Preparation and characterization of toxic Aβ aggregates for structural and functional studies in Alzheimer's disease research

Asad Jan[1], Dean M Hartley[2] & Hilal A Lashuel[1]

[1]Laboratory of Molecular Neurobiology and Neuroproteomics, Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland. [2]Department of Neurological Sciences, Rush University Medical Center, Chicago, Illinois, USA. Correspondence should be addressed to H.A.L. (hilal.lashuel@epfl.ch) or D.M.H. (dean_hartley@rush.edu).

The amyloid cascade hypothesis, supported by strong evidence from genetics, pathology and studies using animal models, implicates amyloid-β (Aβ) oligomerization and fibrillogenesis as central causative events in the pathogenesis of Alzheimer's disease (AD). Today, significant efforts in academia, biotechnology and the pharmaceutical industry are devoted to identifying the mechanisms by which the process of Aβ aggregation contributes to neurodegeneration in AD and to the identity of the toxic Aβ species. In this paper, we describe methods and detailed protocols for reproducibly preparing Aβ aggregates of defined size distribution and morphology, including monomers, protofibrils and fibrils, using size exclusion chromatography. In addition, we describe detailed biophysical procedures for elucidating the structural features, aggregation kinetics and toxic properties of the different Aβ aggregation states, with special emphasis on protofibrillar intermediates. The information provided by this approach allows for consistent correlation between the properties of the aggregates and their toxicity toward primary neurons and/or cell lines. A better understanding of the molecular and structural basis of Aβ aggregation and toxicity is crucial for the development of effective strategies aimed at prevention and/or treatment of AD. Furthermore, the identification of specific aggregation states, which correlate with neurodegeneration in AD, could lead to the development of diagnostic tools to detect and monitor disease progression. The procedures described can be performed in as little as 1 day, or may take longer, depending on the exact toxicity assays used.

INTRODUCTION

Alzheimer's disease (AD), the most common cause of senile dementia[1], is characterized pathologically by the formation of amyloid plaques and neurofibrillary tangles in the brain[2]. The principal components of amyloid plaques are the fibrillar aggregates of amyloid-β (Aβ) peptides[3] (39–43 amino acids), which are produced as a result of enzymatic proteolysis of amyloid precursor protein (APP) in neurons and other cells throughout life[4–6]. Strong circumstantial evidence from genetics, pathology, biochemical studies and animal models led to the emergence of the Aβ amyloid cascade hypothesis of AD[7,8]. This hypothesis stipulates a central role of Aβ production, oligomerization and/or fibrillogenesis (**Fig. 1**) in triggering a cascade of cellular and molecular events, which lead to the development of synaptic failure, synapse loss, neuronal loss and eventually dementia. Missing links between this cascade and AD pathogenesis include the identification of the mechanisms by which the process of Aβ aggregation contributes to neurodegeneration and to the identity of the toxic Aβ species. This knowledge is crucial for developing effective strategies to prevent and/or treat AD. Furthermore, the identification of specific aggregation states, which correlate with neurodegeneration in AD, could lead to the development of diagnostic tools to detect and monitor disease progression. Such tools are crucial for evaluating the efficacy of therapies at different stages of AD pathogenesis.

In solution, Aβ is unstructured, but acquires a β-sheet structure on aggregation and amyloid formation. Amyloid formation by Aβ follows a nucleation polymerization pathway that is characterized by an initial lag phase dominated by monomeric (M) species, followed by formation of low-molecular-weight (LMW) oligomers (dimer, trimers and < 8-mers)[9,10].

A β-sheet–rich oligomeric 'nucleus' subsequently forms from these aggregates, which undergoes further growth to form higher-order aggregates of heterogeneous size and morphology distribution (**Table 1**). These β-sheet–rich oligomeric intermediates are collectively referred to as prefibrillar aggregates and disappear on fibril formation[11–13]. Some of the well-documented prefibrillar morphologies include protofibrils (curvilinear structures less than 200 nm in length and 4–6 nm in diameter)[12–14], Aβ-derived diffusible ligands (ADDLs; spherical aggregates 3–5 nm in diameter)[15], Aβ-annular assemblies (doughnut-like structures; outer diameter 8–12 nm and inner diameter 2–2.5 nm)[16–18] and LMW oligomers[17,19] (**Table 1**). Whether some of these species represent off- or on-pathway intermediates to amyloid fibril formation remains unknown. Some of the key distinguishing features of protofibrillar aggregates are that they (i) contain a mixture of different secondary structure elements with a predominance of β-sheet[20]; (ii) bind amyloid-specific dyes, such as Congo red and Thioflavin-T (ThT), although less strongly than mature fibrils[20–22]; and (iii) are less stable than mature amyloid fibrils and exist in equilibrium with monomeric Aβ[20,21,23]. In the presence of monomeric Aβ, all these species convert to mature amyloid fibrils by monomer addition. Mature amyloid fibrils have been described as insoluble, β-sheet-rich structures that are more than 1 μm long, 8–12 nm in diameter and composed of 2–6 protofilament subunits[24,25]. *In vitro*, synthetic and recombinant forms of Aβ peptides have been shown to form aggregates and amyloid fibrils that are indistinguishable from the ones isolated from AD brain[26–28]. This suggests that the physiological process of amyloid formation can be recapitulated *in vitro* and highlights
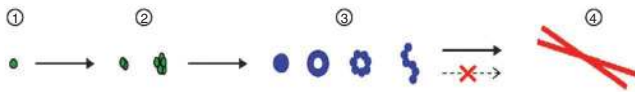
Figure 1 | Aβ fibril formation pathway. (**1**) Aβ monomers, under favorable conditions, can aggregate to form non-fibrillar and fibrillar aggregates. The non-fibrillar aggregates, based on their molecular size and/or morphology, can range from low-molecular-weight (LMW) oligomers (*n* = 2–8) (**2**) to highly ordered structures such as protofibrils and Aβ-derived diffusible ligands (ADDLs) (**3**). The non-fibrillar aggregates can be 'on pathway' (solid arrows) to fibril formation and disappear upon fibril formation (**4**), or 'off-pathway' (dashed arrows with a cross) species (that is, they do not convert into fibrils). Structures are not drawn to scale.

the relevance of *in vitro* studies for elucidating the molecular mechanism(s) of amyloid formation *in vivo*.

Several studies using familial AD (fAD) mutations in animal models and cell culture experiments strongly support the notion that early intermediates in the fibrillization process (i.e., oligomers, including protofibrils) are the primary toxic Aβ species and have a central role in AD pathogenesis. This hypothesis is supported by (i) the lack of a clear correlation between fibrillar amyloid burden in AD patients and cognitive impairment[29,30], whereas such a correlation has been reported for the soluble pool of Aβ (monomers and oligomers including protofibrils) and AD severity[31,32]; (ii) the fact that autosomal dominant mutations in APP or its cleaving enzymes (presenilins), which favor increased production of Aβ42 and/or accumulation of protofibrils, are associated with the development of fAD[33–36]; (iii) the indication that transgenic animals overexpressing fAD mutations in APP exhibit learning deficits and lower performance in memory tasks, compared with control animals, long before the appearance of amyloid plaques[37,38]; (iv) the fact that intracerebral administration of soluble Aβ oligomers in live animals impairs cognitive performance and inhibits long-term potentiation (LTP), a cellular model of memory formation[19], and induces widespread plaque pathology[39]; and (v) the observation that treating cultured neurons with Aβ protofibrils results in impairment of ion channels[40,41], inhibition of LTP[42], loss of synapses and cell death[14]. Finally, anti-Aβ antibodies specifically targeting oligomers, including protofibrils, block neurotoxicity in cultured neurons[43] and reverse behavioral deficits in mice[44]. Therefore, there is a vast academic, medical and commercial interest in identifying therapeutic agents that target specifically soluble Aβ (monomers and oligomers, including protofibrils) and their impact on AD pathogenesis.

Studies using animal models of AD or using cultured neurons suggest that prefibrillar Aβ aggregates, including LMW oligomers and protofibrils, exert their toxic effects by several potentially interrelated mechanisms, including pore formation[45], channel-like activity[46], permeabilization of lipid bilayers[47], inhibition of K+ currents in cortical cultures[40], disruption of neuronal glutamate uptake[48], activation of apoptosis[49], oxidative damage[50] and degradation of postsynaptic density marker PSD-95 (ref. 51). A definitive correlation of any of these biological effects with the development and progression of AD is not straightforward and remains difficult to establish. A detailed molecular understanding of the process of amyloid formation, as well as the structural properties and dynamics of the various prefibrillar intermediates, is crucial for understanding the role of amyloid formation in AD pathogenesis. This requires access to experimental protocols that allow the preparation of sufficient quantities of well-characterized Aβ aggregate states for structural and functional studies.

Various methods have been used to prepare different oligomeric and protofibrillar Aβ species from synthetic Aβ (sAβ) peptides, and each method has its own advantages and disadvantages. Treatment of lyophilized Aβ with strong acids and bases to disrupt preformed aggregates and enhance solubility[52], filtration through LMW cut-off filters[53], photo-induced crosslinking of unmodified proteins[17], density gradient centrifugation[54] and size exclusion chromatography (SEC)[13,14,17] have all been used to prepare soluble aggregates of Aβ and yield preparations that vary in size and morphology distribution. SEC, in particular, offers several advantages: (i) a variety of column matrices with different separation capacities are readily available and can be used in isolation or in combination with other columns to obtain high-resolution separation and fractions containing Aβ aggregates of defined size distribution; (ii) generally, the columns are equipped with filters at the top that allow for the removal of fibrillar (or insoluble) material from the injected sample, thus ensuring that the Aβ fractions are free of fibrillar seeds; (iii) if SEC is coupled to a light-scattering detector, accurate determination of Aβ aggregates' size distribution becomes possible[20]; (iv) in analytical mode, SEC is a valuable tool to monitor early events in amyloid formation and quantification of monomer and/or protofibril loss during the time course of fibril formation[21]; and (v) by choosing proper running conditions (i.e., buffer pH and contents), fractions are obtained in solution conditions suitable for biological systems, free of harmful or undesired substances (e.g., organic solvents and so on). SEC has also been successfully used to isolate Aβ oligomers (such as dimers and trimers) and high-molecular-weight aggregates (including protofibrils and amorphous aggregates) from complex cell culture media[55] or from postmortem AD brain extracts[56].

Although it is one of the most commonly used methods for isolating and purifying Aβ oligomers[13,21,23], SEC suffers from certain drawbacks that should be taken into consideration when using this technique: (i) some aggregates, because of their variable stability, may dissociate on interacting with column matrix and elute as LMW species[21]; (ii) there may be a low resolution of separation if the sample is composed of a continuum of interconverting aggregates of related sizes; (iii) the samples are diluted in concentration after SEC fractionation and may not be suitable for use in certain experiments requiring high Aβ concentration; (iv) long duration of separation may allow interconversion and mask changes taking place within the time scale of minutes or less; (v) interactions with column matrices and separation may also be affected by factors other than size, such as aggregate conformation and tertiary structure; and (vi) nonspecific adsorption of hydrophobic peptides (such as Aβ) to the column matrix might result in a low yield of purified material.

Previous results obtained using this protocol
The early reports showing the appearance of protofibrils during the course of amyloid fibril formation *in vitro*[12] heightened the interest in obtaining these structures in purified form[13]. Using SEC, several groups showed that they can isolate protofibrillar aggregates, thus making it possible to elucidate their structural features and compare their toxicity with purified monomers or fibrils[14,20]. These studies have contributed significantly to (i) our current understanding of the mechanism of amyloid formation and its potential role in the pathogenesis of AD; (ii) our knowledge of the physical and structural properties of different oligomeric and protofibrillar Aβ species; and (iii) the development of important research tools

TABLE 1 | Glossary of Aβ aggregation.

Term	Description	References
Amyloid	Extracellular, insoluble proteinaceous deposits (plaques) that are composed primarily of fibrillar aggregates comprising a single protein (Aβ in the case of AD-linked amyloid). Amyloid is defined by its dye-binding properties: increased fluorescence and green birefringence with Thioflavin-T and Congo red, respectively. In addition to the fibrillar protein, amyloid deposits contain other proteins, glycosaminoglycans and proteoglycans	87,88
Fibril	Filamentous structure, consisting of 2–6 protofilaments, seen in cross-sections from disease-affected tissue and also observed during aggregation of synthetic Aβ peptides *in vitro*. Mature fibrils are characterized by: (i) cross-β X-ray fiber diffraction pattern; (ii) β-sheet rich CD and FTIR spectra; (iii) binding to Congo red and Thioflavin-T and (iv) characteristic filamentous morphology (8–12 nm in diameter, >1 μm long) by AFM and TEM imaging (**Figs. 3f** and **4h**). Depending on the solution and incubation conditions, a given protein sequence can yield fibrils of diverse morphologies	25,27,89,90
Protofilament	Structural subunit of a mature fibril. Appear as twisted thread with a characteristic periodicity and helical arrangement. (Note: different from protofibril (see below))	25,91,92
Oligomers	The term 'oligomers' encompasses a wide range of species, ranging from low-molecular-weight (LMW) species (including dimers, trimers and tetramers) to high-molecular-weight (HMW) oligomeric species (such as spherical, chain-like and annular structures or longer structures (**Fig. 4e–g**)). Fibrils are, by definition, protein oligomers. Different types of oligomeric species have been described and are typically distinguished by their composition, stability and molecular weight	13,15,17–19
LMW oligomers	Generally, LMW oligomer preparations contain a mixture of species that are less than 8-mers and are in equilibrium with monomeric Aβ, as determined by denaturing SDS-PAGE gels or cross-linking studies. These species are generally not detectable by standard TEM techniques. The smallest Aβ oligomer species that could be visualized and its size determined by STEM was of 42–80 kDa. Evidence for the LMW oligomers has been primarily derived from denaturing SDS-PAGE electrophoresis experiments and cross-linking studies. There is no evidence that these species accumulate in a stable form. Furthermore, the possibility that these oligomers could be derived from HMW oligomers has not been ruled out	17–19
SDS-stable LMW oligomers	SDS-resistant LMW oligomers (<8-mers) have been prepared *in vitro* or isolated from natural sources (cell cultures or brain tissues). LMW oligomers that are secreted from CHO cells overexpressing wild-type APP, or APP bearing fAD mutations, exhibit strong stability to SDS and chemical-induced dissociations, whereas the majority of LMW oligomers generated *in vitro* by separation through a SEC column are SDS sensitive, unless stabilized by chemical cross-linking. Naturally secreted LMW oligomers were shown to inhibit LTP and impair cognitive performance when injected into rat brains at much lower concentration than that used to observe toxicity with synthetic Aβ. However, despite extensive efforts by different laboratories to establish the chemical identity and true stoichiometry of naturally formed SDS-stable oligomers/aggregates by mass spectrometry, such data remain lacking and current size estimates are solely based on their mobility in denaturing gels	17,19,68
High molecular weight	Metastable Aβ heterogeneous oligomers that can be visualized by TEM and AFM and range in size from ~42 kDa to >1 mDa	12,13,15,16, 18,20,21,93
•Protofibrils	Metastable, heterogeneous, β-sheet rich prefibrillar structures that have been observed by AFM and TEM during amyloid fibril formation. Different protofibril preparations of Aβ reveal heterogeneous mixtures of discrete β-sheet aggregates of different sizes (50–1,500 kDa) and morphologies (spherical (5–20 nm), annular structures and smooth, curvilinear structures (5 nm in diameter and <200 nm in length)). TEM and AFM studies support a structural link between some of these different aggregates, but the mechanisms by which they form, the dynamics of their interconversion and their relationship with the amyloid fibrils remain poorly understood. Although protofibrils were initially thought to be on the linear pathway to fibrils, in its current usage, this term embraces all prefibrillar aggregates (≥4 nm) that can be detected by EM and AFM. Protofibrils bind to Congo red and Thioflavin, although to a lesser extent than mature fibrils. Therefore, protofibrils here are kinetic intermediates, whereas protofilaments (see definition given above) represent the substructure of the amyloid fibril	12,20,21
•ADDLs	Globular structures of 3–5 nm in diameter. Also referred to as globulomers. Initial reports, using mainly AFM and denaturing SDS-PAGE electrophoresis, suggested that these preparations contain primarily small oligomers ~10–12-mers. However, subsequent solution characterization of the ADDL preparations showed that they consist of a heterogeneous population of Aβ species, encompassing molecular sizes ranging from monomers to aggregates larger than 1 mDa, resembling the distribution observed for protofibrils	15,67,83

TABLE 1 | Glossary of Aβ aggregation (continued).

Term	Description	References
•Annular oligomers	Doughnut-shaped structures that have an outer diameter of 8–12 nm and inner diameter of 2–2.5 nm	16,18
Nucleus	A rare unstable entity (such as LMW oligomers and protofibrils) that influences the rate of a nucleated polymerization process. Nuclei are metastable in nature; they can form and act as seeds (see below) for growth of a fibril or possibly disassemble, thus reducing the fibril growth	21,89,94
Seed	A relatively stable entity (such as fibrils and fragmented fibrils), which, when introduced into a solution containing monomeric subunits, serve as effective nuclei and accelerate fibril formation (by eliminating the lag phase associated with nuclei formation) in a nucleation polymerization process	9,95

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; ADDL, Aβ-derived diffusible ligand; AFM, atomic force microscopy; APP, amyloid precursor protein; CD, circular dichroism; CHO, Chinese hamster ovary; EM, electron microscopy; fAD, familial AD; FTIR, Fourier transform infrared; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STEM, scanning transmission electron microscopy; TEM, transmission electron microscopy.

(such as conformational antibodies) and of novel amyloid-based diagnostic tools and therapeutic strategies.

