Alzheimer´s disease-associated Aβ42 peptide: expression and purification for NMR structural studies

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Running title: A straightforward strategy to obtain Aβ42 for NMR studies

ABSTRACT

The aggregation of the amyloid-beta peptide (Aβ) in the brain is strongly associated with Alzheimer´s disease (AD). However, the heterogeneous and transient nature of this process has prevented identification of the exact molecular form of Aβ responsible for the neurotoxicity observed in this disease. Therefore, characterizing Aβ aggregation is of utmost importance in the field of AD. Nuclear magnetic resonance spectroscopy (NMR) is a technique that holds great potential to achieve this goal. However, it requires the use of specific labels introduced through recombinant expression of Aβ. In this paper, we report on a straightforward expression and purification protocol to obtain [U-^{15}N] and [U-^{2}H,^{13}C,^{15}N] Aβ42. Aβ42 is expressed fused to Small Ubiquitin-like Modifier (SUMO), which prevents Aβ42 aggregation. The solubilizing capacity of SUMO has allowed us to design a purification protocol involving immobilized metal affinity chromatography (IMAC), a desalting step, and two size exclusion chromatography (SEC) purifications. This approach, which does not require the use of costly and time-consuming reversed phase high performance liquid chromatography (RP-HPLC), offers a much straightforward strategy to those previously described to obtain [U-^{15}N] Aβ42 and it is the first protocol through which to achieve [U-^{2}H,^{13}C,^{15}N] Aβ42. The peptides obtained are of high purity and have the required isotope enrichment to support NMR-based structural studies.

KEYWORDS

Alzheimer´s disease, Aβ42 peptide, isotope labeling, NMR, soluble expression, SUMO.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that robs us of our most human qualities. Beyond its devastating effects on patients, AD is also a major burden for patients' families and implies enormous healthcare costs [1]. To date, no disease-modifying treatment is available, and therefore research on the molecular basis of AD must continue. The aggregation of the amyloid-beta peptide (Aβ) in the brain is strongly associated with AD [2]. Aβ is obtained from the transmembrane amyloid precursor protein (APP) through the sequential cleavage of β- and γ-secretase. γ-secretase cleavage is not specific, leading to Aβ peptides of different lengths, Aβ38 to Aβ43, ranging from 38 to 43 residues. Among them, Aβ42 is considered to be the peptide most strongly linked to AD, as levels of this variant are an indication of AD progression [3], and several familial AD (fAD) mutations that give rise to early AD alter the Aβ40/Aβ42 ratio in favor of Aβ42 production [4]. Upon release from the membrane into the extracellular media, the Aβ peptide has a strong tendency to aggregate. This aggregation is a multistep process involving various aggregates that ultimately leads to the formation of amyloid fibrils. Due to the heterogeneity and transient nature of this process, the exact molecular form of Aβ responsible for the neurotoxicity observed in AD is not known. Therefore, characterizing Aβ aggregation is of utmost importance in the AD field. One of the techniques with the potential to characterize this process is nuclear magnetic resonance spectroscopy (NMR) [5-12], which requires the use of specific labels introduced through recombinant expression of Aβ.

A large number of strategies have been developed to produce recombinant Aβ42. However, due to the aggregation-prone nature of Aβ42, initial approaches resulted in the production of modified Aβ42 sequences rather than of the wild type. Modifications

included oxidation on the methionine 35 side chain of Aβ42 to methionine sulfoxide (Met-35(ox)Aβ42) [6,13], Aβ42 fused to a tag [14-16], and Aβ42 containing unnatural mutations in its sequence [6,17] or additional N-terminal residues [18-21]. More recently, several strategies have led to the production of wild-type Aβ42 with satisfactory yields [22-28], as well as isotopically labeled ^{15}N and ^{15}N, ^{13}C Aβ42 [29,30]. One particularly attractive strategy initially proposed by Satakarni and Curtis relies on the expression of Aβ42 fused to Small Ubiquitin-like Modifier (SUMO) [27]. They showed that SUMO solubilizes Aβ42, since the SUMO-Aβ42 fusion protein was obtained from both soluble and insoluble cell lysates. However, in spite of the solubilizing power shown by SUMO, Weber et al. have recently reported on a purification protocol to obtain ^{15}N and ^{15}N, ^{13}C Aβ42 starting from SUMO-Aβ42 accumulated in inclusion bodies, and thus used denaturing buffers for the lysis and initial purification steps [29].

