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Aβ peptide oligomers — potential neurotoxic agents in Alzheimer's disease

Summary — Amyloid beta peptide (Aβ) is recognized as the main constituent of the extracellular amyloid plaques, the major neuropathological hallmark of Alzheimer's disease (AD). Aβ is a small peptide present in normal cells, not toxic in the monomeric form but aggregated Aβ is assumed to be the main if not the only factor causing Alzheimer's disease. Interestingly, the new reports suggest neurotoxicity of soluble Aβ oligomers rather then amyloid fibrils. Due to the fact that fibrils were thought to be the main toxic species in AD, early structural studies focused on fibrils themselves and Aβ monomers, as their building blocks, while there is practically no data on oligomer structure and mechanism of neurotoxicity. Thus a new area of research opened, focusing on Aβ soluble oligomers and the results of the studies will be reviewed here.
Key words: Alzheimer's disease; amyloid beta peptide, A-beta oligomers, neurotoxicity.

Alzheimer's disease (AD) [1] is the most common form of dementia characterized by memory loss and confusion. The major neuropathological hallmarks of this disease are accumulation of intracellular neurofibrillary tangles and extracellular amyloid plaques [2, 3]. The main constituent of these plaques is aggregated peptide, named Aβ peptide [4, 5] derived by proteolytic cleavage of the amyloid precursor protein (APP) [6] performed by β- [7, 8] and γ-secretases [9] (see Fig. 1). Aβ peptide is not a homogeneous species, its length varies, it may consist of 39—43 residues, however 42—43 residue species are less abundant. The longer peptides are more hydrophobic and aggregate most readily. Aβ peptide is constitutively expressed in normal cells, it can be detected in cultured cells [10] and biological fluids of healthy individuals [11]. It was demonstrated that in monomeric form Aβ peptide is not toxic [12—15]. By unknown mechanism this endogeneous material changes its monomeric character, becomes aggregated and neurotoxic.

In 1992 Hardy and Higgins [16] in their amyloid cascade hypothesis postulated that the presence of the aggregated Aβ is the main if not the only factor causing Alzheimer's disease. The hypothesis was confirmed by many years of biochemical, genetic and tissue culture studies and now is widely accepted [17, 18]. Obtained results implicate at least twenty distinct possible pathways of Aβ toxicity [19]. Despite years of studies the nature of the toxic agent itself and the toxicity mechanism is still under debate.

Interestingly, although it was shown beyond doubt that aggregated forms of Aβ are necessary for neurotoxicity, the correlation between aggregated amyloid load and neurological symptoms of AD was reported to be surprisingly weak [20, 21]. AD studies demonstrate patients with amyloid accumulation but presenting no synapse loss or symptoms of dementia. Additionally, for some of the patients it was demonstrated that plaques are localized at sites distant from sites of neuronal loss [22]. Another striking observation came from the studies of transgenic mice bearing overexpressed human APP gene. Mice revealed expected AD symptoms, like loss of synaptic terminals, but no accumulation of amyloid plaques [23]. It became obvious that there must exist a toxin responsible for neurodegeneration, which escaped detection by measurements of solid amyloid.

This seemingly paradoxical situation sparked interest in different structural forms of Aβ, species that are not monomeric but also non-fibrillar and shifted the focus of research groups to oligomeric species, their detection and potential neurotoxicity. This shift has an important practical aspect. Therapeutic strategies should aim at destabilizing neurotoxic forms and until recently fibrils were the primary target. If fibrils are benign their destabilization by therapeutic intervention may lead to increase in the level of toxic species and be therapeutically counterproductive.

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Change of focus lead fast to new results confirming a potential neurotoxicity of oligomeric forms of Aβ. Recently several classes of soluble oligomeric forms of Aβ have been discovered both in *ex vivo* material [24] and cell cultures [25], but also from *in vitro* experiments [24]. Discovered oligomers are divided into three classes: small oligomers, which include dimers to tetramers [26], Aβ-derived diffusible ligands (ADDLs), having molecular weights of 20—40 kD [27], and even larger forms, named protofibrils [28].

Despite debate on which form of aggregated Aβ is fatal there is a consensus that non-monomeric species of Aβ are responsible for Aβ toxicity. Suggested mechanisms of toxicity include: mediation by generation of Aβ-induced oxidative stress, inflammation, disregulation of ionic homeostasis and cerebrovascular degeneration. It has not been elucidated yet which of the many possible ways is primary. For instance Aβ peptide fragments were reported to produce reactive free radicals and reactive oxygen species (ROS) upon incubation in aqueous media [29, 30]. Increase of intracellular peroxide level was observed as a result of exposure of neuronal cell cultures to Aβ species correlating with their toxicity [31]. There is also evidence that Aβ is a potent lipoperoxidation initiator [32] what may lead to neuronal degradation. Some antioxidant agents such as vitamin E [33] as well as estrogens [34] may effectively protect cells against Aβ toxicity. Moreover, Aβ due to its amphiphilic properties may penetrate the membrane and affect the enzymatic pathway causing the leakage of ROS [35]. Also, the interaction of Aβ with membranes may affect ion homeostasis, induce ion pore formation and blockade of K^+ channel [36].

