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A Robust and Efficient Production and Purification Procedure of Recombinant Alzheimer's Disease Methionine-Modified Amyloid-β Peptides

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Abstract

An improved production and purification method for Alzheimer’s disease related methionine-modified amyloid-β 1–40 and 1–42 peptides is proposed, taking advantage of the formation of inclusion body in Escherichia coli. A Thioflavin-S assay was set-up to evaluate inclusion body formation during growth and optimize culture conditions for amyloid-β peptides production. A simple and fast purification protocol including first the isolation of the inclusion bodies and second, two cycles of high pH denaturation/neutralization combined with an ultrafiltration step on 30-kDa cut-off membrane was established. Special attention was paid to purity monitoring based on a rational combination of UV spectrophotometry and SDS-PAGE analyses at the various stages of the process. It revealed that this chromatography-free protocol affords good yield of high quality peptides in term of purity. The resulting peptides were fully characterized and are appropriate models for highly reproducible in vitro aggregation studies.

Introduction

With a worldwide incidence of millions people, Alzheimer’s disease is considered as a major health issue, and much research effort is currently being devoted to better understand the causes and mechanisms of the disease. Diverse therapeutic approaches are being explored, including hormonotherapy [1], immunotherapy [2], genetics [3] or pharmaceutics [4].

Although their implication in the disease remains unclear, the presence of amyloid plaques and oligomers in the synaptic cleft is widely considered as one of the hallmarks of Alzheimer’s...
disease, and were found to be highly deleterious for neuron plasticity [5]. These aggregates are mainly constituted of amyloid-\(\beta\) (A\(\beta\)) peptides, namely A\(\beta\)\(_{1-40}\) and A\(\beta\)\(_{1-42}\), resulting from the abnormal proteolytic cleavage of the amyloid precursor protein. Understanding the mechanism of formation and the behavior of these aggregates could pave the way to new therapeutic approaches against Alzheimer’s disease, promoting multiple investigations on A\(\beta\) peptide aggregation process [6].

For \textit{in vitro} experimentation, disposing of A\(\beta\) samples displaying reproducible behaviours is a key issue. Indeed, aggregation was shown to be highly sensitive to many factors, such as temperature, concentration, agitation, but also storage or sample preparation [7,8]. But most importantly, the presence of both contaminants and pre-aggregated forms of the peptide were shown to strongly alter the course of aggregation, either inhibiting it or yielding no fibrillary material. In this regard, the easy access to pure and homogeneous A\(\beta\) samples is still challenging and essential for \textit{in vitro} experimentation.

Most investigations are usually performed with chemically synthesized peptides. Although convenient and easily accessible, solid phase peptide synthesis can be complex in terms of purification. Indeed, the presence of peptides with altered sequence caused by non-quantitative synthetic steps or of racemized peptides is observed, which can hardly be removed, even by up-to-date purification methods. Traces of salts or metal ions were also reported [9]. Finally, presence of pre-formed aggregates due to storage conditions is frequent. All of this induce significant variations of aggregation properties from batch to batch. This has been recently exemplified in a study, which showed that the recombinant peptide is more aggregation-prone and more neurotoxic than its synthetic analogue, demonstrating a deleterious effect of synthetic procedures on peptide properties [10]. This has encouraged the exploration of various biological routes to access recombinant A\(\beta\), getting rid of such issues.

A\(\beta\) peptide recombinant production was attempted in yeast [11] or by combining recombinant and synthetic procedures [12] but, to date, the most common expression system remains \textit{Escherichia coli}. Most often, peptides are appended to a fusion protein that enhance their solubility and ease their isolation and purification using affinity chromatography. However, such strategies raised several issues. First of all, despite the increased solubility of the fusion proteins, a significant amount of the peptide frequently remains insoluble, thus requiring an extra denaturation step to achieve full recovery [13,14]. Moreover, a cleavage site has to be installed, its position being critical; indeed if not carefully chosen, upon cleavage amino-acids will be introduced at the terminal end of the peptide, potentially modifying its aggregation and metal-binding properties [15,16]. The same drawback is also observed with smaller affinity tags [17,18]. This problem has been solved through the use of proteolytic enzymes whose cleavage sites enable the formation of native A\(\beta\) (Factor Xa, Enterokinase… ) [19,20]. Still such methods are time-consuming and require many purification steps, including long affinity chromatography purification procedures that induce in a loss of peptide. A representative example was described by Zhang and co-workers, showing that only 24% of Glutathione S-transferase-tagged peptide is recovered in the soluble fraction after cell lysis. This yield is weakly affected by affinity chromatography (21%), but drops to 5.1% after thrombin cleavage. To overcome this drawback, it was proposed to express A\(\beta\) in its native form in \textit{E. coli} [21,22]. Notably, A\(\beta\) forms insoluble inclusion bodies (IBs) when expressed in \textit{E. coli} in their native form. This was exploited by Walsh and co-workers to isolate pure recombinant methionine-modified A\(\beta\) peptides MA\(\beta\)\(_{1-40}\) and MA\(\beta\)\(_{1-42}\) in five purification steps [21].

