Supplementary information

Biphasic modulation of tau liquid-liquid phase separation by polyphenols

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1) Materials and methods. All the chemicals and solvents used were acquired from Merck, HiMedia, BioRad, Gibco, and Thermofischer Scientific and were utilized without any additional purification, unless otherwise stated. Ampicillin sodium salt (cat #A022), chloramphenicol (cat #PCT1117), Luria Bertani (LB) powder (cat #GM1151), Phosphate buffered saline powder (PBS) (cat #TS1101), bovine serum albumin powder (BSA) (cat #GRM3151), sodium chloride (NaCl) (cat #MB023) and isopropyl-β-D-thiogalactoside (IPTG) (cat #MB072), (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) (cat #MB186), Thioflavin T (ThT) (cat #RM10365), were purchased from HiMedia. HisPur™ Ni-NTA Resin (cat #88221) for tau protein purification was obtained from Invitrogen Thermofischer Scientific. Various chemicals were procured from Merck including acrylamide, bis-acrylamide (cat #A2792) ammonium persulfate (APS) (cat #A3678), tetramethylethylenediamine (TEMED) (cat #T9281), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (cat #1.10110) , phenylmethanesulfonyl fluoride (PMSF) (cat # P7626) and rhodamine isothiocynate (cat #R1755). 1,6 hexane diol (HD) (cat #0108102), and dimethylformamide (DMF) (cat #110403), were purchased from spectrochem. The full-length wild type Tau 2N4R protein was expressed in *Escherichia coli* (E. coli) and subsequently purified using Ni-NTA affinity chromatograph. α-synuclein (MDL #MFCD06411761) was purchased from sigma Aldrich. Proteinwasay dye reagent concentrate (for Bradford assay) (cat #5000002), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) tricline sample loading buffer (cat #161-0739), and protein ladder (cat #1610374) were obtained from BioRad. Microplate reader (SpectraMax i3x) was utilized to monitor turbidity and MTT assay. Hydrophobic slide marker nCircle was procured from NeoDx. For microscopy imaging, the Olympus Fluoview-3000 confocal laser scanning microscope was employed, and images were processed using the inbuilt cellsense software, data analysis was carried out using ImageJ FIJI (v2) software. Dynamic light scattering (DLS) experiment was conducted using Malvern Zetasizer Pro instrument, while TEM analysis was carried out using the FEI Tecnai T20 instrument. All data plotting and analysis were performed using Origin (Pro) Version 2022 and GraphPad Prism 8.0.

2. Tau protein expression, purification and characterization. The protein expression, purification and characterization were carried out as reported elsewhere.[10] The plasmid construct containing full-length tau was a kind gift to the lab from Dr. Sharad Gupta, IIT Gandhinagar, India. Briefly, the complete tau plasmid construct was introduced into *E. coli* BL21 and the bacteria was

cultured in LB broth supplemented with antibiotics, ampicillin (100 µg/ml) and chloramphenicol (50 µg/ml) at 37 °C under continuous agitation at 185 rpm overnight. Subsequently, a 1% aliquot of the primary culture was inoculated into a 1 L volume of LB broth containing antibiotics and incubated until it reached an optical density (OD) of 0.6. Induction was initiated by adding 1 mM IPTG, and the culture was further incubated for a duration of 4 h. Cell pelleting was done through centrifugation at 7000 rpm at 4 °C, followed by resuspension in a 50 mM phosphate buffer supplemented with 0.3 M NaCl, 5 mM imidazole, and 1 mM PMSF at pH 8. The subsequent step involved subjecting the suspension to boiling for 10-15 min, with intermittent agitation, to facilitate the precipitation of undesired proteins. The supernatant, enriched with the tau protein, was subjected to Ni-NTA affinity column chromatography. Post-purification, the quantification of the tau protein was performed employing the Bradford assay, and its characterization was carried out *via* SDS-PAGE and stored at -80 °C for subsequent applications.

3. Turbidity assay. Tau protein (10 µM) independently and in the presence of the SPs (10-100 µM) was incubated in LLPS buffer (10 mM HEPES, 10 mM NaCl,10% PEG 8000, pH 7.4) at 37 °C for 30 min. Absorbance at 400 nm was measured in well plate using Spectramax i3 (Molecular devices) microplate reader and analysed using GraphPad Prism 8.0.

4. Differential interference contrast (DIC) microscopy studies. Tau protein (5 µM) with varying concentrations of GA (1µM, 5 µM, 7.5 µM, 10 µM, 15 µM, 20 µM, 35 µM and 50 µM) was incubated in HEPES (10mM, pH 7.4) buffer, for 30 min at 37 °C. Post incubation, the sample was drop-cast onto a clean glass slide and mounted with a glass coverslip. The sample was then visualized and captured at 60x using the Olympus Fluoview-3000 DIC microscope. The data was then quantified using FIJI ImageJ(v2) software and plotted using GraphPad Prism 8.0.

