fractions containing pure protein (as assessed by RP-UPLC and mass spectrometry) were lyophilized and kept around -20°C until use. The combined desulfurization and purification yield was 11%, to obtain 0.11 μ mol of pure product **7** (~1.5 mg) obtained.

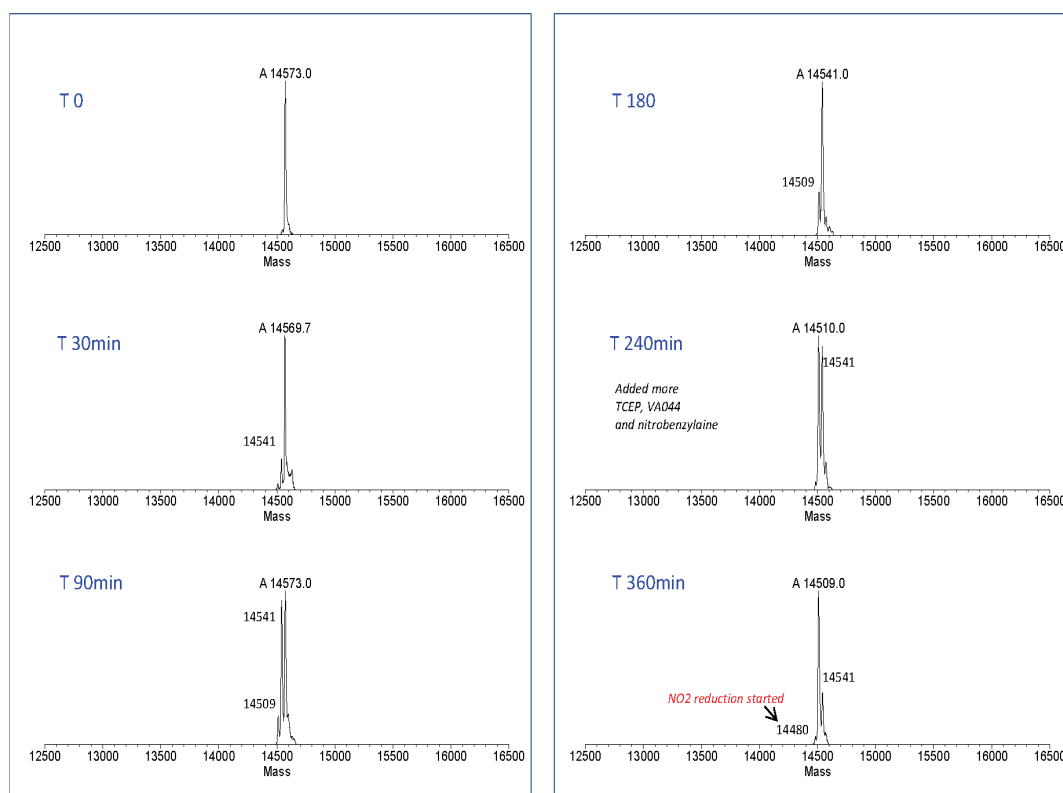


Figure S16. Desulfurization kinetics of nY39 α -syn A30C, A56C

The kinetics of the desulfurization procedure were followed by ESI-MS. The ESI-MS spectra correspond to the mass calculated by deconvolution with MagTran. At 4 h, another 30 mM TCEP, 5 mM VA044 and 4 mM nitrobenzyl

amine were added and the reaction was stopped at $t = 6\text{h}$, when a trace amount of nitro reduced product was first detected.

