3

In Vitro Preparation of Prefibrillar Intermediates of Amyloid-β and α-Synuclein

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Summary

Elucidating the structural properties of early intermediates (protofibrils) on the fibril formation pathway of Aβ and α-synuclein, the structural relationship among the different intermediates and their relationship to the structure of the amyloid fibrils is critical for understanding the roles of amyloid fibril formation in the pathogenesis of Alzheimer's and Parkinson's diseases. In this chapter we discuss several methods, developed by different laboratories, that enable the preparation and stabilization of amyloid-β and α-synuclein protofibrillar species of defined morphologies for biochemical, biophysical and toxicity studies.

Key Words: Alzheimer's disease (AD); Parkinson's disease (PD); amyloid; fibrils; protofibrils; oligomers; amyloid-β (Aβ)-derived diffusible ligand (ADDLS); amylospheriods; annular structures; pores; size exclusion chromatography (SEC); electron microscopy (EM); atomic force microscopy (AFM); analytical ultracentrifugation (AU); scanning transmission electron microscopy (STEM); arctic variant (E22G); wild-type (WT).

1. Introduction

Amyloid fibril formation is a process by which one of 20 distinct proteins undergoes a conformational change either before or coincident with its self-assembly into highly ordered β-sheet rich aggregates known as amyloid fibrils *(1,2)*. Initially, the amyloid hypothesis supported by strong genetic, pathologic, and biochemical evidence implicated amyloid fibrils as the main cause for neurodegeneration and/or organ dysfunction in several human diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, and other amyloidoses. The identification and characterization of potential neurotoxic quaternary structure intermediates, referred to as protofibrils, that precede fibril formation and the finding that several pathogenic mutations promote

protofibril formation suggest that the protofibrils rather than the fibrils are the pathogenic species *(3–5)*. However, the mechanism by which protofibrils contribute to the pathogenesis of these diseases and the nature of the pathogenic species remain the subject of intense investigation and debate.

Biophysical studies have shown that several amyloid forming proteins self-assemble into protofibrils of heterogeneous size and morphology, including spheres, chain-like structures, annular pore-like structures, and large granular structures (**Scheme 1**) *(6–11)*. To date, most of the studies on the biochemical and biological properties of protofibrils employ heterogeneous mixtures of protofibrillar species, making it difficult to decipher which of these species is the toxic species. Therefore, developing solution conditions for stabilizing protofibrils in general and/or protofibrils of defined size and morphology is critical for investigating the relationship between monomer, protofibrillar intermediates, and fibrils in the pathogenesis of neurodegenerative diseases (e.g., AD and PD) and the identification of targets for therapeutic intervention.

Over the past years, several biophysical approaches were developed to stabilize, isolate, and characterize different protofibrillar intermediates on the pathway of amyloid formation. These approaches take advantage of complementary biophysical techniques to guide the preparation, purification, and characterization of protofibrils of defined molecular mass distribution and morphologies *(6,7,12)*. Generally, the first step involves the identification of optimal conditions for protofibril formation. Depending on the protein of interest, these conditions could involve manipulation of solution conditions (pH, temperature, ionic strength, incubation media, or buffer) or use of mutations that are known to promote protofibril formation and/or the formation of particular protofibril morphology. However, one needs to keep in mind that while one can manipulate solution conditions to alter the molecular mass and morphology distribution of protofibrils in favor of a particular protofibrillar species, preparation of protofibrils that are homogeneous in size and morphology remains a challenging task. In this chapter, we will discuss several methods that take advantage of the propensity of disease associated mutation, known to promote oligomerization of the amyloidogenic proteins amyloid-β and α-synuclein, to prepare and stabilize protofibrillar species of different morphologies for biochemical, biophysical, and toxicity studies.