5. Time-dependent DIC microscopy imaging study. Tau protein (5 µM) with 10 µM GA was incubated in HEPES buffer (10mM, pH 7.4), at 37 °C. Small aliquots were taken out at different intervals and were drop cast onto a clean glass slide and mounted with a glass coverslip. The sample was then visualized and captured at 60x using Olympus Fluoview-3000 DIC and processed with the same inbuilt software. The data was then quantified using ImageJ FIJI (v2) software and plotted using GraphPad Prism 8.0.

6. Fluorescent labeling of tau protein. Tau protein tagged with rhodamine B isothiocyanate was used for confocal microscopy analysis. Rhodamine B isothiocyanate labeled tau protein was prepared by dissolving purified tau protein with 1M sodium bicarbonate buffer (pH 9), followed by the addition of Rhodamine isothiocyanate B dye (10 mg/ml in DMSO) while gently stirring the sample at room temperature for 1 h under light protection. After incubation, the labeled protein was purified by removing excess unlabeled dye by dialyzing in PBS (10 mM, pH 7.4) overnight.

7. Fluorescence recovery after photobleaching (FRAP) experiments. Tau protein (5 μM) (labelled: unlabeled, 1:4) with and without GA (10 μM) in HEPES (10 mM, pH 7.4) was incubated at 37 °C for 30 min, following which, the samples were drop cast onto a clean glass slide, sealed with a glass coverslip and viewed under the Olympus Fluoview-3000 confocal laser scanning microscope with 60x magnification. The droplets were imaged with a 561 nm laser and 595/50 nm detection filter. FRAP was used to measure the dynamic exchange of proteins between the droplets and the surrounding solution. At 60x magnification and 10x optical zooming, droplets were outlined with a circular "bleaching" overlay object and an unbleached droplet was circled with a "reference" overlay object. Bleaching was carried out with 35% laser power for 100 μs, and fluorescent intensity data was recorded for over 1 min. All imaging was done with the same acquisition settings (i.e., scan speed, resolution, magnification, optical zoom, gain, offset, laser intensity and bleaching time). The recovery time constant was derived from a single exponential fit of the corrected fluorescence intensities plotted versus time using CellSens software. The FRAP experiments were repeated in three independent replicates. For FRAP, the prepared sample was dropcast onto a confocal dish (cover glass bottom), within the boundary marked by a hydrophobic slide marker (NeoDx-nCircle) and then analyzed using the Olympus Fluoview-3000 confocal laser scanning microscope.

8. Dynamic light scattering (DLS) study. DLS measurements were carried out using Malvern Zetasizer Nano ZS instrument at 25 °C with a scattering angle of 173°. Tau protein (5 μM) was incubated in HEPES buffer(10 mM, pH 7.4) independently and in the presence of GA (10 μM) at 37 °C for 30 min and subjected to measurements. Each measurement consisted of 10–15 runs of 10 s. The data was then plotted using Origin (Pro) Version 2022.

9. Transmission electron microscopy (TEM) imaging. The samples for TEM analysis were prepared on carbon-coated copper mesh grids without the use of staining. Tau (5 μM) was

incubated with GA (10 μM) in HEPES buffer (10 mM, pH 7.4) at 37 °C for 30 min, to monitor tau aggregation, tau (5 μM) was incubated with arachidonic acid (AA) (185 μM) independently and in the presence of GA (10 μM) at 37 °C for 24 h in tau aggregation buffer (10 mM HEPES, 0.1 mM NaCl, 5 mM DTT). The prepared grid was then mounted onto a TEM holder and inserted into the FEI Tecnai T20 TEM instrument for imaging.

10. Cryo-EM sample preparation. Tau (5 μM) was incubated with GA (10 μM) for 1 hour at 37 °C and then dropcasted onto glow discharged Quantifoil holey carbon grids (R 1.2/1.3, Au 300 mesh and R 2/2, Au 300 mesh respectively). The grids were plunge-frozen using a Vitrobot Mark IV (Thermo Fisher Scientific), with settings at 100% humidity and temperature of 20 °C. Grids were blotted with Whatman no. 1 filter paper for 3.5 seconds with a blot force of 0 and then plunge-frozen in liquid ethane. Images of both samples were acquired using a Titan Krios microscope at the National Electron Cryomicroscopy facility in Bangalore, with a Falcon 3 detector at 75000X magnification, corresponding to a pixel size of 1.07Å.

