

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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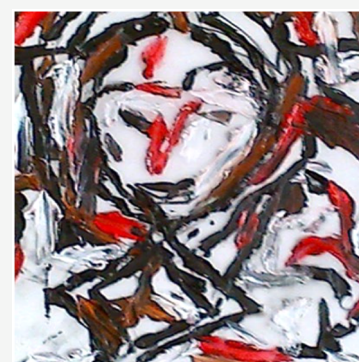


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ABSTRACT: Protein misfolding and aggregation is observed in many amyloidogenic diseases affecting either the central nervous system or a variety of peripheral tissues. Structural and dynamic characterization of all species along the pathways from monomers 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), α -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 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.¹ 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.² 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.³ 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 α -synuclein (αS) protein in PD, the islet amyloid polypeptide (IAPP) or amylin of 37 amino acids in T2D, and the superoxide dismutase 1 of 32 kDa (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 αS proteins to AD and PD were Glenner and Wong,⁴ Goldberg and Lansbury,⁵ and Hardy and Selkoe,⁶ 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.¹⁰

In vitro, the A β , tau, α S, IAPP, TDP-43, and FUS proteins form readily cross- β 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.^{11,12} 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.¹³ 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 β 42 vs A β 40) and genetic risks, including several mutations in A β , α 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).^{14–16}

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 nondisease proteins, short peptides, and even single amino acid homopolymers can form fibrils under appropriate conditions,^{17,18} and changing experimental conditions lead to nanotubes and ordered nanomaterials.^{19,20} 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.²¹

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.²² 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.^{23–25} Even under early earth (prebiotic) conditions, peptides can form amyloid fibrils leading to the current amyloid world hypothesis in the origin of life^{26,27} and the possibility that all globular protein structures may have originated from amyloid fibrils.²⁸

While SOD1 is a globular protein with a well-defined 3D structure, the A β , tau, and α -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.²⁹ 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.³⁰

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.³¹ 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 on 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.^{32–38} 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) prion³⁹ and on cryo-electron microscopy (cryo-EM) experiments.^{40,41} Fibril structures of short amyloid peptides were also determined by X-ray diffraction analysis.⁴²

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.^{12,14,43–45} 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,⁴⁶ i.e., several orders of magnitude less than the time scales of hours and days required for fibril formation *in vitro* at a μ M concentration.¹¹

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 Lansbury⁵ and reviewed more recently.^{47–50} There is also growing evidence from *in vivo* and *in vitro* studies of co-occurring pathologies across common neurodegenerative diseases,⁵¹ indicating cross-talk between the amyloid proteins and interactions and cross-seeding between the A β , tau, and α -synuclein proteins which promote aggregation, generate different strains, and accelerate cognitive dysfunction.^{52–54}

In summary, we provide an in-depth overview of our current knowledge on the biogenesis and domain organizations of A β , tau, α -synuclein, and IAPP related to their aggregation and binding properties, the molecular structures of amyloid

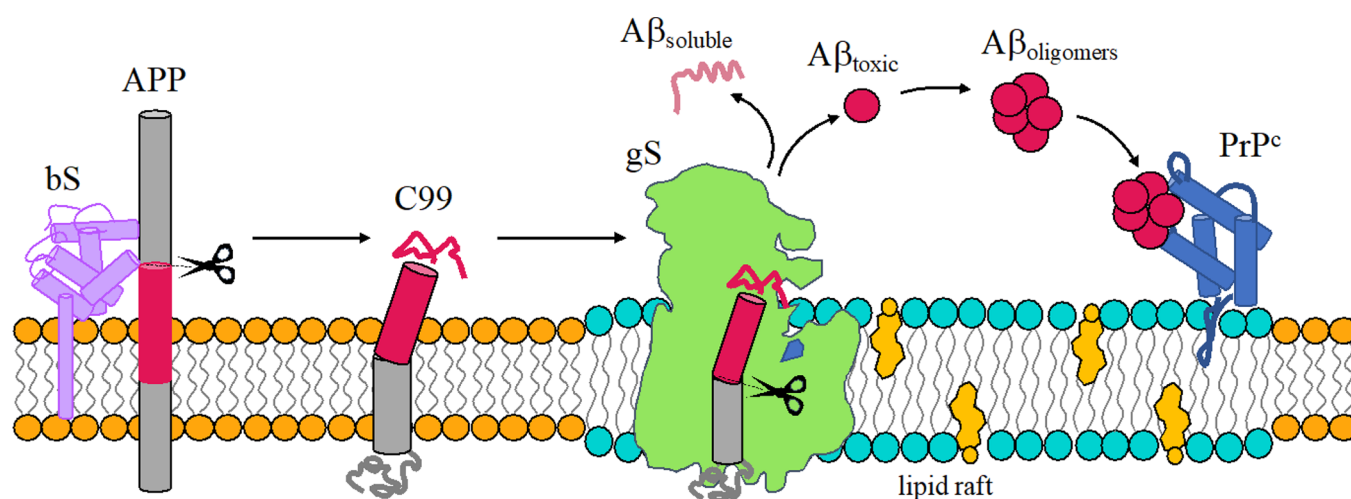


Figure 1. Schematic representation of the amyloidogenic processing of APP. A β is produced by sequential processing of APP by bS and gS to produce either a soluble and nontoxic mixture of A β or a more amyloidogenic A β mixture with a propensity to form oligomers that bind PrP^c and potentially induce AD.

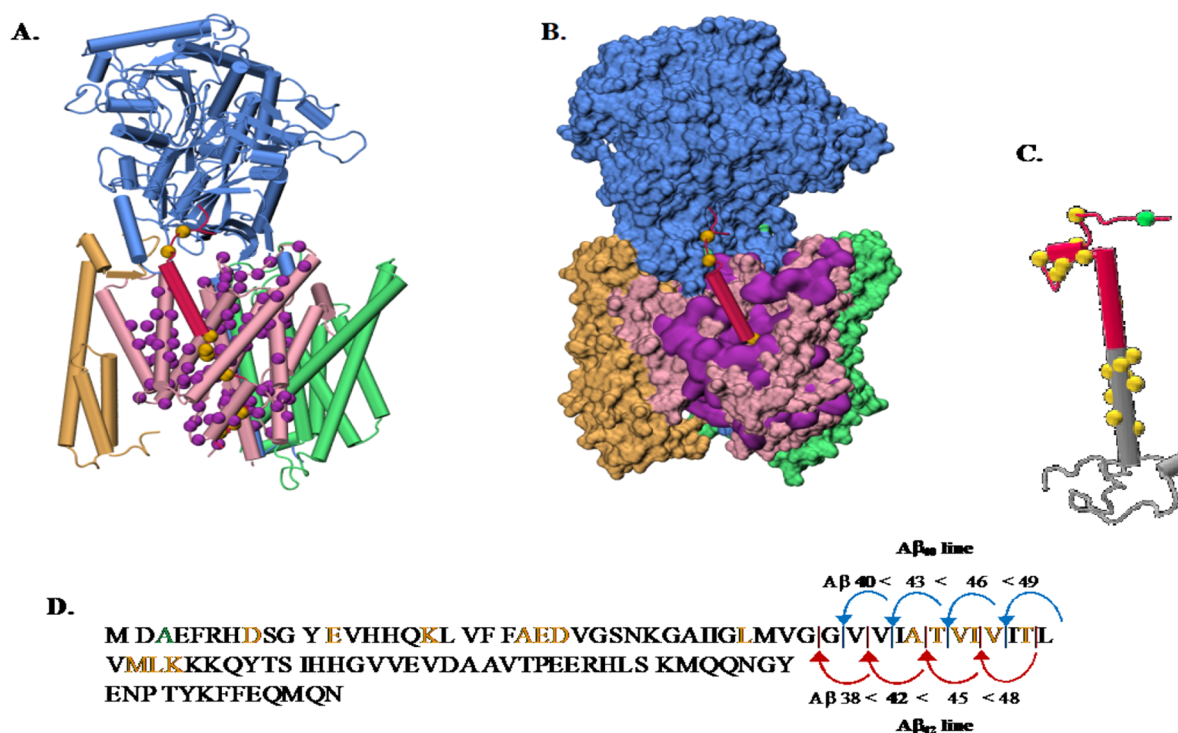


Figure 2. (A) Overview of the gS and APP structures and pathogenic AD mutations. (A) Depiction of the gS complex (PDB 6IYC).⁹⁵ NCT subunit (blue), APH-1A (green), PEN-2 (yellow), and the catalytic PSN subunit (pink) bound to a C99 fragment (red). C-alpha atoms from the PSN disease-causing mutations¹³⁹ 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 part A in a surface representation. (C) Depiction of a C99 structure model (gray) with the A β 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 representation and showing the two main A β production lines. The structures in parts A, B, and C from the PDB were drawn using VMD.¹⁴⁰

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 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 list future directions.

2. A β BIOGENESIS AND DOMAIN ORGANIZATIONS OF TAU, α -SYNUCLEIN AND IAPP

2.1. A β Biogenesis: Role of Pathogenetic and Protective Mutations, Membrane Composition, and Detailed Interaction with γ -Secretase (gS)

2.1.1. Generation of A β Peptides by gS. It is established that amyloid β protein (A β) contributes to the dysfunction and degeneration of neurons and the pathogenesis of AD.^{6,55–57} A β is derived from the proteolytic cleavage of the single-pass transmembrane amyloid precursor protein (APP) by secretases and may be processed along nonamyloidogenic and amyloidogenic pathways. The nonamyloidogenic pathway involves initial cleavage of APP by α -secretase (aS) leading to formation of sAPP α and the 83 amino acid fragment APP-C83. The amyloidogenic pathway involves initial cleavage of APP by β -secretase (bS) leading to formation of sAPP β and the 99 amino acid fragment referred to as CTF99, APP-C99, or simply C99. Cleavage of C99 by γ -secretase is the last step in the production of A β (Figure 1).

Cleavage of C99 by gS lacks fidelity.^{58–60} Cleavage of C99 by gS is initiated at the ϵ -sites (gS endopeptidase activity) to produce the amyloid intracellular domain (AICD) and A β 49 or A β 48 peptides. After the first cut, tri- and tetrapeptides are generated from sequential cleavage (gS carboxypeptidase-like activity) until A β proteins are released to the extracellular environment.⁶¹ Cleavage of C99 results in a variety of A β isoforms with A β 40 being most prevalent and A β 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 β . C99 is produced along two main cleavage lines A β 49 > 46 > 43 > 40 and A β 48 > 45 > 42 > 38 (Figure 2, panel 2D). The first is responsible for the release of the major isoform A β 40 and the second leads to the minor isoforms A β 42 and A β 38.⁶²

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,^{63–66} 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.⁶⁷ The polybasic regions on the C-terminal side of TM helices are globally found in gS substrates⁶⁸ 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.⁶⁹ These observations support the conclusion that the N-terminal 21 amino acids of C99 are not required for gS cleavage.⁷⁰

Evidence suggests that the mature gS complex is active at the plasma membrane and in endosomes^{71–73} and that cleavage of C99 by γ -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 play a

critical role. The actual products of APP cleavage depend on the specific membrane environment.⁷⁴

While A β has long been implicated as a pathogenic agent in AD, the molecular mechanism by which A β induces neuronal dysfunction largely remains a mystery.^{75–79} One focus has been to characterize the structural ensembles of A β 40 and A β 42 monomers using experimental NMR and computational studies^{80,81} with an eye on identifying aggregation prone structures termed N* states.⁸² Given the role of the monomer ensemble in identifying aggregation prone sequences (N* conjecture), one promising putative mechanism for A β cytotoxicity involves the binding of A β oligomers with cellular prion protein PrP^{C83} leading to activation of a kinase and the abnormal phosphorylation of tau (see section 2.2).

2.1.2. 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.^{84–87} 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 β 42 cleavage site that typically have little effect on C99 cleavage. In addition, (3) the transmembrane C-terminal fragment below the A β 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 β 42:A β 40 ratio by distorting the relative efficiencies of the ϵ -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.

2.1.3. Role of Mutations to APP and gS. The majority of pathogenic APP mutations decrease the overall cleavage of C99 by gS, meaning that they are loss-of-function (LOF) mutations.⁶⁸ 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 β -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.^{88–90} (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.⁹¹ (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.⁹² (3) Presenilin (PS1) contains two aspartate residues that form the active site. (4) Presenilin enhancer 2 (PEN-2) is needed for the autocatalytic maturation.^{93,94} The recognition of APP by gS is illustrated in Figure 2A.⁹⁵ 180 mutations occurring in the PS1 subunit have been linked to familial AD.

Pathogenic PSEN-FAD mutations may affect gS endopeptidase activity (and the initial ϵ -cleavage site) and consistently impair the carboxypeptidase-like efficiency. This results in reduced processivity of A β 49 or A β 48 and the release of longer and more toxic A β 43 or A β 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 and B).

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 disperse distribution of the mutation sites suggests that there should be a variety of mechanisms. (1) Disturbed PS1/APP interactions⁹⁶ act by changing the preference of the initial position of the ϵ -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 ϵ -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.⁹⁷ (2) Loss of gS carboxypeptidase activity interferes with the catalytic efficiency, releasing premature intermediate and longer APP products.⁹⁸ (3) Inhibitory effects at the initial ϵ -cleavage sites may lead to changes in the distribution of A β isoforms critically impacting aggregation kinetics and toxic effects.⁹⁹ (4) Finally, catalytic cycle impairment may lead to alterations in the gS product lines.⁹⁸

2.1.4. Role of Membrane in A β Genesis and Aggregation. Early experimental⁶³ and computational¹⁰⁰ 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 co-workers 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 nonhelical structures,¹⁰¹ 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).¹⁰² They observed that at least part of the JM domain assumes a β -strand structure. Recent simulation results provide some support for this intriguing observation.¹⁰³

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 the structure of the TM helices within the dimer.^{100,104} There is evidence that changes in the stability of the C99 dimer influence its cleavage by gS and the resulting overall level of A β protein and its isoform distribution.^{101,104,105} Multhaup et al. first recognized that modifications in sequence that reduced homodimer affinity impacted cleavage of C99 by gS.¹⁰⁶ 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 β formation.^{107,108} 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 β .¹⁰⁹ There is little doubt that C99 homodimer is an essential species in the overall ensemble of C99 structures.^{107–111} More recently, Sanders et al. reported an ensemble of coexisting C99 monomer, dimer, and large-scale oligomers in lipid bicelles.¹¹²

2.1.5. 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.^{103,110,113,114} 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,^{113,114} the first C99 homodimer structure in a bilayer environment,¹¹³ and the ability to “environmentally select” C99 topologically distinct homodimer structures in membrane environments of varying lipid composition.¹¹⁴

Enhanced levels of cholesterol resulting from diet, genetic predisposition, or aging are positively correlated with early onset of AD.^{115–117} A variety of theories have been proposed to explain these observations. Lower levels of cholesterol promote membrane fluidity and nonamyloidogenic cleavage of APP by aS.^{118,119} 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.^{62,120,121} 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,^{109,122} thus enhancing the partitioning of C99 to lipid raft domains and the proteolytic cleavage of C99 by gS to produce A β .

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^{120,123–127} have proposed a role for raft domains in the biogenesis of A β . However, their influence on the mechanism of creation of A β 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 possesses 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 β S and gS formed in cholesterol rich membrane domains might lead to substrate shuttling and enhanced efficiency in the biogenesis of A β .¹²⁸ Taken together, these observations demonstrate the important role played by membrane spatial heterogeneity in A β genesis.

2.1.6. Role of Membrane on gS. gS is accountable for the final step in the regulated intramembrane proteolysis of APP to

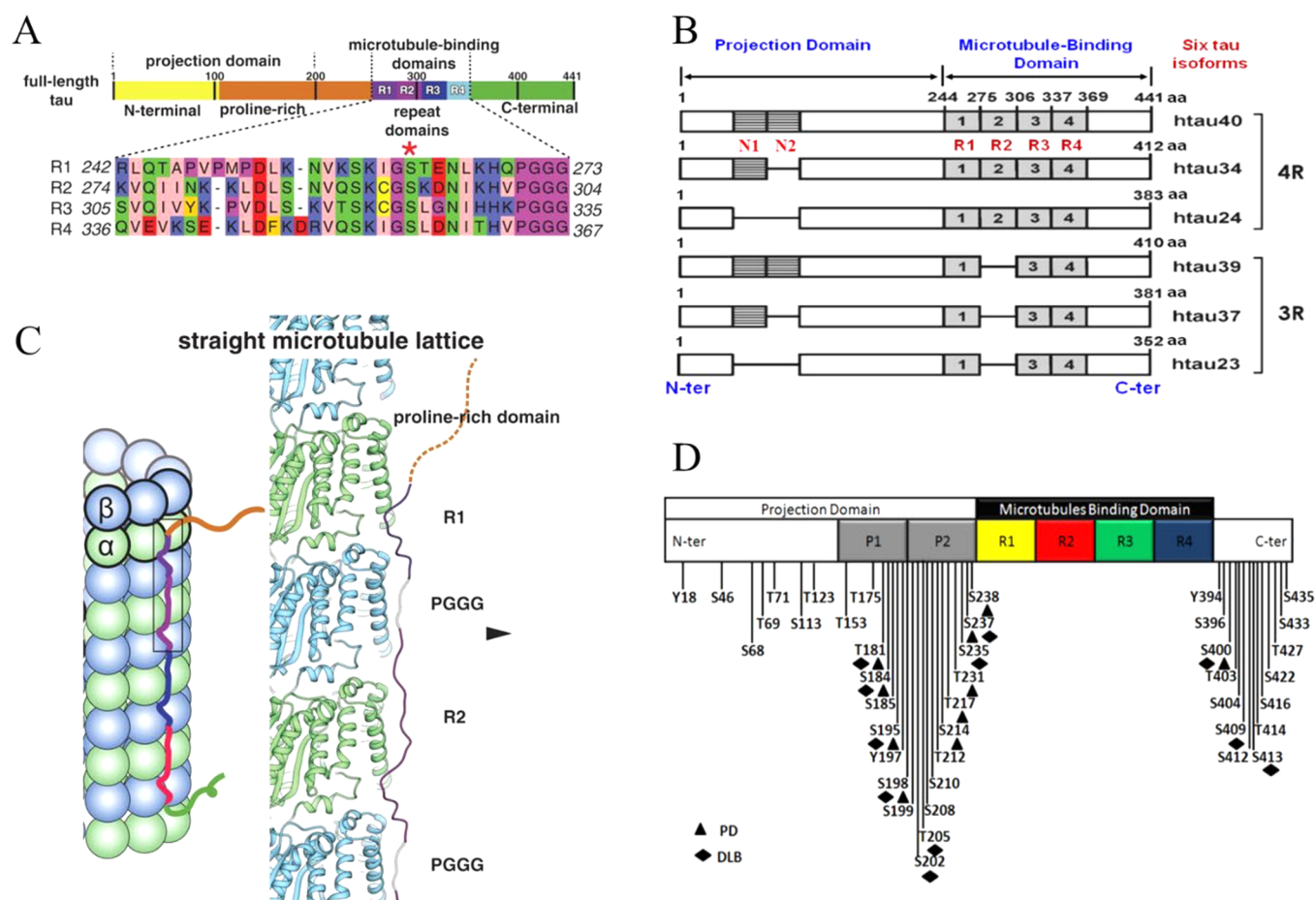


Figure 3. Domain organization, isoforms of tau, and binding to microtubules. (A) Schematic of tau domain architecture and assigned functions. The MT-binding domain of four repeats is defined as residues 242 to 367. The inset shows the sequence alignment of the four repeat sequences, R1 to R4, that make up the repeat domain. Ser262 is marked by the asterisk.¹⁴² (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.¹⁴² (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.

generate A β . 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.^{66,129} Clinical and experimental studies suggest that lipid composition is altered in AD brain tissue¹³⁰ and that the production of A β peptides varies with the membrane composition.¹³¹ 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 β peptides by gS.¹³² 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 β 42:A β 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 β 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 β 42:A β 40 ratios. Similarly, the presence of cholesterol in a membrane leads to a considerable increase in gS activity that decreased at higher concentrations producing a bell-shaped form in the gS activity.¹³²

Computational modeling approaches have made an effort to characterize changes in the gS conformational states while varying the bilayer lipid composition.^{69,133} 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.¹³³ 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 unsaturations of lipids.¹³³ 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.^{134,135}

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.¹³³ 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,¹³³ 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,^{137,138} but their impact on A β biogenesis and the conformational ensemble remains poorly understood.

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 an N-terminal region (with two N-terminal inserts 2N) and a proline-rich region. The MBD has high affinity for microtubules. All 4 repeats end with a PGGG sequence, and there are two hexapeptide regions (PHF6*: ²⁷⁵VQIINK²⁸⁰ and PHF6: ³⁰⁶VQIVYK³¹¹) 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.¹⁴¹

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).¹⁴² 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 α -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.¹⁴²

Besides the six tau isoforms, 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.¹⁴³ 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.¹⁴⁴ For example, tau (297–391) forms filaments that structurally mimic the core of paired helical filaments in AD brain.¹⁴⁵ A fragment from the proteolytically stable core of the PHF, tau 297–391 known as “dGAE”, spontaneously forms cross- β -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.¹⁴⁵ Cleavage of tau by legumain (LGMN) has been proposed to be crucial for aggregation of tau into fibrils.¹³⁶ Using an *in vitro* enzymatic assay and nontargeted 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 uncleaved 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 uncleaved tau. This suggests that LGMN-cleaved tau has a limited role in the progressive accumulation of tau inclusions in AD.¹⁴⁶

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 β fibrillogenesis.¹⁴⁷ However, in the longer tau sequence, tau local structure shields the PHF6 motif.¹⁴⁸ When A β acts as tau aggregation seeds, A β fibril can promote the exposure of these hexapeptide regions.¹⁴⁹ However, the PHF6 peptide lacks the ability to seed aggregation of tau244–372 in cells.¹⁵⁰ But as the hexapeptide is gradually extended to 31 residues, the peptides aggregate more slowly and gain potent activity to induce aggregation of tau244–372 in cells.¹⁵⁰ Further characterizations narrow down the β -forming region to a 25-residue sequence, indicating that the nucleus for self-propagating aggregation of tau244–372 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.¹⁴⁸ Numerous MD simulations have been conducted to study the PHF6 conformation and aggregation. In a recent MD and Markov state model (MSM) study,¹⁵¹ PHF6 can spontaneously

aggregate to form multimers enriched with β -sheet structure, and the β -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, and then a dimer with short β -sheet structure at the N-terminal forms; finally, β -sheets elongate to form an extended parallel β -sheet.¹⁵¹

Many studies have targeted these hexapeptide regions to prevent tau aggregation and toxicity. Using the model peptide, Ac-PHF6-NH₂, the substitution of its amino acids with proline is shown to reduce self-assembly. Two of these modified inhibitors also disassemble preformed Ac-PHF6-NH₂ fibrils, and one inhibits induced cytotoxicity of the fibrils.¹⁵² Inhibitors based on the peptide SVQIVY, shifted by -1 residue compared with PHF6 (VQIVYK), can also block proteopathic seeding by patient-derived fibrils.¹⁵³ 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.¹⁵⁴ 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.¹⁵⁴

Tau constructs can self-aggregate to PHFs directly; however, hyperphosphorylated tau appears to aggregate more readily and may sequester normal tau at lower concentrations.^{155,156} 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.¹⁵⁷ Other PTMs can also regulate tau aggregation; for example, tau truncation may take place after tau hyperphosphorylation with subsequent glycation.¹⁵⁸ Methylation has been shown to suppress tau aggregation propensity whereas glycation and acetylation promote pathological tau aggregation.^{159,160} 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.¹⁶¹ Figure 3D illustrates the known phosphorylation sites in tau.

Cryo-EM structures of tau fibers in four distinct diseases, AD,¹⁴⁰ Pick's disease, chronic traumatic encephalopathy, and Corticobasal degeneration (CBD) have different conformations.^{41,162–164} 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.¹⁶⁵ 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, the tau306–327 R3 fragment is able to self-assemble into large, flat, multistranded ribbons consisting of up to 45 laterally assembled protofilaments.¹⁶⁶

Besides heparin, other polyanions have also been employed to study the structural consequences; aggregation 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.¹⁶⁷ Polyphosphate initiates tau aggregation through intra- and intermolecular scaffolding, most notably breaking long-range interactions between the termini.¹⁶⁸ Different from heparin, polyglutamic acid does not immediately convert tau into oligomers.¹⁶⁹ 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.¹⁷⁰

Using a heparin-immobilized chip, surface plasmon resonance (SPR) revealed that tau K18 and K19 bind heparin with a *K_d* of 0.2 and 70 μ 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.¹⁷¹ However, it was found that heparin-induced tau filaments are structurally heterogeneous and differ from AD filaments,¹⁷² 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.¹⁷³ 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 β 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.¹⁵³ 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.¹⁷⁴ 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 β -strands, which ultimately lead to amyloid fibrils, while the amorphous oligomers are characterized by a near random β -strand stacking, leading to a distinct amorphous phase.¹⁷⁴ The feature

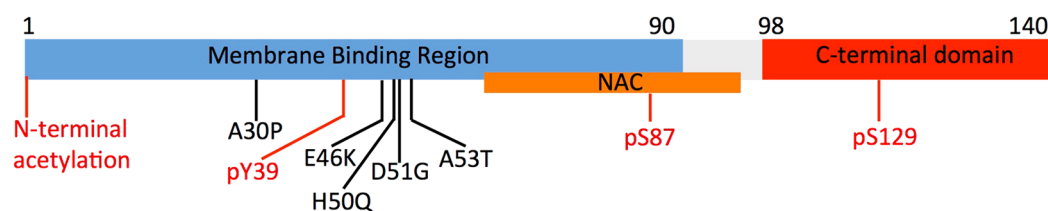


Figure 4. Key α S domains having a role in functional and pathological contexts. The membrane-binding domain (residues 1 to 90), the NAC region (residues 61 to 95), and the C-terminal domain (residues 98 to 140) are shown in blue, orange, and red, respectively. The diagram also shows the main pathological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

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.¹⁷⁵ Kjaergaard et al. observed that the shortest growing filaments only represent a small population of transient oligomers with cross- β 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.¹⁷⁵

In vitro, 0N4R tau fibrils contain a monomorphic β -sheet core enclosed by dynamically heterogeneous fuzzy coat segments.¹⁷⁶ 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 β -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 PHFs¹⁷⁷ and play distinct roles in the stability and consequently neurotoxicity of tau filaments.¹⁷⁸ The N-terminal fragment (residues 1–15) did not affect tau polymerization¹⁷⁹ whereas a fragment consisting of residues 1–196 could inhibit polymerization of full-length tau.¹⁸⁰ The tau C-terminus is crucial in the formation of tau PHFs,¹⁸¹ and it also modulates the cross-seeding barrier between 4R and 3R tau.¹⁸² Xu et al. performed multiscale 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.¹⁸³ 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.

2.3. α -Synuclein and IAPP Domains and Aggregation Properties

α S is a 14 kDa neuronal protein that is predominantly localized at the presynaptic termini.¹⁸⁴ In its physiological form, α S is monomeric and disordered,¹⁸⁵ although some studies have generated a debate¹⁸⁶ on whether it adopts a helical tetramer *in vivo*.¹⁸⁷ The aggregation of α 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.¹⁸⁸ There are also links between the α S-encoding gene and familial forms of PD, with mutations, duplications, and triplications being found in patients affected by early onset forms of PD.¹⁸⁹ Fibrillar aggregates of the relevant aggregation-prone region of α S, the nonamyloid- β

component (NAC), are also found in AD patients¹⁹⁰ and in the context of other neurodegenerative disorders, including dementia with Lewy bodies,¹⁹¹ multiple system atrophy,¹⁹² and other synucleinopathies.¹⁹³

While the pathological relevance of α S is generally acknowledged, its function remains unclear.¹⁹⁴ The abundance of α S at the synaptic termini has suggested that it may be involved in neuronal processes and studies have indicated possible roles in synaptic plasticity¹⁹⁵ and learning.¹⁹⁶ A number of pieces of evidence have been collected on a possible function of α S in the trafficking of synaptic vesicles (SVs) during neurotransmitter release.^{197,198} α S binds SVs *in vitro* and colocalizes with SVs in synaptosomes in a calcium responsive manner.¹⁹⁹ Key evidence indicates that α S has a role of chaperone for the assembly of the SNARE complex;²⁰⁰ the machinery promoting the fusion of SVs with the plasma membrane. α S was indeed shown to rescue the formation of the SNARE complex in knockout mice lacking CSP α ,²⁰¹ whereas knockout mice lacking the three synucleins (α -, β -, and γ -) showed neuropathological phenotypes that are indicative of impaired SNARE activity.²⁰² The interaction with SVs by α S has also been implicated in the promotion of SV-clustering,^{203,204} a key process in the maintenance of pools regulating SV homeostasis during neurotransmitter release.¹⁹⁸ In addition to its interaction with SVs, α S has also been implicated in the regulation of the vesicle trafficking from the endoplasmic reticulum (ER) to the Golgi²⁰⁵ and the mitigation of oxidative stress in mitochondria.²⁰⁶

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

Another domain of α S, the NAC (residues 61–95), has relevance in the context of self-assembly and aggregation. NAC is essential for the kinetics of α S aggregation²⁰⁹ and is the main component of the core of α S fibrils. Fibrils of the NAC region have been isolated in the context of PD as well as in other neurodegenerative diseases.¹⁹⁰ Finally, the negatively charged C-terminal domain of α S spanning residues 99 to 140 (net charge of -9) is implicated in calcium binding¹⁹⁹ and in protein–protein interactions at the surface of synaptic vesicles.^{197,200}

IAPP or amylin is a 37-residue hormone produced by pancreatic β -cells to regulate the response to high glucose levels in the blood in conjunction with insulin, which is cosecreted.²¹⁰ The biogenesis of amylin requires the production of the proIAPP, a peptide composed of 67 residues whose cleavage generates IAPP.²¹¹ 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 fact that the toxicity of IAPP amyloids has been shown *in vitro*,^{212,213} it remains to be established whether its aggregation is a causative factor or a downstream effect of T2D. Indeed, in contrast to A β 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 nondiabetic elderly individuals,²¹⁴ 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 β -cells of healthy individuals.

Among the factors that inhibit the aggregation of IAPP in β -cells are the acidic pH (~ 5.0), shown to be largely unfavorable to the misfolding of IAPP *in vitro*,²¹⁵ and the presence of high levels of Zn(II) in β -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- β arrangement, generating two facing β -sheets stabilized by a steric-zipper dry interface.²¹⁶

3. STRUCTURES OF A β , TAU, α -SYNUCLEIN, IAPP OLIGOMERS, AND FIBRILS FROM EXPERIMENTS

3.1. Experimental Techniques for Fibrils

Amyloids are proteinaceous 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 a few nanometers to microns, and (iii) 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.²¹⁷ 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 toward 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 PDB²¹⁸ using different techniques such as X-ray diffraction, NMR, and EM.²¹⁰ 3D model determination of

amyloid architecture relies on a multistep process to elucidate structural features at various levels:

3.1.1. Length of the Amyloid Core. The amino acid sequence of the amyloid core usually covers only a fraction of the full-length protein; for example, the amyloid core of α -synuclein does not comprise its C-terminal domain.²¹⁹ 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 spectrometry²²⁰ or solution NMR²²¹ is an experimental approach that is commonly used to delineate the amyloid core at a residue-specific resolution.