Other studies implicate receptors as mediators of toxicity. The studies of Aβ effect on activity of nuclear factor-κβ (NF-κβ), a redox sensitive transcription factor inducing cellular stress response were carried out, however the results are not consistent. Either the decrease [37] or increase [38] of NF-κβ levels was observed in rat primary neurons and activation in microglia [37]. Hence, the role of NF-κβ in AD pathology is not clear. It was shown that activation of NF-κβ in familial amyloidotic polyneuropathy may be mediated by the interaction of transthyretin fibrils with the receptor for advanced glycation end products (RAGE) [39]. The first evidences for mediating effects of the Aβ on neurons by RAGE were presented in 1996 by Yan *et al.* [40]. Further studies of RAGE function have shown that RAGE may be involved in a range of chronic disorders associated with the enhanced accumulation of its ligands [41]. Also Aβ activation of microglia can be mediated by RAGE and macrophage colony-stimulating factor [42].

In parallel with the studies on the toxicity mechanism, much work has been carried out on the structure of potential toxic form of Aβ. The dogma that amyloid itself is necessary for neuron death or malfunction observed in AD lead to focusing of structural AD studies on Aβ fibril structure and formation mechanism. Numerous reviews cover this area of research [43—53] and systematic description of work carried out on fibrillar structures of aggregated Aβ will not be attempted. Only recently, with growing recognition of the above mentioned paradox, a new area of research opened, focusing on the structure of Aβ soluble oligomers and their potential neurotoxicity, and the results of these studies will be reviewed here.

NEUROTOXICITY OF OLIGOMERIC FORMS OF Aβ

The first suggestion of amyloid intermediates being involved in neurotoxicity was formulated by Frackowiak *et al.* [54], where the accumulation of nonfibrillar, monomeric, and oligomeric Aβ forms was observed during formation of amyloid in AD vessel walls.

Kuo *et al.* [24] have demonstrated during the studies of AD and normal brains that AD brains contain 6-fold more water-soluble Aβ than control brains. In most AD cases the presence of Aβ1—42 was detected representing 12 times the amount found in control brains. Similarly, Lue *et al.* [55] showed that soluble Aβ level was a very strong predictor of synapse loss and suggested that soluble Aβ level correlated well with Aβ neurotoxicity. Western blot techniques confirmed that the mean level of soluble Aβ was increased threefold in Alzheimer's disease and correlated with markers of disease severity [56, 57]. Increased level of soluble Aβ increases the probability of oligomeric species formation.

Pathogenicity of the dimeric form of Aβ1—40 and 1—42 in cell culture systems was described by Roher *et al.* [26]. Authors reported neuronal killing elicited by the dimer in the presence of microglia in cultures of rat hippocampal neuron glia cells. The neurotoxicity of small oligomers in cultured primary cortical neurons over a period of days was confirmed by Hartley *et al.* [58], however the oligomers did not elicit the electrophysiological response. The studies on the localization of oligomers, mostly dimers, revealed that in neural cells the ratio of intracellular to extracellular oligomers was much higher in brain-derived than in other cells suggesting the possibility that the pathogenically critical process of Aβ oligomerization begins intraneuronally [59]. Recently, oligomers were shown to be formed shortly after peptide synthesis in the cell. Oligomers, mainly dimers and trimers, are assembled in vesicles and then secreted from the cell leading to inhibition of hippocampal long term potentiation (LTP) *in vivo* [60]. LTP is considered a classic model for synaptic plasticity as well as for memory and learning, faculties that are selectively lost in the early stage of AD.

The studies of neuronal dysfunction caused by ADDLs revealed that neurons in the cerebellum are spared while hippocampal CA1 region and entorhinal cortex neurons are selectively damaged by ADDLs [27, 61]. The electrophysiological data on ADDL neurotoxi-

city show that, prior to killing neurons, ADDLs inhibit LTP. This inhibition was observed despite continued capacity of hippocampal neurons for action potentials [27]. Recently these data were confirmed and ADDL-induced plasticity imbalance was shown [62]. It was also suggested that due to selective preservation of long-term depression (LTD) ADDLs might lead to accelerated and pathologic synapse elimination in granule cells. The studies of the kinetics and LTP inhibition specificity lead to conclusion that ADDLs may take aim at signal transduction pathways [61].

