Rapid, Refined, and Robust Method for Expression, Purification, and Characterization of Recombinant Human Amyloid-beta M1-42

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Amyloid plaques found in the brains of Alzheimer’s disease (AD) patients primarily consists of amyloid beta 1-42 (Ab42). Commercially, Ab42 is synthetized using peptide synthesizers. We describe a robust methodology for expression of recombinant human Ab(M1-42) in Rosetta(DE3)pLysS and BL21(DE3)pLysS competent E. coli with refined and rapid analytical purification techniques. The peptide is isolated and purified from the transformed cells using an optimized set-up for reverse-phase HPLC protocol, using commonly available C18 columns, yielding high amounts of peptide (~15-20 mg per 1 L culture) in a short time. The recombinant Ab(M1-42) forms characteristic aggregates similar to synthetic Ab42 aggregates as verified by western blots and atomic force microscopy to warrant future biological use. Our rapid, refined, and robust technique to purify human Ab(M1-42) can be used to synthesize chemical probes for several downstream in vitro and in vivo assays to facilitate AD research.

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Title
Rapid, Refined, and Robust Method for Expression, Purification, and Characterization of Recombinant Human Amyloid-beta M1-42

Short Title
Recombinant Human Amyloid-beta M1-42 from Rosetta(DE3)pLysS and BL21(DE3)pLysS Cells using HPLC

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Amyloid beta; recombinant abeta; HPLC; expression; purification; neuroscience; peptide; neurodegeneration; Alzheimer’s disease

Abstract
Amyloid plaques found in the brains of Alzheimer’s disease patients primarily consists of amyloid beta 1-42 (Aβ42). Commercially, Aβ42 is synthetized using high-throughput peptide synthesizers. The repeated purchase of even a small quantity (~1 mg) of commercial Aβ42 can be expensive for academic researchers. Here, we describe a detailed methodology for robust expression of recombinant human Aβ(M1-42) in Rosetta(DE3)pLysS and BL21(DE3)pLysS competent E. coli using standard molecular biology with refined and rapid analytical purification techniques. The peptide is isolated and purified from the transformed cells using an optimized set-up for reverse-phase HPLC protocol, using commonly available C18 columns, yielding high amounts of peptide (~15-20 mg per 1 L culture) within a short period of time. The recombinant human Aβ(M1-42) forms characteristic aggregates similar to synthetic Aβ42 aggregates as verified by western blotting and atomic force microscopy to warrant future biological use. Our rapid, refined, and robust technique to purify recombinant human Aβ(M1-42) can be used to synthesize chemical probes and in several downstream in vitro and in vivo assays to facilitate Alzheimer’s disease research.
1. Introduction

Amyloid beta 1-42 (Aβ42) is a small ~4 kDa peptide produced when the amyloid precursor protein expressed on neuronal membranes is sequentially cleaved by β-secretase and γ-secretase. Aβ exists as several variants ranging from 36 to 43 amino acid residues. However, the main component of the toxic amyloid plaques found in the brains of Alzheimer’s Disease (AD) patients is composed of the Aβ42 isoform. The extracellular accumulation of Aβ42 in the brain over time contributes to neuronal dysfunction and death leading to progressive memory loss and cognitive decline.

Several therapeutics currently in preclinical and clinical trials for AD are focused on targeting the cellular and molecular mechanisms related to Aβ42. Thus, there is an immediate need for further understanding the biology of Aβ42 function and its effect on both neurons and non-neuronal glial cells. Researchers commonly use commercially-available synthetic Aβ42 for their experiments, however, the repeated purchase of synthetic Aβ42 can be expensive (~ $300 for 1 mg of peptide). Synthesizing Aβ42 in a traditional biochemistry laboratory has additional hurdles such as, 1) being expensive due to high instrument costs and 2) challenging due to the high hydrophobicity of the peptide that can affect the yield and efficiency of the procedure. The C-terminal sequence of Aβ42 in particular is known to be resistant to ready solid-phase peptide synthesis and is therefore called a “difficult sequence” peptide.

Here, we present an alternate and refined approach for the rapid, easy, and low-cost production and purification of recombinant human Aβ42 containing an exogenous N-terminus methionine, denoted as Aβ(M1-42) in the laboratory. Expressing the human Aβ(M1-42) in E. coli is a highly efficient and feasible method to produce large quantities of the peptide in a short period of time (3-5 days). The pET-Sac-Abeta(M1-42) plasmid was developed previously by Walsh et al. and expresses Aβ(M1-42) in E. coli cells. In this original protocol, a combination of anion-exchange chromatography and centrifugal filtration was used to purify the peptide. Further, they demonstrated that Aβ(M1-42) does not affect the kinetics and morphology of the fibrillation process compared to Aβ42. Thus, the recombinant human Aβ(M1-42) and synthetic Aβ42 exhibit similar aggregate-forming properties. Recently, Yoo et al. published a protocol where the pET-Sac-Abeta(M1-42) plasmid was purified using reverse-phase high-performance liquid chromatography (HPLC). We have further expanded and optimized this protocol with the following changes to provide additional versatility to the method: 1) We have expressed the pET-Sac-Abeta(M1-42) plasmid in Rosetta(DE3)pLysS cells (a BL21 derivative designed to enhance the expression of eukaryotic proteins) in addition to the BL21(DE3)pLysS cells, 2) We have performed the purification of the peptide with HPLC using a commonly available C18 column with optimized solvent system conditions, 3) We have provided characteristic details of the peptide with Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) and verified its characteristic aggregate formations in different conditions by western blotting and Atomic Force Microscopy (AFM) to warrant future biological use. We provide all the details required for a complete experimental methodology (Figure 1) to obtain pure Aβ(M1-42) within...
a short period of time by using simple molecular biology and analytical chemistry tools that are commonly available in biochemical and chemical biology laboratories.