11. Atomic force microscopy. The AFM investigations were conducted utilizing a bioscope-resolved AFM instrument from Bruker. For the imaging of LLPS droplets, Peak Force Quantitative Nanomechanical Mapping (PF-QNM) was employed, applying the peak force tapping principle. In this mode, both the height and logarithmic Derjaguin-Muller-Toporov (logDMT) values of the droplets were systematically recorded. The logDMT was calculated using Derjaguin, Muller, Toropov (DMT) model using the formula given below,

$$F_{tip}=4/3E^{*}\sqrt{Rd^{3}} + F_{adh}$$

Where F_{tip}= Force on tip, F_{adh} = Adhesion force, R= Tips radius and d= Tip sample separation.

The investigations were carried out employing an MLCT BIO-F probe, which utilized nonconductive silicon nitride coated with reflective gold. The probe featured a tip radius of 20 nm and a spring constant of 0.6 N/m. Image acquisition was conducted at a scanning speed of 0.5 Hz, with 512 samples per line recorded.

12. Thioflavin-T (ThT) assay. ThT assay was performed to check the ability of GA to modulate AA-induced tau aggregation. Tau (5 μM) and AA (185 μM) were incubated independently and in

the presence of GA for 24 h in tau aggregation buffer. After 24 h, ThT (5 µM) (1:1) was added and fluorescence emission intensity at 484 nm (λ_{ex} = 442 nm) was recorded in triplicates using a microplate reader and the data was processed using GraphPad prism 8.0.

13. Cellular studies. Neuronal rescue from tau-induced toxicity was performed on SH-SY5Y neuroblastoma cell line. MTT assay was performed to demonstrate the neuronal rescue ability of the GA from AA-induced tau aggregates toxicity. The cells were seeded in a 96-well plate (16,000 cells/well) and were allowed to adhere for 24 h. Post 24 h, the cells were treated with preincubated complex of Tau: AA (10 µM: 185 µM) independently and in the presence of GA (20-50 µM) for 24 h at 37 °C under a 5% CO_2 atmosphere. After 24 h, 10 µL MTT solution (5 mg/mL) was added and incubated for 3 h. The media was replaced with 100 µL of DMSO: MeOH (1:1) and the absorbance at 570 nm was recorded using spectramax i3 microplate reader. The data was analyzed and processed with GraphPad Prism 8.0.

Results.

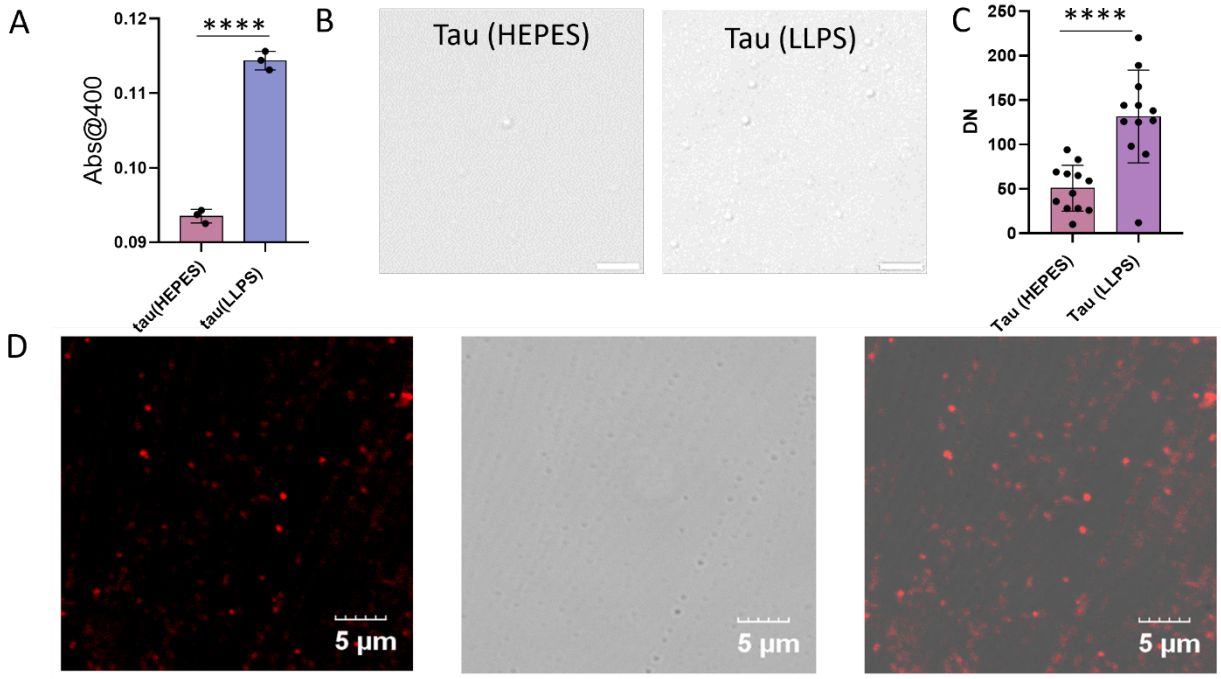

Fig. S1 Characterization of tau LLPS with tau (5 µM) in HEPES and LLPS buffers. (A) Turbidity measurement in HEPES and LLPS buffers. (B) DIC microscopy imaging of Tau LLPS in HEPES buffer