We describe here an alternative and straightforward procedure of recombinant MA\(\beta\)\(_{1-40}\) and MA\(\beta\)\(_{1-42}\) production and purification. First, expression conditions were optimized using Thioflavin-S (Th-S), a known fluorescent probe for \textit{in vivo} imaging of IBs. We subsequently established a rapid purification process based on inclusion body treatment with NaOH followed...
by an ultrafiltration step that has proven remarkably efficient to remove protein and nucleic acid contaminants. Finally, a detailed monitoring of the purification process was conducted, showing that only a combination of characterization techniques can ensure sample reliability.

**Materials and Methods**

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA). pET28a plasmid was purchased from Novagen (Darmstadt, Germany). Ncol, Xhol and BamHI restriction enzymes Antarctic phosphatase and T4 DNA Ligase were purchased from New England Biolabs (Ipswich, USA) and used with the provided buffer according to supplier’s recommendations. pET28a_His-Thromb-Aβ₁₋₄₂ plasmid was synthesized by Genecust (Dudelange, Luxembourg). E. coli TOP10 cells and E. coli BL21 (DE3) cells were obtained from Invitrogen (Carlsbad, USA). QIAprep spin MiniPrep kit was purchased from Qiagen (Hilden, Germany).

Sequence analyses of the constructions were performed by GATC Biotech (Constance, Germany).

**Vectors construction and cloning procedure**

The commercial pET28a plasmid was digested by Ncol and Xhol restriction enzymes, dephosphorylated using Antarctic phosphatase, and gel purified on an agarose 0.8% Tris acetate EDTA (TAE) gel. The pET28a_His-Thromb-Aβ₁₋₄₂ plasmid was used as template for PCR amplification of the Aβ₁₋₄₂ coding sequence using pR_pET28_Ab42: AGCGGTGGCAGCAGCTTTCAGCCCTACC and pF_pET28_Ab42: GACCTACCCATGGACGCTGAATTTCGCCACGACTCGGCTAT as primers. The PCR product was digested by Ncol and Xhol, gel purified on an agarose 1.2% TAE gel and ligated in the Ncol/Xhol digested pET28a yielding the desired pET28a_MAβ₁₋₄₀ plasmid. The construction was used to transform chimiocompetent E.coli TOP10 cells that were grown on solid Luria-Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) containing 50 mg/L kanamycin (1X). One colony was used to inoculate 5 mL LB kanamycin 1X at 37°C. Cells were grown overnight, harvested before plasmid extraction using a MiniPrep kit.

The pET28a_MAβ₁₋₄₀ was obtained from pET28a_MAβ₁₋₄₂ using a similar procedure except that the sequence coding for Aβ₁₋₄₀ was PCR-amplified with pR_pet28_Ab40: AATGGATCCTAATTAAAAACGCCGCGCAACCATCAGACGCCGATG and pF_pet28_Ab40: AATTGTGAGCGGATAAACAATTCCTCTCTAG as primers. The PCR-product was digested using Ncol and BamHI, and ligated into Ncol/BamHI digested and dephosphorylated pET28a.

