Supplementary information

Polycatechols inhibit ferroptosis and modulate tau liquid-liquid phase separation to mitigate Alzheimer's disease

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Methods and Materials

All chemicals, reagents, and solvents were procured from Sigma Aldrich, Spectrochem, or TCI chemicals and used without further purification unless otherwise specified. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F12), penicillin-streptomycin (PS), and Fetal bovine serum (FBS) were purchased from Thermofischer Gibco and used without further purification. Cell culture plastic T25 flasks (Eppendorf), 96 well plates (Thermos Fischer Scientific), and confocal dishes (SPL Lifesciences) were procured and used without further sterilization. Precursor poly(methyl vinyl ether-alt-maleic anhydride) (MVEMA) (Cat. No. 416339) was procured from Sigma and used post-dialysis with methanol. 1S,3R-RSL3 was procured from Sigma and used as per guidelines. GPX4 antibody of rabbit origin was purchased from ELABS Science (Cat no. E-AB-64550). Goat-origin HRP conjugated secondary antibody was procured from Invitrogen and used as per manufacturer guidelines. 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT), metal salts, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from spectrochem and used without any purification. FINO-2 (Cat #HY-129457, MCE) was procured from MedChemExpress and used as per manufactures guidelines. Agilent Cary series UV-Vis-NIR absorption and fluorescence spectrophotometers were used to monitor absorbance and fluorescence. Microplate reader (SpectraMax i3x) was used to monitor well plate experiments. Bruker AV-400 NMR spectrometer was used to record ^{1}H and ^{13}C spectra with tetramethylsilane as internal standard. Live cell fluorescence imaging was carried out in Leica DMi8 fluorescence microscope, processing with Huygen software. The confocal imaging for the immunofluorescence study was performed with the Olympus Fluoview 3000 confocal laser scanning microscope, and the processing was performed with the inbuilt software. The quantification was performed with ImageJ software. All the other data was processed and analyzed using Prism 8 and Origin 8.5.

UV-visible absorbance measurements. Polycatechols of stock 10 mg/mL were prepared in DMSO and the measurements were performed in HEPES buffer (10 mM, pH 7.4) at room temperature. The metal ions like Fe^{3+}, Cu^{2+}, $Zn^{2+,}$ and Al^{3+} were added in increasing concentration from 10 μM to 60 μM with 3 min incubation time. The changes in the absorbance spectra of polycatechols were analysed to calculate the binding parameters. The values were fitted using the Benesi Hilderbrand equation.

$$\frac{1}{|A_0 - A|} = \frac{1}{|A_\alpha - A|} + \frac{1}{|A_\alpha - A| K_B [Q]}$$

Where K_B is the binding constant, A_0 and A_α are the absorbance values of polycatechols without or with metal ions with the highest concentration (60 μM).

Raman spectroscopy characterization.

Raman spectroscopy characterization was performed to solid PDP and PLDP using Renishaw inVia Raman microscope with a laser of 785 nm and a grating of 1200l/mm. The exposure time was set to 20 s, and the power to 0.1%. The data was plotted and processed in OriginPro 8.5.

***In vitro* antioxidant assay**

Fe^{III} ascorbate assay. The ability of polycatechols to redox silence Fe^{III} to prevent or quench ROS generated was monitored using ferric ascorbate assay. Coumarin-3-carboxylic acid (3-CCA, 50 μM), Fe^{III} (10 μM) were incubated independently and in the presence of PDP and PLDP in PBS (10 mM, pH 7.4) at 37 °C with ascorbate (150 μM). The •OH radical generated converted non-fluorescent 3-CCA to 7-OH-CCA which is highly fluorescent (λ_{ex} = 385 nm, λem = 450 nm) The fluorescence emission was monitored at a regular interval upto saturation, and the data was processed with origin 8.5 software.

DPPH radical scavenging assay. The ability of PLDP and PDP to quench ROS was monitored by DPPH assay. DPPH (50 μM) was incubated independently and in the presence of PLDP and PDP of varying concentration in MeOH: H_2O (1:1) at 37 °C for 30 min with ascorbic acid as a positive control. The absorbance at 540 nm was monitored with Spectramax i3 multiplate reader and the data was plotted with Origin 8.5.

DNPH study to monitor protein oxidation. Bovine serum albumin BSA (1 mg/mL), Fe^{3+} (100 μM) and H_2O_2 (2 mM) were incubated in PBS (50 mM, pH 7.4) independently and in the presence of PDP and PLDP for 30 h at 37 °C. 500 μL of DNPH (10 mM in 0.5 M H_3PO_4) was mixed with an equal volume of BSA mixture and incubated in the dark for 20 min followed by the addition of 250 μL of trichloroacetic acid (50% w/v) and allowed to further incubate at 25 °C. Samples were centrifuged for 7 min, the supernatant was discarded and the pellet was washed with 1 mL (3X) with ethanol/ethyl acetate mixture (1:1). The pellet was resuspended in 6M guanidine-HCl with absorbance recorded at 370 nm. Control samples in the absence of polycatechols were normalized to 100%.

Thiobarbituric acid reactive substances (TBARS) assay. Lipid peroxidation assay was performed by monitoring malondialdehyde (MDA), a side product of lipid peroxidation. Thiobarbituric acid (TBA) reacts with MDA to form TBARS which shows absorbance at 532 nm. To a solution of Fe^{3+} (100 μM), α-phosphatidylcholine (10 mM), and polycatechols, Asc

(2 mM) was added, and the samples were incubated for 30 min at 37 °C. Samples were treated with TBA and absorbance at 532 nm was measured. Control samples in the absence of polycatechols were normalized to 100%.

Thioflavin T assay. Thioflavin T (ThT) assay was performed to demonstrate the ability of PDP and PLDP to modulate Aβ42 aggregation. Briefly, freshly prepared Aβ42 (10 μM) was coincubated with ThT independently and in the presence of PLDP and PDP of varying concentrations in phosphate buffer saline (PBS, pH 7.4). The ThT fluorescence was monitored at a regular interval of 2 h up to 48 h to monitor the fibrillation kinetics.

The time-dependent ThT fluorescence data was modelled using the following equation:

$$y = y_0 + \frac{y_{max} - y_0}{1 + e^{\frac{-(t-t_1)k}{2}}}$$

Where y indicates fluorescence intensity corresponding to time t, while y_0 and y_{max} represent fluorescent intensities at the initial and maximum points, respectively. $t_{1/2}$ indicates the time needed for half the maximum fluorescence intensity.

Fluorescent tagged tau. Tau protein was tagged with rhodamine B isothiocyanate for confocal microscopy imaging purposes. Labelled tau was prepared by dissolving purified tau protein (50 μM) with 1M $NaHCO_3$ buffer (pH 9), followed by slow addition of rhodamine isothiocyanate (10 mg/mL), and the reaction was allowed to occur for 3 h, protected from light. Post incubation, the excess unreacted dye was dialyzed in PBS (10 mM, pH 7.4) for 24 h.

Fluorescence Recovery after Photobleaching (FRAP) analysis. Tau protein (5 μM) with (labelled: unlabelled- 1:4) and PLDP (20 μg/mL) were incubated in HEPES (10 mM, pH 7.4) for 30 min at 37 °C following which the samples were dropcasted in confocal dishes. For FRAP, the bleaching was carried out with 35% laser for 100 μs, with the fluorescent recovery monitored over 120 s. The recovery time was derived from a single exponential fit of the fl intensities plot using cell Sens software. The experiments were performed in triplicates.