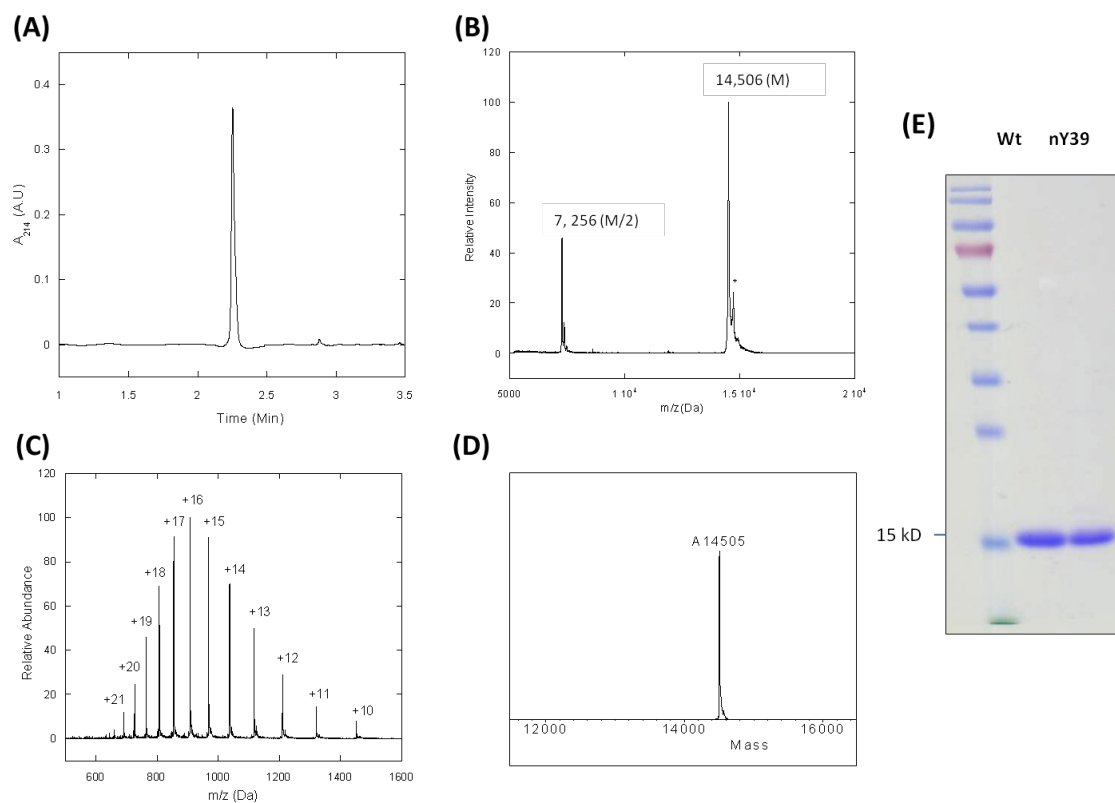


Figure S17. Characterization of nY39 α -Syn

(A) Analytical RP-UHPLC and **(B)** MALDI-TOF MS analysis of nY39 α -syn (observed mass = 14506 Da, expected mass = 14505 Da). **(C)** ESI-LCMS and **(D)** ESI-MS spectra corresponding to the mass calculated by deconvolution with MagTran. **(E)** SDS-PAGE of purified nY39 α -Syn.

Nitration of WT and mutated α -syn by tetranitromethane (TNM)

General nitration procedure by TNM

Following published protocol⁶, nitration of α -syn was done by adding 10 eq. of tetranitromethane (TNM) (10% in EtOH) per tyrosine residue to protein (2mg/mL) in degassed nitration buffer (0.1 M Tris-HCl, 0.1 M KCl, pH 8). The reaction mixture was stirred for 30 min at 30°C, after which an additional 10 eq. of TNM (10% in EtOH) per tyrosine residue was added and reaction was stirred at the same temperature. The nitration reaction was monitored by ESI-LCMS and was generally complete within 2-3 h. The reaction was also monitored by SDS-PAGE.

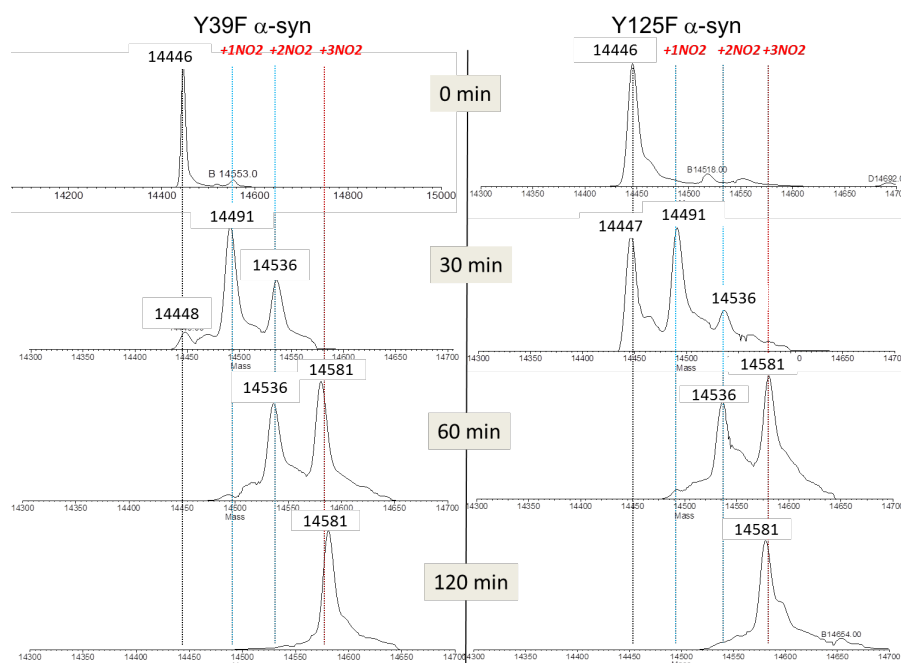


Figure S18. Representative nitration reaction (here of mutated Y39F and Y125F α -syn)
TNM nitration reaction kinetics of α -syn Y39F and Y125F nitration, as followed by ESI-LC/MS