**Expression of MAβ₁₋₄₀ and MAβ₁₋₄₂ and inclusion body isolation**

Chemically competent E. coli BL21 (DE3) cells transformed with pET28a_MAβ₁₋₄₀ or pET28a_MAβ₁₋₄₂ plasmids were used for peptide production. One single colony was used to inoculate 10 mL LB kanamycin 1X medium. The cells were grown for 16 h. 100 mL of LB medium containing kanamycin 1X were inoculated to an OD₆₀₀ of 0.05 with an overnight preculture performed from one single clone in 10 mL LB-Kana medium. Cells were grown at 37°C under agitation to an OD₆₀₀ of 0.8. Expression was induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) during for 4 additional hours of culture at 37°C. Cells were harvested by centrifugation (8,000 g, 10 min) and re-suspended to an OD₆₀₀ of 80 in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8) containing 1% Tergitol-type NP40 detergent. Cells were then lysed by 30s sonication on ice and the lysate was centrifuged (12,000 g, 10 min). The insoluble fraction was re-suspended in 1 mL lysis buffer, containing phenylmethanesulfonyl fluoride (15 mM) and lysozyme (300 μg/mL), and incubated for 1 h at room temperature. IBs were harvested by centrifugation (12,000 g, 10 min), washed with Triton X100 0.5% and twice with phosphate saline buffer (PBS) 1X pH 8. IBs were finally resuspended in PBS buffer and
submitted to 10 cycles of 10 s sonication followed by centrifugation to remove nucleic acid contaminants contained in the supernatant and that could be stacked on their surface, according to a previously reported procedure [23].

**Peptide purification**

IBs were denatured by 2 h incubation in 500 μL of NaOH 50 mM under smooth shaking at room temperature. The resulting mixture was centrifuged (12,000 g, 5 min), and the supernatant neutralized by addition of 500 mM HCl. The formation of a white precipitate was observed. The mixture was centrifuged once again, and the supernatant was collected. To optimize recovery, this denaturation/neutralisation cycle was applied twice. Supernatants were passed through a 30-kDa Amicon-Ultra centrifugal device, to remove high molecular weight contaminant proteins, and then concentrated on a 3-kDa Amicon-Ultra centrifugal device.

**SDS-PAGE analysis**

15 μL protein samples were mixed with 5 μL Laemli Sample buffer (Bio-Rad) containing 10% β-mercaptoethanol, and denatured at 95°C for 10 min. Samples were then loaded on a Mini-protean TGX Stain-Free Any Kd Precast Gel (Bio-Rad) and electrophoresis was run for 30 min at 150 V in Tris/Glycine/SDS buffer (Bio-Rad). Gel was then stained using PageBlue Protein Staining solution (Bio-Rad).

**Thioflavin-S staining**

Samples of Aβ peptide producing cells or purified IBs are centrifuged (12,000 g, 10 min) and resuspended in 200 μL Thioflavin-S 125 μM in PBS 1X pH 8. After 15 min incubation, cells or IBs were harvested by centrifugation, washed with 200 μL PBS, re-suspended in PBS, and submitted to fluorescence experiments.

**Fluorescence microscopy**

50 μL samples of Th-S stained cells or IBs were dispensed on a microscope glass slide and imaged using a Leica microscope with a 63x lens with a DAPI filter (λ<sub>exc</sub> = 340–380 nm, dichroic = 400, λ<sub>em</sub> = 425 nm).

**Fluorescence spectrophotometry**

1 mL samples of Th-S stained cells or IBs were placed in a quartz cuvette and their emission spectra were recorded using a Jasco fluorimeter (λ<sub>exc</sub> = 375 nm). Right after each measurement, OD<sub>600</sub> was measured, to normalize the signal.

**<sup>1</sup>H NMR**

Pure samples of peptides in water were diluted to 100 μM in phosphate buffer 50 mM pH 7 in D<sub>2</sub>O. <sup>1</sup>H NMR spectra were recorded on a 500 MHz NMR (Bruker) at 25°C using solvent as reference. As a control, synthetic Aβ<sub>1-40</sub> was dissolved in NaOD and diluted to 100 μM under the same conditions.

**LC-MS**

Peptide samples were analysed by LC-MS on an Agilent 1200 HPLC fitted with a Macherey Nagel Nucleodur 300–5 C4 ec 250 mm x 2 mm 5 μm at 30°C coupled with a Thermo Fisher Scientific LCQ Fleet mass spectrometer fitted with an H-ESI II Probe.
Samples were eluted at 0.33 mL/min using a gradient of acetonitrile going from 5% to 90% in aqueous formic acid 0.1% in 12 min. Multiply-charged peptides were detected by mass spectrometry (ESI +).