Rhodamine tagging of PLDP. Rhodamine-tagged PLDP (Rh-PLDP) was synthesized by partial functionalization of the carboxylic acid side chain in PLDP. Briefly, PLDP: rhodamine B ethylenediamine (RE) (10:1 equivalent) were dissolved in the presence of EDC-NHS (1 equivalent) coupling in DMSO. Post 24 h, the Rh-PLDP was purified using dialysis in MeOH: DMSO (5:1) for 24 h using a 13 kD dialysis bag to remove the unreacted dye and EDC.

Preparation of Aβ$_{42}$ fibrils. Aβ$_{42}$ peptide (~1 mg) was dissolved in 1.5 mL hexafluoro-2-propanol (HFIP) and incubated for 1 h at room temperature. HFIP was removed with N_2 purging. The processed Aβ42 was dissolved in PBS (10 mM, pH 7.4), 1% DMSO to make a monomeric form. The absolute concentration of Aβ42 was determined using absorption at 280 nm (ε = 1450 cm^{-1} M^{-1}). The fibrillar aggregates were prepared by incubating in PBS buffer (10 mM pH 7.4) for 48 h.

Aβ$_{42}$ expression and purification. Aβ$_{42}$ plasmid construct was gifted by Prof James S. Nowick, University of California, USA. The Aβ$_{42}$ plasmid was transformed into *E. coli* BL21 strain and cultured in Luria-Bertani (LB) broth at 37 °C pretreated with ampicillin (100 μM), with continuous shaking was kept overnight. Next 1% of the primary culture was inoculated to 1L of LB broth pre-treated with ampicillin and was incubated till OD reached 0.43. Next, induction was initiated using isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 4 h. The cells were pelleted by centrifugation at 7000xg at 4 °C and the pellet was resuspended in 10 mM tris HCl in the presence of 1 mM EDTA in water (pH 8.0). This was followed by sonication and centrifugation at 38000x g to obtain pellet, which was subsequently resuspended in TRIS buffer containing 8M urea. Next, the suspension was purified with HPLC and lyophilized to obtain while Aβ42 powder. The protein was characterized with MALDI and LC-MS and stored at -80 °C until further use.

Tau expression and purification. The plasmid construct was gifted generously from Dr. Sharad Gupta, IIT Gandhinagar, India. Tau plasmid transformed into E. coli BL21 strain was cultured in LB broth at 37 °C continuously overnight in the presence of ampicillin (100 μM) and chloramphenicol (50 μM). Primary culture (1%) was inoculated to 1L LB broth pre-treated with antibiotics and was induced with IPTG once the OD reached 0.6. The cells were pelleted by centrifugation at 7000 rpm at 4 °C and boiled to heat precipitate unwanted protein in 20 mM PBS (pH 7.4). The tau protein present in the supernatant was purified by Ni-NTA affinity column chromatography and was measured by Bradford assay and further characterized by SDS-PAGE. The purified protein was stored at -80 °C for further use.

Cell culture. SH-SY5Y cells were cultured using DMEM/F12 with 10% (Fetal Bovine Serum) FBS and 1% (pen-strep) PS under ambient growing conditions (37 °C and 5% CO_2 atmosphere).

Cellular Toxicity assay. SH-SY5Y cells were seeded in the 96 well plate with the cell density of 25,000 cells per well and incubated for 24 h under ambient cell growing conditions. After that the media was changed and the cells were treated with PDP and PLDP in a concentration-dependent manner followed by further incubation for 48 h. After 48 h, 10μL of (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) was added followed by incubation for 4 h. The media was replaced and the formed formazon crystals were redissolved in DMSO:MeOH (1:1) solution. The absorbance was recorded at 540 nm along with 630 nm for background correction.

Cellular Uptake study. The cells were seeded in confocal dishes followed by incubation for 24 h. The polymer tagged with rhodamine (Rh-PLDP) was incubated with cells for 8 h followed by gentle washing thrice with PBS and media replacement and live cell imaging with Leica Microscope (Live cell mode).

Intracellular ROS Measurements. 2′,7′-dichlorofluorescin diacetate (DCFDA) was performed to monitor the ability of PLDP and PDP to quench intracellular ROS under oxidative stress conditions. SH-SY5Y cells were seeded in 48 well plates, followed by incubation for 24 h. The cell media was replaced with low serum (2.5% FBS) DMEM F12 and Aβ42+Fe independently and in the presence PLDP (20 μg/mL) and PDP (20 μg/mL). After 8h, the cells were incubated with DCFDA (20 μM) for 30 min. The cells were gently washed with warm PBS and the total well scan (λem= 530 nm) was performed using a multiplate reader followed by analysis and plotting with GraphPad Prism 6 software.

Mitochondrial membrane potential assay (MMP). To quantify the relative change in MMP upon induction of ferroptosis, SHSY5Y cells were seeded into 96 well plates for 24 h. After 24 h, the cells were treated with RSL3 (1 μM) independently and in the presence of PDP (20 μg/mL) and PLDP (20 μg/mL) of varying concentrations for 30 h. The media was removed followed by staining with MitoGreen (500 nM) for 20 min followed by washing with PBS twice. The fluorescence well scan was performed (λex = 554 nm and λem = 576 nm) using Spectramax i3 and the values were averaged. The relative fluorescence of treatments compared to healthy cells was assessed to monitor the MMP during ferroptosis.

LLPS induction in cells. GFP tau plasmid was transfected with lipofectamine 3000 as per users' guidelines in HEK293T cells. After 24 h, rhodamine-tagged PLDP (Rh-PLDP) (20 μg/mL) was treated for 48 h and cells were counter-stained with Hoechst dye. The tau droplets were visualised in live cells under the confocal microscope.

Mitochondrial ROS measurements. Changes in the mitochondrial ROS under ferroptotic conditions were measured MitoSOX red probe. SH-SY5Y cells were incubated with RSL3 (1 μM) independently and in the presence of PDP (20 μg/mL) and PLDP (20 μg/mL) for 30 h. After 30 h, the cells were stained with MitoSox Red (5 μM) in PBS for 30 min followed by a gentle rinse. Fluorescence well scan was performed (λex = 510 nm and λem = 580 nm) using Spectramax i3 and the data was averaged and plotted using GraphPad Prism 8.0.

Mitochondrial structural change. Changes in the mitochondrial structural fragmentation upon ferroptosis induction, and the ability to rescue in the presence of PDP and PLDP were monitored using Mito-TG probe. SH-SY5Y cells were seeded in confocal dishes, followed by incubation for 24 h. The cells were treated with RSL3 (1μM) independently and in the presence of PDP (20 μg/mL) and PLDP (20 μg/mL) and left for 24 h. After 24 h, the media was replaced and the cells were stained with Mito-TG (250 nM) for 30 min. Cells were gently rinsed after 30 min and imaged under live cell setup in rhodamine channel with fluorescence microscope. The images were processed with inbuilt software.

Lipid peroxidation assay.

In order to monitor lipid peroxidation under ferroptotic conditions we used BODIPY C11 dye. The cells were seeded in confocal dishes and were allowed to settle for 24 h. Post 24 h, the cells were treated with RSL3 (2 μM) independently and in the presence of PDP (20 μg/mL) and PLDP (20 μg/mL) for 6 h. The media was removed and stained with BODIPY C11 dye (1 μM) for 30 mins, followed by live cell imaging under Leica fluorescence microscope. The data was quantified using Graphpad 8.0.

Immunofluorescence Assay.