2. Materials

1. α-Synuclein: Recombinant α-synuclein was expressed and purified as described previously (9).
2. Aβ Peptides: A$\beta40_{WT}$ and the Arctic variant, A$\beta40_{ARC}$ (E22G), were purchased as TFA salts from the Biopolymer Facility at Brigham and Women's Hospital. All peptides were dissolved using their true peptide weight.
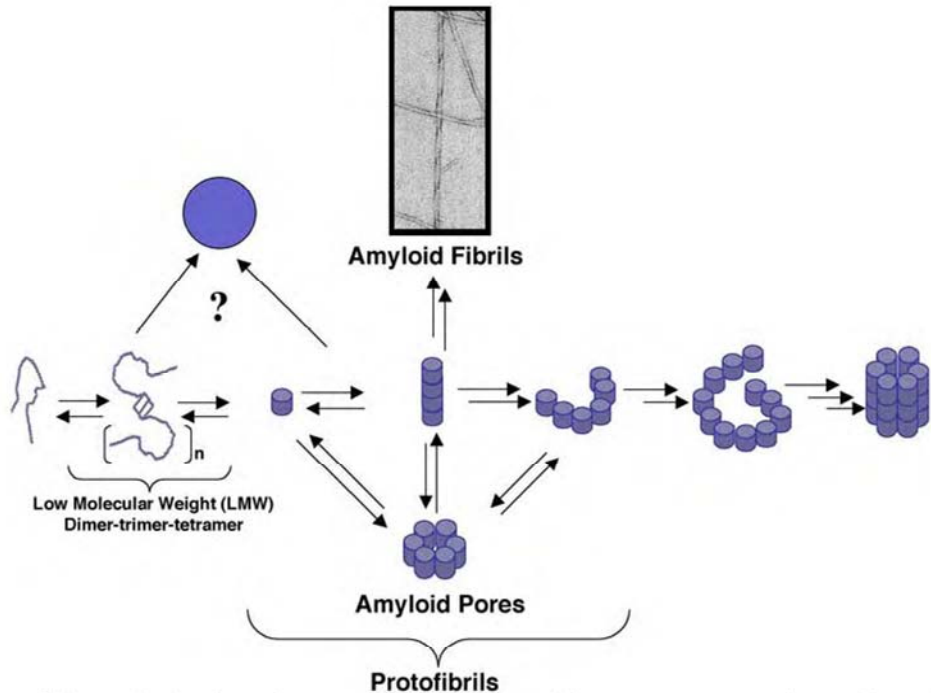
Scheme 1. A schematic representation summarizing our current understanding of the mechanism of oligomerization and amyloid fibril formation of Aβ and α-synuclein in vitro *(5)*.

3. Size exclusion columns.
    a. Superose 6 HR 10/30 (Amersham Pharmacia no. 17-0537-01).
    b. Superdex 75 HR 10/30 (Amersham Pharmacia no. 17-1047-01).
    c. Superdex 200 HR 10/30 (Amersham Pharmacia no. 17-1088-01).
4. Buffer A: 10 mM Tris-HCl, 150 mM NaCl, pH 7.4.
5. Buffer B: 5 mM Tris-HCl, 70 mM NaCl, pH 7.4.
6. Buffer C: 1X PBS (without Ca^{2+} and Mg^{2+}) pH 7.4 (GIBCO no. 70011-044).
7. Hexafluoroisopropanol (HFIP) (Sigma no. H8508).
8. Dry DMSO (Aldrich no. 276855).
9. Ham's F12 media without Phenol Red (Biosource).
10. Phenol Red (Sigma no. P4633).
11. 0.22 μm nylon spin centrifuge tube filter (Costar no. 8160).
12. Ultrafree-15 centrifugal filter device (5 kDa) (Millipore no. UFV2BCC40).

3. Methods

3.1. Preparing α-Synuclein Protofibrils

Genetic, neuropathologic, and biochemical evidence strongly suggest that the process of α-synuclein fibrillization plays a central role in the etiology of (PD).

PD is a neurodegenerative movement disorder that is characterized by the loss of dopaminergic neurons from the *substantia nigra*, and the formation of fibrillar α-synuclein intraneuronal inclusions (called Lewy bodies) *(13,14)*. Two mutations in the α-synuclein gene (A30P and A53T) have been linked to autosomal dominant early-onset PD. Both mutations promote the formation of transient protofibrils (prefibrillar oligomers), suggesting that protofibrils are linked to cytotoxicity. The PD-linked mutations (A30P and A53T) were observed to affect both the morphology and the size distribution of α-synuclein protofibrils (measured by analytical ultracentrifugation and scanning transmission electron microscopy). The A30P variant was observed to promote the formation of annular, pore-like protofibrils, whereas A53T promotes formation of annular and tubular protofibrillar structures. Wild-type (WT) α-synuclein also formed annular protofibrils, but only after extended incubation.

3.1.1. Preparing Crude α-Synuclein Protofibrils

1. To induce protofibril formation, incubate WT α-synuclein or any of the PD-linked variants (A30P and A53T) in 1X PBS buffer (pH 7.4) at concentrations of ≥300 μ*M* for 30 min at 4°C. The amount of protofibrils formed under these conditions varies among the different α-synuclein variants, but the amount of protofibrils formed (based on absorbance values) is usually ≤10% of the total protein in solution. Attempts to populate more protofibrils by further incubation have not been successful.
2. Filter the protein solution through 0.22-μm filters to remove any particles.
3. To separate the protofibrils (5–10%) from the monomer (majority of the sample), inject the α-synuclein sample onto a superdex 200 HR SEC column equilibrated with buffer A. **Figure 1A** shows a typical SEC chromatogram of A53T α-synuclein. Elute at a flow rate of 0.5 mL/min and collect fractions of 0.5 mL. α-Synuclein elutes as two peaks corresponding to protofibrillar oligomeric species and monomer (*see* **Note 1**).


