PEDS Advance Access published May 11, 2011

Protein Engineering, Design & Selection pp. 1–8, 2011
doi:10.1093/protein/gzr020

A standardized and biocompatible preparation of aggregate-free amyloid beta peptide for biophysical and biological studies of Alzheimer's disease

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Received April 7, 2011; revised April 7, 2011; accepted April 8, 2011

Edited by Sir Alan Fersht

We provide a validated and rapid protocol for the solubilization of amyloid β-peptide (Aβ). This procedure involves sequential solubilization using structure-breaking organic solvents hexafluoroisopropanol and DMSO followed by column purification. The low solubility and tendency of Aβ to aggregate considerably impede the *in vitro* handling and biophysical or biological investigation of Aβ, despite the interest in this peptide because of its implication in Alzheimer's disease. The main advantage of the proposed protocol over others is that it results in standardized aggregate-free Aβ peptide samples that are biocompatible for cell culture studies and yield reproducible aggregation kinetics and cytotoxicities. This three-step protocol also enables the co-solubilization of the longer Aβ42 variant with Aβ40 in ratios relevant to Alzheimer's disease.

Keywords: aggregation/amyloid beta peptide/biocompatibility/*in vitro* studies/solubilization

Introduction

The importance of protein aggregation has become increasingly clear in the last decades with the discovery of a link between this phenomenon and a number of human diseases, including scrapie (Prusiner *et al.*, 1983), familial amyloidosis (Costa *et al.*, 1978) and Parkinson's disease (Spillantini *et al.*, 1997). Among the proteins identified in amyloid plaques, amyloid β (Aβ) peptides are special because they constitute what could be considered as the prototype of an intrinsically unstructured sequence with an unusually high amphipathic character. Also, they are directly implicated in Alzheimer's disease, one of the emerging threats of modern society. Aβ peptides are generated from amyloid precursor protein as a result of sequential β-secretase and γ-secretase proteolytic activity (De Strooper *et al.*, 1998; Vassar *et al.*, 1999; Wolfe *et al.*, 1999). Imprecise cleavage specificity by γ-secretase results in the generation of a range of Aβ peptides varying in C-terminal length from 37 up to 46 amino acids (Wiltfang *et al.*, 2002; Lewczuk *et al.*, 2003; Maddalena *et al.*, 2004) although the majority of this pool is composed of Aβ40 and Aβ42 (Suzuki *et al.*, 1994; Scheuner *et al.*, 1996). It is now thought that the Aβ variation pattern in the brains of patients with Alzheimer's disease is reflected by a general elongation of the Aβ peptides through a modulated γ-secretase activity (Wiltfang *et al.*, 2002; Lewczuk *et al.*, 2003; Bentahir *et al.*, 2006). Familial mutations in the gene encoding for the independent composites of the γ-secretase complex all induce the formation of longer Aβ peptides, which appear to be directly involved in plaque formation in the brains of Alzheimer's disease patients (Jarrett *et al.*, 1993; Suzuki *et al.*, 1994; Scheuner *et al.*, 1996). The extra residues in the elongated Aβ peptides are hydrophobic thus drastically influencing the solubility and amyloidogenicity of the peptide. To effectively study the impact of Aβ on toxicity and to elucidate the structural pathway of aggregation and formation of toxic oligomers, it is crucial to initiate the studies with a well-defined aggregate-free Aβ species. It is, however, difficult to deal with Aβ peptides precisely for the very reason that makes them interesting: they are poorly soluble and aggregation-prone in aqueous solutions. Proteins that are prone to aggregate under physiologically relevant conditions are usually characterized by high hydrophobicity and/or low net charge (Chiti *et al.*, 2003; DuBay *et al.*, 2004; Fernandez-Escamilla *et al.*, 2004; Linding *et al.*, 2004). Apart from their tendency to induce pathological conditions and high aggregation propensity, the low solubility of these peptides also results in inherent difficulties in the experimental handling and investigation by biophysical techniques and in cell culture. Nevertheless, high-resolution characterization of these proteins may provide the information required to tackle key questions aiming at therapeutic targeting. In response to these technical difficulties that significantly hamper the research into neurodegenerative diseases, a variety of protocols to aid the solubilization of Aβ are available in the literature. However, these procedures often involve the use of extremely alkaline (Fezoui *et al.*, 2000) or acidic solutions (Ward *et al.*, 2000) or rely on the presence of organic solvents (Bitan and Teplow, 2004), thus introducing biologically incompatible compounds and/or conditions that are toxic to cell cultures. Filtration of freshly prepared Aβ samples is also frequently used to remove pre-existing aggregates, but often leads to significantly reduced recovery of soluble Aβ material (Bernstein *et al.*, 2005). It is therefore essential to develop a robust and reproducible preparation protocol that allows the solubilization of these peptides under biocompatible buffer conditions.

Here, we describe a new and validated procedure to solubilize the amyloid β-peptide that circumvents the above-mentioned problems and allows us to obtain appreciable quantities of virtually aggregate-free material. The method relies on the use of a sequential solubilization procedure with organic solvents, followed by the complete removal of these chemicals to provide a non-toxic environment that is suitable for biophysical characterization. We validated the procedure by demonstrating complete solubilization of the peptide and providing evidence that all chemicals involved are effectively removed from the peptide solution without introducing oxidation of the peptide. By using Thioflavin T fluorescence (Tht) assays and cell culture studies, we show that the resulting solutions exert highly reproducible aggregation kinetics and toxicities. We conclude that our procedure provides monomeric Aβ preparations in a cell-biology-compatible environment, and is suitable for biophysical characterization.