Working under denaturing conditions makes proteins more vulnerable to chemical modifications, leading, for example, to the oxidation of the methionine side chain to methionine sulfoxide. Aβ42 has a methionine in position 35 (Met-35). Indeed, there is much controversy in the literature regarding the role of Met-35(ox)Aβ42 in AD [31,32]. Therefore, in any Aβ42 purification strategy, it is critical to separate Met-35(ox)Aβ42 from Aβ42. Since these molecules differ in hydrophobicity, the most efficient method to separate them relies on the use of costly and time-consuming preparative reversed phase high performance liquid chromatography (RP-HPLC). Indeed, most Aβ42 purification protocols use this technique during the purification steps [22-28] [29,30].

In this paper, and inspired by the work of Satakarni and Curtis [27], we exploit the capacity of SUMO to solubilize Aβ42 when these two molecules are fused and thus establish a new and efficient purification protocol. Rather than using highly denaturing

conditions, our approach requires mild conditions for the lysis and initial immobilized metal affinity chromatography (IMAC) purification steps, which are followed by a desalting step, cleavage of Aβ42 from SUMO using the efficient SUMO protease (Ulp1), and two size exclusion chromatography (SEC) steps. Using this approach, no oxidation of Met-35 was observed and therefore RP-HPLC was not required. This strategy afforded 6 mg of [U-^{15}N] Aβ42 and 2 mg [U-^{2}H,^{13}C,^{15}N] Aβ42 per L of culture. The chemical purity of the final product was assessed by analytical RP-HPLC, and the isotope incorporation of the labeled samples was determined by high-resolution mass spectrometry (HRMS). Both techniques indicated that the strategy provides peptides of sufficiently high purity and isotope enrichment to support NMR-based structural studies.

MATERIALS AND METHODS

Reagents

The following labeled compounds were used: $^{15}NH_4Cl$ (99%, Cambridge Isotope Laboratories), D-glucose-$^{13}C_6$, 1,2,3,4,5,6,6-d_7 (97-98% D, 99% ^{13}C, Euriso-top) and D_2O (99.9%, Euriso-top). All reagents were purchased from Sigma-Aldrich unless otherwise specified.

Cloning

The DNA encoding Aβ42 was synthesized by PCR with KOD polymerase (Novagen) methods and following the modular approach previously described [20], but with the following primers to add the 15 bp on each side for the In-Fusion method:

Fw 5'-GCGAACAGATCGGTGGTGATGCGGAGTTCCGTCATGATTCAG-3' and

Rev 5'-ATGGTCTAGAAAGCTTTATTACGCTATGACAACACCACCCACCATGAGTCCAATGATGGCACC-3'

The amplified fragment was further purified and cloned into a pOPINS vector [33] previously cut with KpnI and HindIII (New England Biolabs) restriction enzymes using the In-Fusion cloning method (Clontech). This yielded a plasmid for expression of Aβ42 in the *Escherichia coli* (*E. coli*) cytoplasm as a fusion protein with an N-terminal hexahistidine SUMO affinity tag (MGSSHHHHHHGSDSE

VNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGK EMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGG↓-Aβ42), where ↓ represents the SUMO protease cleavage site).

Stock solutions

[U-^{15}N] Aβ42 was produced using auto-induction. The following stock solutions were required. The 20x ^{15}N-NPS and 50x 5052 solutions were prepared as previously described [34]. Briefly, the 20x ^{15}N-NPS solution contained 142 g Na_2HPO_4, 136 g KH_2PO_4, 50 g $^{15}NH_4Cl$ and 14.2 g Na_2SO_4 per L and the 50x 5052 solution contained 250 g glycerol, 25 g D-glucose and 100 g α-lactose per L. The 500x trace metal solution was also prepared based on previous descriptions with small adjustments [35]. Briefly, 1 L of 500x trace metal solution contained 8 mL 5 M HCl, 5 g $FeCl_2 \cdot 4H_2O$, 184 mg $CaCl_2 \cdot 2H_2O$, 64 mg H_3BO_3, 18 mg $CoCl_2 \cdot 6H_2O$, 4 mg $CuCl_2 \cdot 2H_2O$, 340 mg $ZnCl_2$, 605 mg $Na_2MoO_4 \cdot 2H_2O$, and 40 mg $MnCl_2 \cdot 4H_2O$. These three solutions were heat-sterilized and stored at room temperature until use. The 100x vitamin solution was prepared by dissolving 50 mg thiamine hydrochloride, 10 mg D-biotin, 10 mg choline chloride, 10 mg folic acid, 10 mg niacin, 10 mg pantothenic acid, 10 mg pyridoxal, and 1 mg riboflavin in 100 mL MilliQ water. This solution was sterilized using a 0.2-µm

filter, wrapped in aluminum foil, and stored at -20ºC until use.