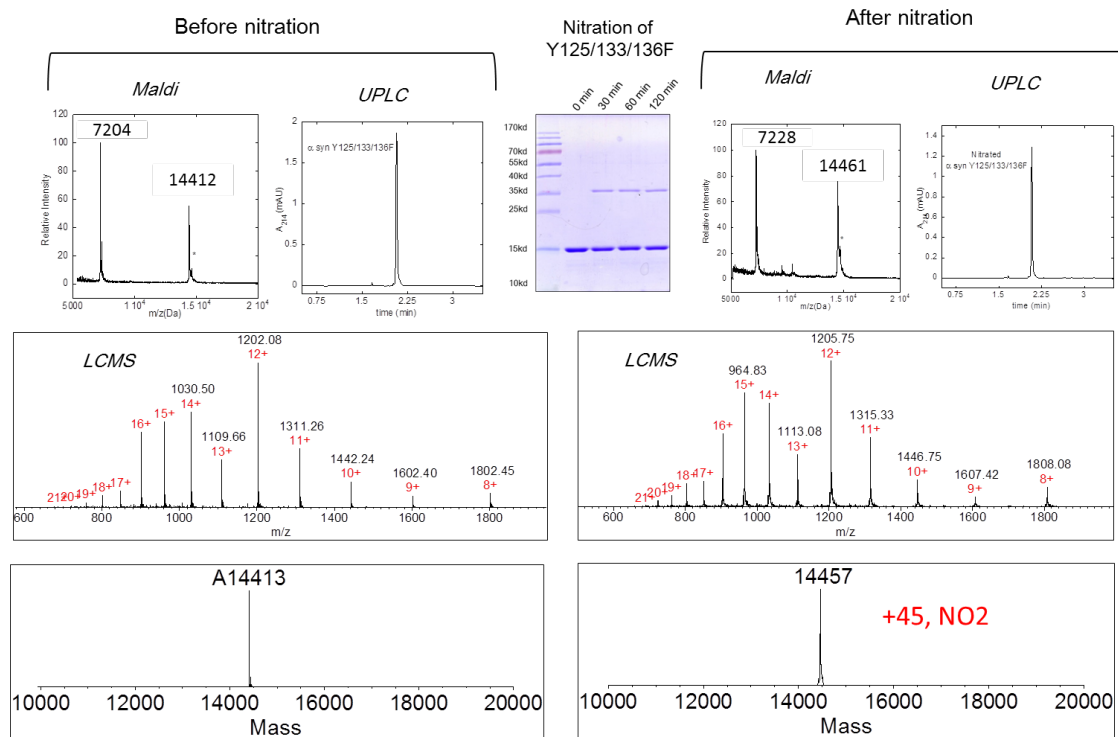


Figure S19. Analysis of nitrated α -syn (here of Y125/133/136F α -syn)

WT and TNM nitrated α -syn Y125/133/136F analysed by Maldi, UPLC, SDS-PAGE and ESI-LC/MS.

Nitration of WT α -syn at different concentration

TNM nitration with increasing protein concentration

Protein used: WT α -Syn
Protein concentration: 5 μ M, 20 μ M, 50 μ M, 100 μ M and 400 μ M
TNM: 10 eq. / tyrosine residue, another 10 eq. / tyrosine residue added after 30 min.
Reaction time: 2 h
Temperature: 30 °C

TNM nitration with increasing TNM concentration

Protein used: WT α -Syn
Protein concentration: 20 μ M
TNM: 5, 10, 50 and 100 eq. / tyrosine residue, another 5, 10, 50 and 100 eq. / tyrosine residue added after 30 min
Reaction time: 2 h
Temperature: 30 °C