Structural and mechanistic studies. The ability to purify protofibrils in sufficient quantities has facilitated biophysical and structural studies aimed at characterizing their structure and toxic properties. Biophysical studies, using multiple techniques, on SEC-purified Aβ protofibrils revealed many important structural and physical features that distinguish these species from monomeric and fibrillar forms of Aβ. Aβ protofibrils were shown to (i) bind amyloid-specific dyes such as Congo red and ThT[18,20,21]; (ii) be enriched with a β-sheet structure[20]; (iii) possess a core structure protected from solvent[20,21,23]; and (iv) exist in equilibrium with monomeric Aβ[20,21,23]. Quasielastic light-scattering measurements revealed that SEC-purified Aβ protofibrils contained aggregates with hydrodynamic radii (R_H) of 10–50 nm, which correspond to lengths of ~30–500 nm for noninteracting rods[13]. The heterogeneity of protofibrils was further elucidated by scanning transmission electron microscopy (TEM)[18] and sedimentation velocity measurements[57]. These studies revealed that protofibrils are a population of aggregates with molecular weight distribution ranging from 80 kDa to ~1 mDa (ref. 23).

Previous studies have established the ratio of Aβ40 to Aβ42 as an important factor in determining the fibrillogenesis, toxicity and pathological distribution of Aβ *in vivo*[58–60]. The ability to obtain monomeric and protofibril preparations of Aβ42 and Aβ40 by SEC allowed us to characterize the interactions between these two peptides at the levels of monomers, protofibrils and fibrils. This approach also enabled us to investigate how these interactions and changes in the relative ratio of Aβ42/Aβ40 influence Aβ oligomerization and fibrillogenesis *in vitro*. We showed that monomeric Aβ40 is a potent inhibitor of monomer Aβ42 amyloid formation at 1:1 ratio, whereas it inhibits fibrillization by Aβ42 protofibrils at substoichiometric amounts[21]. Furthermore, we found that Aβ42 protofibrils seed the fibrillization of monomeric Aβ42 but not of monomeric Aβ40. In contrast, fibrils composed of either Aβ42 or Aβ40 seeded the fibrillization of both Aβ42 and Aβ40 monomers[21]. Using these purified preparations, we were also able to show that monomeric Aβ42 and Aβ40 are constantly recycled and compete for binding to the ends of protofibrillar and fibrillar Aβ aggregates[21].

Finally, using the same monomer and protofibril preparations referred to above, we dissected the mechanism by which the heat shock protein (Hsp104) interferes with Aβ oligomerization and

fibrillogenesis. These studies revealed that Hsp104 inhibits Aβ fibrillization, targets different intermediates on the amyloid pathway and suppresses the seeding capacity of Aβ42 protofibrils and fibrils by blocking their growth[61].

Identifying the toxic Aβ species and elucidating the mechanism of toxicity. A critical challenge in understanding the mechanism of Aβ toxicity, and in the subsequent design of intervention strategies, has been the correlation of a particular aggregate structure or size with biological effects, especially toxicity. Initially, fibrils were reported to be toxic, and inhibition of fibril formation or disruption of fibrils was considered to be a viable strategy for inhibiting Aβ toxicity[62–64]. Subsequent studies, using SEC-purified protofibrils and monomers, showed that protofibrils exhibit stronger toxicity than monomeric and fibrillar Aβ[14,20,21].

Together, these seminal studies showed that Aβ40 protofibrils consistently impaired neuronal metabolism[20], instantly altered neuronal electrical activity and induced cell death[14,21]. SEC-purified monomeric Aβ, in contrast, had no effect on neuronal electrical activity[14] and required 3–5 d of incubation to induce quantifiable cell injury[14]. This observation suggested that some aggregation intermediates, formed during the incubation of monomeric Aβ, were responsible for inducing toxicity. These findings were corroborated by studies in which cultured neurons were treated with monomers of highly amyloidogenic Aβ sequences, Aβ42 and Aβ40-Arctic. The results showed that the treated neurons exhibited loss of processes within 7 h and cell death within 13–24 h of incubation with monomeric Aβ42 and Aβ40-Arctic[65]. Similarly, using SEC-isolated Aβ42 protofibrils, we have shown that monomeric Aβ40 inhibited Aβ42 protofibril toxicity toward cultured neurons by altering their aggregation properties and inhibiting fibril formation. These observations reinforced the importance of an ongoing Aβ-nucleated polymerization as a critical factor for Aβ-induced toxicity[21,66].

Size exclusion chromatography has also been used to characterize the relative toxicity of different Aβ protofibril preparations and has improved our understanding of the nature of toxic species in such preparations. ADDLs are soluble Aβ aggregates that, by atomic force microscopy, appear as spherical structures of 3–5 nm in diameter[15]. SEC was used to fractionate ADDLs and different fractions were compared for binding to the neuronal surface[67]. Results showed that void volume (>70 kDa) fractions selectively bound to the cell surface and processes of neurons. No such binding was reported

for fractions containing monomers to tetramers, suggesting that advanced oligomer states, protofibrils, are more likely to be the culprit toxic species in ADDL preparation[67]. Subsequent solution-state characterization of ADDL preparations revealed that they consist of a heterogeneous population of Aβ species encompassing molecular sizes ranging from monomers to aggregates larger than 1 mDa, resembling the distribution observed for protofibrils.
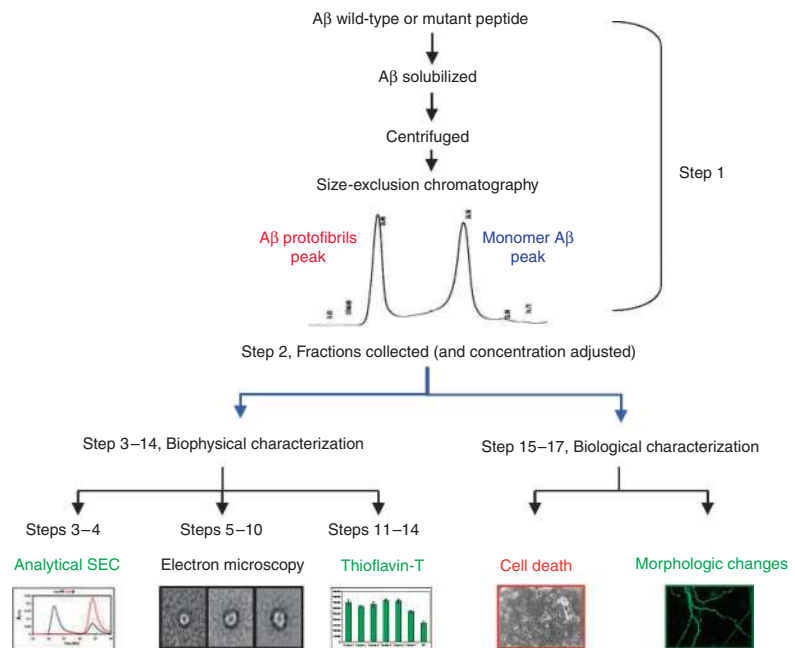
In addition to sAβ oligomers, SEC has also been used to elucidate the nature of toxic Aβ oligomers obtained from natural sources. The conditioned media of Chinese hamster ovary cells, stably expressing Val-717Phe fAD mutation in APP, contain monomeric and LMW oligomeric forms of Aβ[68]. SEC was used to separate the monomers from oligomers and then fractions were used to assess the effects of both species on learning and memory-related tasks in rats. Intracerebroventricular injections into rats with fractions containing oligomers, but not monomers, were associated with significant errors in the behavioral performance of animals[69]. Transgenic (Tg 2576) mice, harboring the Swedish mutation in APP (APPSWE) linked to fAD, exhibit signs of memory deficits at the age of 6–14 months without accumulation of plaques or neuronal loss. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and SEC analysis of brain extracts from 6- to 14-month-old mice revealed that soluble multimers of Aβ, particularly a 56-kDa species and to lesser extent a 40-kDa species, correlated with the onset of memory deficit and remained stable during this time[70]. The 56-kDa species (Aβ*56) was then purified by SEC and injected into the lateral ventricles of young healthy rats. Assessment of spatial memory task performance revealed that Aβ*56 induced transient memory impairment in treated rats without affecting the learning of new tasks[70]. Thus, the use of SEC in the studies cited above allowed the investigators to selectively identify particular Aβ oligomers (dimers and trimers or Aβ*56) as the most likely cause of behavioral deficits observed in animal experiments.

Generation of novel tools and therapeutics. SEC-based methods, or information garnered from these methods, have also been useful for elucidating the mechanism of action of novel diagnostic and therapeutic approaches for AD. Immunizing rabbits with soluble oligomers of synthetic Aβ mimics resulted in the generation of an antibody that selectively reacted with Aβ oligomers in dot blot assays[71]. The antibody did not react with LMW or fibrillar preparations, suggesting conformation specificity for oligomers. SEC fractionation of Aβ oligomers and subsequent dot blot assay with an oligomer-specific antibody showed that the smallest oligomer size detected by the antibody corresponded to a 40-kDa protein[71].

Interestingly, the antibody also showed binding to oligomeric preparations of other amyloidogenic sequences such as α-synuclein and human insulin, suggesting a molecular conformation shared by oligomeric intermediates of these proteins. Most importantly, the antibody was not only able to discriminate between AD-affected and normal brain but also distinguished thioflavin-S–positive (fibrillar) aggregates from non-thioflavin-S–binding (oligomeric) aggregates in AD-affected brain[71]. Similarly, immunization of mice with ADDLs led to the generation of monoclonal antibodies that selectively reacted with oligomeric SEC fractions of ADDLs and not with monomeric fractions. Immunostaining of brain sections or dot blots on brain extracts by these antibodies showed their ability to discriminate AD-affected from normal brain[72].

Experimental design
The general procedure for isolating monomeric and protofibrillar Aβ fractions, and subsequent elucidation of their biophysical and biological properties, is outlined in **Figure 2**. To prepare monomeric Aβ fractions, the lyophilized peptide is dissolved and disaggregated by guanidine hydrochloride treatment[65,73], and then the solution is injected into a suitable SEC column to separate Aβ monomers from guanidine (**Table 2**).

Figure 2 | Flow diagram illustrating steps and approximate timeline for Aβ fractionation by SEC and subsequent biophysical and/or biological characterization.

TABLE 2 | Fractionation range of SEC columns and Aβ42 fractions obtained after SEC.

Serial no.	SEC column	Separation range for globular proteins (kDa)	Protofibrillar fractions (ml)	Monomer fractions (ml)
1	Superdex 75 HR 10/30	3–70	2 (7–9)	2 (11–13)
2	Superose 6 HR 10/30	5–5,000	4–6 (6–11)	2 (14–16)

Abbreviation: SEC, size exclusion chromatography.

This procedure yields fractions that are highly enriched (≥90%) in monomeric Aβ. Although these preparations are free of protofibrillar or fibrillar aggregates, the presence of unstable LMW oligomers (dimers, trimers, and so on) that are in rapid equilibrium with the major monomeric species has been suggested and cannot be ruled out. To prepare protofibrillar Aβ fractions, the peptide is initially dissolved in DMSO[57] and then aggregation is promoted under high-salt conditions. This leads to the generation of a solution enriched with Aβ protofibrils and monomers. The different aggregation states and monomers are separated on a suitable SEC column (**Table 2**). Often, these aggregation parameters can be assessed and customized by monitoring the degree of monomeric versus protofibrillar Aβ elution from the column. After a defined interval of incubation, if needed, the peptide solution is centrifuged to pellet out fibrils or insoluble material. The supernatant is then injected into a suitable SEC column capable of carrying out separation in a defined molecular size range, and Aβ species are eluted from the column under isocratic conditions. To avoid unwanted Aβ aggregation during the separation procedure, it is recommended that SEC fractionation be carried out using a chromatography system placed inside a cold (4 °C) chamber. Alternatively, SEC can be carried out at room temperature (RT; 20–25 °C) provided that Aβ solutions are kept at 4 °C before injection and immediately transferred to 4 °C after eluting from the column. Fractions are then assessed for protein content and subsequently used for biophysical and/or biological studies. Biophysical studies may include further separation by SEC, characterization of the kinetics of fibril formation (such as ThT binding) and the structural and morphological properties of aggregates by imaging techniques such as electron microscopy and atomic force microscopy. For biological experiments, SEC monomeric or protofibrillar fractions are diluted by the addition of cell/tissue culture (TC) components needed for maintaining the osmolarity, pH and nutritive value of the media. Monomeric and protofibril fractions at high concentrations (50–100-μM) are desired for these experiments. This can be achieved by scaling up the peptide preparation or using a modified or concentrated cell/TC media. Preparation of Aβ fractions at higher concentrations, by scaling up the amount of injected peptide, is possible only up to a certain concentration: 2–3 mg ml^{-1} for slow aggregating Aβ sequences (e.g., Aβ40), but not for highly amyloidogenic Aβ peptides such as Aβ42 or the Arctic variant of both Aβ40 and Aβ42. At high concentrations of >1–2 mg ml^{-1}, the latter peptides start to fibrillize within minutes and precipitate, thus reducing the overall yield of monomers and protofibrils. Hence, a modified TC recipe for culturing primary neurons with concentrated components has been devised to minimize the dilution of Aβ fractions (**Table 3**). With tissue components added,

TABLE 3 | Preparation of modified neurobasal components.

Component	Composition
Inorganic salts (100×)	*mg per 10 ml H_2O*
$CaCl_2 \cdot 2H_2O$	264
KCl	400
$MgCl_2 \cdot 6H_2O$	162
NaH_2PO_4	108
Amino acids (100×)	*mg per l H_2O*
Alanine	20
Arginine HCl	840
Asparagine·H_2O	83
Cysteine	121
Glutamine	735
Glycine	30
Histidine·HCl·H_2O	420
Isoleucine	1,050
Leucine	1,050
Lysine HCl	1,460
Methionine	300
Phenylalanine	660
Proline	77.6
Serine	420
Theronine	950
Tryptophan	160
Tyrosine	720
Valine	940
Vitamin solution (100×)	*mg per l H_2O*
NaCl	8,500
D-Ca pantothenate	100
Choline chloride	100
Folic acid	100
I-Inositol	200
Nicotinamide	100
Pyridoxal HCl	100
Riboflavin	10
Thiamine HCl	100

various neurotoxicity studies can be conducted. Lactate dehydrogenase (LDH) release or MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assays can be used to measure overt cell loss (Step 17A) or histological staining for subtle morphological changes using cytoskeletal markers (Step 17B) can also be carried out. Electrophysiological measurements on cultured cells or brain slices were shown to provide a highly sensitive and instantaneous readout of neuronal abnormalities caused by the addition of specific Aβ aggregates, but require expensive equipment and specialized expertise, and will not be discussed in this paper[14]. Finally, biological activity can also be assessed in commercial cell lines of neuronal origin such as PC12 and SHSY5Y cells. In this case, modified commercial TC media (such as 10× Dulbecco's modified Eagle's medium) are commercially available and can be used to avoid further dilution of Aβ fractions.

Although SEC has been used to obtain monomeric and protofibrillar forms of Aβ, the yield of the respective fractions may vary depending on the peptide sequence and the presence of preformed seeds in lyophilized peptide stock. It is known that Aβ peptides from different manufacturers, or even different lots from the same source, may yield inconsistent aggregation and toxicity results[74]. The approach outlined in this protocol ensures that the recovered peptide fractions are free of any fibrillar material and variability among different Aβ preparations is minimized. Finally, to circumvent any potential problems associated with the use of sAβ peptides (such as impurities, racemization, salts or trace metals introduced during peptide synthesis and purification[52]), many laboratories have developed methods for the expression of recombinant Aβ (rAβ) in *E. coli*[75–77]. rAβ peptides have been shown to exhibit similar or higher toxicity compared with sAβ[75,76]. In addition to being free of any impurities associated with sAβ, which might interfere with aggregation or toxicity, rAβ peptides also offer an inexpensive means of obtaining radioisotope-labeled Aβ for structural studies using NMR[75,76].

The present protocol, principally based on the use of SEC, describes methods for generating monomeric, protofibrillar and fibrillar forms of Aβ40 and Aβ42, using sAβ and rAβ peptides and fAD mutants (e.g., Aβ40-Arctic and Aβ40-Dutch) prepared with different counterions (trifluoroacetate (TFA) or sodium hydroxide) and obtained from different commercial suppliers and academic research groups.

