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# **Amyloid Oligomers: A Joint Experimental/Computational Perspective on Alzheimer's Disease, Parkinson's Disease, Type II Diabetes and Amyotrophic Lateral Sclerosis**

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## **ABSTRACT**

Protein misfolding and aggregation is observed in many amyloidogenic diseases affecting either the central nervous system or a variety of peripheral tissues. Structural and dynamic characterization of all species along the pathways from monomers to fibrils is challenging by experimental and computational means because they involve intrinsically disordered proteins in most diseases. Yet understanding how amyloid species become toxic is the challenge in developing a treatment for these diseases. Here we review what computer, *in vitro*, *in vivo* and pharmacological experiments tell us about the accumulation and deposition of the oligomers of the (A $\beta$ , tau),  $\alpha$ -synuclein, IAPP and superoxide dismutase 1 proteins, which have been the mainstream concept underlying Alzheimer's disease (AD), Parkinson's disease (PD), type II diabetes (T2D) and amyotrophic lateral sclerosis (ALS) research, respectively for over many years.

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## **1. Introduction**

The self-assembly of intrinsically disordered proteins (IDPs) into extracellular or intracellular transient oligomers and amyloid fibrils is shared by many human diseases, including Alzheimer's (AD) and Parkinson's (PD) diseases, type II diabetes (T2D), amyotrophic lateral sclerosis (ALS) and prion diseases.<sup>1</sup> It is estimated that there about 50 and 10 million people worldwide living with AD and PD, respectively, and it was recently reported that AD can also be found in old chimpanzees.<sup>2</sup> As citizens, we do not have an idea of its worldwide financial cost, but many of us know exactly the suffering for the patients and their families.

The history of senile dementia is very rich, dating back to the Greco-Roman period.<sup>3</sup> However, AD was first described in 1906 by the German doctor Alois Alzheimer, PD was first described in 1817 by the English doctor James Parkinson, the French medical Professor Etienne Lancereaux is the pathfinder of T2D in 1877, and ALS also named "maladie de Charcot" or "Lou Gehrig's disease (American baseball player)" was first described by Jean-Martin Charcot in Paris in 1874.

The key misfolded proteins are clearly identified for each disease: the A $\beta$  protein (A $\beta$ 40 and A $\beta$ 42 with 40 and 42 amino acids) and the tau protein ranging from 352 to 421 amino acids in AD, the 140 amino acid  $\alpha$ -synuclein ( $\alpha$ S) protein in PD, the islet amyloid polypeptide (IAPP) or amylin of 37 amino acids in T2D, and the superoxide dismutase 1 of 32kDa (SOD1), TAR DNA binding protein 43 (TPD-43) and 526 amino acid fused in sarcoma protein (FUS) in ALS. The first authors to correlate A $\beta$  and  $\alpha$ S proteins to AD and PD, were Glenner and Wong,<sup>4</sup> Goldberg and Lansbury,<sup>5</sup> and Hardy and Selkoe,<sup>6</sup> and the implications of the other proteins are reviewed in Refs. 7-9. Their sequences do not have any homology and are very diverse in length, yet they all share the ability to form amyloid deposits or inclusions in the brain or the tissue (T2D) of patients, the exception being the wild-type SOD1 protein, but the monomeric apo-SOD1 in its disulfide-reduced state forms fibrillar aggregates under near quiescent conditions<sup>10</sup>.

*In vitro*, the A $\beta$ , tau,  $\alpha$ S, IAPP, TDP-43 and FUS proteins form readily cross- $\beta$  structures with an aggregation kinetics profile typically displaying a sigmoidal curve where the proteins assemble into oligomers (lag-phase) prior to fibril elongation (growth phase), and a plateau where the fibrils and free monomers are in equilibrium (saturation phase), as shown in many reviews <sup>11,12</sup>. Amyloid fibrils display an intermolecular hydrogen-bond (H-bond) network parallel to the fibril axis. The molecular mechanisms leading to amyloid fibrils are well described by primary nucleation, (fragmentation and surface-catalyzed) secondary nucleation and elongation growth mechanisms as shown elsewhere.<sup>13</sup> The aggregation kinetics and the lifetimes of the heterogeneous conformations of all oligomers along the amyloid fibril formation pathways are very sensitive to the amino acid length (e.g., A $\beta$ 42 vs. A $\beta$ 40) and genetic risks, including several mutations in A $\beta$ ,  $\alpha$ S and SOD1 proteins and one unique mutation in IAPP, the level of hyperphosphorylation in tau and SOD1, and acetylation and glycosylation in tau. Experimental conditions modulate the self-assembly process, such as pH, T, peptide concentration, external applications resulting from agitation, electric field and shear forces, and the presence of membrane, metal ions, crowding and heparin (for tau, in particular).<sup>14-16</sup>

Until 20 years ago, it was believed that the ability to form amyloid fibrils was restricted to a few proteins involved in diseases. However, there have been many more recent reports that non-disease proteins, short peptides and even single amino acid homopolymers can form fibrils under appropriate conditions,<sup>17,18</sup> and changing experimental conditions leads to nanotubes and ordered nanomaterials.<sup>19,20</sup> Many studies have indicated the strategies and the selection pressures that protein sequences (either alone or helped by chaperones) have followed to avoid undesired aggregation, to adjust the kinetic and thermodynamic stability of their well folded three-dimensional (3D) structures and to optimize the efficiency of their folding pathways <sup>21</sup>.

However, the emergence of amyloid folds is not a surprise for several reasons. They allow a variety of functional roles in both prokaryote and eukaryote organisms.<sup>22</sup> Amyloids are able to replicate and catalyze their own formation, transmit information and provide a scaffold for chemical reactions (e.g., ester hydrolysis) and enzyme-like activities <sup>23-25</sup>. Even under early earth (prebiotic) conditions, peptides can form amyloid fibrils leading to the current amyloid world hypothesis in the origin of life <sup>26,27</sup> and the possibility that all globular protein structures may have originated from amyloid fibrils. <sup>28</sup>

While SOD1 is a globular protein with a well-defined 3D structure, the A $\beta$ , tau and  $\alpha$ -synuclein proteins belong to the class of intrinsically disordered proteins (IDPs). IDPs are also known to play a critical role in many cellular functions such as signal transduction, cell growth, binding with DNA and RNA, and transcription, and are implicated in the development of cardiovascular problems and cancers

<sup>29</sup>. The IDPs involved in neurodegenerative diseases have a few aggregation-prone regions and overall all IDPs have a low mean hydrophobicity and a high mean net charge <sup>30</sup>.

IDPs are structurally flexible and lack stable secondary structures in aqueous solution. When isolated, they behave as polymers in a good solvent and their radii of gyration are well described by the Flory scaling law.<sup>31</sup> The insolubility and high self-assembly propensity of IDPs implicated in degenerative diseases have prevented high-resolution structural determination by solution nuclear magnetic resonance (NMR) and X-ray diffraction experiments. Local information at all aggregation steps can be, however, obtained by chemical shifts, residual coupling constants, and J-couplings from NMR, exchange hydrogen/deuterium (H/D) NMR, Raman spectroscopy; and secondary structure from fast Fourier infrared spectroscopy (FTIR) or circular dichroism (CD). Long-range tertiary contacts can be deduced from paramagnetic relaxation enhancement (PRE) NMR spectroscopy and single molecule Förster resonance energy transfer (sm-FRET), and short-range distance contacts can be extracted by cross linked residues determined by mass spectrometry (MS). Low-resolution 3D information of monomers and oligomers can be obtained by ion-mobility mass-spectrometry data (IM/MS) providing cross-collision sections, dynamic light scattering (DLS), pulse field gradient NMR spectroscopy and fluorescence correlation spectroscopy (FCS) providing hydrodynamics radius, small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS), atomic force microscopy (AFM) and transmission electron microscopy (TEM) providing height features of the aggregates, as reported by some of the first and recent applications of these methods to IDPs <sup>32-38</sup>. However, the information obtained from most experimental observables represents an average over the free energy landscape and gives time- and space-averaged properties. Experiments can also lead to different values of properties, for example the radius of gyration ( $R_g$ ) as a result of the equilibrium between the monomeric and multimeric states of the IDPs under the conditions used. Fibril structures of long amyloid proteins are mainly proposed based on solid-state NMR (ssNMR) with the first high resolution structure of HET-s(218-289) prion<sup>39</sup> and on cryo-electron microscopy (cryo-EM) experiments.<sup>40,41</sup> Fibril structures of short amyloid peptides were also determined by X-ray diffraction analysis.<sup>42</sup>

Computer simulations at different time and length scales can in principle provide the dominant microstates of IDPs using multiple sampling techniques and various representations ranging from all-atom and coarse-grained (CG) to mesoscopic models <sup>12,14,43-45</sup>. However, they are limited by the accuracy of the force field and the size of the energy landscape to be explored. Even on the fastest Anton computer, the simulation time using molecular dynamics (MD) simulations does not exceed 1

ms for a monomeric protein of 76 amino acids<sup>46</sup>, i.e., several orders of magnitude less than the time scales of hours and days required for fibril formation *in vitro* at a  $\mu\text{M}$  concentration<sup>11</sup>.

The most currently accepted hypothesis is that accumulation of oligomers of the key proteins is the primary cause of AD, PD, T2D, and ALS diseases and initiates a series of events leading to neuronal or tissue death, a view pioneered by Golberg and Lansbury<sup>5</sup> and reviewed more recently<sup>47-50</sup>. There is also growing evidence from *in vivo* and *in vitro* studies of co-occurring pathologies across common neurodegenerative diseases,<sup>51</sup> indicating cross-talk between the amyloid proteins and interactions and cross-seeding between the  $\text{A}\beta$ , tau and  $\alpha$ -synuclein proteins which promote aggregation, generate different strains and accelerate cognitive dysfunction<sup>52-54</sup>.

In summary, we provide an in-depth overview of our current knowledge on the biogenesis and domain organizations of  $\text{A}\beta$ , tau,  $\alpha$ -synuclein and IAPP related to their aggregation and binding properties, the molecular structures of amyloid monomers, oligomers and fibrils implicated in AD, T2D and PD from experiments and simulations, as well as the early and final aggregation steps using coarse-grained simulations. We will not provide all the answers to the questions that we are facing, nor describe all the protein cellular partners interacting with these amyloid proteins, but we will discuss what experiments and simulations tell us about the role of liquid-liquid phase separation, the effect of effect of crowding and shear flow, and the role played by the cell membranes and the Zn and Cu metal ions on protein aggregation. Next, we discuss what we know about ALS etiology, and present a pharmacological perspective to cure these diseases considering small compounds, antibodies or physical methods. This is followed by recent findings on crosstalk between amyloid proteins from *in silico* to *in vivo* experiments. We conclude with a series of unanswered questions that can potentially be handled by simulations and experiments, discuss the alternative hypotheses to amyloid oligomers causing human diseases and and list future directions.

## **2. $\text{A}\beta$ biogenesis and domain organizations of tau, $\alpha$ -synuclein and IAPP**

### **2.1. $\text{A}\beta$ biogenesis: role of pathogenetic and protective mutations, membrane composition, and detailed interaction with $\gamma$ -secretase (gS)**

#### **Generation of $\text{A}\beta$ peptides by gS**

It is established that amyloid  $\beta$  protein ( $\text{A}\beta$ ) contributes to the dysfunction and degeneration of neurons and the pathogenesis of AD.<sup>6,55-57</sup>  $\text{A}\beta$  is derived from the proteolytic cleavage of the single-pass transmembrane amyloid precursor protein (APP) by secretases and may be processed along non-amyloidogenic and amyloidogenic pathways. The non-amyloidogenic pathway involves initial



cleavage of APP by  $\alpha$ -secretase (aS) leading to formation of sAPP $\alpha$  and the 83 amino acid fragment APP-C83. The amyloidogenic pathway involves initial cleavage of APP by  $\beta$ -secretase (bS) leading to formation of sAPP $\beta$  and the 99 amino acid fragment referred to as CTF99, APP-C99, or simply C99. Cleavage of C99 by  $\gamma$ -secretase is the last step in the production of A $\beta$  (Figure 1).

Cleavage of C99 by gS lacks fidelity.<sup>58-60</sup> Cleavage of C99 by gS is initiated at the  $\epsilon$ -sites (gS endopeptidase activity) to produce the amyloid intracellular domain (AICD) and A $\beta$ 49 or A $\beta$ 48 peptides. After the first cut, tri- and tetra-peptides are generated from sequential cleavage (gS carboxypeptidase-like activity) until A $\beta$  proteins are released to the extracellular environment.<sup>61</sup> Cleavage of C99 results in a variety of A $\beta$  isoforms with A $\beta$ 40 being most prevalent and A $\beta$ 42 being a minor but more amyloidogenic form. Sequential cleavage by gS at points separated by roughly 0.5 nm, results in a specific isoforms of A $\beta$ . C99 is produced along two main cleavage lines A $\beta$ 49>46>43>40 and A $\beta$ 48>45>42>38 (Figure 2, panel 2D). The first is responsible for the release of the major isoform A $\beta$ 40 and the second leads to the minor isoforms A $\beta$ 42 and A $\beta$ 38.<sup>62</sup>

The observed infidelity in the cleavage of C99 may result from variation in the points of initiation and termination of cleavage, which in turn may be influenced by a variety of factors. Interestingly, gS is also known to process a wide variety of substrates<sup>63-66</sup> some of which are cleaved with high fidelity, suggesting that substrate sequence plays a primary role. A minimal model of the transmembrane domain of C99, Glu22-Lys55, is required for cleavage by gS, and importantly, negative charges on the extracellular side and positive charges on the intracellular side are required.<sup>67</sup> The polybasic regions on the C-terminal side of TM helices, are globally found in gS substrates<sup>68</sup> and are conserved in both C99 and the Notch family. MD simulations have shown that following the first cleavage step the substrate is pulled deeper into the binding cavity of PS1. Negative charges at the N-terminus are observed to remain in place during the processing process.<sup>69</sup> These observations support the conclusion that the N-terminal 21 amino acids of C99 are not required for gS cleavage.<sup>70</sup>

Evidence suggests that the mature gS complex is active at the plasma membrane and in endosomes,<sup>71-73</sup> and that cleavage of C99 by  $\gamma$ -secretase most commonly occurs when the enzyme and the substrate are colocalized in cholesterol-rich lipid raft domains. Therefore, while an important contributing factor to AD is mutation of APP or gS, alterations in the lipid membrane environment in which APP and gS are embedded also plays a critical role. The actual products of APP cleavage depend on the specific membrane environment.<sup>74</sup>

While A $\beta$  has long been implicated as a pathogenic agent in AD, the molecular mechanism by which A $\beta$  induces neuronal dysfunction largely remain a mystery.<sup>75-79</sup> One focus has been to characterize the structural ensembles of A $\beta$ 40 and A $\beta$ 42 monomers using experimental NMR and computational studies<sup>80,81</sup> with an eye on identifying aggregation prone structures termed N\* states.<sup>82</sup> Given the role of the monomer ensemble in identifying aggregation prone sequences (N\* conjecture), one promising putative mechanism for A $\beta$  cytotoxicity involves the binding of A $\beta$  oligomers with cellular prion protein PrP<sup>C</sup><sup>83</sup> leading to activation of a kinase and the abnormal phosphorylation of tau (see Section 2.2).

### **Impact of disease-causing mutations on APP and gS**

APP pathogenic mutations were the first to be recognized to cause early-onset AD which led to the amyloid hypothesis.<sup>84-87</sup> APP mutations are observed to be clustered near the aS, bS, and gS proteolytic cleavage sites and can be categorized according to location in the APP structure, including mutations located in the (1) APP extracellular region and (2) APP transmembrane fragment N-terminal to the A $\beta$ 42 cleavage site that typically have little effect on C99 cleavage. In addition, (3) the transmembrane C-terminal fragment below the A $\beta$ 42 cleavage site is a hotspot of familial AD (FAD) mutation and has been proposed to be important as the main gS recognition site. This latter region has also been proposed to be the region that determines the A $\beta$ 42:A $\beta$ 40 ratio by distorting the relative efficiencies of the  $\epsilon$ -cleavage sites at C99 residues 48 or 49. Mutations that remove the positive charges from the invariant lysine or arginine residue located at the C99 TM junction greatly compromise the cleavage efficiency of gS.

### **Role of mutations to APP and gS**

The majority of pathogenic APP mutations decrease overall cleavage of C99 by gS, meaning that they are loss-of-function (LOF) mutations.<sup>68</sup> Interestingly, the protective Icelandic mutation A673T was discovered in an individual with Down Syndrome (DS) who did not develop early onset AD related degeneration. Individuals with DS carry an extra copy of chromosome 21 and therefore they also carry an extra copy of the gene for APP. This A $\beta$ -lowering APP mutation is located in the APP extracellular region, close to the bS cleavage site and has been identified in the Icelandic population.

PS1 pathogenic mutations modify substrate recognition, enzyme structure, or catalytic activity. These mutations result in partial or total loss-of-function of the enzyme. Before 2015, there was no structure available for gS. At this time, eight human gS structures have been determined by cryo-EM with a

resolution range between 2.6 and 4.4 Å. gS is a transmembrane protein with four components.<sup>88-90</sup> (1) Nicastrin (NCT) has been proposed to be in charge of substrate sorting, acting as a gatekeeper by sterically obstructing the access of proteins with large extracellular domains.<sup>91</sup> (2) Anterior pharynx defective-1 (APH-1A) is required for the gS complex stability. Computational studies showed that it contains a water cavity able to transport water and store cations.<sup>92</sup> (3) Presenilin (PS1) contains two aspartate residues that form the active site. (4) Presenilin enhancer 2 (PEN-2) is needed for the autocatalytic maturation.<sup>93,94</sup> The recognition of APP by gS is illustrated in Figure 2A.<sup>95</sup> 180 mutations occurring in the PS1 subunit have been linked to familial AD.

Pathogenic PSEN-FAD mutations may affect gS endopeptidase activity (and the initial  $\epsilon$ -cleavage site) and consistently impair the carboxypeptidase-like efficiency. This results in reduced processivity of A $\beta$ 49 or A $\beta$ 48 and the release of longer and more toxic A $\beta$ 43 or A $\beta$ 42 isoforms. These mutations result in the partial or total loss-of-function of the gS enzyme. PS1 mutants are dispersed among the whole gS structure, but those located at the gS complex surface may point to a substrate interaction/recognition site (Figure 2A-2B).

While mutations to APP and gS mutations are known to modify C99 processing by gS, the details of how various mutations impact the cleavage process are still unknown. The dispersed distribution of the mutation sites suggests that there should be a variety of mechanisms. (1) Disturbed PS1/APP interactions<sup>96</sup> act by changing the preference of the initial position of the  $\epsilon$ -cleavage site (gS endopeptidase activity). It has been proposed that APP mutations modify the tilting of the TMD helix, thereby altering the presentation of substrate to the proteolytic enzyme changing the initial  $\epsilon$ -cleavage site. Alternatively, the mechanism of substrate docking and displacement toward the catalytic site may be impacted, as there is evidence that the substrate binding site is distinct from the catalytic active site. Once the substrate is docked it is subsequently displaced to the catalytic site of the enzyme.<sup>97</sup> (2) Loss of gS carboxypeptidase activity interferes with the catalytic efficiency, releasing premature intermediate and longer APP products.<sup>98</sup> (3) Inhibitory effects at the initial  $\epsilon$ -cleavage sites may lead to changes in the distribution of A $\beta$  isoforms critically impacting aggregation kinetics and toxic effects.<sup>99</sup> (4) Finally, catalytic cycle impairment may lead to alterations in the gS product lines.<sup>98</sup>

### **Role of membrane in A $\beta$ genesis and aggregation**

Early experimental<sup>63</sup> and computational<sup>100</sup> studies demonstrated that C99 consists of an extracellular region, including asparagine glycosylation sites, an extracellular juxtamembrane (JM) helical domain Q15-V21 (Q686-V695), an TM domain K28-K53 (K699-K724), and an intracellular

C-terminal domain in model membranes (Figure 2C). Using ssNMR experiments, Tycko and coworkers suggested that, for the construct containing 27 residues K28-K55 (K699-K726) in multilamellar vesicles, the TM domain of APP adopts a mixture of helical and non-helical structures<sup>101</sup>, which varies as temperature is altered. Smith and workers reported NMR and FTIR data of the wild type (WT) construct N1-K55 (N672-K726) and the Flemish mutant A21G (A692G).<sup>102</sup> They observed that at least part of the JM domain assumes a  $\beta$ -strand structure. Recent simulation results provide some support for this intriguing observation.<sup>103</sup>

Variations in sequence of C99 impact dimerization of the C99 TM domain, altering not only lateral mobility of the protein, but also its TM helical structure (tilt and kink), C99 dimer structure and stability, and structure of the TM helices within the dimer.<sup>100,104</sup> There is evidence that changes in the stability of the C99 dimer influence its cleavage by gS and the resulting overall level of A $\beta$  protein and its isoform distribution.<sup>101,104,105</sup> Multhaup *et al.* first recognized that modifications in sequence that reduced homodimer affinity impacted cleavage of C99 by gS.<sup>106</sup> Subsequent studies of homodimer formation in WT and mutant C99 congeners supported the view that C99 homodimerization is critical to C99 processing by gS and A $\beta$  formation.<sup>107,108</sup> However, it has been argued that C99 homodimerization is weak and may be largely irrelevant *in vivo*, suggesting that C99 monomer is the sole substrate for gS in the production of A $\beta$ <sup>109</sup>. There is little doubt that C99 homodimer is an essential species in the overall ensemble of C99 structures.<sup>107-111</sup> More recently, Sanders *et al.* reported an ensemble of coexisting C99 monomer, dimer, and large-scale oligomers in lipid bicelles.<sup>112</sup>

### **Role of membrane and cholesterol on APP**

The role of membrane and cholesterol on APP has been examined in computational studies of C99 monomer and dimer structure in membrane and micelle environments as a function of protein sequence and composition of the lipid environment.<sup>103,110,113,114</sup> Many of the observations derived from simulations have been validated using NMR experiments, including the existence of a flexible "hinge" region in the C99 monomer structure,<sup>113,114</sup> the first C99 homodimer structure in a bilayer environment,<sup>113</sup> and the ability to "environmentally select" C99 topologically distinct homodimer structures in membrane environments of varying lipid composition.<sup>114</sup>

Enhanced levels of cholesterol resulting from diet, genetic predisposition, or aging are positively correlated with early onset of AD.<sup>115-117</sup> A variety of theories have been proposed to explain these observations. Lower levels of cholesterol promote membrane fluidity and non-amyloidogenic cleavage of APP by aS.<sup>118,119</sup> In addition, decreased levels of cholesterol diminish both bS and gS activity and deplete cholesterol rich lipid raft microdomains deemed important for colocalization of gS and its

substrate C99.<sup>62,120,121</sup> Finally, site-specific binding of cholesterol to C99 protein has been observed. It has been proposed that elevated levels of cholesterol may increase the population of C99-cholesterol dimers,<sup>109,122</sup> thus enhancing the partitioning of C99 to lipid raft domains and the proteolytic cleavage of C99 by gS to produce A $\beta$ .

Membranes comprising these distinct cellular domains are composed of a mixture of lipids, including glycerolipids, sphingolipids, and cholesterol. The complex lipid mixtures are characterized not by a uniform mixture but by a heterogeneous mosaic of liquid-disordered regions and liquid-ordered microdomains of varying lipid composition, including regions rich in saturated lipids, sphingomyelin, and cholesterol referred to as detergent-resistant membranes or lipid rafts. Many studies<sup>120,123-127</sup> have proposed a role for raft domains in the biogenesis of A $\beta$ . However, their influence on the mechanism of creation of A $\beta$  is not understood.

Current evidence suggests that the mature gS complex is active at the plasma membrane and in endosomes. However, the actual product of APP cleavage depends on the specific membrane environment. In addition, there is substantial evidence that cleavage of C99 by gS most commonly occurs when the enzyme and substrate are colocalized in cholesterol-rich lipid raft domains.

The enzyme bS possess a single transmembrane anchoring domain. It has been observed in the plasma membrane and endosomes colocalized with APP in regions where the membrane is enriched in lipid raft domains. Like bS, APP possesses a single transmembrane domain and is found in a variety of locations in the cell including in lipid raft domains, colocalized with the enzymes bS and gS. It was recently proposed that a complex of  $\beta$ S and gS formed in cholesterol rich membrane domains might lead to substrate shuttling and enhanced efficiency in the biogenesis of A $\beta$ .<sup>128</sup> Taken together, these observations demonstrate the important role played by membrane spatial heterogeneity in A $\beta$  genesis.

### **Role of membrane on gS**

gS is accountable for the final step in the regulated intramembrane proteolysis of APP to generate A $\beta$ . As such, substantial attention has focused on understanding how the lipid environment modulates gS activity, including variations in the membrane lipid composition and the presence of cholesterol and sphingolipids.<sup>66,129</sup> Clinical and experimental studies suggest that lipid composition is altered in AD brain tissue<sup>130</sup> and that the production of A $\beta$  peptides varies with the membrane composition.<sup>131</sup> The mechanism explaining these observations and the exact cause or consequence have not been determined. Holmes *et al.* reported that the composition of lipid bilayers has profound and complicated effects on the production of A $\beta$  peptides by gS.<sup>132</sup> Both, the fatty acyl chains and headgroups of the bilayer can regulate proteolysis of the intramembrane protease. gS has very low activity when

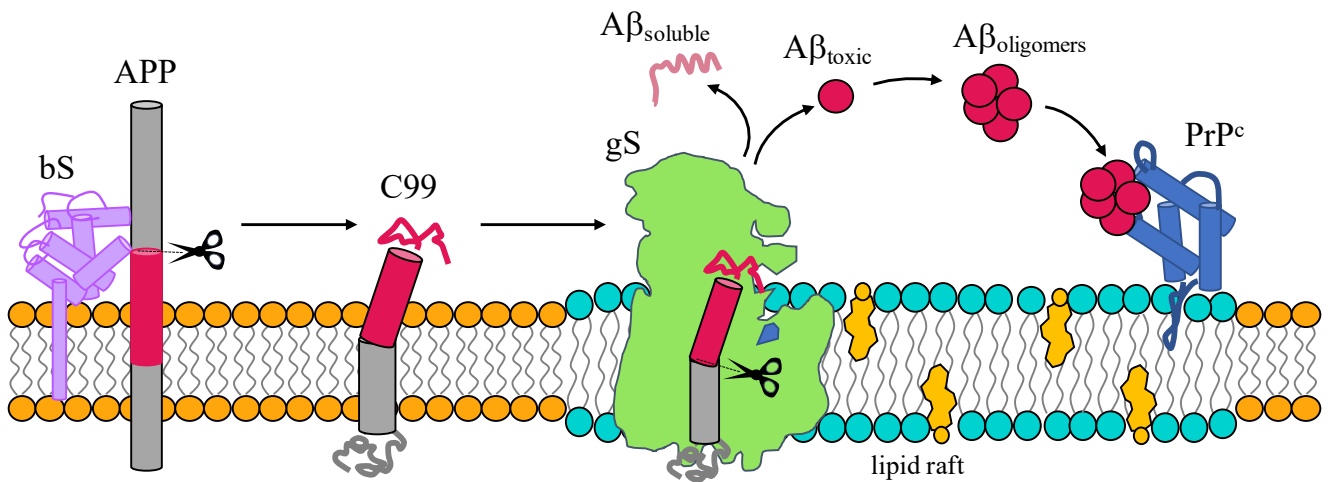
embedded in fatty acyl chain lengths below 14 carbons, then activity rises in a bell-shaped form and decreases again at 22 carbons. It has also been proposed that the A $\beta$ 42: A $\beta$ 40 ratio decreases as the FA chain length increases. Thicker hydrophobic membranes, together with a reduced fluidity induced by modification of the head groups, retain longer A $\beta$  species and allow subsequent gS cleavage to shorter isoforms. In the same study, Holmes *et al.* found that the double bond isomer in a phospholipid fatty acyl chain also influences the gS activity modifying the A $\beta$ 42: A $\beta$ 40 ratios. Similarly, the presence of cholesterol in membrane let do a considerable increase in gS activity that decreased at higher concentrations producing a bell-shaped form in the gS activity. <sup>132</sup>

Computational modeling approaches have made an effort to characterize changes in the gS conformational states while varying the bilayer lipid composition. <sup>69,133</sup> Aguayo *et al.* found that bilayer lipid composition has a large impact on the gS structural ensemble and proposed that lateral pressure across the bilayer and the protein-bilayer hydrophobic mismatch regulate the proteolytic enzyme. <sup>133</sup> Higher transmembrane lateral pressures restrain the gS dynamics and favor active state conformations of the PS1 catalytic subunit, which may explain experimentally observed differences in gS activity when varying, for example, cis/trans saturations of lipids. <sup>133</sup> Thinner bilayers reduce the distances between the PS1 catalytic ASP residues in the active site.

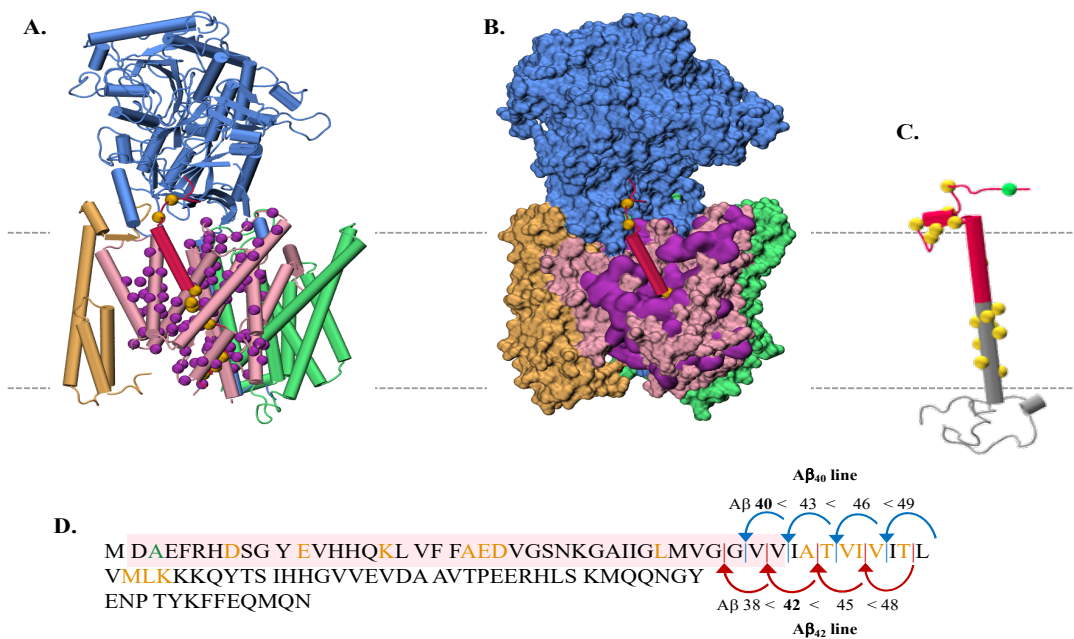
Variations in lipid headgroups can impact the mobility of the enzyme. In particular, a reduced flexibility is observed in transmembrane helix 2 related to substrate entry. Inactivation of gS resulting from the presence of charged lipids has been observed. This may be due to high PS1 structural restriction caused by bilayer rigidity that disturbs substrate recruitment and entry into the gS active site, as proposed by Holmes *et al.* Alternatively, it has been proposed that charged lipids interacting with the catalytic aspartates may lead to inactivation of the enzyme. <sup>134,135</sup>

Since C99 cleavage by gS most commonly occurs when the enzyme and its substrates are colocalized in cholesterol-rich lipid raft domains, Aguayo *et al.* studied the structural properties of gS in the presence of cholesterol rich bilayers and a mixture of lipids mimicking a lipid raft. <sup>133</sup> They found that higher cholesterol concentrations lead to increased lateral pressures favoring active enzyme conformations. Interestingly, gS in the presence of lipid mixtures does not show high lateral pressures but does favor dynamic structural transitions between active and inactive states of the GS complex. These dynamics were also observed in more compact NCT conformations. In that case, lipid headgroups interact with and retain the NCT extracellular domain, <sup>133</sup> which is folded over the gS active site. This allows for the steric sorting of gS substrates, similar to what has been observed in other studies. Of note, the omega-3 and omega-6 polyunsaturated fatty acids are two components of brain

cell membranes, with omega-3 slowing the progression of AD, and omega-6 increasing the AD risk<sup>137,138</sup>, but their impact on A $\beta$  biogenesis and conformational ensemble remains poorly understood.



**Figure 1.** Schematic representation of the amyloidogenic processing of APP. A $\beta$  is produced by sequential processing of APP by bS and gS to produce either a soluble and nontoxic mixture of A $\beta$  or a more amyloidogenic A $\beta$  mixture with a propensity to form oligomers that bind PrP<sup>c</sup> and potentially induce AD. The authors designed the figure.



**Figure 2.** (A) Overview of the gS and APP structures and pathogenic AD mutations. **A.** Depiction of the gS complex (PDB 6IYC<sup>95</sup>, NCT subunit (blue), APH-1A (green), PEN-2 (yellow), and the catalytic PSN subunit (pink) bound to a C99 fragment (red). C-alpha atoms from the PSN disease-causing mutations<sup>138</sup> that affect C99 processing are shown (purple spheres). C99 disease causing mutations available in the 6IYC structure are shown (yellow spheres). **(B)** gS structure with the same color code as (A) in a surface representation. **(C)** Depiction of a C99 structure model (grey) with the A $\beta$  sequence highlighted (red). C-alpha atoms from disease causing mutations are shown in yellow spheres with the protective Icelandic mutant (green). **(D)** C99 sequence with mutations highlighted in the same color code representation and showing the two main A $\beta$  production lines. The structures in A, B, and C from the PDB were drawn using VMD.<sup>140</sup>

## 2.2. Domain organization, isoforms of tau and minimal sequence for tau aggregation and toxicity

The tau protein forms paired helical filaments (PHFs) in neurofibrillary tangles central to the development of AD and other tauopathies. Tau is a microtubule-associated protein that plays an important role in axonal stabilization, neuronal development, and neuronal polarity. Full-length tau (htau40) includes a projection domain, a microtubule-binding domain (MBD) of four imperfect sequence repeats (R1 to R4), and a C-terminal domain (Figure 3A). The projection domain protrudes from the microtubule surface and it contains N-terminal region (with two N-terminal inserts 2N) and proline-rich region. The MBD has high affinity for microtubules. All 4 repeats end with PGGG sequence and there are two hexapeptide regions (PHF6\*: <sup>275</sup>VQIINK<sup>280</sup> and PHF6: <sup>306</sup>VQIVYK<sup>311</sup>) located in repeats 2 (R2) and 3 (R3). The MBD and the proline-rich regions are both positively charged. The N-terminal part and a short region at the C-terminus are acidic. Alternative splicing leads to the generation of six major isoforms of tau in the human brain: htau40(2N4R), htau32(1N4R), htau24(0N4R), htau39(2N3R), htau37(1N3R), and htau23(0N3R); ranging from 352 to 441 amino acid residues in length (Figure 3B). The MBD itself reproduces much of the aggregation behavior of tau in cells and animal models. Therefore, the peptides only encompassing the repeat region including K18 (4R) and K19 (3R) were often used to study tau aggregation to provide important insights into the amyloidogenesis of tau. The K18 and K19 are prone to aggregation since they do not contain the flanking regions that inhibit amyloidogenesis and they correspond to an amyloid core of PHFs. <sup>141</sup>

A recent cryo-EM study revealed different tau constructs on microtubules and complementary atomic models of tau-tubulin interactions were generated by Rosetta modeling (Figure 3C).<sup>142</sup> The conserved tubulin-binding repeats within tau adopt similar extended structures along the crest of the protofilament, spanning both intra- and interdimer interfaces, centered on  $\alpha$ -tubulin and connecting three tubulin monomers. The cryo-EM structures suggest that all four tau repeats are likely to associate with the MT surface in tandem, through adjacent tubulin subunits along a PF. This modular structure explains how alternatively spliced variants can have essentially identical interactions with tubulin but different affinities according to the number of repeats present. <sup>142</sup>

Besides the six tau isomers, other tau fragments were also studied for their roles in tau aggregation. There is considerable interest in discovering the minimal sequence and active conformational nucleus that defines tau aggregation events. Truncation of tau may play a causative role in tauopathies. The long-running research of truncated tau has led to the generation of the first active tau vaccine that has



entered clinical trials.<sup>143</sup> Studies have shown that proteolytic fragments of tau can drive neurodegeneration in a fragment-dependent manner. Proteolytic fragments of tau have been found in the cerebrospinal fluid and plasma of patients with different tauopathies, providing an opportunity to develop these fragments as novel disease progression biomarkers.<sup>144</sup> For example, tau (297-391) forms filaments that structurally mimic the core of paired helical filaments in AD brain.<sup>145</sup> A fragment from the proteolytically stable core of the PHF, tau 297-391 known as 'dGAE', spontaneously forms cross- $\beta$ -containing PHFs and straight filaments under physiological conditions. The comparison of the structures of the filaments formed by dGAE *in vitro* with those deposited in the brains of individuals diagnosed with AD found that they share a similar macromolecular structure.<sup>145</sup> Cleavage of tau by legumain (LGMN) has been proposed to be crucial for aggregation of tau into fibrils.<sup>136</sup> Using an *in vitro* enzymatic assay and non-targeted mass spectrometry, four putative LGMN cleavage sites were identified at tau residues N167-, N255-, N296- and N368. Cleavage at N368 generates variously sized N368-tau fragments that are aggregation prone in the Thioflavin T assay *in vitro*. Both N368-cleaved tau and un-cleaved tau were significantly increased in AD because of the accumulation of pathological tau inclusions. However, most of N368-cleaved tau remains largely soluble and is present only in low proportion in tau insoluble aggregates compared to un-cleaved tau. This suggests that LGMN-cleaved tau has limited role in the progressive accumulation of tau inclusions in AD.<sup>146</sup>

It is well known that two hexapeptide regions, PHF6\* and PHF6, located in R2 and R3 are top amyloidogenic motifs. The AcPHF6 may promote  $A\beta$  fibrillogenesis.<sup>147</sup> However, in the longer tau sequence, tau local structure shields PHF6 motif.<sup>148</sup> When  $A\beta$  acts as tau aggregation seeds,  $A\beta$  fibril can promote the exposure of these hexapeptide regions.<sup>149</sup> However, the PHF6 peptide lacks the ability to seed aggregation of tau<sub>244-372</sub> in cells.<sup>150</sup> But as the hexapeptide is gradually extended to 31 residues, the peptides aggregate more slowly and gain potent activity to induce aggregation of tau<sub>244-372</sub> in cells.<sup>150</sup> Further characterizations narrow down the  $\beta$ -forming region to a 25-residue sequence, indicating that the nucleus for self-propagating aggregation of tau<sub>244-372</sub> in cells is packaged in a remarkably small peptide. Disease-associated mutations, isomerization of a critical proline, or alternative splicing are all sufficient to destabilize this local structure and trigger spontaneous aggregation.<sup>148</sup> Numerous MD simulations have been conducted to study the PHF6 conformation and aggregation. In a recent MD and Markov state model (MSM) study,<sup>151</sup> PHF6 can spontaneously aggregate to form multimers enriched with  $\beta$ -sheet structure and the  $\beta$ -sheets in multimers prefer to exist in a parallel way. The residues Ile308, Val309, and Tyr310 play an essential role in the dimerization. MSM analysis shows that the formation of dimer mainly occurs in three steps. The

separated monomers collide with each other at random orientations, then a dimer with short  $\beta$ -sheet structure at the N-terminal forms; finally,  $\beta$ -sheets elongate to form an extended parallel  $\beta$ -sheet.<sup>151</sup>

Many studies have targeted these hexapeptide regions to prevent tau aggregation and toxicity. Using the model peptide, Ac-PHF6-NH<sub>2</sub>, the substitution of its amino acids with proline is shown to reduce self-assembly. Two of these modified inhibitors also disassemble pre-formed Ac-PHF6-NH<sub>2</sub> fibrils and one inhibits induced cytotoxicity of the fibrils.<sup>152</sup> Inhibitors based on the peptide SVQIVY, shifted by -1 residue compared with PHF6 (VQIVYK), can also block proteopathic seeding by patient-derived fibrils.<sup>153</sup> It was suggested that inhibitors based on the structure of the PHF6 segment only partially inhibit full-length tau aggregation and are ineffective at inhibiting seeding by full-length fibrils, and that the PHF6\* segment is the more powerful driver of tau aggregation.<sup>154</sup> The PHF6\* based-inhibitors not only inhibit tau aggregation but also inhibit the ability of exogenous full-length tau fibrils to seed intracellular tau in HEK293 biosensor cells into amyloid.<sup>154</sup>

Tau constructs can self-aggregate to PHFs directly, however, hyperphosphorylated tau appears to aggregate more readily and may sequester normal tau at lower concentrations.<sup>155,156</sup> The regulation of tau primarily involves post-translational modifications (PTMs) including phosphorylation, truncation, and acetylation. The most common tau PTM is phosphorylation. In the AD brain, tau is excessively phosphorylated, at least ~3-fold over normal brain, leading to the disruption of the MTs and the promotion of filament formation.<sup>157</sup> Other PTMs can also regulate tau aggregation; for example, tau truncation may take place after tau hyperphosphorylation with subsequent glycation.<sup>158</sup> Methylation has been shown to suppress tau aggregation propensity whereas glycation and acetylation promote pathological tau aggregation.<sup>159,160</sup> The distribution of phosphorylation sites along the sequence of full-length tau is uneven. Htau40 has 80 serine/threonine and 5 tyrosine residues that can be phosphorylated. Most of them are in either the N- or C-terminal regions. Mass spectrometry identified around 36 sites in purified PHF-tau from human Alzheimer brain.<sup>161</sup> Figure 3D illustrates the known phosphorylation sites in tau.

ICryo-EM structures of tau fibers in four distinct diseases, AD<sup>140</sup>, Pick's disease, chronic traumatic encephalopathy, and Corticobasal degeneration (CBD) have different conformations.<sup>41,162-164</sup> These structures are discussed in Section 3. The tau protein and its fragments have an intrinsic ability to assemble into amyloid structures of large dimensions in the absence of aggregation-enhancing species, such as heparin or alternative polyanions. Luo *et al.* have shown that heating K18 to a high temperature leads to tau aggregation, but it disassociates to monomeric state reversibly when cooling down.<sup>165</sup>

Their replica exchange molecular dynamics (REMD) simulations predicted that tau proteins could form amyloid fibrils at a high temperature of 343 K, and the tau amyloid fibrils may cold dissociate at 275 K. This intriguing feature was then confirmed by fluorescence experiments and it was observed that K18 fibrils cold dissociate when cooled. They also found that heparin locks the tau fibril and prevents its reversion. Adamcik *et al.* demonstrated that in the absence of heparin, tau306-327 R3 fragment is able to self-assemble into large, flat, multistranded ribbons consisting of up to 45 laterally assembled protofilaments.<sup>166</sup>

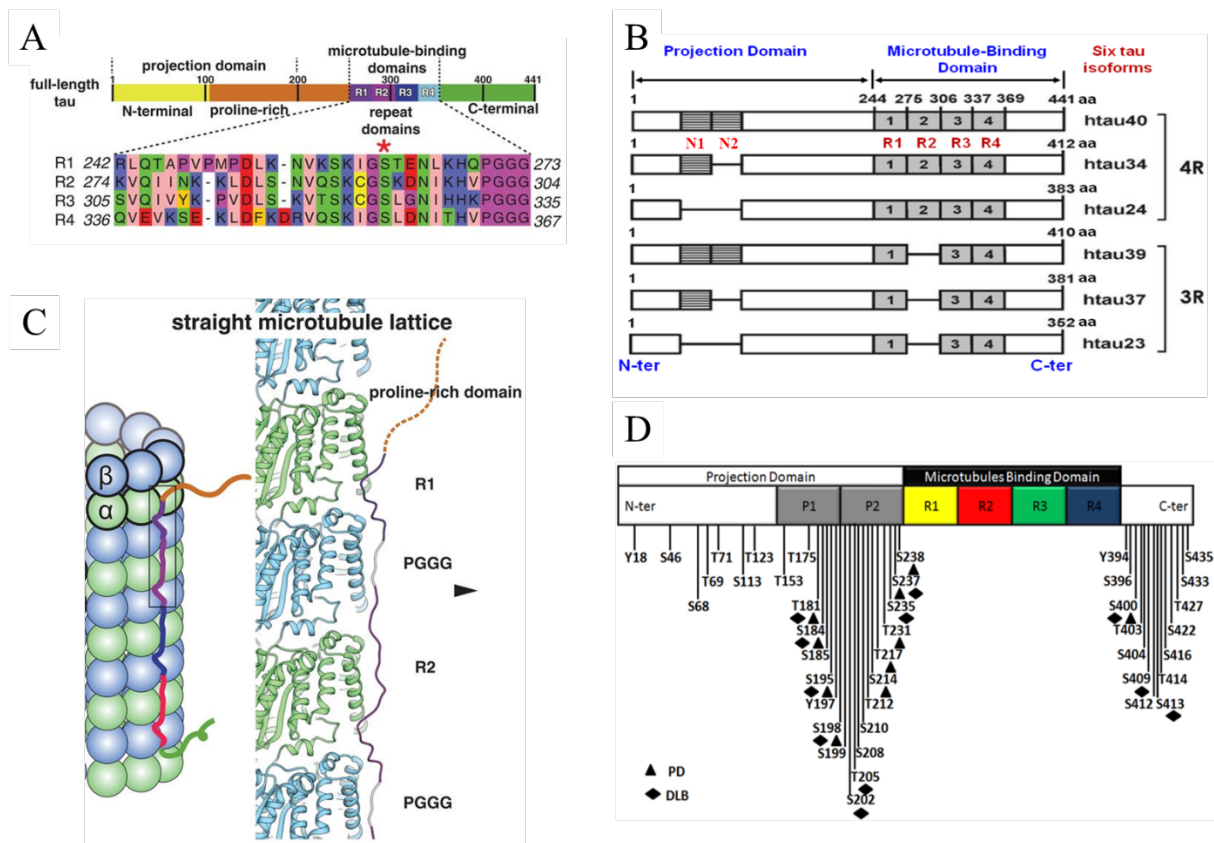
Besides heparin, other polyanions have also been employed to study the structural consequences regulation through interaction with a physiologically relevant aggregation inducer is important. The formation of AD filaments is routinely modeled in vitro by mixing tau with heparin. Heparin promotes tau aggregation and recently has been shown to be involved in the cellular uptake of tau aggregates.<sup>167</sup> Polyphosphate initiates tau aggregation through intra- and intermolecular scaffolding, most notably breaking long-range interactions between the termini.<sup>168</sup> Different from heparin, polyglutamic acid does not immediately convert tau into oligomers.<sup>169</sup> Tau is predominantly monomeric in the presence of polyglutamic acid at low temperature and only aggregates into oligomers and fibrils at higher temperature and longer incubation time. Utilizing this feature, through a combined NMR spectroscopy and molecular ensemble calculation method, Akoury *et al.* examined the conformational ensembles of K18 in the presence and absence of the polyglutamic acid and found that binding of polyglutamic acid to tau remodels the conformational ensemble of tau.<sup>170</sup>

Using a heparin-immobilized chip, surface plasmon resonance (SPR) revealed that tau K18 and K19 bind heparin with a KD of 0.2 and 70  $\mu$ M, respectively. In SPR competition experiments, N-desulfation and 2-O-desulfation had no effect on heparin binding to K18, whereas 6-O-desulfation severely reduced binding, suggesting a critical role for 6-O-sulfation in the tau-heparin interaction. The tau-heparin interaction became stronger with longer-chain heparin oligosaccharides. As expected for an electrostatics-driven interaction, a moderate amount of salt (0.3 M NaCl) abolished binding.<sup>171</sup> However, it was found that heparin-induced tau filaments are structurally heterogeneous and differ from AD filaments,<sup>172</sup> as discussed in Section 3.

The heparin induced tau structures illustrate the structural versatility of amyloid filaments, and raise questions about the relevance of in vitro assembly.<sup>173</sup> Our understanding of this question is that polyanion induced tau structure and those found in patients' brain so far present favorable states in the complex amyloid formation energy landscape. As the structure of A $\beta$  fibril, the tau fibril may exist in

different forms from different patients. This can be supported by the study of PHP6 based inhibitors. Donors with progressive supranuclear palsy exhibited more variation in inhibitor sensitivity, suggesting that fibrils from these donors were more polymorphic and potentially vary within individual donor brains.<sup>153</sup> Exploring the interplay between fibrillization and amorphous aggregation channels on the energy landscapes of 3R and 4R tau provided a global view of polymorphic tau aggregates.<sup>174</sup> A coarse-grained protein force field was used to study the energy landscapes of nucleation of the 3R and 4R fibrils derived from patients with Pick's and Alzheimer's diseases. The landscapes for nucleating both fibril types contain amorphous oligomers leading to branched structures as well as prefibrillar oligomers. These two classes of oligomers differ in their structural details: The prefibrillar oligomers have more parallel in-register  $\beta$ -strands, which ultimately lead to amyloid fibrils, while the amorphous oligomers are characterized by a near random  $\beta$ -strand stacking, leading to a distinct amorphous phase.<sup>174</sup> The feature of the energy landscape connecting the tau oligomers and fibrils was reflected in the sm-FRET studies of oligomer diversity during aggregation of K18, although also in the presence of heparin.<sup>175</sup> Kjaergaard *et al.* observed that the shortest growing filaments only represent a small population of transient oligomers with cross- $\beta$  structure, while the two largest oligomer populations are structurally distinct from fibrils and are both kinetically and thermodynamically unstable. The first electrostatic driven population is in rapid exchange with monomers; the second kinetically more stable one is probably off-pathway to fibril formation.<sup>175</sup>

*In vitro*, 0N4R tau fibrils contain a monomorphic  $\beta$ -sheet core enclosed by dynamically heterogeneous fuzzy coat segments.<sup>176</sup> A variety of experiments indicate that 0N4R tau fibrils exhibit heterogeneous dynamics. Outside the rigid R2-R3 core, the R1 and R4 repeats are semirigid even though they exhibit  $\beta$ -strand character and the proline-rich domains undergo large-amplitude anisotropic motions, whereas the two termini are nearly isotropically flexible. It has been suggested that the N- and C-termini differentially associate with PHFs,<sup>177</sup> and play distinct roles in the stability and consequently neurotoxicity of tau filaments.<sup>178</sup> The N-terminal fragment (residues 1–15) did not affect tau polymerization<sup>179</sup> whereas a fragment consisting of residues 1–196 could inhibit polymerization of full-length tau.<sup>180</sup> Tau C-terminus is crucial in the formation of tau PHFs,<sup>181</sup> and it also modulates the cross-seeding barrier between 4R and 3R tau.<sup>182</sup> Xu *et al.* performed multi-scale MD simulations to study the structure and dynamics of full-length tau filaments, especially the effects of the flanking regions on the stability of the filament core.<sup>183</sup> They found that full-length tau filaments consist of one dense core and two sparse layers, consistent with the structural model derived from the experimental observations. The relative stability of the filaments not only depends on the core morphology, but can also shift by interactions among different domains.



**Figure 3.** Domain organization, isoforms of tau and binding to microtubules. **(A)** Schematic of tau domain architecture and assigned functions. The MT-binding domain of four repeats is defined as residues 242 to 367. The inset shows the sequence alignment of the four repeat sequences, R1 to R4, that make up the repeat domain. Ser262 is marked by the asterisk. **(B)** Schematic representation of the six human tau isoforms and two tau constructs. Tau isoforms differ by the absence or presence of one or two 29-amino acid inserts in the amino-terminal part, in combination with either three (R1, R3 and R4) or four (R1–R4) repeat regions (black boxes) in the carboxy-terminal part. Isoform sizes range from 352 amino acids (aa) to 441 aa. **(C)** Model of full-length tau binding to microtubules and tubulin oligomers. **(D)** Schematic representation of the largest isoform of tau with specific phosphorylation sites. Serine, threonine, and tyrosine residues that can be phosphorylated in AD, PD, and dementia with Lewy bodies (DLB) are indicated. Panels A and C are from<sup>142</sup>, and panels B and D are designed by the authors.