[U-^{2}H,^{13}C,^{15}N] Aβ42 was produced using M9 minimal media. Various stock solutions were required: the 30x salt solution was prepared as a 50-mL aliquot of D_2O containing 10.2 g anhydrous Na_2HPO_4, 4.5 g anhydrous KH_2PO_4, 0.75 g NaCl and 0.37 g $MgSO_4$; the 100x vitamin solution and the 500x trace metal solution were prepared as detailed above using D_2O instead of H_2O.

Media for growth and expression

For the production of [U-^{15}N] Aβ42, ^{15}N-labeled P-5052 medium for auto-induction was prepared from 2 mL of 1 M $MgSO_4$ solution, 50 mL of the 20x ^{15}N-NPS, 20 mL of the 50x 5052, 2 mL of the 500x trace metal solution and 916 mL heat-sterilized MilliQ water. The resulting medium was heat-sterilized and subsequently 10 mL of the previously filtered 100x vitamin solution was added to it.

For the production of [U-^{2}H,^{13}C,^{15}N] Aβ42, M9 minimal media was prepared from 33.3 mL of the 30x salt solution, 10 mL of the 100x vitamin solution, 1 g NH_4Cl, and 4 g D-glucose and brought to 1 L with 50% H_2O/50% D_2O or 100% D_2O for the pre-cultures. For the final culture, 1 g $^{15}NH_4Cl$ and 2 g D-glucose-$^{13}C_6$, 1,2,3,4,5,6,6-d_7 were used in 100% D_2O. The resulting solutions were sterilized by filtering them through a 0.2-μm filter. Afterwards, 2 mL of heat-sterilized 500x trace metal solution, freeze-dried and resuspended in D_2O, were added to each of them.

Protein expression

Rosetta (DE3) pLysS *E. coli* cells (Novagen) were transformed with the expression vector and grown overnight at 37°C on Luria Bertani (LB)-agar plates containing 1% glucose. All cell cultures were also supplemented with 35 μg/mL chloramphenicol and 50 μg/mL kanamycin.

For [U-^{15}N] Aβ42 expression, the following auto-induction procedure was applied,

adapted from a previously described protocol [34]. Single colonies were picked and grown overnight in 2 x 12.5 mL LB, 1% glucose. The pre-cultures were centrifuged at 3,000 *g* for 10 min at 25ºC. Each pellet was transferred to 0.5 L ^{15}N-labeled P-5052 auto-inducing media with the appropriate antibiotics using a 3-L Erlenmeyer flask. The resulting cultures were grown for 6 h at 37ºC and 180 rpm. The temperature was then lowered to 25ºC, and the culture was incubated 22 h more at 180 rpm. The cells were then harvested by centrifugation at 9,000 *g* for 15 min at 4ºC and then frozen at -80°C.

For [U-^{2}H,^{13}C,^{15}N] Aβ42 expression, single colonies were picked and grown overnight in 4 x 3 mL LB, 1% glucose. The LB pre-cultures were centrifuged at 3,000 *g* for 10 min at 25ºC. The pellets were then transferred to 120 mL M9 medium, containing 50% H_2O/50 % D_2O and the corresponding antibiotics. The 50% D_2O pre-culture was grown for 7 h at 37ºC and centrifuged at 2,000 *g* for 20 min. The pellet was re-suspended and inoculated in 240 mL M9 medium in 100% D_2O. The 100% D_2O pre-culture was grown overnight. The next morning, the pre-culture was centrifuged at 2,000 *g* for 20 min, and the pellet was re-suspended and inoculated in 1 L M9 100% D_2O medium, containing 1 g/L $^{15}NH_4Cl$ and 2 g/L D-glucose-$^{13}C_6$, 1,2,3,4,5,6,6-d_7 glucose. The culture was grown at 37°C and 180 rpm and induced at OD_{600} ~0.8 by the addition of IPTG to a final concentration of 0.5 mM. After further growth for 3 h, the cells were harvested by centrifugation at 9,000 *g* for 15 min at 4ºC and then frozen at -80°C.