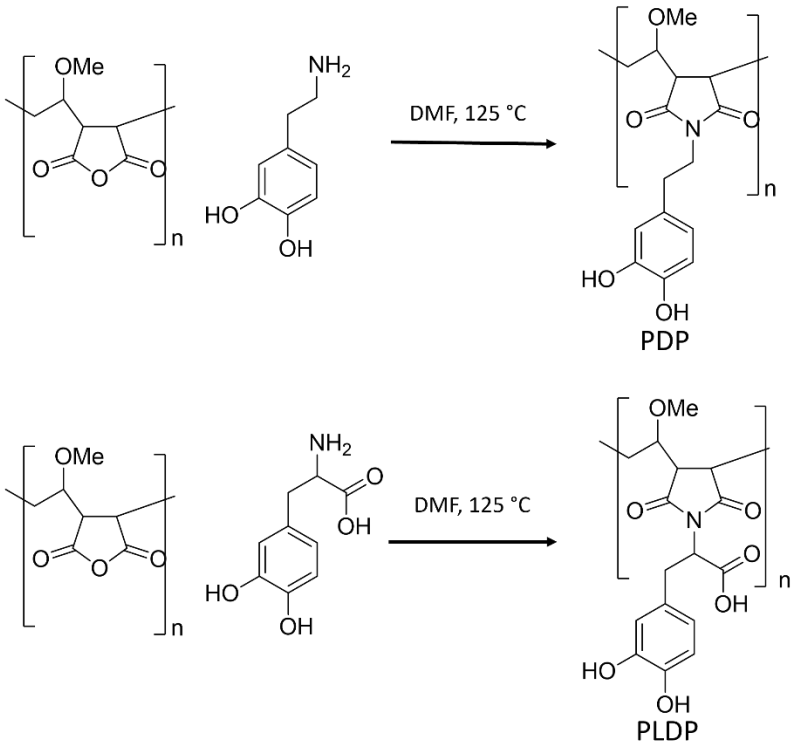
GPX4 and Nrf2 quantification. SH-SY5Y cells were seeded in confocal dishes and incubated till the confluency reached 70%. RSL3 (2 μM) or Aβ+Fe (1:5) were treated independently and in the presence of PDP (20 μg/mL) and PLDP (20 μg/mL) for 30 h. The cells were gently washed thrice with warm PBS and fixed with 4% paraformaldehyde for 20 min. The cells were washed twice for 5 mins followed by treatment with permeabilization buffer (PBS with 0.1% Triton X-100) for 10 mins. The samples were gently washed with PBS twice followed by blocking with 10% goat serum in PBS for 30 min at room temperature. The blocking solution was aspirated followed by treatment with primary antibody with a dilution (1:250) in 4 °C for 24h. The samples were washed with PBS thrice followed by treatment with Alexa Fluor-488 conjugated secondary antibody for 1 h at room temperature. The cells were gently rinsed and

incubated with DAPI (1μM) for 10 min followed by imaging using the confocal microscope. The quantification was performed using ImageJ software.

Total GSH level measurement

The total GSH level has been detected using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent). Briefly, the cells were cultured in 6 well plate with a density of 1,50,000 cells followed by incubation for 24 h. Cells were treated with RSL3 (500 nM) independently and in presence of PLDP (20 μg/mL) for 12 h. Further, the cells were trypsinized and incubated with RIPA lysis buffer for 30 min. The cell lysate was harvested and to the equal concentration of the lysates, NADPH (0.5 mg/mL), Glutathione reductase enzyme and Ellman's reagent (100 μM) were added and allowed for incubation for 10 min in dark, post which absorbance at 412 nm was recorded. The increase/decrease in the absorbance reflect the relative GSH levels present in samples.

Results.



Scheme S1 Synthesis of PDP and PLDP.

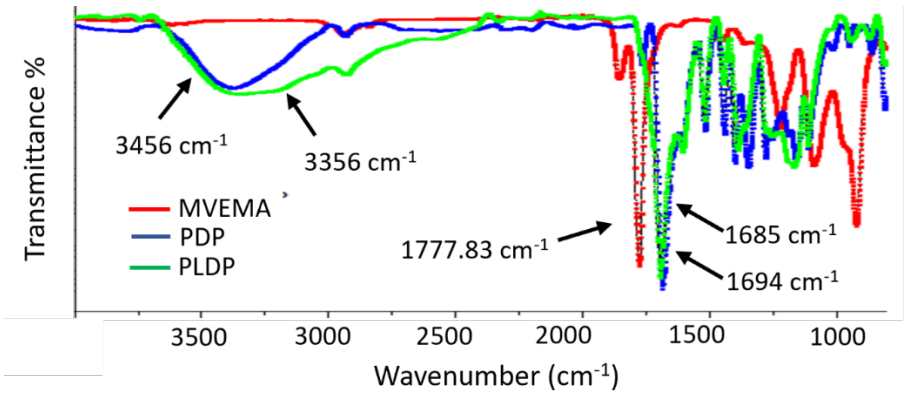


Fig. S1 FTIR spectra of precursor polymer MVEMA, PDP and PLDP indicating polymer functionalization.

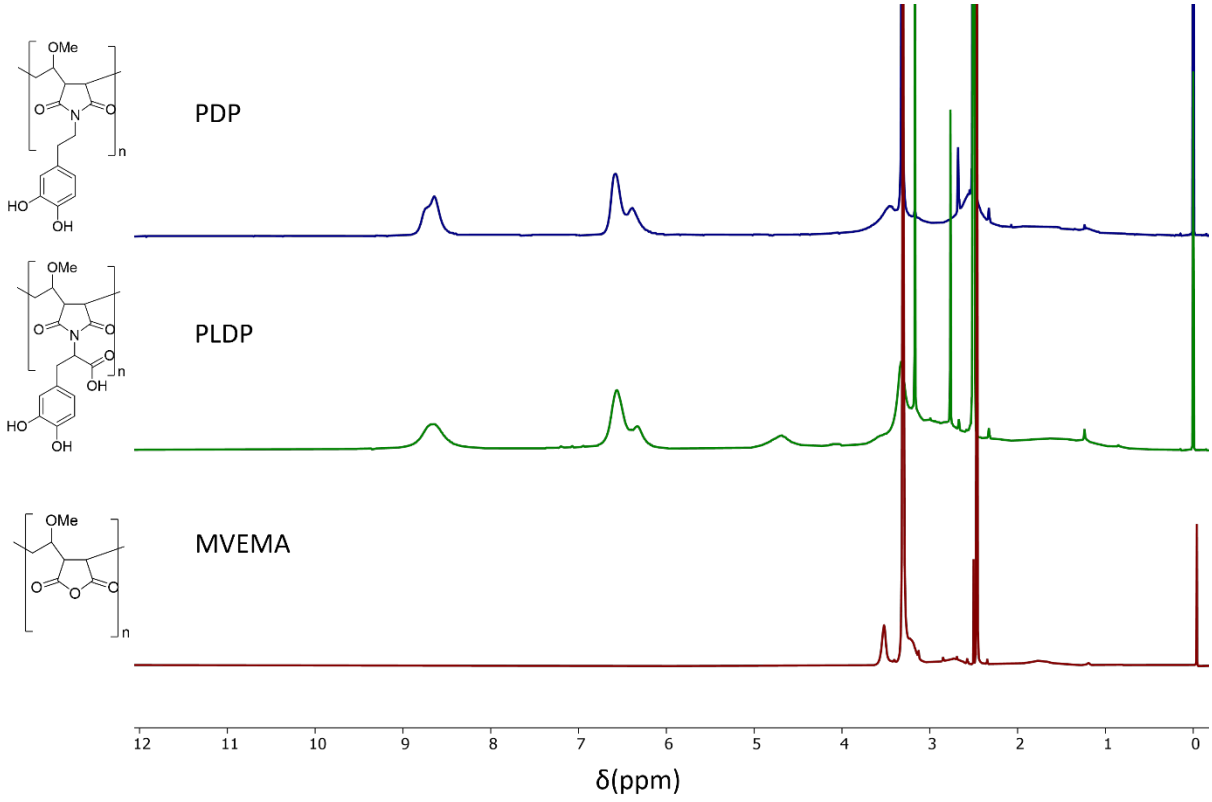


Fig. S2 ^{1}H NMR characterization of PDP, PLDP and MVEMA.