### 2.3. $\alpha$ -Synuclein and IAPP domains and aggregation properties

$\alpha$ S is a 14 kDa neuronal protein that is predominantly localized at the pre-synaptic termini<sup>184</sup>. In its physiological form,  $\alpha$ S is monomeric and disordered<sup>185</sup>, although some studies have generated a debate<sup>186</sup> on whether it adopts a helical tetramer *in vivo*<sup>187</sup>. The aggregation of  $\alpha$ S is inherently connected with PD, as its aggregates are major components of intracellular inclusions known as Lewy bodies forming in dopaminergic neurons of PD patients<sup>188</sup>. There are also links between the  $\alpha$ S-encoding gene and familial forms of PD, with mutations, duplications and triplications being found in

patients affected by early onset forms of PD<sup>189</sup>. Fibrillar aggregates of the relevant aggregation-prone region of  $\alpha$ S, the non-amyloid- $\beta$  component (NAC), are also found in AD patients<sup>190</sup> and in the context of other neurodegenerative disorders, including dementia with Lewy bodies<sup>191</sup>, multiple system atrophy<sup>192</sup> and other synucleinopathies.<sup>193</sup>

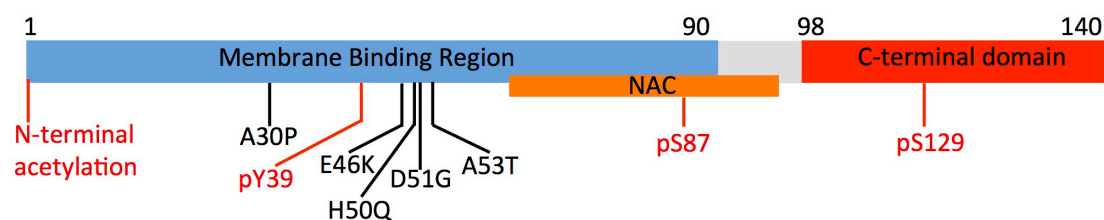
While the pathological relevance of  $\alpha$ S is generally acknowledged, its function remains unclear<sup>194</sup>. The abundance of  $\alpha$ S at the synaptic termini has suggested that it may be involved in neuronal processes and studies have indicated possible roles in synaptic plasticity<sup>195</sup> and learning<sup>196</sup>. A number of evidences have been collected on a possible function of  $\alpha$ S in the trafficking of synaptic vesicles (SVs) during neurotransmitter release<sup>197,198</sup>.  $\alpha$ S bind SVs *in vitro* and colocalizes with SVs in synaptosomes in a calcium responsive manner<sup>199</sup>. Key evidence indicates that  $\alpha$ S has a role of chaperone for the assembly of the SNARE complex<sup>200</sup>, the machinery promoting the fusion of SVs with the plasma membrane.  $\alpha$ S was indeed shown to rescue the formation of the SNARE complex in knock-out mice lacking CSP $\alpha$ <sup>201</sup>, whereas knockout mice lacking the three synucleins ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -) showed neuropathological phenotypes that are indicative of impaired SNARE activity<sup>202</sup>. The interaction with SVs by  $\alpha$ S has also been implicated in the promotion of SV-clustering<sup>203,204</sup>, a key process in the maintenance of pools regulating SV homeostasis during neurotransmitter release<sup>198</sup>. In addition to its interaction with SVs,  $\alpha$ S has also been implicated in the regulation of the vesicle trafficking from the endoplasmic reticulum (ER) to the Golgi<sup>205</sup>, and the mitigation of oxidative stress in mitochondria<sup>206</sup>.

The domain organization of  $\alpha$ S is defined on the basis of its sequence properties and in relation to the biological context (Figure 4). The major biological form of  $\alpha$ S *in vivo* features the binding with cellular membranes<sup>207</sup>. Membrane interactions by  $\alpha$ S are promoted by a lipophilic domain (residues 1 to 90) and featuring 7 imperfect sequence repeats. These 11 residues repeats induce binding via a disorder-to-order transition into amphipathic class A2  $\alpha$ -helical segments that promote the membrane binding<sup>208</sup>. This domain has also genetic links with inherited forms of early onset PD, as it hosts all the PD-related  $\alpha$ S mutations (A30P, E46K, H50Q, G51D and A53T)<sup>189</sup>.

Another domain of  $\alpha$ S, the NAC (residues 61 – 95), has relevance in the context of self-assembly and aggregation. NAC is essential for the kinetics of  $\alpha$ S aggregation<sup>209</sup> and is the main component of the core of  $\alpha$ S fibrils. Fibrils of the NAC region have been isolated in the context of PD as well as in other neurodegenerative diseases<sup>190</sup>. Finally, the negatively charged C-terminal domain of  $\alpha$ S spanning residues 99 to 140 (net charge of -9) is implicated in calcium binding<sup>199</sup> and in protein-protein interactions at the surface of synaptic vesicles<sup>197,200</sup>.

IAPP or amylin is a 37-residue hormone produced by pancreatic  $\beta$ -cells to regulate the response to high glucose levels in the blood in conjunction with insulin, which is co-secreted<sup>210</sup>. The biogenesis of amylin requires the production of the proIAPP, a peptide composed of 67 residues whose cleavage generates IAPP<sup>211</sup>. In addition to his role as a hormone, IAPP is well known for its connection with T2D. Indeed, fibrillar aggregates of IAPP are the major constituents of deposits in pancreatic islets that are found in the majority of patients suffering from this condition. Despite the toxicity of IAPP amyloids has been shown *in vitro*<sup>212,213</sup>, it remains to be established whether its aggregation is a causative factor or a downstream effect of TD2. Indeed, in contrast to A $\beta$  protein where many familial mutations exist, there is only one unique disease-causing mutation in IAPP (S20G), and in the majority of the cases, no specific alterations or mutations of IAPP are found in diabetic individuals, suggesting that other factors, such as the failure of control mechanisms to prevent protein misfolding, may be involved. Islet amyloids are *de facto* found also in small populations of non-diabetic elderly individuals<sup>214</sup>, likely owing to a reduced efficiency in the mechanisms of clearance of protein aggregation. *In vitro* IAPP has been shown to aggregate in a concentration dependent manner, and it rapidly self assembles into amyloids when concentrated in the millimolar range, a condition that by contrast is well managed in the secretory granules of  $\beta$ -cells of healthy individuals.

Among the factors that inhibit the aggregation of IAPP in  $\beta$ -cells are the acidic pH (~5.0), shown to be largely unfavorable to the misfolding of IAPP *in vitro*<sup>215</sup>, and the presence of high levels of Zn(II) in  $\beta$ -cells. The binding to Zn(II) indeed promotes conformations of the otherwise intrinsically disordered IAPP that are aggregation resistant, presumably by shielding the two amyloidogenic sequences of IAPP. Indeed, two regions have been found to be crucial for the fibrillization of IAPP. These are included in the N- and C-strands forming the fibrillar core of IAPP amyloids and spanning respectively residues 8 to 17 and 28 to 37. The two strands form a single stack in the cross- $\beta$  arrangement, generating two facing  $\beta$ -sheets stabilized by a steric-zipper dry interface<sup>216</sup>.



**Figure 4. Key  $\alpha$ S domains having a role in functional and pathological contexts.** Membrane-binding domain (residues 1 to 90), NAC region (residues 61 to 95) and the C-terminal domain (residues 98 to 140) are shown in blue, orange and red, respectively. The diagram also shows the main pathological mutations (black) and key post-translational modifications (red) such as the N-terminal acetylation and phosphorylation of residues Ser87, Ser129 and Tyr39. The authors designed the figure.

### 3. Structures of A $\beta$ , tau, $\alpha$ -synuclein, IAPP oligomers and fibrils from experiments

### 3.1 Experimental techniques for fibrils

Amyloids are proteineous deposits associated with peculiar physical and chemical features: (i) they exhibit a very low degree of solubility, (ii) they form nanoobjects of various ultrastructural shapes and multiple sizes ranging from few nanometers to microns (ii) they can represent objects of heterogeneous morphologies at the macroscopic scale (e.g. aggregates, fibrils or oligomers) and heterogeneous conformation or polymorphic nature at the atomic level.<sup>217</sup> Additionally, amyloid deposits tend to be difficult to isolate, biochemically handle and purify due to their insolubility. Their biochemical features constitute major hurdles for biophysical approaches and structural biology towards determining atomic resolution structural models. The development of innovative methods in structural biology during the past two decades has been very fruitful and more than 100 structures of protein and peptide fibrils (~40 from full length constructs) have been deposited at the PDB<sup>218</sup> using different techniques such as X-ray diffraction, NMR and EM.<sup>210</sup> 3D model determination of amyloid architecture relies on a multi-step process to elucidate structural features at various levels:

**Length of the amyloid core.** The amino acid sequence of the amyloid core usually covers only a fraction of the full-length protein, e.g., the amyloid core of  $\alpha$ -synuclein does not comprise its C-terminal domain.<sup>219</sup> A simple biochemical maneuver to delineate the length of the amyloid core is the enzymatic digestion of protein segments that are not involved in the core region. H/D exchange performed by mass spectrometry<sup>220</sup> or solution NMR<sup>221</sup> an experimental approach that is commonly used to delineate the amyloid core at a residue-specific resolution.

**Secondary structure.** Amyloid deposits are rich in  $\beta$ -sheet secondary structure; this information can be rapidly extracted with CD and FTIR. A key step towards determining the amyloid structure is the identification of the number and delineations of the  $\beta$ -sheets as well as the localization of  $\beta$ -sheet breakers or turns. This can be achieved by high-resolution techniques, i.e. ssNMR and cryo-EM. Using solid-state, the secondary structure can be predicted from chemical shifts using computational routines such as TALOS<sup>222</sup> or by the secondary chemical shifts<sup>223</sup>. Because the chemical exchange of amide hydrogens with the buffer is remarkably slowed down if the involved residue is comprised in the amyloid core, H/D exchange techniques offer a powerful approach to distinguish amino-acids involved in the hydrophobic amyloid core and thus identify  $\beta$ -sheet positioning. Recent advances in cryo-EM methodology, including software developments and new electron detectors, have facilitated structure determination of amyloid fibrils, backbone conformation and secondary structure elements<sup>224</sup>. They can be recognized after single particle EM analysis, recently demonstrated for  $\alpha$ S<sup>225</sup> and A $\beta$ <sup>226</sup>.



**Three-dimensional fold and intermolecular packing.** Although most amyloids share the generic cross- $\beta$  architecture, the number of  $\beta$ -sheet elements and their placement relative to each other can be very diverse. Several generic architectures have been proposed, such as the  $\beta$ -helix,  $\beta$ -sandwich, super-pleated  $\beta$ -sheet structure or the  $\beta$ -roll. The supramolecular arrangement of amyloid fibrils is often regular in a protofilament and has been observed as  $\beta$ -sheets that run antiparallel or parallel in-register along the fibril axis. The  $\beta$ -solenoid fold was the first experimentally determined amyloid fold at high resolution, from the amyloid prion HET-s by ssNMR<sup>39</sup>. This architecture is characterized by specific amino acid sequence patterns composed of hydrophobic residues pointing inside the core that sequentially alternate with polar residues pointing outside. Although originally associated with functional amyloids, this fold has been recently seen in tau fibrils by cryo-EM<sup>40</sup>, suggesting this fold to be generic in the context of pathological and functional amyloids.

The cross- $\beta$  nature of the sample is essentially characterized by X-ray diffraction<sup>227</sup> and additional information relative to high-order symmetry can be derived from scanning transmission EM<sup>228</sup> and tilted-beam transmission EM<sup>229</sup>. These two approaches provide the so-called mass-per-length measurement, a crucial structural parameter to determine how many protein monomers are stacked per fibril layer. 3D structure determination of amyloids using ssNMR relies on the collection of internuclear distance restraints, typically in the range of 2-8 Å.<sup>230</sup> Distinction between intramolecular proximities (i.e. two nuclei in two  $\beta$ -sheets within the same molecule) and intermolecular proximities (i.e., two nuclei in two  $\beta$ -sheets in adjacent molecules) requires the use of strategic isotope labeling<sup>231</sup> and is still a major bottleneck for ssNMR-based structure determination. In analogy to distance measurements by ssNMR, double electron-electron resonance spectroscopy (DEER) and continuous-wave electron paramagnetic resonance (EPR) are powerful approaches to provide residue-residue structural restraints in a distance range not accessible to ssNMR >10 Å.<sup>232</sup>

In case of short amyloid-forming peptides that crystallize, conformational studies at atomic resolution can be performed by X-ray diffraction analysis<sup>42</sup> and micro electron diffraction<sup>233</sup>. Numerous fibril-forming peptides have been crystallized to uncover numerous intermolecular stacking arrangements. Notably, these studies have highlighted the role of steric zipper motifs that consist of complementary side chains interdigitation in a dry interface, resulting in stable packing of high density.

Cryo-EM has opened an avenue to obtain both intramolecular and intermolecular arrangement of amyloid fibrils at atomic resolution<sup>224</sup>. A major advantage of cryo-EM is the ability to obtain high-resolution maps from patient-derived samples, reducing artifacts associated with *in vitro* aggregation

of recombinant proteins. Amyloid fibrils are usually observed as bundle of individual filaments termed protofilaments. Ultrastructural analysis of such bundles has revealed the presence of particular high order architectures, such as twisted or straight morphologies. AFM and EM are used to decipher the morphology of amyloid fibrils, but their resolution is limited. Cryo-EM has recently proven to be a unique technique to characterize the quaternary arrangement of protofilaments, as exemplified by the determination of the staggering of non-planar  $\beta$ -strands in case of A $\beta$ 42<sup>226</sup> and tau<sup>40</sup>.

### 3.2. Diversity of fibril structures in Tau, $\alpha$ -synuclein, A $\beta$ and IAPP

**Tau filaments.** Tau filaments were first observed in paired helical PHF<sup>234</sup> and then a mixing of PHF and straight filaments (SF) by EM<sup>235</sup>. Structure determination of tau filaments has been limited for a long time by the important length of the amyloid core and the particular nature of the filament that is composed of a rigid core and a fuzzy coat lacking structural order. Moreover, *in vitro* aggregation of recombinant tau requires the use of cofactors such as heparin and these cofactors might modulate the details and the obtained polymorphism<sup>236</sup> of the cryo-EM structures of tau in AD and Pick's disease.

In AD, both structures of PHF and SF (Figures 5A-B), are composed of eight  $\beta$ -sheets in a C-shaped fold, although lateral protofilament contacts revealed a different intermolecular organization between the protofilaments, suggesting an important role of ultrastructural polymorphism in the context on *in vivo* tau aggregation. Filament cores are made of two identical protofilaments comprising residues 306-378 of tau protein, which adopt a combined cross- $\beta$ / $\beta$ -helix structure and define the seed for tau aggregation. Paired helical and straight filaments differ in their inter-protofilament packing, showing that they are ultrastructural polymorphs.<sup>40</sup>

In Pick's disease, the tau filament core comprises a distinct fold<sup>41</sup> compared to the structures of AD's PHF and SF. This suggests that a disease-specific amyloid fold might be the hallmark of clinical phenotypes, in analogy with structural strains observed in prion diseases<sup>237</sup>. The structures of tau filaments from several brains of patients with chronic traumatic encephalopathy (CTE) confirmed this observation, revealing the presence of several structural polymorphs (Figure 5B) with a molecular fold and protofilament interfaces different to AD's filaments.<sup>41</sup> Additional density, not connected to tau, was observed in Pick's disease tau filaments, suggesting a tight incorporation of biological cofactors into the structure. Structures of filaments from Pick's disease consist of residues Lys254-Phe378 of 3R tau, explaining the selective incorporation of 3R tau in Pick bodies, and the differences in phosphorylation relative to the tau filaments of AD. Interestingly, novel tau filament fold in chronic traumatic encephalopathy encloses hydrophobic molecules. Importantly, residues K274-R379 of 3R tau and S305-R379 of 4R tau form the ordered core of two identical C-shaped protofilaments,

indicating common driving forces do exist in tau aggregation. The core of a CBD filament comprises residues lysine 274 to glutamate 380 of tau, spanning the last residue of the R1 repeat, the whole of the R2, R3 and R4 repeats, and 12 amino acids after R4.<sup>163</sup> The core adopts a previously unseen four-layered fold, which encloses a large nonproteinaceous density. This density is surrounded by the side chains of lysine residues 290 and 294 from R2 and lysine 370 from the sequence after R4.

Finally, heparin-induced filaments of 2N4R tau have at least four different conformations. Cryo-EM structures of three of these conformations reveal a common, kinked hairpin fold, with differences in kink, helical twist and offset distance of the ordered core from the helical axis. 2N3R tau filaments are structurally homogeneous, and adopt a dimeric core, where the third repeats of two tau molecules pack in a parallel manner.<sup>173</sup>

**$\alpha$ -synuclein fibrils.** Unlike tau, recombinant expression of  $\alpha$ S led to the preparation of homogeneous fibrils amenable to structure determination. A first 3D structure was proposed by ssNMR<sup>238</sup>, revealing a Greek-key topology (Figure 5C). This structure, later classified as the polymorph "1c"<sup>225</sup> contains a parallel in-register arrangement with hydrophobic side chain packing and a steric zipper. Cryo-EM studies have uncovered the high-resolution structures of various polymorphs, including the familial PD mutant H50Q<sup>239</sup>. The inter-protofibril arrangement is characterized by the presence of staggered  $\beta$ -strands, and familial mutations H50, G51 and A53 are localized at the protofilament interface and participate to its stability. Several wild type polymorphs have been solved (Figures 5D-E), revealing different protofilament interfaces. The familial mutations are localized at crucial positions that stabilize either the intramolecular fold or the inter protofilament interface.

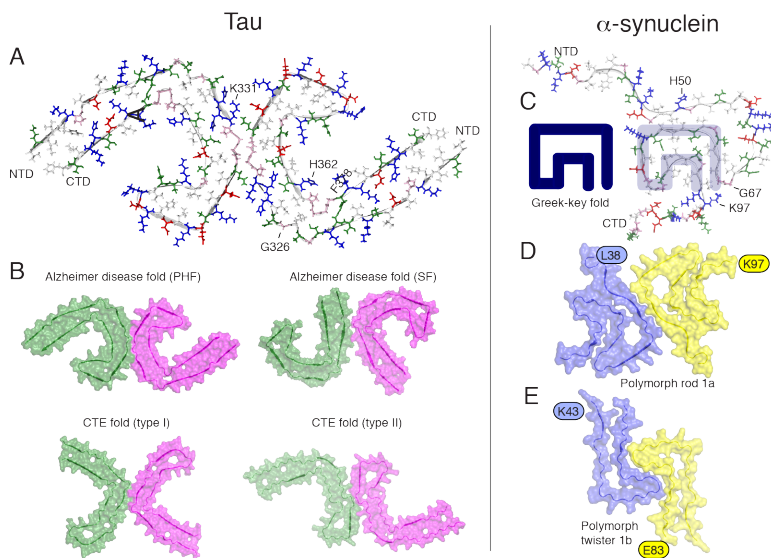
The atomic structures of  $\alpha$ S fibrils extracted from brains of individuals with multiple system atrophy (MSA) have been determined by cryo-EM.<sup>240</sup> Two types of filaments were identified (named type I and II) each composed of two protofilaments. An astonishing feature of type I and type II  $\alpha$ S filaments is the asymmetry of their protofilaments, leading to a different solvent exposure of the critical residues (e.g., K60). Post-translational modifications of only one protofilament have been proposed to explain the different conformations of the two protofilaments in the same fibril<sup>240</sup>. Structures of brain-derived  $\alpha$ -synuclein fibrils are distinct from fibrils obtained by recombinant expression and *in vitro* aggregation, as already observed for tau filaments.

**A $\beta$ 40/42 fibrils.** Because of its small size (compared to tau or  $\alpha$ -synuclein proteins), its production by solid-phase peptide synthesis has offered a convenient way for numerous research groups to investigate its structure by biophysical techniques. To date, most methodological developments in

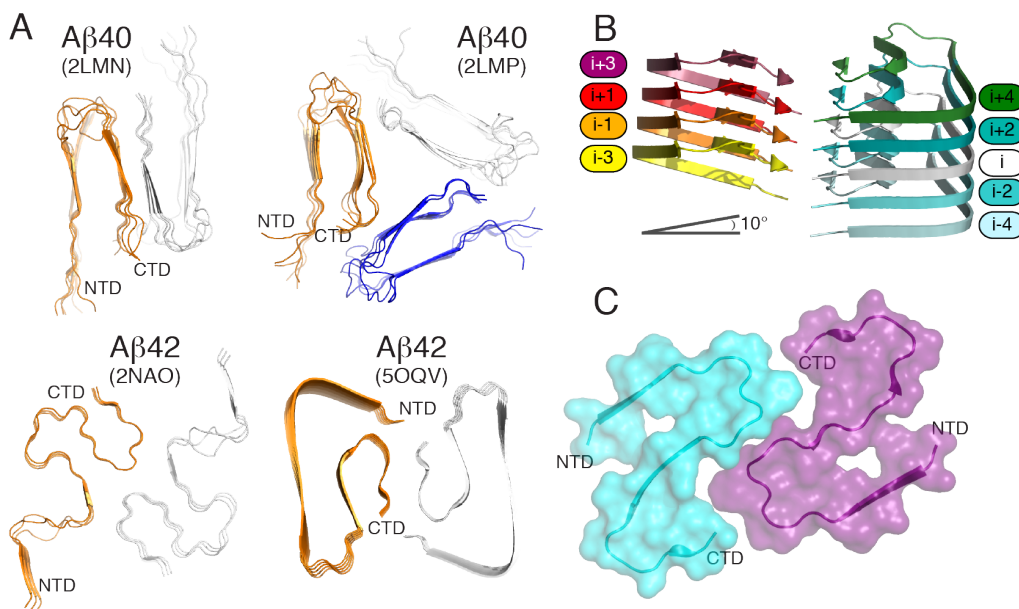
structural biology of amyloid fibrils have been performed on A $\beta$  peptides. Early studies using FTIR and CD have demonstrated the high propensity of  $\beta$ -sheet structure of A $\beta$  fragments<sup>241</sup>. Pioneering work by the Tycko's laboratory using solid-state<sup>242,243</sup> determined the  $\beta$ -turn- $\beta$  (U-shape) fold in an in-register, parallel arrangement for A $\beta$ 40 fibril with residues 12-24 and 30-40 in  $\beta$ -strand conformations based on distance restraints and torsion angle measurements.<sup>244</sup>

Several 3D models of A $\beta$ 40 and A $\beta$ 42 (Figure 6A) have been proposed on the basis on ssNMR and cryo-EM<sup>226,245-247</sup>, showing a diversity of intramolecular fold (U-shape or S-shape) and quaternary arrangement between A $\beta$ 40 and A $\beta$ 42. Small variations in the aggregation conditions can lead to distinct molecular conformation within the same peptide sequence, and even different toxicity level<sup>248</sup>. By combining cryo-EM and ssNMR, Schröder *et al.* presented a structure of A $\beta$ 42 fibrils assembled at low pH which is to date the highest resolution model of A $\beta$  peptides<sup>226</sup>. As already observed for other pathogenic amyloids, the staggering of non-planar molecules (Figure 6B) constitutes a unique feature that has profound implications for fibril growth mechanisms, because the binding site of the two fibril ends are different, implying a polarity of subunit growth. To date no high resolution of A $\beta$ 40 and A $\beta$ 42 fibrils extracted from brains have been solved. Tycko *et al.* have reported conformational studies at high resolution of A $\beta$ 40 and A $\beta$ 42 fibrils seeded from brain extracts<sup>249,250</sup>. Studies of these human brain-derived fibrillar assemblies showed distinct molecular conformation by solid-state from each patient, suggesting the existence of structure-specific conformations depending on the patient AD clinical history. Comparison of A $\beta$ 40 and A $\beta$ 42 in this context revealed a predominant molecular conformation in A $\beta$ 40, while A $\beta$ 42 exhibited a larger degree of structural heterogeneity as revealed by ssNMR chemical shift analysis<sup>250</sup>. It implies a greater structural susceptibility of A $\beta$ 42 brain seeds compared to those for A $\beta$ 40, as already suggested<sup>251</sup>. As suggested for  $\alpha$ -synuclein or tau, the existence of structure-based strains for A $\beta$ 40 and A $\beta$ 42, in analogy to conformational strains known for prion-like proteins, is still a matter of debate.

**IAPP fibrils.** Previous studies based on ss NMR of IAPP place the majority of the 37 residues into the fibril core and a U-shape conformation, the N-terminus being at the periphery, but all models display substantial deviation<sup>216,252</sup>. A very recent study by cryo-EM at physiological pH<sup>253</sup> provides three polymorphs with the dominant one comprising residues 13-37 with two S-shape, intertwined protofilaments (Figure 6C). The high similarity between this model and the A $\beta$ 42 model from Gremer is striking considering the link between T2D and AD.



**Figure 5.** Orthogonal view of the three-dimensional structural models of tau filaments and  $\alpha$ -synuclein amyloid fibrils. NTD and CTD are the N-terminal and C-terminal domain. (A) Solid-state tau paired PHF spanning residues 306-378 in the right protofilament. (B) Cryo-EM PHF and SF tau amyloid cores in AD. In chronic traumatic encephalopathy (CTE), tau filaments contain predominantly the type I (90%) and type II filaments. The inter-protofilament interfaces are different compared to those in AD. (C) Amyloid core of human  $\alpha$ -synuclein amyloid fibrils (PDB entry 2n0), containing a Greek-key fold. (D-E) amyloid core fibril structure of  $\alpha$ -synuclein: polymorph rod 1a (PDB entry 6CU7) and polymorph twister 1b (PDB entry 6CU8). The authors prepared this figure with pymol<sup>254</sup>.



**Figure 6.** (A) Orthogonal view of A $\beta$ 40 structures (PDB entries 2LMN and 2LMP) spanning residues 9-40 and A $\beta$ 42 structures (PDB entries 2NAO and 5OQV) spanning residues 1-42. (B) Cryo-EM structure of A $\beta$ 42 showing the staggered arrangement of non-planar A $\beta$ 42 subunits. (C) Cryo-EM structure of IAPP grown at physiological pH (PDB 6Y1A) spanning residues 13-37. The authors designed this figure with pymol<sup>254</sup>.

### 3.3. Structures of transient oligomers

#### 3.3.1 A $\beta$ and IAPP

The fibrillation of A $\beta$  is preceded by a transition from a random-coil like structure to helical conformation where the latter facilitate the formation of  $\beta$ -sheet structured amyloid filaments.<sup>255</sup> The helical conformation of A $\beta_{1-40}$  monomer determined by NMR in a water-detergent solution adopts a helical structure in the K16-V24 and K28-V36 regions.<sup>256</sup> The bi-helical structure is shown to be disrupted in the oxidized A $\beta_{1-40}$  and adopts a single helical region between residues K16 and V24.<sup>257</sup> Another NMR study trapped a low population of an early intermediate of A $\beta_{1-40}$  characterized by a  $3_{10}$   $\alpha$ -helix (Figure 7) spanning the core hydrophobic regions H13 to D23 in an aqueous medium containing no detergent.<sup>258</sup> The partially folded  $3_{10}$ -helical structure has also been observed in computational <sup>259</sup> highlighting the identification of a potential target species from an on-pathway aggregation to design small-molecule A $\beta$  inhibitors. The phenolic inhibitor EGCG is used to study the ligand-A $\beta$  interaction using the  $3_{10}$   $\alpha$ -helical structure and shows a tendency to bind the hydrophilic N-terminus and the  $\alpha$ -helical (H13 to D23) region.<sup>260</sup>

High-resolution 3D model structure determination of pathologically relevant low- and high-ordered A $\beta$  species being very challenging, chemical cross-linking approach has been employed to homogenize the sample for structural determination. Macrocyclic  $\beta$ -sheets peptides that mimic  $\beta$ -hairpins of amyloid peptides and stabilize both low- and high-ordered oligomers have been developed for X-ray crystallography study.<sup>261</sup> Using A $\beta$   $\beta$ -hairpin mimicking peptides,<sup>262</sup> a twisted  $\beta$ -hairpin triangular A $\beta$  trimer structure has been solved which also form hexamers, dodecamers and annular oligomers (Figure 7) through self-assembling that resemble annular structures reported for A $\beta$  oligomers by EM.<sup>263,264</sup> These A $\beta$  oligomers presents substantial neurotoxicity and thus proposed to be useful molecular models of A $\beta$  oligomer for structure-based inhibitor designing. Stable A $\beta$  dimers and trimers characterized with parallel  $\beta$ -sheets and neurotoxicity have been developed by sequentially varying the position for cross-linking using chemical linkers or disulfide bridges.<sup>265-267</sup> A combination of solution and ss NMR and AFM characterized a highly disordered oligomers of A $\beta$  that were formed in parallel to fibril formation process.<sup>268</sup> A recent study employed pressure-jump NMR to observe the oligomerization of A $\beta$ .<sup>269</sup> By combining solution NMR, DLS, EM and wide-angle X-ray diffraction and cell viability, Melacini *et al.* provide an atomic resolution map of soluble A $\beta$  toxic surfaces, with the exposure of a hydrophobic surface spanning residues 17-28 and the shielding of the N-terminus (Figure 7).<sup>270</sup> While isolation and characterization of A $\beta$  dimers, trimers, tetramers etc. without chemical modification is challenging in experimental conditions, atomistic MD simulations

complemented these limitations and provide high-resolution 3D structures. For example, simulations identified dimer, trimer, tetramer and stable globular-like oligomers starting from disordered monomers with 13-23% of  $\beta$ -sheet content.<sup>271</sup> While self-assembled A $\beta$  oligomers are of interest, recent studies highlighted the formation of A $\beta$  hetero-oligomers through cross seeding or binding to other proteins in the brain. A $\beta_{1-40}$  and A $\beta_{1-42}$  mixed oligomers are reported to have an intermediate structure between those of the self-assembled oligomers and proposed to have a large surface with antiparallel  $\beta$ -sheet structure.<sup>272</sup> A $\beta$  interaction with prion protein reported to trap an antiparallel  $\beta$ -sheet oligomer, but lack a high-resolution structure; on the other hand, A $\beta$  shows a random-coil structure when forming heterooligomers with apolipoprotein-E derived peptide fragments with enhance neurotoxicity.<sup>273-275</sup> Readers are referred to a review that discusses the high-resolution solution and ssNMR approaches to monitor the formation and characterization of A $\beta$  oligomers.<sup>276</sup>

A number of biophysical techniques and different types of sample preparations have been used to investigate the aggregation mechanisms of IAPP. Helical intermediates of human-IAPP have been reported.<sup>277,278</sup> NMR experiments have shown high-resolution structures for full-length human-IAPP<sup>279</sup>, human-IAPP-1-19<sup>280</sup>, full-length rat-IAPP<sup>281</sup> peptides both in solution as well as in a membrane environment. Investigation of the aggregation pathways revealed the formation intermediate species of IAPP,<sup>282-288</sup> and Figure 7 show a structure of membrane (nanodisc)-associated hIAPP oligomers. For additional details on the structure and kinetics of aggregation under various conditions, readers are referred to review articles on IAPP.<sup>289-291</sup>

### 3.3.2. $\alpha$ -synuclein and tau proteins

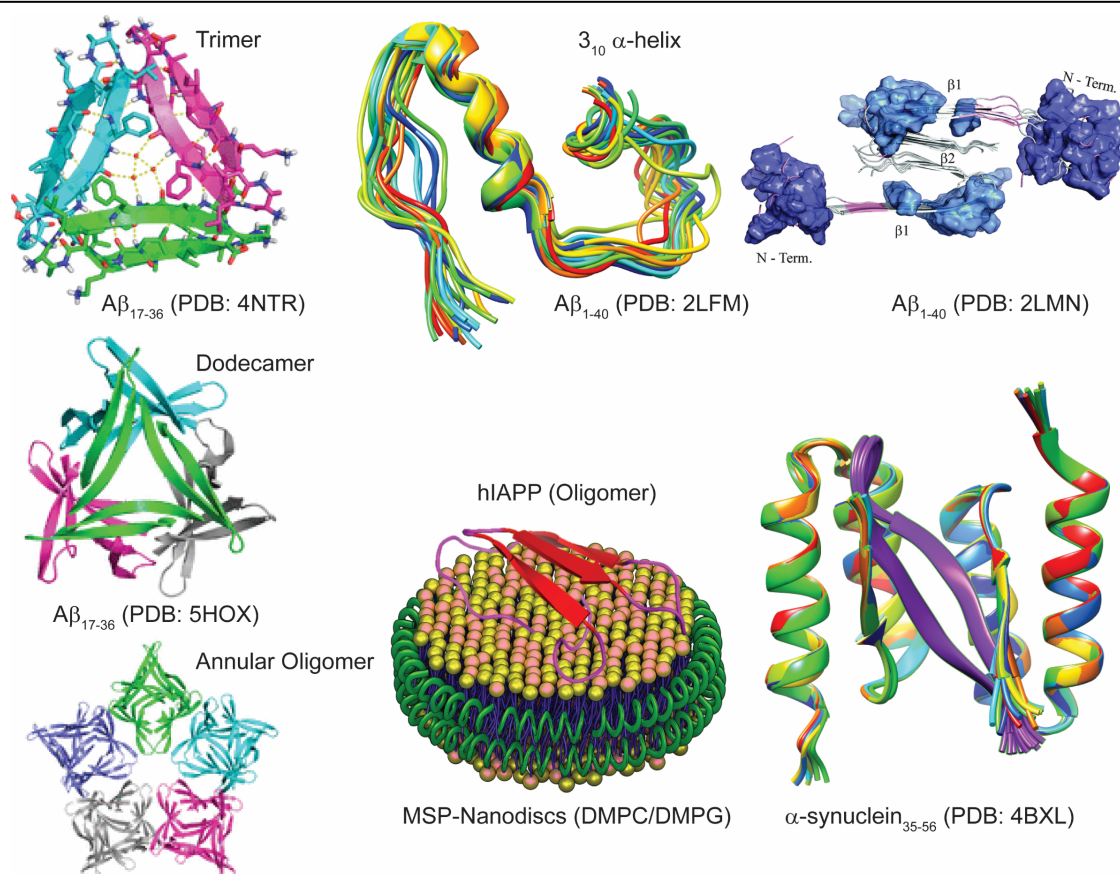
In PD, oligomerization of  $\alpha$ S has been shown to increase in individuals with Lewy pathology. A low-resolution structural evolution of individual  $\alpha$ S oligomers characterized by antiparallel  $\beta$ -sheet structure is detected using AFM-IR.<sup>292</sup> Structural mapping of oligomers at all-stages of aggregation by AFM-IR highlighted the early oligomers (with one day incubation) are spherical in shape and dominated by a  $\alpha$ -helix/random-coil secondary structure, but some of the oligomers showed a mixed parallel and antiparallel  $\beta$ -sheet structures. The size of the spherical oligomers is observed to grow in size on day-2 with an increasing  $\beta$ -sheet and decreasing  $\alpha$ -helix structures. Later on day-3, AFM-IR detected both the presence of spherical oligomers ( $\alpha$ -helix/ $\beta$ -sheet rich structures) and protofibrils with a predominant  $\beta$ -sheet structure.<sup>292</sup> The structure of  $\alpha$ S oligomers observed under cryo-EM is shown to have a cylinder-like appearance and characterized with a predominant  $\beta$ -sheet structure ( $35 \pm 5\%$ ).<sup>293</sup> The formation of early aggregates of  $\alpha$ S and interaction between monomers and stable bioengineered

oligomers are studied using fluorescence spectroscopy by labelling  $\alpha$ S species with differently labeled fluorophores.<sup>294</sup> This study highlighted a comparatively higher binding affinity between oligomers as compared to oligomer-monomer/monomer-monomer interactions suggesting oligomer-oligomer assembly is a major driving molecular process of aggregation in the early stages of the disease progression. Importantly, large oligomers are found to assemble to form aggregates sooner as compared to low-size oligomers (octamer>tetramer>dimer/monomer), but not competently assist nucleation and monomer recruitment.<sup>294</sup> Another study showed  $\alpha$ S oligomers do not seed the fibrillation reaction. This study underlined meta-stable spherical shape (10 nm), with a disordered conformation, for the  $\alpha$ S oligomers by small-angle neutron scattering method.<sup>295</sup> Amyloid oligomers differ in their surface properties (in general hydrophobic residues are solvent exposed) which correlate to its competent membrane binding and toxicity. Hydrophobicity surface landscaping of individual  $\alpha$ S oligomer by selective fluorescent molecules is probed using super-resolution imaging technique.<sup>296</sup> The hydrophobicity of the surface of the oligomers are found comparatively higher than that of aged fibers evidencing  $\alpha$ S aggregation proceeds via generation of toxic intermediates characterized with hydrophobic heterogeneity like that has been reported for amyloid proteins and peptides.<sup>296</sup> A recent study showed that  $\alpha$ S promotes formation of A $\beta$  oligomers and inhibits fibrillation by stabilizing the oligomer structure.<sup>297</sup> Notably, this effect is identified only by the soluble species of  $\alpha$ S (monomers and oligomers), but not by fibers. EM studies revealed globular morphologies for the oligomer mixture of A $\beta$  and  $\alpha$ S. While structural information for the A $\beta$  oligomers induced by  $\alpha$ S has still not been reported, further investigation could provide more details to establish a correlation between AD and PD.<sup>297</sup> The aggregation and folding of  $\alpha$ S is shown to be modulated by the isomeric state of the five proline residues (P108, P117, P120, P128, and P138) located in the disordered C-terminus.<sup>298</sup> Among the five-proline residues, isomerization of P128 is identified to be catalyzed by cyclophilin A (CypA), a protein belonging to the family of peptidylprolyl isomerases. CypA and  $\alpha$ S are co-localized in cells, where CypA interacts with  $\alpha$ S with a micromolar binding affinity.<sup>298</sup> NMR titration experiments, combined with a crystal structure of the  $\alpha$ S- CypA complex, showed CypA binds to the amyloid core preNAC hydrophobic domain (residues 47-56) of  $\alpha$ S<sup>298</sup> and also known to bind membrane.<sup>299</sup> The  $\alpha$ -synuclein complexed by affibody displays a  $\beta$ -hairpin spanning residues 35-56 by NMR (Figure 7).<sup>261</sup>

Finally, we report on recent studies that provided structural and functional details for the tau oligomers. Antibody assay detected an increasing amount of soluble tau oligomers in the limbic regions of human brain with positive NFT, also referred as Braak stages III and IV.<sup>300,301</sup> Clusters of soluble tau multimers (globular) were observed in AFM with a measured height ranging from 10 to 30 nm.<sup>301</sup> TEM imaging analysis reported a similar globular morphology of chemically stabilized tau oligomers



prepared *in vitro* and detected by oligomer specific antibodies.<sup>302</sup> Tau oligomer rich in  $\beta$ -sheet structure is shown to be influenced by both  $A\beta$  and  $\alpha S$  oligomers.<sup>303</sup> A recent NMR study reported tau monomers binding to the C-terminal disordered domain of  $\alpha S$  monomer following the generation of toxic  $\alpha S$  oligomers.<sup>304</sup> Similarly,  $\alpha S$  fibers and monomers are shown to induce tau oligomerization. Tau oligomerization is also shown to be triggered by its interaction with other molecules like  $\beta$ -arrestin and molecular chaperones ubiquitously expressed in cells.<sup>305,306</sup> An increase in tau level shown to increase  $\beta$ -arrestin that impairs tau clearance by stabilizing toxic tau species and promotes the aggregation of tau.<sup>305</sup> Molecular chaperone Hsp90 complex with PPIase FKBP51 is shown to promote tau oligomerization. The ternary complex (Hsp90/FKBP51/tau) formation is proposed to have a synergistic effect where the chaperone and PPIase may influence tau proline isomerization leading to the formation of tau oligomers.<sup>306</sup> Readers are referred to recent reviews on tau oligomers.<sup>307,308</sup>



**Figure 7.** High-resolution structures of amyloid oligomers in solution or membrane-bound state solved using X-ray and NMR methods, the exception being the model of  $A\beta_{40}$  assembly toxic surface (top, right). The figure was designed by the authors.

### 3.4. Reproducibility issue of *in vitro* experiments

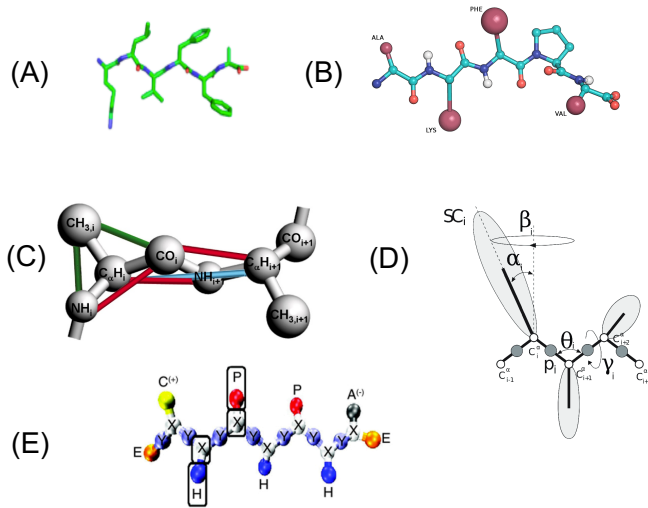
The self-assembly of amyloidogenic peptides and proteins into amyloids is generally considered to work via a nucleation and elongation (a noncovalent polymerization) process. Depending on the amyloidogenic peptide/protein also other processes can be very important, such as secondary nucleation.<sup>309</sup> This self-assembly is a stochastic process and susceptible to a lot of non-specific interactions. Moreover, the process is auto-catalytic and hence the addition of preformed aggregates can dramatically accelerate the self-assembly.

*In vitro* studies have shown that the self-assembly process can be influenced by numerous factors including peptide/protein concentration, salt, pH, temperature, metal ions, lipids, other molecules (such as small molecules as discussed in Section 9) and cofactors as for tau. Thus, for test-tube experiments, reproducibility needs identical starting conditions. As the number and/or type of pre-aggregates are very difficult to control, the self-assembly process should be started with a sample of only monomeric species.<sup>193</sup> Obtaining pure monomeric species can be very difficult, but depends on the peptide/protein under investigation.<sup>310</sup> Some are very strongly aggregating and hence difficult to monomerize.<sup>311</sup> For instance  $\alpha$ S is less aggregating than A $\beta$ . Self-assembly of  $\alpha$ S is often studied at higher concentration or stimulated by effectors, as it is slow.<sup>312</sup> In contrast amyloid- $\beta$  aggregates very fast; particular the A $\beta$ 42 aggregates faster than the more abundant A $\beta$ 40 form. IAPP is another very fast aggregating peptide that is quite sensitive to pH and other conditions.

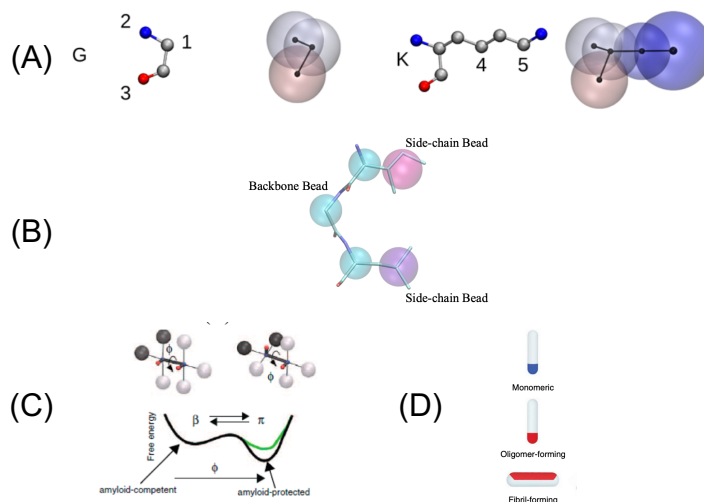
Amyloid- $\beta$  (even A $\beta$ 40) and IAPP are very difficult to monomerize, and this is a reason why self-assembly is often not reproducible even when the same conditions were used. First, different batches of A $\beta$  can vary in purity and in the preaggregation states; and, therefore, reproducible results are more difficult to obtain between different batches. Even an extremely low amount of impurities (for example, metal ions) that cannot be detected by the regular analytical methods sometimes can have significant influence on the reproducibility. In general, biologically (typically via expression from *E. coli*) obtained fresh peptides are higher in purity than synthetically obtained peptides and more likely to provide reproducible results. Then, a lot of other parameters, in addition to those described previously, influence the self-assembly, like ionic strength, surfaces (type and size), shaking, sheering forces etc.).<sup>310,313</sup> So, it is very important to report the complete details of the samples treatments including the monomerization steps and the use several batches is highly recommended, in particular for the very aggregation-prone amyloidogenic peptides (like A $\beta$  and IAPP).<sup>313,314</sup>

#### **4. Multiscale simulations of the early and final aggregation steps of amyloid peptides**

There are various sampling techniques to explore the configuration space of amyloids at different aggregation step. Readers are referred to recent reviews describing their main features [12,14](#). Multiple protein representations are also used throughout this review and are shown in Figures 8 and 9.



**Figure 8.** Protein representations from all-atom to coarse-grained models. (A) All-atom. (B) OPEP. (C) Geometric representation of the protein intermediate-resolution model, PRIME, for polyalanine. Covalent bonds are shown with thick gray lines connecting united atoms for N-H, C=O, C $\alpha$ -H and CH<sub>3</sub> sidechain. At least one of each type of pseudo-bond is shown with a colored line. Pseudobonds are used to maintain backbone bond angles, consecutive C $\alpha$  distances and L-isomerization. The united atoms are not shown full size for ease of viewing. (D) UNRES. (E) Shea's model. The authors designed the figure.



**Figure 9.** Protein representations from coarse-grained to mesoscopic models. (A) SIRAH. (B) SOP-IDP. (C) Caflisch's model. (D) Frenkel's model. The authors designed the figure.

#### 4.1 Thermodynamic phase diagram of short amyloid peptide aggregation from simple to more realistic protein models

Discontinuous molecular dynamics (DMD) is a fast event-driven alternative to conventional molecular dynamics in which particles interact with each other via discontinuous potentials including hard-sphere and square-well potentials.<sup>315</sup> A moderately coarse-grained model, PRIME (PRotein Intermediate resolution Model) was developed for use with DMD simulation to model the folding behavior<sup>316-318</sup> and fibril formation of homo-polypeptides, in particular, polyalanine.<sup>319,320</sup> In the PRIME model, as shown in Figure 8C, each amino acid is represented by three backbone spheres N-H, C $\alpha$ -H, C=O and one sidechain sphere R. The two major non-bonded interactions captured in the PRIME model are the directional hydrogen bonding interactions between backbone N-H and C=O spheres and the non-directional hydrophobic interaction between two sidechain spheres. The other non-bonded excluded volume interactions between backbone spheres and sidechain spheres are all modeled as hard-sphere potentials. The PRIME model was later extended to become the PRIME20 model which contains distinct square-well potential parameters to represent all possible sidechain-sidechain polar, electrostatic and hydrophobic interactions between the twenty amino acids. This was achieved by Cheon *et al.*<sup>321</sup> who used a perceptron learning algorithm to derive knowledge-based sidechain-sidechain square-well potential parameters by fitting to the structures of 711 native-state globular proteins in the PDB and 2 million decoy structures. Later, Wang *et al.* related the reduced temperature and time scale to real units by matching a peptide helical folding profile and a self-diffusion coefficient from DMD/PRIME20 simulation with experimental measurements and atomistic simulations.<sup>322</sup> The validity and efficiency of DMD simulations with the PRIME20 model for studying protein aggregation problems have been demonstrated by extensive investigation of self-assembly systems, including A $\beta$ <sub>16-22</sub><sup>323,324</sup>; hexapeptides<sup>325</sup> designed by Serrano *et al.*<sup>326</sup>; tau fragment<sup>327</sup>; A $\beta$ <sub>17-36</sub><sup>328</sup> and A $\beta$ <sub>17-42</sub><sup>329</sup>, and co-assembly systems of A $\beta$ <sub>16-22</sub> and A $\beta$ <sub>40</sub><sup>330</sup> and the charge complementary peptide pair CATCH (+/-).<sup>331</sup>

Of note, researchers have adopted a number of different geometric and energetic representations of peptides to emphasize those aspects of protein aggregation that they consider most essential and to be compatible with their simulation engine. Examples of prominent CG models include the anisotropic rod-like model by Frenkel<sup>332</sup> (Figure 9D), the lattice-based toy models by Thirumalai<sup>333</sup> and by Frenkel<sup>334</sup>, the mid-resolution off-lattice CG model of Shea<sup>335</sup> (Figure 8E), the UNRES model by Scheraga<sup>336</sup> (Figure 8D), the SOP-IDP model<sup>31</sup> (Figure 9B), the OPEP model by Derreumaux<sup>337,338</sup>

(Figure 8B), the CG DMD model by Urbanc<sup>339</sup>, the SIRAH CG model (Figure 9A) by Pantano,<sup>340</sup> and the Caffisch's model (Figure 9C)<sup>12,341</sup>.

Although one might expect that DMD/PRIME20 and other MD simulations of CG protein models could be used to predict the equilibrium phase behavior of peptide systems, and that by comparing their prediction to experimentally-determined phase diagrams one could test each model's validity, this turns out not to be the case. More precisely, the seemingly-straightforward way to do this--simulate the fibril nucleation event and aggregation of a small system (e.g. <50) of randomly-distributed peptides aggregates into a fibrillar structure on a short time scale (<5 $\mu$ s) at each temperature (T) or equivalently, intermolecular energy ( $\epsilon$ ) and concentration (C), and then plot the boundary between the observed phases in the T-C or  $\epsilon$ -C plane<sup>342-344</sup>--- has two problems. The first problem is that the system will not necessarily equilibrate but will instead become kinetically trapped even if one simulates for a long time. The second problem is that since amyloid formation is a solid-liquid transition, the thermodynamic quantity to calculate is the solubility, the concentration of peptides in solution in equilibrium with the solid phase (the amyloid), not the total concentration as above. In other words, the microscopic "kinetics" phase diagram so derived cannot be used to calculate macroscopic thermodynamic quantities, such as solubility and latent heat, which can be directly measured from *in vitro* experiments. A more rigorous approach is described below.

From a thermodynamic point of view, amyloid formation is a spontaneous nucleation and growth process whose nucleation rate is determined by the driving force associated with the difference in the chemical potentials of the peptides in the fibril and in the solution phases. At solid-liquid phase equilibrium where the chemical potentials of the peptides in the fibril and in the solution phases equal each other, an amyloid fibril neither grows nor dissolves. The equilibrium protein composition in the solution phase is essentially the solubility of the protein, as mentioned before. In the light of classical nucleation theory (CNT), Kashchiev and Auer<sup>345</sup> treated amyloid fibril nucleation as a classical two-dimensional nucleation problem in which fibrils are formed by "one step" direct polymerization. They derived a theoretical description of the work of nucleation, the critical nucleus size and the nucleation rate for fibril formation as explicit functions of the concentration and temperature of a protein solution. Auer and Kashchiev<sup>346</sup> first applied Monte Carlo (MC) simulation with the seeding approach<sup>347</sup> to calculate the liquid-liquid coexistence line for helical-rich oligomers and the solid-liquid coexistence line for  $\beta$ -sheet-rich fibrils using a one-bead-per-residue homo-polypeptide model<sup>348</sup>. Later, Auer<sup>349</sup> applied this amyloid nucleation theory approach and MC simulation to the same protein model to calculate the equilibrium solubility line for an infinite-layer  $\beta$ -sheet fibril which represents a macroscopic fibril.

