# α-synuclein oligomers and fibrils: a spectrum of species, a spectrum of toxicities

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## REVIEW

# α-synuclein oligomers and fibrils: a spectrum of species, a spectrum of toxicities

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### Abstract

This review article provides an overview of the different species that α-synuclein aggregates can populate. It also attempts to reconcile conflicting views regarding the cytotoxic roles of oligomers versus fibrils. α-synuclein, while highly dynamic in the monomeric state, can access a large number of different assembly states. Depending on assembly conditions, these states can interconvert over different timescales. The fibrillar state is the most thermodynamically favored due to the many stabilizing interactions formed between each monomeric unit, but different fibrillar types form at different rates. The end distribution is likely to reflect kinetic partitioning as much as thermodynamic equilibra. In addition, metastable oligomeric species, some of which are on-pathway and others off-pathway, can be populated for remarkably long periods of time. Chemical modifications (phosphorylation, oxidation, covalent links to ligands, etc.) perturb these physical inter-conversions and invariably destabilize the fibrillar state, leading to small prefibrillar assemblies which can coalesce into amorphous states. Both oligomeric and fibrillar species have been shown to be cytotoxic although firm conclusions require very careful evaluation of particle concentrations and is complicated by the great variety and heterogeneity of different experimentally observed states. The mechanistic relationship between oligomers and fibrils remains to be clarified, both in terms of assembly of oligomers into fibrils and potential dissolution of fibrils into oligomers. While oligomers are possibly implicated in the collapse of neuronal homeostasis, the fibrillar state(s) appears to be the most efficient at propagating itself both *in vitro* and *in vivo*, pointing to critical roles for multiple different aggregate species in the progression of Parkinson's disease (https://onlinelibrary.wiley.com/page/journal/14714159/homepage/virtual_issues.htm).

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### Dynamics of α-synuclein: the basis for folding and assembly into oligomeric and fibrillary species

The 140-residue protein α-synuclein (α-syn) is widely considered a central player in the development of Parkinson's disease (PD). Here, we will consider its structural and biophysical properties, which are likely to be critical for its biological function and malfunction, although the precise connection between structure and toxicity remains a subject of intense and controversial debate.

Monomeric αSN is dynamic and populates an ensemble of conformational states (Ferreon *et al.* 2009; Frimpong *et al.* 2010). This is why is considered natively unfolded (Weinreb *et al.* 1996; Eliezer *et al.* 2001; Uversky 2003; Maiti *et al.* 2004; Sandal *et al.* 2008; Anderson *et al.* 2012; Burre *et al.* 2013; Lashuel *et al.* 2013; Theillet *et al.* 2016). If we assume that each of the consecutive amino acid residues within αSN can adopt a limited number of conformations, for example, three (one trans and two gauche) with two torsions each, as the 140 residues constituting αSN are linked by 139 peptide

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bonds, the number of possible conformations would be $3^{139 \times 2}$ or $10^{132}$ which is beyond astronomical. In practice, αSN conformational plasticity is limited by its multiple functions that range from maintaining lipid-packing, sensing, and inducing membrane curvature promoting vesicle fusion to the regulation of synaptic vesicle fusion pore size (Nuscher *et al.* 2004; Ouberai *et al.* 2013) (Varkey *et al.* 2010; Pranke *et al.* 2011; Braun *et al.* 2012; Mizuno *et al.* 2012; Braun *et al.* 2014) (Logan *et al.* 2017). Nonetheless, all the conformations this protein adopt are in equilibrium (Alan 1999; AR 1999).

Each conformation αSN adopts has a life span that is dependent on intramolecular interactions between amino acid residues. Those interactions are stabilized by hydrogen bonds, electrostatic and hydrophobic interactions. The latter depend on the chemical and physical conditions surrounding αSN, for example, the nature of the solvent, αSN hydration, the ions, the viscosity, and the pH in a test tube, the differential interaction with numerous partner molecules such as lipids and proteins in the highly crowded cellular environment.

As is the case for a range of aggregation prone protein such as the proteins Tau, HTTExon1, A-beta etc., monomeric αSN populates conformers exposing amino acid stretches that allow them to establish well-defined interactions with compatible conformations. As with protein crystals, interactions allowing the formation of dimers, trimers, and higher molecular weight species between αSN molecules in different conformations define the stability of the assemblies (Oosawa and Asakura 1975). αSN molecules in conformations incapable of establishing interactions that are sufficiently stable in time and/or highly complementary interaction stay off the assembly pathway, that is they need to regroup (typically dissociate) to reach the final fibrillar state. They may still form long-lived soluble oligomeric species which can be isolated for further studies and only slowly dissociate to monomers upon dilution, but these are essentially metastable species which represent trapped states that αSN can populate while trying to find the most stable fibrillary state. Thus, αSN molecules in different conformations co-exist and the on- and off-fibrillar pathways assemblies which αSN molecules populate are equally important as they represent a continuum of states which can interconvert, although the rates of interconversion will vary widely depending on the exact conditions (the most important of which is the concentration of free monomer, the most dynamic of all the species).

