“Preparing Synthetic Aβ in Different Aggregation States”

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Running Title: Aβ42 Assembly

Abstract

This chapter will outline protocols that produce homogenous preparations of oligomeric and fibrillar amyloid-β peptide (Aβ). While there are several isoforms of this peptide, the 42 amino acid form is the focus because of its genetic and pathological link to Alzheimer’s disease (AD). Past decades of AD research highlight the dependence of Aβ42 function on its structural assembly state. Biochemical, cellular and in vivo studies of Aβ42 usually begin with purified peptide obtained by chemical synthesis or recombinant expression. The initial steps to solubilize and prepare these purified dry peptide stocks are critical to controlling the structural assembly of Aβ. To develop homogenous Aβ42 assemblies, we initially monomerize the peptide, erasing any “structural history” that could seed aggregation, by using a strong solvent. It is this starting material that has allowed us to define and optimize conditions that consistently produce homogenous solutions of soluble oligomeric and fibrillar Aβ42 assemblies. These preparations have been developed and characterized by using atomic force microscopy (AFM) to identify the structurally discreet species formed by Aβ42 under specific solution conditions. These preparations have been used extensively to demonstrate a variety of functional differences between oligomeric and fibrillar Aβ42. We also present a protocol for fluorescently labeling oligomeric Aβ42 that does not affect structure, as measured by AFM, or function, as
measured by a cellular uptake assay. These reagents are critical experimental tools that allow for defining specific structure/function connections.

Keywords
Amyloid-beta, oligomer, fibril, aggregation, atomic force microscopy

1. Introduction

Currently, research is focused on soluble oligomeric assemblies of Aβ42 as the proximate cause of the neuropathology that defines AD. Controlling Aβ assembly is critically important as Aβ structure determines its function (for example, Figures 1 and 2). Numerous experiments have addressed methods to characterize Aβ structure (for review, (1, 2)). These studies demonstrate that peptide conformation and aggregation behavior are highly dependent on initial solvent conditions (Figure 3) and subsequent solution conditions (for example, Figure 1A). Oligomer preparations are defined using a variety of different methods, including neurotoxic activities, isolation techniques (primarily size exclusion chromatography (SEC)), size estimation such as by SDS or native PAGE, imaging techniques, and reactivity with various Aβ conformation-specific antibodies. These multiple operative definitions of oligomeric Aβ have resulted in a literature that is often difficult to interpret and almost impossible to compare. A rigorous approach is particularly important with Aβ42, which aggregates faster and to a significantly greater extent than Aβ40 and other shorter forms of the peptide (for example, Figure 1B).

AFM is particularly well suited to the analysis of amyloidogenic peptides and proteins that can assemble into a variety of structurally discreet species, specifically those like Aβ. Polydispersity of morphologies and sizes often complicates or precludes the use of other biophysical techniques (such as NMR or light scattering methods), or is masked by solvent incompatibilities of the bulk solution (as for secondary structure detected by far-UV circular dichroism). Techniques based on
separation by size (SDS-PAGE, Native PAGE and SEC) may lead to apparent multimers/sizes arising from technical artifacts due to matrix effects. AFM is one of the few techniques that provide direct, high-resolution, 3-dimensional morphological images of the broad range of structures present in a single scan without the need for chemical manipulation of the sample. Numerous studies have demonstrated several advantages of tapping mode AFM for Aβ42 morphological characterization (3-8). We have used AFM for developing conditions that consistently produce homogenous preparations of oligomeric or fibrillar assemblies of Aβ42 (9, 10). We have used these preparations extensively to demonstrate significant functional differences between Aβ42 oligomers and fibrils using a variety of experimental models (for example, Figures 1C and 2B) (9, 11-14).

1.1. Overview of experimental methods to prepare and characterize defined Aβ assemblies (unaggregated, oligomers, fibrils, and “plaques-in-a-dish”) (9, 10)

To directly assess the conformation dependent difference among Aβ assemblies, we have developed protocols for the preparation of homogeneous unaggregated, oligomeric and fibrillar Aβ42 (Figures 1 and 4). Because Aβ42 is the isoform of the peptide associated with AD, we chose to utilize it almost exclusively, with Aβ40 used occasionally as a negative control (Figure 1B and D). Using AFM to image Aβ42, we remove pre-existing aggregates and β-sheet secondary structure from Aβ42 with a strong fluorinated alcohol, hexafluoroisopropanol (HFIP) (Figure 3), followed by solubilization of the now monomerized peptide in dimethylsulfoxide (DMSO). Starting with this monomeric peptide preparation, we further developed two aggregation protocols that consistently produce extensively oligomeric or fibrillar populations of Aβ42 (Figure 1). For the “unaggregated” peptide preparation, the DMSO solubilized peptide is diluted in the experimental solution (for example, culture media) and used immediately (Figure 1A, 0-hours). To grow a “plaque in a dish”, follow the fibril forming procedure, with the addition of salt at physiological
concentrations (Figure 5A-2). Note that AFM is an optimal method for determining the aggregation state of Aβ42 as it is difficult to consistently identify Aβ42 assemblies by Western analysis of SDS-PAGE (for example, Figure 5) (15).