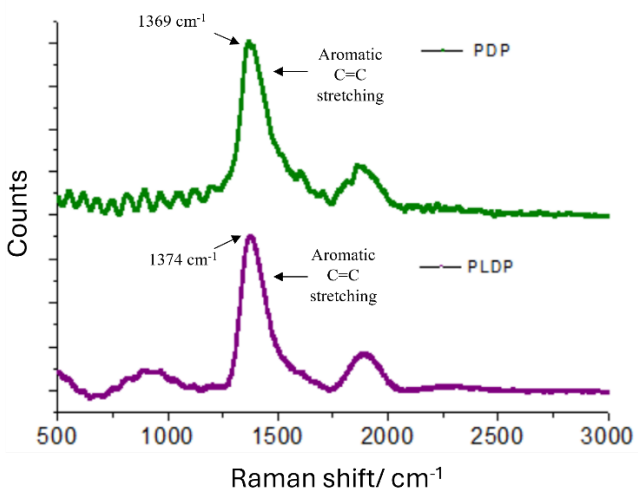


Fig. S3 Raman spectroscopy characterization of PDP and PLDP.

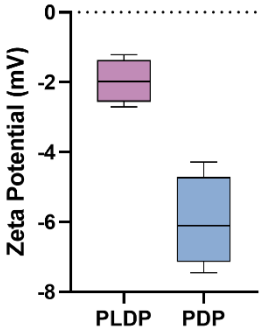


Fig. S4. Zeta potential of PDP and PLDP.

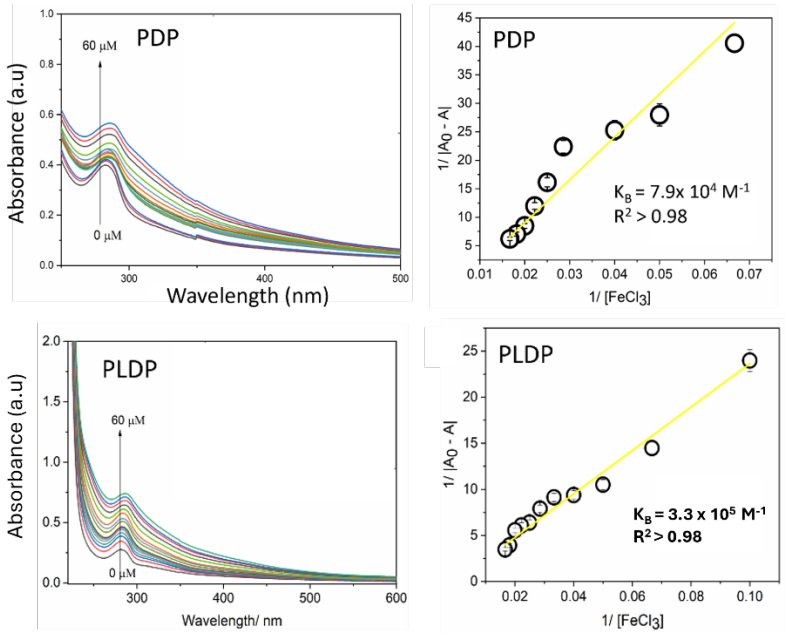


Fig. S5 UV-visible absorption spectra of PDP and PLDP in presence of Fe^{3+}.

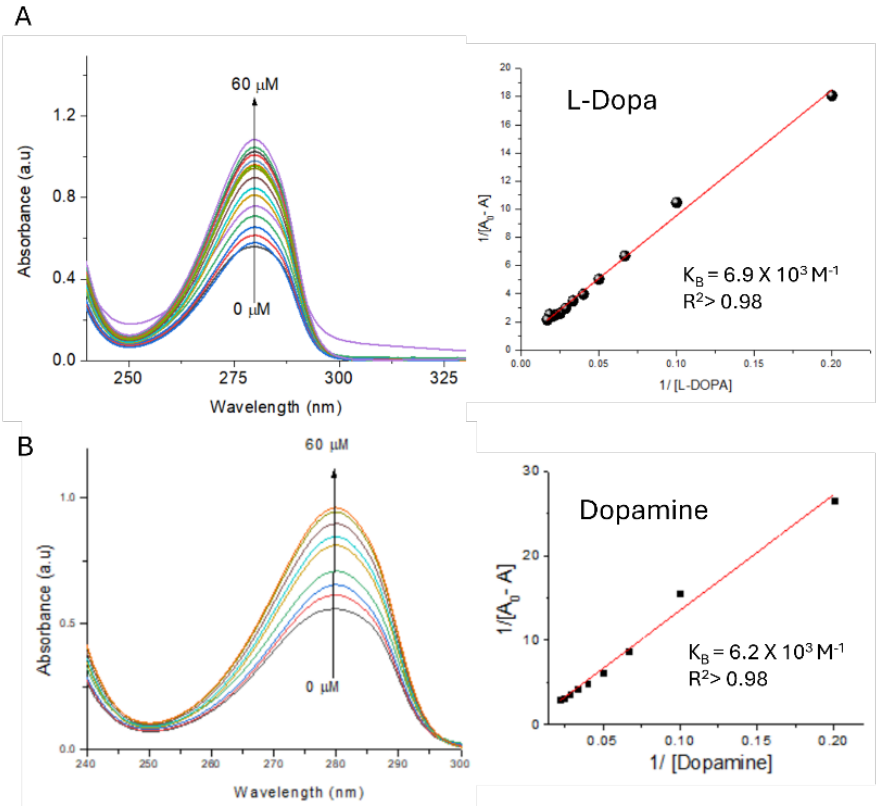


Fig. S6 UV-visible absorption spectra of A) L-Dopa and B) dopamine in presence of Fe^{3+}.

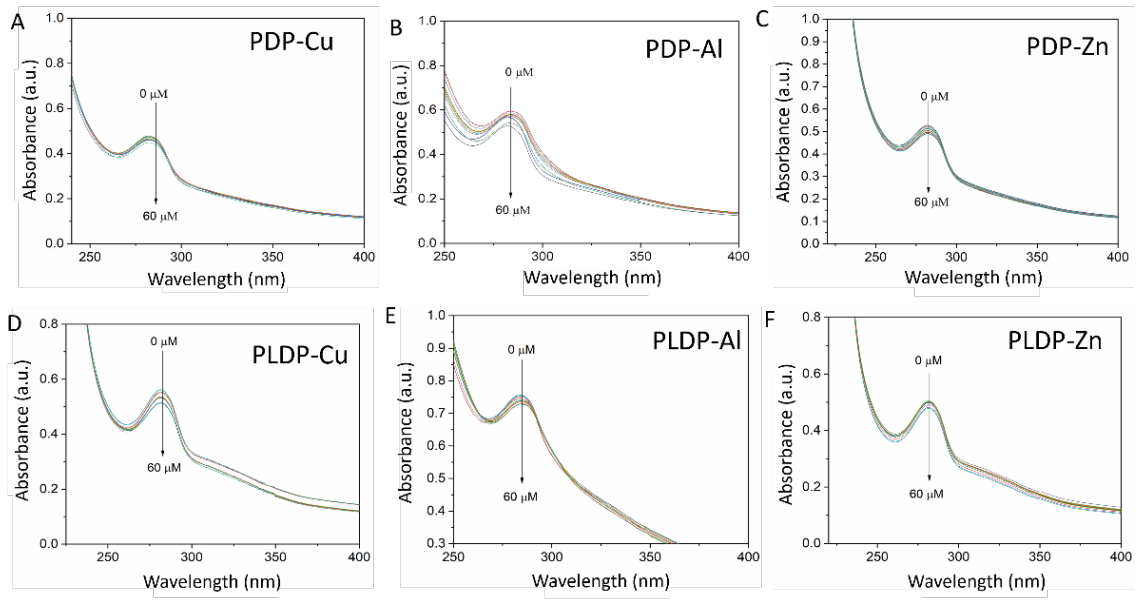


Fig. S7 UV-visible absorption spectra of PDP and PLDP in presence of various biologically relevant metal ions Cu^{2+}, Zn^{2+}, Al^{3+}.