Materials and methods

Reagents

Escherichia coli expressed human recombinant Alzheimer's beta peptide 1–40, Ultra Pure HFIP (cat. A1153), 1–42 Ultra Pure HFIP (cat. A-1163) and their uniformly ^{15}N-isotope-labeled variants (cat. A-1101-2 and A-1102-2, resp.) were purchased from rPeptide. Imperial™ Protein Stain (cat. 24615) was obtained from Pierce. Dimethyl sulfoxide, minimum 99.5% GC (cat. D4540), Trizma base (cat. T6066), Trizma hydrochloride (cat. T5941), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 99+% (cat. 105228) and disodium ethylenediaminetetraacetate (EDTA) (cat. E4884) were obtained from Sigma. Quick Start™ Bradford Dye Reagent (cat. 500–0205) was purchased from BioRad. Invitrogen provided Novex® 10–20% Tricine gels (cat. EC66252), SeeBlue® Plus 2 pre-stained standard (1×) (cat. LC5925), Novex® Tris-Glycine SDS sample buffer (2×) (cat. LC2676) and Novex® Tricine SDS running buffer (10×) (cat. LC1675).

Preparation of Aβ peptide solutions

Various ratios of Aβ42:Aβ40 were prepared starting from material previously stored in a −20°C freezer. The vials containing 0.5 mg Aβ-HFIP films were allowed to defrost at room temperature (~25°C) for 10 min. A quantity of 0.5 mg Aβ40 or Aβ42 was dissolved in 500 μl hexafluoroisopropanol (HFIP). The sample was mixed vigorously using a vortex for 1 min and visually inspected for efficient solubilization. To prepare Aβ42:Aβ40 ratios, a gas tight syringe was used to add together specific volumes of Aβ40 in HFIP and Aβ42 in HFIP to obtain a defined ratio of Aβ42:Aβ40 followed by mixing using a vortex for 1 min. The preparation of specific ratios is further reported by Kuperstein *et al.* (2010). The HFIP was evaporated using a gentle stream of oxygen-free nitrogen gas and, alternatively, argon gas can be used. Based on an HFIP volume of 500 μl in each vial prior to drying with nitrogen gas, the peptide/HFIP films were redissolved in 500 μl dimethyl sulfoxide (DMSO). Samples were mixed using a vortex for 1 min and visually inspected for efficient solubilization. Immediately thereafter, the Aβ peptide solutions were separated from the DMSO by means of a desalting column (HiTrap™ Desalting column (cat. 17-1408-01), GE Healthcare) that was pre-equilibrated with 25 ml 50 mM Tris-HCl, 1 mM EDTA buffer, pH 7.4. Other buffer systems, including phosphate-buffered saline can also be used. The 500 μl sample was applied to the column using a 1 ml syringe followed by additional injection of 1 ml buffer, the flow-through was discarded. Subsequently, the peptide was eluted from the column by the additional injection of 1 ml buffer. Typically, the first 500 μl flow-through contained ~100–120 μM peptide and the second 500 μl contained lower Aβ concentrations (~40–50 μM), which results in a yield of original Aβ peptide of ~80% based on Bradford determination of the peptide concentration. The Aβ-containing samples were collected in pre-cooled low-adhesion resin-coated polypropylene centrifuge tubes (Bioplastics, cat. B74030). After concentration measurement using the Bradford assay for protein determination (Bradford, 1976), both fractions were combined to yield the required concentration of Aβ. Samples were kept on ice directly on elution and further experiments were proceeded with within 20 min after elution from the desalting column.

Oligomerization and fibrillization of Aβ

Oligomer-enriched fractions (1.5–2 h incubation) or amyloid fibrils (>1 week incubation) of Aβ were prepared by incubation of 50–100 μM Aβ at 25°C under quiescent conditions in 50 mM Tris-HCl, 1 mM EDTA, pH 7.4 using low-adhesion resin-coated polypropylene centrifuge tubes.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE was performed by incubating 10 μl Aβ-containing solution in 15 μl Novex Tris-Glycine SDS sample buffer. Samples were then loaded onto a 10–20% Tricine gel (Invitrogen) and the gel was run at 170 V for 40 min followed by staining with Imperial Protein Stain (Pierce).

Thioflavin T (Tht) fluorescence

Aggregate-free Aβ42 solutions at a concentration of 50 μM were prepared as described above by further dilution using 50 mM Tris-HCl, 1 mM EDTA containing buffer and a final concentration of 12 μM Tht. In order to evaluate the effect of DMSO on the aggregation kinetics, various concentrations of DMSO (1–10%) were added to the elution buffer of Aβ. In order to validate the effectiveness of the proposed three-step solubilization procedure, Aβ42 was also directly dissolved into buffer without organic solvent treatment. The fibrillization kinetics of Aβ were monitored *in situ* using a Fluostar OPTIMA fluorescence plate reader at an excitation wavelength of 440 nm and an emission wavelength of 480 nm. Fluorescence readings were recorded every 10 min for a period of 7 h. Measurements were performed in triplicate, the recorded values were averaged and standard deviations were calculated. Background measurements included buffer or buffer containing DMSO and 12 μM Tht.

Transmission electron microscopy (TEM)

Aliquots (5 μl) of the Aβ preparation were adsorbed to carboncoated FormVar film on 400-mesh copper grids (Plano GmbH, Germany) for 1 min. The grids were blotted, washed twice in droplets of Milli-Q water and stained with 1% (wt/vol) uranyl acetate. Samples were studied with a JEOL JEM-1400 microscope at 80 kV (JEOL Ltd, Japan).