The protofibril structures show biological activity very similar to the toxicity caused by fibrils. The MTT assay [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5--diphenyltetrazolium bromide — MTT, a standard test for cell proliferation and viability] in cultures of rat cortical neurons revealed that protofibrils like fibrils perturbed neuronal metabolism leading to neuronal dysfunction and subsequent cell death [63]. Moreover, protofibrils can alter the electrical activity of neurons, cause neuronal loss and reproducibly induce toxicity in mixed brain cultures in a time- and concentration-dependent manner that cannot be explained by conversion to fibrils. Importantly, protofibrils, but not small Aβ oligomers, produced a rapid increase of excitatory postsynaptic potentials (EPSPs — postsynaptic membrane depolarization produced by opening of channels permeable to Na^+) [58] (Fig. 2). Increased level of Aβ leads to self-association and in consequence to oligomers formation. Oligomers are stable *in vivo* and *in vitro* even at small concentrations. With increase in Aβ concentration more oligomers are accumulated and more complicated forms are present. As a result of oligomer presence and accumulation the neurological disfunction is observed.

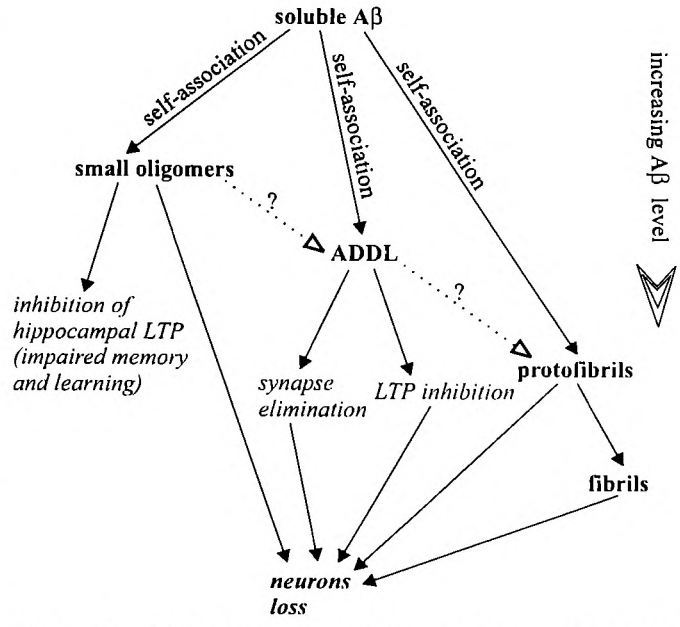

Fig. 2. Graphical presentation of the possible pathways of the Aβ oligomer neurotoxicity

Aβ STRUCTURAL STUDIES

Detailed structural knowledge of all the players in the complicated pathway leading from monomer to fiber is of utmost importance for understanding the pathology. Recent results, suggesting that amyloidogenic oligomers of any protein can be inherently toxic, irrelevant of their aa. sequence due to exposure of heterogeneous hydrophobic patches [64], underscore the need of sound structural characterization of these species. *In vitro* studies of oligomer structure and formation are a necessary step towards understanding pathology *in vivo*.

Due to the fact that fibrils were thought to be the main toxic species in AD, early structural studies focused on fibrils themselves, Aβ monomers, as their building blocks, and the transition between the two forms. In spite of the intense work (the list of published attempts of monomeric Aβ structure elucidation references 26 papers — see Table 1 in [43]), only the most general features both of fibril structure and conformational preferences of monomers have been established. Beyond doubt the aggregated state presents a high content of β-sheet but obtained data does not allow to de-

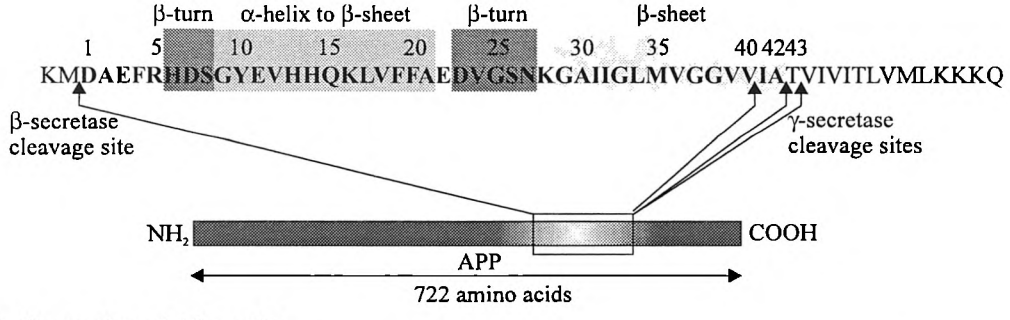

Fig. 1. Schematic illustration of Aβ peptide.
The amyloid β peptides result from the proteolytic cleavage of the amyloid precursor protein (APP). The cleavage sites for β and γ secretases are indicated by arrows. The sequence of Aβ is shown in bold letters. Aβ regions having different secondary structure propensities are indicated by various shadowed areas

cide conclusively if the alignment of strands is parallel or anti-parallel.