and LLPS buffer alongside droplet quantification (Scale bar 5 μM) represented in C. (D) Fluorescent tagged tau in LLPS buffer indicating droplet formation. (Statistics was performed with Student's t-test with ****$p < 0.0001$)

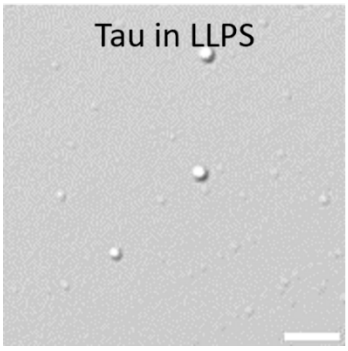

Fig. S2 DIC image of homotypic tau (5 μM) droplets in LLPS buffer (Scale bar 5 μm) indicating phase separation.

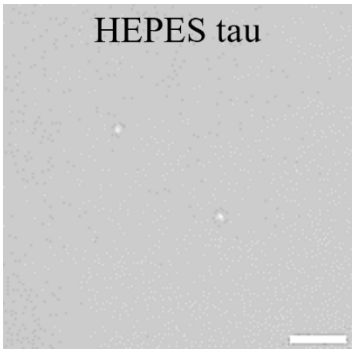

Fig. S3 DIC image of tau (5 μM) in HEPES buffer indicating minimum droplets or phase separation in HEPES buffer (Scale bar 5 μm).

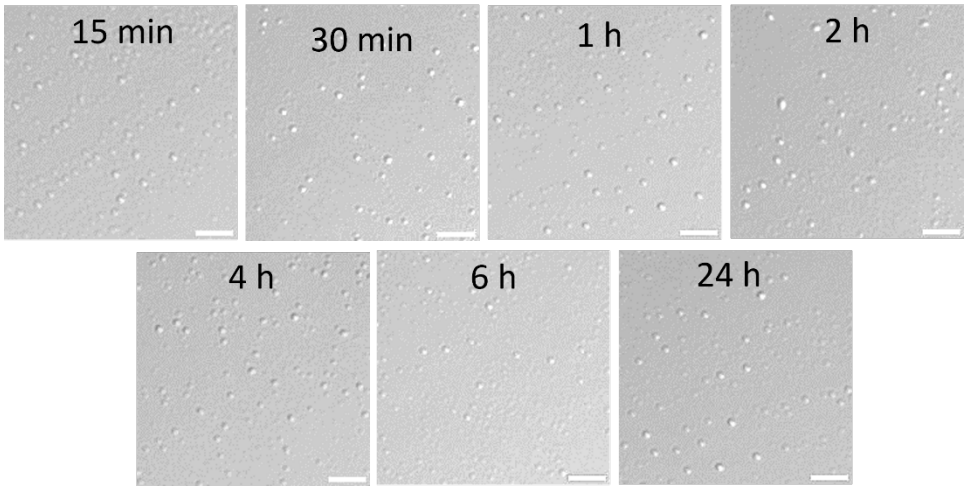

Fig. S4 DIC imaging of time dependent tau (5 µM) LLPS promoted by GA (10 µM) indicating stability of the droplet over 24 h (Scale bar 5 µm).

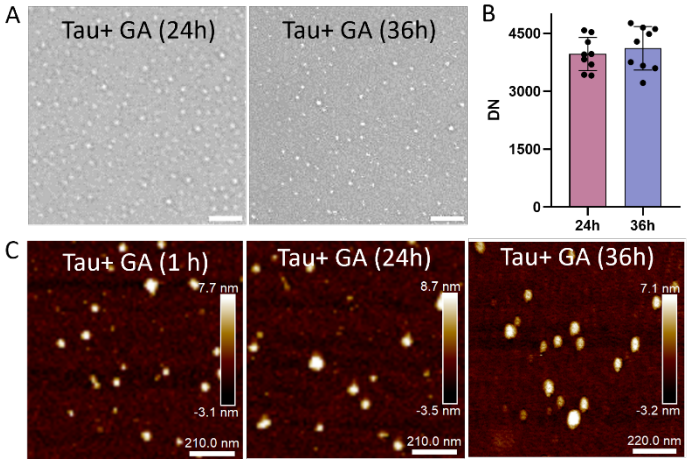

Fig. S5 Time dependent (A) DIC imaging to determine DN alongside its quantification represented in (B). (C) Time dependent AFM study upto 36 h indicates that the stability of the droplets.