We applied DMD/PRIME20 simulation and the nucleation theory approach of Auer to calculate solubilities, and hence the equilibrium phase diagram, of a real fibril-forming peptide, A $\beta_{16-22}$  peptide, the archetypal amyloid former.<sup>350</sup> To do this it was necessary to simulate many assembly and disassembly events for fibrillar aggregates which is difficult as it requires the breaking of many backbone hydrogen bonds. One reason for studying this peptide sequence is that the A $\beta_{16-22}$  fibril structures predicted using DMD/PRIME20 simulation agree well with experiment<sup>323</sup>. Firstly, we applied constrained canonical ensemble DMD simulations and the seeding approach<sup>346,347</sup> to determine the solubilities of a series of small aggregates (disordered oligomers and 2, 3, 4  $\beta$ -sheet A $\beta_{16-22}$  fibrils) formed by the A $\beta_{16-22}$  peptides, as shown in Figures 10A-E. Specifically, the solubilities for the different aggregates at a given temperature were taken to be the equilibrium monomer concentration  $C_e$  at which the aggregate neither grows nor shrinks. During the simulation, the monomer peptides freely attach or detach from the two fibril ends but the creation of a new  $\beta$ -sheet on the preformed fibril is prevented. The latent heats of peptide aggregation into fibril phases from solution can be obtained by fitting the  $C_e(T)$  data to the van't Hoff equation,

$$C_e = C_r \exp\left(-\frac{L}{k_B T}\right) \quad (1)$$

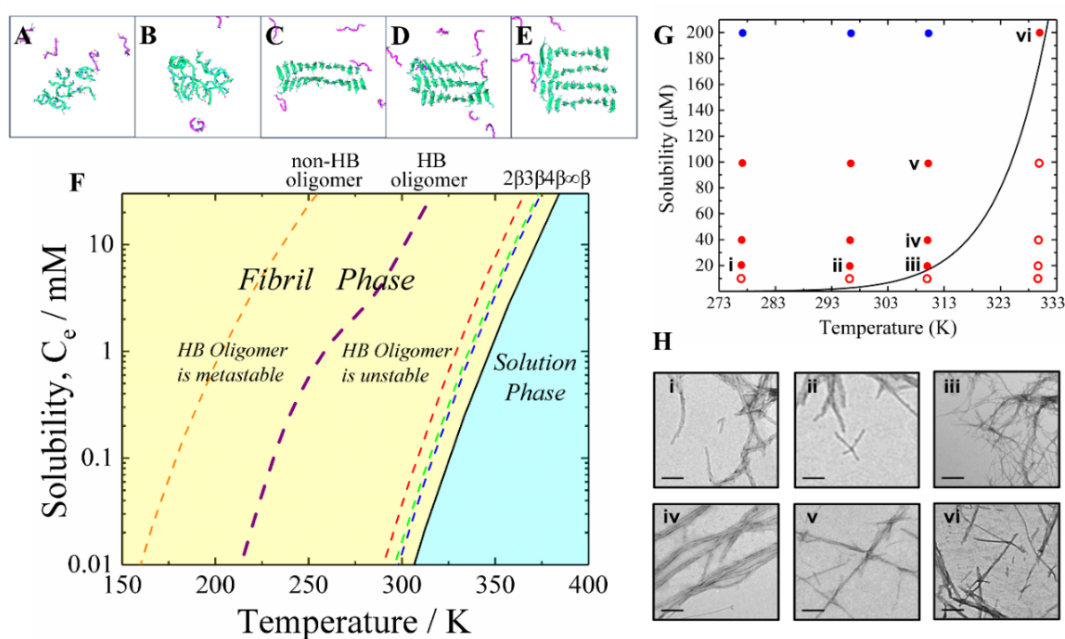
where  $C_e$  is the equilibrium fibril solubility,  $C_r$  is a temperature-independent reference concentration,  $k_B$  is the Boltzmann constant,  $T$  is the simulation temperature, and  $L$  is the latent heat of monomer peptide aggregation into the oligomer or fibril. As shown in Figure 10F, at a given temperature, the solubilities for the 2, 3 and 4  $\beta$ -sheet fibrils decrease with increasing thickness (number of  $\beta$ -sheet layers) of the fibril. This indicates that the stability of the fibril increases with increasing fibril thickness. However, the solubilities directly measured for 2, 3 and 4  $\beta$ -sheet fibrils from simulation are not necessarily consistent with the experimental solubility measurements since the macroscopic fibril formed by short peptides may contain on the order of ten  $\beta$ -sheets.<sup>19,351</sup>

To estimate the solubility line for a macroscopic (i.e. thick) fibril, we adopt Auer's approach<sup>346</sup> and applied a linear fit of the simulation data ( $\ln C_{i\beta}$  vs  $1/i$ ) to Eq. (2) to extrapolate the solubility  $C_{\infty\beta}$  for an infinitely thick fibril. The dependence of the solubility of a fibril with  $i$  layers ( $i=1, 2, 3$ , etc.) of a  $\beta$ -sheet,  $C_{i\beta}$ , on fibril thickness ( $i$ ) at fixed temperature was derived by Kashchiev and Auer<sup>345</sup>:

$$C_{i\beta} = C_{\infty\beta} \exp\left(\frac{2\psi_h}{k_B T} \cdot \frac{1}{i}\right) \quad (2)$$

where  $C_{\infty\beta}$  is the solubility of an infinite thick fibril,  $\psi_h = a_h\sigma_h$  is the fibril surface energy parallel to the fibril thickening axis,  $\sigma_h$  is the specific surface energy of the face perpendicular to the fibril axis, and  $a_h$  is the lateral surface area occupied by each peptide within one  $\beta$ -sheet<sup>349</sup>.

The predicted phase diagram is presented in Figure 10F, which shows that the solution phase and the macroscopic fibrillar phase are thermodynamically stable phases, and that there also exists a hierarchy of metastable phases. To validate the *in silico* prediction of  $A\beta_{16-22}$  solubility at biophysically relevant temperatures, TEM was used to determine whether fibrils had formed after a predetermined time (2-wk incubation). Importantly, our prediction of  $A\beta_{16-22}$  solubility over temperatures from 277 to 330 K agrees well with experimental measurements (Figs. 10G and 10H).



**Figure 10.** (A-E) Simulation snapshots of non-HB oligomer; HB oligomer; and two, three, and four  $\beta$ -sheet fibrils, respectively. The peptides in the five aggregates and in solution are shown in green and magenta, respectively. (F) Phase diagram for  $A\beta_{16-22}$  peptide. Solubility,  $C_e$ , versus temperature data for the oligomer and fibril. The fibril and solution phases are colored yellow and cyan, respectively. (G) Summary of both the simulation-predicted temperature-dependent solubility line,  $C_e(T)$ , for  $A\beta_{16-22}$  peptide (black curve) and the fibrillation experiments performed under given conditions. Red dots and red circles indicate conditions at which fibrils have been found to form and not to form, respectively, via TEM. Blue dots indicate that fibrils have been reported in the literature<sup>352-357</sup> to form under these conditions. (H) Six selected TEM images (i-vi) showing that  $A\beta_{16-22}$  forms fibrils under conditions that correspond to the six red dots labeled i-vi in A. (Scale bar: 200 nm.) Reprinted by permission from National Academy of Science: ref 350. Copyright 2019.

It has traditionally been challenging to create accurate thermodynamic phase diagrams for complex biomolecules such as polypeptides due to the lack of appropriate force fields and limited computational resources. Our work represents a significant milestone in efforts to overcome this challenge. By applying an advanced computational technique to a relatively realistic peptide model, we predicted an equilibrium concentration and temperature phase diagram for a short amyloid  $\beta$  peptide that is in remarkably good quantitative agreement with experiment.

Finally, it is important to point out that the solubility measurement approach adopted here assumes that peptides self-assemble through direct polymerization or “one-step” nucleation, which is mainly applicable for short polypeptides (e.g. <15 amino acids). In contrast, A $\beta$ <sub>42</sub>, amylin and  $\alpha$ S undergo a two-step nucleation process, first forming oligomers (due to the non-negligible intra-chain interactions) that eventually merge rearrange and nucleate to form a fibrillar structure. Thus, the nucleation process may involve oligomer formation<sup>358</sup> which may be accompanied by liquid-liquid phase separation<sup>359</sup>. In this case, a non-classical nucleation theory needs to be developed for *in silico* prediction of protein solubility and the comparison with experimental measurements<sup>360-362</sup>.

#### **4.2. Hydrodynamics, shear and crowding effects on amyloid formation**

Even at *in vitro* conditions where proteins/peptides concentration is at least 3 orders of magnitude higher than *in vivo*, the formation of amyloid fibrils occurs in hours/days. Therefore, in order to get molecular insights on this process via computer simulation it is necessary to make compromises. The first and more important is the use of simplified models representing the proteins and the aqueous environment by simplified representations where CG models and implicit solvent are used.<sup>363</sup> The second compromise concerns the effective concentration that is used in simulations<sup>364</sup> and that can be tuned so to favour the encounter of the molecules when the kinetics of aggregation and elongation is investigated. Finally, even with these simplifications in hands the size of the simulated systems is generally limited, generally with less than 10<sup>3</sup> monomers<sup>365,366</sup>.

The two principal aspects that have been studied via CG models are the early steps of aggregation and the mechanism of fibril elongation.<sup>367</sup> Peptides aggregation can be described as a nucleation process, but whether the primary nucleation is a one-step or two-steps process clearly depends on the amino acid sequence and the experimental condition (Figure 11A). As for many proteins, however, and notably the prion and A $\beta$  proteins, it has been reported experimentally that the proteins first collapse into disordered aggregates that associate and dissociate, eventually producing fibrils that elongate and fragment<sup>12,368-370</sup>, all events involving very long-time scales for each event (Figure 11B).



Simulations based on simplified molecular models have addressed these issues. In seminal works the attention has been posed on how the molecular propensity of the amyloid peptide to sample the aggregation  $\beta$ -prone state influences the path of the initial aggregation. Pellarin and Caflish,<sup>371</sup> and Bellasia and Shea<sup>372</sup> explored the aggregation mechanism by tuning the conformational stability of the ‘fibrillar-like’ state of model peptides. Both studies clearly showed that when the peptide is able to access conformations different from that of fibrillar structures, the aggregation passes through disordered micelles. On the contrary for peptides having strong propensity for the  $\beta$ -configuration, the aggregation is funnelled directly toward structured aggregates. These, as other studies, highlighted the competition among thermodynamic and kinetic selection during aggregation, and the effect of the monomer configurational energetic landscape<sup>371-374</sup>. A nucleation diagram as a function of solution concentration and inter-monomer interaction strength has been reported on the basis of MC simulations of simple stick-like molecules, stressing the important role of the intermediate disordered aggregates, and the predominant contribution of the two-steps primary nucleation process.<sup>365</sup>

Computational studies focused also on the behaviour of a monomer when aggregating. Following the study by Massi and Straub,<sup>375</sup> two idealized pictures can be drawn (Figure 11C). In a first one, the amylogenic protein/peptide acquires very rapidly its monomeric prone-aggregation configuration that drives fast adhesion with other monomers for the creation of a critical structured nuclei or with an existing fibril. In a second scenario, the adhesion and structuring processes of the monomer occur in two distinct phases, referred to as dock and lock. There is now a consensus from experiments and simulations that the dock-lock mechanism is the dominant elongation path in many conditions. There is a clear kinetic separation among the timescales of the dock and lock phases. The first one being limited by the diffusion and the energetics of desolvation, while the second one is controlled by the free energy barriers that separate peptides conformations, and that can be affected by the local packing in the aggregate. According to several estimates, a difference of at least two orders of magnitude for the characteristic kinetics exists,  $\tau_{\text{lock}}/\tau_{\text{dock}} \geq 100$ .<sup>376-379</sup> Experimentally the elongation process results to be entropically driven with an entropy gain associated to the release of interfacial water into the bulk. Clearly in CG simulations such a contribution cannot be accounted explicitly. However, critical information about the change in conformational flexibility of the proteins/peptides upon aggregation has been obtained. The steps from the dock toward the multiple lock states have been related to the size of the proteins and their conformational space, and described by monitoring the shift from intra vs intermolecular interactions.<sup>77,380,381</sup> A very detailed picture of the dock and lock mechanism has been recently drawn by combining atomistic simulations and Markov State Model. It was shown that

for a simple amylogenic peptide, the fragment A $\beta$ <sub>16-22</sub>, the locking phase requires a complex search in a multi-minima landscape characterized by off-register configurations, and with no evident funnel toward the fibrillar configuration.<sup>382</sup>

Simulations have also inquired the global process of elongation, and an asymmetric elongation has been often reported,<sup>367</sup> with one cap of the fibril growing faster, and involving local disorder. Thanks to the simulation of a very large system, a complementary view to the one monomer addition was explored. It was possible to probe that at large length-scale once the first oligomers are formed, multiple fusion events take place to elongate the protofibril with not clear dominant scheme,<sup>365,366</sup> and including lateral branching that use the surface of the growing fibril as a seed.<sup>366,383</sup> This finding confirms the theoretical intuition proposed to describe the kinetics of amyloid formation, and that includes a key secondary nucleation process.<sup>309,384</sup>

When using implicit solvent CG models to explore protein aggregation two issues must alert the reader. The first concerns the estimate of thermodynamics quantities. As anticipated, solvation free energy is not explicitly accounted for, and empirical or approximated approaches are required to estimate relevant thermodynamic contributions like the specific heat variation. Moreover, by reducing the degrees of freedom of a system the quantification of entropy is altered with respect to atomist models, with the risk to unbalance entropy/enthalpy compensation effect. The simplification of the free energy landscape clearly impacts also the barriers separating states, and therefore the resulting kinetics. However, a second aspect must be accounted for when focusing on the dynamic behaviour. In implicit solvent model there is not exchange of momenta between the particles and the solvent, and solvent mediated correlations are not accounted for. This is critical when dealing with many particles systems, and their aggregations since the hydrodynamics interactions (HI) might play an essential role. Several techniques can be used to cure this deficiency<sup>385-387</sup> and have been applied in several contexts, like protein folding<sup>388,389</sup> or protein motion under crowding.<sup>390</sup> More specifically, the Brownian dynamics including HI has been used to investigate lipid aggregation using a simplified dumbbell model.<sup>391</sup> It was shown that HI speeds up the process since, as can be deduced by comparing the Zimm versus Rouse model for polymer motion,<sup>392</sup> when the first oligomers are formed their diffusivity scales more favorable with the aggregate size than when HI are not included in the simulation. A similar result was obtained for amyloid aggregation by Lattice Boltzmann MD simulations with the OPEP model for A $\beta$ <sub>16-22</sub> peptides,<sup>393-394</sup> see Figure 11D. Notably the presence of HI coupled to the high-resolution and flexible OPEP model, enhances size fluctuations of the formed oligomers. This size fluctuations

generate solvent local flows at the nanoscale that contribute to the progress of the aggregation. This is especially important at a large length-scale when very extended protofibrils (each formed by hundreds of monomers) fuse together.<sup>366</sup>

The impact of fluid dynamics on amyloid aggregation is well documented by *in vitro* experiments where the action of stirring or shaking accelerates the kinetics of fibril elongation. For instance, the shearing of a protein solution in a Couette flow device favors the aggregation of beta-lactoglobulin by enhancing the formation of spheroidal seeds in the solution.<sup>395</sup> It was also reported that the action of shear alters the morphology of the formed fibrils.<sup>396,397</sup> The energetic landscape of  $\beta$ 2-microglobulin fibrils was explored by combining isothermal titration calorimetry and stirring.<sup>398</sup> The stirring force modifies the end-point of the aggregation process as measured by the solution enthalpy difference, and the kinetics of aggregation that results enhanced. The action of shear may also perturb formed fibrils. For example, the capability of shear flow to break existing fibrils has been used to explore the different mechanisms co-acting during fibril elongation. Namely shearing is used to enhance the kinetic of the fragmentation contribution over secondary and primary nucleation.<sup>384</sup>

The molecular mechanism of flow action on aggregation is still debated. In fact, for standard globular proteins the initial unfolding that should trigger aggregation can be forced only at very high shear rates,<sup>399-402</sup> values much higher than what found typically in physiological condition that are  $<10^4 - 10^5$   $s^{-1}$ . In a recent study elongational flow was used to perturb and trigger aggregation of several proteins,  $\beta$ 2-microglobulin, bovine serum albumin (BSA), and monoclonal antibodies.<sup>16</sup> It was shown that when exposed several times to an the elongational flow at shear rate  $\sim 10^4 - 10^5$   $s^{-1}$  even a stable protein like BSA can expose to solvent parts of the sequence generally screened in the folded state, and that therefore induce aggregation. It must also be considered that in many neurodegenerative diseases the involved proteins are intrinsically disordered. For these latter ones even a weak fluid perturbation might alter the ensemble of conformational states accessed by the protein, and therefore select the ones prone to aggregate, or force preferential orientations that favour the fibrillar elongation. Moreover, shearing and interactions with a surface might act together and modify the structure of prefibrillar seeds. A possible impact of fluid flow in cerebrospinal and interstitial fluid on the amyloid aggregation has been proposed recently and discussed in detail.<sup>403</sup>

In order to complete the discussion on protein aggregation, we should address an extra problem. In fact, *in vivo* proteins generally move in a very crowded space like the cytoplasm or the membranes. This crowding has multiple impacts on aggregation. The macromolecular crowding generates an

excluded volume effect that locally alters the concentration of the aggregating species, and thus could have a favourable impact on the aggregation with respect to a dilute reference solution. On the other hands, moving in a crowding space is more difficult, the diffusion of a specie is reduced as effect of the reduced dimensionality, and higher viscosity of the solution. The molecular collisions needed to start the nucleation, and the further elongation become rarer. Finally, the macromolecules surrounding the aggregating species are not inert, they might have specific interactions with them that could ease misfolding, or compete with the aggregation.

Experimentally several studies have tackled these issues. *In vitro*, the crowding effect can be reproduced by adding polymers to a solution, e.g., Ficoll, dextran or PEG, or proteins of different sizes, for example BSA proteins generate large excluded volumes. NMR studies reported that depending on the nature of crowders the  $\alpha$ S monomer can adopt more or less compact configurations, indicating that the effect on protein extension is not merely an excluded volume effect but might depend on other features of the crowding agent.<sup>404</sup> The ensemble of configurations explored by  $\alpha$ S can also be tuned by two-dimensional crowding on a lipid surface.<sup>405</sup> Interestingly, when focusing on aggregation it was reported that proteins linked to neurodegenerative diseases have indeed a propensity to aggregate more and faster under crowding conditions.<sup>406</sup> Moreover, the morphology of the formed fibrils is also affected by crowding, for instance Ficoll induces a more packed state of  $\beta$ 2-microglobulin fibrils.<sup>398</sup> However, the heterogeneity of *in vivo* crowding cannot be easily mimicked in *in vitro* sets up, and the effect on protein mobility, conformation and stability is subtle. While excluded volume effect predicts stabilization of proteins under crowding, a variety of studies reported a much more complex response, pointing to the importance of quinary interactions, the fifth degree of organization of a protein within its surrounding environment.<sup>407,408</sup> For the B1 domain of protein G, the stability effect of a single amino-acid mutation was found to be amplified ten-fold by the crowded cytosol of *Escherichia coli*.<sup>407</sup> On the other hand, mutating a single amino-acid residue in SOD1 sufficed to reverse the sign of the stability effect of the intracellular environment.<sup>408</sup> Interestingly, for monomeric  $\alpha$ S, it was shown that under crowding the conformation is flexible and disordered while one would have expected a compact state due to excluded volume.<sup>185</sup>

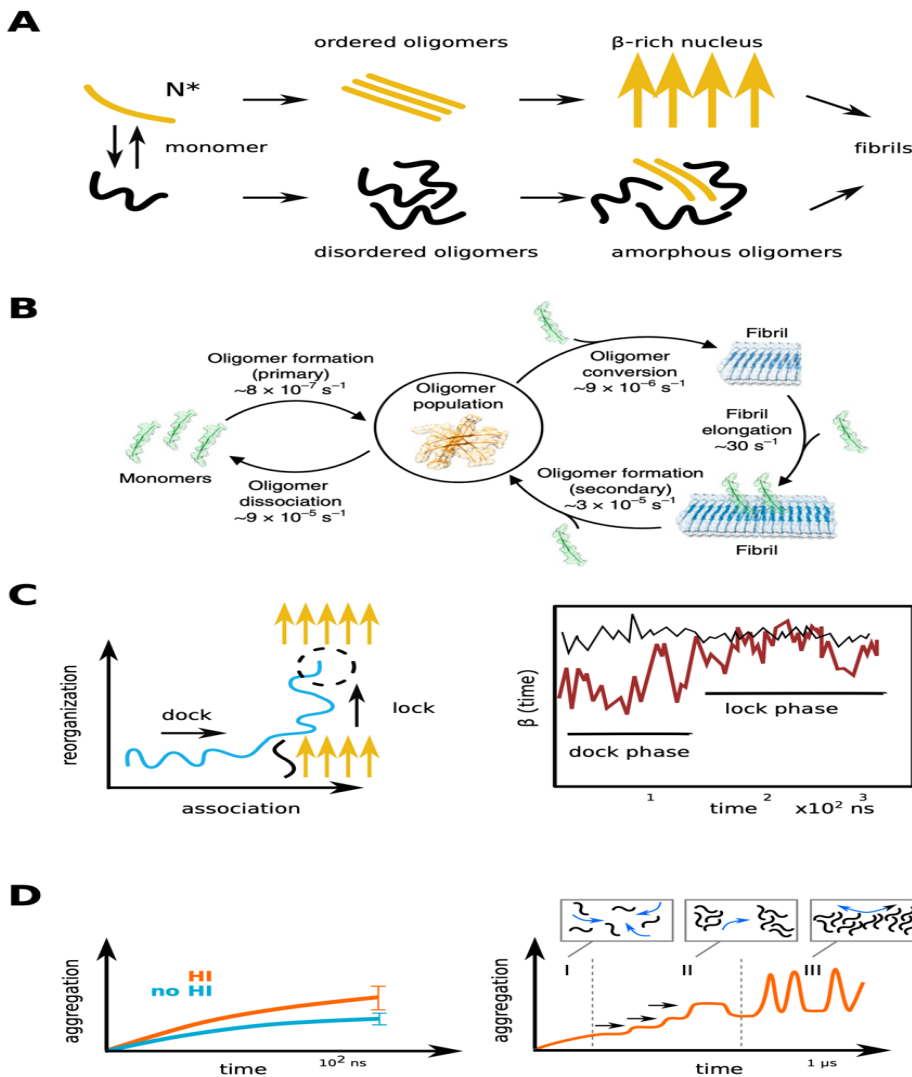
However, concerning the general picture, fibrillar aggregation of SOD1<sup>409</sup> or Huntingtin exon 1 protein<sup>410</sup> in cells has been characterized by means of different techniques, NMR and fluorescence spectroscopy, respectively, and the obtained results are similar to what found *in vitro* experiments. Of

note when discussing the impact of aggregation on neurodegenerative diseases one should also consider the aging of the cells, a difficult condition to reproduce *in vivo* experiments.

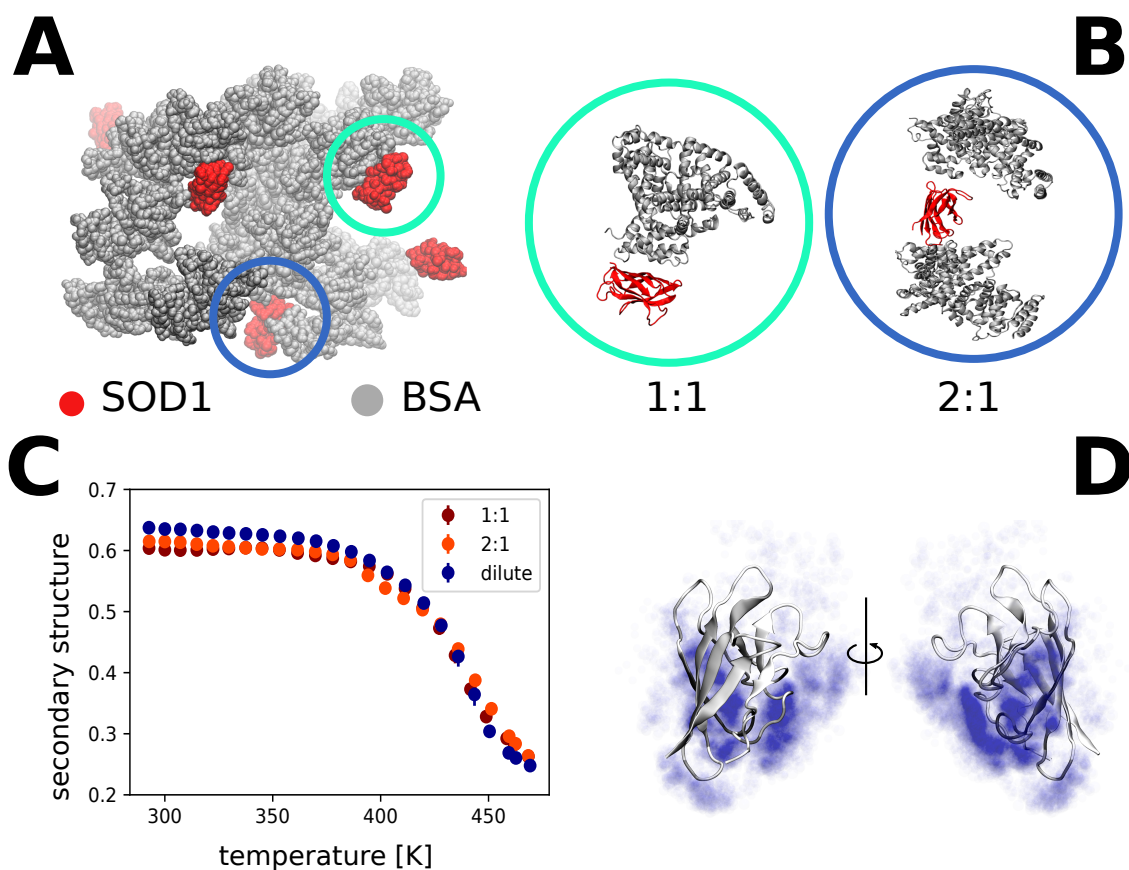
A few simulations have tried to shed light on the mechanism of aggregation under crowding. A first set of studies have focused on the effect of crowding on the conformation sampled by IDPs and using different resolutions models to compare with experiments like SAXS or FRET.<sup>411-413</sup> As an example, we refer to the work by Zhou *et al.*<sup>412</sup> where by using a mixed resolution approach, atomistic for the IDP and CG for the crowders, three IDPs (for the N-terminal domain of HIV-1 integrase, the ACTR, and prothymosin  $\alpha$ ) were investigated in a PEG crowded solution. A good match with experimental FRET data was obtained only when a certain degree of attraction between the proteins and the crowders was incorporated in the model, while a pure repulsive interaction led to too compact states for the IDPs.<sup>412</sup>

In a second class of work the focus was placed on the aggregation kinetics. In the presence of inert crowders modelled as hard spheres it was shown that A $\beta$ <sub>16-22</sub> amyloid peptides simulated by the PRIME-DMD generally collapse quickly in disordered aggregates, and the structuring phase occurs as a second step.<sup>324</sup> However, when the nature of the crowder is specified, e.g., being modelled as hydrophobic particles, the interactions with the peptides become competitive with the inter-peptide interactions, causing the extension of the lag time of fibril elongation.<sup>324</sup> The competitive effects of crowding, local concentration increase and diffusion slow down, were inspected for an ideal peptide considering the different propensity toward different aggregation paths.<sup>414</sup> For peptides with low aggregation affinity, the formation of a fibril is rate limited by the creation of a first nuclei, the crowders tend to stabilize the nuclei and therefore impact favourably aggregation; on the contrary for peptides owning high affinity, the elongation is clearly delayed as an effect of diffusion slowdown. All these studies used simplified or mixed molecular representations. However, CG simulations can be directly complemented by high-resolution atomistic approaches in a multi-scale strategy as recently proposed for investigating the effect of crowding on the stability of the aggregation prone SOD1 protein<sup>408,415</sup>, see Figure 12. Here, a large-scale Lattice Boltzmann MD simulation served to identify states of local packing around SOD1 in a crowded protein solution (BSA), which were then closely examined using atomistic enhanced-sampling simulations. In line with experimental results, the presence of crowders was found to have only a minor effect on the overall thermal stability of SOD1, and crowding did not dramatically perturb the unfolding process. Nevertheless, the simulations identified a fragile region on the beta barrel, susceptible to early unfolding and subsequently showing a strong propensity to interact with the crowder. This semi-unfolded intermediate state, appearing to

be stabilized by the interactions with the crowded environment, was hypothesized to play a role in the aggregation of SOD1 in the crowded cellular conditions.



**Figure 11.** Panel A: Schematic representation of two extreme scenarios, the one-step and the two-step nucleation process for early steps of amyloid aggregation. Panel B: Characteristic steps and kinetics of A $\beta$ 42 amyloid proliferation determined experimentally, with the reaction rates at a concentration of 5  $\mu\text{M}$ <sup>370</sup>. Panel C: Description of the dock and lock mechanism, left chart schematically reproduces the results,<sup>375</sup> where the evolution of the  $\beta$ -structure of a monomer is followed in time during the dock and lock phases. Panel D: Effect of hydrodynamic interactions on the aggregation process of A $\beta$ 16-22 peptides (left) and protofibril elongation due to oligomer fusion (right) as observed in LBMD simulations.<sup>366,394</sup> The authors designed panel A, panels C and D are updated from<sup>366, 375 and 394</sup>. Panel B was taken from<sup>370</sup>.



**Figure 12.** Unfolding and stability of SOD1 in crowded conditions.<sup>408,415</sup> Panel A. Snapshot from a 1  $\mu$ s coarse-grain LBMD simulation of loop-truncated SOD1 monomers immersed in a 200 g/L BSA solution.<sup>408</sup> Panel B. Representative states of local packing around SOD1 extracted from the coarse-grain simulation and converted into a fully all-atom representation. Panel C. Thermal stability—calculated using enhanced-sampling all-atom simulations—of SOD1 in the different states of local packing. The stability of the SOD1 monomer is expressed by means of the secondary-structure content of the protein. Comparison with a result obtained in dilute conditions reveals only a weak effect induced by crowding.<sup>415</sup> Panel D. A semi-unfolded intermediate state observed in the enhanced-sampling all-atom simulations. The blue clouds, representing the spatial distribution of BSA atoms in contact with SOD1, show that the denaturated region has an increased probability to interact with the crowder.<sup>415</sup> The panels are taken from Ref. 408 and 415.

## 5. Extensive simulations on monomers and small oligomers

We discuss A $\beta$ ,  $\alpha$ -synuclein and tau systems. Simulation results on IAPP can be found in Ref. 12.

### 5.1 A $\beta$ 40/42 in solution

There have been numerous computational studies on monomers and oligomers of A $\beta$ 40 and A $\beta$ 42 (Figure 13A) to understand their links with the fibrils with intramolecular U- and S-shapes. We mainly discuss the works published during the last five years (Table 1).

**Monomers.** Simulations showed that the conformational ensemble of both A $\beta$  alloforms depends on the force field, water model and sampling method, but a consensus has been reached.<sup>80,416-422</sup> All-atom REMD simulations with both an implicit<sup>421,422</sup> and explicit solvent<sup>80,419</sup> confirmed that, in agreement with NMR relaxation data<sup>423</sup>, the C-terminus of A $\beta$ 42 is more rigid and has a higher  $\beta$ -strand content than A $\beta$ 40. This fact is often invoked to explain the increased rate of A $\beta$ 42 aggregation. Regardless of the force field and simulation method, for both alloforms the  $\alpha$ -helix content ( $\leq 10\%$ ) is lower than the  $\beta$ -strand content (10-27%), which is consistent with CD data showing the  $\alpha$ -helix content is about 9% and the  $\beta$ -content is between 12 and 25%.<sup>33,424</sup> NMR studies suggested the presence of an antiparallel  $\beta$ -sheet between CHC and residues 29–36 for monomeric A $\beta$ 42,<sup>80</sup> and this was confirmed by REMD simulation using CHARMM36m, FF14SB-IDPs, FF14SB and FF99SB force fields.<sup>418</sup> A small  $\beta$ -hairpin, centered at residues 36–37, was populated in A $\beta$ 42, but not in A $\beta$ 40, and this may contribute to the difference between the two forms in the aggregation rates.<sup>425</sup> Through MD simulations with ten combinations of distinct CHARMM, AMBER and water models, it was shown that helical conformations at the N-terminus of A $\beta$ 42 are stable due primarily to hydrophobic interactions with CHC, and salt bridges with other fragments playing a secondary role.<sup>426</sup> The existence of such helices and the formation of  $\alpha$ -sheets may promote the formation of  $\alpha$ -sheet in the lag phase.<sup>427</sup>

Meng *et al.* performed MD simulations and sm-FRET studies to explore the conformations of A $\beta$ 40 and A $\beta$ 42 at physiological conditions<sup>428</sup>. They found that, similar to recent NMR data,<sup>255</sup> both peptides adopt random coil conformations with A $\beta$ 42 being slightly more compact than A $\beta$ 40. Sm-FRET revealed<sup>435</sup> that some conformations rapidly interconvert and nanosecond fluorescence correlation spectroscopy provided the time scale of the transition of about 35 ns (Figure 13B).<sup>428</sup> The same time scale was also obtained for other IPDs.<sup>429</sup> The application of NMR-guided metadynamic sampling for A $\beta$ 40 monomer revealed that highly populated basins are separated by low barriers, and stability seems to increase with temperature, which contradicts the behavior of ordered proteins.<sup>430</sup>

Both experiment and simulation provided evidence that the aggregation rates of proteins are controlled by hydrophobicity, charge and secondary structure.<sup>431-434</sup> In a pioneering work by Chiti *et al.*<sup>432</sup>, the latter factor was accessed as the propensity to convert from  $\alpha$ -helical to  $\beta$ -sheet conformation in a monomeric state and, therefore, the direct relationship between aggregation rates and secondary



structures remains unclear. In addition, the estimation of the free energy change for this conversion using empirical formulas is not sufficiently accurate.<sup>432-434</sup> To solve this problem, Thu *et al.*<sup>435</sup> calculated the  $\beta$ -content of 19 mutants of A $\beta$ 42 using REMD simulation with the OPLS force field and implicit water model and related it with the experimentally measured aggregation rate  $\kappa$ . They found  $\kappa \sim \exp(c\beta)$ , where  $c=0.071$  and  $\beta$  is the percent of  $\beta$ -content, implying that the higher the  $\beta$ -structure in monomeric state the faster the fibril formation. Thus, the propensity to aggregation is encoded in the conformations accessed by the monomers at equilibrium.

Li *et al.* introduced the concept of the fibril-prone states  $N^*$ , which resemble monomers in the fibril structure.<sup>333</sup> In lattice models  $N^*$  is a single state<sup>431</sup>, while in off-lattice models it comprises an ensemble of states in the same basin.<sup>436,437</sup> Since  $N^*$  can serve as a template for the nucleation step, it is rational to assume that the propensity to aggregate depends on the gap between  $N^*$  and the ground state. Moreover, because the population of the fibril-prone state  $P_{N^*}$  is determined by this gap, the fibril formation time  $\tau_{\text{fib}}$  is related to  $P_{N^*}$  as

$$\tau_{\text{fib}} \sim \exp(-cP_{N^*}), \quad (3)$$

where  $c$  is a constant and  $P_{N^*}$  is expressed as a percentage.<sup>431</sup> Chakraborty *et al.*<sup>437</sup> used Eq. (3) to understand why A $\beta$ 42 aggregates faster than A $\beta$ 40 and to shed light on the kinetics of fibril polymorphism. Using the SOP-IDP model<sup>31</sup> (Figure 9B) for MD simulation and the striated fibril structure (PDB code: 2M4J) as the reference state for  $N^*$ , they estimated  $P_{N^*}(\text{A}\beta 40)$  for A $\beta$ 40 monomer. For A $\beta$ 42 monomer,  $P_{N^*U}(\text{A}\beta 42)$  and  $P_{N^*S}(\text{A}\beta 42)$  were obtained using the U-bend (PDB code: 2BEG) and the S-bend (PDB code: 2NAO, Figure 13B) fibril structures, respectively. Overall,  $P_{N^*}(\text{A}\beta 40) < P_{N^*U}(\text{A}\beta 42) < P_{N^*S}(\text{A}\beta 42)$  and using Eq. (3) with  $c=1$  and the estimated values of  $P_{N^*}$ ,  $\tau_{\text{fib}}$  of S-bend A $\beta$ 42 fibril is around 24 times smaller than A $\beta$ 40.<sup>437</sup> This prediction is consistent with recent experiments showing that the rate of A $\beta$ 42 fibril formation is about an order of magnitude higher than A $\beta$ 40. The higher population of  $N^*$  in A $\beta$ 42 than in A $\beta$ 40 was also confirmed by all-atom simulations.<sup>438</sup> If the U-bend conformations are identified as  $N^*$ , then the aggregation occurs only two times faster than A $\beta$ 40.<sup>437</sup> Therefore, the  $N^*$  theory can capture the fact that formation of various polymorphic structures such as U-bend and S-bend fibrils is time dependent or under kinetic control.<sup>371</sup> Assuming that the A $\beta$  aggregation obeys the Ostwald's rule, which states that the least stable polymorph would form first, followed by a subsequent transition to a more stable form, one can predict that the S-bend A $\beta$ 42 fibril is more stable than the U-bend form, as the latter forms faster.<sup>437</sup>

**Dimers.** The dimer has been subjected to many studies.<sup>439-450</sup> Zhang *et al.*<sup>440</sup> combined MD and MC pulling simulations with AFM experiments to obtain models for A $\beta$ 42 dimers. First, various structures were generated using MD and then an external force was applied to the C $\alpha$  atom of the first Cys residue of each monomer to pull them away at a constant speed. The obtained patterns of the rupture force

were compared with experimental data to select the best models. The A $\beta$ 42 dimer structures are mainly stabilized by the inter-monomer interactions within the CHC regions and do not contain long  $\beta$ -strands that occur in the fibril state. An AFM-based force clamp was also applied for dissociating A $\beta$ 42 dimer and identified two transient states with lifetimes of  $188 \pm 52$  and  $317 \pm 67$  ms.<sup>441</sup>

By performing 9.5  $\mu$ s MD with GROMOS96-53a5 and AMBER99SB-ILDN force fields in SPC solution, Mehrazma and Rauk reported<sup>442</sup> that in addition to CHC, the hydrophobic C-terminal also plays a key role in A $\beta$ 42 dimer stabilization. Unlike other groups,<sup>440,443-445</sup> their models are richer in  $\beta$ -structure than monomers, which may be due to the simulation setup, where the initial conformations were taken from predefined NMR structures. Using OPLS-AA, CHARMM22\*, AMBER99sb-ildn, and AMBERsb14 with the TIP3P water model, Man *et al.* showed that the equilibrium ensembles of A $\beta$ 42 dimers are random coil stabilized by nonspecific interactions.<sup>453</sup> Despite significant differences in the secondary structure, the cross collision sections and small-angle X ray scattering profiles are independent of the force field and are consistent with experimental data. Derreumaux *et al.* conducted REMD simulations to explore the equilibrium ensembles of the A $\beta$ 40 dimer and its mutants in solution.<sup>446-448</sup> They found that the wild type dimer at 315 K is highly disordered, but the secondary structure of  $\beta$ -strand and  $\alpha$ -helix is richer than that of the monomer. Although conformations with an unstructured N-terminus and  $\beta$ -hairpins covering residues 17-21 and 30-36 are transiently populated, the antiparallel and perpendicular orientation of the peptides is preferable to parallel organization.

Combining a hybrid-resolution model and adaptive sampling techniques, Cao *et al.*<sup>449</sup> carried out a 2.7 ms simulation in order to investigate the mechanisms of formation of A $\beta$ 40 dimers. They then developed a Markov state model (MSM) to characterize transition pathways and related kinetics, finding hairpin-containing and parallel in-register structures resembling fibrils (see recent review<sup>450</sup> on the application of MSM to study the aggregation of amyloid peptides). Hairpin-like structures occur through one step nucleation, in which two preformed  $\beta$ -hairpins spanning residues 16–35 occasionally associate, preserving the  $\beta$ -hairpin conformation. This process occurs on a time scale of about 200  $\mu$ s, which is  $\sim$ 100-fold faster than the formation of fibril-like dimers (25.8 ms). Transformation into fibril-like structures includes rapid hydrophobic collapse, followed by a slow configuration rearrangement, which proceeds via different routes, but always requires transient unfolding of complexes. Interactions involving the N-terminal region play a crucial role in the dimerization kinetics.<sup>449</sup>

**Oligomers.** The A $\beta$ 42 tetramers were explored by a multi-scale approach involving REMD simulation with the UNRES force field (Figure 8D) followed by all-atom MD to refine the most representative CG structures.<sup>451</sup> The models obtained are polymorphic and more compact than its fibril counterpart (Figure 13C). In both OPLS-AA/L AMBER99SB-ILDN force fields the calculated collision cross

section ( $\sim 2000 \text{ \AA}^2$ )<sup>63</sup>, which falls into the experimental range<sup>452</sup>, is lower than that of fibril ( $\sim 2600 \text{ \AA}^2$ ). The high population of  $\beta$ -structure in residues 9–14, 17–21, and 30–40 is in agreement with the experiment, and A $\beta$ 42 models are dominated by turn and coil, which is also consistent with experimental observations. Interaction with solvent promotes compactness while the inter-chain electrostatic interaction facilitates the formation of extended structures. As a consequence, when the number of chains becomes large the interplay between these two interactions should lead to fibrillar structures.<sup>451</sup>

Employing the DMD4B-HYDRA force field and DMD simulation, Zhang et al<sup>453</sup> investigated the role of cross-linking via tyrosines in A $\beta$  self-assembly kinetics and morphology of aggregates. They found that cross-linking promotes aggregation, especially that of A $\beta$ 40, and significantly alters the shape of the oligomers by increasing the solvent exposure of hydrophobic residues, which leads to elongated oligomeric structures that differ from more globular structures of noncrosslinked partners. Recent experimental<sup>454</sup> and simulation<sup>455</sup> works confirmed that the oxidative reactivity of Cu-A $\beta$  catalyzes the formation of Tyr–Tyr crosslinks in peptide dimers. Voelker *et al*<sup>456</sup> used the structures obtained in the coarse-grained DMD4B-HYDRA simulations as initial conformations for multiple all-atom MD simulations of monomers and oligomers of 2-5 A $\beta$  chains. They observed water-permeable pores in trimers, tetramers and pentamers of both A $\beta$ 40 and A $\beta$ 42, and found that the tendency to form pores increases with increasing oligomer size.

Combining IM/MS, EM, AFM and computational modeling, the Eisenberg's group<sup>457</sup> demonstrated that cylindrin-like barrels of tandem repeats of A $\beta$  fragments with a length of 11 residues (residues 24–34, 25–35, 26–36) held together by two glycine residues are stable. To check whether a similar structure is possible for A $\beta$  oligomers, Xi et al.<sup>458</sup> built out-of-register A $\beta$ 42 assemblies and also discovered barrel-shaped structures consisting of  $\beta$ 2-turn- $\beta$ 3 domains (residues 27-42). They occurred both in trimers and in tetramers, though the stability in the latter is higher than in the former after at least 200 ns of MD simulations with explicit water.

The stability of tetrameric A $\beta$ 40 and A $\beta$ 42  $\beta$ -barrel structures, which are different from out-of-register barrels,<sup>458</sup> was probed by REMD simulation with four atomistic force fields.<sup>459</sup> In aqueous solution, due to a change in the CHC-CHC and C-end-C-end interfaces, a  $\beta$ -barrel structure, made of eight antiparallel  $\beta$ -strands covering residues 9–40/42 with two distinct  $\beta$ -hairpin types and an inner pore diameter of 0.7 nm, exists transiently and to a greater extent for A $\beta$ 42 than A $\beta$ 40.

Using the CG AWSEM<sup>460</sup> (associative memory, water-mediated, structure, and energy model) force field, Zheng et al.<sup>461</sup> studied the relative stabilities of A $\beta$ 40 monomer and oligomers up to an octamer. A transient hairpin structure populated in a monomer becomes increasingly more stable in oligomers, where hydrogen bonds between adjacent chains can form. Oligomers have either prefibrillar or fibrillar

forms. Prefibrillar oligomers are polymorphic, but typically have a cylindrical-like shape, consisting mainly of antiparallel  $\beta$ -strands, while fibrillar oligomers contain only parallel  $\beta$ -sheets.<sup>461</sup> The aggregation free energy profile of A $\beta$ 42 is more downhill than A $\beta$ 40, and the two terminal residues stabilize the oligomeric structures for A $\beta$ 42 relative to A $\beta$ 40, which greatly facilitates the conversion from prefibrillar trimers to fibrillar tetramers.<sup>462</sup>

While Zheng et al. focused on the thermodynamics of A $\beta$  aggregation, Barz et al. looked at the kinetic aspects by MD simulation of 20 A $\beta$  peptides, which are initially disordered and randomly distributed, using the OPLS-AA force field and GBSA.<sup>463</sup> They developed transition networks to show that both A $\beta$  alloforms can form extended and compact oligomers, which play different roles; the former are apparently engaged in the formation of new aggregates, while the latter are likely metastable, off-pathway and experimentally observable. A $\beta$ 40 and A $\beta$ 42 show distinct propensities for the formation of oligomers, i.e., A $\beta$ 40 primarily populates dimers, trimers, and tetramers, while A $\beta$ 42 predominantly forms dimers, tetramers, and hexamers (Figure 13D), consistent with experiment<sup>452</sup> apart from the absence of dodecamers for A $\beta$ 42. This may be due to either insufficient sampling or the small number of chains used in the simulation. Also, A $\beta$ 42 tetramers appear to be more involved in the formation of bigger oligomers than A $\beta$ 40 tetramers.<sup>463</sup>

Finally, Man et al.<sup>464</sup> performed all-atom MD simulation for A $\beta$ 42 dimers, trimers and tetramers and found that, in accordance with classical nucleation theory, the oligomerization time depends on the monomer concentration by a power of  $-2.4$ . Using this dependence and assuming a concentration of A $\beta$  monomers in the human brain of 0.8 nM, they speculated that it will take 62 years for the formation of toxic A $\beta$ 42 oligomers, a time being equal to the age of AD onset.

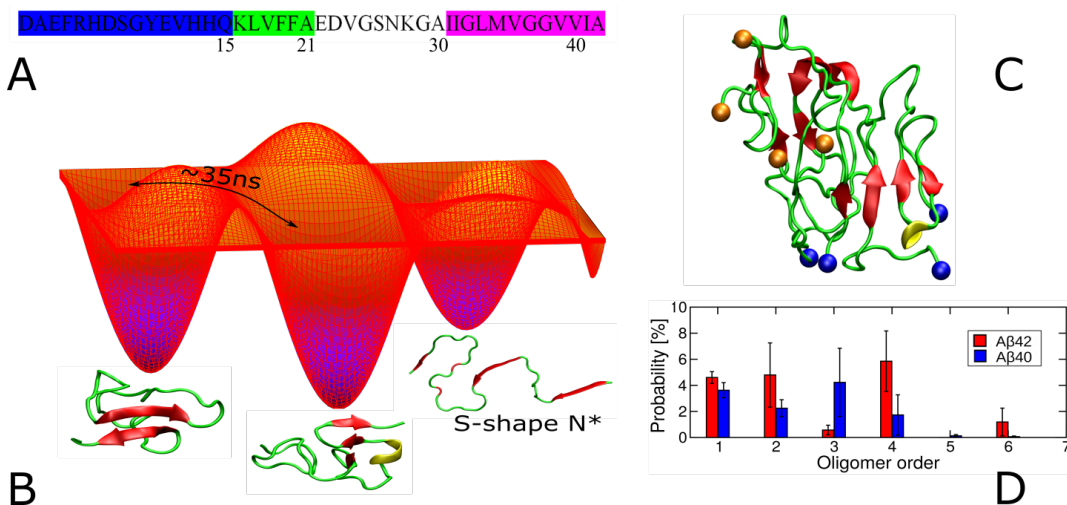
Overall, we must recall that despite a constant improvement of atomistic force fields in explicit solvent,<sup>465</sup> there are divergences between the simulation results on kinetics and thermodynamics,<sup>466</sup> the impact of mutations,<sup>467,468</sup> the population of critical states,<sup>469</sup> and the shapes of oligomers.<sup>470,471</sup>

Table 1. Computational studies on monomers and oligomers of A $\beta$  alloforms in solution after 2015

Ref	force field	Solvent model	Method	Time scale	A $\beta$ alloform	Oligomer size
Rosenman <sup>419</sup>	OPLS-AA/L AMBER99SB-ILDN CHARMM22*	TIP3P-Ew TIP4P TIP3P	REMD	52 $\mu$ s	A $\beta$ 42	1
Weber <sup>416</sup>	AMBER FF99SB CHARMM22/CMAP	TIP3P	REMD	5.28 $\mu$ s	A $\beta$ 42	1
Carballo-Pacheco <sup>476</sup>	OPLS, AMBER99SB, AMBER99SB-ILDN, AMBER99SBILDN- NMR, CHARMM22*	TIP3P TIP4P-Ew	REMD	6.4 $\mu$ s	A $\beta$ 42	1

Meng <sup>428</sup>	AMBER99SB	TIP4P/2005	REMD	740 ns	A $\beta$ 42, A $\beta$ 40	1
Krupa <sup>418</sup>	AMBER14SB AMBER14SB_IDPS AMBER99SB CHARMM36 CHARMM36m	TIP3P	REMD	28.8 $\mu$ s	A $\beta$ 42	1
Thu <sup>435</sup>	OPLS	GB	REMD	6 $\mu$ s	A $\beta$ 42 and 19 variants	1
Frigori <sup>438</sup>	Charmm22*	TIP4P/Ew	REMD	19.2 $\mu$ s	A $\beta$ 42, A $\beta$ 40	1
Aggarwal <sup>434</sup>	AMBER99SBILDN	SP C/E	MD	100 ns	A $\beta$ 42 and 5 variants	1
Li <sup>468</sup>	AMBER99SB	GB	REMD	8 $\mu$ s	A $\beta$ 40, A $\beta$ 40-A2V and tautomer	1
Liu <sup>469</sup>	AMBER99SB-ILDN	TIP3P	MD	0.4-1.8 $\mu$ s	A $\beta$ 42	1
Bhattacharya <sup>426</sup>	CHARMM36, CHARMM22*, CHARMM36m, AmberA03, AmberA03Ws	mTIP3P, TIP4P, TIP4P-D, TIP4P-Ew	MD	33 $\mu$ s	A $\beta$ 42	1
Robustelli <sup>465</sup>	AMBER99SB*- ILDN, CHARMM22* CHARMM36m, AMBER03ws, AMBER99SB-ILDN AMBER99SB-disp	TIP3P TIP4P-D	MD	30 $\mu$ s	A $\beta$ 40	1
Zhang <sup>440</sup>	AMBER99SB-ILDN	TIP3P	MD	4 $\mu$ s	A $\beta$ 42	2
Man <sup>444</sup>	AMBER99SB-ILDN	TIP3P	REMD	25 $\mu$ s	A $\beta$ 42 WT, S8C	2
Man <sup>445</sup>	OPLS-AA, CHARMM22, AMBER99SB-ILDN, AMBERSB14	TIP3P	REMD	36 $\mu$ s	A $\beta$ 42	2
Nguyen <sup>448</sup>	CHARMM22*	TIP3P	REMD	24 $\mu$ s	A $\beta$ 40, A $\beta$ 40-A2V	2
Nguyen <sup>447</sup>	CHARMM22*	TIP3P	REMD	24 $\mu$ s	A $\beta$ 40-A2T	2
Cao <sup>449</sup>	PACE	MARTINI	REMD	2.7 ms	A $\beta$ 40	2
Hashemi <sup>470</sup>	AMBER99SB-ILDN	TIP3P	MD	4 $\mu$ s	A $\beta$ 40	2
Das <sup>443</sup>	OPLS-AA	TIP3P	REMD	51.2 $\mu$ s	A $\beta$ 42, A $\beta$ 42-A2T	2
Sharma <sup>467</sup>	CHARMM36	TIP3P	MD+REMD	1.5 $\mu$ s	A $\beta$ 42-WT, A $\beta$ 42-A2V, A $\beta$ 42-A2T	2

Mehrazmaal <sup>442</sup>	GROMOS96-53a5 AMBER99SB-ILDN	SPC	MD	9.5 $\mu$ s	A $\beta$ 42	2
Press-Sandler <sup>471</sup>	CHARMM36/CMAP	TIP3P	MD	200 ns	A $\beta$ 42	2
Nguyen <sup>41</sup>	UNRES AMBER99SB-ILDN, OPLS-AA/L	TIP3P, TIP4P	MD+REMD	50 $\mu$ s	A $\beta$ 42	4
Xi <sup>458</sup>	CHARMM36	TIP3P	MD	800 ns	A $\beta$ 42	3-4
Nguyen <sup>459</sup>	AMBER99SB-ILDN, OPLS, AMBER99SB, CHARMM36m	TIP3P, TIP3P- modified, DISP	REMD	101.9 $\mu$ s	A $\beta$ 40, A $\beta$ 42	4
Man <sup>464</sup>	AMBER14SB	TIP3P	MD	22.5 $\mu$ s	A $\beta$ 42	2-4
Zhang <sup>453</sup>	DMD4B-HYDRA		DMD	21.6 $\mu$ s	A $\beta$ 40, A $\beta$ 42	32
Voelker <sup>456</sup>	OPLS-AA	TIP3P, SPCE	MD	24.35 $\mu$ s	A $\beta$ 40, A $\beta$ 42	1-5
Barz <sup>463</sup>	OPLS-AA	GBSA	MD	2.5 $\mu$ s	A $\beta$ 42, A $\beta$ 40	20
Zheng <sup>461</sup>	AWSEM		MD, umbrella sampling		A $\beta$ 40	1-8
Zheng <sup>462</sup>	AWSEM		MD, umbrella sampling	1 $\mu$ s	A $\beta$ 42	1-8



**Figure 13.** (A) The N-terminus (blue, 1-15), CHC (green, 16-21), loop (grey, 22-29) and C-terminus (purple, 30-40/42). (B) Schematic free energy landscape of A $\beta$  monomers. Switching between some conformations occurs within 35 ns, as reported by FRET data.<sup>428</sup> The conformation of S-shape N\* from the fibril structure with PDB ID 2NAO was also sampled in CG simulations<sup>437</sup>. (C) Representative structure of the A $\beta$ 42 tetramer, obtained by using multiscale MD simulation<sup>451</sup>. Blue and orange balls refer to the first and last residues, respectively, of monomer subunits. (D) Population of oligomer sizes obtained from simulations of 20-peptides.<sup>463</sup> The authors designed the figure.

