**Betulinic acid exacerbates biomolecular condensation of α-synuclein: Possible role in Parkinson's Disease**

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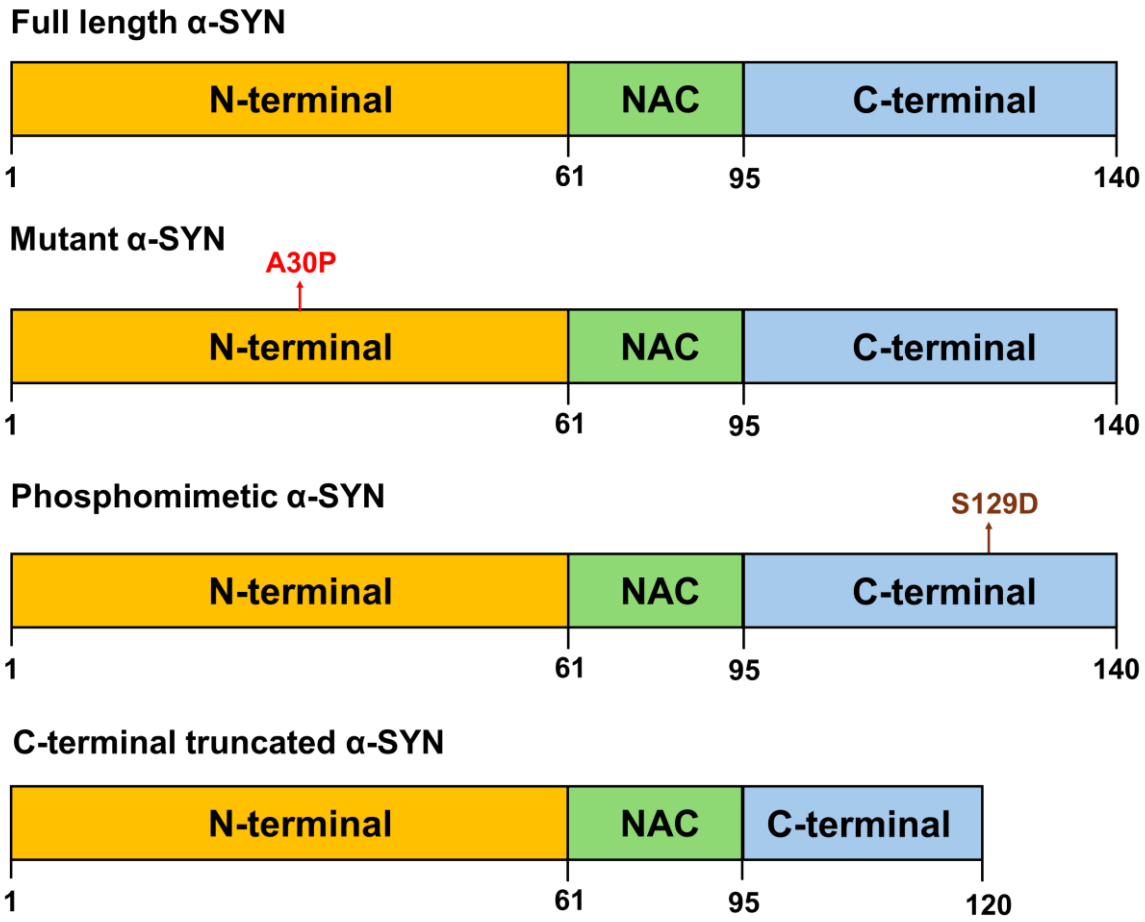


**Figure S1. Schematic representation of α-synuclein (α-SYN) domain organization and constructs used in this study.** Linear schematic illustrating the domain architecture of α-SYN, including the N-terminal amphipathic region (residues 1–60), the central hydrophobic NAC domain (residues 61–95), and the acidic C-terminal region (residues 96–140). The locations of the α-SYN A30P and α-SYN S129 mutations, as well as the CT α-SYN (residues 1–120), are indicated. This schematic is provided to facilitate comparison of construct design and to contextualize the position of mutations and truncations relative to functional domains of α-SYN. NAC: non-amyloid-β component; A30P: alanine-to-proline substitution at position 30; S129D: serine-to-aspartic acid substitution at position 129.