Fourier transform infraRed spectroscopy (FT-IR)

An aggregate-free Aβ42 solution was prepared as described in the '*Preparation of Aβ peptide solutions*'. The Aβ concentration was adjusted to 100 μM by dilution using 50 mM Tris-HCl, 1 mM EDTA containing buffer. Spectra of Aβ42 were recorded directly on elution from the column and after 24 h incubation at 25°C under quiescent conditions. To evaluate the contribution of organic solvent components to the FT-IR spectrum, 1% DMSO or 1% HFIP were directly dissolved into 50 mM Tris-HCl, 1 mM EDTA containing buffer. Buffer was used to record a blank signal. The InfraRed spectra were recorded using a Bruker Tensor 27 infrared spectrophotometer (Bruker Optik GmbH, Ettlingen, Germany) equipped with a Bio-ATR II accessory. Spectra were recorded at a spectral resolution of 4 cm^{-1} and 120 accumulations were performed per measurement at a wavenumber range from 900 to 3500 cm^{-1}. Analysis of the spectra involved blank (buffer) and baseline subtraction from the obtained spectra and rescaling in a wavenumber range from 900 to 1800 cm^{-1}.

Electrospray-ionization mass spectrometry (ESI-MS)

Aβ42 was prepared as described in the '*Preparation of Aβ peptide solutions*', but the column elution step was disregarded. The concentrated Aβ42 solution in DMSO was diluted 100× in acetonitrile:water (1:1) containing 1% acetic acid to a final concentration of 2 μM Aβ. Positive-ion mass spectra were recorded on an orthogonal acceleration quadrupole time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard electrospray probe (Z-spray) and controlled by a datasystem running MassLynx 3.4 (Micromass, Manchester, UK) and cone voltage was set to 30 V, capillary voltage was 3 kV. Spectra were recorded from *m/z* 600 to *m/z* 1600. Scan time was set to 4.9 s with an interscan time of 0.1 s. At least 10 spectra were acquired and averaged. Deconvolution was performed using the MaxEnt algorithm included in the software.

Nuclear magnetic resonance spectroscopy (NMR)

Uniformly ^{15}N-labeled Aβ40 and Aβ42 peptides were treated as described above using 1 ml HFIP and 1 ml DMSO. Peptide concentrations were adjusted to 60 μM for Aβ42 and 100 μM for Aβ40 with 50 mM Tris-HCl, 1 mM EDTA (pH 7.4) buffer and the samples contained 10% (vol/vol) ^2H_2O (Aldrich, cat. 269786). A $^{15}N-^1H$ heteronuclear single quantum coherence (HSQC) spectrum for Aβ42 was recorded on a Bruker Avance spectrometer equipped with a cryoprobe and operating at 700 MHz at 5°C. The Aβ40 $^{15}N-^1H$-HSQC spectrum was recorded at 25°C on a Varian Inova spectrometer operating at 600 MHz. Spectral assignment was verified using ^{15}N-NOESY-HSQC and $^1H,^1H$-TOCSY experiments. Spectra were processed using NMRPipe/NMRDraw (Delaglio *et al.*, 1995) and analyzed by XEASY/CARA software (Bartels *et al.*, 1995).

Cell viability in neuroblastoma cells

Neuroblastoma SH-SY5Y cells (HPA 94030304) were used with a maximum passage number of 15. Cells were cultured in Dulbecco's modified eagle medium (DMEM) 1× ([+] 4.5 g/l glucose, [+] L-glutamine, [−] pyruvate) (Gibco, cat. 41965) and 1% (vol/vol) penicillin/streptomycin (Invitrogen, cat. 15140-122) and 10% (vol/vol) fetal calf serum (Perbio, cat. SH30070.03) at 37°C, 5% CO_2 to a confluency of ∼85% in a 75 cm^2 flask (Greiner Bio-one, cat. 658170). On trypsinization, cells were resuspended at a concentration of 200 000 cells/ml in DMEM/F12 (1:1) [+]L-glutamine, [+] 15 mM HEPES (Gibco, cat. 11039), containing 1% (vol/vol) penicillin/streptomycin (Invitrogen, cat. 15140-122). The resuspended cells were plated at a volume of 100 μl and a cell density of 20 000 cells/well in a 96-well plate (BD Falcon, cat. 353072). The plated cells were incubated for 48 h at 37°C at 5% CO_2. Aβ40 and Aβ42 oligomer-enriched fractions were prepared at a concentration of 100 μM as described under section '*Oligomerization and fibrillization of Aβ*' and incubation for 1.5 h under quiescent conditions at 25°C. After incubation, the Aβ was diluted to final concentrations of 1–50 μM in buffer and then diluted 1:1 with prewarmed (37°C) DMEM/F12 (1:1) [+]L-glutamine, [+] 15 mM HEPES (Gibco, cat. 11039), containing 1% (vol/vol) penicillin/streptomycin (Invitrogen, cat. 15140-122). The Aβ or medium as a control was added at a volume of 100 μl in medium to each well and left to incubate for 48 h. After 48 h, 10 μl Cell Titer-Blue® Cell Viability Assay (Promega, cat. G8080) compound was added to each well and incubated for 4–6 h at 37°C to allow viable cells to convert resazurin into resorufin. Fluorescence intensity of resorufin was measured on a 96-well plate reader (BMG Labtech, Fluostar Optima) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Measurements were performed in three independent experiments and statistical analysis was performed to calculate average values and standard deviations.