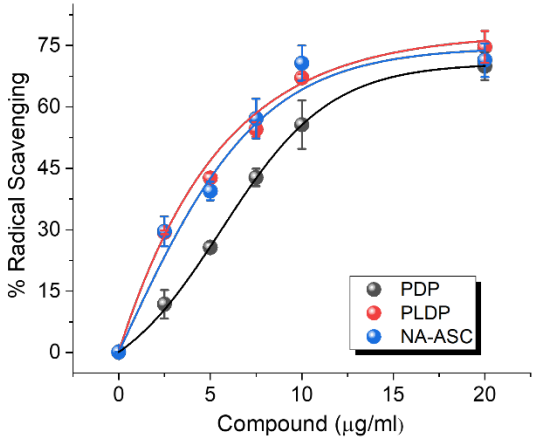


Fig. S8 DPPH assay of PDP and PLDP to monitor ROS scavenging.

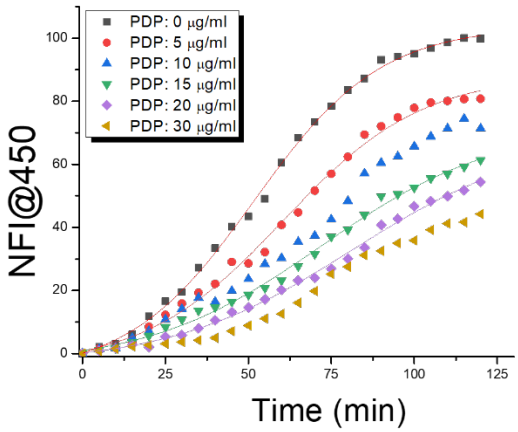


Fig. S9 Fe-ascorbate assay to monitor the ability of PDP to redox silence Fe-ROS.

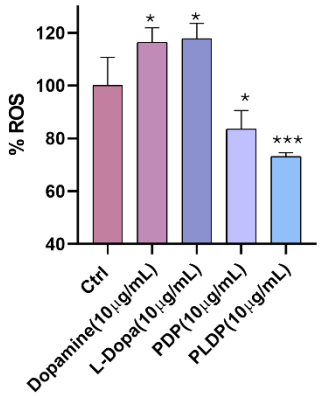


Fig S10. ROS generated from Fe-ascorbate system monitored by the fluorescence of 7 hydroxyl coumarin carboxylic acid in presence of dopamine, L-Dopa, PDP and PLDP.

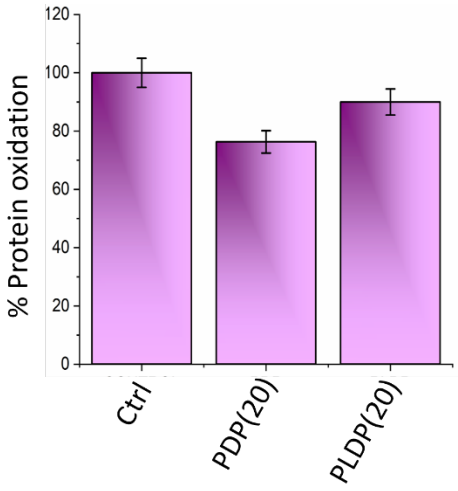


Fig. S11 2,4-Dinitrophenylhydrazine (DNPH) assay to monitor protein oxidation inhibition in presence of PLDP and PDP. The concentration in parenthesis is in µg/mL.

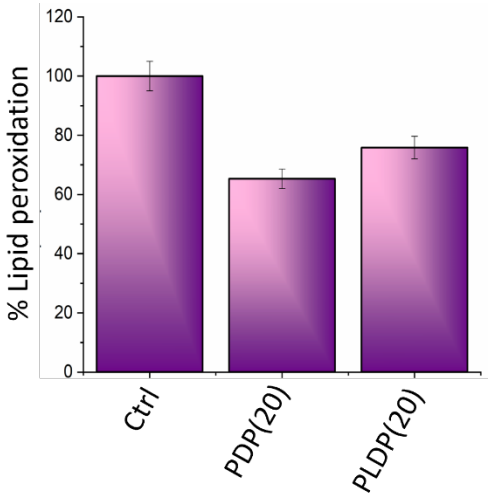


Fig. S12 TBARS assay to monitor lipid peroxidation inhibition in presence of PLDP and PDP. The concentration in parenthesis is in µg/mL.

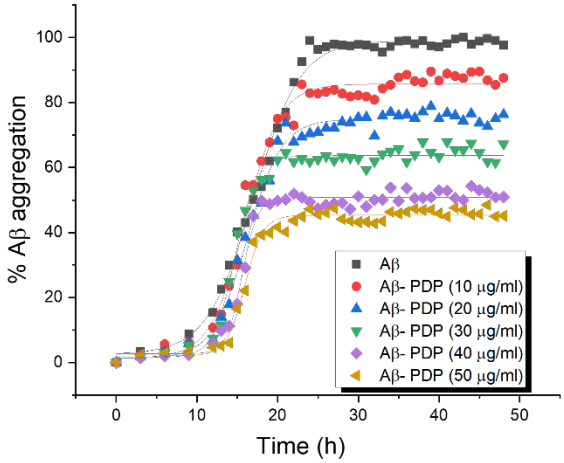


Fig. S13 ThT assay to monitor Aβ aggregation in presence of PDP.

Table S1 Lag time and rate constant of Aβ aggregation in presence of PDP and PLDP.

Aβ (10μM): PDP (μg/mL)	T_{lag} (h)	k (1/h)	Aβ (10μM): PLDP (μg/mL)	T_{lag} (h)	k (1/h)
Aβ	13.47 ± 0.21	1.20 ± 0.089	Aβ	13.21 ± 0.24	1.31 ± 0.071
Aβ:PDP (10)	13.01 ± 0.32	1.15 ± 0.078	Aβ:PLDP (10)	13.17 ± 0.29	1.10 ± 0.065
Aβ:PDP (20)	13.91 ± 0.37	0.96 ± 0.029	Aβ:PLDP (20)	13.92 ± 0.34	0.87 ± 0.043
Aβ:PDP (30)	14.03 ± 0.29	0.81 ± 0.064	Aβ:PLDP (30)	14.31 ± 0.32	0.66 ± 0.037
Aβ:PDP (40)	14.90 ± 0.41	0.75 ± 0.059	Aβ:PLDP (40)	15.13 ± 0.37	0.59 ± 0.021
Aβ:PDP (50)	15.02 ± 0.19	0.58 ± 0.091	Aβ:PLDP (50)	15.99 ± 0.21	0.23 ± 0.019

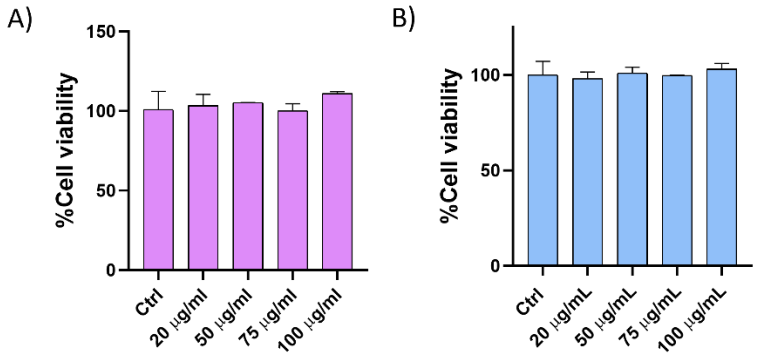


Fig. S14 MTT assay to assess the cytotoxicity of A) PLDP and B) PDP to SH-SY5Y cells post 48 h incubation.