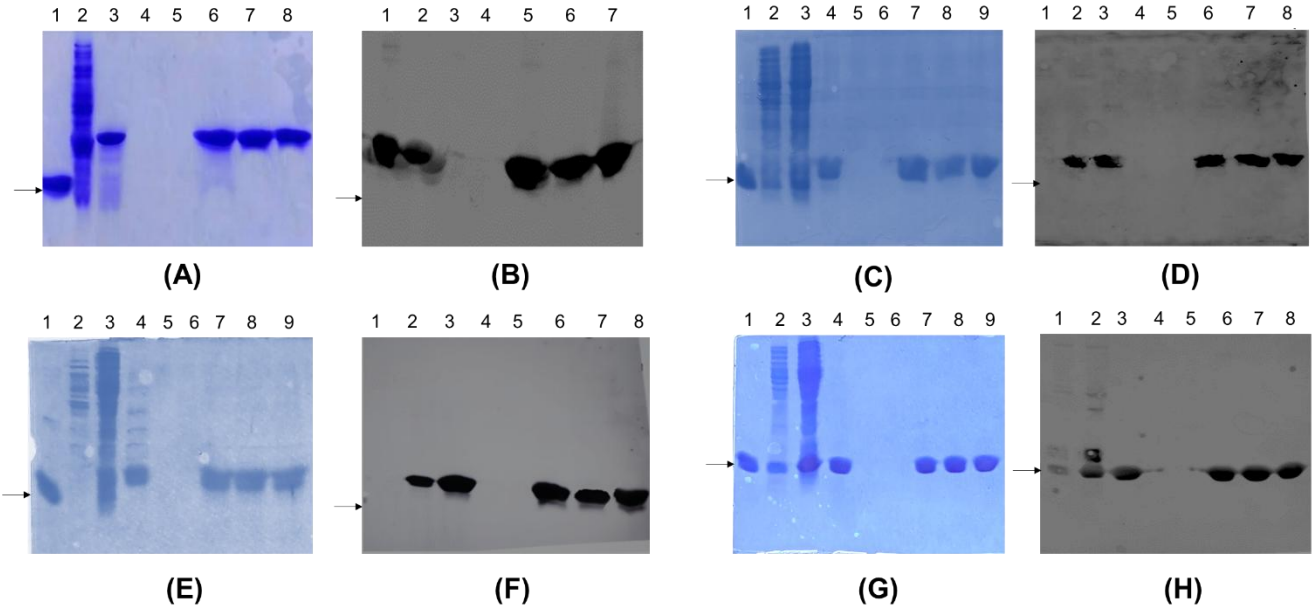


**Figure S2. Expression and purification of α-SYN variants.** (A) SDS PAGE of WT α-SYN, Lane 1: Lysozyme (marker), Lane 2: Lysate 1 (induced), Lane 3: Lysate 2 (induced), Lane 4: Flowthrough, Lane 5: Wash, Lane 6: Eluate 1, Lane 7: Eluate 2, Lane 8: Dialysed protein. (B) Western blot of WT α-SYN using rabbit monoclonal anti-α-SYN antibody, Lane 1: Lysate 1, Lane 2: Lysate 2, Lane 3: Flowthrough, Lane 4: Wash, Lane 5: Eluate 1, Lane 6: Eluate 2, Lane 7: Dialysed protein. (C) SDS PAGE of mutant α-SYN A30P, Lane 1: Lysozyme (marker), Lane 2: Lysate (uninduced), Lane 3: Lysate 1, Lane 4: Lysate 2, Lane 5: Flowthrough, Lane 6: Wash, Lane 7: Eluate 1, Lane 8: Eluate 2, Lane 9: Dialysed protein. (D) Western blot mutant α-SYN A30P using rabbit monoclonal anti-α-SYN antibody, Lane 1: Lysate (uninduced), Lane 2: Lysate 1, Lane 3: Lysate 2, Lane 4: Flowthrough, Lane 5: Wash, Lane 6: Eluate 1, Lane 7: Eluate2, Lane 8: Dialysed protein. (E) SDS PAGE of phosphomimetic α-SYN S129D, Lane 1: Lysozyme (marker), Lane 2: Lysate (uninduced), Lane 3: Lysate 1, Lane 4: Lysate 2, Lane 5: Flowthrough, Lane 6: Wash, Lane 7: Eluate 1, Lane 8: Eluate 2, Lane 9: Dialysed protein. (F) Western blot of phosphomimetic α-SYN S129D using rabbit monoclonal anti-α-SYN antibody, Lane 1: Lysate (uninduced), Lane 2: Lysate 1, Lane 3: Lysate 2, Lane 4: Flowthrough, Lane 5: Wash, Lane 6: Eluate 1, Lane 7: Eluate 2, Lane 8: Dialyzed protein. (G) SDS PAGE of C-terminal truncated α-SYN (CT α-SYN), Lane 1: Lysozyme (marker), Lane 2: Lysate (uninduced), Lane 3: Lysate 1, Lane 4: Lysate 2, Lane 5: Flowthrough, Lane 6: Wash, Lane 7: Eluate 1, Lane 8: Eluate 2, Lane 9: Dialyzed protein. (H) Western blot of CT α-SYN using rabbit polyclonal α-SYN (1-10 amino acid) antibody, Lane 1: Lysate (uninduced), Lane 2: Lysate 1, Lane 3: Lysate 2, Lane 4: Flowthrough, Lane 5: Wash, Lane 6: Eluate 1, Lane 7: Eluate 2, Lane 8: Dialyzed protein. Proteins were resolved on a 15% SDS PAGE, followed by Coomassie Brilliant Blue staining (Figures S2A, S2C, S2E and S2G). For Western blotting, 50 µg of total protein was loaded in each lane (Figures S2B, S2D, S2F and S2H). Arrow shows the position of lysozyme (14 kDa).

