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# Structure–Function Relationships of Pre-Fibrillar Protein Assemblies in Alzheimer's Disease and Related Disorders

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## Abstract

Several neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's and prion diseases, are characterized pathognomonically by the presence of intra- and/or extracellular lesions containing proteinaceous aggregates, and by extensive neuronal loss in selective brain regions. Related non-neuropathic systemic diseases, e.g., light-chain and senile systemic amyloidoses, and other organ-specific diseases, such as dialysis-related amyloidosis and type-2 diabetes mellitus, also are characterized by deposition of aberrantly folded, insoluble proteins. It is debated whether the hallmark pathologic lesions are causative. Substantial evidence suggests that these aggregates are the end state of aberrant protein folding whereas the actual culprits likely are transient, pre-fibrillar assemblies preceding the aggregates. In the context of neurodegenerative amyloidoses, the proteinaceous aggregates may eventuate as potentially neuroprotective sinks for the neurotoxic, oligomeric protein assemblies. The pre-fibrillar, oligomeric assemblies are believed to initiate the pathogenic mechanisms that lead to synaptic dysfunction, neuronal loss, and disease-specific regional brain atrophy.

The amyloid β-protein (Aβ), which is believed to cause Alzheimer's disease (AD), is considered an archetypal amyloidogenic protein. Intense studies have led to nominal, functional, and structural descriptions of oligomeric Aβ assemblies. However, the dynamic and metastable nature of Aβ oligomers renders their study difficult. Different results generated using different methodologies under different experimental settings further complicate this complex area of research and identification of the exact pathogenic assemblies *in vivo* seems daunting.

Here we review structural, functional, and biological experiments used to produce and study pre-fibrillar Aβ assemblies, and highlight similar studies of proteins involved in related diseases. We discuss challenges that contemporary researchers are facing and future research prospects in this demanding yet highly important field.

### Keywords

Amyloid; neurodegeneration; Alzheimer's disease; amyloid β-protein; protein misfolding; pre-fibrillar assemblies; oligomers; toxicity



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## INTRODUCTION

The amyloid-cascade hypothesis [1], suggesting that amyloid β-protein (Aβ) fibril formation and plaque deposition lead to neuronal dysfunction, dementia, and death in Alzheimer's disease (AD), had guided scientific research into discovery of etiologic and pathogenic mechanisms of AD. However, this hypothesis has been contentiously debated because: 1) fibrillar amyloid burden does not correlate well with neurological dysfunction [2], 2) cognitive impairment in transgenic murine models of AD is observed before and/or independently of amyloid plaque formation [3], 3) plaque-independent pathology can be explained by the neurotoxicity of soluble Aβ assembly intermediates, 4) oligomer-induced memory dysfunction occurs before neuronal death, and 5) brain, plasma, and cerebrospinal fluid (CSF) concentrations of soluble Aβ oligomers correlate with neurodegeneration better than those of fibrils [4]. These observations have led to a burgeoning yet encompassing alternative paradigm hypothesizing that soluble pre-fibrillar protein assemblies, rather than mature fibrillar deposits, act as proximate neurotoxins that cause synaptic dysfunction, neuronal loss, dementia, and death [4–11]. This new hypothesis has been supported by discovery of toxic pre-fibrillar protein assemblies involved in other protein-misfolding diseases, such as, Parkinson's disease, Huntington's disease, transmissible spongiform encephalopathies, amyotrophic lateral sclerosis, poly-glutamine diseases, type-2 diabetes mellitus (T2D), and systemic amyloidoses [7,12–15].

Diversity and sometimes inaccuracy in nominal definitions, and in structural/functional descriptions of soluble pre-fibrillar Aβ assemblies, along with different methodologies to generate and study these assemblies, are confounding factors in this already-vast and complex research area. Various forms of soluble pre-fibrillar Aβ assemblies (reviewed in [10,16–18]) including monomeric Aβ conformers [19], secreted cell- and brain-derived low-order oligomers [20–25], Aβ-derived diffusible ligands (ADDLs) [26,27], protofibrils (PF) [28–31], Aβ*56 [32], paranuclei [33–35], amylospheroids [36], annular assemblies [37], amyloid pores [18,37,38], and βamy balls [39] have been described. However, despite a global concerted scientific effort, the relationships amongst these Aβ-derived assemblies and their relevance to AD pathogenesis are unclear and the fundamental quest for a unanimous pathogenic "equivalent" active in AD-afflicted brain is ongoing [9].

We begin our discussion in the first step of studying pre-fibrillar Aβ assemblies—protein preparation.

## SOURCES AND METHODS OF Aβ PREPARATION

The low physiological concentration and the difficulty of procuring highly pure and homogeneous tissue-derived Aβ have precluded its routine use in experimental studies *in vitro*. Therefore, synthetic Aβ preparations have emerged as alternatives. Synthetic Aβ is produced either by standard solid-phase peptide synthesis (SPPS) [40,41] or by recombinant DNA technology [42–45].

The Aβ sequence is recognized as a "difficult" target for SPPS owing to its high hydrophobicity and innate propensity to aggregate [46,47]. To overcome these issues, various deprotecting agents [46], novel solvent systems for coupling [47], and solid-support modifications [48] have been employed to augment synthetic yield and improve purity of crude Aβ peptides. Recently, application of an *O*-*N* acyl migration reaction, also called "*O*-acyl isopeptide" chemistry was proposed as an efficient alternative SPPS route for obtaining Aβ with increased solubility and purity [49,50].

Similarly, tailored recombinant expression systems have been used to produce Aβ in high yields. A strategy to increase solubility and facilitate purification is production of Aβ peptides

as fusions with sequences affording high solubility, followed by cleavage of the fusion protein and purification by high-performance liquid chromatography (HPLC) [42–44,51].

Because self-association of Aβ is believed to be central in the pathogenesis of AD, extensive research has been dedicated to developing methodologies to characterize Aβ assemblies structurally and biologically [52]. Multiple studies have shown that many of the neurotoxic effects of Aβ assemblies can be recapitulated by synthetic Aβ *in vitro* and *in vivo* [53]. However, differences in peptide quality, presence of trace contaminants in Aβ preparations from different sources, and compositional variation of Aβ preparations, even from the same source, have been a serious problem leading to irreproducible or discrepant study outcomes [54–56]. For example, under identical conditions, an Aβ oligomer-specific monoclonal antibody was shown to react only with oligomers derived from recombinant Aβ but not those derived from chemically synthesized Aβ [57]. In our hands, photo-induced cross-linking of unmodified proteins (PICUP) using Aβ obtained from different sources, but prepared identically, yielded distinct results (Fig. (1)).

Neither SPPS nor recombinant methods can produce 100% pure Aβ. Failure sequences, oxidation of $Met^{35}$, and racemization may occur during various SPPS steps [52]. In recombinant preparations where a fusion protein is enzymatically cleaved to release Aβ, it is important to verify that the cleavage product is not contaminated with the uncleaved fusion protein, the cleaving enzyme, or adventitious proteolytic fragments [52]. Practically, it is important that the researcher verifies chemical purity of the preparation and ensures removal of residual components which could complicate solvation and stock preparation, potentially alter the biophysical and biological features of the peptide, and render concentration measurements error-prone [52].

## METHODS USED TO STUDY PRE-FIBRILLAR Aβ ASSEMBLIES

Important biological functions of oligomeric Aβ assemblies have spurred extensive efforts to characterize them structurally. The non-crystalline nature of the oligomers and their slow tumbling time in aqueous solutions preclude high-resolution structural determination by X-ray crystallography and solution-state NMR, respectively. Moreover, the metastable nature of Aβ oligomers and their existence in rapidly changing mixtures have made their structural characterization particularly difficult. To address these issues, multiple low-resolution methodologies have been used to assess various structural features of pre-fibrillar Aβ assemblies. Here, we outline some of the key methods used to provide structural and biophysical information on pre-fibrillar Aβ assemblies.

## ATOMIC-RESOLUTION TECHNIQUES

### NMR Spectroscopy

Solution-state NMR is a powerful high-resolution technique for determining peptide and protein structure in solution. Typically, the structure is calculated based on distances and angles obtained through measurements of nuclear Over-hauser effect and spin-spin scalar coupling interactions as constraints for computer-generated models. As mentioned above, peak broadening due to slow tumbling times currently precludes solving the structure of Aβ oligomers using solution-state NMR. However, multiple NMR studies have assessed structural properties of Aβ monomers. For instance, studies by Lee *et al.* introduced the concept of "plaque competence," which defines the propensity of near-physiological concentrations of soluble Aβ to deposit onto authentic amyloid plaques *in vitro* [58]. The plaque competence assay identified a central 26-residue fragment ($Tyr^{10}$–$Met^{35}$) which was deemed necessary to mimic plaque-deposition characteristics of the full-length Aβ [58]. Preliminary NMR conformational analysis showed that this 26-residue fragment had a different conformation

from a plaque-incompetent fragment ($Asp^{1}$–$Lys^{28}$) [58]. Further NMR studies also confirmed that the central 26 residues of Aβ were sufficient to mimic amyloidogenic properties of Aβ [59]. It was reported that the central hydrophobic cluster of full-length Aβ, and Aβ(10–35), both adopted well-defined, albeit irregular, conformations in solution, whereas the C- and N-terminal flanking regions of the full-length Aβ were partially disordered [59].

NMR studies also have highlighted differences between Aβ40 and Aβ42. Solution-state NMR studies of non-oxidized [60] or Met-oxidized [61,62] Aβ40 and Aβ42 show that the C-terminus of Aβ42 is more rigid compared to that of Aβ40, likely due to the extended hydrophobic C-terminus of Aβ42. Similarly, a study combining molecular dynamics and NMR experiments, showed that the C-terminus of Aβ42 is more structured than that of Aβ40 [63]. NMR studies also revealed that common C-terminal peptide segments within Aβ40 and Aβ42 have distinct structures, which may be relevant to the strong disease-association of elevated Aβ42 production [64].

## X-Ray Crystallography

X-ray crystallography examines atomic structures of crystals by X-ray diffraction techniques (reviewed in [65]). The signal is intensified by the coherent alignment and lattice repeat of the crystal. The wavelength of the light used in X-ray crystallography is usually around 1.5 Å, about the length of a C-C bond. Use of X-rays with this wavelength theoretically allows resolution of individual atoms. Recently, Sawaya *et al*. reported that 33 peptide segments derived from 14 different amyloidogenic proteins formed amyloid-like fibrils, microcrystals, or both [66] and used X-ray crystallography to examine the atomic organization of molecules within microcrystals of these peptides. Microcrystals of 2 Aβ segments were resolved, $Gly^{37}$–$Ala^{42}$ and $Met^{35}$–$Val^{40}$. The authors suggested that the structural organization of these peptides within the crystals is similar to those of Aβ fibrils and concluded that the fundamental unit of amyloid-like fibrils is a steric zipper arrangement formed by two tightly interdigitated β-sheets [66].

## Hydrogen–Deuterium Exchange

Hydrogen–deuterium exchange is a powerful probe of protein structure and dynamics. The method involves the study of exchange rates of labile protons in proteins with deuterons from the solvent, typically $D_2O$. Labile protons are those bonded to nitrogen, sulfur, or oxygen. These protons can exchange with solvent hydrogen or deuterium cations. Labile protons that are solvent-exposed and are not involved in hydrogen bonding exchange rapidly, whereas buried or hydrogen-bonded protons exchange at substantially slower rates. This makes hydrogen–deuterium exchange sensitive to structural rearrangements occurring during protein aggregation. Thus, amide protons buried in the core of oligomeric and higher-order assemblies or hydrogen-bonded in helices and sheets do not exchange readily with solvent deuterons. The exchange rate is detected using NMR and/or mass spectrometry. For study of rapidly changing assemblies, mass spectrometric detection of exchange may be advantageous because NMR requires longer times (hours) to record the spectra, making the study of short-lived oligomers difficult. In addition, NMR requires prior assignment of the protons and is generally limited to proteins smaller than 25 kDa. An additional advantage of mass spectrometric detection is requirement of substantially smaller amounts of protein. However, assignment of specific exchanging protons using mass spectrometry requires tandem mass spectrometry and can be a daunting task, whereas for a previously assigned NMR spectrum, identification of exchanging protons is straight-forward. Hydrogen–deuterium exchange coupled with mass spectrometry was used to map structural differences in Aβ PF and fibrils [67].