MATERIALS

REAGENTS
- Human wild-type (wt) synthetic Aβ42-wt, Aβ40-wt and fAD mutants (Aβ40-Arctic (E22G), Aβ40-Dutch (E22Q) and Aβ40-Flemish (A21G)). These were synthesized and purified as TFA salts by Dr. James I. Elliott, WM Keck Facility, Yale University, New Haven, CT, USA, as previously described[78] ▲ CRITICAL Aliquot the lyophilized peptide in glass vials, seal with a tight wrapping of parafilm and store the aliquots at −20 °C. Before use, bring the peptides to RT by leaving the vial in a dry place for 10–15 min. Do not remove the parafilm wrapping except just before weighing and/or solubilization (see Step 1 of PROCEDURE). It is recommended to store the vials in a box containing a desiccant (EM Sciences, cat. no. 71225-00) for this purpose.
- DMSO (Sigma, cat. no. 41639) ▲ CRITICAL DMSO is hygroscopic and should be stored in a dry place.
- Tris (hydroxymethyl)-aminomethane (Tris-base; Sigma, cat. no. T1503)
- Trizma hydrochloride solution (Tris-HCl, 1 M; Sigma, cat. no. T2663)
- Hydrochloric acid (32% (wt/vol) Merck, cat. no. 1.00319.1000) ❗ CAUTION It is extremely corrosive and toxic and readily produces toxic vapors. Use a fume hood, face mask, eye protection and gloves while handling.
- Sodium hydroxide solution (NaOH, 1 N; Sigma, cat. no. 59223C) ❗ CAUTION It is extremely corrosive and toxic. Use a fume hood, face mask, eye protection and gloves while handling.
- Guanidine hydrochloride (Gn-HCl; Sigma, cat. no. G4630) ❗ CAUTION It is toxic and an irritant.
- Uranyl acetate (UA; Electron Microscopy Sciences, cat. no. 22400) ❗ CAUTION It is a radioactive chemical, toxic if inhaled or ingested and it is categorized as a suspected carcinogen. Wear gloves, eye protection and face mask while handling.
- Thioflavin-T (ThT; Sigma, cat. no. T3516)
- Glycine (Sigma, cat. no. 50046)
- Bicinchoninic acid (BCA) protein assay kit (Pierce, cat. no. 23227)
- DMEM (1×, high glucose without glutamine and sodium pyruvate; Invitrogen, cat. no. 11960-051)
- Hank's buffered salt solution (HBSS; Invitrogen, cat. no. 14185052)
- (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES, 1 M; Invitrogen, cat. no. 15630-080)
- PBS (GIBCO, cat. no. 10010-015)
- Sorensen's phosphate buffer (0.15 M; Electron Microscopy Sciences, cat. no. 11682-10)
- F-12 nutrient mixture (Invitrogen, cat. no. 11765-054)
- B-27 supplement (Invitrogen, cat. no. 10889-038)
- Neurobasal medium (Invitrogen, cat. no. 10888-022)
- FBS (GIBCO, cat. no. 10101145)
- Penicillin-streptomycin (Pen-strep, 100×; GIBCO, cat. no. 15140-122)
- Poly-L-lysine, 331 (MW 331 k; Sigma, cat. no. P-1524)
- Laminin (Sigma, cat. no. L2020)
- Trypsin-EDTA (Invitrogen, cat. no. 25200-056)
- L-Glutamine (200 mM; GIBCO, cat. no. 25030)
- Inorganic salts (100×; **Table 4**)
- Amino acids (100×; **Table 4**)
- Vitamin solution (100×; **Table 4**)
- Lactate dehydrogenase cytotoxicity assay kit (Cayman chemical, cat. no. 10008882). It contains LDH diaphorase, NAD+ (100×), lactic acid (100×), LDH standard, cell-based assay buffer tablet, LDH (tetrazolium salt (INT) (100×)).
- Mouse monoclonal microtubule-associated protein-2 antibody (MAP-2; Sigma, cat. no. M2320)
- Rabbit polyclonal neurofilament-M (NF-M) antibody (Millipore, cat. no. AB1987)
- Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, cat. no. A-11001)
- Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen, cat. no. A-21207)
- 4′,6-Diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen, cat. no. D1306)
- ProLong Gold antifade reagent with DAPI (Invitrogen, cat. no. P36931)

EQUIPMENT
- Tabletop centrifuge (Eppendorf, model no. 5417R) with adjustable temperature and speed controls
- Vortex genie 2 (Scientific Industries, model no. G560E) or other suitable vortexing equipment
- ÄKTAexplorer fast protein liquid chromatography (FPLC; GE Healthcare) or any other chromatography system equipped with a UV detector and a fraction collector
- Carry 100-Bio UV spectrophotometer (Varian) or any other UV-VIS spectrophotometer
- Microcell (10-mm pathlength; Varian, part no. 6610010400) or appropriate microcell for UV-VIS spectrophotometry
- Size exclusion chromatography (SEC) column, Superdex 75 HR 10/30 (GE Healthcare, cat. no. 17-1047-01; 1 column volume (CV) = 24 ml)
- SEC column, Superdex 75 pc 3.2/30 (GE Healthcare, cat. no. 17-0771-01; 1 CV = 2.4 ml)
- SEC column, Superose 6 HR 10/30 (GE Healthcare, cat. no. 17-0537-01; 1 CV = 24 ml)
- SEC column, Superose 6 pc 3.2/30 (GE Healthcare, cat. no. 17-0673-01; 1 CV = 2.4 ml)

- Sample loop, PEEK (1 ml; GE Healthcare, cat. no. 18-1114-01)
- PEEK tubing (i.d. 0.75 mm; GE Healthcare, cat. no. 8-1112-53)
- Standard 1.5-ml plastic micro test tube or equivalent (low protein binding) (Eppendorf 3810×; Eppendorf, cat. no. 0030 125.150)
- Sterile microtubes (Fisherbrand, cat. no. 05-669-27)
- HPLC separation module (2795) with PDA detector (Waters) for analytical SEC (Ana-SEC) or any other chromatography system for high-sensitivity measurements
- A Philips CM10 transmission electron microscope (TEM; Philips) equipped with a Morada CCD camera or other TEM setup with image acquisition equipment
- Formvar/carbon-coated TEM grids (Electron Microscopy Sciences, cat. no. FCF200-Cu-50)
- Grid storage box (Electron Microscopy Sciences, cat. no. 71140)
- High-precision ultrafine tweezers (Electron Microscopy Sciences, cat. no. 78318-3X)
- Parafilm (Milian, cat. no. ARA-994)
- Cell culture plates (96-well; BD Falcon, cat. no. 353072)
- Cell culture plates (24-well; BD Falcon, cat. no. 353047)
- Plates, black (384-well; Nunc, cat. no. 262260)
- Glass coverslips (12-mm) or coverslips that use German-made glass (Carolina Biological Supplies, cat. no. BA-62-4734)
- Microdissection forceps (Carolina Biological Supplies, cat. no. 633009)
- HERAcell incubator (Thermo Scientific, model. no. 150) or any other appropriate cell culture incubator (37 °C, 5% CO$_2$ and 100% humidity)
- Precision incubator (Memmert, model no. INB 400) or any other temperature-controlled incubator
- Fluorescence microplate reader (Analyst AD; Molecular Devices)
- Absorbance microplate reader (Safire 2; TECAN)
- Stericup-GP polyethersulfone (PES) filtration unit (0.22 μm, 500 ml; Millipore, cat. no. SCGPU05RE)
- UV/Vis photometer (Eppendorf, cat. no. 6132000.008)
- Microcuvette (Eppendorf, cat. no. 0030106.300)
- Synthetic Aβ42 (as TFA salt; Bachem, cat. no. H-1368)
- rAβ peptides (rPeptides, cat. no. A-1002-2 (for TFA salt) and cat. no. A-1165-2 (for NaOH salt))
- Syringe filter (PES) (0.22 μm; TPP, cat. no. 99722); PVDF or cellulose filter media can also be used for filtering buffers and solutions

REAGENT SETUP

Running buffer for preparative and Ana-SEC Add 10 ml of 1 M Tris-HCl (pH 7.4) to 990 ml of ultrapure H$_2$O. Filter and degas the solution with a vacuum-driven Stericup-GP 0.22-μm PES filtration unit. ▲ CRITICAL Prepare fresh buffer each time. For most of the biophysical studies, Tris buffer can be used with the addition of 0–70 mM NaCl. However, Tris is toxic to neurons, and thus HEPES is used for cell-based assays (70 mM NaCl + 10 mM HEPES (pH 7.4)).

Guanidine hydrochloride solution (6 M) Dissolve 5.8 g of guanidine hydrochloride (analytical grade) in 5 ml of ultrapure H$_2$O by continuous vortexing until a visibly clear solution is achieved. Bring the solution's final volume to 10 ml and store at 4 °C. ▲ CRITICAL Bring the solution to RT before use by leaving the solution on a bench for ~5–10 min. Wear gloves while handling. ■ PAUSE POINT The solution can be stored at 4 °C for 2–3 months. Presence of precipitates warrants immediate disposal.

Tris base solution (2 M; pH 7.6) Dissolve 2.2 g of Tris base in 10 ml of ultrapure H$_2$O and mix until a visibly clear solution is achieved. Bring the pH to 7.6 with dropwise addition of 32% HCl and with continuous mixing. Filter the solution with a 0.22-μm PES syringe filter. ■ PAUSE POINT The solution can be stored at 4 °C for 2–3 months. The presence of precipitates warrants immediate disposal.

UA solution (2% (wt/vol)) Dissolve 200 mg of UA in 10 ml of ultrapure H$_2$O and vortex until a particulate-free solution is achieved. Filter the solution using a syringe-driven 0.22-μm PES filter and store at 4 °C. ■ PAUSE POINT The solution should be prepared as needed and can be stored at 4 °C for 1 month. ▲ CRITICAL Dispose of the solution as per institutional regulations. Wear gloves while handling.

ThT solution (100-μM) Dissolve 0.32 mg of ThT in 10 ml of ultrapure H$_2$O until a particulate-free solution is achieved. Filter the solution using a 0.22-μm PES syringe filter. ■ PAUSE POINT Aliquot the volume needed for the day (Step 12) and store the rest at 4 °C. The solution can be stored at 4 °C for 2–3 months. The presence of precipitates warrants immediate disposal. ▲ CRITICAL Protect from light by wrapping the tube in aluminum foil.

TABLE 4 | Ordering information of the components for modified Neurobasal media.

Component	Supplier	Catalog no.
Inorganic salts		
CaCl$_2$·2H$_2$O	VWR	EM-CX013-1
KCl	VWR	JT3040-1
MgCl$_2$·6H$_2$O	VWR	JT2444-1
NaH$_2$PO$_4$, anhydrous	VWR	JT3821-1
Amino acids in acid		
Isoleucine	Sigma-Aldrich	I7403
Leucine	Sigma-Aldrich	L8912
Phenylalanine	Sigma-Aldrich	P5482
Tryptophan	Sigma-Aldrich	T8941
Tyrosine	Sigma-Aldrich	T8566
Amino acids in H$_2$O		
Alanine	Sigma-Aldrich	A7469
Arginine HCl	Sigma-Aldrich	A6969
Asparagine·H$_2$O	Sigma-Aldrich	A7094
Cysteine	Sigma-Aldrich	C7352
Glutamine	Sigma-Aldrich	G8540
Glycine	Sigma-Aldrich	G8790
Histidine·HCl·H$_2$O	Sigma-Aldrich	H5659
Lysine·HCl	Sigma-Aldrich	L8662
Methionine	Sigma-Aldrich	M5308
Proline	Sigma-Aldrich	P5607
Serine	Sigma-Aldrich	S4311
Theronine	Sigma-Aldrich	T8441
Valine	Sigma-Aldrich	V0513
Vitamin solution	Invitrogen	1087845
NaCl		
D-Ca pantothenate		
Choline chloride		
Folic acid		
I-Inositol		
Nicotinamide		
Pyridoxal HCl		
Riboflavin		
Thiamine HCl		

Glycine-NaOH solution (500 mM; pH 8.5) Dissolve 376 mg of glycine in 10 ml of ultrapure H$_2$O until clear. Bring the pH to 8.5 by adding 1 N NaOH while continuously mixing. Filter the solution with a 0.22-μm PES syringe filter and store at 4 °C. ■ PAUSE POINT Remove the volume needed for the day (Step 5), and store the rest at 4 °C for up to 2–3 months. The presence of precipitates warrants immediate disposal.

EQUIPMENT SETUP

Equilibration of SEC columns Connect the appropriate SEC column (**Table 2**) to an ÄKTAexplorer FPLC or any other equivalent chromatographic

system, equipped with a UV detector and a fraction collector. Equilibrate the column with 10 mM Tris-HCl (pH 7.4) at a flow rate of 0.5 ml min^{-1}.

■ **PAUSE POINT** The columns should be equilibrated with at least two CVs or more (~50 ml for HR 10/30 columns) until a flat baseline UV signal is achieved. For carrying out Aβ fractionation, it is highly recommended to use a chromatography system placed in a 4 °C chamber. Alternatively, Aβ fractionation can still be carried out at RT, provided fractions are immediately transferred to ice (within 5–10 min of eluting from the column).

▲ **CRITICAL** For toxicity studies, the column is equilibrated with 70 mM NaCl + 10 mM HEPES (pH 7.4). The amount of NaCl used can vary from 0 to 70 mM as desired. Test the pH of the eluant to ensure that a pH between 7.2 and 7.6 is obtained. All Aβ preparations and column solutions used for cell culture experiments should be processed to maximize sterility. For storage and sterilization, columns should always be completely washed with 20% ethanol; for cleaning and sterilizing, the columns should be washed with 0.5 M NaOH solution (refer to the column data sheets provided by the manufacturer, for cleaning procedures). The columns can be operated at a higher flow rate (up to 1 ml min^{-1}), provided the maximum operational pressure, as recommended by the manufacturer, is not exceeded.

Equilibration of Superdex 75 or Superose 6 pc 3.2/30 columns for Ana-SEC
Connect the Superdex 75 pc 3.2/30 or Superose 6 pc 3.2/30 column to the FPLC placed in a 4 °C chamber. Equilibrate the column with two CVs (~5 ml for pc 3.2/30 columns) of 10 mM Tris-HCl (pH 7.4) at a flow rate of 0.05 ml min^{-1}. ▲ **CRITICAL** Set the high pressure limit to 2.4 MPa (Superdex 75 pc 3.2/30) or 1.2 MPa (Superose 6 pc 3.2/30) to avoid damaging the column matrix. Cleaning and storage should be carried out as recommended by the manufacturer. Flow rate can be increased up to 0.1 ml min^{-1}, provided the maximum operational pressure, as recommended by the manufacturer, is not exceeded.

Neuronal toxicity studies Obtain cortical neurons from E16 mouse embryos (**Box 1**). Add the cells in plating medium (**Table 5**) to poly-L-Lysine-coated glass coverslips or plastic 24-well plates (**Box 2**) at a density of ~2.5 × 10^5 cells per 0.4 ml per well. Place the plates in a cell culture incubator under conditions of high humidity, 37 °C and 5% CO$_2$. On day 2, fully replace the media with neuronal growth media (**Table 5**) and return the plates to the 37 °C incubator. ■ **PAUSE POINT** Healthy cells should start to develop processes within 24 h of plating and interconnectivity should be observed 2–3 d after plating.

PROCEDURE

1| Fractionation of Aβ by SEC (**Fig. 2**) ● **TIMING** ~ 3–4 h

(A) Obtaining the monomeric form of various Aβ peptides, including Aβ40 and Aβ42

(i) Dissolve 1 mg of Aβ in 1 ml of 6 M guanidine hydrochloride and mix until a visibly clear solution is achieved.
▲ **CRITICAL STEP** By careful visual inspection, ensure that no precipitates are present and almost all of the lyophilized peptide has been dissolved in the solvent.
■ **PAUSE POINT** The vial/tube with the peptide can be centrifuged (3,000*g* at RT for 1 min) before solubilization to bring the lyophilized peptide to the bottom of the vial/tube and minimize peptide loss.

(ii) Centrifuge the solution (16,000*g* for 10 min at 4 °C) in a tabletop centrifuge.

(iii) Carefully draw the supernatant into a 1-ml syringe for subsequent injection for SEC (see below).
▲ **CRITICAL STEP** Avoid taking pelleted material into the syringe, as it will cause pressure problems if injected into the SEC column.

(iv) Inject the supernatant, obtained from Step 1A(iii), into a Superdex 75 HR 10/30 SEC column, using a 1-ml sample loop.
▲ **CRITICAL STEP** Ensure that the column is equilibrated with appropriate running buffer, as outlined in EQUIPMENT SETUP above.

(v) Fractionate Aβ at a flow rate of 0.5 ml min^{-1} with the individual fraction size being 1 ml.
▲ **CRITICAL STEP** Set upper operational pressure limit to 1.8 MPa.
? TROUBLESHOOTING

(vi) Collect and combine the two fractions under elution peak at 11–13 ml (0.45–0.55 CV of the Superdex 75 HR 10/30 column). The combined fractions represent monomeric Aβ.
▲ **CRITICAL STEP** Immediately transfer the fractions to ice or keep at 4 °C, but do not freeze or leave at RT (if SEC is carried out at RT).

(B) Obtaining protofibrillar Aβ42 (or Aβ40-Arctic and Aβ40-Dutch mutants)

(i) Dissolve 1 mg of Aβ in 50 μl of 100% DMSO. Carefully mix with pipette to avoid introducing air bubbles.
▲ **CRITICAL STEP** By careful visual inspection, ensure that no precipitates are present and that all of the lyophilized peptide has been dissolved in the solvent. To avoid Aβ fibrillization, do not spend more than 30–60 s on this step.
■ **PAUSE POINT** To ensure maximal peptide recovery, the peptide vial/tube can be centrifuged (3,000*g* at RT for 1 min) before adding the DMSO, collecting all of the lyophilized peptide at the bottom of the vial/tube.

(ii) Add 800 μl of ultrapure H$_2$O and carefully mix with a pipette.

(iii) Immediately add 10 μl of 2 M Tris-base solution (pH 7.6) and vortex briefly (2–5 s).
■ **PAUSE POINT** This procedure leads to the formation of a mixture of monomer and protofibrillar Aβ42 in solution; however, the amount of protofibrils formed varies from 50–80% depending on the batch of the peptide and solubilization efficiency. To maximize the amount of protofibrils, incubate the solution at RT for 1–5 min.

(iv) Carry out centrifugation and SEC fractionation as described in Steps 1A(ii–v) above.
■ **PAUSE POINT** Aβ protofibrils elute in the void volume (~7–9 ml of elution volume for HR 10/30 columns) and monomeric fractions elute similarly as mentioned in Step 1A(vi).

(v) Combine the two fractions under void volume elution peak (7–9 ml of elution volume) and label as protofibrils. Separately combine the two fractions under elution peak at 11–13 ml (0.45–0.55 CV of the Superdex 75 HR 10/30 column) and label as monomeric Aβ.

BOX 1 | DISSECTING CEREBRAL CORTEX NEURONS

1. Purchase Swiss Webster, time-pregnant mice (Taconic or Charles River) ~E15–16.

■ **PAUSE POINT** This age is optimal for removing meninges, and neurons are predominately postmitotic. Younger and older animals are more difficult to dissect because of problems in removing meninges.

2. Prepare three 60-mm sterile Petri dishes with 3 ml of ice-cold dissection medium (**Table 2**) and place on ice.

■ **PAUSE POINT** Keeping the brain ice cold during dissection improves the recovery of healthy neurons and keeps the brain architecture intact, facilitating microdissection.

▲ **CRITICAL STEP** Prepare in tissue culture (TC) hood.

3. Prepare two 35-mm sterile Petri dishes with 1 ml of dissection medium (**Table 2**) and place on ice.

▲ **CRITICAL STEP** Prepare in TC hood.

4. Mice are killed under the guidelines of the Institution's Animal Care Committee and according to local governmental regulations.

5. After killing the pregnant mouse, place the animal on a clean disposable dipper, ventral side up, and wet down the animal's fur with 70% ethanol.

6. Using a pair of sterile scissors and forceps, cut the abdominal skin along the midline and retract skin to the right and left side.