## Polymorphism and the race to fibrillate in vitro and in vivo

A subset of αSN molecules coalesces transiently or stably into more or less ordered assemblies in a stochastic manner throughout life (Fig. 1). Those assemblies can be trapped by the use of cross-linking agents to reveal a ladder of intermediate species (Pieri *et al.* 2016). The ladder is seen with simple cross-linkers such as glutaraldehyde but is particularly prominent if αSN is encouraged to self-assemble through compounds such as dequalinium and dopamine (Lee *et al.* 2006). Detection of multiple species by cross-linking does not, however, necessarily implies that they are equally stable. The stable assembly competent conformations populated by αSN monomers expose distinct polypeptide chain structures. The different conformations of solvent-exposed polypeptide chains define their different sets of intermolecular interactions with compatible amino acid stretches. This yields αSN stacks that possess different intrinsic architectures (Fig. 2). Each of these structurally distinct αSN stacks can grow indefinitely by incorporation of monomeric αSN molecules in a conformation compatible with the stacks ends. As all αSN conformers are in equilibrium in solution, the recruitment of one particular conformer by one stable on-fibrillar assembly unit displaces the equilibrium toward the formation of this particular conformer until all molecules are in a one given fibrillar conformation. Since the concentration and life span of different assembly-competent αSN molecules depend on the environment of those molecules, structurally distinct αSN stacks form and grow at different rates under different environmental conditions, leading to mixed fibrillar polymorphs. This represents a case of kinetic



**Different fibrillar stacks** **Oligomers**
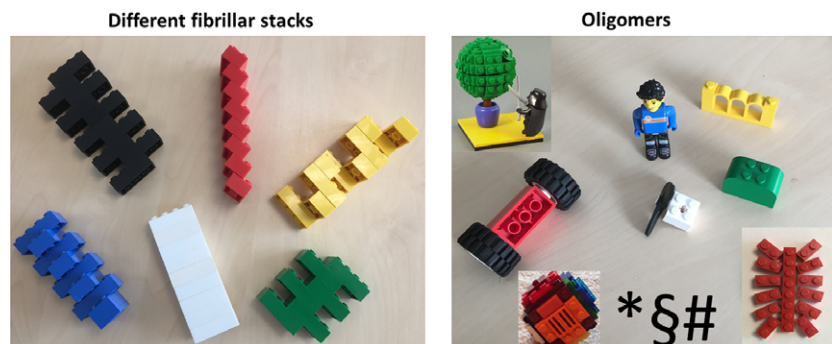
**Fig. 1** A schematic depiction of αSN aggregation into distinct high-molecular weight polymorphs, including both fibrils of indefinite length and more self-contained oligomers of limited size. *§# refers to oligomers too fluid to be represented by a simple and well-defined structure.
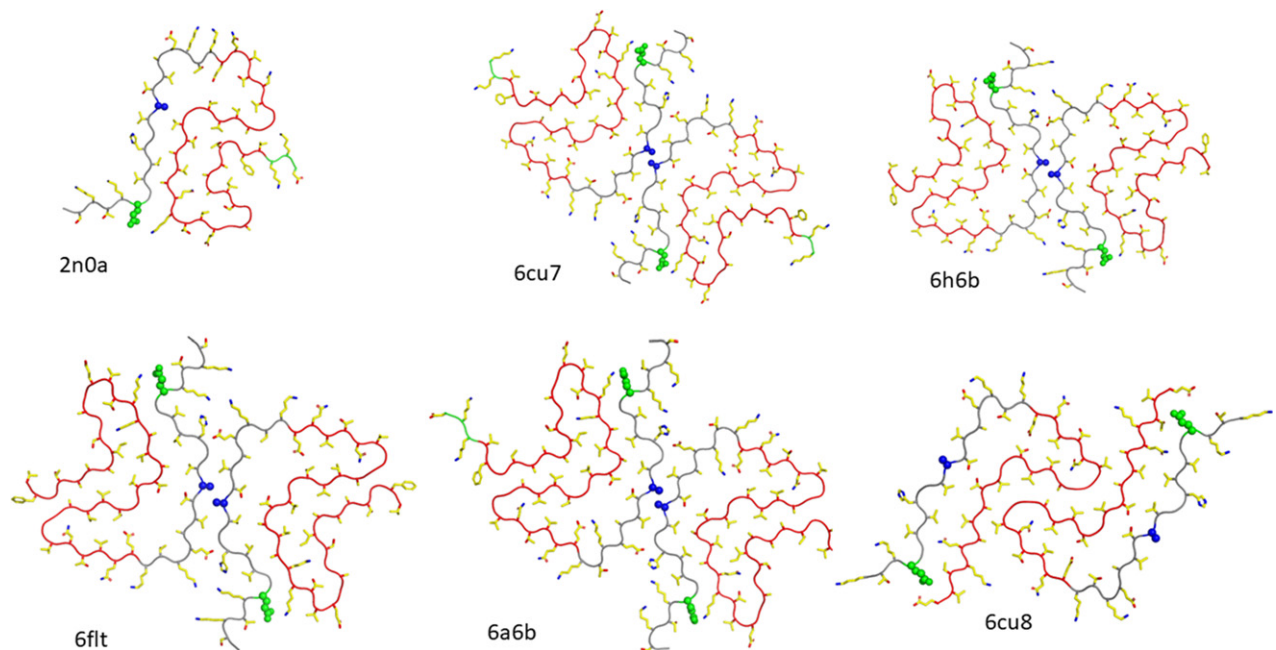
**Fig. 2** Structure of the different fibrillar polymorphs αSN forms. The structures of fibrillar αSN obtained by solid-state NMR [pdb ID# 2n0a (Tuttle *et al.* 2016)] or Cryo-Electron Microscopy [PDB id# 6cu7 (Li *et al.* 2018a), 6h6b (Guerrero-Ferreira *et al.* 2018), 6flt (Guerrero-Ferreira *et al.* 2018), 6a6b (Li *et al.* 2018b), and 6cu8 (Li *et al.* 2018a)] are represented with their respective pdb identity. The amino acid residues located to the N-terminal side of residue 60 are colored in gray. Amino acid residues 60–95 that constitute the NAC region are colored in red. To allow the reader to orient the polypeptide chains, residue E46 is represented in space fill and colored in green. Similarly, residue A53 is represented in space fill and colored in blue. Amino acid residues' side chains are in yellow.

partitioning rather than true thermodynamic distributions, where the dominating species reflects the αSN conformation that is most populated or the stack that can grow the fastest.