3.1.2. Secondary Structure. Amyloid deposits are rich in β -sheet secondary structure; this information can be rapidly extracted with CD and FTIR. A key step toward determining the amyloid structure is the identification of the number and delineations of the β -sheets as well as the localization of β -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 TALOS²²² or by the secondary chemical shifts.²²³ 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 β -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.²²⁴ They can be recognized after single particle EM analysis, recently demonstrated for α S²²⁵ and A β .²²⁶

3.1.3. Three-Dimensional Fold and Intermolecular Packing. Although most amyloids share the generic cross- β architecture, the number of β -sheet elements and their placement relative to each other can be very diverse. Several generic architectures have been proposed, such as the β -helix, β -sandwich, superpleated β -sheet structure, or β -roll. The supramolecular arrangement of amyloid fibrils is often regular in a protofilament and has been observed as β -sheets that run antiparallel or parallel in-register along the fibril axis. The β -solenoid fold was the first experimentally determined amyloid fold at high resolution, from the amyloid prion HET-s by ssNMR.³⁹ 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,⁴⁰ suggesting this fold to be generic in the context of pathological and functional amyloids.

The cross- β nature of the sample is essentially characterized by X-ray diffraction,²²⁷ and additional information relative to high-order symmetry can be derived from scanning transmission EM²²⁸ and tilted-beam transmission EM.²²⁹ 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.²³⁰ Distinction between intramolecular proximities (i.e., two nuclei in two β -sheets within the same molecule) and intermolecular proximities (i.e., two nuclei in

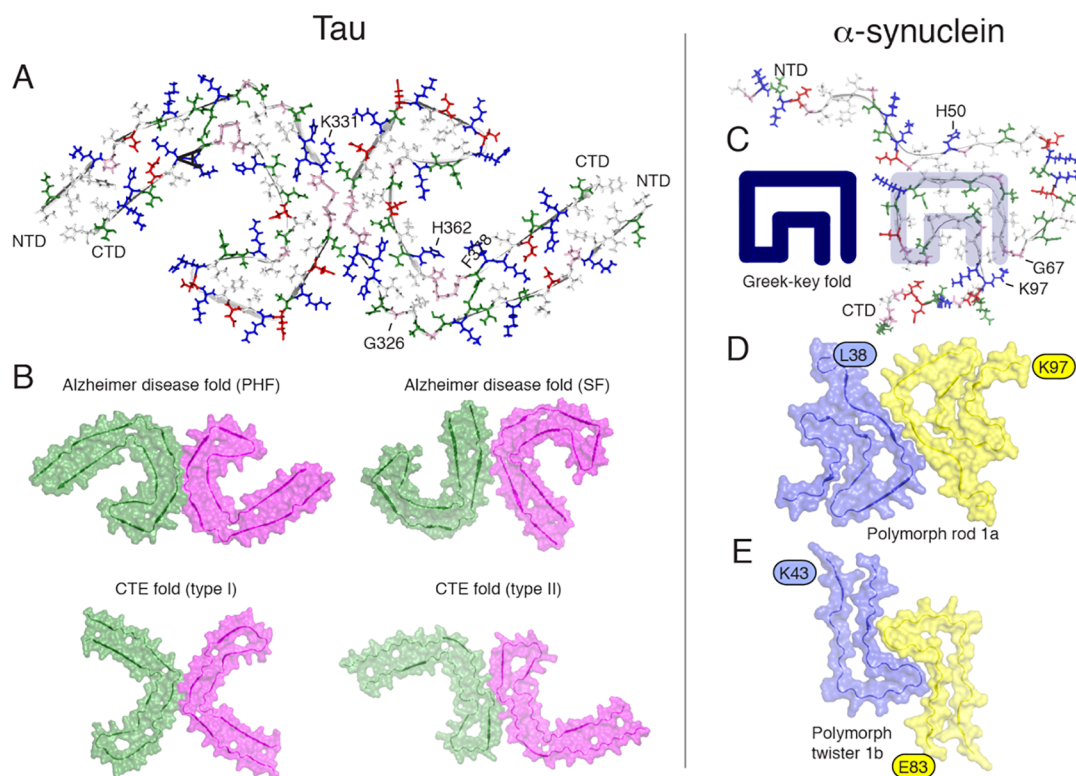


Figure 5. Orthogonal view of the three-dimensional structural models of tau filaments and α -synuclein amyloid fibrils. NTD and CTD are the N-terminal and C-terminal domains. (A) Solid-state tau paired PHF spanning residues 306–378 in the right protofilament. (B) Cryo-EM PHF and SF tau amyloid cores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

two β -sheets in adjacent molecules) requires the use of strategic isotope labeling²³¹ 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.²³²

In the case of short amyloid-forming peptides that crystallize, conformational studies at atomic resolution can be performed by X-ray diffraction analysis⁴² and microelectron diffraction.²³³ 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.²²⁴ 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 bundles 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 nonplanar β -strands in the case of A β 42²²⁶ and tau.⁴⁰

3.2. Diversity of Fibril Structures in Tau, α -Synuclein, A β , and IAPP

3.2.1. Tau Filaments. Tau filaments were first observed in paired helical PHF²³⁴ and then a mixing of PHF and straight filaments (SF) by EM.²³⁵ 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 polymorphism²³⁶ of the cryo-EM structures of tau in AD and Pick's disease.

In AD, both structures of PHF and SF (Figures 5A and B) are composed of eight β -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- β / β -helix structure and define the seed for tau aggregation. Paired helical and straight filaments differ in their interprotofilament packing, showing that they are ultrastructural polymorphs.⁴⁰

In Pick's disease, the tau filament core comprises a distinct fold⁴¹ compared to the structures of AD's PHF and SF. This

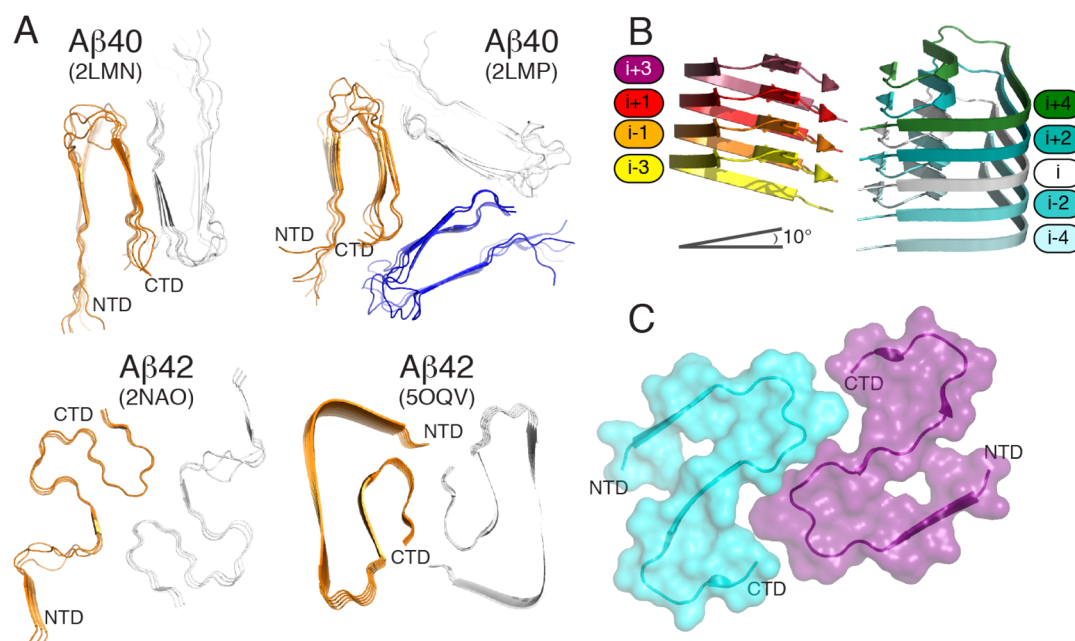


Figure 6. (A) Orthogonal view of A β 40 structures (PDB entries 2LMN and 2LMP) spanning residues 9–40 and A β 42 structures (PDB entries 2NAO and 5OQV) spanning residues 1–42. (B) Cryo-EM structure of A β 42 showing the staggered arrangement of nonplanar A β 42 subunits. (C) Cryo-EM structure of IAPP grown at physiological pH (PDB 6Y1A) spanning residues 13–37. The authors designed the figure with pymol.²⁵⁴

suggests that a disease-specific amyloid fold might be the hallmark of clinical phenotypes, in analogy with structural strains observed in prion diseases.²³⁷ 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.⁴¹ 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 R4.¹⁶³ 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.¹⁷³

3.2.2. α -Synuclein Fibrils. Unlike tau, recombinant expression of α S led to the preparation of homogeneous fibrils amenable to structure determination. A first 3D structure was

proposed by ssNMR,²³⁸ revealing a Greek-key topology (Figure 5C). This structure, later classified as the polymorph “1c”,²²⁵ 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.²³⁹ The interprotofibril arrangement is characterized by the presence of staggered β -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 and 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 α S fibrils extracted from brains of individuals with multiple system atrophy (MSA) have been determined by cryo-EM.²⁴⁰ 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 α 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.²⁴⁰ Structures of brain-derived α -synuclein fibrils are distinct from fibrils obtained by recombinant expression and *in vitro* aggregation, as already observed for tau filaments.

3.2.3. A β 40/42 Fibrils. Because of its small size (compared to tau or α -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 β peptides. Early studies using FTIR and CD have demonstrated the high propensity of the β -sheet structure of A β fragments.²⁴¹ Pioneering work by the Tycko's laboratory using solid-state^{242,243} determined the β -turn- β (U-shape) fold

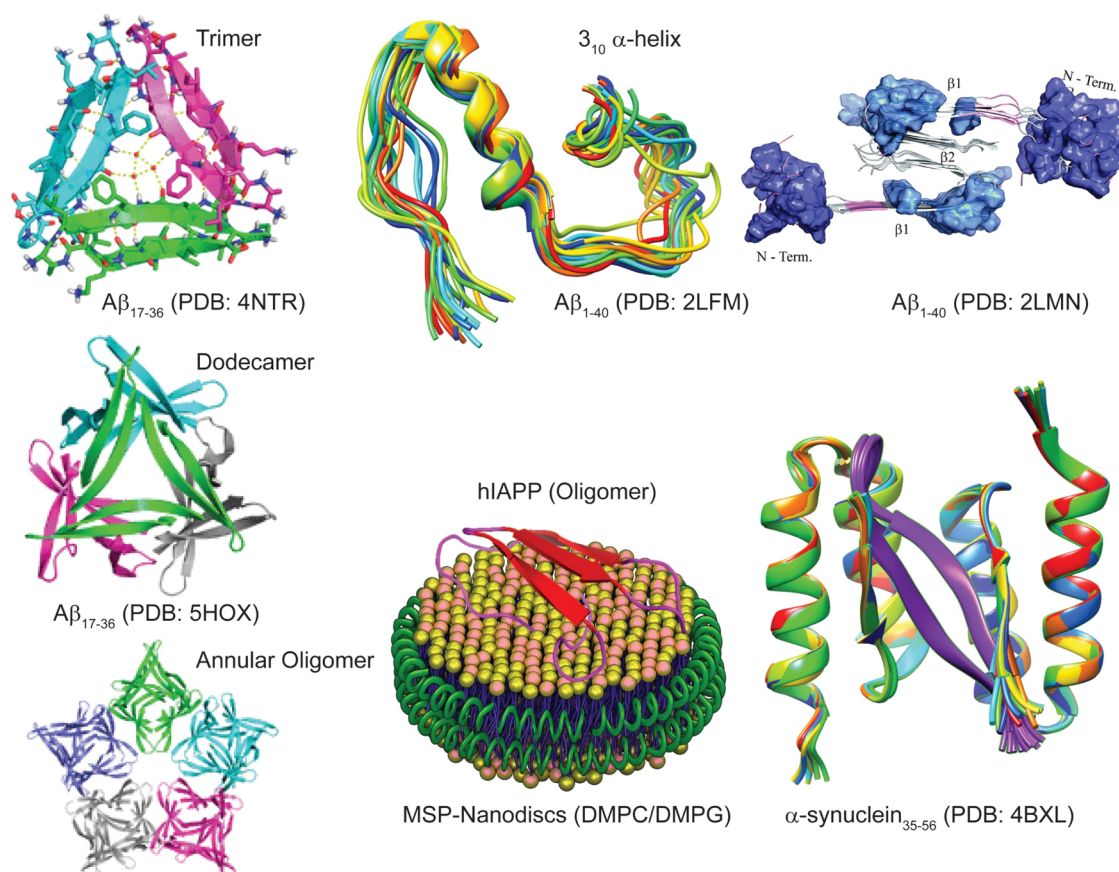


Figure 7. High-resolution structures of amyloid oligomers in solution or membrane-bound state solved using X-ray and NMR methods, with the exception being the model of A β 40 assembly toxic surface (top, right).

in an in-register, parallel arrangement for A β 40 fibril with residues 12–24 and 30–40 in β -strand conformations based on distance restraints and torsion angle measurements.²⁴⁴

Several 3D models of A β 40 and A β 42 (Figure 6A) have been proposed on the basis of ssNMR and cryo-EM,^{226,245–247} showing a diversity of intramolecular fold (U-shape or S-shape) and quaternary arrangement between A β 40 and A β 42. Small variations in the aggregation conditions can lead to distinct molecular conformation within the same peptide sequence and even different toxicity level.²⁴⁸ By combining cryo-EM and ssNMR, Schröder et al. presented a structure of A β 42 fibrils assembled at low pH which is to date the highest resolution model of A β peptides.²²⁶ As already observed for other pathogenic amyloids, the staggering of nonplanar molecules (Figure 6B) constitutes a unique feature that has profound implications for fibril growth mechanisms, because the binding sites of the two fibril ends are different, implying a polarity of subunit growth. To date no high resolution of A β 40 and A β 42 fibrils extracted from brains has been solved. Tycko et al. have reported conformational studies at high resolution of A β 40 and A β 42 fibrils seeded from brain extracts.^{249,250} 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 β 40 and A β 42 in this context revealed a predominant molecular conformation in A β 40, while A β 42 exhibited a larger degree of structural heterogeneity as revealed by ssNMR chemical shift analysis.²⁵⁰ It implies a greater

structural susceptibility of A β 42 brain seeds compared to those for A β 40, as already suggested.²⁵¹ As suggested for α -synuclein or tau, the existence of structure-based strains for A β 40 and A β 42, in analogy to conformational strains known for prion-like proteins, is still a matter of debate.

3.2.4. 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, with the N-terminus being at the periphery, but all models display substantial deviation.^{216,252} A very recent study by cryo-EM at physiological pH²⁵³ provides three polymorphs with the dominant one comprising residues 13–37 with two S-shaped, intertwined protofilaments (Figure 6C). The high similarity between this model and the A β 42 model from Gremer is striking considering the link between T2D and AD.

3.3. Structures of Transient Oligomers

3.3.1. A β and IAPP. The fibrillation of A β is preceded by a transition from a random-coil like structure to helical conformation where the latter facilitates the formation of β -sheet structured amyloid filaments.²⁵⁵ The helical conformation of A β _{1–40} monomer determined by NMR in a water-detergent solution adopts a helical structure in the K16–V24 and K28–V36 regions.²⁵⁶ The bihelical structure is shown to be disrupted in the oxidized A β _{1–40} and adopts a single helical region between residues K16 and V24.²⁵⁷ Another NMR study trapped a low population of an early intermediate of A β _{1–40} characterized by a 3₁₀ α -helix (Figure 7) spanning the core hydrophobic regions H13 to D23 in an aqueous medium containing no detergent.²⁵⁸ The partially folded 3₁₀-helical

structure has also been observed in computations²⁵⁹ 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} α -helical structure and shows a tendency to bind the hydrophilic N-terminus and the α -helical (H13 to D23) region.²⁶⁰

Since high-resolution 3D model structure determination of pathologically relevant low- and high-ordered $A\beta$ species is very challenging, a chemical cross-linking approach has been employed to homogenize the sample for structural determination. Macrocyclic β -sheet peptides that mimic β -hairpins of amyloid peptides and stabilize both low- and high-ordered oligomers have been developed for X-ray crystallography study.²⁶¹ Using $A\beta$ β -hairpin mimicking peptides,²⁶² a twisted β -hairpin triangular $A\beta$ trimer structure has been solved which also forms hexamers, dodecamers, and annular oligomers (Figure 7) through self-assembling that resembles annular structures reported for $A\beta$ oligomers by EM.^{263,264} These $A\beta$ oligomers present substantial neurotoxicity and thus are proposed to be useful molecular models of $A\beta$ oligomer for structure-based inhibitor designing. Stable $A\beta$ dimers and trimers characterized with parallel β -sheets and neurotoxicity have been developed by sequentially varying the position for cross-linking using chemical linkers or disulfide bridges.^{265–267} A combination of solution and ssNMR and AFM characterized highly disordered oligomers of $A\beta$ that were formed in parallel to the fibril formation process.²⁶⁸ A recent study employed pressure-jump NMR to observe the oligomerization of $A\beta$.²⁶⁹ 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).²⁷⁰ 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 β -sheet content.²⁷¹ 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 β -sheet structure.²⁷² $A\beta$ interaction with prion protein was reported to trap an antiparallel β -sheet oligomer but lacks 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 enhanced neurotoxicity.^{273–275} 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.²⁷⁶

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.^{277,278} NMR experiments have shown high-resolution structural structures for full-length human-IAPP,²⁷⁹ human-IAPP-1-19,²⁸⁰ and full-length rat-IAPP²⁸¹ peptides both in solution as well as in a membrane

environment. Investigation of the aggregation pathways revealed the formation intermediate species of IAPP,^{282–288} and Figure 7 shows 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.^{289–291}

3.3.2. α -Synuclein and Tau Proteins. In PD, oligomerization of α S has been shown to increase in individuals with Lewy pathology. A low-resolution structural evolution of individual α S oligomers characterized by antiparallel β -sheet structure is detected using AFM-IR.²⁹² Structural mapping of oligomers at all-stages of aggregation by AFM-IR highlighted the early oligomers (with 1 day incubation) are spherical in shape and dominated by a α -helix/random-coil secondary structure, but some of the oligomers showed mixed parallel and antiparallel β -sheet structures. The size of the spherical oligomers is observed to grow on day-2 with an increasing β -sheet and decreasing α -helix structures. Later on day-3, AFM-IR detected the presence of both spherical oligomers (α -helix/ β -sheet rich structures) and protofibrils with a predominant β -sheet structure.²⁹² The structure of α S oligomers observed under cryo-EM is shown to have a cylinder-like appearance and be characterized with a predominant β -sheet structure ($35 \pm 5\%$).²⁹³ The formation of early aggregates of α S and interaction between monomers and stable bioengineered oligomers are studied using fluorescence spectroscopy by labeling α S species with differently labeled fluorophores.²⁹⁴ 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.²⁹⁴ Another study showed α S oligomers do not seed the fibrillation reaction. This study underlined metastable spherical shape (10 nm), with a disordered conformation, for the α S oligomers by a small-angle neutron scattering method.²⁹⁵ 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 α S oligomers by selective fluorescent molecules is probed using a super-resolution imaging technique.²⁹⁶ The hydrophobicity of the surface of the oligomers is found to be comparatively higher than that of aged fibers evidencing α S aggregation proceeds via generation of toxic intermediates characterized with hydrophobic heterogeneity like that which has been reported for amyloid proteins and peptides.²⁹⁶ A recent study showed that α S promotes formation of $A\beta$ oligomers and inhibits fibrillation by stabilizing the oligomer structure.²⁹⁷ Notably, this effect is identified only by the soluble species of α S (monomers and oligomers) but not by fibers. EM studies revealed globular morphologies for the oligomer mixture of $A\beta$ and α S. While structural information for the $A\beta$ oligomers induced by α S has still not been reported, further investigation could provide more details to establish a correlation between AD and PD.²⁹⁷ The aggregation and folding of α 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.²⁹⁸ Among the five-proline residues, isomerization

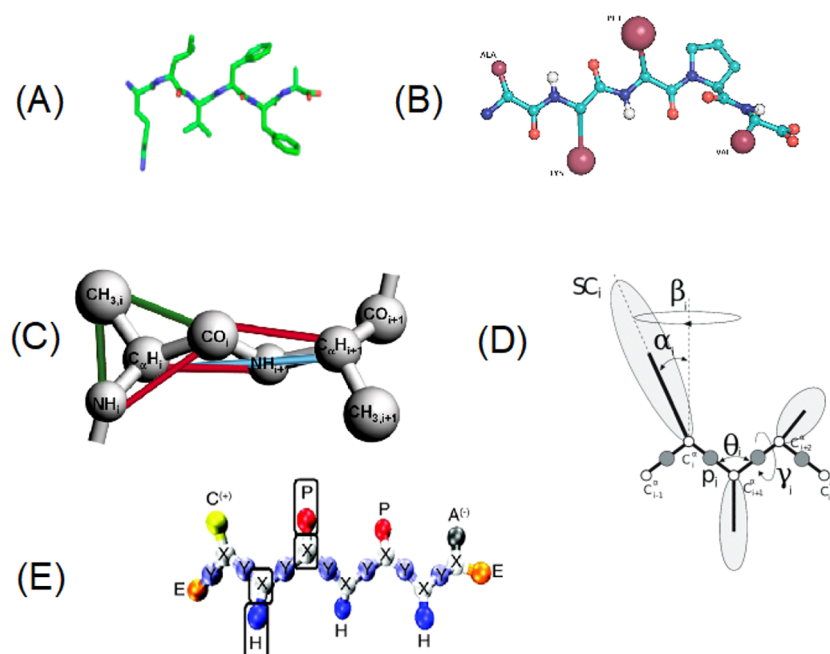


Figure 8. Protein representations from all-atom to coarse-grained models. (A) All-atom. (B) OPEP. (C) Geometric representation of the protein intermediate-resolution model, PRIME, for polyaniline. Covalent bonds are shown with thick gray lines connecting united atoms for N–H, C=O, C α –H, and CH₃ side chain. At least one of each type of pseudobond is shown with a colored line. Pseudobonds are used to maintain backbone bond angles, consecutive C α distances, and L-isomerization. The united atoms are not shown full size for ease of viewing. (D) UNRES. (E) Shea's model.

of P128 is identified to be catalyzed by cyclophilin A (CypA), a protein belonging to the family of peptidylprolyl isomerases. CypA and α S are colocalized in cells, where CypA interacts with α S with a micromolar binding affinity.²⁹⁸ NMR titration experiments, combined with a crystal structure of the α S–CypA complex, showed CypA binds to the amyloid core preNAC hydrophobic domain (residues 47–56) of α S²⁹⁸ and also is known to bind membrane.²⁹⁹ The α -synuclein complexed by affibody displays a β -hairpin spanning residues 35–56 by NMR (Figure 7).²⁶¹

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 to as Braak stages III and IV.^{300,301} Clusters of soluble tau multimers (globular) were observed in AFM with a measured height ranging from 10 to 30 nm.³⁰¹ TEM imaging analysis reported a similar globular morphology of chemically stabilized tau oligomers prepared *in vitro* and detected by oligomer specific antibodies.³⁰² Tau oligomer rich in β -sheet structure is shown to be influenced by both A β and α S oligomers.³⁰³ A recent NMR study reported tau monomers binding to the C-terminal disordered domain of α S monomer following the generation of toxic α S oligomers.³⁰⁴ Similarly, α 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 β -arrestin and molecular chaperones ubiquitously expressed in cells.^{305,306} An increase in tau level shown to increase β -arrestin impairs tau clearance by stabilizing toxic tau species and promotes the aggregation of tau.³⁰⁵ 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.³⁰⁶ Readers are referred to recent reviews on tau oligomers.^{307,308}

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.³⁰⁹ This self-assembly is a stochastic process and susceptible to a lot of nonspecific interactions. Moreover, the process is autocatalytic 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 such as for tau. Thus, for test tube experiments, reproducibility needs identical starting conditions. As the number and/or type of preaggregates are very difficult to control, the self-assembly process should be started with a sample of only monomeric species.¹⁹³ Obtaining pure monomeric species can be very difficult but depends on the peptide/protein under investigation.³¹⁰ Some are very strongly aggregating and hence difficult to monomerize.³¹¹ For instance α S is less aggregating than A β . Self-assembly of α S is often studied at higher concentration or stimulated by effectors, as it is slow.³¹² In contrast amyloid- β aggregates are very fast; particularly the A β 42 aggregates are faster than the more abundant A β 40 form. IAPP is another very fast aggregating peptide that is quite sensitive to pH and other conditions.

Amyloid- β (even A β 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,

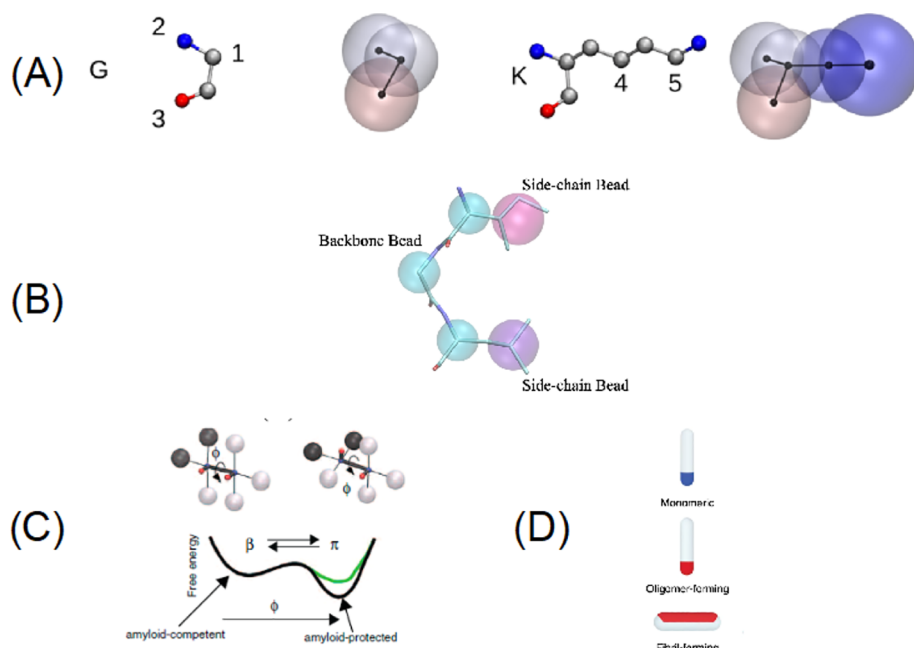


Figure 9. Protein representations from coarse-grained to mesoscopic models. (A) SIRAH. (B) SOP-IDP. (C) Caflisch's model. (D) Frenkel's model.

different batches of A β 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 a 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.).^{310,313} So, it is very important to report the complete details of the sample treatments including the monomerization steps and the use of several batches that are highly recommended, in particular for the very aggregation-prone amyloidogenic peptides (such as A β and IAPP).^{313,314}

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 steps. 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.