2. Material and methods

The methodology of obtaining recombinant human Aβ(M1-42) is outlined in Figure 1 and explained in detail in the following subsections. A complete list of materials and instruments are provided in the Supporting Information.

2.1. Chemicals and reagents

The pET-Sac-Abeta(M1-42) plasmid was purchased from Addgene. Tryptone, sodium chloride, yeast extract, Luria broth (LB) Agar (Miller, granulated), ampicillin, chloramphenicol, tris hydrochloride, isopropyl β-D-1-thiogalactopyranoside (IPTG), hydrochloric acid, acetonitrile (HPLC-grade), urea, and other chemicals were purchased from Thermo Fisher Scientific. The plasmid miniprep kit was from Promega and the Rosetta™(DE3)pLysS cells were from Millipore-Sigma. The sonicator ultrasonic homogenizer (125 W) equipped with ¼” probe was from Qsonica, Inc. The centrifuge used was a Sorvall LYNX 6000 with a swinging bucket rotor from Thermo Fisher Scientific. The equipment for preparative HPLC were as follows: RediSp Prep C18 100Å, 5 μm column and Guard column were from Teledyne ISCO. The AFM instrument was Veeco Multimode with NanoScope V controller and NanoScope Software with tapping mode. The probes used were Silicon AFM probes with TAP300 Aluminum reflex coating (details in Supporting Information).

2.2. Preparation of solutions

Liquid LB was prepared by dissolving 10 g tryptone, 10 g NaCl, and 5 g yeast extract in 1 L ultrapure water. The liquid LB was autoclaved and brought to room temperature before adding the antibiotics. Solid LB was prepared as 3.2 g of LB agar mix in 100 mL water. The media was autoclaved and allowed to cool before adding the antibiotics (100 mg/L ampicillin for plasmid culture and 100 mg/L ampicillin plus 34 mg/L chloramphenicol for transformed bacterial culture). The media with the antibiotics was poured on to the petri dishes and kept in the biosafety cabinet with lids open to allow for the media to solidify. These plates can be prepared in bulk and refrigerated at 4 °C for future use, as it will save time during the protocol. For peptide purification, two buffers were made: (i) Buffer A containing 10 mM Tris/HCl and 1 mM EDTA in water (pH set to 8.0), (ii) Buffer B containing 8 M urea, 10 mM Tris/HCl, and 1 mM EDTA in water (pH set to 8.0). For preparative HPLC, Solvent A was prepared as 0.1% TFA in ultrapure water and Solvent B was prepared as 0.1% TFA in acetonitrile.

2.3. Isolation of the pET-Sac-Aβ(M1-42) plasmid

The pET-Sac-Aβ(M1–42) plasmid from Addgene arrived as a bacterial agar stab culture and was streaked onto a solid LB agar plate containing 100 mg/L ampicillin using a sterile loop. The plate was placed overnight (12-18 h) in the incubator at 37 °C for the colonies to grow. The next day, a single colony was picked from the plate (Figure 2A) using a sterile loop or a sterile 10 µL
20 min intervals starting from 3 h before proceeding to the next step. The next day, we isolated the plasmid (Figure 1, Part 1) from the culture using the plasmid isolation miniprep kit following instructions per the user manual. The concentration of the isolated pET-Sac-Aβ(M1-42) plasmid was measured at 260 nm absorbance using a spectrophotometer for nucleic acid quantification.

2.4. Transformation of pET-Sac-Aβ(M1-42) plasmid into competent E. coli by heat shock method

To transform the Aβ(M1-42) plasmid into the E. coli (Figure 1, Part 2), frozen vials of Rosetta(DE3)pLysS cells or BL21(DE3)pLysS competent cells (20-50 µL) were thawed on ice. Once thawed, around 1-2 µL of the isolated plasmid (50-100 ng) was added to the cells and the tube was gently flicked a few times to mix the plasmid with the cells. The cells plus the plasmid mixture were incubated on ice for 20 mins after which the tube was placed in a 42 °C water bath for 45 s to facilitate the transformation of plasmid into the cells via heat shock method. After heat shock, the tubes were immediately placed on ice for 2 mins. The transformed bacteria was then inoculated into 500 µL of liquid LB media without any antibiotics. Finally, the tube was kept in the 37 °C shaker for 1 h at 220 rpm to allow the bacteria to express the antibiotic resistance proteins necessary for future steps.

