alzheimer's research&therapy

REVIEW

Amyloid β-protein oligomers and Alzheimer's disease

Eric Y Hayden[1] and David B Teplow[1,2*]

Abstract

The oligomer cascade hypothesis, which states that oligomers are the initiating pathologic agents in Alzheimer's disease, has all but supplanted the amyloid cascade hypothesis, which suggested that fibers were the key etiologic agents in Alzheimer's disease. We review here the results of *in vivo*, *in vitro* and *in silico* studies of amyloid β-protein oligomers, and discuss important caveats that should be considered in the evaluation of these results. This article is divided into four sections that mirror the main approaches used in the field to better understand oligomers: (1) attempts to locate and examine oligomers *in vivo in situ*; that is, without removing these species from their environment; (2) studies involving oligomers extracted from human or animal tissues and the subsequent characterization of their properties *ex vivo*; (3) studies of oligomers that have been produced synthetically and studied using a reductionist approach in relatively simple *in vitro* biophysical systems; and (4) computational studies of oligomers *in silico*. These multiple orthogonal approaches have revealed much about the molecular and cell biology of amyloid β-protein. However, as informative as these approaches have been, the amyloid β-protein oligomer system remains enigmatic.

Introduction

Alzheimer's disease (AD) is a disease of aging that is characterized in part by progressive loss of memory and executive function, as well as aphasia, agnosia and difficulties with the activities of daily living. These losses of function are attributed to synaptic damage and neuronal loss in the hippocampus, cerebral cortex and other brain regions. A crucial unanswered question is, 'what causes this damage?' Genetic studies have revealed a central role for the amyloid β-protein (Aβ), as well as for the enzymes responsible for the processing of the amyloid β-protein precursor (APP) into Aβ. What remains unclear is which forms of neurotoxic Aβ are most disease relevant and what the structures and structural dynamics (formation pathways and equilibria) of these forms are.

Our current understanding of AD is based in large part on more than a century of study of amyloid plaques, the extracellular deposits of fibrillar Aβ that are pathognomonic for AD. Advances in magnetic resonance imaging and positron emission tomography imaging, the latter using amyloid-specific imaging agents, have revealed the formation of amyloid deposits decades before clinical signs of disease [1,2]. Considered together with the concentrations of tau and Aβ in the cerebrospinal fluid (CSF), these metrics serve as useful biomarkers for AD [3]. However, the predominant working hypotheses of AD etiology now focus upon Aβ oligomers. Although plaques and tangles remain the most trusted identifiers and predictors of AD, a clear paradigm shift has occurred that emphasizes the primacy of Aβ oligomers in disease causation [4].

Is this paradigm shift warranted? Some would argue 'no', based on failures of recent clinical trials. However, clinical trial design may be flawed by the selection of cohorts that are too advanced in their disease state [5]. It also is possible that metabolites of APP other than Aβ may be pathogenic [6-8]. Determining the temporal involvement of Aβ oligomers in human disease is crucial to elucidating the etiology of AD and the involvement of oligomers in it. As we shall discuss, this is very challenging.

What implicates Aβ oligomers?

Considerable evidence has accumulated over the last 10 to 15 years that oligomers play a central role in AD pathogenesis. Experiments have shown that oligomers are toxic entities *in vivo* [9] and *in vitro* [10], and that

* Correspondence: dteplow@ucla.edu
[1]Department of Neurology, 635 Charles E. Young Drive South, Room 455, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
[2]Brain Research and Molecular Biology Institutes, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA

learning and memory deficits caused by oligomers in transgenic mouse models can be reduced when oligomer levels are decreased by accelerating fibril formation [11]. Early studies of a mouse model using the FAD APP Indiana mutation (V717F) found that Aβ-induced neurotoxicity does not require Aβ deposition in plaques [12]. Deficits in synaptic transmission between hippocampal CA3 and CA1 cells, as measured by the slope of the excitatory postsynaptic potential, were found prior to and independent of plaque formation [12,13]. Furthermore, in animal models of AD, animals that lacked brain amyloid plaques, but did have oligomers present, displayed disease symptoms [14]. Interestingly, these studies showed that even with increased levels of the Aβ-degrading enzyme neprilysin, the levels of two types of oligomers, Aβ trimers and Aβ*56 (dodecamers [15]), did not change, nor did the severity of memory impairments [14]. The amount of oligomer extracted from human AD brain tissue correlated better with disease symptoms than did the number of amyloid plaques [16,17]. These early findings in animal models are consistent with recent findings that human brain contains Aβ oligomers up to two decades prior to disease onset [18]. While animal models may be imperfect, these studies are still informative.

Indeed, preclinical stages of AD have recently been described that involve the development of brain pathology well before the clinical presentation of AD [19]. In CSF samples from AD and control patients, concentrations of oligomers of size 40-200 kDa (10 to 50 monomers) distinguished controls from AD patients and from patients with mild cognitive impairment who converted to AD within 3 years [20]. In a study of plasma from AD and control patients, oligomer levels declined over time [21], suggesting they may have been sequestered in plaques in the AD brain. Establishing the temporal relation between oligomer formation and disease state is imperative if disease mechanism and the involvement of oligomers in it are to be determined.