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.³¹⁵ A moderately coarse-grained model, PRIME (PRotein Intermediate resolution Model), was developed for use with DMD simulation to model the folding behavior^{316–318} and fibril formation of homopolypeptides, in particular, poly-

alanine.^{319,320} In the PRIME model, as shown in Figure 8C, each amino acid is represented by three backbone spheres N–H, C α -H, and C=O and one side chain sphere R. The two major nonbonded interactions captured in the PRIME model are the directional hydrogen bonding interactions between backbone N–H and C=O spheres and the nondirectional hydrophobic interaction between two side chain spheres. The other nonbonded excluded volume interactions between backbone spheres and side chain 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 side chain-side chain polar, electrostatic, and hydrophobic interactions between the 20 amino acids. This was achieved by Cheon et al.,³²¹ who used a perceptron learning algorithm to derive knowledge-based side chain-side chain 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.³²² 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 β _{16–22},^{323,324} hexapeptides³²⁵ designed by Serrano et al.;³²⁶ tau fragment;³²⁷ A β _{17–36},³²⁸ and A β _{17–42},³²⁹ and coassembly systems of A β _{16–22} and A β ₄₀,³³⁰ and the charge complementary peptide pair CATCH ($\pm$).³³¹

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 rodlike model by Frenkel³³² (Figure 9D), the lattice-based toy models by Thirumalai³³³ and by Frenkel,³³⁴

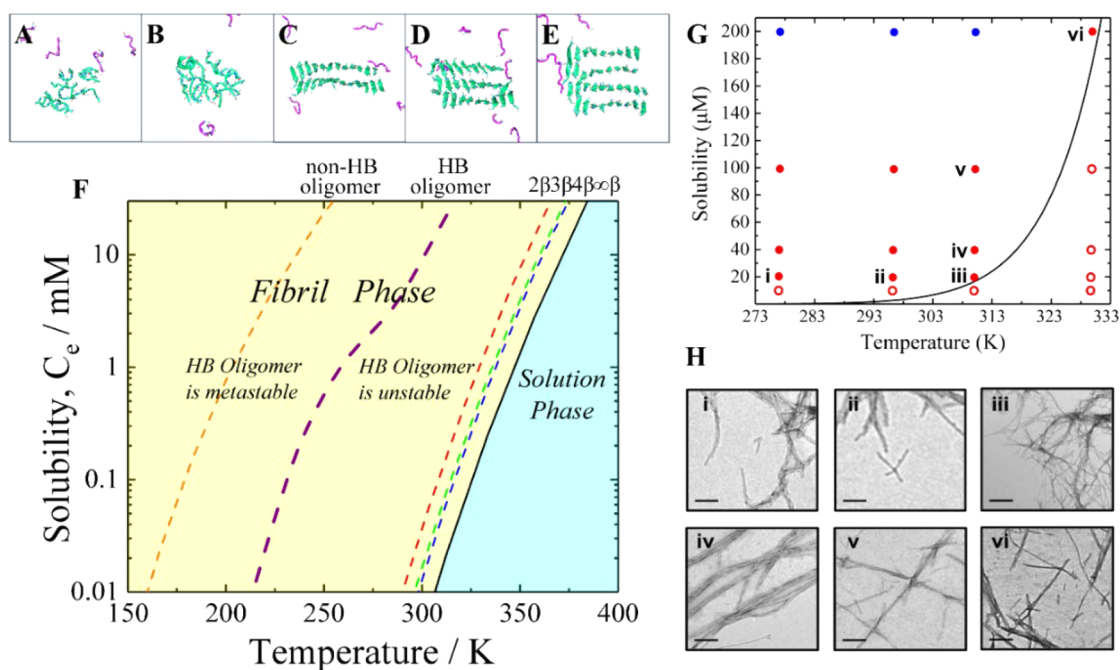


Figure 10. (A–E) Simulation snapshots of non-HB oligomer; HB oligomer; and two, three, and four β -sheet fibrils, respectively. The peptides in the five aggregates and in solution are shown in green and magenta, respectively. (F) Phase diagram for $A\beta_{16-22}$ peptide. Solubility, C_e , versus temperature 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^{352–357} 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 part A. (Scale bar: 200 nm.) Reprinted with permission from ref 350. Copyright 2019 National Academy of Science.

the midresolution off-lattice CG model of Shea³³⁵ (Figure 8E), the UNRES model by Scheraga³³⁶ (Figure 8D), the SOP-IDP model³¹ (Figure 9B), the OPEP model by Derreumaux^{337,338} (Figure 8B), the CG DMD model by Urbanc,³³⁹ the SIRAH CG model (Figure 9A) by Pantano,³⁴⁰ and the Caflisch's model (Figure 9C).^{12,341}

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 μ s) at each temperature (T), or equivalently, intermolecular energy (ϵ) and concentration (C), and then plot the boundary between the observed phases in the T–C or ϵ –C plane^{342–344}—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 Auer³⁴⁵ 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 Kashchiev³⁴⁶ first applied Monte Carlo (MC) simulation with the seeding approach³⁴⁷ to calculate the liquid–liquid coexistence line for helical-rich oligomers and the solid–liquid coexistence line for β -sheet-rich fibrils using a one-bead-per-residue homopolymer model.³⁴⁸ Later, Auer³⁴⁹ applied this amyloid nucleation theory approach and MC simulation to the same protein model to calculate the equilibrium solubility line for an infinite-layer β -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.³⁵⁰ To do this it was necessary to simulate many assembly and disassembly

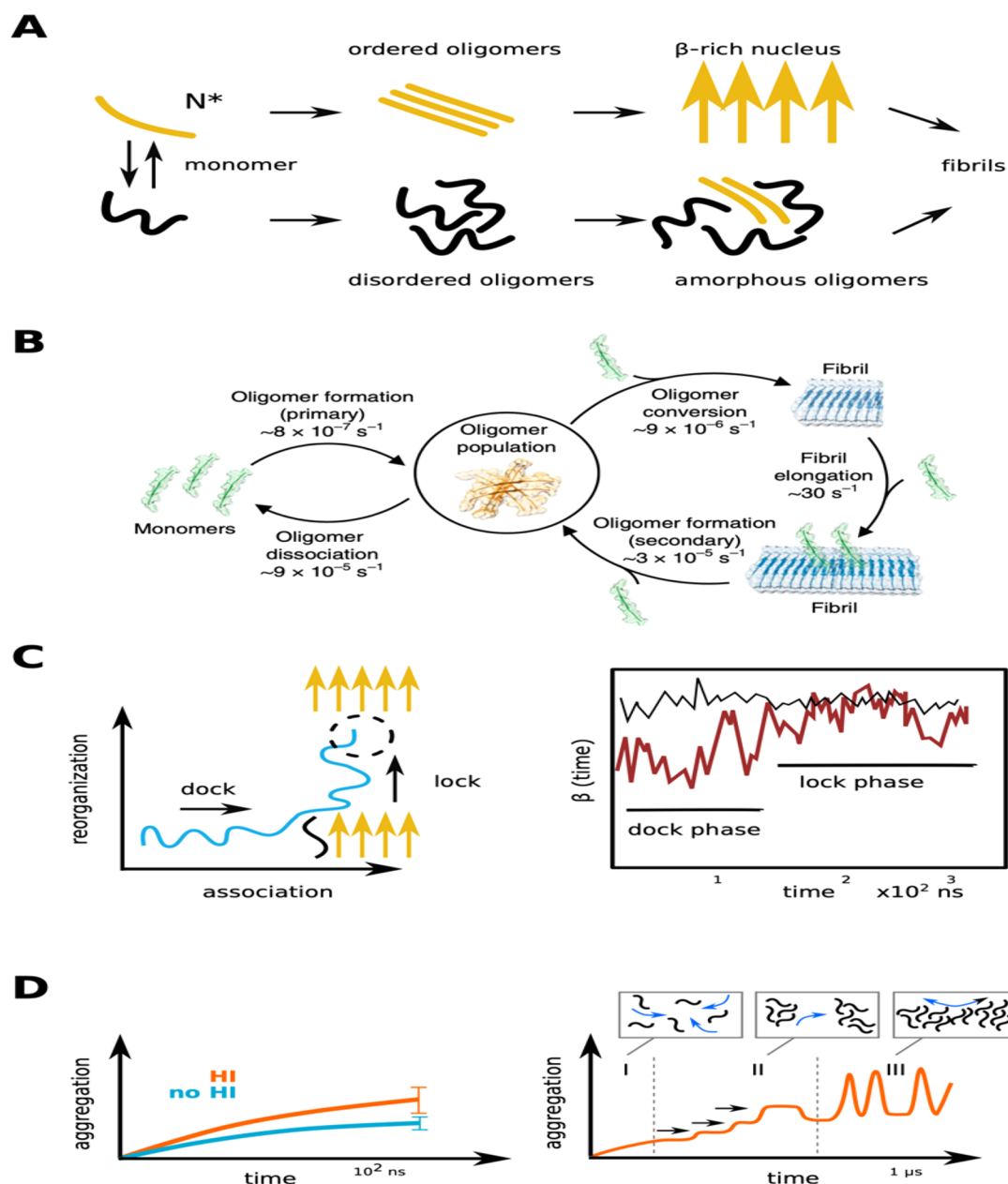


Figure 11. (A) Schematic representation of two extreme scenarios, the one-step and the two-steps nucleation process for early steps of amyloid aggregation.³⁶⁶ (B) Characteristic steps and kinetics of A β 42 amyloid proliferation determined experimentally, with the reaction rates at a concentration of 5 microM.³⁷⁰ (C) Description of the dock and lock mechanism, left chart schematically reproduces the results,³⁷⁵ where the evolution of the β -structure of a monomer is followed in time during the dock and lock phases. (D) Effect of hydrodynamic interactions on the aggregation process of A β _{16–22} peptides (left) and protofibril elongation due to oligomer fusion (right) as observed in LBMD simulations.^{366,394}

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 β _{16–22} fibril structures predicted using DMD/PRIME20 simulation agree well with experiment.³²³ First, we applied constrained canonical ensemble DMD simulations and the seeding approach^{346,347} to determine the solubilities of a series of small aggregates (disordered oligomers and 2, 3, 4 β -sheet A β _{16–22} fibrils) formed by the A β _{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 β -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 β -sheet fibrils

decrease with increasing thickness (number of β -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 β -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 β -sheets.^{19,351}

To estimate the solubility line for a macroscopic (i.e., thick) fibril, we adopt Auer's approach³⁴⁶ 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 β -sheet, $C_{i\beta}$, on fibril thickness (i) at fixed temperature was derived by Kashchiev and Auer:³⁴⁵

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

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, σ_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 β -sheet.³⁴⁹

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 (Figures 10G and 10H).

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 β 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, $A\beta_{42}$, amylin, and αS undergo a two-step nucleation process, first forming oligomers (due to the non-negligible intrachain interactions) that eventually merge, rearrange, and nucleate to form a fibrillar structure. Thus, the nucleation process may involve oligomer formation³⁵⁸ which may be accompanied by liquid–liquid phase separation.³⁵⁹ In this case, a nonclassical nucleation theory needs to be developed for *in silico* prediction of protein solubility and the comparison with experimental measurements.^{360–362}

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.³⁶³ The second compromise concerns the effective concentration that is used in simulations³⁶⁴ and that can be tuned so to favor the encounter of the molecules when the kinetics of aggregation and elongation is investigated. Finally, even with these simplifications in hand the size of the simulated systems is generally limited, generally with less than 10^3 monomers.^{365,366}

The two principal aspects that have been studied via CG models are the early steps of aggregation and the mechanism of fibril elongation.³⁶⁷ Peptide aggregation can be described as a nucleation process, but whether the primary nucleation is a one-step or two-step 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,^{12,368–370} with 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 attention has been posed on how the molecular propensity of the amyloid peptide to sample the aggregation β -prone state influences the path of the initial aggregation. Pellarin and Caflish³⁷¹ and Bellasia and Shea³⁷² 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 β -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.^{371–374} A nucleation diagram as a function of solution concentration and intermonomer 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.³⁶⁵

Computational studies focused also on the behavior of a monomer when aggregating. Following the study by Massi and Straub,³⁷⁵ two idealized pictures can be drawn (Figure 11C). In the 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 the 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 time scales of the dock and lock phases. The first one is limited by the diffusion and the energetics of desolvation, while the second one is controlled by the free energy barriers that separate peptide conformations and that can be affected by the local packing in the aggregate. According

to several estimates, a difference of at least 2 orders of magnitude for the characteristic kinetics exists, $\tau_{\text{lock}}/\tau_{\text{dock}} \geq 100$.^{376–379} Experimentally the elongation process results to be entropically driven with an entropy gain associated with 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.^{77,380,381} A very detailed picture of the dock and lock mechanism has been recently drawn by combining atomistic simulations and the Markov state model. It was shown that for a simple amylogenic peptide, the fragment A β _{16–22}, the locking phase requires a complex search in a multim minima landscape characterized by off-register configurations and with no evident funnel toward the fibrillar configuration.³⁸²

Simulations have also inquired the global process of elongation, and an asymmetric elongation has often been reported,³⁶⁷ 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^{365,366} and including lateral branching that uses the surface of the growing fibril as a seed.^{366,383} This finding confirms the theoretical intuition proposed to describe the kinetics of amyloid formation and that includes a key secondary nucleation process.^{309,384}

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 such as 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 the 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 behavior. In an 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^{385–387} and have been applied in several contexts, such as protein folding^{388,389} or protein motion under crowding.³⁹⁰ More specifically, the Brownian dynamics including HI has been used to investigate lipid aggregation using a simplified dumbbell model.³⁹¹ It was shown that HI speeds up the process since, as can be deduced by comparing the Zimm versus Rouse model for polymer motion,³⁹² when the first oligomers are formed their diffusivity scales more favorably with the aggregate size than when HI is not included in the simulation. A similar result was obtained for amyloid aggregation by lattice Boltzmann MD simulations with the OPEP model for A β _{16–22} peptides,^{393,394} see Figure 11D.

Notably the presence of HI coupled to the high-resolution and flexible OPEP model enhances size fluctuations of the formed oligomers. These 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.³⁶⁶

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.³⁹⁵ It was also reported that the action of shear alters the morphology of the formed fibrils.^{396,397} The energetic landscape of β 2-microglobulin fibrils was explored by combining isothermal titration calorimetry and stirring.³⁹⁸ 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 is 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 coacting during fibril elongation. Namely shearing is used to enhance the kinetics of the fragmentation contribution over secondary and primary nucleation.³⁸⁴

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,^{399–402} values much higher than what was found typically in physiological conditions that are $<10^4$ – 10^5 s^{−1}. In a recent study elongational flow was used to perturb and trigger aggregation of several proteins, β 2-microglobulin, bovine serum album (BSA), and monoclonal antibodies.¹⁶ It was shown that when exposed several times to an elongational flow at shear rate $\sim 10^4$ – 10^5 s^{−1} even a stable protein such as BSA can expose to solvent parts of the sequence generally screened in the folded state and can 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 favor 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.⁴⁰³

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 such as 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 favorable impact on the aggregation with respect to a dilute reference solution. On the other hand, moving in a crowded space is more difficult, and the diffusion of a species is reduced as an effect of the reduced dimensionality and higher viscosity of the solution. The molecular collisions needed to start the nucleation, and the further elongation becomes rarer. Finally, the macromolecules surrounding the aggregating species are

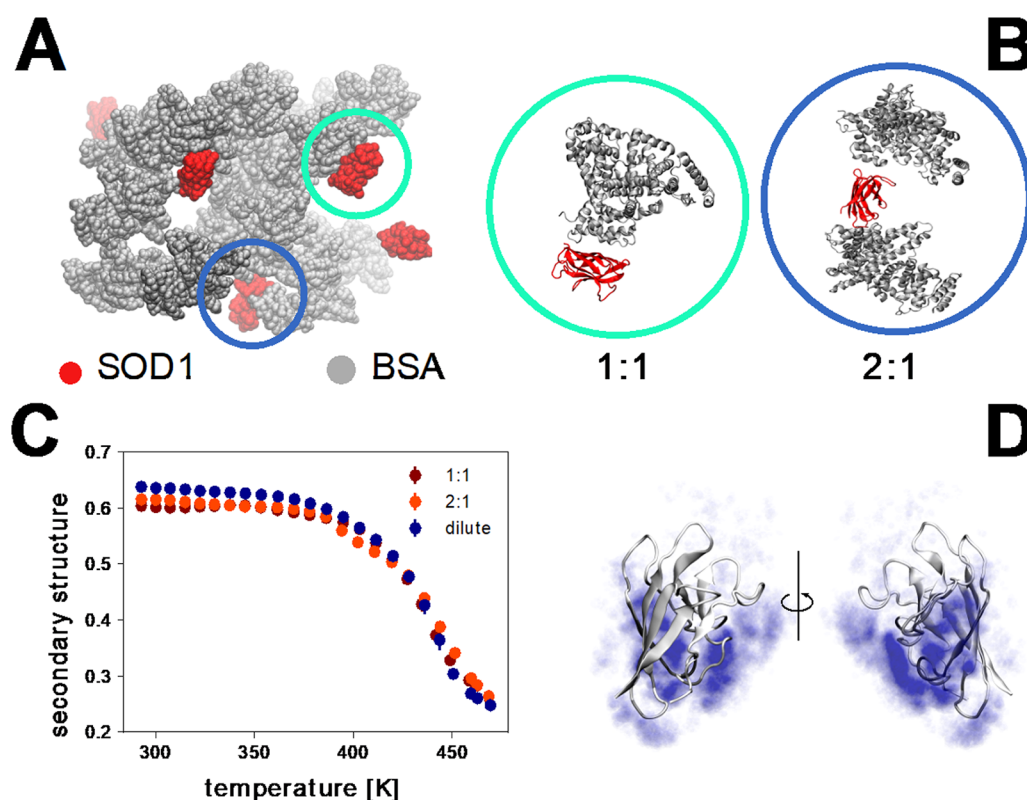


Figure 12. Unfolding and stability of SOD1 in crowded conditions.^{408,415} (A) Snapshot from a 1 μ s coarse-grain LBMD simulation of loop-truncated SOD1 monomers immersed in a 200 g/L BSA solution.⁴⁰⁸ (B) Representative states of local packing around SOD1 extracted from the coarse-grain simulation and converted into a fully all-atom representation. (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.⁴¹⁵ (D) Semiunfolded 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 denatured region has an increased probability to interact with the crowder.⁴¹⁵

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 (polyethylene glycol), or proteins of different sizes, for example BSA proteins generate large excluded volumes. NMR studies reported that depending on the nature of crowders the α 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.⁴⁰⁴ The ensemble of configurations explored by α S can also be tuned by two-dimensional crowding on a lipid surface.⁴⁰⁵ 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.⁴⁰⁶ Moreover, the morphology of the formed fibrils is also affected by crowding, for instance Ficoll induces a more packed state of β 2-microglobulin fibrils.³⁹⁸

However, the heterogeneity of *in vivo* crowding cannot be easily mimicked in *in vitro* setups, and the effect on protein mobility, conformation, and stability is subtle. While the 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.^{407,408} For the B1 domain of protein

G, the stability effect of a single amino-acid mutation was found to be amplified 10-fold by the crowded cytosol of *Escherichia coli*.⁴⁰⁷ 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.⁴⁰⁸ Interestingly, for monomeric α 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.¹⁸⁵

However, concerning the general picture, fibrillar aggregation of SOD1⁴⁰⁹ or Huntingtin exon 1 protein⁴¹⁰ in cells has been characterized by means of different techniques, NMR and fluorescence spectroscopy, respectively, and the obtained results are similar to what was found in *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 *in vivo* experiments.

A few simulations have tried to shed light on the mechanism of aggregation under crowding. A first set of studies has focused on the effect of crowding on the conformation sampled by IDPs and using different resolution models to compare with experiments such as SAXS or FRET.^{411–413} As an example, we refer to the work by Zhou et al.⁴¹² 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 α) were investigated in a PEG crowded solution. A good match with experimental FRET data was obtained only when a certain

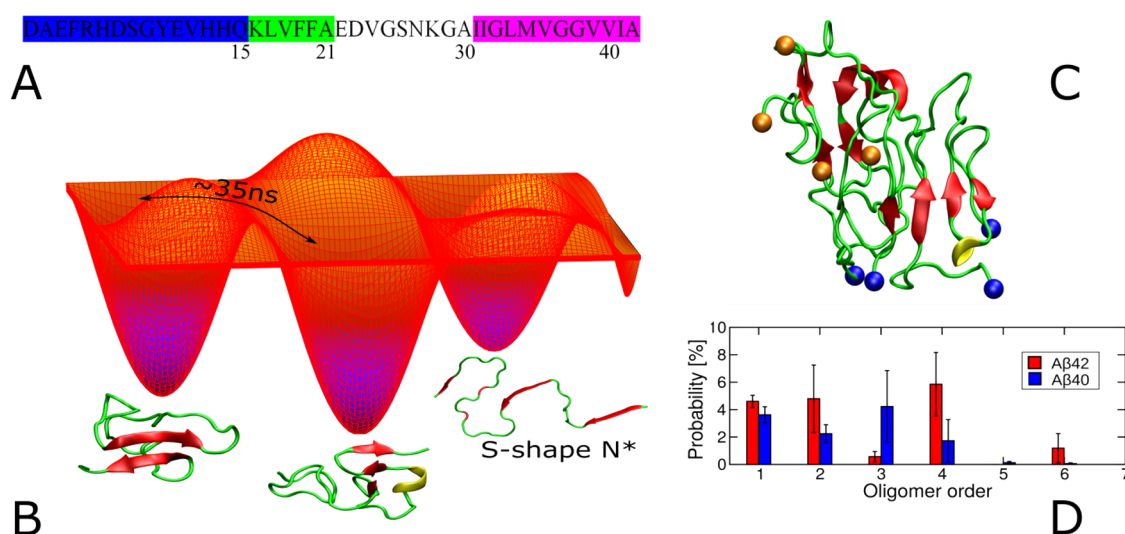


Figure 13. (A) N-terminus (blue, 1–15), CHC (green, 16–21), loop (gray, 22–29), and C-terminus (purple, 30–40/42). (B) Schematic free energy landscape of $A\beta$ monomers. Switching between some conformations occurs within 35 ns, as reported by FRET data.⁴²⁸ The conformation of S-shape N* from the fibril structure with PDB ID 2NAO was also sampled in CG simulations.⁴³⁷ (C) Representative structure of the $A\beta$ 42 tetramer, obtained by using multiscale MD simulation.⁴⁵¹ 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.⁴⁶³

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.⁴¹²

In a second class of work the focus was placed on the aggregation kinetics. In the presence of inert crowders modeled as hard spheres it was shown that $A\beta_{16-22}$ amyloid peptides simulated by the PRIME-DMD generally collapse quickly in disordered aggregates, and the structuring phase occurs as a second step.³²⁴ However, when the nature of the crowder is specified, e.g., being modeled as hydrophobic particles, the interactions with the peptides become competitive with the interpeptide interactions, causing the extension of the lag time of fibril elongation.³²⁴ 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.⁴¹⁴ For peptides with low aggregation affinity, the formation of a fibril is rate limited by the creation of a first nuclei, and the crowders tend to stabilize the nuclei and therefore impact favorably 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 multiscale strategy as recently proposed for investigating the effect of crowding on the stability of the aggregation prone SOD1 protein;^{408,415} 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 was 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 β 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.

5. EXTENSIVE SIMULATIONS ON MONOMERS AND SMALL OLIGOMERS

We discuss $A\beta$, α -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).

5.1.1. 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.^{80,416–422} All-atom REMD simulations with both an implicit^{421,422} and explicit solvent^{80,419} confirmed that, in agreement with NMR relaxation data,⁴²³ the C-terminus of $A\beta$ 42 is more rigid and has a higher β -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 α -helix content ($\leq 10\%$) is lower than the β -strand content (10–27%), which is consistent with CD data showing the α -helix content is about 9% and the β -content is between 12 and 25%.^{33,424} NMR studies suggested the presence of an antiparallel β -sheet between CHC and residues 29–36 for monomeric $A\beta$ 42,⁸⁰ and this was confirmed by REMD simulation using CHARMM36m, FF14SB-IDPs, FF14SB, and FF99SB force fields.⁴¹⁸ A small β -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.⁴²⁵ 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 due to salt bridges with other fragments playing a secondary role.⁴²⁶

Table 1. Computational Studies on Monomers and Oligomers of A β Alloforms in Solution after 2015

ref	force field	Solvent model	Method	Time scale	A β alloform	Oligomer size
Rosenman ⁴¹⁹	OPLS-AA/L AMBER99SB-ILDN CHARMM22*	TIP3P-Ew TIP4P TIP3P	REMD	52 μ s	A β 42	1
Weber ⁴¹⁶	AMBER FF99SB CHARMM22/CMAP	TIP3P	REMD	5.28 μ s	A β 42	1
Carballo-Pacheco ⁴⁷⁶	OPLS, AMBER99SB, AMBER99SB-ILDN, AMBER99SBILDN-NMR, CHARMM22*	TIP3P TIP4P-Ew	REMD	6.4 μ s	A β 42	1
Meng ⁴²⁸	AMBER99SB	TIP4P/2005	REMD	740 ns	A β 42, A β 40	1
Krupa ⁴¹⁸	AMBER14SB AMBER14SB_IDPS AMBER99SB CHARMM36 CHARMM36m	TIP3P	REMD	28.8 μ s	A β 42	1
Thu ⁴³⁵	OPLS	GB	REMD	6 μ s	A β 42 and 19 variants	1
Frigori ⁴³⁸	Charmm22*	TIP4P/Ew	REMD	19.2 μ s	A β 42, A β 40	1
Aggarwal ⁴³⁴	AMBER99SBILDN	SP C/E	MD	100 ns	A β 42 and 5 variants	1
Li ⁴⁶⁸	AMBER99SB	GB	REMD	8 μ s	A β 40, A β 40-A2 V and tautomer	1
Liu ⁴⁶⁹	AMBER99SB-ILDN	TIP3P	MD	0.4–1.8 μ s	A β 42	1
Bhattacharya ⁴²⁶	CHARMM36, CHARMM22*, CHARMM36m, AmberA03, AmberA03Ws	mTIP3P, TIP4P, TIP4P-D, TIP4P-Ew	MD	33 μ s	A β 42	1
Robustelli ⁴⁶⁵	AMBER99SB*-ILDN, CHARMM22* CHARMM36m, AMBER03ws, AMBER99SB-ILDN AMBER99SB-disp	TIP3P TIP4P-D	MD	30 μ s	A β 40	1
Zhang ⁴⁴⁰	AMBER99SB-ILDN	TIP3P	MD	4 μ s	A β 42	2
Man ⁴⁴⁴	AMBER99SB-ILDN	TIP3P	REMD	25 μ s	A β 42 WT, S8C	2
Man ⁴⁴⁵	OPLS-AA, CHARMM22, AMBER99SB-ILDN, AMBERSB14	TIP3P	REMD	36 μ s	A β 42	2
Nguyen ⁴⁴⁸	CHARMM22*	TIP3P	REMD	24 μ s	A β 40, A β 40-A2 V	2
Nguyen ⁴⁴⁷	CHARMM22*	TIP3P	REMD	24 μ s	A β 40-A2T	2
Cao ⁴⁴⁹	PACE	MARTINI	REMD	2.7 ms	A β 40	2
Hashemi ⁴⁷⁰	AMBER99SB-ILDN	TIP3P	MD	4 μ s	A β 40	2
Das ⁴⁴³	OPLS-AA	TIP3P	REMD	51.2 μ s	A β 42, A β 42-A2T	2
Sharma ⁴⁶⁷	CHARMM36	TIP3P	MD+REMD	1.5 μ s	A β 42-WT, A β 42-A2 V, A β 42-A2T	2
Mehrazmaal ⁴⁴²	GROMOS96–53a5 AMBER99SB-ILDN	SPC	MD	9.5 μ s	A β 42	2
Press-Sandler ⁴⁷¹	CHARMM36/CMAP	TIP3P	MD	200 ns	A β 42	2
Nguyen ⁴⁵¹	UNRES AMBER99SB-ILDN, OPLS-AA/L	TIP3P, TIP4P	MD+REMD	50 μ s	A β 42	4
Xi ⁴⁵⁸	CHARMM36	TIP3P	MD	800 ns	A β 42	3–4
Nguyen ⁴⁵⁹	AMBER99SB-ILDN, OPLS, AMBER99SB, CHARMM36m	TIP3P, TIP3P-modified, DISP	REMD	101.9 μ s	A β 40, A β 42	4
Man ⁴⁶⁴	AMBER14SB	TIP3P	MD	22.5 μ s	A β 42	2–4
Zhang ⁴⁵³	DMD4B-HYDRA		DMD	21.6 μ s	A β 40, A β 42	32
Voelker ⁴⁵⁶	OPLS-AA	TIP3P, SPCE	MD	24.35 μ s	A β 40, A β 42	1–5
Barz ⁴⁶³	OPLS-AA	GBSA	MD	2.5 μ s	A β 42, A β 40	20
Zheng ⁴⁶¹	AWSEM		MD, umbrella sampling		A β 40	1–8
Zheng ⁴⁶²	AWSEM		MD, umbrella sampling	1 μ s	A β 42	1–8

The existence of such helices and the formation of α -sheets may promote the formation of an α -sheet in the lag phase.⁴²⁷

Meng et al. performed MD simulations and sm-FRET studies to explore the conformations of A β 40 and A β 42 at physiological conditions.⁴²⁸ They found that, similar to recent NMR data,²⁵⁵ both peptides adopt random coil conformations with A β 42 being slightly more compact than A β 40. Sm-FRET revealed⁴³⁵ that some conformations rapidly interconvert, and

nanosecond fluorescence correlation spectroscopy provided the time scale of the transition of about 35 ns (Figure 13B).⁴²⁸ The same time scale was also obtained for other IPDs.⁴²⁹ The application of NMR-guided metadynamic sampling for A β 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.⁴³⁰

Both experiment and simulation provided evidence that the aggregation rates of proteins are controlled by hydrophobicity, charge, and secondary structure.^{431–434} In a pioneering work by Chiti et al.,⁴³² the latter factor was accessed as the propensity to convert from α -helical to β -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.^{432–434} To solve this problem, Thu et al.⁴³⁵ calculated the β -content of 19 mutants of A β 42 using REMD simulation with the OPLS force field and implicit water model and related it with the experimentally measured aggregation rate κ . They found $\kappa \sim \exp(c\beta)$, where $c = 0.071$ and β is the percent of β -content, implying that the higher the β -structure in the 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.³³³ In lattice models N^* is a single state,⁴³¹ while in off-lattice models it comprises an ensemble of states in the same basin.^{436,437} 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 τ_{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.⁴³¹ Chakraborty et al.⁴³⁷ used eq 3 to understand why A β 42 aggregates faster than A β 40 and to shed light on the kinetics of fibril polymorphism. Using the SOP-IDP model³¹ (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 β 40 monomer. For A β 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^*} , τ_{fib} of S-bend A β 42 fibril is around 24 times smaller than A β 40.⁴³⁷ This prediction is consistent with recent experiments showing that the rate of A β 42 fibril formation is about an order of magnitude higher than A β 40. The higher population of N^* in A β 42 than in A β 40 was also confirmed by all-atom simulations.⁴³⁸ If the U-bend conformations are identified as N^* , then the aggregation occurs only two times faster than A β 40.⁴³⁷ 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.³⁷¹ Assuming that the A β 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 β 42 fibril is more stable than the U-bend form, as the latter forms faster.⁴³⁷

5.1.2. Dimers. The dimer has been subjected to many studies.^{439–450} Zhang et al.⁴⁴⁰ combined MD and MC pulling simulations with AFM experiments to obtain models for A β 42 dimers. First, various structures were generated using MD and then an external force was applied to the C α 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 β 42 dimer structures are mainly stabilized by the intermonomer interactions within the CHC regions and do not contain long β -strands that occur in the fibril state. An AFM-based force clamp was also applied for dissociating A β 42 dimer and identified two transient states with lifetimes of 188 ± 52 and 317 ± 67 ms.⁴⁴¹

By performing 9.5 μ s MD with GROMOS96-53a5 and AMBER99SB-ILDN force fields in SPC solution, Mehrzama and Rauk reported⁴⁴² that in addition to CHC, the hydrophobic C-terminal also plays a key role in A β 42 dimer stabilization. Unlike other groups,^{440,443–445} their models are richer in β -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 β 42 dimers are random coil stabilized by nonspecific interactions.⁴⁵³ 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 β 40 dimer and its mutants in solution.^{446–448} They found that the wild type dimer at 315 K is highly disordered, but the secondary structure of the β -strand and α -helix is richer than that of the monomer. Although conformations with an unstructured N-terminus and β -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.⁴⁴⁹ carried out a 2.7 ms simulation in order to investigate the mechanisms of formation of A β 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 review⁴⁵⁰ on the application of MSM to study the aggregation of amyloid peptides). Hairpin-like structures occur through one step nucleation, in which two preformed β -hairpins spanning residues 16–35 occasionally associate, preserving the β -hairpin conformation. This process occurs on a time scale of about 200 μ s, which is ~ 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.⁴⁴⁹

5.1.3. Oligomers. The A β 42 tetramers were explored by a multiscale approach involving REMD simulation with the UNRES force field (Figure 8D) followed by all-atom MD to refine the most representative CG structures.⁴⁵¹ 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 ($\sim 2000 \text{ \AA}^2$), which falls into the experimental range,⁴⁵² is lower than that of fibril ($\sim 2600 \text{ \AA}^2$). The high population of the β -structure in residues 9–14, 17–21, and 30–40 is in agreement with the experiment, and A β 42 models are dominated by turn and coil, which is also consistent with experimental observations. Interaction with solvent promotes compactness while the interchain electrostatic interaction facilitates the

Table 2. Experimentally Guided Molecular Dynamics Simulations of α -Synuclein Monomer

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

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.⁴⁵¹

Employing the DMD4B-HYDRA force field and DMD simulation, Zhang et al.⁴⁵³ 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 experimental⁴⁵⁴ and simulation⁴⁵⁵ works confirmed that the oxidative reactivity of Cu- $A\beta$ catalyzes the formation of Tyr–Tyr cross-links in peptide dimers. Voelker et al.⁴⁵⁶ 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 group⁴⁵⁷ 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.⁴⁵⁸ built out-of-register $A\beta$ 42 assemblies and also discovered barrel-shaped structures consisting of β 2-turn- β 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 β -barrel structures, which are different from out-of-register barrels,⁴⁵⁸ was probed by REMD simulation with four atomistic force fields.⁴⁵⁹ In aqueous solution, due to a change in the CHC–CHC and C-end-C-end interfaces, a β -barrel structure, made of eight antiparallel β -strands covering residues 9–40/42 with two distinct β -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 AWSEM⁴⁶⁰ (associative memory, water-mediated, structure, and energy model) force field, Zheng et al.⁴⁶¹ 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 cylindrin-like shape, consisting mainly of antiparallel β -strands, while fibrillar oligomers contain only parallel β -sheets.⁴⁶¹ 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.⁴⁶²

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 (generalized Born solvent area).⁴⁶³ 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; that is, $A\beta$ 40 primarily populates dimers, trimers, and tetramers, while $A\beta$ 42 predominantly forms dimers, tetramers, and hexamers (Figure 13D), consistent with experiment⁴⁵² 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.⁴⁶³

Finally, Man et al.⁴⁶⁴ 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,⁴⁶⁵ there are divergences between the simulation results on kinetics and thermodynamics,⁴⁶⁶ the impact of mutations,^{467,468} the population of critical states,⁴⁶⁹ and the shapes of oligomers.^{470,471}

5.2. α -Synuclein in Solution

α S can form oligomers that are toxic to neuronal cells and cause cell death.^{472,473} To understand the aggregation mechanism and develop promising neuroprotective strategies, knowledge of the conformational characteristics of monomeric α S may be critical. The structural characterization of α S by traditional biophysical experiments or MD simulations has been challenging.^{474,475} Force fields developed for folded

proteins in MD simulations tend to predict more compact structures of IDPs that contradict experimental measurement.⁴⁷⁶ To address these challenges, scientists modified force fields to improve the simulations of IDPs.^{477–481} Robustelli et al.⁴⁶⁵ 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 modeling. Pietrek et al.⁴⁵ 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 α S captured the local structures from NMR and the overall dimension from SAXS data.⁴⁵ Experimentally constrained computational simulations have been widely applied to predict the α S atomistic structures (Table 2).^{482–488} Brodie et al. used a novel approach that integrates protein cross-linking constraints and discrete molecular dynamics (CL-DMD) simulations.^{488–491} Adding experimental data to DMD simulations reduces the conformational space and allows the simulations to achieve accurate protein folding on a reasonable time scale.^{489–494}

Computationally predicted ensembles of α S conformations revealed the interactions that stabilize the monomer and the structures that drive the early stages of aggregation.⁴⁹⁵ The interaction between NAC and C-terminal was proposed to protect the highly hydrophobic NAC region from aggregation.^{482,483} However, the interaction between the N- and C-terminals places the NAC region in a solvent exposed orientation, which might trigger α S aggregation.⁴⁸⁴ Secondary structures, such as folded stable helices within the N-terminal and the NAC region,^{496,497} slow down the fibrillation rate by inhibiting the formation of partially structured helices.^{433,498} On the other hand, β -sheets and α -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.^{488,499–503} Interestingly, transient trefoil knots were formed in an all-atom simulation with explicit solvent,⁵⁰⁴ and the authors deduced that the knots should increase accumulation of the protein and, hence, induce multimeric aggregation.