The sequence of Aβ (Fig. 2) can be divided into several regions: (*i*) hydrophobic *C*-terminal domain (from residue 28) of high β-sheet propensity, (*ii*) an *N*-terminal domain within region 9—21 of lower probability of forming β-sheet structure and more likely displaying α-helical structure and (*iii*) two segments of possible β-turns between residues 6—8 and 23—27 [65]. Charged residues are localized at the *N*-terminus with only three of them beyond position 16 and nine preceding Lys16.

The studies of the structure of Aβ peptides were complicated by their limited solubility in aqueous solutions and lack of crystal form, which rendered them intractable by high resolution techniques such as solution phase NMR and X-ray crystallography [5]. Fibrils were studied by fiber diffraction, small angle neutron scattering, electron and atomic force microscopy, solid state NMR, Fourier transform infrared spectroscopy (FT-IR), *etc.* Only recently technical improvements finally yielded NMR results for non-fibrillar forms in aqueous solution, previously organic solvents such as DMSO, and detergents such as SDS were used as solubilizing agents. Moreover, proper monomerization procedures proved necessary for obtaining reproducible results of Aβ structural studies [66].

The use of detergents, originally stemmed from of the difficulties in maintaining peptides in solution at NMR concentrations for appropriate time, was rationalized by assuming that the presence of detergent or organic solvent mimics membrane environment. The results of many experiments on various length fragments of Aβ indicate that in organic solvents Aβ generally shows α-helical preferences [67—78]. The NMR analysis of Aβ1—40 in aqueous sodium dodecyl sulfate (SDS) micelles, pH 5.1, revealed α-helical conformation between residues 15 and 36 [75] or 35 [77] with a kink or hinge at 25—27 [75] or loop at 24—30 [77], while the other study showed Aβ1—40 and Aβ1—42 containing helices at 10—24 and 28—42 [78]. The differences in the localization of α-helix and kink or loop can be attributed to the differences in pH and solvent composition.

In spite of early opinion, Aβ1—40 proved to be quite soluble and after proper monomerization procedure, which provides reproducible starting conditions, it can be maintained in solution even at moderately high concentrations. In aqueous buffers or in water circular dichroism (CD) and NMR studies suggest the mixture of random coil and short β-structure elements, strongly dependent on pH, concentration and incubation time [79—83]. NMR studies of Aβ1—28 have shown the conformational change from α-helix *via* random coil to β-sheet structure as a result of pH and temperature changes [68]. In contrast, the hydrophobic segment 29—42 in the *C*-terminal domain of Aβ always exists as a β-sheet, regardless of alteration in solvents, pH or temperature, suggesting that this segment directs the complete peptide folding [67, 84]. Benzinger *et al.* [85] studied Aβ10—35 fragment by NMR in aqueous solvent, pH 5.6, and found no traces of β-helix or β-sheet, but evidence of folded structure with conformational restrictions in the hydrophobic region Leu17-Phe20 and salt bridges involving Glu22 and His13 or 14. Also recent NMR studies of Aβ structure [86] or Aβ1—40 and 1—42 oxidized at methionine 35 [87] in water milieu indicate a stable structure of a collapsed coil without definite secondary structure. Structural differences between highly aggregation prone variant 1—42 and mildly aggregating 1—40 could not be detected in this study.

Spontaneous ordered association of monomeric Aβ peptides leads to the formation of amyloid fibrils, which are insoluble, not susceptible to proteolysis and display specific tinctorial properties [43]. Aβ fibrils obtained *ex vivo* are indistinguishable from those formed by synthetic peptides. Amyloid fibrils of different aggregated peptides or proteins are characteristic rods of the diameter 70—100 Å and unrestricted length. Fibrils are composed of 5-6 aligned protofilaments [88]. Monomers forming protofilaments have β-sheet conformation and are aligned perpendicular to the fiber long axis.