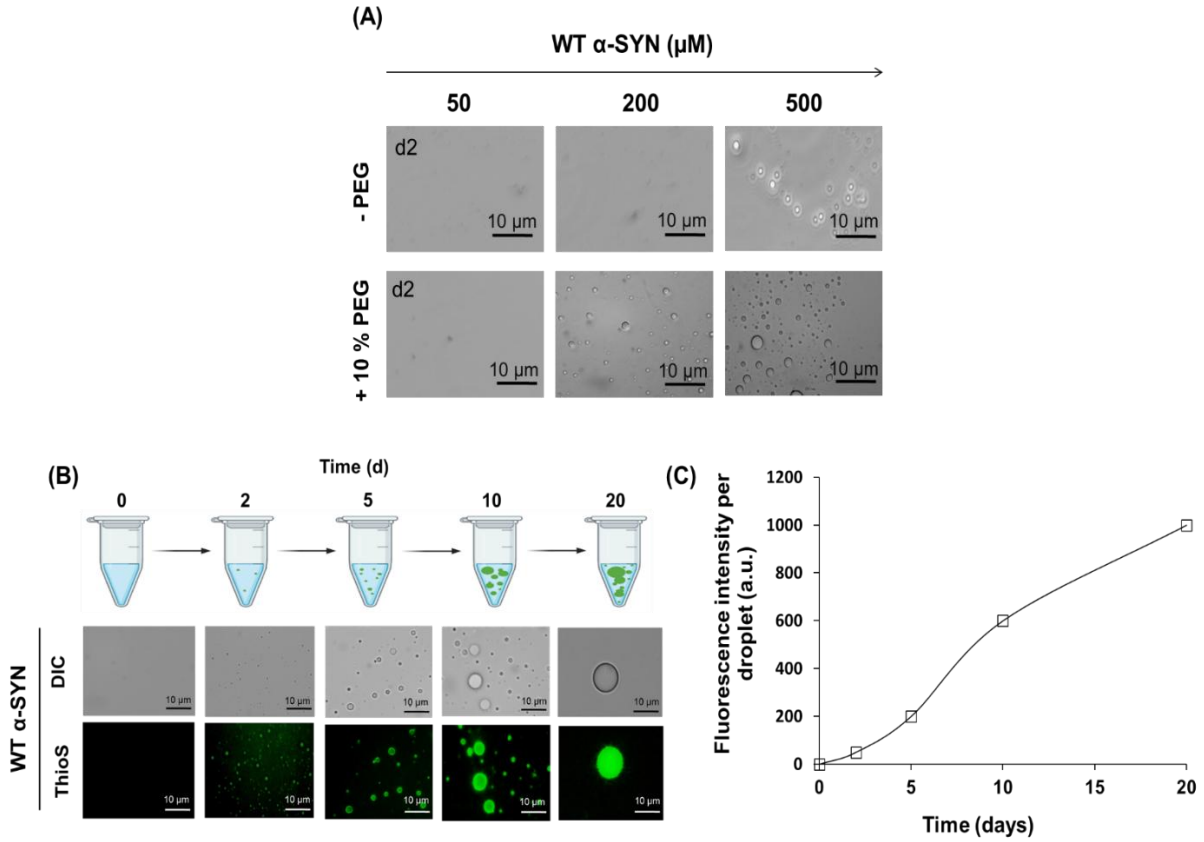


**Figure S3. Phase separation of WT α-SYN.** (A) DIC images showing phase-separated droplets of WT α-SYN at varying protein concentrations, both with and without PEG8000, on day 2 (d2). The images were captured using a 100X objective lens and 10X eyepiece. Scale bar represents 10 μm. (B) ThioS staining of WT α-SYN over time. 20 μM of ThioS (w/v) prepared in 20 mM Tris HCl (pH 7.8) was mixed with LLPS reaction mixture (200 μM WT α-SYN protein and 10% PEG8000) and was incubated at 37 °C. At various time points (days), ThioS fluorescence was observed using a Nikon Eclipse 600 microscope under a 100X objective and 10X eyepiece. (C) ThioS kinetics of WT α-SYN per droplet over time. The fluorescence intensity of WT α-SYN per droplet at all time points were quantified using ImageJ software. Scale bar represents 10 μm. All experiments were repeated three times with similar observations.

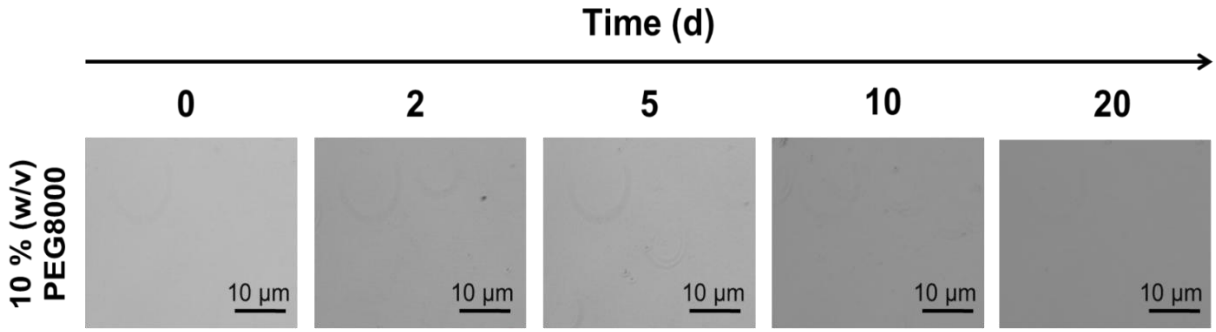


**Figure S4. Phase separation of PEG8000.** Representative DIC images showing 10% (w/v) PEG 8000 prepared in 20 mM Tris HCl buffer (pH 7.8), in the absence of α-SYN protein and incubated at 37 °C for the indicated time points. All images were acquired using identical imaging parameters and magnification (100X). Scale bar: 10 µm.

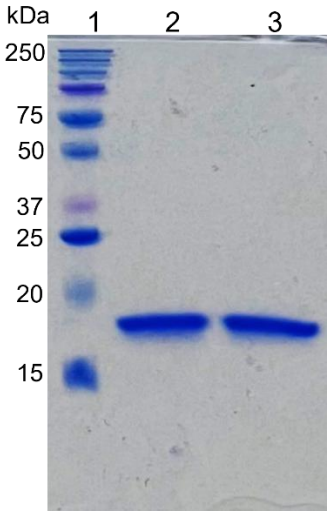


**Figure S5. Evaluation of WT α-SYN stability after LLPS at 37 °C by SDS-PAGE.** Recombinant WT α-SYN was incubated at 37 °C for 20 days in Tris HCl, pH 7.8, as described in Methods section. Lane 1: Protein ladder, Lane 2: WT α-SYN (day 0), Lane 3: WT α-SYN (day 20). Equal amounts of WT α-SYN protein (20 µg per lane) were loaded on the gel and analyzed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue to visualize the protein bands.