7. Rinse any fur from the abdominal muscles with 70% ethanol and use a second pair of sterile scissors to cut the abdominal muscles and peritoneum along the midline. Retract the abdominal walls to expose the uterus carrying the embryos, which will be readily observable.

8. Pull out the uterus after cutting the connective tissue and vasculature and transfer to a sterile 100-mm Petri dish placed on ice.

▲ **CRITICAL STEP** When picking out the uterus containing embryos, do not grab and puncture the intestines, which might pose a risk of infecting the embryos with bacteria.

■ **PAUSE POINT** The intact uterus can be removed in a chain-like manner containing all embryos (on an average 8–12 embryos in a Swiss Webster mouse). In the following steps, transfer brain tissue to successive sterile dishes to reduce the possibility of bacterial contamination.

9. Dissect out the individual embryos by cutting open the uterus and amniotic sacks.

10. Using scissors, cut the head off at the neck region directly into the prepared 60-mm dish containing ice-cold dissection medium placed on ice.

11. Under a dissection microscope at ×2 magnification, cut the skin and the developing cranium (membranous and not calcified at this stage) along the midline.

▲ **CRITICAL STEP** Do not cut into the underlying neocortical surfaces.

■ **PAUSE POINT** Less distortion of the brain increases the success of the microdissection.

12. With the brain exposed, scoop out the brain using fine forceps starting at the olfactory bulbs and moving posteriorly.

■ **PAUSE POINT** A pair of forceps is used to disconnect the cranial nerves and lift the brain out as one moves posteriorly. The brain is removed intact and placed into the next clean 60-mm dish containing ice-cold dissection medium. Repeat this process for each of the remaining brains.

13. Transfer one brain into a 35-mm dish with ice-cold dissection medium and remove the meninges. The meninges (easily visible because of their vascularity) are removed as a continuous sheet by grabbing with the tips of the fine forceps and peeling over the front of the brain and then back toward the brainstem.

▲ **CRITICAL STEP** Removing the meninges is an important step to avoid fibroblast contamination of cultures.

14. Using forceps and starting at the fissure between the two hemispheres, roll out the medial part of one hemisphere to reveal the hippocampus.

■ **PAUSE POINT** The hippocampus is at the medial interface of the upper surface of the neocortex and appears as a rostral–caudal curved tubule.

15. Using fine forceps, cut along the neocortex–hippocampus interface and remove the hippocampus.

■ **PAUSE POINT** The hippocampus removed at this stage can be used to prepare hippocampal cultures.

16. With the hippocampus removed and the neocortex rolled a little further laterally, cut with the fine forceps at the underlying neocortex (light, lateral area)–striatum (dark, medial, striated structure) interface. Repeat this step for each side and place the two cortical hemispheres into new 35-mm dishes with ice-cold dissection medium.

■ **PAUSE POINT** The process of isolating each cerebral cortex from each brain is continued and all hemispheres are combined.

17. Carefully remove as much of the dissection medium as possible and then add 1 ml of Trypsin-EDTA, mince with forceps and incubate for 10 min at 37 °C to activate Trypsin.

■ **PAUSE POINT** Trypsin is kept cold before addition to minimize self-inactivation. In addition, Trypsin is frozen as 1-ml aliquots, allowing only the amount needed to be thawed for each dissection.

▲ **CRITICAL STEP** The repeated freezing and thawing of Trypsin, especially at 37 °C, will continually reduce the effectiveness of this protease.

18. While trypsinizing tissue, narrow the opening of a sterile glass Pasteur pipette by flaming the tip.

▲ **CRITICAL STEP** Do not narrow it too much.

19. Slowly triturate the trypsin-containing cortices through the pipette until a homogenous suspension is achieved.

20. Transfer the triturated tissue (should be around 1 ml) to a sterile 15-ml tube and add 1 ml of plating medium containing serum to inhibit the trypsin. The tissue and medium solutions are mixed with the trituration pipette.

BOX 1 | CONTINUED

21. Count the cells using a hemocytometer.
■ **PAUSE POINT** Cells can be incubated with trypan blue before counting to determine the ratio of live to dead cells. Trypan blue is excluded from viable cells.
22. Finally, dilute the cells for plating in the plating media (**Table 2**) at a density of 2.5×10^5 cells per 0.4 ml per well, then add to poly-L-lysine-coated 24-well plates or poly-L-lysine + laminin-coated coverslips (**Box 2**). Place plated cells into an incubator at 37 °C.

▲ **CRITICAL STEP** Immediately transfer the monomeric and protofibrillar fractions to ice or keep at 4 °C, but do not freeze or leave at RT (if SEC is carried out at RT).

(C) Obtaining protofibrillar Aβ40

(i) Dissolve Aβ40 at a concentration of 1 mM (4.3 mg ml^{-1}, based on the total weight of the peptide) in 1 mM NaOH plus sterile phenol red (0.1 mg ml^{-1} to monitor the pH of the solution). The final pH of this solution is ~3.
▲ **CRITICAL STEP** By careful visual inspection, ensure that no precipitates are present and that all of the lyophilized peptide has been dissolved in the solvent.
■ **PAUSE POINT** The vial/tube with the peptide can be centrifuged (3,000g at RT for 1 min) before solubilization to bring the lyophilized peptide to the bottom of the vial/tube and minimize peptide loss.
(ii) Add 140–155 µl of 10 mM NaOH per mg of peptide.
▲ **CRITICAL STEP** To minimize isoelectric precipitation of Aβ (pI ~5.5), which can vary between manufacturers and specific lots, 10 mM NaOH is added to achieve a rapid transition to a pH of ~7.0–7.5. This can be visualized by orange to red color change, when phenol red is used as the pH indicator.
■ **PAUSE POINT** Additional acid or base can be added to achieve the proper pH.
(iii) Dilute the peptide with sterile water and then with 10× PBS to yield a final concentration of 500 µM peptide in 1× PBS (70 mM NaCl, 1.35 mM KCl and 5 mM NaH$_2$PO4/Na$_2$HPO4 (pH ~7.4)).
(iv) Incubate the solution at RT for 2–3 d to generate protofibrils and monomers of Aβ40.
■ **PAUSE POINT** The progression of the aggregation can be checked periodically by injecting small amounts of the mixture to observe the monomer to protofibril ratio.
(v) Carry out centrifugation and SEC fractionation as described in Step 1A(ii–v) above.
(vi) Combine the two fractions under void volume elution peak (~7–9 ml of elution volume for the HR 10/30 columns) and label as protofibrils. Separately combine the two fractions under elution peak at 11–13 ml (0.45–0.55 CV of the Superdex 75 HR 10/30 column) and label as monomeric Aβ.
▲ **CRITICAL STEP** Immediately transfer the monomeric and protofibrillar fractions to ice or keep at 4 °C, but do not freeze or leave at RT (if SEC is carried out at RT).

(D) SEC for subfractionating protofibrils

(i) Centrifuge the Aβ peptide solution (16,000g for 10 min at 4 °C) obtained from Step1B(iv) and inject the supernatant, using a 1-ml sample loop, into a Superose 6 HR 10/30 SEC column.
(ii) Fractionate at a flow rate of 0.5 ml min^{-1}, with the individual fraction size being 1 ml.
▲ **CRITICAL STEP** Set upper pressure limit to 1.5 MPa.
(iii) Collect four 1-ml fractions, corresponding to the elution volume of 6–11 ml, which contain the total protofibril peak, and do not combine. The monomer fractions (elution volume 14–16 ml) can be combined.
▲ **CRITICAL STEP** Immediately transfer the fractions to ice or keep at 4 °C, but do not freeze or leave at RT (if SEC is carried out at RT).
■ **PAUSE POINT** Fractionation efficiency can also be increased by connecting two SEC columns in series (such as two Superose 6 HR 10/30 columns or a

TABLE 5 | Media for dissection and culture of neuronal cells.

Component	Volume (ml)
Dissection medium	500
5 ml of 1 M HEPES	5
5 ml of 1 M glucose	5
5 ml of 1 M sucrose	5
50 ml of 10× Hank's salt	50
Cell culture–grade H$_2$O	435
Plating medium	500
1× DMEM (without glutamine)	50
FBS	50
F-12	50
1 M HEPES	10
Glutamine	5
Pen-strep (100×)	5
H$_2$O	330
Growth medium for neuronal cultures	100
Neurobasal medium	98
B-27	2

Abbreviations: HEPES, (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; Pen–Strep, Penicillin–streptomycin.

BOX 2 | COATING COVERSLIPS AND 24-WELL PLASTIC PLATES

Neurons are plated onto either 24-well plastic plates for biochemical evaluation of cell viability or onto glass coverslips for histological observations.

1. Sterilize coverslips with 100% ethanol for a minimum of 2 h or overnight in a sealed sterile dish.
▲ **CRITICAL STEP** Do not use 70% ethanol, which will lead to coverslips being difficult to separate and potential breakage.
2. After sterilization, suction off ethanol and air-dry the coverslips in a sterile environment (e.g., in a TC hood).
3. Place one coverslip per well; a 12-mm coverslip fits into the well of a 24-well plate.
▲ **CRITICAL STEP** Low evaporation plates are necessary to minimize the liquid loss in the outside wells.
4. Prepare substrate solution to coat glass coverslips by combining poly-L-lysine (PLL) (MW 331K) + laminin solution. To obtain 10 ml of substrate solution, add 1 ml of PLL solution (1 mg ml^{-1}) and 0.1 ml of laminin solution (1 mg ml^{-1}) to 8.9 ml sterile, high-quality water.
■ **PAUSE POINT** Laminin is not needed if coating is carried onto plastic surfaces.
5. Add 300 µl to each well containing a coverslip (requiring 7.2 ml per 24-well plate). Wrap plates in aluminum foil and incubate overnight at RT.
6. Next day, wash plates three times with sterile water and then air-dry in TC hood.
■ **PAUSE POINT** Plates that are not used immediately can be stored at 4 °C, properly wrapped in aluminum foil.

TSK4000 SEC column connected in series to Superose 6 HR 10/30). However, in the latter case, to obtain Aβ fractions at concentrations above 20 µM, 1.5–2 mg ml^{-1} peptide should be solubilized for fractionation.

Concentration determination

2| The protein concentration of the fractions can be determined by different methods depending on the instrumentation available and desired accuracy. Aβ concentration can be determined by measuring absorbance at 280 nm using a UV spectrophotometer or any of the commercially available protein assays (such as BCA assay). Both of these methods are readily available, but may contain marginal error. For measurements requiring high precision, amino-acid analysis (AAA) is recommended. The latter method requires sending the samples to a specialized facility, and requires a few hours/days to complete. If information regarding the absolute concentration of Aβ fractions is critically needed, we recommend using one of the methods described below to determine the concentration for short-term reference, and sending some of the original solution for AAA. If the concentration determined by AAA is different from that determined by UV or BCA, the latter is then corrected accordingly. We have carried out a comparison of these methods for measuring Aβ concentrations and the findings are summarized in **Box 3**. **Box 3** also contains some assay-related information for UV A_{280} nm and BCA, in the context of Aβ concentration estimations.

(A) Using UV spectrophotometry ● **TIMING** ~10 min

   (i) Stabilize the light source of the spectrophotometer by turning on the lamp for ~10–15 min before its use. Obtain the UV absorbance of SEC running buffer (10-mM Tris-HCl (pH 7.4)), at wavelength (λ) 280 nm (A_{280}), by placing 100 µl of the buffer in a 10-mm-path-length cell. Set the reading of the spectrophotometer to zero using the unadulterated running buffer. Thereafter, obtain UV absorbance at λ280 nm for the different Aβ fractions.

   (ii) Calculate the molar concentration of Aβ fractions by dividing A_{280} values by the factor of 0.0015 (**Box 4**).

   (iii) Adjust the concentration of individual fractions as appropriate for desired biophysical studies (10–20 µM for Steps 3, 5 and 11) or as appropriate for biological assays (Step 15) in SEC running buffer.
     ■ **PAUSE POINT** For neuronal toxicity studies, refer to **Table 6** for sample preparation.

(B) Using BCA method ● **TIMING** ~45 min

   (i) For generation of a standard curve, dissolve 1 mg ml^{-1} of lyophilized Aβ40 (200 µM, based on dry weight) in high-purity H$_2$O by continuous vortexing.
     ▲ **CRITICAL STEP** Aliquot 200 µl of this stock Aβ solution per tube and store at −20 °C until needed. Use sterile tubes (Fisherbrand, cat. no. 05-669-27). Avoid repeated freezing and thawing of the peptide solution. Thaw the stock solution on ice.
     ■ **PAUSE POINT** Most of the sAβ is provided as peptide content of ~80% by dry weight. Check the peptide content from the Aβ supplier and adjust accordingly.

   (ii) Prepare the 'working reagent' (WR) provided in the BCA kit (follow manufacturer's instructions).

   (iii) Using Aβ40 stock (200 µM), prepare 50 µl of Aβ40 protein at standard dilutions of 5, 10, 20, 30, 40, 50, 75, 100, 150 and 200 µM in high-purity H$_2$O for generating a standard curve.

   (iv) In a clear-bottomed 96-well microplate, add 200 µl of WR to wells that will receive either unknown Aβ samples or Aβ standards.

   (v) Add 25 µl of each Aβ standard to the appropriate well already containing WR.
     ▲ **CRITICAL STEP** Use H$_2$O as blank control.
     ■ **PAUSE POINT** Each standard concentration should be tested with a minimum of duplicates.

BOX 3 | COMPARISON OF UV $A_{280\,nm}$, BICINCHONINIC ACID (BCA) ASSAY AND AMINO-ACID ANALYSIS (AAA) FOR DETERMINATION OF Aβ CONCENTRATION

We have outlined two commonly available methods of protein concentration determination, namely UV A_{280} and BCA, in Step 2 of this protocol. However, the following points are worth mentioning in the context of Aβ concentration estimations:

1. *UV A_{280}*: The method relies on the molar absorbance coefficient for a particular protein (**Box 4**). Theoretically, the concentration of monomeric protein can be reliably assessed; however, the concentration of protofibrils contains a marginal error (as protofibrils do not have the same molar extinction coefficient as monomer). Therefore, another assay such as BCA can be used to increase the reliability of protofibril concentration determined by UV A_{280}. In addition, we have also observed that Aβ concentrations below ≤20 μM are underestimated by the UV A_{280} method than by BCA (see below). The most likely reason for this is the scarcity of aromatic residues in the Aβ amino-acid sequence.

2. *BCA*: The majority of commercial kits for determining protein concentrations (such as the PIERCE BCA kit) include BSA for generating a standard curve. The concentration of protein in unknown samples is determined using this standard curve. We found that a BSA standard curve results in ~40–50% underestimation of Aβ concentration in the fractions, compared with a standard curve based on Aβ40 serial dilutions (**Supplementary Fig. 4b**). Moreover, there was close correlation between Aβ concentration estimations by two assays, when we used a standard curve based on Aβ40 serial dilutions. Therefore, we encourage the use Aβ40 standard curve for Aβ concentration estimations and not BSA. In contrast to Aβ42, Aβ40 is soluble in many aqueous media and less prone to aggregation. A 200-μM Aβ40 stock solution can be used for 10 serial dilutions (5–200 μM) with only ~200 μl of Aβ40 stock needed for one assay. Accordingly, repeated freeze and thaw cycles can be avoided by aliquoting the stock solution (200 μl per aliquot) and thawing it on ice. Freezing and thawing Aβ may also introduce unwanted artifacts and interfere with the protein determination. To address this, we also carried out BCA assay on Aβ40 solutions, which were either freshly prepared or aliquoted and frozen. Data show that both fresh and frozen Aβ40 solutions yield comparable results by BCA (**Supplementary Fig. 4c**). However, repeated freezing and thawing of the stock Aβ solution may introduce greater variablility and should be avoided.

3. *Comparing AAA, BCA and UV$_{280}$*: Aβ concentration estimations by UV A_{280} or BCA may contain a marginal error due to some limitations posed by the basic principles of each of these assays. AAA, however, is free of such limitations and provides a quantitative estimate of concentration and is more reliable. A comparative analysis of the three methods mentioned is summarized in **Supplementary Figure 4a**. Briefly, (i) BCA measurements were found to be ~10–20% higher than UV measurements (Aβ40 standard curve was used for BCA); and (ii) UV A_{280} measurements were ~10% higher than AAA measurements. This applied to both monomeric and protofibrillar fractions.

(vi) Add 25 μl of each Aβ sample of unknown protein concentration to the appropriate well already containing WR.
   ▲ **CRITICAL STEP** Use SEC running buffer as a blank control.
   ■ **PAUSE POINT** Each sample should be tested using a minimum of duplicates.

(vii) Cover the plate and incubate at 37 °C for 30 min.

(viii) Read the absorbance at λ562 nm.
   ■ **PAUSE POINT** If a microplate reader is not available, a suitable UV/Vis photometer (for example Eppendorf, cat. no. 6132000.008) can also be used. For this purpose, use a microcuvette (such as Eppendorf, cat. no. 0030106.300) to read each sample and proceed as outlined below.

(ix) Subtract H_2O absorbance values from the standards and SEC running buffer absorbance from Aβ samples.
   ■ **PAUSE POINT** This step is required only if BCA is performed using a microplate reader.

(x) Determine the concentration of Aβ in each fraction (μM) from the standard curve using Microsoft Excel or any other suitable software.

(xi) Adjust the concentration of individual fractions as appropriate for desired biophysical studies (e.g., 10–20 μM for Steps 3, 5 and 11) or as appropriate for biological assays (Step 15) in SEC running buffer.
   ■ **PAUSE POINT** For neuronal toxicity studies, refer to **Table 6** for sample preparation.

Analytical SEC ● **TIMING** ~1 h for one sample
3| Inject 50 μl of each Aβ fraction (protofibrils and monomer) into a Superdex 75 pc 3.2/30 SEC column connected to a Waters 2,795 separation module or any other chromatography system with high-sensitivity detectors.