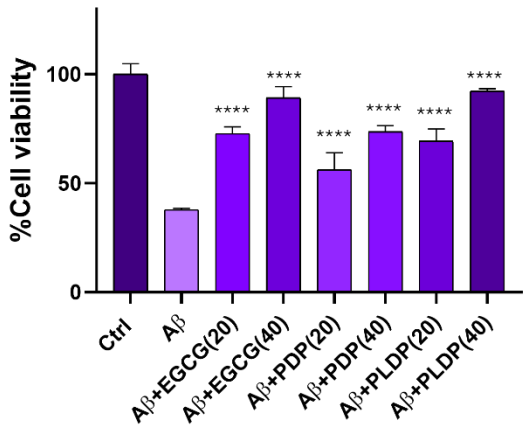


Fig. S15 Comparative neuronal cell rescue assay from Aβ42 induced toxicity by EGCG, PDP and PLDP. The concentration in parenthesis is in μg/mL.

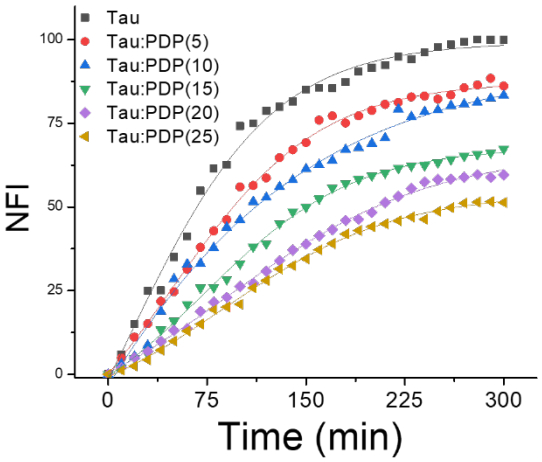


Fig. S16 ThT assay to monitor tau aggregation in presence of PDP. The concentration in parenthesis is in µg/mL.

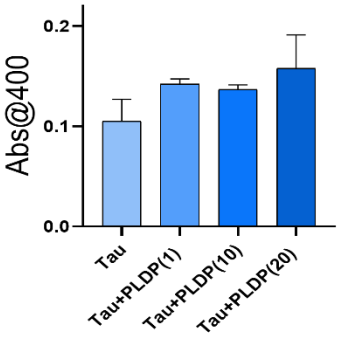


Fig. S17 Turbidity measurement of tau in presence of PLDP in HEPES. The concentration in parenthesis is in µg/mL.

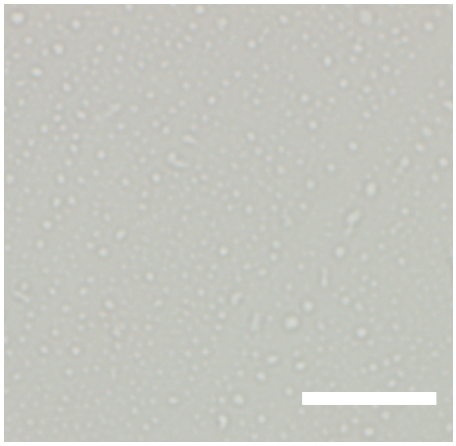


Fig. S18 DIC imaging of phase separated tau droplet promoted by PLDP (scale bar 20 μm).

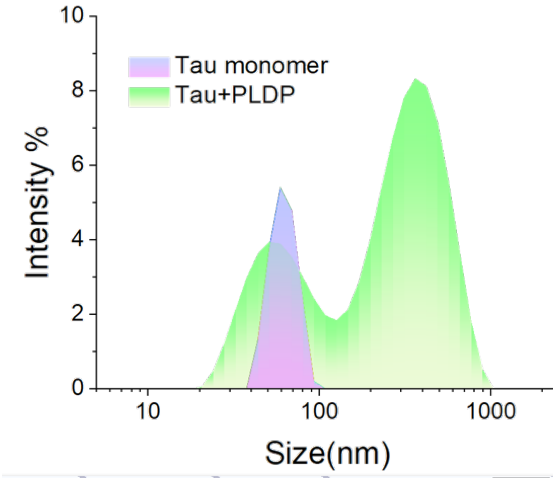


Fig. S19 DLS study to investigate PLDP promoted tau LLPS.

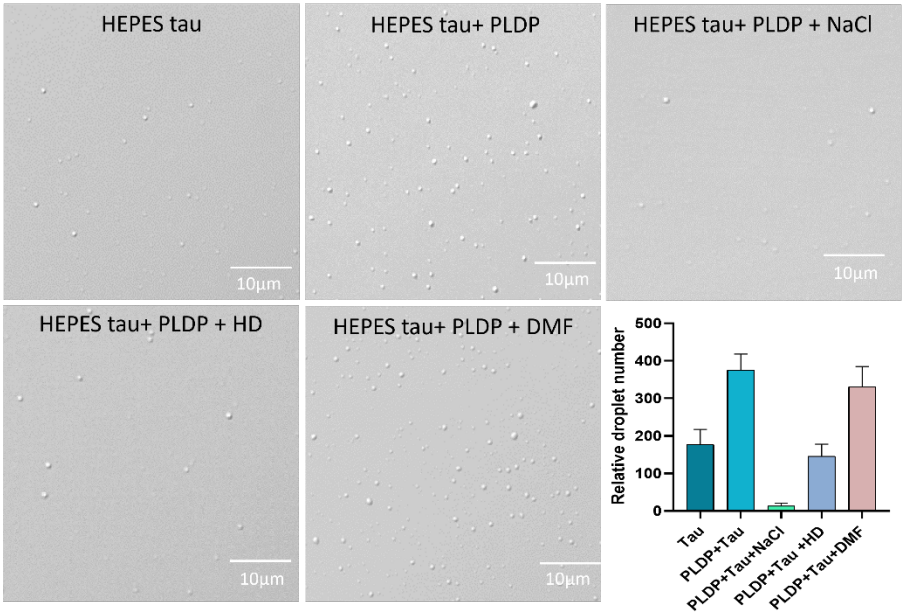


Fig. S20 Mechanistic insights into complex coacervation between tau and PLDP (scale bar 10 μm).

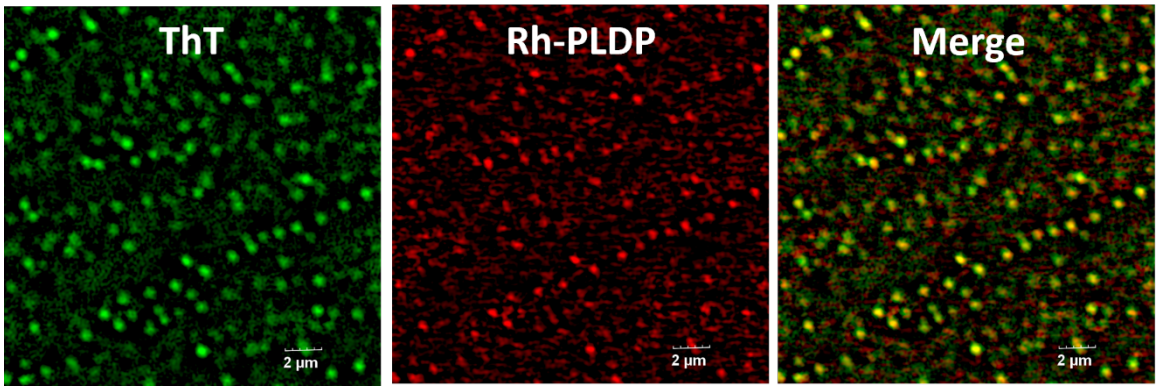


Fig. S21 Colocalization of ThT and Rh-PLDP fluorescence confirm PLDP induce tau LLPS (scale bar 2 μm).

