

# In vitro aggregation assays for the characterization of $\alpha$ -synuclein prion-like properties

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Aggregation of  $\alpha$ -synuclein plays a crucial role in the pathogenesis of synucleinopathies, a group of neurodegenerative diseases including Parkinson disease (PD), dementia with Lewy bodies (DLB), diffuse Lewy body disease (DLBD) and multiple system atrophy (MSA). The common feature of these diseases is a pathological deposition of protein aggregates, known as Lewy bodies (LBs) in the central nervous system. The major component of these aggregates is  $\alpha$ -synuclein, a natively unfolded protein, which may undergo dramatic structural changes resulting in the formation of  $\beta$ -sheet rich assemblies. In vitro studies have shown that recombinant  $\alpha$ -synuclein protein may polymerize into amyloidogenic fibrils resembling those found in LBs. These aggregates may be uptaken and propagated between cells in a prion-like manner. Here we present the mechanisms and kinetics of  $\alpha$ -synuclein aggregation in vitro, as well as crucial factors affecting this process. We also describe how PD-linked  $\alpha$ -synuclein mutations and some exogenous factors modulate in vitro aggregation. Furthermore, we present a current knowledge on the mechanisms by which extracellular aggregates may be internalized and propagated between cells, as well as the mechanisms of their toxicity.

## Introduction

Human  $\alpha$ -synuclein is a natively unfolded 140-amino-acid residue protein widely expressed in neurons, found predominantly in presynaptic terminals.<sup>1–3</sup> It was originally identified at the nuclear level, accounting for the designation “syn-nuclein”,<sup>4</sup> but subsequently described as a predominantly cytoplasmic protein.<sup>5–7</sup> While an ultimate definition of  $\alpha$ -synuclein function has not been provided yet, different studies have shown its role in mediating dopamine synthesis, release and reuptake in dopaminergic neurons, as strongly suggested by the protein presynaptic location and its interaction with membranes.<sup>8–13</sup> Additional studies reported  $\alpha$ -synuclein upregulation in response to oxidative stress and excitotoxicity, suggesting a role

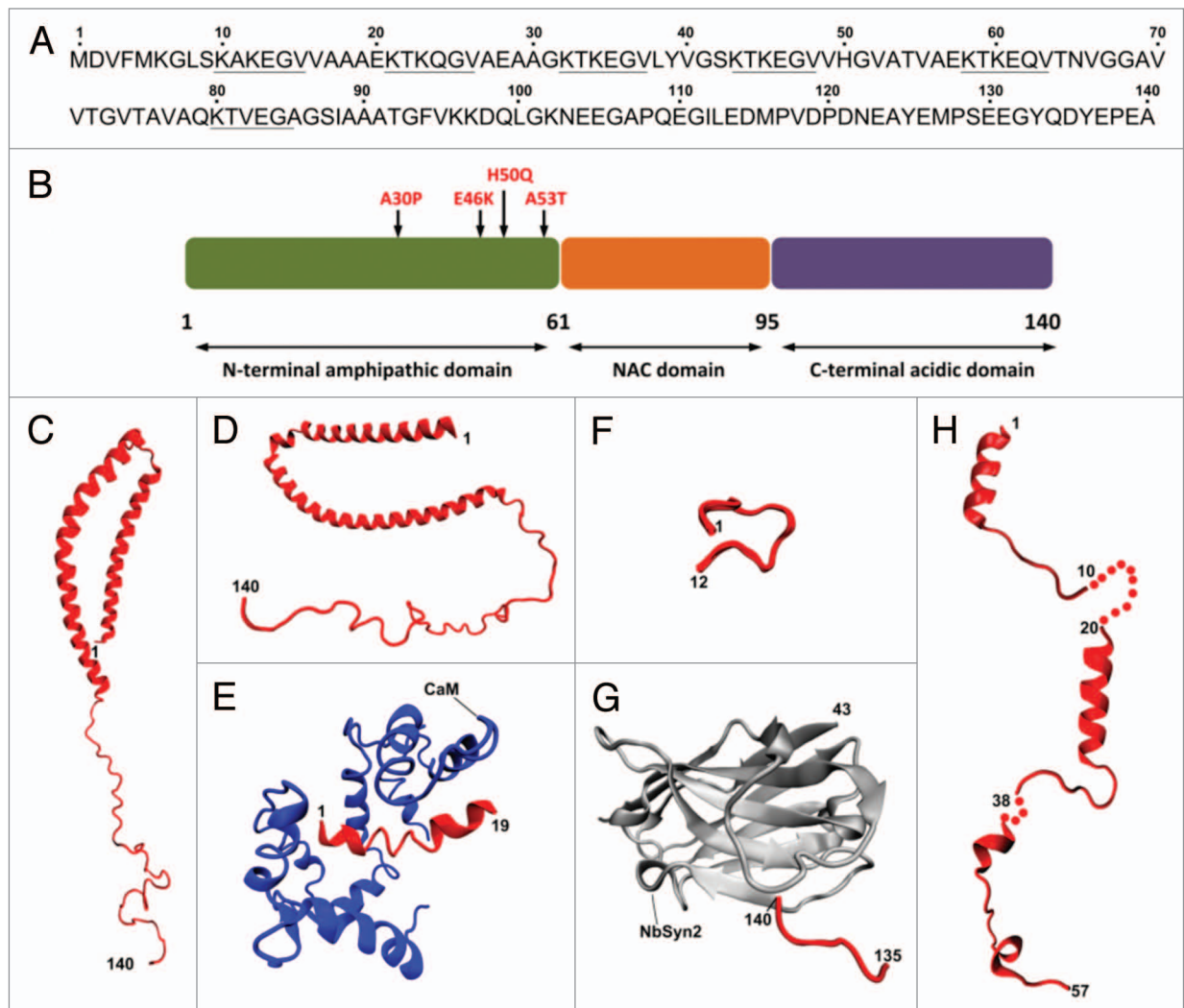
for the protein in protecting neuronal cells against damage.<sup>14</sup> In particular,  $\alpha$ -synuclein may cooperate with the cysteine-string protein- $\alpha$  (CSP $\alpha$ )—a synaptic vesicle protein with co-chaperone activity—in preventing neurodegeneration.<sup>15</sup>

Structurally,  $\alpha$ -synuclein features an N-terminal domain (residues 1–60), a central hydrophobic portion denoted as non-A $\beta$  component of Alzheimer diseases amyloid (NAC, residues 61–95) and a C-terminal negatively charged region (residues 96–140). Both the N-terminal and NAC domains contain six highly conserved hexameric (KTKEGV) motifs (Fig. 1A and B).<sup>16</sup> In its native monomeric state,  $\alpha$ -synuclein behaves as an intrinsically unstructured protein, characterized by the lack of a stable tertiary structure.<sup>17,18</sup> However, it may adopt a secondary structure upon binding to protein interactors or lipid membranes. Early studies reported that the N-terminal domain of  $\alpha$ -synuclein displays  $\alpha$ -helical folding in the presence of artificial membranes such as sodium dodecyl sulfate micelles.<sup>19–22</sup> Solution state NMR studies on the structures of micelle-bound  $\alpha$ -synuclein have shown that the protein contains two distinct  $\alpha$ -helices in the region encompassing residues 1–92, while the C-terminal results predominantly unstructured (Fig. 1C and D).<sup>23,24</sup> Other studies have attempted to obtain structural information about  $\alpha$ -synuclein segments complexed with physiological partners such as calmodulin<sup>25</sup> or synphilin-1,<sup>26</sup> with a nanobody<sup>27</sup> or the maltose binding protein<sup>28</sup> (Fig. 1E–H). A recent work has reported that natively cell-extracted  $\alpha$ -synuclein may be present as a tetramer formed by  $\alpha$ -helical folded monomers with no binding to lipid membranes,<sup>29</sup> but a further work has shown that  $\alpha$ -synuclein in mammalian cells exists as unfolded monomer when dissociated from lipid membranes.<sup>30</sup>

Overall the structural studies on the  $\alpha$ -synuclein monomer suggest a model where the lack of secondary structure motifs is the first event leading to its misfolding and aggregation in pathological and disease-related amyloid assemblies found in Parkinson disease (PD) and other synucleinopathies.