The conformational ensemble of monomeric α S predicted by CL-DMD revealed interaction between the NAC region and the C-terminal portion and secondary structures, such as α -helix near the N-terminus and β -sheet in the NAC domain, which are in agreement with the predictions from the other simulations.⁴⁸⁸ The contents of α -helix ($\sim 2.4\%$), β -structure ($\sim 29.1\%$), and other secondary structures ($\sim 68.5\%$) are consistent with the data from other simulations and CD studies of the monomer (α -helix $3 \pm 1\%$, β -sheet $23 \pm 8\%$, and random coil $74 \pm 10\%$).^{477,488,505,506} The structures of the cluster centroids (Figure 14), however, are more compact than those determined by simulations in combination with PRE-NMR, FRET, or SAXS data.^{482,485,488} This might be due to the short-range cross-linking data used in the study. A total of 44 cross-links were used for CL-DMD simulations, including the cross-linkers EDC, TATA, SDA, and ABAS with spacer lengths of 0, 5, 5, and 7 Å, respectively. The maximal sequence separation by cross-linkers is from the N-terminus to E126, and the minimal sequence separation is from D121 to Y125 or

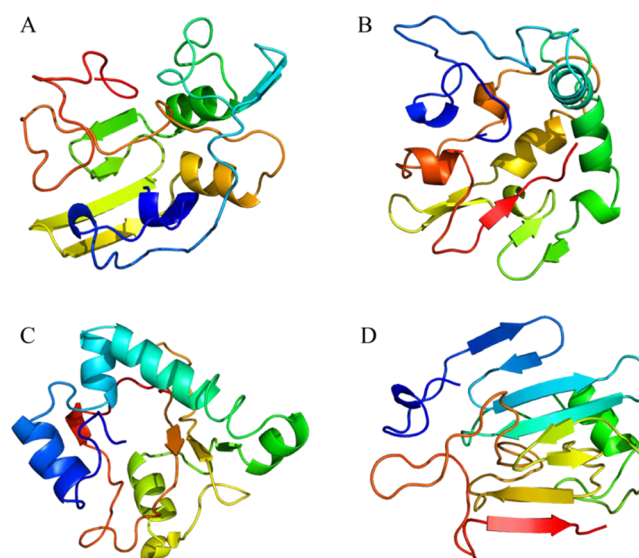


Figure 14. Representative structures of the α S conformational ensemble obtained by short-distance cross-linking constraint-guided DMD simulations.⁴⁸⁸ Conformers A, B, C, and D represent the first four clusters. Structures are colored from blue (N-terminus) to red (C-terminus). The figure was created using Pymol.²⁵⁴

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 α -synuclein.⁴⁸⁸ 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.^{476,477,485,507,508} This inconsistency and the large R_g values might be attributed to the multimeric states of α S under the experimental conditions. Moreover, the lower energy conformations are verified by the long-distance cross-linking, hydrogen–deuterium exchange, surface modification, and CD data, suggesting that the predicted compact structural ensemble by CL-DMD is convincing.

Apart from the monomeric state of α S, MD simulations have been used to study dimeric and tetrameric conformations. In dimeric conformations, stable parallel or antiparallel β -sheets are formed within the NAC region through hydrophobic interactions.^{495,496,509} Dimer structures with β -sheets could generate stronger interactions between the monomers driving the oligomerization process toward fibrillar aggregation.⁵¹⁰ Experiments revealed that α S can form a tetramer within the native cell environment.¹⁸⁷ Simulations exhibit that the tetramer has less stable interchain β -sheets than the dimers, while the helices are more stable in the tetramer.⁵⁰⁹ 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.^{484,511}

Overall, simulations of α S predicted its heterogeneous conformational ensemble comprising metastable states.⁵⁰³ 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.

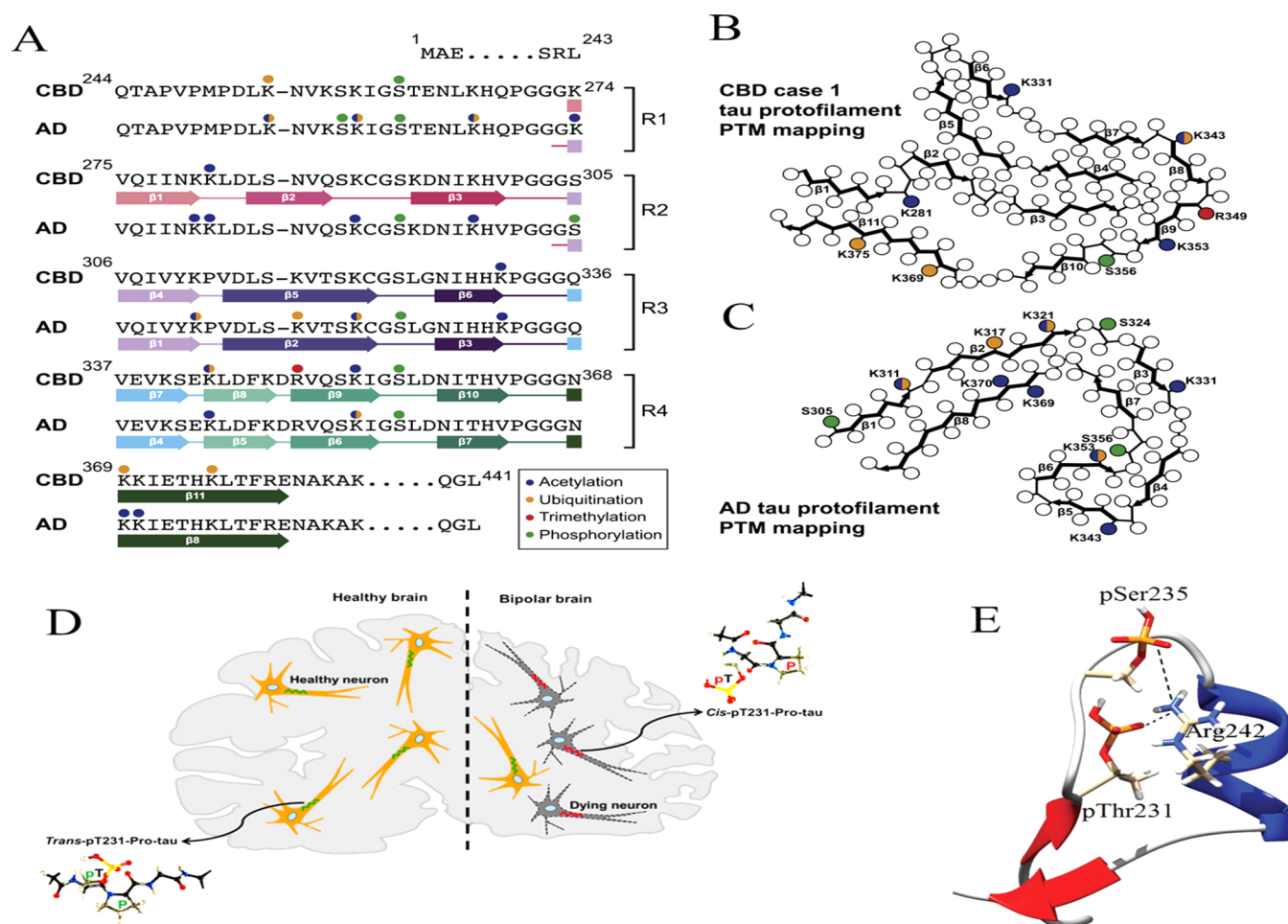


Figure 15. (A) Sequence alignment of the R1–R4 repeats and the sequence after R4 that are part of the CBD and AD fibril cores. The positions of filamentous β strands in both diseases are shown. PTMs detected by MS in tau fibrils from CBD case 1 and AD. The cryo-EM structures are shown with acetylation, ubiquitination, trimethylation, and phosphorylation sites marked with blue, orange, red, and green balls, respectively. Side chains with multiple PTMs detected are shown with two colors.⁵¹⁸ (B and C) PTMs 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.⁵¹⁸ (D) *cis* pT231-tau is highly neurotoxic and acts as an early driver of tauopathy, with bipolar illustrated here.⁵²⁵ (E) Snapshot from the simulation showing the α -helix and two salt bridge interactions (pThr231-Arg242 and pSer235-Arg242) of peptide htau_{225–250}.⁵²¹

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).^{159,160,512} 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).¹⁶¹ Furthermore, AD P-tau seeds hyperphosphorylated tau to form aggregates, which resist the dephosphorylation by PP2A, resulting in hyperphosphorylation and pathology of tau.⁵¹³ 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.⁵¹⁴ 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.⁵¹⁵ 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 increased deposition of pSer-324-positive tau has been observed in both 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.⁵¹⁵

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.⁵¹⁶ Heparin-induced tau and *in vitro* phosphorylated tau have different conformations, properties, and activities.⁵¹⁷ 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.^{518–523}

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).⁵¹⁸ By comparing the structures and PTMs of tau filaments from CBD and AD, it is found that phosphorylation occurs largely in the fuzzy coat. Previously, Xu et al. explored the conformational consequences of hyperphosphorylation on tau and also primarily in the fuzzy coat region.¹⁸³ 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.¹⁸³

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 the 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/Thr205.⁵¹⁹ Using the time-resolved FRET method, Chin et al. studied the property of tau173–183 (AKTPAPKTPP)⁵²⁴ 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.^{520,531} Lyons et al. modeled 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 β -sheet pattern (²²⁶VAVVR²³⁰ and ²⁴⁴QTAPVP²⁴⁹). 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 α -helix (²³⁹AKSRLQ²⁴⁵). They found that pThr231/pSer235 forms a salt bridge with Arg242 (Figure 15E).⁵²⁰ Another study provided a 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.⁵²¹ Two different phosphorylation patterns were investigated: 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 β -sheet structure observed in Tau225–246. They also observed the formation of a transient α -helix (²³⁸SAKSRLQ²⁴⁴) when Tau225–246 is phosphorylated at four sites.⁵²¹

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. A 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.⁵²⁵

Antibodies have been developed to specifically recognize this unique phosphor-epitope in tau. The Fab fragment forms a complex with the tau peptide ²²⁴KKVAVVR(pT²³¹)PPK-(pS²³⁵)PSSAKC²⁴¹.⁵²⁶ In the Fab-peptide cocrystal structure, 10 amino acids (²²⁵KVAVVR(pT)PPK²³⁴) are visible, of which six (²²⁵KVAVVR(pT²³¹)) 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,⁵²⁶ 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 nonphosphorylated peptides with the same sequence.⁵²⁶

Configuration specific antibodies were developed to distinguish cis- and trans-tau.^{527,528} 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.⁵²⁷

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, Tau208–324.⁵²⁹ 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.⁵²⁹ 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.⁵³⁰ 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.⁵³¹ The results show that intramolecular electrostatic interactions are coupled to the isomeric state of the peptidyl prolyl bond. Intramolecular electrostatic interactions are better formed in the trans isomer; however, the loss of intramolecular interactions and the more restricted conformational ensemble of the cis isomer could favor self-aggregation.⁵³²

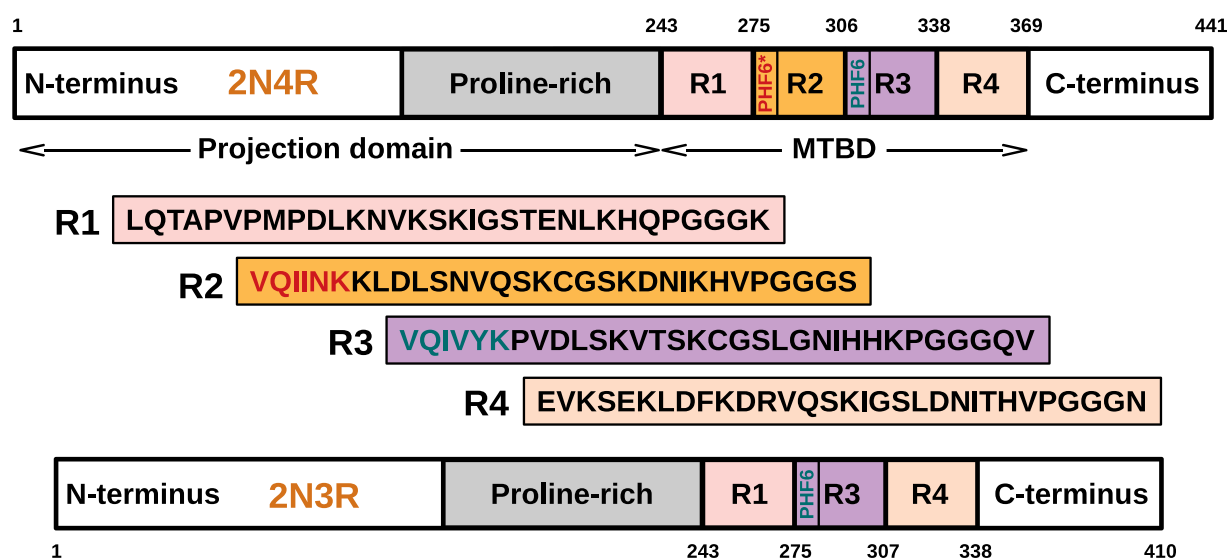


Figure 16. Schematic diagram of 4-repeat (2N4R) and 3-repeat (2N3R) human tau isoforms. The microtubules binding region (MBD) is highlighted, and the sequences for the repeat units are shown along with the hexapeptides PHF6* and PHF6.

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.⁵³² 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.⁵³² As can be seen in Figure 15, the Ser305 can be buried inside the fibril core (as in the case of CBD) or exposed on the fibril surface in AD fold. Therefore, depending on the diseases, we expected pSer305 may have a different outcome. Ser305 is not phosphorylated in CBD, since pSer305 would clearly disrupt the fibril core. In the AD fold, Ser305 could be phosphorylated since it is on the outside of the 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.⁵²² The inhibition increased with increasing the number of phosphorylated sites, with pSer262 having the strongest effect.⁵²² 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.⁵³³ 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.⁵³⁴

Both A β monomer and oligomers are able to affect tau phosphorylation.^{523,535–537} Distinct A β assemblies activate neuronal signaling pathways in a selective manner and thus trigger tau phosphorylation.⁵³⁵ Soluble phosphorylated tau can happen even in initial increases of aggregate A β as early as two decades before the development of aggregated tau pathology.⁵²³ A β monomer can affect tau phosphorylation either through changing signaling pathways or directly interacting with tau protein. A β monomer induced phosphorylation of tau at Ser214 through both β 2AR-cAMP/PKA-JNK and β 2AR-GRK signaling pathways.⁵³⁶ Intracellular binding of soluble A β to soluble nonphosphorylated tau promoted tau phosphorylation and A β nucleation.⁵³⁷ However, phosphorylation of Thr212, Ser214, Ser356, and Ser396 completely blocks A β 42 binding.⁵³⁷ Ser356 phosphorylation also contributes to tau stabilization when PAR-1/MARK activity is elevated.⁵³⁸ Using 5 μ s MD simulation of tau R3–R4 dimer with and without Ser356 phosphorylated, pSer356 not only perturbs the population of the β -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.⁵³⁹

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 IDPs (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.⁵⁴⁰ 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.⁵⁴¹ 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.

5.3.2.1. Monomeric and Early Oligomeric Conformations of Tau in Solution. In comparison to other IDPs such as A β and α -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 they 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 MC⁴⁸⁵ and MD⁵⁴² 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 coexists 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 co-workers 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.³¹ 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 β -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, Δ 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.^{543–545} Simulations on dimers confirmed the hypothesis that structure encoded in the monomer could be transferred to oligomers. R2(273–284), R2(273–284, Δ K280), and the R3(306–317) homodimers were found to

assume a mixture of compact and extended structures.^{543,546} 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 nonfibril competent conformations to further the aggregation process.⁵⁴⁷

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 (α -helix and β -sheet) conformers⁵¹⁴ and suggested that enhanced β -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).^{548,549}

A number of simulations have focused not on the monomer structure but rather on elucidating the structures of stable oligomers and fibrils.^{327,543,546,550–554} Early MC study of the PHF6 domain predicted a fibril structure with mixed parallel and antiparallel β -strands, with an increase in the number of parallel strands for larger fibrils.⁵⁵⁰ Subsequent REMD simulations of the R3(306–317) domain (which contains PHF6) also predicted stable β -sheet structures for dimers with parallel orientation of the monomers.⁵⁴⁶ 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).⁵⁴⁷ Antiparallel stacking of the sheets was 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*.^{543,546} Further REMD simulations by Bolhuis showed that PHF6 can form fibrils in a two-step process, while PHF6* remains as amorphous aggregates.⁵⁴⁴

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 birepeat.⁴⁰ MD simulations of these filaments consisting of different birepeats 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.⁵⁵⁵ However, a recent REMD study of an R3–R4 dimer did not identify C-shaped structures,⁵³⁹ 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

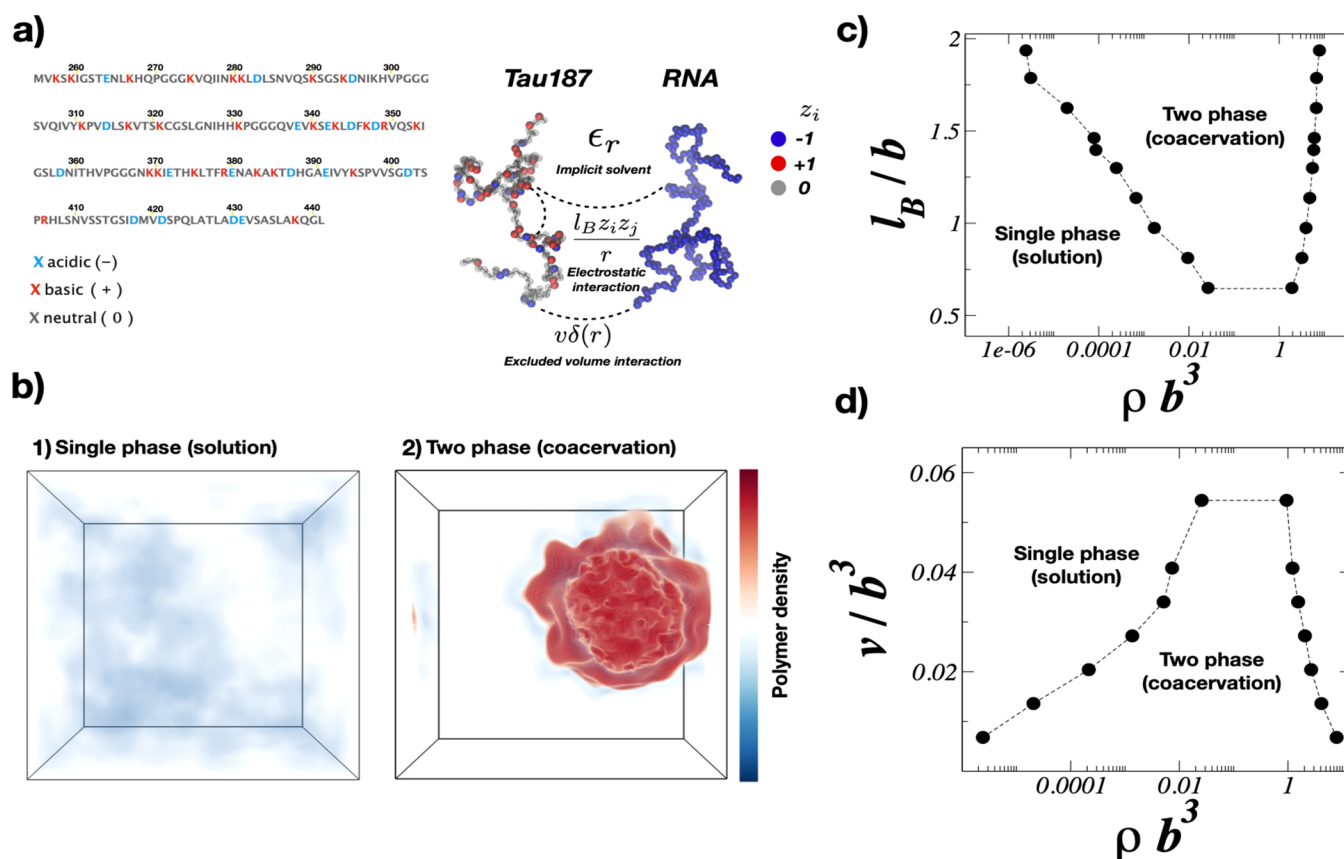


Figure 17. (a) Schematic representation of Tau and RNA models. Tau and RNA are modeled as chains of bonded monomeric units with size b in implicit solvent. The charge of each monomer is determined from the corresponding amino acid in the tau sequence at pH = 7. RNA is modeled as a uniformly charged polyanion. (b) 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 $l_B = 1.79 b$.⁵⁷⁰

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 co-workers.⁵⁵⁶ 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 β -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 co-workers.⁵⁵⁷

5.3.2.2. 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.⁵⁵⁸ 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 a quasi-static dense liquid phase known as a coacervate, which is in coexistence with the surrounding supernatant-tau solution.^{559–561} The intramolecular 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.⁵⁶⁰ Favored by lower interfacial energy, the dense microphase of tau is in equilibrium, forming pseudospherical droplets.⁵⁶² Recent *in vitro* experiments suggest that in some instances, coacervation, in the next stage of collective dynamics, might initiate and accommodate tau fibrillization.^{541,562–565} The mechanism of LLPS and the influence of various experimental parameters, namely, tau concentration, ionic strength, and temperature modulation, are largely unknown. Establishing the phase behavior of tau in physiological conditions 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.^{566–568} 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.^{540,569,570} 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 parametrizes the strength of the repulsive potential at contact, and the Bjerrum length l_B that parametrizes the strength of long-range 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 homogeneous phase.⁵⁷⁰ By performing an *in vitro* study of the N-terminus truncated isoform of human 4R, residues 255–441 (Figure 17a), 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 were investigated under different solvent conditions (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 17b, the instantaneous snapshots of the tau density profiles from FTS in two regimes are shown: (1) a homogeneous 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 single phase to a stable state in which tau phase separates into a dilute tau-depleted domain (white) and a condensed tau coacervate (red).

The first complete phase diagram of tau condensation studied in the literature is shown in Figure 17c, as a function of l_B and tau density at fixed solvent conditions (constant v); similarly in Figure 17d, the phase behavior of tau solution is probed by varying the solvent quality at fixed l_B . As is evident from Figures 17c and 17d, 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 homogeneous phase. The FTS phase diagrams shown in Figures 17c and 17d 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.⁵⁴⁰ By using the typical analytical polymer theories such as the Flory–Huggins–Voorn–Overbeek theory⁵⁷¹ (with no charge sequence), one can adjust an effective temperature dependent χ 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.⁵⁷⁰ Using FTS with a proper polymer model that includes the polypeptide spatial charge-sequence (Figure 18) and by considering reasonable values of the model

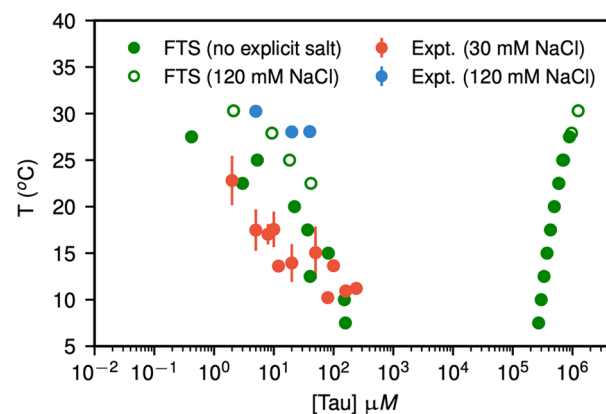


Figure 18. Coexistence points obtained from parametrized FTS at low salt (filled green circles) and at 120 mM NaCl (open green circles) as compared to experimental cloud point temperature as measured in turbidity experiments performed at 20 mM NaCl (filled red circles) and 120 mM (filled blue circles). 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.⁵⁷⁰

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 also shedding insight into the *in vivo* phase separation process.

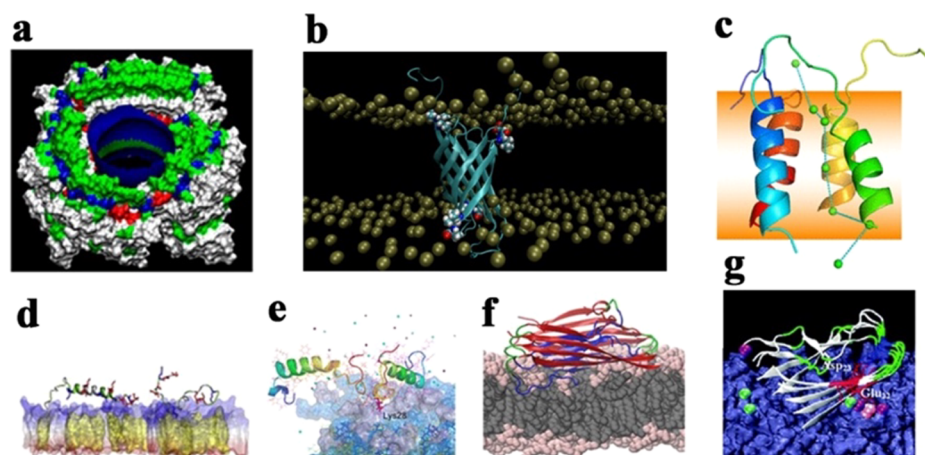


Figure 19. Different interaction models of full-length $A\beta_{40/42}$ peptides with lipid membranes. (a) $A\beta$ transmembrane pores with high Ca^{2+} permeability and selectivity.⁵⁷⁹ (b) Tetrameric $A\beta_{42}$ β -barrel pores in PC/PS/cholesterol/sphingomyelin and DPPC bilayers.⁵⁹¹ (c) Tetrameric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

6. INTERACTIONS OF AMYLOID PEPTIDES WITH CELLULAR MEMBRANES FROM SIMULATIONS AND EXPERIMENTS

Interactions of amyloid oligomers have been studied using different model membrane mimetic systems that include liposomes, bicelles, and nanodiscs by experiments and simulations.^{263,572–580} On one hand, amyloid proteins (e.g., $A\beta$, tau, hIAPP, α -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 has been used to obtain insights into these mechanisms of membrane disruption, which is also shown to vary with the lipid composition and 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), and 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, and cell calcium imaging have consistently shown that $A\beta$ peptides can penetrate into the cell membrane to form ion-permeable, transmembrane amyloid pores.^{581–585} $A\beta$ interaction with liposomes containing ganglioside (GM1), cholesterol, and

sphingomyelin has been shown to form in-register parallel and two-residue-shifted antiparallel β -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 to clustering by interacting with its headgroup.⁵⁷³ $A\beta$ is shown to interact and form oligomers at the membrane interface at nanomolar concentrations and is transient in nature as membrane-association and dissociation of oligomers are detected. An increase and decrease in the contents of β -sheet and α -helix, respectively, are 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 β -sheet structure.²⁶³

With the exception of the very recent NMR structure of an $A\beta_{42}$ oligomer formed in a membrane mimicking environment,⁵⁸⁶ namely an $A\beta_{42}$ tetramer, which comprises a six stranded β -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 β -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 β -strand-turn- β -strand amyloid monomers derived from $A\beta$ fibrils as building block,⁵⁸⁷ $A\beta$ pores were computationally constructed by preinserting 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 β -strands facing the solvated pore or contacting with lipids) into different model lipid bilayers, including zwitterionic DOPC, DPPC, POPE, and mixed DPPC/DOPS bilayers.^{579,588–590} 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 β pores are structurally stable: (1) the lipid bilayers did not support too small 12-mer A β pores or too large 36-mer A β pores, and only 18- or 24-mer A β pores can retain their pore-like morphology across the lipid bilayers; (2) A β pores with CNpNC pore topology (i.e., hydrophobic C-terminal β -strands are in contact with lipids and hydrophilic N-terminal β -strands face the pore) are stable (Figure 19a), while A β pores with NCpCN are collapsed due to unfavorable peptide–lipid interactions.