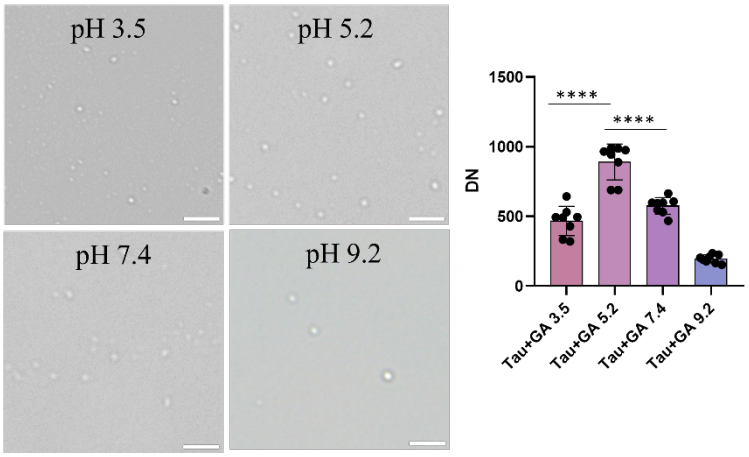

Fig. S6. Effect of pH on GA promoted tau DN after 30 min. of incubation (Scale bar 5µm) (Statistical analysis conducted using ordinary one-way ANOVA, Tukey's post hoc test *p = 0.05).

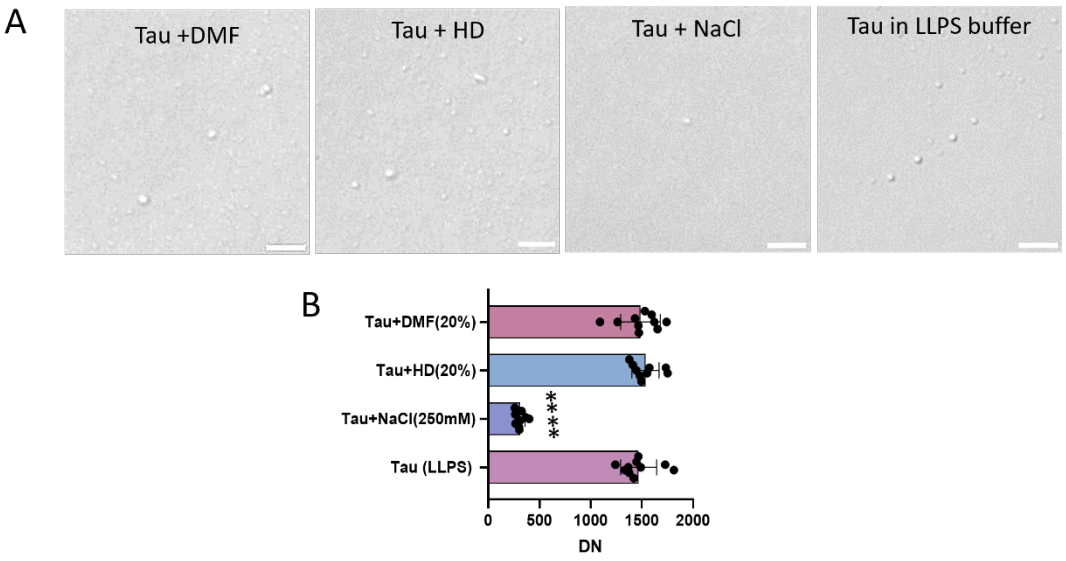

Fig. S7 (A) DIC imaging shows tau (5 µM) droplet modulation in presence of 20% DMF, 20% HD and 250 mM NaCl along with its droplet quantification represented in B (Scale bar 5µm). (Statistical analysis conducted using ordinary one-way ANOVA, Tukey's post hoc test *p = 0.05)

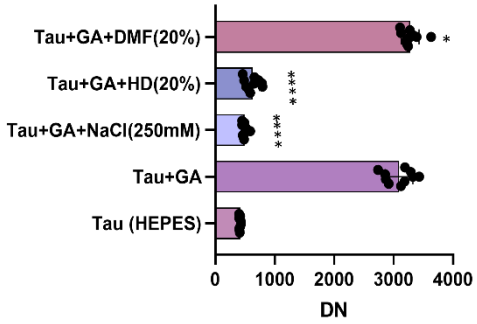

Fig. S8 Quantification of GA (10 µM) promoted tau (5 µM) LLPS in presence of 20% DMF, 20% HD and 250 mM NaCl (quantification of data from Fig 3A). (Statistical analysis conducted using ordinary one-way ANOVA, Tukey's post hoc test *p = 0.05).