In the next sections, we present an overview of the strategies currently used to produce synthetic  $\alpha$ -synuclein assemblies and their possible implications for the future of PD research.

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**Figure 1.** Structural features of monomeric  $\alpha$ -synuclein. Primary amino acidic sequence (UniProtKB: P37840) of human  $\alpha$ -synuclein with the imperfect exapeptide repeats<sup>16</sup> underlined (A) and schematic representation of the biochemically different domains including the currently identified PD-related mutations (B). Currently available NMR and X-ray crystal structures of  $\alpha$ -synuclein segments (in red) bound to micelles (C and D) PDB: 1XQ8 and 2KKW, respectively, calmodulin, CaM (E) PDB: 2M55, synphylin-1 (F) PDB: 2JN5, nanobody, NbSyn2 (G) PDB: 2X6M and maltose binding protein (H) PDB: 3Q25, 2Q26, 3Q27.

### In Vitro Aggregation Assays of $\alpha$ -Synuclein

Deposition of Lewy bodies (LBs) and Lewy neurites (LNs) in the brain is a major pathological hallmark of several neurodegenerative diseases, including PD, dementia with Lewy bodies (DLB), diffuse Lewy body disease (DLBD), and multiple system atrophy (MSA). LBs are spherical cytoplasmic inclusions, 5–25  $\mu\text{m}$  in diameter, formed by aggregated proteins, with a dense eosinophilic core and a clearer surrounding halo. The major component of the proteinaceous filaments of LBs and LNs is  $\alpha$ -synuclein.<sup>31</sup>

In vitro studies have revealed that recombinant  $\alpha$ -synuclein can assemble into fibrils with morphologies and staining characteristics similar to those extracted from disease-affected brains. Wild-type (WT)  $\alpha$ -synuclein fibrils formed in vitro are 8–10 nm in height as determined by atomic force microscopy (AFM) and

about 10 nm in width (electron microscopy, EM), while fibrils of PD LBs observed in tissue slices are reported to be about 10 nm in width.<sup>32–34</sup> Moreover, the presence of “twisted” fibrils as well as some small filaments of 5 nm width is a common feature of fibrils extracted from brains and some fibrils formed in vitro.<sup>31,32</sup>

Aggregation of  $\alpha$ -synuclein plays a critical role in PD and related neurodegenerative disorders; hence in vitro aggregation assays are powerful molecular tools for studying the mechanisms of neurodegeneration. Understanding the mechanisms by which a soluble  $\alpha$ -synuclein is transformed into insoluble  $\beta$ -rich fibrils, as well as describing the structural features of these fibrils are still questions to be addressed in future research. Additionally,  $\alpha$ -synuclein aggregation assays may be feasible tools for the development of new drug compounds capable of interfering with  $\alpha$ -synuclein fibril formation. Increasing evidence demonstrates that in vitro formed fibrils are internalized by cells and may be

propagated in a prion-like manner leading to cytotoxicity. Thus, in vitro aggregation of  $\alpha$ -synuclein may yield crucial components for studying molecular mechanisms of aggregate uptake, spreading and toxicity.

#### Strategies for the purification of recombinant $\alpha$ -synuclein

In vitro fibrillization assays require high amounts of highly pure  $\alpha$ -synuclein protein. Recombinant  $\alpha$ -synuclein is usually produced in *E. coli* cells and purified from either whole cell extract or periplasmic extract. Traditionally, the protein is purified from the whole cell extract by sonication or boiling, or ammonium sulfate precipitation followed by anion exchange and size exclusion chromatography purifications.<sup>35-37</sup> Ren and collaborators have shown that *E. coli*-expressed  $\alpha$ -synuclein without signal sequence mostly localizes in the bacterial periplasm.<sup>38</sup> The protein translocation appeared to be mediated by the signal recognition particle (SRP)-dependent pathway facilitated by the  $\alpha$ -synuclein C-terminal domain (residues 90–140) as a major part responsible for the translocation.<sup>38</sup> Therefore, a new efficient and fast method has been developed for  $\alpha$ -synuclein extraction and purification from periplasm.<sup>39</sup> Briefly, the protein is first extracted from bacterial periplasm by osmotic shock, followed by anion-exchange chromatography purification. One L of *E. coli* LB medium culture yields about 80 mg of highly pure  $\alpha$ -synuclein.<sup>39</sup>

#### Tools for monitoring $\alpha$ -synuclein fibril formation in vitro

In vitro fibrillization assays require powerful tools for detecting fibril formation and monitoring the fibrillization kinetics in real time. Two common histological dyes, Thioflavin T (ThT) and Congo red (CR), are used to this end. The mechanism of interaction between both dyes and amyloid fibrils remains poorly understood; both compounds seem nevertheless to interact with the cross- $\beta$  structures, exhibiting changes in fluorescence. ThT is a benzothiazole dye that exhibits enhanced fluorescence upon binding to amyloid fibrils and constitutes the mostly used extrinsic probe for monitoring fibrillization kinetics in vitro. In the presence of fibrils, ThT gives rise to an excitation maximum of 450 nm and enhanced emission at 482 nm, whereas unbound ThT is not fluorescent at these wavelengths.<sup>40</sup> ThT is a reliable and specific fibril-detecting tool, which does not interfere with the kinetics of  $\alpha$ -synuclein fibrillization.<sup>41</sup> However, despite its specificity toward fibrils, a slight increase in ThT fluorescence has been detected after binding to amorphous  $\alpha$ -synuclein aggregates.<sup>42</sup> The use of ThT for monitoring fibril formation should be optimized and negative controls are needed, as spectral properties of this dye may vary under different conditions. Additionally, being ThT a charged and hydrophobic molecule, it may non-specifically bind to some proteins by electrostatic and hydrophobic interactions.<sup>43</sup>

CR seems unsuitable for monitoring  $\alpha$ -synuclein aggregation, since it exhibits high affinity binding to  $\alpha$ -synuclein and has an inhibitory effect on the fibrillization assay.<sup>44</sup> Some studies have indicated that CR may interfere with protein misfolding and aggregation by stabilizing native protein monomers or partially folded intermediates.<sup>45</sup>

Since the sensitivity and specificity of ThT and CR have been questioned, several efforts have been made to design

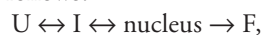
extrinsic probes and spectroscopic techniques for monitoring the fibrillization process. Celej and coworkers reported that N-arylamino-naphthalenesulfonate (NAS) and its derivatives act as non-covalent, highly sensitive probes of  $\alpha$ -synuclein fibril formation.<sup>46</sup> Recently, it has been shown that protein aggregation reactions can be spectroscopically monitored without the need for extrinsic labels. Some studies have reported that amyloid-like structure is associated with an intrinsic fluorescent phenomenon that occurs in the visible range.<sup>47-49</sup> The physical mechanism underlying the intrinsic fluorescence emission remains unknown. It has been suggested that the hydrogen-bonded water molecules within the cross- $\beta$  structure might play a key role in the fluorescence emission of the fibrils. Direct excitation of the fibrils could induce electronic transitions in peptides because of a partial delocalization of peptide electrons elicited by the presence of hydrogen bonds.<sup>47</sup> Pinotsi and collaborators have shown that this phenomenon can be used for monitoring the aggregation of proteins with no need for extrinsic labels, thus avoiding potential interference with the aggregation process.<sup>50</sup> This method appears feasible and reproducible; it requires a confocal microscope and it has been successfully used for monitoring  $\alpha$ -synuclein fibrillization in vitro.<sup>50</sup>