Protein isolation from the soluble and insoluble fractions

20 mg of cell pellet was resuspended in 1 mL buffer A (300 mM NaCl, 50 mM sodium phosphate, 20 mM imidazole, 1% Tween-20 and 1 mM tris(2-carboxyethyl)phosphine (TCEP) pH 8.0), supplemented with half a pill of ethylenediaminetetraacetic acid (EDTA)-free Complete protease inhibitor (Roche) and

1 mg DNAse (Roche). The resuspended cells were lysed using a 3-mm tapered microtip sonicator (VCX 750 Ultrasonic Processor, Sonics) for 10 min in an ice water bath. The cell extract was diluted with buffer A and then centrifuged at 15,000 *g* for 10 min at 4ºC, and the supernatant corresponding to the soluble fraction was kept. The pellet was resolubilized in buffer A containing 8 M urea using the same volume of the previously collected supernatant. The resulting solution was then sonicated in a bath (Bransonic® Ultrasonic cleaner B1510E) for 5 min and then centrifuged at 15,000 *g* for 10 min at 4ºC. The supernatant obtained, corresponding to the insoluble fraction, as well as that corresponding to the soluble fraction, were analyzed by Western Blot (WB).

Protein purification

The cell pellet was resuspended in 6 mL buffer A per g of cells, supplemented with 1 EDTA-free Complete protease inhibitor pill (Roche) and 1 spatula of DNAse (Roche) per 50 mL of buffer. The resuspended cells were lysed using a cell disruptor (Constant Systems Ltd. U.K.) operating at 20,000 psi. The cell extract was then centrifuged at 30,000 *g* for 30 min at 4ºC, the supernatant filtered using a 0.45 μm and subsequently purified by IMAC. The supernatant was loaded at 1 mL/min onto a HisTrap HP 5-mL Ni column (GE Healthcare), which was previously equilibrated with 5 column volumes of buffer A. After the loading step, the resin was washed with buffer B (300 mM NaCl, 50 mM sodium phosphate, 40 mM imidazole, 0.05% Tween-20 and 1 mM TCEP pH 8.0) for 10-15 column volumes, until UV absorbance was stable. The fusion protein was eluted at 2-5 mL/min using the following 3-step elution method: (a) a 15 mL linear gradient from 0 to 15% of buffer C (300 mM NaCl, 50 mM sodium phosphate, 500 mM imidazole, 0.05% Tween-20 and 1 mM TCEP pH 8.0), followed by (b) a 20 mL isocratic step at 15% buffer C and (c) a second isocratic step at 100% buffer C until UV absorbance was stable. IMAC fractions were analyzed by sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE), and those containing the fusion protein were pooled in batches of 10 mL. Subsequently, buffer was exchanged using a HiPrep 26/10 desalting column (GE Healthcare) equilibrated with 50 mM ammonium carbonate and 1 mM TCEP. Afterwards, the concentration and purity of protein was determined by nanodrop and RP-HPLC, respectively. Afterwards, to cleave Aβ42 from the SUMO fusion tag, samples were incubated overnight at 4ºC with SUMO protease (Ulp1) [33] in a 1:50 protease:protein ratio. The concentration of Aβ42 peptide after the cleavage was determined by RP-HPLC analysis. Subsequently, aliquots containing 3.75 mg Aβ42 were prepared and freeze-dried. Each of these aliquots was solubilized with 6.8 M guanidinium thiocyanate (GdnSCN) to 2.5 mg Aβ42/mL and sonicated for 5 min in an ice bath. Afterwards, the sample was further diluted with MilliQ water to 1.5 mg Aβ42/mL and 4 M GdnSCN, and centrifuged at 10,000 g for 6 min at 4ºC. Finally, 2.5 mL of the 1.5 mg Aβ42/mL was injected into a HiLoad Superdex 30 prep grade column (GE Healthcare), previously equilibrated with 50 mM ammonium carbonate, and eluted at 4ºC at a flow rate of 1 mL/min. The peaks corresponding to SUMO and monomeric Aβ42 were collected separately and their purity and concentration were determined by RP-HPLC. Both pools were freeze-dried, and the Aβ42 pool was subjected to the same GdnSCN solubilization protocol and SEC fractionation, as described. Pure Aβ42 was obtained after the two SEC steps. It was then aliquoted in the desired amounts, freeze-dried, and kept at -20ºC until use.