# DETERMINATION OF OLIGOMER-SIZE DISTRIBUTION

## Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a routine and inexpensive method enabling separation of proteins based on their electrophoretic mobility which is affected by a combination of the primary, secondary, tertiary, and quaternary structures of proteins. In this method, protein mixtures are electrophoresed after treatment with SDS. SDS binds proteins *via* its hydrophobic dodecyl tail, leaving its sulfate group solvent-exposed, and thus creating a negatively-charged envelope that "coats" protein molecules [68]. In most cases, SDS binding denatures secondary and non-disulfide-linked tertiary structures, negatively charging proteins approximately uniformly and proportionally to their mass. Under these conditions, electrophoretic migration of proteins through the gel matrix is governed directly by the molecular mass of the protein. Without SDS, different proteins of the same mass may electrophorese distinctly due to differences in overall charge (different isoelectric points) and folding.

Importantly, the effect of SDS on all proteins is not equivalent. In many cases, SDS can induce or stabilize secondary and quaternary structures. SDS may cause dissociation of some oligomers or conversely induce oligomerization and aggregation. Therefore, resolution of apparently monomeric or low-molecular-weight (LMW) oligomeric components in a protein mixture does not necessarily indicate existence of such components under native conditions, i.e., without SDS. Aβ is an amphipathic protein known to form SDS-stable oligomers [30]. Indeed, SDS-induced assembly of Aβ into insoluble aggregates has been capitalized on to purify Aβ from brain homogenates [69]. When treated with SDS, Aβ assembles rapidly into high-molecular-mass aggregates [57]. During electrophoresis of Aβ40, these aggregates dissociate completely and only a monomer is observed following staining, whereas electrophoresis of Aβ42 yields apparent trimeric and tetrameric components [33]. Essentially identical monomer-trimer-tetramer distributions are observed when different preparations of Aβ42, including "monomeric", oligomeric, and fibrillar Aβ42 are analyzed by SDS-PAGE [70]. Thus, despite its wide use, SDS-PAGE is not a reliable method for characterization and size determination of non-covalently associated Aβ oligomers.

## Photo-Induced Cross-Linking of Unmodified Proteins (PICUP)

PICUP is a method originally developed to study stable protein homo- and hetero-oligomers [71]. PICUP was used by us and others to study oligomer size distribution of Aβ [72] and a variety of other proteins, including amyloidogenic proteins [73]. PICUP generates covalent bonds between closely interacting polypeptide chains within ≤1 *s* exposure to visible light without *pre facto* chemical modifications of the native sequence and without using spacers. The cross-linking is induced by rapid photolysis of a tris-bipyridyl Ru(II) complex in the presence of an electron acceptor. Illumination causes generation of a Ru(III) ion, which subsequently abstracts an electron from, and produces a carbon radical within, the polypeptide. The radical reacts rapidly with adjacent susceptible groups and forms covalent bonds. Therefore, PICUP stabilizes oligomer populations by covalent cross-linking, and "freezes" molecular interactions that exist before cross-linking. The mechanism, protocol, and limitations of PICUP were discussed in detail elsewhere [71,73].

## Size-Exclusion Chromatography (SEC)

SEC (gel permeation chromatography) fractionates solutes based on their Stokes (hydrodynamic) radii. When solutes of different sizes pass through an SEC column packed with porous material, larger molecules spend less time interacting with the solid phase and elute faster, whereas smaller molecules diffuse into the pores and therefore spend more time interacting with the solid phase and elute later. SEC affords an SDS-independent separation mechanism and covers a molecular mass range of ~$10^3$–$10^6$ Da. However, SEC provides lower

resolution than SDS-PAGE and molecular-mass estimations of polypeptides can be inaccurate if the elution profiles are sensitive to the protein conformations. SEC analysis of Aβ assemblies does not resolve LMW oligomers but can distinguish between PF and small oligomers [30]. At this resolution, SEC may be useful for studying the kinetics involved in conversion of LMW Aβ to PF (or dissociation of PF into LMW Aβ). In addition to its use as an analytical method [39], SEC has been used extensively to purify fractions of particular Aβ assemblies [30,33, 38,74–76]. Description of the basic instrumentation and utilization of SEC for preparation of aggregate-free Aβ was published previously [77].

## Analytical Ultracentrifugation (AU)

AU is a versatile technique used to characterize the hydrodynamic and thermodynamic properties of proteins or macromolecules. AU combines an ultracentrifuge and an optical detection system capable of measuring the sample concentration inside the centrifuge cell during sedimentation. Coupled with data-analysis software, AU can determine sample purity and molecular mass in the native state, measure sedimentation and diffusion coefficients, characterize assembly–disassembly mechanisms of complex analytes, determine subunit stoichiometry, detect and characterize macromolecular conformational changes, and measure equilibrium constants and thermodynamic parameters for self- and hetero-associating assemblies. Two types of experiments are commonly performed using ultracentrifugation–sedimentation-velocity [78,79] and sedimentation-equilibrium [80], Sedimentation-equilibrium experiments can analyze a mixture of moieties of various molecular masses. After each analyte reaches its equilibrium, high-molecular-mass species locate towards the bottom of the cell, whereas low-molecular-mass species dominate at the top. The equilibrium data can be fitted to calculated models for the distribution of the solutes. Using this type of analysis, Huang *et al.* have reported that Aβ40 existed as an equilibrium mixture of monomers, dimers and tetramers at neutral pH [81]. However, other equilibria, including monomer–dimer, monomer–trimer, or monomer–tetramer, produced equivalent residuals [81] hindering precise determination of the oligomerization state of the peptide.

## Dynamic Light Scattering (DLS) Spectroscopy

DLS, also known as quasielastic light scattering or photon-correlation spectroscopy, is a non-invasive analytical method for determination of diffusion coefficients of particles undergoing Brownian motion in solution. DLS measures the temporal dependence of light scattering emanated from an analyte in solution over $10^{-7}$–1 *s*. Fluctuations in the intensity of the scattered light relate to the rate of the Brownian motion which is correlated to the diffusion coefficients of the particles. In a mixture of analytes, a distribution of diffusion coefficients is obtained. The data are processed to determine the particle hydrodynamic radii which relate to the diffusion coefficients using the Stokes-Einstein equation. DLS has an intrinsic bias for large aggregates because the intensity of the scattered light is proportional to the square of the particle size [82], Therefore, DLS is well suited to measure minute amounts of aggregated proteins (<0.01% by weight) on the background of monomers and small oligomers. Because DLS allows monitoring protein assembly without manipulation or consumption of the analyte, it has been used widely to study Aβ aggregation and assembly processes [33,75,83–87].

For proteins larger than ~500 kDa or for extended proteins (rod-like/unfolded), scattering varies significantly with angle. Determining scattering at additional angles (multi-angle laser light scattering or MALLS) allows direct measures of mass (≤MDa) and radius (a measure of geometric size). Because the light-scattering signal is directly proportional to protein concentration and molecular mass, a combination of the DLS signal and concentration measurements using refractive index or absorbance, allows calculation of the molecular mass of each component when proteins are fractionated chromatographically. DLS can resolve the monomeric or dimeric state of a protein, but it cannot distinguish among small oligomers when

their hydrodynamic radii differ by less than a factor of 2 [88]. Consequently, DLS is less useful for analyzing individual small oligomers than SEC-MALLS, PICUP coupled with SDS-PAGE, or sedimentation velocity. Detailed accounts of the theory and practice of DLS and its application to study of Aβ were given by Lomakin *et al.* [82,89,90]. Solution state and size distribution of ADDLs has been assessed recently by Hepler *et al.* using SEC coupled with MALLS [70].

### Ion-Mobility Spectrometry-Mass Spectrometry (IMS-MS)

IMS-MS is a mass-spectrometric method that can resolve molecules of identical mass-to-charge (m/z) values which differ by assembly state or conformation. In IMS-MS, ions are carried by a weak, uniform electric field through a drift cell in which they collide at low velocity with a low-pressure inert gas (typically helium). The analyte ions quickly reach equilibrium resulting in a constant drift velocity. At equilibrium, the mobility of the ions is inversely proportional to their collisional cross-section. Thus, ions with compact structures drift fast through the cell, whereas ions with large cross-sections move more slowly. The ions exit the drift cell, pass through a mass filter, and are detected as a function of time, producing an "arrival-time" distribution. Protein oligomers often have identical m/z ratio (i.e., a singly-charged monomer would have the same m/z as a doubly-charged dimer, triply-charged trimer, etc.). IMS-MS analysis can resolve these species yielding an oligomer size distribution. IMS-MS studies of AB have shown that freshly prepared LMW Aβ40 contained monomers, dimers, trimers, and tetramers, whereas similarly prepared solutions of Aβ42 comprised oligomers up to a dodecamer [91]. These results accord with earlier observations of distinct oligomer size distributions of Aβ40 and Aβ42 by PICUP [33] and may explain differences in neurotoxic effects of the two Aβ alloforms.

## ANALYSIS OF SECONDARY STRUCTURE

### Circular Dichroism (CD) Spectroscopy

CD is the change in the absorption of circularly polarized light as a function of wavelength exhibited by optically active molecules. Because secondary structural elements such as α-helices, β-strands, β-turns and disordered regions display characteristic wavelength-dependent dichroism, CD is a useful method to determine protein secondary structure. Secondary structure analysis by CD spectroscopy uses "far-UV" spectra (190–250 nm), in which the chromophores are peptide bonds. The CD signal reflects an average of the entire molecular population. Therefore, CD can only determine the overall proportion of secondary structural elements, but not the amino acid residues involved or the fraction of molecules that have a particular conformation. CD has been used extensively to investigate secondary structure of Aβ peptides and to monitor structural transitions of Aβ during its oligomerization and aggregation [31, 92–95].

### Fourier-Transform Infrared (FTIR) Spectroscopy

Complementary to CD, FTIR enables determination of the secondary structure of protein samples as thin films, as solids, or in solution. Characteristic bands in IR spectra of proteins and polypeptides include predominantly amide I and amide II. The amide I band corresponds to the absorption in the vibrational spectrum of the C=O component of the amide bond, whereas the amide II band corresponds to the absorption of the N–H bond. Because C=O and N–H bonds are involved in hydrogen bonding, the absorption wavelength of both the amide I and amide II bands are sensitive to the secondary structure content of proteins. In many cases, instead of a series of well-resolved peaks for each type of secondary structure, one broad peak is observed. However, for proteins that cannot be studied by high-resolution methods, FTIR provides useful structural information. It is thought that FTIR is more sensitive to β-sheet content whereas CD measurements generally tend to underestimate β-sheet relative to α-helix

content [96]. FTIR has been used extensively to study the conformational changes of Aβ during assembly [97–101].

## MORPHOLOGICAL ANALYSIS

### Transmission Electron Microscopy (TEM)

TEM uses a cathode ray which emits a high-voltage electron beam focused by electrostatic and electromagnetic lenses. When the electron beam passes through a thin, electron-transparent specimen, it carries information about the inner structure of the sample as it reaches the TEM imaging system. There, the spatial variation in this information, which creates the image, is magnified by a series of electromagnetic lenses and detected by a fluorescent screen, photographic plate, or a light-sensitive sensor, e.g., a camera. TEM has been used extensively to examine the morphology of pre-fibrillar Aβ assemblies, including LMW Aβ [33,34], small oligomers [102–105], paranuclei [33,34], PF [31,75,76,87] and spheroids [36,39].

### Scanning Transmission Electron Microscopy (STEM)

In STEM, an electron beam scans a specimen and scattered electrons are collected by detectors behind the specimen. In a thin proteinaceous specimen, the image intensity is directly proportional to the mass of the irradiated region. Therefore, following background subtraction and calibration, the protein mass and mass-per-length unit (MPL) can be determined quantitatively [106]. STEM has been used to characterize the MPL ratios of Aβ fibrils and PF [107–109].