2.5. Expression of Aβ(M1-42) peptide by the transformed E. coli

After 1 h, 25-30 µL out of 500 µL of the transformed cells was spread onto solid LB agar plates containing 100 mg/L ampicillin and 34 mg/L chloramphenicol using a sterile glass spreader. The plates were let to sit on the bench or in the hood for 5 min to allow the cells to absorb on the solid LB. Next, the plate was placed overnight in the incubator at 37 °C for the transformed colonies to grow. Note that it is best to use a lower volume of the transformed cells (25 to 30 µL) as this results in more single colonies that are easier to pick and prevent the overcrowding of the agar plate. The next day, a single colony was picked from the plate using a sterile glass spreader or a sterile 10 µL pipet tip and inoculated into 5 mL of liquid LB containing 100 mg/L ampicillin and 34 mg/L chloramphenicol (first culture). At the same time, another single colony of the transformed bacteria was picked and inoculated into a second 5 mL liquid LB media containing 100 mg/L ampicillin and 34 mg/L chloramphenicol for overnight growth (second culture). These cultures were placed in the shaking incubator at 220 rpm at 37 °C overnight (12-18 h) (Figure 2B).

The following day, after the growth of the overnight culture, the first 5 mL culture was inoculated into 1 L of liquid LB media containing 100 mg/L ampicillin and 34 mg/L chloramphenicol (Figure 2C). The 1 L culture was kept at 220 rpm and 37 °C until the cell density reached an optical density (OD) value of approximately 0.45 at 600 nm (OD₆₀₀). The BL21(DE3)pLysS cells reached an OD of 0.45 in 3 h time, whereas the Rosetta(DE3)pLysS cells required around 3.5-4 h to reach an OD of 0.45. We measured the OD of the culture at regular 20 min intervals starting from 3 h before proceeding to the next step.
The second 5 mL culture of transformed bacteria was used to make 25% glycerol stocks by adding 500 μL of 50% glycerol to 500 μL bacterial culture and frozen at -80 °C for future use. The -80 °C frozen glycerol stocks of the transformed bacteria were thawed for use in the future for inoculating 5 mL liquid LB containing 100 mg/L ampicillin and 34 mg/L chloramphenicol to grow additional cultures of the transformed bacteria containing the Aβ(M1-42) plasmid. This step served as a starting point for all future experiments performed for the isolation of the Aβ(M1-42) peptide (Figure 1, Part 3). It is important to note that this step is critical for reducing the time taken for the entire protocol along with saving the reagents used for expression.

Once the OD$_{600}$ of the 1 L culture reached 0.45, protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to obtain a final concentration of 0.1 mM in 1 L of the liquid LB media. The culture was again kept on the shaking incubator at 220 rpm and 37 °C for additional 4 h in the presence of IPTG to allow the cells to express the Aβ(M1-42) peptide. After 4 h, the 1 L culture was centrifuged at 7068 x g for 25 min. The supernatant liquid LB was discarded and the pelleted cells was resuspended in 25 mL of 1x PBS and the thick cell suspension was transferred to a 50 mL falcon tube using a 10 mL pipet. The cells were then centrifuged at 7068 x g for 25 min and the 1x PBS supernatant was discarded. The pelleted cells were stored at -80 °C until the next day or when ready for cell lysis.

2.6. Cell lysis and resuspension

To lyse the cells (Figure 1, Part 4), the frozen cell pellet was resuspended in 25 mL Buffer A. A 1 mL pipette was used with the tip cut off to dissociate the pellet in the buffer. The pellet was mechanically disrupted by mixing the cells with Buffer A. The tube was placed in an ice bucket and the sonicator probe was introduced into the cell mixture. The cells were sonicated at 30 second pulse with an amplitude of 100% for 2 min until the lysate appeared homogenous. To increase the lysis efficiency, the cell pellet obtained from 1 L cultures was sonicated in the original 50 mL falcon tube as transferring the cell mixture to containers with a large surface area reduced the lysis efficiency. The cell lysate was centrifuged at 7068 x g for 25 min at 4 °C, the supernatant was discarded, and the pellet was resuspended in 25 mL Buffer A. The solution was sonicated on ice and centrifuged at 7068 x g for 25 min at 4 °C. The sonication and centrifugation steps were repeated three more times. After discarding the supernatant from the last (fourth) step, the pellet was resuspended in 15 mL of freshly prepared Buffer B and sonicated until the solution appeared clear. Due to the inconsistencies of mechanical lysis using the sonicator, the solution may appear to be cloudy. The turbidity of the solution can be resolved by further centrifugation at the same or higher speeds. Finally, the lysate was filtered through a 0.22 μm non-sterile hydrophilic PVDF syringe filter using a 30 mL syringe and appeared as a clear solution.

2.7. Equipment set-up for peptide purification

A water bath containing reverse osmosis water was heated using a commercially-available sous vide to around 80 °C (Figure 4A). Both the guard and the primary columns were completely submerged in the water bath (Figure 4B). It is recommended to set up the water bath with the submerged columns at least 4 hours prior to purification to allow the water bath to reach 80 °C
and the temperature of the columns to equilibrate. The solvent lines from the CombiFlash HPLC instrument were placed into the Solvent A and Solvent B bottles. Next, the system was auto primed with 75 mL of Solvent B followed by 75 mL Solvent A to clear the solvent lines of any residual solvents. The column was then cleaned by injecting 4 mL of Buffer B into the 5 mL injection loop (Figure 4C) and running the solvent gradient for the cleaning protocol provided in Table 1.