BOX 4 | DETERMINATION OF Aβ CONCENTRATION BY UV $A_{280\,nm}$

Aβ concentration determination by UV spectrophotometry is based on the Beer–Lambert law: $c = A \div \varepsilon \times d$

In the equation above, 'c' denotes the molar concentration, 'A' is the UV absorbance at a particular wavelength, 'ε' is the molar extinction coefficient at that wavelength and 'd' is the path length of the UV cell. Keeping the path length as 1 cm (10 mm) and the molar extinction coefficient at 280 nm ($\varepsilon 280 = 1490$ M^{-1} cm^{-1})[86], the A_{280} of 1 mg ml^{-1} Aβ is predicted to be 0.33. In terms of molar concentration, this corresponds to ~221 μM Aβ42 and ~231 μM Aβ40.

■ **PAUSE POINT** Protofibrillar fractions can also be analyzed by injection into a Superose 6 pc 3.2/30 column. The elution of protofibrils on a Superdex 75 pc 3.2/30 column provides a measure of protofibrillar content rather than size distribution. In contrast, on a Superose 6 pc 3.2/30, protofibrils elute as a broad peak in the void volume + included volume, thus allowing for analysis of heterogeneity and progression of aggregation.

4| Elute the fraction at a flow rate of 0.05 ml min^{-1} for 60 min.

▲ **CRITICAL STEP** Set the high pressure limit to 2.4 MPa (Superdex 75 pc 3.2/30) or 1.2 MPa (Superose 6 3.2/30).

■ **PAUSE POINT** Although the PDA detector monitors sample elution at a range of wavelengths (200–400 nm), for analytical purposes, $A_{210-214}$ nm is adequate. Samples can also be eluted at flow rates of up to 0.1 ml min^{-1}, provided the maximum pressure limit recommended by the manufacturer is not exceeded.

? TROUBLESHOOTING

Morphological characterization by TEM ● TIMING ~20 min for one sample

5| Deposit 2–10 μl of a sample containing Aβ on a formvar-coated TEM grid and let the sample droplet settle for ~60 s.

▲ **CRITICAL STEP** Place Aβ samples on TEM grids as soon as possible after fractionation to be able to assess the aggregation state of the starting material.

■ **PAUSE POINT** If the sample is ≥20 μM, deposit between 2 and 5 μl; for samples <20 μM, deposit 5–10 μl. The grids can also be glow discharged, which improves subsequent sample deposition on the surface.

6| Remove excess solution by wicking the edge of the grid on a piece of blotting paper.

7| Deposit a 10-μl drop of 2% UA on the grid and let it settle for ~60 s

8| Remove excess solution by wicking the edge of the grid on a piece of blotting paper.

9| Gently vacuum dry the grid from the edges.

▲ **CRITICAL STEP** Do not touch the grid with vacuum probe to avoid physical damage to the grid. Use the vacuum to produce an air current over the grid to evaporate the remaining liquid.

■ **PAUSE POINT** Grids can be placed in a storage box in a dry place. Storing in a desiccating chamber or box will help prevent moisture accumulation and sample degradation.

10| Acquire images on a CM10 TEM (or equivalent TEM), equipped with a CCD camera, operated at an acceleration voltage of 80–100 kV.

? TROUBLESHOOTING

Assessment of fibril formation kinetics by ThT binding ● TIMING ~10 min for one time point

11| For fibrillization studies, aliquot 350–500 μl of each Aβ fraction into two 1.5-ml sterile microtubes.

▲ **CRITICAL STEP** The sample volume should be ≥300 μl in a 1.5 ml polypropylene sterile microtube. Smaller volumes generate more errors during different aggregation experiments. Tubes larger than 1.5 ml also pose a problem because of increased evaporation.

12| For ThT-binding measurements, mix 80 μl of 10 μM Aβ fraction with 10 μl of 100 μM ThT and 10 μl of 500 mM glycine–NaOH (pH 8.5) in one well of a 384-well black plate.

▲ **CRITICAL STEP** Avoid generating air bubbles as they will interfere optically with fluorescence signal acquisition.

■ **PAUSE POINT** This step is described for a final concentration of Aβ of ~10 μM, a ThT:Aβ ratio ≥1 and a final glycine–NaOH concentration of 50 mM. The step can be modified to accommodate different concentrations of Aβ. For example, for a sample

TABLE 6 | Preparation of Aβ fractions for neuronal toxicity assessment.

Components	Stock	Volume (μl)
Aβ fraction	—	893.8
NaCl	2M	3.6
Amino acids in H$_2$O	100×	10.0
Vitamins	100×	10.0
Amino acids in HCl	100×	10.0
Inorganic salts	100×	10.0
HEPES	100×	10.0
Pen-strep	100×	10.0
Glucose	1 M	25.0
Phenol red	0.5%	1.6
NaOH	1 M	7.0 (final pH 7.3–7.5)
Sodium bicarbonate	1 M	26.0
Total volume		1,000 μl

Abbreviations: HEPES, (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; Pen–Strep, Penicillin–streptomycin.

of 50-µM Aβ, either dilute the sample with sample buffer to 10-µM and proceed as above or use 70 mM glycine–NaOH stock-solution (20 µl Aβ + 70 µl glycine–NaOH + 10 µl ThT (100 µM)). Each sample should be assayed at least twice.

13| Acquire ThT fluorescence values using an Analyst AD fluorometer or a comparable fluorescence plate reader using an excitation wavelength of λ450 nm and an emission wavelength of λ485 nm.

14| Close the lids of microtubes and incubate at 37 °C without agitation in a Precision incubator.
■ **PAUSE POINT** Acquire ThT fluorescence data for 96 h at 24 h intervals or as desired.
▲ **CRITICAL STEP** Briefly vortex the fractions before acquiring ThT fluorescence at each time point.
? TROUBLESHOOTING

Assessment of toxicity in primary neuronal cultures ● TIMING ~1 h–5 d (depending on the assays used)
15| On days 5–7 after cell plating, Aβ fractions from SEC are mixed with components of a modified neurobasal media (**Table 6**). Cultured neurons are exposed to various Aβ aggregates by removing all the media and adding 0.4 ml per well of the SEC fraction mixed with modified neurobasal media.
■ **PAUSE POINT** The eluate from the SEC will not sustain the viability of the cultured brain cells. Therefore, TC components need to be mixed with SEC fractions to correct sodium and potassium levels, provide energy and provide the correct pH. Standard TC medium can be purchased, but SEC fractions might be low in Aβ concentration, thus necessitating minimal further dilution of the SEC fraction by TC media. To accommodate this, modified neurobasal media are used to add TC components in minimal volumes (**Table 3**). Neurobasal medium was developed to specifically support neurons[79]. Using this formulation, TC components can be varied for experimental purposes. The TC components should be added to SEC fractions shortly before application to cells.

16| Remove media from neuronal cultures and replace with Aβ fractions containing TC components and place the cells back in a 37 °C incubator with 5% CO_2.

17| After 1 h to 5 d, assess the effects of Aβ treatment on neurons using biochemical methods such as LDH release assay and/or immunocytochemistry methods. LDH is a cytosolic enzyme that is primarily released on loss of integrity of the cell membrane and is a reliable indicator of cell death. The LDH release is proportional to the number of dead cells that can be measured in the culture medium. Alternatively, neurons plated on glass coverslips can be fixed and stained with antibodies to detect changes in neuronal architecture. The LDH release assay cannot detect cell loss, nor can it detect minor structural changes that may have important repercussions for the function of the neuron. Loss of synaptic connections would render a neuron as dysfunctional as neuronal death. The antibodies used here can also distinguish between changes in the dendritic (MAP2) versus axonal (NF-M) compartments.
(A) LDH release assay to determine cell death
  (i) Prepare 100 ml of buffer by adding one buffer tablet to 100 ml of distilled water.
  (ii) Prepare LDH diaphorase solution by adding 150 µl of buffer and placing it on ice.
    ▲ **CRITICAL STEP** Extra solution can be frozen, but aliquoted to go through only one freeze–thaw cycle.
 (iii) Prepare LDH reaction solutions by adding 100 µl of the following four components to 9.6 ml of buffer.
    (a) LDH diaphorase (see Step 17A(ii))
    (b) NAD 100× (provided in the kit)
    (c) Lactic acid 100× (provided in the kit)
    (d) INT 100× (tetrazolium salt) (provided in the kit)
  (iv) Prepare the LDH standard by diluting the vial included in the kit with 1.8 ml of buffer and placing immediately on ice.
    ▲ **CRITICAL STEP** The LDH standard is not stable and the remaining solution should be stored at −80 °C. The enzyme should only go through one freeze–thaw cycle.
  (v) Dilute LDH standard in the ratio 1:20, 1:40, 1:80, 1:160 and 1:320, thus giving a standard curve of 10, 5, 2.5, 1.25 and 0.75 mU ml^{-1}, respectively.
    ■ **PAUSE POINT** 1 U of standard converts 1 µmol of substrate per min.
  (vi) Set up a standard curve in a 96-well plate by adding 100 µl of standard to two or three wells for each concentration. Include a zero concentration by adding 100 µl of the buffer prepared in the first well.
    ▲ **CRITICAL STEP** If serum is used in an experiment, it will contain LDH and could interfere with the assay.
 (vii) Remove 100 µl of the medium from the experimental wells and transfer to the plate containing standards.
(viii) Using the reaction solution prepared in Step 17A(iii), add 100 µl to each of the standard and experimental wells.
  (ix) Shake for 30 min at RT. Using a 96-well microplate reader (TECAN safire 2 or any other suitable microplate reader), measure absorbance at λ490 nm. A standard curve of the LDH activity versus absorbance can be generated. The absorbance of the experimental sample can be used to determine LDH activity.

■ **PAUSE POINT** The standard curve should be linear in the range of standards and the experimental samples should fall into this range. If not, higher samples must be diluted to bring them into the linear range of the standard curve.

■ **PAUSE POINT** The assay is based on Cayman Chemical Company's LDH assay and is outlined for measuring all wells in a 96-well plate. The assay for all standards and samples should be carried out either in duplicate or in triplicate. If more than one plate is needed, it is recommended to add standards to all plates that are being used.

(B) Immunocytochemical staining of neurons

(i) Fix cells in 4% paraformaldehyde in 0.15 M PO_4 (Sorenson's phosphate buffer plus 0.1% sodium azide) for 20 min.

  ! CAUTION Paraformaldehyde is toxic.

(ii) Wash cells three times for 5 min each with phosphate buffer.

(iii) Block the neurons with 10% FBS + 0.3% Triton X-100 in 0.15 M PO_4 for 1 h at RT.

(iv) Wash three times for 5 min each with phosphate buffer.

(v) Dilute both primary antibodies in the ratio 1:500 in 0.15 M PO_4, apply to cells and incubate for 1 h at RT. The MAP2 and NF-M antibodies can be incubated together, as one is a monoclonal (raised in mouse) and the other is a polyclonal antibody (raised in rabbit).

(vi) Wash three times for 5 min with phosphate buffer.

(vii) Dilute fluorescent secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 donkey anti-rabbit IgG) in 0.15 M PO_4. Apply them onto the cells and incubate for 1 h at RT.

  ▲ **CRITICAL STEP** To avoid photobleaching the dye, cells should be kept in the dark during incubation and after washing.

(viii) Mount coverslips on slides with ProLong Gold mounting medium containing antifade reagent and the nuclei stain, DAPI.

  ■ **PAUSE POINT** Antifade reagent helps suppress photobleaching. Coverslips are allowed to dry overnight, whereas plates can be assayed directly.

  ■ **PAUSE POINT** Cultures can be visually monitored to determine when the experiment should be stopped depending on the response(s) of interest (for example, neuronal changes versus cell death). Small volumes of the medium can be tested from living cultures to determine whether cell death is occurring (such as by the release of LDH (Step 17A)). If the morphological changes in neurons are important, the entire medium at that time point is collected and the neurons are immediately fixed and stained (Step 17B). Morphological changes can be seen using the Aβ40-Arctic peptide as early as 5 h, whereas Aβ40 wt peptide-induced cell loss could require 3–5 d.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 7**.

TABLE 7 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1, 3 and 4	High column pressure	1. Precipitates or particulate matter	1. Ensure adequate peptide solubility and proper centrifugation to remove precipitates
		2. Column not properly equilibrated and contains traces of ethanol or other buffer with high viscosity	2. Ensure proper column equilibration (2–3 CVs)
		3. Aβ, or other hydrophobic peptides, sticking to the column from previous runs	3. Try extensive cleaning with 0.5 M NaOH
		4. Very long (>10 cm) or narrow diameter PEEK tubing	4. Use tubing of minimal length and 0.75 mm internal diameter
		5. Column matrix is degenerating	5. Use new columns or try extensive cleaning with 0.5 M NaOH
5–10	Clustering of Aβ aggregates on TEM grid	Decreased hydrophilicity of carbon coating; contamination with organic molecules etc.	The TEM grids should be treated with glow discharge before sample application
11–14	ThT values become stationary or decrease after initial rise[a]	1. Lower than critical Aβ concentration for fibrillization	1. Check the Aβ concentration in samples and use a higher concentration, if too low, than that initially estimated
		2. Increased evaporation from the tube, or low sample volume	2. Use a sample volume that leaves at least 100–200 μl at the end of measurement
		3. Clustering of fibrils, reducing the access of ThT to binding sites on fibrils	3. Vortex and/or sonicate in a water bath briefly (~30 s) before the assay

Abbreviations: Aβ, amyloid-β; CV, column volume; TEM, TEM, transmission electron microscopy; ThT, Thioflavin-T.
[a]This can also be a normal phenomenon, if free Aβ monomers are scarce and no further elongation of fibrils is taking place.

● **TIMING**

Step 1, Fractionation of Aβ by SEC: ~3–4 h (~1–2 h column equilibration, ~15 min solubilization and centrifugation and ~1–2 h fractionation)

Step 2, Aβ concentration determination: ~10 min by UV spectrophotometry and ~ 45 min by BCA

Steps 3 and 4, Analytical SEC: ~1 h for one sample

Steps 5–10, TEM: ~20 min for one sample (~5 min for preparing the grid and ~15 min for image acquisition)

Steps 11–14, ThT binding: ~10 min for one time point

Steps 15–17, Assessment of toxicity in neuronal cultures: ~ 1 h–5 d (depending on the assays used)

ANTICIPATED RESULTS

This paper describes a step-by-step method, principally based on SEC, for generating preparations of Aβ monomers and protofibrils, as well as fibrils, from various Aβ peptide sequences. This method has been used extensively by our group and by many other researchers in the field to elucidate Aβ amyloid formation, investigate the nature of the toxic Aβ species and develop amyloid-based diagnostic tools and therapeutic strategies for AD. This paper provides a convenient reference for the AD research community for reliable preparation and isolation of these different Aβ aggregate states for structural and functional studies.

Preparation and characterization of monomeric Aβ

Fractionation. Lyophilized Aβ peptides, obtained from different sources, are easily solubilized using 6-M guanidine hydrochloride, and the resultant solution contains predominantly monomeric Aβ (**Fig. 3**). Insoluble preexisting aggregates, if present, are effectively removed with brief centrifugation (≥16,000g, ~10 min). When this guanidine hydrochloride solution of Aβ is injected into a Superdex 75 HR 10/30 SEC column, the peptide elutes as a single peak in the included volume of SEC column (0.45–0.55 CV for Superdex 75 HR 10/30; elution volume 11–13 ml) corresponding to predominantly monomeric Aβ (**Fig. 3a**—red line). Generally, 1 mg of Aβ40 or Aβ42 (~80% net weight) dissolved in 1 ml of guanidine hydrochloride yields ~2 ml at final concentrations of ~50–80 μM. Previous translational diffusion measurements of these Aβ elution fractions, by pulse field gradient solution phase NMR, showed that Aβ predominantly elutes as a monomer[80]. The presence of a small amount of LMW oligomers (dimers, trimers, and so on), which exist in rapid equilibrium with the monomer, has been suggested and cannot be ruled out[53,73]. However, these oligomeric species do not seem to accumulate or exist in stable forms, unless stabilized by chemical crosslinking[17].