#### Mechanism and kinetics of fibril formation in vitro

During the fibrillization process, a soluble protein is converted into insoluble highly ordered aggregates exhibiting  $\beta$ -sheet enriched structures. Fibrillization of globular proteins requires the presence of partially unfolded conformations in order to adopt a structure competent for self-assembly into fibrils.<sup>51</sup> Partial unfolding exposes the hydrophobic regions of the protein, thus facilitating the interactions between exposed amyloidogenic regions of two proteins and subsequent formation of higher order species. Several factors destabilizing the native fold of the protein, such as mutations, denaturants, changes in pH, or elevated temperature, have been shown to enhance fibril formation.<sup>51</sup>

Fibrillization of natively unfolded proteins such as  $\alpha$ -synuclein presents intriguing issues, since it remains unclear how an intrinsically unstructured protein may form highly organized  $\beta$ -structured fibrils. Partial folding of the protein seems to represent a crucial step in the fibrillization pathway.<sup>51</sup> Natively unfolded proteins are characterized by low hydrophobicity and a large net charge.<sup>52</sup> It is supposed that any factors increasing protein hydrophobicity or decreasing protein net charge may lead to its partial folding and thus promote the fibrillogenesis pathway. The overall hydrophobicity of a protein increases with higher temperature.<sup>53</sup> It has been reported that either a decrease in pH or an increase in temperature leads  $\alpha$ -synuclein to adopt a partially folded conformation, a change correlated with the enhanced formation of fibrils.<sup>41</sup>

The model for  $\alpha$ -synuclein fibrillization has been proposed as follows:



where U corresponds to natively unfolded conformation; I, partially folded intermediate; nucleus, fibril nucleus; and F, fibril. This model describes the two key kinetic steps in the fibrillization process. The first step is the conformational

transformation of  $\alpha$ -synuclein into the aggregation-competent, partially folded intermediate; the second is the formation of the assembly-competent oligomers (nuclei), which is followed by oligomer growth and fibril formation. The model suggests that any factor that promotes intermediate formation (for example point mutations, non-polar molecules, cations, and others) will also promote  $\alpha$ -synuclein fibrillization.<sup>41</sup>

The kinetics of  $\alpha$ -synuclein fibrillization is commonly described as a sigmoidal curve, composed of an initial lag phase (nucleation) when the amount of fibrils is too low to be detected, a subsequent exponential phase (elongation) when fibril concentration increases rapidly, and a final plateau phase indicating the end of fibril formation. The length of lag phase and the fibril growth rates depend on several factors, including initial protein concentration, pH, temperature, agitation and other factors that will be discussed below.

Moreover, the fibrillization process can be catalyzed by adding preformed fibrils (seeds), which act as nuclei.<sup>54-56</sup> This effect has been termed “nucleation-dependent” fibrillization.” The added seeds may act as catalytic sites that induce conformational changes in  $\alpha$ -synuclein and accelerate the reaction rates.<sup>54</sup>

To compare the effects of different reaction conditions on fibrillization kinetics, ThT fluorescence is commonly plotted as a function of time and fitted to a sigmoidal curve using the equation:

$$Y = y_i + m_i x + \frac{y_f + m_f x}{1 + e^{-\frac{x - x_0}{\tau}}}$$

where  $Y$  is the fluorescence intensity,  $x$  is the time, and  $x_0$  is the time to 50% of maximal fluorescence. Thus, the apparent rate constant,  $k_{app}$ , for fibril growth is given by  $1/\tau$  and the lag time is given by  $x_0 - 2\tau$ .<sup>57</sup>

To test the effects of different factors on fibrillization kinetics, usually the lag phase, the apparent rate constant and/or the maximum intensity of ThT emission intensity at the end of fibril formation are calculated. These values obtained from tested samples are compared using commonly available statistic tests.<sup>58</sup> The fibrillization kinetics has been shown to be fairly similar among different amyloidogenic proteins. However, the kinetic profiles do not display always the typical sigmoidal shape since multiple steps may be present, thus affecting the interpretation of the correct lag phase and the apparent rate constant. Optimizing the reproducibility of protein fibrillization is crucial to obtain reliable results.<sup>58</sup>

Various data confirmed that in vitro aggregation of  $\alpha$ -synuclein may yield different final products, such as fibrils, soluble oligomers, or insoluble “amorphous aggregates.” Aggregation assays resulted in the formation of several oligomeric species with different morphologies, including spherical, chain-like, and annular oligomers.<sup>59</sup> However, the mechanism of conversion among different oligomers is not fully understood.

The generation of different  $\alpha$ -synuclein conformations has also been associated with the pathogenesis of PD and other synucleinopathies; fibrillar forms seem nevertheless to be the main component of LBs.<sup>31</sup>