Fig. 1. (*Opposite page*)   (**A**) Crude preparation of α-synuclein protofibrils. Size exclusion chromatogram showing separation of protofibrils (heterogeneous mixture) (V) from monomer (M) using a superdex 200 HR 10/30 SEC column. Circular dichroism (CD) spectra of the purified fractions demonstrate that the protofibrils are rich in β-sheet structures, whereas the monomeric fraction exhibits a CD signal characteristic of a random coil. The presence of protofibrils in the void fraction (V) is verified by AFM imaging, which reveals heterogeneous distribution of spherical aggregates. **Figure 1A** was adapted from *(5)*. (**B**) α-Synuclein annular protofibril preparation. Relative separation of α-synuclein protofibrils by fractionation of the protofibril's peak using a superose 6 HR 10/30 SEC column. Size exclusion chromatogram of α-synuclein (A53T) stock solution (350 μ*M*) in buffer A, inset, shows how the oligomeric peaks were fractionated. (**C**) Negative stain electron microscopy (EM) images of the protofibril fractions in the inset of **Fig. 1B. Figure 1B** was adopted from **ref. 6.**
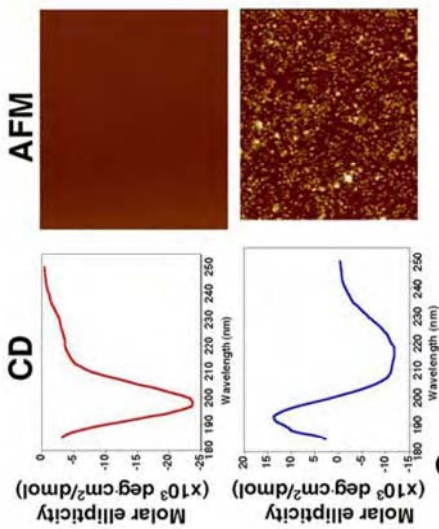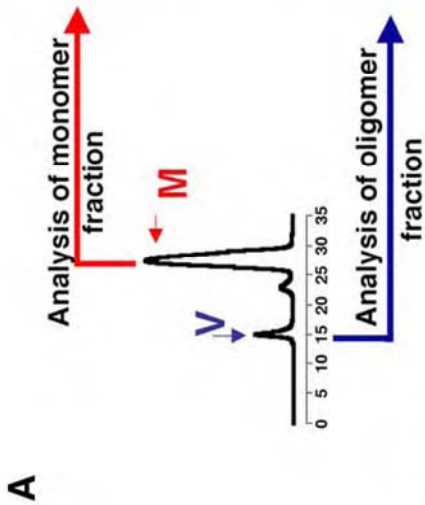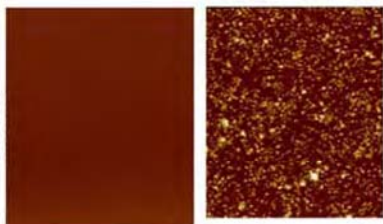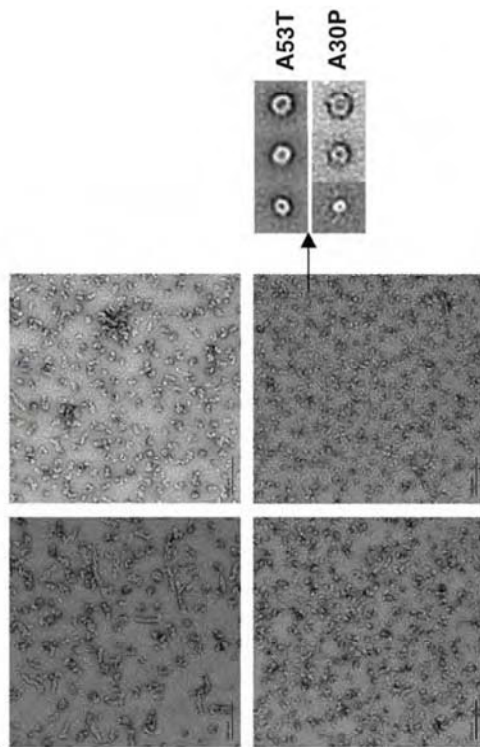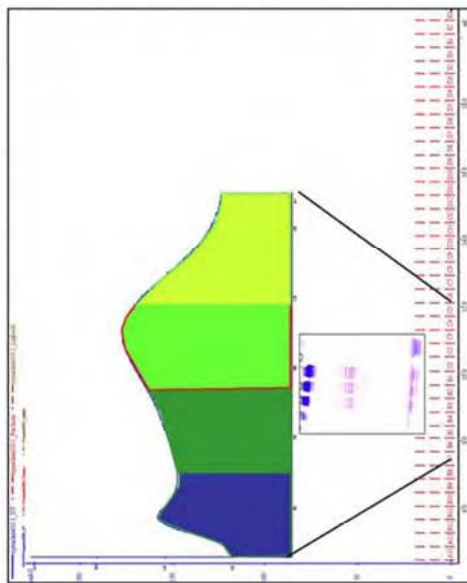
4. Collect the fractions corresponding to α-synuclein monomers and protofibrils separately.
5. Concentrate each species to the desired concentration using an ultrafree-15 centrifugal filter device (5 kDa). For storage purposes, we recommend that the monomer and oligomer concentrations should not exceed 100 μM and 30 μM, respectively, unless future experiments demand so.
6. Filter the samples through 0.2-μm nylon membrane spin filter.
7. Protein concentration should be determined by amino acid analysis (AAA) or by optical absorbance at 280 nm (OD_{280} for 1 mg/mL = 0.354).