**Thioflavin-T aggregation assay**

To ensure the homogeneity of the starting sample, monomeric peptides were isolated by steric exclusion chromatography (SEC). An Aktä Purifier system equipped with a Superdex 75 10/300 column was equilibrated with 2 column volumes of 15 mM NaOH. Peptide samples in NaOH 50 mM were then eluted using NaOH 15 mM at 1 mL/min, with UV monitoring at 220 and 293 nm. Monomeric MAβ peptides display retention times around 9 min. Fractions containing peptides were collected, centrifuged, and their concentration was assessed by UV-Vis at pH 12 (λ = 293 nm, ε = 2400 L.mol⁻¹.cm⁻¹).

In a 384-wells microplate (Greiner) were added phosphate buffer (50 mM), Thioflavin-T (10 μM) and freshly SEC-purified peptide (20 μM or 50 μM). Fluorescence intensity was recorded every 5 min for a total duration of 4 days using a ClarioStar plate reader (BMG Labtech) set at 37°C. Fluorescence parameters were set as follows: λ_exc = 440 nm, λ_em = 490 nm, gain = 650, shaking at 200 rpm for 12 s before each measurement. Aggregation curves were fitted using KaleidaGraph software.

**Results and Discussion**

**Optimization of MAβ₁–₄₀ and MAβ₁–₄₂ expression**

The genes encoding MAβ₁–₄₀ and MAβ₁–₄₂ were first cloned in pET28. They were then expressed by growing *E. coli* BL21 (DE3) transformed with the recombinant plasmids on LB broth supplemented with kanamycin. To determine optimal conditions for peptide expression, we envisioned a procedure based on Th-S monitoring.

Indeed, Th-S is usually used for staining amyloid plaques but was also recently shown to stain bacterial IBs formed by MAβ peptides, both in vivo and in vitro. Hence, Th-S was used to detect protein aggregation in bacteria [22], or to screen in vivo aggregation inhibitors [24]. In order to use Th-S staining as a tool for the optimization of peptide expression, the correlation between IB content and the Th-S fluorescence level had to be established. For this purpose, cultures expressing MAβ₁–₄₂ were stopped at different induction times. The cells were sampled, incubated with Th-S, and their emission spectra were recorded. As shown in Fig 1A, the fluorescence level increased during the first 4 hours of induction and then reached a plateau. The corresponding cultures were lysed and peptide was purified and analysed by SDS-PAGE. A good correlation was observed between the amount of purified peptide and the fluorescence level (Fig 1B). Similar results were obtained with MAβ₁–₄₀, showing that Th-S staining is very useful to estimate MAβ IB content in cells. Based on these results, a 4 h induction was considered as adapted and applied throughout the rest of the study.

Th-S monitoring was then used to screen for the optimal concentration of IPTG inducer. No significant variations of IB production was observed with IPTG concentration comprised between 0.5 and 1.5 mM (Fig 1C). Contrariwise, increased levels of protein contaminants were observed when using 1 or 1.5 mM IPTG, which could be due to the overexpression of *E. coli* chaperones [25]. Induction at 0.5 mM IPTG was thus found optimal. Finally, the optimal temperature of cell growth was investigated. As shown in Fig 1D, the best temperature allowing the formation of a maximal amount of IBs was 37°C.

This preliminary work allowed us to establish the optimal conditions of MAβ peptide recombinant production as corresponding to recombinant cell cultures carried out at 37°C and
induced for 4 h with 0.5 mM IPTG. Under these conditions, both visible and fluorescence microscopy confirmed the presence of IBs located at the poles of the cells (Fig 1E).

Purification of MAβ1–40 and MAβ1–42

As Aβ peptides were produced as IBs, the first step of the purification procedure consisted in isolating and purifying IBs. The main challenge of this approach was to succeed in removing proteins and nucleic acids that could be trapped in the IBs or stacked on their surface.

First, the previously reported “soni-removal” method, which consists in multiple sonication and centrifugation cycles, was performed to roughly eliminate nucleic acids and proteins stacked at the IB surface [23]. At this stage, Th-S fluorescence visualization of isolated IBs confirmed that their average size remained unchanged, showing no deterioration due to sonication.

