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Review Article

ALPHA-SYNUCLEIN STRUCTURE, AGGREGATION AND MODULATORS

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Abstract: Alpha-synuclein is an intrinsically unstructured protein, involved in various neurodegenerative disorders. *In vitro/in vivo* experiments, as well as genetic mutation studies establish a direct link between alpha-synuclein and synucleinopathies. Due to its natively unfolded state, alpha synuclein can adopt numerous conformations upon interaction with its partners and cellular factors, offering explanation for its diverse interactions. Aggregated form of alpha-synuclein has been observed in the brain of patients with synucleinopathies, a hallmark of neurodegeneration, and cell death has been attributed to aggregation induced toxicity. The process of aggregation involves nucleation, followed by intermediate oligomeric states, and finally the fibrillar amyloids. Of the various conformations/species that alpha-synuclein assumes before it transforms into mature amyloid fibrils, the oligomeric species is the most toxic. Thus, an effective way to limit disease progression is by modifying/slowing down protein aggregation/deposition in the brain. Various small natural products, synthetic chemicals, peptides and antibodies specific to alpha-synuclein have been designed/identified to reduce its rate of aggregation. Unfortunately, not even a handful of the molecules have cleared the clinical trials. Even today, medications available for Parkinson's patients are mostly the drugs that adjust for loss of dopamine in the brain, and hence do not stop the progression of the disease or cure the symptoms. Thus, more molecular level studies are warranted to fully elucidate the process of alpha-synuclein aggregation, which in turn could help in identifying novel therapeutics and preventives. The present review summarizes the insights gained into the structure, *in vitro* aggregation and inhibitors/modulators of alpha-synuclein aggregation, that can be used to design better and effective inhibitors against the diseases.

Keywords: Alpha-synuclein; Amyloid; Protein aggregation; Parkinson's disease

Introduction

Historically, synucleins were first identified by the expression screening of cDNA clones prepared from the electric lobe of the pacific ray (*Torpedo californica*) that reacted towards antiserum against cholinergic vesicles (Bennet, 2005; Maroteaux *et al.*, 1988). Subsequently, the Torpedo clone was used to screen a rat brain cDNA library. A homologous protein of 140 amino acids, displaying 85% homology to the Torpedo protein was identified in rat. A primary antibody raised against a fusion protein from the Torpedo cDNA clone, detected the protein subcellularly on regions of nuclear membrane and presynaptic terminals of the nervous system, and hence the nomenclature synuclein, syn for synapse and nuclein for nucleus (Bennet, 2005). Rat alpha-synuclein displays 95% identity to the non-Aβ component of Alzheimer's disease amyloid precursor protein (NACP) (Ueda *et al.*, 1993). Two major isoforms of synucleins have been isolated, purified and sequenced from the human cerebral cortex, a) the alpha-synucleins, and b) the beta-synucleins, respectively, corresponding to 140 and 134 amino acids. The identification of mutations in alpha-synuclein gene leading to Parkinson's disease, raised widespread interest in understanding the

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structure and function of this protein (Bennet MC, 2005). Subsequently, alpha-synucleins were identified in the Lewy bodies from the post-mortem brain tissue of Parkinson patients. These findings fuelled further research, leading to the identification of alpha-synuclein in several other neurodegenerative disorders *viz.* amyloid plaques from the frontal cortex of Alzheimer's disease patients, amyotrophic lateral sclerosis (ALS) (Mezey *et al.,* 1998), multiple system atrophy (Arima *et al.,* 1998; Tu *et al.,* 1998), Hallervorden-Spatz syndrome *etc*. Since then, alpha-synuclein has been extensively studied *in vitro,* as well *in vivo,* using cells and animal models. This review focuses on the structure, *in vitro* aggregation and inhibitors/modulators of alpha-synuclein that can intervene in its process of aggregation.

Alpha-synuclein and its synucleinopathies

The role of alpha-synuclein in Parkinson's disease came to light soon after the identification of the A53T mutation, also known as Contursi kindred mutation, named after the small town Contursi Terme of Italy, where it was identified for the first time. This finding provided the first evidence that Parkinson's disease is hereditary and associated with the *SNCA* gene (Golbe *et al.,* 1990). Later, genetic mutations were identified in three other families of Greek origin, that displayed autosomal dominant inheritance of Parkinson's disease (Polymeropoulos *et al.,* 1997). As the A53T mutation naturally exists in the rodent homologue in low frequency, the role of alpha-synuclein in Parkinson's disease was still a debate (Chan *et al.,* 1998; Vaughan *et al.,* 1998). Finally, the identification of alpha-synuclein in Lewy bodies from the post-mortem brain tissue of sporadic, as well as familial Parkinson's disease patients, established its direct connection with the disease (Baba *et al.,* 1998; Spillantini *et al.,* 1997). A review on the role of alpha-synuclein in Parkinson's disease has been published before (Breydo *et al.,* 2012). Loss of dopaminergic neurons of the substantia nigra pars compacta (SNpc) in the midbrain is the main characteristic of Parkinson's disease, and this is caused by the accumulation of misfolded alpha-synuclein. The surviving neurons show the presence of intraneuronal proteinaceous cytoplasmic inclusions, called the Lewy bodies (Bourdenx *et al.,* 2014). More than 90 proteins have been identified in Lewy bodies till date, though alpha-synuclein is the most abundant of all (Wakabayashi *et al.,* 2013). Another point mutation A30P, was identified in a German kindred (Kruger *et al.,* 1998). Several other mutations have been identified since then, namely, A18T, A29S, E46K, H50Q, G51D, and A53E. Of all the known mutations, A30P, E46K and A53T have been extensively studied *in vitro*, and *in vivo*.