Characterization. Reinjection of purified monomers into a Superdex 75 pc 3.2/30 SEC analytical column showed a single elution peak and confirmed that the fractions corresponded to monomeric (M)
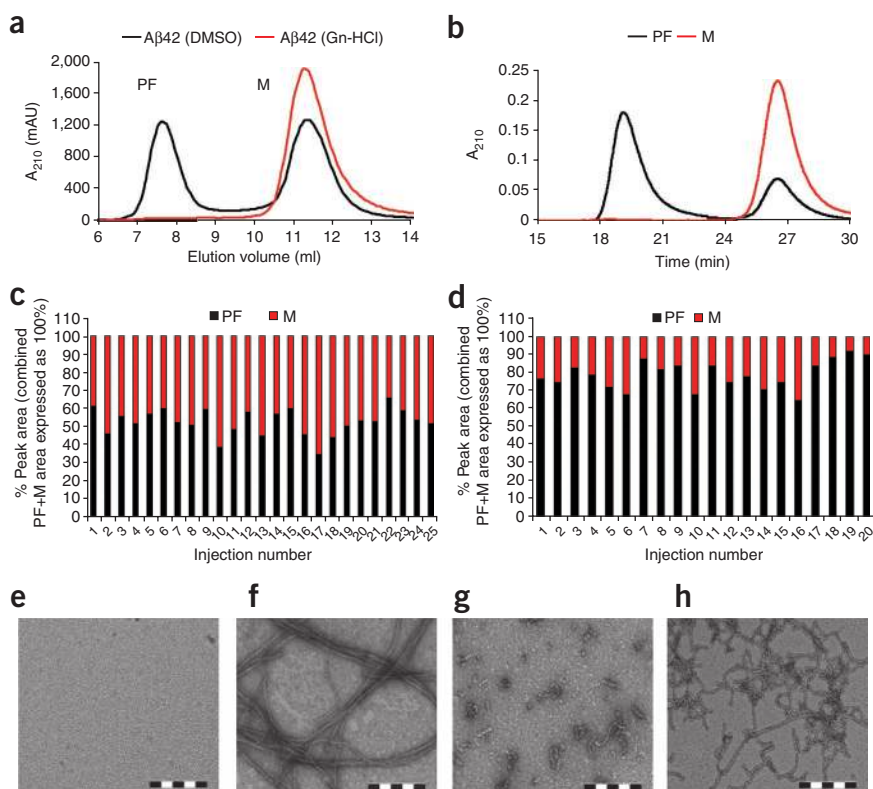
Figure 3 | SEC fractionation and biophysical characterization of Aβ42 monomers and protofibrils. (**a**) Fractionation of Aβ42 into protofibrillar (black line—using DMSO solubilization method) or monomeric (M) fractions (red line; using 6 M guanidine hydrochloride solubilization method) on a Superdex 75 HR 10/30 SEC column. (**b**) Analytical SEC (Ana-SEC) of protofibrillar (black line) and monomeric Aβ42 (red line; using 6 M guanidine hydrochloride solubilization method) on a Superdex 75 pc 3.2/30 SEC column. (**c**) Statistical analysis of the mean peak area for the elution of protofibrils and monomeric Aβ42 (using DMSO solubilization method) on a Superdex 75 HR 10/30 SEC column from previous 25 injections. For each injection, the total (PF + M) peak area was expressed as 100% (n = 25; PF:M ratio = 52:48; % s.d. from the mean = PF (0.8–18) and M (0.1–13)). (**d**) Statistical analysis of the mean peak area for the elution of monomer from SEC purified protofibrils, after re-injection into a Superdex 75 pc 3.2/30 for Ana-SEC, from previous 20 injections. For each injection, the total (PF + M) peak area was expressed as 100% (n = 20; PF:M ratio = 79:21; % s.d. from the mean = PF (0.95–14) and M (0.05–13)). (**e,f**) Representative TEM images of monomeric Aβ42 fractions soon after SEC (**e**) and fibrils formed by monomeric Aβ42 after 96 h of incubation at 37 °C (10 μM Aβ42, without agitation) (**f**). (**g,h**) Representative TEM images of Aβ42 protofibrils soon after SEC (**g**) and fibrils formed by Aβ42 protofibrils after 96 h of incubation at 37 °C (10 μM Aβ42, without agitation) (**h**). PF, protofibrils; M, monomeric Aβ; scale bar, 200 nm.

elution (**Fig. 3b** red line; peak around ~26 min). TEM analysis of monomeric fractions, soon after fractionation, confirmed the absence of any aggregated Aβ in these preparations (**Fig. 3e**) (monomeric Aβ is not detectable by TEM). Circular dichroism (CD) analysis followed by spectra deconvolution using CCA+ software Lincomb algorithm[81,82] was carried out to assess the secondary structure elements of the Aβ fractions. CD analysis of this preparation showed a spectrum with minima at ~195–205 nm, consistent with a predominantly random coil conformtion (**Supplementary Fig. 1a**). Deconvolution of the spectrum suggested a mixture of predominanly (60%) random coil conformation and 39% β-turn structure. SDS-PAGE and the subsequent silver staining and/or western immunoblotting with 6E10 antibody showed that SEC-isolated monomeric Aβ40 migrated as a single band. In contrast, monomeric Aβ42 fractions migrated as a mixture of monomers, dimers and trimers, with monomer being the predominant species under similar conditions of electrophoresis (**Supplementary Fig. 1b**). Previous studies have suggested that this anomalous migration of Aβ42 on SDS gels is a result of SDS-induced Aβ oligomerization because of the effect of SDS micelle formation and high local concentration of Aβ in micelles[73]. In addition, it is also possible that Aβ42 at higher than critical concentrations, and because of its high aggregation tendency, exists in equilibrium with LMW oligomers (dimers, trimers and low n-mers) seen on the gels[53,73].

Briefly, the SEC method outlined above ensures that the starting material of the experiment is predominantly monomeric Aβ, free of protofibrillar or fibrillar aggregates and can be obtained in solutions free of organic solvents and compatible with cell culture manipulations. The extent of oligomerization and the amount of LMW oligomers formed is strongly dependent on the Aβ peptide sequence, the Aβ concentration and the incubation temperature and time. Therefore, we recommend the immediate use of SEC fractions or the maintenance of samples at 4 °C until use. Although we have not observed significant protofibril formation by TEM and ThT binding, when these solutions are stored at 4 °C for up to 24 h (**Supplementary Fig. 3c,i**), we cannot rule out that the oligomerization and formation of LMW oligomers take place under these conditions. This is especially critical when Aβ42 and Aβ42/Aβ40-Arctic mutant (E22G) are used and stored at concentrations >50 μM. Therefore, we recommend that peptide solutions be prepared freshly before performing the experiments, maintained at 4 °C and used within a few hours (<3–5 h). If the experimental setup and conditions do not allow for rapid preparation and the immediate use of the monomeric Aβ fractions, we recommend that the samples be stored at 4 °C. To ensure the absence of fibrillar or protofibrillar aggregates at the time of using a stored sample, a small aliquot can be centrifuged and evaluated by Ana-SEC, ThT and TEM.

Preparation and characterization of Aβ42 protofibrils

Fractionation. To generate protofibrils of Aβ42, or Aβ sequences with a high aggregation tendency (like Aβ-Arctic), the peptide is solubilized using the DMSO method[18] (Step 1B). This method is very effective in yielding a solution enriched with protofibrils of broad size distribution[18]; however, this preparation also contains a significant amount of monomeric Aβ that must be separated from the protofibrils. Fractionating this preparation on a Superdex 75 HR 10/30 SEC column yields two elution peaks separated by ≥2 ml of elution volume. The first peak corresponding to the void volume (~7–9 ml of elution volume for the HR 10/30 columns) is enriched with protofibrils (PF), whereas the second elution peak corresponds to monomeric (M) Aβ42 (**Fig. 3a**, black line). When 1 mg of Aβ42 (~80% net weight) is solubilized using the DMSO method (860 μl of total solution) and fractionated as mentioned, the typical yield is as follows: PF ~2 ml, ~40–60 μM of final Aβ concentration and M ~2 ml, ~40–50 μM of final Aβ concentration.

Reproducibility of fractionation. Generally, when Aβ42 is solubilized in DMSO and fractionated, the PF peak area and height are almost always greater than the M peak, but the absolute values are not identical from one injection to another. To illustrate this, analysis of Aβ42 protofibrillar preparations (using the DMSO method), carried out by three different individuals in our group over the past 2–3 years, is presented in **Figure 3c**. The data show that there is occasional variation between the extent of PF and M elution. For each injection, the combined (PF + M) peak area was expressed as 100%. The percentage (%) mean peak area for PF and M elution was calculated and the results confirmed that the usual trend of Aβ42 elution is PF > M (the mean peak area ratio of PF to M = 52:48; % s.d. from mean: PF = 0.8–18, M = 0.1–13; $n = 25$). For Aβ40 or other less-aggregation-prone Aβ sequences, the M elution almost always predominates (**Supplementary Fig. 1f**). Despite these slight variations, structural analysis of Aβ42 PF and M fractions did not exhibit any drastic differences (data not shown). However, these different elution patterns produce variations in the concentration of Aβ fractions.

Characterization. Protofibrils were obtained as described above and subjected to further analysis. TEM analysis of the protofibril fractions showed aggregates of various morphologies including curvilinear aggregates (<200 nm long) and small spherical structures (**Fig. 3g**). These observations were consistent with previously described morphologies for protofibrils[12,13]. Protofibrils, on reinjection into an Ana-SEC (a Superdex 75 pc 3.2/30 column), elute predominantly in the void elution volume (**Fig. 3b**, black line; peak height around ~19 min). Interestingly, in addition to void volume elution, we consistently observe an additional small peak corresponding to the monomer elution. This could be due to either the reestablishment of monomer-protofibril equilibrium, monomer dissociation due to interactions with the chromatography matrix or a combination of both. To determine whether this monomer peak was consistent and also whether there was a variation in this peak from one protofibril preparation to another, we carried out statistical analysis of 20 Ana-SEC elution profiles of Aβ42 protofibrils.

The analysis was based on reinjection of purified protofibrils on independent occasions and using different preparations. For each reinjection, the combined (PF + M) peak area was expressed as 100% and the percentage (%) mean peak area for PF and M elution was calculated. The data presented in **Figure 3d** show that SEC-purified protofibrils, when reinjected into a Superdex 75 pc 3.2/30 column, predominantly elute in the void volume, with a small elution peak corresponding to monomeric elution (mean peak area PF:M = 79:21; % s.d. from mean: PF = 0.95–14, M = 0.05–13; n = 20).

The fractions were then subjected to CD spectroscopy for analysis of secondary structure elements. CD spectra deconvolution, carried out using the CCA+ software Lincomb algorithm[81,82], showed that the protofibrils, obtained using this protocol, consisted of 43% β-sheet, 38% α-helix and 5% random coil as secondary structure elements (**Supplementary Fig. 1a**). These values for the secondary structure elements were similar as reported previously for Aβ40 protofibrils and show that protofibrils consist of mixed α-helical-β-sheet structural elements[14,20].

Similar to protofibrils of synthetic Aβ40 (ref. 14), Aβ42 protofibrils are SDS-labile and run as multiple bands (monomer to trimer), along with a high-molecular-weight smear, by SDS-PAGE (**Supplementary Fig. 1b**), despite the fact that these species elute in the void volume of Superdex 75 (>70 kDa), which should only contain PFs. This anomalous migration on SDS gels by protofibrils and oligomers of sAβ has also been reported by other groups[13,83]. There are different possibilities to explain these experimental observations; (i) some of these LMW oligomers may exist in rapid equilibrium with protofibrils; (ii) these oligomers may not exist in solution but form as a result of SDS-induced disassociation of protofibrils; or (iii) formation of these species may be induced by the interaction of Aβ with SDS micelles as described above[73].

SDS-stable Aβ oligomers have been consistently observed in naturally secreted Aβ preparations obtained from the conditioned media of Chinese hamster ovary cells, overexpressing wt APP or APP-bearing fAD mutations[19,55]. These species migrate predominantly as dimers and trimers on SDS-PAGE and have been proposed as *bona fide* oligomers[19]. However, these species are distinct from the oligomeric forms described above, because they were shown to be stable and did not disassociate after treatment with formic acid, heating or incubation with chemical denaturants (guanidine hydrochloride). Furthermore, naturally secreted oligomers have not been shown to undergo further oligomerization and fibril formation. Although chemical modifications and/or covalent cross-linking of monomeric Aβ species could explain the kinetic stability and lack of fibrillization by these oligomers, direct evidence for such modifications by mass spectrometry or by other tools remains lacking.

Fibrillization studies

To study the aggregation and kinetics of fibril formation, purified Aβ42 PF and M fractions were separately incubated (~10 μM) at 37 °C without agitation and allowed to fibrillize. Consistent with previous reports[13], we observed that PF fractions showed a less pronounced rise in ThT binding over time, compared with M fractions (**Supplementary Fig. 1c**). TEM analysis, after 96 h of incubation, revealed that although in the case of monomeric fraction elongated intertwining fibrils were formed (**Fig. 3f**), the PF fraction contained a substantial amount of elongated protofibrils interspersed among fibrils (**Fig. 3h**). The slow fibrillization of protofibrils is consistent with the proposed mechanism of fibril formation by monomer addition, rather than annealing and/or self-assembly of protofibrillar aggregates[20,21]. Indeed, addition of monomeric Aβ to protofibrillar fractions accelerates their fibrillization[21].

Preparation and characterization of Aβ40 protofibrils

Aβ40 protofibrils can be generated in a consistent manner by dissolving the peptide in NaOH and rapidly moving the highly acidic TFA salt peptide through its isoelectric point to a pH of 7.2–7.6 (ref. 14). Incubating the peptide at 500 μM in PBS (pH 7.4) for 2–3 d at RT generates monomers and protofibrils, which vary around a PF:M ratio in the range of 40–60% (ref. 14). The progression of protofibril formation can be quantitatively monitored on a daily basis by injecting small aliquots of the stock solution into a Superdex 75 HR 10/30 SEC column or into an analytical Superdex 75 pc 3.2/30 column. Before injecting into an SEC column, the sample is briefly centrifuged (16,000g for 10 min at 4 °C) to remove any fibrils that might have formed during the incubation. This is usually indicated by the appearance of a pellet after the centrifugation. TEM analysis of the pellet obtained in this procedure revealed that they are predominately made of fibrils[14]. Regarding the SEC elution profile, the protofibril peak appears first in the void volume (~7–9 ml for HR 10/30 columns) and can be collected in 0.5–1-ml fractions with Aβ concentrations ranging from 10 to 40 μM. TEM images of this protofibril peak showed small spheres, ~4–6 nm in diameter with a variation in length, and curvilinear structures <200 nm in length and ~4–6 nm in width[14]. The CD spectrum of these fractions showed a broad negative ellipticity between 225 and 208 nm, consistent with the presence of a mixture of random coil, α-helix and β-sheet structures[14]. Estimation of the secondary structure contents of the CD spectra, performed using the CDANAL program[82] and the Brahms and Brahms reference library[84], showed that these protofibril preparations contain 47% β-sheet, 40% random coil and 13% α-helix secondary structure elements[20]. In addition, the protofibril peak was found to bind Congo red and ThT similarly, but to a lesser extent, than mature fibrils[14,20]. The protofibril fractions can be combined (~1–2 ml) or analyzed separately depending on the concentration of peptide and solution volume needed. The monomer peak emerges in the included volume, as mentioned above, and can yield similar concentrations as the protofibril peak. TEM analysis of monomer fractions confirmed the aggregate-free nature

of the preparation[14,20]. SDS-PAGE, followed by western blotting analysis, for monomer fractions, showed a single 4-kDa band; however, the protofibrillar fractions migrated as a mixture of multiple bands with molecular weights corresponding to species from monomer to tetramer[14]. The latter observation suggested that Aβ40 protofibrils were not SDS stable and migrated as LMW bands on SDS gels.
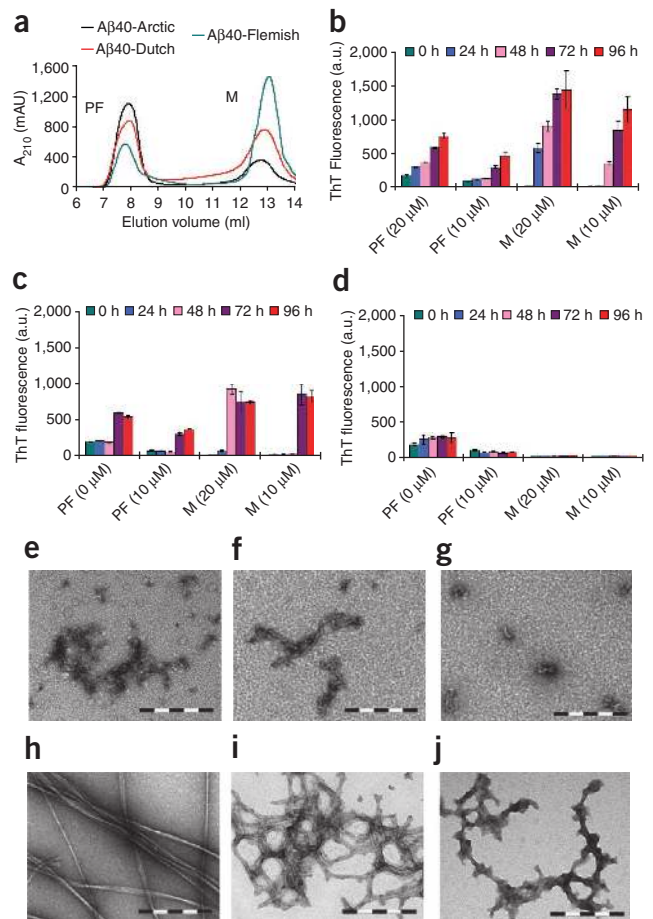
Application to Aβ fAD mutants

The methods described above for wt Aβ (Aβ40 and Aβ42) solubilization and fractionation are also useful for preparation of different monomeric and protofibril fractions of fAD Aβ mutants. We have used this protocol to isolate the monomeric and protofibrillar forms of Aβ40-Arctic (E22G), Aβ40-Dutch (E22Q) and Aβ40-Flemish (A21G) peptides. **Figure 4** summarizes the results of these studies. Aβ40-Arctic (E22G) exhibits faster aggregation kinetics than does wt Aβ40 and has a higher tendency to form and accumulate protofibrils[18,36,85]. Accordingly, we found that dissolving synthetic Aβ40-Arctic in DMSO (Step 1B) resulted in a solution that is highly enriched with protofibrils and monomeric Aβ. Fractionation of this solution on a Superdex 75 HR 10/30 column showed that the predominant elution peak was in the void volume (**Fig. 4a**, black line) and contained aggregates of heterogeneous morphologies including curvilinear and spherical assemblies (**Fig. 4e**). The fibrillization studies revealed that Aβ40-Arctic formed high ThT-binding fibrils, both from solution containing monomeric or protofibrillar Aβ40-Arctic (**Figs. 2h** and **4b**, only fibrils from the monomeric fraction are shown). Using the DMSO solubilization method, the yield of Aβ40-Arctic is similar to that of Aβ42 protofibrils (2 ml; ~40–60 μM). Previous efforts to generate Aβ40-Arctic protofibrils, using the NaOH solubilization method, resulted in only 10–40 μM for similar volumes[18]. Therefore, we recommend using the DMSO solubilization method (Step 1B) for generating Aβ40-Arctic protofibrils. The Aβ40-Dutch (E22Q) mutant exhibited a similar SEC elution pattern as the wt Aβ42 (**Fig. 4a**, red line); however, these protofibrils formed fibrils only at concentrations ≥20 μM (**Figs. 2i** and **4c**). Finally, Aβ40-Flemish (A21G) also eluted as two peaks corresponding to a protofibrillar and monomeric species (**Fig. 4a**, green line), but neither was shown to form fibrils over the time course of the experiment (96 h incubation at 37 °C, without agitation) (**Figs. 2j** and **4d**). These observations are in accordance with previously published reports showing that Arctic (E22G) and Dutch (E22Q) mutations in Aβ significantly increase fibril nucleation and growth, whereas the Flemish (A21G) mutation decreases fibril growth[85].

