O-GlcNAc modification blocks the aggregation and toxicity of the Parkinson’s disease associated protein α-synuclein

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Supplemental References
**General:** All solvents and reagents were purchased from commercial sources (Sigma-Aldrich, Fluka, EMD, Novagen, etc.) and used without any further purification. All aqueous solutions were prepared using ultrapure laboratory grade water (deionized, filtered, sterilized) obtained from an in-house ELGA water purification system and filter sterilized with 0.45 μm syringe filters (VWR) before use. Growth media (LB broth, Miller, Novagen and S.O.C. broth, Sigma) were prepared, sterilized, stored, and used according to the manufacturer. Antibiotics were prepared as stock solutions at a working concentration of 1000x (ampicillin sodium salt, EMD 100 mg mL\(^{-1}\), kanamycin sulfate, EMD, 50 mg mL\(^{-1}\)) and stored at -20 °C. All bacterial growth media and cultures were handled under sterile conditions under open flame. All silica gel column chromatography was performed using 60 Å silica gel (EMD) and all thin-layer chromatography performed using 60 Å, F254 silica gel plates (EMD) with detection by ceric ammonium molybdate (CAM) and/or UV light. Reverse phase high performance liquid chromatography (RP-HPLC) was performed using an Agilent Technologies 1200 Series HPLC with Diode Array Detector. Unless otherwise stated the HPLC buffers used were buffer A: 0.1% TFA in H\(_2\)O, buffer B: 0.1% TFA, 90% ACN in H\(_2\)O. Mass spectra were acquired on an API 3000 LC/MS-MS System (Applied Biosystems/MDS SCIEX). \(^1\)H NMR spectra were acquired on either a Varian Mercury 400 MHz or Varian VNMRS 500 MHz magnetic resonance spectrometer.

**Plasmid Construction:** A pRK172 construct was generated containing wild-type human α-synuclein inserted into Nde I and Hind III restriction sites using standard molecular cloning techniques, as has been described previously\(^1\). The C-terminal fragment of α-synuclein 2 (aa 76-140, A76C) was introduced into a pET42b vector using Nde I and Spe I restriction sites and standard molecular cloning techniques. The N-terminal fragment of α-synuclein 3 (aa 1-68) was introduced into a modified pTXB1 construct containing the Ava-DnaE N137A intein\(^2\) using Nde I and Bpu10I restriction sites and standard molecular cloning techniques.

**Expression of recombinant α-synuclein:** BL21(DE3) chemically competent *E. coli* (VWR) were transformed with the pRK172 construct containing wild-type human α-synuclein or α-synuclein(T72A) by heat shock, plated on selective LB agar plates containing 100 μg mL\(^{-1}\) ampicillin (LB-amp), and incubated at 37 °C for 16 h. Single colonies were selected and used to inoculate two 5 mL LB-amp liquid cultures, which were grown at 37 °C with shaking at 250 rpm for 16 h. Each 5 mL culture was used to inoculate a 1 L LB-amp culture. These cultures were grown to an OD600 of 0.6-0.7 at 37 °C shaking at 250 rpm, and then expression was induced with IPTG (final concentration: 0.5 mM) at 25 °C shaking at 250 rpm for 18 h. Bacteria were harvested by centrifugation (8,000 x g, 30 min, 4 °C), and the cell pellets were lysed by three freeze thaw cycles, using liquid N\(_2\) and a 37 °C water bath. Cell lysates were resuspended, on ice, in 10 mL (per 1 L of culture) of lysis buffer (500 mM NaCl, 100 mM Tris, 10 mM β-mercaptoethanol (βME), 1 mM EDTA, pH 8.0). Cell lysates were boiled at 80 °C for 10 min, allowed to cool to room temp, and then placed on ice. Protease inhibitor cocktail (mini complete EDTA free, Roche) was added and lysates were incubated on ice for 20 min and then cleared by centrifugation (42,000 x g, 30 min, 4 °C). The resulting supernatant was acidified, on ice, to pH 3.5 with HCl and then incubated on ice an additional 20 min before centrifuging again (42,000 x g, 30 min, 4 °C). The resulting supernatant was dialyzed against 3 x 1 L of 1% acetic acid in water (degassed with N\(_2\), 1 h per L). The dialyzed protein solution was then purified by RP-HPLC over a C4 semi-preparative column (Vydac). Purified material was flash frozen in liquid N\(_2\) and lyophilized. Pure α-synuclein was characterized by C4 analytical RP-HPLC column (Vydac) and ESI-MS (M+H\(^{+}\)) and yield was determined by Pierce BCA assay (Thermo Scientific).