### Structural features of human $\alpha$ -synuclein fibrils

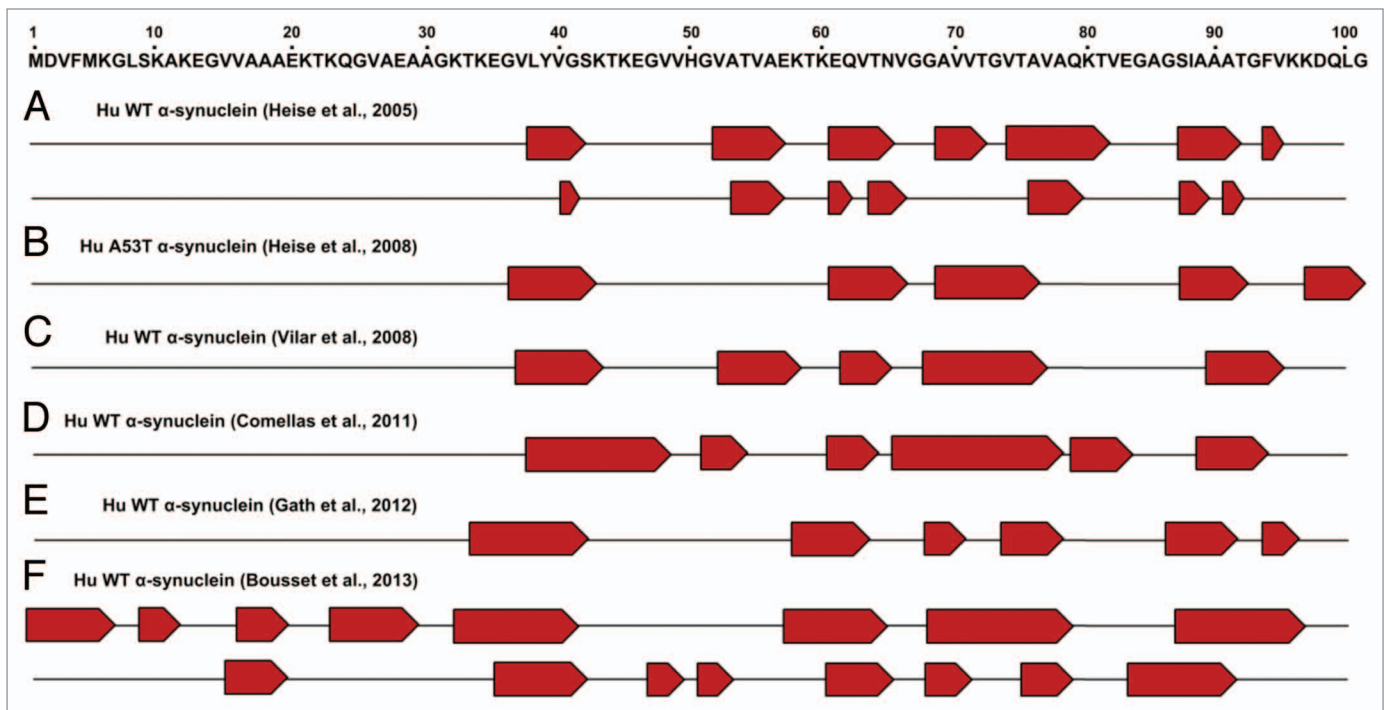
The assembly of physiologically expressed proteins into oligomeric and fibrillar aggregates with cross- $\beta$  structure is an essential event of different human neurodegenerative diseases, such as PD, Alzheimer disease, and prion diseases. It is generally accepted that aggregation takes place from partially structured states of proteins. However, the role of the residual structures present in such conformational states is not well understood.  $\alpha$ -synuclein fibrillar aggregates are pathological hallmarks of PD and other related synucleinopathies. The structural organization of  $\alpha$ -synuclein amyloid fibrils has been researched extensively. Amyloid fibrils are composed of several protofilaments arranged into the classical cross- $\beta$  conformation, with individual  $\beta$ -strands running perpendicular to the fiber axis.<sup>34,60,61</sup> Findings from electron paramagnetic resonance (EPR)<sup>62,63</sup> and magic-angle spinning solid-state NMR (ssNMR) have shown that the amyloidogenic  $\beta$ -sheet core is mostly located within the central NAC domain.<sup>64-71</sup> **Figure 2** illustrates an updated view of the  $\beta$ -sheet organization into the  $\alpha$ -synuclein amyloid fibrils as determined by ssNMR. Although the fibril core spans approximately residues 38 to 100, structural variations of local  $\beta$ -strands have been reported inside this region. A recent report has expanded current knowledge of the  $\alpha$ -synuclein segments involved in amyloid structural reorganization. In particular, Bousset and collaborators identified two novel  $\alpha$ -synuclein assemblies characterized by ordered  $\beta$ -sheet organization starting from residue 1 to 38; this region appeared unstructured in all previous ssNMR studies.<sup>65</sup> Interestingly, the same group has reported that the two  $\alpha$ -synuclein polymorphs showed marked differences in their biological functions. Overall the EPR and ssNMR studies highlight the remarkable conformational plasticity of  $\alpha$ -synuclein, which can adopt different monomeric, oligomeric, or fibrillar assemblies depending on the environment.

### Factors affecting the kinetics of $\alpha$ -synuclein fibrillization in vitro

Oligomers and fibrils produced in vitro under diverse conditions may result in different structural properties and consequently exert different biological functions.<sup>72</sup> As mentioned above, several factors including the initial concentration of monomeric protein, molecular crowding, agitation, pH, temperature, and ionic strength, have been shown to greatly affect the kinetics of  $\alpha$ -synuclein fibrillization.<sup>32,41,73-75</sup> Thus, developing a reliable and reproducible aggregation assay is necessary to screen the effects of exogenous factors on the fibrillization process, as well as to produce different aggregate species for structural studies and test their biological functions. Here, we characterize crucial factors that determine the kinetics of fibril formation in vitro.

#### $\alpha$ -synuclein concentration

Initial concentration of monomeric  $\alpha$ -synuclein is a crucial factor determining the kinetics of fibril formation. Commonly, 0.5 to 1 mg/mL (35–70  $\mu$ M) of  $\alpha$ -synuclein is used during fibrillization assays.<sup>39,74,76,77</sup> Other studies have used higher concentrations, namely 5 to 7 mg/mL.<sup>78,79</sup> The minimal  $\alpha$ -synuclein concentration, below which fibrils are not formed, has been



**Figure 2.**  $\alpha$ -synuclein fibril core regions as determined by ssNMR studies. Upper panel: schematic representation of the  $\alpha$ -synuclein segment from residue 1 to 100 involved in structural changes. Lower panel: proposed  $\beta$ -strand (indicated as red pentagons) organization of different human (Hu)  $\alpha$ -synuclein fibrillar assemblies determined by different groups: (A) reference 68 (B) reference 67 (C) reference 69 (D) reference 70 (E) reference 71 (F) reference 65.

estimated to be approximately 0.2 mg/mL.<sup>80</sup> The kinetics of fibril formation is a concentration-dependent process; however, the concentration dependence almost disappears in the presence of higher protein concentrations. Above the supercritical concentration, nucleation is no longer rate-limiting and the fibril growth rate is almost independent of the protein concentration.<sup>81</sup> In fact, increasing  $\alpha$ -synuclein concentration has been reported to decrease the lag-phase of fibrillization.<sup>82</sup> Supercritical concentration of  $\alpha$ -synuclein has been estimated to be about 8.2 mg/ml ( $\approx$  574  $\mu$ M); however this may vary according to the conditions used for the fibrillization reaction.<sup>42</sup>

#### Molecular crowding

Macromolecular crowding is another factor significantly affecting aggregation, as it may dramatically accelerate  $\alpha$ -synuclein fibril formation.<sup>74,83</sup> The effect of molecular crowding on protein aggregation *in vitro* has been examined by using concentrated solutions of a model “crowding agent” such as polyethylene glycols (PEG), polysaccharides (dextrans or Ficolls), or proteins (for example, lysozyme and bovine serum albumin). All these molecules accelerated  $\alpha$ -synuclein fibrillization *in vitro*.<sup>83</sup> The crowding agents had little effect on the conformation of  $\alpha$ -synuclein, as shown by far-UV CD spectroscopy.<sup>74</sup> It seems that the mechanism by which crowding agents affect fibrillization depends on their concentrations. It has been suggested that at lower crowding agent concentrations, the major effect of macromolecular crowding on protein aggregation is due to excluded volume effect, which favors self-association of  $\alpha$ -synuclein following the increase of the effective protein concentration. At

a higher concentration, viscosity effects of the polymer solutions may become dominant.<sup>74</sup>

#### Agitation

As reported by several studies, agitation is yet another crucial factor impacting the kinetics of the fibrillization process. Agitation of  $\alpha$ -synuclein solutions leads to a dramatic increase in the rate of fibrillization, shortening the lag phase from weeks to hours.<sup>32,84</sup> Uversky and collaborators have reported that vigorous agitation was the major determinant of the fibrillization rate. Very vigorous agitation attenuated the effects of ionic strength, protein concentration and various additives on the rate of fibril formation.<sup>85</sup> The exact mechanism by which agitation induces fibrillization is unknown, however it could increase the air-water interface, thus leading to a partial folding of  $\alpha$ -synuclein and the formation of the fibrillization intermediate.<sup>82</sup> Moreover, agitation may increase the number of fibril ends through fragmentation or increase the collision of monomeric and oligomeric species interacting with fibril ends.<sup>86</sup>

Agitation is commonly performed either in a glass vial with a mini stir bar or in microplates on a fluorescence plate reader. With the former method, termed single time-point dilution ThT assay,<sup>39,41,75,87,88</sup> aliquots are taken out at given time intervals for ThT fluorescence measurement, whereas in the latter approach (in situ real-time assay) ThT fluorescence is monitored in real-time at regular intervals.<sup>58,73,74</sup> Agitation speed in different assays ranges from 120 rpm<sup>89</sup> to 1000 rpm.<sup>78</sup> Agitation with glass beads has been shown to significantly decrease the lag time of the fibrillization and to increase the reproducibility of the assay.<sup>58</sup>