Results and discussion

Overview of the protocol and comparison with other methods

The proposed protocol provides for a method to dissolve amyloid beta (Aβ) peptide via a rapid three-step procedure. Dissolving recombinant produced Aβ42 directly into buffer shows that a Tht curve to follow the kinetic aspects of the aggregation curve lacks the typical sigmoidal shape commonly observed for aggregating peptides and proteins (Fig. 1a). No lag phase is apparent and also the Tht fluorescence intensity is already increased on starting the assay, both indicative for the presence of pre-formed seeds or aggregates in the solution. FT-IR is used for a wide range of applications and also has shown its applicability in the field of protein conformation determination (Miyazawa and Blout, 1961). Figure 1b shows that Aβ42 that has been prepared using the proposed three-step dissolving procedure is intrinsically unstructured directly after solubilization in aqueous solution, characterized by a broad peak centered around 1654 cm^{-1}, but assumes a β-sheet aggregate fold (peak at 1627 cm^{-1}) on incubation for 24 h. Figure 1c shows that directly in buffer dissolved Aβ42 already exerts a high degree of β-sheet structure, while monomeric dissolved Aβ is an intrinsically disordered peptide. These results underline the necessity to develop an alternative procedure to dissolve Aβ.

We first exploited the structure-promoting properties of HFIP. From a number of tested alcohols, HFIP was shown to most effectively denature the model protein β-lactoglobulin

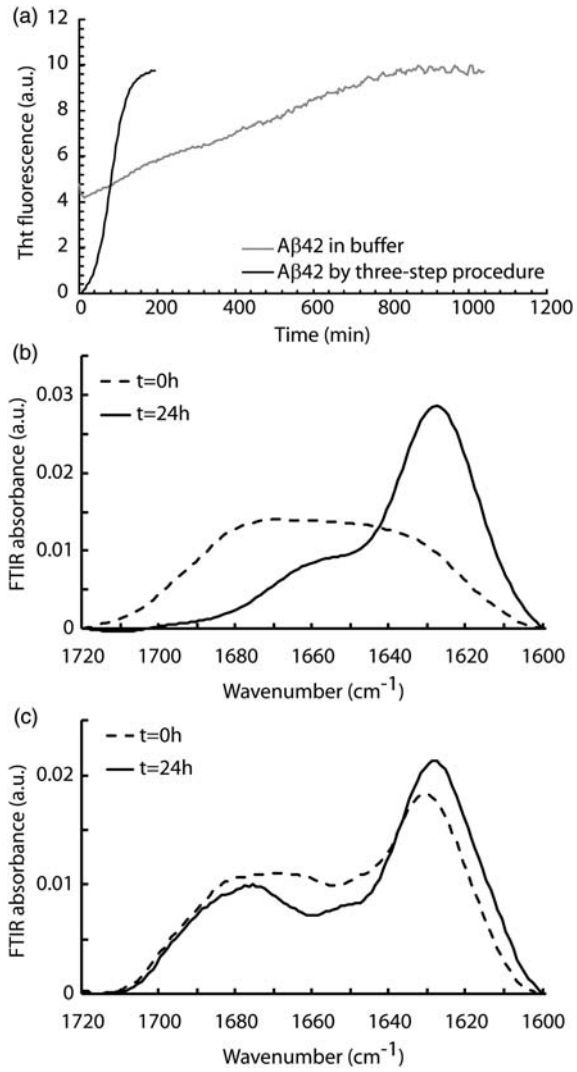

Fig. 1 Solubilization of Aβ42 directly into buffer indicates the presence of pre-formed aggregates. (**a**) Comparison of aggregation kinetics probed by Tht fluorescence of Aβ dissolved directly into buffer and the novel proposed three-step procedure. A concentration of 50 μM Aβ was either dissolved directly into buffer or solubilized using the proposed three-step procedure. (**b**) FT-IR spectroscopy of Aβ42 dissolved into buffer using the three-step dissolving procedure. Directly on elution off the column the broad FT-IR spectrum is characteristic for an unstructured peptide while after 24 h incubation at a concentration of 100 μM at 25°C the intensity at 1627 cm^{-1} increases characteristic for β-sheet amyloid aggregation. (**c**) FT-IR spectroscopy of Aβ42 dissolved directly into buffer. Directly on solubilization the spectrum is characteristic for a mixture of unfolded peptide and β-sheet aggregation with a main intensity at 1627 cm^{-1} and a broad shoulder at 1654 cm^{-1}. After 24 h incubation at a concentration of 100 μM at 25°C the intensity at 1627 cm^{-1} increased slightly and the shoulder at 1654 cm^{-1} is smaller.

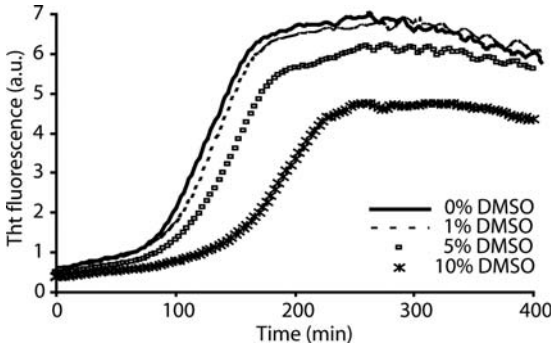

Fig. 2 Different solubilization procedures affect the aggregation of Aβ. The presence of traces of DMSO affects the aggregation kinetics of Aβ42. Dilution of concentrated Aβ stocks in DMSO into buffer followed by monitoring of the aggregation rate of Aβ by Tht fluorescence. The aggregation of Aβ is linearly inhibited with increasing concentrations of DMSO.