SDS-PAGE and WB

Samples for Fig. 1 were analyzed as follows: 10 µL of 3X sample buffer (SB) was added to 20 µL of supernatant and reconstituted pellet, boiled for 5 min at 95ºC. 20 µL of each of the resulting samples was electrophoresed using Mini-protean tetracell® system (Bio-Rad) in 0.75 mm-thick SDS-PAGE containing 15% acrylamide. Gels were

run at 120 V and stained with Coomassie Blue. For WB protein samples, SDS-PAGE gels were transferred to a 0.22 μm nitrocellulose (GE Healthcare) at 100 V for 2 h at 4ºC. Next, the membranes were washed in Tris-buffered saline and 0.1% Tween 20 (TBST), blocked in 5% (w/v) non-fat dried milk overnight at 4ºC, and incubated with primary antibody 6E10 (Covance) in 5% (w/v) non-fat dried milk overnight at 4ºC. Blots were treated with secondary horseradish peroxidase-conjugated mouse secondary antibody (GE Healthcare) using the chemiluminescence Immobilon ECL detection system (Millipore) and exposed to X-ray films (Super RX Medical X-Ray, Fujifilm), which were developed using the Hyper processor automatic film developer (Amersham Pharmacia Biotech).

Samples for Fig. 3A were analyzed as follows: 5μL of NuPAGE® LDS Sample Buffer (4X) were added to 15μL of both uncleaved and cleaved samples, and boiled for 2 min at 95ºC. 15μL of each of the resulting samples were electrophoresed using NuPAGE® Novex™ 10% Bis-Tris Midi Protein SDS-PAGE gels using NuPAGE® 2-(*N*-morpholino)ethanesulfonic acid (MES) SDS running buffer supplemented with NuPAGE® antioxidant (all NuPAGE® products are from Thermo Fisher Scientific). Gels were run at 200 V and stained with Coomassie Blue.

RP-HPLC

10-50 μL of sample obtained after desalting, Aβ42 cleaved from SUMO-Aβ42, and different fractions obtained after either of the two SEC steps were injected into a RP-HPLC (Waters Alliance 2695 equipped with 2998 photodiode array detector). Samples were analyzed using a Symmetry 300 C_4 column (4.6 × 150 mm, 5 μm, 300 Å; Waters) at a flow rate of 1 mL/min and a linear gradient from 0 to 60% B in 15 min (A = 0.045% trifluoroacetic acid (TFA) in water, and B = 0.036% TFA in acetonitrile) at 60ºC. The concentration of monomeric Aβ42 was determined by RP-HPLC using the

above-described conditions. A calibration curve was generated on the basis of an Aβ42 solution that had previously been quantified by amino acid analysis.

Mass spectrometry

The retention time of Met-35(ox)Aβ42 relative to that of Aβ42 in RP-HPLC was determined by analyzing a mixture of them by liquid chromatography coupled to MS (LC-MS). The sample was examined using a BioSuite pPhenyl 1000 RPC analytical column (10 μm, 2 × 75 mm; Waters) at a flow rate of 100 μl/min comprising a linear gradient running from 5 to 80% B in 60 min (A= 0.1% formic acid (FA) in water, B= 0.1% FA in acetonitrile). The column outlet was directly connected to an Advion TriVersa NanoMate, which was used as a splitter and as the nanospray source (250 nl/min) of an LTQ-FT Ultra mass spectrometer (Thermo Scientific). Positive polarity was used with a spray voltage in the NanoMate source set to 1.7 kV. The capillary voltage, capillary temperature, and tube lens on the LTQ-FT were tuned to 44 V, 200°C, and 100 V, respectively.