Point mutations (A30P, E46K, H50Q, G51D, or A53T) within *SNCA*, the gene encoding αSN, associated with familial early onset forms of PD, increase the number of possible conformations which αSN can adopt and possibly the life span of assembly competent conformations, thus favoring αSN aggregation. Duplication and triplication of *SNCA* also favor aggregation simply by increasing the concentration of assembly-competent conformers. This is why certain point mutations and gene duplication/triplication are associated with increased aggregation propensity and early onset PD.(Polymeropoulos *et al.* 1997; Kruger *et al.* 1998; Singleton *et al.* 2003; Zarranz *et al.* 2004; Lesage *et al.* 2013; Proukakis *et al.* 2013) Post-translational modifications of αSN occurring prior to oligomerization would also affect the conformational landscape it populates (see below for more details). Thus, the different tertiary structures adopted by monomeric αSN dictate both the intermolecular interactions and the surface characteristics of different assembly polymorphs (Fig. 2). The surfaces of structurally distinct assemblies define (i) growth of assemblies by recruitment of monomeric molecules in conformations that can establish thermodynamically stable interactions with assembly tips, (ii) their lateral stacking into bundles, (iii) their ability to interact with phospholipids, membranous, and cytosolic partner proteins, and (iv) their post-translational modification and/or processing by the ubiquitin-proteasome system. Thus, the surfaces of αSN assemblies dictate αSN functional properties such as their seeding propensity, resistance to the cellular clearance machinery, tropism for different neuronal cells, and potential toxicity. The specific types of interactions stabilizing fibrils may differ between polymorphs; studies describing, for example, the cold denaturation of α-synuclein fibrils and the increasing viscosity of fibrillary networks at higher temperatures suggest that some fibrillar polymorphs are stabilized by hydrophobic interactions and actually destabilized by internal electrostatic repulsion. However, the balance of forces is likely to be different in different polymorphs and its elucidation still awaits systematic thermodynamic studies done on different polymorphs in parallel (Ikenoue *et al.* 2014) (Semerdzhiev *et al.* 2018).

The physicochemical composition and conditions within the different cellular microenvironments and compartments are diverse. As in test tubes, those conditions favor or disfavor formation of assembly-competent αSN monomers that establish inter-molecular interactions with molecules in the same conformations, yielding αSN stacks of different molecular weights and structures (Oosawa and Asakura 1975). In addition, each cell type has a well-defined level of cellular proteostasis at the different cell cycle phases. Stress conditions

imbalance normal proteostasis leading to pathological situations (Morimoto 2011; Brehme *et al.* 2014). While the assembly competent conformers populated by αSN is independent of cellular proteostasis, their life span and concentration depend on cellular proteostasis. This is either due to the processing of well-defined conformations or their stabilization or destabilization by partner proteins, including molecular chaperones, whose expression increases or decreases upon stress. Neuronal proteostasis is constantly challenged by exogenous stresses in regions where they are exposed to the environment, for example, the olfactory bulb and the intestinal wall. Interestingly, several studies suggest that αSN pathogenic assemblies initially form in those regions. This may be due to the fact that ubiquitin-proteasome system, which represents a line of cell defense against protein misfolding and aggregation, is solicited by stress in those neurons to an extent which allows the persistence of misfolded αSN conformers and their aggregation into pathogenic assemblies (Morimoto 2011). All of the events listed above are time dependent and account for the age-dependent progression of synucleinopathies (Ho *et al.* 2018).

## α–syn Oligomers: a spectrum of toxic states

The scientific literature describes an extremely large variety of early prefibrillar αSN oligomeric species, coined "oligomers" (αSOs), differing in structure, molecular weight, and morphology (Lashuel *et al.* 2002; Cremades *et al.* 2017). Operationally, these oligomers are sometimes termed soluble as opposed to insoluble fibrils, but a more accurate description would be to provide the sedimentation coefficient, since all proteins can be pelleted down if centrifuged sufficiently rapidly for sufficiently long. Some oligomers are on-fibrillar assembly pathway, others are off-fibrillar assembly pathway as they eventually form amorphous, non-fibrillar assemblies.

Whether αSN oligomers or fibrils are more toxic remains a subject of intense discussion. Both classes of species have been shown to be toxic in different contexts, but this is complicated by the fact that both oligomers and fibrils come in many different sizes and shapes with ensuing differences in reactivity and toxicity and this is a source of confusion in the literature. Furthermore, it is vital to consider how to measure toxicity and relate it to the concentration of the active species.