The overall Aβ fibril structure is often referred to as a "cross-β fibril", which is a historical term based on the X-ray diffraction analysis [89—91], however the precise alignment of β-strands in fibrils is still discussed. X-ray diffraction of Aβ11—28 fibril [92] suggests antiparallel strand alignment. Antiparallel hairpin with turn at positions 18—19 has been suggested as the building block of Aβ11—25 fibrils based on X-ray diffraction [93]. However, X-ray fiber diffraction data are not detailed enough to allow to distinguish the parallel or antiparallel alignment and support of antiparallel alignment is based mainly on FT-IR data [94]. Solid state NMR of fibrils from short Aβ fragments Aβ34—42 [95] and Aβ16-22 [96] supports an antiparallel arrangement. For longer fragments (Aβ10—35) however, solid state NMR [85, 97] and cross-linking studies [97] of fibrils indicate a parallel arrangement. The parallel arrangement was observed also in solid-state multiple quantum NMR of Aβ1-40 fibrils [98]. Based on available data no unequivocal consensus has been reached pertaining to the alignment of strands in fibrils. Partial digestion and mass spectrometry (MS) studies show that the *N*-terminal part of Aβ peptide is not incorporated into protofilament structure and can be cleaved off by proteases [99].

Mutational analysis of fibril formation was widely applied to localize amino acids responsible for stabilization of the aggregated state [100]. Collected data indicates that the fragment 1—9 of Aβ sequence is of no importance for fibril formation, fibril morphology or its neurotoxicity [12, 99, 101]. In general, the hydrophilic *N*-terminal part at positions 1—28 presents a wider spectrum of structural states, depending on the conditions [67, 69, 102, 103]. *N*-terminally truncated versions of Aβ seem to aggregate more easily and retain neurotoxicity [104].

A highly hydrophobic segment Leu17-Val18-Phe19-Phe20-Ala21 seems to be the core segment for aggregation and has been shown to be indispensable for polymerization [105]. Replacement of these hydrophobic residues by hydrophilic ones destabilizes aggregated forms [82, 101, 106—110]. Single site mutation F19T is sufficient to block aggregation [107], although threonine is a strong β-sheet former.

In vitro studies of mutations linked to familial or sporadic forms of the disease, where the onset is faster, like Flemish substitution (A21G) [111] or substitution associated with hereditary cerebral hemorrhage and Dutch-type amyloidosis (E22Q) [112] show that these substitutions increase the tendency to aggregate. On the other hand, rat Aβ with substitutions R5G, F10Y, H13R does not form aggregates [113]. Strong pH dependence of aggregation indicates the role of charged residues [83, 114]. Fraser *et al.* [115, 116] proposed that charge-charge interactions function together with non-ionic interactions in stabilizing the β-sheet conformation and assembly of AD amyloid fibers. Important factors have been catalogued, they are dispersed along Aβ sequence with *C*-terminal part being more critical.

Homeostasis of zinc, copper and iron has been shown to be distorted in the AD affected brain tissue [117]. Amyloid plaques accumulate high concentrations of copper and iron [118]. It has been agreed that metals play important role in AD pathogenesis. Aβ peptide binds zinc [119] *via* histidines [120], some data indicate even two binding sites [119]. The impact of zinc binding on aggregation is unclear. Some authors conclude that zinc accelerates aggregation [121] the others find support for its destabilizing role [122]. Also copper is bound by Aβ [123, 124], accelerates aggregation [125] and can mediate neurotoxicity [126—128].

AD onset is slow, it affects people over 60, so kinetics of amyloid fibril assembly is of great importance for understanding of the disease. Several groups have studied the time course of fibril formation. Naiki and Nakakuki [129] have developed a first order kinetic model for beta-amyloid fibril formation and proposed that fibril formation occurs *via* association of beta-amyloid 1—40 molecule one after the other. The first order kinetics was observed for Aβ monomers deposition on preexisting tissue plaques while in the absence of the template the process showed higher order kinetics [130]. The rate-determining step of *de novo* fibrilization process is slow formation of the nucleus, which could be accelerated or even passed up by 'seeding' [131]. The seeds can consist of longer Aβ peptide variants, which include the critical *C*-terminal residues [131], fibrils of Aβ [132] or non-amyloid component peptide of senile plaque core [133]. The consequences of this kinetic model are following: a lag phase, during which no fibrils are formed, and critical concentration, above which fibrilization can proceed [131]. The critical concentration for Aβ1—40 has been estimated to be *ca.* 10 μM [132] and for Aβ1—42 — *ca.* 2 μM [134] for *in vitro* fibrillization at neutral pH. Aβ concentration in body fluids of healthy individuals is subnanomolar (0.2—0.6 nM) what indicates that spontaneous nucleation of fibrils in normal brain is unlikely to occur [135—137]. However, the critical concentration *in vivo* could be effectively lowered by association of Aβ with chaperones such as apoE [138].