Duplication (Chartier-Harlin *et al.,* 2004) and triplication (Singleton *et al.,* 2003) of alpha-synucleins is also very common, associating the cellular levels of alpha-synuclein with toxicity. Alpha-synuclein mutations are in general less common, compared to its multiplicity, observed in more than 31 families (Ibanez *et al.,* 2004). Genome wide studies have also associated single nucleotide polymorphisms with alpha-synuclein gene (SNCA gene), as a common risk factor for Parkinson's disease (Wakabayashi *et al.,* 2013). *In vitro* studies have shown a) increased rates of aggregation of alpha-synuclein mutants that cause disease in yeast and mammalian cell lines (Greenbaum *et al.,* 2005; Li *et al.,* 2001), b) neurotoxicity of alpha-synuclein (Fernandes *et al.,* 2014; Zhou *et al.,* 2000) and c) toxicity of alpha-synuclein in animal models (Feany and Bender, 2000; Jackson-Lewis and Przedborski, 2007). Apart from Alzheimer's and Parkinson's disease, alpha-synuclein is associated with multiple system atrophy, Parkinson's disease with dementia, dementia with Lewy bodies, pure autonomic failure, neuroaxonal dystrophy, and Lewy body variant of Alzheimer's disease (Bendor *et al.,* 2013). Disparity in phenotype of these diseases is primarily due to the differences in the degree of neuronal loss, age of onset, and the type of cells that are affected (McCann *et al.,* 2014). There are other diseases as well in which the pathological role of this protein is not yet established, yet there is sighting of alpha-synuclein aggregation *viz.* essential tremor, Gaucher disease and other lysosomal storage disorders, neurodegeneration with brain iron accumulation (Pantothenate kinase-associated neurodegeneration) (Puschmann *et al.,* 2012). A recent study has suggested that the absence of β-glucocerebrosidase enzyme, the marker of

Gaucher disease, is possibly responsible for the cell to cell transmission of alpha-synuclein aggregates, associating Parkinson's disease with Gaucher disease (Bae *et al.,* 2014). A chaperone widely used for Gaucher disease, AT2101, has been shown to be effective in slowing down Parkinson's disease (Richter *et al.,* 2014). Prion like transmission of alpha-synuclein has been observed in several studies, and has been considered important for disease transmission (Masuda-Suzukake *et al.,* 2013; Tyson *et al.,* 2016).

Function and cellular localization of alpha-synuclein

Exact function of alpha-synuclein still remains unclear. It is considered a presynaptic protein, that plays a role in endoplasmic reticulum and golgi vesicle trafficking (Cooper *et al.*, 2006). In dopamine homeostasis, alpha-synuclein is involved in regulating synaptic neurotransmission *via* effects on vesicular dopamine (DA) storage (Bellani *et al.,* 2010). It is also involved in transmembrane formation, modulation of the activity of phospholipase 2 when membrane bound, and neuronal survival (Chandra *et al.,* 2005; Quilty *et al.*, 2006). Alpha-synuclein can bind to the cytoplasmic chaperone 14-3-3 protein, with which it shares ~40% homology. Structural similarities point out that akin to 14-3-3, alpha-synuclein is possibly an inhibitor of tyrosine hydroxylase, the rate limiting enzyme for dopamine synthesis (Bennet, 2005; Perez *et al.,* 2002). When alpha-synuclein is sequestered into aggregates, and its cellular concentration goes down, abnormally high activity of tyrosine hydroxylase occurs, which in turn leads to an increase in the dopamine synthesis and dopamine neurotoxicity (Zigmond *et al.,* 2002). This aspect has been discussed in detail in another review (Bendor *et al.*, 2013; Eschbach and Danzer, 2013).

In humans, alpha-synuclein gene has been mapped to chromosome 4q21(Spillantini *et al.,* 1995) and is found presynaptically. However, it has been detected in perikarya within several brainstem structures, including raphe, hypoglossal, and arcuate nuclei (Giasson *et al.,* 2001a; Bennet, 2005). Alpha-synuclein expression has been observed in human and rat brain somata, and dendrites of neurons and glia (Bennet, 2005; Mori *et al.,* 2002). Within the brain, alpha-synuclein has been observed in soluble, as well as membrane associated fractions (Jensen *et al.,* 1998; Maroteaux *et al.,* 1988).

Structure of alpha-synuclein

Alpha-synuclein is a natively unfolded protein, with a molecular weight of 14.46 kDa. Circular Dichroism spectra suggests 2% alpha-helical content, and 70% random coil. FTIR spectroscopy displays a broad peak at 1650 cm^{-1}, indicative of random coil structure. Due to its natively unstructured conformation, alpha-synuclein can withstand extreme conditions *i.e.* boiling temperatures, and low pH. Its molecular mass is inconsistent with its hydrodynamic radius calculated using gel-filtration, indicating an extended structure, rather than a globular fold (Weinreb *et al.,* 1996). On SDS-PAGE, it has a molecular weight of 19 kDa, owing to the high proportion of negatively charged residues present at the C-terminal end, and low SDS binding ability (Ueda *et al.,* 1993). NMR spectroscopy also supports a natively unstructured conformation of alpha-synuclein. Figure 1A displays a $^{1}H^{15}N$ HSQC spectrum of alpha-synuclein. The low spectral dispersion in the ^{1}H dimension suggests that the protein is unstructured.

The amino acid sequence of alpha-synuclein can be divided into three regions a) the N-terminal region comprising of residues 1-60, which contains 4 imperfect KTKEGV motif repeats, b) central region consisting of residues 61-95 that are hydrophobic, also called the non-amyloid-β component (NAC) as it was observed in the brain of an Alzheimer's disease patient along with Aβ and c) C-terminal region comprising of residues 96-140, rich in acidic residues as shown in Figure 1B. The central region is crucial for aggregation, as its deletion results in reduced aggregation (Giasson, 2001b). Twelve residues in the central region are considered important for aggregation and are absent in β and γ synuclein (that do not form fibrils). The C-terminal region has chaperone activity and facilitates interaction with other proteins (Rekas *et al.*, 2012; Uversky and Eliezer, 2009).