3.1.2. Preparing Annular Protofibrils of α-Synuclein

1. To obtain protofibrillar preparations of more defined molecular size distribution and morphology, inject the α-synuclein samples from step 2 onto a Superose 6 HR 10/30 SEC column. Unlike the Superdex 200, where the protofibrils elute as a single peak in the excluded volume, in Superose 6, the majority of α-synuclein protofibrils is eluted as a broad peak in the included volume (**Fig. 1B**).
2. To achieve relative separation of α-synuclein protofibrils, fractionate the protofibril peaks into 3 to 4 fractions. While the first and second fractions remain heterogeneous, the third and fourth fractions tend to be more homogeneous and dominated by annular protofibrils (>85%) of a narrow size distribution as determined by electron microscopy (EM), analytical ultracentrifugation (AU), and scanning transmission electron microscopy (STEM) (**Fig. 1B**) *(6)*.
3. Concentrate each fraction using an ultrafree-15 centrifugal filter device.
4. Fractions 3 and 4 are rich in annular (pore-like) protofibrils having an average diameter of 11 ± 1 nm, and an inner core of 2 to 3 nm, whereas fractions 1 and 2 are heterogeneous and contains significant amounts of ring and tube-like protofibrils (width = 10–12 nm, length = 13–24 nm).

3.2. Amyloid-β (Aβ) and Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is characterized by the presence of extracellular amyloid plaques and intraneuronal neurofibrillary tangles in the brain *(15–17)*. Biochemical analysis of amyloid plaques revealed that the main constituent is fibrillar aggregates of a 39–42 residue peptide referred to as the amyloid-β protein (Aβ) *(18)*. Several lines of evidence point toward a central role for the process of Aβ fibril formation in the etiology of AD. Transgenic animals overexpressing mutant forms of its precursor, the amyloid precursor protein (APP), develop amyloid plaques comprising fibrillar Aβ *(19)*. Several pathogenic AD mutations have been shown to affect the processing of APP resulting in increased Aβ levels, in particular, the more amyloidogenic variant Aβ42 *(20)*. Finally, active or passive vaccination against Aβ reduces the amyloid burden and reverses behavioral and cognitive deficits in AD transgenic animals *(21,22)*.

3.2.1. Solubilization of Aβ and NaOH Method Aβ Preparation

Several methods have been described for preparing protofibrils of Aβ, Herein, we will focus on the NaOH method used in our laboratory to generate protofibrils of Aβ40 (WT and Arctic). Based on our experience, the NaOH method results in accelerated protofibril formation by Aβ peptides. Synthetic Aβ peptides, prepared as trifluoroacetic acid (TFA) salts, are highly acidic, and care must be excersized to properly buffer the peptide in order to stabilize the effects of pH on morphology and neurotoxicity.

1. Dissolve Aβ40 (TFA salt) at a concentration of 1 mM (4.3 mg/mL, based on total weight of peptide) in 1 mM NaOH plus phenol red (0.1 mg/mL to monitor pH), pH approx 3.0.
2. To minimize isoelectric precipitation of Aβ (pH 5.5), add 10 mM NaOH (140–155 μL NaOH/mg of peptide, this can vary between manufactures and specific lots) to achieve a rapid transition to a pH of approx 7.0 to 7.5 (orange to red). This is the value of the phenol red, you can monitor the pH (yellow = too acidic and purple = too basic).
3. Additional acid or base can be added to achieve the proper pH.
4. Dilute the peptide solution to 500 μM in water and PBS (final concentration: 70 mM NaCl, 1.35 mM KCl, 5 mM NaH_2PO_4/Na_2HPO_4).