Autosomal dominant APP mutations that result in early onset AD and increased oligomer production support a role for oligomers in AD *in vitro* [22]. The English (H6R) and the Tottori (D7N) substitutions, both located at the peptide N-terminus, and in both the Aβ40 and Aβ42 systems, produce oligomer size distributions skewed to higher order [22,23]. Importantly, the mutant forms of both native and chemically stabilized oligomers are significantly more toxic in assays of cell physiology and death [22]. Altering assembly of Aβ at its earliest stages thus could be important in disease onset and progression in these familial forms of AD [22,24].

The *in vivo* and *in vitro* studies just discussed support the involvement of Aβ oligomers in disease pathogenesis. However, contradictory studies also have been published. A recent study points out the complex relation between oligomer levels and cognitive impairment in a mouse model in which new production of a mutant APP/Aβ could be suppressed [25]. This study found that even with significant amyloid pathology, when new APP/Aβ production was lowered, there was a rapid improvement in both long- and short-term memory despite unchanged amounts of oligomeric Aβ. Another recently examined mouse model revealed that if APP expression levels remained normal, and extracellular Aβ40, Aβ42 or both together were highly expressed, the mice developed amyloid pathology but stable cognitive performance before and after amyloid plaque formation [7]. More studies are needed to establish a consensus (if this is even possible).

Aβ is enzymatically cleaved from the transmembrane protein APP by β- and γ-secretase and is released extracellularly in lengths ranging from approximately 37 to 43 amino acids. Aβ40 is the most abundant species and exists in an approximately 10:1 concentration ratio with Aβ42 [26]. The structure of Aβ *in vivo* remains unknown, though *in vitro* evidence suggests it exists in a largely disordered form that occasionally forms partially folded structures [27-29]. The Aβ sequence is amphipathic, with the first 28 amino acid residues containing both hydrophobic and hydrophilic groups, whereas the remaining residues all are apolar and uncharged [30]. In AD brains, as well as in some cognitively normal brains, Aβ is found in the form of amyloid fibers. These fibers have a characteristic cross-β sheet core secondary structure.

Aβ is a member of the class of proteins known as intrinsically disordered proteins (IDPs) – proteins that lack a stable tertiary structure in physiological conditions. IDPs are known to have many binding partners due to their flexibility of conformation [31]. Importantly, in many of these cases, and particularly so for amyloidogenic proteins, monomeric units interact non-covalently to form oligomers [32,33]. Many different oligomeric forms of Aβ can exist simultaneously in a dynamic equilibrium. This lack of a native fold results in Aβ occupying a large conformational space. This space is highly dependent on environment [34], making the oligomer states very sensitive to perturbation by the procedures used for analysis. Figure 1 illustrates different low-order oligomer states and how the simplest oligomer, the dimer, and progressively larger and structurally diverse oligomers, may be formed from smaller subunits.

Challenges for oligomer study

A number of reports have pointed out that one of the most common methods for defining oligomer distributions, SDS-PAGE, may produce misleading results [24,35]. SDS-PAGE or Western blotting are simple methods for detecting the apparent molecular weights (M_r) of proteins, but they cannot reveal what the distribution of oligomer sizes was prior to electrophoresis [36]. For