**Expression of α-synuclein C-terminal fragment (2):** BL21(DE3) chemically competent *E. coli* (VWR) were transformed with the pET42b construct containing 2 by heat shock and plated on selective LB agar plates containing 50 μg mL\(^{-1}\) kanamycin (LB-kan). Expression and purification of 2 was carried out as described above for recombinant α-synuclein.

**Expression of α-synuclein N-terminal thioester (3):** BL21(DE3) chemically competent *E. coli* (VWR) were transformed with the modified pTXB1 construct containing 3 by heat shock, plated on selective LB agar plates containing 100 μg mL\(^{-1}\) ampicillin (LB-amp), and incubated at 37 °C for 16 h. Bacteria were
cultured and induced as described above. After harvesting bacteria by centrifugation (8,000 x g, 30 min, 4 °C), the cell pellet was resuspended on ice in 10 mL (per 1 L of culture) cold lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 5 mM imidazole, 2 mM TCEP HCl, pH 8.0) plus protease inhibitor cocktail and lysed by tip sonication (35% amplitude, 30 sec pulse duration, 30 sec rest for 12 min) while on ice. The crude cell lysate was cleared by centrifugation (42,000 x g, 30 min, 4 °C) and the supernatant was loaded onto a Ni-NTA purification column (HisTrap FF Crude, GE Healthcare). The column was washed with 5 column volumes (CV) of lysis buffer, 5 CV of wash buffer 1 (lysis buffer, 20 mM imidazole), 3 CV of wash buffer 2 (lysis buffer, 50 mM imidazole), and then eluted in 4 x 1 CV of elution buffer (lysis buffer, 250 mM imidazole). Elution fractions were dialyzed against 3 x 1 L (100 mM NaH₂PO₄, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP HCl, pH 7.2) and then concentrated approximately 5-fold in spin-column concentrators (Amicon Ultra 3 kDa MW cut-off, Millipore). Sodium mercaptoethane sulfonate (MESNa) was added to a final concentration of 200 mM along with fresh TCEP (2 mM final concentration), and the thiolysis reaction was incubated at room temperature to generate the protein thioester. Reaction progression was monitored by analytical RP-HPLC. Upon completion, the thiolysis reaction was purified over a C4 semi-prep column and stored as a lyophilized solid. Pure thioester 3 was characterized by analytical RP-HPLC and ESI-MS.

**Solid phase synthesis of thioester peptides 1 and 6:** All solid-phase peptide syntheses were conducted manually using unprotected Rink amide ChemMatrix® resin, (PCAS BioMatrix) with an estimated loading of 0.45 mmol g⁻¹ using a 4-amino benzoic acid linker³. Commercially available N-Fmoc and side chain protected amino acids (10 eq, Novabiochem) were activated for 20 min with HBTU (10 eq, Novabiochem) and DIEA (20 eq, Sigma) and then coupled to the resin for 1 h, bubbling with N₂ to mix. Reaction completion was checked using the Kaiser test. Briefly, a small amount of resin was incubated with equal volumes of 5% w/v ninhydrin in EtOH, 80% w/v phenol in EtOH, and 20μM KCN in pyridine and heated to 99 °C for 5 min in a sealed tube. If necessary, a second coupling was conducted with 10 eq amino acid, 10 eq HOBt (Novabiochem) and 12 eq DCC (Sigma) for 2 h, with N₂ mixing. After successful coupling, the terminal Fmoc group was removed with 20% v/v piperidine in DMF for 5 min with N₂ mixing, and then for an addition 15 min with fresh 20% piperidine in DMF. When peptides were completed, peptide 7 was deprotected with hydrazine hydrate (80% v/v in MeOH) twice for 30 min, with N₂ mixing. Both peptides were then acetylated at the free amine of the Dbz linker with p-Nitrophenylchloroformate (5 eq in DCM, N₂ mixing) followed by treatment with excess DIEA (0.5 M in DMF) for 15 min to cyclize the Dbz linker. Peptides were then cleaved from the resin by incubating in cleavage cocktail (95:2.5:2.5 TFA/H₂O/Triisopropylsilane) for 3.5 h at room temperature. The peptide was then diluted ~1/10 in cold diethyl ether and precipitated over night (-80 °C). The resulting suspension was centrifuged (5,000 x g, 30 min, 4 °C) and the pellet was resuspended in fresh Et₂O and centrifuged again (5,000 x g, 30 min, 4 °C). The pellet was then resuspended in H₂O, flash frozen, and lyophilized. This crude lyophilized material was resuspended in thiolysis buffer (150 mM NaH₂PO₄, 150 mM MESNa, pH 7.0) and incubated at room temperature for 24 h before being purified by RP-HPLC (0-50% buffer B over 60 min) over a C18 semi-preparative column (Vydac). Purified peptides were characterized by RP-HPLC (0-70% B gradient over 60 min) over an analytical C18 column (Vydac) and ESI-MS.