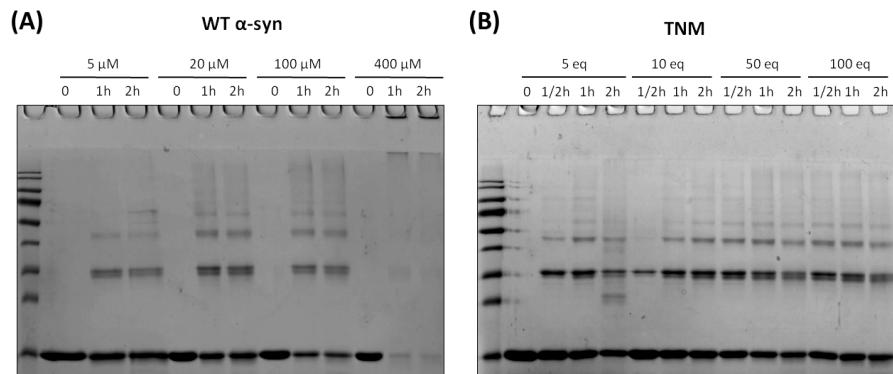


Figure S20. Nitration and cross linking pattern of WT α -syn with increasing protein or TNM concentration
(A) SDS-PAGE analysis of TNM nitration of WT α -syn at 5, 20 100 and 400 μ M. (B) SDS-PAGE analysis of TNM nitration of WT α -syn with increasing TNM equivalents.

TNM-nitration of C-terminal α -syn fragments

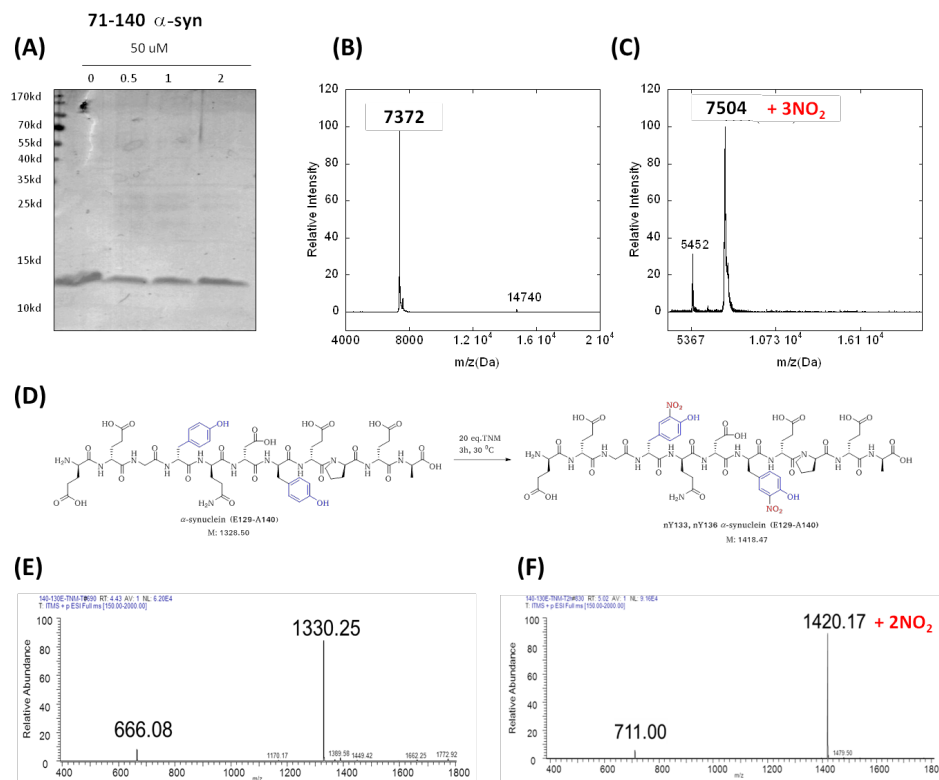


Figure S21. TNM nitration C-terminal α -syn fragments 71-140 and 129-140

(A) SDS-PAGE analysis of TNM nitration of truncated α -syn (71-140) at 50 μ M. **(B)** MALDI-TOF MS analysis of truncated α -syn (71-140) before nitration (observed mass = 7372 Da, expected mass = 7373 Da) **(C)** MALDI-TOF MS analysis of truncated α -syn (71-140) after nitration (observed mass = 7504 Da, expected mass = 7508 Da) **(D)** TNM nitration of truncated α -syn (129-140) at 100 μ M **(E)** ESI-LCMS analysis of truncated α -syn (129-140) before nitration (observed mass $[\text{M}+\text{H}]^+ = 1330.25$ Da, expected mass $[\text{M}+\text{H}]^+ = 1329.5$ Da) **(F)** ESI-LC/MS analysis of truncated α -syn (129-140) after nitration (observed mass $[\text{M}+\text{H}]^+ = 1420.17$ Da, expected mass $[\text{M}+\text{H}]^+ = 1419.47$ Da]