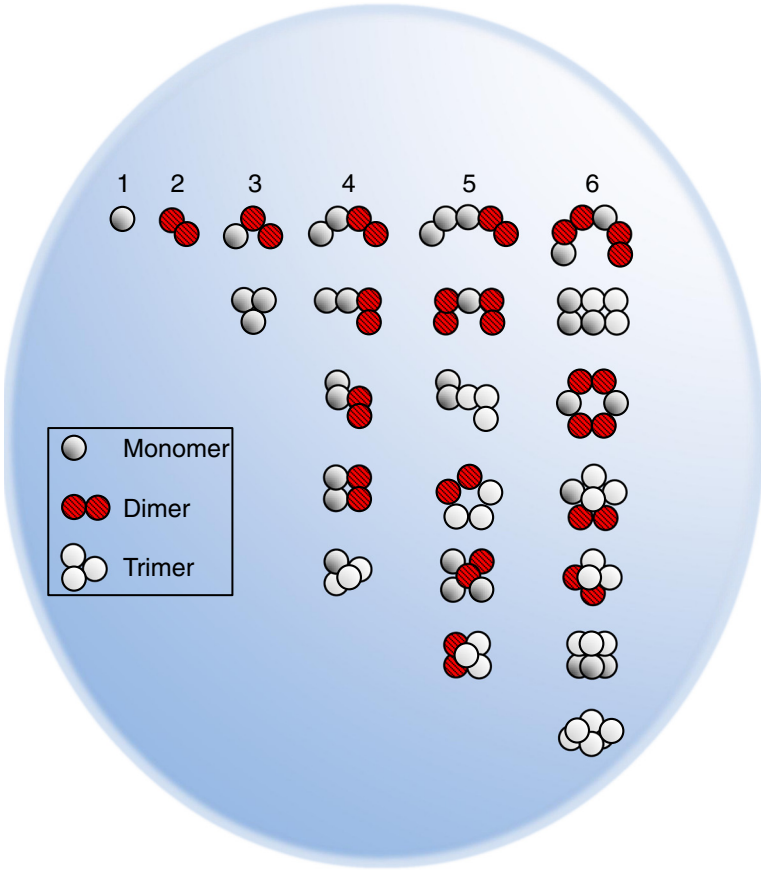

Figure 1 The diverse nature of oligomers. Oligomeric assemblies ranging from two to six monomers. Each sphere represents a monomeric unit. Monomers (grey), dimers (red, diagonal lines) and trimers (white) can combine in different combinations comprising up to six monomers. Each small oligomer also could be a building block for larger oligomers. For simplicity and clarity, we only display basic units up to trimer. We note that the assemblies shown here, and others, are in rapid equilibrium, one that can be perturbed easily during experimental studies.

example, Watt and colleagues compared the Aβ in samples from human cortical tissue using SDS-PAGE, xMAP multiplex immunoassay and surface enhanced laser desorption/ionization time-of-flight mass spectrometry (MS) [35]. Oligomers were not detected with MS, though they were observed using SDS-PAGE, and, surprisingly, monomer and dimer levels increased with increasing SDS concentration in the sample buffer. Bitan and colleagues earlier reported that dimers and trimers could be induced to form by SDS [24,37]. Determining the presence and size distribution of oligomeric assemblies in biologic fluids and tissues using techniques involving SDS thus must be interpreted with the understanding that artifactual dissociation and formation of oligomers can occur.

Similarly, size exclusion chromatography can be used to fractionate extracts of Aβ, but the distribution and abundance of peaks that elute may not accurately represent the species present prior to analysis. When the sample is injected into the column, retardation of monomers and low-order oligomers perturbs the equilibrium of the original sample. Large oligomers dissociate to re-establish equilibria with smaller species and monomers [38]. Equilibrium is continuously being re-established, unless and until a stable oligomer species is produced. The result, as with techniques involving SDS, is that artifactual oligomer distributions may be observed.

It is crucial to determine if the oligomerization state within cell cultures or animal extracts is the same at the beginning of an experiment and at the end of it. When extracting oligomers and subsequently testing their activity, oligomers may aggregate further or dissociate on the timescales of many experiments. For example, Aβ oligomers extracted from the cerebral cortices of AD subjects have been reported to be dimers. These dimers were found to inhibit long-term potentiation (LTP), enhance long-term depression (LTD) and reduce dendritic spine density in the normal rodent hippocampus [39]. However, subsequent investigation of the methods used in these studies revealed that the effects previously attributed to dimers were, in fact, caused by the aggregation of the 'dimers' into protofibrils [40]. A study using the APP J20 transgenic mouse – bearing the Swedish

and Indiana mutations and displaying increased β-secretase cleavage and increased Aβ42/Aβ40 concentration ratios [13] – revealed the presence of SDS-stable monomers through tetramers of Aβ throughout the life of the mice [41]. The simultaneous presence of monomers through tetramers makes it challenging to attribute synaptotoxicity *in vivo* to a single Aβ species.

The isolation of cellular Aβ may include a variety of tissue homogenization, cell lysis and extraction techniques (some including detergents), many of which change the environment and concentration of Aβ and its distribution of oligomers [17]. Even when care is taken to employ experimental conditions thought least likely to alter the native oligomerization process of Aβ, one cannot guarantee that some perturbation of the process does not occur.

Furthermore, recent findings have pointed out that oligomers can be formed in the fluid phase not only by monomer accretion, or coalescence of monomers or small oligomers, but also through secondary nucleation [42] on fibril surfaces. Secondary nucleation is a rare event, because its critical concentration of 10 nM is lower than that for oligomerization in the fluid phase. Nevertheless, an accurate system description must consider this process.

Oligomer characterization *in vivo*[1]

Several groups have developed 'anti-oligomer' or 'anti-amyloid' antibodies that are reported to recognize oligomers but not fibrils [43-46]. Key questions about these antibodies are 'to what epitopes do they bind?' and 'with what affinity?' Detection of 'Aβ oligomers' by immuno-electron microscopy (EM) with an antibody to an oligomer mimetic, 'oligomer-specific polyclonal antibody', [47] has been reported in both APP transgenic mice and AD brain [48]. It remains unclear if these studies, utilizing a single oligomer-selective antibody, are actually detecting Aβ oligomers, another Aβ assembly, or simply cross-reacting with other proteins. Antibodies developed thus far have been useful in distinguishing oligomeric species that exhibit fibril-like folds versus oligomers that do not [44]. Details of Aβ oligomer antibodies are summarized in supplementary Table 1 of Benilova and colleagues [49] and a discussion of caveats in immunological studies of Aβ oligomers may be found in [50].