Reproducibility is an important issue in in vitro fibrillization assays, as it may vary significantly across sample replicates and among experiments.<sup>58</sup> Giehm and Otzen have tested factors that influence the reproducibility of the fibrillization of  $\alpha$ -synuclein in plate reader assays. The authors have determined that increased reproducibility is correlated with decreased lag time. Orbital agitation using a small glass bead in each well significantly increased lag time and was crucial to obtain good reproducibility.<sup>58</sup>

#### *Temperature, pH, and ionic strength*

Several data have indicated that decreasing the pH or increasing the temperature substantially accelerated the kinetics of  $\alpha$ -synuclein fibrillization, affecting both the lag time and the elongation rate. Elevated temperatures have been shown to strengthen hydrophobic interactions whereas lower pH decreased the net charge of the protein.<sup>41,53</sup> Both factors induced some structural changes in  $\alpha$ -synuclein, as shown by far-UV CD and FTIR spectroscopies, and consequently contributed to a reversible transformation of  $\alpha$ -synuclein into a partially folded intermediate, whose formation is crucial for the fibrillization process.<sup>41,75</sup>

The rate of fibril formation has been shown to increase dramatically with increasing ionic strength, reaching the maximal effect by 300 mM NaCl. Anions have been suggested to induce partial folding of  $\alpha$ -synuclein at neutral pH, thus increasing the population of the amyloidogenic intermediate. CD spectra confirmed that at 20 mM concentration, different salts induced partial folding of  $\alpha$ -synuclein.<sup>73</sup>

#### **Exogenous factors modulating $\alpha$ -synuclein fibrillization in vitro**

Although the specific etiology of PD is unknown, several factors, both genetic and environmental, may increase the risk of developing the disease.<sup>90</sup> In vitro aggregation assays are easy, fast, and powerful tools for screening potential factors that may affect  $\alpha$ -synuclein aggregation and thus contribute to PD pathogenesis. Several studies have reported that  $\alpha$ -synuclein aggregation may be triggered by environmental and other exogenous factors, such as pesticides, heavy metals, polycations, organic solvents, and some small chemical compounds.<sup>91,92</sup> Here, we briefly characterize factors that have been shown either to accelerate or inhibit  $\alpha$ -synuclein aggregation in vitro. **Table 1** summarizes exogenous factors that have been involved in the modulation of  $\alpha$ -synuclein aggregation.

#### *Pesticides and herbicides*

Uversky and collaborators have reported that certain pesticides and herbicides (rotenone, dieldrin, and paraquat) dramatically accelerated the rate of fibril formation in vitro.<sup>85</sup> It has been suggested that the relatively hydrophobic pesticides may bind to the partially folded  $\alpha$ -synuclein intermediate, induce its conformational changes and consequently lead to fibril formation.<sup>85</sup> In fact, epidemiological studies have shown increased risk of PD in populations exposed to pesticides.<sup>93,94</sup>

#### *Polycations*

Polycations, such as spermine, polylysine, polyarginine, and polyethylenimine, dramatically accelerated  $\alpha$ -synuclein fibrillization in vitro.  $\alpha$ -Synuclein-polycation complex formation was not accompanied by significant structural changes in  $\alpha$ -synuclein.<sup>77</sup> Electrostatic interactions between polycations and  $\alpha$ -synuclein

have been suggested to lead to neutralization of the protein net negative charge and to the partially folded amyloidogenic intermediate that initiates fibril formation.<sup>77</sup>

#### *Metal ions*

Several works have described how some metal ions affected  $\alpha$ -synuclein fibrillization in vitro. Di- and trivalent ions (e.g., copper, iron, cobalt, and manganese) accelerated the rate of fibril formation. Metal ions stimulated  $\alpha$ -synuclein conformational change and led to the development of a partially folded intermediate, a possible critical precursor of fibrils.<sup>76,95</sup> Metal exposure has been suggested as one of the factors that may increase the risk of PD. Several epidemiological studies have shown that exposure to some metals, including manganese, iron, aluminum, and copper, was associated with a higher risk of PD.<sup>96-99</sup> Moreover, elevated levels of iron, zinc, and aluminum have been reported in the *substantia nigra* of PD patients, thus confirming their involvement in the pathogenesis.<sup>100-102</sup>

#### *Histones*

Some histones have been shown to form a tight complex with  $\alpha$ -synuclein and accelerate its fibrillization in a dose-dependent manner.<sup>103</sup> This effect may be physiologically important, as the translocation of  $\alpha$ -synuclein into the nucleus and its binding to histones may play a large role in  $\alpha$ -synuclein pathology.

#### *Small chemical compounds*

Many therapeutic strategies for PD aim at developing chemical compounds and small molecules capable of inhibiting fibril formation. Masuda and collaborators have tested 79 small compounds belonging to different chemical classes (including polyphenols, benzothiazoles, phenothiazoles, steroids, Congo red and its derivatives) for their ability to inhibit the assembly of  $\alpha$ -synuclein into filaments in vitro.<sup>44</sup> Several of these compounds inhibited  $\alpha$ -synuclein assembly; among them, polyphenols proved to be an especially effective class of anti-aggregation compounds. The mechanism underlying the polyphenol-induced inhibition is unclear, but these compounds seem to stabilize soluble, prefibrillar intermediates.<sup>44</sup> Meng and coworkers have tested 48 compounds representative of different classes of flavonoids for their ability to inhibit  $\alpha$ -synuclein fibril formation in vitro. A variety of flavonoids inhibited  $\alpha$ -synuclein in vitro fibrillization to varying extents: some of them (myricetin, tricetin, 6-HP and baicalein) completely inhibited fibril formation and many were able to disaggregate preformed fibrils.<sup>104</sup>

#### *Heat shock proteins*

Heat shock proteins (Hsp) have been reported to interact with  $\alpha$ -synuclein and inhibit its aggregation in vitro.<sup>105-108</sup> The small Hsp  $\alpha$ B-crystallin, a molecular chaperone, inhibited  $\alpha$ -synuclein fibrillization. The interaction of  $\alpha$ -synuclein with  $\alpha$ B-crystallin resulted in large irregular aggregates, suggesting that the chaperone protein redirected  $\alpha$ -synuclein from a fibril-formation pathway toward an amorphous aggregation pathway.<sup>105</sup> Hsp70 protein inhibited  $\alpha$ -synuclein filament formation by binding to soluble prefibrillar intermediates.<sup>106,107</sup> Moreover, different Hsp proteins (HspB8, Hsp27, HspB2B3, and  $\alpha$ B-crystallin) inhibited the  $\alpha$ -synuclein aggregation process, as shown by ThT assay and AFM imaging.<sup>108</sup>