The molecular weight of the labeled Aβ42 samples was determined by LC-MS. Briefly, aliquots of pure [U-^{15}N] Aβ42 and [U-^{2}H,^{13}C,^{15}N] Aβ42 obtained after SEC fractionation were diluted 1/10 with H_2O and 1% FA. 10 μL of the resulting samples (20 pmols in column) were used for LC-MS analysis. Samples were injected to a BioSuite pPhenyl 1000 column (10 μm RPC 2.0 mm x 75 mm; Waters) at a flow rate of 100 μl/min using an Acquity UPLC system (Waters) provided with a Binary Solvent Manager and an autosampler. Peptides were eluted using a linear gradient from 5% to 80% B in 60 min (A = 0.1% FA in water, B = 0.1% FA in acetonitrile). The column outlet was directly introduced into the electrospray ionization (ESI) source of an LCT-Premier XE mass spectrometer (Waters). Capillary voltage, cone voltage, cone gas flow and desolvation gas flow were set to 3000 V, 100 V, 50 L/h and 600 L/h, respectively.

Desolvation and source temperatures were set to 350ºC and 120ºC, respectively. The mass spectrometer acquired full MS scans (400-4000 m/z) working in positive polarity and TOF-V mode. Data was acquired with MassLynx software, V4.1. (Waters). MS spectra corresponding to the chromatographic peak were summed. Charged protein species in the resulting spectrum were deconvoluted to their zero charged average masses using the integrated MaxEnt1 (maximum entropy) algorithm.

NMR spectroscopy

2D ^{1}H-^{15}N Heteronuclear Single Quantum Coherence (HSQC) spectra were recorded on 400 μM [U-^{15}N] SUMO dissolved in 50 mM sodium phosphate, pH 7.4, 90% H_2O/10% D_2O at 25ºC and on 150 μM [U-^{15}N] Aβ42 dissolved in 95% dimethylsulfoxide-d_6 (DMSO-d_6) (Euriso-top), 5% D_2O at pH* 4.6 (adjusted with dichloroacetic acid-d_2) and 25ºC on a Bruker 800 and 600 MHz spectrometer, respectively, equipped with a cryogenic probe head. All data were processed and analyzed using TopSpin software from Bruker.

RESULTS AND DISCUSSION

SUMO solubilizes Aβ42 when the two molecules are fused

The expression of [U-^{15}N] SUMO-Aβ42 fusion protein was accomplished through two steps, including an LB pre-culture and a final culture in ^{15}N-labeled P-5052 auto-inducing media. Approximately 7-10 g (wet weight) of cells was obtained per L of culture. The use of auto-induction to produce [U-^{2}H,^{13}C,^{15}N] Aβ42 required lactose-d_{14} and glycerol-d_5 as metabolic precursors. The cost of these reagents is prohibitively expensive so we opted for IPTG induction, which required the less expensive D-glucose-$^{13}C_6$, 1,2,3,4,5,6,6-d_7. The expression of [U-^{2}H,^{13}C,^{15}N] SUMO-Aβ42 required four steps to adapt the cells to D_2O media. These included a first pre-culture in LB, a second pre-culture in M9 minimal media containing 50% H_2O/50% D_2O, a third pre-

culture in 100% D_2O, and a final culture in 100% D_2O containing 1 g/L $^{15}NH_4Cl$ and 2 g/L D-glucose-$^{13}C_6$, 1,2,3,4,5,6,6-d_7 glucose. We obtained approximately 3-5 g (wet weight) of cells per L of culture.

Satakarni and Curtis previously reported on the capacity of SUMO to solubilize Aβ42 when fused to it [27]. To examine this property under our expression conditions, we analyzed the supernatant corresponding to the soluble fraction, as well as the insoluble pellet resuspended in the same volume as that of the previously collected supernatant, by WB using 6E10, an antibody that recognizes residues 3 to 8 of the Aβ sequence (Fig. 1). Our results indicated that SUMO-Aβ42 was predominantly expressed in the soluble fraction. This finding contrasts with that described by Satakarni and Curtis, who reported obtaining the same volumetric productivity of SUMO-Aβ42 after IMAC purification of the soluble supernatant and the insoluble pellet [27]. These differences can be explained by the fact that we produced SUMO-Aβ42 using minimal media while they used richer LB media. Given that protein expression yields are usually lower in the former media and that aggregation is highly dependent on protein concentration, it is likely that the concentrations of SUMO-Aβ42 produced in minimal media are not high enough to promote extensive aggregation and accumulation of the fusion protein in inclusion bodies. This explanation is supported by a report describing the expression and purification of the 50-amino acid protein medin [36]. Similar to Aβ42, medin forms amyloid fibrils, which are the major protein component of Aortic Medial Amyloid. In the aforementioned report, the authors describe the successful expression and purification of [U-^{13}C,^{15}N] medin fused to His_6-SUMO. As in the case of SUMO-Aβ42, the SUMO-medin construct expressed in minimal media gives rise to a high level expression in the soluble fraction (90%). Notably, the final volumetric yield

of [U-^{13}C,^{15}N] medin obtained from the soluble fraction is the same as the one we report here for [U-^{15}N] Aβ42.