2.8. Peptide purification using reverse-phase HPLC

After running the solvent gradient in the cleaning protocol, the solvent gradient outlined in Table 2 was programmed into the CombiFlash and a rack of clean and dry 18 x 150mm glass test tubes (Figure 4D) was placed in the instrument. The protocol was initiated (Figure 1, Part 5) and the column was allowed to equilibrate with the starting solvent system. After equilibration, 4 mL of the filtered bacterial cell lysate was injected into the HPLC injection loop for separation (Figure 4C). The instrument was set to collect peaks detected at 214nm. Upon completion of the purification protocol, the column was cleaned with the solvent gradient described in Table 1. The cleaning and purification steps were repeated three more times or until all the lysate was used. The collected Aβ(M1-42) fractions that eluted at 26 mins were then combined together (Figure 5). The acetonitrile was evaporated off under reduced pressure at 65 °C using a rotary evaporator until a cloudy aqueous solution remained. The solution was frozen at -80 °C and then submerged in liquid nitrogen for 5 mins prior to overnight lyophilization at -90 °C at 0.003 mbar pressure to obtain a white powder (Figure 5).

2.9. Characterization of Aβ(M1-42) using MALDI-TOF MS and High-Resolution LC-MS

The Aβ(M1-42) was dissolved and diluted in water until the sample was approximately 100 µg/mL. 1 µL of analyte solution was thoroughly mixed with 1 µL of CHCA matrix solution (10 mg/mL a-cyano-4-hydroxycinnamic acid in 0.1% TFA). The analyte/matrix mixture was spotted on a MALDI target plate and allowed to dry. A MALDI-TOF mass spectra was obtained on a Voyager-DE PRO (Applied Biosystems) from 1000-22000 Da in the positive ion mode with an accelerating voltage of 25000V. The analyte solution prepared for the MALDI-TOF MS was further diluted and injected into the Agilent 6550 iFunnel Q-TOF LC-MS in positive mode and fragmented using electrospray ionization (ESI) with a fragmentor voltage of 175V.

3.0. Western blot characterization of the purified peptide

To perform western blotting, the purified peptide from HPLC was lyophilized and dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol-D2 (HFIP) to prepare monomeric Aβ(M1-42) using previously established protocols13,14. Once the peptide was dried overnight in the chemical hood, 1 mg of it was dissolved in 221 µL DMSO to obtain a concentration of ~ 1.0 mM. The peptide was loaded on 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) at different concentrations (10, 20, and 40 µg) and the SDS-PAGE gel was run at 115 V for 1 h 20 mins. The gel was then transferred to a nitrocellulose membrane at 10 V for 35 mins. After transfer, the membrane was boiled in PBS for 5 mins, then blocked with blocking buffer (5% milk
in Tris-buffered saline, 0.1% Tween 20 (TBST)) for 1 h. The membrane was incubated in the blocking buffer containing the 6E10 mouse monoclonal antibody for the human abeta peptide overnight on a rocker at 4 °C. The next morning, the membrane was washed 3 times with TBST, incubated with the secondary antibody (HRP-conjugated goat anti-mouse antibody) for 1 h, washed 3 times with TBST, and developed in the dark room using chemiluminescence reagents as per the manufacturer’s protocol. The same protocol was followed for synthetic Aβ(1-42) for comparison (Figure 7).

3.1. Characterization of Aβ(M1-42) oligomers by Atomic Force Microscopy

The synthetic Aβ(1-42) and the lyophilized Aβ(M1-42) powder from section 2.8. were taken to prepare 1 mM solution with 215 μL of 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP). HFIP was handled carefully using a 1 mL glass Hamilton syringe with a Teflon plunger and a sharp needle. The clear Aβ(M1-42) and synthetic Aβ(1-42) solution were incubated at room temperature for 30 min. Next, 100 μL aliquots (~0.45 mg) were transferred to microcentrifuge tubes. The tubes were left open in the fume hood overnight for the HFIP to evaporate and then dried under high vacuum for 1 h without heating to remove any remaining traces of HFIP and moisture. This resulted in thin clear films of monomeric peptides at the bottom of the tubes which were stored at -20 °C until further use.

To evaluate the aggregation property of the recombinant Aβ(M1-42) with the synthetic Aβ(1-42), the protocol established by Stine et al. was used to prepare Aβ oligomers. In brief, the tubes containing monomeric peptide films were allowed to equilibrate at room temperature for a few minutes and 5 mM of synthetic Aβ(1-42) and recombinant Aβ(M1-42) DMSO stocks were prepared by adding 20 μL of cell-grade DMSO to each tube containing ~0.45 mg of the peptide. The solution was pipetted thoroughly by scraping down the sides of the tube and was vortexed for ~30 s followed by bath sonication for 10 min to ensure complete resuspension of the peptide film. This stock solution was used immediately as the starting material for oligomeric Aβ preparation. To prepare Aβ oligomers, the freshly resuspended 5 mM of synthetic Aβ(1-42) and recombinant Aβ(M1-42) in DMSO were used to make 100 μM solution with 1x PBS (for example, 2 μL of 5 mM Aβ(M1-42) in DMSO and 98 μL of 1x PBS buffer). (Note: 1x PBS buffer was filtered through a sterile 0.22 um filter). The solution was vortexed for 15 s and incubated at 4 °C for 24 h.

