Details of the research work duly signed by the applicant, for which the Sun Pharma Science Foundation Research Award is claimed, including references and illustrations.

α-Synuclein misfolding and aggregation are directly implicated in Parkinson's disease (PD) pathogenesis, which is the second most prevalent progressive neurodegenerative disorder after Alzheimer's disease (AD). The pathological hallmark of the disease is the accumulation of misfolded α-Syn protein in the form of amyloid aggregates inside the cytoplasmic inclusions, named Lewy bodies (LBs) and Lewy neuritis (LN) (Spillantini *et al*., Nature, 1997, 2000; Spillantini and Goedert, Annals of the New York Academy of Sciences, 2000; Spillantini *et al*., Cell and tissue research, 2018). Interestingly α-Syn aggregation is also involved in an array of other neurodegenerative disorders, such as Dementia with Lewy bodies (DLB), Parkinson's disease dementia (PDD), and multiple system atrophy (MSA) that are collectively designated as synucleinopathies. Although most of the PD cases are sporadic in nature, the identification of various familial mutations of α-Syn, gene duplications/ triplications in the early onset familial form of PD (<10%). Observation of α-Syn rich aggregates in LB of all forms of synucleinopathies strongly supports that α-Syn aggregation is indeed a pathogenic mechanism causing neuronal cell death in PD and other diseases. Several cell, and animal models and *in vitro* studies indeed suggest that α-Syn aggregation into amyloid fibrils could be a toxic mechanism leading to neurodegeneration. However which species of α-Syn cause neurodegeneration is largely unknown. In recent years it has been however suggested that early-formed soluble oligomers are the most possible toxic species causing dopaminergic cell death in PD and other synucleinopathies (Mehra *et al.*, Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 2019; Mukherjee *et al.,* Journal of Molecular Biology, 2022). Therefore, understanding the α-Syn misfolding and aggregation is very important for designing therapeutics against PD. α-Syn aggregation proceeds through nucleation-dependent polymerization mainly involving three steps. (1) This includes the lag phase, where the soluble monomeric protein polymerizes and forms growth-competent nuclei, (2) growth or exponential phase, where the nuclei constantly grow and rapidly convert to oligomers, protofibrils, and fibrils and the (3) stationary phase where a steady state equilibrium is achieved between the monomers and the fibrils (Morris *et al*., 2009, Gadhe *et al*., Biophysical Chemistry, 2021) (Figure 1).

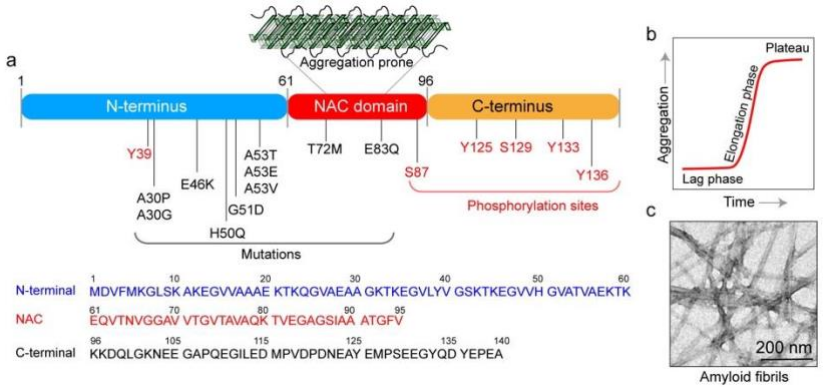

Figure 1: Structure and sequence of α-Syn and its aggregation. (a) Schematic representation of three distinct domains of α-Syn showing the amphipathic N-terminal domain (residue 1-60) hydrophobic and aggregation-prone NAC domain (residue 61-95), and acidic C-terminal domain (residue 96-140). The N-terminus is prone to mutations while the C-terminus harbors the site for various post-translational modifications. (b) Schematic representation showing the sigmoidal growth kinetics of α-Syn aggregation. α-Syn undergoes structural transition by nucleation-dependent aggregation having a slow lag phase, exponential elongation phase, and an equilibrium phase. (c) The electron micrograph images show fibrillar morphology of α-Syn.