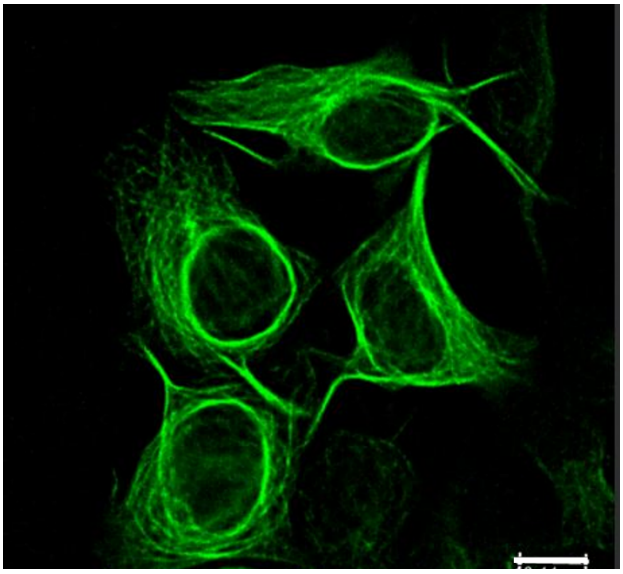


Fig. S22 Tau GFP transfected in HEK293T in the absence of PLDP. (scale bar 10 μM).

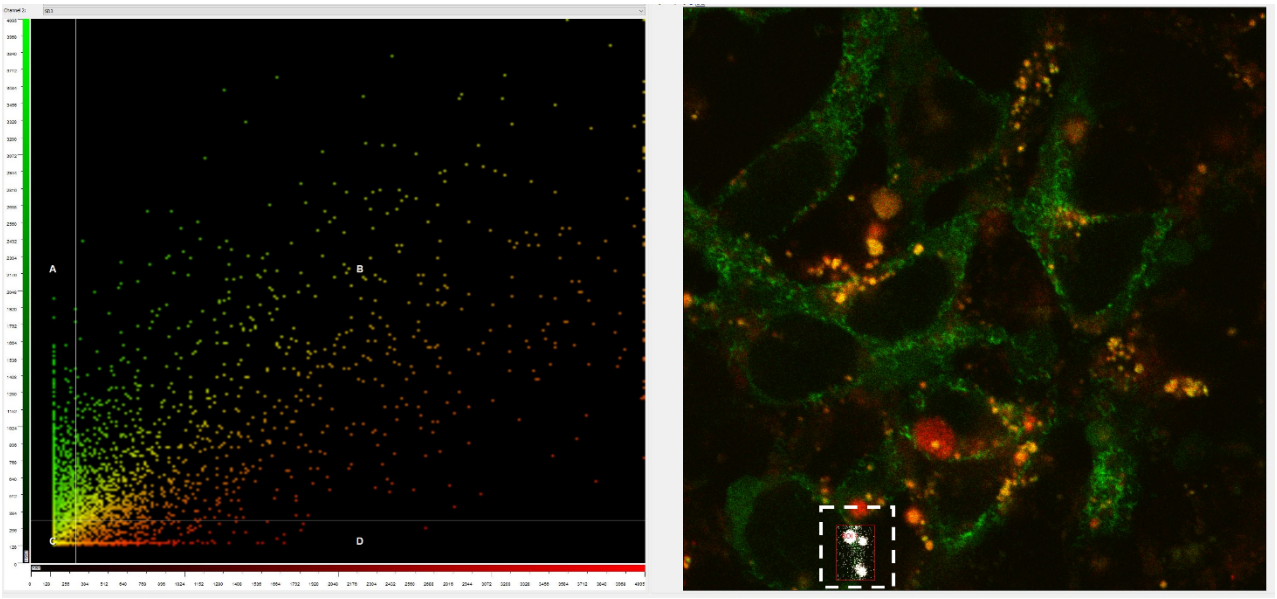


Fig. S23 Colocalization study to monitor tau LLPS in presence of R-PLDP (red) and tau GFP (green).

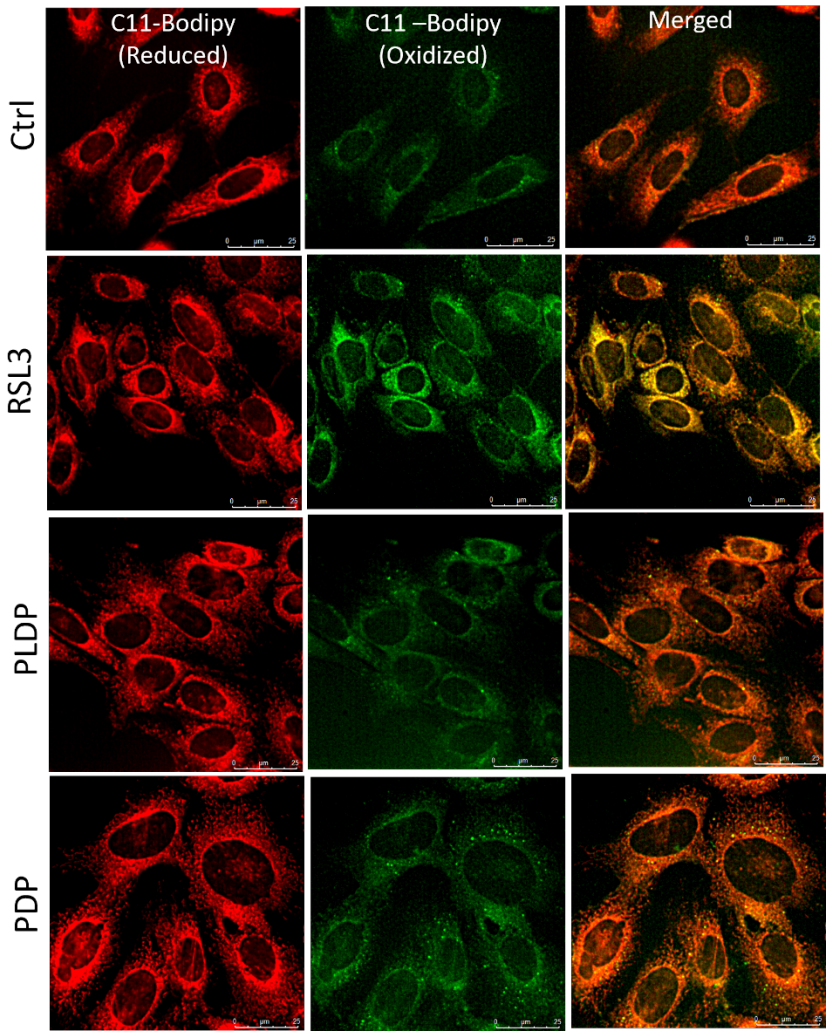


Fig. S24 C11-bodipy imaging for monitoring lipid peroxidation in presence of RSL3, PDP and PLDP.

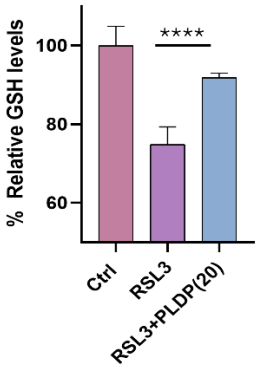


Fig. S25 Cellular Glutathione (GSH) levels monitored in presence of RSL (500 nM) alone and in presence of PLDP (20 µg/mL) using Elman's reagent.