Apart from the organization of A β strands parallel or antiparallel to lipids, another class of A β β -barrel pores were also proposed and modeled computationally with titled A β strands relative to lipids as assisted with structural data from experimental and theoretical results. Computational construction and MD simulations of tetrameric β -barrel transmembrane pores by A β_{42} or A β_{40} peptides in membrane bilayers of PC/PS/cholesterol/sphingomyelin and DPPC showed the higher structural stability of A β_{42} β -barrels with different barrel topologies at the same diameter of 0.7 nm (Figure 19b), in sharp contrast to unstable A β_{40} β -barrels.⁵⁹¹ Mutations of D23N (more toxic mutant) or A2T (more protective mutant) did not largely affect the stability of A β_{42} β -barrels in DPPC bilayers and thus appeared not to strongly correlate their structures with the known both mutant-induced toxicity.⁵⁹² Computational A β_{42} β -barrel pores, but not A β_{40} pores, also exhibited specific Zn²⁺ binding at the pore entrance, consistent with several experimental observations that A β_{42} , not A β_{40} , can (i) assemble into β -barrels in OG/DM/DHPC/LDAO/DPC membranes with varied pore diameters of 0.7 nm⁵⁹³ and 1.7–2.4 nm⁵⁹⁴ and (ii) induce ionic currents in planar lipid bilayers by electric recordings.⁵⁹³ Moreover, different from small A β β -barrel pores, they could also exist with the much larger sizes formed by six parallel A β hexamers (36 monomers) with N-terminus segments facing the pores in the POPE bilayer.^{588,589} A recent study from native ion mobility-mass spectrometry revealed that A β_{42} formed a diverse set of hexameric β -barrel pores in a membrane-mimicking environment, including tilted hexameric barrels, star shaped hexamers, dimers of trimers, and trimers of dimers,⁵⁹⁵ all of which shared the similar peptide organization by folding and assembling hydrophobic C-terminal ends of the A β -peptide into a pore center. In parallel to β -structure-based transmembrane pores of A β , α -helix bundles of the A β_{17-42} tetramer and trimer can also form transmembrane pore-like structures, which were stable enough to transport Ca²⁺ ions across the DPPC bilayer⁵⁹⁶ (Figure 19c). Ca²⁺ transport and binding sites were similar to a classical voltage-gated calcium pore but not an M2 proton pore. The α -helix pores of A β provide additional possible pathways for Ca²⁺ homeostasis.

While these membrane-supported A β 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 β pores are made of four to six, small, dynamic oligomers (each oligomer contains 4–6 A β monomers), which are loosely associated with each other through some β -strand interactions to prevent pore dissociation. Such irregular and polymorphic A β pores suggest the possible pore formation pathways; that is, small A β aggregates (monomers or oligomers) first insert into the membranes, followed by the self-assembly of these inserted A β aggregates into different A β pores. From a membrane permeability viewpoint, these transmembrane A β pores indeed exhibited specific cation

selectivity (particularly for Ca²⁺ and Zn²⁺) and characteristic stepwise ion permeability, presumably due to specific side chain orientations and organizations of A β .⁵⁹⁷ Ca²⁺-permeable A β pores are an attractive target to develop inhibitory compounds, and thus prevention of A β -induced Ca²⁺ influx is expected to restore the Ca²⁺ balance, cycling, and bioenergetics in neurons that would generate a potential therapeutic strategy for AD treatment. Taken together, A β pores were found in different lipid membranes, suggesting that the formation of a pathogenic, ion conducting pore by A β is considered as a general membranotropic mechanism of AD.⁵⁹³ In principle, amyloid polymorphism in solution and the cell membrane allows formation of a wide variety of amyloid pores, but only stable A β 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 β pore formation by altering membrane structure and A β –membrane interactions.^{598–600}

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

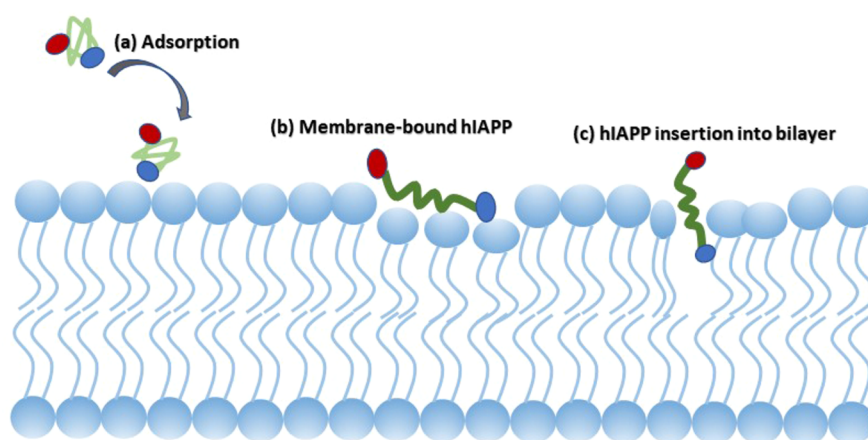


Figure 20. Typical interaction model of hIAPP monomer with lipid bilayers via a three-step approaching–adsorption–insertion process. The hIAPP monomers with the disordered structures approach the cell membrane to establish initial contacts with their N-terminal residues via electrostatic interactions,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

reveal the important role of $A\beta$ concentration and initial oligomerization in promoting the transformation into a β -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 the POPE bilayer over the POPC bilayer.^{608,609} 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 of 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 the 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.⁶¹⁰ More importantly, Ca^{2+} 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^{2+} – PO_4^- complexes (Figure 19g). Further SPR and AFM data also confirmed that Ca^{2+} ions and the lipid bilayer concertedly accelerate the conformational change or misfolding of $A\beta$.⁶¹¹ Intensive Ca^{2+} bound to the lipid bilayer and Ca^{2+} ionic bridges may explain Ca^{2+} homeostasis responsible for neuronal dysfunction and death. $A\beta$ oligomers also affect neuronal Ca^{2+} homeostasis by modulating the activity of NMDA receptors. Ca^{2+} bridges have also been shown to mediate the interaction between the membrane and the acidic tail of αS and hIAPP.^{612,613}

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 toward a β -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 a crucial driving force for modulating the anchoring and insertion of $A\beta$ peptides into lipid bilayers. Third, Ca^{2+} promotes membrane interactions of $A\beta$ aggregates to facilitate their aggregation, adsorption, and insertion to form Ca^{2+} -permeable pores in membranes or to increase membrane conductance. Blocking of $A\beta$ pores by small-molecule compounds enabled reduction of the Ca^{2+} conductivity of $A\beta$ pores and rescue of synaptic plasticity and memory function for amyloid pathology in a mouse.⁶¹⁴ 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.⁶¹⁵

6.2. Tau and IAPP

6.2.1. 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 β -structure and aggregation kinetics toward amyloid fibrils.

hIAPP and $A\beta$ have very similar properties with regard to membrane interactions, and both peptides adopt an α -helical conformation when interacting with lipid membranes prior to amyloid formation. Similar to other amyloid proteins, the interaction of hIAPP monomer with the lipid membrane is considered as the first step to initiate hIAPP aggregation on the

cell membrane. A number of MD simulations have been performed to study the interaction of hIAPP monomers with different conformations (e.g., α -helix, β -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.^{613,616–619} Such adsorption was largely driven by electrostatic interactions between positively charged residues from the N-terminal region of hIAPP and negatively charged headgroups of lipid membranes^{613,620–622} (Figure 20a). Upon initial adsorption, specific hIAPP–lipid interaction would drive hIAPP monomers to adopt α -helical⁶²³ or coil⁶¹⁶ structure and orientations for better establishing stable and strong contacts with lipid membranes⁶²⁴ (Figure 20b), followed by subsequent full or partial insertion of hIAPP monomers into lipid membranes⁶¹⁸ (Figure 20c). During this adsorption–insertion process, structural transition of hIAPP peptides toward β -sheet-rich aggregates would occur at any stage of the hIAPP aggregation process both in solution and on the cell membrane.⁶²⁴ All-atom MD simulations of hIAPP monomer with α -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.⁶¹⁸ Moreover, hIAPP monomers on anionic POPG bilayers experienced the faster dynamics to orient their N-terminal toward the membranes than those on neutral POPC bilayers.^{620,621,625}

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, α -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 β -structure.⁶¹⁶ Another simulation strategy was applied to preinsert hIAPP monomer into POPG lipid bilayer, in which hIAPP monomer liked to bind to POPG bilayer with α -helical structure. Moreover, REMD simulation of a hIAPP_{1–19} fragment with a POPG bilayer confirmed that hIAPP_{1–19} favored to adopt random coils with a parallel orientation to the POPG surface at the adsorption state but changed to helical structure inside the POPG bilayer at the insertion state. In addition, the presence of cholesterol in DPPC/DPPS bilayers promoted hIAPP_{1–19} aggregation, while the absence of cholesterol alleviated the insertion tendency of hIAPP_{1–19} into in DPPC/DPPS bilayer.⁶¹⁷ 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 the cell membrane encounters a substantial energy barrier, which is typically beyond the time scale 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 the umbrella sampling protocol, to directly measure the free

energy barrier required to transfer the peptides from the bulk water phase to the water–membrane interface to the bilayer interior. PMF profiles from the ABF-based simulations of hIAPP monomer insertion into a 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 lower energy penalty by ~ 11 kcal/mol.⁶¹³ Different from PC, PG, and PE-based bilayers that do not present in the outer leaflet of islet β -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-enriched DOPC (GM3/DOPC) bilayers.⁶²⁶ Simulations showed that GM3 lipids tended to induce conformational changes of hIAPP from α -helix to β -hairpin structure, in contrast to DOPC bilayers to retain the α -helix of hIAPP. The ratio of GM3/DOPC components determines not only the conformational 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 α -helical conformations and oriented their hydrophilic residues toward lipid head groups, but their hydrophobic residues toward lipid tail groups, and such orientation would facilitate hIAPP–hIAPP interaction to promote their oligomerization and fibrillization on lipid membranes.

Numerous computational studies of the interaction of hIAPP oligomers with lipid membranes have also been reported, which are more biologically relevant to hIAPP oligomer-induced cell toxicity, particularly to the membrane disruption mechanism.^{617,627,628} hIAPP dimers as the smallest oligomers and building blocks have been computationally modeled with initial α -helical structures to interact with and penetrate into POPG bilayers.⁶²¹ It was found that the interaction of hIAPP dimers with the POPG bilayer is mostly driven by the N-terminal positively charged residues via electrostatic interactions, and upon adsorption/insertion of hIAPP on/into the POPG bilayer, hIAPP dimers had a larger disturbance on the ordering of head groups of the POPG bilayer than hIAPP monomer, but both hIAPP dimers and monomers still largely retained their α -helical structures (Figure 21a). Later, a relatively large size of hIAPP pentamer with β -sheet conformation was selected to interact with both POPC and POPC/POPE (3:1) bilayers to examine the β -structure effect of hIAPP pentamer on the adsorption, orientation, and interactions of hIAPP on both bilayers.⁶¹³ 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,⁶²⁹ hIAPP pentamer had a shallow insertion depth of ~ 1.62 nm and a small insertion title angle

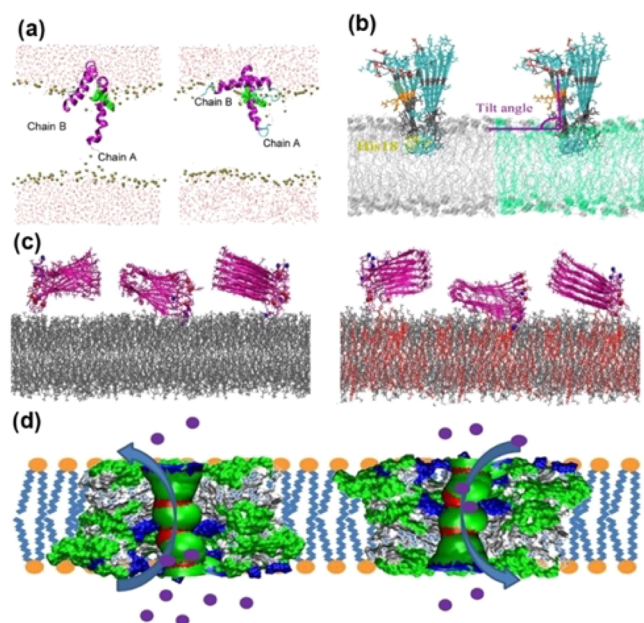


Figure 21. Different interaction models of full-length hIAPP_{1–37} oligomers with lipid membranes. (a) hIAPP dimers with α -helical structure insert into POPG bilayers with different insertion depths, strongly depending on interactive contacts of hIAPP dimers with POPG bilayers.⁶²¹ (b) hIAPP pentamer with U-bent β -structures to be partially inserted into (left) DPPG and (right) DPPC/DPPG bilayers via the turn region.⁶²⁹ (c) hIAPP pentamer with U-bent β -structures to be adsorbed on (left) POPC and (right) POPG/POPE bilayers with different hIAPP orientations.⁶¹³ (d) Double hIAPP pores with different (left) “turn-to-tail” and (right) “tail-to-turn” orientations in DOPC bilayers to show nonselective, ion-permeable activity.⁶³²

$\sim 52^\circ$ into DPPG bilayer, as compared to a deeper insertion of 1.75 nm and a larger tilt angle of $\sim 77^\circ$ into the DPPC/DPPG bilayer (Figure 21b).⁶²⁹

Besides adsorption and insertion models, the 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 β -strand–turn– β -strand hIAPP monomers de-

rived from amyloid fibrillar structure as a building block, a series of hIAPP pore models with different sizes and orientations were constructed and preinserted in the presence of a DOPC bilayer.^{630,631} It was found that all individual hIAPP pores displayed high selectivity for Cl^- ions over other cations (Na^+ , K^+ , and Ca^{2+}), but the coexistence of “turn-to-tail” and “tail-to-turn” double hIAPP pores resulted in the nonionic selectivity. Double hIAPP pore simulations explain experimental observation that hIAPP forms nonselective, ion-permeable channels in planar phospholipid bilayer membranes, due to the coexistence of “turn-to-tail” and “tail-to-turn” channels in the membrane (Figure 21d).⁶³² Computationally modeled hIAPP pores showed similar structural morphologies to AFM-identified hIAPP pores,⁵⁸¹ 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 the 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 fibrillation 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 β -strands in the regions A8–L12, F15–H18, and I26–S29.²⁸⁵

6.2.2. 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.^{633–637} Purified PHFs were found to contain different cell membrane components,

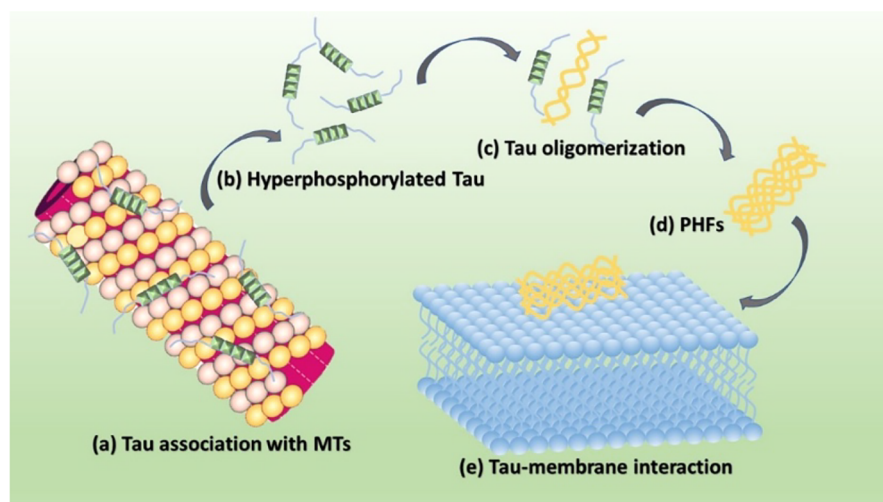


Figure 22. Pathological process of tau production, hyperphosphorylation, oligomerization, fibrillization, and interaction with cell membranes.

including phosphatidylcholine (PC), cholesterol (CH), galactocerebrosides (GC), and sphingomyelin (SM), supporting the pathology mediated by tau–membrane interactions in AD.⁶³⁸ 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.⁶³⁹ In addition, tau also interacts with other ionic vesicles and micelles to promote tau oligomerization and PHFs formation.^{635,636,639–642} Tau proteins have the tendency to form porelike 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,⁶⁴³ which mimic the membrane-disrupting properties of pore-forming protein toxins, consistent with A β , hIAPP, and α S pores.⁶⁴⁴ 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.^{639,645,646} 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.

6.3. α -Synuclein Membrane Interaction

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

The key membrane interaction of α S *in vivo* is established with synaptic vesicles.^{194,200,201,204} In addition α S has been shown to bind a variety of other cellular membranes, including mitochondrial membranes and the presynaptic membrane,¹⁸⁹ as well as nonphysiological interfaces such as detergent micelles⁶⁵¹ and the water–air interface.⁶⁵² In general, the membrane-binding affinity of α S has been shown to be favored by negative charges and high curvature of the membrane.⁶⁵³

Structural studies have clarified the topology of the amphipathic helical segments forming in α S upon membrane binding, indicating that these adopt a parallel orientation with respect to the membrane,²⁹⁹ with most of the protein remaining bound at the membrane surface while the first 12 residues of α S experience a mild insertion into the hydrophobic region of the bilayer.⁶⁵⁴ 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,⁶⁵¹ whereas structural studies of the membrane-bound state indicated mostly a single helix ranging from residues 1 to 97.⁶⁵⁵ But the key feature governing the biological properties of the membrane-bound state of α S appears to be the dynamical content enabling a multiplicity of binding modes to occur by the same protein.⁶⁵⁶ Conformational dynamics in the membrane-bound state differ significantly across the α 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)²⁹⁹ (Figure 23). Of these regions, the most enigmatic

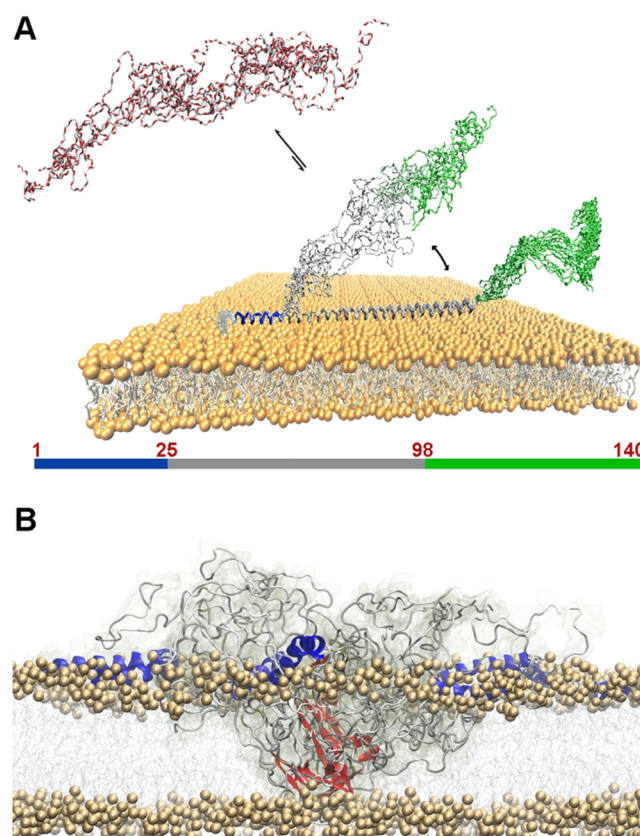


Figure 23. Membrane binding by monomeric and oligomeric α S. (A) Monomeric α S is disordered in solution (red) and binds the membrane with three regions having distinct structural and dynamical properties. The N-terminal region (blue) acts as a rigid membrane anchor. The central region (gray) is in conformational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

is certainly the central that, in addition to hosting the amyloidogenic NAC segment, has been shown to influence the binding affinity to acidic lipid bilayers as well as the ability of α S to bridge multiple membranes in a so-called “double-anchor” mechanism.^{199,203} The equilibrium between membrane-bound and detached states of the region 65 to 97, however, may also lead to aberrant aggregation of α 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 α S are inextricably connected to the equilibrium between ordered and disordered conformations of the region 65 to 97 at the surface of biological membranes.⁶⁴⁷ 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

	Cu(II)	Cu(I)	Zn(II)
Amyloid- β (1-16/40/42)	<i>component I</i> <i>component II</i>		
Amyloid- β (4-16/40/42)		main form: His13&14 minor forms: His 6&13 and His6&14	
α -Synuclein: (free N-terminus).			
α -Synuclein (N-terminus acetylated)			
IAPP		—	
τ_{full} length	—	—	—
$\tau_{323-335}$			—
$\tau_{244-372}$	—	—	

Figure 24. Coordination spheres of A β , α 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 β 4-40/2 which has a well-defined coordination sphere. Abbreviations: NH₂, N-terminal amine; N^{Im}, nitrogen of imidazole ring of a His; N⁻, nitrogen of an amide; N, any nitrogen of unknown ligand. Review⁶⁶⁸ and references.^{678–680}

of residues Ser 87,⁶⁵⁷ Ser 129,⁶⁵⁸ and Tyr 39⁶⁵⁹ that also regulate the equilibrium between membrane-bound and detached states of α S (Figure 23). A key posttranslational modification for the membrane binding of α S is the N-terminal acetylation,^{650,660} which is believed to define the predominant form of the protein *in vivo* and that enhances the α -helical propensity of the N-terminal residues to increase the membrane affinity.⁶⁶¹

As expected, the binding modes of the physiological monomers of α S were found to differ significantly from those of its neurotoxic oligomers. In particular it was found that prefibrillar toxic α S oligomers bind neuronal membranes via a lipophilic element, which is composed of several copies of the N-terminal region of α S molecules from the oligomers, and insert their structured hydrophobic core into the lipid bilayer by disrupting its integrity.⁶⁵⁰ 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.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 colocalized along with amyloid fibrillary aggregates in the senile plaques and with amyloid- β in blood vessels from the brains of AD patients.⁶⁶² Specifically, zinc with its highest content in islets (millimolar)⁶⁶³ 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 and morphological and pathological phenotypes of several amyloidogenic peptides/proteins has

been tested *in vitro*.⁶⁶⁴ Here, we cover the reported studies on the effects of metal ions on the amyloid aggregation and toxicity of amyloid- β , α 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 β , α 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 refs 15, 313, and 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 to 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.^{670,671} 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 β , α 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.⁶⁷² 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 β , followed by α S and IAPP, with tau being the amyloid protein with the 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 the more

comprehensive and recent reviews on these interactions, see the reviews^{668,669,673} 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 β 1–16. However, it should be noted that for the shorter peptides A β and IAPP, a lot of studies have been performed not only on the metal-binding domain but also on the full-length peptides. Similarly, for the longer α 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.

7.2.1. General Features of Cu(I/II) and Zn(II) Binding to A β , α 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 the 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 log K 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.^{673–675} 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.⁶⁷³ In Figure 24, only the most populated coordination sphere for each of the metal ion–amyloid peptide/protein combinations 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.⁶⁷³

An exception to this feature is the Cu(II)-binding to an abundant form of A β , where the first 3 amino acids are truncated (called A β 4-40/2). Here, Cu(II) is bound to the amino acids 4 to 6 (Phe-Arg-His) by the N-terminal NH₂, the two N⁻ of the two amides (from peptide bonds between Phe-Arg and Arg-His), and the N ^{π} of the His. This binding site belongs to a well-known N-terminal binding motif with the general sequence NH₂-Xxx-Zzz-His (Xxx-Zzz are any amino acid, but Pro for Zzz) with high affinities of log K 13–15 at pH 7.4 and more inert Cu(II)-binding compared to other peptides.^{676,677}

7.3. Reactivity of Cu-Amyloid Peptides with O₂ and Reducing Agent to Produce ROS

Oxidative stress is a well-recognized factor in several amyloid-related diseases, although a cause or a consequence is not clear as reported in the review.⁶⁸¹ 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 nonspecifically 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 control of the reactivity of Cu.⁶⁷⁰

Thus, it is possible that Cu bound to amyloidogenic peptides (A β , α S, etc.) can contribute to oxidative stress via its catalytic redox activity and formation of ROS (Figure 25). The

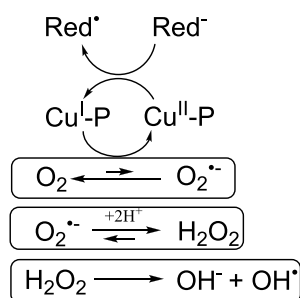


Figure 25. Proposed mechanism of ROS production by a Cu-peptide (Cu-P) in the presence of a reducing agent (Red⁻) and dioxygen.

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.⁶⁸² An exception here is again the truncated A β 4-40/42,⁶⁷⁶ in which Cu(II) is rigidly bound to the N-terminal motif Xxx-Zzz-His and is thus inefficient in ROS production.⁶⁸³

As outlined above, a general feature of these peptides (apart from Cu(II)-A β 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 β including both experimental and theoretical work (e.g.,^{684,685}) 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 review⁶⁸⁸). These low populated states (also called in-between states), which are accessible due to the flexibility of A β and are responsible for the redox chemistry, likely also exist in the other amyloid peptides/proteins IAPP, α S, and the so far studied portions of tau, as the key features are also present there.^{678,686}

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.⁶⁸⁷ 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 β , α 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 β 4-40/42.⁶⁸⁸ 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 β , α 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 β

High-resolution structural information on 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 β under various conditions would not only shed light on the mechanism of conversion of nontoxic 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 β peptides and their influence on the structure have been investigated to obtain atomic-resolution information using NMR techniques and MD simulations. In the following subsections, we limit the focus to briefly cover the structural interactions of Zn(II) and copper ions with A β .

7.4.1. NMR Studies. The three major histidine residues (H6, H13, and H14) in A β 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}N relaxation NMR experiments.⁶⁸⁹ A second, but lower affinity $\text{Zn}(\text{II})$ binding site is with D23 and K28 that disrupts the D23–K28 salt bridge, which otherwise is known to favor the nucleation of the $\text{A}\beta$ aggregation process.^{690,691} Zinc binding has exhibited no significant effects on the longitudinal (or spin–lattice, T_1) relaxation time and the diffusion rate, suggesting that the $\text{Zn}(\text{II})$ – $\text{A}\beta$ complex is present as monomers. Diffusion NMR and size-exclusion chromatography have identified an increase in the compactness of monomeric $\text{A}\beta$ bound to $\text{Zn}(\text{II})$ with a hydrodynamic radius of 1.5 nm compared to 1.7 nm for $\text{A}\beta$ alone.⁶⁹² This was later confirmed by obtaining a contraction upon Zn binding from 1.66 to 1.59 nm using pulsed field gradient diffusion measurements.^{693,694} Transverse relaxation rate analysis further highlighted that the $\text{Zn}(\text{II})$ binding increased the structural order in the N-terminal region while the C-terminus remained flexible.⁶⁸⁹ This observation is supported by a thermodynamic analysis model that suggested chaperonin activity of $\text{Zn}(\text{II})$ and induction of a short-lived folded $\text{A}\beta$ conformation around the $\text{Zn}(\text{II})$ -binding site at the N-terminus.⁶⁹³

$\text{Zn}(\text{II})$ interaction with $\text{A}\beta$ has been shown to generate nonfibrillary $\text{A}\beta$ aggregates, and the underlying molecular mechanism has been probed using NMR experiments. $\text{Zn}(\text{II})$ -binding induces a rigid turn-like structure in the V24–K28 region of $\text{A}\beta$ allowing the C-terminus to be highly dynamic.⁶⁹⁶ Investigation of the Zn – $\text{A}\beta$ complex is limited by (i) structural plasticity of $\text{A}\beta$, (ii) multiple zinc coordination sites in $\text{A}\beta$, (iii) precipitation of sample at high $\text{Zn}/\text{A}\beta$ concentration required for NMR detection, and (iv) inactive spectroscopic nature of zinc (d^{10} electronic configuration). Alies et al. addressed these limitations using a combination of approaches that include ^1H NMR, X-ray absorption spectroscopy, and affinity measurements. They reported $\text{Zn}(\text{II})$ ions are tetrahedrally bound to $\text{A}\beta$ with the Zn coordination sphere comprised of two histidine residues and two carboxylate side chains (Figure 26).⁶⁹⁵ The binding of zinc to $\text{A}\beta$ is pH and concentration dependent as revealed by NMR.^{696–698} At substoichiometric concentration of $\text{Zn}(\text{II})$ (i.e., 0.2 equiv), line-broadening was observed for

H6, S8, V12, H13, and H14. Further increase in $\text{Zn}(\text{II})$ concentration (i.e., above 0.5 equiv) 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.^{697,698}

Zinc binding has been shown to induce $\text{A}\beta$ oligomerization and its pathological phenotype. NMR experiments have been used to probe the high-resolution structural details of zinc induced $\text{A}\beta$ oligomers by using an $\text{A}\beta$ fragment involving residues 1–16.⁶⁹⁹ $\text{A}\beta$ 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 $\text{A}\beta$ fragments derived from the Taiwanese mutation D7H. A dimer model structure shows the coordination of $\text{Zn}(\text{II})$ 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).⁷⁰⁰ In another study, zinc binding to phosphorylated $\text{A}\beta$ (at S8), as detected in AD brain, showed the H6 imidazole ring and phosphate group of S8 to form three of the four $\text{Zn}(\text{II})$ coordination bonds promoting $\text{A}\beta$ dimerization.^{699,701} Lee et al. reported $\text{Zn}(\text{II})$ induced $\text{A}\beta$ oligomers are spherical with a diameter of ~ 12 – 14 nm and used homonuclear ($^{13}\text{C}/^{13}\text{C}$) magic angle spinning (MAS) solid-state NMR to study the Zn – $\text{A}\beta$ aggregates where $\text{A}\beta$ was selectively labeled with ^{13}C at V24, A30, I31, G33, and L34.⁷⁰² This study identified A30 in a rigid β -sheet that differed from its conformation in aged fibers, whereas L34 showed no conformational change highlighting the Zn – $\text{A}\beta$ aggregates adopting a β -sheet structure but is relatively disordered when compared with aged $\text{A}\beta$ fibers.⁷⁰² In another MAS NMR study, Mithu et al. revealed a structural distortion in Zn – $\text{A}\beta$ aggregates due to the disruption of the D23–K28 salt bridge but maintained a cross- β structure.⁶⁹¹ 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 – $\text{A}\beta$ aggregates they observed a single chemical shift for D23 and K28 and two distinct chemical shifts for A30 and V36⁶⁹¹ suggesting the Zn – $\text{A}\beta$ aggregates are morphologically distinct from aged fibers.