There is multiple evidence for αSO toxicity (Ingelsson 2016) which cannot be described in detail here. For example, induction of αSOs *in vivo* by over-expressing of oligomer-inducing αSN mutants is more toxic than the corresponding up-regulation of fibrils (Winner *et al.* 2011) and antibodies targeting αSOs rescue cells against αSN-induced toxicity (Lindstrom *et al.* 2014). αSOs are reported to exert many different pathogenic effects, including cytoskeletal alterations, membrane permeabilization (plasma, mitochondria, endoplasmatic reticulum, vesicle), increased $Ca^{2+}$ influx, increased reactive oxygen species (ROS) production, and synaptotoxicity in the form of decreased neuronal excitability and decreased synaptic firing (Danzer *et al.* 2007; Parihar *et al.* 2009; Colla *et al.* 2012; Choi *et al.* 2013). αSOs also impair two major protein degradation systems, the autophagy-lysosomal and the ubiquitin-proteasomal system (Lindersson *et al.* 2004; Vekrellis *et al.* 2011). Different mechanisms of αSO toxicity for different αSOs have been reported some of them disrupt the lipid bilayer that leads to increased permeability and influx of ions from extracellular space whereas some other directly enter cells and cause increased protein aggregation (Volles and Lansbury 2002; Danzer *et al.* 2007). Danzer *et al* reported that in vitro-generated α-syn oligomers induce transmembrane seeding of α-syn aggregation in time and a dose-dependent manner in primary neuronal cultures (Danzer *et al.* 2009). αSOs also cause alterations in the protein synthesis machinery in the
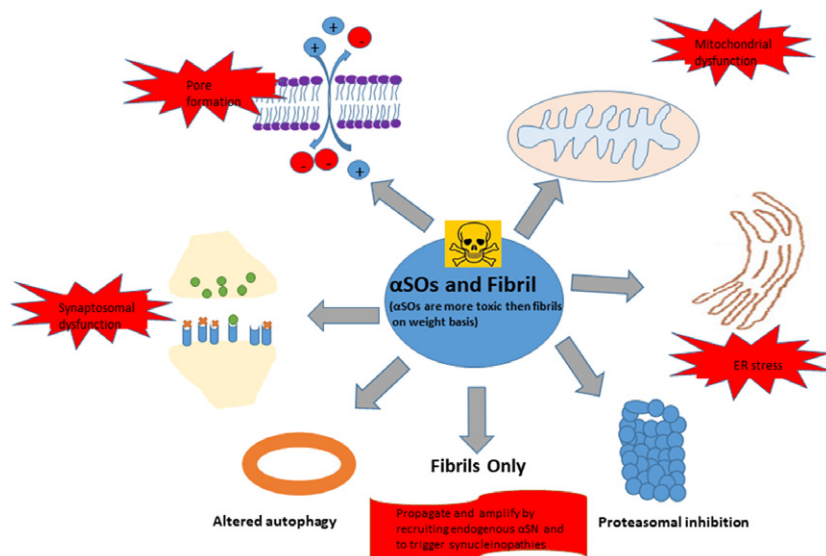


**Fig. 3** Various harmful effects exerted by αSN oligomers and fibrils.

brains of PD patients including alterations in expression of several mRNAs encoding ribosomal proteins and altered level of transcription factor eIF2 and eIF2 (Garcia-Esparcia *et al.* 2015).

It is very likely that αSOs exerts their toxic effects through a combination of multiple pathways. The various harmful effects of αSOs are summarized in Fig. 3.

We will start by providing an overview of the diversity of structures and mechanistic properties of αSOs, followed by a comparison with the toxic properties of the fibrillar species.

## Oligomer structure and shape

Depending on the method of preparation, αSOs come in a wide range of molecular weights, with variable levels of secondary structure (ranging from mainly α-helical to β-sheet with different amounts of disordered regions) and exposed hydrophobic regions (Cremades *et al.* 2017). Simple incubation of monomeric αSN under conditions which eventually lead to fibrillation (typically 1–12 mg/mL protein while shaking at 37°C) will convert 1–5% of the αSN population to oligomers within a few hours (Paslawski *et al.* 2016); these can be purified by gel filtration and remain stable under a variety of conditions (Paslawski *et al.* 2014a). However, various physical (solvent conditions) or chemical (reactive small molecules) conditions can increase the αSO yield. Thus, oligomer formation can be induced by lipids, metal ions, chemical modification of αSN, alcohol, and small molecules which block fibrillation of the protein. Oligomers formed under different conditions may or may not have altered biological properties. Oligomers formed and stabilized by lipid peroxidation products show much the same neuronal binding and toxicity properties as their unmodified counterparts, whereas more radical changes in preparation procedures (*e.g.*, use of iron salts, ethanol, and/or different shaking regimes) can have substantial impact on their cellular activity profiles (Van Diggelen *et al.* 2019),(Danzer *et al.* 2007; Qin *et al.* 2007; Ehrnhoefer *et al.* 2008; Cremades *et al.* 2012).

Due to somewhat differences in the way oligomers are prepared and purified, they are reported to form a large range of different structures (Stockl *et al.* 2013). However, a prevailing type of shape, revealed by both atomic force microscopy and electron microscopy, is a spheroid ~ 30 nm in diameters and 2 and 10 nm in height (Conway *et al.* 2000a; Conway *et al.* 2000b; Ding *et al.* 2002). The most unbiased way to study oligomer structure and formation is to monitor the population of different aggregate species as they evolve over time. Small-angle X-ray scattering (SAXS) offers such an opportunity: provided there is only a relatively small number of different species, one can tease out the contributions from the different species using start and end point populations. In one such study, the αSN oligomer was proposed to be ellipsoidal (Fig. 4a) (Giehm *et al.* 2011). This

model was consolidated in a subsequent study on the purified oligomer where SAXS was combined with Multi-Angle Light Scattering to arrive at a stoichiometry of 29 monomers per oligomer (Lorenzen *et al.* 2014a). Here, SAXS also revealed that the αSOs consist of a rigid core surrounded by a 5-nm thick layer of disordered polypeptides (Fig. 4b). HDX-MS (Hydrogen deuterium exchange mass spectrometry) identified a core consisting of residues 39–76, intermediate protection in regions 19–38 and 77–90 and highly exposed N- and C-terminal regions (Paslawski *et al.* 2014b). The prevailing secondary structure is antiparallel β-sheet (Celej *et al.* 2012). In good agreement with these approaches, Subramaniam and coworkers calculated the number of monomers in αSN oligomer to be around 31 with an elegant combination of substoichiometric labeling and single-molecule photo bleaching (Zijlstra *et al.* 2012). By introducing Trp into individual positions of the otherwise Trp-free αSN molecule, they also conclude that both the N-terminus and NAC (non-amyloid β component) region are part of the oligomer core whereas the C-terminus remains disordered (van Rooijen *et al.* 2009). Nevertheless, this conclusion should be weighed against a site-specific fluorescence study which concluded that the N- and C-termini also form intermolecular interactions (Gallea and Celej 2014).