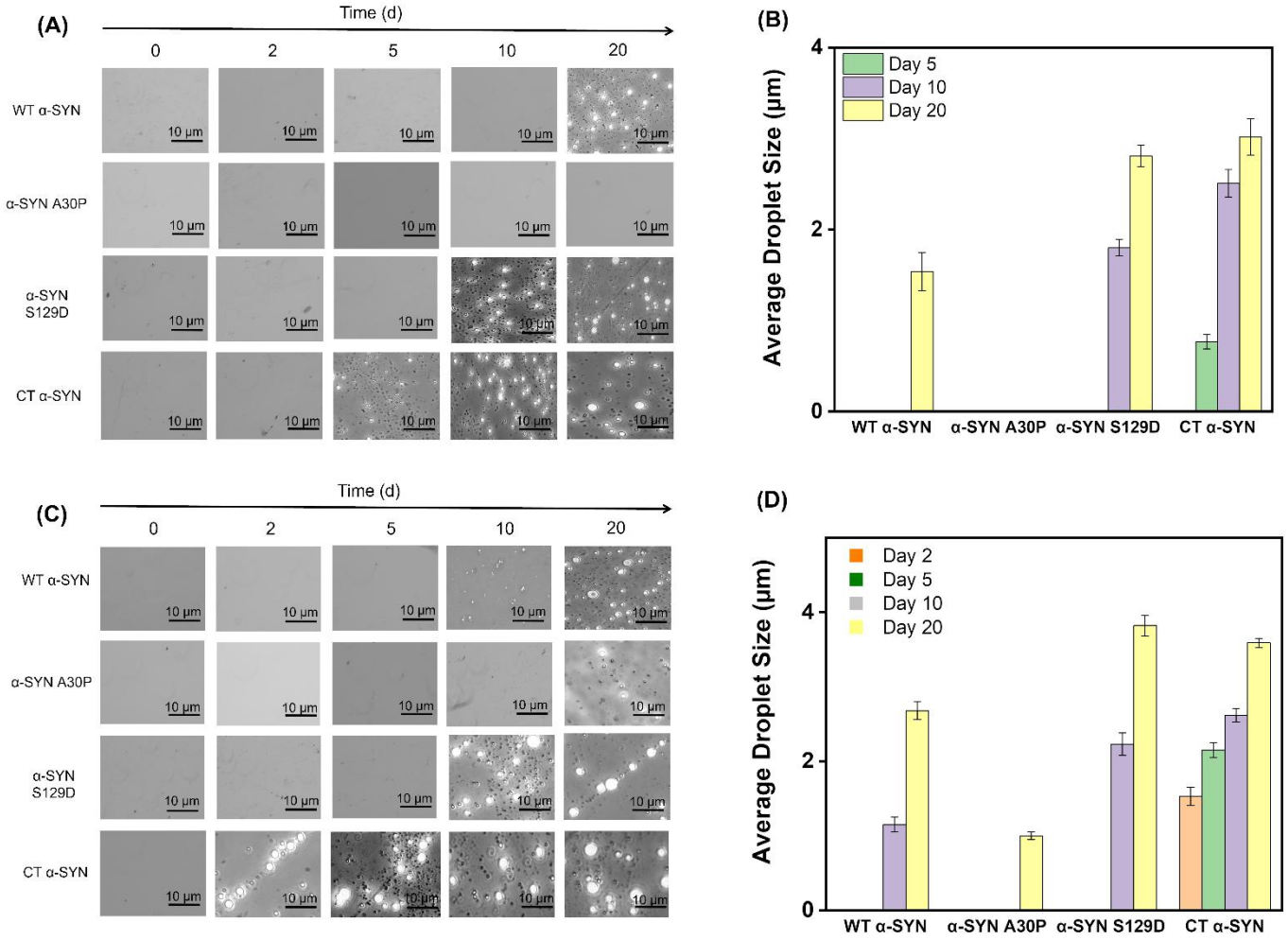


**Figure S6. Phase separation of α-SYN variants in the presence of betulinic acid and absence of PEG8000.** α-SYN variants (200 µM) were incubated in the presence of different concentrations of betulinic acid at 37 ºC. No PEG8000 was used in this experiment. At different time intervals, microscopy images were taken and the average droplet size were quantified by ImageJ software. (A) DIC image showing the formation of phase-separated condensates of α-SYN variants in the presence of 10 µg/mL betulinic acid. Scale bar 10 µm. The size of droplets was counted from ten different microscopic fields (100x magnification). (B) Average droplet size of α-SYN variants in the presence of 10 µg/mL betulinic acid at different time intervals. (C) DIC image showing the formation of phase-separated condensates of α- SYN variants in the presence of 40 µg/mL betulinic acid. Scale bar 10 µm. The size of droplets was counted from ten different microscopic fields (100x magnification). (D) Average droplet size of α-SYN variants in the presence of 40 µg/mL betulinic acid at different time intervals. Values shown are mean ± sem for n=3 independent experiments with similar observations.