3.2.2. Preparation of Crude Aβ Protofibrils

1. To prepare Aβ protofibrils, dissolve the lyophilized synthetic Aβ (WT, ARC, or equimolar mixture of WT and ARC) as described previously to obtain a total concentration of 100 μM. To induce protofibril formation, incubate the peptide solution prepared by the NaOH method at room temperature (RT) for 16 to 24 h. The incubation time required for protofibril formation varies depending on the batch of peptide. Generally, we perform analytical SEC experiments to probe the kinetics of protofibril formation every time we receive a new batch of material. The protofibrils formed under these conditions are heterogeneous in terms of their size (80–1000 kDa) and morphology (spheres, chain-like protofibrils, granular aggregates are usually observed) (*see* **Note 2**).
2. Centrifuge the samples at 13,000g for 5 min to remove any insoluble particle(s).
3. Filter the samples through 0.2-μm filter.
4. Load the supernatants onto either a superdex 75 HR (Amersham Pharmacia) SEC column (used to separate protofibrils from LMW Aβ species [monomers-dimers]) equilibrated with protein buffer B or a superdex 6 HR column (used to fractionate Aβ protofibrils). Elute proteins at a flow rate of 0.5 mL/min and collect fractions of 0.5 mL volume size.
5. Collect the fractions corresponding to the oligomeric and LMW peaks. The amount of protofibrils formed ranges from 30 to 60% depending on the incubation time and concentration of the peptide. Using 1 mg of peptide, we generally collect 1.5 mL of protofibrillar Aβ at concentrations ranging form 15 to 20 μM. In the case of

Aβ, we strongly recommend that samples in this concentration range should not be concentrated further.

6. Store purified protofibril fractions at 4°C. Samples should be used within 1 to 10 h.

3.2.3. Preparing Annular Protofibrils of Aβ40 (Amyloid Pores)

The increased propensity of the Arctic variant (E22G) of Aβ (Aβ40$_{ARC}$), which was shown to lead to early onset AD *(12,23)*, to form protofibrils presents researchers with an opportunity to generate significant quantities of protofibrils very rapidly. We have reported that the arctic variant (ARC) seems to more rapidly form annular and pore-like protofibrillar structures ("amyloid pores"), reminiscent of those observed for α-synuclein and bacterial pore-forming toxins *(6,7)*. We have discovered that mixing wild-type Aβ with the arctic variant alters the distribution of Aβ protofibrils and enhances greatly the stability of the formed protofibrils. Purified Aβ$_{ARC}$ protofibrils formed in the presence of WT are stable for 1 to 4 d at 4°C (as judged by SEC and EM) *(12)*, providing sufficient time to study and characterize these intermediates. Therefore, a 1:1 mixture of Aβ$_{ARC}$ and Aβ$_{WT}$ (100 μM) is used to prepare annular protofibrils of Aβ$_{ARC}$ (**Fig. 2**). Attempts to separate Aβ protofibrils prepared from Aβ40 or Aβ40$_{ARC}$ alone on superose 6 have failed, as the protofibrils have been observed to interact with the column matrix and/or undergo dissociation to monomers.

1. Prepare a mixture of Aβ40$_{WT}$ and Aβ40$_{ARC}$ at 100 μM using the NaOH method described in **Subheading 3.2.1.** To generate sufficient amount of protofibrils for biophysical and toxicity studies, 2.5 mg of each variant is used.
2. Incubate at room temperature for 16 to 20 h.
3. Centrifuge at 16000g and filter the supernatant through a 0.22-μm membrane. Generally, 15 to 25% of the peptides is converted into amyloid fibrils under these conditions with remaining peptides existing as mixture of protofibrils and low molecular weight species.
4. To separate the protofibrils, inject the peptide solution from step 3 onto a superose 6 column and elute with buffer A. Use a flow rate of 0.5 mL/min and collect fractions of 0.5 mL volume size (**Fig. 2**). To achieve optimal separation, load no more than 800 μL of the Aβ mixtures per run.