## 5.2 $\alpha$ -synuclein in solution

$\alpha$ S can form oligomers that are toxic to neuronal cells and cause cell death<sup>472,473</sup>. To understand the aggregation mechanism and develop promising neuroprotective strategies, knowledge of the conformational characteristics of monomeric  $\alpha$ S may be critical. The structural characterization of  $\alpha$ S

by traditional biophysical experiments or MD simulations has been challenging<sup>474,475</sup>. Force fields developed for folded proteins in MD simulations tend to predict more compact structures of IDPs that contradict experimental measurement<sup>476</sup>. To address these challenges, scientists modified force fields to improve the simulations of IDPs<sup>477-481</sup>. Robustelli *et al.*<sup>465</sup> performed MD simulations of 21 systems that contain folded and disordered proteins using six force fields and a 2.5-fs time step. Based on the results, they modified parameters and developed a force field, a99SB-disp, which is competent for accurate simulations of both ordered and disordered proteins. Other methods have been integrated with standard MD simulations to guide accurate modelling. Pietrek *et al.*<sup>45</sup> used a hierarchical approach that explores all possible structures of IDPs by assembling protein fragments modeled by 100 ns all-atom simulations with the Amber99SB\*-ILDN-q force field and the TIP3P water model. The generated ensemble of  $\alpha$ S captured the local structures from NMR and the overall dimension from SAXS data.<sup>45</sup> Experimentally-constrained computational simulations have been widely applied to predict the  $\alpha$ S atomistic structures (Table 2)<sup>482-488</sup>. Brodie *et al.* used a novel approach that integrates protein crosslinking constraints and discrete molecular dynamics (CL-DMD) simulations<sup>488-491</sup>. Adding experimental data to DMD simulations reduces the conformational space and allows the simulations to achieve accurate protein folding on a reasonable time scale.<sup>489-494</sup>

Computationally-predicted ensembles of  $\alpha$ S conformations revealed the interactions that stabilize the monomer and the structures that drive the early stages of aggregation<sup>495</sup>. The interaction between NAC and C-terminal was proposed to protect the highly hydrophobic NAC region from aggregation<sup>482,483</sup>. However, the interaction between the N- and C- terminals places the NAC region in a solvent exposed orientation, which might trigger  $\alpha$ S aggregation<sup>484</sup>. Secondary structures, such as folded stable helices within N-terminal and the NAC region<sup>496,497</sup>, slow down the fibrillation rate by inhibiting the formation of partially structured helices<sup>433,498</sup>. On the other hand,  $\beta$ -sheets and  $\alpha$ -helix form frequently within the NAC domain and exposure of these secondary structures in solvent may initiate the formation of toxic oligomers and promote fibril formation.<sup>488,499-503</sup> Interestingly, transient trefoil knots were formed in an all-atom simulation with explicit solvent,<sup>504</sup> and the authors deduced that the knots should increase accumulation of the protein and hence, induce multimeric aggregation.

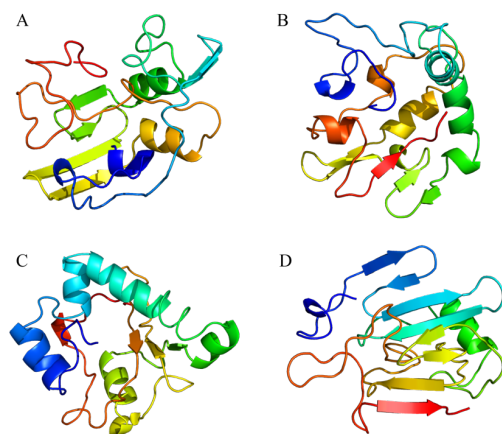
The conformational ensemble of monomeric  $\alpha$ S predicted by CL-DMD revealed interaction between NAC region and C-terminal portion and secondary structures, such as  $\alpha$ -helical near the N-terminus and  $\beta$ -sheet in the NAC domain, which are in agreement with the predictions from the other simulations<sup>488</sup>. The content of  $\alpha$ -helix (~2.4%),  $\beta$ -structure (~29.1%), and other secondary structures (~68.5%) are consistent with the data from other simulations and CD studies of the monomer ( $\alpha$ -helix  $3 \pm 1\%$ ,  $\beta$ -sheet  $23 \pm 8\%$ , and random coil  $74 \pm 10\%$ ).<sup>477,488,505,506</sup> The structures of the cluster centroids

(Figure 14), however, is more compact than those determined by simulations in combination with PRE-NMR, FRET, or SAXS data.<sup>482,485,488</sup> This might be due to the short-range crosslinking data used in the study. A total of 44 crosslinks were used for CL-DMD simulations, including crosslinkers EDC, TATA, SDA, and ABAS with spacer lengths of 0, 5, 5, and 7 Å, respectively. The maximal sequence separation by crosslinkers is from N-terminus to E126 and the minimal sequence separation is from D121 to Y125 or E28 to K32. The structures with lowest 10% of the energies selected for clustering, which eliminated the unfolded conformations, might also lead to the compact conformation of  $\alpha$ -synuclein.<sup>488</sup> Although the radii of gyration ( $R_g$ , 14.1 Å) of the centroids are relatively small, the  $R_g$  determined by different techniques (NMR, PRE, SAXS, and smFRET) display significant disagreement ranging from 22.6 to 50 Å.<sup>476,477,485,507,508</sup> This inconsistency and the large  $R_g$  values might be attributed to the multimeric states of  $\alpha$ S under the experimental conditions. Moreover, the lower energy conformations are verified by the long-distance crosslinking, hydrogen-deuterium exchange, surface modification, and CD data, suggesting that the predicted compact structural ensemble by CL-DMD is convincing.

Apart from monomeric state of  $\alpha$ S, MD simulations have been used to study dimeric and tetrameric conformations. In dimeric conformations, stable parallel or antiparallel  $\beta$ -sheets are formed within the NAC region through hydrophobic interactions.<sup>495,496,509</sup> Dimer structures with  $\beta$ -sheets could generate stronger interactions between the monomers driving the oligomerization process towards fibrillar aggregation<sup>510</sup>. Experiments revealed that  $\alpha$ S can form a tetramer within the native cell environment<sup>187</sup>. Simulations exhibit that the tetramer has less stable interchain  $\beta$ -sheets than the dimers, while the helices are more stable in the tetramer<sup>509</sup>. The tetramer was stabilized by hydrophobic interactions mediated by helices in the N-terminal and NAC region and the salt bridge along with KTKEGV repeat motifs.<sup>484,511</sup>

Overall, simulations of  $\alpha$ S predicted its heterogeneous conformational ensemble comprising of metastable states.<sup>503</sup> Analysis of key structural elements revealed the possible mechanisms that trigger oligomerization. The proposed mechanisms can be validated by future experiments and the essential structural elements can be targeted to develop new therapies for neurodegenerative diseases.





**Figure 14.** Representative structures of the  $\alpha$ S conformational ensemble obtained by short-distance crosslinking constraint-guided DMD simulations.<sup>488</sup> Conformers A, B, C, and D represent the first four clusters. Structures are coloured from blue (N-terminus) to red (C-terminus). The figure was created using Pymol.<sup>254</sup>

Table 2. Experimentally guided molecular dynamics simulations of  $\alpha$ -synuclein monomer

Simulation method	Force field	Water model	Experimental data used
Ensemble MD <sup>482</sup>	CHARMM	Implicit solvent	Paramagnetic relaxation enhancement nuclear NMR
REMD <sup>483</sup>	OPLS-AA	AGBNP implicit solvent	
REMD <sup>484</sup>	CHARMM	EEF1 implicit solvent	NMR chemical shifts, NMR residual dipolar couplings (RDCs), and SAXS
Monte Carlo <sup>485</sup>	Repulsive Lennard Jones interactions and harmonic restraints derived from smFRET measurements	Not applicable	Single-molecule Förster resonance energy transfer (smFRET)
REMD <sup>486</sup>	OPLS-AA	AGBNP implicit solvent	RDCs and PREs data
Monte Carlo <sup>487</sup>	Rosetta score function and the constraint energy	Not applicable	Ensemble FRET data
Discrete molecular dynamics <sup>488</sup>	Medusa force field	Implicit solvent	Short-distance crosslinking data by mass spectrometry

### 5.3. Tau in all its states

#### 5.3.1. Impact of phosphorylation and other PTM on tau aggregation

Phosphorylation and acetylation change tau aggregation and toxicity (Figure 15).<sup>159,160,512</sup> While each kinase can phosphorylate 10-28 sites in tau, the combination of the four kinases can produce 32 (out of total of 52 phosphorylation sites when using the kinases separately).<sup>161</sup> Furthermore, AD P-tau seeds hyperphosphorylated tau to form aggregates, which resist to the dephosphorylation by PP2A, resulting in hyperphosphorylation and pathology of tau.<sup>513</sup> To understand the self-acetylation activity and aggregation propensity of tau, Luo et al investigated the conformational ensembles of K18 and K19 using REMD simulations.<sup>514</sup> The simulation results revealed dynamically ordered conformations with close lysine–cysteine distances essential for tau self-acetylation. The “order in disorder” property in conformations provides the structural basis for tau self-acetylation. Interestingly, an acetylation-phosphorylation switch can regulate tau aggregation propensity and function.<sup>515</sup> The acetylation on Lys-321 (within a KCGS motif) is both essential for acetylation-mediated inhibition of tau aggregation *in vitro* and a molecular tactic for preventing phosphorylation on the downstream Ser-324 residue. Phosphorylation of Ser-324 (pSer-324) has not previously been evaluated in the context of tauopathy, and it has been observed increased deposition of pSer-324-positive tau both in mouse models of tauopathy and AD patients. These findings uncover a novel acetylation-phosphorylation switch at Lys-321/Ser-324 that coordinately regulates tau polymerization and function.<sup>515</sup>

Determining the functional relationship between tau phosphorylation and aggregation has proven a challenge owing to the multiple potential phosphorylation sites and their clustering in the tau sequence. For this reason, actual phosphorylation is often mimicked by mutating the selected amino acid into glutamate or aspartate. It has been shown that the two methods may produce a similar ensemble of conformations, even though the kinetic and chemical details that lead to it are quite different.<sup>516</sup> Heparin-induced tau and *in vitro* phosphorylated tau have different conformations, properties, and activities.<sup>517</sup> Decades of studies using the traditional methods and recent approaches of cryo-EM, specific kinases, and simulations provided unprecedented insights into phosphorylation effects on tau aggregation and toxicities.<sup>518-523</sup>

Cryo-EM and mass spectrometry of tau filaments from corticobasal degeneration (CBD) reveal that the CBD conformer is heavily decorated with PTMs, making it possible to map PTMs directly onto the structures (Figures 15A-C).<sup>518</sup> By comparing the structures and PTMs of tau filaments from CBD and AD, it is found phosphorylation occurs largely in the fuzzy coat. Previously, Xu et al. explored the conformational consequences of hyperphosphorylation on tau, also primarily in the fuzzy coat region.<sup>183</sup> The presence of the phosphorylated terminal domains alters the relative stabilities of

conformations in the K18 ensemble. The hyperphosphorylation of the two terminal domains decreases the attractive interactions among the N- and C-terminus and repeat domains. However, the structure with the straight repeats in the core region is still the most stable, and the exposure of the repeat domains upon hyperphosphorylation could enhance tau filament aggregation.<sup>183</sup>

The effects of phosphorylation are coupled with conformational and electrostatic contributions. In vitro kinase assays can generate well-characterized phosphorylated tau samples with combined phosphorylation at the Ser202/Thr205/Ser208 sites, together with absence of pSer262. This phosphorylated tau readily forms fibers. Based on analysis of synthetic phosphorylated peptides, it was found that aggregation correlates with destabilization of the turn-like structure defined by phosphorylation of Ser202/Thr205.<sup>519</sup> Using the time-resolved FRET method, Chin et al. studied the property of tau173-183 (AKTPPAPKTPP),<sup>524</sup> and found that phosphorylation extends the end-to-end distance and increases the effective persistence length of this tau-derived peptide. However, the peptide extension is independent of salt concentration, indicative of a nonelectrostatic origin. These data indicate that geometric extension and stiffening at the peptide scale may be an important conformational consequence of phosphorylation in disordered proteins.

Two independent simulations found that the effect of phosphorylation on proline-rich domains of tau is mostly electrostatic.<sup>520,531</sup> Lyons et al. simulated the phosphorylation induced conformational change on a peptide of htau225-250 with different phosphorylation patterns of the peptide (pThr231 and/or pSer235). All patterns were found to disrupt a nascent terminal  $\beta$ -sheet pattern (<sup>226</sup>VAVVR<sup>230</sup> and <sup>244</sup>QTAPVP<sup>249</sup>). The double pThr231/pSer235 phosphorylation pattern at experimental ionic strength resulted in the best agreement with NMR structural characterization, with the observation of a transient  $\alpha$ -helix (<sup>239</sup>AKSRLQT<sup>245</sup>). They found that pThr231/pSer235 form salt bridge with Arg242 (Figure 15E).<sup>520</sup> Another study provided similar picture. Metadynamics simulations have been used to investigate the phosphorylation-induced conformational effects on a Tau segment (Tau225-246) from the proline-rich domain, 4 residues less than the first study.<sup>521</sup> Two different phosphorylation patterns were investigated in this work: group 1 with phosphorylation at Thr231 and Ser235, and group 2 with Thr231, Ser235, Ser237, and Ser238 phosphorylated. Phosphorylation leads to the formation of strong salt-bridge contacts with adjacent lysine and arginine residues, which disrupts the native  $\beta$ -sheet structure observed in Tau225-246. They also observed the formation of a transient  $\alpha$ -helix (<sup>238</sup>SAKSRLQ<sup>244</sup>) when Tau225-246 is phosphorylated at four sites.<sup>521</sup>

The phosphorylation site Thr231 discussed above is followed by Pro232. Thr231 can be phosphorylated by various proline-directed kinases. Since that proline can exist in either trans- or cis-conformation defined by the prolyl bond, the coupling of the Thr231 phosphorylation and Pro232 isomerization generated a unique phenomenon and terminology in tauopathy: trans-tau and cis-tau.

(Figure 15D). Therefore, depending on the relative conformation of pThr231, the trans-tau and cis-tau may have different functions. Some study found that cis pT231-tau is highly neurotoxic and acts as an early driver of tauopathy in several neurodegenerative diseases. Examination of bipolar and healthy human brain samples also detected cis p-tau in the patients' brains.<sup>525</sup>

Antibodies have been developed to specifically recognize this unique phosphor-epitope in tau. The Fab fragment forms complex with the tau peptide <sup>224</sup>KKVAVVR(pT<sup>231</sup>)PPK-(pS<sup>235</sup>)PSSAKC<sup>241</sup>.<sup>541</sup> In the Fab-peptide co-crystal structure, 10 amino acids (<sup>225</sup>KVAVVR(pT)PPK<sup>234</sup>) are visible, of which six (<sup>225</sup>KVAVVR(pT<sup>231</sup>)) interact directly with the Fab fragment, and the remaining eight residues of the peptide are disordered. The segment 224-241 is in the N-terminal side of the tau repeat domains, which starts at residue 243. Since the antibody recognizes the phosphorylated epitope in the intact molecule,<sup>526</sup> the segment 224-241 must be exposed in the full-length tau. The critical phosphorylation site (pThr-231) is exclusively recognized by CDR-H2, which forms a positively charged pocket to accommodate the phosphate. The highly specific phosphate recognition explains why the antibody does not bind to non-phosphorylated peptides with the same sequence.<sup>526</sup>

Configuration specific antibodies were developed to distinguish cis- and trans-tau.<sup>527,528</sup> It was found that cis, but not trans, p-tau appears early in mild cognitive impairment neurons and further accumulates in neurofibrillary degenerated neurons as AD progresses, localizing to the dystrophic neurites, an early hallmark change that correlates with synaptic and cognitive deficits. Unlike trans p-tau, the cis not only cannot promote microtubule assembly, but also is more resistant to dephosphorylation and degradation, and prone to aggregation.<sup>527</sup>

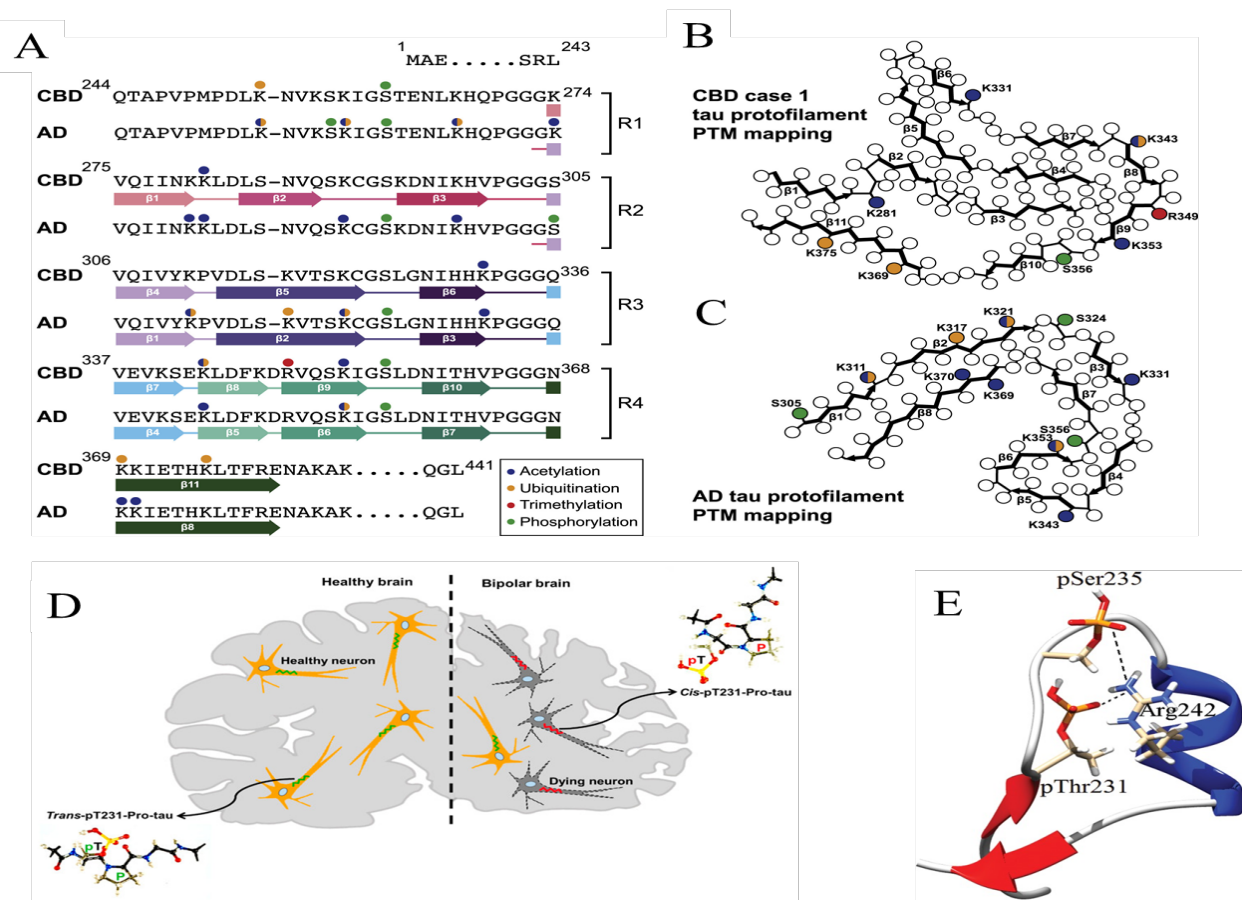
While the trans proline is thermodynamically more stable, the cis proline may form presumably by the help of peptidyl-prolyl isomerase. In order to examine the conformational preference of the Pro232, NMR was used to examine the conformation of all prolines in a functional tau fragment, Tau208-324.<sup>529</sup> Although they detect and identify some minor conformers in the cis form, all prolines are for over 90% in the trans conformation. Phosphorylation by Thr231 specific kinase does not change preference of trans-configuration. The results hence disagree with the notion that specific prolyl bonds in tau would adopt preferentially the cis conformation.<sup>529</sup> Other conflicting results were also observed in the proneness of aggregation. One study suggested that the trans isomer of tau peptide is prone to aggregate, and the WW domain of Pin1 drastically decreases its aggregation.<sup>530</sup> It could be possible that both trans-tau and cis-tau are able to aggregate, depends in the environment in the experimental conditions to change their energy landscape. Accelerated MD were used to explore the conformational landscape of the tau segment containing the phosphorylated-Thr(231)-Pro(232) motif.<sup>531</sup> The results show that intra-molecular electrostatic interactions are coupled to the isomeric state of the peptidyl prolyl bond. Intra-molecular electrostatic interactions are better formed in the trans isomer; however,

the loss of intra-molecular interactions and the more restricted conformational ensemble of the cis isomer could favor self-aggregation.<sup>532</sup>

Some phosphorylation sites in tau inhibit, rather than enhance tau aggregation. It is within expectation when a phosphorylation site is in MBD. The effects of phosphorylation of two unique residues within MBD, Ser305 and Ser320, were examined in the context of established aggregation and seeding models.<sup>532</sup> It was found that the S305E phosphomimetic significantly inhibited both tau seeding and tau aggregation in this model, while S320E did not. To further explore Ser305 phosphorylation in vivo, a monoclonal antibody (2G2) specific for tau phosphorylated at Ser305 was generated and characterized. Consistent with inhibition of tau aggregation, pSer305 was not detected in pathological tau inclusions in AD brain tissue.<sup>532</sup> As can be seen in Figure 15A, the Ser305 can be buried inside the fibril core (as in the case of CBD) or exposed on fibril surface in AD fold. Therefore, depending on the diseases, we expected pSer305 may have different outcome. Ser305 is not phosphorylated in CBD, since pSer305 would clearly disrupt fibril core. In the AD fold, Ser305 could be phosphorylated since it is on the outside of fibril core. However, the N-terminal or C-terminal fuzzy coat may prevent its binding with antibody 2G2. The second example of phosphorylation inhibition of tau aggregation also happens for the MBD sites. A total chemical synthetic approach to site-specifically phosphorylate the MBD of tau (K18) at single (pSer356) or multiple (pSer356/pSer262 and pSer356/pSer262/pSer258) residues was used to show that hyperphosphorylation of K18 inhibits 1) its aggregation in vitro, 2) its seeding activity in cells, 3) its binding to microtubules, and 4) its ability to promote microtubule polymerization.<sup>522</sup> The inhibition increased with increasing the number of phosphorylated sites, with pSer262 having the strongest effect.<sup>522</sup> The third example of phosphorylation effects is indirect. Proteolytic truncation of microtubule associated human (h) Tau protein by caspase-3 at the carboxy (C) terminus has been linked to the pathogenesis of AD. Ser422 phosphorylation blocks human tau cleavage by caspase-3 and suggests that the kinase associated with this Ser-phosphorylation may protect tau from aggregation.<sup>533</sup> While the majority of phosphorylation sites in tau are threonine and serine, there are five tyrosine residues in Tau (Tyr-18, -29, -197, -310, and -394). As expected, phosphorylation of Tyr-310 would prevent tau aggregation, it is interesting to see that phosphorylation at multiple N-terminal tyrosine residues (Tyr-18, -29, and -197) also abolishes tau aggregation and inhibits its microtubule- and lipid-binding properties.<sup>534</sup>

Both A $\beta$  monomer and oligomers are able to affect tau phosphorylation.<sup>523,535-537</sup> Distinct A $\beta$  assemblies activate neuronal signaling pathways in a selective manner thus trigger tau phosphorylation.<sup>535</sup> Soluble phosphorylated tau can happen even in initial increases of aggregate A $\beta$  as early as two decades before the development of aggregated tau pathology.<sup>523</sup> A $\beta$  monomer can affect tau phosphorylation either through change signaling pathways or directly interact with tau protein. A $\beta$

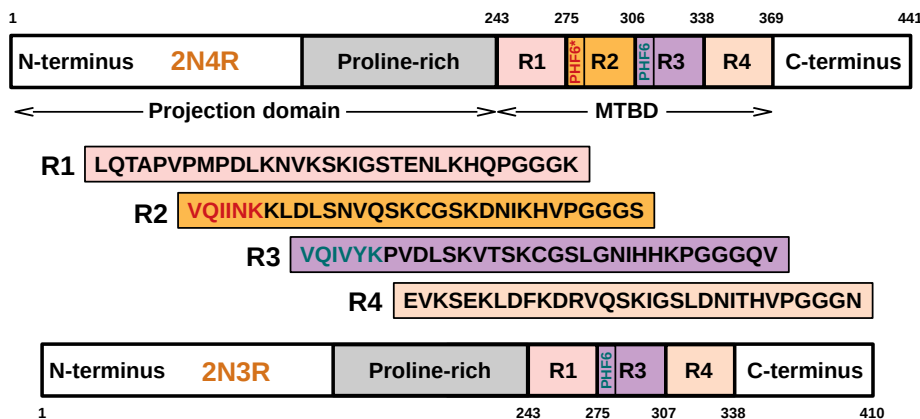
monomer induced phosphorylation of tau at Ser214 through both  $\beta$ 2AR-cAMP/PKA-JNK and  $\beta$ 2AR-GRK signaling pathways.<sup>536</sup> Intracellular binding of soluble A $\beta$  to soluble nonphosphorylated tau, thus promoting tau phosphorylation and A $\beta$  nucleation.<sup>537</sup> However, phosphorylation of Thr212, Ser214, Ser356, and Ser396 completely blocks A $\beta$ 42 binding.<sup>537</sup> Ser356 phosphorylation also contributes to tau stabilization when PAR-1/MARK activity is elevated.<sup>538</sup> Using 5  $\mu$ s MD simulation of tau R3–R4 dimer with and without Ser356 phosphorylated, pSer356 not only perturbs the population of the  $\beta$ -helix motif spanning residues 336–354 but also induces distinct heterogeneous interfaces between the two chains compared to its WT counterpart. Also, pSer356 modulates the population of distinct architectures by increasing the number of globular shapes.<sup>539</sup>



**Figure 15.** (A) Sequence alignment of the R1–R4 repeats and the sequence after R4 that are part of the CBD and AD fibril cores. The positions of filamentous  $\beta$  strands in both diseases are shown. PTMs detected by MS in tau fibrils from CBD case 1 and AD, the cryo-EM structures are shown with acetylation, ubiquitination, trimethylation, and phosphorylation sites marked with blue, orange, red, and green balls, respectively. Sidechains with multiple PTMs detected are shown with two colors. (B and C) PTMs are mapped onto schematics of the protofilament structures from (B) CBD case 1, and (C) AD. The same color scheme as described above is used to depict PTMs. (D) *cis* pT231-tau is highly neurotoxic and acts as an early driver of tauopathy, with bipolar illustrated here. (E) Snapshot from the simulation showing the  $\alpha$ -helix and two salt bridge interactions (pThr231-Arg242 and pSer235-Arg242) of peptide htau<sub>225–250</sub>. The authors designed the figure with panels A–C from Ref. 518, panel D from Ref. 525, and panel E from Ref. 521.

### 5.3.2. Aggregation-prone conformation of tau in solution and coacervation of tau under cellular conditions

In addition to forming fibrillar aggregates, tau has the ability to phase separate in the presence of RNA into liquid droplets. This process of liquid-liquid phase separation (also known as coacervation) into a polymer rich and a polymer depleted phase is observed in vivo for a number of ODP (for example in the formation of membraneless organelles such as cajal bodies), and this process can be readily recapitulated in in vitro experiments. The role of tau coacervation in vivo is hotly debated. Coacervation has been proposed as a means of concentrating tau to facilitate microtubule binding. Its role could be a protective one, in which tau uses the droplets to store free tau and prevent its aggregation into fibrillar species. This premise is supported by experiments that show reversible formation of tau droplets.<sup>540</sup> However, evidence of droplet “aging” in the context of hyperphosphorylated or disease-mutant peptides suggests that in these pathological constructs, droplets may transition to a toxic, fibrillar state, thereby playing a role in neurodegeneration.<sup>541</sup> We review recent literature focusing on simulations of the aggregation and coacervation of tau, with a focus on the MBD region (Figure 16). This region which consists of 4 repeat regions (R1, R2, R3, R4) is of particular interest as it can not only form fibrillar aggregates, but also form liquid droplets in the presence of RNA.



**Figure 16.** Schematic diagram of 4-repeat (2N4R) and 3-repeat (2N3R) human Tau isoforms. The microtubules binding region (MBD) is highlighted and the sequences for the repeat units are shown along with the hexapeptides PHF6\* and PHF6. The authors design the figure.

#### Monomeric and Early Oligomeric Conformations of Tau in Solution

In comparison to other IDPs such as A $\beta$  and  $\alpha$ -synuclein, the number of computational studies on tau is still fairly small. The primary reason is that full length tau is 441 amino acids in length, rendering its study computationally challenging. Hence much of the effort on the computational front has focused on studying fragments of tau rather than the full-length constructs. The smallest

aggregating fragments of tau are PHF6\* located near the R2 region and PHF6 located near the R3 region (Figure 16), and serve as model systems for understanding the aggregation of this protein. The majority of tau simulation studies focus on PHF6/6\* and on larger constructs encompassing these segments.

The earliest computational studies of tau involved MC<sup>485</sup> and MD<sup>542</sup> simulations aimed at understanding the conformational states sampled by tau in its monomeric form. An important outcome of these monomeric simulations has been to reveal that tau co-exists between disordered structures with no detectable secondary structure along with structures that retain some elements of secondary structure. In addition, both extended and compact conformations were sampled in simulation. More recent simulations by Thirumalai and coworkers using the coarse-grained SOP-IDP model (Figure 9B) confirm that tau peptides do not behave simply as random coil chains, but can adopt locally compact structures.<sup>31</sup> The authors studied fragments of tau ranging from 99 to 441 amino acids in length and showed that the K25 fragment of tau populated an equilibrium ensemble of compact conformations determined not only by entropy, but also from energetic interactions that are sequence dependent. Interestingly, a locally compact segment observed in the SOP-IDP simulations of the K25 fragment was also present in full length tau, highlighting the fact that the study of fragments can shed significant insights into the behavior of larger tau constructs.

Simulations support a picture in which monomers of small fragments can adopt a structure in the monomeric state that strongly resembles the structure adopted in the fibrillar state. For example, an aggregation-competent extended  $\beta$ -strand conformation was directly observed in simulations of the R2(273-284) tau fragment. In the case of the R2(273-284) tau fragment and its disease-implicated mutant R2(273-284,  $\Delta$ K280), REMD simulations showed a net difference in the population of extended (aggregation-competent) structures in the case of the mutant over compact hairpin-like conformations, suggesting that the propensity to aggregate is encoded in the monomeric state.<sup>543-545</sup> Simulations on dimers confirmed the hypothesis that structure encoded in the monomer could be transferred to oligomers. R2(273-284), R2(273-284,  $\Delta$ K280), and the R3(306-317) homodimers were found to assume a mixture of compact and extended structures.<sup>543,546</sup> However, the disease-related mutant showed a greater stability and a much greater probability of adopting extended homodimers than the less aggregation-prone variants of tau. Of interest is that the compact tau monomer conformations showed a gradual extension when the monomers formed dimers, higher order oligomers, and fibrils. Hence not only is fibril-structure encoded in the monomer, but oligomers can change the shapes of non-fibril competent conformations to further the aggregation process.<sup>547</sup>

Insights into the combined roles of the PHF6 and PHF6\* peptides in promoting aggregation of tau can be gleaned from replica-exchange simulations of the longer fragment K18(244-372), which



includes all the four repeat units. Simulations revealed a mixture of dynamically disordered and structured ( $\alpha$ -helix and  $\beta$ -sheet) conformers<sup>514</sup> and suggested that enhanced  $\beta$ -sheet conformations of the key aggregation-prone hexapeptides (PHF6 and PHF6\*) and their relatively higher hydrophobic surface exposure could lead to nucleation and aggregation of tau. This conjecture about the role of the hydrophobic effect and secondary structure in driving aggregation of tau is supported by experimental studies that show that increased temperature induces compaction in tau and that furthermore, changes in secondary structure contribute to the thermal collapse (with entropic collapse as a driving factor).<sup>548,549</sup>

A number of simulations have focused not on the monomer structure, but rather on elucidating the structures of stable oligomers and fibrils.<sup>327,543,546,550-554</sup> Early MC study of the PHF6 domain predicted a fibril structure with mixed parallel and antiparallel  $\beta$ -strands, with an increase in the number of parallel strands for larger fibrils.<sup>550</sup> Subsequent REMD simulations of the R3(306-317) domain (which contains PHF6) also predicted stable  $\beta$ -sheet structures for dimers with parallel orientation of the monomers.<sup>546</sup> In contrast, the R2(273-284) (containing PHF6\*) dimers were found to be consisting of antiparallel monomers. Using these antiparallel dimer structures as building blocks, fibrils were constructed using MD simulations by stacking two sheets made out of strands of R2(273-284).<sup>547</sup> Antiparallel stacking of the sheets were shown to lead to more stable fibrils than parallel stacking, and this work provided a computational prediction of fibril structure of the R2(273-284) peptide. Comparing the stability of the dimers involving R2 (which includes PHF6\*) and R3 (which includes PHF6), PHF6 was found to be significantly more aggregation-prone than PHF6\*.<sup>543,546</sup> Further REMD simulations by Bolhuis showed that PHF6 can form fibrils in a two-step process, while PHF6\* remains as amorphous aggregates.<sup>344</sup>

Cryo-EM studies observed paired helical filaments of the R3-R4(306-378) domain of tau (extracted from the brain of an AD patient) and predicted C-shaped motifs for this bi-repeat.<sup>40</sup> MD simulations of these filaments consisting of different bi-repeats of R1, R2, R3 and R4 showed that the C-shaped motif was only stable for R3-R4, while the R1-R2 repeat assumed a linear structure.<sup>555</sup> However, a recent REMD study of an R3-R4 dimer did not identify C-shaped structures,<sup>539</sup> which potentially indicates that the C-shaped motifs of this domain may only form in the context of the entire brain-derived filament, hence bearing the signature of pathogenesis.

All the simulations mentioned in this subsection were performed in water (with implicit or explicit description). The presence of small molecules and cosolvents in water can dramatically modulate the aggregation propensity of tau. The effect of the presence of osmolytic cosolvents, such as urea and methylamines in water on the aggregation propensity of tau-fragments was studied by Shea and coworkers.<sup>556</sup> ThT assay experiments showed that the protein-protective osmolyte trimethylamine N-

oxide (TMAO) significantly enhances aggregation of R2(273-284), while protein-denaturant urea reduces it. Extensive REMD simulations showed that none of the osmolytes induces any new peptide conformation, otherwise absent without the osmolytes. However, urea was found to promote extended conformations of the peptide by directly binding to the peptide. In pure water, extension of the peptide would incur enhanced aggregation, as discussed above. Interestingly, since urea binds to the peptide, it reduces the number of available hydrogen bonding sites in the peptide which are crucial for the oligomerization process. Hence, urea inhibits the aggregation. In contrast to urea, TMAO was found to promote compact helical dimers of R2 that could further rearrange into  $\beta$ -rich structures. A TMAO-induced rearrangement of the water molecules around the amino acids was observed in simulations, and the importance of the dynamics of the surface water around Tau in the fibrillation process was further suggested in a combined neutron scattering and MD study by Weik and coworkers.<sup>557</sup>

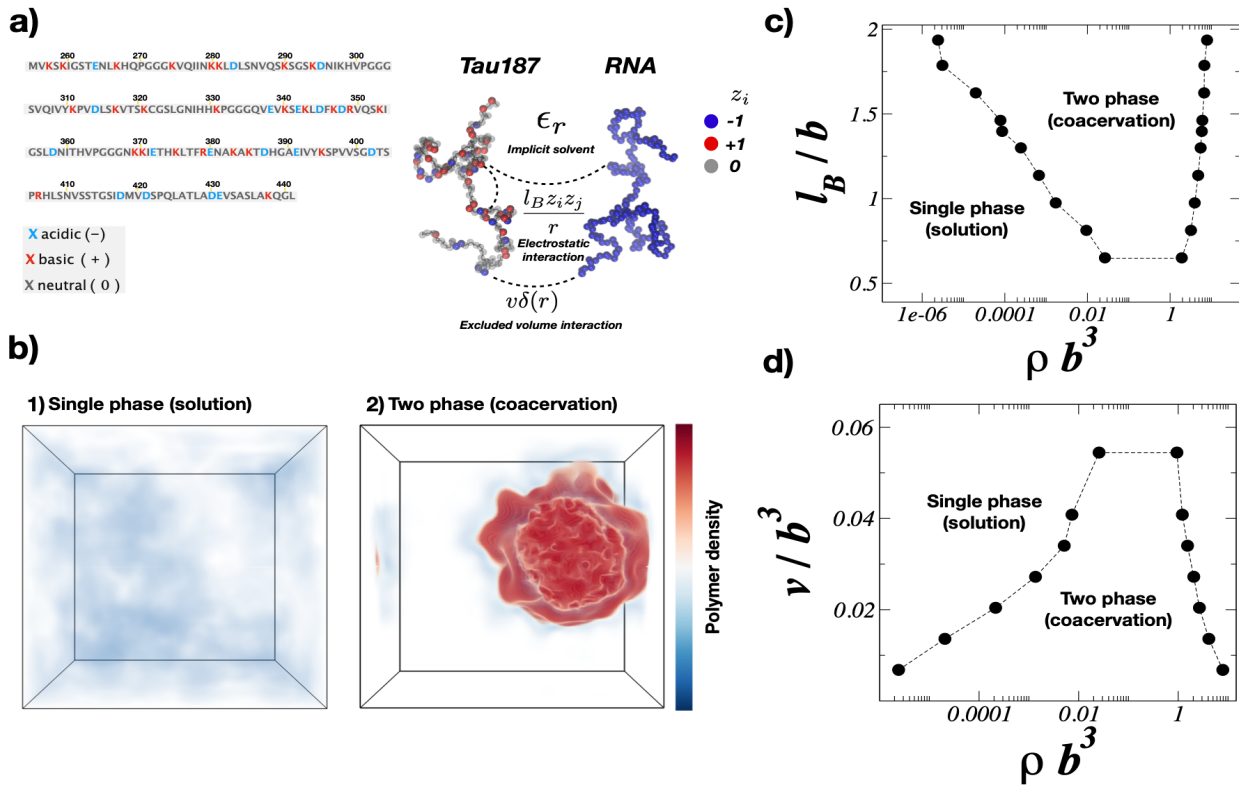
### **Tau coacervation**

The intrinsically disordered nature of tau, with its lack of a well-defined native state and of canonical secondary structure, predisposes it to exhibit a rich phase behavior.<sup>558</sup> The balance between underlying entropic and molecular-level chemical interactions leads to the emergence of coherent mesoscale stable or metastable tangles with important biological implications. Tau liquid-liquid phase separation (LLPS) is a process in which the proteins, through a delicate interplay between solvation entropy and conformational energy, spontaneously assemble into quasi-static dense liquid phase known as a coacervate, which is in coexistence with the surrounding supernatant-tau solution.<sup>559-561</sup> The intra-molecular energy scale of tau coacervation is typically of the order of the thermal energy fluctuation, and thus, the thermodynamic driving forces of LLPS can be affected by the conformational and compositional fluctuations.<sup>560</sup> Favored by lower interfacial energy, the dense micro-phase of tau in equilibrium, forming pseudo-spherical droplets.<sup>562</sup> Recent in vitro experiments suggest that in some instances, coacervation, in the next stage of collective dynamics, might initiate and accommodate tau fibrillization.<sup>541,562-565</sup> The mechanism of LLPS and the influence of various experimental parameters, namely, tau concentration, ionic strength, and temperature modulation is largely unknown. Establishing the phase behavior of tau in physiological condition is critical for understanding whether the thermodynamic state of tau coacervates in cellular environments is a stable mesophase or a transitional-intermediate state toward fibrillization.

In spite of recent advances in computational approaches and methodologies, many large-scale biological processes cannot be simulated with atomically detailed models. The computational limitations motivate the use of CG models that enable efficient simulations of complex systems. Mesoscopic physics models have been used to describe phase separation in the context of polymer models and such approaches can be adapted to proteins.<sup>566-568</sup> A promising computational approach,

which can be used to understand sequence-dependent phase separation, is Field-Theoretic simulation (FTS) with Complex-Langevin sampling. FTS is an approximation-free numerical method that fully accounts for inherent thermal fluctuations of the system. In FTS, by using the Hubbard-Stratonovich transformation, a particle-based model is exactly transformed to a statistical field theory through a self-consistent approach that decouples many-body interactions in the particle based model by introducing complex-valued auxiliary fields.<sup>540,569,570</sup> Thus, by employing a CG model that properly describes the physics of tau polypeptide with its specific charge-sequence, the phase diagram and the relevant equilibrium properties can be computed accurately and efficiently. In this CG model, the solvent is treated implicitly, and the interactions are generalized into two types of potentials: long-range Coulomb interactions mediated by a solvent dielectric constant, and a short-range repulsive interaction that reflects the solvent quality. Each amino acid of tau polypeptide is represented by a single monomeric unit, and the connectivity of the successive monomers of the chain is enforced by a simple harmonic potential. In this polymer physics model, the relevant thermodynamic state variables are an excluded volume parameter  $v$ , which parameterizes the strength of the repulsive potential at contact, and the Bjerrum length  $l_B$  that parameterizes the strength of long-ranged electrostatic interactions.

Phase diagrams associated with biological phase separation have been the subject of a number of reviews but to date, there has only been a single computational study of the phase diagram of tau, and we focus on this study below. A phase diagram maps out how the system behaves given a combination of state variables. Lin, McCarty et al. employed FTS in conjunction with experiment to scrutinize the phase behavior of Tau-RNA LLPS and determine the conditions in which tau can undergo LLPS from a homogenous phase.<sup>570</sup> By performing an in vitro study of N-terminus truncated isoform of human 4R, residues 255–441 (Figure 17-a), they showed that tau-RNA LLPS is reversibly stable within a narrow range of biologically accessible conditions. The thermodynamic state and the phase diagram of tau-RNA solution was investigated under different solvent condition (as described by the excluded volume parameter  $v$ ), ion concentration (that can implicitly be regulated by the Bjerrum length  $l_B$ ), and tau density. The results suggest that slight changes of the stimulus present inside the cellular environment (in other words, small changes in conditions inside the neuron) might be sufficient to induce LLPS. In Figure 17-b, the instantaneous snapshots of the tau density profiles from FTS in two regimes are shown: (1) a homogenous dilute solution phase in weak electrostatic strength and good solvent conditions; (2) a two-phase region in low salt concentration (or relatively large  $l_B$ ) and poor solvent quality, where a dilute supernatant and dense coacervate coexist. In both cases, the tau density is fixed and identical, however, due to the local density evolution, the uniformly distributed tau proteins throughout the solution gradually assemble and transform the conformational state from a

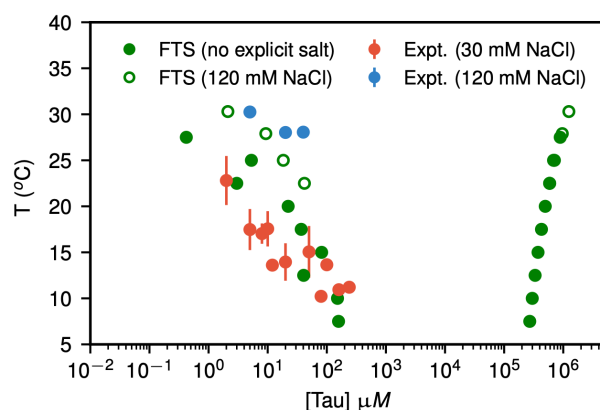


**Figure 17.** a) Schematic representation of Tau and RNA models. Tau and RNA are modeled as chains of bonded monomeric units with size  $b$  in implicit solvent. The charge of each monomer is determined from the corresponding amino acid in tau sequence at pH = 7. RNA is modeled as a uniformly charged polyanion. b) The Tau solution phases at fixed total density: b-1) single phase solution in weak electrostatic strength and good solvent conditions ( $l_B = 0.16 b$ ,  $v = 0.02 b^3$ ). b-2) two phase coacervate in relatively strong electrostatic interaction and low solvent quality ( $l_B = 3.25 b$ ,  $v = 0.0068 b^3$ ). c) Coexistence phase boundary determined from FTS as a function of the Bjerrum length and tau density at fixed excluded volume of  $v = 0.0068 b^3$ . d) Binodal points as a function of the excluded volume at fixed Bjerrum length  $l_B = 1.79 b$ . The authors design the figure from Ref. 570.

single phase to a stable state in which tau phase separate into a dilute tau-deplete domain (white) and a condensed tau coacervate (red).

The first complete phase diagram of tau condensation studied in the literature is shown in Figure 17-c, as a function of  $l_B$  and tau density at fixed solvent condition (constant  $v$ ); similarly in Figure 17-d, the phase behavior of tau solution is probed by varying the solvent quality at fixed  $l_B$ . As is evident from Figures 17-c and 17-d, by increasing the solvent quality (or reducing  $v$ ), the tendency of tau coacervation decreases. However, the increment of the electrostatic screening (or reduction of  $l_B$ ), results in inhibition of tau coacervation and promotes the appearance of a single homogenous phase. The FTS phase diagrams shown in Figures 17-c and 17-d, suggest that, it is possible for cells to drive tau-RNA complex coacervation in vivo.

Experimentally, tau-RNA complex coacervation exhibits a lower critical solution temperature (LCST) phase diagram.<sup>540</sup> By using the typical analytical polymer theories such as the Flory-Huggins-Voorn-Overbeek theory<sup>571</sup> (with no charge sequence), one can adjust an effective temperature dependent  $\chi$  parameter that enables an empirical fit to experiment; however, this approach provides limited insight due to the number of approximations involved. Instead, Lin, McCarty et al. computed the phase diagram for Tau-RNA complex coacervation using FTS with a linear temperature-dependent excluded volume.<sup>570</sup> Using FTS with a proper polymer model that includes the polypeptide spatial charge-sequence (Figure 18), and by considering reasonable values of the model parameters to match the experimental conditions, one can provide direct approximation-free insights into the mechanism of tau LLPS in vivo. FTS simulations are emerging as a powerful tool to study the process of phase separation in vivo and hold the promise of not only guiding experimental studies, but of shedding insight into the in vivo phase separation process.



**Figure 18.** Coexistence points obtained from parameterized FTS at low salt (filled green circles) and at 120 mM NaCl (open green circles) as compared to experimental cloud point temperature as measured in turbidity experiments performed at 20 mM NaCl (filled red circles) and 120 mM (filled blue circles). The experimentally determined cloud point temperature corresponds to the low-density branch of the phase diagram. FTS simulations also predict the corresponding high-density branch of the binodal curve. The authors design the figure from ref. 570.

## 6. Interactions of amyloid peptides with cellular membranes from simulations and experiments

Interactions of amyloid oligomers has been studied using different model membrane mimetic systems that include liposomes, bicelles, and nanodiscs by experiments and simulations<sup>263,572-580</sup>. On one hand, amyloid proteins (e.g., A $\beta$ , tau, hIAPP,  $\alpha$ -synuclein, and prion protein), regardless of their sequences, structures, and normal functions, have a general ability to strongly interact with cell membranes. On the other hand, cell membranes serve as catalytic sites to facilitate the misfolding and formation of toxic amyloid oligomers, which in turn disrupt cell membranes via a two-step mechanism for A $\beta$ , IAPP, and maybe others. A combination of ThT based fluorescence, dye-leakage fluorescence, AFM, solution NMR and ssNMR experiments have been used to obtain insights into these mechanisms of membrane disruption, which is also shown to vary with the lipid composition, metal ions (such as Ca(II), Zn(II)). In what follows the following abbreviations are used: Dipalmitoylphosphatidylcholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), dodecylphosphocholine (DPC), octyl glucoside (OG), dodecylmaltoside, decylmaltoside (DM), lauryldimethylamine-*N*-oxide (LDAO).

### 6.1. A $\beta$ 40/42 and its mutants

Numerous studies have shown that A $\beta$  can adsorb on, insert into, and destabilize cell membranes to induce cell membrane disruption via the direct and physical interactions between amyloid oligomers and cell membranes.

Experimental studies from AFM, electrophysiology, cell calcium imaging have consistently shown that A $\beta$  peptides can penetrate into cell membrane to form ion-permeable, transmembrane amyloid pores.<sup>581-585</sup> A $\beta$  interaction with liposomes containing ganglioside (GM1), cholesterol, and sphingomyelin has been shown to form in-register parallel and two-residue-shifted antiparallel  $\beta$ -sheet structures that are tested with enhanced toxicity. By comparing the interaction of human and rodent A $\beta$  with GM1 clusters in membrane, the formation of toxic fibers by human A $\beta$  was identified; whereas rodent fibers are shown to be less toxic. Using MD simulations, it is shown that the side-chains of H13 and H14 residues of A $\beta$  drive GM1 the clustering by interacting with its head group.<sup>573</sup> A $\beta$  is shown to interact and form oligomers at the membrane interface at nanomolar concentrations and are transient in nature as membrane-association and dissociation of oligomers are detected. An increase and decrease in the contents of  $\beta$ -sheet and  $\alpha$ -helix, respectively, is observed for membrane bound preformed oligomers. Nanodiscs are shown to trap amyloid-intermediates in a near-native environment. Nanodisc trapped A $\beta$  intermediates are shown to have a predominant  $\beta$ -sheet

structure.<sup>263</sup>

With the exception of the very recent NMR structure of an A $\beta$ 42 oligomer formed in a membrane mimicking environment,<sup>586</sup> namely an A $\beta$ 42 tetramer, which comprises a six stranded  $\beta$ -sheet core and reveals a mechanism of membrane disruption in which water permeation occurred through lipid-stabilized pores mediated by the hydrophilic residues located on the core  $\beta$ -sheets edges of the oligomer, a number of A $\beta$  pores have been computationally constructed in different lipid bilayers using low-resolution structural data. Using NMR-determined  $\beta$ -strand-turn- $\beta$ -strand amyloid monomers derived from A $\beta$  fibrils as building block<sup>587</sup>, A $\beta$  pores were computationally constructed by pre-inserting annular A $\beta$  oligomers with different numbers of peptides (12 to 36 monomers), pore sizes (2-4 nm inner diameter and 7-12 nm outer diameter), and pore topology (different  $\beta$ -strands facing the solvated pore or contacting with lipids) into different model lipid bilayers, including zwitterionic DOPC, DPPC, POPE, and mixed DPPC/DOPS bilayers.<sup>579,588-590</sup> MD simulations showed some very interesting atomic details to describe pore structures/dynamics and ion permeability/selectivity. From a structural viewpoint, not all computationally constructed A $\beta$  pores are structurally stable: (1) the lipid bilayers did not support too small 12-mer A $\beta$  pores or too large 36-mer A $\beta$  pores, and only 18- or 24-mer A $\beta$  pores can retain their pore-like morphology across the lipid bilayers; (2) A $\beta$  pores with CNpNC pore topology (i.e., hydrophobic C-terminal  $\beta$ -strands are in contact with lipids and hydrophilic N-terminal  $\beta$ -strands face the pore) are stable (Figure 19a), while A $\beta$  pores with NCpCN are collapsed due to unfavorable peptide-lipid interactions.