Biophysical characterization of nY125 and nY39 α -synuclein

***In vitro* protein fibrillization and sedimentation**

Lyophilized semisynthetic proteins were dissolved in 50 mM Tris, 150 mM NaCl, pH 7.5 and filtered through a 100 KDa MW-cut-off filter. Fibril formation was induced by incubating the protein at a final concentration of 10 μ M under agitation (1000 rpm) on an orbital shaker at 37°C, for 170 h. Amyloid formation was monitored by Thioflavin T (ThT) binding, soluble protein content and transmission electron microscopy (TEM). ThT fluorescence reading was performed in triplicates with a ThT concentration of 100 μ M and a protein concentration of 1 μ M in 50 mM glycine pH 8.5, using a Bucher Analyst AD plate reader (excitation: 450 nm, emission: 485 nm). To determine the remaining soluble protein content, aliquots of samples at each time point were centrifuged at 20'000 g for 10 min at 4°C to pellet insoluble fibrils, and the supernatant run on 15% SDS-PAGE, subsequently stained with a Coomassie R-450 solution. The relative amounts of soluble protein with respect to the initial conditions were determined by densitometry analysis (ImageJ).

Lipid vesicle preparation

Phospholipids in chloroform (1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol), (POPG) from Avanti Polar Lipids, Inc. were dried using an argon stream to form a thin film on the wall of a glass vial. Potential remaining of chloroform was removed by placing the vial under vacuum for 2 h. The phospholipids were then resolubilized in 20 mM sodium phosphate pH 7.5 to the desired stock concentration by freeze and thaw cycles (10X). The solution was then extruded through the Avestin LiposoFastTM (Avestin Inc.), according to manufacturer's instructions.

Circular dichroism

CD data collection was performed on a Jasco J-815 CD spectrometer operated at 20°C. CD spectra were acquired in the range of 195–250 nm using a 1.0 mm optical path length quartz cuvette. The following parameters were used for data recording: data pitch, 0.2 nm; bandwidth, 1 nm; scanning speed, 50 nm/min, digital integration time, 2 s. For each sample, ten spectra were averaged and smoothed using binomial approximation.

Transmission electron microscopy

3.5 μ l of sample was applied onto glow-discharged Formvar/carbon-coated 200-mesh copper grids (Electron Microscopy Sciences) for 1 min. The grids were then blotted with filter paper, washed twice with ultrapure water, once with uranyl formate 0.7% (w/V), stained for 30s, and then blotted off. Imaging was carried out on a Tecnai Spirit BioTWIN electron microscope equipped with a LaB₆ gun and a 4K x 4K FEI Eagle CCD camera (FEI), and operated at 80kV.

***In vitro* phosphorylation of nitrated α -syn by PLK3**

Nitrated proteins (WT α -Syn, nitrated WT α -Syn, nitrated Y39F α -syn and nitrated Y125F α -Syn, each 100 μ g) were dissolved in 100 μ L of reaction buffer (50 mM HEPES, 10 mM MgCl_2 , 10 mM DTT, 4 mM ATP and 2 mM EGTA, pH 7.4) to a final protein concentration of 70 μ M. To this, PLK3 (0.1 μ g) was added and incubated at 30 °C without shaking⁷. After 15 h, the reaction was analysed by ESI-MS and MALDI-TOF-MS. Complete phosphorylation was observed in all cases (+ 80 Da mass shift due to addition of phosphate group).

Western-Blot Analysis

Protein samples were separated on 1 mm-thick 15% polyacrylamide gels for 1 h at 180 V. The proteins were then transferred onto nitrocellulose membranes (Omnilab SA), using a semidry transfer system (Bio-Rad) under constant current (0.5A) and a maximum tension of 25 V. The membranes were then blocked for 1 h at RT in 1:2 Odyssey blocking buffer (Li-COR Biosciences):PBS. The membranes were probed with the relevant primary antibodies overnight at 4 °C (Supplemental Figure S26). After three washes with PBS buffer containing 0.1% (V/V) Tween 20 (Fluka), the membranes were incubated with secondary goat antimouse or antirabbit antibodies conjugated to Alexa 680 (dilution 1:5000; Invitrogen). The membranes were then washed three times with PBS buffer containing 0.01% (V/V) Tween 20, once with PBS buffer and scanned on a LiCOR scanner at 700 nm.