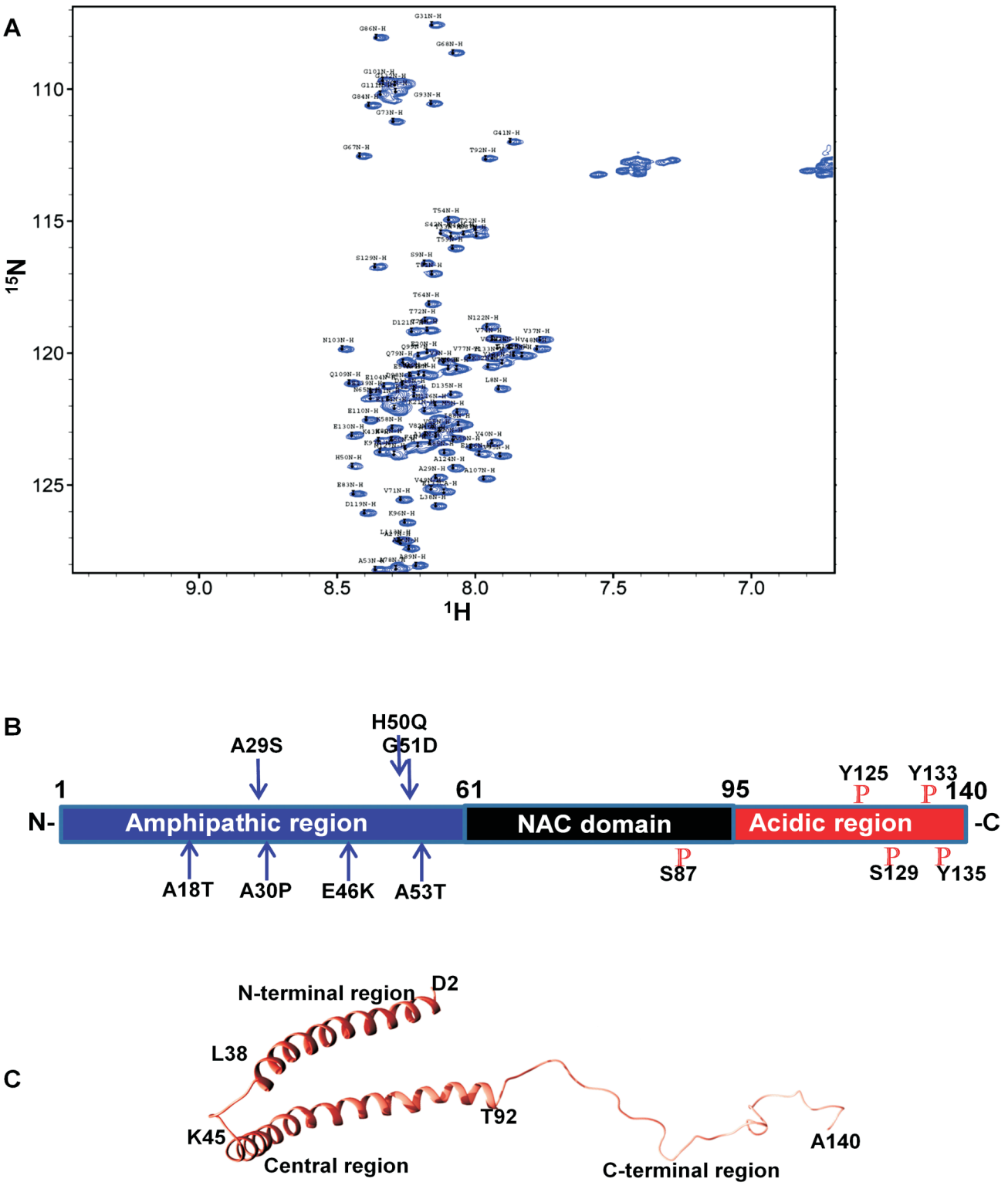

Figure 1: **A) A^{1}H^{15}N HSQC spectrum of á-synuclein in 20 mM Sodium phosphate buffer, pH 6.0 containing 0.002% sodium azide. The spectrum was acquired at 10°C on a Bruker Avance III 700 MHz NMR spectrometer, equipped with a cryoprobe, installed at the National Institute of Immunology, India. B) The three regions of alpha-synuclein *i.e.* the N-terminal amphipathic repeat region, the central hydrophobic non-amyloid component (NAC) region, and the C-terminal acidic region. Mutations associated with alpha-synuclein are shown with arrows, and phosphorylation sites are shown as P in red. C) The NMR solution structure of human alphasynuclein bound to miscelle displayed as ribbons (PDB 1XQ8).**

Some studies argue that alpha-synuclein is not completely unfolded as previously thought, but has some structure due to the presence of long range electrostatic interactions between the C terminus/NAC domain and the N-terminus (Bertoncini *et al.*, 2005; Brodie *et al.*, 2014; Dedmon *et al.*, 2005b). Deletion of the C-terminus leads to increased aggregation compared to the wild type protein, underscoring the importance of long range interactions in aggregation prevention (Hoyer *et al.*, 2004). Five prolines in the C-terminal domain also decrease aggregation (Zhou *et al.*, 2000).

In the presence of lipids/micelles, alpha-synuclein monomer exists in a helical conformation (Davidson *et al.*, 1998; Eliezer *et al.*, 2001). Lipid binding of alpha-synuclein and its structural rearrangement has been discussed before (Alderson and Markley, 2013). Likewise, when purified under non-denaturing conditions, it co-purifies with small amounts of lipids and attains a helical conformation, that forms a tetramer (Bartels *et al.*, 2011; Luth *et al.*, 2014). Figure 1C shows the helical conformation of alpha-synuclein in the presence of micelles. Three distinct regions are observed; the N-terminal helical region that acts as a membrane anchor, an unstructured C-terminal region that weakly associates with the membrane, and a central region that acts as a lipid sensor as shown in Figure 1C (Fusco *et al.*, 2014). Circular Dichroism spectroscopy suggests 65% helix, 17% turn and 8% disordered conformation. NMR spectroscopy also confirms the presence of transient helices in presence of micelles.

In cell $^{1}H^{15}N$ HSQC (Binolfi *et al.*, 2012) and other NMR experiments confirm that alpha-synuclein is monomeric and unstructured in non-neuronal as well as neuronal cells. Under physiological conditions, it is N-terminal acetylated and the aggregation prone NAC region is shielded from the cytoplasm, preventing it from aggregation (Theillet *et al.*, 2016). Acetylation does not seem to influence the disordered structure, though slightly increases its helicity (Fauvet *et al.*, 2012; Kang *et al.*, 2012; Maltsev *et al.*, 2012).