Apart from the organization of A $\beta$  strands parallel or anti-parallel to lipids, another class of A $\beta$   $\beta$ -barrel pores were also proposed and modelled computationally with titled A $\beta$  strands relative to lipids as assisted with structural data from experimental and theoretical results. Computational construction and MD simulations of tetrameric  $\beta$ -barrel transmembrane pores by A $\beta$ 42 or A $\beta$ 40 peptides in membrane bilayers of PC/PS/cholesterol/sphingomyelin and DPPC showed the higher structural stability of A $\beta$ 42  $\beta$ -barrels with different barrel topologies at the same diameter of 0.7 nm (Figure 19b), in sharp contrast to unstable A $\beta$ 40  $\beta$ -barrels.<sup>591</sup> Mutations of D23N (more toxic mutant) or A2T (more protective mutant) did not largely affect the stability of A $\beta$ 42  $\beta$ -barrels in DPPC bilayers and thus appeared not to strongly correlate their structures with the known both mutant-induced toxicity<sup>592</sup>. Computational A $\beta$ 42  $\beta$ -barrel pores, but not A $\beta$ 40 pores, also exhibited specific Zn<sup>2+</sup> binding at the pore entrance, consistent with several experimental observations that A $\beta$ 42, not A $\beta$ 40, can (i) assemble into  $\beta$ -barrels in OG/DM/DHPC/LDAO/DPC membranes with varied pore diameters of 0.7 nm<sup>593</sup> and 1.7-2.4 nm<sup>594</sup> and (ii) induce ionic currents in planar lipid bilayers by electric recordings<sup>593</sup>. Moreover, different from small A $\beta$   $\beta$ -barrel pores, they could also exist with the much larger sizes formed by six

parallel A $\beta$  hexamers (36 monomers) with N-terminus segments being facing the pores in POPE bilayer.<sup>588,589</sup> A recent study from native ion mobility-mass spectrometry revealed that A $\beta$ <sub>42</sub> formed a diverse of hexameric  $\beta$ -barrel pores in a membrane-mimicking environment, including tilted hexameric barrels, star shaped hexamers, dimers of trimers, and trimers of dimers<sup>595</sup>, all of which shared the similar peptide organization by folding and assembling hydrophobic C-terminal ends of the A $\beta$ -peptide into a pore center. In parallel to  $\beta$ -structure-based transmembrane pores of A $\beta$ ,  $\alpha$ -helix bundles of the A $\beta$ <sub>17-42</sub> tetramer and trimer can also form transmembrane pore-like structures, which were stable enough to transport Ca<sup>2+</sup> ions across DPPC bilayer<sup>596</sup> (Figure 19c). Ca<sup>2+</sup> transport and binding sites were similar to classical voltage-gated calcium pore, but not M2 proton pore. The  $\alpha$ -helix pores of A $\beta$  provide additional possible pathways for Ca<sup>2+</sup> homeostasis.

While these membrane-supported A $\beta$  pores have a wide variety of conformational heterogeneities and polymorphisms, they all adopt some common structural characteristics, including pore sizes, irregular shapes, and assembly patterns. Briefly, stable A $\beta$  pores are made of four-six, small, dynamic oligomers (each oligomer contains 4-6 A $\beta$  monomers), which are loosely associated with each other through some  $\beta$ -strand interactions to prevent pore dissociation. Such irregular and polymorphic A $\beta$  pores suggest the possible pore formation pathways, i.e. small A $\beta$  aggregates (monomers or oligomers) first insert into the membranes, followed by the self-assembly of these inserted A $\beta$  aggregates into different A $\beta$  pores. From a membrane permeability viewpoint, these transmembrane A $\beta$  pores indeed exhibited specific cation selectivity (particularly for Ca<sup>2+</sup> and Zn<sup>2+</sup>) and characteristic stepwise ion permeability, presumably due to specific sidechain orientations and organizations of A $\beta$ <sup>597</sup>. Ca<sup>2+</sup>-permeable A $\beta$  pores are an attractive target to develop inhibitory compounds and thus prevention of A $\beta$ -induced Ca<sup>2+</sup> influx is expected to restore the Ca<sup>2+</sup> balance, cycling, and bioenergetics in neurons that would generate potential therapeutic strategy for AD treatment. Taken together, A $\beta$  pores were found in different lipid membranes, suggesting that the formation of pathogenic, ion conducting pore by A $\beta$  is considered as a general membranotropic mechanism of AD<sup>593</sup>. In principle, amyloid polymorphism in solution and cell membrane allows to form a wide variety of amyloid pores, but only stable A $\beta$  oligomers that adopted a specific structure and incorporated into membranes as pores are more biologically linked to neurotoxicity. On the other hand, it is known that membrane components such as anionic lipids, gangliosides, and cholesterol could influence A $\beta$  pore formation by altering membrane structure and A $\beta$ -membrane interactions.<sup>598-600</sup>

Apart from A $\beta$ -induced ion permeable pores, numerous simulations have studied the interaction

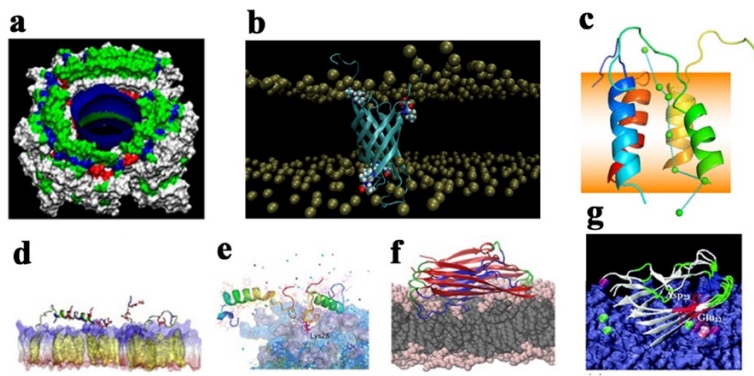


of A $\beta$  monomers (A $\beta$ <sub>40</sub> or A $\beta$ <sub>42</sub>) and oligomers of different sizes and conformations with different lipid bilayers and observed the adsorption/insertion of A $\beta$  peptides on/into cell membranes could also increase membrane conductance. On the other hand, some controversial results were also reported for membrane-induced A $\beta$  conformations and A $\beta$ -induced membrane thinning effects, probably due to different experimental/computational setting-up and conditions and different structural ensemble in a complex A $\beta$ -membrane energy landscape. For instance, A $\beta$ <sub>42</sub> monomers with the disordered or  $\beta$ -hairpin structures can be adsorbed on both anionic DOPS and zwitterionic DPPC bilayers, but A $\beta$ <sub>42</sub> conformations were well retained on DOPS bilayer as compared to DPPC bilayer, due to the stronger electrostatic interactions between A $\beta$ <sub>42</sub> and DOPS.<sup>601</sup> A $\beta$ <sub>40</sub> also behaved similarly to strongly adsorbed on anionic DLPG liposomes, with spontaneous structural transition from where it initially adopts mixtures of disordered and helical structures.<sup>602</sup> Furthermore, increased cholesterol in POPC bilayer promoted A $\beta$ <sub>42</sub> monomer with different conformations (i.e.  $\alpha$ -helical and  $\beta$ -hairpin) to be adsorbed on the POPC bilayer, which further facilitated A $\beta$  aggregation and membrane insertion (Figure 19d).<sup>603</sup> For a more realistic and complex POPC bilayer containing ganglioside (GM), sphingomyelin (SM), and cholesterol (Chol), A $\beta$ <sub>42</sub> monomer adsorption behavior was strongly depended on the ratios of A $\beta$ :membrane components<sup>604</sup>, i.e., A $\beta$ <sub>42</sub> monomers favored  $\alpha$ -helical conformation at a lower ratio of A $\beta$ :GM, but changed to  $\beta$ -strand-rich conformation when this ratio was high (Figure 19e). At the same ratio, A $\beta$ <sub>42</sub> dimerization significantly promotes  $\beta$ -structure formation.<sup>605</sup> Similar simulation results were presented where A $\beta$ <sub>40</sub> remained inserted in POPC, POPS, POPC/POPE, and raft membranes, and A $\beta$ -GM1 interactions promote the structural conversion of A $\beta$  from  $\alpha$ -helix to  $\beta$ -strand.<sup>606</sup> In parallel, numerous experiments from Z-scan fluorescence spectroscopy, cross-correlation spectroscopy, fluorescence lifetime Förster resonance energy transfer have confirmed that sphingomyelin triggers oligomerization of A $\beta$ <sub>40</sub> and that GM1 is counteractive thus preventing oligomerization.<sup>607</sup> These findings not only explain why A $\beta$  is favorably bound to the GM1/SM/Chol membrane, but also reveal the important role of A $\beta$  concentration and initial oligomerization in promoting the transformation into a  $\beta$ -sheet structure in the presence of raft-like membrane in the brains of patients with AD.

Membrane-bound A $\beta$  oligomers also showed preferential adsorption and insertion into POPE bilayer over POPC bilayer.<sup>608,609</sup> While both POPE and POPC bilayers are electrostatically neutral, different structural morphologies between zwitterionic groups in both lipids lead to strong differences in electrostatic attraction between the proximal charged residues of A $\beta$  oligomers and lipid headgroups (Figure 19f). Furthermore, comparison the interactions between A $\beta$  pentamer with neutral POPC and anionic POPC/POPG (3:1) bilayers revealed that A $\beta$  pentamer had much stronger interactions with anionic POPC-POPG lipids than neutral POPC lipids, and such strong interaction with and adsorption on anionic POPC/POPG bilayer were mainly driven by electrostatic interactions, consistent with

experimental observation that A $\beta$  adsorption and fibrillation are enhanced on anionic lipid bilayers.<sup>610</sup> More importantly, Ca<sup>2+</sup> ions are found to form ionic bridges to associate negatively charged residues of A $\beta$  with anionic headgroups of the lipid bilayer, resulting in A $\beta$ -Ca<sup>2+</sup>-PO<sub>4</sub><sup>-</sup> complexes (Figure 19g). Further SPR and AFM data also confirmed that Ca<sup>2+</sup> ions and the lipid bilayer concertedly accelerate the conformational change or misfolding of A $\beta$ .<sup>611</sup> Intensive Ca<sup>2+</sup> bound to the lipid bilayer and Ca<sup>2+</sup> ionic bridges may explain Ca<sup>2+</sup> homeostasis responsible for neuronal dysfunction and death. A $\beta$  oligomers also affect neuronal Ca<sup>2+</sup> homeostasis by modulating the activity of NMDA receptors. Ca<sup>2+</sup> bridges have also been shown to mediate the interaction between the membrane and the acidic tail of  $\alpha$ S and hIAPP.<sup>612,613</sup>

While different and somewhat conflicting data have been reported due to notoriously more complex A $\beta$ -membrane systems, some common observations regarding the interaction of the various A $\beta$  species with membranes could still be achieved. First, upon adsorption of A $\beta$  on different lipid membranes, A $\beta$  aggregates usually undergo structural transition towards  $\beta$ -sheet-rich structure, which appears to be a prerequisite step for facilitating A $\beta$  aggregation and insertion to induce membrane damage. Second, electrostatic and hydrogen bonding interactions are often observed and considered as crucial driving force for modulating the anchoring and insertion of A $\beta$  peptides into lipid bilayers. Third, Ca<sup>2+</sup> promotes membrane interactions of A $\beta$  aggregates to facilitate their aggregation, adsorption, and insertion to form Ca<sup>2+</sup>-permeable pores in membranes or to increase membrane conductance. Block of A $\beta$  pores by small molecule compounds enabled to reduce the Ca<sup>2+</sup> conductivity of A $\beta$  pores and rescues synaptic plasticity and memory function for amyloid pathology in a mouse.<sup>614</sup> This may explain a possible link between calcium homeostasis and amyloid hypotheses. Finally, membrane components (e.g. lipids, cholesterol, sphingomyelin, ganglioside) with different structure, hydrophobicity, and charges play an important role in determining their interactions with A $\beta$  (i.e. adsorption, orientation, and insertion) and the fate of cell membranes, but the exact mechanisms of membrane-induced A $\beta$  aggregation and toxicity are still far more complex than what we expected. In a broader view, these types of membrane disruptions have been shown to be the molecular basis of cytotoxicity for other amyloid proteins and even antimicrobial peptides, including bacterial pore-forming toxins.<sup>615</sup>



**Figure 19.** Different interaction models of full-length A $\beta_{40/42}$  peptides with lipid membranes. (a) A $\beta$  transmembrane pores with high Ca $^{2+}$  permeability and selectivity.<sup>579</sup> (b) Tetrameric A $\beta_{42}$   $\beta$ -Barrel pores in PC/PS/cholesterol/sphingomyelin and DPPC bilayers<sup>591</sup>. (c) Tetrameric A $\beta_{42}$   $\alpha$ -helix-bundle pores in DPPC bilayer with Ca $^{2+}$  transport across the bilayer<sup>596</sup>. (d) A $\beta_{42}$  monomer with both  $\alpha$ -helical and  $\beta$ -structure conformations being adsorbed on cholesterol-rich POPC bilayers, where increase of cholesterol promotes A $\beta$ -membrane interactions and adsorption<sup>603</sup>; (e) A $\beta$  dimers on the GM1-clustering membrane, with C-terminal residues be inserted into membrane<sup>604</sup>; (f) A $\beta$  tetramers with typical U-bent  $\beta$ -structure being preferentially adsorbed on and inserted into POPE bilayer over POPC bilayer, as driven by electrostatic interactions<sup>609</sup>; (g) A $\beta_{42}$  pentamer being adsorbed onto POPC/POPG bilayer via Ca $^{2+}$  ionic bridges between Glu22 and Asp23 and anionic headgroups of the lipid bilayer.<sup>610</sup> The authors design the figure from the above references.

## 6.2. Tau and IAPP

### Interactions of hIAPP with lipid bilayers

In general, hIAPP-membrane interactions have mutual effects on the structure and dynamics of both hIAPP and cell membrane. On one hand, hIAPP aggregates of different sizes and structures would disrupt the integrity, permeability, and functions of cell membranes, leading to cell death. On the other hand, the presence of cell membranes also modulates the aggregation kinetics, pathways, and structures of hIAPP, in most cases, cell membranes accelerate the structural transition to  $\beta$ -structure and aggregation kinetics towards amyloid fibrils.

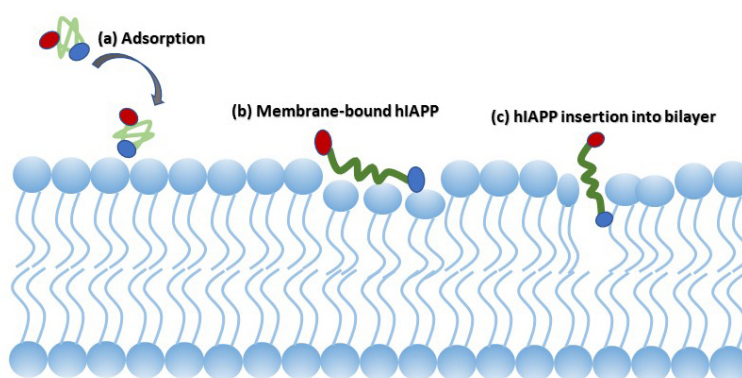
hIAPP and A $\beta$  have very similar properties with regard to membrane interactions, and both peptides adopt an  $\alpha$ -helical conformation when interacting with lipid membranes prior to amyloid formation. Similar to other amyloid proteins, the interaction of hIAPP monomer with lipid membrane is considered as the first step to initiates hIAPP aggregation on cell membrane. A number of MD simulations have been performed to study the interaction of hIAPP monomers with different conformations (e.g.  $\alpha$ -helix,  $\beta$ -hairpin, and coil structure) with lipid membranes (e.g. pure POPC, POPG, and DOPS and mixed DOPC/DOPS, POPC/POPE, DPPC/DPPS, and DVPC/DVPS). These MD simulations consistently showed that independent of initial hIAPP monomer conformations and membrane components, hIAPP monomers were always adsorbed on these lipid membranes.<sup>613,616-619</sup> Such adsorption was largely driven by electrostatic interactions between positively charged residues from N-terminal region of hIAPP and negatively charged headgroups of lipid membranes<sup>613,620-622</sup> (Figure 20a). Upon initial adsorption, specific hIAPP-lipid interaction would drive hIAPP monomers

to adopt  $\alpha$ -helical<sup>623</sup> or coil<sup>616</sup> structure and orientations for better establishing stable and strong contacts with lipid membranes<sup>624</sup> (Figure 20b), followed by subsequent fully or partially insertion of hIAPP monomers into lipid membranes<sup>618</sup> (Figure 20c). During this adsorption-insertion process, structural transition of hIAPP peptides towards  $\beta$ -sheet-rich aggregates would occur at any stage of the hIAPP aggregation process in both solution and on cell membrane<sup>624</sup>. All-atom MD simulations of hIAPP monomer with  $\alpha$ -helical structure on DOPC, DOPS, and a mixed DOPC/DOPS (7:3) showed that the helicity of hIAPP monomers was reduced on DOPS bilayers, rearranged on DOPC bilayers, and enhanced on DOPC/DOPS bilayers.<sup>618</sup> Moreover, hIAPP monomers on anionic POPG bilayers experienced the faster dynamics to orient their N-terminal towards the membranes than those on neutral POPC bilayers.<sup>620,621,625</sup>

Moreover, different lipid membranes lead to different insertion dynamics of hIAPP monomers. Unstructured hIAPP monomer tended to insert into POPC bilayers by establishing an initial contact of amphiphilic F23-F25, followed by a subsequent insertion of T4-T9. Differently,  $\alpha$ -helical hIAPP monomer used its N-terminal residues (C7-Q10) to insert into POPG bilayers, and further insertion of F15-H18 led to the conformational change of hIAPP monomer to  $\beta$ -structure<sup>616</sup>. Another simulation strategy was applied to pre-insert hIAPP monomer into POPG lipid bilayer, in which hIAPP monomer liked to bind to POPG bilayer with  $\alpha$ -helical structure. Moreover, REMD simulation of hIAPP<sub>1-19</sub> fragment with POPG bilayer confirmed that hIAPP<sub>1-19</sub> favored to adopt random coils with a parallel orientation to POPG surface at the adsorption state, but change to helical structure inside POPG bilayer at the insertion state. In addition, the presence of cholesterol in DPPC/DPPS bilayers promoted hIAPP<sub>1-19</sub> aggregation, while the absence of cholesterol alleviated the insertion tendency of hIAPP<sub>1-19</sub> into in DPPC/DPPS bilayer.<sup>617</sup> This indicates that cholesterol may play a double-edged sword to accelerate hIAPP aggregation and prevent membrane damage from hIAPP insertion.

From a computational viewpoint, spontaneous insertion of any protein or peptide into cell membrane encounters substantial energy barrier, which is typically beyond the timescale explored by MD and REMD simulations. To overcome this issue, a hybrid simulation method combining the adaptive biasing force (ABF) method with the MARTINI CG force field is often used to probe a complete membrane insertion process of proteins or peptides with different insertion pathways. Potential mean force (PMF) is calculated, using umbrella sampling protocol, to directly measure the free energy barrier required to transfer the peptides from bulk water phase to water-membrane interface to bilayer interior. PMF profiles from the ABF-based simulations of hIAPP monomer insertion into 7:3 DOPS/DOPC bilayer showed new observation that hIAPP monomers were more energetically

favorable to insert their tails into the lipid membranes than their turn regions at a less energy penalty by  $\sim 11$  kcal/mol.<sup>613</sup> Different from PC, PG, and PE-based bilayers that do not present in the outer leaflet of islet  $\beta$ -cell membranes, anionic ganglioside lipids (G-lipids) are the main component of the pancreas. A combination of CG and all-atom MD simulations were applied to study the interactions of hIAPP monomers with GM3-enrich DOPC (GM3/DOPC) bilayers.<sup>626</sup> Simulations showed that GM3 lipids tended to induce conformation changes of hIAPP from  $\alpha$ -helix to  $\beta$ -hairpin structure, in contrast to DOPC bilayers to retain  $\alpha$ -helix of hIAPP. The ratio of GM3/DOPC components determines not only the conformation population of hIAPP, but also the aggregation kinetics (acceleration or inhibition) of hIAPP. Membrane binding was driven by electrostatic interactions between positively charged N-terminal residues and the anionic elements of the membrane. It is also interesting to observe from different simulations of hIAPP monomers with lipid membranes that the adsorbed hIAPP monomers, regardless of different conformations, often adopted highly populated  $\alpha$ -helical conformations and oriented their hydrophilic residues towards lipid head groups, but their hydrophobic residues towards lipid tail groups, and such orientation would facilitate hIAPP-hIAPP interaction to promote their oligomerization and fibrillization on lipid membranes.



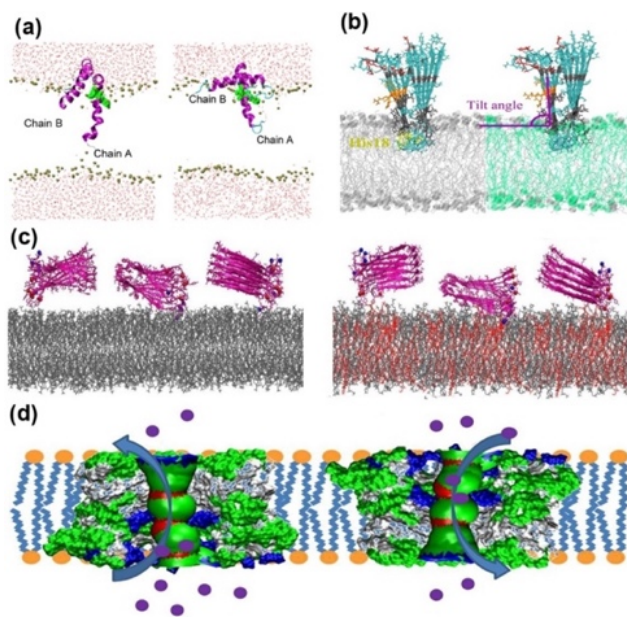
**Figure 20.** Typical interaction model of hIAPP monomer with lipid bilayers via a three-step approaching-adsorption-insertion process. The hIAPP monomers with the disordered structures approach to cell membrane to establish initial contacts with their N-terminal residues via electrostatic interactions, then adjust their orientation parallel to membrane surfaces with hydrophobic residues facing towards hydrophobic tails of lipids, finally tend to insert partially or fully into cell membranes. During the whole process, hIAPP monomers always involve the conformational changes from bulk phase to membrane surface to membrane interior for inducing their potential membrane-disruption activity. The authors design the figure.

Numerous computational studies of the interaction of hIAPP oligomers with lipid membranes have also been reported, which are more biologically relevant to hIAPP oligomers-induced cell toxicity, particularly to the membrane disruption mechanism.<sup>617,627,628</sup> hIAPP dimers as the smallest oligomers and build blocks have been computationally modelled with initial  $\alpha$ -helical structures to interact with

and penetration into POPG bilayers.<sup>621</sup> It was found that the interaction of hIAPP dimers with POPG bilayer is mostly driven by the N-terminal positively-charged residues via electrostatic interactions, and upon adsorption/insertion of hIAPP on/into POPG bilayer, hIAPP dimers had a larger disturbance on the ordering of head groups of POPG bilayer than hIAPP monomer, but both hIAPP dimers and monomers still largely retained their  $\alpha$ -helical structures (Figure 21a). Later, a relatively large size of hIAPP pentamer with  $\beta$ -sheet conformation was selected to interact with both POPC and POPC/POPE (3:1) bilayers to examine the  $\beta$ -structure effect of hIAPP pentamer on the adsorption, orientation, and interactions of hIAPP on both bilayers.<sup>613</sup> hIAPP pentamer requires a specific orientation to be adsorbed on both lipid bilayers via N-terminal residues, as driven by electrostatic interactions. Because dominant driving forces mainly stem from electrostatic interactions, hIAPP pentamer showed stronger interactions with mixed POPC/POPE lipids than pure POPC lipids, but no membrane disruption effect was observed (Figure 21c). Differently, when partially inserting hIAPP oligomers (e.g. trimer, tetramer, and pentamer) into DPPG or mixed DPPC/DPPG (7:3) bilayers, while all hIAPP oligomers disturbed the lipid ordering and the local bilayer thickness around hIAPP.<sup>629</sup> hIAPP pentamer had a shallow insertion depth of  $\sim 1.62$  nm and a small insertion title angle  $\sim 52^\circ$  into DPPG bilayer, as compared to a deeper insertion of 1.75 nm and a larger title angle of  $\sim 77^\circ$  into DPPC/DPPG bilayer (Figure 21b).<sup>629</sup>

Besides adsorption and insertion models, hIAPP pore model was also proposed and computationally studied in the presence of different lipid membranes, with special focus on ion selectivity and permeability of hIAPP pores. Using NMR-determined  $\beta$ -strand-turn- $\beta$ -strand hIAPP monomers derived from amyloid fibrillar structure as building block, a series of hIAPP pore models with different sizes and orientations were constructed and pre-inserted in the presence of DOPC bilayer.<sup>630,631</sup> It was found that all individual hIAPP pores displayed high selectivity for  $\text{Cl}^-$  ions over other cations ( $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$ ), but the co-existence of “turn-to-tail” and “tail-to-turn” double hIAPP pores resulted in the non-ionic selectivity. Double hIAPP pores simulations explains experimental observation that hIAPP forms nonselective, ion-permeable channels in planar phospholipid bilayer membranes, due to the co-existence of “turn-to-tail” and “tail-to-turn” channels in the membrane (Figure 21d).<sup>632</sup> Computationally modelled hIAPP pores showed similar structural morphologies to AFM-identified hIAPP pores<sup>581</sup>, including inner diameters of 1-2 nm and outer diameters of 8-12, irregular pore shapes assembled by multiple, small oligomers, and U-turn region orientation. Despite different simulations of hIAPP with lipid membranes, some general interaction modes between hIAPP and cell membranes are observed at molecular level. Regardless of hIAPP sizes/conformations and lipid components, hIAPP peptides have high probability for its N-terminal residues to be adsorbed on

and inserted into cell membranes as driven by electrostatic interactions. Consistently, hIAPP also favors to interact with anionic cell membranes over neutral ones, further highlighting the importance of electrostatic interactions in hIAPP-membrane interactions and potential membrane disruption mechanisms, consistent with experimental observation that hIAPP adsorption and fibrililation are enhanced on anionic lipid bilayers. A high-resolution structure of membrane-associated hIAPP was solved using NMR distance constraints and MD simulations. The structural model shows that each monomer subunit in the hIAPP oligomer is characterized with three antiparallel  $\beta$ -strands in the regions A8-L12, F15-H18, and I26-S29.<sup>285</sup>



**Figure 21.** Different interaction models of full-length hIAPP<sub>1-37</sub> oligomers with lipid membranes. (a) hIAPP dimers with  $\alpha$ -helical structure insert into POPG bilayers with different insertion depths, strongly depending on interactive contacts of hIAPP dimers with POPG bilayers<sup>621</sup>; (b) hIAPP pentamer with U-bent  $\beta$ -structures to be partially inserted into (left) DPPG and (right) DPPC/DPPG bilayers via the turn region<sup>629</sup>; (c) hIAPP pentamer with U-bent  $\beta$ -structures to be adsorbed on (left) POPC and (right) POPG/POPE bilayers with different hIAPP orientations<sup>613</sup>; (d) double hIAPP pores with different (left) a “turn-to-tail” and (right) a “tail-to-turn” orientations in DOPC bilayers to show nonselective, ion-permeable activity<sup>632</sup>. The authors design the figure.

### Interaction of tau with lipid bilayers

Figure 22 illustrates the pathological process of tau production, hyperphosphorylation, oligomerization, fibrillization, and interaction with cell membranes. Several lines of evidence have shown that tau is able to directly interact with plasma membrane via multiple binding modes, including involving the N-terminal projection domain and R1-R4 repeats.<sup>633-637</sup> Purified PHFs were found to contain different cell membrane components, including phosphatidylcholine (PC), cholesterol (CH), galactocerebrosides (GC), and sphingomyelin (SM), supporting the pathology mediated by tau-

membrane interactions in AD.<sup>638</sup> Tau exhibited the stronger interactions with anionic DMPG bilayers than neutral DPPC bilayer, leading to complete structural disruption of DMPG bilayers in sharp contrast to intact DPPC bilayer.<sup>639</sup> In addition, tau also interacts with other ionic vesicles and micelles to promote tau oligomerization and PHFs formation.<sup>635,636,639-642</sup> Tau proteins have the tendency to form pore-like amyloid structures in brain tissue from patients with progressive supranuclear palsy (PSP) and dementia with Lewy bodies (DLB), as well as in the P301L mouse model<sup>643</sup>, which mimic the membrane-disrupting properties of pore-forming protein toxins, consistent with A $\beta$ , hIAPP, and  $\alpha$ S pores<sup>644</sup>. These studies from different aspects suggest that tau-membrane interactions not only mediate conformational change of tau, but also accelerate nucleated seed formation, both of which modulate the aberrant aggregation of tau into mature fibrils and induce adverse effects on the structural integrity and biological functions of cell membranes.<sup>639,645,646</sup> Considering its large size and its disorder, no molecular simulations have been conducted to study the interaction of full-length tau with lipid membranes. Thus, this remains as a major challenge to be overcome by continuous and fast progress in computational hardware technologies, simulation algorithms, and even machine learning methods.

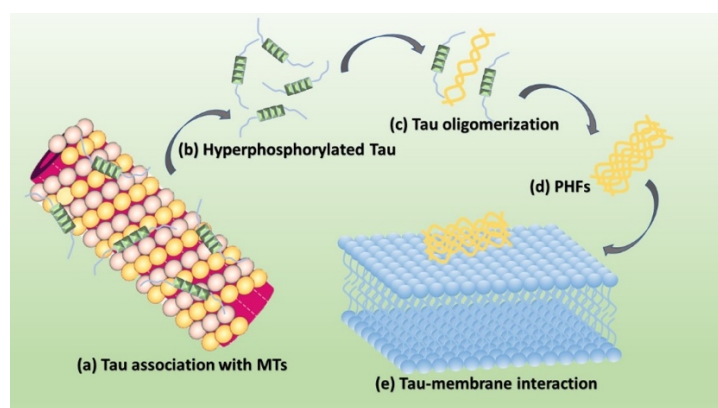


Figure 22. Pathological process of tau production, hyperphosphorylation, oligomerization, fibrillization, and interaction with cell membranes. The authors design the figure.

### 6.3. $\alpha$ -synuclein membrane interaction

The large majority of the putative functions of  $\alpha$ S are based on a fundamental step requiring the binding to biological membranes.<sup>189,647</sup> The interaction with the membrane is indeed a signature of the biological active form of  $\alpha$ S *in vivo*<sup>206</sup> and plays a central role under pathological conditions by influencing the kinetics of  $\alpha$ S aggregation, with both enhancement or inhibition being observed depending on the experimental conditions,<sup>648,649</sup> and the toxicity of its oligomers.<sup>650</sup>

The key membrane interaction of  $\alpha$ S *in vivo* is established with synaptic vesicles.<sup>194,200,201,204</sup>

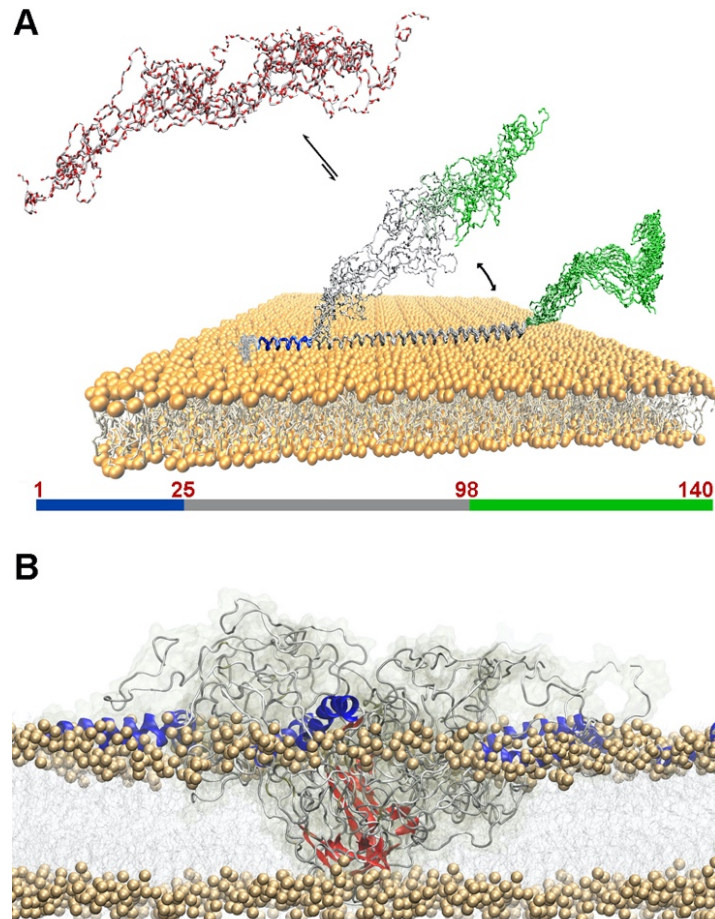


In addition  $\alpha$ S has been shown to bind a variety of other cellular membranes, including mitochondrial membranes and the presynaptic membrane,<sup>189</sup> as well as non-physiological interfaces such as detergent micelles<sup>651</sup> and the water-air interface.<sup>652</sup> In general, the membrane-binding affinity of  $\alpha$ S has been shown to be favored by negative charges and high curvature of the membrane.<sup>653</sup>

Structural studies have clarified the topology of the amphipathic helical segments forming in  $\alpha$ S upon membrane binding, indicating that these adopt a parallel orientation with respect to the membrane<sup>299</sup>, with most of the protein remaining bound at the membrane surface while the first 12 residues of  $\alpha$ S experience a mild insertion into the hydrophobic region of the bilayer.<sup>654</sup> In the micelle-bound state, a structure of a 'broken helix' with two helical segments (residues 3-37 and 45-92) followed by a disordered C-terminal region was refined,<sup>651</sup> whereas structural studies of the membrane-bound state indicated mostly a single helix ranging from residues 1 to 97.<sup>655</sup> But the key feature governing the biological properties of the membrane-bound state of  $\alpha$ S appears to be the dynamical content enabling a multiplicity of binding modes to occur by the same protein.<sup>656</sup> Conformational dynamics in the membrane-bound state differ significantly across the  $\alpha$ S sequence, with a rigid N-terminal region (residues 1-25), serving as a primary binding site that anchors the protein to the membrane, a central region featuring an equilibrium between bound and unbound states (residues 26-97) and a disordered-unbound C-terminal region (residues 98-140)<sup>299</sup> (Figure 23). Of these regions, the most enigmatic is certainly the central that, in addition to host the amyloidogenic NAC segment, has been shown to influence the binding affinity to acidic lipid bilayers as well as the ability of  $\alpha$ S to bridge multiple membranes in a so called 'double-anchor' mechanism.<sup>199,203</sup> The equilibrium between membrane-bound and detached states of the region 65 to 97, however, may also lead to aberrant aggregation of  $\alpha$ S at the membrane surface as it determines the level of exposure of the NAC region. A view is emerging, therefore, that the balance between functional and pathological roles of  $\alpha$ S are inextricably connected to the equilibrium between ordered and disordered conformations of the region 65 to 97 at the surface of biological membranes.<sup>647</sup> This balance can be influenced by external factors such as pathological mutations, particularly A30P, G51D and E56K having significant effects on the membrane binding, and PTMs such as phosphorylation of residues Ser 87,<sup>657</sup> Ser 129<sup>658</sup> and Tyr 39<sup>659</sup> that also regulate the equilibrium between membrane-bound and detached states of  $\alpha$ S (Figure 23). A key posttranslational modification for the membrane binding of  $\alpha$ S is the N-terminal acetylation,<sup>650,660</sup> which is believed to define the predominant form of the protein *in vivo* and that enhances the  $\alpha$ -helical propensity of the N-terminal residues to increase the membrane affinity.<sup>661</sup>

As expected, the binding modes of the physiological monomers of  $\alpha$ S were found to differ

significantly from those of its neurotoxic oligomers. In particular it was found that pre-fibrillar toxic  $\alpha$ S oligomers bind neuronal membranes via a lipophilic element, which is composed of several copies of the N-terminal region of  $\alpha$ S molecules from the oligomers, and insert their structured hydrophobic core into the lipid bilayer by disrupting its integrity.<sup>650</sup> This key mechanism of membrane interaction and disruption is followed by imbalance in calcium and metal ions, leading to downstream processes of cellular toxicity such as the generation of reactive oxygen species and disruption of the mitochondrial function.<sup>650</sup>



**Figure 23.** Membrane binding by monomeric and oligomeric  $\alpha$ S. A) Monomeric  $\alpha$ S is disordered in solution (red) and binds the membrane with three regions having distinct structural and dynamical properties. The N-terminal region (blue) acts as a rigid membrane anchor. The central region (grey) is in conformational exchange between membrane-bound (helical) and detached (disordered) conformations. The C-terminal region (green) remains essentially unbound from the membrane. B) The membrane binding by toxic  $\alpha$ S oligomers involves N-terminal regions (blue) of  $\alpha$ S molecules from the oligomer, strongly anchoring the oligomers to the membrane surface in a cooperative manner, and the prefibrillar  $\beta$ -sheeted rigid core (red), inserting into the lipid bilayer and disrupting its integrity. The figure was created from Ref. 299 and 650.

## 7. Role of metal ions in amyloid diseases

Metal ions such as Cu, Zn, Ca and Fe have been thought to play important roles in the development of a variety of amyloid diseases including AD and T2D. For example, high concentrations of metal ions have been found to be co-localized along with amyloid fibrillary aggregates in the senile plaques and with amyloid- $\beta$  in blood vessels from the brain of AD patients' brains.<sup>662</sup> Specifically, zinc with its highest content in islets (millimolar)<sup>663</sup> stored with insulin and IAPP is shown to have a direct correlation with the progression and severity of T2D. To this end, the effect of metal ions in modulating the aggregation kinetics, morphological and pathological phenotypes of several amyloidogenic peptides/proteins have been tested *in vitro*.<sup>664</sup> Here, we cover the reported studies on the effects of metal ions on the amyloid aggregation and toxicity of amyloid- $\beta$ ,  $\alpha$ S, tau and IAPP.

### 7.1. Biophysical probing the effects of metal ions on amyloid aggregation

In general, the effects of metal ions on A $\beta$ ,  $\alpha$ S, IAPP, and tau are very difficult to reconcile. The self-catalyzing aggregation process is very difficult to control and hence the effect of metal ions is very condition dependent, ranging from aggregation promoting effects, inhibition effects, change in structure of aggregates, more toxic, less toxic etc. One robust result obtained is that Zn(II) and Cu(II) modulate the aggregation behavior, and this always in a metal-specific way. For recent reviews, see Ref. [15,313,665-669](#).

The metal ions that were mostly investigated are the essential metal ions, in particular Cu and Zn ions, but also ions of Fe, Ca, etc. Here we concentrate on the most active research on Cu and Zn. In the case of Cu it is important to realize that its main redox state is different in the cytosol compared that in the extracellular environment. In the thiol-rich cytosol and nucleus Cu is mainly present in its reduced form, Cu(I). In contrast, in extracellular spaces Cu(II) is predominant.<sup>670,671</sup> So depending where the amyloidogenic peptide occurs, one or the other redox state is more relevant.

To better understand the potential roles of these metal ions in the pathology of amyloid diseases, a multitude of *in vitro* studies investigated the effects of metal ions on the structures of amyloidogenic peptides/proteins, the kinetics of amyloid aggregation, the mechanisms of the formation of toxic intermediates, off-pathway intermediates, and the final fibrillary assemblies of A $\beta$ ,  $\alpha$ S, tau etc. To overcome the many challenges posed by the complexities of the effects of metal ions on amyloid aggregation, a combination of various biophysical and biochemical approaches is employed to systematically dissect the complicated molecular processes and structural changes underlying the metal-induced effects on amyloid aggregation. The kinetics of amyloid aggregation is most commonly

measured using ThT based fluorescence experiments, while the structural changes during the course of amyloid aggregation are typically investigated by using a combination of CD and NMR as well as MD simulations, which yield atomic-resolution structures of amyloid species. The morphologies of metal-amyloid-protein aggregates are usually obtained from TEM and AFM images. Recent studies have highlighted the value of HS (high speed)-AFM, which is able to monitor the real-time aggregation process of amyloid proteins as reported in a review.<sup>672</sup> Other techniques such as EPR, ITC and FTIR have also been used to study the interactions between metal ions and amyloidogenic peptides/proteins. Finally, chemical tools have been applied to control the effects of metal-protein interactions on amyloid aggregation and toxicity, and also to probe the aggregation pathways.

## 7.2 Structure of copper- and zinc-peptide complexes

The coordination sites of Cu(I/II) and Zn(II) have been studied for several amyloidogenic peptides and their variants, but not to the same degree. The most intensely studied peptide is A $\beta$ , followed by  $\alpha$ S and IAPP, and tau being the amyloid protein with least information on its interactions with metal ions. The so far best supported models of the binding modes between these peptides/proteins and Cu(I/II) or Zn(II) are summarized in Figure 24. For more comprehensive and recent reviews on these interactions see the reviews <sup>668,669,673</sup> and references cited therein. The elucidation of these binding modes was often realized with the relevant fragments of the respective amyloid peptide/protein harboring the metal-binding domain, such as on A $\beta$ 1-16. However, it should be noted that for the shorter peptides A $\beta$  and IAPP, a lot of studies have not only been performed on the metal-binding domain, but also on the full-length peptides. Similarly, for the longer  $\alpha$ S protein, several studies were also performed on the full-length form. This is, however, not the case for tau as it is much longer and occurs under different phosphorylated states. Phosphates are potential coordinating groups and could be very important in interactions with the harder (according to Pearson's) metal ions, such as Fe(III). This is more likely restricted to the extracellular space, as in the thiol rich intracellular environment, Cu and Fe are mainly present in the reduced forms, Cu(I) and Fe(II). These metal ions are soft or intermediate and hence have less relative affinity compared to the oxidized Cu(II) and Fe(III), respectively.

### General features of Cu(I/II) and Zn(II) binding to A $\beta$ , $\alpha$ Syn, IAPP and tau:

The binding of metal ions, especially Cu(I/II) and Zn(II) to the various amyloid peptides/proteins is characterized by certain similarities, which can be summarized as following:

i) Importance of His: In most of the cases one or more His residues are involved in the binding of metal ions to amyloid peptides/proteins. Histidine is one of the most common metal coordinating ligands among the amino acids. They are intermediate bases (according to Pearson's hard and soft acid base concept) and hence have a certain selectivity for intermediate metal ions, like Cu(II) and Zn(II).

Though soft Cu(I) has also been found bound to His, but this is likely only relevant in the absence of or in combination with cysteines. Thus, Cu(I) binding to His only is unlikely to occur intracellularly.

ii) Moderate affinities: In the cases where the metal ion binding affinities have been measured, they were found to be moderate and generally lower than affinities to metalloproteins. For Cu(II) and Cu(I) the logK values at pH 7.4 are classically between 7 and 10, and for Zn(II) they are even lower with values in the range of 4-7.<sup>673-675</sup> This can be explained by the fact that the amyloid peptides/proteins are mainly intrinsically disordered and hence binding metal ions with different ligands bears an entropic penalty. This is an indication that the interaction of these peptides with metal ions is not physiological, but rather pathophysiological. It further implies that an accumulation of peptides and/or metal ions, possibly originating from dysregulation in the respective disease, is needed before the interaction can occur.

iii) Dynamic coordination: Again unlike metalloproteins, which can be usually characterized by binding of one or more metal ions to specific sites in the protein, there are no rigid, very well defined metal-binding sites in amyloid peptides. In general, each metal ion can bind with different coordination spheres to the peptides as reported in the review.<sup>673</sup> In Figure 24, only the most populated coordination sphere for each of the metal ion-amyloid peptide/protein combination is shown, but in general several coordination modes exist, which are pH dependent and in fast exchange with each other. The existence of multiple metal binding modes is in line with the IDP signature, which is not inhibited by the binding of metal ions.<sup>673</sup>

An exception to this feature is the Cu(II)-binding to an abundant form of A $\beta$ , where the first 3 amino acids are truncated (called A $\beta$ 4-40/2) Here, Cu(II) is bound to the amino acids 4 to 6 (Phe-Arg-His) by the N-terminal NH<sub>2</sub>, the two N<sup>-</sup> of the two amidates (from peptide bonds between Phe-Arg and Arg-His) and the N <sup>$\pi$</sup>  of the His. This binding site belongs to a well-known N-terminal binding motif with the general sequence NH<sub>2</sub>-Xxx-Zzz-His (Xxx-Zzz are any amino acid, but Pro for Zzz) with high affinities of logK 13-15 at pH 7.4 and more inert Cu(II)-binding compared to other peptides.<sup>676,677</sup>

	Cu(II)	Cu(I)	Zn(II)
Amyloid- (1-16/40/42)			
Amyloid- 4-16/40/42)			
-Synuclein: (free N-terminus).			
-Synuclein (N-terminus acetylated)			
IAPP		—	
Tau <sub>full length</sub>	—	—	—
Tau <sub>323-335</sub>			—
Tau <sub>244-372</sub>	—	—	

**Figure 24.** Coordination spheres of A $\beta$ ,  $\alpha$ S, IAPP and tau peptides and some variants with Cu(I), Cu(III) and Zn(II). Generally, the most populated states at pH 7.4 are shown. Often several coordination spheres (states) are present and differently populated. They are in fast equilibrium. An exception is Cu(II)-A $\beta$ 4-40/2 which has a well-defined coordination sphere. Abbreviations: NH<sub>2</sub>: N-terminal amine, N<sup>Im</sup>: Nitrogen of imidazole ring of a His; N<sup>-</sup>: Nitrogen of an amidate; N: any nitrogen of unknown ligand. Review<sup>668</sup> and References<sup>678-680</sup>. The authors design the Figure.

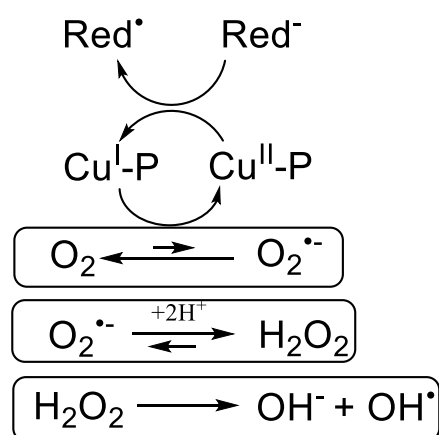
### 7.3 Reactivity of Cu-amyloid peptides with O<sub>2</sub> and reducing agent to produce ROS

Oxidative stress is a well-recognized factor in several amyloid-related disease, although if a cause or a consequence is not clear as reported in the review.<sup>681</sup> Oxidative stress is an imbalance between ROS (reactive oxygen species) production and scavenging, leading to an accumulation of ROS, which can damage all types of biomolecules. Cu can be very potent in catalyzing the production of ROS from dioxygen and a reductant, such as ascorbate. In particular “loosely” or non-specific bound Cu is often more prone to ROS production. Cu coordinated to proteins and enzymes is normally

strongly bound and in a well-controlled protein environment, which allows to control the reactivity of Cu.<sup>670</sup>

Thus it is possible that Cu bound to amyloidogenic peptides (A $\beta$ ,  $\alpha$ S, etc.) can contribute to oxidative stress via its catalytic redox activity and formation of ROS (Figure 25). The importance of this ROS production *in vivo* is not clear, but test tube experiments showed that these Cu-peptide complexes are quite competent in the catalysis of ROS production, at least more than the other tested Cu-peptide/protein complexes.<sup>682</sup> An exception here is again the truncated A $\beta$ 4-40/42<sup>676</sup>, in which Cu(II) is rigidly bound to the N-terminal motif Xxx-Zzz-His and is thus inefficient in ROS production.<sup>683</sup>

As outlined above, a general feature of these peptides (apart from Cu(II)-A $\beta$ 4-40/2) is the moderate affinity for Cu(II) and Cu(I) and the quite different ligands in the coordination sphere between the two redox states. This can be explained by the conformational flexibility of the peptide that adapts to the very different coordination preferences of Cu(II) and Cu(I). This flexibility enables the redox reaction between Cu(I) and Cu(II), but the structural rearrangement between the two states makes it slow. Very detailed investigation on Cu-A $\beta$  including both experimental and theoretical work (e.g., <sup>684,685</sup>) suggested that the redox chemistry and ROS production is performed by a low populated state(s), which is different from the highest populated states shown in Figure 24 (see review <sup>668</sup>) These low populated states (also called in-between states), which are accessible due to the flexibility of A $\beta$  and are responsible for the redox chemistry, likely also exist in the other amyloid peptides/proteins IAPP,  $\alpha$ S and the so far studied portions of tau, as the key features are also present there.<sup>678,686</sup>



**Figure 25.** Proposed mechanism of ROS production by a Cu-peptide (Cu-P) in the presence of a reducing agent (Red<sup>-</sup>) and dioxygen. The authors design the Figure.

The high structural flexibility and the presence of several metal binding modes with different structures speak against a controlled reactivity. First, the peptide is too flexible for selective substrate binding. Second, a tight control of structure is necessary to control the reaction. Thus, the flexibility is

more in line with unspecific and not controlled reactions and Cu bound to these peptides seems potentially dangerous and not for physiological purposes.

In test tube studies of ROS production ascorbate was mostly used as reducing agent. Ascorbate is of physiological relevance and occurs at concentrations of around  $10^{-4}$  M and  $10^{-3}$  M extra- and intracellularly, respectively as reported in a review.<sup>687</sup> Other reducing agents have also been tested, but are often much less potent than ascorbate, such as catechols. The intracellularly most abundant reducing agent, glutathione (GSH), at relevant concentrations is a stronger Cu(I)-chelator than A $\beta$ ,  $\alpha$ S, etc. GSH first reduces Cu(II) to Cu(I) by forming GSSG (oxidized GSH) and second GSH (present in large excess) withdraws the Cu(I) from the amyloid peptides/proteins, which even occurs for the strongest complex, Cu(II)-A $\beta$ 4-40/42.<sup>688</sup> Consequences are that, first, GSH cannot serve as a reducing agent to fuel the ROS production by Cu-peptide like ascorbate (as GSH withdraws Cu(I) from amyloids), and second, A $\beta$ ,  $\alpha$ S, IAPP and tau (at least to so far studied peptides with Cu) are most likely not strong enough Cu-binders to exist in a mM GSH environment like in the cytosol or nucleus.

#### **7.4. Structural studies probing metal ion interactions with A $\beta$**

High-resolution structural information of amyloid proteins under various conditions is important to better understand the underlying molecular mechanism of the pathological progression of amyloid diseases as explained in other sections of this review. Such structural details would also provide insights into the roles of metal ions and other molecules on amyloid aggregation and also aid in the rational design of potential therapeutics to treat amyloid diseases. Therefore, the high-resolution metal bound structures of A $\beta$  under various conditions would not only shed light on the mechanism of conversion of non-toxic to toxic amyloid species but also be helpful for the rational designing of compounds to suppress the metal induced toxicity. To this end, the interaction of metal ions (such as Zn(II), Cu(I/II), Cd(II), Ag(I) etc.) with A $\beta$  peptides and their influence on the structure have been investigated to obtain atomic-resolution information using NMR techniques and MD simulations. In the following sub-sections, we limit the focus to briefly cover the structural interactions of Zn(II) and copper ions with A $\beta$ .