Other metal ions such as $\text{Cu}(\text{II})$ share similar but not identical binding sites and modes with $\text{A}\beta$. $\text{Cu}(\text{II})$ binds in a pH-dependent fashion to $\text{A}\beta$, 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, and 703). These two forms are often called components I and II. In contrast $\text{Cu}(\text{I})$ seems to be less pH dependent, at least from pH 6.5 to 9, and bound in a diagonal fashion to two His residues. $\text{Cu}(\text{I})$ bound to H13 and H14 is the dominant form but is in rapid exchange with $\text{Cu}(\text{I})$ bound to H6/H13 and H6/H14.⁷⁰³ NMR spectra of $\text{Cu}(\text{II})$ – $\text{A}\beta$ (1:1) showed significant line-broadening for the N-terminal residues spanning the region E3–V18.⁷⁰⁴ It should be noted that both $\text{Cu}(\text{I})$ and $\text{Cu}(\text{II})$ are involved in the interaction with $\text{A}\beta$. 2D TOCSY NMR experiments identified $\text{Cu}(\text{I})$ binding to all three histidine residues in $\text{A}\beta$ 1–16. $\text{Cu}(\text{I})$ has been shown to bind to the imidazole nitrogen (H6–N δ and H13–N ϵ , H14–N ϵ) that induced significant chemical shift perturbation for the side chain protons from all His residues.⁷⁰⁵ Proton NMR experiments revealed that $\text{Zn}(\text{II})$ does not alter the copper binding environment in $\text{A}\beta$ in line with the known stronger binding of $\text{Cu}(\text{II})$ when compared to $\text{Zn}(\text{II})$. This is in contrast to EPR and XAS measurements, which showed that

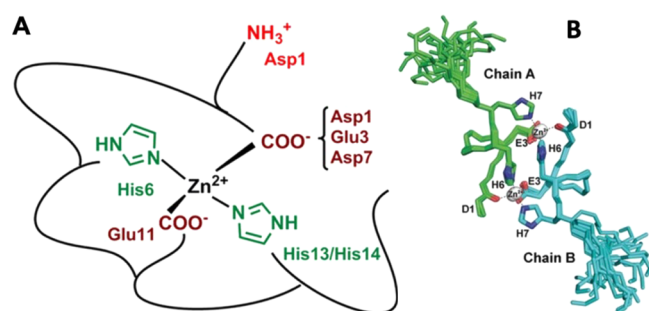


Figure 26. High-resolution structural characterization of zinc binding sites to Alzheimer's $\text{A}\beta$. (A) At physiological pH (7.4), zinc was proposed to tetrahedrally bind with $\text{A}\beta$ where the coordination sphere is contributed from two of three histidine (H6, H13, and H14) residues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

Zn slightly pushes the Cu(II) from component I to II.^{706,707} 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 β .⁷⁰⁸ 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 β 40 fibrils mixed with copper, but no change in the structure was identified.⁷⁰⁸ Relaxation experiments under MAS further identified that the paramagnetic Cu(II) binding to A β 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 β 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 1:1.⁶⁹⁸ Similarly, Ag(I) interaction studies with A β , and an A β mutant having no histidine, revealed interaction with the N-terminal residues reducing the NMR signal intensities of all histidine residues by ~75–80%.⁷⁰⁹

7.4.2. 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 β . 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 β . The results of these QM calculations are usually compared to experimental data and help to decide which of the possible binding modes best agrees with 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 β . Different coordination spheres were constructed and their geometry optimized using DFT calculations. The optimized structures were then utilized in the EXAFS spectral 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.⁷¹¹ Moreover, a pentacoordinated structure with Y10 involved^{712,713} 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,^{714,715} in agreement with the findings from NMR, potentiometry, and electrochemistry that never identified Y10 as a main ligand for A β .

Studies on metal–A β 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 β 1–16 were probed by CPMD simulations,⁷¹⁶ reporting

H6, E11, H13, and H14 as most likely ligands. Possible Zn(II)-bridged A β dimer structures were also suggested from CPMD simulations.⁷¹⁷ 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 study⁷¹⁸ that assessed possible dimerization scenarios of A β involving Zn(II) concluded that the Zn(II) ion is likely chelated by E11 and H14, which is in agreement with the NMR results mentioned above⁶⁹⁹ and was also supported by ITC experiments of the sequence A β 11–14.⁷¹⁸ 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.⁷¹⁸ CPMD was also employed to probe the binding of Cu(I) to A β , testing whether two or three His residues coordinate with Cu(I).⁷¹⁹ 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 H14.⁷¹⁹

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 β . 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, is needed to observe relevant conformational transitions in these peptides. From the multitude of MD simulations that studied conformational changes in monomeric or oligomeric A β 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 β , 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,⁷²⁰ using previous QM/MM calculations and experimental findings as input.⁷²¹ In general, similar observations were made by most of these simulation studies. Many of them found that both Zn(II)^{722,723} and Cu(II)^{720,724} binding increases the formation of β -sheets in the C-terminal residues ~30–40/42 of monomeric A β . In the N-terminal residues, on the other hand, where the metal ion binds, the amount of helix and β -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 β ,

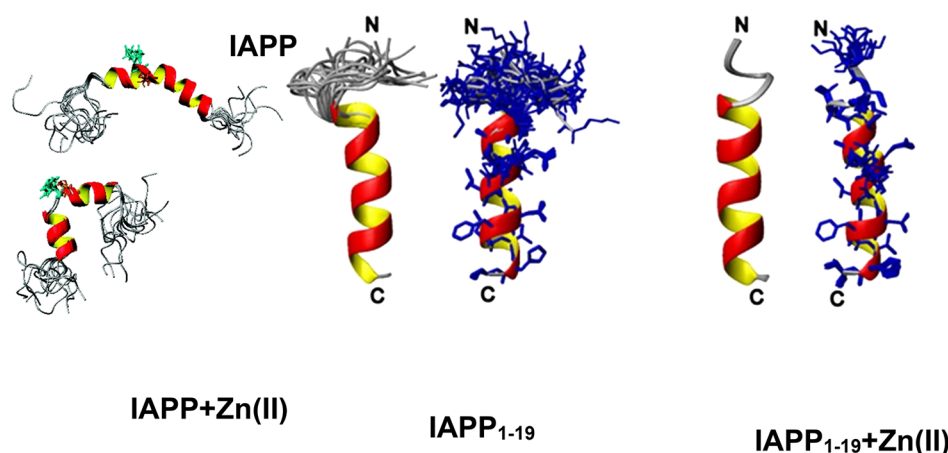


Figure 27. High-resolution NMR model structure of IAPP in the absence or presence of zinc. Full-length NMR structure of IAPP (top) and Zn-IAPP complex (bottom). Zinc binding to His18 (green sticks) induces helical perturbation and formation of a helix–kink–helix conformation. NMR model structures of IAPP_{1–19} and IAPP_{1–19} obtained from 2D ¹H/¹H TOCSY and NOESY experiments. Zinc binding transforms the disordered N-terminus (left) to an ordered structure (right).^{727,728}

explaining the reduction of the hydrodynamic radius of A β upon Zn(II) binding as observed experimentally,^{692,693} 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 β -sheet formation. An REMD study of the Cu(II)-bridged A β 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 β -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.⁷²⁵ It was suggested that the increased aggregation tendency will rather lead to amorphous aggregates as Cu(II) binding enhances the probability of closed A β structures with more intramolecular hydrogen bonds, including intramolecular β -sheets, which are different from the extended monomeric structures prone to fibril formation.⁷²⁶

7.5. Structural Studies on Metal Induced IAPP Aggregation

7.5.1. 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 has been linked to β -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 nontoxic 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 β -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 toward β -cells.

7.5.2. 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.⁷²⁷ Zinc binding showed a significant chemical shift perturbation for H ϵ (0.208 ppm) and H δ (0.106 ppm) from His18's imidazole side chain. The binding of zinc (10 equiv) 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 α -helices (Figure 27).⁷²⁷ 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.⁷²⁷ 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 reason for the conformational unwinding of IAPP helical structure.⁷²⁷

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.⁷²⁸ 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 (¹H/¹⁵N) SOFAST-HMQC and homonuclear (¹H/¹H) were carried

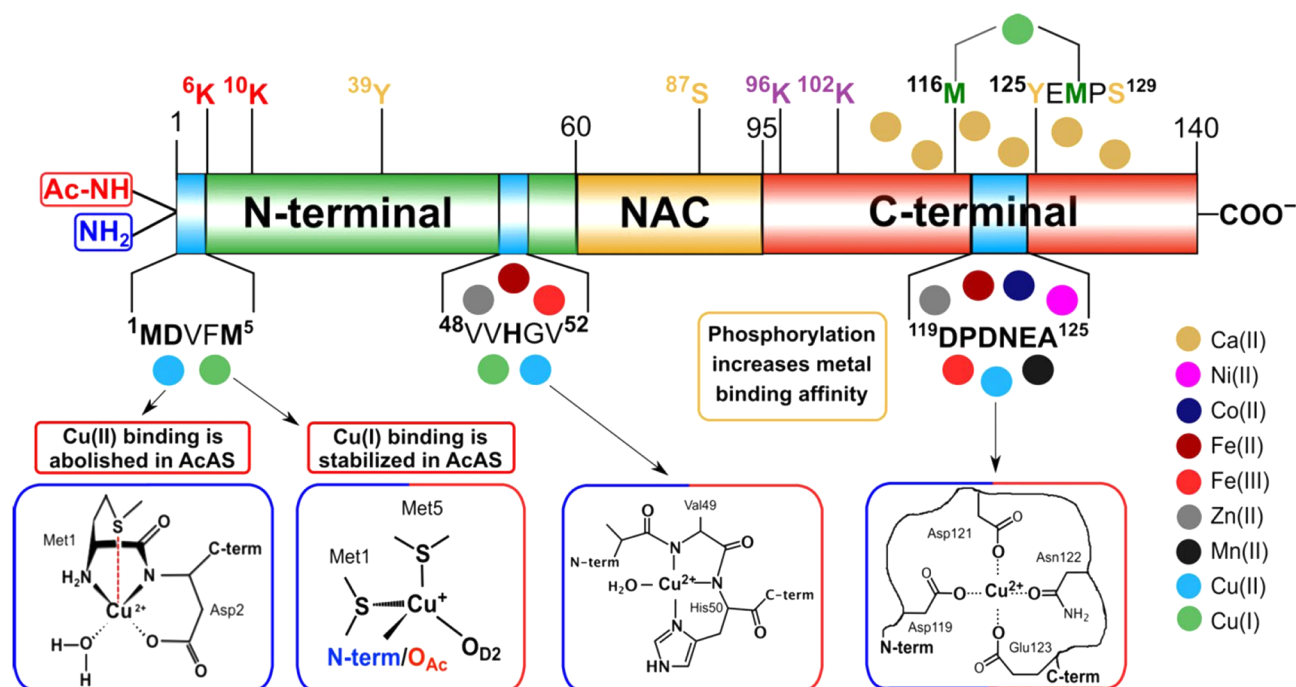


Figure 28. Metal binding sites and post-translational modifications of α S. α S harbors three binding sites for metal ions (displayed with colored circles): the low affinity, nonspecific metal binding site at the acidic DPDNEA segment in the C-terminal, the His50 site, and the first five residues at 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 α S is a high affinity and highly specific site for Cu ions. Reprinted with permission from ref 734. Copyright 2021 John Wiley and Sons.

out on the full-length (human-IAPP-1-37) and the N-terminal truncated (human-IAPP-1-19) peptides.⁷²⁸ 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 the $^1\text{H}/^1\text{H}$ correlation spectrum of IAPP(1–19) in the absence and presence of zinc.⁷²⁸ 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 changes in the IAPP monomer–oligomer equilibrium upon zinc binding by analyzing the depletion in ^1H signal intensities within the limit of NMR detection.⁷²⁹ 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.⁷²⁹

The binding of Zn(II) to IAPP fibrils was probed by MD simulations.^{730,731} In these simulations IAPP was modeled in a preassembled 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. α -Synuclein and Metal Ions

Like A β and IAPP, α 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 α S, possibly by leading to the formation of a partially folded state that is more prone to aggregation than the monomeric protein in solution.⁷³² 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 μM concentrations. The major finding of these studies was that under these conditions only Cu(II) is able to accelerate the formation of amyloid fibrils.⁷³³ Further characterizations of the metal binding revealed that the metal ions generally have a high tendency to interact with α S through its C-terminal domain, which contains several Asp and Glu residues that are strong attractors of cations and positively charged molecules.⁷³³ The DPDNEA sequence, located at the C-terminal domain (Figure 28), is a nonspecific 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.⁷³⁴ For Cu(II) three binding sites in α S have been elucidated (Figure 28).⁷³⁴ 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, via 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 NH₂ 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.^{735,736} Copper has been shown to be one of the most potent modulators of α S structure as well as a very effective accelerator of α S fibrillation *in vitro*.⁷³² AFM-based experiments revealed that the presence of Cu(II) significantly enhanced the relative abundance of the β -sheetlike structure in the otherwise conformationally heterogeneous monomeric α S.⁷³⁷ Even at physiologically relevant concentrations, Cu(II) ions were effective in the acceleration of α S aggregation without affecting the resultant fibrillary structures.⁷³⁸

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.⁷³⁹ Rebalancing metal dyshomeostasis could be a feasible therapeutic strategy for AD.⁷⁴⁰

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.^{741,742} 5–10% of cases are considered familial (fALS), i.e., genetic mutations can be traced back to relatives, while 90–95% of cases are considered sporadic (sALS).⁷⁴³ 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.^{744–746} 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.⁷⁴⁷ 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.⁷⁴⁸ 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 that showed a loss of motor function in the SOD1^{G93A} mice but not in the SOD1 deficient mice.^{749,750} These findings led to the theory that SOD1 mutations cause a toxic gain of function.

Similar to tau, amyloid β , and α S, misfolded SOD1 aggregates are seen in large inclusion bodies in cells both *in*

vivo and *in vitro*.^{751,752} 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 nontoxic to cells but actually protective.^{753,754} Other proteins associated with ALS, mainly TAR DNA-binding protein 43 kDa (TDP-43) and Fused in Sarcoma (FUS), are also found in large inclusion bodies during disease progression.⁷⁵⁵ TDP-43 and FUS are both important for RNA transport and are natively found in the nucleus, and mutations cause both proteins to mislocalize to the cytoplasm and aggregate into stress granules (large inclusion bodies).^{756–758} Yet, overexpression of either mutant is not sufficient to trigger the appearance of stress granules in healthy cells without a stress stimulus.⁷⁵⁹ Overexpression of misfolded SOD1 has been shown to cause mislocalization and aggregation of both mutant and nonmutant TDP-43 and FUS,^{760,761} and mutant TDP-43 and FUS, in a stressed environment, have been shown to coexist with misfolded wild-type SOD1 in cells.^{762–764} 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.^{765–767} Multiple other genetic mutations in the RNA processing and protein trafficking/degradation categories could act similarly to TDP-43 and FUS or contribute to these cell death pathways.⁷⁶⁸

Motor neurons are unique from other cells due to the length of their axons. Due to how large an area a neuron can span, the cytoskeleton system is especially crucial to deliver organelles and materials throughout the entire cell.^{769,770} 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.⁷⁷¹ Coat protein complex II (COPII) vesicles transport proteins between the ER and Golgi; loss of transport leads to ER stress and Golgi fragmentation.^{772,773} Expression of COPII with G93A or alanine-4 to valine substitution (A4 V) mutant SOD1 prevents stress granules and cell death, suggesting mutant SOD1 affects ER to Golgi transport by interfering with COPII vesicles.^{774,775} Misfolded SOD1 has been shown to interact with p38 MAP kinase (p38 MAPK).⁷⁷⁶ P38 MAPK phosphorylates kinase-1 preventing it from moving along microtubules.⁷⁷⁷ Disrupted functioning of p38 MAPK could cause excessive kinesin-1 phosphorylation which would inhibit microtubule transport; inhibition of p38 MAPK in mutant SOD1^{G93A} cell models reduced cell death.⁷⁷⁸ 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^{G93A} mouse models.⁷⁷⁹ Another pathological hallmark of ALS is the accumulation of neurofilaments;^{780,781} any defects in kinesin or microtubule stability could cause this accumulation.^{782,783} 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.^{784,785} *In vitro* ALS models expressing SOD1^{G93A} showed a significant reduction in the number of axonal mitochondria, and the mitochondria present were spaced further apart than in the

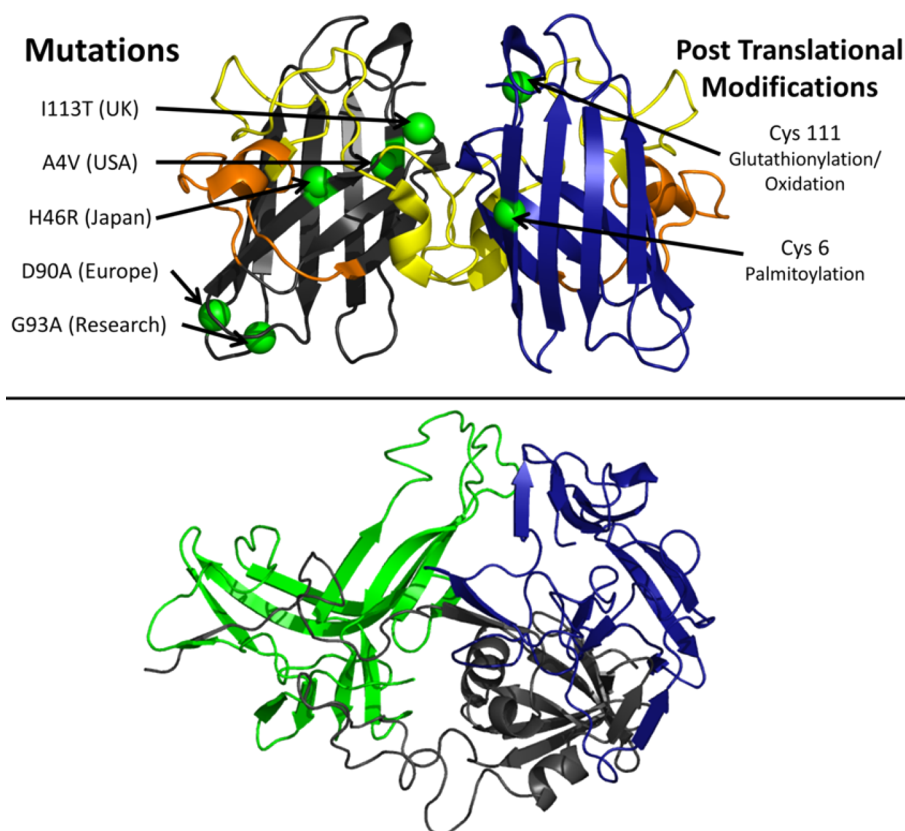


Figure 29. Mutations, modifications, and structural elements of SOD1. The crystal structure of SOD1^{WT} is shown in the top panel (pdb: 2V0A)⁸³⁰ with the zinc binding loop, loop IV, spanning residues 49–84 (yellow) and the electrostatic loop, loop VII, spanning residues 122–143 (orange). The most prevalent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

controls; the same was seen in SOD1^{G93A} transgenic mice and rats.^{786,787} SOD1^{G93A} has also been shown to interfere with the activity of voltage-dependent anion channel 1 (VDAC1) on the outer mitochondrial membrane.⁷⁸⁸ VDAC1 is necessary for the exchange of ATP and ADP across the mitochondrial membrane; in SOD1^{G93A} mouse models depressed mitochondrial respiration rates and impaired ATP synthesis were seen in the brain and spinal cord well before disease onset.^{789,790} Bcl-2 proteins regulate the release of apoptotic cytochromes by VDAC1 and have also been shown to interact with misfolded SOD1.⁷⁹¹

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.^{792,793} 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.^{794–796} Misfolded SOD1 has been shown to reduce expression of EAAT2 leading to glutamate induced excitotoxicity.^{797–799} Riluzole, the first FDA approved drug to treat ALS, slows disease progression by increasing EAAT2 activity.^{800,801} 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.^{802–804} 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.⁸⁰⁵ For toxic SOD1 trimers to form, the stable dimer must dissociate into monomers and lose its metals. In 2004 Khare et al. showed SOD1^{A4V} dimers have a 10,000 fold increase in dimer dissociation equilibrium constant compared to SOD1^{WT}.⁸⁰⁶ SOD1^{A4V} 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).⁸⁰⁷ 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.^{808,809} 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 β -sheet region of the monomer. Loss of metals exposes the β -sheet region, destabilizing the monomer and making it prone to aggregation.^{810–813} Simulations of the SOD1 barrel, without metals, found that the β -strands β 5 and β 6 are the weak spots for thermal unfolding. In simulations in a

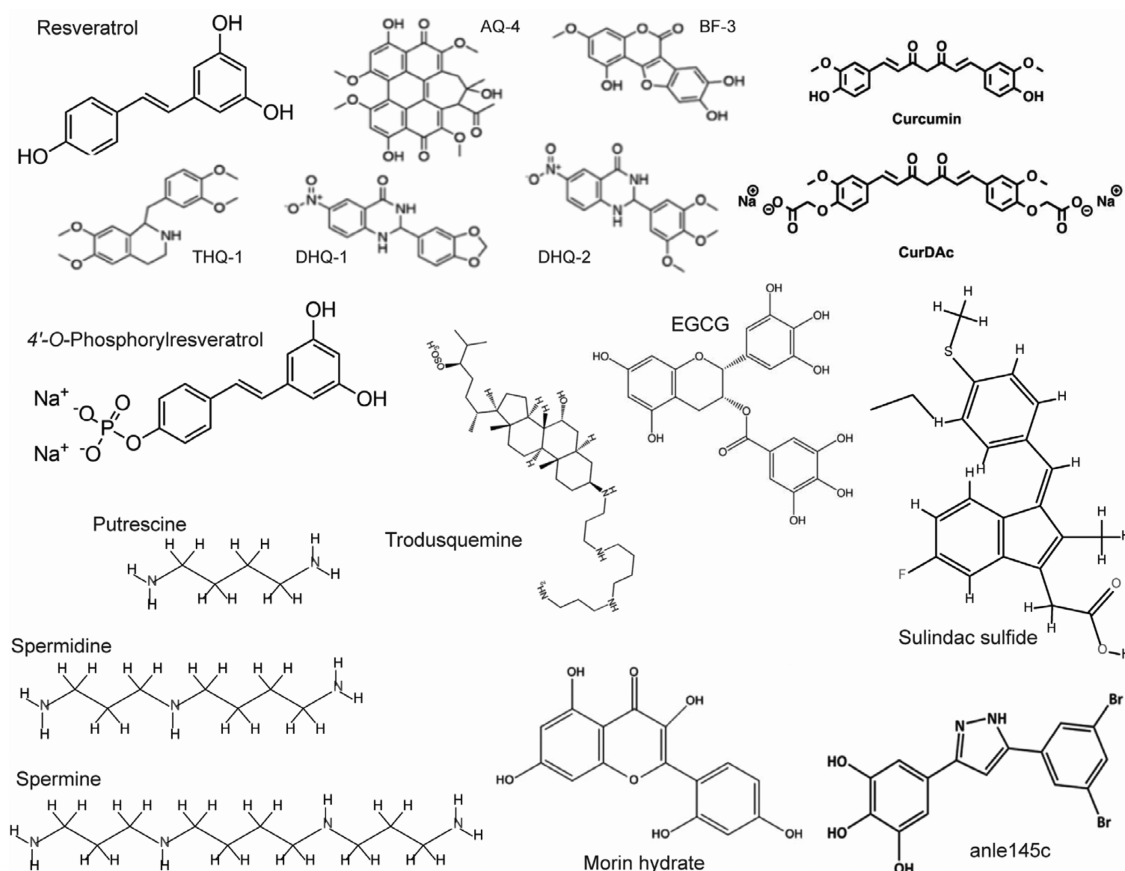


Figure 30. Chemical structures of a few selected small molecules that are shown to inhibit amyloid aggregation, remodel aged fibers, and/or promote A β or IAPP fibrillation.

crowded environment, oscillations of the VII electrostatic loop region expose the same β -strands correlating with increased interactions with the crowder. Mutations of histidine 46 stabilized the VII loop in the compact state, thus protecting the β 5 and β 6 strands similar to how metal ions protect the barrel.⁴¹⁵

Multiple studies have shown that PTMs can destabilize the dimer interface, increasing the chance of misfolding.^{814,815} In 2011 Redler showed that glutathionylation of dimeric SOD1^{WT} 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.^{816,817} Oxidation of Cysteine 111 to sulfonic acid in SOD1^{WT} in neurons led to similar phenotypes of familial ALS, misfolded SOD1 aggregates, and inhibition of kinesin-based fast axonal transport.⁸¹⁸ 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.^{814,819} 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.⁸²⁰ Antinone et al. found that misfolded SOD1 in SOD1^{G93A} cell culture and mice spinal cords showed increased palmytoylation compared to SOD1^{WT}.⁸²¹ 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.⁸²² Fay et al. found that a phosphor-mimicking aspartic acid substitution at Threonine-2 stabilizes SOD1 A4 V dimers, suggesting that phosphorylation of SOD1 could be an innate cellular protection against SOD1 destabilization.⁸²³

Mutations from environmental toxins have also been shown to destabilize the dimer interface of SOD1.^{824–826} Most notably, exposure to β -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.⁸²⁷ 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.⁸²⁸ 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, show the aggregation of SOD1 into toxic trimers leads to a toxic gain of function in both sporadic and familial ALS.

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,^{193,831} and designing new

strategies to reduce the toxicities by targeting A β , tau, α S, and IAPP.^{302,832–846}

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 complicates 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 the 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 β aggregation in a membrane environment.⁸⁴⁷ This study reported five small molecules as efficient inhibitors of A β 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 nonfibrillary A β species in the presence of large unilamellar vesicles composed of mixed (zwitterionic/anionic) lipids.⁸⁴⁷ The mechanism of the action of these compounds to inhibit A β 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 β . Another approach that promotes A β 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 β aggregation by suppressing the amount of toxic A β species.^{848,849} Recently, Dobson et al. discovered a natural product aminosterol trodusquemine (Figure 30) isolated from shark that enhanced A β aggregation following the suppression of neurotoxicity.⁸⁵⁰ Promotion of A β aggregation has also been observed in the presence of poly amino acids.⁸⁵² Nonsteroidal anti-inflammatory drug (NSAID) sulindac sulfide is another small molecule that forms colloid particles and catalyzes A β fibrillation (Figure 30). Solid-state NMR results probing the action of NSAID have identified a homogeneous distribution of amyloid-fibers characterized by a canonical β -strand–turn– β -strand with reduced neurotoxicity.⁸⁵³

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 such as EGCG present in green tea, curcumin present in turmeric, and resveratrol present in red wine inhibit IAPP amyloidogenesis (Figure 30).⁸⁵⁵ Curcumin is a planar biphenolic compound that delays IAPP aggregation when mixed with peptide monomers. The mechanism of action is proposed to be mediated by curcumin assisted disassembly of an α -helical intermediate of IAPP, that has been reported to nucleate the aggregation.⁸⁵⁶ 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 β -cells and partially protect cells from IAPP induced apoptosis.⁸⁵⁷ 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).⁸⁴⁶ Pithadia et al. tested the effect of CurDac in rescuing the IAPP induced membrane damage using a variety of biophysical techniques.⁸⁵⁸ 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.⁸⁵⁸ 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 β , IAPP, and human calcitonin).⁸⁴⁶ Toxicity measurements of the disintegrated peptide species from aged fibers, or monomers, incubated with CurDac were found to enhance the cytotoxicity of IAPP.⁸⁴⁶ 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 β and IAPP aggregation, remodeling toxicity of oligomers and disaggregate aged fibers in solution (Figure 30).^{859,860} In addition, a seeding reaction identified that EGCG bound IAPP species do not bind IAPP monomers and thus restrict fibrillation.^{859,861} 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.⁸⁶² Morin hydrate is another good example of a small-molecule compound that is also shown to inhibit IAPP aggregation and disaggregates the preformed IAPP fibers (Figure 30).⁸⁶³ IAPP oligomers are also targeted by small molecules to reduce their cytotoxicity. For example, sulfonated triphenyl methane derivative acid fuchsin⁸⁶⁴ and EGCG⁸⁵⁹ 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.⁸⁶⁵ Saravanan et al. reported a diphenylpyrazole derived inhibitor anle145c to effectively bind and generate nontoxic IAPP oligomers (Figure 30).⁸⁴² Computational study of an analogous compound anle145c shows remodeling of preformed A β and IAPP oligomers.⁸⁶⁶ Polyphenols and their effect on IAPP aggregation in the membrane interface have also been tested. Derivatives of resveratrol with improved solubility (Figure 30) are shown to abolish IAPP fibrillation in the presence of liposomes.⁸⁶⁷ In addition, suppression of IAPP induced membrane damage is revealed by conjugating resveratrol with lipids containing a dimyristoylphosphatidyl moiety.⁸⁶⁷ Lolicato et al. provided mechanistic insights into rescue of IAPP induced membrane damage. The simulation shows resveratrol disrupts IAPP interaction with an anionic membrane by binding to the hydrophobic region (23–25 and 32–34) of the peptide.⁸⁶⁸

Many compounds were synthesized to prevent amyloid aggregation.^{842,869} Of note, the compound anle138b also ameliorates tau pathology.⁸⁶⁹ Studies have also attempted to determine potential inhibitors from natural compounds^{870,871} since they normally show low side effects, well-known pharmacological properties, and high availability. Several

Table 3. List of Seven Inhibitors for AD, PD, T2D, and ALS

N ^o	Compound	Alzheimer	Parkinson	Type II Diabetes	ALS
1	Asaxanthin (Experiments/Computations)	Inhibits A β (cell viability assay, ThT, TEM, neurite outgrowth assay/docking, MD, REMD, linear interaction energy)	Inhibits α 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 β fibrils (cell viability assay, CD, ThT assay, TEM/docking, MD)	Inhibits α 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 β oligomers (cell viability assay, free radical assay, TEM/docking, MD, free energy perturbation)	Prevents formation of α 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 (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 β fibrils (AFM, light scattering, TEM, size-exclusion HPLC, MS/MD, REMD)	Inhibits A β and α S (AFM, light scattering, TEM, size-exclusion HPLC, MS, /MD)	Inhibits hIAPP protofibrils (ESI-MS, 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 β oligomers (aggregation assay, cell viability assay, CD, dot blot assay, DLS, nitroblue tetrazolium staining, size-exclusion chromatography, ThT, EM, /MD, REMD)	Inhibits α 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-MS, fibril depolymerization, ThT assay, TEM, /MD, REMD)	Inhibits SOD1 aggregation (ANS assay, collision induced unfolding, ESI-MS, ThT assay, VC50 experiment, /docking, steered MD simulations)
6	Resveratrol (Experiments/Computations)	Reduces A β aggregation (aggregation assay, Bio-Rad protein assay/DMD)	Inhibits α 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 β oligomerization (AFM, cell viability assay, oligomer size distributions, electrophysiology, NMR, size-exclusion chromatography/docking)	Inhibits α 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)