3.2.4. Preparation of Aβ Amylospheriod

Recently, Hoshi et al. described procedures for reproducible preparation of spherical oligomers of Aβ40 or Aβ42, termed amylospheriod *(24)*. These spherical aggregates of Aβ (with average diameters ranging from 3 to 20 nm) can be prepared reproducibly by slow rotation of Aβ40 or Aβ42 peptide solution. These aggregates form in the absence of chain-like or pore-like Aβ aggregates. Purification of spherical aggregate of various diameters can be accomplished by using glycerol-gradient (15–30%) centrifugation (86,000g) (**Fig. 3**). Hosi et
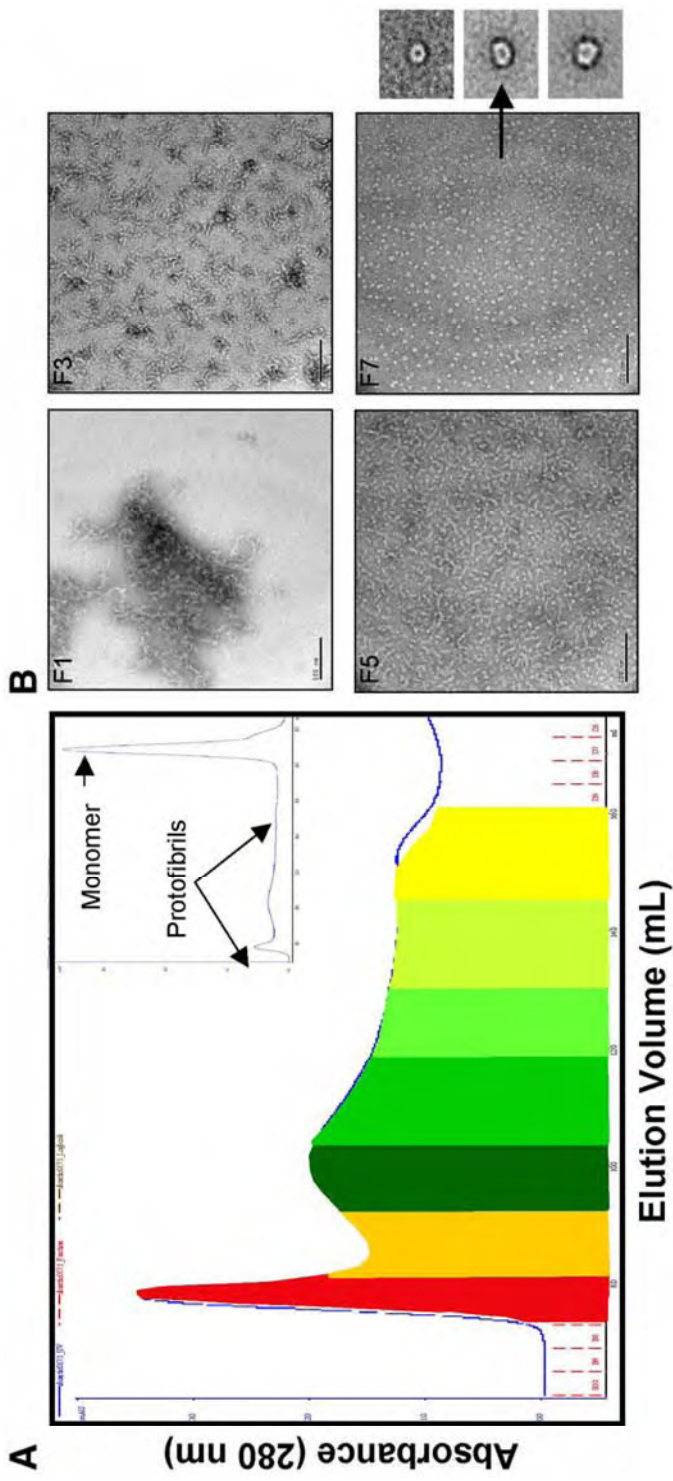
Fig. 2. Aβ Annular protofibril preparation. Separation of Aβ (mixture of WT and Arctic) protofibrils by fractionation of the protofibril peak on a superose 6 HR 10/30 SEC column. (**A**) SEC chromatogram illustrating how the Aβ protofibril peak, which was obtained by incubating Aβ40$_{ARC}$ and Aβ40$_{WT}$ at equimolar concentration (100 μM) for 16 h at room temperature, was divided into seven fractions (F1–F7). The inset shows illustrates the amount of protofibrils and monomers present after the 16 h incubation (RT). (**B**) Negative stain EM images of the protofibril fractions F1, F3, F5, and F7. **Figure 2B** was adapted from *ref. 12.*

al. demonstrated that toxicity was highly dependent on the average diameter of the spherical aggregate. Spherical oligomers with an average diameter >10 nm were shown to exhibit significantly higher toxicity than those with diameter <10 nm. Aβ42 was shown to form spherical aggregates of similar size (d >10 nm) more rapidly (8–10 h rotation vs 5–7 d for Aβ40) and at lower concentrations (0.01–1 u*M*) than Aβ40. Spherical aggregates (d >10 nm) formed by Aβ42 exhibited a 100-fold greater toxicity to neuronal cultures than those formed by Aβ40. The structural and molecular basis for this apparent difference in toxicity of identical morphologies of Aβ40 and Aβ42 remains unknown. The procedure for preparing amyloispheriod is outlined in **Fig. 3**.