Therefore, understanding the early molecular events of α-Syn aggregation and identifying the associated early-stage species are important for deciphering the disease onset and pathogenesis. In this direction, we have shown that α-Syn undergoes a helix-rich intermediate state at the onset of nucleation into growth-competent fibril nucleation. We showed that at the beginning, α-Syn poses mostly random coil structure but with time it converts to an α-helix rich state, which subsequently converts to β-sheet-rich fibrils (Ghosh *et al*., International Journal of biological macromolecules, 2018). Moreover, we also showed that at extremely high crowding conditions (very high α-Syn concentration), it can convert to a gel-like state which entraps various toxic α-Syn species such as oligomers and fibrils (Kumar *et al*., Angewandte Chemie International Edition, 2018). This study from our lab substantially enriches how α-Syn might undergo toxic oligomer formation. To understand in more detail the possible conformational change of the protein in cytoplasmic crowding, we performed α-Syn aggregation in the presence of PEG and discovered a completely new state of α-Syn, which is liquid-liquid phase separation of the protein (Figure 2). In this direction, it has been shown that similar to the de-mixing of oil in water, a plethora of biomolecules (e.g. proteins, RNA and DNA) inside the cells can undergo phase separation and execute spatiotemporal compartmentalization, thus leading to the formation of a dense phase rich in a biomolecule (refer to review Shin and Brangwynne., Science, 2017). This type of spatial

segregation results in the formation of membrane-less cellular organelles, which play a significant role in performing a variety of native functions of the cells/host organisms (Li *et al*., Nature, 2012, Ray *et al*., Nature Chemistry, 2020). Previous studies have highlighted that the unstructured, intrinsically disordered regions and low complexity domain mediate multivalent interactions facilitating protein LLPS. We hypothesized that the globular proteins/small peptide models can also undergo condensate formation and demonstrated that all proteins/polypeptides (23 in our case) irrespective of their diverse sequences, structures and properties can form dynamic liquid assemblies in the presence and absence of molecular crowder (Poudyal *et al*., Nature Communications, 2023). This suggests that the cellular system finely tunes the phase behaviour of proteins for cellular fitness. Further, we also demonstrated that electrostatic, hydrophobic and H-bonding interactions either alone or in combinations play an essential role in protein LLPS (Poudyal *et al*., Nature Communications, 2023).

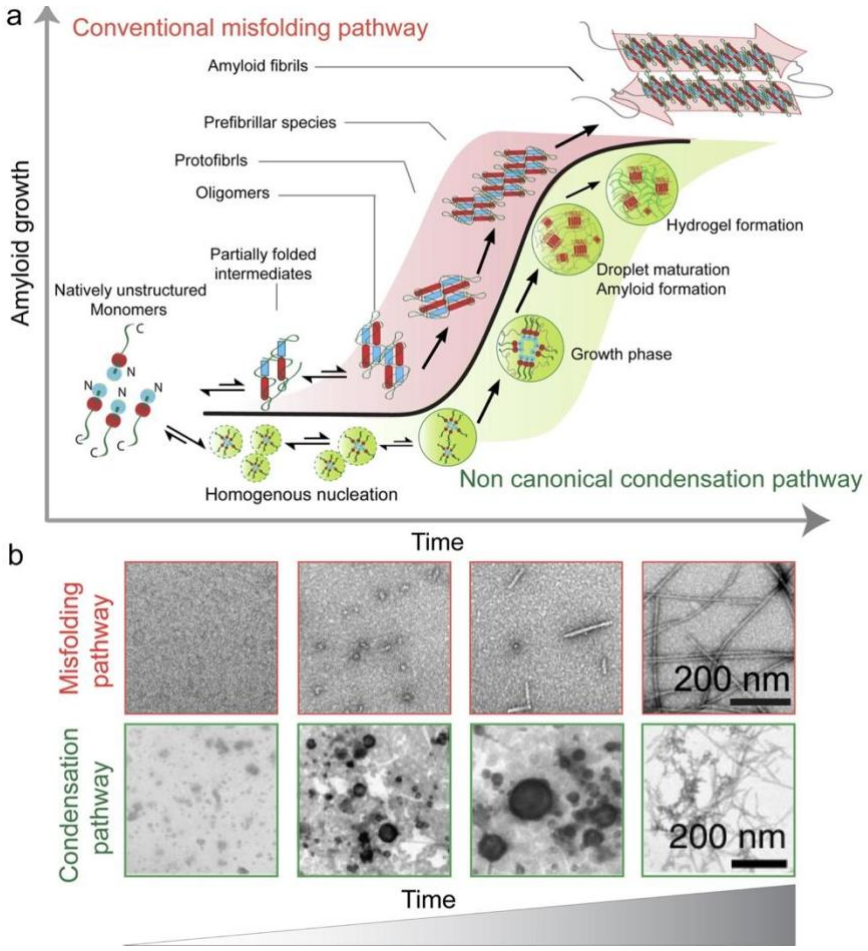

Figure 2: (a) Two distinct pathways for α-Syn aggregation and amyloid formation are depicted in a scheme, where one shows the conventional misfolding pathway in which monomers interact together and form intermediates like oligomers, protofibrils, pre-fibrillar species before forming beta-sheet amyloid fibrils. The other pathway is the alternate non-canonical condensation pathway, where primary nucleation processes require a high local concentration of protein in a crowded environment. (b) The lower panel depicts the TEM images showing the morphology of the intermediates formed in both the aggregation pathways. *The image has been adapted from Maji and Co-workers, Mukherjee et al., 2022, Journal of Molecular Biology*).