**Unmodified α-synuclein synthesis:** Lyophilized WT thioester peptide 1 (4 mM) and α-synuclein C-terminal fragment 2 (2 mM) were dissolved in ligation buffer (300 mM NaH₂PO₄, 5.5 M guanidine HCl, 100 mM MESNa, 1 mM TCEP, pH 7.8) and allowed to react at room temperature. The reaction pH was readjusted with concentrated NaOH as needed after dissolving both components. Reaction progress was monitored by RP-HPLC over an analytical C18 column with a gradient of 0-70% B over 60 min. The reaction was complete after 48 h and purified by HPLC to yield pure α-synuclein fragment (69-140) 4. Product was confirmed by ESI-MS. The N-terminal thiazolidine (NThz) protecting group of 4 was then removed with methoxylamine to yield the free N-terminal cysteine fragment 5. Specifically, lyophilized 4 (2 mM) was dissolved in deprotection buffer (100 mM NaAcO, 5.5 M guanidine HCl, 100 mM NaCl, 250 mM MeONH₂ HCl, pH 5.0) and heated to 37 °C for 48 h. Upon completion, βME was added to reduce protein disulfides and the reaction was purified by RP-HPLC and lyophilized. Product was confirmed by ESI-MS. Lyophilized 5 (2 mM) was then dissolved in freshly prepared ligation buffer. This solution was then added to lyophilized α-synuclein (1-68) thioester 3 (8 mM) and the pH of the solution was adjusted
back to 7.8 with conc. NaOH. The reaction was complete after 72 h at room temperature and fresh TCEP was added to reduce any MES disulfides. The reaction was purified by RP-HPLC to yield full-length α-synuclein 6. Finally, radical desulfurization was used to convert the two cysteines in 6 to native alanines using the radical initiator VA-061 (Wako). Briefly, protein 6 was dissolved in buffer (200 mM NaH₂PO₄, 6 M guanidine HCl, 300 mM TCEP, pH 7.0) to which was added 2% v/v ethanethiol, and 10% v/v tertbutylthiol. VA-061 (as a 0.2 M stock in MeOH) was then added to a final concentration of 2 mM (final protein concentration was 0.75 mg mL⁻¹). The reaction was heated to 37 °C for 15 h and then purified by RP-HPLC to yield synthetic, full-length WT α-synuclein.