The protocol shown in Fig 2A was then applied. Aβ peptides are soluble at high pH, since their isoelectric point is about 5–6. The IBs were thus first incubated with 50 mM sodium hydroxide, to solubilize MAβ. Upon pH neutralization, a large amount of proteins is removed by precipitation, while MAβ peptides remain in the supernatant (Fig 2B, lanes S1 and P2). The high pH denaturation/neutralization cycle was applied twice to maximize MAβ recovery (Fig 2B, lane S2). Further completion of contaminant elimination was carried out by ultrafiltration of the solubilized peptides followed by concentration, respectively on a 30-kDa and a 3-kDa molecular weight cut-off membranes. As shown on the electrophoresis gel (Fig 2B, lane F), the
ultrafiltration step allowed getting one single band around 5 kDa, the presence of any protein contaminant being excluded.

In parallel, the MAβ₁₋₄₂ IBs were also solubilized using 8M urea and then passed through a DEAE Sepharose ion-exchange resin to remove NA traces. As seen in Fig 3, this protocol did not allow the elimination of a 13 kDa protein contaminant, regardless of IPTG concentration used for induction. Furthermore, this protein could not be eliminated by ultrafiltration indicating that the urea treatment did not allow breaking the interaction probably existing between the 13 kDa protein and the peptide (Fig 3 lanes 4 and 5). This clearly showed the limitation of the denaturation with urea and emphasizes the interest of the NaOH treatment with to eliminate proteins and nucleic acids.
At this point, one important issue was the monitoring of the sample purity at the various stages of the purification procedure. The Bradford protein assay was eliminated because it does not give any information on sample purity and only allows the determination of a global protein content. Combination of UV-Vis spectrophotometry with gel electrophoresis was clearly preferred. Indeed, SDS-PAGE permits to visualize the presence of protein contaminants while UV-Vis analyses inform on the predominant presence of either nucleic acids ($\lambda = 260$ nm) or specific amino acids, namely tryptophan ($\lambda = 280$ nm), tyrosine/tyrosinate ($\lambda = 274$ nm/$\lambda = 293$ nm), phenylalanine ($\lambda = 257$ nm) [26]. Both methods were thus combined to assess the purity level of the peptide samples depending on the purification steps as shown in Fig 2C. After denaturation (Fig 2C, spectrum D), a lot of species are evidenced. The supernatant harvested after neutralization (Fig 2C, spectrum S1+S2), still contain proteins but is already devoid of nucleic acids. After ultrafiltration, the sample was too diluted to give an informative UV spectrum (Fig 2C, spectrum F). Finally, the concentration step yielded a pure sample showing a UV signature with the typical UV band at $\lambda = 293$ nm corresponding to the tyrosinate form (Fig 2C, spectrum C). This typical profile is conserved after size exclusion chromatography, which was performed to isolate only monomeric peptide before aggregation assays (Fig 2C, spectrum SEC). Overall, these analyses confirmed the high level of purity of the peptide preparation.
As the sample was devoid of any protein contaminants, the pH-dependent absorption of Tyr was used to estimate the peptide yield. Absorption spectra of the peptides were thus recorded at pH 12 (showing the absorption band of tyrosinate at 293 nm) and at pH 2 (displaying no tyrosinate band). The precise quantification of the tyrosine content of the sample was performed by subtracting spectra. This enabled the estimation of the peptide yield as being around 4 mg/L of culture, which is in the same range as those previously reported, comprised between 3 and 7 mg/L [13,14,19,27,28].

Characterization of MA\(\beta\)\(_{1-40}\) and MA\(\beta\)\(_{1-42}\)

To further characterize the peptides, LC-MS and NMR analyses were conducted right after the concentration step on both peptides. MA\(\beta\)\(_{1-40}\) displays a clean chromatographic trace with a major peak at 10.8 min. The mass spectrum associated to this peak is in good agreement with the expected masses (Fig 4A and 4C). MA\(\beta\)\(_{1-42}\) chromatogram is less resolved, the main peak being shouldered (Fig 4B and 4D). The masses detected for both the shoulder and the peak are consistent with the calculated masses of MA\(\beta\)\(_{1-42}\), indicating that oligomeric forms of MA\(\beta\)\(_{1-42}\) could be present. This is consistent with the high propensity of A\(\beta\)\(_{1-42}\) to aggregate.