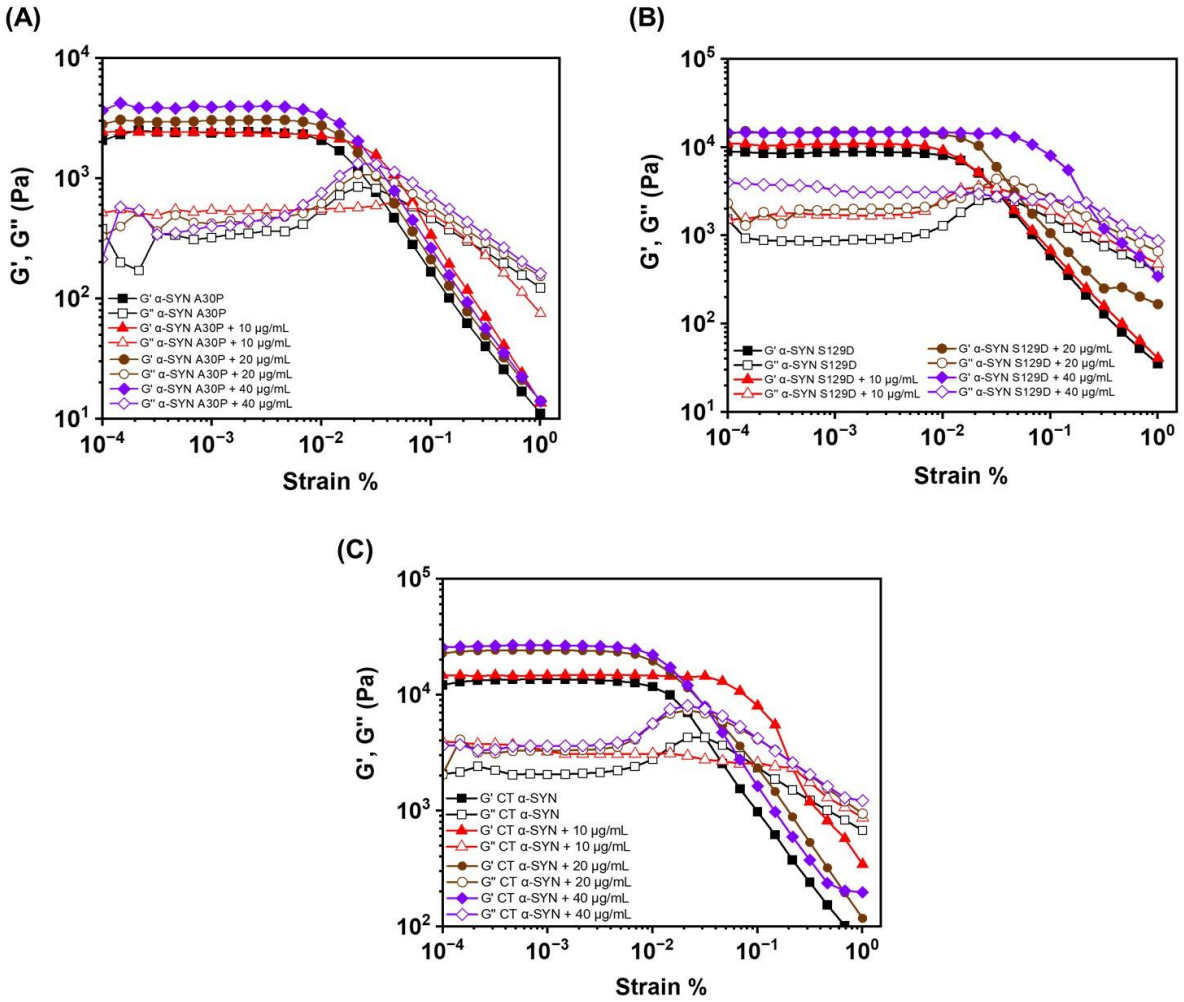


**Figure S7. Rheological characterization of α-SYN variants in the absence or presence of different concentrations of betulinic acid.** (A) Amplitude sweep experiment of α-SYN A30P gels with a strain ramp (γ = 0.01–100%) under a constant frequency with G′ (filled markers; solid lines) and G″ (empty markers; dotted lines). (B) Amplitude sweep experiment of α-SYN S129D gels with a strain ramp (γ = 0.01–100%) under a constant frequency with G′ (filled markers; solid lines) and G″ (empty markers; dotted lines). (C) Amplitude sweep experiment of CT α-SYN gels with a strain ramp (γ = 0.01–100%) under a constant frequency with G′ (filled markers; solid lines) and G″ (empty markers; dotted lines).