Experimental data unequivocally demonstrate that low femtomolar levels of Aβ and other protein oligomers can affect neuronal synapses in culture and in hippocampal brain slices (for example, by attenuation of LTP, induction of LTD, or dendritic spine loss) [9,51-53]. Similarly, acute effects of exogenous oligomeric assemblies on memory and hippocampal LTP in rodents have been reported *in vivo* (reviewed in [54]). The apparent potency of exogenously applied oligomers supports a role for oligomers in AD. An Arctic kindred that develops a familial form of AD has a mutation within Aβ (E22G) that leads to increased amounts of protofibrils [55]. The Arctic mutation has been studied in a mouse model [56,57], and recent studies have reported that reducing levels of the enzyme responsible for APP cleavage into Aβ (β-site APP cleaving enzyme) can prevent cognitive decline and reduce tau accumulation and phosphorylation in the model [58]. Photochemical cross-linking and SDS-PAGE showed that the Arctic form of Aβ produces greater numbers of Aβ40 heptamers through nonamers, and more Aβ42 heptamers [59]. More recent studies examined the ΔGlu22 mutant of Aβ, found in an AD kindred in Osaka, Japan [60]. This Osaka mutation increased oligomer formation *in vivo*. *In vitro* studies of the Osaka forms of Aβ40 and Aβ42 revealed that the Glu22 deletion resulted in increased dodecamer and octadecamer formation [61].

A very interesting animal model of AD is that of the rodent *Octodon degus*, which naturally produces oligomers, develops Aβ plaques and shows tau phosphorylation. *O. degus* has a sequence that differs from human Aβ by only a single amino acid, H13R, whereas *Mus musculus* has three differences relative to human Aβ (R5G, Y10F and H13R). Interestingly, in the *O. degus* model, age-related increases in both Aβ oligomers and tau phosphorylation are observed. These increases occur concomitantly with decreases in spatial and object recognition, postsynaptic function and synaptic plasticity [62].

The neurotoxic effects of synthetic Aβ oligomers have been reported to be greater than those of fibrils, with toxicity *in vitro* typically observed at low micromolar concentrations [63-65]. In addition, Aβ42 has been observed to reduce neuronal viability to a greater extent than Aβ40 [63]. Aβ42 thus seems to be most toxic, but both Aβ40 and Aβ42 form oligomers, and through distinct mechanisms [37]. Cell-derived oligomers are found to be toxic at low nanomolar concentrations [66,67]. We also know that fibrils accumulate in large amounts in the AD brain and that oligomers are likely present at low concentrations. This suggests that if acute neurotoxic effects of oligomers contribute to the disease process, they must do so potently. It is important to consider whether the oligomers formed *in vivo* are the same as oligomers formed by synthetic Aβ *in vitro*. Interestingly, there are reports that brain- or cell-derived Aβ oligomers are more potent than synthetic oligomers [68]. While it is possible that the dynamic nature of oligomers is different when comparing physiologically produced versus synthetic material, it is also possible that post-translational modifications could cause different behaviors. Factors present in biological systems could increase the toxicity of physiologically produced material. It is conceivable that factors also exist that could do the opposite. Ikeda and colleagues tested the ability of CSF to inhibit Aβ oligomerization [69]. They found that CSF from cognitively normal subjects

inhibited oligomerization to a greater extent than did CSF from AD patients. However, the study did not examine oligomerization of Aβ in CSF *per se*, nor did it address oligomerization in the brain parenchyma or intracellularly in neurons.

Ex vivo studies of oligomers extracted from human and animal tissue

Oligomers have been detected in brain samples at concentrations as low as 40 pg/ml, though these studies did not detect oligomers in the CSF, potentially due to the very low concentration present [70,71]. In the ELISA system employed, the same antibody used to capture the assembly was used to detect it. In theory, any oligomer of order 2 or greater thus could be quantified. Such ELISAs have been shown to detect oligomers. However, competition by monomer could skew the results, yielding artifactually low oligomer concentrations, depending on the oligomer/monomer number concentration ratio. Further, this ELISA design cannot discriminate among oligomers of different sizes, and thus determination of the oligomer size distribution is difficult to accomplish. Oligomers composed of different numbers of subunits will present different numbers of epitopes, resulting in differences in the avidity of different oligomers for the same solid-phase immunoglobulin.

Using the Tg2576 mouse model containing the Swedish APP K670N/M671L mutation [72,73], a correlation was found between memory deficits and the amounts of Aβ nonamers and dodecamers (Aβ*56) extracted from the forebrain [15]. Interestingly, these extracted oligomer preparations could be injected into the lateral ventricles of the brains of young rats, causing memory impairments, as determined using a Morris water maze. These data support the hypothesis that oligomers are sufficient to cause memory deficits.