The structure of the oligomers of an alpha-synuclein peptide (residues 36-55) has recently been solved by X-ray crystallography (PDB ID 5F1T and 5F1W). The peptide attains a β-hairpin structure, and three β-hairpins form a triangular trimer. Three trimers associate to form a basket shaped nonamer, and two nonamers associate to form octadecamers (Salveson *et al.*, 2016). A ribbon representation of the crystal structure of the alpha-synuclein octadecamer and nonamer are shown in Figure 2 A&B.

Amyloid fibrils of alpha-synuclein have also been studied. Like other amyloid fibrils, they possess a cross-β sheet structure, as revealed from solid state NMR studies (PDB 2N0A). The hydrogen bonded cross beta-sheet structure is protease resistant (Lv *et al.*, 2012). Figure 2C shows the structure of the amyloid fibril of full length alpha synuclein, displaying a Greek key topology (Figure 2D), formed from parallel beta sheets. An intermolecular salt bridge is observable between Glu 46 and Lys 80, that stabilizes the fibril (Figure 2D). The structure was also validated by microscopy and fibre diffraction (Tuttle *et al.*, 2016).

Amyloid fibrils bind dyes like THT, congo-red, and ANS (Nilsson, 2004). Figure 3A shows the changes in fluorescence intensity of an alpha-synuclein sample as a function of time, upon binding THT. Upon binding congo-red, a slight shift in absorbance maxima is observed. Figure 3B shows a difference spectrum for the changes in absorbance of alpha-synuclein as a function of time, upon binding congo-red.

Mechanism of alpha-synuclein aggregation

As alpha-synuclein has the propensity to aggregate naturally, understanding the conditions that favor or disfavor this process, and the molecular mechanism underlying its aggregation are of considerable interest. Long incubation times, low pH, and high protein concentration, are some of the conditions that promote aggregation (Hashimoto *et al.*, 1998). Nucleation dependent chain polymerization occurs, converting the soluble unstructured monomeric species into a partially soluble oligomeric nuclei (the rate limiting step), followed by rapid elongation and assembly into insoluble mature fibrils (Tappel and Tappel, 2004; Pallito and Murphy, 2001). The formation of protofibrils is the intermediate step, which is followed by the

formation of amyloid fibrillar aggregates. Another mechanism of fibril formation can occur in a nucleation independent manner, by lateral association of the preformed granules, simultaneously inducing structural distortion of preformed structures (Bhak *et al.*, 2009; Wood *et al.*, 1999). Dynamic association of oligomeric granules occurs to form fibrils.

Alpha-synuclein mutants reported in synucleinopathies show remarkable differences in their aggregation kinetics *in vitro*, influencing two steps in particular, either nuclei formation or

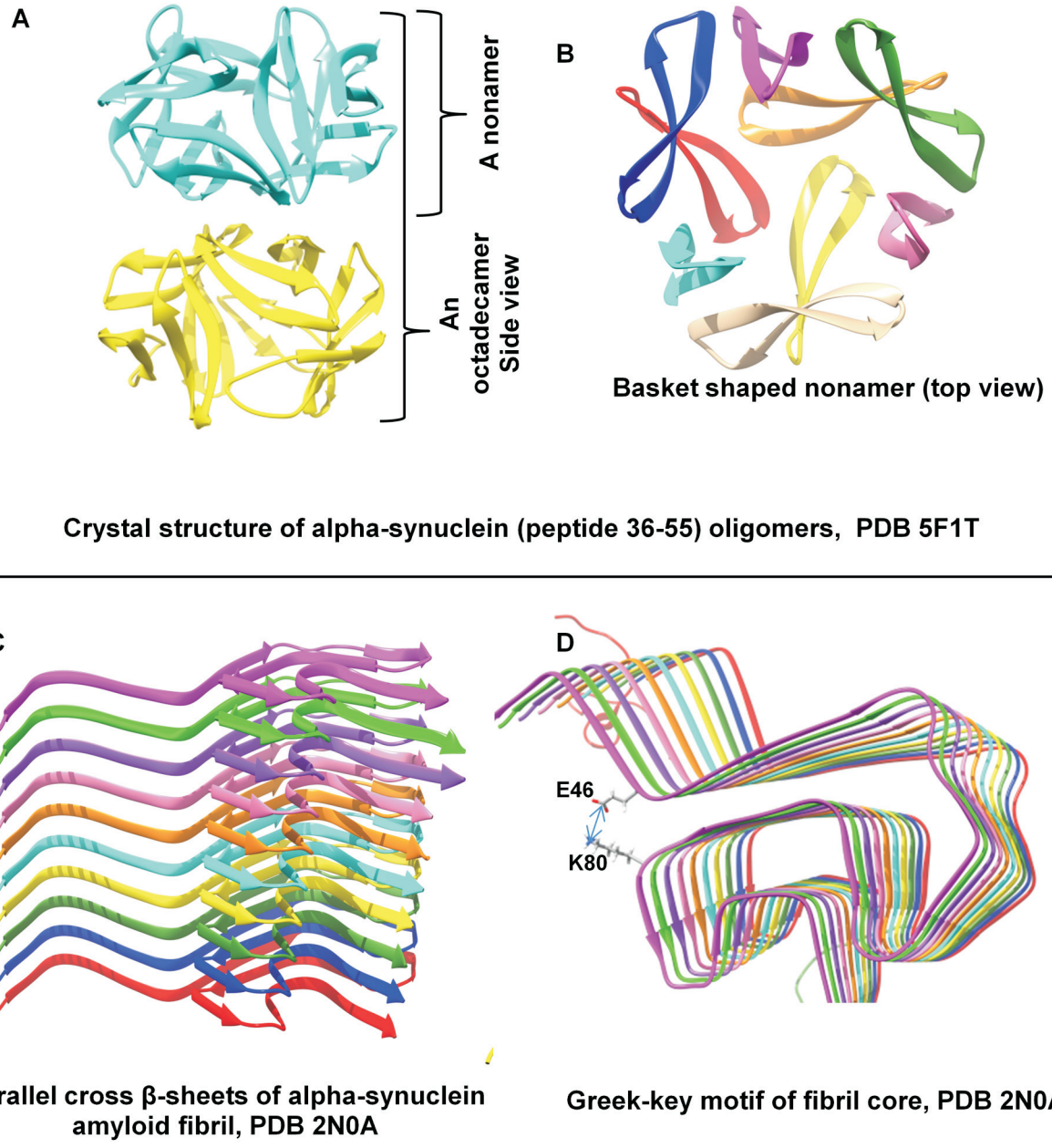

Figure 2: **A ribbon representation of A) the alpha-synuclein octadecamer B) nonamer, formed from the peptide fragment 36-55 (PDB 5F1T). C) The solid state NMR structure of the amyloid fibril of alpha-synuclein (PDB 2N0A). D) The amyloid fibril of alpha-synuclein displaying Greek motif.**