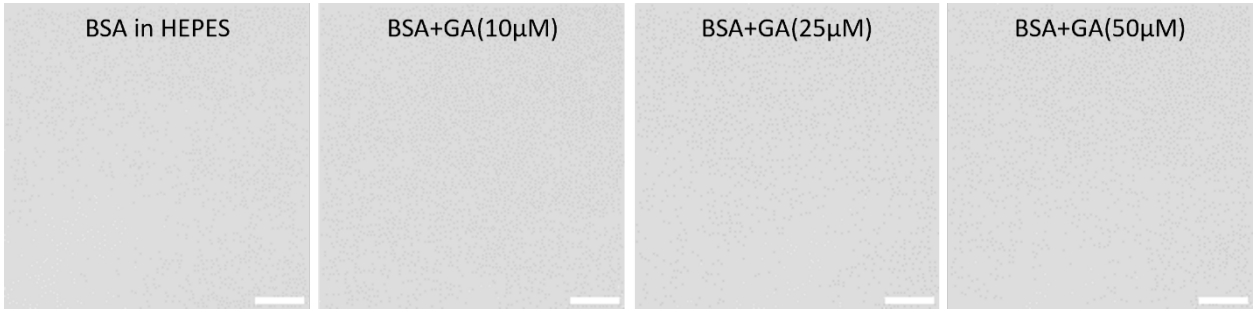

Fig. S9 DIC images of BSA (10 µM) in the presence of various concentrations of GA incubated in HEPES buffer for 30 min indicating no phase separation of BSA in the presence of GA (Scale bar 5 µm).

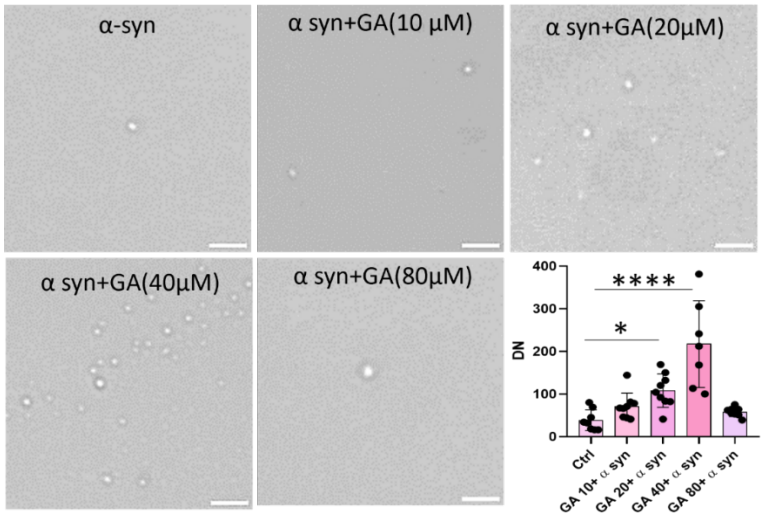

Fig. S10 DIC imaging of LLPS of α-Synuclein (10 µM) promoted by varying concentration of GA and its respective quantification (Scale bar 5 µm). (Statistical analysis conducted using ordinary one-way ANOVA, Tukey's post hoc test *p = 0.05).

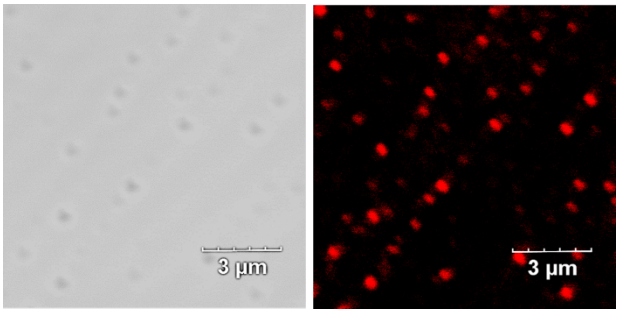

Fig. S11 Confocal microscopy image of GA (10 µM) induced LLPS of fluorescent-tagged Tau (5 µM) (Scale bar 3 µm).