Figure 4 | Application to fAD-associated Aβ mutant peptides. (**a**) SEC fractionation of DMSO solubilized Aβ40-Arctic (black line), Aβ40-Dutch (red line) and Aβ40-Flemish (green line) on a Superdex 75 HR 10/30 column. (**b–d**) Fibril formation by protofibrillar and monomeric Aβ40-Arctic (**b**), Aβ40-Dutch (**c**) and Aβ40-Flemish (**d**) assessed by ThT binding assay (10 μM Aβ; 96 h, 37 °C without agitation). (**e–j**) TEM analysis of SEC isolated protofibrils of Aβ40-Arctic (**e**), Aβ40-Dutch (**f**) and Aβ40-Flemish (**g**). (**h–j**) TEM analysis of fibrils or non-fibrillar aggregates formed by 10 monomeric Aβ40-Arctic (**h**), Aβ40-Dutch (**i**) and Aβ40-Flemish (**j**) after 96 h incubation (10 μM Aβ, 37 °C, without agitation). PF, protofibrils; M, monomeric Aβ; a.u., arbitrary units; the error bars in (**b–d**) represent s.d. in duplicate samples; scale bar, 200 nm.

Application to rAβ peptides and synthetic peptides from other sources

Next, we sought to determine whether this protocol for generation of monomeric and protofibrillar Aβ fractions is equally applicable to synthetic (sAβ) and recombinant forms (rAβ) of Aβ obtained from different sources. For this purpose, we obtained synthetic Aβ42 (as TFA salt) from Bachem (Switzerland, cat. no. H-1368). rAβ peptides were purchased from a commercial source (rPeptides, Bogart, GA, USA) and were also donated generously by two independent research groups (Carl Frieden, Washington University, St. Louis, USA and Rudolf Glockshuber, ETH, Zurich, Switzerland). Further, the rAβ42 peptides from rPeptides were provided as either TFA (cat. no. A-1002-2) or NaOH (cat. no. A-1165-2) salts, thus allowing us to determine whether different counterions affect the extent of protofibril formation. All sAβ and rAβ peptides were solubilized using the DMSO method and fractionated on a Superdex 75 HR 10/30 column. Synthetic and recombinant Aβ42 eluted as protofibrillar and monomer peaks (**Fig. 5a,b**), and TEM analysis of the protofibrillar fractions showed predominantly curvilinear and spherical morphologies (**Fig. 5c–f**).

Recombinant Aβ40 peptides also eluted in an identical manner as synthetic Aβ40 (Keck facility), as a predominant single elution peak in the included volume and as a small peak in the void volume (**Supplementary Fig. 1f–g**). These observations show that the protocol outlined above is applicable to Aβ peptides from synthetic and recombinant sources. However, it is noteworthy that protofibrils are a heterogeneous population of aggregates and it is quite likely that the size distribution among protofibrils might not be identical for Aβ prepared in different methods (synthetic and recombinant).

Subfractionation of Aβ42 protofibrils and aggregated Aβ42
Further separation of protofibrils of different sizes can be achieved using columns with different separation capacities or combining multiple columns with extended separation ranges (**Table 2**) (for example, the Superose 6 HR 10/30 column by itself (**Supplementary Fig. 2**) or a Superose 6 HR 10/30 column connected in series to a TSK-GEL 4,000 SEC column). Generally, this procedure yields from four to six protofibril fractions (1 ml each), but only the first and last fractions differ significantly in terms of size and morphology distribution. For example, we previously showed that fractionation of the protofibril peak, obtained from a 1:1 solution mixture of Aβ-Arctic and Aβ40 wt, using a Superose 6 column, yielded seven protofibril fractions[18]. Only fraction 7 was highly enriched in annular Aβ structures, with a molecular weight distribution centered around 150–250 kDa. The remaining fractions were heterogeneous and exhibited a molecular weight distribution of 80–880 kDa[18]. We have observed that the addition of monomers of a slow aggregating sequence (e.g., Aβ40) to a highly amyloidogenic sequence (e.g., Aβ42 (ref. 21) and Aβ40-Arctic[18]) results in kinetic stabilization of the protofibrils formed by the more amyloidogenic sequence. This strategy can be exploited to stabilize protofibrils and facilitate their purification[18].

Figure 5 | Application to synthetic and recombinant Aβ peptides. (**a**) Comparison of recombinant Aβ42 (rAβ42; black line) and synthetic Aβ42 (sAβ42; red line) fractionation into protofibrillar and monomeric Ab fractions on a Superdex 75 HR 10/30 SEC column. The peptides were solubilized using DMSO at concentrations of: 0.5 mg ml^{-1} (rAβ42) and 1 mg ml^{-1} (sAβ42). (**c,e**) Representative TEM images of rAβ42 protofibrils (**c**) and sAβ42 protofibrils (**e**). (**e**) Comparison of recombinant rAβ42, provided as TFA (red line) or NaOH (black line) salts. The peptides were solubilized with DMSO at 1 mg ml^{-1}, and fractionated on a Superdex 75 HR 10/30 SEC column. (**d,f**) Representative TEM images of rAβ42-TFA protofibrils (**d**) and rAβ42-NaOH protofibrils (**f**). Scale bar, 200 nm.

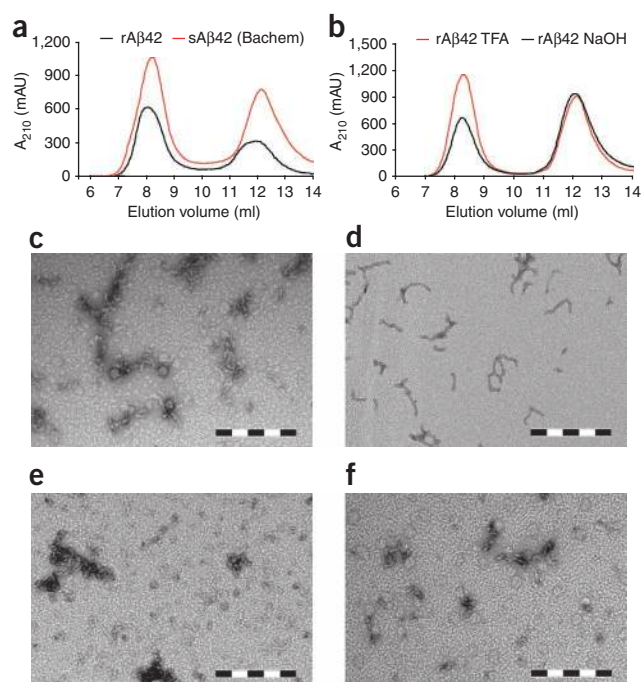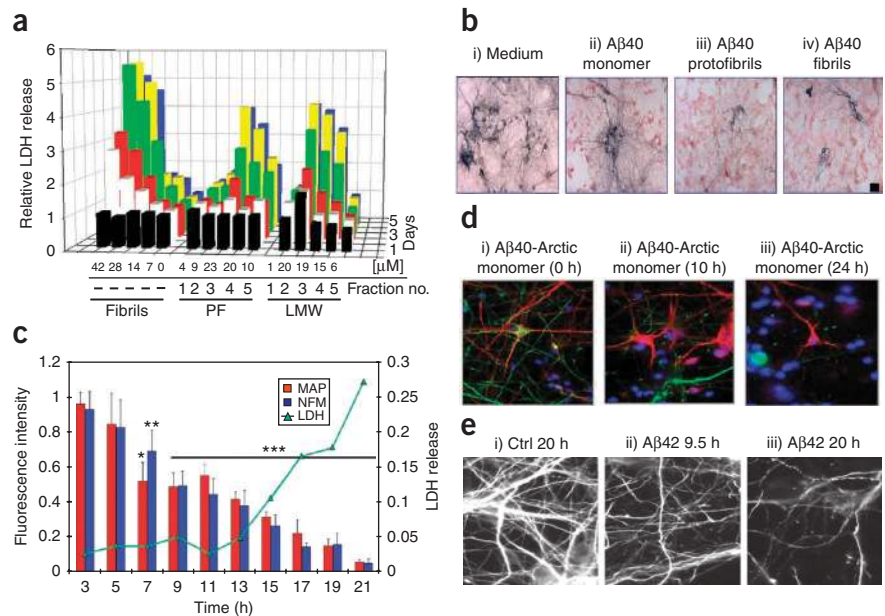**Stability of the preparation**
Considering the situations in which fractionation is not immediately possible, or when protofibrils are not used within a day after fractionation or samples need to be sent to another location, we sought to determine the stability of the protofibrillar preparation over time. To study this, we solubilized synthetic Aβ42 (1 mg ml^{-1}, using the DMSO method) and either injected (250 µl) into a Superdex 75 column immediately after solubilization (and centrifugation) or left the solution at 4 °C for a duration of up to 24 h. At 4 and 24 h, the solution was centrifuged and injected (250 µl). We observed that the protofibrillar peak area increased by ~10–15%, as compared with time 0, with a corresponding decline in the monomer peak area (**Supplementary Fig. 3a**). A TEM analysis of the crude (CR) stock solution after 4 h of incubation at 4 °C revealed predominantly protofibrillar structures and did not show any fibrils (**Supplementary Fig. 3d,g**). Although the peptide loss due to fibril formation, if any, was minimal, we do not recommend storage of the stock solution for more than 2–4 h.

Similarly, SEC-isolated protofibril fractions (1 ml, ~50 µM) were also incubated at 4 °C for 24 h. Comparison of sample reinjection, soon after fractionation and after 24 h of incubation at 4 °C, showed that the predominant elution was in the void volume (% total peak area: PF = 80–86, M = 14–20) (**Supplementary Fig. 3b**). TEM analysis, after 24 h of incubation at 4 °C, did not show any mature fibrils; however, protofibrils seemed to cluster with extended incubation (**Supplementary Fig. 3e,h**).

Preparation of Aβ fibrils
The majority of structural and toxicity studies have compared the properties of monomeric and protofibrillar Aβ with that of Aβ fibrils. Aβ fibrils can be generated using purified monomeric preparations or CR Aβ preparations, i.e., those that contain a mixture of monomeric and protofibrillar species, depending on the quantity of fibrils desired and the experimental conditions used. Both methods yield fibrils of similar ThT binding and morphologies.

For highly aggregation-prone sequences (e.g., Aβ42 wt or Aβ40-Arctic), fibrils can be obtained by incubating monomeric Aβ (10–50 µM) at 37 °C under mild agitation for 24–48 h (**Figs. 3f** and **4h,** respectively, for Aβ42 WT or Aβ40-Arctic). Aβ40 fibril

formation, from SEC-isolated monomeric Aβ40, requires concentrations ≥50 μM and agitation for 24–48 h[21]. These conditions result in the formation of mature amyloid fibrils with an average diameter of 8–12 nm. The fibrils are characterized by high ThT binding and TEM analysis, and shows an extensive network of intertwining >1-μm-long filamentous morphologies.

Fibrils can also be prepared from high concentrations of CR Aβ preparations (1–2 mg ml^{−1} of Aβ solutions containing monomers, protofibrils and a small amount of fibrils) obtained using one of the methods described for generating protofibrils (the DMSO or NaOH method). Concentrated stock solutions are incubated at 37 °C, under mild agitation, for 24 h. This method generates high ThT-binding fibrils with morphologies similar to those obtained when using purified monomeric Aβ (**Supplementary Fig. 1d,e**). Preparing fibrils from CR mixtures allows for obtaining fibrils in a short time span and for the generation of large quantities of fibrils for use in high-throughput screening or *in vivo* studies. Therefore, we recommend the latter method only if experiments demand substantial quantities of fibrils.

SEC-isolated Aβ fractions are toxic to cultured neurons

Initial studies showing the neurotoxic nature of protofibrils used Aβ40 protofibrils isolated by SEC using a Sephadex G75 column[14,20]. Applications of ~20 μM protofibrils produced massive cell loss over a 5 d period, as measured by LDH (**Fig. 6a**)[14]. This was

Figure 6 | Neurotoxicity of SEC-purified Aβ monomers and protofibrils. (**a,b**) Time course of wild-type Aβ40–induced injury: Aβ40 was incubated at 500 μM in PBS for 3 d at RT, and then centrifuged to remove fibrils. The monomeric and protofibril fractions were isolated by SEC using a Superdex 75 10/30 column. The monomeric (LMW) and protofibril (PF) peaks were collected in 0.5-ml fractions to which modified neurobasal medium was added, and then placed on primary cerebral cortical cultures. (**a**) Neuronal loss was measured by LDH release into the culture medium every 24 h. Each monomeric and protofibril peak was collected in 5 individual fractions and Aβ concentration was measured in each. Control LDH release (no Aβ added) is listed as "0" under fibrils and given a value of 1. (**b**) Neurons were exposed to medium only (i), monomeric Aβ (18 μM) (ii), protofibrils (21 μM) (iii), or fibrils (20 μM for 5 d) (iv), and then cultures were preserved and stained with microtubule associated protein-2 (MAP2) to identify neurons (dark blue staining). (**c,d**) Time course of Aβ40-Arctic–induced injury: Aβ40-Arctic was solubilized using 6 M guanidine hydrochloride and fractionated on a Superdex 75 10/30 column. Modified neurobasal medium was added to the monomeric fractions (Aβ diluted to 20-μM concentration). (**c**) Quantitative results of the immunocytochemical staining and biochemical measurements of LDH release. Statistically significant loss of neuronal process is observed by 7 h, whereas the release of LDH was not significant until 15 h. Data is reproduced with copyright approval[65]. (**d**) Immunocytochemical staining of primary cerebral cortical neurons treated with monomeric Aβ40-Arctic at time 0 (i), show a progressive neurodegeneration with initial loss of dendrites by MAP2 staining (red) and axons by NF-M staining (green) at 10 h (ii) followed by cell loss at 24 h (iii). (**e**) Time course of Aβ42-induced injury: Aβ42 was solubilized using 6 M guanidine HCl solubilization method and fractionated on a Superdex G75 10/30 column. Modified Neurobasal media was added to the monomeric fractions. Neurons, on coverslips, were treated with Aβ42 (22.5 μM). At 9.5- and 20-h time periods, coverslips were removed, fixed and stained for Neurofilament N, an axon-specific protein. (i) Healthy neurons receiving no Aβ42 for 20 h; (ii) Neurons losing neuronal process as early as 9.5 h; (iii) Further neuronal loss observed at 20 h. The time course of neurite loss with Aβ42 was very similar to the findings with Aβ40-Arctic in **d**.

supported by cytological observations showing loss of neuronal processes and cell bodies (**Fig. 6b**)[14]. A concern with cell culture experiments is that the aggregation state of Aβ can change with extended incubation periods (>2–6 h), depending on the concentration of the peptide. Cell culture media were collected after 5 d and no fibrils could be observed in either the monomer or protofibril-treated cultures[14]. This suggested that fibrils were not killing the neurons and some Aβ species preceding fibril formation were toxic. Interestingly, similar neuronal death was observed with monomeric fractions after 10–24 h of treatment as the protofibril preparation (**Fig. 6a**)[14], suggesting that monomer fractions can form nonfibrillar Aβ aggregates during the course of the experiment, which might have contributed and/or caused toxicity. Instantaneous readout such as the MTT assay or electrophysiology has confirmed that protofibrils have their own inherent biological activity[14,20]. In support of the soluble Aβ aggregates causing neurotoxicity, preparations of disaggregated Aβ40-Arctic peptide purified by SEC were found to produce rapid toxicity[65]. Aβ40-Arctic induced neuronal loss, but observations at earlier time points (within 5 h) showed morphological changes in neuronal dendrites and axons, which preceded the rise in LDH (**Fig. 6c**)[65]. Similarly, isolating monomeric Aβ42 and applying it to primary neocortical cultures produced a loss of neuronal process (**Fig. 6e**) that was almost identical to that of Aβ40-Arctic (**Fig. 6d**). These findings support the concept that prefibrillar aggregates of Aβ induce synaptic failure, which precedes overt cell loss. These data show the value and versatility of SEC to generate different Aβ preparations in order to probe the pathophysiological relevance of various aggregation states in AD.