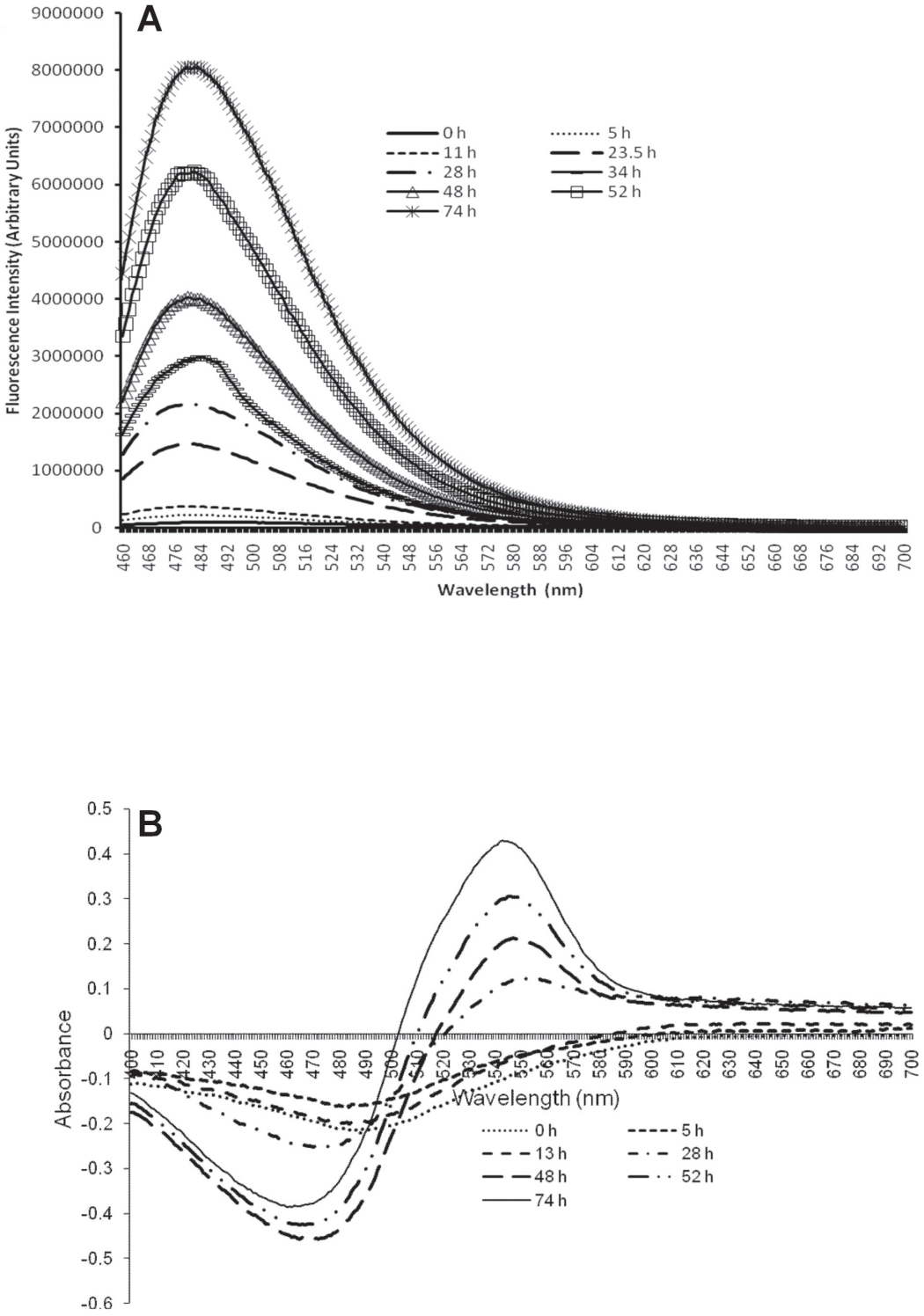

Figure 3: **Alpha-synuclein aggregation measured by following the changes in A) Thioflavin T fluorescence. Alpha-synuclein aggregation was performed by incubating α-synuclein (3 mg/ml) in phosphate buffer at 37 °C for 200 rpm. 50 µl was withdrawn at regular intervals and diluted to 200 µl with phosphate buffer. 10 µl of 200 µM Thioflavin T was added in the protein sample, and fluorescence was measured by exciting at wavelength 440 nm. Excitation slit width and Emission slit width was kept at 5 nm and 10 nm, respectively. B) Congo red absorbance. Congo red (7 mg/ml) was prepared in 5 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4. A 5 µl sample of 7 mg/ml Congo red was taken and diluted to 200 µl with potassium phosphate buffer, and scanned between 400 to 700 nm. To this solution, 10 µl of alpha-synuclein (3 mg/ml) incubated at 37°C, 200 rpm was added. At regular intervals, protein sample was withdrawn and congo red absorbance was measured. Absorbance peak at 540 nm is characteristic of amyloid formation.**

fibril growth (Ono *et al.*, 2011). The mutations A53T and E46K form fibrils faster than the wild-type (WT) protein, and A30P forms slowly, despite the rapid formation of smaller oligomeric species by this mutant (Bruinsma *et al.*, 2011; Conway *et al.*, 1998, 2000). Rate of nucleation, as opposed to the rate of elongation is responsible for the differences in fibrillation kinetics.