3.2.5. Preparation of 3–5 nm Globular Oligomers of Aβ42

Using atomic force microscopy (AFM), several groups have demonstrated that small globular oligomers of Aβ42 with an average height of 3–5 nm could be prepared reproducibly using the protocols developed by Lambert et al. and Stine et al. *(25–27)*. The procedure for preparing 3 to 5 nm globular oligomers is summarized in **Fig. 4** as described by Dahlgren et al. Similar structures with a slightly higher average height of 5.0 + 0.3 nm, referred to as Aβ-derived diffusible ligand (ADDLS) were first described by Lambert et al. *(25,28)*. The exact molecular mass distribution of these 3 to 5 nm structures in solution has not been determined, but on denaturing SDS-PAGE, ADDLS exhibits a distribution of oligomers ranging in size from trimer to tetramer and, in some cases, higher oligomers (6–12 mer) *(25,27,28)* (*see* **Note 3**). However, it remains to be determined whether such globular oligomers of similar height, but formed under different conditions, are structurally and biologically (toxicity) equivalent. The protocol in **Fig. 4** has been shown to yield preparation of Aβ42 oligomers (3–5 nm) with reproducible structural and neurotoxic properties *(27,29–31)*.

3.3. Preparation of Aβ and α-Synuclein Fibrils

Fibrillar Aβ can be generated by incubation of monomeric Aβ fractions from SEC at 37°C for 1 to 4 d, depending on the concentration of the protein and the Aβ variant used. α-Synuclein fibrils can also be prepared by incubation of monomeric α-synuclein (100–200 μ*M*) from SEC at 37°C for 1 to 2 wk or for 1 to 4 d by agitating the sample or placing the tube containing the sample in a rotator at 37°C. The fibrils formed under these conditions are identical to those formed under stagnant conditions (based on EM and AFM). The fibrillar solutions are spun at 15,000*g* during which the fibrils pellet to the bottom of the tube. The supernatant is removed and the fibrils are washed several times with buffer (10 m*M* Tris-HCl, pH 7.4) to remove soluble protein. Prior to use, resuspend the fibrils in the desired buffer. EM should be used to verify the structure of the fibrils and the absence of soluble oligomers in these preparations.
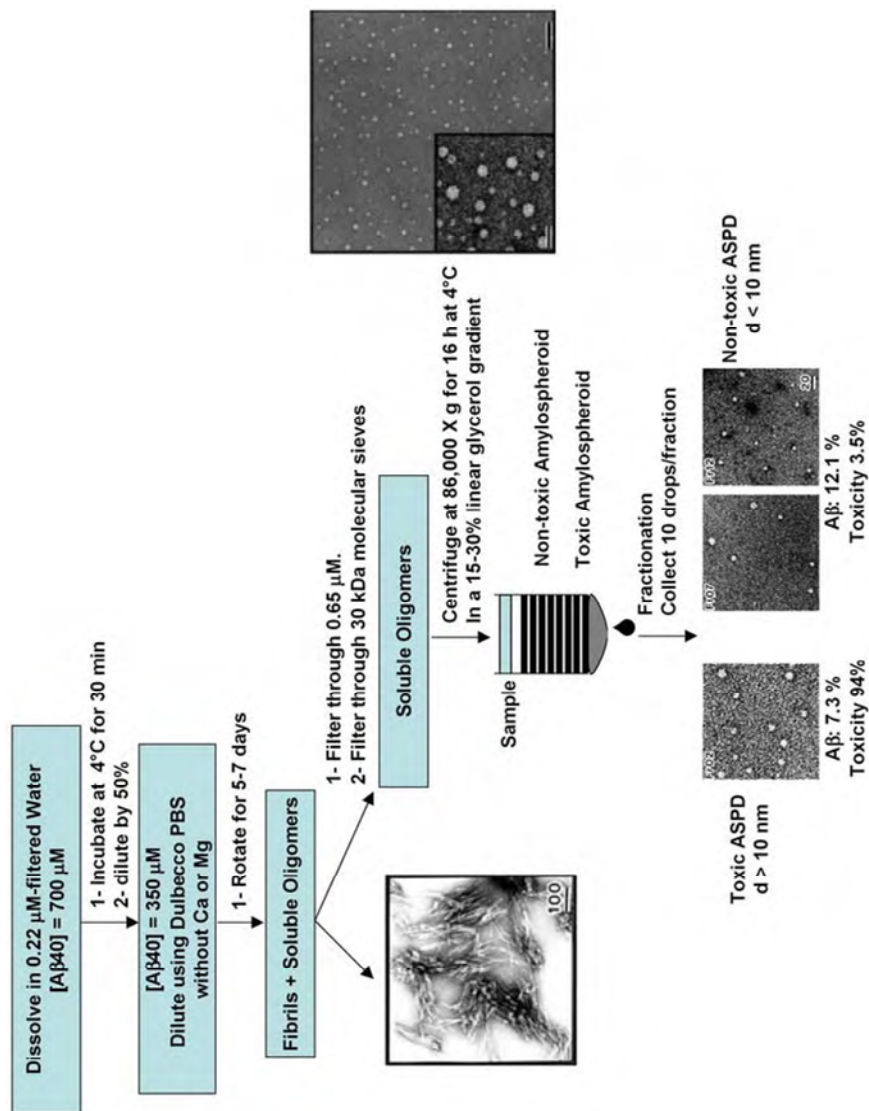
Fig. 3. Amylospheriods preparation. A schematic diagram summarizing the procedures used to prepare Aβ-Amylospheriods of various diameters. Adapted from Hoshi et al. (*24*) with permission from proceedings of the national academy of science.
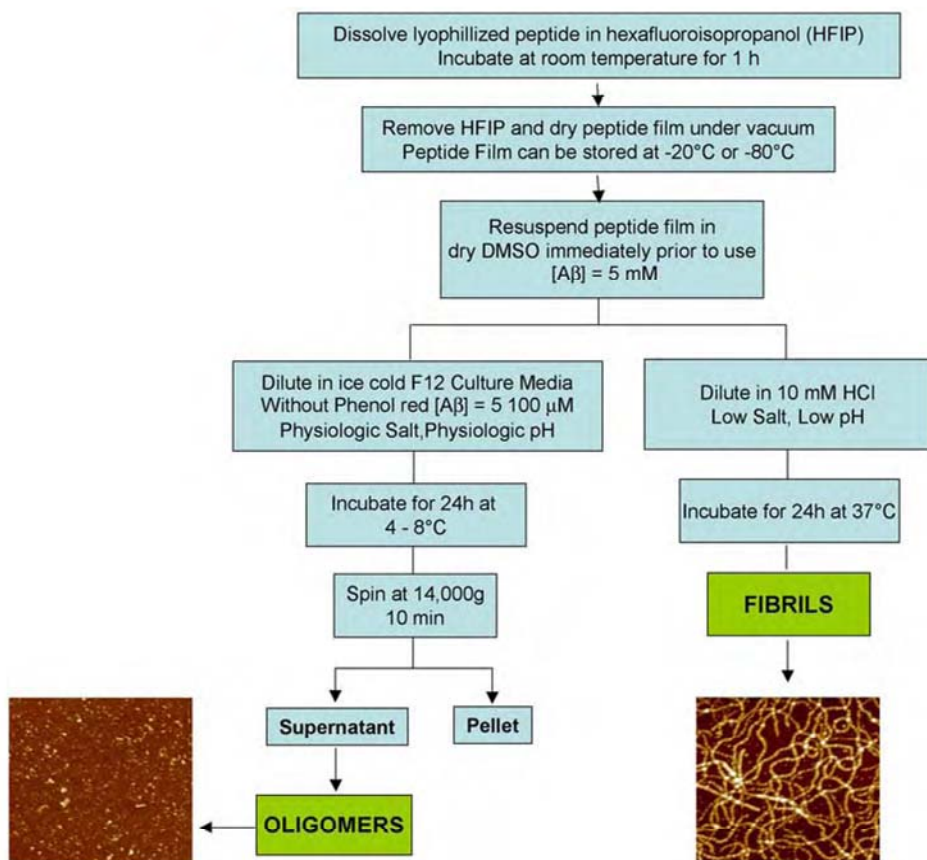
Fig. 4. Preparation of 3 to 5 nm globular oligomers of Aβ42. A schematic diagram summarizing the procedures used to prepare globular oligomers (3–5 nm) of Aβ42 as described by Lambert et al. *(25,28)*, and Dahlgren et al. *(27)*. AFM images were adopted from Stein et al. *(26)*.