After 24 h, samples for AFM were prepared with proper sterile technique in the hood as follows. The 100 μM samples were diluted to a concentration of 10–30 μM in filtered water. The mica sheet was mounted on 15 mm stainless steel pucks. Immediately before sample plating, a few layers of the mica sheet were removed using adhesive tape to reveal a featureless surface for the absorption of the peptide. Next, the mica surface was pretreated with ~5-8 μL of filtered 1M HCl for 30 s and rinsed with 2-3 drops of ultrapure water using the 1 mL Hamilton syringe. (Note: The mica plate was held at a 45° angle to washed with water.) Immediately after cleaning, the peptide sample was spotted onto mica and incubated for 3 min. The mica was then gently rinsed with 2-3 drops of water using the 1 mL syringe and dried with several gentle pulses of clean compressed air or nitrogen gas. Samples were incubated at room temperature for a few
hours until imaging. The AFM imaging was performed using a multimode AFM equipped with aluminum-coated silicon probes with \( \sim 300 \text{ kHz} \) resonant frequency and 40 N/m force constant under the tapping mode. Image analysis was performed using the NanoScope Analysis software.

3. Results and discussion

A handful of methodologies have been published in recent years that show expression of Aβ42 in *E. coli* using standard molecular biology techniques, such as, transformation and bacterial cell culture. The major differences among these methodologies have been the isolation and purification methods used to obtain purified peptide samples. The purification of the peptides expressed from cells are challenging due to many different techniques that are proposed combined with limited resources available in a traditional biochemistry or chemical biology laboratory. Previously, nickel affinity chromatography was used to purify Aβ42 fusion proteins with N-terminal His-affinity tag\(^{15}\) or as an extended polypeptide of His-tagged ubiquitin\(^{16}\). Both these methods require additional steps from which Aβ42 has to be eventually cleaved requiring extra time, reagents, as well as affecting yield. Another method used NaOH treatment followed by ultracentrifugation to isolate and purify the insoluble inclusion bodies expressed in the *E. coli*\(^{17}\). This purification method reduces the peptide purity and yield (\( \sim 4 \text{ mg of peptide} \)). Recently, Walsh et al.\(^{16}\) used exchange chromatography and Yoo et al.\(^{12}\) modified the original protocol by using preparative HPLC equipped with a C8 column for Aβ(M1-42) purification.

To address these limitations, we developed a robust and fast method for Aβ(M1-42) expression and purification. Specifically, we used BL21(DE3)pLysS and Rosetta(DE3)pLysD *E. coli* cells to express human Aβ42 peptide containing an exogenous methionine (Aβ(M1-42)). MALDI-TOF MS was performed on the crude lysate to confirm the presence of Aβ(M1–42) (not shown), followed by purification using preparative reverse-phase HPLC equipped with a commonly used C18 semi-preparative column. The samples were lyophilized to give a fine white powder (Figure 5) and the purity and the identity of the sample was determined using high-resolution LC-MS. The purified Aβ(M1-42) was further characterized by MALDI-TOF MS (Figure 3A), western blotting using a monoclonal antibody, and AFM was compared with the properties of synthetic Aβ42.

An important point of consideration during the cell lysis and resuspension step is to prevent leaving the peptide in 8M urea after cell lysis for a long period of time. Exposing the lysate to a high concentration of urea solution is known to cause carbamylation of the lysine residues\(^{18}\). Carbamylation can be seen in the MALDI-TOF MS by the presence of a secondary peak \( \text{m/z} \) 43 higher than the peak \( \text{m/z} \) 4642.50 corresponding to [M+H]\(^+\) (Figure 3B). Our HPLC system has a 5 mL injection loop, which limits the amount of sample that can be loaded onto the column. In Yoo et al. method, the cell lysate in 8 M urea was further diluted before injecting in the column.\(^{8}\) We decided not to dilute the cell lysate in our protocol due to the smaller volume of the injection loop allowing for fewer batch runs thereby reducing the time taken for purification without reducing the purity of the peptide. Also, in the previous method\(^{12}\), the cell lysate was centrifuged at 15000 x g during the wash step. Our centrifuge allowed for a maximum speed of 7068 x g, which is typically available in any laboratory setting. We show that a lower speed of 7068 x g, is
sufficient to obtain similar yields of the final peptide product (~15-20 mg). The mass characterization of the final peptide shows that the final peptide obtained with very few impurities (Figure 6).