### Atomic-Force Microscopy (AFM)

AFM images high-resolution (≤1 nm) topography of a sample, adsorbed on an atomically flat smooth surface, typically mica. A cantilever tip samples the surface and when the tip contacts a spot with adsorbed sample, an ionic repulsive force bends the cantilever upwards. The extent of bending, measured by a laser reflected onto a split photo detector, is translated to force units. By keeping the force constant while scanning across the surface, the vertical movement of the tip generates the surface contour which is recorded as the topography of the sample. AFM has been modified for specific applications and can be used in different modes. In "tapping mode" (commonly referred to as "intermittent-contact" or "dynamic-force mode"), a stiff cantilever oscillates close to the sample. Part of the oscillation extends into the repulsive regime so that the tip intermittently touches, or "taps," the surface. This mode provides good resolution on soft samples and therefore is useful for investigation of pre-fibrillar Aβ species.

An advantage of AFM over TEM is that it allows continuous monitoring of the growth of oligomers in solution [110]. Multiple studies on the structure of soluble oligomers have used conventional tapping-mode AFM [26,30,31,111–114]. The smallest size of individual oligomers that could be observed by AFM corresponded to a height of ~1–4 nm [101].

### Scanning Tunneling Microscopy (STM)

STM is a non-optical microscopic technique which employs principles of quantum mechanics. The electron cloud of atoms on the surface of a sample extends a minute distance above the surface. When a probe, with a tip as sharp as a single atom, is brought sufficiently close to such a surface and a small voltage is applied, a strong interaction occurs between the electron cloud on the surface and the tip leading to an electric tunneling current. The magnitude of the current depends exponentially on the distance between the probe and the surface. The tunneling current rapidly increases as the distance between the tip and the surface decreases. This rapid alteration in the current due to changes in distance results in construction of an atomically resolved image when the tip scans the structure. The feedback signal, applied to a piezoelectric element provides a measure of molecular surface contour. STM was used to examine the structure of

Aβ40 monomers, dimers and oligomers on a surface of atomically flat gold [115]. At low concentrations (0.5 μM) small globular structures were observed. High-resolution STM measurements of Aβ samples, both immediately following preparation and after 24 h aging, found structures of ~3–4 nm in diameter corresponding to oligomeric Aβ. These results suggested that oligomer formation could potentially proceed through a mechanism involving linear association of monomers [115].

## STRUCTURAL AND BIOLOGICAL STUDIES OF PRE-FIBRILLAR Aβ ASSEMBLIES

The use of proper terminology to describe soluble pre-fibrillar Aβ assemblies is crucial to forming consensus in the literature. However, achieving this goal is difficult because various oligomeric forms of Aβ have been described structurally, functionally, or both and the relationship amongst these is unclear. Although all these structures are oligomeric, the use of the term "oligomer" to describe all the assemblies may be misleading for at least three reasons, as discussed before: 1) the structure of each assembly is unique; 2) the pathways leading to the formation of the assemblies or the ultimate path they may take towards fibrillization may differ; and 3) the biological activities of each assembly may differ or similar activities may be mediated through different mechanisms [57]. In the following section, we describe pre-fibrillar Aβ species ranging from monomers to PF (Table (1)). In most cases, we begin with structural characterization of each species followed by discussion of its biological activity.

## "ACTIVATED MONOMERIC CONFORMER" OF Aβ

### Structural Characterization

Monomer activation denotes a conformational change preceding Aβ self-assembly that may render monomers toxic, or cause them to nucleate further aggregation, or both. Based on concepts taken from actin polymerization [116] and a kinetic model of Aβ aggregation induced by constant rotary shaking, Taylor *et al.* introduced the idea of "activated monomeric conformers" of Aβ40 [19], also called "intermediate aggregated species" [117]. It was postulated that this moiety was an oxidative or hydrolytic derivative, or a slowly-folding conformer of intact Aβ40 [19,117]. It was proposed that the "inactive" monomer slowly converted into the "activated" monomeric conformer, several of which might cooperate to form a growing nidus for oligomerization and progression of aggregation [19]. In these studies, the presence of the active monomeric Aβ conformer was tested by HPLC using acetonitrile and trifluoroacetic acid, which might have caused structural alterations in Aβ sequence by increasing its β-sheet content [118]. Lee *et al.* have provided evidence that Aβ aggregation intermediates and final structures formed under slowly agitated or quiescent conditions at 37° C differed in their toxicity, stability to denaturant, and apparent morphologies [119], emphasizing that parametrical consideration of methodologies used to prepare Aβ for structural or biological studies and proper methods to assess the assembly state of the resulting preparation are paramount [52]. Similarly, NMR studies emphasize the importance of performing structural studies under physiological conditions [61] rather than "structure-inducing" milieus as reported by Taylor *et al.* [19].

Other studies also have shown presence of Aβ intermediates, however, the assembly state of these was not determined unambiguously. A study by Chimon *et al.* described Aβ40 intermediates that contained a β-sheet-rich character and were thought to originate from a monomeric state, preceding PF and fibril formation [120]. Filtration experiments showed that these intermediates were not monomeric but were likely larger than decamers, indicating that unambiguous determination of the assembly intermediates is difficult. By electron microscopy, these intermediates had a spherical morphology similar to ADDLs [121], amylospheroids

[36] and βamy balls [39]. NMR studies showed that the intermediate species was well ordered in the hydrophobic core and the C-terminal region [120]. Aβ40 was used in this study at higher concentrations than those found in biological specimens and the possibility that the intermediate could have undergone fibril formation during preparation for NMR studies was not excluded. Similarly, Lim *et al*. provide evidence for presence of monomeric intermediates using CD and NMR studies of Aβ40 and Aβ42 under both non-amyloidogenic (<5°C) and amyloid-promoting conditions (>5°C) at physiological pH [94]. CD studies of the Aβ peptides suggested that the initially unfolded Aβ peptides at low temperature gradually transformed to β-sheet-containing monomeric intermediates at stronger amyloidogenic conditions (higher temperatures) [94]. However, exclusive presence of monomers after dialysis of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)-treated Aβ species against phosphate buffer was not confirmed [94]. Providing formal proof for the presence of Aβ monomers exclusively is difficult and has not been achieved unambiguously in the studies mentioned above. Therefore, whether a critical conformational change in the monomer occurs before self-assembly, or the conformational change and the assembly occur concurrently and co-dependently remains an open question.

### Biological Activity

In cell biological studies, toxicity and apoptotic activity were enhanced when the concentration of the "activated monomeric conformer" described by Taylor *et al.* was maximal during the aggregation continuum [19]. Similarly, in electrophysiological experiments, Aβ species described as monomeric conformers obtained between ~60–120 min during rotary-shaken aggregation inhibited neuronal action potentials [122]. This neurotoxicity was attributed only to the "active" monomeric conformers detected at 60 min after the initiation of aggregation but not to inactive monomers or fully formed aggregates [19,117,122]. In these studies, the kinetics of aggregation was monitored by turbidity and the loss of the low-molecular-mass starting material was monitored by HPLC [19]. The sensitivity of turbidity measurements for detection of small particles is substantially lower than that of DLS and it is therefore unlikely that the method can distinguish monomers from small oligomers. Also of note is that biological activities similar to those obtained by Taylor *et al.* [19] have been described for Aβ oligomers [53], thus it is unclear that these toxicity data can be ascribed to monomeric Aβ.

## BRAIN- AND CELL-DERIVED LOW-ORDER Aβ OLIGOMERS

### Structural Characterization

Dimeric and trimeric assemblies of Aβ have been isolated from amyloid plaque cores [20], cerebrospinal fluid (CSF) [22], and human cortical homogenates [21], and produced by cells transfected with amyloid precursor protein (APP) [23]. Dimeric and trimeric Aβ (apparent SDS-PAGE mobility corresponding to ~9 and ~13.5 kDa) purified from neuritic plaques and leptomeningeal mural amyloid, were characterized by SEC, AFM, and electron microscopy [104]. Matrix-assisted laser-desorption ionization (MALDI) mass spectrometry was used to determine the mass of the "dimeric" components [104]. However, to extract Aβ from tissue sources, SDS was included in extraction buffers and the possibility that these Aβ species could form during the extraction process was not excluded. Immunoaffinity purification, HPLC, and MALDI analyses of CSF from patients suffering from meningitides and other neurological conditions including dementia, revealed Aβ species of various length, including two truncated trimer species, $(Asp^{1}–Met^{35})_3$ and $(His^{6}–Ala^{42})_3$ and an Aβ40 dimer, $(Asp^{1}–Val^{40})_2$ [22]. Aβ in CSF was shown to be associated with high-density lipoprotein particles in an apparent monomeric form detected by SDS-PAGE [123,124]. Similarly, 24 and 27% of brain Aβ40 and Aβ42, respectively, were shown to be concentrated in lipid-raft extracts as dimers determined by SDS-PAGE in the *Tg2576* murine model of AD [125]. However, as discussed above, SDS-PAGE is not a reliable method for Aβ size determination [57].

Walsh *et al.* have reported "natural" Aβ oligomers that are "SDS-stable" low-order oligomers (dimers, trimers, and tetramers) detected in the conditioned media and/or lysates of cells [23, 24,126–128]. These oligomers are produced by Chinese hamster ovarian cells that express human mutant (V717F, V717I, or E693Q, cells referred to as 7PA2), or wild-type APP [23, 129]. Low-abundance species of masses ~10, 14 and sometimes 16 kDa, which were reactive with antibodies against Aβ also were detected in the culture media of these cells [23], The nature of these low-order oligomers is not fully understood but the observations that they are not disassembled by several types of denaturants suggests that they may be covalently linked [24].

## Biological Activity

Initially, biological effects of oligomeric Aβ fractions purified from neuritic plaques and leptomeningeal mural deposits were investigated in an astroglial-neuronal co-culture system believed to approximate *in vivo* conditions [104]. Neuronal viability was compromised by AD-derived Aβ only when microglial cells were co-cultured, indicating that toxicity was mediated through a microglia-dependent mechanism [104].

Ease of maintenance and fast growth-rate of 7PA2 cells facilitated investigations of the biological activities of "natural", cell-derived Aβ oligomers. Intracerebroventricular microinjection of small volumes (~1.5 μL containing ~3 ng/mL Aβ) of SEC-fractionated cell-culture media to anesthetized wild-type rats was shown to inhibit hippocampal long-term potentiation (LTP) [25,130]. This inhibition was predominantly mediated by Aβ trimers in wild-type murine hippocampal brain slices, whereas dimers and tetramers had intermediate potencies, and monomers were apparently ineffective [128]. Cell-derived oligomers were shown to interfere primarily with the induction of LTP, but not its expression, once the signaling cascades responsible for LTP were initiated [128]. These results suggested that cell-derived Aβ oligomeric assemblies altered certain aspects of hippocampal synaptic plasticity both *in vivo* and *in vitro* (reviewed in [131]).

The validity of LTP as an electrophysiological paradigm for learning and memory composition has been debated [132], To test the effect of the "natural Aβ oligomers" in a non-LTP paradigm, Cleary *et al.* used an *in vivo* behavioral model in rats that were injected with cell-conditioned media containing Aβ assemblies into the dorsal lateral cerebral ventricles. The treatment was found to cause a transient interruption of pre-learned behaviors [133]. This was attributed to Aβ oligomers because SEC fractions containing oligomers caused the deficits, whereas monomer-containing fractions were ineffective [133].

Actin-based cytoskeletal network dynamics is critical for the regulation of neuronal spine morphology and function [134]. Alterations in spine morphology and actin-regulatory mechanisms recently have emerged as a sensitive measure of early neuronal functional deficits and neurotoxicity [135–137], Because loss of synaptic termini strongly correlates with the severity of dementia, Shankar *et al.* assessed the effect of cell-derived soluble oligomers on synapses [138]. They showed that the density of dendritic spines decreased substantially in neurons treated with sub-nanomolar levels of cell-derived Aβ oligomers [138]. This effect was shown to be Aβ-oligomer-specific, i.e., SEC monomer fractions alone were ineffective. The decrease in spine density was reverted by monoclonal immunodepletion of Aβ, by transfer of cells back into the control media, and by *scyllo*-inositol [138], a molecule thought to stabilize synthetic Aβ as nontoxic species, possibly preventing Aβ interaction with neuronal target proteins [139,140].