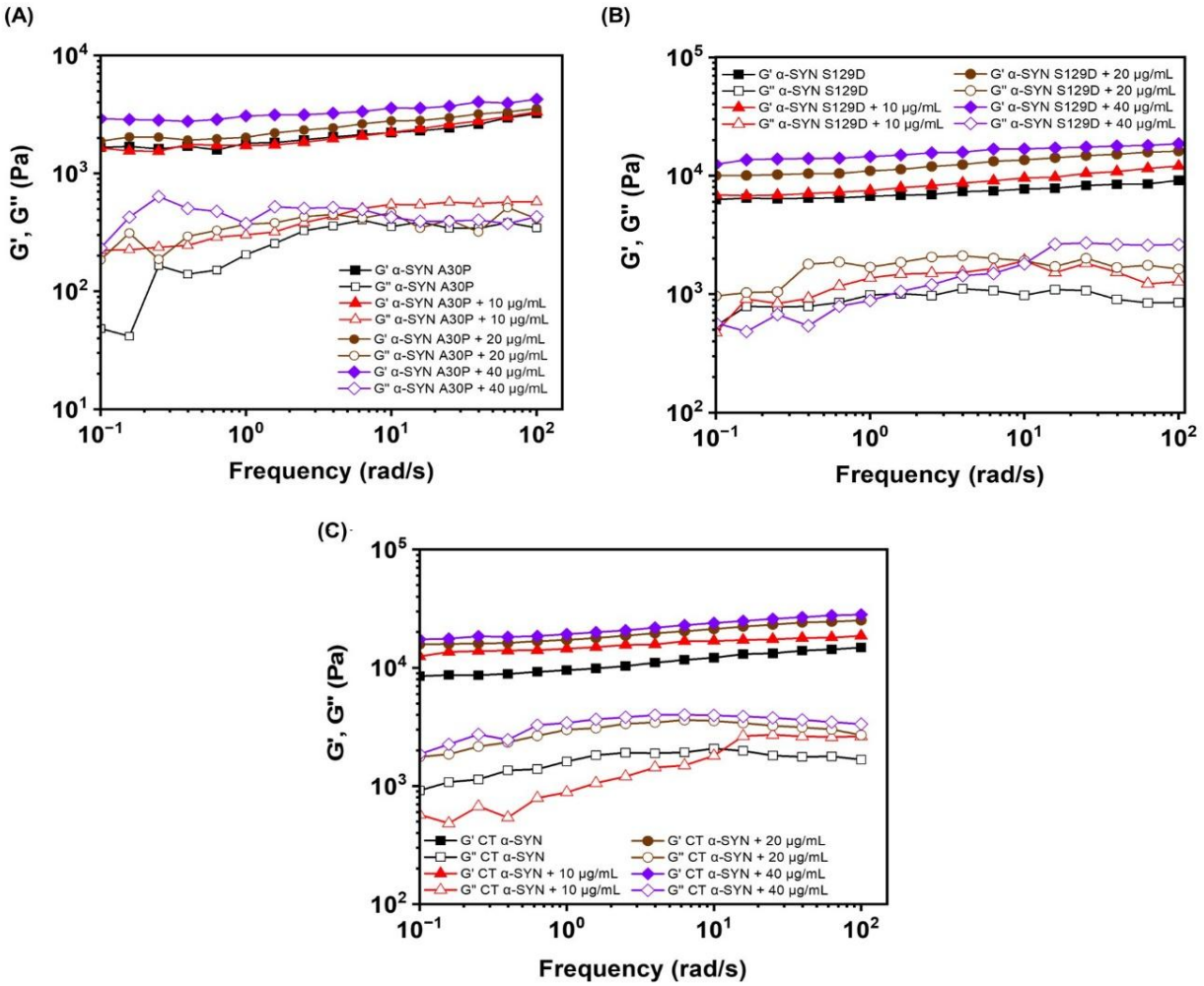


**Figure S8. Rheological characterization of α-SYN variants in the absence or presence of different concentrations of betulinic acid.** (A) Frequency sweep of α-SYN A30P gels showing G′ (filled markers; solid lines) and G″ (empty markers; dotted lines) at a strain of 0.5% over a frequency range of 0.1–100 rad/s. (B) Frequency sweep of α-SYN S129D gels showing G′ (filled markers; solid lines) and G″ (empty markers; dotted lines) at a strain of 0.5% over a frequency range of 0.1–100 rad/s. (C) Frequency sweep of CT α-SYN gels showing G′ (filled markers; solid lines) and G″ (empty markers; dotted lines) at a strain of 0.5% over a frequency range of 0.1–100 rad/s.

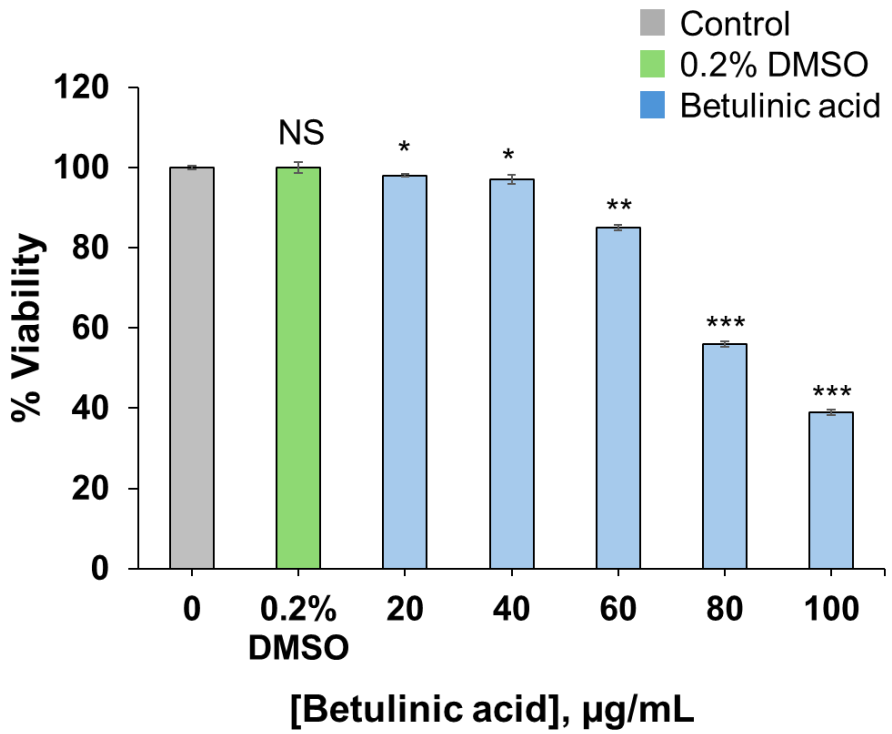


**Figure S9. Effect of betulinic acid on untransformed SH-SY5Y cells.** Untransformed SH-SY5Y cells were incubated in the absence or presence of different concentrations of betulinic acid. Cell viability was assessed by MTT assay after 48 h. The cell viability measured after 48 h in the absence of betulinic acid was defined as 100%. ***p<0.001, **p<0.01, *p<0.05 and NS, non- significant against corresponding untreated SH-SY5Y cells. Data are presented as mean ± sem from three independent experiments and were analyzed using one-way ANOVA for multiple comparisons.