These distinct assemblies are derived from chemically identical and structurally homogeneous starting materials and are thus particularly well suited for comparative structure-function studies. We have demonstrated that in vitro, oligomeric Aβ42 is ~10-fold more neurotoxic than the fibrillar (plaque-forming) assembly, and ~40-fold more toxic than the unaggregated peptide, with oligomeric Aβ42-induced toxicity significant at 10 nM (Figure 1C). Under Aβ42 oligomer- and fibril-forming conditions, Aβ40 remains predominantly as unassembled monomer (Figure 1B) and had significantly less effect on neuronal viability than preparations of Aβ42 (Figure 1D). We applied the aggregation protocols developed for wild type (WT) Aβ42, to Aβ42 with the Dutch (E22Q) or Arctic (E22G) mutations (Figure 2). Oligomeric preparations of the mutant peptides exhibited extensive protofibril and fibril formation, respectively, but were not consistently different from WT Aβ42 in terms of inhibition of neuronal viability. However, fibrillar preparations of the mutants appeared larger in diameter and induced significantly more inhibition of neuronal viability than WT Aβ42 fibril preparations. These data demonstrate that protocols developed to produce oligomeric and fibrillar Aβ42 are useful in distinguishing the structural and functional differences between Aβ42, Aβ40, and Aβ containing known genetic mutations.

1.2. Preparation and use of fluorophore-labeled Aβ42 assemblies

As researchers become increasingly conscientious of utilizing structurally uniform, well-characterized Aβ preparations, the same criteria need to be applied to fluorophore-labeled-Aβ, prior to their widespread use as experimental tools. Numerous recent studies utilizing fluorophore-labeled Aβ42 peptides demonstrate this need for defined methods of consistently preparing well-characterized fluorescent Aβ assemblies (16-31). The fluorescent Aβ42 reagents used to date are prepared from different sources of Aβ assemblies, in many cases using Aβ42 preparations that
have not yet been structurally/morphologically characterized. Thus, structural comparisons between the unlabeled and labeled Aβ assemblies are not possible. Establishing the specific structural form of the assemblies, by AFM and other methods, is necessary to be able to interpret and compare results from the various fluorescent Aβ42 species. We present a method for preparing Alexa Fluor®488-labeled Aβ oligomers, extending our structural and functional characterization to fluorophore-labeling of Aβ42 oligomers. Structural characterization by AFM establishes a method for labeling uniform oligomeric assemblies that is comparable to unlabeled oligomeric Aβ42 (Figure 6A). To compare function, we demonstrate that the uptake of labeled and unlabeled oligomeric Aβ42 by neurons in vitro is also similar (Figure 6B) (see Note 1). These well-characterized fluorophore-Aβ42 oligomers are an exciting new reagent for use in a variety of studies designed to elucidate critical cellular and molecular mechanisms underlying the functions of this Aβ42 assembly form in AD.

2. Materials

2.1. Preparation of HFIP treated Aβ peptide stocks (Figures 3 and 4)

2. 1,1,1,3,3,3-Hexafluoro-2-Propanol (HFIP, Sigma-Aldrich Fluka Cat. 52512 ≥ 99.8%)
3. 2.5mL glass syringe with Teflon plunger (Hamilton Cat. 81430) with sharp non-coring needle tip (point style 5, Hamilton Cat. 7780-03)
4. Chemical fume hood
5. Bath Sonicator (Branson benchtop ultrasonic cleaner)
6. Needle (such as Becton-Dickinson Precision Glide 16 or 18 gauge needle; Fisher Scientific, Cat. 305195)
7. 0.65mL and 1.7mL microcentrifuge tubes (untreated e.g., not siliconized; VWR, Cat. 20170-293 and 20170-355)
8. Eppendorf Repeat Pipettor (Eppendorf Repeater Plus, cat. 022260201) and Combitips
9. SpeedVac (Thermo Model SPD2010 or equivalent)
10. Dessicant (Drierite anhydrous calcium sulfate, Fisher Scientific, Cat. 7778-18-9)
11. Plastic screw-top containers

2.2. Unaggregated Aβ42 Preparation (for example, Figure 1, t = 0h)

1. Dimethylsulfoxide (DMSO, Sigma-Aldrich Sigma Hybri-Max, Cat. D-2650). (see Note 3).
2. Ultrapure 18.2 MΩ H₂O (from a water purification system like the Milli-Q Biocel System, Millipore)

2.3. Oligomeric Aβ42 Preparation (for example, Figure 1A)

1. Ham’s F-12, phenol-red free cell culture media (Promocell cat. C-72117) supplemented with 146 mg/L L-Glutamine (Invitrogen, Gibco®, cat. 25030). (see Note 4)

2.4. Fibrillar Aβ42 Preparation (for example, Figure 1A)

1. 10mM hydrochloric acid solution (prepared in ultrapure H₂O from a 1M HCl stock; Sigma-Aldrich, Cat. H9892)

2.5. Plaques in a dish Preparation (Figure 5A-2)

1. 10mM HCl containing 150mM NaCl

2.6. Fluorophore-labeled Aβ42 oligomer Preparation (Figure 6A)

1. 1X PBS, pH 7.4 (Invitrogen, Gibco® Cat. 70011)
2. Alexa Fluor® 488 TFP Ester Microscale Protein labeling kit (Invitrogen, Molecular Probes, Cat. A30006)