Aggregation can be induced by a variety of biological and environmental factors. Lipid membranes, low pH (Lv *et al.*, 2016), SDS (Basak *et al.*, 2015), Fe_3O_4 particles (Joshi *et al.*, 2015), molecular crowding, metals and pesticides can induce aggregation. Nitration, which leads to formation of di-tyrosine bonds *via* the oxidation of tyrosine residues, can induce stable aggregates (Souza *et al.*, 2000). A direct link has been observed between heavy metal concentration in the environment and neurodegenerative diseases. Therefore, metals such as manganese, copper, lead, iron, mercury, zinc, and aluminum were screened for their role in aggregation, and most of them were found to promote aggregation (Uversky *et al.*, 2001; Yamin *et al.*, 2003). Iron exists at high concentrations in substantia nigra, and ferrous iron catalyzes the formation of insoluble alpha-synuclein aggregates with amyloidogenic properties in the presence of hydrogen peroxide by fenton reaction *in vitro* (Hashimoto *et al.*, 1999; Hare and Double, 2016). Interaction of metals with alpha-synuclein has been discussed separately (Santner and Uversky, 2010).

Pesticide exposure has also been associated with increased risk for Parkinson's Disease (Caudle *et al.*, 2012; Noyce *et al.*, 2012). Pesticides such as paraquat, rotenone and menab can accelerate the rate of alpha-synuclein fibril formation *in vitro*. Occupational exposure to organophosphorous insecticide like chlorpyrifos, has also been shown to increase blood alpha-synuclein expression (Searles *et al.*, 2015). Presumably, the hydrophobic nature of the pesticides stabilizes the partially folded conformation of alpha-synuclein *via* binding to the non-polar patches of the latter (Uversky *et al.*, 2002a).

Various post-translational modifications have also been shown to enhance aggregation in alpha-synuclein (Xuan *et al.*, 2016). Phosphorylation has been observed in 90% of alpha-synuclein found in Lewy bodies. It has been observed at several positions; S87, Y125, S129, Y133 and Y135 (Lu *et al.*, 2011). S129 phosphorylation has been observed in alpha-synuclein isolated from the post-mortem brain tissue of Parkinson's disease patients (Anderson *et al.*, 2006; Oueslati, 2016). Approximately 90% of alpha-synuclein in Lewy body is serine-129 phosphorylated, suggesting that serine-phosphorylated cytoplasmic species is susceptible to pathological alteration and aggregation. Upregulated levels of S87 are also associated with other synucleinopathies (Paleologou *et al.*, 2010), while Y125, Y133 and Y135 have been inversely correlated with aggregation (Bill *et al.*, 1989). Other post-translational modifications are oxidation, ubiquitination, truncation and splicing (Beyer and Ariza, 2012; Oueslati *et al.*, 2010). These modifications not only affect the aggregation of alpha-synuclein, but also its charge, oligomer conformation, and binding to lipids or other biomolecules. Because of its chimeric nature, alpha-synuclein has also been exploited for metal detection (Lee *et al.*, 2011), and controlled drug delivery (Lee *et al.*, 2014).

As alpha-synuclein binds lipids, studies have been conducted to understand its aggregation behavior in their presence. Membrane-bound alpha-synuclein aggregates faster than unassociated form, and can further seed the aggregation of monomers (Lee *et al.*, 2002). Prevention of aggregation upon membrane binding has also been studied (Narayanan and Scarlata, 2001). The results are mixed on the interaction of lipids with alpha-synuclein and their effect on aggregation. Various factors seem to play, *i.e.* membrane composition, its curvature, aggregation state of alpha-synuclein, and experimental conditions. Lipid bilayers are disrupted upon binding of oligomeric form of alpha-synuclein. The formation of annular-shaped protofibrils (ring, spherical or tubular) can create pores in the cell-membrane as a result of which, cellular contents can leak, intracellular levels of potential cytotoxins, calcium and dopamine are elevated (Volles *et al.*, 2001). Amyloid pore-channel hypothesis states that the annular protofibrils get embedded in the lipid bilayers forming channels (Lashuel *et al.*, 2002).

Another school of thought believes that rearrangement of the protein in the lipid bilayer results in the disruption of lipid packing (Stockl *et al.*, 2013). The A30P mutant of alpha-synuclein has less lipid affinity, and binds membranes weakly (Jo *et al.*, 2002; Kruger *et al.*, 1998).

Dopamine neurotransmitter can also alter the kinetics of alpha-synuclein aggregation. Dopamine modifies the aggregation of alpha-synuclein, and oligomeric aggregates are observed. It mediates its effect *via* its oxidation product, dopaminochrome that interacts with the 125YEMPS129 motif of alpha-synuclein (Norris *et al.*, 2005). Similarly, another study has shown that DOPAC, the oxidation product of dopamine, inhibits fibril formation by binding non-covalently, and stabilizing the protofibrils or oligomers (Zhou *et al.*, 2009).

β-synuclein and γ-synuclein, other two members of alpha-synuclein family can also inhibit the aggregation of alpha-synuclein *in vitro* (Uversky *et al.*, 2002b) by oligomer stabilization, blocking the inter-conversion to fibrils, and promoting the amorphous aggregates. These results motivate researchers to explore β-synuclein and γ-synuclein based gene therapy as therapeutics (Hashimoto *et al.*, 2004; Shaltiel-Karyo *et al.*, 2010). FK506 binding proteins and peptide prolyl isomerases (PPIases) have been shown to induce aggregation of alpha-synuclein by acting on the prolines of the C-terminal region (Gerard *et al.*, 2006). Mutation of these prolines to alanine resulted in the loss of aggregation inducing ability of the molecules (Meuvis *et al.*, 2010).

Hexane induced fast formation of fibrils from granules has also been observed. Approximately 3% hexane leads to amyloid fibril formation, while 10% to amorphous adduct formation (Bhak *et al.*, 2009). Hexane or shear force induced structural rearrangement of granules can lead to fast fibril formation.

Toxicity

Alpha-synuclein transforms into a neurotoxic species in the presence of metals, dopamine and its metabolites, oxidative stress, endogenous toxins, and mitochondrial insufficiency (Rajagopalan and Andersen, 2001; Winklhofer *et al.*, 2008). Tissue transglutaminase induced cross-linking of alpha-synuclein leads to the formation of homopolymers or heteropolymers, which are assumed to be responsible for the insolubility of Lewy bodies (Segers-Nolten *et al.*, 2008). The long standing notion that the suprastructured fibrillar amyloid form is toxic to the cell is beginning to fade, and has been superseded by the concept that the amyloid form is non-toxic, neuroprotective and is formed to evade the toxic oligomeric form. The alpha-synuclein protofibrils are capable of disrupting membrane bilayers and induce toxicity. Identification of ubiquitin in Lewy bodies hints towards the possible role of ubiquitin-proteasome system in disease pathology. Impairment of degradation of alpha-synuclein by proteasome system could lead to the accumulation of alpha-synuclein (McNaught *et al.*, 2002). Association of duplication and triplication of alpha-synuclein with familial Parkinson's disease suggests that toxicity is to some extent also associated with the abundance of alpha-synuclein (Singleton *et al.*, 2003).