##### **NMR studies**

The three major histidine residues (H6, H13 and H14) in A $\beta$  are shown to coordinate with zinc based on 2D heteronuclear ( $^1\text{H}/^{13}\text{C}$  and  $^1\text{H}/^{15}\text{N}$ ) chemical shift correlation (HSQC) and  $^{15}\text{N}$  relaxation NMR experiments.<sup>689</sup> A second, but lower affinity Zn(II) binding site is with D23 and K28 that disrupts the D23-K28 salt bridge, which otherwise is known to favor the nucleation of the A $\beta$  aggregation process.<sup>690,691</sup> Zinc binding has exhibited no significant effects on the longitudinal (or spin-lattice,  $T_1$ ) relaxation time and the diffusion rate, suggesting that the Zn(II)-A $\beta$  complex is present as monomers. Diffusion NMR and size-exclusion chromatography have identified an increase in the compactness of

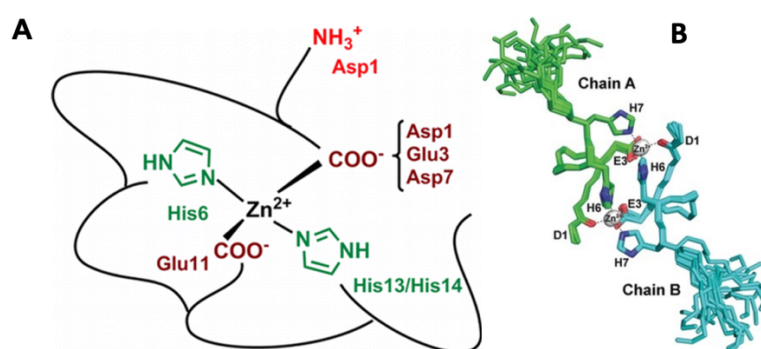


monomeric A $\beta$  bound to Zn(II) with a hydrodynamic radius of 1.5 nm compared to 1.7 nm for A $\beta$  alone.<sup>692</sup> This was later confirmed by obtaining a contraction upon Zn binding from 1.66 nm to 1.59 nm using pulsed field gradient diffusion measurements.<sup>693</sup> Transverse relaxation rate analysis further highlighted that the Zn(II) binding increased the structural order in the N-terminal region while the C-terminus remained flexible.<sup>689</sup> This observation is supported by a thermodynamic analysis model that suggested chaperonin activity of Zn(II) and induction of a short-lived folded A $\beta$  conformation around the Zn(II)-binding site at the N-terminus.<sup>693</sup>

Zn(II) interaction with A $\beta$  has been shown to generate non-fibrillary A $\beta$  aggregates and the underlying molecular mechanism has been probed using NMR experiments. Zn(II)-binding induces a rigid turn-like structure in the V24-K28 region of A $\beta$  allowing the C-terminus to be highly dynamic.<sup>696</sup> Investigation of the Zn-A $\beta$  complex is limited by (i) structural plasticity of A $\beta$ , (ii) multiple zinc coordination sites in A $\beta$ , (iii) precipitation of sample at high Zn/A $\beta$  concentration required for NMR detection, and (iv) inactive spectroscopic nature of zinc (d<sup>10</sup> electronic configuration). Alies et al., addressed these limitations using a combination of approaches that include <sup>1</sup>H-NMR, X-ray absorption spectroscopy, and affinity measurements. They reported Zn(II) ions are tetrahedrally bound to A $\beta$  with the Zn coordination sphere comprised of two histidine residues and two carboxylate side chains (Figure 26).<sup>695</sup> The binding of zinc to A $\beta$  is pH and concentration dependent as revealed by NMR.<sup>696-698</sup> At substoichiometric concentration of Zn(II) (i.e. 0.2 equivalents), line-broadening was observed for H6, S8, V12, H13 and H14. Further increase in Zn(II) concentration (i.e. above 0.5 equivalents) broadened the signals from many N-terminal residues (E3, F4, R5, D7, S8, E11, V12, Q15 and K16) including all histidine residues beyond detection by solution NMR.<sup>697,698</sup>

Zinc binding has been shown to induce A $\beta$  oligomerization and its pathological phenotype. NMR experiments have been used to probe the high-resolution structural details of zinc induced A $\beta$  oligomers by using an A $\beta$  fragment involving residues 1-16.<sup>699</sup> A $\beta$ 1-16 forms a dimer in the presence of zinc and E11-H14 was identified as the key interface region to stabilize the dimer. Similarly, a binuclear zinc interaction is shown to form dimers for A $\beta$  fragments derived from the Taiwanese mutation D7H. A dimer model structure shows the coordination of Zn(II) with residues D1 and H6 of one peptide and E3 and H7 from another peptide in the coordination sphere, bringing the H6 residues of both peptides to a close proximity for stacking interactions (Figure 26).<sup>700</sup> In another study, zinc binding to phosphorylated A $\beta$  (at S8), as detected in AD brain, showed the H6 imidazole ring and phosphate group of S8 to form three of the four Zn(II) coordination bonds promoting A $\beta$  dimerization.<sup>699,701</sup> Lee et al. reported Zn(II) induced A $\beta$  oligomers are spherical with a diameter of ~12-14 nm, and used homonuclear (<sup>13</sup>C/<sup>13</sup>C) magic angle spinning (MAS) solid-state NMR to study the Zn-A $\beta$  aggregates where A $\beta$  was selectively labeled with <sup>13</sup>C at V24, A30, I31, G33, and L34.<sup>702</sup>

This study identified A30 in a rigid  $\beta$ -sheet that differed from its conformation in aged fibers, whereas L34 showed no conformational change highlighting the Zn-A $\beta$  aggregates adopting a  $\beta$ -sheet structure, but are relatively disordered when compared with aged A $\beta$  fibers.<sup>702</sup> In another MAS NMR study, Mithu et al. revealed a structural distortion in Zn-A $\beta$  aggregates due to the disruption of the D23-K28 salt bridge, but maintained a cross- $\beta$  structure.<sup>691</sup> In aged fibers, they observed two different chemical shifts for D23, K28, A30, and V36 indicating the presence of a heterogeneous structure. But in Zn-A $\beta$  aggregates they observed a single chemical shift for D23 and K28 and two distinct chemical shifts for A30 and V36<sup>691</sup> suggesting the Zn-A $\beta$  aggregates are morphologically distinct from aged fibers.



**Figure 26.** High-resolution structural characterization of zinc binding sites to Alzheimer's A $\beta$ . **A:** At physiological pH (7.4), zinc was proposed to tetrahedrally bind with A $\beta$  where the coordination sphere is contributed from two of three histidines (H6, H13 and H14) residues and two carboxylate sidechain (E11 and one from D1, E3 or D7) **B:** Superimposition of 20 NMR model structures of Taiwanese mutant A $\beta$  fragment (D7H-A $\beta$ <sub>1-10</sub>) showing zinc (grey sphere) induced formation of a homodimer. The dimer is stabilized by two zinc ions where one coordinates to D1 and H6 of one A $\beta$  subunit and the other to H7 and E3 orienting the H6 residue of both subunits to form stacking interactions. This figure is designed from Ref. 695 and Ref. 700.

Other metal ions like Cu(II) share similar, but not identical binding sites and modes with A $\beta$ . Cu(II) binds in a pH-dependent fashion to A $\beta$ , with two main forms present at pH 6-8 as shown by EPR, X-ray absorption, CD, and potentiometry (Figure 24) (reviewed in Refs. 668,674,703). These two forms are often called components I and II. In contrast Cu(I) seems to be less pH dependent, at least from pH 6.5 to 9, and bound in a diagonal fashion to two His residues. Cu(I) bound to H13 and H14 is the dominant form, but is in rapid exchange with Cu(I) bound to H6/H13 and H6/H14.<sup>703</sup> NMR spectra of Cu(II)-A $\beta$  (1:1) showed significant line-broadening for the N-terminal residues spanning the region E3-V18.<sup>704</sup> It should be noted that both Cu(I) and Cu(II) are involved in the interaction with A $\beta$ . 2D TOCSY NMR experiments identified Cu(I) binding to all three histidine residues in A $\beta$ <sub>1-16</sub>. Cu(I) has been shown to bind to the imidazole nitrogen (H6-N $\delta$  and H13-N $\epsilon$ , H14-N $\epsilon$ ) that induced significant chemical shift perturbation for the sidechain protons from all His residues.<sup>705</sup> Proton NMR

experiments revealed that Zn(II) does not alter the copper binding environment in A $\beta$  in line with the known stronger binding of Cu(II) when compared to Zn(II). This is in contrast to EPR and XAS measurements, which showed that Zn slightly pushes the Cu(II) from component I to II.<sup>706,707</sup> In addition, it is also shown that unlike the formation of amorphous aggregates upon zinc binding, copper binding does not alter the fibrillary state of A $\beta$ .<sup>708</sup> The paramagnetic effect of copper measured using MAS experiments has been used to identify the selective quenching of NMR peaks from the N-terminal residues (E3, E11, H13 and H14), and C-terminal V40 in A $\beta$ 40 fibrils mixed with copper, but no change in the structure was identified.<sup>708</sup> Relaxation experiments under MAS further identified that the paramagnetic Cu(II) binding to A $\beta$  changed to a diamagnetic Cu(I) which remained attached to the fiber. Interaction of other metal ions such as Ag(I) and Cd(II) with A $\beta$  has also been studied using NMR spectroscopy. Cd(II) showed interaction with the N-terminal histidine residues, similar to Zn(II), with a binding stoichiometry of 1:1.<sup>698</sup> Similarly, Ag(I) interaction studies with A $\beta$ , and an A $\beta$  mutant having no histidine, revealed interaction with the N-terminal residues reducing the NMR signal intensities of all histidine residues by ~75-80%.<sup>709</sup>

### **Molecular simulations**

Molecular simulations complement the experimental studies of the interactions between amyloid peptides and metal ions as they provide atomic-level details of the metal binding and the resulting effects on the structure and dynamics of the peptide with femtosecond resolution. Different simulations ranging from quantum mechanical (QM) calculations and *ab initio* MD simulations to quantum mechanics/molecular mechanics (QM/MM) and pure classical MD simulations have been conducted that studied the binding of Zn(II) and Cu(I) or Cu(II) to A $\beta$ . A comprehensive review summarizing the findings from a large set of these different simulation approaches is provided in Ref. 710. The aim of the simulations involving a QM approach is to shed light on the coordination spheres around the metal ions bound to A $\beta$ . The results of these QM calculations are usually compared to experimental data and help to decide which of the possible binding modes best agree to experimental signatures. Moreover, the stability of the different binding modes can be estimated based on the calculated molecular energies. To give an example, DFT calculations helped in the interpretation of extended X-ray absorption fine structure (EXAFS) spectra of Cu(II) in complex with A $\beta$ . Different coordination spheres were constructed and their geometry optimized using DFT calculations. The optimized structures were then utilized in the EXAFS spectra data refinements, based on which a distorted six-coordinated arrangement with three histidine (H6, H13 and H14) residues and a carboxylate oxygen (from either E11 or D1) in an approximately equatorial planar arrangement and a water molecule and another carboxylate oxygen also from E11 or D1 as axial ligands was reported.<sup>711</sup> Moreover, a pentacoordinated structure with Y10 involved<sup>712,713</sup> could be ruled out. Several other DFT studies that

also assessed the likelihood of Y10 as Cu(II) ligand found a preference of N-terminal E and D residues over Y10,<sup>714,715</sup> in agreement with the findings from NMR, potentiometry and electrochemistry that never identified Y10 as a main ligand for A $\beta$ .

Studies on metal-A $\beta$  complexes using ab initio MD simulations usually employed the Car–Parrinello molecular dynamics (CPMD) method. They also aimed at making predictions regarding the prevalence of different coordination spheres by testing their stability in short CPMD simulations. For example, different binding modes of Zn(II) in complex with A $\beta$ 1-16 were probed by CPMD simulations,<sup>716</sup> reporting H6, E11, H13, and H14 as most likely ligands. Possible Zn(II)-bridged A $\beta$  dimer structures were also suggested from CPMD simulations.<sup>717</sup> Here, the most stable complex was found to be one in which two peptides are bridged by a Zn(II) ion in a four-histidine coordination mode involving H13 and H14 of both peptides. Another QM/MM study<sup>718</sup> that assessed possible dimerization scenarios of A $\beta$  involving Zn(II) concluded that the Zn(II) ion is likely chelated by E11 and H14, which is in agreement with NMR results mentioned above<sup>699</sup> and was also supported by ITC experiments of the sequence A $\beta$ 11-14.<sup>718</sup> When replacing H13 with an arginine, the mutated peptide binds with the same affinity Zn(II) as the original peptide EVHH, indicating that H13 is not involved in Zn(II) binding.<sup>718</sup> CPMD was also employed to probe the binding of Cu(I) to A $\beta$ , testing whether two or three His residues coordinate with Cu(I).<sup>719</sup> The coordination sphere with three His residues turned out not to be stable as one of them detached from Cu(I) during the CPMD simulation. This result is in accord with findings from XAS and NMR experiments. It was further found that H6 binding to Cu(I) is possible, but the preferred coordination involves H13 and H14.<sup>719</sup>

Classical MD simulations allow studying the impact of metal ion binding on the structural preferences of amyloid peptides. Here, special care has to be taken when modeling transition metal ions at the classical level. The most common approach is to describe the metal ion as a sphere with a partial charge and van der Waals parameters. However, this simplistic approach often fails to model the specific interactions between transition metals and the coordinating ligands so that the coordination site geometry is not retained during an MD simulation, especially if the protein is as flexible as A $\beta$ . An approach to overcome this problem is provided by so-called bonded models, where bonds between the transition metal ion and ligated amino acid residues are explicitly modeled, enabling a stable coordination sphere geometry during MD simulations. To elucidate the effects of metal ion binding on amyloidogenic peptides, MD simulations on the microsecond time scale or enhanced sampling, such as REMD simulations are needed to observe relevant conformational transitions in these peptides. From the multitude of MD simulations that studied conformational changes in monomeric or oligomeric A $\beta$  upon metal ion binding (as comprehensively reviewed in Ref. 710) only those that used a bonded model for the coordination site and performed sufficient MD sampling are discussed here.

As binding modes for Zn(II) and Cu(II) those previously reported from NMR spectroscopy and/or DFT calculations were generally adopted. In the case of monomeric A $\beta$ , most of the simulations used a coordination sphere including H6, H13 and H14 plus D1 or E11 as fourth ligand. In one study, which employed REMD simulations, the most populated coordination sphere constituted by H6 and H13 as well as the amine and carbonyl groups of D1 (Figure 24) was considered,<sup>720</sup> using previous QM/MM calculations and experimental findings as input.<sup>721</sup> In general, similar observations were made by most of these simulation studies. Many of them found that both Zn(II)<sup>722,723</sup> and Cu(II)<sup>720,724</sup> binding increases the formation of  $\beta$ -sheets in the C-terminal residues ~30-40/42 of monomeric A $\beta$ . In the N-terminal residues, on the other hand, where the metal ion binds, the amount of helix and  $\beta$ -sheets is reduced as the coordination geometry counteracts the formation of these secondary structures. Moreover, metal ion binding causes a compaction of the N-terminal half of A $\beta$ , explaining the reduction of the hydrodynamic radius of A $\beta$  upon Zn(II) binding as observed experimentally,<sup>692,693</sup> which in turn interacts less with the C-terminal half of the peptide. The C-terminal residues instead interact with each other, leading to increased  $\beta$ -sheet formation. An REMD study of the Cu(II)-bridged A $\beta$ 42 dimer, where the metal ion was coordinated by a pair of H13 and H14 residues from the two peptides, also found a considerable increase in  $\beta$ -sheet in the C-terminal residues, along with a gain in the exposed hydrophobic surface area, which is expected to increase the aggregation tendency as compared to the metal-free peptide.<sup>725</sup> It was suggested that the increased aggregation tendency will rather lead to amorphous aggregates as Cu(II) binding enhances the probability of closed A $\beta$  structures with more intramolecular hydrogen bonds, including intramolecular  $\beta$ -sheets, which are different from the extended monomeric structures prone to fibril formation.<sup>726</sup>

## **7.5. Structural studies on metal induced IAPP aggregation**

### **Roles of zinc on IAPP aggregation**

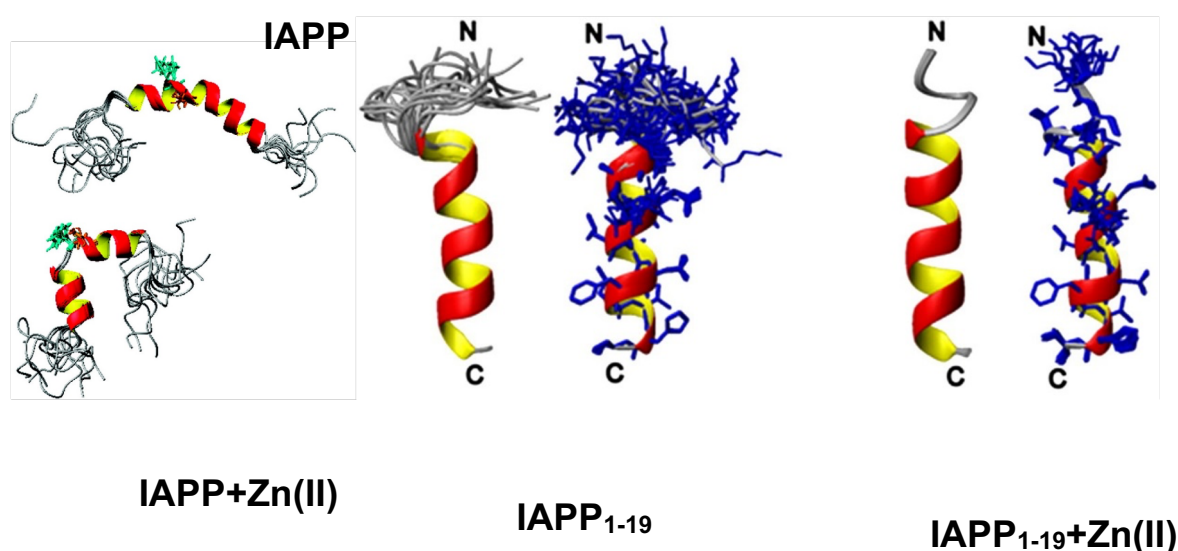
Human IAPP has been shown to aggregate to form amyloid fibrils at nanomolar concentrations under *in vitro* conditions. While the formation of both the amyloid fibers and smaller oligomeric species by hIAPP have been linked to  $\beta$ -cell destruction, it is safely stored in the secretory granule at millimolar concentrations. Given that hIAPP in isolation spontaneously aggregates at concentrations 2 to 3 orders of magnitude lower than those present in the secretory granule where it is stored, it is reasonable to look for other factors that act as chaperones to stabilize hIAPP in a non-toxic form in normal individuals. In contrast to other amyloid proteins for which high-affinity metal binding sites have been identified, the influence of metal binding on hIAPP has been almost entirely unexplored. Zinc is of particular interest as the zinc content of pancreatic  $\beta$ -cells is among the highest in the body and several clinical and epidemiological studies suggest zinc deficiency is a common symptom of type II diabetes. The recent discovery between the genetic linkage between the SLC30A8 gene, which transports zinc

into the secretory granule where insulin and hIAPP are stored, and type II diabetes suggests zinc could have an impact on hIAPP cytotoxicity towards  $\beta$ -cells.

### **NMR and MD simulation studies on Zn-IAPP complexes**

Structural characterization of zinc-bound IAPP complex is important to delineate the biological activity of IAPP and the role of zinc in type-2 diabetes. Poor understanding and the lack of high-resolution structural knowledge of the zinc bound IAPP species have been a major roadblock to designing structure-based therapeutics. Here we briefly present the advances in studying Zn-IAPP complex at a molecular level using NMR. Solution NMR experiments, 2D TOCSY and 2D NOESY, have been carried out to probe the interaction of zinc with human-IAPP. Based on the NMR results, a structural model reported for the Zn-IAPP complex identified His-18 to be the key residue in zinc binding.<sup>727</sup> Zinc binding showed a significant chemical shift perturbation for H $\epsilon$  (0.208 ppm) and H $\delta$  (0.106 ppm) from His18's imidazole side chain. The binding of zinc (10 equivalents) to human-IAPP neutralized the overall peptide charge and induces a local disruption of the secondary structure in the vicinity of His-18 spanning two short  $\alpha$ -helices (Figure 27).<sup>727</sup> On the other hand, under identical NMR conditions, a regular kinked helix-spanning region Arg11-Thr30 was found for IAPP in the absence of zinc, while the rest of the peptide regions were found to be disordered similar to that observed from the Zn-IAPP complex.<sup>727</sup> Taken together, the comparative structural model analysis revealed the major structural difference between IAPP and Zn-IAPP was found to be centered at the His18 residue which has been shown to induce a profound kink in the Zn-IAPP complex with a pattern of helix-kink-helix conformation (Figure 27). Atomic insights further showed that a loss of Phe15-His18 and Leu16-Ser19 nuclear Overhauser enhancements (NOEs) due to zinc binding to IAPP is the reasons for the conformational unwinding of IAPP helical structure.<sup>727</sup>

The intermediates of IAPP (oligomers) have the most pathological relevance and zinc plays a crucial role in the formation of IAPP intermediates. In a follow-up study, Salamekh et al. showed the inhibition of dimers or fibers, but the formation of IAPP hexamers upon zinc binding.<sup>728</sup> Importantly, a second zinc-binding site in human-IAPP has been observed only at a high concentration. To obtain high-resolution structural details, 2D heteronuclear ( $^1\text{H}/^{15}\text{N}$ ) SOFAST-HMQC and homonuclear ( $^1\text{H}/^1\text{H}$ ) were carried out on the full-length (human-IAPP-1-37) and the N-terminal truncated (human-IAPP-1-19) peptide.<sup>728</sup> These experiments showed a significant chemical shift change for His18 upon zinc binding in the  $^1\text{H}/^{15}\text{N}$  spectrum that correlates to the previous observation, and also chemical shift perturbation for the N-terminal residues including the disulfide bonded Cys residues that are disordered in the absence of zinc suggesting a structural transition. These studies further verified the structural change by comparing  $^1\text{H}/^1\text{H}$  correlation spectrum of IAPP(1-19) in the absence and presence of zinc.<sup>728</sup> The observed results highlighted the zinc binding to be inducing an ordered conformation in the N-terminal as compared to the zinc free IAPP peptide (Figure 27). Another study probed the



**Figure 27.** High-resolution NMR model structure of IAPP in the absence or presence of zinc. Full-length NMR structure of IAPP (top) and Zn-IAPP complex (bottom). Zinc binding to His18 (green sticks) induces helical perturbation and formation of a helix-kink-helix conformation. NMR model structures of IAPP<sub>1-19</sub> and IAPP<sub>1-19</sub> obtained from 2D  $^1\text{H}/^1\text{H}$  TOCSY and NOESY experiments. Zinc binding transform the disordered N-terminus (left) to an ordered structure (right). This Figure is designed from Refs. <sup>727</sup> and <sup>728</sup>.

changes in the IAPP monomer-oligomer equilibrium upon zinc binding by analyzing the depletion in  $^1\text{H}$  signal intensities within the limit of NMR detection.<sup>729</sup> Zinc binding to IAPP monomers did not show appearance of new peaks or substantial line broadening of the His18 imidazole peak. Time-lapse measurements and comparison of lag-times of aggregation between IAPP and Zn-IAPP complex using

2D SOFAST-HMQC ( $^1\text{H}/^{15}\text{N}$ ) showed zinc to affect the monomer-oligomer equilibrium and generate prefibrillary IAPP aggregates that are not visible in NMR.<sup>729</sup>

The binding of Zn(II) to IAPP fibrils was probed by MD simulations.<sup>730,731</sup> In these simulations IAPP was modeled in a pre-assembled fibril state and Zn(II) was placed at different potential binding sites including H18, but also C2 and C7 which were modeled without a disulfide bridge between them. The most stable binding was observed for Zn(II) being coordinated to two C2 and two C7 of two neighboring IAPP peptides in the fibrillary structure. In general it was found that Zn(II) ions increase the number of polymorphic states of the fibrillary IAPP by inducing conformational changes. However, these changes are overall minor and the fibrillary models are still very similar to each other so that they are unlikely to be captured as being different from each other by experimental means.

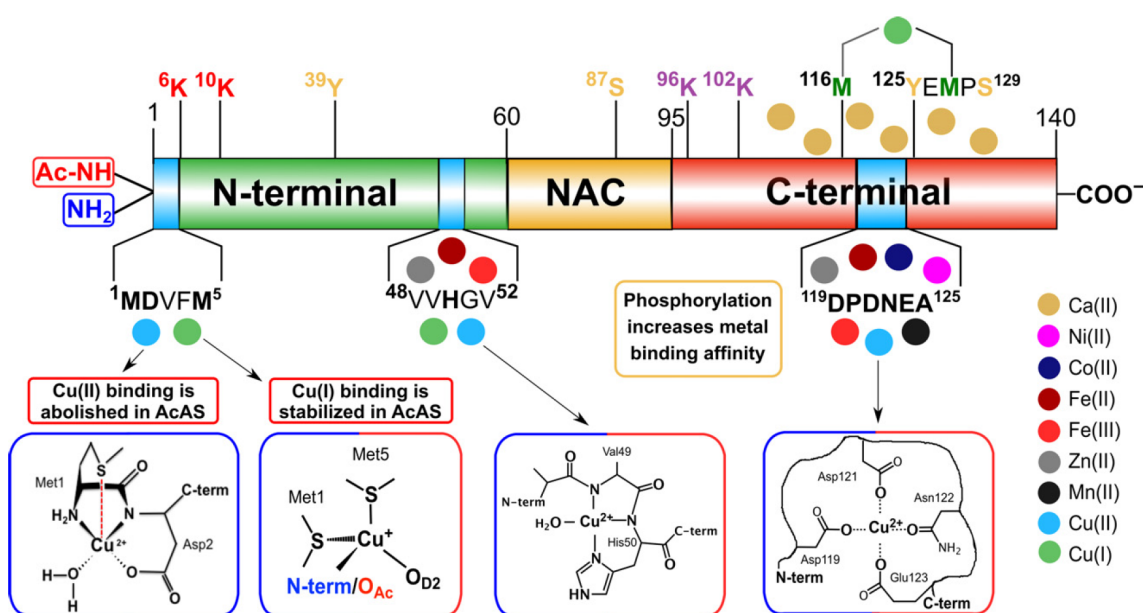
### 7.6. $\alpha$ -synuclein and metal ions

Like A $\beta$  and IAPP,  $\alpha$ S can bind metal ions. A seminal study by Uversky et al. showed that metal ions, in particular Al(III) and Cu(II), can impact the fibrillation rate of  $\alpha$ S, possibly by leading to the formation of a partially folded state that is more prone to aggregation than the monomeric protein in solution.<sup>732</sup> However, the metal concentration used in this pioneering study was in the millimolar range, i.e. greater than those normally occurring in cerebral tissues. Subsequent studies investigated the impact of divalent metal ions (Fe(II), Cu(II), Zn(II), Mn(II), Co(II) and Ni(II)) using physiologically relevant  $\mu\text{M}$  concentrations. The major finding of these studies was that under these conditions only Cu(II) is able to accelerate the formation of amyloid fibrils.<sup>733</sup> Further characterizations of the metal binding revealed that the metal ions generally have a high tendency to interact with  $\alpha$ S through its C-terminal domain, which contains several Asp and Glu residues that are strong attractors of cations and positively charged molecules.<sup>733</sup> The DPDNEA sequence, located at the C-terminal domain (Figure 28), is a non-specific binding site (site 3) for many di- and trivalent metal ions. This site shows conditional dissociation constants in the order of  $\sim 10^{-6}$  M, which is indicative of a low binding affinity for different cations.<sup>734</sup> For Cu(II) three binding sites in  $\alpha$ S have been elucidated (Figure 28).<sup>734</sup> Copper coordination in site 3, which has the lowest Cu(II) affinity, is mediated by the backbone carbonyl groups of D122 and by the carboxylate groups of D119, D121 and D123. Cu(II) binding to H50 (site 2) is pH-dependent and at physiological pH the metal ion is anchored via the imidazole ring of H50, the deprotonated backbone amides of H50 and V49 (or by the carbonyl group of V48) and by one water molecule. In site 1, which has the highest affinity for Cu(II), the cation is coordinated by the N-terminal NH<sub>2</sub> group of M1, the deprotonated backbone amide, the carboxylic groups of D2 and a water molecule. However, it can also involve H50 depending on pH and concentration.<sup>735,736</sup> Copper has been shown to be one of the most potent modulators of  $\alpha$ S structure as well as a very effective accelerator of  $\alpha$ S fibrillation *in vitro*.<sup>732</sup> AFM-based experiments revealed that



the presence of Cu(II) significantly enhanced the relative abundance of the  $\beta$ -sheet like structure in the otherwise conformationally heterogeneous monomeric  $\alpha$ S.<sup>737</sup> Even at physiologically relevant concentrations, Cu(II) ions were effective in the acceleration of  $\alpha$ S aggregation without affecting the resultant fibrillary structures.<sup>738</sup>

We would like to conclude this section by indicating that lysosomes are responsible for degradation of biomolecules and stores for metal ions, and several forms of lysosomal dependent cell death have been identified in diseases, including cancers and neurodegenerative diseases.<sup>739</sup> Rebalancing metal dyshomeostasis could be a feasible therapeutic strategy for AD.<sup>740</sup>



**Figure 28.** Metal binding sites and post-translational modifications of  $\alpha$ S.  $\alpha$ S harbors three binding sites for metal ions (displayed with colored circles): the low affinity, non-specific metal binding site at the acidic DPDNEA segment in the C-terminal, the His50 site, and the first five residues at the N-terminal. His50 can anchor transition-metal ions such as Zn(II), Fe(III), and Fe(II), but the highest affinity is found for Cu(II) and Cu(I) ions. The N-terminus of  $\alpha$ S is a high affinity and highly specific site for Cu ions. This figure is reproduced from Ref. 734.

## 8. What do we know about ALS etiology ?

ALS causes motor neuron death leading to paralysis and eventual respiratory failure. Symptoms typically begin later in life with disease onset typically beginning around ages 40-60. The majority of patients die within 3 to 5 years of their first symptoms. More than 5,600 people worldwide are newly diagnosed with ALS each year and the disease accounts for 2 deaths per 100,000 people <sup>741,742</sup>. 5-10% of cases are considered familial (fALS), genetic mutations can be traced back to

relatives, while 90-95% of cases are considered sporadic (sALS)<sup>743</sup>. Despite a lack of family history in sALS, a pattern of genetic mutations is beginning to emerge across all cases.

Since 1993 over 46 different genetic mutations have been linked to ALS and more continue to be discovered each year. Most of the mutations fall into four main functional categories: RNA processing, protein trafficking/degradation, cytoskeletal/axonal dynamics, and mitochondria function<sup>744-746</sup>. All these mutations could increase cell's vulnerability to an overarching toxic effect.

Cytosolic antioxidant enzyme Cu, Zn superoxide dismutase (SOD1) is a dimeric protein responsible for the conversion of superoxide radicals into oxygen and hydrogen peroxide. Mutations in SOD1 lead to destabilization of the dimeric form and accumulation of toxic aggregates<sup>747</sup>. SOD1 mutations are the second most commonly occurring fALS mutations, found in 10-20% of patients, and have also been found in 1-2% of sALS cases<sup>748</sup>. Unlike the majority of other genetic mutations tied to ALS, normal SOD1 function does not fit into any of the previously mentioned functional categories. It was originally thought that SOD1 mutations prevented the protein from functioning as it should, leading to a buildup of superoxide radicals in cells; this hypothesis was disproved in studies comparing SOD1 deficient mice to SOD1 Glycine-93 to Alanine (G93A) mutant mice showed a loss of motor function in the SOD1<sup>G93A</sup> mice but not in the SOD1 deficient mice<sup>749,750</sup>. These findings led to the theory that SOD1 mutations cause a toxic gain of function.

Similar to tau, amyloid  $\beta$ , and  $\alpha$ S, misfolded SOD1 aggregates are seen in large inclusion bodies in cells both *in vivo* and *in vitro*<sup>751,752</sup>. Despite this, in 2016 Proctor et al. showed that trimeric SOD1 was the most toxic species in NSC-34 cells, and in 2018 Zhu et al. further showed that large aggregates of SOD1 were not only non-toxic to cells but actually protective<sup>753,754</sup>. Other proteins associated with ALS, mainly TAR DNA-binding protein 43kDa (TDP-43) and Fused in Sarcoma (FUS), are also found in large inclusion bodies during disease progression<sup>755</sup>. TDP-43 and FUS are both important for RNA transport and are natively found in the nucleus, mutations cause both proteins to mislocalize to the cytoplasm and aggregate into stress granules (large inclusion bodies)<sup>756-758</sup>. Yet, overexpression of either mutant is not sufficient to trigger the appearance of stress granules in healthy cells without a stress stimulus<sup>759</sup>. Overexpression of misfolded SOD1 has been shown to cause mislocalization and aggregation of both mutant and non-mutant TDP-43 and FUS<sup>760,761</sup>, and mutant TDP-43 and FUS, in a stressed environment, have been shown to coexist with misfolded wild-type SOD1 in cells<sup>762-764</sup>. Stress granules containing TDP-43 and FUS aggregates can lead to cell death in two main ways; either through the unfolded protein response from the cell's inability to break down the large insoluble aggregates, or through loss of function and RNA dysfunction through a lack of correctly folded TDP-43 and FUS in the nucleus<sup>765-767</sup>. Multiple other genetic mutations<sup>768-770</sup> in the RNA

processing and protein trafficking/ degradation categories could act similarly to TDP-43 and FUS or contribute to these cell death pathways<sup>768</sup>.

Motor neurons are unique from other cells due to the length of their axons. Due to how large an area neuron can span, the cytoskeleton system is especially crucial to deliver organelles and materials throughout the entire cell<sup>769,770</sup>. Mutant SOD1 has been shown to alter cytoskeleton transport in two main areas: inhibiting anterograde trafficking between the ER and Golgi and affecting microtubule transport<sup>771</sup>. Coat protein complex II (COPII) vesicles transport proteins between the ER and Golgi, loss of transport leads to ER stress and Golgi fragmentation<sup>772,773</sup>. Expression of COPII with G93A or Alanine-4 to Valine substitution (A4V) mutant SOD1 prevents stress granules and cell death, suggesting mutant SOD1 affects ER to Golgi transport by interfering with COPII vesicles<sup>774,775</sup>. Misfolded SOD1 has been shown to interact with p38 MAP kinase (p38 MAPK)<sup>776</sup>. P38 MAPK phosphorylates kinase-1 preventing it from moving along microtubules<sup>777</sup>. Disrupted functioning of p38 MAPK could cause excessive kinesin-1 phosphorylation which would inhibit microtubule transport, inhibition of p38 MAPK in mutant SOD1<sup>G93A</sup> cell models reduced cell death<sup>778</sup>. HDAC6 expression is also affected in mutant SOD1 models; HDAC6 is a histone deacetylase which can destabilize microtubules. Deletion of HDAC6 slowed disease progression in SOD1<sup>G93A</sup> mouse models<sup>779</sup>. Another pathological hallmark of ALS is the accumulation of neurofilaments<sup>780,781</sup>, any defects in kinesin or microtubule stability could cause this accumulation<sup>782,783</sup>. Genetic mutations in the cytoskeletal/ axonal dynamics functional category have been linked to either ER-Golgi transport or microtubule transport, making it likely that these two systems are crucial to ALS pathology.

Kinesin-1 and microtubules are also responsible for the transport of mitochondria throughout the cell<sup>784,785</sup>. *In vitro* ALS models expressing SOD1<sup>G93A</sup> showed a significant reduction in the number of axonal mitochondria, and the mitochondria present were spaced further apart than in the controls, the same was seen in SOD1<sup>G93A</sup> transgenic mice and rats<sup>786,787</sup>. SOD1<sup>G93A</sup> has also been shown to interfere with the activity of voltage-dependent anion channel 1 (VDAC1) on the outer mitochondrial membrane<sup>788</sup>. VDAC1 is necessary for the exchange of ATP and ADP across the mitochondrial membrane, in SOD1<sup>G93A</sup> mouse models depressed mitochondrial respiration rates and impaired ATP synthesis was seen in the brain and spinal cord well before disease onset<sup>789,790</sup>. Bcl-2 proteins regulate the release of apoptotic cytochromes by VDAC1 and have also been shown to interact with misfolded SOD1<sup>791</sup>.

Dysfunction at multiple points throughout the cell can lead to problems with calcium signaling, ER stress can lead to depletion of calcium stores and VDAC1 controls calcium signaling in mitochondria<sup>792,793</sup>. Motor neurons trigger calcium release through glutamate signaling. Excess glutamate in the synaptic cleft is taken up by excitatory amino acid transporter 2 (EAAT2) which is

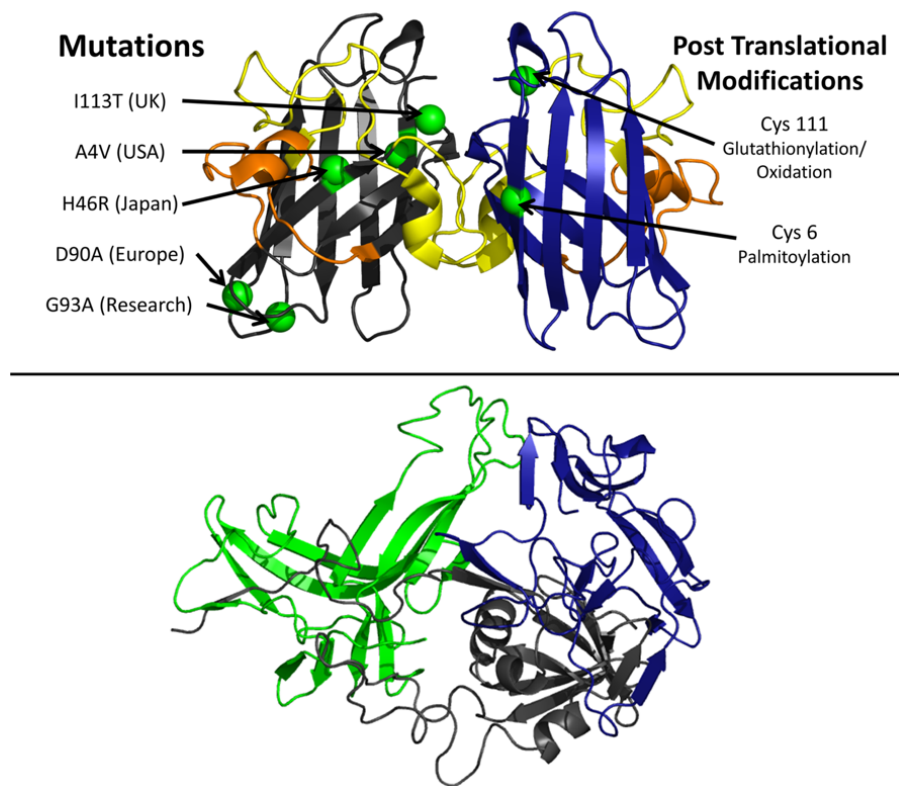
expressed on glial cells<sup>794-796</sup>. Misfolded SOD1 has been shown to reduce expression of EAAT2 leading to glutamate induced excitotoxicity<sup>797-799</sup>. Riluzole, the first FDA approved drug to treat ALS, slows disease progression by increasing EAAT2 activity<sup>800,801</sup>. Since Riluzole only slows disease progression and there are so many different mechanisms that can alter calcium homeostasis within neurons, it is likely that EAAT2 dysfunction is not a direct cause of cell death in ALS.

Although SOD1 is the most prevalent ALS genetic mutation that spans all the other categories of mutations, the role of misfolded SOD1 in sALS is debated. Studies using patient tissue samples conflict on whether misfolded SOD1 is present, even when using the same antibodies<sup>802-804</sup>. One of the limitations of these patient studies is that most only look for misfolded SOD1 in stress granules instead of investigating its presence throughout the cell<sup>805</sup>. For toxic SOD1 trimers to form, the stable dimer must dissociate into monomers and lose its metals. In 2004 Khare et al. showed SOD1<sup>A4V</sup> dimers have a 10,000 fold increase in dimer dissociation equilibrium constant compared to SOD1<sup>WT</sup>.<sup>806</sup> SOD1<sup>A4V</sup> is only one out of more than 100 structurally diverse point mutations tied to SOD1 aggregation in ALS, many of which are not located in the dimer interface (Figure 29). The full database of mutant SOD1 variants tied to ALS can be found on the ALS online database (alsod.ac.uk)<sup>807</sup>. In 2005 Khare and Dokholyan showed that the monomers of SOD1 have coupled motion when a dimer is formed, specifically in the movement of the distal metal-binding loops<sup>808,809</sup>. Mutations within the dimer interface and on the outside of the monomers shared the effect of disrupting the coupled motion of the dimer and destabilizing it. Beyond destabilization of the dimer, loss of metals in monomeric form is also crucial to SOD1 aggregation. Metal ions stabilize the Glutamic acid-49 to Asparagine-53 region of the Zinc loop, protecting the  $\beta$ -sheet region of the monomer. Loss of metals exposes the  $\beta$ -sheet region, destabilizing the monomer and making it prone to aggregation<sup>810-813</sup>. Simulations of the SOD1 barrel, without metals, found that the  $\beta$ -strands  $\beta 5$  and  $\beta 6$  are the weak spots for thermal unfolding. In simulations in a crowded environment, oscillations of the VII electrostatic loop region expose the same  $\beta$ -strands correlating with increased interactions with the crowder. Mutations of histidine 46 stabilized the VII loop in the compact state, thus protecting the  $\beta 5$  and  $\beta 6$  strands similar to how metal ions protect the barrel.<sup>415</sup>

Multiple studies have shown that PTMs can destabilize the dimer interface, increasing the chance of misfolding<sup>814,815</sup>. In 2011 Redler showed that glutathionylation of dimeric SOD1<sup>WT</sup> increased the equilibrium dissociation constant 100,000 fold. Glutathionylation is a natural protective measure in response to oxidative stress, and could propagate the SOD1 misfolding as neighboring cells are stressed<sup>816,817</sup>. Oxidation of Cysteine 111 to sulfonic acid in SOD1<sup>WT</sup> in neurons led to similar phenotypes of familial ALS, misfolded SOD1 aggregates and inhibition of kinesin-based fast axonal transport<sup>818</sup>. SAPH-ire, a machine learning program that can predict post translational modifications

to a protein, indicated that the 31 amino acids between Serine-98 and Lysine-128 had the highest likelihood to affect SOD1 protein function.<sup>814,819</sup> That region of residues encompasses part of both the Zinc binding loop VI and electrostatic loop VII (Figure 29), providing further evidence that alterations in these loops may affect the stability of dimeric SOD1. Palmitoylation of SOD1 at Cysteine-6 impairs nuclear localization leading to mislocalized SOD1 accumulating in the cytoplasm<sup>820</sup>. Antinone et al. found that misfolded SOD1 in SOD1<sup>G93A</sup> cell culture and mice spinal cords showed increased palmytoylation compared to SOD1<sup>WT</sup>.<sup>821</sup> Other PTMs have been shown to protect SOD1. Cysteinylation of SOD1 could prevent oxidation of cysteine 111, the crystal structure of cysteinylated SOD1 showed a slight change at loops VI and VII<sup>822</sup>. Fay et al. found that a phosphor-mimicking aspartic acid substitution at Threonine-2 stabilizes SOD1 A4V dimers, suggesting that phosphorylation of SOD1 could be an innate cellular protection against SOD1 destabilization<sup>823</sup>.

Mutations from environmental toxins have also been shown to destabilize the dimer interface of SOD1<sup>824-826</sup>. Most notably, exposure to  $\beta$ -methylamino-L-alanine (BMAA) is linked to 100 times increase in ALS incidence in the indigenous Chamorro population on Guam. A new study shows that substituting any serine for BMAA in SOD1 has the potential to destabilize the dimer interface and promote SOD1 aggregation<sup>827</sup>. Post translational modifications and mutations from environmental toxins could explain why there is such a large amount of sALS cases with no known genetic cause<sup>828</sup>. The toxic effects of mutant SOD1 on RNA processing, protein trafficking/ degradation, cytoskeletal/ axonal dynamics, and mitochondria function, along with the decrease in dimer stability from modifications and mutations shows the aggregation of SOD1 into toxic trimers leads to a toxic gain of function in both sporadic and familial ALS.



**Figure 29.** The mutations, modifications, and structural elements of SOD1. The crystal structure of SOD1<sup>WT</sup> is shown in the top panel (pdb: 2V0A)<sup>830</sup> with the Zinc binding loop, loop IV, spanning residues 49-84 (yellow) and the electrostatic loop, loop VII, spanning residues 122-143 (orange). The most prevalent ALS associated mutations are shown on the left monomer (green)<sup>829</sup> and three SOD1 destabilizing post translational modifications are shown on the right monomer (green)<sup>814</sup>. The bottom panel shows a possible trimeric structure of SOD1 modeled by Procter et al.<sup>753</sup>. The authors design the figure with Pymol.<sup>254</sup>

## 9. AD, PD and T2D therapies

### 9.1. Small molecules, polymer-based molecules and carbon nanoparticles

Understanding the mechanistic determinants of amyloid-inhibitor interaction is a prerequisite for developing new drugs, as discussed in recent reviews,<sup>193,831</sup> and designing new strategies to reduce the toxicities by targeting A $\beta$ , tau,  $\alpha$ S and IAPP.<sup>302,832-846</sup>

#### 9.1.1. Small molecules

While a plethora of small molecules has been shown to be efficient in solution, the lipid membrane has been shown to catalyze the formation of toxic amyloid intermediates and influence

amyloid aggregation. The amphipathic lipid membrane not only complicate the amyloid aggregation process but will also modulate the action of amyloid inhibitors. Therefore, to efficiently suppress amyloid aggregation, the action of small molecule compounds must be examined in the presence of lipid membrane. In a recent study, Cox et al. developed an approach for high-throughput screening of a library of >1800 molecules that inhibited A $\beta$  aggregation in a membrane environment.<sup>847</sup> This study reported five small molecules as efficient inhibitors of A $\beta$  aggregation both in solution and in the presence of membrane. Using a combination of fluorescence and dot-blot assay, they validated the inhibitory efficacy of these five molecules (AQ-4, THQ-1, BF-3, DHQ-1 and DHQ-2, Figure 30) that generated non-fibrillary A $\beta$  species in the presence of large unilamellar vesicles composed of mixed (zwitterionic/anionic) lipids.<sup>847</sup> The mechanism of the action of these compounds to inhibit A $\beta$  fibrillation in the membrane interface, as compared to widely studied natural phenolic compounds (EGCG, curcumin etc.), needs to be explored. However, a suggested mechanism of action is the ability of the screened compounds to interact and adsorb on the membrane without affecting its interaction with A $\beta$ . Another approach that promotes A $\beta$  aggregation and reduces the formation of toxic intermediates has also been considered to treat AD. In this category, the major players are the cellular polyamines (Figure 30), present intracellularly with millimolar concentration, which have been shown to promote A $\beta$  aggregation by suppressing the amount of toxic A $\beta$  species.<sup>848,849</sup> Recently, Dobson et al. discovered a natural product aminosterol trodusquemine (Figure 30) isolated from shark that enhanced A $\beta$  aggregation following the suppression of neurotoxicity.<sup>850</sup> Promotion of A $\beta$  aggregation has also been observed in the presence of poly-amino acids.<sup>852</sup> Nonsteroidal anti-inflammatory drug (NSAID) sulindac sulfide is another small-molecule that form colloid particles and catalyze A $\beta$  fibrillation (Figure 30). Solid-state NMR results probing the action of NSAID have identified a homogenous distribution of amyloid-fibers characterized by a canonical  $\beta$ -strand-turn- $\beta$ -strand with reduced neurotoxicity.<sup>853</sup>

Small-molecule inhibitors of IAPP have been reviewed in Ref. 854. We report on recent studies on a few of these small-molecules that modulate IAPP aggregation, morphology and pathological phenotypes. Natural polyphenols like EGCG present in green tea, curcumin present in turmeric and resveratrol present in red wine inhibit IAPP amyloidogenesis (Figure 30).<sup>855</sup> Curcumin is a planar biphenolic compound that delays IAPP aggregation when mixed to peptide monomers. The mechanism of action is proposed to be mediated by curcumin assisted disassembly of an  $\alpha$ -helical intermediate of IAPP, that has been reported to nucleate the aggregation.<sup>856</sup> Daval et al. highlighted a less likely therapeutic use of curcumin due to its toxicity to cells and proposed chemical modifications. This study showed curcumin to be toxic at micromolar concentration to  $\beta$ -cells and

partially protect cells from IAPP induced apoptosis.<sup>857</sup> Moreover, the poor water solubility of curcumin is a major limitation for its potential use to protect cells from amyloid toxicity. A chemically modified curcumin derivative (CurDac) was recently shown to significantly modulate IAPP aggregation (Figure 30).<sup>846</sup> Pithadia et al. tested the effect of CurDac in rescuing the IAPP induced membrane damage using a variety of biophysical techniques.<sup>858</sup> Using fluorescence and NMR, they observed a significant delay in IAPP aggregation when peptide monomers are mixed with CurDac. In addition, they identified the interaction of CurDac with aged IAPP fibers to be mediated by the aromatic ring atoms. More interestingly, they demonstrated that CurDac inhibits the membrane catalyzed IAPP fibrillation.<sup>858</sup> A very recent study on CurDac interaction with IAPP fibers by Cox et al. presented some surprising results highlighting the limitations of the use of CurDac as a therapeutic compound. This study reported the ability of CurDac to disaggregate amyloid fibers as tested on three different amyloidogenic peptides (A $\beta$ , IAPP and human calcitonin).<sup>846</sup> Toxicity measurement of the disintegrated peptide species from aged fibers, or monomers, incubated with CurDac were found to enhance the cytotoxicity of IAPP.<sup>846</sup> Together, these studies direct small-molecule intervention of IAPP aggregation could be lethal and need further attention and chemical optimization. Like CurDac, EGCG and its stereoisomer (GCG) have been shown to be an effective inhibitor of monomeric A $\beta$  and IAPP aggregation, remodeling toxicity of oligomers and disaggregates aged fibers in solution (Figure 30).<sup>859,860</sup> In addition, a seeding reaction identified that EGCG bound IAPP species do not bind IAPP monomers and thus restrict fibrillation.<sup>859,861</sup> Unlike curcumin, EGCG is shown to rescue cells from IAPP induced toxicity. Another recent study reported the combined effects of EGCG and zinc to suppress IAPP aggregation and toxicity.<sup>862</sup> Morin hydrate is another good example of small-molecule compound that is also shown to inhibit IAPP aggregation and disaggregates the preformed IAPP fibers (Figure 30).<sup>864</sup> IAPP oligomers are also targeted by small-molecules to reduce their cytotoxicity. For example, sulfonated triphenyl methane derivative acid fuchsin<sup>864</sup> and EGCG<sup>859</sup> are shown to effectively inhibit IAPP aggregation and suppress toxicity when added to the peptide during the lag-phase of growth where toxic intermediates are formed. In addition to *in vitro* observation, the effectiveness of EGCG was tested on IAPP transgenic mice.<sup>865</sup> Saravanan et al., reported a diphenylpyrazole derived inhibitor anle145c to effectively bind and generate non-toxic IAPP oligomers (Figure 30).<sup>842</sup> Computational study of an analogous compound anle145c shows remodeling of preformed A $\beta$  and IAPP oligomers.<sup>866</sup> Polyphenols and their effect on IAPP aggregation in membrane interface has also been tested. Derivatives of resveratrol with improved solubility (Figure 30) is shown to abolish IAPP fibrillation in the presence of liposomes.<sup>867</sup> In addition, suppression of IAPP induced membrane damage is revealed by conjugating resveratrol with lipids containing dimyristoylphosphatidyl moiety.<sup>867</sup> Lolicato et al. provided mechanistic insights into



rescue of IAPP induced membrane damage. The simulation shows resveratrol disrupt IAPP interaction with anionic membrane by binding to the hydrophobic region (23-25 and 32-34) of the peptide.<sup>868</sup>

Many compounds were synthesized to prevent amyloid aggregation.<sup>842,869</sup> Of note, the compound anle138b also ameliorates tau pathology.<sup>869</sup> Studies have also attempted to determine potential inhibitors from natural compounds<sup>870,871</sup> since they normally show low side effects, well-known pharmacological properties, and high availability. Several compounds with high antioxidant and anti-inflammatory properties were found to be able to remodel or inhibit the neurotoxic conformations of amyloidogenic peptides via various pathways.<sup>872</sup> The inhibition probably activates through direct binding with the peptides<sup>873</sup> and/or interfering with  $\pi$ - $\pi$  stacking interaction between aromatic rings of amyloidogenic residues according to the observation via microscopic and quantum chemical analyses.<sup>874</sup> Interestingly, some natural compounds such as astaxanthin,<sup>875-879</sup> brazilin,<sup>880-882</sup> curcumin,<sup>883-887</sup> dopamine,<sup>888-894</sup> EGCG,<sup>283,860,895-900</sup> resveratrol,<sup>328,901-904</sup> and rosmarinic acid<sup>905-910</sup> can simultaneously target the proteins involved in AD, PD, T2D and ALS where all experimental and computational studies are reported in Table 3. Interestingly, although the inhibitory mechanism of these ligands to the diseases are probably different, they all adopt a similar binding pose to A $\beta$  peptides,  $\alpha$ -synuclein, hIAPP, insulin, and SOD1 via molecular docking simulations (Figure 31). As seen in Table 3, the knowledge of the interaction of some drugs with the amyloid proteins is still lacking.