**α-Synuclein(gT72) synthesis:** Lyophilized O-GlcNAc thioester peptide 7 (2 mM) and α-synuclein C-terminal fragment 2 (1.6 mM) were dissolved in ligation buffer (300 mM NaH₂PO₄, 5.5 M guanidine HCl, 100 mM MESNa, 1 mM TCEP, pH 7.8) and allowed to react at room temperature. The reaction pH was readjusted with concentrated NaOH as needed after dissolving both components. Reaction progress was monitored by RP-HPLC. The reaction proceeded very slowly and was allowed to continue for 192 h, after which it was purified by HPLC to yield pure O-GlcNAcylated α-synuclein fragment (69-140) 8. Product was confirmed by ESI-MS. The N-terminal thiazolidine (NThz) protecting group of 8 was removed with methoxylamine to yield the free N-terminal cysteine fragment 9. Specifically, lyophilized 8 (2 mM) was dissolved in deprotection buffer (100 mM NaAcO, 5.5 M guanidine HCl, 100 mM NaCl, 250 mM MeONH₂ HCl, pH 5.0) and heated to 37 °C for 72 h. Upon completion the reaction was purified by RP-HPLC and lyophilized. Product was confirmed by ESI-MS. Lyophilized 9 (2 mM) was dissolved in freshly prepared ligation buffer. This solution was then added to lyophilized α-synuclein (1-68) thioester 3 (8 mM) and the pH of the solution was adjusted back to 7.8 with concentrated NaOH. The reaction was complete after 96 h at room temperature and fresh TCEP was added to reduce any MES disulfides. The reaction was purified by RP-HPLC to yield full-length α-synuclein 10. Radical desulfurization was used to convert the two cysteines in 10 to native alanines using VA-061. Protein 10 was dissolved in buffer (200 mM NaH₂PO₄, 6 M guanidine HCl, 300 mM TCEP, pH 7.0) to which was added 2% v/v ethanethiol, and 10% v/v tertbutylthiol. VA-061 (as a 0.2 M stock in MeOH) was added to a final concentration of 2 mM (final protein concentration was 0.75 mg mL⁻¹). The reaction was heated to 37 °C for 16 h and then purified by RP-HPLC to yield synthetic, full-length O-GlcNAc α-synuclein (α-synuclein (gT72)).

**Aggregation reactions:** Synthetic or recombinant wild-type or α-synuclein(gT72) were aliquoted and lyophilized. Lyophilized proteins were resuspended in reaction buffer (10 mM NaH₂PO₄, 0.05% NaN₃, pH 7.4) to the appropriate concentration (50 μM), and all material was dissolved using a bath sonicator. For mixtures of recombinant α-synuclein and α-synuclein(gT72), 50, 25, or 10% α-synuclein(gT72) material was aliquoted into the same tube prior to lyophilization and resuspended the same as pure samples. Reactions were centrifuged for 15 min. at 14,000 x g to remove any debris or aggregated material. The supernatant was then split into triplicate reaction in 1.5 mL conical centrifuge tubes (VWR). Reactions were then incubated in a Thermomixer F1.5 orbital shaker at 1000 rpm, 37 °C for seven days. Sample aliquots for analysis at t₀ were removed prior to heating at 37 °C.

**Circular Dichroism:** Circular dichroism spectra were collected on a Jasco J-815 CD Spectrometer. Samples were diluted to 7.5 μM α-synuclein with reaction buffer containing no NaN₃. Spectra were collected from 250-195 nm with a 0.1 nm data pitch, 50 nm min⁻¹ scanning speed, data integration time of 4 sec, 1 nm bandwidth, 1 mm path length with 3 accumulations, at 25 °C.

**Dynamic light scattering:** Light scattering data was collected with a Dynapro Titan temperature controlled microsampler (Wyatt). Samples taken directly from aggregation reactions (50 μM) at time 0 h were analyzed with ten 10 s acquisition, at 25 °C, with laser power adjusted to give an intensity of 2.0E⁶ counts sec⁻¹. Radii were calculated using a Raleigh sphere approximation.

**Thioflavin T fluorescence:** The degree of α-synuclein aggregation was quantified by Thioflavin T fluorescence. Samples were prepared by diluting samples from aggregation reaction (final α-synuclein concentration = 1.25 μM) in 20 μM Thioflavin T in reaction buffer (above). Samples were diluted, vortexed briefly, and then incubated 2 min before analyzing. Spectra were collected using a NanoLog Spectro-
fluorometer (Horiba), $\lambda_{\text{ex}} = 450 \text{ nm}$, ex slit = 4 nm, $\lambda_{\text{em}} = 482$, em slit = 3 nm, 10 mm path length, integration time = 0.1 sec, 3 averaged accumulations. Data was measured in triplicate for all aggregation reaction conditions.

**Transmission electron microscopy:** A 10 µL droplet from each sample was deposited on formvar coated copper grids (150 mesh, Electron Microscopy Sciences) and allowed to sit for 5 min and then excess liquid was removed with filter paper. Grids were then negatively stained for 2 min with 1% uranyl acetate, washed three times with 1% uranyl acetate, each time removing excess liquid with filter paper. The grids were desiccated for 48 h in a vacuum desiccator. Grids were imaged using a JOEL JEM-2100F transmission electron microscope operated at 200 kV, 60,000x magnification, and an Orius Pre-GIF CCD.