There also is evidence that monomers through trimers derived from the Chinese hamster ovary cell line 7PA2 (which is stably transfected with the 751 amino acid isoform of APP containing the V717F mutation) decreased hippocampal synapse density in the mouse brain at a critical time during memory consolidation [74]. A study that extracted oligomers from the frontal cortex of human postmortem brain tissue into three fractions, soluble (in Tris buffered saline), detergent soluble (in Tris buffered saline with Tween 20), and insoluble (in guanidine-HCl), reported the predominant oligomeric Aβ assemblies were pentamers, decamers, and dodecamers, as detected by the 'NU' antibody cocktail developed against Aβ-derived diffusible ligands (ADDLs). Oligomer concentration distinguished between early onset AD patients and late onset AD patients [75].

Recently, an intriguing discovery was made that linked Aβ to the mammalian prion protein (PrP). Synthetic Aβ oligomers were found to bind with membrane-associated PrP^C, the normal cellular form of PrP, and this interaction blocked long-term potentiation in mouse hippocampal slices [52]. This finding was supported using oligomers extracted from the human AD frontal or temporal cortex [76]. Larson and colleagues used coimmunoprecipitation to find that human- and mouse-extracted SDS-stable Aβ dimers and trimers interact with PrP^C at neuronal dendritic spines *in vivo* and *in vitro* [77]. This interaction involves complex formation between PrP^C and Fyn (a membrane-associated tyrosine kinase important in signaling), resulting in the activation of the kinase [77]. Furthermore, oligomers extracted from the AD brain caused dendritic spine loss in hippocampal neurons, and lactate dehydrogenase release from primary cortical cultures. Both of these effects depended on the presence of PrP^C and Fyn [78].

Studies of oligomers *in vitro*

Oligomers are formed in the laboratory using chemically or recombinantly produced Aβ and any of a number of recipes that specify particular solvent conditions, incubation times, temperatures and agitation conditions. Physiologic conditions cannot be duplicated in the laboratory because no one knows formally what the milieu of Aβ is in its different locations (for example, intracellularly in the cytosol or in a specific organelle, or extracellularly in CSF, plasma, saliva, and so on). PBS is used as a proxy for the Aβ milieu, and a poor one at that, but at least the use of PBS allows comparison of experimental results among many different laboratories. Temperature (37°C) is easily mirrored *in vitro*. Aβ can be found at low nanomolar or high picomolar concentrations *in vivo*, but *in vitro* studies often are performed at micromolar concentrations to enable monitoring of assembly and accelerate the process [79]. Physiologic pH is generally considered to be close to neutral (7 to 7.4), but many experiments are done at different pHs. Acidic pH favors fibril formation [43] or rapid aggregation, especially if the pH is near the pI of Aβ (approximately 5.4) [80]. It is important to emphasize that physiologic Aβ concentration and pH vary, depending on which compartment in the body Aβ is found. In late endosomes and lysosomes, acidic pH and higher peptide concentration may exist [81]. At synapses, different conditions may exist [82], including those involving high metal concentrations [83]. Ionic strength also has strong effects on fibril assembly rates [43].

Each recipe for producing oligomers can yield different oligomer types, including ADDLs [10], globulomers [84], oligomers <4 nm [47], oligomers 4 nm to 10 nm in diameter [85], β-amyballs [86], amylospheroids [87], or annular protofibrils [45] (for a review, see [88]). The oligomers produced using these procedures can continue aggregating during experiments. Importantly, these techniques do not produce a homogeneous preparation of one oligomer

species, but rather a mixture of oligomers in equilibrium. To address this problem, some have taken the approach of 'trapping' oligomers in specific states through chemical cross-linking.

A benefit of studying synthetic oligomers is that they are pure in the sense that no contaminating factors are present, a situation that may not exist using oligomers extracted from cells, tissues or biological fluids. Such factors can affect the behavior of the oligomers in significant ways. Of course, establishing the similarity of the synthetically produced material to that obtained *in vivo* is crucial, but may or may not be possible [50]. The ability to rigorously define the oligomer under study, in terms of the number of monomers, the relative abundances of the different oligomers in the preparation, as well as the biophysical, structural and biological properties of each of the oligomers present, is arguably just as important.