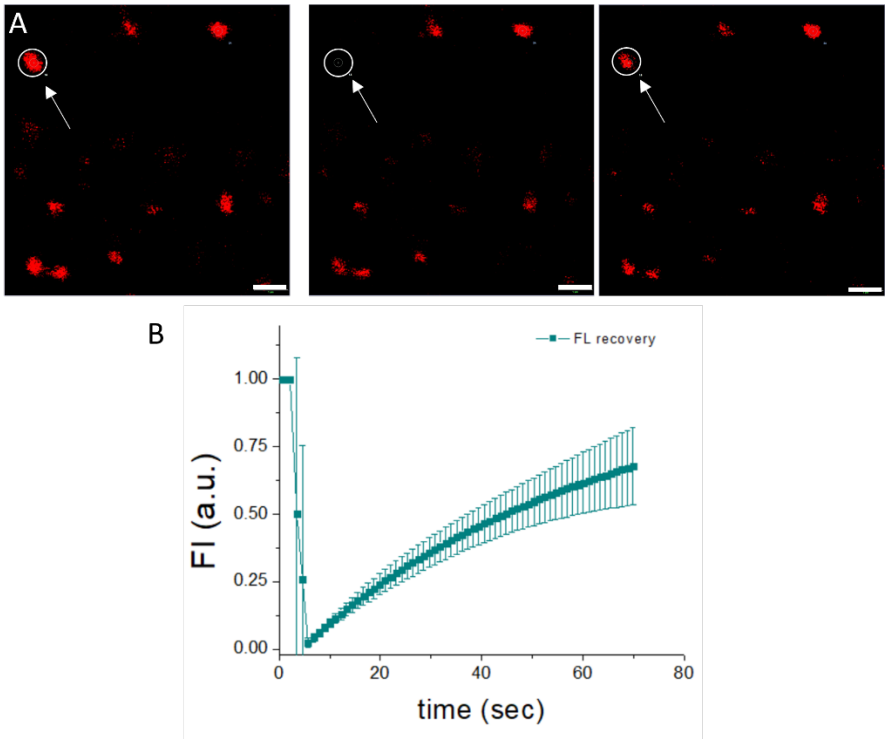

Fig. S12 (A) FRAP experiment of homolytic tau LLPS droplet and its recovery quantification represented in B (Scale bar 1 µm).

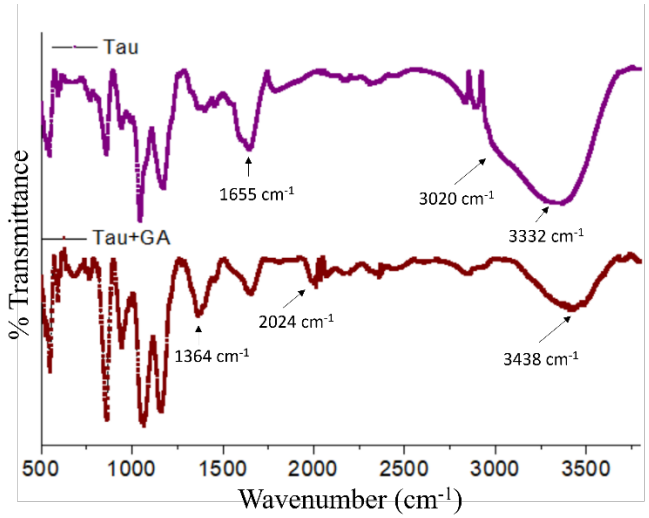

Fig. S13 FTIR spectra of tau independently and GA promoted tau droplet.

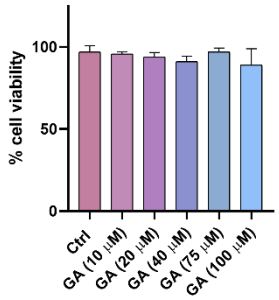

Fig. S14 MTT assay of GA to check the cell viability of SH-SY5Y neuronal cell line.

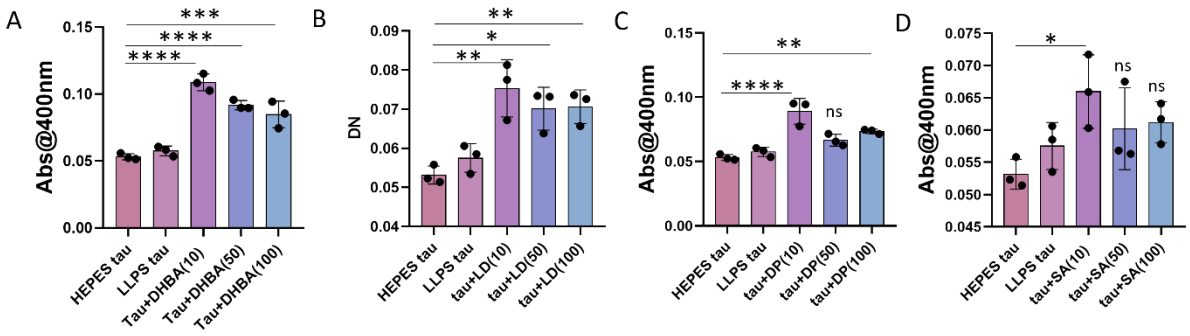

Fig. S15 Evaluation of commercially available SPs for biphasic modulation of tau coacervates. Data presents the turbidity assay used to screen the following SPs: A) DHBA, B) LD, C) DP, and D) SA (concentration in the parenthesis is in μM) (Statistical analysis conducted using ordinary one-way ANOVA, Tukey's post hoc test *p = 0.05).