To predict the presence of disordered regions and low complexity regions, we examined the protein sequence of α-Syn. We found α-Syn comprises two LCDs, one at the N-terminal region and another in the NAC domain with an intrinsically disordered C-terminal region (Fig. 3a). The presence of such domains might enhance the propensity of the protein to undergo phase separation (Fig. 3b). We showed for the first time that soluble, monomeric WT α-Syn forms phase-separated spherical liquid-like droplets in the presence of molecular crowder such as PEG-8000 (Polyethylene Glycol), which mimics the crowding of a cellular microenvironment (Fig. 3c). The droplets at the initial time point are highly dynamic, mobile, can fuse (Fig 3d) and gradually grow and increase in size, and also exhibit Ostwald's ripening in which larger droplets grow at the expense of smaller droplets. The dynamic characteristic of the droplet can be attributed to the translational motion of the protein molecules inside them and therefore show a rapid fluorescence recovery after photobleaching (FRAP), confirming their liquid-like behaviour (Fig. 3e). In addition to this, the rotational dynamics of the molecules were probed by the fluorescence anisotropy study that indicated the rotational correlational times of α-Syn inside the liquid droplets (Fig. 3f). The critical concentration of α-Syn for LLPS turned out to be very high (200 uM in presence of 10% Peg at pH 7.4) compared to the physiological concentration of the protein at the presynapse that indicates the LLPS property of α-Syn might be associated with the pathological overexpression of the protein. Interestingly, we also demonstrated that several PD-associated conditions, such as familial mutations (A53T, E46K, and phosphomimetic mutant S129E), environmental factors (metal ions, pH, different buffer conditions, lipids), and PTM's tend to drastically decrease the critical concentration of LLPS with faster kinetics for phase separation. In contrast, the N terminal acetylation, which maintains high protein solubility in physiological conditions, and the presence of dopamine have been shown to inhibit α-Syn LLPS (Ray *et al*., Nature Chemistry, 2020; Sawner *et al*., Biochemistry, 2021). All of these observations suggest that α-Syn LLPS might be a crucial event associated with PD pathogenesis.

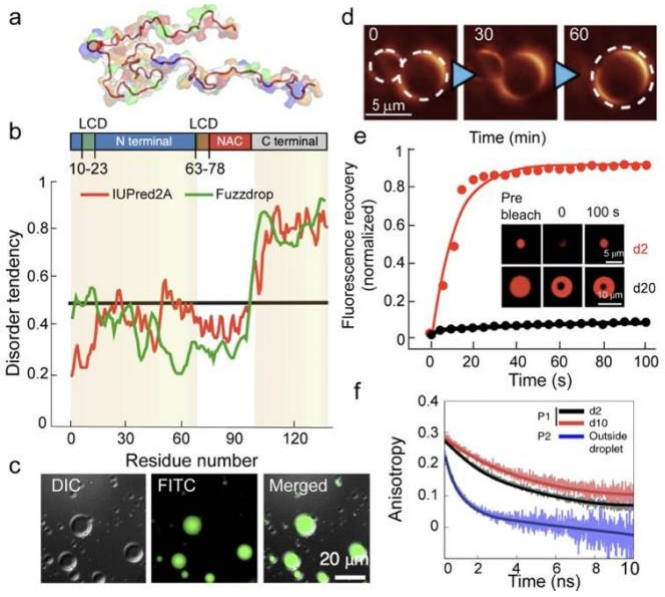

Figure 3: (a) A 3D surface representation of WT α-Syn superimposed with its secondary structure shown in dark red. Amino acids marked in blue, red and green colors depict positive, negative, and hydrophobic amino acids respectively. (b) The primary sequence of WT α-Syn has been analyzed by using different in silico techniques, namely SMART (for low complexity domain), IUPred2 (for the intrinsically disordered region), and Fuzdrop (to predict LLPS propensity). (c) Representative differential interference contrast (DIC) microscopy images, fluorescence images using FITC label, and corresponding merged image of WT α-Syn showing spherical droplets upon LLPS in the presence of 10% PEG. (d) Time-lapse images showing the fusion of two droplets to form a single large droplet over time. (e) FRAP analysis depicting complete fluorescence recovery of droplets at an early time point and decreased recovery at a later time point, showing solidification over time. (f) Slower rotational dynamics of α-Syn inside the droplet as shown by the fluorescence anisotropy decay study. (*This figure has been adapted from Maji and co-workers, Ray et al., 2020, Nature Chemistry and Mukherjee et al., 2022, Journal of Molecular Biology*).