**SEC-MALS:** Recombinant, wild-type $\alpha$-synuclein or $\alpha$-synuclein(gT72) were aggregated at 50 µM as described above. After seven days, aggregation reactions were centrifuged (20,000 x g, 25 °C, 1 h) to remove fibrils and other large aggregates. The supernatants were then separated using a Shodex KW-802.5 size exclusion chromatography column (mobile phase 50 mM HEPES, 200 mM Na$_2$SO$_4$, pH 7.5) and light scattering and differential refractive index were measured with an in-line Wyatt Dawn Heleos and Optilab rEX detectors respectively.

**Circular Dichroism of $\alpha$-synuclein in the presence of lipids:** CD spectra were obtained using a Jasco J-810 spectropolarimeter at room temperature at a 1:100 protein:lipid molar ratio with vesicles of different degrees of negative charge. Lipid vesicles were prepared through mixing different ratios of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or using 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-RAC-(1-glycerol)] (POPG). Dried lipid films were resuspended in 10 mM sodium phosphate pH 7.4 buffer. Spectra were acquired using a scan rate of 50 nm/minute, bandwidth of 1 nm, 1 sec time response and step resolution of 1 nm. The final spectra were obtained by subtracting away the appropriate blanks.

**Transmission electron microscopy of membrane tubulation:** O-GlcNAc modified and wild type $\alpha$-synuclein were incubated with POPG vesicles in a 1:20 protein:lipid molar ratio as previously described. 10 µL of the sample was loaded onto carbon-coated formvar films on copper grids (Electron Microscopy Sciences) and subsequently stained with 1% uranyl acetate. Negative stain transmission electron microscopy was performed on a JEOL 1400 transmission electron microscope accelerated to 100 kV.

**Cellular toxicity assay:** Cortices from E17 Sprague-Dawley rat embryos were dissociated in a 0.25 % trypsin 1 mM HEPES, Hank’s balanced salt solution (HBSS) for 15 min; the tissue was then washed 3 times in fresh HEPES HBSS. At 10 days prior to treatment, the dissociated neurons were then plated in poly-D-lysine (Sigma) and laminin (Sigma) pre-treated 96-well plates (Costar, black plate, clear bottom with lid) at a density of 2.5x10$^4$ cells per well in supplemented Neurobasal medium (Invitrogen). The Neurobasal medium was supplemented with 10 ml L$^{-1}$ Glutamax (Invitrogen), 1 mg L$^{-1}$ gentamicin solution (Invitrogen), 20 ml L$^{-1}$ B-27 supplement (Invitrogen), and 50 ml L$^{-1}$ fetal bovine serum (Invitrogen) and grown at 37 °C under humidified condition in 5% CO$_2$ atmosphere. Four hours after the neurons were plated, the medium was diluted 1:3 with serum-free supplemented Neurobasal medium and again diluted 1:2 with fresh serum-free supplemented Neurobasal medium after 7 days in vitro. SH-SY5Y cells were grown at 37 °C under humidified condition in 5% CO$_2$ atmosphere using DMEM/F12K 1:1 supplemented with 10% FBS. At 48 h prior to the treatment, 1.25 x 10$^4$ cells per well were plated into 96-well assay plate (Costar, black plate, clear bottom with lid). Recombinant $\alpha$-synuclein or $\alpha$-synuclein(gT72) were subjected to aggregation conditions (shaking at 1000 rpm, 37 °C) at 50 µM for 7 days. The reactions were then centrifuged (2,000 x g, 25 °C, 1 h) and the supernatants were removed and lyophilized to dryness. The aggregates/supernatant were resuspended with the appropriate growth medium, followed by bath sonicication (20 min) and tip-sonication (20% amplitude, 1 sec pulse duration, 1 sec rest for 14 sec) and then added to the cells. Cells were monitored using microscope after the treatment. The ethidium homodimer toxicity assay was performed at t = 60 h. Specifically, 100 µL of 1X DPBS solution containing 3 µM of Ethidium homodimer (VWR) were added into each well. The plate was incubated in tissue culture incubator for 40 min before taking readings. The plate was shaken for 10 sec at the highest intensity be-
fore fluorescent signal was measured using Synergy H4 Hybrid reader (BioTek) Excitation = 530 nm, Emission = 620 nm, Gain = 100, Band width = 20 nm, read height of 5 mm). For SH-SY5Y cells, the cells were then trypsonized and counted using a Countess II Automated Cell Counter (Life Technologies) according to the manufacturer’s procedure.