and to induce a non-native α-helix conformation (Hirota *et al.*, 1997). Dilute solutions of HFIP (1–4%) promote fibril formation of the islet amyloid polypeptide (Padrick and Miranker, 2002) but concentrated HFIP solutions have been shown to actively remove pre-formed aggregate seeds from solutions containing amyloidogenic proteins (Buck, 1998; Zagorski *et al.*, 1999; Nichols *et al.*, 2005). Later studies confirmed similar observations for Aβ (Nichols *et al.*, 2005). HFIP owes its activity due to its high degree of fluorination and induces helical formation (Hirota-Nakaoka *et al.*, 2003; Tomaselli *et al.*, 2006). Even though HFIP has been shown to dissociate fibrils, β2-microglobulin aggregates can resist the application of this solvent (Hirota-Nakaoka *et al.*, 2003). The insufficient ability of HFIP to completely dissociate amyloid fibrils has been ascribed to its non-polar character that weakens hydrophobic interactions but cannot dissolve rigid fibrils (Hirota-Nakaoka *et al.*, 2003). Therefore, in the presented procedure HFIP solubilization is followed by DMSO, as this polar compound has been shown to completely dissociate amyloid fibrils based on its ability to destruct a hydrogen bond network (Kosower, 1958). HFIP can be removed using oxygen-free nitrogen gas or, alternatively, argon gas as methionine-35 of the Aβ peptide is susceptible to oxidation (Butterfield and Bush, 2004). A molecular dynamics simulation of the effects of DMSO on the structure and function of subtilisin confirmed that DMSO acts as a highly effective hydrogen bond acceptor and, hence, can strip away water from the protein surface (Zheng and Ornstein, 1996). Many published methods to solubilize Aβ suggest preparation of concentrated Aβ stock solutions in DMSO followed by a 10- to 100-fold dilution in buffer to induce aggregation. However, as a result of the effect of DSMO on the hydrogen network of proteins, the kinetics and mechanism of the aggregation of Aβ are adversely affected by the presence of small (~5%) concentrations of DMSO (Fig. 2, and Shen and Murphy, 1995) in a non-physiologically relevant manner. We therefore apply a column-exchange step to remove all traces of DMSO. Other published methods often employ the effects of HCl and NaOH to aid Aβ solubility at a pH far removed from its isoelectric point (~pH 5.3). In order to adjust the pH of the solution to approach more physiologically relevant pH values from an acidic pH requires transition through the isoelectric point at which the peptide is most prone to aggregate. Also the addition of these pH-affecting compounds introduces an additional factor that could induce artifacts in the aggregation mechanism of Aβ.

Experimental design

The protocol for Aβ solubilization that provided best results consists of sequential treatment using HFIP and DMSO followed by exchange of DMSO into buffer using a desalting column (Fig. 3a). The use of a high concentration (~100%) of HFIP, a fluorinated alcohol, provides a means of