\(^1\)H NMR spectra of both peptides were then recorded, and compared with commercially available synthetic A\(\beta\)\(_{1-40}\) (Fig 5). They display the typical signals usually observed for A\(\beta\)\(_{1-40}\) peptide. In the aromatic region, signals corresponding to the H\(\delta\) and H\(\varepsilon\) of aromatic residues are well defined. As such, Phe 19 and 20 (ca. 7.2 ppm and ca. 7.3 ppm) and Tyr 10 (ca. 7.95 ppm and ca. 6.8 ppm) can be distinguished. Slight shifts are observed for His 6, 13 and 14.
(ca. 7.8 ppm and ca. 7.1 ppm), which can be attributed to pH variations. The absence of signal ca. 7.5 ppm confirms the absence of Trp residue in the peptides. Expectedly, signals corresponding to Hα (4.5–3.8 ppm) and Hβ (3.2–2.6 ppm and 2.3–1.5 ppm) are more difficult to attribute in details. In the aliphatic region, signals corresponding to Lys 16 and 28 (1.4 ppm) and to Val and Leu (1.0–0.7 ppm) are observed. Finally, the signal at 3.9 ppm reveals the presence of a methionine residue for the recombinant peptides as expected.

Because most in vitro studies on Aβ peptides consist in studying their aggregative properties under various conditions, it was essential to ensure that the auto-assembling properties of the peptides are conserved after this purification process. Prior to this, it was important to remove traces of oligomeric species, to ensure a homogeneous aggregation process. The two peptides were thus submitted to SEC. To assess the apparent molecular weight of targeted peptides, a calibration curve was established using other unstructured peptides. It confirmed that the apparent molecular weight of the two peptides was about 4 kDa (S1 Fig). Fractions corresponding to monomeric peptides were then submitted to Th-T fibrillization assay. In 26 h, both peptides afford a characteristic sigmoidal aggregation curve, with aggregation half-times around 15 h (Fig 6). The MAβ peptides obtained through our purification procedure thus conserved their auto-assembling properties. The same results were obtained with peptide batches from different cultures, showing the high robustness of the purification procedure in terms of reproducibility. This purification protocol could thus be applied for routine lab-scale MAβ production. This also confirms that the presence of a N-ter Met residue does not affect the aggregation properties of MAβ peptides as previously reported [16]. Removing Met residue could be beneficial for specific studies such as metal coordination, but does not seem mandatory for classical aggregation assays.

**Conclusion**

We have here reported a rapid, straightforward and highly reproducible recombinant production and purification of MAβ1–40 and MAβ1–42. First, we showed that Th-S staining was reliable to monitor IB production during cell growth. Purification monitoring provided evidence that only the combination of UV-Vis spectrophotometry and gel electrophoresis analyses could properly allow the assessment of the peptide purity along the purification process.
In addition, we established that pH treatment of IBs is an efficient way to get rid of most nucleic acid and protein contaminants, contrasting with commonly used chaotropic agents. Additional purification steps consisting in simple ultrafiltrations, providing clean peptide preparations, as revealed by UV-Vis spectrophotometry, SDS-PAGE, LC-MS and 1H NMR. With this protocol, up to 4 mg of peptides are produced within 2 days. In addition, the preparations are suitable for aggregation assays, which were highly reproducible from batch to batch, accounting for a robust purification procedure. Supplementary investigation might now be engaged to remove the N-ter methionine residue, in order to obtain the native Aβ, as already proposed by Walsh and co-workers and further improve the peptide yield [21].

Supporting Information

S1 Fig. SEC calibration curve with unstructured peptides. The different peptides were dissolved in NaOH 50 mM to 10 mg/mL and injected on a Superdex 75 10/300 GL. Samples were eluted with NaOH 15 mM at 1 mL/min monitoring at 220 nm and 293 nm. The arrows indicate the elution volume found for synthetic Aβ1-40 and the two recombinant MAβ1-40 and MAβ1-42 peptides.

(TIFF)
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Author Contributions

Conceptualization: CH PF EG IA MRS.
Funding acquisition: EG CH IA MRS.
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Methodology: MH YM RI MRS.
Project administration: EG MRS.
Supervision: EG MRS.
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Visualization: MH.
Writing - original draft: MH.
Writing - review & editing: MH YM RI CH PF EG IA MRS.

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