

● PERSPECTIVE

Study of alpha-synuclein fibrillation: state of the art and expectations

Since the discovery of the presence of fibrillary forms of α -synuclein (α -syn) in Lewy bodies (LB) and Lewy neurites in the brain of patients affected by Parkinson's disease (PD) and dementia with LB, great effort has been dedicated to study the features of α -syn fibrillation. In parallel, the pathological relevance of the different toxic forms of α -syn has been also matter of investigation. In the last twenty years, scientists have been able to single out that α -syn fibrillation initiates pathological mechanisms that by contributing to or triggering neurodegeneration/neuroinflammation, may lead to PD pathogenesis. This notwithstanding, we still ignore the reasons why α -syn shifts from its natively unfolded conformation to toxic oligomeric and fibrillary forms. The chameleonic nature of monomeric α -syn, and the extremely polymorphic characteristics of aggregated strains, renders it difficult to picture the real nature of α -syn fibrils, their exact composition and formation dynamics. Recently, sophisticated biophysical methods and microscopy techniques have been exploited to study α -syn fibrillation. Here, we provide an overview of the most relevant advancement in our understanding of α -syn fibrils formation and conformation. Nonetheless, numerous techniques and patient-derived experimental models still need to be optimized to actively disclose causes and characteristics of α -syn fibrillation in disease-specific cellular milieus.

The presence of fibrillary α -syn in LB of the brain of patients affected by PD and dementia with LB was first described by Spillantini et al. (1997). Since then, understanding the features and dynamics of the formation of α -syn aggregates has become compelling in light of the central role that α -syn pathological deposition plays in PD pathogenesis. α -Syn represents one of the most plastic and interacting-prone proteins at the presynaptic space. It is involved in different pathways such as synaptic vesicles trafficking, and interacts with both proteins and lipid membranes to exert its function. In particular, at synaptic level, α -syn behaves as a hub within the protein interaction networks coordinating synaptic activity. There, α -syn interacts with synaptic vesicle-associated proteins such as synapsin III, synaptic vesicle glycoprotein 2C, cistein string protein α or Rab GTPases such as Rab 3a, and acts as a chaperone for soluble N-ethylmaleimide-sensitive factor-attachment protein receptors complex assembly. In addition, α -syn can regulate synaptic vesicle transport by interacting with cytoskeletal components such as actin. Of note, the protein finely tunes release of neurotransmitters, in particular dopamine, by modulating the enzymes that are responsible of its synthesis (tyrosine hydroxylase), vesicular storage (vesicular monoamine transporter 2) and reuptake (dopamine transporter), respectively (Longhena et al., 2019).

α -Syn is small (140 amino acids) and encompasses an N-terminal region which can adopt α -helical folding and bind high-curvature membranes, an amyloid-like fibril-prone central domain, and a C-terminal disordered region acting like a shield to prevent aggregation of the central domain (Longhena et al., 2019) (Figure 1). α -Syn conformational plasticity can be affected by environmental changes such as reactive oxygen species, pH alterations or interacting proteins. These factors can lead to the formation of different toxic α -syn species such as oligomers

or fibrils. Despite more than twenty years of intensive research, only recently, by using advanced microscopy tools and correlation studies, researchers have been able to elucidate the inner structure of α -syn fibrils and the process of LB formation (Moors et al., 2018). This notwithstanding, studying α -syn fibrillation is intrinsically difficult because of the insoluble, dense and polymorphic nature of these structures (Longhena et al., 2019). Tuttle and colleagues recently described the structure of pathogenic fibrils of human α -syn by using solid-state nuclear magnetic resonance (Tuttle et al., 2016). In particular, they observed that these fibrils adopt a Greek-key- β -sheet topology, where the innermost core was composed of α -syn central aminoacids 71–82. Later, cryo-electron microscopy, a technique that is able to give insights about the organization of α -syn fibrils at near-atomic resolution, also allowed detection of an α -syn fibril structure collimating with Greek-key (Guerrero-Ferreira et al., 2018). These studies have disclosed that applying biophysical methods or structure-solving microscopy to the investigation of fibril morphology holds significant potentialities and can produce relevant advancements in our understanding on the molecular basis of PD. On this line, techniques such as small angle X-ray scattering or atomic force microscopy have been successfully used to investigate α -syn fibrils at an ultrastructural level. In particular, atomic force microscopy was used to study the toxicity of α -syn fibrils in the cellular context (Gao et al., 2017). Other biophysical methods such as circular dichroism-dependent approaches have been significantly refined to allow a better understanding of fibrils properties. These are of particular interest in order to study α -syn supramolecular organization in the presence of different interactors, such as lipid membranes. Since the binding with phospholipids-enriched membranes is fundamental to α -syn in order to exert its functions at the synapse, studying the effects of α -syn structural dynamics in presence of lipids can help to understand the toxic effects of α -syn multimers on membrane stability and *vice versa*.