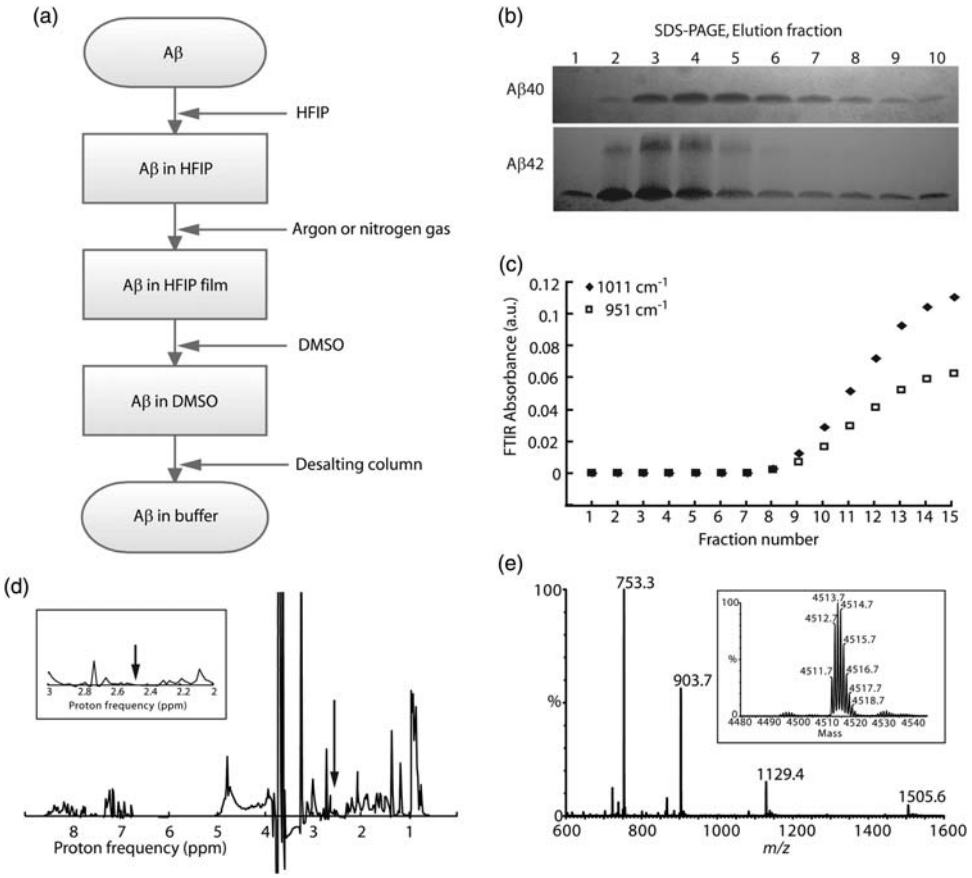

Fig. 3 Novel solubilization procedure for Aβ leads to primarily monomeric Aβ and absence of contaminating chemicals. (**a**) Treatment of the Aβ peptide to dissolve it into buffer involves sequential dilution in HFIP, and DMSO followed by buffer exchange by means of a desalting column. Details of the procedure are described in the text. (**b**) Non-reducing SDS-PAGE analysis of fractions of 150 μl each eluted off the desalting column shows that fractions 2–5 (300–750 μl) are enriched in Aβ peptide. Aβ40 is primarily monomeric on elution while Aβ42 is primarily monomeric with a small fraction of apparently trimeric peptide. (**c**) Up to and including fraction 8 (1200 μl) can be eluted off the desalting column without contamination by DMSO. The intensities of FT-IR peaks characteristic for the presence of DMSO (1011 and 951 cm^{-1}) are plotted against eluted fraction number. (**d**) One-dimensional ^{1}H NMR spectrum of an Aβ42 preparation confirms the complete removal of DMSO because of the absence of characteristic solvent signal at 2.5 p.p.m., as indicated by the arrow. The inset shows a zoomed-in view of the region from 2 to 3 p.p.m. proton frequency. (**e**) Electrospray mass spectrum of Aβ42 in positive mode with the deconvoluted spectrum as inset.

interaction with the backbone and removal of water from the surface. Subsequently, the HFIP is evaporated off by the use of oxygen-free nitrogen gas or argon gas that protects the peptide from oxidative modification. The hydrophobic methyl groups of DMSO have the ability to then interact with hydrophobic side chains of the Aβ peptide while the polar S=O group interacts with water molecules. DMSO is then removed by buffer exchange using a desalting column resulting in Aβ virtually free from aggregating seeds in buffer (Fig. 3b). The electrophoretic analysis of the elution of Aβ from the column shows that fractions 2–5 are enriched with Aβ (150–750 μl). Aβ40 elutes as a pure monomeric solution while Aβ42 elutes as a primarily monomeric solution containing small quantities of an SDS-resistant species with an apparent molecular weight resembling trimeric Aβ. No higher-molecular-weight aggregates were observed. As the presence of small amounts of HFIP and DMSO can modify the aggregation rates of Aβ (Nichols *et al.,* 2005, and Fig. 2), it is important to validate their complete removal. We use FT-IR that provides characteristic and sensitive fingerprints for the presence of trace amounts of HFIP (Czarnik-Matusewicz *et al.*, 2008) or DMSO. FT-IR spectra of the various elution fractions after column elution show that with increasing elution fraction the concentration of DMSO increases (Fig. 3c). It is also shown that HFIP has been effectively removed during the procedures supported by the lack of characteristic intensity in the FT-IR spectrum between 1300 and 1100 cm^{-1}. A 1% DMSO solution in buffer has two characteristic peaks: at 1011 cm^{-1} and one at 951 cm^{-1}. Plotting the intensity at these two wavelengths against the fraction number shows that only from fraction 9 (1350 μl) up the concentration of DMSO starts to increase (Fig. 3c). In addition, the absence of a detectable signal at 2.5 p.p.m., which is characteristic of DMSO, in the one-dimensional ^{1}H NMR spectrum of the Aβ preparations, confirms that elution of the first 1 ml of Aβ from the column yields DMSO-free Aβ (Fig. 3d). The electrospray ionization mass spectrum shows that Aβ42, using the protocol, eluted free of contaminants and without apparent modifications (Fig. 3e), such as dityrosine-formation that is commonly observed for Aβ peptides as a function of oxidation (Yoburn *et al.,* 2003).

We tested the reproducibility of the behavior of the prepared Aβ solutions and their performance in a variety of assays related to aggregate formation or cell viability commonly used to study the effects of Aβ. Commonly used