## Off–pathway rather than on–pathway αSN oligomers?

Amyloid formation is an intrinsic property for all proteins, but their tendency to form amyloid and the mechanism of formation varies between proteins depending on the solution conditions (Dobson 2003; Chiti and Dobson 2017). Oligomers that take part directly in the fibril formation as precursors are known as on-fibrillar pathway species; in contrast, off-fibrillar pathway oligomers may exhibit a degree of stability leading to fibril formation inhibition (Fig. 5). Sophisticated Förster Resonance Energy Transfer (FRET) studies (Cremades *et al.* 2012) and meticulous cross-linking studies have proposed schemes in which different αSO species accumulate *en route* to fibrils as on-fibrillar pathway schemes. Also, αSOs constituted of ~ 10 monomeric αSN molecules have been shown to promote fibrillation (Pieri *et al.* 2016). In fact, some αSOs formed during the early stages of fibrillation and purified by gel filtration (weakly) inhibit the conversion of monomeric αSN into fibrils, indicating that they are off-fibrillar pathway (Lorenzen *et al.* 2014a). Although stable to extremes of pH and temperature (Paslawski *et al.* 2014a), these oligomers dissociate in high concentrations of urea or simple dilution (*e.g.*, repeated runs on a gel filtration column); furthermore, they eventually transform into fibrils in the presence of monomeric αSN, probably due to simple dissociation. However, off-pathway species are readily prepared in different ways. Thus, stable off-pathway oligomers can be
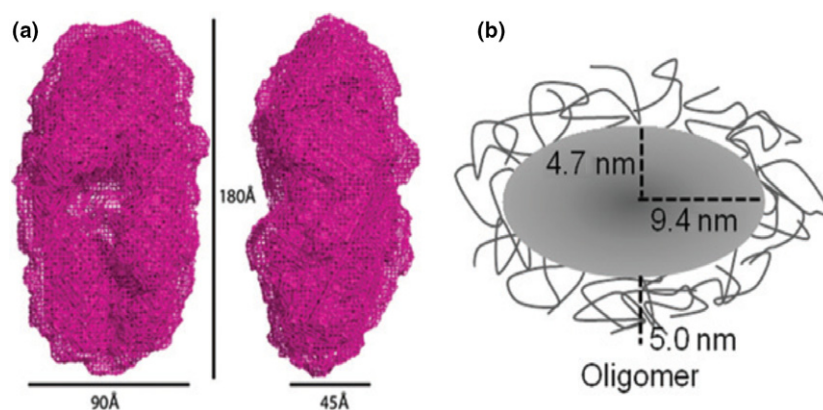
**Fig. 4** Low-resolution structural models of the αSN oligomer based on Small Angle X-ray scattering. (a) *Ab initio* model of a αSN oligomer observed during fibril formation (Giehm et al PNAS 2011. (b) Schematic model of the structure of purified αSN oligomers with a β-sheet core builtup of the N-terminus and NAC region and a disordered brush shell outer layer consisting of the C-terminus. (Lorenzen *et al* JACS 2014) The core region corresponds by and large to the *ab initio* model in panel A where co-existence of other species made it difficult to model the disordered outer layer.

formed by addition of small molecules such as the polyphenol epigallocatechin gallate (EGCG) which block fibril formation (Ehrnhoefer *et al.* 2008; Lorenzen *et al.* 2014b) or naphthalenyl sulfonamides (Kurnik *et al.* 2018). Both groups of compounds also chemically modify αSN (predominantly by forming Schiff bases with Lys residues), but this seems to be a collateral effect which is not critical for oligomer formation and fibrillation inhibition (Palhano *et al.* 2013; Kurnik *et al.* 2018). Interestingly, EGCG and sulfonamides target different parts of monomeric αSN *en route* to the formation of oligomers, indicating that there are multiple binding sites on the αSN chain (Kurnik *et al.* 2018). However, there is no indication that this in itself leads to different types of oligomers. Nevertheless, according to recent solid-state NMR (nuclear magnetic resonance) studies, the oligomer formed in the presence of EGCG is overall less structured than the "pristine" oligomer isolated at early stages in the fibrillation process (Fusco *et al.* 2017). Rather surprisingly, the "pristine" oligomer showed higher mobility in the N-terminal region despite its overall higher level of structure and also bound with higher affinity and more deeply in the phospholipid membrane. Given the observation that EGCG reduces the membrane affinity of preformed oligomer, this implies that EGCG can remodel one type of oligomer to another (Lorenzen *et al.* 2014b).

It is important to emphasize that multiple αSOs can co-exist under the same conditions, testimony to the general plasticity of this type of aggregate. Thus, HDX-MS analysis of an αSO population purified by gel filtration identified two co-existing oligomers. It was not possible to separate these oligomers, so their different roles in the aggregation mechanism could not be assessed directly. However, based on exchange kinetics, it was proposed that Oligomer I (a minor and transient species) exists in equilibrium with αSN monomers and forms straight fibrils. In contrast, the dominant species named oligomer II is not in (rapid) equilibrium with monomers and does not elongate to form fibrils (Paslawski *et al.* 2014b). There is a reasonable correspondence between these results and the aforementioned FRET studies which proposed two different co-existing oligomers (Cremades *et al.* 2012). However, in the latter case, type A oligomers (which are just as sensitive to proteinase K as monomeric αSN) are proposed to convert to type B oligomers, which are significantly more resistant to proteinase K and also generate more ROS in neuronal cells than type A oligomers. Interestingly, αSN fibrils disaggregated by dilution released oligomeric species with a FRET profile similar to the type B oligomers, consistent with (but not proving) type B oligomers being on-pathway to fibrillation. This discrepancy with data indicating an off-pathway αSO remains unresolved. Properties and methods of preparation of different types of oligomers are described in Table 1.

## Effect of chemical modifications on αSN oligomers

αSN is subject to several kinds of post-translational modifications (PTMs) which affect its aggregation and toxicity both in vivo and vitro. PTMs that have been extensively found in Lewy bodies in PD patients including acetylation, oxidation, nitration, ubiquitination, etc. However, the exact contribution of PTMs to PD pathology remains elusive.