The consequence of seeding model was the prediction that in solution, both *in vivo* and *in vitro*, practically only monomers and fibrils of Aβ peptide will be present. The intermediates as metastable should not accumulate. However, during *in vitro* studies in solution as well as in tissue cultures *in vivo* various forms of Aβ such as mono-, di-, tetra- and oligomers in equilibrium with fibrils are observed. Either the oligomers are off-pathway to fibrils or the transition of monomeric Aβ to fibrils is a multistep process with many intermediate states, rather then the direct co-operative process of transition from monomer to fibril. The necessity of a conformational switch from α-helix to β-sheet structure is postulated to be the key step in Aβ fibrillogenesis [139, 140], although α-helical content is usually not detected for Aβ in the absence of additives. However, Kirkitadze *et al.* [140] during incubation of monomerized 1—40 and 1—42 Aβ fibrils observed rise of significant α-helical CD signal at some moment of incubation, which disappears upon transition to fibrils. This α-helical signal was attributed to oligomers, as it was retained after filtration through 10 kDa cutoff membrane. As Aβ α-helix destabilizing mutations were found which retard fibrillization, these helical oligomers were suggested to be a necessary intermediate on-pathway of fibrillization. Other authors, however, find that helix stabilizing factors inhibit fibrillization and conclude that helical intermediates are off-pathway [79]. The above contradiction has not been resolved yet and it may be of importance for design of proper therapeutic strategies.

The discovery of stable oligomeric forms of Aβ coincided with doubts as to the toxicity of the amyloid senile plaques. Discovery of neurotoxicity of the oligomeric forms both *in vitro* and *in vivo* studies lead to vivid interest in characterization of their structures and the mechanism of formation.

SMALL OLIGOMERS — STRUCTURAL ASPECTS

Different methods give different answers to the question of the oligomeric nature of *in vitro* Aβ preparations. Size exclusion chromatography (SEC) consistently indicates that Aβ at all ranges of a concentration migrates as a dimer [106, 119, 134, 141—144] or higher oligomers [26, 84]. This conclusion has been challenged however by arguing that monomeric Aβ may run anomalously on SEC column [142] and elute earlier. Indeed, Walsh *et al.* [141] checking different SEC conditions showed that Aβ could elute as species of weights from 7 000 Da to 18 000 Da, but not below 5 000 Da. On the other hand Aβ1—40

runs as mass 5.3—6.6 kDa in 8M urea, whereas it shifts to 9.5 kDa upon dilution of chaotropic agent [145]. Upon incubation of synthetic Aβ1—42 at pH 7.4 dimeric and trimeric species of molecular mass 9.0 and 13.5 kDa were observed by SEC and mass spectrometry [26].

Ultracentrifugation studies give contradictory results. Whereas Huang *et al.* [146] find trimers for 10 μM Aβ 1—40 at pH 7 or at least the oligomer-monomer equilibrium, the others [80, 147] find mass corresponding to monomers or predominantly monomers at 25 μM. Snyder *et al.* [148] working at 450 μM sample state coexistence of high MW oligomers.

The results of NMR analyses of Aβ1—40, 1—42 or Aβ10—35 in water or aqueous solutions, without fluoroalcohols or detergents added, have been interpreted as indicative of monomeric species [142, 149]. Tseng *et al.* [142] measured translational diffusion of 0.2 mM Aβ1—40 at pH 7.5 by NMR and found agreement with peptide being monomeric in these conditions. Due to time averaging of the signal a dynamic equilibrium of different oligomers cannot be excluded based on these studies.

Fluoresence energy transfer (FRET) studies of Aβ9—25 and 1—40 [114, 143, 146] indicate the presence of intramolecular fluorescence transfer between chromofores introduced at termini of different Aβ variants. These studies implicate at least dimers, stable in nanomolar to micromolar concentration (with the 25 μm critical concentration for massive aggregation) at neutral pH. Subunit exchange for Aβ1—40 is slow in these oligomers, indicating their high stability [143]. Structural differences were found for oligomers formed at different pH values [114].

Stabilization of Aβ oligomeric intermediates may be achieved by chemical cross-linking. Pentameric or hexameric complexes in aqueous solution were demonstrated by SDS-PAGE following treatment with glutaraldehyde and borohydride reduction while without such treatment no SDS stable oligomers were observed. Photoinduced cross-linking studies were also carried out and the results were indicative of small Aβ1—40 oligomers such as dimers, trimers and tetramers in rapid equilibrium with monomer [150].

Dynamic light scattering data were interpreted as indicating a compact dimer or an extended monomer [151]. The presence of small oligomers of Aβ1—40 was also revealed by quasielastic light scattering analysis, however the differentiation between monomer and dimer could not be done [63, 141, 152].

Circular dichroism spectra do not suggest any definite secondary structures of oligomers of Aβ1—40 present at neutral pH and β-structure at low pH [146]. Other authors [140] observe transient build-up of α-helical signal upon incubation of Aβ.