4. Notes

1. Various buffers (pH 7.4) can be used in the SEC experiments, including PBS, 10 m*M* Tris-HCl (pH 7.4), and 10 m*M* HEPES. All buffers used need to contain 100 to 150 m*M* NaCl.

2. We did observe some variation in protofibril stability between different preparations. In some cases, a shift in the size distribution of the protofibrils toward higher MW protofibrillar species was observed during the 16 to 24 h procedure. Shortening both the incubation time for inducing protofibril formation and the fractionation time could minimize these changes. Partial separation of the protofibrillar species was possible, in particular, separation of the chain-like protofibrils from the spheres and annular species.

3. These globular oligomers resemble, based on size and morphology, pseudo-spherical oligomers (3.9 ± 0.5 nm diameter) observed by EM and AFM as the first forming oligomers during the in vitro fibrillization of Aβ40 *(10,12,32–34)*.

4. During our studies of Aβ and α-synuclein (both form spherical and annular protofibrils *[7]*), we have observed that spherical oligomers of both Aβ and α-synuclein seem to have the highest affinity for the mica surface, whereas annular protofibrils have the lowest affinity; very few could be detected using enriched annular fractions (unpublished data). In contrast, most of Aβ protofibrillar species appear to adsorb equally to carbon-coated grids used for EM and STEM studies. These findings underscore the importance of supplementing imaging data (AFM or EM) with data from complementary techniques (SVAU and STEM).

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