### 9.1.2. Polymer-based molecules

Recently bio-inspired peptides containing natural and unnatural amino acids, synthetic polymers and polymer-based particles are developed to control amyloid aggregation. Selectivity, specificity, toxicity, rational designing, membrane permeability, and proteolytic stability are the advantages of these molecules to effectively target amyloidogenic proteins/peptides. Sahoo et al. developed a cationic PMAQA polymer and proposed its multifunctional use against AD and T2D.<sup>851</sup> It is shown that PMAQA<sup>917</sup> accelerates the fibrillation (~ 1 min) of A $\beta$  and delays IAPP aggregation (~3 days). In another study, star-shaped poly(2-hydroxyethyl acrylate) polymers are shown to accelerate the fibrillation of IAPP thus reducing the formation of toxic intermediates.<sup>918</sup> Recently, short anionic and cationic styrene-based copolymers (~2.2 kDa) are shown to catalyze IAPP aggregation by quickly inducing the formation of non-toxic fibers; whereas cationic styrene copolymers inhibit IAPP aggregation and induce generation of non-toxic globulomers.<sup>919</sup> Smith et al. recently developed polymer-peptide conjugates to control the molecular ordering of A $\beta$  structure and stability, but the pathological phenotypes of these nanostructure are yet to be tested.<sup>920</sup> Thermoresponsive polymers

varying in hydrophobicity/hydrophilicity ratio is shown to selectively modulate A $\beta$  aggregation kinetics.<sup>921</sup> Poly(p-phenylene vinylene) derived polymer is shown to degrade A $\beta$  fibers that are non-toxic and to clear the A $\beta$  plaques from the brain tested *ex vivo*.<sup>922</sup> Polymer nanocomposites have shown to eliminate toxic amyloid species under *in vitro* and *in vivo* conditions. Zhao et al., developed a nanocomposite that wrapped a protein with a cross-linked A $\beta$  fragment “KLVFF” polymer layer. The nanocomposite is shown to reduce toxic oligomers by trapping A $\beta$  following the formation of co-assembled nanoclusters and its efficacy is measure *in vivo* using Alzheimer’s mice.<sup>923</sup> Dendrimers designed using poly(propylene imine) core and maltose-histidine shell shown to enhance its ability to cross blood-brain-barrier (BBB) and protect from the progression of AD in transgenic mice.<sup>924</sup> Poly (amidoamine) dendrimer and N-isopropylacrylamide:N-tert-butylacrylamide copolymeric nanoparticles are shown to effectively abolish IAPP aggregation and its toxicity.<sup>925,926</sup> Lipid-nanodiscs prepared by conjugating synthetic polymers or peptides with natural phospholipids are recently tested on A $\beta$ .<sup>263,274</sup> Polymer nanodiscs are shown to strongly bind A $\beta$  following a quick induction of fibers that are tested non-neurotoxic.<sup>263</sup> Protein and peptide lipid-nanodiscs show promising application in trapping amyloid intermediates and delaying aggregation of A $\beta$  or IAPP.<sup>274,285</sup> Apolipoprotein J conjugated nanoparticles have shown to reduce the amount of cerebral A $\beta$  in transgenic mice.<sup>927</sup> Cationic fluorescent conjugated polymer nanoparticles are shown to inhibit A $\beta$  aggregation.<sup>928</sup> Nanoliposomes decorated with A $\beta$  binding surface containing benzothiazolyl are proposed to be an effective therapeutic systems for AD treatment.<sup>929</sup> For other peptide-based inhibitors, readers are referred to a recent review<sup>930</sup> and recent articles.<sup>931-933</sup>

### 9.1.3. Carbon nanoparticles

Carbon nanoparticles (CNPs, including fullerene, carbon nanotube, graphene, and their derivatives) have received great interest due to their exceptional physicochemical properties (such as large surface-to-volume ratio and capability of translocation across BBB), as discussed in two recent reviews.<sup>836,838</sup> As early as 1997, it was reported by Dugan et al that C<sub>60</sub> derivatives (i.e. carboxyfullerenes) can function as neuroprotective drugs *in vivo*.<sup>934</sup> In the following years, a few of research groups investigated the influence of carbon nanoparticles on the aggregation of amyloidogenic proteins. For example, in 2003, a ThT study by Kim et al showed that fullerenes inhibit strongly the amyloid aggregation of A $\beta$ <sub>1-40</sub> at the early stage.<sup>935</sup> In 2007, TEM and *in vivo* investigations by Podolski et al demonstrated that hydrated fullerenes impede the fibrillization of A $\beta$ <sub>25-35</sub> peptide and improve the performance of the cognitive task in control rats.<sup>936</sup> In the same year, Linse et al. reported that carbon nanotubes (CNTs) enhance the fibril formation of human  $\beta$ 2-microglobulin by accelerating the nucleation process.<sup>937</sup> Those early experimental studies revealed

the distinct roles of CNPs on the aggregation of different proteins and inspired more research in this direction. In order to determine the physicochemical determinants of CNPs in modulating protein aggregation and to find more effective inhibitors against protein aggregation, a growing number of computational and experimental studies have been carried out over the past decade. In what follows, we mostly describe the most recent simulation studies aimed at understanding the inhibitory mechanisms of CNPs against the fibrillization of A $\beta$  fragments, full-length A $\beta$ , hIAPP and  $\alpha$ S fragment (Table 4).

#### **Inhibition of CNPs against fibrillization of A $\beta$ /A $\beta$ -fragment and shape/surface curvature effects of CNPs.**

In 2011, Li et al. investigated the effects of carbon nanotubes on the oligomerization of A $\beta$ <sub>16-22</sub> peptide by atomistic REMD simulations.<sup>938</sup> Their simulations demonstrated that CNTs not only inhibit the formation of  $\beta$ -sheet-rich octamers (with an average  $\beta$ -sheet content of 7.9% in the presence of CNT relative to 44.5% in the absence of CNT), but also destabilize fibrillar  $\beta$ -sheets through hydrophobic and  $\pi$ - $\pi$  stacking interactions. The authors proposed that CNT is likely to impede the fibrillation of A $\beta$ <sub>16-22</sub> and of the full-length A $\beta$ , as  $\beta$ -sheet is the dominant secondary structure content of A $\beta$  fibril and A $\beta$ <sub>16-22</sub> is the central hydrophobic core of A $\beta$ . Two years later, Xie et al. examined the influence of a hydroxylated carbon nanotube (CNT-OH) on A $\beta$ <sub>16-22</sub> aggregation using a combined simulation and experimental method.<sup>939</sup> REMD simulations revealed that a hydroxylated CNT, with the same diameter and length as the pristine CNT used in Ref. 978, can also prevent the formation of ordered  $\beta$ -sheet-rich octamers (with a  $\beta$ -sheet content of 17.5% in the presence of CNT-OH). Hydrophobic,  $\pi$ - $\pi$  stacking and electrostatic interactions between CNT-OH and A $\beta$ <sub>16-22</sub> play important roles in inhibiting  $\beta$ -sheet formation. Their AFM and thioflavin ThT fluorescence experiments confirmed the inhibitory effect of both CNT and CNT-OH on A $\beta$ <sub>16-22</sub> fibrillization, in support of the REMD simulation predictions.<sup>938,939</sup> Next, Xie et al investigated the effect of fullerenes with different surface curvatures (C<sub>60</sub> and C<sub>180</sub>) on the  $\beta$ -sheet formation of A $\beta$ <sub>16-22</sub> octamers by REMD simulations.<sup>940</sup> They found that C<sub>60</sub> significantly reduces the  $\beta$ -sheet probability (25.7% with C<sub>60</sub> relative to 44.5% without C<sub>60</sub>) and increase the coil propensity, thus retarding the fibril formation of A $\beta$ <sub>16-22</sub>. The simulation-predicted inhibitory effect of C<sub>60</sub> on A $\beta$ <sub>16-22</sub> fibrillization was confirmed by AFM and ThT experiments.<sup>940</sup> Interestingly, it was found that C<sub>180</sub> (with the same total mass as C<sub>60</sub>) exhibits a stronger inhibitory effect on the  $\beta$ -sheet formation (with a  $\beta$ -sheet content of 18.1%) than C<sub>60</sub>. This observation can be explained by the strong hydrophobic and aromatic stacking interactions between fullerene hexagonal rings and the Phe rings of A $\beta$ <sub>16-22</sub> as C<sub>180</sub> contains more hexagonal rings than C<sub>60</sub>. This result highlights the significant role of fullerene hexagonal rings in the inhibition of A $\beta$ <sub>16-22</sub> fibrillation. By comparing the  $\beta$ -sheet probability of A $\beta$ <sub>16-</sub>

22 oligomers in the presence of CNT, C<sub>60</sub> and C<sub>180</sub> (with almost the same total mass) obtained by the two REMD studies,<sup>938,940</sup> it was found that CNT displays the strongest inhibitory effect on the  $\beta$ -sheet formation, followed by C<sub>180</sub> and then C<sub>60</sub>. The different inhibitory ability is attributed to the delicate balance of hydrophobic and aromatic stacking interactions dictated by the shape and/or surface curvature of CNPs. Consistent with REMD results, AFM experiments by Wang et al demonstrated that GO nanosheets have the strongest inhibitory effect on A $\beta$ <sub>33-42</sub> aggregation, followed by nanotubes and nanodots.<sup>941</sup> These results reveal the important role of shape and surface curvature of CNP in inhibiting A $\beta$ <sub>16-22</sub>/A $\beta$ <sub>33-42</sub> fibrillation. Meanwhile, Yang et al examined the influence of graphene nanosheets on preformed A $\beta$ <sub>16-21</sub> fibrils by performing MD simulations. They found that graphene nanosheets can penetrate amyloid fibrils, extract a large number of peptides from them, and destruct A $\beta$ <sub>16-21</sub> fibrils.<sup>942</sup>

In order to reveal at atomistic level the molecular mechanism behind the experimental observations showing that both C<sub>60</sub> and graphene oxide (GO) nanosheet can inhibit the fibrillization of full-length A $\beta$  peptide,<sup>935,942-944</sup> Sun et al and Jin et al performed REMD simulations on A $\beta$ <sub>1-42</sub> dimer in the absence and presence of four C<sub>60</sub> molecules or four GO nanosheets.<sup>945,946</sup> The GO nanosheet contains the same number of carbon atoms as C<sub>60</sub>, which is denoted as GO<sub>60</sub>. These REMD simulations showed that A $\beta$ <sub>1-42</sub> peptides form diverse  $\beta$ -hairpin-containing dimeric conformations, whereas those conformations are significantly reduced in the absence of C<sub>60</sub> or GO<sub>60</sub>. C<sub>60</sub> suppresses the  $\beta$ -sheet formation of A $\beta$ <sub>1-42</sub> mostly by binding to the central hydrophobic motif LVFFA and the C-terminal hydrophobic region I31~V40, while GO<sub>60</sub> inhibits the  $\beta$ -sheet formation by mainly binding to charged residues and hydrophobic residues. GO<sub>60</sub> displays a better inhibitory effect on the  $\beta$ -sheet formation of A $\beta$ <sub>1-42</sub> dimer than C<sub>60</sub>,<sup>946</sup> indicating that the shape of CNP may play a role in the inhibition of A $\beta$ <sub>42</sub> aggregation. *In silico* and *in vitro* experiments showed that water-soluble fullereneol C<sub>60</sub>(OH)<sub>16</sub> tightly binds to negatively charged residues of A $\beta$ <sub>1-40</sub> monomers and effectively reduces the formation of amyloid fibrils.<sup>947</sup> The interactions of C<sub>60</sub>/C<sub>60</sub>OH with U-/LS-shaped A $\beta$  protofibrils were studied by explicit-solvent MD simulations.<sup>948,949</sup> It was found that C<sub>60</sub> and C<sub>60</sub>OH exhibit different binding mechanisms and the binding of C<sub>60</sub> to A $\beta$  protofibril results in disruption of D23-K28 salt bridge. More recently, a combined MD simulation and experimental study by Liu et al.<sup>950</sup> reported that hydroxylated carbon nanotubes (CNT-OHs) also inhibit A $\beta$ <sub>42</sub> fibrillization, disaggregate mature fibrils, and reduce A $\beta$ <sub>42</sub>-induced cytotoxicity. The six residues H13, H14, Q15, V36, G37, and G38 contribute most to the interactions between CNT-OH and A $\beta$ <sub>11-42</sub> protofibril.

### **Size effect of carbon nanoparticles on the inhibition of A $\beta$ <sub>33-42</sub> aggregation**

CD spectra and AFM experiments by Dong's group reported that GO interferes with the aggregation pathway of A $\beta$ <sub>33-42</sub> peptide, leading to a reduced  $\beta$ -sheet content and considerably short fibrils, and large-size GO displays a strong capacity in impeding A $\beta$ <sub>33-42</sub> aggregation.<sup>951,952</sup> In order to unravel the mechanistic basis of the inhibition and size effect of GO nanosheets on the fibrillization of A $\beta$ <sub>33-42</sub> peptide, Chen et al.<sup>953</sup> investigated the oligomerization of A $\beta$ <sub>33-42</sub> peptide in the absence and presence of four GO<sub>60</sub> nanosheets or two GO<sub>120</sub> nanosheets by performing all-atom REMD simulations starting from four extended A $\beta$ <sub>33-42</sub> peptide chains. Their simulations showed that in the absence of GO nanosheets, four A $\beta$ <sub>33-42</sub> chains can form  $\beta$ -barrel-like structures and antiparallel  $\beta$ -sheets. Inter-peptide hydrophobic interactions among residues M35, V36, V39 and V40 and backbone hydrogen bonding interactions play crucial roles in the formation of  $\beta$ -barrels/ $\beta$ -sheets. GO retards A $\beta$ <sub>33-42</sub> oligomerization by interfering with peptide-peptide interactions. Consisting of the same total carbon atoms and oxidation groups as GO<sub>60</sub>, GO<sub>120</sub> exhibits a stronger capacity in inhibiting A $\beta$ <sub>33-42</sub> aggregation than GO<sub>60</sub>. The oxidation groups of GO<sub>120</sub>, especially the hydroxyl groups, can form more hydrogen bonds with A $\beta$ <sub>33-42</sub> than those of GO<sub>60</sub>. Moreover, GO<sub>120</sub> has a larger hydrophobic contact surface area with A $\beta$ <sub>33-42</sub> than GO<sub>60</sub>. These results reveal that GO<sub>120</sub> displays stronger hydrogen-bonding and hydrophobic interactions with A $\beta$ <sub>33-42</sub> than GO<sub>60</sub>, thus leading to a better inhibitory effect on A $\beta$ <sub>33-42</sub> aggregation. These simulation results provide mechanistic insights into the inhibition and size effects of GO nanosheets on the aggregation of A $\beta$ <sub>33-42</sub> peptide.<sup>953</sup>

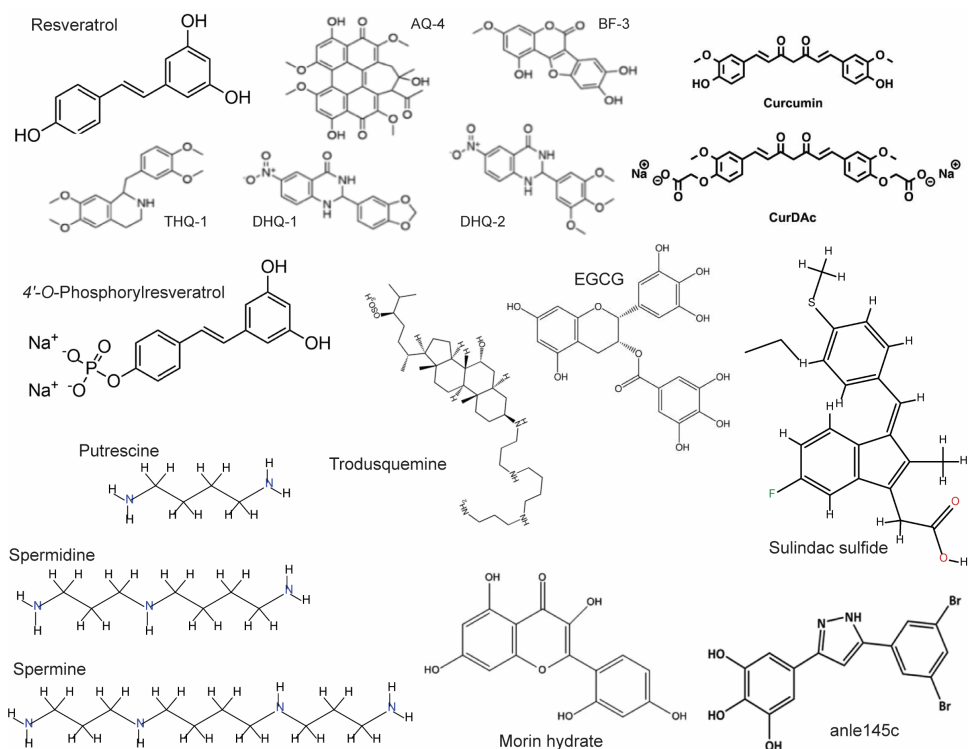
### **Inhibition of CNPs against hIAPP/ $\alpha$ S aggregation and effect of surface hydroxylation extents of CNPs**

The influence of zero-, one- and two-dimensional carbon nanoparticles on the amyloid fibrillization of other amyloid proteins has also been reported in recent five years. Nedumpully-Govindan et al investigated the effect of GO nanosheets on hIAPP fibrillization and cytotoxicity by combining computational modeling, biophysical characterization, cell toxicity measurements.<sup>954</sup> DMD simulations showed a strong binding of hIAPP monomers/oligomers on the surface of GO nanosheets. TEM images and cell toxicity assays indicated that small GO nanosheets inhibit hIAPP aggregation and reduce hIAPP toxicity. Following that work, Wang et al.<sup>955</sup> demonstrated the use of graphene quantum dots (GQDs: a single- or few-layer graphene with a tiny size less than 100 nm) as inhibitors against the aggregation and toxicity of hIAPP using DMD simulations, *in vitro* ThT and  $\beta$ -cell viability assays, and an embryonic zebrafish model. Their recent study<sup>956</sup> reported that GQDs can rescue protein dysregulation of pancreatic  $\beta$ -cells exposed to hIAPP, pointing to the potential of using GQDs for *in vivo* mitigation of T2D amyloidosis.

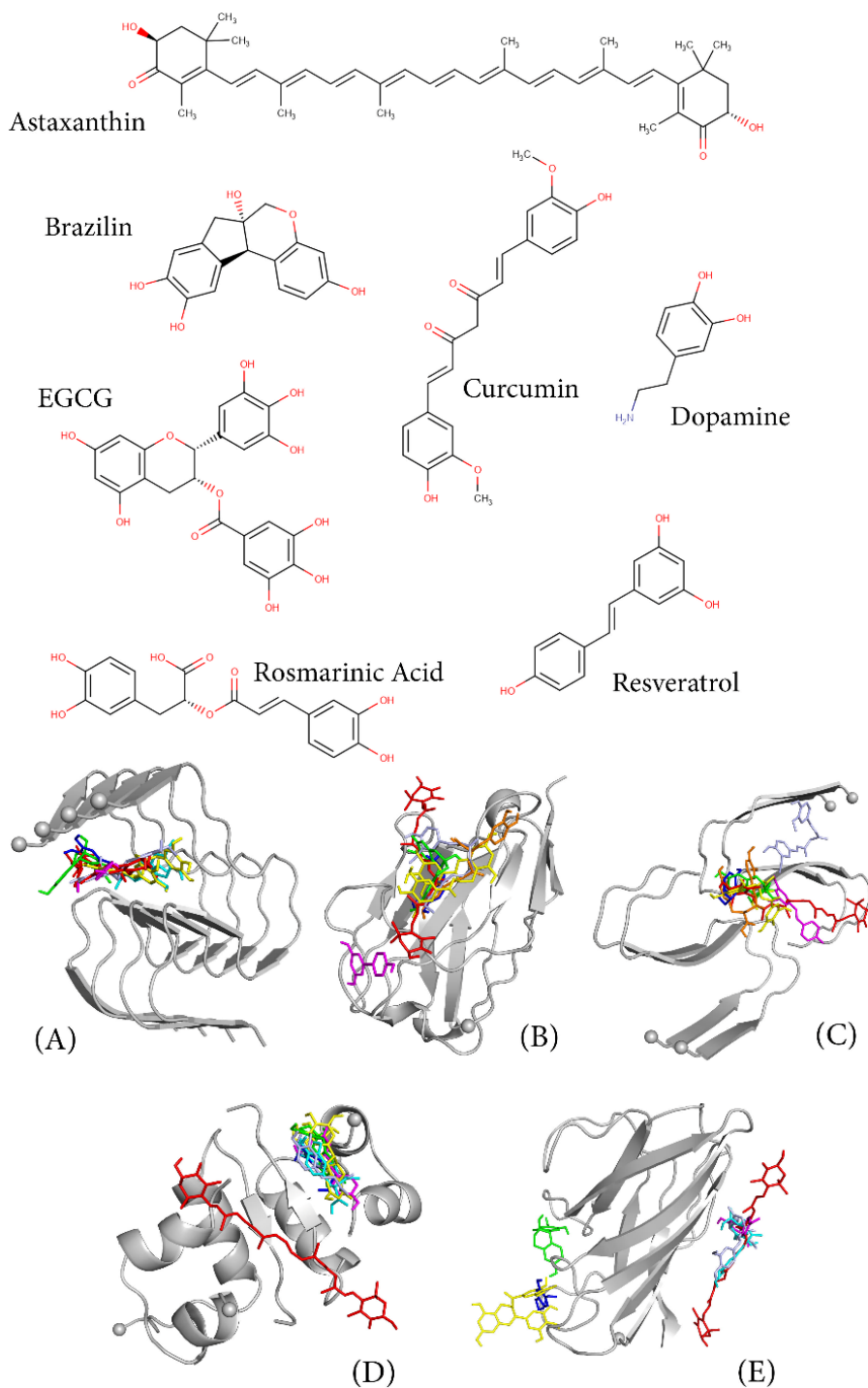
By combining all-atom REMD and MD simulations, AFM and TEM images, ThT and cell toxicity

assays, Mo et al.<sup>957-959</sup> examined the influences of hydroxylated carbon nanotubes and fullerenes on the fibrillization of hIAPP. REMD and MD simulations demonstrated that both CNT-OHs and C<sub>60</sub>OHs not only suppress the formation of  $\beta$ -sheet and  $\beta$ -hairpin-containing amyloidogenic precursor of hIAPP, but also remodel or disassemble preformed hIAPP protofibrils/fibrils. Hydrogen-bonding, hydrophobic, cation- $\pi$  and  $\pi$ - $\pi$  stacking interactions between hIAPP and CNT-OH/C<sub>60</sub>OH are the dominant driving forces in inhibiting hIAPP aggregation. Turbidity analyses, ThT fluorescence assays, CD microscopies, TEM and AFM images provide direct evidence for the simulation-predicted  $\beta$ -sheet inhibition and protofibril-disruption by hydroxylated CNTs/fullerenes. SH-SY5Y cell viability assays demonstrated the rescue effect of C<sub>60</sub>OHs on hIAPP-induced cytotoxicity.<sup>958</sup> The REMD simulations by Bai et al.<sup>958,959</sup> showed that C<sub>60</sub>, C<sub>60</sub>(OH)<sub>8</sub> and C<sub>60</sub>(OH)<sub>24</sub> possess different inhibitory effects on the  $\beta$ -sheet formation of hIAPP: the  $\beta$ -sheet content of hIAPP dimer in the presence of C<sub>60</sub>, C<sub>60</sub>(OH)<sub>8</sub> and C<sub>60</sub>(OH)<sub>24</sub> is respectively 1.8, 4.2, and 5.6% (the  $\beta$ -sheet content of hIAPP dimer alone is 10.8%). These data reveal that hydroxylation extents modulate the inhibition capacity of C<sub>60</sub>OHs against hIAPP aggregation. Recently, Sun et al.<sup>960</sup> explored the effects of hydroxylation extents of C<sub>60</sub> on  $\beta$ -sheet formation of the nonamyloidogenic core (NAC) fragment (residues 68-78) of  $\alpha$ S by DMD simulations. Their simulation data showed that the  $\alpha$ S<sub>68-78</sub> peptide can assemble into cross- $\beta$  aggregates and  $\beta$ -barrel intermediates. Hydroxylated C<sub>60</sub> (C<sub>60</sub>(OH)<sub>4n</sub> with n = 1~5) significantly inhibit  $\beta$ -sheet formation and aggregation of  $\alpha$ S<sub>68-78</sub> peptide, while C<sub>60</sub>(OH)<sub>40</sub> displays very weak inhibitory impact. These results suggest the necessity of the amphiphilic surface chemistry of hydroxylated fullerenes in hindering the amyloid aggregation of  $\alpha$ -Syn<sub>68-78</sub> peptide. The influence of GQDs on the aggregation of  $\alpha$ -synuclein ( $\alpha$ -Syn) protein was investigated by Kim et al using a combination of *in silico*, *in vitro* and *in vivo* methods.<sup>961</sup> It was shown that GQDs not only inhibit  $\alpha$ -Syn fibrillization and disaggregate fibrils, but also penetrate the BBB and possess unique neuroprotective effects against the neuropathological  $\alpha$ S aggregates/fibrils.<sup>961</sup> A recent study demonstrated the anti-aggregation effect of fullerlenols (C<sub>60</sub>(OH)<sub>30</sub> and C<sub>70</sub>(OH)<sub>30</sub>) on  $\alpha$ S protein and the neuroprotective activity in *Drosophila* PD models.<sup>962</sup> The observed neuroprotective effects of CNPs point to carbon-based nanomedicine as a new frontier against human amyloid diseases although challenges of CNPs in the application of biomedicine still exist, as discussed in a recent review.<sup>963</sup>

Taken together, all studies demonstrate that CNPs/small-molecules can effectively interfere with the fibrillization process and reduce the amyloid-induced toxicity, providing new clues for de-novo design of anti-amyloid inhibitors. The schematic diagram in Figure 32 illustrating the inhibitory mechanisms of CNPs/small-molecules against protein aggregation.



**Figure 30.** Chemical structures of a few selected small-molecules that are shown to inhibit amyloid aggregation, remodel aged fibers and/or promote A $\beta$  or IAPP fibrillation. The authors designed the figure.



**Figure 31.** Chemical structures and the binding poses of the seven natural ligands to (A) 4Aβ<sub>11-42</sub> peptides (PDB 2MX),<sup>911</sup> (B) αS (2X6M)<sup>912</sup>, (C) hIAPP (6Y1A)<sup>253</sup>, (D) insulin (1GUJ)<sup>913</sup> and (E) SOD1 (6FLH).<sup>914</sup> Binding poses were obtained by Autodock vina<sup>915</sup> with exhaustiveness of 8 and a grid of 60 × 60 × 60 Å covering the whole receptor.<sup>916</sup> Astaxanthin, brazilin, curcumin, dopamine, EGCG, resveratrol, and rosmarinic acid are highlighted in red, green, light blue, blue, yellow, magenta, and orange colors, respectively. The authors designed the figure.

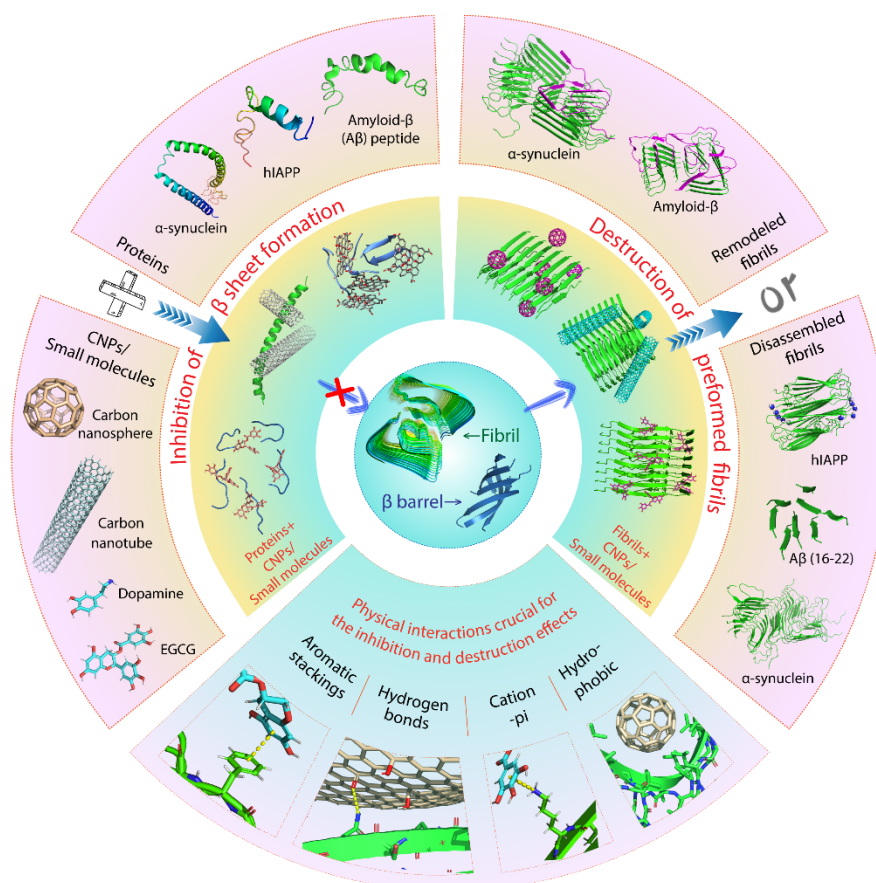


**Table 3.** List of seven inhibitors for AD, PD, T2D and ALS.

N <sup>o</sup>	Compound	Alzheimer	Parkinson	Type II Diabetes	ALS
1	Astaxanthin (Experiments /Computations)	Inhibits A $\beta$ (Cell viability assay, ThT, TEM, neurite outgrowth assay /Docking, MD, REMD, Linear Interaction Energy)	Inhibits $\alpha$ S (Cell viability assay, indirect immunofluorescence microscopy, mitochondrial membrane potential, immunohistochemistry, immunoblot analysis /N/A)	Increases insulin concentration (Immunodetection, 2-deoxyglucose uptake, ROS detection, /N/A)	Inhibits SOD1 (Immunocytochemistry, neurite outgrowth assay, photomicrographs /N/A)
2	Brazilin (Experiments /Computations)	Remodels A $\beta$ fibrils (Cell viability assay, CD, ThT assay, TEM/ Docking, MD)	Inhibits $\alpha$ S (AFM, cell viability assay, Fluorescein-Propidium iodide double staining assay, ThT assay /MD, binding energy)	Inhibits hIAPP (cell viability assay, CD, ThT assay, TEM, /MD)	N/A
3	Curcumin (Experiments /Computations)	Blocks A $\beta$ oligomers (cell viability assay, free radical assay, TEM, /Docking, MD, free energy perturbation)	Prevents formation of $\alpha$ S oligomers and fibrils (Aggregation assay, apoptosis study, cell uptake study, cell viability assay, <i>in vivo</i> bioavailability study, immunofluorescence, immunohistochemistry, western blot analysis, /N/A)	Inhibits hIAPP <sup>929</sup> (DLS/DMD)	Inhibits SOD1 (AFM, ATR-FTIR, DLS, ThT assay, TEM, MTT metabolic assay, Rayleigh scattering /Docking, Steered MD simulations)
4	Dopamine (Experiments /Computations)	Inhibits A $\beta$ fibrils (AFM, Light scattering, TEM, Size-exclusion HPLC, MS /MD, REMD)	Inhibits A $\beta$ and $\alpha$ S (AFM, Light scattering, TEM, Size-exclusion HPLC, MS, /MD)	Inhibits hIAPP protofibrils (ESI-IMS-MS, ThT assay, TEM, /MD, REMD)	Inhibits SOD1 (Aggregation assay, X-ray crystallography, small-angle X-ray scattering, /Docking, MD)
5	EGCG (Experiments /Computations)	Remodels A $\beta$ oligomers (Aggregation assay, cell viability assay, CD, dot blot assay, DLS, nitroblue tetrazolium staining, Size-exclusion chromatography, ThT, EM, /MD, REMD)	Inhibits $\alpha$ S (Aggregation assay, cell viability assay, CD spectroscopy, dot blot assay, DLS, nitroblue tetrazolium staining, EM, Size-exclusion chromatography, NMR, ThT /Docking, REMD)	Remodels hIAPP fibrils (ESI-IM-MS, Fibril depolymerization, ThT assay, TEM, /MD, REMD)	Inhibits SOD1 aggregation (ANS assay, collision induced unfolding, ESI-IM-MS, ThT assay, VC50 experiment, /Docking, Steered MD simulations)
6	Resveratrol (Experiments /Computations)	Reduces A $\beta$ aggregation (Aggregation assay, Bio-Rad protein assay, /DMD)	Inhibits $\alpha$ S (Cell viability assay, fibril formation assay, size-exclusion chromatography, CD, TEM /MD)	Inhibits hIAPP (DLS /DMD)	Inhibit SOD1 (Aggregation assay, ESI-MS, MS, ThT assay, /Docking, DMD simulations, quantum mechanics calculation)
7	Rosmarinic Acid (Experiments /Computations)	Blocks A $\beta$ oligomerization (AFM, cell viability assay, oligomer size distributions, electrophysiology, NMR, size-exclusion chromatography /Docking)	Inhibits $\alpha$ S oligomerization (AFM, CD, chemical cross-linking, size-exclusion, ThT, EM, chromatography, NMR, electrophysiology /MD)	Inhibits Insulin (CD, NMR, ThT assay, TEM, /Docking)	Extends survival of rats having SOD1 (G93A)

**Table 4.** Summary of atomistic simulation of the inhibition of carbon nanoparticles (CNPs) on the fibrillization of amyloid peptides, starting from oligomers or protofibrils. Abbreviations, GO for graphene oxide; CNT for carbon nanotube; and GQD for graphene quantum dot.

Ref	Peptide	Carbon nanoparticle	Force field	Solvent model	Method	T (K)	Time (ns)	Oligomer or Protofibril
938	A $\beta$ <sub>16-22</sub>	CNT	Gromos96 43a1	SPC	REMD	310~420	110 ns×40	8mer
939	A $\beta$ <sub>16-22</sub>	CNT-(OH) <sub>30</sub>	Gromos96 43a1	SPC	REMD	310~420	200 ns×40	8mer, fibrillar $\beta$ - sheet
940	A $\beta$ <sub>16-22</sub>	C <sub>60</sub> , C <sub>180</sub>	Gromos96 43a1	SPC	REMD	310~420	200 ns×40	8mer
942	A $\beta$ <sub>16-21</sub>	Graphene nanosheet	OPLS/AA	SPC	MD	310	1000 ns×5	Protofibril
945	A $\beta$ <sub>42</sub>	C <sub>60</sub>	Gromos96 53a6	SPC	REMD	306~418	340 ns×54	2mer
946	A $\beta$ <sub>42</sub>	GO <sub>60</sub>	Gromos96 53a6	SPC	REMD	306~418 308~419	410 ns×54 500 ns×54	2mer
950	A $\beta$ <sub>11-42</sub>	CNT-(OH) <sub>30</sub>	OPLS/AA	TIP4P	MD	310	100 ns×3	Protofibril
953	A $\beta$ <sub>33-42</sub>	GO <sub>60</sub> , GO <sub>120</sub>	Amber99sb- ILDN	TIP3P	REMD	307~437	400 ns×54	4mer
954	hIAPP	GO nanosheet	Medusa	Implicit solvent EEF1	DMD	290	50 ns×10	2mer, 4mer, 6mer
955	hIAPP	GQD	Medusa	Implicit solvent EEF1	DMD	300	400 ns×2	2mer
957	hIAPP	CNT-(OH) <sub>30</sub>	OPLS/AA	TIP4P	REMD MD	306~409 305~445 310	400 ns×48 400 ns×48 200 ns×3	2mer, Protofibril
958,959	hIAPP	C <sub>60</sub> , C <sub>60</sub> (OH) <sub>8</sub> C <sub>60</sub> (OH) <sub>24</sub>	OPLS/AA	TIP4P	REMD MD	306~409 310	360 ns×48 300 ns×6	2mer, Protofibril
960	$\alpha$ S <sub>68-78</sub>	C <sub>60</sub> (OH) <sub>4n</sub> (n=0~5, 10)	Medusa	Implicit solvent EEF1	DMD	300	200 ns×20	10mer



**Figure 32.** Schematic representation of the molecular mechanisms by which carbon nanoparticles (CNPs) and small-molecules inhibit the aggregation of proteins/peptides discussed in this review. Amyloid proteins can self-assemble into toxic  $\beta$ -sheet-rich oligomers (such as  $\beta$ -barrels) and protofibrils/fibrils. CNPs/small-molecules can prevent  $\beta$ -sheet formation and disassemble preformed protofibrils/fibrils through physical interactions, and finally lead to the inhibition of protein amyloid formation. CNPs include graphene, fullerene, carbon nanotube (CNT), and their derivatives (such as hydroxylated CNT/fullerene, graphene oxide, and graphene quantum dot). Many small molecule inhibitors have been reported in the literature, such as dopamine, EGCG, curcumin and resveratrol. Due to space limitations, only fullerene, carbon nanotube, dopamine and EGCG are illustrated in the diagram. The authors designed the figure.

## 9.2 Active and passive immunizations as Alzheimer's and Parkinson's therapies

**Active immunisation as AD therapy.** Active immunisation is where the exposure of the body to an antigen generates an adaptive immune response. While the response can take days or weeks to fully develop, immunisation is long-lasting, and potentially even lifelong. Vaccines that work in this way are a triumph of medicine, providing protection against diphtheria, tetanus, whooping cough, smallpox, polio, measles, mumps, rubella and numerous other major diseases. Vaccine antigens are either live attenuated, killed inactivated, toxin mimics, or virus subunits. Subunits are made either using recombinant DNA technology or normal bacteriological growth processes. Vaccines also contain

excipients, present to stabilise the vaccine, help with delivery to the right part of the body or improve the immune response (adjuvants)<sup>964,965</sup>.

The success of vaccines in general suggested the tantalising prospect of developing a vaccine for AD. The pioneering study in this field was AN-1792: synthetic A $\beta$ (1-42) coupled with a glycosidic adjuvant called QS-21<sup>966</sup>. While it might seem paradoxical to use A $\beta$  therapeutically if excess A $\beta$  leads to AD, the idea was that A $\beta$  might beneficially stimulate antibody production, rather than neurodegeneration, if given early. In a mouse model for AD that overexpressed mutant human APP, administration of AN-1792 to young mice prevented the later development of A $\beta$  plaques, neuritic dystrophy and astrogliosis. Even older animals benefitted from treatment, showing reduced AD pathology<sup>966</sup>. These encouraging results led to a Phase 2a trial in 273 patients with mild to moderate AD. The trial had to be halted after 6% of patients developed brain inflammation. Follow up studies of recipients showed mixed results: Post-mortems showed that while plaque could be cleared, neurofibrillary tangles were not<sup>967</sup>. A minority of patients developed antibodies against the N-terminal region of A $\beta$ .<sup>968</sup> Some patients showed functional benefit several years later<sup>969</sup>. Phospho-tau was reduced<sup>970</sup>.

The AN-1792 results prompted various studies aimed at developing an AD vaccine. Most designs use a fragment of A $\beta$  or repeats of a fragment, enough to stimulate an immune response while avoiding any potential problems from using the full-length, aggregation-prone sequence. The N-terminal region of A $\beta$  is most often used, as most antibodies target this region of the peptide. Various other excipients are included to help with delivery and stability, or to help stimulate an immune response. Most active AD vaccine candidates have not progressed beyond Phase 1 trials or have been discontinued, presumably due to lack of efficacy. A few trials in AD patients are ongoing, however:

AB-vac40 consists of multiple repeats of a short C-terminal fragment of A $\beta$ 40 conjugated to the keyhole limpet cyanine carrier protein and formulated with the adjuvant alum hydroxide<sup>971</sup>. Using the C-terminal end of A $\beta$  is designed to minimise binding to the parent APP when APP is within a membrane. It will also be unaffected by N-terminal reactions of A $\beta$ . Coupling to the keyhole limpet protein boosts the immune response. A pilot study in 12 participants found that 11 successfully developed antibodies after three injections of ABvac40. There were no safety issues. A Phase 2 trial of AB-vac40 is currently underway, with 120 participants at the earliest stage of AD or MCI, monitored over 2 years using amyloid PET (position emission tomography) scans, biomarkers, and cognitive and quality-of-life measures.

UB-311 is two A $\beta$ (1-14) peptides linked to T-cell peptide epitopes, formulated in a proprietary delivery system, biased to Th2 cells<sup>972</sup>. It is safe and well-tolerated, and successfully generates an immune response in almost all patients. After encouraging pilot data, Phase 2 study continues.

Naturally, there is considerable interest in targeting tau as well as A $\beta$  in AD<sup>973</sup>. Since tau aggregates are intracellular, it seemed doubtful whether antibody administration could be beneficial. However, promising results in tau transgenic mice<sup>974,975</sup> gave some encouragement. The AADvac1 vaccine is amino acids 294 to 305 of tau (KDNIKHVPGGGS), coupled to keyhole limpet hemocyanin with the aluminium hydroxide adjuvant. In transgenic rats it reduced tau pathology and improved function; it reduced AD-type hyperphosphorylation of tau by approximately 95%.<sup>976</sup> In patients, 98% generated anti-tau antibodies and showed reduced hyperphosphorylation<sup>977</sup>. Patterns of tau phosphorylation are distinct markers of disease progression over decades<sup>523</sup>.

Tau-targeting antibodies may enter neurons using Fc $\gamma$  receptors or endocytosis, after which they can promote intracellular clearance of pathological tau<sup>978</sup>. Alternatively, clearing extracellular tau may be all that is needed, as this could stop transmission between cells<sup>979</sup>. Intracellular tau oligomers are known to be secreted and taken up by nearby cells<sup>980</sup>.

GV001 is a 16 amino acid peptide with a sequence from the human enzyme telomerase reverse transcriptase, an enzyme commonly upregulated in cancer. It has anti-inflammatory and anti-oxidative stress properties. It was first developed as an unsuccessful immunotherapy for pancreatic cancer. However, in rat neuronal stem cells, GV1001 reduced A $\beta$  oligomer-induced toxicity, and protected against oxidative stress<sup>981,982</sup>, so it is being repurposed as an AD vaccine. A small phase 2 study showed promising effects at halting cognitive decline<sup>983</sup>.

**Passive immunisation against AD.** The elderly people typically show a weak response to antigens, making active immunisation a challenge. Alternatively, passive immunisation involves creating antibodies to AD targets outside the body and using them as a therapy. Antibodies can be generated in an animal either against antigens of A $\beta$  or tau fragments, or against aggregates to achieve specificity against pathogenic forms of A $\beta$ . The monoclonal antibodies are collected and given to a patient with AD. Their own immune system then clears the antigens bound to the antibodies<sup>984</sup>. A big problem for this approach is that few immunoglobulins dosed into blood will enter the brain. Crossing the BBB might not be necessary, however. The peripheral sink hypothesis holds that A $\beta$  can slowly leave the brain, down a concentration gradient, if it is mopped up in the blood<sup>985</sup>. Alternatively, passive

immunisation may work by antibodies crossing the BBB, activating microglia and triggering phagocytosis of antibody-bound antigens, perhaps using strategies to facilitate this process<sup>984</sup>.

A second concern with passive immunisation is initiating unwanted inflammation in the brain, already recognised as a pathological feature of AD. Using IgG1 and IgG4 immunoglobulins and avoiding proinflammatory subclasses such as IgG3 can reduce this risk<sup>986</sup>. Examples of promising antibodies currently in clinical trials for AD include the following:

Aducanumab shows a strong preference for binding to aggregated forms of A $\beta$  over monomers, a highly desirable property given that oligomers are likely to be the most toxic form of the peptide. It has more than 10,000-fold selectivity for aggregates over monomers<sup>987,988</sup>, binding to A $\beta$  residues 3-7 in an extended conformation<sup>989</sup>. In a mouse model, the antibody reduced A $\beta$  deposits by 70%, mostly likely by microglia-mediated phagocytosis<sup>987</sup>. A phase 1b trial of 165 prodromal or mild AD patients showed that aducanumab could clear A $\beta$  plaques with no toxicity, and there were hints of cognitive benefits and decreased inflammation<sup>987</sup>. Large phase 2 trials, called ENGAGE and EMERGE were therefore initiated.

In March 2019, both trials were stopped early, due to an apparent inability to slow cognitive decline, the primary endpoint of the trial<sup>990,991</sup>. Most unusually, however, in October 2019, Biogen announced that this conclusion was premature: later analysis of a larger EMERGE dataset showed that patients on the highest dose of aducanumab (10 mg/kg), given to ApoE4 carriers, had a significant reduction in cognitive decline; the low dose group also showed benefit, but this was not statistically significant. Similarly, the ENGAGE trial showed that a subgroup of people receiving the higher dose declined more slowly, though without meeting its primary endpoint. In addition, patients showed dose-dependent reduction in brain amyloid and phosphor-tau in CSF<sup>992</sup>. Biogen claims that this is the first time that a phase 3 study has shown that clearing A $\beta$  deposits can reduce cognitive decline in AD. As such, this would be a great boost to the amyloid hypothesis of AD, the immunotherapy approach, and AD drug discovery in general. Caution is needed however: the data is not yet published in detail in a peer-reviewed journal. Differences between the ENGAGE and EMERGE outcomes also need further investigation. In addition, positive claims based on retrospective analyses of subsets of participants are notoriously unreliable. Release of complete data and analyses is needed<sup>993</sup>.

Similarly to aducanumab, the IgG1 antibody BAN2401 shows a strong preference for binding to soluble protofibrils of A $\beta$ , rather than the monomeric peptide, binding to the N-terminal region. In transgenic mice, a murine version of the antibody reduced A $\beta$  protofibrils, while leaving monomers and insoluble plaques untouched<sup>994</sup>. In phase 1 trials, the humanised antibody was non-toxic<sup>995</sup>. An

18-month phase 2 study of 856 early AD patients showed statistically significant and dose-dependent slowing of amyloid deposition in the brain and cognitive function. Larger trials are underway<sup>996</sup>.

Solanezumab is a humanised monoclonal IgG1 antibody that binds the central region of monomeric A $\beta$ . The IgG1 antibody Gantenerumab binds to N-terminal and central amino acids of A $\beta$ , preferentially interacting with A $\beta$  aggregates<sup>997</sup>. Both reached phase 3 trials for mild AD, though were unsuccessful<sup>998,999</sup>. Nevertheless, as both have good safety records and some promising activity, they are now being tested in asymptomatic carriers of dominant mutations in APP, PSEN1 and PSEN2. If their phase 3 failures are because they are given too late in the disease progression, they may still work if given at an earlier stage to people sure to be on the path to AD. This long-term trial will first track AD biomarkers before adding the earliest cognitive effects<sup>1000</sup>.

A common form of A $\beta$  in plaques is A $\beta$ (p3-42), which has pyroglutamate at its N-terminus<sup>1001</sup>. Donanemab is a humanised IgG1 monoclonal antibody that binds to this plaque-specific form. It is effective at clearing plaques from transgenic mice<sup>1002</sup> and in patients with AD and a positive amyloid-PET scan<sup>1003</sup>. A phase 2 trial is underway.

Work on antibodies that target tau antigens<sup>979</sup> is less advanced than those that target A $\beta$ , though phase 2 trials are in progress for a few. Gosuranemab is a humanized IgG4 monoclonal antibody that binds to extracellular N-terminal fragments of tau. These secreted forms of tau may cause neuronal hyperactivity, which leads to increased A $\beta$  production<sup>1004</sup>, one of many positive feedback loops in AD<sup>1005</sup>. The antibody was tested in patients with progressive supranuclear palsy, and showed >90% reduction in secreted tau fragments, while CSF total tau and phosphorylated tau were unchanged<sup>1006</sup>. Semorinemab is an anti-tau IgG4 antibody that targets extracellular human tau, binding to the N-terminal region in both monomeric and oligomeric conformations, for all six tau isoforms and independent of phosphorylation<sup>1007</sup>. Zagotenemab is a humanised antibody that is selective for soluble tau aggregates, binding to the N-terminal region of tau<sup>1008</sup>. Phase 2 trials for participants with MCI or mild AD are ongoing for all three of these antibodies.

**Antibody therapies for synucleinopathies.** To date, work on antibodies that targets  $\alpha$ S is still little explored. PRX002 is a humanized IgG1 monoclonal antibody that binds to epitopes near the C-terminus of  $\alpha$ S. In mouse models of PD and DLB, it reduces  $\alpha$ S accumulation and counteracts behavioural deterioration<sup>1009</sup>. In humans, PRX002 had a good safety profile. It nearly entirely removed free  $\alpha$ -synuclein in the blood, though did not reduce  $\alpha$ S levels in CSF<sup>1010</sup>. BIIB054 is a human IgG1 monoclonal antibody directed at an epitope near the N-terminus of  $\alpha$ S, selective for aggregated forms<sup>1011</sup>. In a pilot study in PD patients published in 2019, BIIB054 formed plasma complexes with

$\alpha$ S. Phase 2 trials for both antibodies are underway. Recently, antibodies targeting the N-terminal domain of  $\alpha$ S to neutralize the toxicity of oligomers has been attempted under *in vitro* and *in vivo* conditions. A primary antibody designed from the highly lipophilic region present in the N-terminus of  $\alpha$ S (residues 1-25) showed promising activity in rescuing the neuronal cell damage induced by  $\alpha$ S oligomers.<sup>1012</sup>

### 9.3. Infrared laser, ultrasound, electromagnetic and electric fields

**Infrared laser.** The low-level near-infrared laser irradiation with wavelength 600-1000 nm is widely used because it is able to deliver low energy, non-heating infrared light into tissues and nerves deeply<sup>1013</sup>. So far, the first clinical trial with near-infrared at 810 nm wavelength pulsed at 10 Hz and power of 14.2 mW/cm<sup>2</sup> was performed on five patients with mild-moderate AD for 12 weeks of active treatment, and no treatment in the four follow-up weeks. Significant improvements were obtained in cognition after 12 weeks of active treatment. Also, increased function, better sleep, fewer angry outbursts, less anxiety, and wandering were also reported. There were no side effects such as diarrhea, nausea, vomiting, anorexia, or dizziness<sup>1014</sup>.