Supplemental results

Remaining soluble fraction following aggregation of the semisynthetic proteins

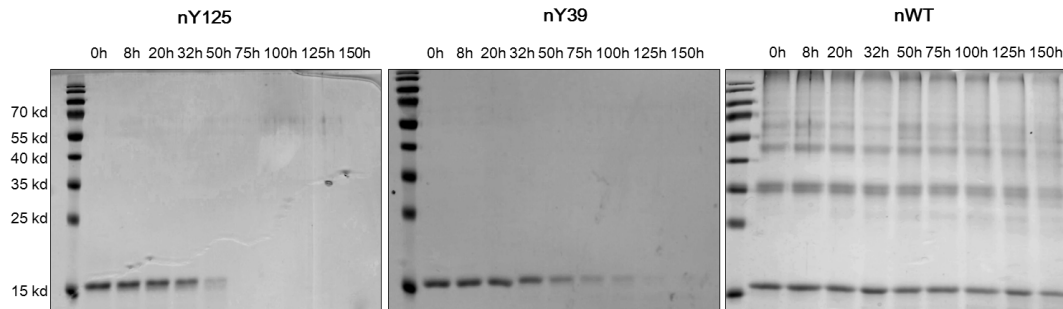


Figure S22: SDS-PAGE of the remaining soluble protein at different time points during the time course of aggregation

Remaining soluble protein at various incubation time points, as assessed by SDS-PAGE. Compared to nY125 (left panel) and nY39 α -syn (middle panel), which show a gradual lowering of the soluble protein fraction with time, TNM nitrated WT α -syn (right panel) presents a pattern of oligomeric species that does not significantly change with time.

WT and semisynthetic α -syn binding to membranes

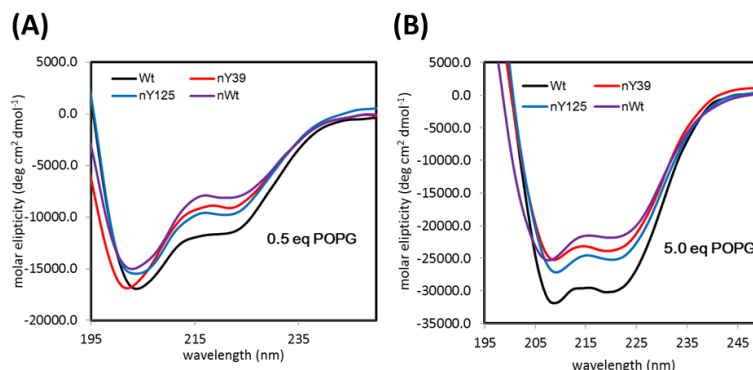


Figure S23: Circular dichroism of WT α -syn semisynthetic nY39 and nY125 α -syn and TNM-nitrated α -syn mixed with POPG vesicles at protein: lipid weight ratio of 1: 0.5 and 1:5.

Circular dichroism of WT α -syn (black), semi-synthetic nY39 (red), nY125 α -syn (blue) and TNM-nitrated α -syn (purple) mixed with POPG vesicles at protein: lipid weight ratio of (A) 1:0.5 and (B) 1:5.

α -syn Y39/125F and 133/136F dimer characterization

5 mg of Y39/125F α -syn was nitrated with TNM as previously described⁶. After the reaction was completed, as assessed by ESI- LCMS, excess TNM was removed using a PD10 column and the protein was lyophilized. The lyophilized powder was then dissolved in 500 μ L of C buffer (50mM Tris, 150mM NaCl, pH 7.5) and loaded on superdex 200 10/300 GL. The dimer was eluted with SEC buffer with a flow rate of 0.4 mL/min. The purified dimer was dissolved in 50

mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.5 to a final dimer concentration 2 ug/uL. Trypsin (1:20 weight ratio to dimer) was added and incubated at 37°C for 15 h. The trypsin digested dimers were purified by semi-preparative RP-HPLC C8 analytical column and the fractions were analyzed by Maldi-TOF and Orbitrap XL MS.