2.7. Structural characterization of Aβ42 preparations
2.7.a. Western blot analysis of SDS-PAGE (For example, Figure 5B)

1. NuPAGE 4 – 12% BisTris gels, MES running buffer, LDS sample buffer (Invitrogen Cat. NP0315, NP0002, NP0008, respectively) and the PowerEase® 500 Power Supply (Cat. EI0001)

2. Molecular weight standards (such as SeeBlue®Plus2 Pre-Stained Standard, Invitrogen Cat. LC5925).

3. PVDF membrane and filter paper packs and Transfer buffer (Invitrogen Cat. LC2005, NP0006, respectively)

4. Methanol

5. Tween-20 (Fisher Scientific Cat. BP337-500)

6. Tris-buffered saline (TBS; 25mM Tris-HCl, pH 7.4, 137mM NaCl, 2.7mM KCl)

7. Carnation Instant Non-fat dry milk (NFDM)

8. Anti-\(\beta\) antibodies (6E10, Covance Cat # SIG-39320; 4G8, Covance Cat # SIG-39245)

9. ECL Western blotting substrate (Pierce®, Cat. 32106)

10. Imaging system to detect chemiluminescence (such as Kodak Image Station 4000R, Carestream Health, Cat. IS4000R)

2.7.b. Atomic force microscopy (AFM) structural analysis (Figures 1, 2, 3, 5, 6)

1. Mica sheets (Ted Pella Cat. 52) die-punched into 7/16” to ½” discs using a punch and die set (Precision Brand, Cat. 40105) and mounted on 12mm stainless steel pucks (Ted Pella Cat.16208) with 2-ton epoxy adhesive (Devcon Cat. 14310).

2. Adhesive tape (such as Scotch® Magic™ Tape)

3. Magnetic sample disc holder, sample disc grippers, and cantilever tweezers (Ted Pella Cat. 16220, 1668, and 5599, respectively)

4. 1M HCl (Sigma Cat. H9892)

5. 0.02µm syringe filter (Whatman Anotop 10, Cat. 6809-1102)

6. 10mL Luer lock non-siliconized syringe (HSW by Sigma Cat. Z248029)
7. Ultrapure H₂O
8. Tetrafluoroethane (CleanTex MicroDuster III, VWR Cat. 58019-540).
9. Veeco Multimode with Nanoscope IIIa controller equipped with a MultiMode head using a Vertical Engage EV piezoceramic scanner (Veeco, Santa Barbara, CA).
10. AFM probes: Al-coated Si cantilevers (42N/m spring constant; ~300kHz resonance frequency; tetrahedral tip with 7nm radius (Olympus, Cat. OMCL-AC160TS-W2)
11. NanoScope Software v. 5.31R1.

2.8. Functional characterization of Aβ42 preparations

2.8a. Neurotoxicity assay (for example, Figures 1C and D, 2B). (See Note 5)

1. Neuro-2a (N2A), mouse neuroblastoma cells (ATCC, CCL 131)
2. White CulturePlate-96 (PerkinElmer, Cat. 6005280)
3. EMEM (Invitrogen, GIBCO® 10370)
4. Liquid Penicillin-Streptomycin (Invitrogen, GIBCO® 15140-122)
5. 0.05% Trypsin solution with EDTA (Invitrogen, GIBCO® 25300-054)
6. FBS (Invitrogen, GIBCO® 16000)
7. N2 supplement (Invitrogen, GIBCO® 17502-048)
8. Unaggregated (section 3.2), oligomeric (section 3.3) and fibrillar (section 3.4) Aβ42
9. DPBS (Invitrogen, GIBCO® 14040)
11. Fluoroskan Ascent® FL (Thermo Scientific)

2.8b. Neuronal uptake assay (Figure 6B)

1. Cellware 8-well culture slides (BD Biosciences, Cat. No. 354632)
2. 16% paraformaldehyde solution (Electron Microscopy Sciences, Cat. No. 15710)
3. Blocking buffer (DPBS, 50mM of NH4Cl, 10 mM of glycine, 3% BSA)
4. Alexa Fluor® 488 donkey anti-mouse IgG (Invitrogen, A21202)
5. VectaShield mounting medium for fluorescence (Vector Laboratories, H-1000)
3. Methods

3.1 Preparation of HFIP treated Aβ peptide stocks (Figures 3 and 4)

Step 3.1-1 through Step 3.1-7 need to be done in a fume hood

1. Prepare a 1mM Aβ solution by adding HFIP directly to the vial containing lyophilized powder through the rubber septum using a 2.5mL glass Hamilton syringe with a Teflon plunger and sharp (not blunt-end) needle. For Aβ 1–42, add 2.217mL to 10 mg peptide. (see Note 6).

2. After the peptide is completely dissolved, pierce the septum with a syringe needle to release the vacuum. (see Note 7).

3. Incubate the Aβ - HFIP solution at RT for at least 30 minutes. (see Note 8).

4. Decap the glass vial (pliers work well) and remove the rubber septum being careful not to allow the HFIP to come in contact with the septum. Have a rack of 0.5mL or 1.7mL microcentrifuge tubes ready.