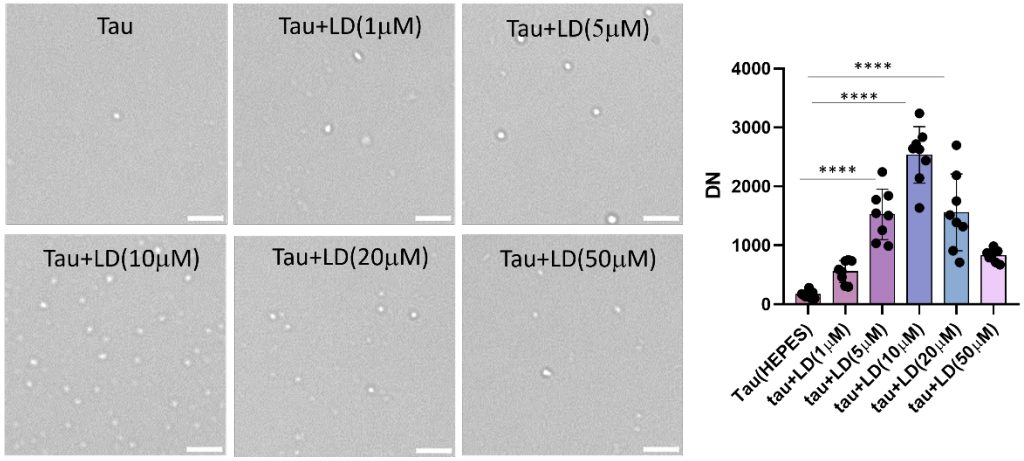

Fig. S16 Representative DIC microscopic images of the tau (5 μM) droplet modulated by varying concentrations of LD and its respective droplet quantification. (Statistical analysis conducted using ordinary one-way ANOVA, Tukey's post hoc test *p = 0.05)

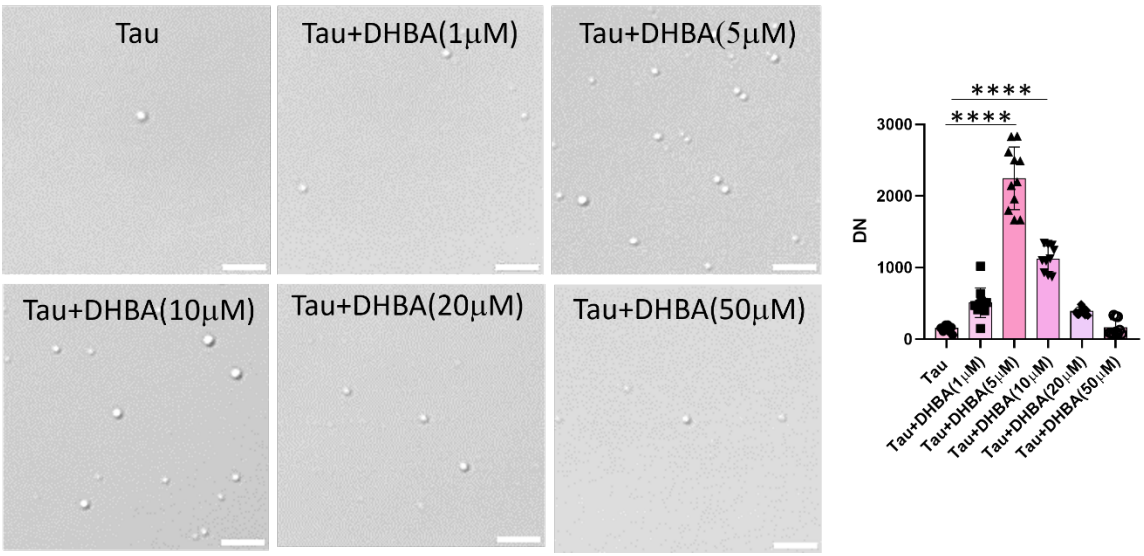

Fig. S17 Representative DIC microscopic images of the tau (5 μM) LLPS modulated by varying concentrations of DHBA and respective droplet quantification. (Statistical analysis conducted using ordinary one-way ANOVA, Tukey's post hoc test *p = 0.05)