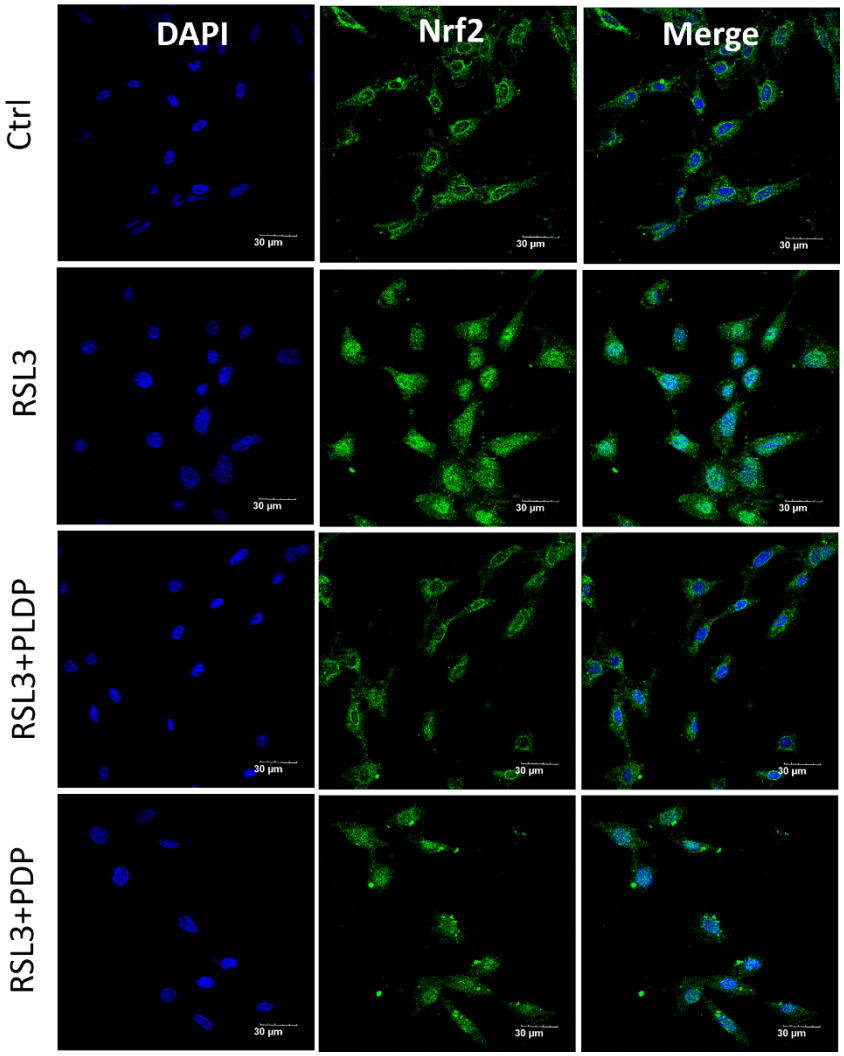


Fig. S26 IF assay to monitor Nrf2 activation under RSL3 treatment independently and in presence of PLDP and PDP (Scale bar 30 µm)

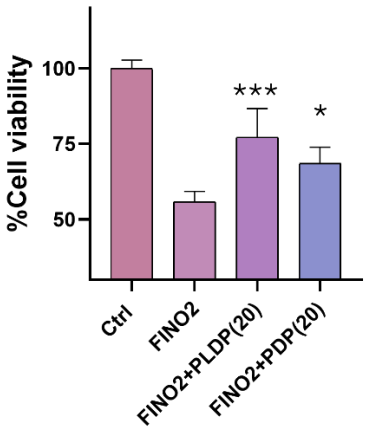


Fig. S27 Cell viability assessment of PLDP (20 µg/mL) and PDP (20 µg/mL) from FINO-2 (20 µM) induced ferroptosis.

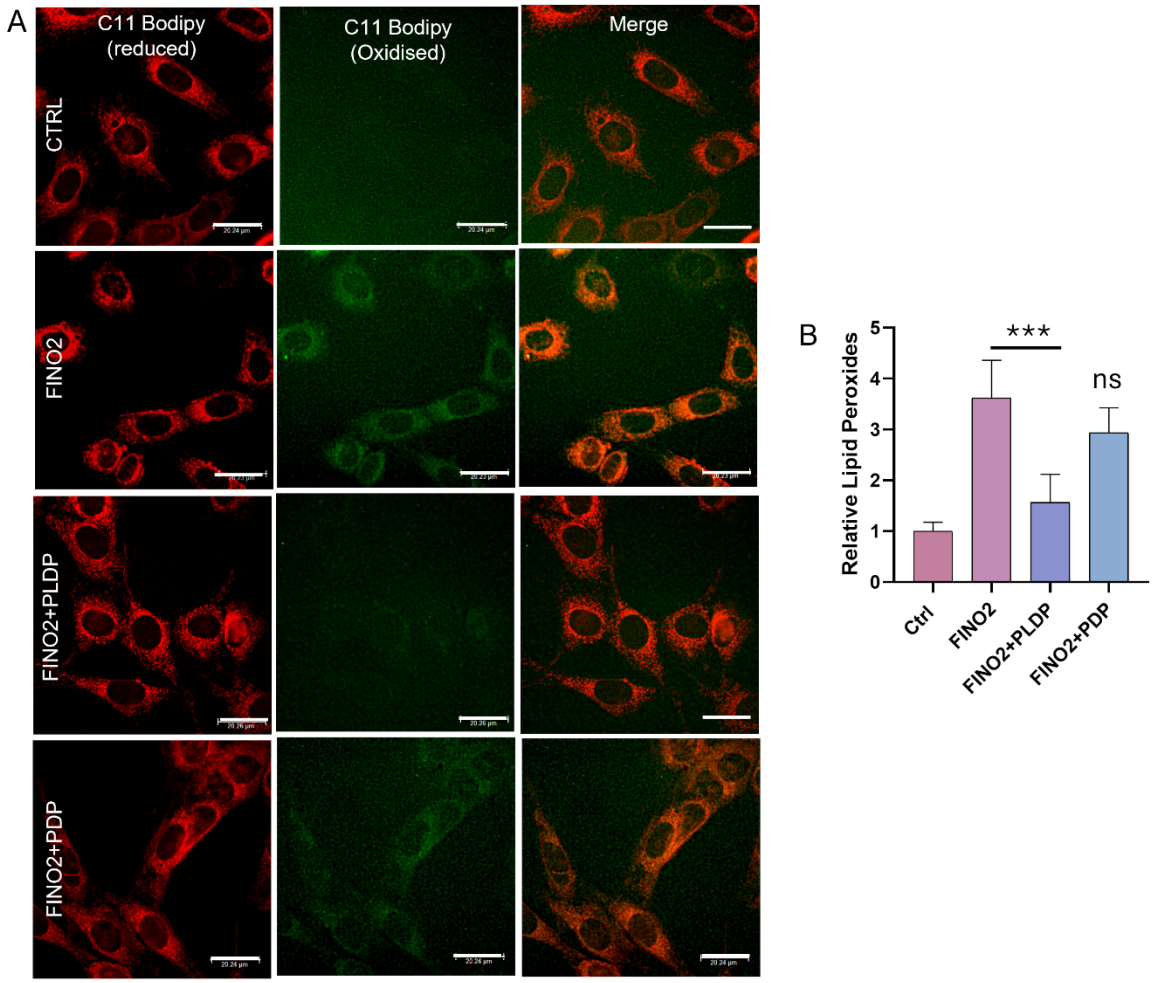


Fig. S28 C11-bodipy imaging for monitoring lipid peroxidation in presence of FINO-2, PLDP and PDP (Scale bar 20 µm).