A successful approach to enable rigorous examination of oligomer structure and toxicity has been photo-induced cross-linking of unmodified proteins (PICUP) (for a review, see [89]). This technique circumvents the complication of metastability by using rapid, zero-length, *in situ* chemical cross-linking to 'freeze' the oligomer population, allowing quantitative determination of the oligomer size frequency distribution using SDS-PAGE. This technique has been used to produce stable Aβ40 oligomers of defined order. This enabled determination of the secondary structure, morphology and toxicity of monomers through tetramers [64]. Circular dichroism spectroscopy showed a direct correlation between oligomer order and β-sheet content [64]. Aβ assembly morphology, determined using electron microscopy and atomic force microscopy (AFM), revealed a direct, but non-linear, relation between oligomer order and size. Dimers were approximately twice as large as monomers, but trimers and tetramers were larger than would have been predicted for three or four monomers, respectively. Oligomer toxicity *in vitro* followed a rank order of tetramer > trimer > dimer > monomer. Importantly, consistent with the non-linear relation between oligomer order and size, dimers were approximately three-fold more toxic than monomers, whereas trimers and tetramers were approximately eight-fold and approximately 13-fold more toxic, respectively [64].

Oligomers have been studied using ion mobility spectrometry coupled with MS (IMS-MS). Aβ40 formed dimers and tetramers, whereas Aβ42 formed dimers, tetramers, hexamers, and dodecamers [90]. This study revealed that the primary oligomer observed for Aβ40 was a tetramer, whereas Aβ42 formed hexamers and dodecamers that could convert to a structure capable of rapid monomer addition.

IMS-MS also revealed that the Tottori (D7N), Flemish (A21G) and Arctic (E22G) forms of Aβ displayed different oligomer distributions [23]. Wild-type Aβ40 only formed monomers through tetramers. However, the Tottori Aβ40 mutant also formed hexamers. Dodecamers were the predominant species formed by [D7N]Aβ42. [A21G]Aβ42 predominantly formed hexamers or smaller oligomers, whereas [E22G]Aβ40 formed decamers and dodecamers, which were not observed in the [D7N]Aβ42 sample. While there appears to be some correlation between oligomer distribution and disease pathology, the data extant do not make clear a definitive mechanistic connection [23].

Aβ has been shown to interact directly with phospholipid bilayers. In addition, membrane insertion, ion channel formation, dysregulation of intracellular calcium levels and mitochondrial depolarization all have been observed (for a review see [91]). Studies in model membrane systems comprising planar lipid membranes and liposomes have shown that anionic phospholipids are essential for Aβ membrane binding and insertion [92,93]. Further, voltage-dependent and -independent single channel ion conductances have been measured for annular Aβ oligomers. These conductances are hypothesized to correlate with the number of monomers per oligomer [94,95]. Evidence suggests a heterodisperse population of Aβ oligomers can insert into membranes [96]. An annulus geometry has been determined for the AD-linked Aβ40 Arctic mutant (E22G). This was done by Superose-6 fractionation of the peptide assemblies that form normally. The lowest molecular weight fraction was examined by transmission EM and contained many annular species. Their outer diameters were 7 nm to 10 nm and their inner diameters were 1.5 nm to 2.0 nm. The relative molecular mass was 150,000 to 250,000 (40 to 60 Aβ molecules) [97,98].

Data produced using chemically synthesized or glutaraldehyde cross-linked oligomers of Aβ40 or Aβ42 generally agree with observations from experiments using PICUP cross-linked oligomers [99]. Although the oligomers were not isolated in this study, oligomer size and β-sheet content were directly proportional [99]. Pore formation was maximal after 2 to 3 days of incubation, and it correlated with toxicity as measured by human neuroblastoma SH-SY5Y cell death. Prangkio and colleagues performed multivariate analyses of the oligomer populations and showed oligomers from tetramers to tridecamers formed pore structures in an artificial membrane bilayer and contributed to cytotoxicity [99]. On the other hand, this analysis suggested that monomers, dimers, trimers, and oligomers >210 kDa, did not contribute substantially to either pore formation or toxicity of SH-SY5Y cells [99].

Recent work using total internal reflection fluorescence microscopy has allowed visualization of individual Aβ species on the surface of murine hippocampal neurons, allowing the determination of the oligomerization state of Aβ on the membrane [100]. Oligomers preferentially interacted (relative to monomers) with these membranes, even at physiologically relevant nanomolar concentrations,

and these oligomers become immobilized on the cell surface [100]. Membrane disruption has been suggested as a mechanism by which Aβ might inflict damage to neuronal cells. This study supports possible toxic membrane disruption by demonstrating membrane binding, an obligate first step in such a process.

Without atomic level resolution of oligomer structure, designing therapeutic drugs specifically targeting one or more oligomers remains a challenge. Using an 11-residue segment of αB crystallin, a peptide that forms amyloid fibers, albeit more slowly than Aβ, Laganowsky and colleagues recently solved a crystal structure that revealed a hexameric cylinder with a β-barrel-like structure [101]. This structure was termed 'cylindrin' and was postulated to be a structure that could be formed by many different amyloid proteins, including Aβ [101]. Because αB crystallin forms amyloid fibers more slowly than do Aβ or islet amyloid polypeptide, it was hypothesized that its oligomeric state may be trapped before the onset of fibril formation, allowing cylindrin formation. Indeed, αB crystallin formed an oligomer with many amyloid properties, including a β-sheet-rich structure, cytotoxicity and recognition by the oligomer-specific antibody A11. Importantly, Goldschmidt and colleagues used the Rosetta–Profile method to determine if other amyloid protein segments could be threaded onto the cylindrin structure [102]. This cylindrin comprises three units. In the Aβ case, each unit is an anti-parallel β sheet formed by two peptide segments, 26–40 and 28–42. This observation does not mean that the structure of an Aβ toxic species is a cylindrin-like fold. However, it is intriguing that Aβ42 readily forms oligomers of order 6 [37,90]. While the cylindrin model may or may not be relevant to AD, its determination is progress toward solving an oligomer crystal structure. The relation of cylindrin structures to previously observed annular oligomers [95] remains unclear.