5. Using an Eppendorf positive-displacement repeating pipette, aliquot the solution into 10µL (0.045mg for 1–42) or 100µL (0.45mg for 1–42) in either 0.5mL or 1.7mL microcentrifuge tubes. (see Note 9)

6. Allow HFIP to evaporate in the open tubes overnight in the fume hood.

7. Transfer tubes to a SpeedVac and dry down for 1 hour without heating to remove any remaining traces of HFIP and moisture.

8. Remove tubes and the resulting peptide should be a thin clear film at the bottom of the tubes. (see Note 10).


10. Prior to use, remove peptide film from –20°C freezer and allow sample to come to room temperature (RT).
11. Prepare a 5mM Aβ DMSO stock by adding 20µL fresh dry DMSO to 0.451mg Aβ42 peptide (2µL to 0.045mg Aβ42). Pipette thoroughly, scraping down the sides of the tube near the bottom to ensure complete resuspension of peptide film. (see Note 12)

12. Vortex well (~30 seconds) and pulse in a microcentrifuge to collect solution at the bottom of the tube. (see Note 13).

13. Sonicate 5mM Aβ DMSO solution for 10 minutes in a bath sonicator

3.2. Unaggregated Aβ Preparation (for example, Figure 1A, t =0). (see Note 14).

1. Dilute freshly resuspended 5mM Aβ42 in DMSO at RT in the same tube with ice-cold H2O to a final concentration of 100µM Aβ. (see Notes 15).

2. Vortex for 15 seconds and use immediately.

3.3 Oligomer Aβ Preparation (for example, Figure 1A)

1. Dilute freshly resuspended 5mM Aβ42 in DMSO at RT (Do not keep 5mM Aβ stock on ice because the DMSO will solidify) in the same tube with cold phenol-free F-12 cell culture media to a final concentration of 100µM Aβ. (For example, 2µL 5mM Aβ in DMSO + 98µL cold F-12) Remember to use proper sterile technique. When using F-12 media, avoid prolonged exposure to light and keep F-12 solutions on ice.

2. Vortex for 15 seconds, transfer to 4°C and incubate for 24 hrs.

3.4 Fibril Aβ Preparation (for example, Figure 1A)

1. Dilute freshly resuspended 5mM Aβ42 in DMSO at RT (Do not keep 5mM Aβ stock on ice because the DMSO will solidify) in the same tube with 10mM HCl at RT to 100µM final Aβ (for example 2µL 5mM Aβ in DMSO + 98µL 10mM HCl).

2. Vortex for 15 seconds, transfer to 37°C and incubate for 24 hrs.

3.5. “Plaque in a Dish” Preparation (Figure 5A-2)

1. Dilute freshly resuspended 5mM Aβ42 in DMSO at RT in the same tube with 10mM HCl + 150mM NaCl to 100µM final Aβ.
2. Vortex for 15 seconds, transfer to 37°C and incubate for 24 hrs.

3.6. Fluorophore-labeled Aβ42 oligomer Preparation (Figure 6)

1. Use a 0.045mg Aβ42 film. Dilute freshly resuspended 5mM DMSO Aβ42 in the same tube with cold 1X PBS buffer, pH 7.4 to a final concentration of 100µM Aβ.

2. Incubate under oligomer-forming conditions (4°C, 24h as in 3.3).

3. Prepare solution for labeling using the Alexa Fluor® 488 Microscale Protein labeling kit (Molecular Probes/Invitrogen) according to the manufacturer instructions by adding 10% volume of 1M NaHCO₃.

4. Dissolve the provided Alexa Fluor® 488 TFP Ester into 10µL ultrapure H₂O immediately before adding to Aβ oligomers. Use 8µL dye for every 100µL oligomer solution.

5. Incubate the labeling reaction for 15 minutes in the dark at RT.

6. In the meantime, prepare the spin columns by delivering 425µL of the kit-provided Bio-Gel P-6 fine resin slurry into the provided spin column tubes. Prepare two spin columns for every 100µL of oligomers. One minute before labeling incubation is up, centrifuge the spin columns at RT for 15 seconds at 16,000xg per the manufacturer recommendations.

7. Add 55µL of the crude labeling reaction to the top of the resin and centrifuge for 1 minute at 16,000xg to removed unincorporated fluorophore. The leftover crude reaction solution can be analyzed by gel.

8. Store the labeled product at 4°C protected from light. Avoid prolonged storage.

3.7. Structural Characterization of Aβ42 preparations

3.7.a. Western analysis of SDS-PAGE (for example, Figure 5B) (see Note 16)

1. Prepare dilutions of Aβ solutions in LDS sample buffer without reducing agent to deliver 50–200pmoles Aβ per lane.

2. Perform electrophoresis on NuPAGE 4–12% Bis-Tris gels in 1X MES running buffer until the dye front reaches the bottom of the gel (see Note 17).
3. Prepare the gel for transfer to PVDF membrane in the transfer cassette using filter paper, pads and membrane pre-equilibrated in chilled 1X transfer buffer containing 10% MeOH.

4. Set power supply limits for the transfer for 20–30 minutes at very low current (such as 15 mA) followed by 25V, 160mA for 1 h.