In cultures of dissociated cortical neurons, synthetic Aβ was shown to activate nicotinic acetylcholine receptors (nAchRs) and trigger internalization of *N*-methyl-D-aspartate (NMDA) receptors (NMDARs) [141]. However, Shankar *et al.* found that an irreversible

nAchR antagonist did not affect Aβ-oligomer-mediated spine loss, indicating that nAchR activity was unnecessary for this effect [138]. Signaling cascades involving NMDARs, cofilin, and calcineurin were found to be involved in Aβ-induced spine loss as determined by inhibition studies [138]. Together these data demonstrated that cell-derived low-order Aβ oligomers could cause reduction of synapse density and loss of electro-physiologically active synapses in hippocampal pyramidal neurons, suggesting that their deleterious effects may be important mechanistic contributors to synaptic dysfunction in AD *in vivo* [131].

## Aβ-DERIVED DIFFUSIBLE LIGANDS (ADDLS)

### Structural Characterization

ADDLs are exclusively Aβ42-derived soluble pre-fibrillar assemblies that morphologically appear as 3–8-nm globules by AFM [26,121] and have estimated masses between 17–42 kDa [26] (reviewed in [142]). It was first observed that apolipoprotein J (apoJ, also called clusterin, is a ubiquitous multifunctional glycoprotein co-localizing with fibrillar deposits in systemic/localized amyloid disorders [143]) partially inhibited Aβ aggregation and caused formation of "slowly-precipitating" Aβ42 complexes of >200 kDa [144]. Follow-up studies showed that ADDLs with the same biochemical and neurotoxic characteristics could be produced in clusterin-free solutions by incubating aggregate-free Aβ42 in phenol-red-free F-12 medium at 4–8°C for 24 h, in clusterin-free brain-slice culture media at 37°C for 24 h [26,142], or even in phosphate-buffered saline [145].

By SEC, ADDL preparations contained two distinct peaks-an early-eluting high-mass component, which exhibited punctate binding to primary neurons, and a late-eluting low-mass component (13 kDa), which lacked this property [121]. These peaks are similar to those reported by Walsh *et al.* for protofibrillar and LMW preparations of Aβ, respectively (see below) [30]. However, Walsh *et al.* showed by TEM that the early-eluting peak contained abundant PF whereas the late-eluting peak was reported later to contain a mixture of monomer and small oligomers detected using PICUP [74]. In a recent study of ADDLs, when SEC coupled with MALLS and AU was used to determine the size distribution of ADDLs, the SEC peaks corresponding to 75 and 13 kDa showed oligomer masses ranging from 150–1,000 kDa, and a monomeric component of 4.5 kDa by MALLS [70]. This study suggested that previous reports identifying low-molecular-mass components as a composite of low-number oligomers were misrepresentations of what may actually be a monomeric Aβ42 fraction [70], ADDLs found in the early-eluting peak were shown to be in a dynamic equilibrium comprising a polydisperse population of oligomers [70]. Multiple parameters such as peptide concentration, temperature, pH, storage duration, and excipient addition were shown to affect this equilibrium dramatically [146].

Importantly, Aβ40 does not form ADDLs [70]. NMR studies have shown that the C-terminus of Aβ42 is more rigid compared to that of Aβ40 [60–62] potentially due to the extended hydrophobic C-terminus of Aβ42. This C-terminal difference and potentially the different monomeric Aβ42 conformers generated due to this structural difference may account for the increased toxicity and plaque competence of Aβ42 compared to Aβ40 [60,61]. Indeed, some oligomeric moieties (ADDLs, paranuclei, and globulomers, see below) were shown to form by Aβ42 only. In fact, the exclusive Aβ42 derivation of ADDLs and paranuclei, and the fact that ADDLs and paranuclei are indistinguishable morphologically (compare [26,121] with [33]), suggests that ADDLs and paranuclei may be related to each other or even be the same species obtained under different conditions [26,33].

ADDLs have been shown to resist dissociation by low SDS concentrations (0.01%) [144]. However, when supramicellar SDS concentrations were used, ADDLs and fibrils migrated with the same electrophoretic profile yielding monomeric, trimeric and tetrameric moieties

[70]. A similar profile was observed for LMW Aβ42 [33] produced by SEC or filtration [77]. At submicellar concentrations of SDS, oligomers were detected both by denaturing electrophoresis and SEC [70], When Aβ42 was electrophoresed in the presence of submicellar or supramicellar concentrations of SDS, high-molecular-mass aggregates and intermediate-sized assemblies formed, respectively [57]. During SDS-PAGE, these aggregates may partially dissociate as diffuse Aβ42 trimer/tetramer components [57] as observed for ADDLs [70], These observations re-emphasize that visualization of SDS-stable oligomeric Aβ may be misrepresenting the actual assembly state.

## Biological Activity

Initial ApoJ-induced ADDL preparations (0.34 mg/mL) were neurotoxic [144], Later studies showed that ADDLs selectively targeted the principal neurons in the hippocampal strata pyramidale and granulosum in organotypic murine brain slices [26] and inhibited LTP in rat hippocampal brain slices [147,148]. ADDLs also were shown to augment the negative synaptic plasticity of long-term depression (LTD) [149]. Prolonged maintenance of LTD along with LTP inhibition leads to an overall synapse-inhibitory effect (reviewed in [53]).

When cultured hippocampal neurons were incubated with synthetic ADDLs, F-12-prepared soluble brain extracts, or crude human CSF, and probed with ADDL-specific antibodies a punctate binding pattern reminiscent of synaptic termini was observed [150] (Fig. (2)). The antibodies used were shown to be 100-fold more sensitive to ADDLs (fmol levels) than to monomeric Aβ [27,136,150–153]. The ADDL-binding sites were demonstrated to coincide with dendritic spines at postsynaptic termini of excitatory synapses [150]. ADDL binding also overlapped with NMDAR subunit NR1 on highly arborized neurons positive for α calcium-calmodulin kinase II [150] which accumulates in postsynaptic termini of neurons involved in memory function [150]. In addition, ADDLs specifically bound to excitatory pyramidal, but not GABAergic, neurons [136], and to neurons positive for NMDAR subunits NR1 and NR2B [136]. Similarly, ADDLs did not bind astrocytes or inhibitory neurons expressing glutamic acid decarboxylase [136]. Preferential binding of ADDLs to excitatory synapses at postsynaptic sites was consistent with the inhibitory impact of ADDLs on NMDAR-dependent LTP [26,149] and NMDAR-mediated phosphorylation of cAMP response-element binding-protein [154], demonstrating that ADDLs could impact crucial receptors involved in synaptic plasticity.

Defective neuronal actin-regulatory machinery is an underlying factor in dendritic and synaptic dysfunctions in many neurological disorders accompanied by cognitive deficits, including AD and Down syndrome (reviewed in [134]). ADDLs were shown to affect spine shape, which, like receptor expression is a facet of spine cell biology with ramifications for signaling and plasticity [155,156]. ADDL-induced alterations of spine morphology resembled the morphology of immature and diseased spines associated with mental retardation and prionoses [157].

ADDL binding to neuritic spines was reported to induce expression of the activity-regulated cytoskeleton-associated protein (Arc), a synaptic immediate-early gene [150,158]. Proper expression of Arc is essential for LTP but its ectopic and aberrant expression causes failure of long-term memory formation [159]. ADDLs generated a rapid and sustained increase in synaptic Arc protein expression [150], which interfered with long-term memory formation [159] and was hypothesized to lead to synapse failure and memory loss. De Felice *et al.* showed that ADDL treatment of mature primary hippocampal neurons led to reproducible and dose-dependent generation of reactive oxygen species (ROS) in the vicinity of synaptic ADDL-binding sites [160]. It was shown that ADDL binding to the NR1 subunit of NMDAR and NMDA-mediated $Ca^{2+}$ influx led to ROS generation, further delineating the mechanisms of ADDL neurotoxicity [160]. Interestingly, ADDL treatment was shown to induce τ

hyperphosphorylation in neuroblastoma cells and rat hippocampal primary neurons before neuronal death occurred [145]. Intrahippocampal injection of an oligomer-specific antibody was sufficient to reverse the effect of amyloid and τ pathologies, providing an additional insight into ADDL-mediated neurotoxicity [161].

Although *in vitro* experiments demonstrated that ADDLs interfered specifically with memory-associated experimental phenomena, a crucial question is whether ADDLs exist, and cause the same toxic effects, *in vivo*. Conformation-specific polyclonal [152] and monoclonal antibodies [162] shown to discriminate between ADDLs and Aβ monomers have been used to address this question. Dot-blot assays have detected ADDL immunoreactivity in transgenic mice and in AD brains which were extracted without detergents or harsh chemicals precluding extraction-induced alterations of the assembly structures [27,153]. ADDL concentrations were 70-fold higher in AD brains compared to controls [27]. In the nontransgenic mouse brain, no ADDLs (detection limit <10 fmol/μg) were detected by dot-blotting brain extracts using an ADDL-specific antibody [153]. However, in brains of transgenic mice, ADDL concentrations varied from ~20–250 fmol/μg depending on the brain region tested [153]. Soluble brain proteins extracted in F-12 culture medium by ultracentrifugation contained ADDL immunoreactivity that correlated with presence of AD [27,150] (reviewed in [53]). These findings support the hypothesis that ADDLs may be an important component in the amyloid cascade, as opposed to the poor correlation between insoluble amyloid deposits and cognitive impairment [53].

## PROTOFIBRILS

### Structural Characterization

PF are Aβ40- and Aβ42-derived curvilinear, soluble assemblies, which were described originally by Walsh *et al.* [30] and Harper *et al.* [28]. Walsh *et al.* reported studies using SEC, DLS, and TEM examining initial stages of Aβ oligomerization and characterizing Aβ intermediates during fibrillogenesis. By SEC, PF had apparent mass >100 kDa. They predominantly comprised curved, fibril-like structures of 6–8 nm in diameter and ≤200 nm long as observed by TEM [30]. Harper *et al.* detected the same protofibrillar assemblies of Aβ during polymerization by AFM [28]. They noted that Aβ40 PF, which appeared during the first week of incubation, had diameters of 3.1 nm and were 20–70 nm long [28]. The PF showed a periodic diametrical variation every 20 nm. In contrast, Aβ42 PF formed within the first day of incubation and had larger diameters (4.2 nm) than those of Aβ40. Aβ42 PF elongated overtime with diametrical periodicity similar to Aβ40 [28].

TEM examination of Aβ PF with rotary shadowing [31] demonstrated flexible rods up to ~200 nm long, with larger diameters than those observed by regular TEM or AFM. PF appeared more beaded with a periodicity of 3–6 nm, and the proportion of small PF (<10 nm) was higher, suggesting that these smaller structures might have been overlooked using TEM with routine negative staining or that the preparations were simply different [31]. The beaded structures were shown to be typical of early PF, whereas at later time points, PF appeared smoother [163]. High-resolution AFM studies have demonstrated that Aβ40 PF had a diameter of ~4.3 nm, with periodicity of ~20 nm, and coexisted with spherical species of the same diameter [112]. Spheres similar to those have been hypothesized by us and others to be precursors which join together to form PF [33,110].

PF have a high β-sheet content, a characteristic similar to that of mature amyloid fibrils [31] and are recognized by a conformation-sensitive antibody, WO1, which reacts with the fibrillar form of various amyloid proteins [164]. Thus, PF are the latest precursors on the pathway of fibril formation described to date. Nevertheless, apparently a substantial conformational rearrangement occurs upon maturation of PF into fibrils as evidenced by the observations that

PF can be readily disassembled into LMW Aβ, whereas mature fibrils do not typically disaggregate back into PF [31]. Proline substitution experiments showed that Aβ40 PF were "less structured" in the region $Glu^{22}$–$Gly^{29}$ compared to mature fibrils [164]. Hydrogen–deuterium exchange data demonstrated that the C-terminal $Met^{35}$–$Val^{40}$ and the N-terminal $Asp^{1}$–$Phe^{19}$ regions of Aβ40 were highly exposed to solvent both in fibrils and PF. In contrast, the $Phe^{20}$–$Leu^{34}$ segment was highly protected from hydrogen–deuterium exchange in fibrils but much less so in PF [67]. The β-structure (β-sheet and β-turn) content of PF was similar to that of fibrils as assessed by CD studies [30]. Collectively, these data suggested that the β-sheet elements comprising the amyloid fibrils were already present in PF. These elements could be expanded into adjacent residues and other elements, such as lateral association of filaments may contribute to the maturation of amyloid fibrils.