Oligomers characterized *in silico*

In silico (computational) studies can provide insights into the structure, conformational dynamics, thermodynamics and kinetics of amyloid protein assembly, including those of Aβ oligomers. Early discrete molecular dynamics (DMD) studies of Aβ40 aggregation were done by Peng and colleagues using a two-bead peptide model with Gō interactions (Gō interactions favor native-like contacts) [103]. They based these simulations on the Aβ40 structure in a membrane-like environment. Peng and colleagues showed that molecules assemble into fibril-like aggregates with parallel, in-register organization.

Folding and dimer formation of Aβ40 and Aβ42 were studied by Urbanc and colleagues using a combination of DMD and all-atom molecular dynamics (MD) simulations [104]. The explicit solvent MD method was applied to estimate the free energies of different dimer conformations of both Aβ40 and Aβ42. Previous simulations suggested a planar β-sheet dimer conformation [105], but Urbanc and colleagues showed that all planar β-sheet dimers had higher free energies than did the corresponding monomeric states, and that there was no significant free energy difference between Aβ40 and Aβ42 dimers. This finding corroborated the experimental observation of assembly differences between Aβ40 and of Aβ42 [37] and suggested that dimer conformations other than planar β-sheets are responsible for experimentally observed differences in oligomerization. At the molecular level, the data of Urbanc and colleagues emphasize the importance of addressing hydrogen bond interactions and other enthalpic properties in the study of Aβ oligomer formation and stability.

Oligomer formation by Aβ40 and Aβ42 was further studied using DMD and a four-bead protein model incorporating hydrogen bond and amino acid-specific interactions [106]. Initially, the separated Aβ peptides folded into collapsed coil structures and then assembled into oligomers of different sizes. Interestingly, the respective Aβ40 and Aβ42 size distributions differed substantially. Aβ42 formed more pentamers than did Aβ40, and Aβ40 formed significantly more dimers than did Aβ42. These results showed that the effective hydrophobic interactions of I41 have a significant impact on Aβ42 oligomer formation [107]. Figure 2 shows the structure of one of the Aβ40 hexamers determined in these studies.

Simulations with fully atomistic MD using explicit water also have been performed [108]. Prior studies had revealed a turn at Gly37–Gly38 in Aβ42 and suggested its importance in pentamer formation [106]. In the new studies, a large ensemble of DMD-derived Aβ40 and Aβ42 monomers and dimers were the starting conformers used in subsequent all-atom analyses. These analyses showed that the conformers were slightly larger and had a lower β-strand propensity, but similar turn propensity, compared to predictions by DMD [108].

Ma and colleagues studied four models of Aβ dodecamers using MD and a TIP3P water box with sodium ions [109]. Orthogonal β-sheets appeared to be the most stable conformation for Aβ dodecamers, and the exposure or shielding of Met-35 was critical in controlling fibril formation [109]. The validity of these *in silico* findings was supported by prior experimental studies showing that Met35 oxidation, to its sulfoxide or sulfone form, strongly inhibited Aβ assembly [110,111]. Informed by experiments that showed a Phe19Pro substitution eliminated ion conductance in a planar lipid bilayer, Connelly and colleagues performed MD simulations that predicted a channel-like octadecamer with a collapsed pore [112]. AFM measurements did indeed reveal a collapsed pore. Further studies of planar lipid bilayers confirmed that a Phe19Pro substituted Aβ42 inhibited channel conductance [113].