In contrast to the studies mentioned above, loss of alpha-synuclein also leads to neurotoxicity. There are reports suggesting that both mRNA and protein from the mutant allele are down-regulated, which leads to haploinsufficiency (Kobayashi *et al.*, 2003). Alpha-synuclein has been considered a tyrosine hydroxylase inhibitor, which causes an increase in the dopamine levels and associated toxicity. The loss of alpha-synuclein also results in an increase in phospholipase D2 activity, that hydrolyzes membrane phosphatidylcholine, thereby increasing membrane permeability.

Inhibitors against alpha-synuclein aggregation

Alpha-synuclein is an attractive target for inhibitor design, owing to the synucleinopathies induced by it. Thus, inhibitors are being designed against the (i) monomers (ii) intermediate oligomeric aggregates to convert them into nontoxic, off pathway aggregates, and (iii) fibrillar forms (Sultana *et al.*, 2011). Seed clearance occurs through the prevention of the formation of partially folded alpha-synuclein. â-synuclein and FKBP inhibitors act in a similar manner to inhibit the aggregation of alpha-synuclein (Gerard *et al.*, 2006; Hashimoto *et al.*, 2004).

Antibodies can be used to selectively target the protein. Monoclonal antibodies against the C-terminus of alpha-synuclein have been shown to be effective in clearing alpha-synuclein and improve the symptoms in Lewy body disease models (Masliah *et al.*, 2005; Masliah *et al.*, 2011). Specific antibodies have been designed which can prevent aggregation at lower concentrations, unlike normal antibodies. In this case, the antibodies were designed by grafting small amyloidogenic part of alpha-synuclein sequence (69-78) into the complementarity-determining regions of a single-domain (VH) antibody, called Grafted Amyloid-Motif Antibodies (gammabodies) (Ladiwala *et al.*, 2012). A vaccine has been successful in the phase I clinical trials *i.e.* AFFITOPE vaccine candidate PD01, which could help lower the level of alpha-synuclein levels by immunogenic response (Schneeberger *et al.*, 2012). This vaccine is made of a peptide carrier, that induces antibody that specifically recognizes alpha-synuclein. As Parkinson's disease is associated with the elevation of iron levels, deferiprone, iron chelators have also been developed (in phase II clinical trial, clinicaltrials.gov identifier: NCT00943748, NCT01539837, NCT00907283) (Mounsey and Teismann, 2012). Hairpin peptide, which has cross strand Trp-Trp and Tyr-Tyr pairs at non-H-bonded strand sites prevent alpha-synuclein aggregation by stimulating the formation of nontoxic aggregates. However, these peptides face significant challenges of crossing the blood-brain barrier (Huggins *et al.*, 2011). Modulation of oligomers to enhance Lewy body formation is also one of the strategies as the oligomeric form is toxic (Beyer and Ariza, 2008).

Chaperones such as heat shock proteins can also modulate the aggregation of alpha-synuclein. Alpha B-crystallin, a heat shock protein found in Lewy body inhibits fibril formation by interacting with the fibril and oligomeric species (Waudby *et al.*, 2010). Hsp70, another heat shock protein acts on the prefibrillar form of alpha-synuclein when incubated *in vitro*. (Dedmon *et al.*, 2005a). Similarly, small heat shock proteins (sHsp) Hsp27, Hsp20, HspB8, and HspB2B3 were tested *in vitro* for their ability to inhibit the aggregation of alpha-synuclein. All sHsps interact with the monomeric alpha-synuclein transiently (Cox *et al.*, 2014). HspB2B3 can inhibit aggregation of wild type and the A30P mutant, but not of E46K and A53T, suggesting that it inhibits the aggregation of slowly aggregating proteins. On the contrary, Hsp20 reduced fibril formation of only E46K and A53T, but not that of wild type and A30P (Bruinsma *et al.*, 2011). Another small heat shock protein, Hsp27 reduces the aggregation of alpha-synuclein *in vivo* (Outeiro *et al.*, 2006; Huggins *et al.*, 2011). Hsp90 inhibitor, which upregulates Hsp70 levels, also prevents alpha-synuclein oligomerization and associated toxicity (Putcha *et al.*, 2010). However, Hsp104 had deleterious effects on yeast cells expressing wild type and A53T mutant cells (Gade *et al.*, 2014). A recent review discusses the role of heat shock proteins in preventing alpha-synuclein associated toxicity in detail (Jones *et al.*, 2014).

Various peptides of alpha-synuclein have also been employed to strategically inhibit alpha-synuclein aggregation. Fragment 68-75, in which Gly73 was replaced with sarcosine (N-methyl glycine) to make it N-methylated peptide was used (Bodles *et al.*, 2004). Since N-methylated peptides do not form beta-strands and disturb aggregation, this peptide along with non-methylated peptide was able to prevent fibril formation and reduce toxicity. Synuclein peptides that bind strongly, and the N-terminal region from NAC region (after modification of hydrophilic residues at both N-terminal and C-terminals) were able to inhibit alpha-synuclein aggregation (Paleologou *et al.*, 2005). Similarly, a peptide fragment of residues 77-82 (VAQKTmV), where valine was N-methylated, inhibited alpha-synuclein aggregation (Madine *et al.*, 2005). Interestingly, incubation of any self-fibrillation defective alpha-synuclein mutant such as V66S, V66P, T72P, V74E, V74G and T75P to wild type alpha-synuclein led to the inhibition of aggregation (Koo *et al.*, 2009). A peptide PGVTAV, which can inhibit the aggregation of alpha-synuclein *in vitro* (Kim *et al.*, 2009), also inhibited toxicity in cell-lines (Choi *et al.*, 2011).