## Biological Activity

Initial studies to assess the biological activity of PF were performed in cultured primary rat cortical neurons over a time scale of minutes to hours, presumably before PF convert to fibrils [31]. In these experiments, compromise of cell viability by SEC-isolated PF was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [165]. It was found that PF and fibrils altered the normal physiology of cultured neurons, whereas LMW Aβ did not [31], To explore and compare the toxic effects of LMW Aβ, PF and fibrils further, Hartley *et al.* used rat cerebral primary mixed cultures (containing neurons, astroglia and microglia) and showed that PF caused neuronal injury and altered electrophysiological activities, ultimately causing cell death [29]. Although LMW Aβ caused a rapid but transient increase in excitatory post-synaptic currents (EPSCs), PF or fibrils (~3 μM) invariably produced rapid and sustained increases in electrical activity, six-fold greater than that induced by LMW Aβ [29]. Similarly, PF and fibrils significantly increased the frequency of action potentials and augmented the frequency and size of membrane depolarization compared to LMW Aβ preparation [29]. Substantial neuronal loss (80% as opposed to 10% non-treated cells) was observed consistently using the lactate dehydrogenase assay [166] and immunostaining against neuron-specific, microtubule-associated protein-2, after 5 days exposure to LMW and protofibrillar Aβ [29]. It was hypothesized that LMW Aβ could either convert to PF that cause the neurotoxicity or it could induce neurotoxicity by a mechanism independent of PF [29].

Important insight into the clinical relevance of PF came from investigation of a family in Northern Sweden, members of which carry a mutation in the *app* gene that leads to a single amino acid substitution in the Aβ region, E22G (dubbed the Arctic mutation) [167]. Surprisingly, even though carriers of this mutation have decreased plasma levels of Aβ40 and Aβ42 compared to non-carriers, they develop early-onset AD [167]. *In vitro* fibrillization studies showed that Aβ containing the E22G substitution formed PF faster and in larger quantities than wild-type Aβ, suggesting that PF may be the main disease-causing agents in carriers of the Arctic mutation [167]. These findings, along with the observations of PF formation by most other amyloidogenic proteins, have positioned PF as a likely primary pathogenic assembly state and an important target for drug development efforts for amyloidosis [168].

Concentrations of 4-hydroxy-2-nonenal (HNE), a metabolite of oxidative stress resulting from fatty-acid peroxidation, and of HNE-derived lipid peroxidation products, have been shown to increase in AD [169]. Recently, HNE has been shown to modify Aβ by 1,4 conjugation to primary amino groups and Schiff base formation, which could result in putative covalent intermolecular cross-linking of Aβ monomers [170]. Aβ40 prepared after a 285-h incubation with HNE predominantly had a PF-like, curved morphology when examined by AFM, whereas preparations formed in the absence of HNE comprised straight fibrils predominantly [170].

Both the long, straight fibrils formed in the absence of HNE and the curved fibrillar aggregates formed in the presence of HNE were rich in β-sheet structure, based on their CD spectra. In the presence of HNE, accelerated formation of protofibrillar Aβ40 species was observed, whereas generation of mature straight fibrils even upon extended incubations was inhibited [170]. These data suggested that HNE could cause accumulation of toxic Aβ PF by preventing their maturation to the less toxic fibrils [169]. Similarly, docosahexaenoic acid was shown to stabilize soluble Aβ42 PF and hinder their conversion to insoluble fibrils, thus leading to a sustained Aβ-induced neurotoxicity measured using cultured PC12 cells [171]. These observations suggest that toxic effects of PF could be promoted by molecular interactions that prevented downstream fibril formation.

## Aβ*56

Lesné and colleagues have identified an oligomeric Aβ species, termed Aβ*56, that caused cognitive deficits in middle-aged transgenic *Tg(APPSWE)2576Kahs (Tg2576)* mice [32]. The *Tg2576* mice express high levels of a human APP variant, which carries a familial AD-linked double mutation originating in a Swedish lineage (K670N, M671L) [172,173]. Aβ concentrations in these mice increase rapidly at 6 months of age, and abundant amyloid plaques are apparent between 9–12 months [172]. *Tg2576* mice recapitulate many other neurological features of AD, including neuroinflammation and oxidative stresses, dystrophic neurites, and significant cognitive deficits (reviewed in [172]). However, other important features, such as neurofibrillary tangles and significant neuronal loss are not found in this model [174].

Because in these and other APP transgenic mice administration of Aβ-specific antibodies has been shown to rapidly ameliorate memory decline, and because memory deficits were thought to precede plaques, Lesné *et al.* reasoned that a particular Aβ species could promote cognitive decline preceding plaque maturation [32]. They found that insoluble Aβ accumulated over 7 months without noticeable spatial memory decline. In contrast, certain soluble and extracellular Aβ species, which migrated as LMW bands in SDS-PAGE/Western blots, correlated strongly with memory deficits at 6 months of age, suggesting that these oligomers, particularly those with apparent gel mobility of Aβ dodecamers, were important neurotoxins in this model. Based on these data, it was proposed that similar species could be causing AD [32]. In support of this hypothesis, when pathophysiologically relevant concentrations of Aβ*56 (8.5 pmol) were administered into the lateral cerebral ventricles of healthy young rats pre-trained in the Morris water maze, the rats developed defective long-term spatial memory. These are important findings supporting the central role of oligomeric Aβ in the disease mechanism of AD.

The structural characterization of Aβ*56 as a putative dodecamer should be interpreted cautiously because the apparent electrophoretic migration of this oligomer, corresponding to 56 kDa, may not represent accurately its *in vivo* mass given the artifactual effects of SDS [57], which was included in the initial steps of the extraction protocol. Of note, Jacobsen *et al.* have found that cognitive deficits in *Tg2756* mice occurred before the reported time of appearance of Aβ*56 [175].

Recently, longitudinal water-maze spatial training was reported to reduce Aβ and τ neuropathology transiently but significantly, and improve later learning performance in a triple transgenic (3×Tg) murine model of AD [176]. The 3×Tg-AD mice harbor human APP containing the Swedish mutations (KM670/671NL), human τ containing the AD-associated mutation, P301L, and a human presenilin 1 gene (*PSEN1*) containing the AD-linked mutation, PS1M146V [176]. The improvement in performance in 3×Tg-AD mice occurred at 6–12 months and depended strongly on spatial training [176]. To achieve this effect, pre-training was required before development of overt neuropathology, presumably because it delayed Aβ redistribution to extracellular plaques and reduced the concentration of Aβ oligomers, including one with an apparent SDS-PAGE mobility similar to Aβ*56 [176]. These findings

suggest that Aβ*56 is a neurotoxic form of Aβ that may be important in the etiology of AD. Currently, the structural relationships of this species to PF and ADDLs are unknown, although under certain conditions, ADDLs also display electrophoretic migration corresponding to a dodecamer [27].

## Aβ PORES

Aβ pores are channel-like structures believed to disrupt cell membranes and cellular ionic homeostasis [177]. In lipid bilayers *in vitro*, Aβ was shown to form uniform pore-like structures with 8–12 nm outer and 2 nm inner diameters [177,178]. These are thought to serve as $Ca^{2+}$ channels and thus have been hypothesized to cause excitotoxicity and mediate Aβ-induced neurotoxicity in AD [179,180]. Reports of various models including artificial phospholipid membrane bilayers, excised neuronal membrane patches, whole-cell patch-clamp experiments, and phospholipid vesicles support a channel-forming property of Aβ [177,180–192]. In these studies, imaging techniques [177,184,186,187], electro-physiological experiments [180,181, 183–185,187,188,191,192] or cation-sensitive dyes [187] were used to assess channel-like properties of Aβ. However, other studies have reported general disruption of the plasma membrane homeostasis without channel formation [193–195]. In a study by Kayed *et al.* the effect of spherical Aβ42 oligomers on membrane conductivity was assessed using planar lipid bilayers [195]. It was found that these Aβ42 oligomers specifically increased the conductance of the bilayer in a concentration-dependent manner whereas no increase in conductance was observed for LMW Aβ species (monomer or dimer) or for Aβ fibrils [195]. The increase in membrane conductance in response to spherical oligomers occurred in the absence of evidence for discrete ion-channel or pore formation [195]. It was postulated that soluble oligomers enhanced movement of ions through the lipid bilayer by a channel-independent mechanism [195]. High sensitivity recording has indicated that there was little change in the noise level of the current trace as the current increased from 0 to ~100 pA after oligomer addition.

Aβ42 was reported to be more prone to forming channels than Aβ40 [18]. High-resolution examination of individual Aβ42 channel-like structures revealed two subunit arrangements: rectangular and hexagonal structures, putatively comprising tetramers and hexamers, respectively [177]. The disease-associated mutant E22G form of Aβ40 was shown to form pore-like structures akin to those formed by Aβ42 [37,196]. Treatment of the hypothalamic neuronal cells GT1-7 with Aβ40 has led to simultaneous formation of $Ca^{2+}$ channels and increased intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) as determined by fluorometric measurements, suggesting that Aβ40 also could disrupt biological and artificial membranes, possibly *via* formation of pores [182,197].

## PARANUCLEI

Using PICUP followed by SDS-PAGE analysis, Aβ40 and Aβ42 were shown to form distinct oligomer size distributions, suggesting that the two Aβ alloforms oligomerized through distinct pathways [33]. In those experiments, Aβ42 preferentially formed pentamer/hexamer units termed paranuclei, which self-associated into larger assemblies, including dodecamers and octadecamers [33]. In contrast, Aβ40 formed a roughly equimolar, quasi-equilibrium mixture of monomers, dimers, trimers, and tetramers [74].

A systematic study using PICUP assessed oligomerization of 34 Aβ alloforms [35], including those containing familial AD-linked amino acid substitutions, N-terminal truncations found in AD plaques, and modifications that altered the charge, hydrophobicity, or conformation of Aβ [35]. C-terminal length was found to be the most important structural determinant in early oligomerization, and the side-chain of $Ile^{41}$ in Aβ42 was found to be important both for effective formation of paranuclei and for their self-association [35]. Thus, Aβ41 and longer alloforms formed abundant paranuclei whereas Aβ40 and shorter alloforms did not [33]. The

side-chain of $Ala^{42}$, and the C-terminal carboxyl group, affected paranucleus self-association [35]. In a related study, oxidation of $Met^{35}$ in Aβ42 was found to preclude paranucleus formation and led to generation of oligomers indistinguishable from those produced by Aβ40 [34]. These data demonstrated that modification of even a single atom could induce dramatic effects on Aβ paranucleus formation and downstream assembly, providing important insights into mechanisms of Aβ assembly into neurotoxic oligomers potentially relevant to AD pathogenesis. As discussed above, the difference in toxicity between Aβ40 and Aβ42 [198] correlates with observations that certain oligomeric Aβ forms, such as paranuclei and ADDLs, are produced by Aβ42 only, emphasizing strong correlation of the latter to the pathogenic process of AD.

## βAMY BALLS

βamy balls are Aβ40-derived structures that form spontaneously when high concentrations of Aβ40 (60–600 μM) are incubated in phosphate-buffered saline at 30°C for 8–13 days [39]. βamy balls, have diameters of 20–200 μm and were shown to be composed of birefringent 6–10-nm diameter Aβ fibrils with random orientation [39]. Although such high Aβ concentrations used to generate βamy balls are unlikely to occur *in vivo* [22,199], it was argued that these concentrations could possibly occur locally at microfoci circumscribing the amyloid plaques in AD brain [39]. Interestingly, *in vivo* extracellular retinal deposits called drusen have an apparent similarity to βamy balls. Drusen are Aβ-containing macromolecular assemblies and are a pathologic sign in age-related macular degeneration [200]. However, these structures have larger diameters and unlike βamy balls, which are produced from synthetic Aβ40 *in vitro* in the absence of other proteins, the retinal deposits contain other Aβ-binding proteins [200].