**In vitro phosphorylation reaction and Western blotting:** Either recombinant α-synuclein or α-synuclein(gT72) was subjected to in vitro phosphorylation with one of three kinases; casein kinase 1 (CK1), polo-like kinase 3 (PLK3), or G protein-coupled receptor kinase 5 (GSK5). Pure, lyophilized proteins were dissolved at 28 μM in buffer (10 mM dithiothreitol (DTT), 1 mM MgCl₂, 50 mM Tris, 1 mM ATP, pH 7.5). These solutions were prepared and split into 49 μL triplicates to which 1 μL of the corresponding kinase was added. Reactions were incubated at 30 °C, shaking at 500 rpm for 16 h. Reactions were quenched by boiling in 4X SDS loading buffer for 10 min. Samples were loaded at 2.5 ng per well on an 18% Criterion TGX precast gel (BioRad) and separated by SDS-PAGE at 190 V for 1 h. Proteins were transferred to PVDF membranes (BioRad) in a Transfer Blot semi-dry transfer cell (BioRad) at 20 V for 1 h. Membranes were blocked in 5% non-fat milk in Tris buffered saline plus Tween-20 (TBST) for 1 h at room temperature and washed with 3 x 10 mL TBST (10 min). Membranes were then incubated overnight at 4 °C with primary antibodies for either α-synuclein p-S87 (Ser 87-R, Santa Cruz Biotech., 1:200 dilution) or α-synuclein p-S129 (P-syn/81A, Covance, 1:1000 dilution) in 5% milk in TBST. For a loading control, an anti-α-synuclein antibody (syn 211, Invitrogen, 1:5000 dilution) was used. Membranes were washed with 3 x 10 mL TBST (10 min) and then incubated with the appropriate secondary antibodies at room temperature for 1 h followed by another 3 x 10 mL TBST wash (10 min). Finally, membranes were incubated with ECL (BioRad) for 3 min and imaged using a ChemiDoc XRS imaging system (BioRad).

**Analysis of aggregates by SDS-PAGE and/or Western blotting:** Recombinant α-synuclein (25 μM) alone or in the presence of α-synuclein(gT72) (25 μM) underwent aggregation for 7 days (shaking at 1000 rpm at 37 °C). Reactions were then centrifuged (20,000 x g, 4 °C, 1 h) and the supernatants were removed and lyophilized. Both the pellets and the lyophilized supernatants were sonicated in freshly prepared 8 M urea, 20 mM HEPES, pH 8.0, followed by boiling for 10 min with 4X SDS loading buffer. Samples were loaded onto an 18% gel (5 ng/well, BioRad), separated by SDS-PAGE as described above, and stained with Coomassie brilliant blue 2 hrs, followed by destaining in 4:5:1 acetic acid, methanol, water overnight. The same samples were also separated by SDS-PAGE before being transferred to PVDF membrane (Bio-Rad) using standard Western blotting procedures. Anti-O-GlcNAc blots were blocked in TBST containing 5% bovine serum albumin (BSA) for 1 h at rt and incubated overnight at 4°C, then incubated with anti-O-GlcNAc (RL2; Sigma, #MA1-072) at 1:1000 dilution in blocking buffer for 24 h at 4 °C. The blots were then washed three times in TBST for 10 min and incubated with the HRP-conjugated anti-mouse (Jackson ImmunoResearch, #715-035-150) for 1 h in the appropriate blocking buffer at RT. After being washed three more times with TBST for 10 min, the blots were developed using ECL reagents (Bio-Rad) and the ChemiDoc XRS+ molecular imager (BioRad).