A variety of small natural and synthetic chemicals have also been used to inhibit the aggregation of alpha-synuclein (Masuda et al., 2006). Polyphenol compounds baicalein, delphinidin, dopamine chloride, epigallocatechin

gallate, exifone, (-)-gallocatechin, (-)-gallocatechin gallate, gossypetin, hinokiflavone, hypericin, procyanidin B1, procyanidin B2, rosmarinic acid and theaflavine strongly inhibit alpha-synuclein aggregation with IC50 value less than 10 μM. Other compounds such as the porphyrin ferric dehydroporphyrin IX, Congo red, and its derivative 1-bromo-2,5-bis(3-carboxystyryl) benzene (BSB), vitamin E (α-tocopherol) also exhibited a significant reduction of alpha-synuclein aggregation. Majority of compounds belonging to class polyphenols, phenothiazines, polyene macrolides (antifungal antibiotics), porphyrins, and rifamycin inhibited amyloid fiber formation (Masuda et al., 2006; Shahpiri et al., 2016). Studies on polyphenols have also reported that two adjacent phenolic –OH groups are required for the inhibition of alpha-synuclein filament *via* covalent modifications (Conway *et al.,* 2001). Phenothiazine derivatives have been synthesized and optimized to find the potent molecule which can inhibit the aggregation of alpha-synuclein (Yu et al., 2012). Entacapone and Tolcapone drugs, that function as catechol-O-methyl transferase (COMT) inhibitors in Parkinson's disease also worked as aggregation inhibitors (Di Giovanni *et al.,* 2010). When these drugs were tested with other catechol containing compounds such as dopamine, pyrogallol, gallic acid, caffeic acid and quercetin; due to the common catechol moiety, they displayed anti-amyloidogenic activity. The drugs act on the prefibrillar state, and convert it into off-pathway non-toxic aggregate (Di Giovanni *et al.,* 2010).

The polyphenol (-)-epigallocatechin gallate (EGCG) present in green tea binds the monomeric alpha-synuclein and modulates its aggregation by converting it into nontoxic, off-pathway aggregate (Ehrnhoefer *et al.*, 2008). Similarly, black tea has some components such as theaflavins, which can also modulate aggregation by converting it into nontoxic aggregates (Grelle *et al.*, 2011). Congo red and lacmoid, phenothiazine derivatives also inhibit aggregation by interacting with the N-terminal and the middle region of alpha-synuclein, thereby preventing self-association (Lendel *et al.*, 2009). Tetrapyrrole phthalocyanine tetrasulfonate and its metal substituted derivatives inhibit alpha-synuclein aggregation by specifically interacting with aromatic residues of the protein (both aromatic rings of phthalocyanine moiety and peripheral negatively charged tetrasulfone group) (Lamberto *et al.*, 2011). Effect of various Vitamin K forms phylloquinone (K1), menaquinone (K2) and menadione (K3) on the aggregation of alpha-synuclein was followed, and the moiety 1,4-napthaquinone was found responsible for the prevention of aggregation. It interacts with Gly31 and Lys32 of the N-terminal domain of alpha-synuclein, and produces amorphous aggregates (da Silva *et al.*, 2013).

Gallic acid, present in the grape skin extract, gallnuts and tea leaves, also inhibits the aggregation of alpha-synuclein by stabilizing the monomer as confirmed by NMR and ion-mobility mass spectroscopy techniques (Liu *et al.*, 2014). A detailed mechanistic study shows that apart from inhibition of fibrillation, it can disaggregate preformed fibrils. Gallic acid was found to bind the soluble non-toxic oligomers of alpha-synuclein. Structure-activity relationship studies suggest that the presence of three vicinal hydroxyl groups or three homo vicinal groups with one-hydroxy group at the 4^{th} position makes the compound capable of inhibiting alpha-synuclein fibrillation (Ardah *et al.*, 2014). Cinnamon extract is another natural compound that inhibits both oligomeric and fibril form of alpha-synuclein, depending upon the concentration used (Shaltiel-Karyo *et al.*, 2012). Lysine coated Fe_3O_4 nanoparticles were observed to inhibit the early events of alpha-synuclein aggregation, in contrast to the bare Fe_3O_4 nanoparticles that increased the rate of aggregation (Joshi *et al.*, 2015).

Polyamidoamine (PAMAM) dendrimer, consisting of an ethylenediamine core and branched units derived from methyl acrylate and ethylenediamine can prevent the aggregation of alpha-synuclein, by a mechanism that remains unclear (Milowska *et al.*, 2011). Fusion of napthaquinone and tryptophan analogues, as both compounds are inhibitors of amyloid formation inhibited the aggregation of alpha-synuclein (Scherzer-Attali *et al.*, 2012). Mannitol, a sugar derivative, has also been found to prevent the aggregation of alpha-synuclein. Being a blood-brain-barrier breaker, it has the potential for use in therapeutics and is protective in

Drosophila and the mouse model of Parkinson's disease (Shaltiel-Karyo *et al.*, 2013). Curcumin and its analogues are excellent amyloid aggregation inhibitors (Marchiani *et al.*, 2013; Singh *et al.*, 2013). Rifampicin has been recently shown to be effective in reducing neurotoxic oligomers (Umeda *et al.*, 2016). The use of molecular tweezer CLR01, to prevent alpha-synuclein from clumping or aggregation and to break toxic aggregates, is yet another approach being used (Prabhudesai *et al.*, 2012).

Conclusions

Various studies conducted so far suggest that alpha-synuclein aggregation is a complex process, influenced by a variety of cellular, as well as environmental factors. Molecular details pertaining to how this process occurs has been uncovered by a plethora of techniques in the past two decades. Majority of the research work considers the oligomeric species as the primary cause of alpha-synucleinopathies. Molecules ranging from antibodies, peptides, to small molecules, have been tried and tested, to halt/slow down the aggregation process. Most of the molecules are non-specific aggregation inhibitors, while a very few are specific. Apart from antibody based vaccine, hardly any other vaccine/inhibitor specific for alpha-synuclein has reached the clinical trials. Thus, there is need for more in depth studies, to fully understand the mechanism of aggregation, which would help in identifying targets/epitopes, useful pharmacophores, and consequently more effective medicine.

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