In this section, we will discuss different types of biologically relevant chemical modifications of αSN can impact the formation of αSOs. Overall, these modifications compromise fibril formation, most likely by introducing new functional groups into αSN which are incompatible with a fibrillar packing arrangements. Although different strains of αSN fibrils exist which will vary in their molecular structure to some extent, it is remarkable that they all rely on a "pristine"
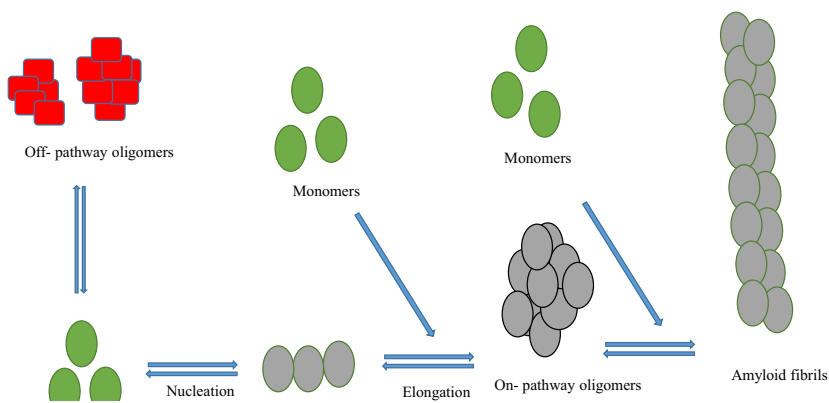
**Fig. 5** On-pathway and off-pathway oligomers.

αSN molecule which is not modified by the introduction of new chemical functionalities. In contrast, the oligomer state accommodates these states quite readily, indicating a more plastic structure. It remains unclear whether such modifications lead to increased oligomer yields because they directly stabilize the oligomeric state or simply block formation of the fibrillar state, thus allowing the oligomer to accumulate without siphoning off αSN monomers into growing fibrils. The chemically modified αSOs constitute interesting drug targets, though their very diversity (see below) makes it questionable whether there can be a combined strategy to target them all.

## Lipid peroxidation products

In aging brains, oxidative stress generates ROS, which can initiate lipid peroxidation of polyunsaturated fatty acids (PUFA) (Finkel and Holbrook 2000). In turn, PUFA and their peroxidation products can affect αSN oligomerization and aggregation. One of the main PUFA in the brain is docosahexaenoic acid localized at synapses (Bazinet and Laye 2014). docosahexaenoic acid-induced αSN oligomers contain a mixture of α-helical and random structure, resist sodium dodecyl sulfate and urea, do not binds to ThT, and lack seeding properties, demonstrating that they are off-pathway oligomers (De Franceschi *et al.* 2011). The peroxidation of lipids also leads to the formation of reactive aldehydes such as ONE (4-oxo-2-nonenal), 4-hydroxyl-2-nonenal (HNE), malondialdehyde, and acrolein (Esterbauer *et al.* 1991; Lee and Blair 2000). These aldehydes are cytotoxic by themselves and covalently modify proteins, altering their normal structure and function. Increased level of HNE- and acrolein-modified proteins have been found in the brain of PD patients (Yoritaka *et al.* 1996; Castellani *et al.* 2002).

Besides triggering α-syn cross-linking with many partner proteins *in vivo*, ONE and HNE both stimulate formation of α-syn oligomers with β-sheet rich structure, but ONE's carbonyl group at position C4 makes it a more potent cross-linker than HNE which has a hydroxyl group in that position (Nasstrom *et al.* 2011). ONE and HNE modify the same amino acids through Schiff base formation (via Lys residues, of which there are 15 in α-syn) or Michael addition (via the single His at position 50 or one of the 15 Lys residues). Neither oligomer forms amyloid fibrils even after prolonged incubation, implying that they are off-pathway and thermodynamically stable (Nasstrom *et al.* 2011). The ONE induced oligomers are mostly amorphous, while HNE induced oligomers with shapes ranging from protofibrillar to annular and globular. Compared to HNE oligomers, ONE oligomers are less protease-resistant but more stable against dissolution by urea and formic acid. This implies that ONE oligomers have a greater amount of dynamic regions but nevertheless higher stability, possibly thanks to the cross-links formed by ONE (Nasstrom *et al.* 2011). Camilla B. Andersen and D.E.O., unpublished observations) implying that ONE have a greater degree of dynamic regions but the intermolecular contacts have higher stability (possibly thanks to the cross-links). Both oligomers are cytotoxic toward SH-SY5Y cells and primary neurons. We reported that they also share common epitopes and an ability to impair long-term potentiation, permeabilize lipid vesicles and colocalize with glutamatergic synapse as well as unmodified oligomers, indicating an overall retention of (unwanted) biological properties.

## Nitration

Besides ROS, oxidative stress can combine with nitric oxide to generate peroxynitrile and other compounds which in turn can nitrate (and cross-link) the 4 Tyr residues in α-syn. Nitrated αSN is found in the brains of PD patients using anti-nitroTyr antibodies (Yamin *et al.* 2003). *In vitro*-nitrated αSN is unable to fibrillate by itself and also inhibits fibrillation of non-nitrated αSN (Hodara *et al.* 2004). Probably due to formation of small soluble oligomers, largely dominated by octamers along with some dimers and tetramers (Yamin *et al.* 2003). Given that nitration of the 3-Tyr αSN variant Y39F reduces vesicle affinity, this would indicate that nitration could actually reduce aggregate toxicity. While nitration also reduces the rate of degradation of monomeric αSN by 20S proteasome and calpain I (Hodara