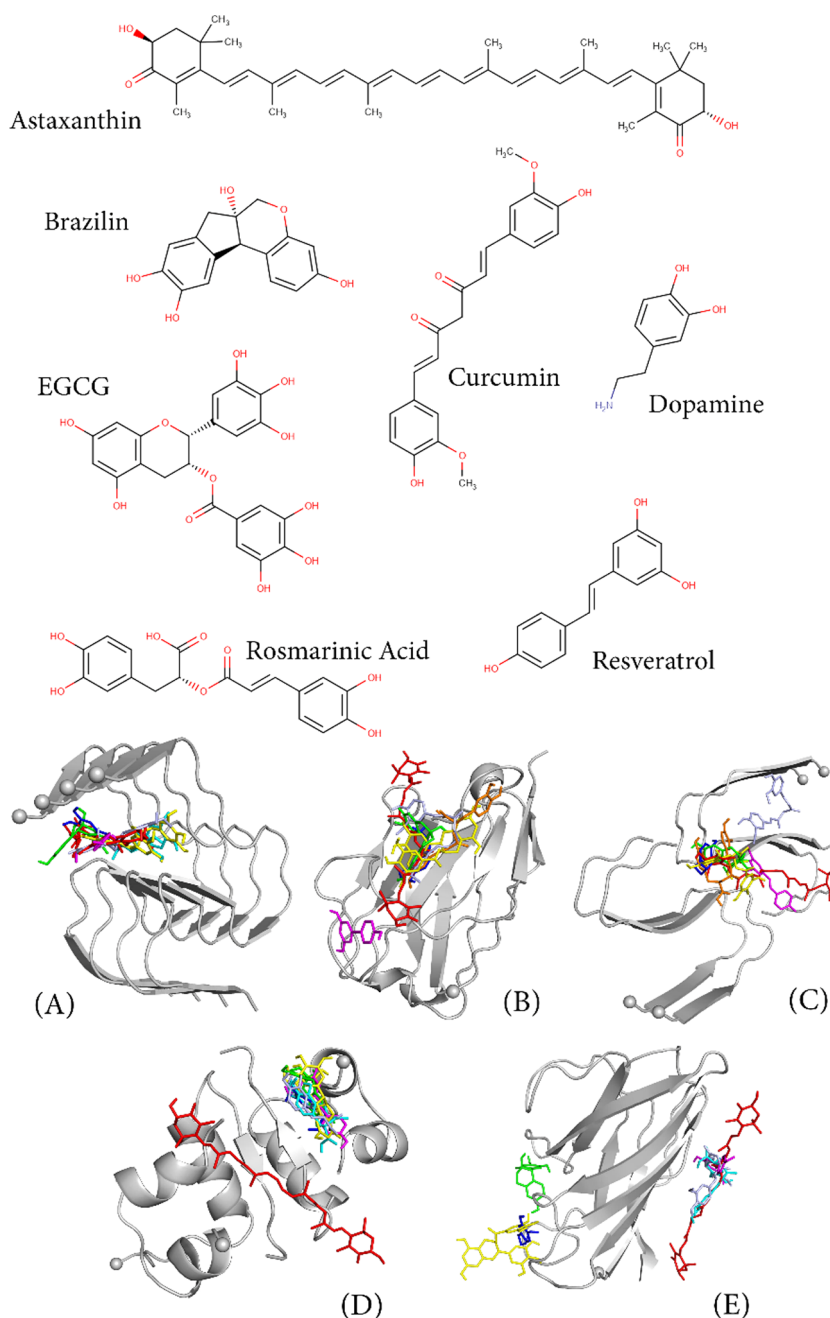


Figure 31. Chemical structures and the binding poses of the seven natural ligands to (A) $4A\beta_{11-42}$ peptides (PDB 2MXU),⁹¹¹ (B) αS (2X6M),⁹¹² (C) hIAPP (6Y1A),²⁵³ (D) insulin (1GUJ),⁹¹³ and (E) SOD1 (6FLH).⁹¹⁴ Binding poses were obtained by Autodock vina⁹¹⁵ with exhaustiveness of 8 and a grid of $60 \times 60 \times 60$ Å covering the whole receptor.⁹¹⁶ Astaxanthin, brazilin, curcumin, dopamine, EGCG, resveratrol, and rosmarinic acid are highlighted in red, green, light blue, blue, yellow, magenta, and orange, respectively.

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.⁸⁷² The inhibition probably activates through direct binding with the peptides⁸⁷³ and/or interfering with π - π stacking interaction between aromatic rings of amyloidogenic residues according to the observation via microscopic and quantum chemical analyses.⁸⁷⁴ Interestingly, some natural compounds such as taxanthin,⁸⁷⁵⁻⁸⁷⁹ brazilin,⁸⁸⁰⁻⁸⁸² curcumin,⁸⁸³⁻⁸⁸⁷ dopamine,⁸⁸⁸⁻⁸⁹⁴ EGCG,^{283,860,895-900} resveratrol,^{328,901-904} and rosmarinic acid⁹⁰⁵⁻⁹¹⁰ 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 mechanisms of these ligands to the diseases are probably different, they all adopt a similar binding pose to $A\beta$ peptides, α -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 bioinspired peptides containing natural and unnatural amino acids, synthetic polymers, and polymer-based particles have been developed to control amyloid aggregation. Selectivity, specificity, toxicity, rational designing, membrane permeability, and proteolytic stability are the advantages of these molecules to

Table 4. Summary of Atomistic Simulation of the Inhibition of Carbon Nanoparticles (CNPs) on the Fibrillization of Amyloid Peptides, Starting from Oligomers or Protofibrils^a

ref	Peptide	Carbon nanoparticle	Force field	Solvent model	Method	T (K)	Time (ns)	Oligomer or Protofibril
938	A β _{16–22}	CNT	Gromos96 43a1	SPC	REMD	310–420	110 ns × 40	8mer
939	A β _{16–22}	CNT-(OH) ₃₀	Gromos96 43a1	SPC	REMD	310–420	200 ns × 40	8mer, fibrillar β -sheet
940	A β _{16–22}	C ₆₀ , C ₁₈₀	Gromos96 43a1	SPC	REMD	310–420	200 ns × 40	8mer
942	A β _{16–21}	Graphene nanosheet	OPLS/AA	SPC	MD	310	1000 ns × 5	Protofibril
945	A β ₄₂	C ₆₀	Gromos96 53a6	SPC	REMD	306–418	340 ns × 54	2mer
946	A β ₄₂	GO ₆₀	Gromos96 53a6	SPC	REMD	306–418 308–419	410 ns × 54 500 ns × 54	2mer
950	A β _{11–42}	CNT-(OH) ₃₀	OPLS/AA	TIP4P	MD	310	100 ns × 3	Protofibril
953	A β _{33–42}	GO ₆₀ , GO ₁₂₀	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) ₃₀	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 ₆₀ , C ₆₀ (OH) ₈ C ₆₀ (OH) ₂₄	OPLS/AA	TIP4P	REMD MD	306–409 310	360 ns × 48 300 ns × 6	2mer, Protofibril
960	α S _{68–78}	C ₆₀ (OH) _{4n} ($n = 0–5, 10$)	Medusa	Implicit solvent EEF1	DMD	300	200 ns × 20	10mer

^aAbbreviations: GO, graphene oxide; CNT, carbon nanotube; GQD, graphene quantum dot.

effectively target amyloidogenic proteins/peptides. Sahoo et al. developed a cationic PMAQA polymer and proposed its multifunctional use against AD and T2D.⁸⁵¹ It is shown that PMAQA⁹¹⁷ accelerates the fibrillation (~ 1 min) of A β 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.⁹¹⁸ Recently, short anionic and cationic styrene-based copolymers (~ 2.2 kDa) are shown to catalyze IAPP aggregation by quickly inducing the formation of nontoxic fibers, whereas cationic styrene copolymers inhibit IAPP aggregation and induce generation of nontoxic globulomers.⁹¹⁹ Smith et al. recently developed polymer–peptide conjugates to control the molecular ordering of A β structure and stability, but the pathological phenotypes of these nanostructure are yet to be tested.⁹²⁰ Thermoresponsive polymers varying in hydrophobicity/hydrophilicity ratio are shown to selectively modulate A β aggregation kinetics.⁹²¹ Poly(p-phenylenevinylene) derived polymer is shown to degrade A β fibers that are nontoxic and to clear the A β plaques from the brain tested *ex vivo*.⁹²² Polymer nanocomposites have been 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 β fragment “KLVFF” polymer layer. The nanocomposite is shown to reduce toxic oligomers by trapping A β following the formation of coassembled nanoclusters, and its efficacy is measured *in vivo* using Alzheimer’s mice.⁹²³ Dendrimers designed using a poly(propyleneimine) core and a maltose-histidine shell have been shown to enhance its ability to cross the blood–brain-barrier (BBB) and protect from the progression of AD in transgenic mice.⁹²⁴ Poly(amidoamine) dendrimer and *N*-isopropylacrylamide:*N*-*tert*-butylacrylamide copolymeric nanoparticles are shown to effectively abolish IAPP aggregation and its toxicity.^{925,926} Lipid-nanodiscs prepared by conjugating synthetic polymers or peptides with natural phospholipids were recently tested on A β .^{263,274} Polymer nanodiscs are shown to strongly bind A β following a quick induction of fibers that tested non-neurotoxic.²⁶³

Protein and peptide lipid-nanodiscs show promising application in trapping amyloid intermediates and delaying aggregation of A β or IAPP.^{274,285} Apolipoprotein J conjugated nanoparticles have been shown to reduce the amount of cerebral A β in transgenic mice.⁹²⁷ Cationic fluorescent conjugated polymer nanoparticles are shown to inhibit A β aggregation.⁹²⁸ Nanoliposomes decorated with A β binding surface containing benzothiazolyl are proposed to be an effective therapeutic systems for AD treatment.⁹²⁹ For other peptide-based inhibitors, readers are referred to a recent review⁹³⁰ and recent articles.^{931–933}

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 the BBB), as discussed in two recent reviews.^{836,838} As early as 1997, it was reported by Dugan et al. that C₆₀ derivatives (i.e., carboxyfullerenes) can function as neuroprotective drugs *in vivo*.⁹³⁴ In the following years, a few 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 β _{1–40} at the early stage.⁹³⁵ In 2007, TEM and *in vivo* investigations by Podolski et al. demonstrated that hydrated fullerenes impede the fibrillization of A β _{25–35} peptide and improve the performance of the cognitive task in control rats.⁹³⁶ In the same year, Linse et al. reported that carbon nanotubes (CNTs) enhance the fibril formation of human β 2-microglobulin by accelerating the nucleation process.⁹³⁷ 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 β fragments, full-length A β , hIAPP, and α S fragment (Table 4).

9.1.3.1. Inhibition of CNPs against Fibrillization of A β /A β -Fragment and Shape/Surface Curvature Effects of CNPs. In 2011, Li et al. investigated the effects of carbon nanotubes on the oligomerization of A β _{16–22} peptide by atomistic REMD simulations.⁹³⁸ Their simulations demonstrated that CNTs not only inhibit the formation of β -sheet-rich octamers (with an average β -sheet content of 7.9% in the presence of CNT relative to 44.5% in the absence of CNT) but also destabilize fibrillar β -sheets through hydrophobic and π – π stacking interactions. The authors proposed that CNT is likely to impede the fibrillation of A β _{16–22} and of the full-length A β , as β -sheet is the dominant secondary structure content of A β fibril and A β _{16–22} is the central hydrophobic core of A β . Two years later, Xie et al. examined the influence of a hydroxylated carbon nanotube (CNT-OH) on A β _{16–22} aggregation using a combined simulation and experimental method.⁹³⁹ 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 β -sheet-rich octamers (with a β -sheet content of 17.5% in the presence of CNT-OH). Hydrophobic, π – π stacking and electrostatic interactions between CNT-OH and A β _{16–22} play important roles in inhibiting β -sheet formation. Their AFM and thioflavin ThT fluorescence experiments confirmed the inhibitory effect of both CNT and CNT-OH on A β _{16–22} fibrillization, in support of the REMD simulation predictions.^{938,939} Next, Xie et al. investigated the effect of fullerenes with different surface curvatures (C₆₀ and C₁₈₀) on the β -sheet formation of A β _{16–22} octamers by REMD simulations.⁹⁴⁰ They found that C₆₀ significantly reduces the β -sheet probability (25.7% with C₆₀ relative to 44.5% without C₆₀) and increases the coil propensity, thus retarding the fibril formation of A β _{16–22}. The simulation-predicted inhibitory effect of C₆₀ on A β _{16–22} fibrillization was confirmed by AFM and ThT experiments.⁹⁴⁰ Interestingly, it was found that C₁₈₀ (with the same total mass as C₆₀) exhibits a stronger inhibitory effect on the β -sheet formation (with a β -sheet content of 18.1%) than C₆₀. This observation can be explained by the strong hydrophobic and aromatic stacking interactions between fullerene hexagonal rings and the Phe rings of A β _{16–22} as C₁₈₀ contains more hexagonal rings than C₆₀. This result highlights the significant role of fullerene hexagonal rings in the inhibition of A β _{16–22} fibrillation. By comparing the β -sheet probability of A β _{16–22} oligomers in the presence of CNT, C₆₀, and C₁₈₀ (with almost the same total mass) obtained by the two REMD studies,^{938,940} it was found that CNT displays the strongest inhibitory effect on the β -sheet formation, followed by C₁₈₀ and then C₆₀. The different inhibitory abilities are 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 β _{33–42} aggregation, followed by nanotubes and nanodots.⁹⁴¹ These results reveal the important role of shape and surface curvature of CNP in inhibiting A β _{16–22}/A β _{33–42} fibrillation. Meanwhile, Yang et al. examined the influence of graphene nanosheets on preformed A β _{16–21} 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 β _{16–21} fibrils.⁹⁴²

In order to reveal at the atomistic level the molecular mechanism behind the experimental observations showing that both C₆₀ and graphene oxide (GO) nanosheet can inhibit the fibrillization of full-length A β peptide,^{935,942–944} Sun et al. and Jin et al. performed REMD simulations on A β _{1–42} dimer in the absence and presence of four C₆₀ molecules or four GO nanosheets.^{945,946} The GO nanosheet contains the same number of carbon atoms as C₆₀, which is denoted as GO₆₀. These REMD simulations showed that A β _{1–42} peptides form diverse β -hairpin-containing dimeric conformations, whereas those conformations are significantly reduced in the absence of C₆₀ or GO₆₀. C₆₀ suppresses the β -sheet formation of A β _{1–42} mostly by binding to the central hydrophobic motif LVFFA and the C-terminal hydrophobic region I31 ~ V40, while GO₆₀ inhibits the β -sheet formation by mainly binding to charged residues and hydrophobic residues. GO₆₀ displays a better inhibitory effect on the β -sheet formation of A β _{1–42} dimer than C₆₀,⁹⁴⁶ indicating that the shape of CNP may play a role in the inhibition of A β ₄₂ aggregation. *In silico* and *in vitro* experiments showed that water-soluble fullereneol C₆₀(OH)₁₆ tightly binds to negatively charged residues of A β _{1–40} monomers and effectively reduces the formation of amyloid fibrils.⁹⁴⁷ The interactions of C₆₀/C₆₀OH with U-/LS-shaped A β protofibrils were studied by explicit-solvent MD simulations.^{948,949} It was found that C₆₀ and C₆₀OH exhibit different binding mechanisms and the binding of C₆₀ to A β protofibril results in disruption of the D23–K28 salt bridge. More recently, a combined MD simulation and experimental study by Liu et al.⁹⁵⁰ reported that hydroxylated carbon nanotubes (CNT-OHs) also inhibit A β ₄₂ fibrillization, disaggregate mature fibrils, and reduce A β ₄₂-induced cytotoxicity. The six residues H13, H14, Q15, V36, G37, and G38 contribute most to the interactions between CNT-OH and A β _{11–42} protofibril.

9.1.3.2. Size Effect of Carbon Nanoparticles on the Inhibition of A β _{33–42} Aggregation. CD spectra and AFM experiments by Dong's group reported that GO interferes with the aggregation pathway of A β _{33–42} peptide, leading to a reduced β -sheet content and considerably short fibrils, and large-size GO displays a strong capacity in impeding A β _{33–42} aggregation.^{951,952} In order to unravel the mechanistic basis of the inhibition and size effect of GO nanosheets on the fibrillization of A β _{33–42} peptide, Chen et al.⁹⁵³ investigated the oligomerization of A β _{33–42} peptide in the absence and presence of four GO₆₀ nanosheets or two GO₁₂₀ nanosheets by performing all-atom REMD simulations starting from four extended A β _{33–42} peptide chains. Their simulations showed that in the absence of GO nanosheets, four A β _{33–42} chains can form β -barrel-like structures and antiparallel β -sheets. Inter-peptide hydrophobic interactions among residues M35, V36, V39, and V40 and backbone hydrogen bonding interactions play crucial roles in the formation of β -barrels/ β -sheets. GO retards A β _{33–42} oligomerization by interfering with peptide–peptide interactions. Consisting of the same total carbon atoms and oxidation groups as GO₆₀, GO₁₂₀ exhibits a stronger capacity in inhibiting A β _{33–42} aggregation than GO₆₀. The oxidation groups of GO₁₂₀, especially the hydroxyl groups, can form more hydrogen bonds with A β _{33–42} than those of GO₆₀. Moreover, GO₁₂₀ has a larger hydrophobic contact surface area with A β _{33–42} than GO₆₀. These results reveal that GO₁₂₀ displays stronger hydrogen-bonding and hydrophobic interactions with A β _{33–42} than GO₆₀, thus leading to a better inhibitory effect on A β _{33–42} aggregation. These simulation results provide mechanistic insights into the inhibition and size

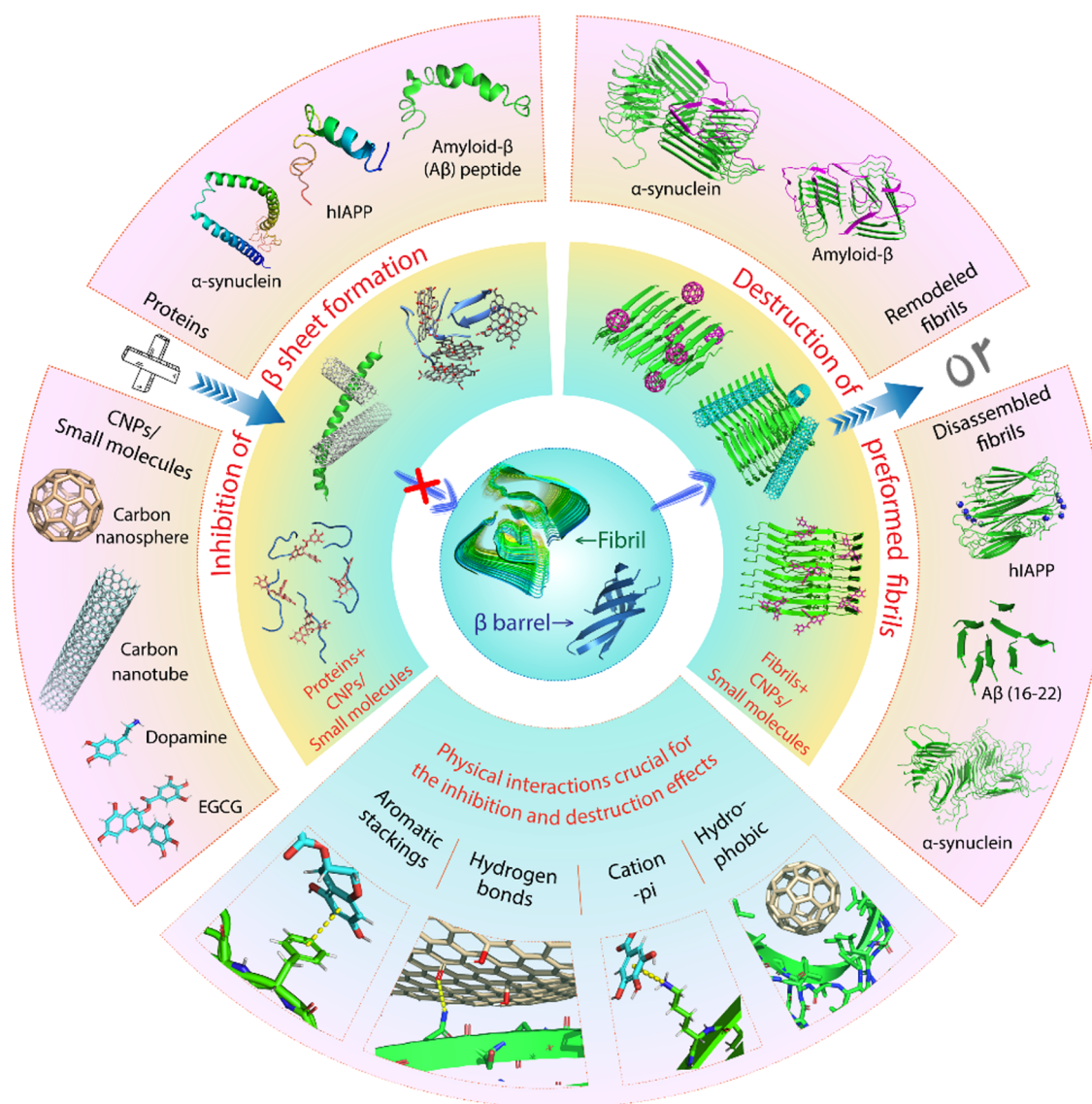


Figure 32. Schematic representation of the molecular mechanisms by which carbon nanoparticles (CNPs) and small molecules inhibit the aggregation of proteins/peptides discussed in this review. Amyloid proteins can self-assemble into toxic β -sheet-rich oligomers (such as β -barrels) and protofibrils/fibrils. CNPs/small molecules can prevent β -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.

effects of GO nanosheets on the aggregation of $A\beta_{33-42}$ peptide.⁹⁵³

9.1.3.3. Inhibition of CNPs against hIAPP/ α 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 the last five years. Nedumpully-Govindan et al. investigated the effect of GO nanosheets on hIAPP fibrillization and cytotoxicity by combining computational modeling, biophysical characterization, and cell toxicity measurements.⁹⁵⁴ 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.⁹⁵⁵ 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 β -cell viability assays, and an embryonic zebrafish model. Their recent study⁹⁵⁶ reported that GQDs can rescue protein dysregulation of pancreatic β -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.^{957–959} 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_{60} OHs not only suppress the formation of β -sheet and β -hairpin-containing amyloidogenic precursors of hIAPP but also remodel or disassemble preformed hIAPP protofibrils/fibrils. Hydrogen-bonding, hydrophobic, cation- π , and π - π stacking

interactions between hIAPP and CNT-OH/C₆₀OH are the dominant driving forces in inhibiting hIAPP aggregation. Turbidity analyses, ThT fluorescence assays, CD microscopies, and TEM and AFM images provide direct evidence for the simulation-predicted β -sheet inhibition and protofibril-disruption by hydroxylated CNTs/fullerenes. SH-SY5Y cell viability assays demonstrated the rescue effect of C₆₀OHs on hIAPP-induced cytotoxicity.⁹⁵⁸ The REMD simulations by Bai et al.^{958,959} showed that C₆₀, C₆₀(OH)₈, and C₆₀(OH)₂₄ possess different inhibitory effects on the β -sheet formation of hIAPP: the β -sheet content of hIAPP dimer in the presence of C₆₀, C₆₀(OH)₈, and C₆₀(OH)₂₄ is respectively 1.8, 4.2, and 5.6% (the β -sheet content of hIAPP dimer alone is 10.8%). These data reveal that hydroxylation extents modulate the inhibition capacity of C₆₀OHs against hIAPP aggregation. Recently, Sun et al.⁹⁶⁰ explored the effects of hydroxylation extents of C₆₀ on β -sheet formation of the nonamyloidogenic core (NAC) fragment (residues 68–78) of α S by DMD simulations. Their simulation data showed that the α S_{68–78} peptide can assemble into cross- β aggregates and β -barrel intermediates. Hydroxylated C₆₀ (C₆₀(OH)_n with n = 1–5) significantly inhibits β -sheet formation and aggregation of α S_{68–78} peptide, while C₆₀(OH)₄₀ displays very weak inhibitory impact. These results suggest the necessity of the amphiphilic surface chemistry of hydroxylated fullerenes in hindering the amyloid aggregation of α -Syn_{68–78} peptide. The influence of GQDs on the aggregation of α -synuclein (α -Syn) protein was investigated by Kim et al. using a combination of *in silico*, *in vitro*, and *in vivo* methods.⁹⁶¹ It was shown that GQDs not only inhibit α -Syn fibrillization and disaggregate fibrils but also penetrate the BBB and possess unique neuroprotective effects against the neuropathological α S aggregates/fibrils.⁹⁶¹ A recent study demonstrated the antiaggregation effect of fullerenols (C₆₀(OH)₃₀ and C₇₀(OH)₃₀) on α S protein and the neuroprotective activity in *Drosophila* PD models.⁹⁶² 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.⁹⁶³

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 illustrates the inhibitory mechanisms of CNPs/small molecules against protein aggregation.

9.2. Active and Passive Immunizations as Alzheimer's and Parkinson's Therapies

9.2.1. Active Immunization as AD Therapy. Active immunization 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, immunization 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 stabilize the vaccine, help with delivery to the right part of the body, or improve the immune response (adjuvants).^{964,965}

The success of vaccines in general suggested the tantalizing prospect of developing a vaccine for AD. The pioneering study in this field was AN-1792: synthetic A β (1–42) coupled with a glycosidic adjuvant called QS-21.⁹⁶⁶ While it might seem paradoxical to use A β therapeutically if excess A β leads to AD, the idea was that A β 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 β plaques, neuritic dystrophy, and astrogliosis. Even older animals benefitted from treatment, showing reduced AD pathology.⁹⁶⁶ 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.⁹⁶⁷ A minority of patients developed antibodies against the N-terminal region of A β .⁹⁶⁸ Some patients showed functional benefit several years later.⁹⁶⁹ Phospho-tau was reduced.⁹⁷⁰

The AN-1792 results prompted various studies aimed at developing an AD vaccine. Most designs use a fragment of A β 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 β 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 β 40 conjugated to the keyhole limpet cyanine carrier protein and formulated with the adjuvant alum hydroxide.⁹⁷¹ Using the C-terminal end of A β is designed to minimize binding to the parent APP when APP is within a membrane. It will also be unaffected by N-terminal reactions of A β . 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 ABvac40 is currently underway, with 120 participants at the earliest stage of AD or MCI, monitored over 2 years using amyloid PET (positron emission tomography) scans, biomarkers, and cognitive and quality-of-life measures.

UB-311 is two A β (1–14) peptides linked to T-cell peptide epitopes, formulated in a proprietary delivery system, biased to Th2 cells.⁹⁷² 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 β in AD.⁹⁷³ Since tau aggregates are intracellular, it seemed doubtful whether antibody administration could be beneficial. However, promising results in tau transgenic mice^{974,975} gave some encouragement. The AADvac1 vaccine is amino acids 294 to 305 of tau (KDNIKHVPGGGS), coupled to keyhole limpet hemocyanin with the aluminum hydroxide adjuvant. In transgenic rats it reduced tau pathology and improved function; it reduced AD-type hyperphosphorylation of tau by approximately 95%.⁹⁷⁶ In patients, 98% generated antitau antibodies and showed reduced hyper-

phosphorylation.⁹⁷⁷ Patterns of tau phosphorylation are distinct markers of disease progression over decades.⁵²³

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

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 antioxidative stress properties. It was first developed as an unsuccessful immunotherapy for pancreatic cancer. However, in rat neuronal stem cells, GV1001 reduced Aβ oligomer-induced toxicity and protected against oxidative stress,^{981,982} so it is being repurposed as an AD vaccine. A small phase 2 study showed promising effects at halting cognitive decline.⁹⁸³

9.2.2. Passive Immunization against AD. Elderly people typically show a weak response to antigens, making active immunization a challenge. Alternatively, passive immunization 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 Aβ or tau fragments, or against aggregates to achieve specificity against pathogenic forms of Aβ. The monoclonal antibodies are collected and given to a patient with AD. Their own immune system then clears the antigens bound to the antibodies.⁹⁸⁴ 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 Aβ can slowly leave the brain, down a concentration gradient, if it is mopped up in the blood.⁹⁸⁵ Alternatively, passive immunization may work by antibodies crossing the BBB, activating microglia, and triggering phagocytosis of antibody-bound antigens, perhaps using strategies to facilitate this process.⁹⁸⁴

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

Aducanumab shows a strong preference for binding to aggregated forms of Aβ 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,^{987,988} binding to Aβ residues 3–7 in an extended conformation.⁹⁸⁹ In a mouse model, the antibody reduced Aβ deposits by 70%, mostly likely by microglia-mediated phagocytosis.⁹⁸⁷ A phase 1b trial of 165 prodromal or mild AD patients showed that aducanumab could clear Aβ plaques with no toxicity, and there were hints of cognitive benefits and decreased inflammation.⁹⁸⁷ 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 end point of the trial.^{990,991} Most unusually, however, in October 2019, Biogen announced that this conclusion was premature: later analysis of a larger EMERGE data set 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 end point. In addition, patients showed dose-dependent reduction in brain amyloid and phosphor-tau in CSF.⁹⁹² Biogen claims that this is the first time that a phase 3 study has shown that clearing Aβ 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.⁹⁹³

Similarly to aducanumab, the IgG1 antibody BAN2401 shows a strong preference for binding to soluble protofibrils of Aβ, rather than the monomeric peptide, binding to the N-terminal region. In transgenic mice, a murine version of the antibody reduced Aβ protofibrils, while leaving monomers and insoluble plaques untouched.⁹⁹⁴ In phase 1 trials, the humanized antibody was nontoxic.⁹⁹⁵ 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.⁹⁹⁶

Solanezumab is a humanized monoclonal IgG1 antibody that binds the central region of monomeric Aβ. The IgG1 antibody Gantenerumab binds to N-terminal and central amino acids of Aβ, preferentially interacting with Aβ aggregates.⁹⁹⁷ Both reached phase 3 trials for mild AD, though they were unsuccessful.^{998,999} 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.¹⁰⁰⁰

A common form of Aβ in plaques is Aβ(p3–42), which has pyroglutamate at its N-terminus.¹⁰⁰¹ Donanemab is a humanized IgG1 monoclonal antibody that binds to this plaque-specific form. It is effective at clearing plaques from transgenic mice¹⁰⁰² and in patients with AD and a positive amyloid-PET scan.¹⁰⁰³ A phase 2 trial is underway.¹⁰⁰³

Work on antibodies that target tau antigens⁹⁷⁹ is less advanced than those that target Aβ, 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 Aβ production,¹⁰⁰⁴ one of many positive feedback loops in AD.¹⁰⁰⁵ 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.¹⁰⁰⁶ Semorinab is an antitau 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.¹⁰⁰⁷ Zagotenemab is a humanized antibody that is selective for soluble tau aggregates, binding to the N-terminal region of tau.¹⁰⁰⁸ Phase 2 trials for participants with MCI or mild AD are ongoing for all three of these antibodies.