**Table 1.** Exogenous factors affecting  $\alpha$ -synuclein fibril formation in vitro

Factor	Description	References
<b>Factors accelerating fibrillization</b>		
Agrochemicals	Pesticides (rotenone, dieldrin, paraquat) bound to the partially folded intermediate conformation of $\alpha$ -synuclein and induced its conformational changes to $\beta$ -sheet enriched structures.	85,89
Polycations	$\alpha$ -synuclein bound to different polycations (spermine, polylysine, polyarginine, polyethyleneimine) forming stable complexes. Complex formation did not cause significant changes in $\alpha$ -synuclein secondary structures, as shown by far-UV CD spectroscopy.	77
Histones	Bovine histone H1 and bovine core histones (a mixture of H2a, H2b, H3 and H4) accelerated $\alpha$ -synuclein fibrillization in a dose-dependent manner. The formation of $\alpha$ -synuclein-histone complexes was not accompanied by significant changes in either secondary structure (CD) or globular structure (SAX).	103
Metal ions	Several divalent and trivalent ions (copper, iron, cobalt and manganese) accelerated $\alpha$ -synuclein fibrillization. Metal ions induced a conformational change of $\alpha$ -synuclein and led to the formation of a partially folded intermediate.	76
Glycosaminoglycans (GAGs)	Some glycosaminoglycans (heparin, heparin sulfate, agrin) and other highly sulfated polymers (dextran sulfate) significantly accelerated the formation of $\alpha$ -synuclein fibrils in vitro. Binding to $\alpha$ -synuclein induced conformational changes of the protein.	87,157
Sodium dodecyl sulfate	SDS stimulated $\alpha$ -synuclein fibrillization in a highly reproducible manner. SDS-induced fibrils did not exhibit the classical rod-like structure. Fibrillization in the presence of SDS seems to present an alternative fibrillization pathway.	158
Organic solvents	Organic solvents affected fibrillization kinetics in a concentration-dependent fashion. Low concentrations of alcohols accelerated the rate of fibrillization, whereas at high concentrations the effect was dependent on the type of alcohol—simple alcohols induced $\beta$ -helix-rich conformation, fluorinated alcohols promoted $\alpha$ -helix-rich species. All solvents induced folding of $\alpha$ -synuclein, as shown by far-UV CD and FTIR spectra.	73,111
<b>Factors inhibiting fibrillization</b>		
Small chemical compounds	Different chemical classes of compounds have been shown to inhibit $\alpha$ -synuclein fibrillization in vitro (polyphenols, benzothiazoles, phenothiazoles, steroids, Congo red and its derivatives, flavonoids). Some of them were able to disaggregate the preformed fibrils.	44,104
Heat shock proteins (Hsp)	Small Hsp proteins ( $\alpha$ B-crystallin, HspB8, Hsp27, HspB2B3) inhibited $\alpha$ -synuclein fibrillization. The formation of aggregates suggested that Hsp proteins may redirect $\alpha$ -synuclein from a fibril-formation pathway toward an amorphous aggregation pathway. Hsp70 inhibited $\alpha$ -synuclein filament formation by binding to soluble prefibrillar intermediates.	105-108
PAMAM dendrimers	PAMAM dendrimers (generations G3, G4, and G5) inhibited fibrillization of $\alpha$ -synuclein and promoted the breakdown of pre-existing fibrils. They induced structural changes in $\alpha$ -synuclein and redirected the protein to an amorphous aggregation pathway.	109
$\beta$ - and $\gamma$ -synucleins	$\beta$ - and $\gamma$ -synucleins increased the lag time and dramatically reduced the elongation rate of $\alpha$ -synuclein. $\beta$ - and $\gamma$ -synucleins could be incorporated into oligomeric intermediates leading to their stabilization.	159
Catecholamines	Several catecholamines, including L-DOPA and dopamine, were able to inhibit $\alpha$ -synuclein fibrillization and to dissolve the preformed fibrils in vitro.	160
Phospholipids	Acidic phospholipids induced $\alpha$ -helical structure in $\alpha$ -synuclein, thus preventing the formation of fibrils.	161
Rifampicin	Rifampicin inhibited $\alpha$ -synuclein fibrillization and disaggregated existing fibrils by stabilizing monomeric and soluble oligomeric forms.	162
Trehalose	Trehalose, a disaccharide, inhibited $\alpha$ -synuclein fibrillization and stimulated the formation of large amorphous aggregates.	163
Oxidative modifications	Oxidation of $\alpha$ -synuclein methionines, N-terminal acetylation, and Tyr-nitration have been reported as protective factors against fibrillization.	164-166

#### *PAMAM dendrimers*

Another class of compounds exerting anti-aggregation effects includes polyamidoamine (PAMAM) dendrimers—highly

branched polymers possessing a well-defined spherical geometry.<sup>109</sup> PAMAM dendrimers (generations G3, G4, and G5) inhibit fibrillization of  $\alpha$ -synuclein and promote the breakdown

of pre-existing fibrils. They have been shown to induce structural changes in  $\alpha$ -synuclein and redirect the protein to an amorphous aggregation pathway.

#### *Organic solvents*

Some factors affecting  $\alpha$ -synuclein fibril formation have been reported to inhibit or accelerate the fibrillization depending on their concentration or reaction conditions. Munishkina and collaborators have tested the effect of different organic solvents on  $\alpha$ -synuclein fibrillization kinetics.<sup>73</sup> Far-UV CD, FTIR and MS spectra confirmed that they induced folding of  $\alpha$ -synuclein.<sup>73,95</sup> The concentration and composition of different alcohols (ethanol, methanol, and propanol) differently affected the fibrillization rate of  $\alpha$ -synuclein.<sup>73</sup> Notably, recent studies have established that overexpression of  $\alpha$ -synuclein and the consequent higher risk for developing PD is modulated by chronic long-term alcohol exposure.<sup>110,111</sup>

#### **Effect of PD-related mutations on $\alpha$ -synuclein fibrillization in vitro**

Three autosomal dominant missense point mutations in the SNCA gene have been identified in early-onset PD: A53T, A30P, and E46K.<sup>112-114</sup> Recently, two independent groups have discovered the novel pathogenic mutation H50Q.<sup>115,116</sup> Several works have shown that PD-linked mutations A53T, A46K, and H40Q promoted  $\alpha$ -synuclein aggregation in vitro, yet neither of them affected the overall structure of the  $\alpha$ -synuclein monomer.<sup>32,75,115-120</sup>

Mutations A53T and E46K have been shown to significantly accelerate fibrillization in vitro when compared with WT protein.<sup>117</sup> Although the A53T and E46K mutated  $\alpha$ -synuclein fibrils exhibited distinct morphologies, the rate of amyloid formation of both mutants was similar.<sup>121,122</sup> A53T fibrils were about 5–14 nm in width and appeared twisted, with a crossover spacing of about 100 nm; E46K fibrils had often a twisted appearance too, with width varying between about 5 and 14 nm, but with shorter crossover spacing of about 43 nm.<sup>121</sup> Recently, Lemkau and collaborators have found by ssNMR that both mutated fibrils possessed site-specific perturbations in their secondary structures, which could alter the overall structural arrangement of the fibril.<sup>123</sup>

Similarly to WT  $\alpha$ -synuclein protein, both mutants generated also prefibrillar intermediates with pore-like activity.<sup>60,118,124</sup>

The effect of the A30P mutation on fibrillization is not clear, as some data reported that it formed fibrils slightly faster than WT protein,<sup>37</sup> whereas others showed that this mutation decreased fibril formation.<sup>60,125</sup> Conway and collaborators have observed that A30P had a slower propensity to form fibrils than WT, and it preferentially adopted a soluble, protofibrillar conformation, whereas the WT protein readily progressed to mature fibrils.<sup>60</sup> In spite of that, some data have shown that A30P fibril morphologies were similar to those formed from WT protein.<sup>34,121</sup> Both WT and A30P fibrils had a filament width of 6–9 nm, which varied slightly along the length of the filaments.<sup>121</sup>

The recently identified H50Q mutant has been shown to aggregate more readily than WT protein, but more slowly than A53T and E46K.<sup>120</sup> AFM studies have shown that, similarly to

WT protein, the H50Q protein oligomerized through small oligomers, from protofibrils to mature fibrils.<sup>120</sup>