5. After transfer, incubate the membrane in TBST for 5 minutes

6. Block for 30–60 minutes in TBST + 5% NFDM.

7. Perform incubation with primary antibody (1:5000 dilutions of mouse monoclonal antibody 4G8 (anti-Aβ 17–24) or 6E10 (anti-Aβ 1–16) prepared in TBST + 5% NFDM) overnight at 4°C.

8. Perform 3 x 10 minute washes in TBST + 5% NFDM

9. Incubate in secondary antibody for 1 hour at RT (1:10,000 dilutions of rabbit-anti-mouse IgG-HRP conjugate).

10. Wash 3 x 10 min in TBST + 5% NFDM

11. Rinse in TBST

12. Incubate in ECL substrate for 1 min followed by immediate detection of chemiluminescence on the imaging system.

3.7.b. Atomic Force Microscopy (AFM). (Figures 1, 2, 3, 5, and 6)

1. Fill a 10mL syringe with ultrapure water and equip with a 0.02 μm filter. Discard the initial 1–2mL syringe filter output. All subsequent steps use 0.02 μm-filtered water.

2. Prepare samples for spotting on mica by diluting to final concentrations of 10–30μM in water.

3. Immediately before sample delivery, cleave away the top 1–4 layers of mica using adhesive tape to reveal a clean, flat, featureless surface.
4. For mica pre-treatment, add ~3µL (enough to cover the surface) of 1M HCl to mica for 30 seconds and rinse with 2 drops of water by letting water out of the syringe filter roll over the mica, held at a 45° angle on a magnetic surface. (see Note 18).

5. Immediately deliver sample onto mica and incubate for 3 min.

6. Rinse with 3 drops of water and blow dry with several gentle pulses of compressed air.

7. Let sit on benchtop for a few minutes to hours (covered to protect from dust) at RT until analysis. (see Note 19).

8. When AFM tip engages, optimize instrument parameters for each scan keeping contact force at a minimum, with scan rates between 1–2 Hz, drive amplitude between 20–100 mV (depending on cantilever), and amplitude set point between 1.4 and 1.5V.

9. Process data to remove vertical offset between scan lines by applying zero order flattening polynomials using NanoScope Software v. 5.31R1.

3.8. Functional characterization of Aβ42 preparations

3.8a. Neurotoxicity/viability assay (Figures 1C and D, 2B) (see Note 5)

Cell Culture

1. Mouse neuroblastoma Neuro-2a (N2A) cells are routinely grown in 100-mm tissue culture dish containing 10mL of complete growth medium consisting of EMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cells are maintained in a humidified incubator at 37°C with a 5% CO₂ atmosphere.

2. Cells are split such that 0.5 x 10⁶ cells were seeded in a new dish each time the cells reached 80–90% confluency. Briefly, when the cells reach this confluency (approximately once a week), the old growth medium is removed, and DPBS is used to wash cells. 1.5mL of 0.05% Trypsin-EDTA solution is added to cells and the dish is returned to the incubator for 2–5 minutes without disturbance. When cells are detached from the dish, a 6mL of the complete growth medium is added to halt the trypsinization of cells. Cells are collected in a
sterile 15mL conical tube, and pelleted in a clinical centrifuge with the setting of 168 x g and 2 minutes. The supernatant is decanted and cells are resuspended in 6mL of complete growth medium. The cell density in a 12µL aliquot of this cell suspension is counted using a hemocytometer. Based on the counted cell density, an appropriate volume to deliver 0.5 x 10^6 cells is then added to a new 100 mm dish containing 10mL of complete growth medium. The dishes are then placed back into the incubator.

The neurotoxicity assay (see Note 5)

Day 1: Preparing cells and Aβ (24 h prior to cell treatment)

3. Start fresh oligomeric or fibrillar Aβ preparations so that it will be ready to use the next day.

4. Prepare 96 well plate culture of cells to be treated. N2A cells from a healthy growing dish are seeded at 5,000 cells/well on an all-white 96-well plate in the complete growth medium. An accurate multi-channel pipette is ideal to dispense cells. In experimental planning, calculate the minimal number of wells required. For example, within a 96 well plate, we typically perform 5–6 replicate treatments of the same dose of Aβ assembly (e.g., 10µM oligomers). Do not use corner wells in the plate as these wells do not have consistent luminescence values. Also included are control wells containing medium without cells to measure the background luminescence of the wells. Cells are allowed to grow for 24 hours before the start of treatments for the neurotoxicity assay.

Day 2: Cell treatment

5. Prepare the 96 well plate cell cultures for treatment. Cells are washed twice with 90µL of pre-warmed plain EMEM medium using a 12-channel pipette. Take care to not touch the well bottom to minimize cell loss during this washing step. 90µL of fresh EMEM medium supplemented with 1% N2 Supplement is added to each well.

6. Add the appropriate volumes of prepared oligomeric or fibrillar Aβ to the wells being treated according to the desired experimental design (e.g., comparing oligomers vs. fibrils at 10µM
Aβ concentration). The final volume of medium is 100µL. Add the same volume of F-12 or 10 mM HCl + DMSO to other wells to serve as the vehicle control. Cells are returned to the incubator for 24h.