**Table 1** Different types of oligomers and their properties

| Method of preparation and analysis | Type of oligomer | Properties |
|---|---|---|
| Equimolar concentrations of differently labeled A90CαSN (1 mg/mL) were mixed at pH 7.4 and incubated at 37°C for 4–8 days with shaking at 200 rpm. Populations were identified single-molecule fluorescence and fluorescence resonance energy transfer (FRET) measurements (Cremades *et al.* 2012) | Type A | These oligomers form first. They are just as sensitive to Proteinase K as monomeric αSN and lack persistent secondary structure. They generate lower amount of ROS in cells |
| | Type B | Formed by conversion of type A oligomers. Resistant to Proteinase K, contains significant amount of beta sheet structure. Generates higher levels of ROS in cells. Conversion from A to B oligomers is the key step in aggregation of αSN |
| 12 mg/mL αSN was incubated in PBS at 37°C with 900 rpm shaking for 5 hrs. Insoluble material was removed by centrifugation and soluble species were separated by gel filtration. Purified oligomers were analyzed by HDX-MS (Paslawski *et al.,* 2014b) | Type I | Same protection pattern as αSN fibrils (protected core in Y39-A89 region; N-terminus also protected while C-terminus is unprotected). Exists in equilibrium with αSN monomers and forms long straight fibrils upon prolonged incubation |
| | Type II | Less protected than Type I oligomers and fibrils. Residues Y39-T75 are most protected while the two small fragments, A18–L38 and A76–A89 represents dynamic flanking regions of the core. Is not in equilibrium with monomers and cannot form long fibrils. Forms worm-like structures upon prolonged incubation |

FRET, Förster resonance energy transfer; ROS, reactive oxygen species.

*et al.* 2004). It is unclear how nitration affects oligomer quality control. It has also been suggested that nitration may be toxic not because of the formation of more cytotoxic oligomers, but rather by modulating the integrin iNOS/FAK signaling pathway (Liu *et al.* 2011).

## Phosphorylation

Phosphorylation plays an important role in α-syn oligomerization, Lewy body formation, and neurotoxicity (Barrett and Greenamyre 2015). α-syn phosphorylated at S129 is a hallmark of both Lewy Bodies and soluble α-syn in PD tissue (Fujiwara *et al.* 2002) (Anderson *et al.* 2006). Phosphorylation also occurs *in vivo* on S87, Y125, Y133, and Y136 (Oueslati *et al.* 2010). Phosphorylation at S87 is up-regulated in synucleinopathies; this inhibits α-syn fibrillation and reduces α-syn-membrane interactions (Paleologou *et al.* 2010). However, phosphorylation of even very closely placed residues can have remarkably different consequences: in fly brain homogenates, levels of oligomeric α-syn were increased by phosphorylation at S129 but decreased by phosphorylation at Y125 (Chen *et al.* 2009). However, this region of the oligomer is highly mobile and therefore unlikely to be involved in intermolecular contacts (Lorenzen *et al.* 2014b).

## Metal ions

The healthy brain has an active metal homeostasis, but free metal ions stimulate ROS with subsequent indirect detrimental effects on α-syn properties (Carboni and Lingor 2015). In fact, ROS production can be completely blocked by addition of metal chelators (Deas *et al.* 2016). Furthermore, aberrant interactions between metal ions and α-syn can trigger formation of oligomers and fibrils, in particular $Al^{3+}$,

$Cu^{2+}$, $Cd^{2+}$, $Ca^{2+}$, and $Fe^3$ (Uversky *et al.* 2001). Thus, $Cu^{2+}$ stimulates formation oligomers in the presence of coupling reagents, probably via the acidic C-terminal region (since proteolytic removal of this region suppresses aggregation) (Hyun-Ju *et al.* 1999), and $Cu^{2+}$ chelators redistribute the α-syn toward the membrane while reducing the extent of aggregation (Wang *et al.* 2010). $Fe^{3+}$ can induce formation of pore forming, sodium dodecyl sulfate-resistant α-syn oligomers (Kostka *et al.* 2008). Similarly, $Ca^{2+}$ binds to the C-terminal region of α-syn to form annular oligomers 70–90 nm in diameter; this effect was abolished by truncating the C-terminal 15 residues.

## Toxicity of fibrillar α–syn species

Apart from α-syn oligomers, other conformers associated with PD include protofibrils and fibrils. The fibrillar form of α-syn is mainly located within LBs inside the cell body of neurons and these intracellular structures are a hallmark of PD (Wakabayashi *et al.* 2007). Here, we will discuss these fibrillary species. Both toxic and non-toxic α-syn fibrillar species have been reported and various toxic effects of α-syn fibrils are summarized in Fig. 3 (Danzer *et al.* 2007; Qin *et al.* 2007; Ehrnhoefer *et al.* 2008; Cremades *et al.* 2012). Those fibrillar α-syn species have been proposed to contribute to neurodegeneration by perturbing cellular ion homeostasis, by seeding the assembly of soluble α-syn into higher molecular weight aggregates (Bousset *et al.* 2013) by imbalancing cellular proteostasis (Morimoto 2011; Brehme *et al.* 2014) and/or by compromising the integrity or function of cytosolic organelles such as the endoplasmic reticulum, the Golgi, the mitochondria, and the lysosomes.(Flavin *et al.* 2017). α-syn fibrillar assemblies also trigger

neurodegeneration through chronic inflammation (Gustot *et al.* 2015; Hoffmann *et al.* 2016; Peralta Ramos *et al.* 2019; Gribaudo *et al.* 2019).