## AMYLOSPHEROIDS

Amylospheroids, were described as Aβ40- and Aβ42-derived assemblies with 10–15 nm diameters [36]. Aβ40 amylospheroids formed by incubating 350 μM of the peptide in phosphate-buffered saline under slow rotation for 5–7 days at 37°C [36]. Aβ42 amylospheroids were produced by incubating 0.01–1 μM peptide in the same buffer for 8–10 h at 4°C [36]. Indeed, it was observed that Aβ42 amylospheroids, which formed faster and at substantially lower concentration than those of Aβ40, were also more toxic than Aβ40 amylospheroids [36], correlating with the higher toxicity and pathogenicity of Aβ42 in AD compared to Aβ40. Although these Aβ42 assemblies are spherical [36], they are morphologically distinct from ADDLs in their diameter (2.5 nm by AFM [70] *vs.* 10 nm by TEM, respectively).

## GLOBULOMERS

Globulomers are Aβ42 oligomers produced by incubating 400 μM Aβ42 in phosphate-buffered saline in the presence of 0.2% SDS at 37°C for 6 h [201]. These species also have been produced by incubation of Aβ42 with lauric, oleic, or arachidonic acids [201], suggesting that they are generated by interaction of Aβ42 with micelles of SDS or fatty acids. Aβ42 globulomers were neurotoxic to rat brain slices and antibodies generated against globulomers detected immunoreactive epitopes in tissue sections [201]. Similar globular structures of Aβ40 were reported to form after 18 h incubation in 25 mM 2-morpholinoethanesulfonic acid buffer (pH 4.5) in a "hanging-drop" environment [202]. Hanging-drop environment is used extensively for protein crystallization and provides a static, low-convection environment with a markedly increased hydrophobic air-buffer interfacial area compared to that of the microfuge-tube environment [202].

## OLIGOMERS OF DISEASE-RELATED AMYLOIDOGENIC PROTEINS OTHER THAN Aβ

Over twenty human amyloidoses are caused by aberrant protein folding and aggregation [203–205]. As Aβ often is considered an archetypal protein in studies of these diseases, the discoveries of toxic pre-fibrillar Aβ assemblies and their centrality in AD have led to a search for similar assemblies of other amyloidosis-related proteins. To date, at least 24 different proteins have been identified as causative agents of amyloidoses [206], In essentially all cases, such assemblies have been found and had adverse biological effects similar to those of Aβ oligomers [5,7,11,207]. Because this review focuses on pre-fibrillar assemblies of Aβ, we do not intend to cover assemblies of other amyloidogenic proteins in detail but rather to highlight a few examples and discuss current features that are common to all or most of these assemblies.

The structures reported for amyloidogenic protein oligomers, in general have been similar to those described for Aβ, namely PF, annular (pore-like) PF, and spherical oligomers. In several cases, annular PF have been the predominant structures found. However, as discussed elsewhere [57], it is important to note that in many cases the term PF has been used even though the morphologies of the assemblies under study were distinct from those originally defined as PF.

One of the most studied amyloidogenic proteins is α-synuclein, the function of which is not clear although it is believed to be part of the ubiquitin system that marks proteins for proteasomal degradation [208,209], α-synuclein is the predominant component in Lewy bodies, the pathological hallmarks in Parkinson's disease brain, and has been implicated in other degenerative disorders (synucleinopathies), including dementia with Lewy bodies and multiple system atrophy [210]. Similar to Aβ, α-synuclein belongs to a growing family of "intrinsically unstructured" proteins [211,212], a characteristic that perhaps renders these proteins more prone to undergoing amyloidogenic assembly. Mutant α-synuclein alloforms linked to familial Parkinson's disease were found to oligomerize faster than the wild-type protein, whereas the rate of fibril formation did not correlate with the presence of disease-causing mutations [213]. Pre-fibrillar assemblies of both wild-type and mutant α-synuclein included spherical oligomers, protofibrillar structures, and most abundantly, annular PF [196,214]. The latter morphology suggested that the mechanism by which α-synuclein induces toxicity is pore formation in cell membranes. In agreement with this idea, protofibrillar α-synuclein was found to permeabilize synthetic vesicles [215]. Interestingly, this effect was increased by the familial PD-linked mutants A30P and A53T [216] but not by the mutant E46K [217]. Thus, although pore formation may be involved in α-synuclein-induced toxicity, other mechanisms also have been implicated, but these are not well understood [218].

Two amyloidogenic proteins involved in sugar metabolism are insulin and islet amyloid polypeptide (IAPP, also called amylin). Insulin aggregation is not associated with disease but has been studied by multiple groups as a convenient *in vitro* model [219–222]. Biophysical investigation of insulin fibrillogenesis has identified oligomeric populations with conformations distinct from those of natively folded insulin dimer and hexamer [223]. Taking advantage of the relative stability of insulin oligomers and using special instrumentation, Robinson and co-workers have provided one of the first examples of mass spectrometric investigation of amyloidogenic protein oligomers, and demonstrated the power of this experimental approach for studying the effects of pH and metal ion binding on oligomerization [224]. In a recent study combining structural characterization and cytotoxicity experiments, Grudzielanek *et al.* found no toxicity for low-order insulin oligomers whereas substantial toxicity was measured for high-order, β-sheet-rich aggregates that displayed either fibrillar or amorphous morphology [225].

In contrast to insulin, IAPP aggregation is believed to be causative in T2D. IAPP is a 37-residue peptide hormone produced in pancreatic β-cells and co-secreted with insulin. Early stages of T2D are characterized by insulin resistance followed by increased insulin and IAPP secretion. Elevated IAPP concentrations lead to its assembly into toxic oligomers and insoluble aggregates [14]. Oligomeric and protofibrillar IAPP were shown to interact with synthetic membranes [226], a characteristic that decreases with further aggregation, providing a clue for the mechanism of IAPP toxicity [227]. The interaction with biological membranes may induce a transient α-helical conformation in IAPP, presumably facilitating penetration of the oligomers into the membrane resulting in solute leakage across the membrane [228,229]. Strong evidence for the neurotoxic role of IAPP oligomers in T2D was given in a study in which rifampicin, an inhibitor of IAPP fibril, but not oligomer formation, did not protect pancreatic β-cells against apoptosis induced by either endogenously expressed or externally applied IAPP [230]. More recent data have suggested that *in vivo*, toxic IAPP oligomers are formed intracellularly and therefore, oligomer-specific antibodies do not prevent cell death *in vitro* and *in vivo* [231].

Numerous other examples have demonstrated the important role of oligomers of amyloidogenic proteins as disease-causing agents. Before the focus in the amyloid field shifted from fibrils to oligomers, it had been known that although no sequence similarity was found among amyloidogenic proteins, the fibril structures of all were highly similar, characterized by fibrillar morphology with periodic helical twist and cross-β structures [232,233]. The realization that the precursor oligomers may be the proximate disease-causing agents in the amyloidoses related to these proteins raised the question whether oligomer structures also were similar. High-resolution microscopic studies of oligomeric structures, mostly by TEM and AFM, have demonstrated that in most cases the morphologies observed were spherical, annular, or protofibrillar (worm-like). Conformational studies of oligomers have been difficult because the oligomers typically are metastable and exist in mixtures. Structural insight has been offered by Glabe and co-workers who developed antibodies that showed specificity for oligomers of proteins with unrelated sequences but did not bind the monomeric or fibrillar forms of these proteins [234]. The first polyclonal antibody, A11, and similar antibodies developed in follow-up studies showed remarkable ability to bind to oligomers formed by proteins as diverse as Aβ, α-synulein, IAPP, lysozyme, insulin, poly Q, and prion fragments [234]. These observations strongly suggested that a predominant mechanism by which these oligomers injure cells is through permeabilization of the plasma membrane because toxic oligomers sharing a common structure formed by both intracellular and extracellular proteins [194,195]. As discussed above, oligomers may interact with membranes by several mechanisms, including pore-formation and shallow penetration under the surface resulting in thinning of the membrane and a net increase in its permeability. Further delineation of the specific mechanisms governing these interactions requires additional studies and will be highly important for designing reagents that block them.

## PRE-FIBRILLAR ASSEMBLIES OF DISEASE-UNRELATED PROTEINS

In his 1972 Nobel-Prize acceptance speech, Anfinsen stated that "the native conformation [of proteins] is determined by the totality of inter-atomic interactions and hence by the amino acid sequence, in a given environment" which does not always favor the normally functional and folded state of proteins. Consistent with Anfinsen's theory, the conformational-change hypothesis postulates that one of 17 normally soluble and functional human proteins could undergo structural alterations under partially denaturing conditions leading to self-assembly and amyloid fibril formation [235]. Besides disease-associated amyloid-forming proteins, and proteins that naturally form non-pathological, functional amyloid-like fibrils (reviewed in [236]), disease-unrelated proteins [237] and artificially designed peptides [238–240] have been found to form amyloid under particular non-native conditions. To the best of our knowledge, the ability of disease-unrelated peptides and proteins to form amyloid fibrils was first reported

by Guijarro *et al.* [241] and Litvinovich *et al.* [242]. The src-homology 3 (SH3) domain of bovine phosphatidyl inositol 3-kinase (PI3K), an 85-residue, β-structured protein, was shown to aggregate slowly and form amyloid fibrils under acidic pH [241]. Thenceforth, the disease-unrelated SH3 domain has served as an excellent model for systematic studies examining structural properties of amyloid fibrils and molecular mechanisms of amyloid formation [243–246]. The PI3K-SH3 was shown to adopt a compact denatured state under acidic conditions before formation of amyloid fibrils [247]. Limited proteolysis studies showed that PI3K-SH3 at low pH had a partially folded conformation [247] and progressively displayed enhanced susceptibility to proteolysis, suggesting that the protein became more unfolded in the early stages of aggregation [247]. In contrast, the amyloid fibrils that formed over longer periods of time were resistant to proteolysis [247]. It was suggested that the protein aggregates formed initially were relatively dynamic species and this flexibility allowed dynamic species and this flexibility allowed for the particular interactions leading to formation of the highly ordered fibrils [247].

After Litvinovich *et al.* demonstrated formation of amyloid-like fibrils by self-association of murine fibronectin type III module [242], others reported that similar conversions in a number of disease-unrelated proteins could be induced *in vitro* by a deliberate, rational choice of excipient conditions [248,249], Examples (reviewed in [237]) include human apolipoprotein CII, ADA2H, amphoterin, stefin B and endostatin, murine VI domain, equine acylphosphatase (AcP) and apomyoglobin, monellin (*Dioscoreophyllum camminsii*), and yeast phosphoglycerate kinase. Fezoui *et al.* reported *de novo* design of a monomeric α-helix-turn-α-helix peptide (αtα) which converted to β-sheet-rich amyloid-type, protease-resistant, 6–10-nm fibrils at 37°C in a neutral aqueous buffer [238]. Formation of fibrils from full-length proteins requires solution conditions that partially or completely disrupt the native structure of the protein but not completely disturb hydrogen bonds [248]. It was observed that proteins with as few as four residues, and amino-acid homopolymers that are unable to fold into stable globular structures, form fibrils readily [237,250,251]. Therefore, it has been suggested that the ability to form amyloid fibrils could be a generic property of polypeptide chains [237].

In one study of pre-fibrillar assemblies of disease-unrelated proteins, tapping-mode AFM was used to follow the process of HypF-N aggregation which was induced by incubating the protein in the presence of trifluoroethanol [252], HypF-N was shown to aggregate hierarchically through a number of distinct steps with morphologically different intermediates [252]. Initially, globular assemblies appeared, which subsequently self-assembled into beaded chains, similar to those found for amyloidogenic proteins [253–255]. Subsequently, these organized into crescents, large annular and ribbon-like structures (Fig. (3)), and eventually assembled into mature fibrils of different sizes [252]. The globule height was measured to be 2.8–3.0 nm [252]. Although HypF-N and AcP are similarly prone to conversion from a predominantly α-helical conformation to one rich in β-sheet, HypF-N aggregation rate was found to be dramatically higher (~1,000-fold) than AcP, possibly due to the higher hydrophobicity and lower net charge of HypF-N compared to AcP [256].