Day 3: Measuring toxicity

7. At the end of the 24 hour treatment, the plate is left at the bench for 10 minutes to allow it to equilibrate to RT. The neurotoxicity assay is performed by measuring the cellular ATP value with CellTiter-Glo® reagent according to the manufacturer’s instruction. Briefly, the reagent is thawed to reach RT and equal volume (100µL) of the reagent is added directly into cells. The plate is then gently rotated in an orbital shaker for 10 minutes. The luminescent intensity is then measured in a luminescent plate reader such as Ascent® Fluoroskan FL.

8. To calculate the neurotoxicity, the data set is normalized to the vehicle-treated cells, which is set to 100% for viability, and the relative values of treated cells vs. control cells is calculated accordingly as cell viability for the treatments.

3.8b. Neuronal uptake assay (Figure 6B)

1. N2A cells are seeded at 30,000 cells/well on poly-D-lysine coated Cellware 8-well culture slides for 8 hours in phenol-red free DMEM + 10% FBS.

2. Cells are washed with plain DMEM medium. Alexa Fluor® 488-labeled (1–2µM) or unlabeled (10µM) synthetic Aβ42 oligomers are added to cells in the fresh media of DMEM supplemented with 1% N2, and incubated for 2–16 hours at 37°C.

3. At the end of the treatment, cells are washed extensively with blocking buffer without BSA, and then fixed in 4% paraformaldehyde for 20 min at RT.

4. Cells treated with unlabeled Aβ42 oligomers are permeabilized with 0.2% Triton X-100 in DPBS for 5 minutes, and then blocked for 15 min with 3% BSA in the blocking buffer. Cells are then incubated overnight with anti-Aβ(1–16) antibody 6E10 (1:500) at 4°C. After several
washing with DPBS, cells are incubated 1 hr at RT with Alexa488-labeled donkey anti-mouse IgG (1:500). Cells are further washed several rounds with DPBS. Wells are mounted with VectaShield mounting medium and covered with glass coverslips.

5. Immunofluorescence images are acquired on a Zeiss LSM 510 META, Axiovert 200M laser scanning confocal microscope using a Plan-Apochromate Zeiss 40X/1.3 oil immersion objective. To visualize the 488 nm excited fluorophores from the directly fluorophore-labeled Aβ42 oligomers, or the Alexa488-immunolabeled Aβ42 oligomers, 488 nm laser light (krypton-argon laser), a 488/543 two notch dichroic excitation mirror, and a 505–530 nm bandpass emission filter are used with optimized PMT parameters.

4. Notes

1. Cellular uptake by neurons is reported only for oligomeric Aβ42 as treatment with fibrillar Aβ42 does not result in any detectable uptake in the model described (data not shown).

2. In-house synthetic peptide or peptide from other vendors will also work but it must be of very high purity and quality. The TFA salt (as opposed to the acetate or ammonium salt) is preferred. In-house material should be accurately weighed in clean glass vials with a HFIP resistant closure.

3. Dry DMSO stocks can be made by transferring DMSO from a freshly opened ampule to a 1–2 mL glass vial with a DMSO-resistant closure (such as Teflon – VWR, Cat. No. 66009-556). Store vials containing the dry DMSO in a dessicated glass jar in the dark at RT and discard after 2 weeks.

4. The glutamine supplementation is to match the composition of the Promocell phenol-red free F-12 media to the media used in the original oligomer protocol (10) Biosource, which is no longer available.

5. Originally, we used the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche Molecular Biochemicals) as a measure of neurotoxicity (9, 11).
(Figures 1C and D, and 2B). This method is based on the reduction of internalized MTT tetrazolium to a colored formazan compound by cellular redox potential. The formazan production is proportional to viable cells in culture. However, MTT reduction does not necessarily reflect cellular metabolic activity, as some Aβ assemblies may also enhance exocytosis of MTT formazan (32). This assay also requires relatively long staining and extraction times. Therefore, we now use CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Cat. No. G7572) as a measure of in vitro neurotoxicity. This has resulted, as one would predict, in less toxicity for comparable doses of Aβ and so higher doses of Aβ42 are now required to achieve the same toxicity (12).

6. HFIP is corrosive and very volatile; avoid contact and work in the fume hood; take care not to contact septum or other surfaces during solubilization.

7. Peptide comes stored under vacuum and the peptide in the bottom of the vial needs to be in solution before the vacuum is broken. After the peptide is in solution, pierce the septum with a syringe needle to release the vacuum. For other peptides, add enough HFIP such that the final peptide concentration is 1mM. Use proper sterile technique to avoid any bacterial contamination when the peptide stocks are resuspended in culture media or buffer.

8. Solution should be clear and colorless. Any trace of yellow color or cloudy suspension indicates poor peptide quality and should not be used. Some peptides may require brief ~5 minute bath sonication.

9. Do not use silinized tubes for preparation of HFIP stocks. Be careful when dispensing HFIP solution and watch for bubbles. Leave tubes open when evaporating HFIP overnight.

10. The peptide should not be white or chunky. An even clear film is a strong indicator of good peptide quality.
11. These stocks should be stable for several months to years.

12. DMSO stock should be clear and colorless. Remember to use proper sterile technique.

13. Do not store peptide as a DMSO stock for more than one hour to avoid protofibril formation.

14. While the “unaggregated “ prep is an ideal control for conformation, it is most useful in assays that require either a very low concentration of peptide (for example, (9)) or a short incubation period (for example, (14)). Prolonged incubation at higher concentrations result in the uncontrolled aggregation of the peptide and unpredictable functional activity

15. Do not keep 5mM Aβ stock on ice because the DMSO will solidify.

16. Western analysis of SDS-PAGE is not a method for assessing the conformation/assembly of Aβ42 (Figure 5) (15). However, it is useful for visualizing the relative amount of peptide for comparison between samples.