The electron microscopy of the Aβ ultrastructures formed during incubation of Aβ1—42 at 37°C revealed oligomeric globular structures with radius of 4—5 nm in diameter. The calculated molecular weight of these structures was *ca.* 14 kDa suggesting trimeric to tetrameric forms. These structures after 0.5 to 2 h of incubation formed short protofilaments of diameter 8.7—11.3 nm and length 30 to 100 nm of V or Y shape. The protofilaments assemble into long and straight fibrils, which continue to grow [153]. The studies of Aβ fibril assembly with small angle neutron scattering revealed that under acidic conditions micelle-like aggregates of Aβ are in rapid equilibrium with Aβ monomers or low molecular weight oligomers. These structures were found to be the centers of fibril nucleation. The elongated micellar assemblies were shown to comprise 30—50 Aβ monomers and exhibit hydrodynamic radius of 7 nm [154].

Kinetic studies of the small oligomeric intermediates *i.e.* dimers to hexamers were recently initiated. Bitan *et al.* [150] showed that a dynamic process of oligomer formation and dissociation preceded Aβ aggregation and proposed that Aβ fibrillogenesis pursued more complex pathway than simple two-step nucleation-elongation models.

These results can be reconciled by assuming that Aβ in aqueous solvents exists as a mixture of monomeric and oligomeric species, which do not show structural preferences for particular secondary structure elements, but are nevertheless partially structured. Oligomers do not seem to be thermodynamically stable in experiments *in vitro*, although some data point to the contrary. Another question remains whether these species do have any correspondence to the ones obtained *ex vivo*.

SDS-stable oligomers were detected by gel electrophoresis in isolated blood vessels [155] and in the conditioned culture media of the specific cell lines [25]. The same technique was used for investigation of human cerebrospinal fluid. Small amounts of SDS-stable dimers with a characteristics similar to that of he oligomers obtained from cell cultures were detected. The results of the intracelluar:extracellular oligomers ratio evaluation suggests that Aβ oligomerization begins inside neurons [59]. Kuo *et al.* [24], using ultracentrifugation and graded molecular sieving for analysis of AD and normal brains demonstrated the presence of water-soluble, non-filamentous Aβl—40 and Aβl—42 forms having molecular weight from below 10 kDa to above 100 kDa. Ward *et al.* [156] using noncontinuos gradient centrifugation for preparative fractionation of Aβ1—40 were unable to evaluate the size distribution of oligomers, though low molecular weight fraction, containing peptide oligomers, was detected. Roher *et al.* [26] studied the composition of AD neuritic plaques and vascular Aβ and found dimeric and trimeric species even in the presence of 80% formic acid or 5M GuHCl, pH 7.4, as determined by SEC and mass spectrometry. The characteristic feature of all *ex vivo* oligomeric species is their SDS stability, which differs these species from obtained *in vitro*. The source of this unusual stability is still unclear and as yet has not been reproduced *in vitro*.

ADDL

Larger neurotoxic species, of molecular weight from 17 000 to 42 000 Da, or even larger in some preparations, have been named ADDL's (Aβ-derived diffusible ligands) [27]. AFM size characterization of ADDLs indicated the predominant species of globular shape *ca.* 4.8 to 5.7 nm in diameter [27]. Depending on the conditions, ADDL preparations could contain predominantly trimers—hexamers, with larger forms of up to 10 monomers [27] or 24-mers [61].

Oda *et al.* [157] were the first who reported toxicity of non-fibrillar oligomers derived from synthetic Aβ. Using clusterin (apoliprotein J or ApoJ) as an inhibitor of fibril formation they observed slowly sedimenting Aβ complexes, which caused the death of mature neurons. They suggested that soluble complexes show a potential for diffusing to damage distal neurons [158]. Using atomic force microscopy Klein's group showed that ApoJ/Aβ1—42 toxic preparations are free of protofibrils and other large structures [61]. The toxic oligomers also form *in vitro* without ApoJ, but at reduced temperature (4—8°C) or closer to physiological concentration of below 50 nM. On this basis Lambert *et al.* [27] postulated that ApoJ might act as a chaperone, decreasing fibril growth from small "seeds" while enabling monomers to form semi-stable oligomers. These oligomers were shown to pass through support filters to reach the culture, showing a diffusible nature consistent with their small size. The binding studies revealed binding of ADDLs to cell surfaces at trypsin-sensitive domains and blocking this binding by tryptic peptides obtained from cell surfaces. The obtained data for ADDLs led to conclusion that ADDLs may be formed only from Aβ1—42 monomer but not from Aβ1—40 one [159].

Huang *et al.* [114] identified narrow distribution of Aβ1—40 particles stable at pH 3 of molecular weight *ca.* 1 MDa. The circular dichroism studies revealed that the particles contain β-structure and similarly to ADDLs are spherical. The observed diameter of these particles was larger then for ADDLs, being in the range of 8 to 18 nm, as observed using electron microscopy or atomic force microscopy [146].