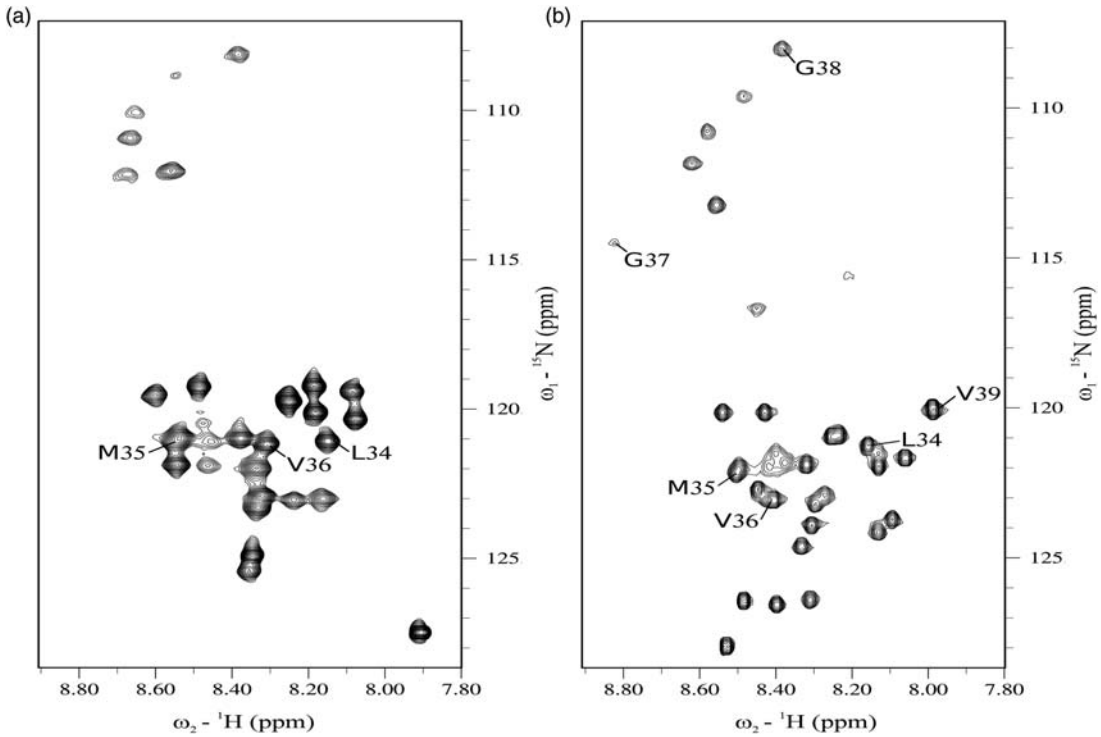

Fig. 4 NMR ^{15}N-^{1}H HSQC spectra of (**a**) Aβ40 and (**b**) Aβ42 recorded at 600 MHz and 25 and 5°C, resp. Oxidation of Met35 residue in Aβ is prevented by the novel solubilization procedure. Spectrum assignment is shown only for those crosspeaks that would undergo a chemical shift displacement as a function of the redox state of Met35 (i.e. Leu34-Met35-Val36 in Aβ40 and Leu34-Met35-Val36-Gly37-Gly38 in Aβ42) (Hou *et al.*, 2004).

assays to study the aggregation of Aβ *in vitro* consist of thioflavin T fluorescence (Hayashi *et al.,* 2004), electron microscopy (Hayashi *et al.,* 2004; Martins *et al.,* 2008; Nerelius *et al.,* 2009) and FT-IR (Martins *et al.,* 2008; Cerf *et al.,* 2009) and the effect of Aβ on the toxicity is usually assayed by viability assays on neuroblastoma cells (Chromy *et al.,* 2003; Nerelius *et al.,* 2009) or neuronal cultures (Lambert *et al.,* 1998; Hayashi *et al.,* 2004; Martins *et al.,* 2008).

Nuclear magnetic resonance

NMR analysis shows that Aβ40 and Aβ42 prepared by our procedure are mainly monomeric species as shown by the sharp resonances in 2D HSQC experiments (Fig. 4). NMR analysis also assures us that the protocol does not induce oxidation of Met35: oxidation of this residue is known to result in a notable chemical shift that affects several crosspeaks in the HSQC (Hou *et al.*, 2004). Our spectra of Aβ40 and Aβ42 are in excellent agreement with literature data reporting NH of Met35 in the reduced state at ∼8.5 p.p.m. for the ^{1}H-frequency, while the NH of Met35 of the oxidized state appeared upfield at ∼8.8 p.p.m. (Fig. 4a and b, and Hou *et al.*, 2004). This observation is an important point to note because pre-treatment with HFIP might occasionally promote oxidation of the Aβ peptide (Hou *et al.*, 2004).

Thioflavin T fluorescence

Tht fluorescence is a useful probe to assay the aggregation of proteins. The quantum yield for fluorescence of this weakly fluorescent dye increases strongly on complexation with amyloid fibrils (Naiki *et al.,* 1989). Fig. 5a shows that differences in aggregation rates between the aggregating Aβ42 and less aggregation-prone Aβ40 can be distinguished using *in situ.* Tht fluorescence. Reproducibility of the obtained results is an important parameter to evaluate the quality of the developed procedures. Figure 5a shows Tht fluorescence curves obtained for three independent experiments. The standard error between the experiments is ∼7–10% and the differences between the aggregation rates of Aβ40 and Aβ42 are therefore significant.

Transmission electron microscopy

We used TEM to characterize fibril morphology of aggregates formed by Aβ40 and Aβ42 on incubation at a concentration of 100 μM for 24 h at 25°C. Amyloidogenic Aβ42 (Fig. 5b) forms aggregates characterized by short fibrils which intertwine and appear rigid. Aβ40 (Fig. 5c) on the other hand forms long semi-flexible negatively stained fibrils with a characteristic periodic twist that is regularly found in fibrils of other origin, such as insulin (Jiménez *et al.,* 2002).

Toxicity on SH-SY5Y cells

The toxicity of 1.5 h incubated 100 μM Aβ40 and Aβ42 preparations was tested using neuroblastoma SH-SY5Y cells plated at a cell density of 20 000 cells/well in serum-deprived medium. To this end, Aβ preparations were diluted to various concentrations ranging from 1 to 50 μM in serum-deprived medium and 100 μl was added to each well in 6-fold. After 48 h incubation cells were tested for cell death using Cell Titer-Blue viability assay (Promega). Figure 5d shows that cell viability is Aβ concentration dependent and that Aβ42 aggregates induce cell death at lower concentrations (from 7.5 μM) compared with Aβ40 aggregates (from 30 μM). The results in Fig. 5d are obtained from three independent