Oxidation of methionine 35 is not observed during purification

Next, to design the purification strategy, we took into account various considerations. First, under the minimal media, SUMO-Aβ42 is predominantly expressed in the soluble fraction. Second, the use of highly denaturing conditions makes proteins vulnerable to chemical modifications, including methionine oxidation. Third, the most widely reported undesired side product during Aβ purification is oxidized Met-35. Fourth, Met-35(ox)Aβ42 and Aβ42 differ mainly in terms of hydrophobicity, so the most efficient method to separate them is via costly and time-consuming preparative RP-HPLC. Given these considerations, we chose to focus on the purification of the soluble SUMO-Aβ42 fraction of the cell lysates, which allowed us to design a purification protocol that did not require the use of highly denaturing conditions in the stages of the purification when Aβ42 was still fused to SUMO. In doing so, our aim was to avoid Met-35 oxidation and thus the use of RP-HPLC. To ensure that Met-35 was not oxidized during the different stages of the Aβ42 purification, we used analytical RP-HPLC. Analysis of a mixture of Met-35(ox)Aβ42 and Aβ42 by analytical RP-HPLC and LC-MS revealed that Met-35(ox)Aβ42 elutes earlier than the reduced form (Fig. 2). This result supports the capacity of this method to detect the presence of Met-35(ox)Aβ42 in Aβ42 samples.

The first step of the purification relied on the $(His)_6$ tag present at the N-terminal of SUMO, which allowed a simple purification of SUMO-Aβ42 by IMAC using non-denaturing, degassed buffers in the presence of TCEP. Next, to cleave the SUMO-Aβ42 construct, the IMAC buffer was replaced by 50 mM ammonium carbonate and 1 mM TCEP at pH 9.0. This buffer allowed us to resolve the following two issues, namely to cleave Aβ42 from SUMO under basic pH—conditions reported to slow down Aβ42

aggregation [37], and to subsequently lyophilize the sample, yielding a lyophilized powder free of insoluble salts. When present, the latter can promote Aβ42 aggregation upon subsequent resuspension. Under the 50 mM ammonium carbonate and 1 mM TCEP buffer, the cleavage of SUMO-Aβ42 using SUMO protease (Ulp1) [33] was highly effective, leading to SUMO and Aβ42, as analyzed by SDS-PAGE (Fig. 3A) and RP-HPLC (Fig. 3C). The two bands detected in the SDS-PAGE analysis of SUMO-Aβ42 (Fig. 3A, lane 1) were assigned to two populations of SUMO-Aβ42 caused by either an incomplete denaturation of SUMO-Aβ42 prior to sample separation or subsequent partial renaturation during the separation. Products of incomplete translation during synthesis, and/or partial degradation during lysis, and/or sample processing of both SUMO and Aβ42 moieties were excluded as these would be evident in both the SDS-PAGE gels and the RP-HPLC profiles of the downstream SUMO protease-cleaved products (Fig. 3A and 3C).

Since the molecular weight of SUMO (12.4 kDa) is almost three-fold larger than that of Aβ42 (4.5 kDa), we proceeded with their separation by means of SEC (Fig. 3B). To obtain the best yield for monomeric Aβ42, it was critical to ensure the absence of Aβ42 aggregates at the time of injection. To this end, we resuspended the lyophilized powder obtained after SUMO protease cleavage at 2.5 mg/mL Aβ42 in 6.8 M GdnSCN. This strong chaotropic reagent is able to solubilize plaque cores from the brains of AD patients [2]. Therefore, performing this step ensured complete solubilization of the sample containing Aβ42 and SUMO. To minimize Aβ42 aggregation during elution from the column, SEC was performed at 4ºC. At this temperature, 6.8 M GdnSCN precipitates. To avoid this, the sample containing Aβ42 and SUMO was diluted to 4 M GdnSCN and 1.5 mg/mL Aβ42 before subjecting it into the SEC apparatus. To prevent the oxidation of Met-35, SEC was carried out using carefully degassed 50 mM