PROTOFIBRILS

Protofibrils were described for the first time in 1997 [28, 141]. Harper *et al.* [28] used AFM to follow Aβ1—40 amyloid fibril formation *in vitro*. During these studies they observed small ordered aggregates that grew slowly and then rapidly disappeared, while prototypical amyloid fibrils of two discrete morphologies appeared. They proposed the metastable intermediate to be called Aβ amyloid protofibrils. The studies of protofibril morphology carried with negative staining and EM [141] or AFM [28, 46] showed curved fibrils, 6—8 nm in diameter [141] and up to 200 nm in length [28, 141]. Further AFM examination of Aβ1—40 protofibril gave protofibril diameter *ca.* 4.4±0.5 nm and periodicity of *ca.* 20±4.7 nm [160]. The electron microscopic examination of protofibrils prepared by rotary shadowing revealed beaded chains with periodicity 3—6 nm [63]. The kinetics of protofibril formation and disappearance was consistent with protofibrils being a transient intermediate in Aβ fibril assembly [141].

The protofibril initiation may be a nucleation-dependent event and may require fewer than 20 Aβ molecules, judging from the approximate volume of the earliest protofibrils. This step was shown by Walsh *et al.* [141] (using gel filtration chromatography for separating Aβ monomer and protofibril) to be accelerated for Aβ1—42 as compared to Aβ1—40. The studies of elongation of protofibrils carried for Aβ1—40 and Aβ1—42 variants showed that elongation rate increased with increasing Aβ concentration and temperature. Aβ protofibrils elongation was accelerated by increasing concentration of sodium chloride and promoted by glycerol. The pH values influenced the elongation process, giving different protofibril morphology at different pH. Dynamic light scattering analysis revealed rapid formation of long (1 μm) filaments of Aβ1—40 at pH 1 and association of large masses of material resembling protofibrils at pH 4.5 and 5.8 (up to 5 μm) that were morphologically different from fibrils obtained at pH 7.4 [160]. The elongation of Aβ1—40 and Aβ1—42 protofibrils progresses with comparable rates [161], although it should be emphasized that a slight difference, undetectable by *in vitro* AFM method could be critical *in vivo* [160]. Protofibril elongation could involve coalescence of smaller protofibrils [162]. Protofibril assembly was reversible in dilution and disasembly could occur by a different process than protofibril growth. Once formed, protofibrils were in equilibrium with low molecular weight Aβ (monomeric or dimeric) [63]. Protofibril-to-fibril transition appeared to be a cooperative step involving protofibril association, winding and possibly some conformational change. The faster rate of Aβ1—42 amyloid fibril formation compared to Aβ1—40 observed by Harper *et al.* [163] could by caused by increased initiation of Aβ1—42 protofibrils and/or acceleration of the subsequent conversion to fibrils. The step of fibril formation was not easily reversible, *i.e.* once protofibrils disappeared during fibril formation they did not reappear, even during dilution of fibrils. Fibril elongation was observed to be nucleation dependent, in which preformed fibrils, but not protofibrils, effectively seeded this transition [161, 163].

Additional support for fibril formation *via* protofibrils came from studies designed to elucidate the structural relationships among small oligomers of Aβ, protofibrils and fibrils. The data of dye binding experiments showed clearly that protofibrils bind both Congo red and thioflavin T, a property of amyloid fibrils not observed in small Aβ oligomers. This suggested that protofibrils contained significant amounts of β-sheet struc-

ture and thus had to evolve following significant conformational changes in small Aβ oligomers. The CD data were consistent with these observations showing 47% β-structure, 40% random coil and 13% α-helix. β-sheet content of protofibrils was similar to that of fibrils [63]. Recent cryoelectron microscopic studies revealed prominent inhomogenities within protofibrils, which in some samples appear to derive from globular subunits. These subunits may represent a structural unit from which protofibrils are assembled [153].

Protofibrils occur in cerebrospinal fluid (CSF), according to a preliminary analysis of individuals with AD [164]. Recently, it was demonstrated that mutation E to G at codon 693 of amyloid precursor protein, located within Aβ sequence, leads to increased rate of protofibril formation and their increased level [165].

Only a first glimpse on the properties of putative neurotoxic Aβ oligomers has been achieved by now. Many basic questions still remain unanswered. First, the major neurotoxic species have to be firmly established and further their correspondence with the oligomers obtained *in vitro* assessed. Next the detailed structural knowledge at atomic detail is necessary allowing to design a rational drug. None of the above has been achieved and much further work to converge *in vivo* and *in vitro* studies is indispensable.

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