17. Depending on the age of the electrode/power supply equipment, voltage and current settings may affect the pattern and abundance of bands typically observed for Aβ (monomer, dimer, trimer and tetramer). We have found that power supply limits set at 90–100V, 80mA for 80–90 minutes for electrophoresis yield consistent results.

18. For Aβ preparations in F-12, the HCl pretreatment of the mica improves consistent and uniform peptide adsorption to the mica. For Aβ preparations in HCl or PBS, including the Alexa Fluor® 488-labeled oligomers, no mica pretreatment is performed.

19. Dried sample discs can be stored in a helium-purged desiccator for several months.

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References


FIGURE LEGENDS

Figure 1. Structure and neurotoxicity of oligomeric or fibrillar Aβ42 and Aβ40 assemblies.

(a, b) Aβ42, but not Aβ40, forms oligomeric and fibrillar assemblies. 5 mM HFIP-treated Aβ42 (a) or Aβ40 (b) in DMSO was diluted to 100 μM in ice-cold F-12 culture media for oligomers, or 10 mM HCl for fibrils. Oligomer and fibril preparations were incubated for 24h at 4°C and 37°C, respectively. Samples before (0h) and after incubation (24h) were mounted for AFM analysis at 10 μM. Representative 2 x 2 μM x-y, 10 nm total z-range AFM images. Inset images 200 x 200 nm x-y, 2 nm total z-range. Reprinted from Stine et al., JBC 2003, with permission from ASBMB. (c, d) Oligomeric Aβ42 reduces neuronal viability significantly more than fibrillar and unaggregated species. Further, under the same preparation conditions for unaggregated, oligomeric, and fibrillar for Aβ42 assemblies, Aβ40 has much less toxicity than Aβ42 assemblies. Unaggregated, oligomeric, and fibrillar preparations of Aβ42 (c) or Aβ40 (d) were incubated with N2A cells for 20h. Oligomeric and fibrillar preparations of Aβ were prepared as described above. For unaggregated peptide preparations, the 5 mM Aβ in DMSO was diluted directly into cell culture media. The MTT assay was used as an indicator of cell viability. Graph represents the mean ± SEM for n≥8 from triplicate wells from at least 2 separate experiments using different Aβ preparations. * Significant difference between Aβ assemblies prepared in oligomers and fibrils conditions (p<0.01). ** Significant difference between unaggregated and both Aβ assemblies prepared oligomers and fibrils conditions (p<0.01). Reprinted from Dahlgren et al., JBC 2002, with permission from ASBMB.

Figure 2. Structure and neurotoxicity of oligomeric or fibrillar wildtype, Dutch (E22Q), and Arctic (E22G) Aβ42.

Panel A: Both E22Q and E22G exhibit enhanced fibril formation, even under oligomer-forming conditions. Representative 2x2μm, 10nm total z-range AFM images of 100μM Aβ. Oligomeric and fibrillar preparations of Aβ42 wildtype, E22Q (rPeptide, Athens, GA.), and E22G (California Peptide,
Napa, CA). Oligomeric and fibrillar preparations of Aβ were prepared as described in the legend to Figure 1A (and Figure 4) and imaged at 10µM. Reprinted from Dahlgren et al., JBC, 2002, with permission from ASBMB.

Panel B: The “toxic fibrils” formed by E22Q and E22G are significantly more toxic than even WT oligomers. Changes to structural assembly states of mutant Aβ42 observed by AFM (above) translate into changes in function as measured by cellular toxicity. N2A cells were treated for 20h with 0.1µM of WT Aβ42 oligomers and fibrils, mutant E22Q Aβ42 or mutant E22G Aβ42 assemblies from oligomer and fibril-forming conditions. MTT assay was used as an indicator of cell viability. The data represent n ≥ 8 triplicate wells from at least 2 separate experiments using different Aβ preparations. * = significant difference between oligomers and fibrils (p < 0.01). Reprinted with modifications from Dahlgren et al., JBC, 2002, with permission from ASBMB.

Figure 3. AFM analysis of Aβ42 solubilized in HFIP and H₂O.

Lyophilized synthetic Aβ42 was solubilized to 5mM in 100% HFIP or deionized H₂O. 5mM stock solutions were incubated for 24 hours at RT. Samples before (0 hours) and after incubation (24 hours) were mounted for AFM analysis at 10µM. Representative 1x1µm x-y, 5nm total z-range AFM images. Inset image 390x390nm x-y, 5nm total z-range. Reprinted with modifications from Stine et al., JBC, 2003, with permission from ASBMB.

Figure 4. Schematic diagram summarizing the solubilization and aggregation conditions developed for preparing oligomeric and fibrillar Aβ42.