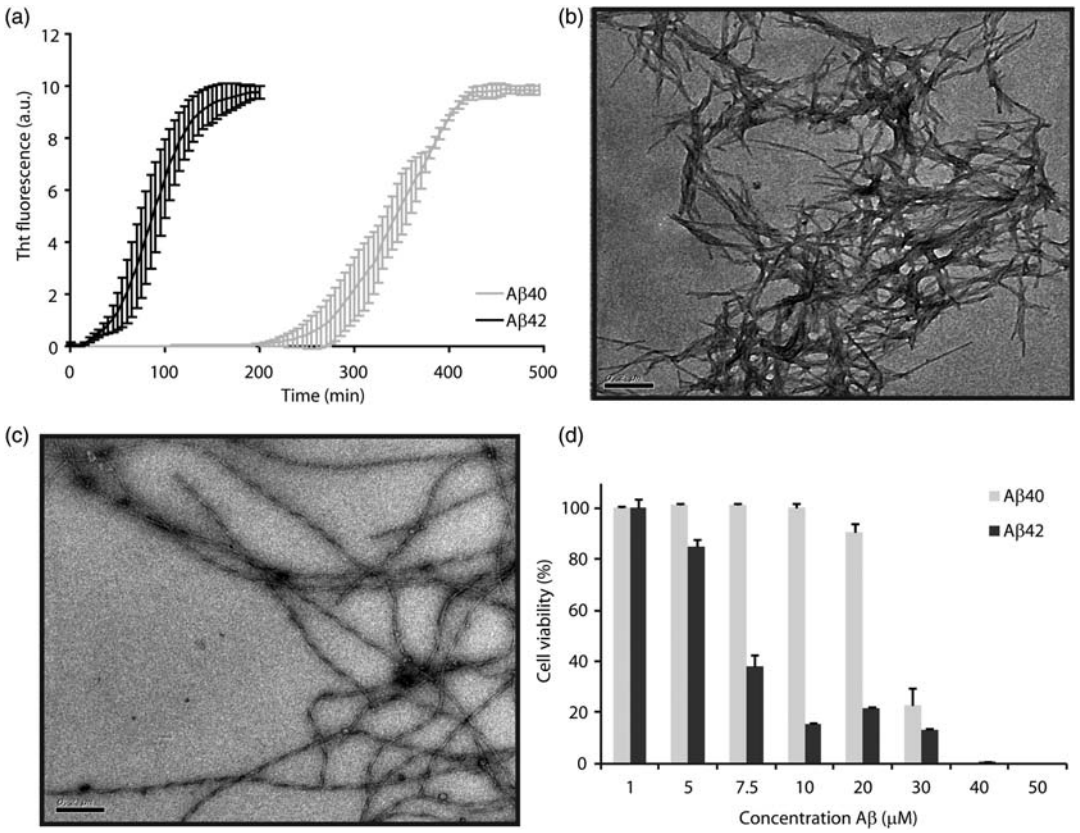

Fig. 5 Anticipated results for commonly used biophysical and cell biological characterization methods of Aβ dissolved by the HFIP/DMSO/column procedure. (**a**) Aβ40 (gray) and Aβ42 (black) aggregation monitored by Tht fluorescence: the nucleation phase for Aβ40 is extended significantly compared with Aβ42. The rate of polymerization is faster for Aβ40 than for Aβ42. Reproducibility of Tht curves shown by error bars of Aβ40 and Aβ42 prepared at three different occasions from three different batches of Aβ peptide. The limited variability in the obtained results illustrates that differences in aggregation rates between different Aβ peptides are significant. (**b**) Transmission electron microscopy (TEM) image for Aβ42 aggregates matured at 100 μM at 25°C for 24 h. The fibrils are short and intertwined. (**c**) Aggregates of Aβ40 matured at 100 μM at 25°C for 24 h and imaged by TEM show long, semi-flexible and twisted fibrils. The length of the segment is 0.2 μm for both micrographs depicted in (**b**) and (**c**). (**d**) Toxicity of Aβ matured for 1.5 h at 100 μM at 25°C and then added at various final concentrations in medium to SH-SY5Y neuroblastoma cells. Cell viability is measured using Cell Titer Blue (Promega) assay. The cell viability of cells incubated with Aβ42 (black bars) is lost between 7.5 and 10 μM while cells incubated with Aβ40 (gray bars) only lose cell viability at an Aβ concentration of 30 μM.

experiments and the standard error is maximum 12% (for 30 μM Aβ40) but in most cases >5%.

Conclusions

A robust and validated three-step procedure is presented to prepare biocompatible virtually aggregate-free solutions with an efficient recovery of amyloidogenic Aβ42 implicated in Alzheimer's disease. The procedure involves the sequential use of HFIP, DMSO and a column exchange step. While the use of organic solvents also allows the reliable preparation of biologically relevant quantities of Aβ42 and Aβ40 and their mixtures, the incorporation of a desalting column in the solubilization procedure makes it possible to obtain the Aβ peptide in any desirably buffer system. The resulting Aβ preparation shows highly reproducible biophysical and cell biological behavior. The use of this protocol may be extended to the solubilization of other highly amphipathic or hydrophobic polypeptides.

Acknowledgments

We thank Dr A.Ganesan for instructive discussions on the chemical basis of HFIP and DMSO-induced protein aggregate dissociation. We acknowledge the MRC biomedical NMR centre for technical support.

Funding

This work was supported by the Alzheimer Research Trust (UK) [grant number ART/PG2007/3]; the FWO Odysseus program [grant number G.0903.09]; the Interuniversity Attraction Poles (IAP) program [grant number P6/43] of the Belgian Science Policy Office (BELSPO); and the Stichting voor Alzheimer Onderzoek (SAO) [grant number 09005]. A.V. was supported by the Agency for Innovation by Science and Technology (I.W.T.) Belgium [grant number 083052]. K.P. was supported by an EMBO Long-term fellowship (grant number ALTF-512-2008).

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