**Aggregation reactions with pre-formed fibers:** Lyophilized wild-type α-synuclein was resuspended in reaction buffer (10 mM phosphate, 0.05% sodium azide, pH7.4) to a concentration of 50 μM. After 15 min of bath sonication, the resuspended protein was incubated at 37 °C under continuous shaking (1000 rpm) in an Eppendorf thermomixer for 7 days. This aggregate reaction was then bath sonicated for 20 min, and subsequently tip-sonicated (8 X 1 sec pulses separated by 1 sec, 20 % amplitude). Sonicated aggregates were aliquoted into three sets of triplicates. To each set, the equal volume of either the reaction buffer, unmodified α-synuclein monomer, or α-synuclein(gT72) monomer were added to give a final concentration of 25 μM fibers and 25 μM monomer. The resulting mixtures were incubated under continuous shaking (1000 rpm) at 37 °C in the Eppendorf thermomixer for indicated times. At each time point, Thioflavin T fluorescence was measured as described above.
Supplementary Figure 1. Characterization of full-length, recombinant α-synuclein and α-synuclein(T72A). a, Analytical RP-HPLC trace and ESI-MS of purified, recombinant WT α-synuclein. b, Analytical RP-HPLC trace and ESI-MS of purified, recombinant α-synuclein(T72A).

Supplementary Figure 2. α-Synuclein(T72A) displays reduced aggregation compared to wild-type α-synuclein. a, α-Synuclein or α-synuclein(T72A) (50 μM) were subjected to aggregation conditions (agitation at 37 °C) before analysis by ThT fluorescence (λ\text{ex} = 450 nm, λ\text{em} = 482 nm) at the indicated time points. y-Axis is fold-change in fluorescence compared to unmodified α-synuclein at t = 0 h. b, The same reactions were analyzed by TEM after 7 days; scale bar: 500 nm.
Supplementary Figure 3. Characterization of thioester peptide 1. Analytical RP-HPLC trace and ESI-MS of purified thioester peptide 1 (* = guanidine HCl used for solubilization).

Supplementary Figure 4. Characterization of protein fragment 2. Analytical RP-HPLC and ESI-MS trace of purified C-terminal protein fragment 2.

Supplementary Figure 5. Ligation of peptide 1 and protein 2 to give protein fragment 4. a, Monitoring of the ligation reaction of 1 and 2 by RP-HPLC. b, Analytical RP-HPLC trace and ESI-MS of purified product 4.
Supplementary Figure 6. N-terminal deprotection of the thiazolidine of protein 4 to yield protein fragment 5. a, Monitoring the thiazolidine (NThz) deprotection reaction of 4 by RP-HPLC. b, Analytical RP-HPLC trace and ESI-MS of purified product 5.

Supplementary Figure 7. Expression and characterization of protein-thioester fragment 3. a, Monitoring the intein-fusion thiolysis to generate protein thioester 3 by RP-HPLC. b, Analytical RP-HPLC trace and ESI-MS of purified product 3.
Supplementary Figure 8. Ligation of protein-thioester 3 and protein 5 to give full-length α-synuclein 6. a, Monitoring the ligation of fragments 3 and 5 by RP-HPLC. b, Analytical RP-HPLC trace and ESI-MS of purified product 6.

Supplementary Figure 9. Desulfurization of α-synuclein 6 to give synthetic α-synuclein. a, Monitoring the desulfurization reaction of 6. b, Analytical RP-HPLC trace and ESI-MS of purified full-length, synthetic α-synuclein.
Supplementary Figure 10. Characterization of O-GlcNAcylated thioester-peptide 7. Analytical RP-HPLC trace and ESI-MS of purified thioester peptide 7.

Supplementary Figure 11. Ligation of protein 2 and glycopeptide 7 to give O-GlcNAcylated protein fragment 8. a. Monitoring the ligation reaction of 2 and 7. The reaction proceeded rapidly at first but was slow to reach completion. b. Analytical RP-HPLC trace and ESI-MS of purified product 8. MS signal strength was decreased by ionization of the GlcNAc moiety and cleavage of the scissile glycosidic bond.
Supplementary Figure 12. N-terminal deprotection of the thiazolidine of protein 8 to yield glycoprotein fragment 9. a, Monitoring of the thiazolidine (NThz) deprotection reaction of 8. b, Analytical RP-HPLC trace and ESI-MS of purified product 9. MS signal strength was decreased as with 8.

Supplementary Figure 13. Ligation of protein-thioester 3 and glycoprotein 9 to give full-length O-GlcNAcylated α-synuclein 10. a, Monitoring the ligation reaction of 3 and 9. b, Analytical trace from t_{96} of the same reaction. b, Analytical RP-HPLC trace and ESI-MS of purified product 10.
Supplementary Figure 14. Desulfurization of α-synuclein 10 to give O-GlcNAcylated α-synuclein [α-synuclein(gT72)]. a, Monitoring the desulfurization reaction of 10. b, Analytical RP-HPLC trace and ESI-MS of purified full-length, synthetic α-synuclein(gT72).