In contrast to fibrils of disease-causing amyloidogenic proteins, those formed by disease-unrelated proteins did not cause cytotoxicity in cell-culture experiments. For example, fibrils formed by the aforementioned αtα peptide displayed no neurotoxicity, even though they were morphologically indistinguishable from Aβ and IAPP fibrils, which were toxic [238]. It was therefore unexpected that the pre-fibrillar assemblies of PI3K-SH3 and HypF-N were shown to be highly toxic to PC12 cells and murine fibroblasts *in vitro* [257]. The extent of cellular injury caused by the cytotoxic oligomers was comparable to that of Aβ42 oligomers, whereas the corresponding fibrils of both PI3K-SH3 and HypF-N were benign.

Early pre-fibrillar HypF-N assemblies were shown to permeabilize artificial phospholipid membranes more efficiently than mature fibrils, indicating that this disease-unrelated protein displayed the same toxic properties as pre-fibrillar assemblies of pathological peptides and proteins [252]. Further investigation of the cellular effects of HypF-N oligomers revealed that they entered the cytoplasm and caused an acute rise in ROS levels and $[Ca^{2+}]_i$, leading to cell death [258]. In a study where murine fibroblasts and endothelial cells were treated with pre-fibrillar HypF-N assemblies, the two cell types underwent two different death mechanisms—fibroblasts exposed for 24 h to 10 μM HypF-N oligomers underwent necrosis, whereas endothelial cells treated similarly sustained apoptosis [259]. A similar study comparing cytotoxic effects of pre-fibrillar and fibrillar HypF-N assemblies using a panel of normal and pathological cell-lines showed that cells were variably affected by the same amount of pre-fibrillar aggregates, whereas mature fibrils showed little or no toxicity [260]. This difference in the extent of compromise of cell viability was significantly related to the cell-membrane cholesterol content and to different cellular $Ca^{2+}$-buffering and antioxidant capacities of the various cell types [260]. Recently, it has been shown that microinjection into rat brain nucleus basalis magnocellularis of PI3K-SH3 or HypF-N assemblies, but not the corresponding mature fibrils, compromised neuronal viability dose-dependently [8]. Taken together, these data clearly demonstrate that the pre-fibrillar assemblies of disease-unrelated proteins are highly toxic whereas the corresponding mature fibrils are not [8]. The toxic effect of the oligomers may arise when these assemblies assume a "misfolded" conformation which may expose hydrophobic residues that are natively entombed within the core structure. Such aggregation-prone regions may interact with membranes and other cellular components modifying their structural/functional homeostasis.

Dobson and co-workers have proposed that evolutionary mechanisms may have been in force to ensure propagation of proteins that resist aggregation for efficient function [261]. However, genetic, environmental and metabolic factors that decrease the solubility or increase the concentration of susceptible proteins *in vivo* may act against those forces and induce protein misfolding disorders including neurodegenerative diseases [261].

The fact that aggregates of some disease-unrelated proteins could function similarly to those formed by amyloidogenic, disease-related peptides and proteins, has profound implications for understanding the mechanistic fundamentals of abnormal protein deposition in amyloidoses. These observations facilitate investigation and discovery of the general mechanistic features underlying protein misfolding and aggregation [237] and help defining likely targets for drug design.

## CONCLUSION

Since the amyloid-cascade hypothesis was formulated [1], intensive research has led to an exponential accumulation of data elucidating the pathogenic mechanisms of AD. In the end of August, 2007, PubMed database searches, using the queries "Alzheimer's disease" or "amyloid" returned 53,767 and 32,576 hits, respectively. Importantly, as our understanding of the devastating *morbus* Alzheimer, and other neurodegenerative and protein misfolding diseases has been growing, an alternative, encompassing paradigm has emerged. This paradigm postulates that pre-fibrillar protein assemblies rather than mature amyloidogenic fibrils likely are the key neurotoxins responsible for most of the pathogenic mechanisms in protein-misfolding and neurodegenerative diseases, including AD. Accordingly, oligomeric species with different degrees of structural order are thought to mediate various pathogenic mechanisms that may lead to cytotoxicity and cell loss eventuating in organic and systemic morbidity. With the progressive use of classical techniques, and the advent of novel, sophisticated methodologies, several different forms of pre-fibrillar assemblies of Aβ and those of other amyloidogenic proteins have been described. This has led to considerable progress in

the elucidation of structural and functional features and fundamentals of the assembly of these proteins at the molecular level. Overall, it is postulated that the pre-fibrillar amyloidogenic proteins are on path to fibrillogenesis. The resulting protein fibrils are thought to be the end-stage sinks for the toxic pre-fibrillar species. Fibrillar assemblies accumulate progressively into intra- and/or extracellular proteinaceous amyloid aggregates generating the disease-specific lesions *in vivo*.

In AD research, various forms of Aβ pre-fibrillar assemblies, including activated monomeric conformers, ADDLs, PF, cell-derived dimers and trimers, and annular assemblies have been described (Table 1). In many cases, the structural and functional interrelationships amongst these assemblies are still elusive. Nevertheless, some have been shown to be pathogenic through one or several common pathways, suggesting that they may share structural features and possibly mechanisms of action. Understanding these intricate structure-function correlations will decipher a complex and interconnected array of pathogenic mechanisms.

Global research efforts have established a framework for understanding the fundamentals of Aβ assembly [7,10,11]. A remaining challenge is to assess how these fundamental structural principles are linked to cellular and tissular microenvironments during progression of AD. Many experimental conditions have been used to study the structure and function of pre-fibrillar assemblies, but it is difficult to regenerate and scrutinize the actual *in vivo* milieus and conditions in which protein assembly, oligomerization, fibrillization, and deposition occur. Similarly, it is extremely difficult to assess all the possible interactions these assemblies may have with various cellular components and organelles. A multitude of detrimental mechanisms, including disruption of cellular metabolism, synapse structure and function deregulation, membrane damage, ionic imbalance, oxidative/inflammatory stress, apoptotic, and other cytotoxic effects, have been shown to be mediated by pre-fibrillar Aβ assemblies, emphasizing that a single therapeutic approach likely will be insufficient to prevent or treat the progression of AD. By inference, this likely is true also for other amyloidoses. The intricacy of the pathogenic mechanisms in these diseases calls for rational diagnostic and therapeutic approaches that would target not only a single assembly or a single mechanism but a multitude of assemblies and mechanisms. It is important to design and discover therapeutic agents that could target various assemblies that potentially become active early or are continuously active throughout the course of disease. Successful targeting of pre-fibrillar assemblies *in vitro* and in animal models could have crucial diagnostic and prognostic implications for amyloid-related diseases.

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## ABBREVIATIONS

| | |
|---|---|
| αtα | α-helix-turn-α-helix |
| AcP | Acylphosphatase |
| AD | Alzheimer’s disease |
| ADDLs | Aβ-derived diffusible ligands |
| AFM | Atomic-force microscopy |
| apoJ | Apolipoprotein J |
| APP | Amyloid β-protein precursor |

| | |
|---|---|
| Arc | Activity-regulated cytoskeleton-associated protein |
| AU | Analytical ultracentrifugation |
| Aβ | Amyloid β-protein |
| $[Ca^{2+}]_i$ | Intracellular $Ca^{2+}$ concentration |
| CD | Circular dichroism |
| CSF | Cerebrospinal fluid |
| DLS | Dynamic light scattering |
| EPSC | Excitatory post-synaptic current |
| FTIR | Fourier-transform infrared spectroscopy |
| HFIP | 1,1,1,3,3,3-hexafluoro-2-propanol |
| HNE | 4-hydroxy-2-nonenal |
| HPLC | High-performance liquid chromatography |
| HypF-N | Hydrogenase maturation-factor |
| IMS-MS | Ion-mobility spectrometry-mass spectrometry |
| LMW | Low-molecular weight |
| LTD | Long-term depression |
| LTP | Long-term potentiation |
| MALDI | Matrix-assisted laser-desorption ionization |
| MALLS | Multi-angle laser light scattering |
| MPL | Mass-per-length |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| nAchR | Nicotinic acetylcholine receptor |
| NMDA | *N*-methyl-D-aspartate |
| NMDAR(s) | NMDA receptor(s) |
| PF | Protofibrils |
| PI3K | Phosphatidyl inositol 3-kinase |
| PICUP | Photo-induced cross-linking of unmodified proteins |
| ROS | Reactive oxygen species |
| SDS | Sodium dodecyl sulfate |
| SEC | Size-exclusion chromatography |
| SH3 | Src-homology 3 |
| SPPS | Solid-phase peptide synthesis |
| STEM | Scanning transmission electron microscopy |
| STM | Scanning tunneling microscopy |
| T2D | Type-2 diabetes mellitus |
| TEM | Transmission electron microscopy |

TFE Trifluoroethanol

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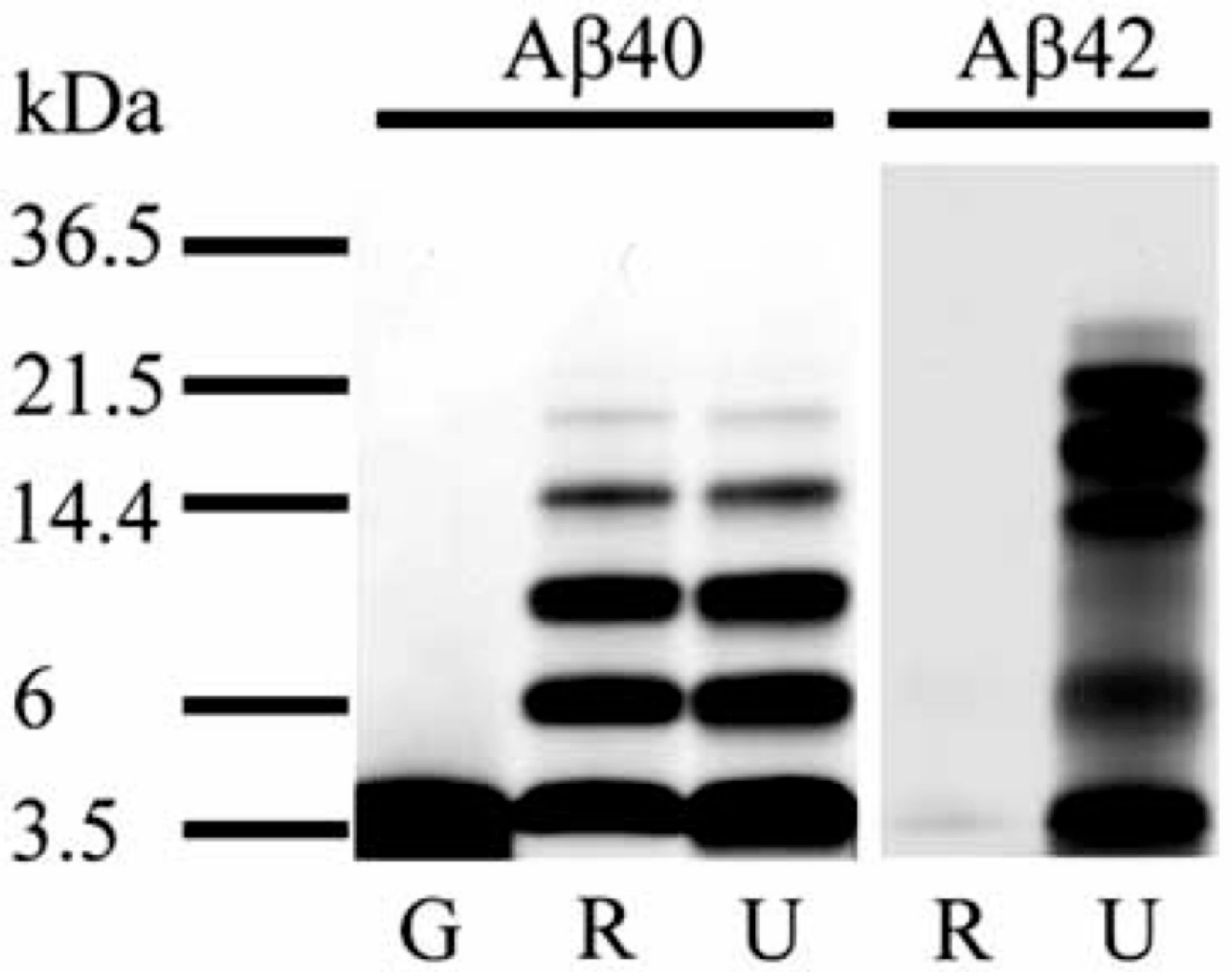


**Fig. 1. Comparison of photo-cross-linking using Aβ peptides from different sources**

Synthetic Aβ from Global Peptide (G) and the UCLA Biopolymers Laboratory (U), and recombinant Aβ from rPeptide (R) were prepared in 10 mM sodium phosphate, pH 7.4 at 2 mg/ml nominal concentration and filtered through a 10-kDa molecular-weight cut-off filter [77]. Each filtered peptide was cross-linked using PICUP [73]. The resulting cross-linked oligomers were subjected to SDS-PAGE and silver-staining. The data suggest that Aβ40 from Global peptide contained contaminants that prevented cross-linking and that Aβ42 from rPeptide aggregated during the filtration step and was hardly detectable.