### **Current Knowledge on Internalization and Propagation of $\alpha$ -Synuclein Aggregates**

Increasing evidence argues that  $\alpha$ -synuclein may transfer from cell to cell, suggesting that prion-like propagation may mediate the pathological progression of PD. Braak and colleagues have proposed that  $\alpha$ -synuclein pathology begins in the lower brainstem and olfactory bulb and spreads into the limbic system and eventually to the cortex.<sup>126</sup> Two independent studies have revealed host-to-graft propagation of  $\alpha$ -synuclein-positive Lewy-like pathology, providing further evidence for prion-like spreading of the pathology.<sup>127,128</sup> Embryonic mesencephalic neurons grafted into the neostriatum of PD patients have been shown to accumulate LB pathology. Moreover,  $\alpha$ -synuclein was transmitted via endocytosis to neighboring neurons, forming Lewy-like inclusions.  $\alpha$ -synuclein was also transmitted from the affected neurons to engrafted neuronal precursor cells in a transgenic (Tg) model of PD-like pathology.<sup>129</sup>

Several recent studies have investigated whether  $\alpha$ -synuclein aggregates formed in vitro from recombinant protein may be internalized by cells and eventually recruit endogenous  $\alpha$ -synuclein to form LB-like pathology. Increasing evidence suggests that misfolded  $\alpha$ -synuclein aggregates, both fibrils and oligomers, may be uptaken and propagated among cells in a prion-like manner and induce pathology in healthy cells.<sup>78,79,129,130</sup>

Luk and collaborators have shown that addition of in vitro preformed  $\alpha$ -synuclein fibrils into culture medium induces intracellular  $\alpha$ -synuclein aggregation in different cell lines overexpressing this protein.<sup>78</sup> The fibrils were shown to rapidly recruit endogenous soluble protein converting this into detergent-insoluble inclusions, while monomeric and oligomeric  $\alpha$ -synuclein showed only a negligible effect. Moreover,  $\alpha$ -synuclein inclusions undergo several modifications characteristic for LBs, such as hyperphosphorylation, ubiquitination and accumulation of cytoplasmic vesicles around the periphery of the inclusions.<sup>78</sup> Morphological and biochemical similarities between intracellular inclusions and LBs suggest that fibrillar seeds may have a central role in the initial formation of LBs and other disease-associated inclusions. Luk et al. also tested whether exogenous aggregates could induce aggregation of endogenous  $\alpha$ -synuclein expressed by primary cells not overexpressing the protein.<sup>131</sup> In fact, preformed fibrils entered primary hippocampal neurons and promoted recruitment of soluble  $\alpha$ -synuclein into insoluble LB-like aggregates. Consistent with previous results, endogenous aggregates were hyperphosphorylated and ubiquitinated, confirming their LB-like properties. Moreover, formation of endogenous aggregates resulted in the decrease of some synaptic proteins, impairments in neuronal excitability and connectivity and, eventually, neuron loss.<sup>131</sup>

Another study has shown that intracerebral inoculations of symptomatic mouse brain lysates or preformed  $\alpha$ -synuclein fibrils into Tg asymptomatic mice overexpressing the human A53T mutant triggered PD-like pathology and dramatically reduced



the survival time of animals.<sup>132</sup> PD-like pathology was shown to spread to distal CNS regions. The most severe  $\alpha$ -synuclein pathology was detected in brain regions that project to, or receive input from the inoculation sites, suggesting that the propagation of pathology occurs between associated neuronal populations.<sup>132</sup> Furthermore, intrastriatal inoculation of synthetic  $\alpha$ -synuclein fibrils into wild-type non-Tg mice also led to cell-to-cell transmission of pathologic  $\alpha$ -synuclein and LB-like pathology. The  $\alpha$ -synuclein pathology caused progressive loss of dopamine neurons in the *substantia nigra pars compacta* and impairment in motor coordination.<sup>130</sup>

Injections of in vitro preformed human or mouse  $\alpha$ -synuclein fibrils in the *substantia nigra* of non-Tg mice have also induced LB-like pathology, while mice injected with soluble  $\alpha$ -synuclein did not exhibit pathological symptoms.<sup>133</sup> Exogenously injected fibrils could be detected for less than one week, but induction of  $\alpha$ -synuclein pathology was observed after three months. The pathology induced by exogenous fibrils was similar to that triggered by intracerebral injection of sarcosyl-insoluble  $\alpha$ -synuclein from brains of patients with DLB.<sup>79</sup>

Sacino and collaborators have obtained similar results.<sup>134</sup> They have observed that neonatal cerebral injections of exogenous  $\alpha$ -synuclein fibrils induced pathology after prolonged incubation times. The injected  $\alpha$ -synuclein was rapidly cleared within a few days and inclusion pathology, which arose from endogenous protein, was developed after months. Tg mice over-expressing  $\alpha$ -synuclein were more prone to develop pathology. Moreover, the authors have shown that injections of the non-amyloidogenic form of  $\alpha$ -synuclein ( $\Delta 71-82$ ) induced similar pathology, suggesting that the pathogenesis might be induced not solely by conformation-dependent templating events.<sup>134</sup>

Altogether these data strongly confirm that exogenous  $\alpha$ -synuclein amyloid fibrils produced from recombinant protein have prion-like properties, as they are sufficient to seed and induce pathology in vivo.

A typical hallmark of prion diseases is the existence of different "strains," arising from the same proteins but having different incubation periods, PrP<sup>Sc</sup> distribution and lesion profiles in the brain, as well as different protease cleavage patterns.<sup>135</sup> The existence of different strains has not been identified so far for other neurodegenerative diseases. Recently, Bousset and collaborators have generated in vitro two different high-molecule assemblies of  $\alpha$ -synuclein, prepared in buffers with different salt concentrations.<sup>65</sup> These assemblies, designated fibrils and ribbons, adopted different conformations, as confirmed by transmission electron microscopy (TEM), FTIR, X-ray diffraction, and ssNMR. Moreover, proteinase-K digestion revealed different cleavage profiles. Fibrils and ribbons exhibited marked differences in their propensity to bind and penetrate cells, toxicity and seeded aggregation of endogenous  $\alpha$ -synuclein in vivo. Moreover, they imprinted their intrinsic structure to endogenous  $\alpha$ -synuclein, suggesting that different strain-like assemblies may account for the development and progression of different synucleinopathies.<sup>65</sup>

The mechanism by which exogenous aggregates may be internalized by cells is not clear. Previous studies on fibrils

comprised of polyglutamine repeats and tau proteins have shown that exogenous fibrils can be internalized by cells by simple addition into culture medium.<sup>136,137</sup> Some studies have reported that a similar internalization process occurs also for  $\alpha$ -synuclein,<sup>72,129,138</sup> while others have shown that fibrils were efficiently introduced into cells only in the presence of a transduction reagent.<sup>56,78</sup> Transduction was performed using cationic-liposomal protein transduction reagent or lipofectamine reagent.<sup>56,139</sup>

Thus the ability of  $\alpha$ -synuclein aggregates to enter into cells and induce aggregation of endogenous protein may depend on the precise structure of the aggregated protein. Danzer and collaborators have observed that, depending on in vitro aggregation conditions,  $\alpha$ -synuclein oligomers formed distinct populations that differed in their biophysical properties and cellular effects.<sup>72,140</sup> Only some types of oligomers were internalized by primary neuronal cells and neuronal cell lines, and induced endogenous  $\alpha$ -synuclein aggregation, while others induced cell death via disruption of cellular ion homeostasis.<sup>72,140</sup>