Supplementary Figure 15. Structural characterization of synthetic and recombinant proteins using circular dichroism (CD) and dynamic light scattering (DLS). a, CD spectra were collected for freshly dissolved samples of recombinant α-synuclein, synthetic α-synuclein, or α-synuclein(gT72) at 7.5 μM concentration. All samples show similar spectra that are consistent with a random-coil secondary structure. b, The indicated proteins were analyzed using DLS at 50 μM concentration. All three preparations showed a single peak with a Stoke’s Radius of approximately 4 nm, and no significant peaks in the 10-100 nm range, consistent with monomeric protein.
Supplementary Figure 16. Large-scale representation of the transmission electron microscopy (TEM) images in Figure 3c.

Supplementary Figure 17. Transmission electron microscopy (TEM) images of the amorphous protein deposited from the α-synuclein(gT72) aggregation reactions. α-Synuclein(gT72) (50 μM) was subjected to aggregation conditions (agitation at 37 °C) for 7 days, after which samples were deposited on formvar coated copper grids and stained with 1% uranyl acetate. These samples were largely devoid of protein aggregates, consistent with the sample being predominantly monomeric. When these grids were scanned for the presence of any larger protein structures, only amorphous deposits, and not fibrils or oligomers, were observed.

Supplementary Figure 18. Large-scale representation of the transmission electron microscopy (TEM) images in Figure 4b.
Supplementary Figure 19. O-GlcNAcylation affects the subsequent phosphorylation of α-synuclein. Unmodified or O-GlcNAcylated α-synuclein were incubated with the indicated kinases for 16 h. Phosphorylation status was then visualized by SDS-PAGE followed by Western blotting. Results are representative of two biological replicates.

Supplementary Figure 20. Analysis of cellular toxicity samples by transmission electron microscopy (TEM). Unmodified or O-GlcNAcylated α-synuclein were subjected to aggregation conditions for 7 days. At this time, the aggregated and soluble material were separated by centrifugation and resuspended by sonication in water followed by analysis by TEM.

Supplementary Figure 21. Large-scale representation of the transmission electron microscopy (TEM) images in Figure 6.
Supplementary Figure 22. O-GlcNAcylation inhibits aggregation by preventing incorporation of α-synuclein into aggregates. Recombinant α-synuclein (25 μM) alone or in the presence of α-synuclein(gT72) (25 μM) underwent aggregation for 7 days, followed by centrifugation and analysis by SDS-PAGE and Western blotting of the aggregate and soluble fractions.

Supplementary Figure 23. O-GlcNAcylation does not strongly inhibit aggregation in the presence of pre-formed fibers. a, Unmodified α-synuclein (50 μM) was subjected to aggregation for 7 days. At this time, these pre-formed fibers were sonicated and mixed with either buffer or unmodified α-synuclein monomers or O-GlcNAcylated α-synuclein monomers to give a mixture of fibers (25 μM) and monomers (25 μM). These mixtures were subjected to aggregation conditions (agitation at 37 °C) before analysis by ThT fluorescence (λ<sub>ex</sub> = 450 nm, λ<sub>em</sub> = 482 nm) at the indicated time points. y-Axis is fold-change in fluorescence compared to only pre-formed fibers at t = 0 h. b, The same reactions were analyzed by TEM after 60 h; scale bar: 500 nm. All results are the mean ±s.e.m. of three separate experiments. Statistical significance (two-tailed, t-test): *P < 0.05, **P < 0.01.
**Supplementary Figure 24. Model of α-synuclein aggregation.**

**a.** In the α-synuclein fiber are protein monomers composed of 5 β-strands (residues ~35-90). These monomers stack to give parallel, in-register structure that is conserved in other amyloid-forming proteins. O-GlcNAcylation is located at the core of the fiber structure in a region (residues 71-82) that are critical to aggregate formation.

**b.** Aggregation of α-synuclein into fibers is a concentration dependent process where individual monomers are added to the growing fiber structure.

**c.** Our model for inhibition of aggregation by O-GlcNAcylation at T72.
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