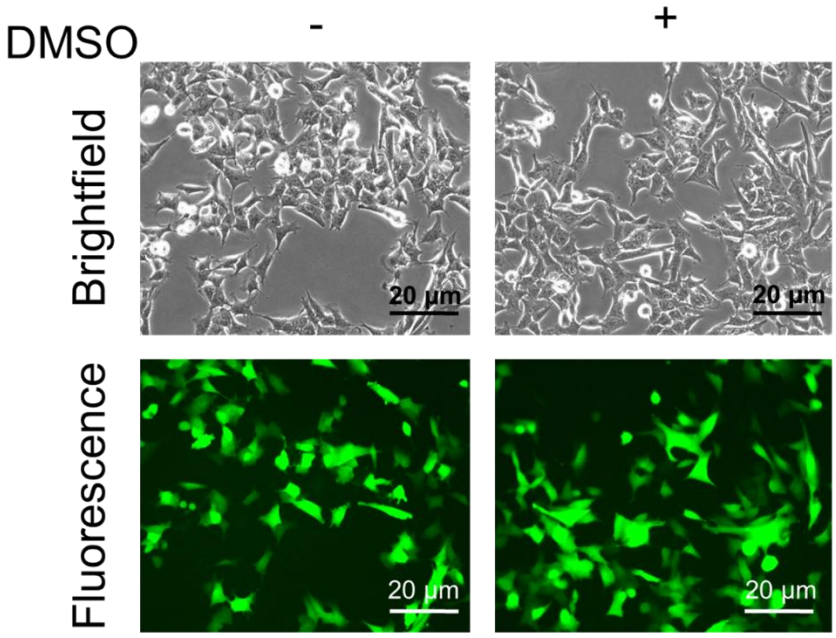


**Figure S10. Effect of DMSO in SH-SY5Y cells expressing p-EGFP-α-SYN.** To evaluate solvent-specific effects, SH-SY5Y cells expressing p-EGFP-α-SYN were treated with and without 0.2% DMSO for five days under identical experimental conditions. Betulinic acid was not added in this experiment. Representative images were acquired using a 40X objective. Scale bar 20 µm.

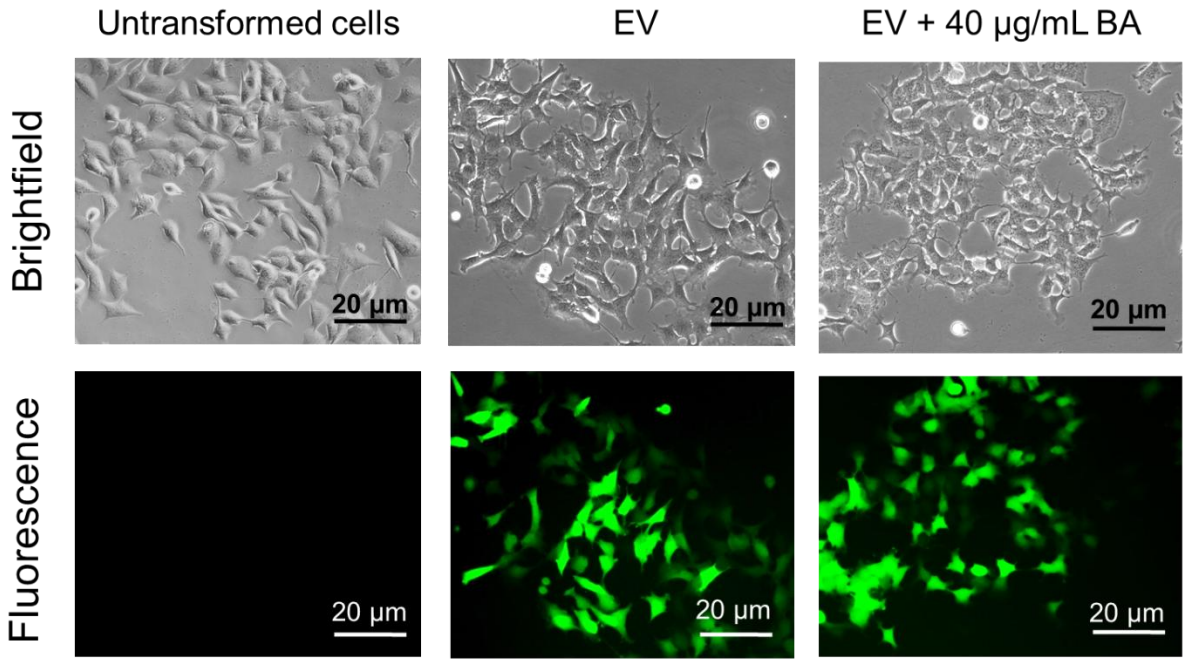


**Figure S11. Effect of betulinic acid on EGFP (empty vector) expressing SH-SY5Y cells**. Representative fluorescence microscopy images of SH-SY5Y cells transiently transfected with an EGFP (empty vector) plasmid and allowed to express EGFP protein for 24 h. BA (40 µg/mL) was then added and cells were incubated for 5 days under identical conditions. Images were acquired using a 40X objective lens. Scale bar: 20 µm. EV: empty vector.