Infrared spectroscopy has also been exploited to study α -syn fibrils. This method, in combination with atomic force microscopy was applied to analyze the intramolecular conformation of β -sheets in α -syn fibrils (Roeters et al., 2017). The methodologies described above allowed the visualization and the study of mature α -syn fibrils but their intermediate precursors, such as oligomers and proto-fibrils, resulted more difficult to analyze, although they can also trigger or contribute to neurodegeneration. This supports that studying the dynamics of fibrillary α -syn aggregation may help to elucidate which are the most toxic α -syn species. Interestingly, recent advances in biophysical techniques have enabled the identification the molecular steps involved in this process, partially explaining its kinetics and the role of oligomers. A widely used approach to verify the presence of amyloid fibrils, examine fibrillation kinetics as well as to quantify fibrils, is to use Thioflavin T, whose fluorescence intensity is enhanced upon binding to amyloid fibrils. Of note, Thioflavin T fluorescence measurement allows to follow the process of fibril formation and to assess whether a particular milieu or compound perturbs the fibrillation kinetics. However, Thioflavin T does not provide any information about the presence of oligomers, and should be implemented by using dynamic light scattering methods, that are very sensitive also to low concentration of aggregates (Li et al., 2019). In addition, Thioflavin T binding is not sufficient to assure fibril formation, so that other ultrastructural methods such as X-ray fiber diffraction are necessary to confirm the disposition of the β -strands. These tools result optimal for the investigation of the dynamics of the fibrillation in pure solution, but they are not recommended for investigating the kinetics of

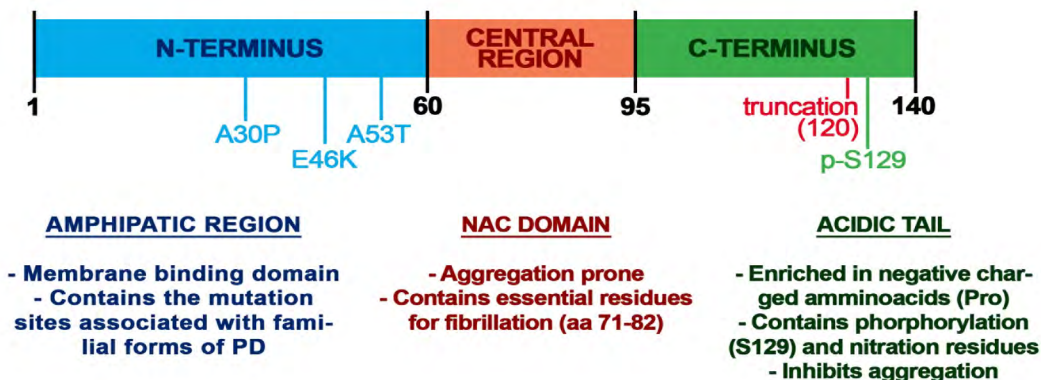


Figure 1 Schematic representation of the amino acid sequence of α -synuclein highlighting the different domains of the protein.

aa: Amino acids; PD: Parkinson's disease.