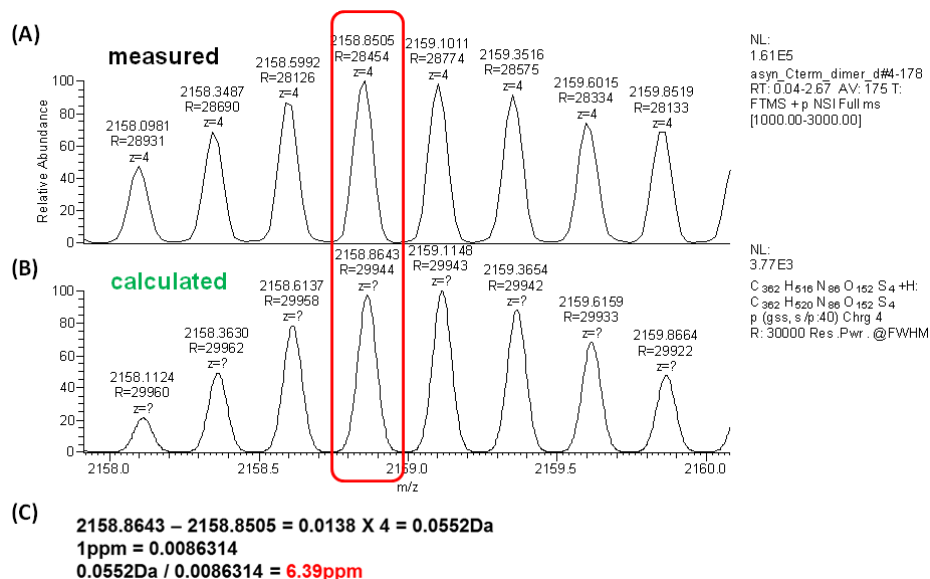


Figure S24: Mass analysis of trypsin digested Y39/125F α-syn dimer

(A) Orbitrap XL MS analysis (infusion) of trypsin digested Y39/125F α-syn dimer. **(B)** Theoretical mass calculation of quadruply charged dimer and **(C)** its deviation from the measured mass.

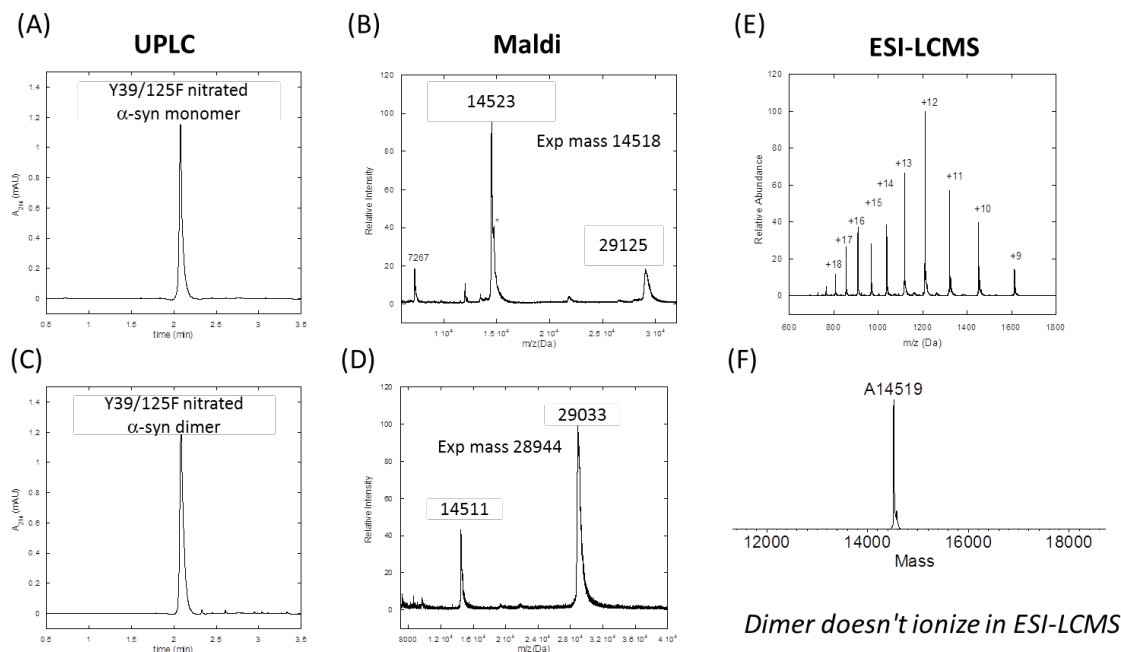


Figure S25. Characterization of nitrated Y39/125F α-syn monomer and dimer

(A) Analytical RP-UHPLC nitrated Y39/125F α-syn monomer **(B)** MALDI-TOF MS analysis of nitrated Y39/125F α-syn monomer (observed mass = 14,523 Da, expected mass = 14,518 Da). **(C)** Analytical RP-UHPLC of nitrated Y39/125F α-syn dimer **(D)** MALDI-TOF MS analysis of nitrated Y39/125F α-syn dimer (observed mass = 29,033

Da, expected mass = 28944 Da) (E) ESI-MS spectra of nitrated Y39/125F α -syn monomer (F) ESI-MS spectra correspond to the mass calculated by deconvolution with MagTran.