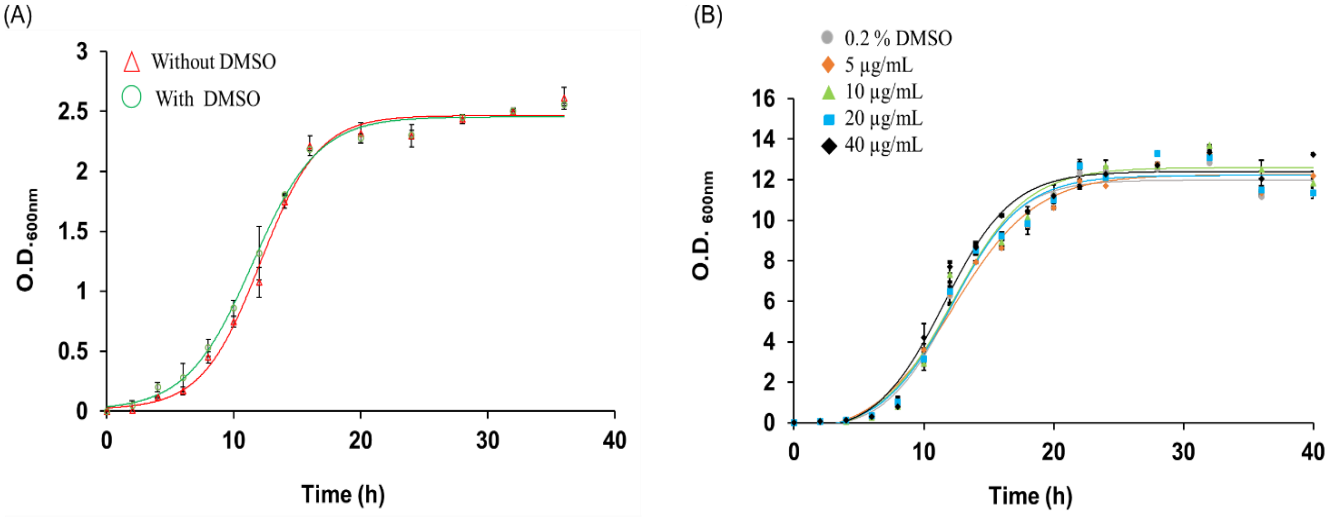


**Figure S12. Growth curves of untransformed *Saccharomyces cerevisiae* W303 cells.** (A) DMSO (B) Betulinic acid. Values shown are mean ± sem of two independent experiments.

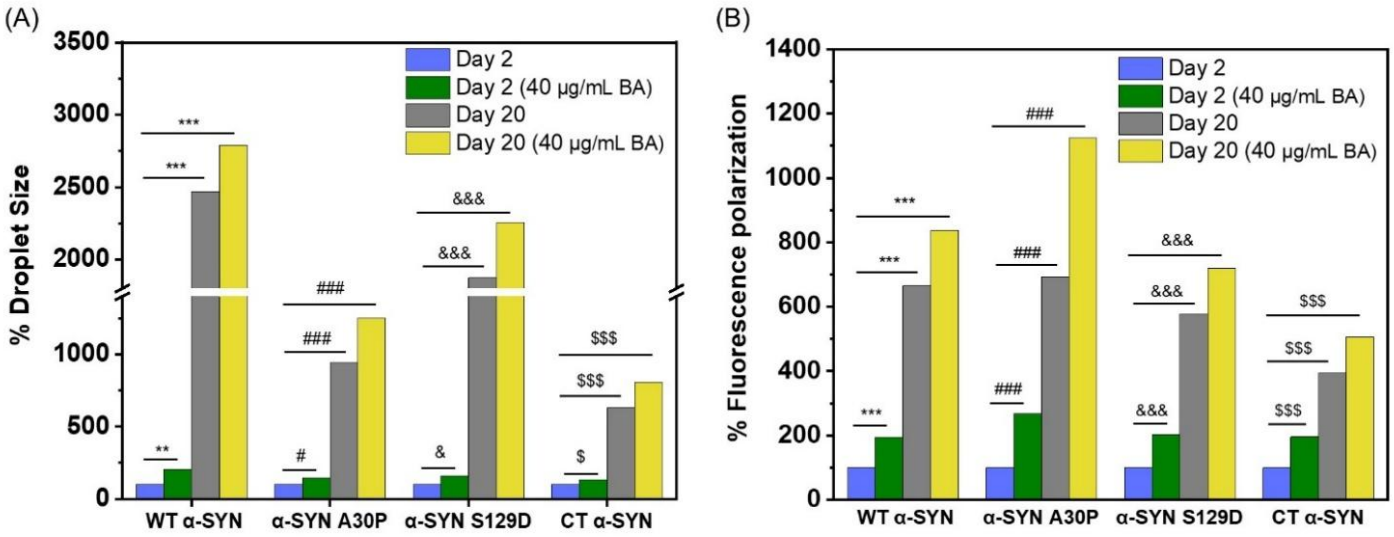


**Figure S13. Effect of betulinic acid on droplet size and fluorescence polarization for α-SYN variants at different time points.** (A) For size distribution; ***p< 0.001 and **p< 0.01 against WT α-SYN in the absence of 40 µg/mL betulinic acid at day 2; $^{###}$p< 0.001 and $^{##}$p< 0.05 against α-SYN A30P in the absence of 40 µg/mL betulinic acid at day 2; $^{\&\&\&}$p< 0.001 and $^{\&\&}$p< 0.05 against α-SYN S129D in the absence of 40 µg/mL betulinic acid at day 2; $^{\$\$\$}$p< 0.001 and $^{\$}$p< 0.05 against CT α-SYN in the absence of 40 µg/mL betulinic acid at day 2. The size of droplets was counted from ten different microscopic fields (100X magnification). (B) Percentage change in the rotational state of α-SYN variants were plotted indicating that FP increases significantly following assembly; ***p< 0.001 against WT α-SYN in the absence of 40 µg/mL betulinic acid at day 2; $^{###}$p< 0.001 against α-SYN A30P in the absence of 40 µg/mL betulinic acid at day 2; $^{\&\&\&}$p< 0.001 against α-SYN S129D in the absence of 40 µg/mL betulinic acid at day 2; $^{\$\$\$}$p< 0.001 against CT α-SYN in the absence of 40 µg/mL betulinic acid at day 2. The significance of the data is analyzed with the help of one-way ANOVA followed by Tukey's multiple comparison test with a 95% confidence interval.