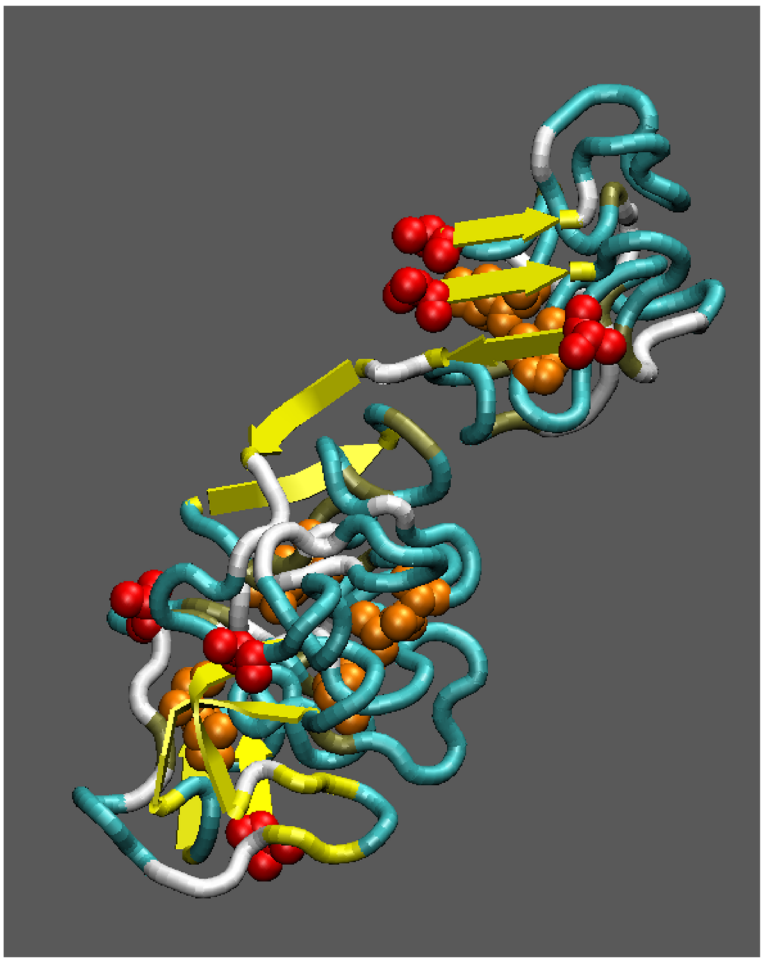

Figure 2 Simulation of amyloid β-protein hexamer formation. Formation of an amyloid β-protein (Aβ)40 hexamer from a tetramer (lower left) and a dimer (upper right). β-strands are depicted as yellow ribbons, turns as light blue ribbons and random coils as white ribbons. The N-termini are represented by red spheres. The C-terminal V39 and V40 are represented by orange spheres. Adapted with permission from [107], copyright 2010 American Chemical Society.

Summary

We have briefly described here the use of antibodies to detect Aβ oligomers in transgenic animal and human brains, animal models with increased oligomer formation, and AD mutations within the Aβ coding region of APP that may result in changes to oligomer distributions and concentrations. *Ex vivo* studies have examined oligomers produced physiologically and determined their abundance, toxicity, and location in tissues. *In vitro* studies have revealed a wide variety of oligomer structures and have determined the toxic properties of a number of these species. Computational studies have simulated the conformational dynamics of many of the structures observed experimentally and have provided atomistic structural details enabling the experimental testing of hypotheses regarding oligomer formation mechanism(s). This combination of methods, and new methods to be developed in the future, must be integrated into a coordinated, multi-disciplinary approach if the molecular biology of Aβ and its metastable oligomers is to be elucidated.

We have emphasized that oligomers are extremely dynamic (see [50]), thus finding them *in vivo* or studying them *in vitro* requires careful control of experimental conditions so that the native state characteristics of the oligomer populations are preserved. A caveat is that the native state characteristics may be impossible to determine. Studies of α-synuclein, another IDP, illustrate the difficulty in preserving the native (physiological) state of non-covalently linked protein oligomers. Substantial prior work has supported the widely held notion that α-synuclein normally exists as a statistical coil *in vitro* and *in vivo* [114-116]. However, Bartels and colleagues recently argued that α-synuclein derived from neuronal and non-neuronal cell lines, brain tissue or human cells, if extracted under non-denaturing conditions, is an α-helical

tetramer [117]. This has been a controversial idea [118,119]. In fact, the Aβ oligomer field continues to produce ever greater numbers of controversies. The resolution of these controversies depends on the field moving away from descriptive science and much closer to mechanistic science. In doing so, one can be hopeful that the information thus obtained will guide future development of effective therapeutic agents.

Endnote

[1] *In vivo* is used here to describe experiments carried out in a live animal, whereas *ex vivo* is used to describe experiments that use tissues collected after death.

Abbreviations

AD: Alzheimer's disease; ADDL: Amyloid β-protein derived diffusible ligand; AFM: Atomic force microscopy; APP: Amyloid β-protein precursor; Aβ: Amyloid β-protein; CSF: Cerebrospinal fluid; DMD: Discrete molecular dynamics; ELISA: Enzyme-linked immunosorbent assay; EM: Electron microscopy; IDP: Intrinsically disordered protein; IMS-MS: Ion mobility spectrometry – mass spectrometry; LTD: Long-term depression; LTP: Long-term potentiation; MD: Molecular dynamics; MS: Mass spectrometry; PBS: Phosphate-buffered saline; PICUP: Photo-induced cross-linking of unmodified proteins; PrP: Prion protein.

Competing interests

The authors declare that they have no competing interests.

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