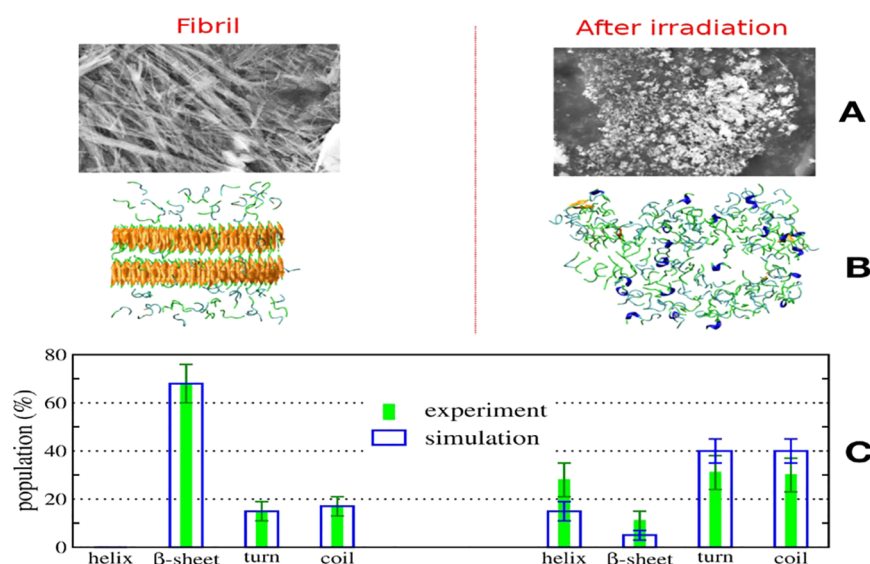


Figure 33. Images of the GNNQQNY fibril sample prior to and after laser irradiation of the GNNQQNY obtained from scanning EM experiment (A) and simulation (B). Their secondary structures are in (C).¹⁰²⁷

9.2.3. Antibody Therapies for Synucleinopathies. To date, work on antibodies that targets α S is still little explored. PRX002 is a humanized IgG1 monoclonal antibody that binds to epitopes near the C-terminus of α S. In mouse models of PD and DLB, it reduces α S accumulation and counteracts behavioral deterioration.¹⁰⁰⁹ In humans, PRX002 had a good safety profile. It nearly entirely removed free α -synuclein in the blood, though it did not reduce α S levels in CSF.¹⁰¹⁰ BIIB054 is a human IgG1 monoclonal antibody directed at an epitope near the N-terminus of α S, selective for aggregated forms.¹⁰¹¹ In a pilot study in PD patients published in 2019, BIIB054 formed plasma complexes with α S. Phase 2 trials for both antibodies are underway. Recently, antibodies targeting the N-terminal domain of α S to neutralize the toxicity of oligomers have been attempted under *in vitro* and *in vivo* conditions. A primary antibody designed from the highly lipophilic region present in the N-terminus of α S (residues 1–25) showed promising activity in rescuing the neuronal cell damage induced by α S oligomers.¹⁰¹²

9.3. Infrared Laser, Ultrasound, and Electromagnetic and Electric Fields

9.3.1. 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, nonheating infrared light into tissues and nerves deeply.¹⁰¹³ So far, the first clinical trial with near-infrared at 810 nm wavelength pulsed at 10 Hz and power of 14.2 mW/cm² was performed on five patients with mild to 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, and less anxiety and wandering were also reported. There were no side effects such as diarrhea, nausea, vomiting, anorexia, or dizziness.¹⁰¹⁴

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 β protein precursor transgenic mouse model. After six months, the numbers of A β plaques were significantly reduced in the brain as indicated by reduction in the plasma A β peptide levels of 17.7–39.8%, depending on the

laser parameters.¹⁰¹⁵ 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 β 42 plaques (approximate 15%) was observed in the cerebral cortex.¹⁰¹⁶ Near-infrared laser was also applied to APP/PS1 transgenic mouse brain as well. Results show that A β 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 nontreated mice.^{1017,1018} 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 β fibrils and oligomers. Li et al. used graphene oxide covalently connected to thioflavin-S, which can bind to A β aggregates.¹⁰¹⁹ The graphene oxide plays a role as a local heater, which absorbs strongly the near-infrared laser energy, to dissociate A β fibrils. Recently, Kawasaki and colleagues have demonstrated that the mid-infrared laser can be used to disassembly amyloid fibrils.^{1020–1023} 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 is shifted compared to single proteins, the laser-irradiation targeted to the amide I bands of fibrils should minimize the damage to surrounding molecules.¹⁰²⁴ 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.¹⁰²⁵ 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 the

far-infrared laser could disrupt the hydrogen-bond network inside of fibril more effectively.¹⁰²⁵

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 β 17–42, 5-mer β -solenoid HET-s, and 200-mer GNNQQNY.^{1026,1027} Samples containing amyloid fibrils, DNA duplexes, and globular proteins are also considered, and the simulations show that the surrounding protein and DNA molecules are not affected, demonstrating therefore laser frequency selectivity for dissociation.¹⁰²⁶ 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 shows the results obtained from a joint experimental/simulation study of the dissociation of the GNNQQNY amyloid fibril.¹⁰²⁷ 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.¹⁰²⁷

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 is 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.¹⁰²⁸

9.3.2. Ultrasound. Ultrasound waves are mechanical waves which create oscillations with frequency between 20 kHz and 10 THz in the media through which they pass, generating thermal and nonthermal 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.¹⁰²⁹ The nonthermal effects include acoustic radiation force, acoustic streaming, shock waves, and bubble cavitation.¹⁰³⁰ Ultrasound waves are already used to treat certain types of cancer and glaucoma.

By *in vivo* studies, most works use focused ultrasound to open temporarily the blood–brain-barrier and thus facilitate the transport of molecules from the blood to the brain.¹⁰³¹ 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 4 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.¹⁰³² Leinenga et al. utilized 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.¹⁰³³ The histological and biochemistry analysis of brain tissues after treatment reveals a reduction of about 50% in the dense core amyloid plaque and soluble species of A β 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 enhanced A β phagocytosis.¹⁰³³ However, it could also be possible that ultrasound increases the activation of A β -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 β load.¹⁰³⁴ In the first clinical trial, Beisteiner et al. developed a technique using single ultrashort (3 μ s) ultrasound pulses with typical energy levels of 0.2–0.3 mJ mm^{−2} 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 have no major side effects.¹⁰³⁵

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/cm² can dissociate soluble A β peptides from fibrils.¹⁰³⁶ The A β 42 is more resistant to dissociation from fibrils to monomers and/or low molecular weight soluble oligomers than A β 40. This is consistent with the fact that A β 42 peptides aggregate faster than A β 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.¹⁰³⁶ Goto et al. studied the effects of low frequency ultrasound (7–20 kHz) irradiation on both aggregation and dissociation of A β 40 peptide. They determined a critical peptide concentration of 0.7–0.9 μ M, below which the ultrasound accelerates the dissociation of fibrils into monomers.¹⁰³⁷ 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 mono-dispersed fibrils.^{1038,1039} 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 to be 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 α S and transthyretin.¹⁰⁴⁰ 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.¹⁰⁴⁰

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 β 17–42 fibril of various sizes under high ultrasound pressure of 200 MPa and very fast period of 1

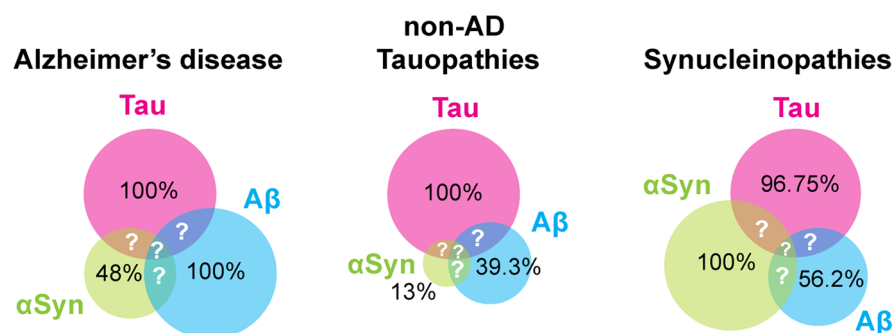


Figure 34. Co-occurring pathologies across common neurodegenerative diseases. Schematic illustrating the relative proportions (numbers within colored disks) of amyloid pathologies formed of Aβ (blue), tau (pink), and αS (green) within each disease class. Question marks indicate unknown proportions for 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 1062.

ns.¹⁰⁴¹ 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,^{1042,1043} where a bubble is represented by a particle with low mass and 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 Aβ17–42 amyloid fibril. The simulation shows that after 5 ns of excitation by 100 stable bubble cavitation periods, the β-structure in the initial fibril structure is reduced to 12%, and significant amounts of turn (30%) and coil (57%), with ≤1% of α-helices being 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.^{1042,1043} 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 marked difference in the kinetics of destabilization of fibrils having different shapes.¹⁰⁴⁴

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

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 Aβ16–22 and the Aβ42 peptides.¹⁰⁵⁰ Using electric field strengths of 150–300 V/cm, Aβ-elicited toxicity of EF-treated samples in two neuroblastoma cell lines and human embryonic kidney cell line was 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.¹⁰⁵⁰

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.¹⁰⁵¹ Zerbetto et al. simulated the interaction of Aβ peptide with electric field of varying strengths and showed that the electric field can switch the Aβ peptide from helical to β-sheet conformation.¹⁰⁵² 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 β-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.^{1053,1054}

10. CROSSTALK AND CROSS-SEEDING BETWEEN AMYLOID PROTEINS

10.1. What Do We Know from *In Vitro* and *In Vivo* Conditions?

Despite intrinsic differences among 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.^{193,1055–1061} 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 ε4 status constituting risk factors.¹⁰⁶² These observa-

tions 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.^{1063–1066} 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 α S inclusions known as Lewy bodies (LB) and Lewy neurites (LN) compared to subjects with AD without α S pathology.^{51,1067} The co-occurrence of these proteinopathies thus provides support to accumulating observations documenting potential molecular cross-talks among 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

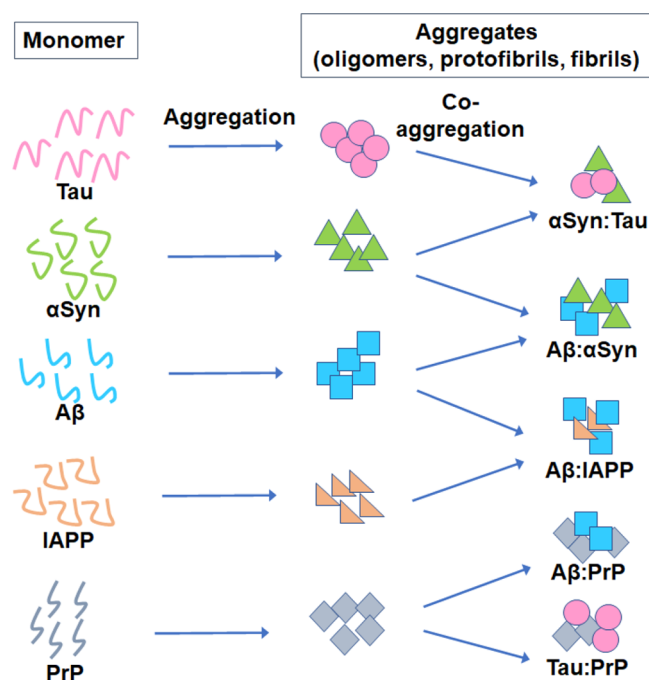


Figure 35. Cross-seeding and coaggregation of amyloid proteins. Schematic illustrating the *in vitro* formation of amyloids (depicted by different shapes) including oligomers, protofibrils, and fibrils from tau (pink), α S (green), $A\beta$ (blue), IAPP (orange), and PrP (gray) proteins. This scheme also summarizes the *in vitro* formation of coaggregates (α S:tau, $A\beta$: α S, $A\beta$:IAPP, $A\beta$:PrP, tau:PrP) by cross-seeding/coaggregation 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.

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.^{193,1055–1059,1061,1068}

10.1.1. $A\beta$ and α S. Cross seeding between aggregated $A\beta$ and α S has been observed *in vitro*,¹⁰⁶⁹ along with hybrid oligomer formation.¹⁰⁷⁰ Many studies in fact have suggested that $A\beta$ and α S are capable of direct and indirect cross-talks in the brain, along with hybrid oligomer formation.^{1069–1075} 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 α S aggregation *in vitro*. Colocalization of the two $A\beta$ species with

α S was also shown in the APP-transgenic Tg2576 mouse brain sections, suggesting an *in vivo* occurrence of the $A\beta$ and α S coaggregates.¹⁰⁷⁶ To assess a potential crosstalk between fibrillar α S and fibrillar $A\beta$, Basil and co-workers in 2020 injected mouse α S preformed fibrils (α S-PFFs) into young adult SXFAD mice harboring amyloid plaques. The presence of $A\beta$ deposits enhanced α S pathology and spreading throughout the brain. These findings have led the authors to suggest a “feed-forward” mechanism whereby $A\beta$ plaques potentiate α S seeding and spreading over time.¹⁰⁷⁷

10.1.2. α Syn and Tau. Lately, the relationship between α S and tau has received much attention, which has also been supported by GWAS studies showing strong association between the genes encoding α S and tau proteins (SNCA and MAPT, respectively) in PD and dementia with Lewy body (DLB) pathologies.^{1078,1079} The *in vivo* association of the two proteins was reported in several studies from post-mortem brain tissues showing their co-occurrence and even coaggregation in PD, DLB, ALS/parkinsonism-dementia complex pathologies.^{1080–1083} Robinson and co-workers recently characterized the presence of co-occurring pathologies of four major amyloid proteins, i.e. $A\beta$, tau, α S, and TDP-43, in several neurodegenerative diseases.¹⁰⁶² Nearly all synucleinopathies including DLB and multiple system atrophy defined by α 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 copathology was identified in 0–22% of cases. It is tempting to hypothesize that this quasi-universal co-occurrence of α S and tau pathologies observed in synucleinopathies might result from a potential cross-seeding between these proteins.¹⁰⁶²

Several groups have shown *in vitro* cross-seeding among α S and tau, which occasionally was a synergistic effect on both the proteins forming aggregates.^{1084–1089} *In vitro* aggregation studies indicate that monomeric α 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 the absence of the flanking regions,¹⁰⁹⁰ thereby promoting tau fibrillization.¹⁰⁸⁹ This interaction is mediated by the C-terminus of α S and is exacerbated by phosphorylation of α S at serine 129, a characteristic PTM of α S pathology.¹⁰⁸⁹ 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 α Syn (TgA53T, M83 line) or E46K mutant α S (TgE46K, M47 line).^{1085,1091} 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 α S^{E46K} is less efficient than wild-type α S (α S^{WT}) at promoting tau inclusions in cultured QBI293 cells.¹⁰⁹¹ 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 α S were not only reported in neurons from M83 TgA53T mice but also exacerbated in oligodendrocytes from a bigenic CNP-Tau^{P301L}/ α S^{WT} mouse model overexpressing P301L mutant tau and human α S^{WT} driven by the murine 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) promoter, in which

inclusions were positive to Thioflavin S.¹⁰⁸⁵ Because these seminal studies focused on fibril formation as opposed to oligomerization per se, the functional role of α 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.¹⁰⁹² By contrast, work from Singh and colleagues suggests that the role of tau in young and middle-aged adult G2.3 TgA53T mice is independent from α S/tau crosstalks because no differences in o- α Syn nor o-Tau were found in the absence or presence of MAPT deletion.¹⁰⁹³ 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 α S and tau. Most notably, α S was shown to regulate a key kinase for tau phosphorylation and glycogen synthase kinase-3 β (GSK3 β), in several mouse models of PD.^{1094–1098} Another comprehensive study from the Sidhu group has shown the colocalization of active GSK3 β (p-GSK3 β -Y216) with phosphorylated tau (p-tau) or α S (p- α S) in the brain tissues of a transgenic mouse model expressing human GSK3 β with a point mutation (S9A). Furthermore, the authors have shown that in addition to tau, GSK3 β also phosphorylates α S *in vitro* and that both tau and α S cooperate with each other to increase the extent of their phosphorylation *in vitro*.¹⁰⁹⁹ Exogenously added α S was shown to modulate the GSK3 β -mediated hyperphosphorylation of tau causing microtubule destabilization in rat pheochromocytoma (PC12) cells.¹¹⁰⁰ These observational studies suggest an indirect association between tau and α S.

10.1.3. A β and IAPP. Accumulating evidence of a potential link between T2D and AD pathologies has led to the investigation of the molecular interaction between A β and IAPP. Studies have shown *in vitro* that IAPP promotes the aggregation of A β 42.¹¹⁰¹ Additionally, cross-seeding between IAPP and A β 42 resulted in hybrid aggregate formation, which could associate with an artificial lipid membrane, thus reducing its fluidity.^{1102–1105} Earlier work had reported IAPP deposition in the brain tissues of AD patients without any diabetes history, indicating the presence of brain insulin resistance.¹¹⁰⁶ Plaques of IAPP were found individually or embedded in the A β 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 β and IAPP.¹¹⁰³ More recently, colocalization of IAPP and A β was also confirmed by proximity ligation assay in both cerebral and vascular A β deposits in AD brain tissues.¹¹⁰⁷ Work from the Soto group reported that intracerebral injection of IAPP aggregates derived pancreatic homogenates into APP transgenic mice exacerbated A β deposition compared to the nontransgenic animals injected with the same inoculum, suggesting an *in vivo* cross-seeding mechanism between the two proteins.¹¹⁰⁸

10.1.4. α Syn and IAPP. The *in vitro* cross-seeding of IAPP has been shown to promote α S aggregation. The two proteins formed coaggregates when incubated together in their monomeric forms, suggesting a possible reason for T2D to be a risk factor for PD.¹¹⁰⁹

10.1.5. PrP, A β , and Tau. Cellular prion protein (PrP^C) was shown to be a receptor for A β oligomers with high affinity, thus facilitating oligomer-induced synaptic dysfunction in

mice.^{1110,1111} Several groups have reported the coimmunoprecipitation of human PrP^C with A β from the brain tissues of AD patients.^{1112,1113} In another study, intraperitoneal inoculation of misfolded PrP aggregates (PrP^{Sc}) in the Tg2576 AD mouse model resulted in the accumulation of both A β and pathogenic PrP^{Sc} with concomitant histopathological features of prion disease.¹¹¹⁴ The same authors also showed that protein misfolding can be enhanced by a cross-seeding mechanism *in vitro*.¹¹¹⁴

Overexpression of the longest human tau isoform was shown to regulate cellular trafficking of PrP^C and reduce its expression on the cell surface bound with concomitant accumulation of insoluble PrP^C in primary cortical neurons.¹¹¹⁵ Additionally, abundant tau pathology was reported in inherited prion diseases.^{1116,1117} Finally, oligomeric assemblies of A β , PrP, α S, and TDP-43 proteins were shown to colocalize in AD pathology,¹¹¹⁸ indicating a possible cross-talk between these amyloid proteins.

10.2. What Have We Learned from Simulations?

10.2.1. IAPP/A β . The attempt to investigate the coaggregation 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 A β (PDB ID: 1Z0Q) and IAPP (PDB ID: 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 β aggregation via interactions of IAPP with residues 16–22 of A β . In addition, two IAPP fibrils with U-shape structures were solved, by ssNMR¹¹¹⁶ and by X-ray crystallography.¹¹¹⁹ Applying these IAPP fibril structures, further polymorphic IAPP fibrils were investigated via computational tools.¹¹²⁰ The interactions of each polymorphic IAPP fibril with A β fibril were investigated using MD simulations with CHARMM FF and TIP3P water.¹¹²¹ Different interactions and orientations between polymorphic IAPP fibrils and A β fibril were studied for single-layer and double-layer conformations.^{1105,1121}

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 coaggregation will be presented. In some double-layer conformations, this phenomenon does not occur or is less preferred to occur.¹¹²¹ The mechanisms that lead to coaggregation between these two amyloids indicate a strong tendency to form single-layer conformations.

It has been shown experimentally that the toxicity of A β oligomers to neuronal cells has been demonstrated to occur via a two-step mechanism of membrane disruption.¹¹²² Yet, the cross-seeding between A β and IAPP aggregates has not been investigated at the atomic resolution in the membrane environment via experimental tools. The interactions between the U-shape structures of IAPP fibrils and A β fibrils associated with different types of membranes were investigated using MD simulations with CHARMM FF and TIP3P water by Zheng's group.¹¹⁰⁵ It has been shown that the cross-seeding fibrils more strongly interact with the 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 β 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 β induces the disruption of the cell membrane via altering calcium homeostasis and the cell membrane phase.

10.2.2. A β /NAC(α S). The study of the cross-seeding between A β fibrils and α S fibrils is more challenging, due to the relatively large number of amino acids in α S. The short time scale of MD simulations with CHARMM FF and TIP3P water was performed to investigate the interactions between A β monomer and two α S monomers associated with a membrane.¹⁰⁷⁰ The interactions of these two amyloids in the membrane indicated a promotion of the formation of stable ringlike oligomers. These oligomers were found to be composed of both A β and α S that are docked in the membrane. To simplify the study of the interactions between A β fibrils and α S fibrils, the interactions between A β fibrils and NAC fibrils were investigated using MD simulations in water solution. While the structures of A β 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.¹¹²³ Then, the interactions between the NAC fibril and the U-shape A β fibril structure were studied by MD simulations.¹¹²⁴ Various associations between NAC fibril and A β were investigated to form polymorphic single and double-layer conformations in the cross-seeding states.

The simulations demonstrated that the polymorphic A β 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 β fibrils. It is well-known that A β fibrils consist of two β -strands connected by one U-turn. Interestingly, the NAC fibrils in the cross-seeding aggregates induce the formation of a third β -strand and further U-turn in the A β fibril. The NAC fibril consists of three β -strands connected by two U-turn domains. Therefore, the cross-seeding between this NAC fibril and A β fibrils initiates the formation of a similar fibrillary structure for A β with three β -strands connected by two U-turn domains. A further effect of the NAC fibril on A β fibrils is related to the stabilization of the cross- β 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 β fibrils is decreased, i.e. forming a more compact stable cross- β structure due to the strong hydrophobic interactions in the inner core domain.¹¹²⁴

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

10.2.3. NAC(α S)/IAPP. The cross-seeding between the U-shaped structure of IAPP aggregates and the S-shaped structure of NAC aggregates was investigated by MD simulations using the CHARMM FF and TIP3P water in Miller's group.¹¹²⁵ In the case of the NAC fibril interacting with polymorphic A β 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 β -sheets in the fibrillary states of IAPP is increased, compared to the separated IAPP fibrils. Moreover, the distance in the inner core of the cross- β structure of IAPP fibrils is decreased, indicating a compact cross- β structure.

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

10.2.4. Tau/Tau Isoforms. The coaggregation between K18 and K19 was investigated by MD simulations.⁵⁵² Simulations showed that coaggregation of some of the K18 or K19 with the tau R3 repeat exhibited a stable L-shaped fibril. The coaggregation of other K18 or K19 with tau R3 repeat demonstrated straight-line shaped fibrils. The aggregation of K18 is strongly initiated by both R2 and R3 repeats, while the aggregation of K19 is induced by only a 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.

10.2.5. A β -Tau/Mutated Tau. There is a synergy between the tau and A β pathology in the mitochondria,^{1126–1130} and it was suggested that A β may accelerate tau neurofibrillary tangles.^{1131–1133} Experimental studies proposed that coaggregation of tau with A β occurs via interactions between the β strands of A β and β -stands of tau.^{1134–1138} Yet, there is a challenge to investigate the interactions between the full-length A β 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 the A β 25–35 fragment and the Tau273–284 fragment were investigated using REMD simulations and the OPLS-AA FF and TIP3P water.¹¹³⁹ It was shown that these interactions initiate cross-seeding between these fragments.

The tau repeats R2, R3, and R4 are known to form β -strands. The fibrillary structures of these U-shape tau repeat fibrils were studied by MD with CHARMM FF and TIP3P water molecules.¹¹⁴⁰ The interactions between each one of these tau repeat fibrils with the full-length A β U-shaped fibrils were studied also by simulations with CHARMM FF and TIP3P water.¹¹⁴⁰ Simulations showed that there are fewer hydrogen bond interactions between a neighboring monomer of A β fibril and a monomer of tau repeat R3 or R4 fibrils. Furthermore, conformational energy analyses illustrated that the “reaction coordinate” of A β fibrils with tau repeat R2 fibril presents exothermic reaction. The “reaction coordinate” of A β fibrils with tau repeats R3 or R4 fibrils demonstrated endothermic reaction. Therefore, it was proposed that tau repeats R3 and R4 are less preferred to interact with A β and

tau repeat R2 has a strong tendency to coaggregate with A β . Furthermore, it was suggested that there is a synergistic effect between tau R2 repeat and A β .

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 Δ 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 β forms aggregates both with itself and with wild-type tau. By analogy, in FTDP-17, it is likely that there are interactions between A β and mutated tau. Yet, it is challenging to investigate the interactions between A β and mutated tau at the atomic resolution via both experimental techniques and computational modeling tools. The molecular mechanisms underlying such interactions between U-shaped A β fibrils and mutated tau Δ K280 R2 repeat U-shaped fibrils were studied by MD simulations with CHARMM FF and TIP3P water molecules.¹¹⁴¹

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

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) to membrane mimetic systems to *in vivo*. The crosstalk and cross-seeding between the A β , tau, α S, and IAPP proteins and the failed and ongoing 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 β , tau, and α S fibrils isolated from the brain extracts of hundreds of patients would be an important step

toward 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 coaggregation of human brain-derived A β , pyroglutamate A β s, and α S proteins interacting with mitochondrial membranes would be useful as mitochondria are playing a pivotal role in AD and PD.^{1142,1143} 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 brains¹¹⁴⁴ 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 multibody 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 the membrane phase, (c) understand the role of membrane microdomains in partitioning and colocalizing secretases and substrate during A β genesis, (d) determine whether and how liquid–liquid phase and other biomolecules can induce pathogenic tau conformations *in vivo*,^{1145,1146} (e) understand how the balance between functional and pathological interactions is regulated in both α S and IAPP and how the exact function of α S is connected with its interaction at the surface of synaptic membranes, and (f) investigate the formation of amyloid oligomer/phospholipid complexes.^{1147,1148}

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 among 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 181,⁵²³ as advanced studies on blood sample analysis reported that detection of this phosphorylated tau could differentiate healthy participants from those with AD.^{1149,1150} What are the

effects of these two phosphorylations on the tau energy landscape? Simulations could also investigate the circulation of A β or α 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,¹⁶ synucleopathies alter nano-scale organization and diffusion in the brain extracellular space,¹¹⁵¹ and the geometry of the brain interstitial system undergoes alterations with aging and the accumulation of A β and tau.^{1152–1154}

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.^{44,1155} Similarly, it is not clear yet which atomistic force field is the most reliable for describing the monomeric and oligomeric structures of A β , tau, and α S in and their aggregation pathways. Despite extensive research toward 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.^{445,1156–1158} 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 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 a 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.^{324,365,371,462,1159} Coarse-grained simulations, which allow longer time- and length-scales at the expense of a reduced energy accuracy, are continuously improving.^{31,321,337,1160} 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.^{111,451,1161} 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 modeling approaches is becoming popular at least on small amyloid peptides and aggregate sizes.^{450,1162} 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 toward 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.¹¹⁶³

Developing disease-modifying therapies for AD presents an immense challenge, with little success despite intense work over decades.^{314,1164–1167} Some of the reasons why drug development for AD continues to fail include the following: (a) *in vitro* experiments poorly mimic *in vivo* conditions, (b) A β 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) animal models are limited.³¹⁴ Among 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.¹¹⁶⁸ First, 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.^{1169,1170} Second, human and mice brains differ in neuroanatomy, in brain biochemistry, and in blood–brain-barrier (BBB) permeability.^{1169,1171–1173} Considering the differences in respective life spans 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.¹¹⁶⁹ The push to generate models that rapidly progressed disease phenotype often resulted in either worsening of the pathology to nonphysiological levels or inducing aberrant early lethality in mice.¹¹⁷⁴ Third, 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.¹¹⁷⁵ Fourth, 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.^{1169,1176} Finally, to observe the desired pathology and phenotypes, the commonly used transgenic mouse models carry multiple mutations, such as in both A β and tau, which rarely happens in human brains.¹¹⁷⁶ 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 β oligomers is the cause or consequence of AD.^{1177,1178} Other researchers in the field place tau aggregates upstream of A β 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.¹¹⁷⁹ 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”¹¹⁷⁷ for several reasons.

First, clinical trials take place too late in the disease process.^{314,1180,1181} 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.^{251,1182}

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 β 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.¹¹⁸³ Many proteins play a significant role in developing AD, such as the prion protein,¹¹⁸⁴ APOE4,^{1185,1186} and the lipoprotein receptor related protein 1 (LRP1), with the latter discovered recently to be a master regulator of tau uptake and spread.¹¹⁸⁷ 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.^{1178,1188,1189} Notably, the LRP1 protein, a component of the BBB, contributes to the clearance of A β from the central nervous system. The pivotal role of mitochondria and their dynamics in the brain¹¹⁴² and changes in microtubule structure and dynamics¹¹⁹⁰ 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 α S aggregates and membrane organelles involving mitochondria and the autophagosome, inducing mitochondrial damage and deficits and synaptic dysfunctions.¹¹⁴⁷ 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. Copathologies 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 copathologies and the failure of all previous monotherapies suggest that multitherapies, targeting simultaneously A β , tau, and α S, could become the norm, as discussed already in 2000.¹⁰⁶⁷ Clearly, the creation of the 2019 EU-US clinical trials on Alzheimer's disease is good news for AD, PD, T2D, and ALS.¹¹⁹¹

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