TNM-nitrated α -syn WT and mutants antibody screening

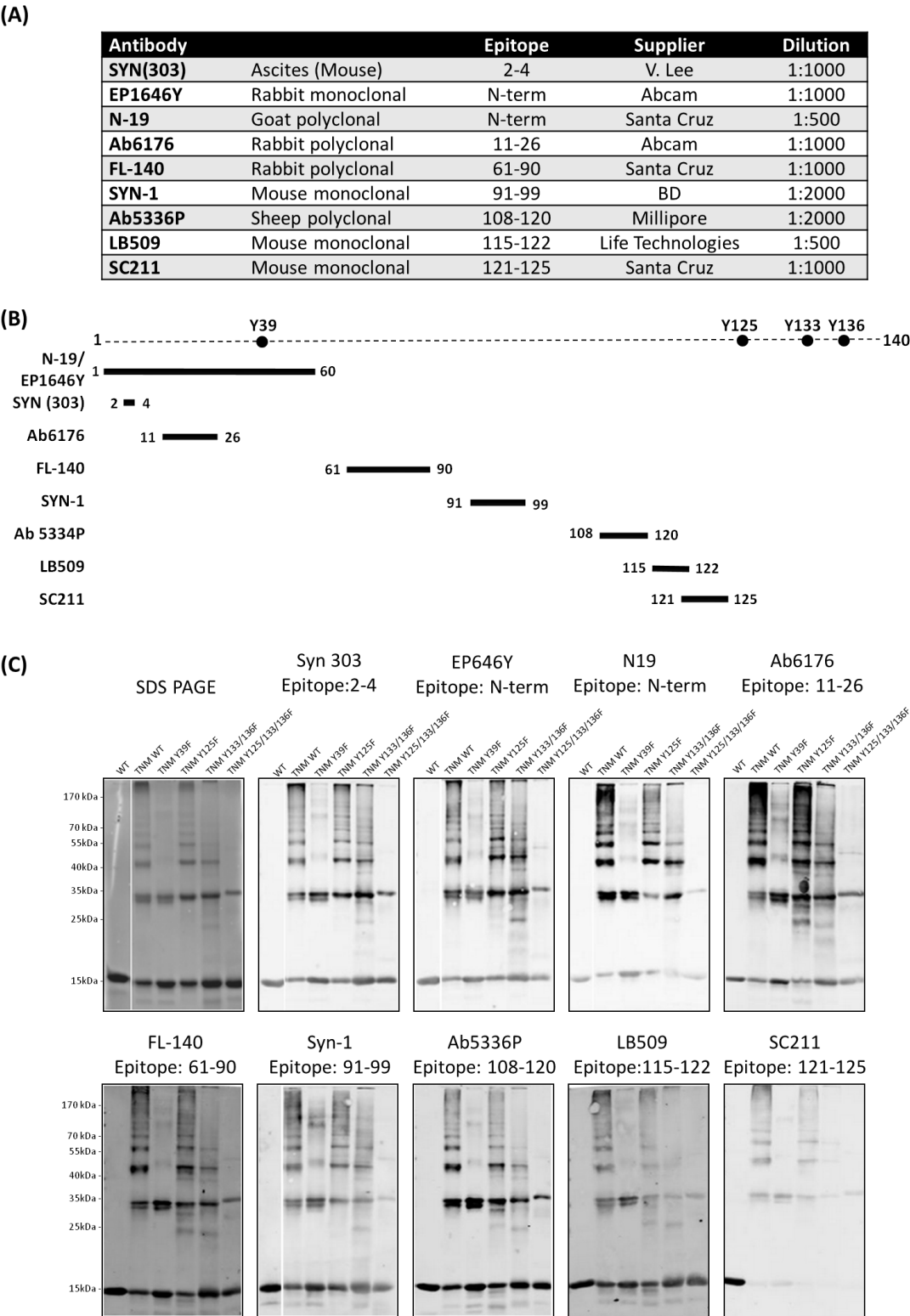


Figure S26. Antibodies list, references, working dilutions, epitopes and recognition of TNM-nitrated α -syn WT and mutants (A) A list of the antibodies used in this study and working dilutions. (B) Map of α -syn and epitopes for the antibodies listed in A. (C) Antibody recognition of TNM-nitrated α -syn WT and mutated at different Tyr residues. Nitration and dityrosine crosslinking influences the recognition of anti N-term α -syn antibodies, and antibodies whose epitope is close or includes C-term Tyr residues. From left to right: R-250 Coomassie blue stained SDS PAGE, syn 303, EP646, N19, Ab6176, FL-140, Syn-1, Ab5336P, LB509 and SC211 immunoblots of WT and TNM-nitrated WT and mutants (Y39F, Y125F, Y133/136F, Y125/133/136F).

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