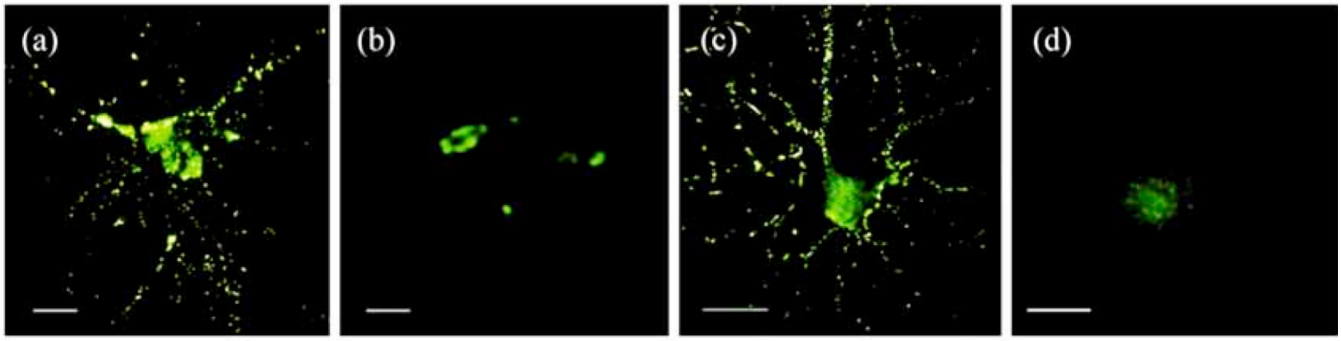


**Fig. 2. Punctate ADDL-binding to neurons**

ADDLs isolated from AD brain or prepared *in vitro* show identical punctate binding to neuronal cell-surface proteins. Cultured hippocampal neurons were incubated with soluble extracts of human brain or synthetic ADDLs. Immunoreactivity against ADDLs was visualized by microscopy using M93 antibody. Soluble AD-brain proteins (a), soluble control-brain proteins (b), synthetic ADDLs (c), and synthetic ADDLs pre-treated (1 h) with oligomer-specific antibody M71 (d) are shown. Small puncta distributed along neurites, are evident for AD extracts and synthetic ADDLs, but not for control extracts or antibody-preadsorbed ADDLs (Scale bar = 10 μm). Adopted with permission from [27].

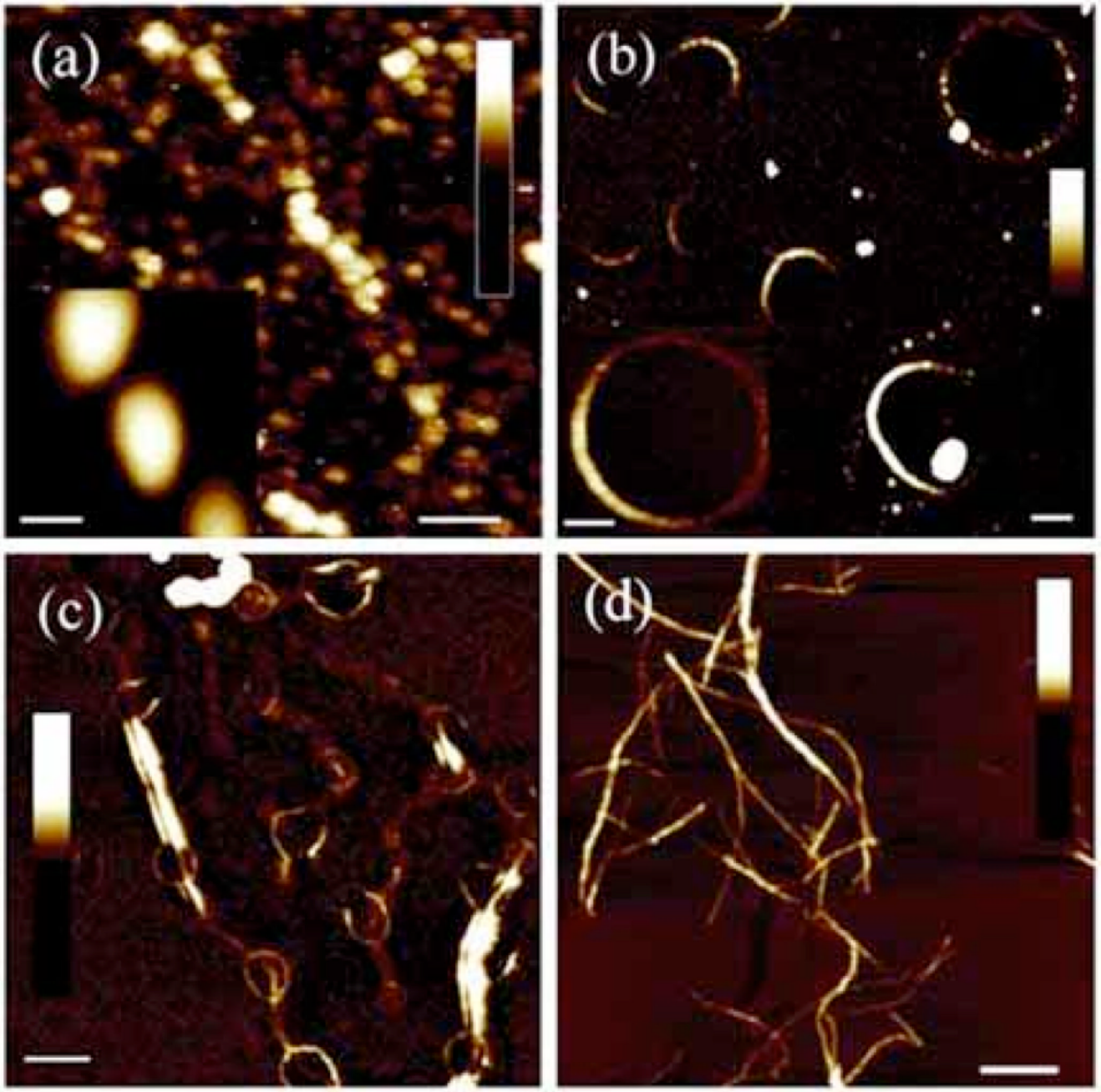


**Fig. 3. Hierarchical aggregation process of HypF-N**

(a) Tapping AFM images taken under liquid (height data, scan size 670 nm, Z range 20 nm) of HypF-N globular aggregates observed a few hours after the onset of the aggregation process in the presence of 30% trifluoroethanol (TFE). Scale bar = 100 nm. Inset, STM image (height data, scan size 42 nm, Z range 15 nm) of globular aggregates obtained under the same conditions, but diluted 1:50 prior to deposition onto the substrate; the globules are apparently asymmetric and tend to form a defined mutual orientation. Scale bar = 10 nm. (b) HypF-N crescents and a ring observed after 3 days of incubation in 30% TFE (scan size 5.3 μm, Z range 45 nm). Inset, high resolution image of a ring (scan sizel.9 μm, Z range 120 nm), revealing its globular components, taken after 5 days of incubation in 30% TFE. Scale bars = 400 nm. (c)

Tapping-mode AFM image taken in air (height data) after three days of incubation in 30% TFE showing the co-existence of annular structures with thin and wide ribbon-like fibrils. Scan size 4.8 μm, Z range 40 nm. Scale = 500 nm. (d) Tapping-mode AFM images taken in air (height data) of mature fibrils obtained in 30% TFE showing supercoiled fibrils after eight days (scan size 3.6 μm, Z range 80 nm). Adopted with permission from [252].

## Table 1

Summary of Structural and Biological Characteristics of Pre-Fibrillar Aβ Assemblies

| Assembly | Structural characteristics/production | Biological activity |
|---|---|---|
| "Activated monomeric conformer" of Aβ | • Produced during aggregation of Aβ40 by constant rotary shaking<br>• Measured by turbidity assay and HPLC<br>• Postulated to be an oxidative or hydrolytic derivative of Aβ [19] [117]<br>• The monomeric nature of these conformers is not confirmed unambiguously. | • Forms 2–3-fold more complexes with apoE4 than apoE2 or apoE3 [117]<br>• Enhances toxicity and apoptotic activity in neurons [19]<br>• Inhibits action potential by blocking fast, inward tetrodotoxin-sensitive $Na^+$ channels [122] |
| Cell-derived Aβ oligomers | • Predominantly dimers, trimers, and tetramers, produced by CHO cells transfected with mutated or wild-type APP [23]<br>• Resistant to SDS denaturation and to cleavage by insulin degrading enzyme [25] | • Inhibit LTP *in vivo* [25]<br>• Impair short-term memory in rats [133]<br>• Cause dendritic spine loss [138]<br>• Affect synaptic structure and function |
| Aβ-derived diffusible ligands (ADDLs) | • Synthetic species formed in the presence of apoJ [144] in F-12 media [142] or in PBS [145]<br>• Small globules 3–8 nm in diameter measured by AFM [121]<br>• Polydisperse mixtures of 150–1,000 kDa determined by MALLS [70] | • Highly neurotoxic [26, 162]<br>• Inhibit LTP [26]<br>• Bind to neuronal surfaces [53] co-localizing with neuronal proteins at postsynaptic punctate sites [136,150] and NR1 and NR2B subunits of NMDAR [136,150]<br>• Promote oxidative stress and increased $[Ca^{2+}]_i$ [160]<br>• Induce τ phosphorylation [145]<br>• Induce IL-1β, iNOS, NO and TNF-α in astrocytes [262] |
| Protofibrils (PF) | • Curvilinear fibril-like structures 6–8 nm in diameter and ≤200 nm long by TEM [30]; a periodicity of 20 nm and diameter of 4.3 nm were determined by AFM [112]<br>• Rich in β-sheet structure<br>• Bind Congo red and Thioflavin T[10] | • Increase EPSCs [29], and cause cell death [29,31]<br>• May activate NMDAR in contrast to fibrils which activate non-NMDA glutamate receptors [263].<br>• Patch-clamping using Aβ42 PF induces reversible, $Ca^{2+}$-dependent increase in spontaneous action potentials and membrane depolarizations. |

| Assembly | Structural characteristics/production | Biological activity |
|---|---|---|
| | | • Can inhibit the A- and D-type $K^+$ currents, but not other outward/inward rectifying $K^+$ channels.<br>• PF-induced membrane activity increases $[Ca^{2+}]_i$ spikes [264].<br>• Aβ40 containing the E22G substitution forms PF faster and in larger quantities than wild-type Aβ *in vitro*, suggesting that PF may be the main disease-causing agents in carriers of the Arctic mutation [167]. |
| Aβ*56 | • Extracted from brains of *Tg2576* mice and isolated by immunoaffinity and SEC [32] | • Concentration correlates with degree of cognitive deficits<br>• Cause defects in long-term spatial memory in rats [32] |
| Amyloid pores | • Channel-like structures of synthetic Aβ, having outer diameter of 8–12 nm and inner diameter of 2–2.5 nm, associated with artificial membrane bilayers [18,37] | • Pore- and channel-forming capacity that may lead to membrane leakage and increased $[Ca^{2+}]_i$ [18,37] |