We further demonstrated that the phase-separated α-Syn droplets gradually undergo liquid-to-solid transition where the translational and rotational dynamics of the molecules get compromised over time and result in amyloid-rich entities containing oligomers and fibrils. Such liquid-to-solid transition of the droplet results in the structural transition of monomeric α-Syn inside the droplet from random coil to β-sheet-rich amyloids over time as suggested by fluorescence enhancement of amyloid-specific Thioflavin T dye. Using an array of biophysical techniques, we simultaneously, showed that LLPS of α-Syn occur at the beginning of the lag phase of aggregation. With time, these liquid droplets mature to form a solid-like state that nucleates oligomers and fibrils. Interestingly we demonstrated the direct emergence of amyloid fibril from the matured condensates due to high amyloid load under an electron microscope (Ray *et al*., Nature Chemistry, 2020). Interestingly, the *in vitro* LLPS solution of α-Syn gradually transitions into a porous

hydrogel upon prolonged incubation, which essentially comprises solidified droplets rich in various oligomers and aggregated species. This observation is in line with our previous findings where our lab showed that α-Syn amyloid hydrogel can entrap toxic oligomers and short fibril (Kumar *et al*., Angewandte Chemie International Edition, 2018). Moreover, the hydrogel formation from protein LLPS has been implicated in other proteins associated with neurodegenerative diseases as well, thus providing a basis for our hypothesis that LLPS-mediated liquid-to-solid transition of proteins to a hydrogel state might act as a repository for accumulating amyloid aggregates and toxic oligomers.

In addition to the *in vitro* phase separation system, we also demonstrated the LLPS of α-Syn in a mammalian cell model system. Upon treatment with two very common metal ion toxicants for PD (Fe3+, and Cu2+ ions), more than 95 % of the cells showed the formation of cytoplasmic spherical liquid droplets (Fig. 4a). We also demonstrated that these liquid droplets transport into perinuclear space of the cells through microtubule network and eventually matured into solid like state of aggresome, similar to pathogenic inclusion bodies (Fig. 4d) (Ray *et al*, Nature Chemistry, 2020).

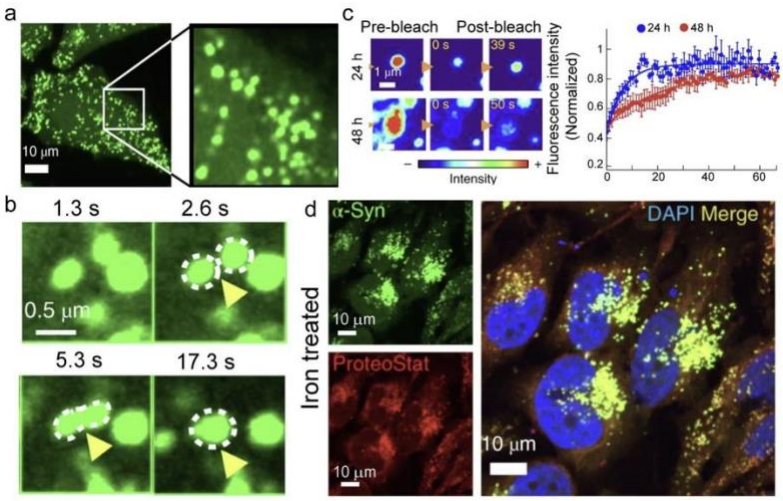

Figure 4: α-Syn LLPS in mammalian cell model: a) Representative confocal images of stable HeLa cells expressing tetra-cysteine tagged α-Syn showing the formation of highly mobile cytoplasmic droplets post 24 hours of stressor treatment. b) The Liquid-like nature of cytoplasmic condensates is seen by a time-lapse series of fusion events. (c) FRAP experiments depicting a slower fluorescence recovery of 48h α-Syn droplets compared to 24h. d) α-Syn condensates (green) localize at the perinuclear region and also show colocalization with ProteoStat dye (red), an aggresome marker after maturation and rigidification.