Using Western Blotting of recombinant Aβ(M1-42) and synthetic Aβ(1-42), we demonstrated that the purified peptide mixture contains large quantities of monomers as seen in the 4 kDa region (Figure 7). Higher concentration of the peptide shows oligomeric forms of the peptide that appear between 14-17 kDa. Overall, we show that the recombinant Aβ(M1-42) peptide epitope can be recognized by the monoclonal 6E10 antibody that is specific for human Aβ42, suggesting future biological use of our recombinant Aβ(M1-42). Interestingly, the recombinant Aβ(M1-42) peptide showed higher molecular weight (HMW) oligomeric bands in the 38-49 kDa region that were not visible with synthetic Aβ42. Increased levels of such HMW oligomers are seen in the cerebrospinal fluid samples from Alzheimer’s patients. Thus, HMW oligomers are of huge importance for understanding the etiology of Alzheimer’s disease. We further characterized the aggregation property of recombinant Aβ(M1-42) using atomic force microscopy (AFM). The recombinant peptide formed large oligomers of different sizes (some greater than 20nm, not shown) compared to the synthetic peptide that formed mostly uniform oligomers during the same time (Figure 8). It is previously known that the recombinant amyloid-beta Abeta1-42 aggregates faster and is more neurotoxic than synthetic Abeta1-42.

Overall, the recombinant Aβ(M1-42) formed characteristic oligomers under similar aggregation conditions as the synthetic Aβ(1-42).

4. Conclusion

We present a rapid, robust, and refined method for the expression and purification of human Aβ(M1-42) peptide in E. coli cells using inexpensive and non-specialized instrumentation. Aβ42 is an important peptide utilized in studies related to neuronal health and non-neuronal glial immune response in Alzheimer’s disease related preclinical and clinical research. Our protocol can be completed within a week’s time (~ 5 days if starting from the beginning or 3 days if using the transformed bacteria for generating more cultures for peptide purification) and a yield of around 15-20 mg of the peptide per one liter of the liquid culture can be obtained. The equipment utilized in this protocol is readily available in not only biological labs, but also in synthetic chemistry and chemical biology labs, therefore enhancing widespread use of the methods. The final Aβ(M1-42) peptide may be used for several downstream in vitro and in vivo applications such as cell-based drug screening, neuroinflammation cell culture and in animal models, etc., as well as, for the development and synthesis of novel Aβ-related biorthogonal chemical and florescent probes to facilitate the advancement of neurological disease research.
5. Tables and figures

| % Solvent A | % Solvent B | Elapsed Time (min) |
|-------------|-------------|--------------------|
| 90          | 10          | 0                  |
| 90          | 10          | 5                  |
| 10          | 90          | 10                 |
| 10          | 90          | 20                 |

Solvent A = H$_2$O with 0.1% TFA
Solvent B = Acetonitrile with 0.1% TFA

*Table 1. Solvent gradient for the cleaning protocol done before and after peptide purification*

| % Solvent A | % Solvent B | Elapsed Time (min) |
|-------------|-------------|--------------------|
| 90          | 10          | 0                  |
| 90          | 10          | 9                  |
| 5           | 95          | 19                 |
| 5           | 95          | 27                 |

Solvent A = H$_2$O with 0.1% TFA
Solvent B = Acetonitrile with 0.1% TFA

*Table 2. Solvent gradient for peptide purification from crude bacteria cell lysate*
Figure 1: Schematic diagram illustrating the experimental protocol for the expression and isolation of recombinant human Aβ(M1-42) peptide from competent cells. The protocol can be divided into 5 main parts: Part 1. Isolation of the pET-Sac-Aβ(M1-42) plasmid from the glycerol stock, Part 2. Transformation of BL21(DE3)pLysS and Rosetta(DE3)pLysS competent cells with the isolated pET-Sac-Aβ(M1-42) plasmid, Part 3. Expression of the Aβ(M1-42) peptide in 1 L liquid LB culture, Part 4. Harvesting and lysis of the cells using a probe sonicator followed by resuspension of the cell lysate in 8M urea, and, Part 5. Purification of the Aβ(M1-42) peptide with preparative HPLC.
Figure 2. Transformed cells as colonies on the LB plate and the growing culture in liquid LB. A. Colonies of transformed E. coli on solid LB. Red circles represent single colonies. B. Single colony is picked from the plate and inoculated into 5 mL liquid LB and shaken at 37 °C overnight that makes a cloudy solution after incubation. C. The next morning, this culture is inoculated into 1 L LB to grow the cells for the next 3 to 3.5 h until the OD reaches 0.45.
Figure 3: MALDI-TOF mass spectra of Aβ(M1-42). A. The MALDI-TOF mass spectra of lyophilized Aβ(M1-42) in the range of m/z 500 to 8000. The Aβ(M1-42) corresponds to the m/z 464.27 peak. B. MALDI-TOF mass spectra of lyophilized carbamylated Aβ(M1-42) in the range of m/z 500 to 8000. Carbamylation peak (m/z 4685.34) appears ~ m/z 43 higher than the Aβ(M1-42) at m/z 4642.50. A Voyager De-Pro MALDI-TOF mass spectrometer in positive linear mode was used with sinapinic acid as the matrix for each spectra.
Figure 4. HPLC set-up. A. The water bath containing filtered reverse osmosis water is heated to around 80 °C. B. Both columns are completely submerged in the water bath. One end of the guard column attached to the injection valve with metal tubing and the other end of the guard column attached to the inlet of the primary C18 100 Å 5 µm 10 mm x 250 mm preparative column. The primary column is fed into the Combiflash. C. 5 mL injection loop. D. Collection of Aβ(M1-42) fractions eluted at 26 mins.
Figure 5. HPLC Chromatogram of Crude Lysate during Purification