Synthetic Aβ42 was dissolved to 1mM in 100% HFIP, HFIP was evaporated, and the dry peptide was stored at −20°C. For the aggregation protocols, the peptide was first resuspended in dry DMSO to 5mM. For oligomeric conditions, F-12 (without phenol red) culture media was added to bring the peptide to a final concentration of 100µM, and incubated at 4°C for 24 hours. For fibrillar conditions,
10mM HCl was added to bring the peptide to a final concentration of 100μM, and incubated for 24 hours at 37°C. Reprinted from Dahlgren et al., JBC, 2002, with permission from ASBMB.

Figure 5. Diverse Aβ42 assemblies imaged by AFM are not preserved by SDS-PAGE.


5mM Aβ42 in DMSO stocks were diluted to 100μM in either 10mM HCl (low ionic strength, acidic pH), 10mM HCl + 150mM NaCl (acidic pH, physiologic ionic strength), 10mM Tris, pH 7.4 (neutral pH), or 10mM Tris, pH 7.4, + 150mM NaCl (neutral pH, physiologic ionic strength). Samples were prepared after a 2h incubation at 37°C. Representative 2x2μm x-y, 10nm total z-range AFM images are shown, except for the image of the acidic pH, physiologic ionic strength condition, which is scaled to 2x2μm x-y, 25nm total z-range. Reprinted from Stine et al., JBC, 2003, with permission from ASBMB.

Panel B: Western analysis of SDS-PAGE does not produce an immunoreactive pattern that correlates with AFM images in Panel A. Representative Western blots of Aβ42 assemblies prepared as described above, separated by SDS-PAGE on a 12% NuPAGE Bis-TRIS gel, and probed with the monoclonal antibody 6E10 (recognizing residues 1-16 of Aβ). Samples were visualized by enhanced chemiluminescence. Lanes: HCl (lane 1), HCl + NaCl (lane 2), Tris (lane 3), and Tris + NaCl (lane 4). Reprinted from Stine et al., JBC, 2003, with permission from ASBMB.

Figure 6. Structure and neuronal uptake of Alexa Fluor® 488-labeled Aβ42 oligomers compared to unlabeled Aβ42 oligomers.

Panel A: AFM analysis shows that oligomer assemblies are preserved after fluorophore-labeling.

Aβ42 oligomers were prepared from unlabeled synthetic Aβ42 HFIP films (100μM, PBS pH 7.4, 4°C) and analyzed by AFM (Panel A.1). Panel A.2: Fluorophore-labeling of the oligomers with Alexa Fluor® 488 was performed using the Microscale Protein Labeling Kit and analyzed by AFM.

Unlabeled oligomers were diluted to 20μM for analysis and Alexa-labeled oligomers were analyzed...
without dilution (estimated concentration of 25µM). All AFM images shown are 2x2µm x-y, 10nm total z-range.

Panel B: Following uptake, Alexa Fluor® 488-labeled oligomers (B2) appear as punctate fluorescence within the cell, similar to immunodetection of unlabeled Aβ42 oligomers (B1). Following 16h treatment, Aβ uptake in N2A cells was analyzed using confocal laser scanning microscopy. Panel B.1 shows the image of cells treated with unlabeled Aβ42 oligomers, immunodetected with anti-Aβ monoclonal antibody 6E10 and Alexa488-rabbit-anti-mouse antibody. Panel B.2 shows N2A cells treated for 16h with 2µM Alexa Fluor® 488-labeled Aβ42 oligomers. Scale bar = 44µm. The insets show a single-cell magnification, scale bar = 12µm. Reprinted from Jungbauer et al., Preparation of fluorescently-labeled amyloid-beta peptide assemblies: the effect of fluorophore conjugation on structure and function, J. Mol. Recog., 2009, with permission from Wiley.
Figure 1

A

Oligomer-forming conditions
Fibril-forming conditions

0-h

24-h

B

Oligomer-forming conditions
Fibril-forming conditions

C

Concentration of Aβ1-42 (µM)
Survival (% of control)

D

Concentration of Aβ1-40 (µM)

Figure 2

A

<table>
<thead>
<tr>
<th>Oligomer-forming conditions</th>
<th>Fibril-forming conditions</th>
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<tbody>
<tr>
<td>WT</td>
<td></td>
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<tr>
<td>E22Q</td>
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<tr>
<td>E22G</td>
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B

Toxicity of corresponding WT oligomers or fibrils

% Change from WT

Oligomers Fibrils

Toxic Fibrils

↑ toxicity of “oligomers”

↓ toxicity of “oligomers”

Dutch (E22Q) Arctic (E22G)
Figure 3
Dissolve lyophilized peptide in hexafluoroisopropanol (HFIP)

Remove HFIP and dry peptide film under vacuum

Resuspend peptide film in dry DMSO immediately prior to use

Dilute in F12 Culture Media
Physiologic Salts
Physiologic pH
Incubate 24h at 4°C
OLIGOMERS

Dilute in 10mM HCl
Low Salt
Low pH
Incubate 24h at 37°C
FIBRILS

Low Salt
Low pH

Physiologic pH
Figure 5

A

Low

Physiologic Ionic Strength

Acidic pH

Neutral pH

B

large oligomers and aggregates

tetramer

tramer

monomer

200kDa

1 2 3 4
Figure 6

A

Unlabeled Aβ42 oligomers

Alexa488-labeled Aβ42 oligomers

B

Alexa488-labeled Aβ42 oligomers

Unlabeled Aβ42 oligomers