this process in the cellular habitat, as they are not applicable to a crowded environment. To this purpose, advanced microscopy tools, such as super-resolution microscopy, have been used to characterize both oligomers and fibrils formed by α -syn. For instance, single molecule fluorescence resonance energy transfer is an interesting method born to study protein-protein interaction and used to investigate α -syn fibrillation, also for what concerns the fibrillation dynamics of PD-related α -syn mutants. Recently, stochastic optical reconstruction microscopy has revealed highly heterogeneous elongation rates of individual fibrils, potentially due to fibril polymorphisms or the spatial arrangement of fibril ends (Pinotsi et al., 2014). This method was also used to study fibrillation kinetics on cells incubated with pre-formed fibrils (Pinotsi et al., 2016). More recently, stimulated emission depletion microscopy has been successfully adopted to study the spatial distribution of α -syn post-translational modifications in human brains with and without LB pathology, revealing a lamellar distribution of different forms of α -syn and lipids in nigral LB (Moors et al., 2018). These techniques seem to be the most promising to study α -syn fibrillation in cells and tissues. Reproducing α -syn fibrillation starting from an aggregation-prone template in a tube has been exploited to follow the fibrillation process as described above, or to test the effect of fibrils on cells and tissue, but also to measure the presence of α -syn seeds in blood, cerebrospinal fluid and saliva. Indeed, only amyloid structures associated with a pathologic state of α -syn, are able to self-replicate. Shahnawaz and colleagues used protein misfolded cyclic amplification, a technique previously developed in order to study prion aggregation, to determine the presence of amyloid seeds in cerebrospinal fluids of patients affected by PD (Shahnawaz et al., 2017). Although this method allows efficient amplification of α -syn fibrillation, questions may be raised about the effective reliability of these protocols in producing disease-specific strains. Indeed, fibrils have been found to contain also other proteins beside α -syn, such as 14-3-3 chaperone and the synaptic protein synapsin III (Longhena et al., 2019), thus supporting that we may have been reproducing just one piece of the puzzle. Moreover, since nucleation and elongation of fibrils are stochastic events, aggregated α -syn preparations could be very diverse one from another and difficult to reproduce within and between laboratories although strong effort has been dedicated to the standardization of this method. These considerations should be taken into account also for the production of conformation-specific antibodies. Nonetheless, some of the conformation-specific antibodies seem to successfully distinguish PD samples from the controls, opening the way to the development of a sensitive and specific α -syn biomarker to assays for PD diagnosis and/or progression. Collectively, all these findings support that in the last ten years our knowledge of α -syn fibrillation has made significant advancements, though it is worth to taken into account that most of them were carried out on pre-formed fibrils obtained by using protocols which own an intrinsically high variability in terms of yield and fibril maturation. Moreover, insoluble α -syn fibrils seem to be disease-specific (Peng et al., 2018) and contain also other proteins (Longhena et al., 2019), thus supporting that other cell-specific players may be involved in the fibrillation process. This highlights the importance of the microenvironment in which fibrillation occurs, which could impact on the process itself and on the fibrillary product. Consistently, the cellular milieu has been found to influence α -syn fibrils conformation and toxicity (Peng et al., 2018). Furthermore, α -syn is mainly present at the synaptic site, a crowded surrounding where the protein interacts with many other players that could alter the conformation of α -syn and cooperate or counteract the process of fibrillation. Since α -syn-dependent neuronal damage may start from the synapse, which is the site where α -syn is most abundant, it became crucial not to underestimate the influence of the synaptic microenvironment on fibrillation.

The implementation of biophysical methods and disease-specific models thus become compelling in order to study patient-derived α -syn fibrils and also to observe cell-specific α -syn ability to form oligomers and fibrils. These studies could allow a better understanding of the precise molecular signature of fibril strains. In addition to this, we need extremely standardized guidelines in order to obtain reliable data and avoid the risk of collecting insightful information on many different α -syn fibril conformers, which however may not occur in the human brain, and thus would not be helpful for the design of effective translational approaches disrupting α -syn fibrillation. The development and standardization of new highly-yielding methods for the purification of α -syn fibrils from the human brain and the implementation of correlative studies based on different biophysical techniques, and possibly advanced microscopy, could certainly contribute to deal with the questions that still remain unanswered. Oscar Wilde thought "The truth is rarely pure and never simple" resumes in our view that much work still needs to be done to reach the core of the matter in α -syn fibrillation.

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