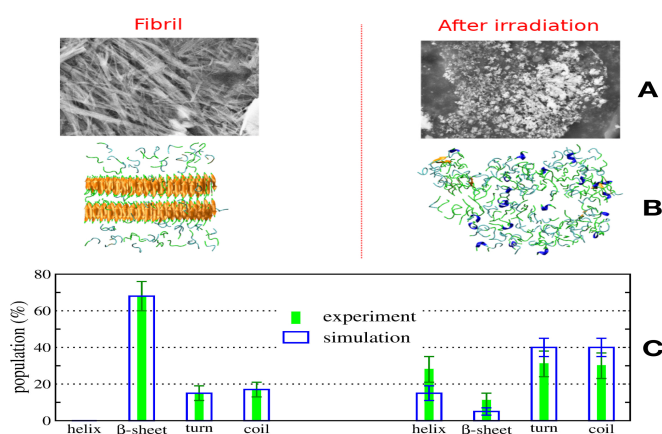
By *in vivo* studies, De Taboada et al. performed a near-infrared (808 nm) laser experiment to determine the effect of laser irradiation in an A $\beta$  protein precursor transgenic mouse model. After six months, the numbers of A $\beta$  plaques were significantly reduced in the brain as indicated by reduction in the plasma A $\beta$  peptide levels of 17.7 - 39.8%, depending on the laser parameters<sup>1015</sup>. Grillo et al. used near-infrared laser at 1072 nm wavelength to treat female TASTPM mice - an AD mouse model, and after 7 months, significant reduction in A $\beta$ 42 plaques (approximate 15%) was observed in the cerebral cortex<sup>1016</sup>. Near-infrared laser was also applied to APP/PS1 transgenic mouse brain as well. Results show that A $\beta$  plaques were reduced by more than 30%, neurofibrillary tangles and the hyperphosphorylation of tau were attenuated in the neocortex, hippocampus and cerebellum as compared to that of non-treated mice<sup>1017,1018</sup>. The authors suggested that the reduction in amyloid plaques is likely due to the enhanced mitochondrial function. It is unclear whether the reduction is also due to the direct interaction between the laser and amyloid fibrils.

By *in vitro* studies, several approaches using infrared laser have been developed to dissociate directly A $\beta$  fibrils and oligomers. Li et al. used graphene oxide covalently connected to thioflavin-S, which can bind to A $\beta$  aggregates<sup>1019</sup>. The graphene oxide plays a role as a local heater, which absorbs strongly the near infrared laser energy, to dissociate A $\beta$  fibrils. Recently, Kawasaki and colleagues have demonstrated that the mid-infrared laser can be used to disassembly amyloid fibrils<sup>1020-1023</sup>. They have developed a free-electron laser having specific oscillation characteristics of a picosecond pulse



structure, a tunable wavelength within infrared frequencies and a high photon density. Tuning the laser frequency to that of the amide I bands, they were able to dissociate various amyloid fibrils, including amyloid fibrils of lysozyme and of short peptide of the thyroid hormone into soluble monomers. Because the amide I frequency of amyloid fibrils are shifted compared to single proteins, the laser-irradiation targeted to the amide I bands of fibrils should minimise the damage to surrounding molecules.<sup>1024</sup> In addition to near- and mid-infrared techniques, Kawasaki et al. also employed the far-infrared laser to dissociate a fibril formed by calcitonin hormone peptide<sup>1025</sup>. The far-infrared laser exhibits high penetration against the biological substance even under the low radiation energy. The results showed that the far-infrared irradiation changes the fibril structure more remarkably than the mid-infrared laser, suggesting that deep penetration of far-infrared laser could disrupt the hydrogen-bond network inside of fibril more effectively.<sup>1025</sup>

At the computational level, we developed a laser-induced nonequilibrium MD simulation method, and applied it to study the dissociation process of various fibrils, made of 5-mer U-shape A $\beta$ 17-42, 5-mer  $\beta$ -solenoid HET-s, and 200-mer GNNQQNY.<sup>1026,1027</sup> Samples containing amyloid fibrils, DNA duplexes and globular proteins are also considered to and the simulations show that the surrounding protein and DNA molecules are not affected, demonstrating therefore laser frequency selectivity for dissociation<sup>1026</sup>. Simulations showed that the primary step in the dissociation process is due to the strong resonance between the fibril amide I vibrations and the tuned laser frequency, and not just the deposited infrared thermal energy.



**Figure 33.** Images of the GNNQQNY fibril sample prior and after laser irradiation of the GNNQQNY obtained from scanning EM experiment (A) and simulation (B). Their secondary structures in (C). The authors designed the figure from <sup>1027</sup>.

Figure 33 shows the results obtained from a joint experimental/simulation study of the dissociation of the GNNQQNY amyloid fibril.<sup>1027</sup> We first constructed the fibril sample so that its 2D and 3D structures are similar to those of experiment before FEL irradiation. By tuning the laser frequency to

the amide I band of the fibril, the resonance takes place and dissociation occurs. The calculated and observed wide-angle X-ray scattering profiles and secondary structures before and after laser irradiation being identical, we proposed a dissociation mechanism with high confidence from our simulations. We find that dissociation starts in the core of the fibrils by fragmenting the intermolecular H-bonds and separating the peptides, and then propagates to the fibril extremities leading to the formation of unstructured expanded oligomers. This should be a generic mechanism of the laser-induced dissociation of amyloid fibrils.<sup>1027</sup>

We find that the effects of the laser irradiation are determined by a balance between fibril formation and dissociation. Understanding the factors that are responsible for this balance are important. The method was also employed to study the relative stability of polyQ and PolyN fibrils. The results show polyQ is more stable than polyN because the enthalpic contributions to the free energy favor polyQ over PolyN<sup>1028</sup>.

**Ultrasound.** Ultrasound waves are mechanical waves which create oscillations with frequency lies between 20 kHz - 10 THz in the media through which they pass, generating thermal and non-thermal effects which are the basis of various therapeutic applications. The thermal effect arises from the absorption of ultrasound energy that creates heat in the system if the rate of heat production is greater than the rate of heat removal<sup>1029</sup>. The non-thermal effects include acoustic radiation force, acoustic streaming, shock waves and bubble cavitation<sup>1030</sup>. Ultrasound waves are already used to treat certain types of cancer and glaucoma.

By *in vivo* studies, most works use the focused ultrasound to open temporarily the blood-brain-barrier, thus facilitates the transport of molecules from the blood to the brain<sup>1031</sup>. However, several studies using ultrasound alone also show positive results. Jordao et al. demonstrate that transcranial focused ultrasound (frequency of 0.5 MHz, pressure of 0.3 MPa) application leads to a significant reduction (20%) in mean plaque size four days after a single treatment in the TgCRND8 mouse model of AD. This is presumably due to the treatment facilitating endogenous antibodies to reach the brain and/or activation of microglial cells<sup>1032</sup>. Leinenga et al. utilised the repeated scanning ultrasound (0.7-MPa peak rarefactional pressure, 10-Hz pulse repetition frequency, 10% duty cycle, and 6-s sonication time per spot) in combination with microbubble injection to treat APP23 mice for several weeks.<sup>1033</sup> The histological and biochemistry analysis of brain tissues after treatment reveal a reduction of about 50 % in the dense core amyloid plaque and soluble species of A $\beta$  peptides, and no evidence of neuronal death, edema, erythrocyte extravasation or ischemic changes. The memory deficit is successfully recovered. The authors postulate that the ultrasound somehow triggers microglial activation and

enhance A $\beta$  phagocytosis<sup>1033</sup>. However, it could also be possible that ultrasound increases the activation of A $\beta$ -degrading enzymes, such as neprilysin, insulin degrading enzyme, and plasmin. It is unclear whether the ultrasound has any direct impact on the amyloid plaques. With a study on dogs with AD, O'Reilly et al. showed, however, that there is no significant reduction in A $\beta$  load<sup>1034</sup>. In the first clinical trial, Beisteiner et al. developed a technique using single ultrashort (3  $\mu$ s) ultrasound pulses with typical energy levels of 0.2–0.3 mJ mm<sup>-2</sup> and pulse frequencies of 1–5 Hz to stimulate the human brain. The treatment of 35 patients with AD shows neuropsychological scores are improved significantly, last up to three months, and no major side effects<sup>1035</sup>.

By *in vitro* studies, several experiments have been carried out to study the direct interaction of ultrasound with amyloid fibrils. Sato et al. showed that ultrasound with frequency of 1 MHz and power of 2.5 W/cm<sup>2</sup> can dissociate soluble A $\beta$  peptides from fibrils.<sup>1036</sup> The A $\beta$ 42 is more resistant to dissociation from fibrils to monomers and/or low molecular weight soluble oligomers than A $\beta$ 40. This is consistent with the fact that A $\beta$ 42 peptides aggregate faster than A $\beta$ 40 counterparts. Because of low ultrasound power, the thermal effect is excluded in the interpretation of results. The authors suggested that the microstreaming generated by bubble cavitation may change the structure of fibrils<sup>1036</sup>. Goto et al. studied the effects of low frequency ultrasound (7–20 kHz) irradiation on both aggregation and dissociation of A $\beta$ 40 peptide. They determined a critical peptide concentration of 0.7–0.9  $\mu$ M, below which the ultrasound accelerates the dissociation of fibrils into monomers<sup>1037</sup>. Above this critical concentration, ultrasound can promote nucleation of fibrillation by lowering the energy barrier agitating effects. However, the newly formed fibrils can also be broken. These two processes lead to the production of minimal and monodispersed fibrils<sup>1038,1039</sup>. The small homogeneous fibrils will be useful for characterizing the structure and dynamics of amyloid fibrils. Again, the shearing forces of liquid flows induced by ultrasound are believed the causes of fibril formation and dissociation. Similar to the ultrasound, Foguel et al. used a cyclic high-pressure technique to dissociate the fibrils of  $\alpha$ S and transthyretin.<sup>1040</sup> Here, the fibrils are compressed at a pressure of 3000 bar for 30 min at 37°C at pH 5, and then released. When the changes in the light scattering leveled off, pressure is applied again to evaluate its effects on the fibril structure. The authors observe that fibrils undergo rapid disassembly upon compression and aggregation after decompression. The mechanism could be due to the pressure induced disruption of hydrophobic interactions and eliminate water-excluded cavities<sup>1040</sup>.

A few nonequilibrium MD simulations have been carried out to understand the molecular mechanism of the ultrasound induced dissociation of amyloid fibrils. Okumura et al. carry out simulations of the A $\beta$ 17-42 fibril of various sizes under high ultrasound pressure of 200 MPa and very fast period of 1 ns.<sup>1041</sup> The simulation shows that the inertial cavitation of bubbles, which are formed during the ultrasound rarefaction phase, is responsible for the fibril disruption. This mechanism could

support the in-vitro results reviewed above. However, in many *in vivo* applications, the stable cavitation of bubbles is more preferable, because the inertial cavitation could damage biomolecules. To simulate such bubble stable oscillation, we developed a new nonequilibrium MD method<sup>1042,1043</sup>, where a bubble is represented by a particle with low mass, no charge and interacts with surrounding waters by a time-dependent Lennard-Jones potential. The time-dependent potential changes harmonically during the simulation, pushing and pulling waters back and forth, respectively, mimicking the bubble stable cavitation. The method was applied to study the effect of the stable cavitation on the pentamer A $\beta$ 17-42 amyloid fibril. The simulation shows that after 5 ns of excitation by 100 stable bubble cavitation periods, the  $\beta$ -structure in the initial fibril structure is reduced to 12%, and significant amounts of turn (30%) and coil (57%), with  $\leq 1\%$  of  $\alpha$ -helices are formed. The simulation shows that the harmonic fluctuation in the water pressure induced by the stable bubble cavitation is the origin of the fibril dissociation.<sup>1042,1043</sup> The method has also been used to study the stability of fibrils having U-shaped and S-shaped motifs exposed to ultrasound stable cavitation. It is shown that there is a markedly difference in the kinetics of destabilisation of fibrils having different shapes.<sup>1044</sup>

**Electromagnetic and electric fields.** The transcranial electromagnetic treatment (TEMT) has emerged as a promising and safe approach to treat AD<sup>1045</sup>. This method uses electromagnetic field to provide stimulatory/inhibitory effects on neuronal activity. With the frequencies in the radio frequency range (around 1GHz), TEMT easily penetrates deep human brain areas and all neurons to impact intraneuronal pathologic processes, such as A $\beta$  and tau. The method has been used in a number of pre-clinical studies involving AD mice, and the prevention as well as reversal of cognitive impairment at multiple ages of mice were obtained. It has been shown that TEMT can prevent/reverse A $\beta$  oligomers/fibrils both inside and outside neurons, disaggregate tau oligomers, enhance mitochondrial function and increase neuronal activity<sup>1046-1048</sup>. So far, a first clinical trial of TEMT has been carried out on eight patients with mild-moderate AD, and the results show that TEMT enhances cognitive performance of AD patients without behavioral/physiological side effects or brain abnormalities<sup>1049</sup>.

By *in vitro* study, Saikia et al. investigated the influence of the external electric field and magnetic field of varying strengths on the fibrillogenesis of the A $\beta$ 16–22 and the A $\beta$ 42 peptides.<sup>1050</sup> Using electric field strengths of 150-300 V/cm, A $\beta$ -elicited toxicity of EF-treated samples in two neuroblastoma cell lines and human embryonic kidney cell line were found to be 15–38% less toxic than the electric field untreated ones under identical conditions. However, the magnetic field around

0.8 T has little ability to induce a conformational switch. A magnetic-field-based therapy may be difficult to implement and, hence, has minimal therapeutic value <sup>1050</sup>.

Nonequilibrium all-atom MD simulations have been carried out. The simulation by English et al. showed that under external static electric field the total dipole moment of the hen egg white lysozyme aligns with the field, and this induces changes in the protein secondary structure relative to the zero-field state<sup>1051</sup>. Zerbetto et al. simulated the interaction of A $\beta$  peptide with electric field of varying strengths and showed that the electric field can switch the A $\beta$  peptide from helical to  $\beta$ -sheet conformation.<sup>1052</sup> For the amyloidogenic apoC-II(60-70) peptide, the simulation shows that high strength electromagnetic field can align the peptide dipole, resulting in the disruption of the inherent  $\beta$ -hairpin conformation known to be the intermediate state for fibril formation. Weaker field-strength can accelerate dynamics which leads to the increased population of structures with fibril-inhibiting characteristics<sup>1053,1054</sup>.

## 10. Crosstalk and cross-seeding between amyloid proteins

### 10.1. What do we know from *in vitro* and *in vivo* conditions?

Despite intrinsic differences amongst the protein misfolding disordered diseases, notably the underlying neural circuit affected by pathology, they share a common molecular pathological mechanism: the misfolding, aggregation, tissue accumulation of a protein whose protein sequences differ greatly, and cell-to-cell propagation <sup>193,1055-1061</sup>. In principle, these shared mechanistic and pathological characteristics also suggest that protein misfolding processes occurring simultaneously might synergistically interact among each other thereby accelerating disease pathogenesis. Recent studies are indeed providing new evidence for prevalent mixed proteinopathies across neurodegenerative diseases, with aging and APOE  $\epsilon$ 4 status constituting risk factors<sup>1062</sup>. These observations are reminiscent of prior studies documenting that mixed neuropathologies are the most common cause of the clinical syndrome of dementia and are also common among persons with mild cognitive impairment or cognitive decline<sup>1063-1066</sup>. Furthermore, these findings support earlier observations indicating that a more rapid rate of cognitive decline is observed in 30-40% of AD cases presenting with  $\alpha$ S inclusions known as Lewy bodies (LB) and Lewy neurites (LN) compared to subjects with AD without  $\alpha$ S pathology<sup>51,1067</sup>. The co-occurrence of these proteinopathies thus provides support to accumulating observations documenting potential molecular cross-talks amongst amyloid proteins (Figure 34).

Here, we define molecular cross-talk by molecular interactions and cross-seeding between aggregates of amyloid proteins with functional consequences for disease pathogenesis or disease

progression (Figure 35). Cross-seeding can occur within the same cell or through cell-to-cell transmission of pathological amyloid aggregates, which is intrinsically part of spreading. Many excellent reviews on this subject are available. <sup>193,1055-1059,1061,1068</sup>

**A $\beta$  and  $\alpha$ S.** Cross seeding between aggregated A $\beta$  and  $\alpha$ S has been observed *in vitro* <sup>1069</sup>, along with hybrid oligomer formation<sup>1070</sup>. Many studies in fact have suggested that A $\beta$  and  $\alpha$ S are capable of direct and indirect cross-talks in the brain, along with hybrid oligomer formation<sup>1069-1075</sup>. Similar findings were shown in a recent study, where A $\beta$ 42 and pyroglutamate A $\beta$ 3-42 (pGlu-A $\beta$ 3-42) peptides accelerated  $\alpha$ S aggregation *in vitro*. Colocalization of the two A $\beta$  species with  $\alpha$ S was also shown in the APP-transgenic Tg2576 mouse brain sections, suggesting an *in vivo* occurrence of the A $\beta$  and  $\alpha$ S co-aggregates<sup>1076</sup>. To assess a potential crosstalk between fibrillar  $\alpha$ S and fibrillar A $\beta$ , Bassil and coworkers in 2020 injected mouse  $\alpha$ S preformed fibrils ( $\alpha$ S-PFFs) into young adult 5XFAD mice harboring amyloid plaques. The presence of A $\beta$  deposits enhanced  $\alpha$ S pathology and spreading throughout the brain. These findings have led the authors to suggest a “feed-forward” mechanism whereby A $\beta$  plaques potentiate  $\alpha$ S seeding and spreading over time<sup>1077</sup>.

**$\alpha$ Syn and tau.** Lately, the relationship between  $\alpha$ S and tau has received much attention, which has also been supported by GWAS studies showing strong association between the genes encoding  $\alpha$ S and tau proteins (*SNCA* and *MAPT*, respectively) in PD and dementia with Lewy bodies (DLB) pathologies<sup>1078,1079</sup>. The *in vivo* association of the two proteins were reported in several studies from post-mortem brain tissues showing their co-occurrence and even co-aggregation in PD, DLB, ALS/parkinsonism-dementia complex pathologies<sup>1080-1083</sup>. Robinson and coworkers recently characterized the presence of co-occurring pathologies of four major amyloid proteins, i.e. A $\beta$ , tau,  $\alpha$ S and TDP-43, in several neurodegenerative diseases<sup>1062</sup>. Nearly all synucleinopathies including DLB and multiple system atrophy defined by  $\alpha$ S inclusions invariably exhibited tau proteinopathy (92-100% of cases). By contrast, A $\beta$  deposits were found in 38-80% of cases while TDP-43 co-pathology was identified in 0-22% of cases. It is tempting to hypothesize that this quasi-universal co-occurrence of  $\alpha$ S and tau pathologies observed in synucleinopathies might result from a potential cross-seeding between these proteins<sup>1062</sup>.

Several groups have shown *in vitro* cross-seeding among  $\alpha$ S and tau, which occasionally was a synergistic effect on both the proteins forming aggregates<sup>1084-1089</sup>. *In vitro* aggregation studies indicate that monomeric  $\alpha$ S directly interacts with two synthetic forms of tau including the K19 construct formed from the aggregation-prone repeat domain of the microtubule-binding domain due to its ability to aggregate faster in absence of the flanking regions<sup>1090</sup>, thereby promoting tau fibrillization<sup>1089</sup>. This interaction is mediated by the C-terminus of  $\alpha$ S and is exacerbated by phosphorylation of  $\alpha$ S at serine 129, a characteristic PTM of  $\alpha$ S pathology<sup>1089</sup>. Additional work is needed to examine the possible

heterologous nature of the assemblies formed and to establish the relevance of this *in vitro* study using artificial recombinant proteins.

Abundant tau pathology was shown in transgenic mouse models of PD expressing either A53T mutant  $\alpha$ Syn (TgA53T, M83 line) or E46K mutant  $\alpha$ S (TgE46K, M47 line)<sup>1085,1091</sup>. It is interesting to note that while TgE46K mice displayed a greater number of tau inclusions compared to TgA53T mice, *in vitro* studies demonstrated that mutant  $\alpha$ S<sup>E46K</sup> is less efficient than wild-type  $\alpha$ S ( $\alpha$ S<sup>WT</sup>) at promoting tau inclusions in cultured QBI293 cells<sup>1091</sup>. The reason for the accumulation of hyperphosphorylated tau inclusions in TgE46K mice is unclear and could result from environment, host and cell type differences between the *in vitro* and *in vivo* paradigms used. Additionally, co-occurring aggregates of tau and  $\alpha$ S were not only reported in neurons from M83 TgA53T mice but also exacerbated in oligodendrocytes from a bigenic CNP-Tau<sup>P301L</sup>/ $\alpha$ S<sup>WT</sup> mouse model overexpressing P301L mutant tau and human  $\alpha$ S<sup>WT</sup> driven by the murine 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) promoter, in which inclusions were positive to Thioflavin S<sup>1085</sup>. Because these seminal studies focused on fibril formation as opposed to oligomerization per se, the functional role of  $\alpha$ S/tau cross-talks was further investigated where passive immunotherapy against tau oligomers prevented brain protein pathology and cognitive and motor deficits in M83 TgA53T mice<sup>1092</sup>. By contrast, work from Singh and colleagues suggest that the role of tau in young and middle-aged adult G2.3 TgA53T mice is independent from  $\alpha$ S/tau crosstalks because no differences in o- $\alpha$ Syn nor o-Tau were found in absence or presence of *MAPT* deletion<sup>1093</sup>. With aging, it is likely that this molecular crosstalk would strengthen and have an impact on the phenotype of this PD mouse model.

Several studies have also documented the existence of a functional coupling between  $\alpha$ S and tau. Most notably,  $\alpha$ S was shown to regulate a key kinase for tau phosphorylation, glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ), in several mouse models of PD<sup>1094-1098</sup>. Another comprehensive study from the Sidhu group has shown the colocalization of active GSK3 $\beta$  (p-GSK3 $\beta$ -Y216) with phosphorylated tau (p-tau) or  $\alpha$ S (p- $\alpha$ S) in the brain tissues of a transgenic mouse model expressing human GSK3 $\beta$  with a point mutation (S9A). Furthermore, the authors have shown that in addition to tau, GSK3 $\beta$  also phosphorylates  $\alpha$ S *in vitro* and that both tau and  $\alpha$ S cooperate with each other to increase the extent of their phosphorylation *in vitro*<sup>1099</sup>. Exogenously added  $\alpha$ S was shown to modulate the GSK3 $\beta$ -mediated hyperphosphorylation of tau causing microtubule destabilization in rat pheochromocytoma (PC12) cells<sup>1100</sup>. These observational studies suggest an indirect association between tau and  $\alpha$ S.

**A $\beta$  and IAPP.** Accumulating evidence of a potential link between T2D and AD pathologies has led to the investigation of the molecular interaction between A $\beta$  and IAPP. Studies have shown *in vitro* that IAPP promotes the aggregation of A $\beta$ 42<sup>1101</sup>. Additionally, cross-seeding between IAPP and A $\beta$ 42 resulted in hybrid aggregate formation, which could associate with artificial lipid membrane, thus

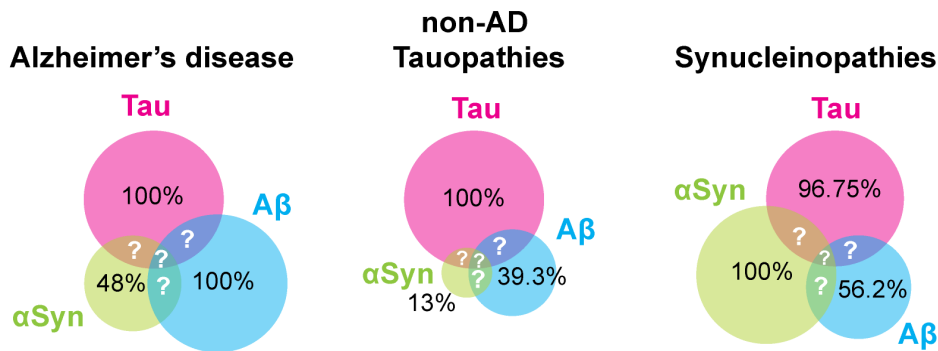
reducing its fluidity <sup>1102-1105</sup>. Earlier work had reported IAPP deposition in the brain tissues of AD patients without any diabetes history, indicating the presence of brain insulin resistance. Plaques of IAPP were found individually or embedded in the A $\beta$  plaques in these AD brain tissues to the extent that they were almost indistinguishable by the staining procedures specific for these amyloids. The findings from this study are supportive of a co-occurrence of these pathologies in AD lesions and suggestive of a potential *in vivo* cross-talk between A $\beta$  and IAPP<sup>1103</sup>. More recently, co-localization of IAPP and A $\beta$  was also confirmed by proximity ligation assay in both cerebral and vascular A $\beta$  deposits in AD brain tissues<sup>1107</sup>. Work from the Soto group reported that intracerebral injection of IAPP aggregates derived pancreatic homogenates into APP transgenic mice exacerbated A $\beta$  deposition compared to the non-transgenic animals injected with the same inoculum, suggesting an *in vivo* cross-seeding mechanism between the two proteins<sup>1108</sup>.

**$\alpha$ Syn and IAPP.** The *in vitro* cross-seeding of IAPP has been shown to promote  $\alpha$ S aggregation. The two proteins formed co-aggregates when incubated together in their monomeric forms, suggesting a possible reason for T2D to be a risk factor for PD<sup>1109</sup>.

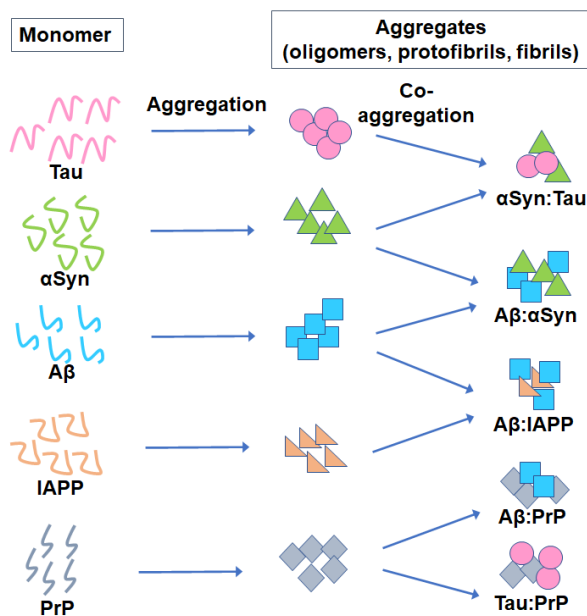
**PrP, A $\beta$  and tau.** Cellular prion protein (PrP<sup>C</sup>) was shown to be a receptor for A $\beta$  oligomers with high affinity, thus facilitating oligomer-induced synaptic dysfunction in mice<sup>1110,1111</sup>. Several groups have reported the coimmunoprecipitation of human PrP<sup>C</sup> with A $\beta$  from the brain tissues of AD patients<sup>1112,1113</sup>. In another study, intraperitoneal inoculation of misfolded PrP aggregates (PrP<sup>Sc</sup>) in the Tg2576 AD mouse model resulted in the accumulation of both A $\beta$  and pathogenic PrP<sup>Sc</sup> with concomitant histopathological features of prion disease<sup>1114</sup>. The same authors also showed that protein misfolding can be enhanced by a cross-seeding mechanism *in vitro*<sup>1114</sup>.

Overexpression of longest human tau isoform was shown to regulate cellular trafficking of PrP<sup>C</sup> and reduce its expression on the cell surface bound with concomitant accumulation of insoluble PrP<sup>C</sup> in primary cortical neurons<sup>1115</sup>. Additionally, abundant tau pathology was reported in inherited prion diseases<sup>1116,1117</sup>. Finally, oligomeric assemblies of A $\beta$ , PrP,  $\alpha$ S and TDP-43 proteins were shown to co-localize in AD pathology<sup>1118</sup>, indicating a possible cross-talk between these amyloid proteins.





**Figure 34.** Co-occurring pathologies across common neurodegenerative diseases. Schematic illustrating the relative proportions (numbers within colored disks) of amyloid pathologies formed of Aβ (blue), tau (pink) and αS (green) within each disease class. Question marks indicate unknown proportions for hybrid assemblies (i.e. Aβ/tau, Aβ/αS, αS/tau and Aβ/αS/tau). The numbers inserted within the colored disks are taken from Ref. <sup>1062</sup>. The authors design the Figure.



**Figure 35.** Cross-seeding and co-aggregation of amyloid proteins. Schematic illustrating the *in vitro* formation of amyloids (depicted by different shapes) including oligomers, protofibrils and fibrils from tau (pink), αS (green), Aβ (blue), IAPP (orange) and PrP (grey) proteins. This scheme also summarizes the *in vitro* formation of co-aggregates (αS:tau, Aβ:αS, Aβ:IAPP, Aβ:PrP, tau:PrP) by cross-seeding/co-aggregation mechanism demonstrated in various studies. Different shapes, colors and their numbers used here arbitrarily represent amyloid aggregates; they do not indicate aggregates of any specific size or shape. The authors design the Figure.

## 10.2 What have we learnt from simulations ?

**IAPP/Aβ.** The attempt to investigate the co-aggregation between amyloids via computational tools requires the structures of the amyloid aggregates that were solved by ssNMR or crystal structures. The cross-seeding between disordered monomers of Aβ (PDB ID: 1Z0Q) and IAPP (PDB 2L86) using REMD simulations and DMD simulations with CHARMM force field (FF) and TIP3P water has been

investigated. It was found that IAPP promotes A $\beta$  aggregation via interactions of IAPP with the residues 16-22 of A $\beta$ . In addition, two IAPP fibrils with U-shape structures were solved, by ssNMR<sup>216</sup> and by X-ray crystallography.<sup>1119</sup> Applying these IAPP fibril structures, further polymorphic IAPP fibrils were investigated via computational tools.<sup>1120</sup> The interactions of each polymorphic IAPP fibrils with A $\beta$  fibril were investigated using MD simulations with CHARMM FF and TIP3P water.<sup>1121</sup> Different interactions and orientations between polymorphic IAPP fibrils and A $\beta$  fibril were studied for single-layer and double-layer conformations.<sup>1105,1121</sup>

It has been suggested that in water solution the cross-seeding between these two amyloid fibrils is preferred to form polymorphic single-layer conformations (in-register interactions) rather than double-layer conformations. It is thus expected that when two amyloid fibrils will interact in-register to form a single layer conformation, a synergistic effect will be produced and a promotion of the co-aggregation will be presented. In some double-layer conformations, this phenomenon does not occur or less preferred to be occurred.<sup>1121</sup> The mechanisms that lead to co-aggregation between these two amyloids indicates a strong tendency to form single-layer conformations.

It has been shown experimentally, that the toxicity of A $\beta$  oligomers to neuronal cells has been demonstrated to occur via a two-step mechanism of membrane disruption.<sup>1122</sup> Yet, the cross-seeding between A $\beta$  and IAPP aggregates has not been investigated at the atomic resolution in membrane environment via experimental tools. The interactions between the U-shape structures of IAPP fibrils and A $\beta$  fibrils associated with different types of membranes were investigated using MD simulations with CHARMM FF and TIP3P water by Zheng's group.<sup>1105</sup> It has been shown that the cross-seeding fibrils more strongly interact with POPC/POPG bilayer than the POPC bilayer. It was therefore concluded that the electrostatic interactions between the cross-seeding fibrils and the membrane are the crucial interactions that stabilize peptide-lipid interactions. In such interactions, the N-termini of A $\beta$  fibrils associate with the membrane and stabilize the contacts between the cross-seeding fibrils and the membrane. Finally, it has been shown that the cross-seeding of IAPP-A $\beta$  induces the disruption of the cell membrane via altering calcium homeostasis and cell membrane phase.

**A $\beta$ /NAC( $\alpha$ S).** The study of the cross-seeding between A $\beta$  fibrils and  $\alpha$ S fibrils is more challenging, due to the relatively large number of amino acids in  $\alpha$ S. Short time scale of MD simulations with CHARMM FF and TIP3P water were performed to investigate the interactions between A $\beta$  monomer and two  $\alpha$ S monomers associated with a membrane.<sup>1070</sup> The interactions of these two amyloids in the membrane indicated a promotion of the formation of stable ring-like oligomers. These oligomers were found to be composed of both A $\beta$  and  $\alpha$ S that are docked in the membrane. To simplify the study of the interactions between A $\beta$  fibrils and  $\alpha$ S fibrils, the interactions between A $\beta$  fibrils and NAC fibrils

were investigated using MD simulations in water solution. While the structure of A $\beta$  fibrils were solved experimentally, the NAC fibrils were not solved. The NAC fibril S-shape structure was solved for the first time by molecular modeling tools.<sup>1123</sup> Then, the interactions between the NAC fibril and the U-shape A $\beta$  fibril structure were studied by MD simulations.<sup>1124</sup> Various associations between NAC fibril and A $\beta$  were investigated to form polymorphic single and double-layer conformations in the cross-seeding states.

The simulations demonstrated that the polymorphic A $\beta$  fibrils prefer to interact with NAC fibril to form double-layer than single-layer conformations. The hydrophobic and electrostatic interactions are the driving forces that stabilize the cross-seeding double-layer conformations. In the single layer conformations, the NAC fibril affects the structure of A $\beta$  fibrils. It is well known that A $\beta$  fibrils consist of two  $\beta$ -strands connected by one U-turn. Interestingly, the NAC fibrils in the cross-seeding aggregates induce the formation of a third  $\beta$ -strand and further U-turn in A $\beta$  fibril. The NAC fibril consists of three  $\beta$ -strands connected by two U-turn domains. Therefore, the cross-seeding between this NAC fibril and A $\beta$  fibrils initiates the formation of a similar fibrillary structure for A $\beta$  with three  $\beta$ -strands connected by two U-turn domains. A further effect of the NAC fibril on A $\beta$  fibrils is related to the stabilization of the cross- $\beta$  structure. While the inner core of NAC in the single-layer conformations of the cross-seeding aggregates does not change, the inner core of A $\beta$  fibrils is decreased, i.e. forming a more compact stable cross- $\beta$  structure due to the strong hydrophobic interactions in the inner core domain.<sup>1124</sup>

One can conclude that there is a lack of a synergistic effect in the cross-seeding NAC-A $\beta$  fibrils. While the NAC fibril strongly affects the polymorphic A $\beta$  fibrils, these polymorphic A $\beta$  fibrils do not affect the structure of NAC fibril. This event is probably due to the compact structure of polymorphic NAC that consists relatively large number of hydrophobic interactions in the inner core, compare to the inner core of the polymorphic A $\beta$  fibrils.

**NAC( $\alpha$ S)/IAPP.** The cross-seeding between U-shape structure of IAPP aggregates and S-shape structure of NAC aggregates were investigated by MD simulations using the CHARMM FF and TIP3P water in Miller's group.<sup>1125</sup> In the case the NAC fibril interacts with polymorphic A $\beta$  fibrils, the double-layer conformations of the cross-seeding states are more preferred. Here, in the case where NAC fibril interacts with polymorphic IAPP fibrils, the single-layer conformations of the cross-seeding states are more preferred. Extensive structural analyses have shown that the NAC fibril stabilizes the structural properties of the polymorphic IAPP fibrils. For instance, the percentage of the hydrogen bond interactions between the  $\beta$ -sheets in the fibrillary states of IAPP is increased, compare

to the separated IAPP fibrils. Moreover, the distance in the inner core of the cross- $\beta$  structure of IAPP fibrils is decreased, indicating on a compact cross- $\beta$  structure.

Finally, both for the case of the cross-seeding between NAC and A $\beta$  and the case of the cross-seeding between NAC and IAPP fibrils, the IAPP and A $\beta$  in the cross-seeding states do not affect the structural features of NAC. Therefore, there is a lack of synergistic between these amyloids. The ability of NAC fibril to enhance the stability of amyloids, such as IAPP and A $\beta$  is due to its hydrophobic properties of the sequence in NAC.

**Tau/tau isoforms.** The co-aggregation between K18 and K19 was investigated by MD simulations.<sup>552</sup> Simulations showed that co-aggregation of some of the K18 or K19 with the tau R3 repeat exhibited a stable L-shape fibril. The co-aggregation of other K18 or K19 with tau R3 repeat demonstrated straight-line shape fibrils. The aggregation of K18 is strongly initiated both by R2 and R3 repeats, while the aggregation of K19 is induced by only R3 repeat. Therefore, these tau repeats are critical for aggregation of K18 and K19. Interestingly, it was proposed that the polymorphic core units of K18 and K19 yield to a cross-seeding barrier for K18 to trigger K19 fibril growth, because R2 is not available in K19. The polymorphic nature in amyloids may impede or initiate fibril formation, and may affect the differences in barriers between K18 and K19.

**A $\beta$ -tau/mutated tau.** There is a synergy between the tau and A $\beta$  pathology in the mitochondria,<sup>1126-1130</sup> and it was suggested that A $\beta$  may accelerate tau neurofibrillary tangles.<sup>1131-1133</sup> Experimental studies proposed that co-aggregation of tau with A $\beta$  occurs via interactions between the  $\beta$  strands of A $\beta$  and  $\beta$ -stands of tau.<sup>1134-1138</sup> Yet, there is a challenge to investigate the interactions between the full-length A $\beta$  aggregates and the full-length tau aggregates or mutated tau aggregates at the atomic resolution both by experimental techniques and by computational modeling tools. The interactions between A $\beta$ 25-35 fragment and Tau273-284 fragment were investigated using REMD simulations and the OPLS-AA FF and TIP3P water.<sup>1139</sup> It was shown that these interactions initiate cross-seeding between these fragments.

The tau repeats R2, R3 and R4 are known to form  $\beta$ -strands. The fibrillary structures of these U-shape tau repeats fibrils were studied by MD with CHARMM FF and TIP3P water molecules.<sup>1140</sup> The interactions between each one of these tau repeats fibrils with the full-length A $\beta$  U-shape fibrils were studied also by simulations with CHARMM FF and TIP3P water.<sup>1140</sup> Simulations showed that there are less hydrogen bond interactions between a neighboring monomer of A $\beta$  fibril and monomer of tau repeat R3 or R4 fibrils. Furthermore, conformational energy analyses illustrated that the “reaction coordinate” of A $\beta$  fibrils with tau repeat R2 fibril presents exothermic reaction. The “reaction

coordinate” of A $\beta$  fibrils with tau repeats R3 or R4 fibrils demonstrated endothermic reaction. Therefore, it was proposed that while tau repeats R3 and R4 are less prefer to interact with A $\beta$ , and tau repeat R2 has strong tendency to co-aggregate with A $\beta$ . Furthermore, it was suggested that there is a synergistic effect between tau R2 repeat and A $\beta$ .

One of the main hallmarks of the fronto-temporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) is the accumulation of neurofibrillary tangles in the brain as an outcome of the aggregation of mutated tau protein. This process occurs due to a number of genetic mutations in the MAPT gene. One of these mutations is the  $\Delta$ K280 mutation in the tau R2 repeat domain, which promotes the aggregation vis-à-vis that for the wild-type tau. Experimental studies have shown that A $\beta$  forms aggregates both with itself and with wild-type tau. By analogy, in FTDP-17, it is likely that there are interactions between A $\beta$  and mutated tau. Yet, it is challenging to investigate the interactions between A $\beta$  and mutated tau at the atomic resolution both via experimental techniques and computational modeling tools. The molecular mechanisms underlying such interactions between U-shape A $\beta$  fibrils and mutated tau  $\Delta$ K280 R2 repeat U-shape fibrils was studied by MD simulations with CHARMM FF and TIP3P water molecules.<sup>1141</sup>

Two predicted polymorphic mutated tau  $\Delta$ K280 R2 repeat fibrils were investigated. Each one of these two mutated tau fibrils were interacted with A $\beta$  fibril, producing single- and double-layer conformations. The interactions of one of the mutated tau polymorph fibrils with A $\beta$  illustrated that thermodynamically the double-layer conformations are more preferred than the single-layer conformations. Interestingly, the polymorphic cross-seeding mutated tau-A $\beta$  fibrils are more preferred than the separated mutated tau fibrils and A $\beta$  fibrils. The mutated tau fibrils and A $\beta$  fibrils synergistically stabilize each other, leading to polymorphic states. The synergistic stabilization was confirmed by the formation of well-defined  $\beta$ -sheet structures for both A $\beta$  fibrils and mutated tau fibrils along the fibril axis. Finally, interactions between trimers of A $\beta$  alternating with trimers of mutated tau in the cross-seeding fibrils form complexes that are thermodynamically less preferred, but structurally A $\beta$  trimers and mutated tau trimers stabilize each other to form similar inter-sheet distances in the core domain along the fibril axis.<sup>1141</sup>

## 11. Conclusions and Perspectives

This review overviews the state of the art in computer, *in vitro*, *in vivo* and pharmacological (small molecule and antibody) experiments related to AD, PD, T2D and ALS. We have reported on the most recent experimental and computational findings on the monomers, oligomeric intermediates

and fibrils of the key amyloid proteins implicated in these diseases ranging from aqueous solution (free or with metal ions and inhibitors), membrane mimetic systems to *in vivo*. The crosstalk and cross-seeding between the A $\beta$ , tau,  $\alpha$ S, and IAPP proteins and the failed and on-going therapy assays and developments are also discussed. Over the last two decades, a huge amount of data has been gathered from different standpoints due to the investments and continued efforts by researchers from various disciplines, and this is very important to achieve a superior understanding.

Which are the next steps and promising directions that can potentially be handled by experiments? We will list some of them here: (a) The application of cryo-EM analysis, in combination with solid-state NMR and other biophysical techniques, on A $\beta$ , tau and  $\alpha$ S fibrils isolated from the brain extracts of hundreds of patients would be an important step towards the atomic structure and dynamics determination of these proteins in the context of the disease propagation. (b) Determination of the high-resolution structures of toxic oligomers resulting from the co-aggregation of human brain-derived A $\beta$ , pyroglutamate A $\beta$ s and  $\alpha$ S proteins interacting with mitochondrial membranes would be useful as mitochondria are playing a pivotal role in AD and PD.<sup>1142,1143</sup> Probing the many roles of the cell membrane by utilizing novel membrane mimetics such as lipid-nanodiscs that offer several advantages such as stability, solubility, monodispersity, size tunability and lipid heterogeneity would also be useful. (c) Knowledge of the structures of SOD1 oligomers upon mutations and PTMs could be a real breakthrough to better understand the molecular events underlying the ALS pathology. Of note a strong challenge is imposed by the limitation for isolating amyloid aggregates in their native conditions, which will accurately mimic the species in human brains<sup>1144</sup> and availability of reagents and tools to study these protein aggregates in their most stable forms. (d) Consider how propagation might proceed in the context of co-occurring proteinopathies, how multiple cell populations or circuits might be affected, and how spreading occurs through multiple pathways (e.g., exosome or prion-like seeding). Of note there is an urgent need for novel experimental approaches to resolve amyloid cross-toxicity, at the neuro-histochemical and organismal levels, to make progress in understanding the cross-seeding.

Which are the next steps and promising directions that can potentially be handled by both experiments and simulations? Some of them are : (a) Investigate the role of multi-body interactions involving oligomers of different amyloid proteins, cellular proteins and membranes, (b) Elucidate the role played by cholesterol in direct interactions with APP and gamma-secretase as well as indirect influences through changes in membrane phase, (c) Understand the role of membrane microdomains in

partitioning and colocalizing secretases and substrate during A $\beta$  genesis, (d) Determine whether and how liquid-liquid phase and other biomolecules can induce pathogenic tau conformations *in vivo* <sup>1145,1146</sup> (e) How the balance between functional and pathological interactions is regulated in both  $\alpha$ S and IAPP, and how the exact function of  $\alpha$ S is connected with its interaction at the surface of synaptic membranes, and (f) Investigate the formation of amyloid oligomer/phospholipid complexes. <sup>1147,1148</sup>

What are the next steps and promising directions that can potentially be handled by simulations? We know that PMTs mediate the structural diversity of tauopathy strains. The similarities and differences amongst amyloid formation of different tau isoforms therefore provide excellent opportunities to understand the driving forces leading to different pathological diseases. Computational simulations are fully appropriate to explore the initial nucleation of aggregation, the energy landscape connecting different fibril structures and possible related oligomers, and conformational effects of phosphorylation and other PTMs. One of the particularly interesting problems is the phosphorylation effects in cis- and trans- tau, which is difficult to examine experimentally. Another interesting problem involves phosphorylation at residue 217, a tau site closely linked to the first stage of AD, and at residue 181, <sup>523</sup> as advanced studies on blood sample analysis reported that detection of this phosphorylated tau could differentiate healthy participants from those with AD <sup>1149,1150</sup> What are the effects of these two phosphorylations on the tau energy landscape? Simulations could also investigate the circulation of A $\beta$  or  $\alpha$ S proteins in a crowded environment and in a constricted geometry with AD and PD aging conditions. Indeed, it is known that shear flows in microfluidic devices accelerate aggregation kinetics, <sup>16</sup> synucleopathies alter nanoscale organization and diffusion in the brain extracellular space <sup>1151</sup>, and the geometry of the brain interstitial system undergoes alterations with aging and the accumulation of A $\beta$  and tau. <sup>1152-1154</sup>

Of note, we are only at the beginning of the road to adapt the computational methods that generate confidence in the field of globular protein science to IDPs. <sup>44,1155</sup> Similarly, it is not clear yet which atomistic force field is the most reliable for describing the monomeric and oligomeric structures of A $\beta$ , tau and  $\alpha$ S in and their aggregation pathways. Despite extensive research towards better atomistic force fields, the aggregation pathways and the dominant microstates in bulk solution are still more sensitive to the choice of the force field than a change in the IDP sequence. <sup>445,1156-1158</sup> The same limitation holds for coarse-grained simulations and mesoscopic approaches, but it is important to recall that these simulations are designed to capture qualitative rather than quantitative properties of the aggregation process. Coarse-grained simulations and mesoscopic approaches, due to the elimination

of many unimportant degrees of freedom and no explicit solvent interactions, are appropriate to study the dependence of fibril formation and the primary and secondary nucleation mechanisms as function of amino acid sequence, temperature, protein concentration, the ratio between intramolecular and intermolecular interactions, and the presence of simplified crowders or cell membranes for instance.<sup>324,365,371,462,1159</sup> Coarse-grained simulations, which allow longer time- and length-scales at the expense of a reduced energy accuracy, are continuously improving.<sup>31,321,337,1160</sup> They are complementary to all-atom simulations, and in some cases, their results are further explored by all-atom simulations, referred to as multiscale approaches.<sup>111,451,1161</sup> We emphasize that there is no emerging state-of-the-art simulation protocol for the aggregation of IDPs, even though coupling all-atom simulations with Markov state chain modelling approaches is becoming popular at least on small amyloid peptides and aggregate sizes.<sup>450,1162</sup> Overall, we face multiple key issues such as the force field accuracy and the sampling and concentration bottleneck over a wide range of time scales towards efficient elucidation of IDPs aggregation kinetics and thermodynamics and accurate predictions from atomistic and coarse-grained simulations. Whether combining deep learning and statistical mechanics through Boltzmann generators can help solve the sampling issue for amyloid aggregation remains to be determined.<sup>1163</sup>

Developing disease-modifying therapies for AD presents an immense challenge, with little success despite intense work over decades.<sup>314,1164-1167</sup> Some of the reasons why drug development for AD continues to fail include: (a) *in vitro* experiments poorly mimic *in vivo* conditions, (b) A $\beta$  and tau exist in multiple covalent and aggregated forms of uncertain relevance to pathology and we have a poor knowledge of their structures, and (c) the limitations of animal models.<sup>314</sup> Amongst animal models, genetically-modified mouse lines are invaluable and widely used models for understanding genes, proteins, and biological pathways, evaluating disease progression, and assessing safety of molecules for therapeutic purposes in many diseases including neurodegenerative diseases, cancer and diabetes. However, there are several significant intrinsic limitations in using *in vivo* animal studies to replicate the pathological environment occurring in the human brain undergoing neurodegeneration<sup>1168</sup>. Firstly, there is an obvious cross-species comparability factor between human and rodents, importantly including aging for which aging in mice does not accurately reflect aging in humans<sup>1169,1170</sup>. Secondly, human and mice brains differ in neuroanatomy, brain biochemistry and in blood-brain-barrier (BBB) permeability<sup>1169,1171-1173</sup>. Considering the differences in respective lifespans for humans and mice, it is naive to take at face value the development of phenotypes in young mice for symptoms that are usually developed in elderly human subjects<sup>1169</sup>. The push to generate models that rapidly progressed disease phenotype often resulted in either worsening of the pathology to non-physiological levels or inducing



aberrant early lethality in mice<sup>1174</sup>. Thirdly, the reliance on inbred mouse models has been widely documented as a translational issue whereby specific alleles can predispose the animals to certain phenotypes prior to genetic manipulation<sup>1175</sup>. Fourthly, the phenotypes observed in mice can also be influenced by potential artifacts resulting from the overexpression of transgenes, from the location of the transgene insertion or from environmental factors<sup>1169,1176</sup>. Finally, to observe the desired pathology and phenotypes, the commonly used transgenic mouse models carry multiple mutations, such as in both A $\beta$  and tau, which rarely happens in human brains<sup>1176</sup>. Thus, challenges are faced while attempting to recapitulate the features of human diseases in mice.

After more than 20 years, we do not know whether the accumulation of A $\beta$  oligomers is the cause or consequence of AD.<sup>1177,1178</sup> Other researchers in the field place tau aggregates upstream of A $\beta$  in AD. As a matter of fact, all promising drugs and antibodies have failed in phase III clinical, with the majority of these compounds even unable to reach such a step. Caution is needed until the full analyses from the phase III aducanumab trials are released. A few weeks ago, the results from the TAURIEL phase II clinical trial of semorinemab in early AD were announced.<sup>1179</sup> This antibody, which binds the N-terminus of all six isoforms of human tau independently of the phosphorylation status, did not show any benefit over placebo. Should we move away from the amyloid cascade hypothesis or the series of events starting from tau for efficient AD treatment? Undoubtedly, the answer is no. As quoted by Karran and De Strooper, 'it is one thing to test a drug and quite another to test a hypothesis'<sup>1177</sup> for several reasons.

First, clinical trials take place too late in the disease process.<sup>314,1180,1181</sup> However, diagnosing AD before the onset of symptoms, where drugs might have a beneficial effect, is not easy, though substantial progress is being made using biomarkers and imaging. Alternatively, drugs can be tested on people with dominant mutations for AD while they are asymptomatic. We do not know much however about the asymptomatic phase of AD and there is a lot of heterogeneity in AD in terms of its presymptomatic and symptomatic phases and pathological features.<sup>251,1182</sup>

Second, approaches such as Genome Wide Association Studies have found over 50 loci significantly associated with AD onset. Pathway and functional genomic analyses show that in addition to A $\beta$  and tau processing, immunity, endocytosis, cholesterol transport and ubiquitination are involved in AD. Inflammation, oxidative stress, infection, diabetes, loss of protein degradation and metal deposition are also likely to play roles in AD.<sup>1183</sup> Many proteins play a significant role in developing AD, such as the prion protein,<sup>1184</sup> APOE4<sup>1185,1186</sup> and the lipoprotein receptor related protein 1 (LRP1), the latter discovered recently to be a master regulator of tau uptake and spread<sup>1187</sup>. There is also increasing evidence that cerebral vascular disease, the BBB and the cerebrospinal fluid environment play an

integral role in the development of AD.<sup>1178,1188,1189</sup> Notably, the LRP1 protein, a component of the BBB, contributes to the clearance of A $\beta$  from the central nervous system. The pivotal role of mitochondria and its dynamics in the brain<sup>1142</sup> and changes in microtubule structure and dynamics<sup>1190</sup> have also been discussed. It is very interesting that Lashuel et al. recently demonstrated that the process of Lewy Body formation involves a complex interplay between  $\alpha$ S aggregates and membrane organelles involving mitochondria and the autophagosome, inducing mitochondrial damage and deficits, and synaptic dysfunctions.<sup>1147</sup> Improving our understanding of all these crucial topics should aid drug development.

Finally, there is accumulating pathological evidence that many neurodegenerative diseases are mixed proteinopathies. Co-pathologies and cross-talks are likely a common feature of aging and neurological disorders, whereby the prevalence of these pathologies ultimately determines the type of disorder development in the aging brain. Therefore, the amyloid hypothesis in AD needs to be modified and to integrate cross-talk with other significant pathologies. This realization of co-pathologies and the failure of all previous monotherapies suggest that multi-therapies, targeting simultaneously A $\beta$ , tau and  $\alpha$ S, could become the norm, as discussed already in 2000.<sup>1067</sup> Clearly, the creation of the 2019 EU-US clinical trials on Alzheimer's disease task force is good news for AD, PD, T2D and ALS.<sup>1191</sup>

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TOC

AD, PD, T2D, ALS