While purified αSOs are more toxic toward SHSY5Y cells than fibrils on a weight basis, fibrils win out on a particle number basis as determined by a combination of analytical ultracentrifugation and quantitative length distribution measurements (Pieri *et al.* 2012; Lorenzen *et al.* 2014b). Besides the higher per-particle activity, fibrillar species possess one additional advantage over oligomers in terms of toxicity: their ability to propagate and amplify by seeding the aggregation of endogenous α-syn. Indeed, on-fibrillar assembly pathway species made of over 10 monomeric α-syn molecules have been shown to possess seeding activities indistinguishable from those of fibrils made of thousands of monomers. While all species are important, only the fibrillar assemblies have been shown to propagate, amplify by recruiting endogenous α-syn, and to trigger synucleinopathies when injected to animal models. This is a very important distinction. There is no evidence that non-fibrillar oligomers can propagate in a similar manner. Thus, while α-syn oligomers are both functional and long-lived (they possess toxic properties and can be more robust than fibrils toward extremes of pH and temperature), there is no convincing evidence that they can spread *in vivo* rather than be formed as a collateral effect of the overall aggregation process (Paslawski *et al.* 2014a).

## α–syn mega–Dalton assemblies with propagation and seeding propensities

When brain homogenates from model animals where α-syn forms deposits resembling Lewy bodies and Lewy neurites and from patients developing PD are injected into the CNS (central nervous system) of naïve model animals ranging from rodents to non-human primates (whether over-expressing human α-syn or not), lesions reminiscent of PD form in their central nervous system (Mougenot *et al.* 2012; Luk *et al.* 2012a; Luk *et al.* 2012b; Watts *et al.* 2013; Masuda-Suzukake *et al.* 2013; Recasens *et al.* 2014; Prusiner *et al.* 2015; Shimozawa *et al.* 2017). No such lesions are observed with equivalent control samples lacking the characteristic Mega-Dalton α-syn assemblies (for comparison, the major oligomeric species of α-syn is around 0.4 MDa in size). The lesions, initially confined to the injected brain region, spread to neighboring and/or axonally connected regions within the brain over several months, suggesting oriented traffic through neuronal transport processes and amplification of pathogenic α-syn within those brain homogenates during propagation. Importantly, α-syn aggregation leads not only to PD but also to additional synucleinopathies in man: LBD (Lewy body dementia) and MSA (multiple system atrophy). In addition, brain homogenates from MSA or PD cases fractionated by ultracentrifugation over sucrose gradients

faithfully induced lesions characteristic of each synucleinopathies upon injection in the CNS of model rodents and/or non-human primates (Mougenot *et al.* 2012; Luk *et al.* 2012a; Luk *et al.* 2012b; Watts *et al.* 2013; Masuda-Suzukake *et al.* 2013; Recasens *et al.* 2014; Prusiner *et al.* 2015; Shimozawa *et al.* 2017).

Importantly, α-syn fibrillar assemblies made *de novo* from recombinant protein induce lesions and trigger the aggregation of endogenous α-syn several months after injection into the CNS in a manner similar to brain homogenates from PD or MSA cases.(Luk *et al.* 2009; Volpicelli-Daley *et al.* 2011; Rey *et al.* 2013; Peelaerts *et al.* 2015; Rey *et al.* 2016) Furthermore, α-syn fibrillar polymorphs made under different experimental conditions yield PD or MSA pathological hallmarks in rodents. Thus, fibrillar polymorphism generated *in vitro* can faithfully propagate *in vivo* (Peelaerts *et al.* 2015).

## Origin of α–syn fibrillar polymorphs deleterious effects in distinct synucleinopathies

The multitude of conformations α-syn adopts yields numerous fibrillar polymorphs. Both the tips and sides of *in vivo* or *in vitro* fibrillar mega-Dalton α-syn assemblies play a critical role in their ability to trigger different synucleinopathies and to propagate in a prion-like manner. The rate at which functional α-syn is recruited by the tips of the elongating fibrillar polymorphs contribute to disease phenotype, in particular, the rate of synucleinopathies progression as a function of time/age. The amino acid stretches exposed on the sides of α-syn fibrillar assemblies define (i) to what extent they can bundle laterally through hydrophobic or electrostatic interactions or via interaction with bridging cellular proteins, (ii) their ability to form macromolecular structures alone or through partner proteins within neuronal cells at the neuronal plasma membrane, (iii) their resistance to degradation and the cellular clearance machinery, (iv) their ability to be post-translationally modified, (v) their interactomes at neuronal plasma membrane and by consequences their tropism for distinct neuronal cell populations and networks, (vi) their interactomes within neuronal cells cytosol, (vii) the rate at which they escape from the endolysosomal compartment after their entry within neuronal cells, (viii) their capacity to interact directly or indirectly, for example, through vesicles or partner proteins with different molecular motors and be transported anterogradely and retrogradely within neurons, and (ix) their export, naked, or within extracellular vesicles that define their spread from cell to cell.

## Fibrillar α–syn structure and shape

Several structures for fibrillar α-syn polymorphs generated under different experimental conditions have been published. Some studies suggest that the fibrils are made of one protofilament while other indicate that they are made of

two protofilaments (Tuttle *et al.* 2016; Guerrero-Ferreira *et al.* 2018; Li *et al.* 2018a). At a first glimpse, α-syn has apparently a similar fold within the different structures resembling a Greek key (Fig. 2). This impression is wrong. The amino acid residues located to the N-terminal side of residue 60 and colored in gray constitute the interface between the two protofilaments in four represented structures with residue A53, represented in space fill and colored in blue, from one protofilament facing the same residue from the second protofilament (Fig. 2). The NAC region of α-syn, amino acid residues 60–95, colored in red, constitutes the inter-protofilament interface in one structure (Fig. 2, bottom right). In all structures, the side chains of residues A53 and E46, represented in space fill, blue, and green, respectively, are oriented on each sides of the polypeptide chain but one (Fig. 2, top right).

## Summary

Synucleinopathies are multifactorial diseases. The numerous on- and off-fibrillar assembly pathway oligomers and the structurally distinct fibrillar polymorphs α-syn populates may either exhibit distinct deleterious effects through their ability to target multiple neuronal cell processes or target different neuronal cell populations leading to different pathologies. While fibrillar assemblies have been demonstrated to play a central role in disease progression, oligomeric species are certainly the trigger factor leading to neuronal homeostasis collapse.

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