The Aβ(M1-42) peak elutes at 26 mins at 95% acetonitrile with 0.1 % TFA, 5 % H₂O with 0.1 % TFA. The urea salt from solution B elutes as a cluster of peaks between 5-7 mins in 10% acetonitrile with 0.1 % TFA, 90 % H₂O with 0.1 % TFA. The absorbance was taken at 214nm. Inserted image of the white powder is the lyophilized peptide corresponding to the Aβ(M1-42) fractions collected at 26 min.
Figure 6. High-resolution Liquid Chromatography Mass Spectrum (LC-MS) of \( \text{A}\beta(\text{M1-42}) \). The high-resolution mass spectra was obtained using an Agilent 6550 iFunnel Q-TOF LC-MS in positive ion mode using electrospray ionization (ESI) with a fragmentor voltage of 175V. **A** The chromatogram from the LC-MS showed a significant peak between 5-7 mins while using 0.1% formic acid and methanol as the solvent system. **B** The averaged mass spectrum of each time point between 5 – 7 mins resulting in the corresponding peaks: \([\text{M+3H}]^{3+} \) (m/z 1549.0789), \([\text{M+4H}]^{4+} \) (m/z 1162.0623), \([\text{M+5H}]^{5+} \) (m/z 929.8515), \([\text{M+6H}]^{6+} \) (m/z 775.0439), and \([\text{M+7H}]^{7+} \) (m/z 664.463). **C** The deconvolution of the mass spectrum in panel B was performed showing the peak corresponding to \([\text{M+H}]^{+} \) at m/z 4644.22. (calculated \([\text{M+H}]^{+} \) of m/z was done using the PEPTIDEMASS program available via https://web.expasy.org/peptide_mass/)
Figure 7: Western blot characterization of the recombinant human Abeta (M1-42) peptide. A. 10, 20, and 40 µg of the recombinant Abβ(M1-42) peptide was run on an SDS-PAGE gel and bands visualized using the 6E10 antibody with Western Blot. Lower concentrations of 10 and 20 µg show monomeric bands at 4 kDa while the 40 µg lane shows oligomeric bands at 14-17 kDa along with the monomeric band. All 3 concentrations show a slight amount of high molecular weight bands at 38-49 kDa suggesting a few aggregated forms of the peptide in the mixture. B. Synthetic human amyloid beta 1-42 for reference shows similar monomeric bands at 10, 20, and 50 µg concentrations. More oligomers are present in the synthetic peptide.
Figure 8. AFM characterization of the recombinant human Aβ (M1-42) peptide. The recombinant Aβ(M1-42) and the synthetic Aβ(1-42) peptide oligomers were prepared from HFIP-treated peptide films in 1x PBS buffer (pH 7.4) at 4°C and analyzed by AFM. Image is 2×2 µm x-µm scale. **Left.** 2-D image. **Right.** 3-D image.

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Disclosures

The authors have nothing to disclose.