PROTOCOL

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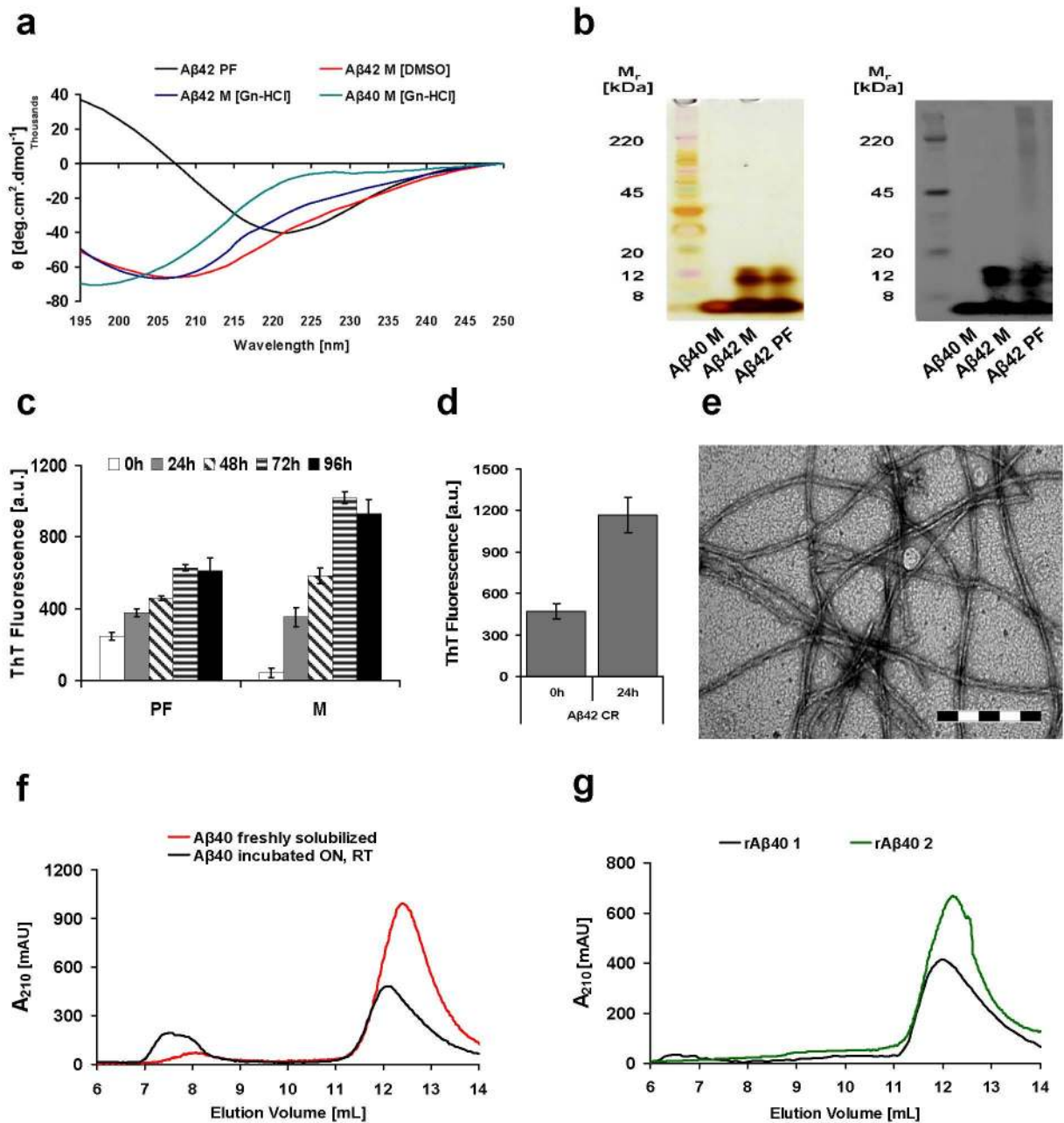
Supplementary Figure 1 │ **(a)** Circular dichroism (CD) spectroscopy analysis of SEC fractions from Superdex 75 HR 10/30 (Aβ40 and Aβ42) in a Jasco-810 CD Spectrometer using a 10 mm pathlength cell (black line = Aβ42 protofibrils; red line = Aβ42 monomer obtained using DMSO method; blue line = Aβ42 monomer obtained using the 6 M guanidine-HCl method; green line = Aβ40 monomer obtained using the 6 M guanidine-HCl method). **(b)** Aβ40 (monomeric = M) and Aβ42 (monomeric = M and protofibrillar = PF) SEC fractions were obtained from a Superdex 75 HR 10/30 column and were electrophoresed on NuPAGE 4-12% Bis Tris gels (Invitrogen, cat. No. NP0336BOX). The proteins were detected by (left) silver staining (SilverXpress, Invitrogen Cat. No. LC6100) and also, after having been transferred on a Nitrocellulose membrane (Whatman, Cat. No. 10401396), immunoblotting with anti-Aβ antibody 6E10 (Signet, Cat. No. 9320). **(c)** Thioflavin-T (ThT) binding over time by protofibrillar and monomeric Aβ42 fractions (10 μM Aβ42; 96 h, 37°C without agitation). **(d-e)** Fibril formation by Aβ42 crude (stock solution obtained after DMSO solubilization method): sAβ42 was solubilized using DMSO (1 mg ml^{-1}) and incubated at 37°C with gentle agitation (4,000 rpm) (d) ThT binding and (e) representative TEM image of resultant fibrils after 24 h of incubation. (f) SEC fractionation of synthetic Aβ40 (Keck facility), solubilized using DMSO method (1 mg ml^{-1}), on a Superdex 75 HR 10/30 SEC column into protofibrils and monomeric Aβ fractions. Aβ40 was solubilized and fractionated, without incubation (red line), or fractionated after incubation at room temperature, overnight (black line). (g) SEC fractionation of recombinant rAβ40 from independent research groups, solubilized using DMSO method (0.5-1 mg ml^{-1}), on a Superdex 75 HR 10/30 SEC. rAβ40 1 (black line; 0.5 mg ml^{-1}) was a generous gift from Prof. Rudolf Glockshuber, ETH, Zurich, Switzerland and rAβ40 2 (green line; 1 mg ml^{-1}) was generously provided by Prof. Carl Frieden, Washington University, St. Louis, USA. (Abbreviations: ON = Overnight; RT= Room temperature; PF= Aβ Protofibrils; M= Monomeric Aβ; CR= Crude Aβ42; a.u. = arbitrary units; the error bars in (c and d) represent STD in duplicate samples; scale bar= 200 nm).
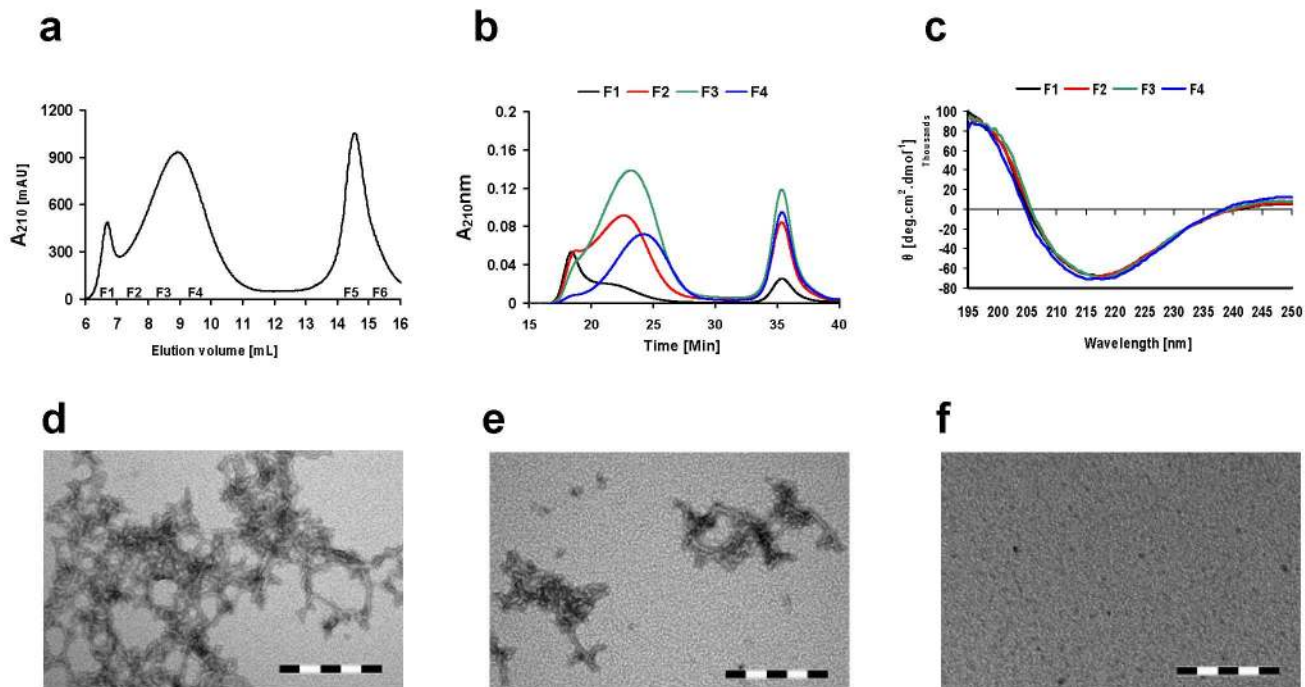
Supplementary Figure 2 │ SEC sub-fractionation of Aβ42 protofibrils on a Superose 6 HR 10/30 SEC column. (a) Aβ42 was solubilized using DMSO method (2 mg ml^{-1}) and fractionated into protofibrillar (F1-F4) and monomeric (F5-F6) fractions on a Superose 6 HR 10/30 SEC column. **(b)** Analytical SEC of F1-F4 on Superose 6 pc 3.2/30 SEC column. **(c)** CD spectroscopy analysis of Aβ 42 protofibrillar fractions from Superose 6 HR 10/30 as in Supplementary Figure 1a. **(d-f)** Representative TEM images of (d) F1, (e) F3 and (f) F5. (Abbreviations: F= Fraction; scale bar= 200 nm).
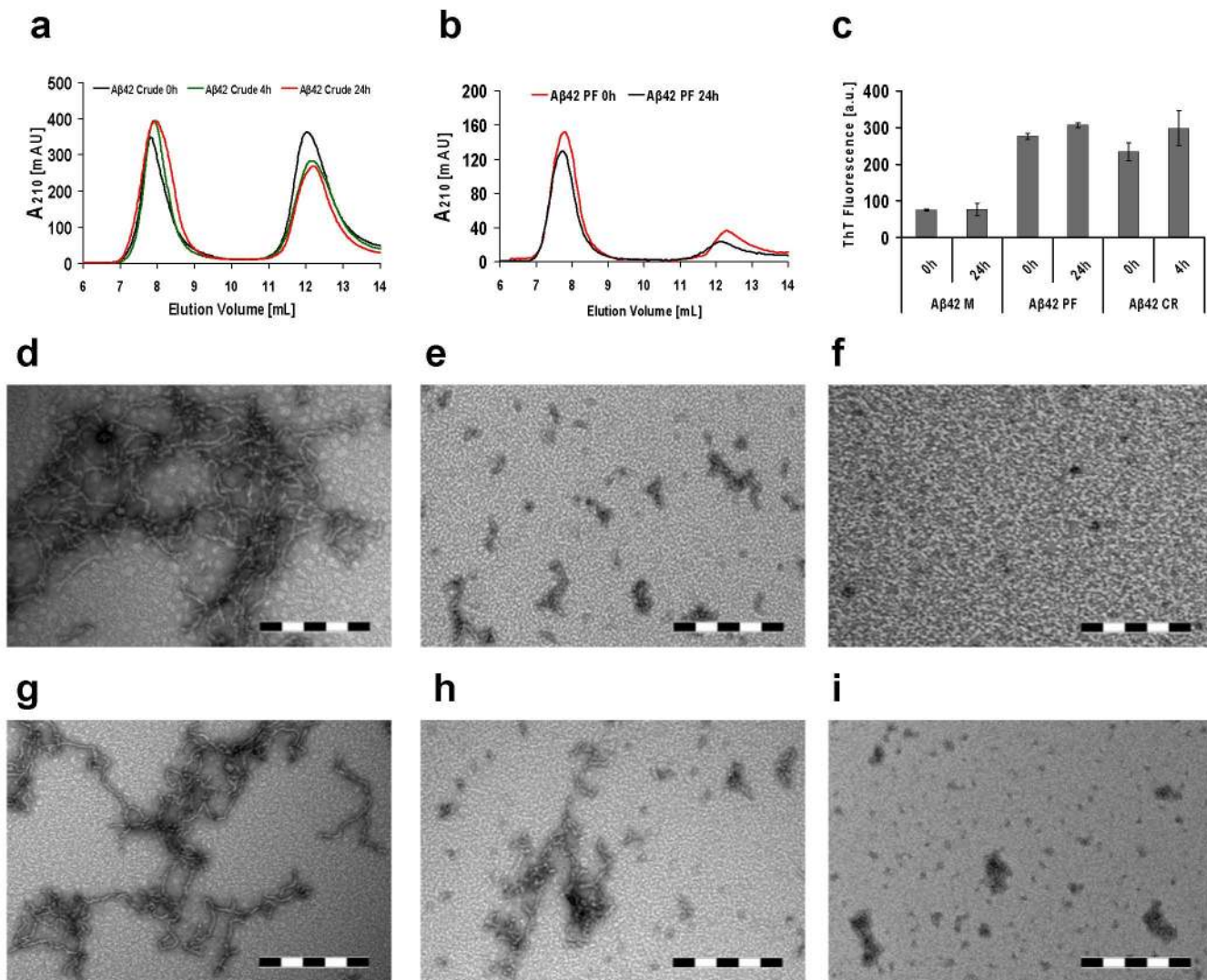
Supplementary Figure 3 | Stability of crude Aβ42 solution and SEC isolated protofibrillar and monomeric Aβ42 fractions. (a) Aβ42 was solubilized (DMSO method, 1 mg ml^{-1}) and split into three aliquots (~ 300 µl/aliquot). The first aliquot was injected immediately into a Superdex 75 HR 10/30 column. The second and third aliquots were incubated at 4°C for 4 and 24 h respectively, and then injected. At each time point, the solution was centrifuged (16,000 g, 4°C, 10 min) and 250 µl of the supernatant were injected. **(b)** Aβ42 protofibrils were obtained by SEC and the fraction (50 µM, 1 ml) was split in two aliquots (500 µl/aliquot). The first aliquot was re-injected immediately into a Superdex 75 HR 10/30 column. The second aliquot was incubated at 4°C for 24 h then re-injected as above. At each time point, the solution was centrifuged (16,000 g, 4°C, 10 min) and 400 µl of supernatant were injected. **(c)** ThT binding by crude, protofibrillar and monomeric Aβ42 after incubation at 4°C. **(d-i)** Representative TEM images: (d and g) Aβ42 crude soon after solubilization and after 4 h of incubation at 4°C, (e and h) Aβ42 protofibrils soon after fractionation and after 24 h of incubation at 4°C and (f and i) monomeric Aβ42 soon after fractionation and after 24 h of incubation at 4°C. (Abbreviations: PF= Aβ42 Protofibrils; M= Monomeric Aβ42; CR= Crude Aβ42; a.u. = arbitrary units; the error bars in (c) represent STD in duplicate samples; scale bar= 200 nm).
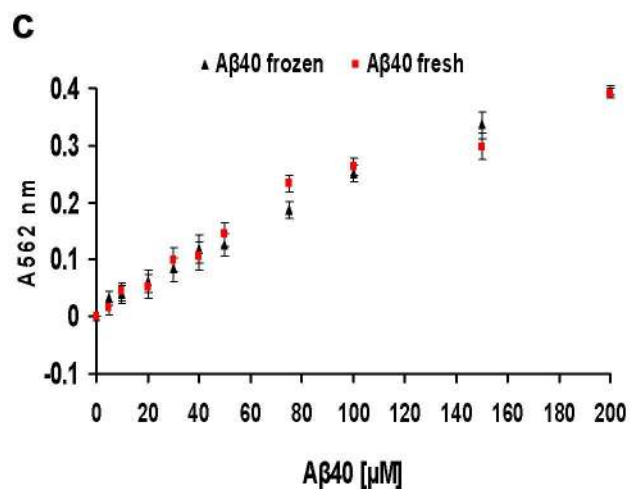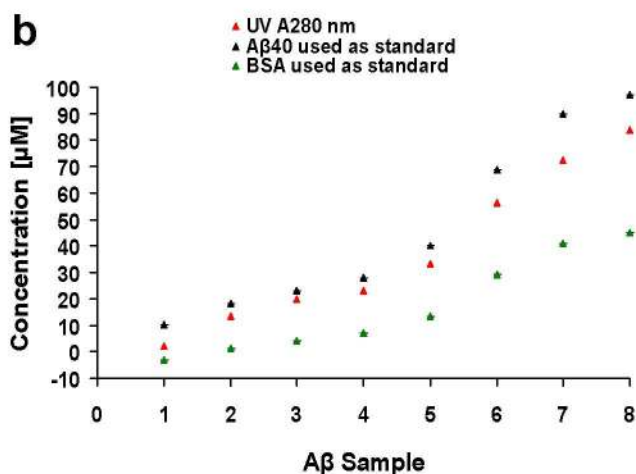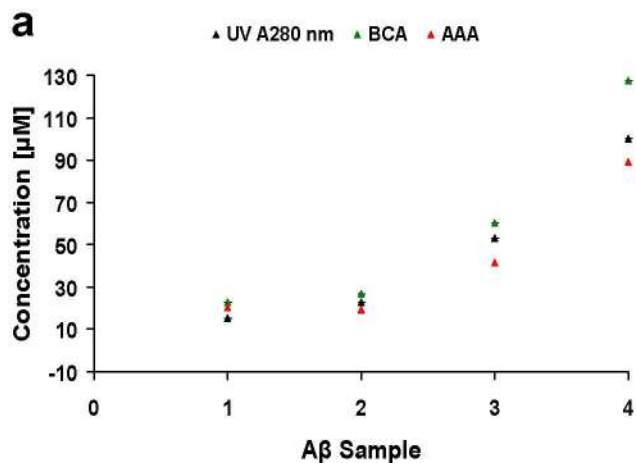
Supplementary Figure 4 | Comparative analysis of Aβ concentration determination by amino acid analysis (AAA), BCA method and UV A_{280} nm method. (a) The protein concentration of four Aβ samples, from different fractionation experiments, was determined by AAA, BCA kit (Pierce Cat. No. 23227) and UV A_{280} nm methods. AAA was carried out in FGCZ, ETH, Zurich, Switzerland. Aβ standard curve, obtained from serial dilutions of Aβ40 in H_2O, was used for BCA. UV A_{280} nm for different fractions was determined in a 10 mm path-length cuvette using a Cary spectrophotometer. **(b)** Protein concentration of eight Aβ samples, from different fractionation experiments, was determined by BCA kit and UV A_{280} nm methods. Aβ concentration by BCA method was determined using a standard curve generated from both Aβ40 and bovine serum albumin (BSA) serial dilutions. UV A_{280} nm was carried out as in (a) above. BSA was provided as part of the PIERCE BCA kit. **(c)** Generation of standard curve of Aβ40 for BCA assay. Aβ40 was freshly solubilized in double distilled H_2O (1 mg ml^{-1}; 200 μM by net weight; 80% peptide content) and serial dilutions (5 - 200 μM) were prepared (in H_2O) for generating a standard curve for BCA assay. The stock (200 μM) Aβ40 solution was aliquoted into sterile tubes (Fisherbrand, Cat. No. 05-669-27; 200 μl/tube) and frozen at -20°C. After 24 h of freezing, one of the aliquots was thawn on ice, and serial dilutions (5-200 μM) were carried out in H_2O. Protein concentration for both series of dilutions was determined by the BCA kit. Synthetic Aβ (Keck facility) was used for all measurements outlined in (a-c).