Another study has confirmed that the mechanism of internalization and propagation may depend on the assembly state of the protein: oligomers and fibrils were uptaken into cells via endocytosis, while the monomeric protein passively crossed the plasma membrane.<sup>138</sup> Internalization of fibrillar  $\alpha$ -synuclein required physiological temperatures and dynamin-1 activity, which were necessary for endocytosis. Moreover, it has been suggested that extracellular fibrils were internalized by receptor-mediated endocytosis. Internalized aggregates moved through the endosomal pathway and were degraded by lysosomes.<sup>138</sup> Internalization of  $\alpha$ -synuclein preformed fibrils was increased by the presence of wheat germ agglutinin, which bound N-acetyl-glucosamine and sialic acids at the cell surface and induced adsorptive-mediated endocytosis, suggesting that some glycoproteins might be important players in fibril internalization.<sup>131</sup>

Once  $\alpha$ -synuclein aggregates reach acceptor cells, they could seed aggregation of endogenous protein in a prion-like fashion and eventually the newly formed aggregates may be released into the extracellular space. Vesicle-mediated exocytosis could be responsible for release of  $\alpha$ -synuclein aggregates. Multiple forms of  $\alpha$ -synuclein have been detected in the cerebrospinal fluid, blood plasma and saliva, suggesting an underlying secretory process.<sup>141</sup> It has been shown that cultured neuronal cells secreted  $\alpha$ -synuclein monomers and aggregates by non-classical vesicle-mediated exocytosis. Moreover, the secretion was elevated in response to proteasomal and mitochondrial dysfunctions associated with PD pathogenesis.<sup>142</sup>

Recently, it has been reported that fibrillar  $\alpha$ -synuclein was transferred along axons through anterograde axonal transport, released, and subsequently uptaken by additional neurons. The axonal transfer of fibrils may explain the spread of LB between anatomically connected brain areas.<sup>143</sup>

Moreover, tunneling nanotubes (TNTs) have been suggested to play a major role in the propagation of  $\alpha$ -synuclein pathology. TNTs are tubular membrane bridges interconnecting cells over long distances, thus enabling the exchange of molecules and

cytoplasmic content between cells.<sup>144</sup> TNTs could be involved in the intracellular propagation of PrP<sup>Sc</sup>.<sup>145</sup> Till now, TNTs have not been implicated in PD but their involvement in the spread of  $\alpha$ -synuclein pathology cannot be ruled out.

### Toxicity of $\alpha$ -Synuclein Aggregates

An increasing number of studies, both *in vitro* and *in vivo*, have suggested that prefibrillar oligomers and protofibrils, rather than mature fibrils, may feature toxic species of  $\alpha$ -synuclein and may cause neuronal injury and cell death.<sup>146-149</sup> The process of fibril formation, rather than the fibrils themselves, has been suggested as a key element in  $\alpha$ -synuclein toxicity. The presence of some  $\alpha$ -synuclein prefibrillar species may indicate an attempt by neurons to convert toxic oligomers to fibrils, which are more stable, less dynamic structures and exhibit reduced toxicity.<sup>147</sup>

Membrane permeabilization by oligomeric species represents a potential mechanism of  $\alpha$ -synuclein mediated cytotoxicity. Monomeric  $\alpha$ -synuclein was found to bind negatively charged phospholipid membranes and undergo the structural transition into  $\alpha$ -helix.<sup>150,151</sup> It has been shown that the protofibrillar  $\alpha$ -synuclein species binds negatively charged vesicles more strongly than the monomer. Moreover, protofibrillar species, but not monomeric protein or fibrils, have permeabilized negatively charged vesicles to calcium.<sup>150</sup> Two PD-linked variants, A53T and A30P, show greater permeabilizing activity than WT protein, while this activity is reduced for E46K.<sup>118,124</sup>

The mechanism of membrane permeabilization is not clear, but protofibrillar oligomers have been suggested to permeabilize membranes in a pore-like fashion.<sup>124</sup> They may integrate into the membrane, creating pores or channel-like structures causing uncontrolled membrane permeability. Alternatively, oligomers or protofibrils could increase the conductivity of the membrane without the formation of pores.<sup>152</sup>

Membrane permeabilization may initiate several pathological events leading to changed ion homeostasis, induction of oxidative stress, altered signaling pathways, dysfunction of mitochondria, inflammation and eventually cell death.<sup>153</sup> In fact, several works have found that exogenous oligomers led to an increase in intracellular Ca<sup>2+</sup> level, diminished levels of some synaptic proteins, production of reactive oxygen species and cell death.<sup>131,149,150,154</sup> Moreover, in mice intracerebro-ventricular injections of  $\alpha$ -synuclein oligomers increased the activity of calcineurin, a key negative regulator of synaptic plasticity and memory function, thus resulting in a decrease of synaptic plasticity and memory deficits.<sup>149</sup>

It seems that toxicity of  $\alpha$ -synuclein oligomers may depend on their structure. Danzer and collaborators developed novel methods for generating different  $\alpha$ -synuclein oligomeric species, which have been tested for their biological effects on SH-SY5Y cells.<sup>72</sup> Only spherical oligomers, 2 to 6 nm in height, increased intracellular calcium level and led to caspase-3 activation, suggesting that some structural properties of oligomeric  $\alpha$ -synuclein

may be important to induce toxicity.<sup>72</sup> Moreover, Bousset and collaborators have tested the toxicity of two  $\alpha$ -synuclein assemblies (fibrils and ribbons) with different structural properties:<sup>65</sup> fibrils resulted more toxic than ribbons. In fact, fibrils induced activation of caspase-3, production of ROS and permeabilized lipid vesicles to a higher extent than ribbons.<sup>65</sup>

Although large sets of data suggest that prefibrillar assemblies represent toxic species of  $\alpha$ -synuclein, it has been shown that a homogenous population of fibrils, rather than their precursor on-assembly pathway oligomers, is highly toxic to cells.<sup>155</sup> Fibrils have been shown to permeabilize membrane vesicles and to alter calcium homeostasis. Moreover, cells exposure to increasing concentrations of fibrils resulted in the activation of caspase-3 in a concentration-dependent manner and cell death, whereas oligomeric  $\alpha$ -synuclein did not affect cell viability and did not activate caspase-3. The authors argued that the discrepancy with previous results on oligomer toxicity originates from the comparison of protein particles at different concentrations. In previous reports, the concentration of different aggregates was usually expressed as a function of the initial soluble protein concentration and not as a real concentration of particles in solution.<sup>155</sup>

### Conclusions

Increasing evidence indicates that aggregation of  $\alpha$ -synuclein is a critical factor in the etiology of PD and other synucleinopathies. Several studies have focused on explaining the structural features of  $\alpha$ -synuclein and the mechanism leading to its aggregation and fibrillization. These investigations may explain molecular mechanisms occurring in living cells and leading to disease development. *In vitro* aggregation assays are powerful tools for studying the mechanisms responsible for the formation of different aggregate species of  $\alpha$ -synuclein, their structural properties and biological functions. Moreover, aggregation assays are crucial to develop new drug compounds or novel therapy strategies to prevent disease development and progression. Recent studies clearly indicate that misfolded  $\alpha$ -synuclein aggregates may be internalized by cells and propagated in a prion-like manner, leading to toxicity. In fact, Watts and collaborators have shown that  $\alpha$ -synuclein aggregates formed in the brains of MSA patients are transmissible in a prion-like fashion.<sup>156</sup>

Prion-like transmission and propagation of misfolded proteins seems to be a process common to several neurodegenerative disorders, not only PD, but also Alzheimer's disease, Huntington's disease, amyloid lateral sclerosis, and others.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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