Our lab also explored several genetic and environmental factors modulating α-Syn LLPS in the subsequent publication (Sawner *et al*., Biochemistry, 2021). We have shown that α-Syn LLPS kinetics and critical concentration are largely dependent on the state of the protein, the presence of salts, buffer, surface, pH, and other several factors (Sawner *et al*., Biochemistry, 2021). α-Syn is highly water soluble due to the long-range electrostatic interaction between the positively charged N-terminal and negatively charged C-terminal domains, thereby existing in an auto-inhibitory form and discouraging NAC region against aggregation (Bertoncini *et al*., Proceedings of the National Academy of Sciences, 2005). This might explain why α-Syn does not form liquid droplets under physiologically relevant conditions. Interestingly, charge screening due to high salt buffer, low pH, and metal ion binding disrupts the autoinhibitory conformation, therefore allowing the N-terminal and NAC domain of α-Syn to undergo interaction supporting LLPS (Mukherjee *et al*, Journal of Molecular Biology, 2022). We have also shown the effect of two familial mutants (E46K, A53T), and phosphorylation (using S129E phosphomimetic mutant) on the LLPS behavior of α-Syn (Ray *et al*., Nature Chemistry, 2020). Similar to their effect on *in vitro* and *in vivo* protein aggregation, these modifications facilitate α-Syn LLPS and liquid-to-solid transition. Thus, our *in vitro* and in-cell studies strongly suggest that the liquid droplet formation of α-Syn occurs might be a crucial step for pathogenic α-Syn aggregation, which can be targeted for effective treatment and therapeutic strategies for PD.

Exploration of research based on our work on α-Syn LLPS.

Apart from our pioneering research on α-Syn LLPS, other research groups have cited our work and further characterized the properties of α-Syn LLPS. Vendruscolo group has also shown α-Syn LLPS in PD animal model of *C elegans* where early nematode shows liquid-like α-Syn condensate whereas aged nematode shows amyloid-rich ubiquitinated α-Syn condensates (Hardenberg *et al*., Journal of Molecular Cell Biology, 2021). The observation is particularly interesting as ubiquitinated α-Syn is heavily accumulated in LB (Hardenberg *et al*., Journal of Molecular Cell Biology, 2021). In another study, the group has shown the dose-dependent seeding effect of α-Syn in LLPS and subsequent solidification that indicates a secondary nucleation mechanism during the liquid-to-solid transition event (refer to review, Mukherjee *et al*., *Journal of Molecular Biology* 2022, Mukherjee *et al.,* Chemical Society Reviews, 2024). The role of various transition metal ions such as $Ni^{2+,}$ $Co^{2+,}$ $Zn^{2+,}$ $Al^{3+,}$ $Cr^{2+,}$ and Mn^{2+} has been investigated by other groups that

demonstrate faster LLPS kinetics in the presence of metal ions. However, LLPS was completely abrogated in the presence of $Mo^{5+,}$ possibly due to the imbalance of local charges. The intermolecular interaction and conformation change during α-Syn LLPS have been recently investigated by another group which supports our hypothesis of elongated conformation during α-Syn LLPS (refer to review, Mukherjee *et al*., Journal of Molecular Biology, 2022). The multicomponent LLPS formation of α-Syn with other neurodegenerative disease-associated proteins, such as Tau, and prion protein has been reported that imply the pathological significance of multiple disease occurrences simultaneously (*For this section please refer Mukherjee et al., 2022, Journal of Molecular Biology*).

The implication of our research of α-Syn LLPS in PD drug design

Since PD is a complex and multifactorial disease it becomes quite challenging to design and develop drugs as the actual target(s) is not fully understood due to the presence of numerous α-Syn intermediates, and polymorphs. As of now, unfortunately, there is no cure for PD except for the drugs that provide symptomatic treatment. We demonstrated that LLPS might serve as a potential therapeutic approach, which creates opportunities for drug discoveries against PD. For example, chaperone or designer chaperone homologs can be targeted to inhibit α-Syn LLPS and subsequent solidification. Indeed, Shorter and co-workers recently demonstrated that a variant of Hsp104 chaperone inhibits α-Syn condensation and associated toxicity in yeast, *C elegans*, and mammalian cells. Based on the fact that antimicrobial peptide inhibits aberrant liquid-to-solid transition of α-Syn liquid droplet, there is huge potential for developing small molecular inhibitors of α-Syn LLPS. Moreover, Proteolysis targeting chimera (PROTAC) can also be designed which might target α-Syn LLPS in cells. Our lab indeed actively working on several aspects of α-Syn LLPS and the LLPS-mediated aggregation mechanism including LLPS property of familial mutants, identifying the oligomers, and establishing the structure-toxicity relationship of LLPS-generated oligomers of α-Syn.

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