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| Name of Material                                                | Company                     | Catalog Number | Comments/Description |
|----------------------------------------------------------------|-----------------------------|----------------|----------------------|
| pET-Sac-Abeta(M1-42) plasmid                                  | Addgene                     | 71875          |                      |
| Tryptone                                                      | Fisher                      | BP1421-500     |                      |
| Sodium Chloride                                               | Fisher Chemical             | S271-1         |                      |
| Yeast Extract                                                 | Fisher                      | BP9727-500     |                      |
| LB Agar (Miller, Granulated)                                 | Fisher                      | BP9724-500     |                      |
| Ampicillin                                                    | VWR                         | 80055-786      |                      |
| Chloramphenicol                                               | Fisher                      | AAJ67273AB     |                      |
| Plasmid Miniprep System                                       | Promega                     | A12222         | Any plasmid miniprep kit will do |
| Rosetta™(DE3)pLysS Competent Cells                            | Millipore-Sigma             | 70956-3        |                      |
| Isopropyl β-D-1-thiogalactopyranoside (IPTG)                  | Fisher                      | 15-529-019     |                      |
| Tris hydrochloride (Tris-HCl)                                | Fisher                      | BP153-500      |                      |
| Ethylenediaminetetraacetic acid                              | Tokyo Chemical Industry     | E0084          |                      |
| Urea                                                          | Invitrogen                  | 15505-035      |                      |
| Hydrochloric Acid                                            | Fisher                      | A1445I-212     |                      |
| Sodium Hydroxide                                              | Sigma-Aldrich               | 221465-500G    |                      |
| 0.22 μm non-sterile hydrophilic PVDF syringe filter           | Fisher                      | 09-719-000     |                      |
| Trifluoroacetic acid (TFA)                                   | Sigma-Aldrich               | T6508-100mL    |                      |
| HPLC grade Acetonitrile                                       | Fisher                      | A998-4         |                      |
| 1,1,1,3,3,3-Hexafluoroisopropyl alcohol                       | Chem-Impex International    | 00080          |                      |
| Dimethyl sulfoxide                                            | Corning                     | MT 25-950-CQC  |                      |
| Beta-Amyloid (1 - 42), Human (synthetic)                      | AnaSpec                     | AS-20276       |                      |
| Nitrocellulose membrane, 0.2 um                               | Bio-Rad                     | 1620112        |                      |
| Nonfat dry milk from bovine                                  | Sigma-Aldrich               | M7409          |                      |
| Purified anti-β-Amyloid, 1-16 Antibody                        | BioLegend                   | 803001         | 6E10 clone           |
| HRP Goat anti-mouse IgG (minimal x-reactivity) Antibody       | BioLegend                   | 405306         | Goat Polyclonal IgG, Clone Poly4053 |
| Name of Equipment                                           | Company     | Catalog Number | Comments/Description |
|------------------------------------------------------------|-------------|----------------|----------------------|
| SuperSignal West Pico Chemiluminescent Substrate           | Thermo Fisher Scientific | 34080          |                      |
| Bolt™ 12% Bis-Tris Plus Gels, 10-well                     | Invitrogen  | NW00120BOX     |                      |
| 20X Bolt™ MES SDS Running Buffer                           | Invitrogen  | B0002          |                      |
| 4X Bolt™ LDS Sample Buffer                                 | Invitrogen  | B0007          |                      |
| 10X Bolt™ Sample Reducing Agent                            | Invitrogen  | B0009          |                      |
| SeeBlue™ Plus2 Pre-stained Protein Standard                | Invitrogen  | LC5925         |                      |
| Syringe, 1 mL                                              | Hamilton    | 81343          |                      |
| Mica sheet                                                 | Ted Pella   | 50             |                      |
| 15 mm stainless steel pucks                                | Ted Pella   | 16218          |                      |
| Aluminum-coated silicon probes for AFM                    | Ted Pella   | TAP300AL-G-10  |                      |
| Benchtop incubator shaker (New Brunswick™ Excella® E24)    | Eppendorf   | M1352-0000     |                      |
| Sonicator Ultrasonic Homogenizer (125W) with 1/4" Probe    | Osonica     | Q700-110 and 4435 |                    |
| CO2 Incubator (New Brunswick™ Galaxy® 48S)                 | Eppendorf   | CO48S-120-0000 |                      |
| Centrifuge (Sorvall LYNX 6000) with a Swinging Bucket Rotor (BIOFlex™ HC) | Thermo Scientific™ | 75006591 and 75003000 |          |
| UV-Visible Spectrophotometer                               | JASCO       | V-730          |                      |
| Microplate reader for nucleic acid quantification (Take3™ Micro-Volume plate) with Gen5 Software | BioTek Instruments |                |                      |
| Combiflash EZ prep UV/ELSD                                 | Teledyne Isco | 218J00936     |                      |
| Name of Solutions                          | Company              | Catalog Number | Comments/Description                                                                 |
|-------------------------------------------|----------------------|----------------|--------------------------------------------------------------------------------------|
| Liquid LB                                 |                      |                | Recipe: 10 g Tryptone, 10 g NaCl, 5 g Yeast Extract in 1 L diH2O. Autoclave before use and add antibiotics once cooled. |
| Solid LB                                  |                      |                | Recipe: 3.2 g of LB agar mix in 100 mL of water. Autoclave and add antibiotics once cooled. Pour on petri dishes immediately. |
| Buffer A for cell lysis                   |                      |                | Recipe: 10 mM Tris/HCl, 1 mM EDTA, pH 8.0                                             |

| RediSep Prep 10 x 250mm C18 100A, 5 um column | Teledyne Isco | 692203809 |
| RediSep Prep Guard 20 x 30mm, C18Aq, 100A, 5 um | Teledyne Isco | 692203805 |
| GenPure UV/UF x CAD plus Ultrapure Water Purification System | Thermo Scientific | 41956240 |
| Rotary evaporator and water bath | Eyela | N-1110 and SB-1200 |
| Dry Bath with heating block | Thermofisher | 88870002 |
| Labconco Freezone 12 Liter Console Freeze Dry System (Lyophilizer) | Labconco | 710612000 |
| Veeco Multimode with NanoScope V controller with NanoScope Software (AFM instrument) |                      |                |
| Silicon AFM probes, TAP300 Aluminum reflex coating (~250 kHz resonant frequency and ~40 N/m force constant) | Ted Pella, Inc | TAP300AL-G-10 |
| Buffer B for cell lysis | Recipe: 8 M urea, 10 mM Tris/HCl, 1 mM EDTA, pH 8.0 |
|------------------------|---------------------------------------------------|
| Solvent A for HPLC    | 0.1 % TFA in ultrapure water (1 mL in 1000 mL water) |
| Solvent B for HPLC    | 0.1 % TFA in acetonitrile (1 mL in 1000 mL acetonitrile) |