ammonium carbonate at pH 9.0. Again, we chose this buffer because its basic pH has been reported to minimize Aβ42 aggregation [37] and because its volatility allowed subsequent lyophilization of the fractions containing Aβ42 without leaving any insoluble salts in the lyophilized powder. To completely separate Aβ42 from SUMO, a second SEC purification was required (Fig. 3B). After this second SEC, the purity of the peptide and the absence of Met-35(ox)Aβ42 (compare to Fig. 2A) was confirmed by RP-HPLC (Fig. 3C). This expression and purification strategy allowed us to obtain 6 mg [U-^{15}N] Aβ42 and 2 mg [U-^{2}H,^{13}C,^{15}N] Aβ42 per L of culture. The purity of labeled peptides was determined by RP-HPLC and found to be > 98% (Fig. 4A). Moreover, their identity and label incorporation was determined by HRMS analysis (Fig. 4B) and reported in Table 1.

The labeled Aβ42 is amenable to NMR-based structural studies

Notably, apart from obtaining pure [U-^{15}N] Aβ42 and [U-^{2}H,^{13}C,^{15}N] Aβ42, the expression and purification strategy simultaneously allowed us to obtain pure [U-^{15}N] SUMO and [U-^{2}H,^{13}C,^{15}N] SUMO after the first SEC purification. Soluble and well-folded proteins are useful as standards to set up NMR experiments and are usually purchased from commercial sources. For example, 5 mg [U-^{15}N] ubiquitin costs more than 1,000 € and 550 µL 0.5 mM [U-^{2}H, ^{13}C, ^{15}N] maltose binding protein more than 6,000 €. Since SUMO is a soluble, well-folded protein, the [U-^{15}N] SUMO and [U-^{2}H,^{13}C,^{15}N] SUMO obtained from this strategy could be useful for setting up NMR experiments (Fig. 5A).

[U-^{15}N] Aβ42 and [U-^{2}H,^{13}C,^{15}N] Aβ42 peptides will be useful to characterize Aβ42 aggregation by means of NMR spectroscopy. As an example of a possible application, we measured ^{1}H-^{15}N HSQC NMR spectrum of monomeric Aβ42, obtained after dissolving [U-^{15}N] Aβ42 in 95% DMSO-d_6, 5% D_2O at pH* 4.6 (Fig. 5B). This buffer

has been reported to disaggregate Aβ into its constituent monomers while preserving hydrogen deuterium exchange (HDX) information [6,38-42]. HDX experiments are among the techniques most used in the literature to obtain structural information about amyloid fibrils [6,38,39,41] and also about aggregates formed during fibril formation [42]. Under these conditions, we observed at least 37-38 N-H cross-peaks corresponding to the amides of the Aβ42 backbone. Among them, six peaks appeared at the characteristic chemical shifts of glycines, consistent with the six glycines present in the Aβ42 sequence. Moreover, we expect that the [U-^{2}H,^{13}C,^{15}N] Aβ42 sample will pave the way for NMR studies of Aβ42 in the form of high molecular weight complexes, including those formed in a membrane environment.

Conclusions

We have produced [U-^{15}N] Aβ42 and [U-^{2}H,^{13}C,^{15}N] Aβ42 using a novel and efficient expression and purification protocol (Figure 6). Our purification protocol involves an IMAC, a desalting step, and two SEC purifications. The ease of the purification strategy is based on the expression of Aβ42 fused to SUMO, which prevents Aβ42 aggregation. This strategy circumvents the requirement of denaturing conditions in the initial stages of purification, thus preventing the formation of Met-35(ox)Aβ42 and the need for costly and time-consuming preparative RP-HPLC purification. Consequently, recombinant expression of labeled Aβ42 is now accessible to many protein laboratories, including those that do not have access to preparative-RP-HPLC. Indeed, all the purification steps reported can be performed with the generally accessible fast protein liquid chromatography system (FPLC). Indeed, the cost of the reagents to produce 6 mg [U-^{15}N] Aβ42 using this protocol is negligible; in this regard, we calculated it to be less than €15-20 per mg, thus being much cheaper than commercially available recombinant [U-^{15}N] Aβ42, which is sold at $695 per mg

(https://www.rpeptide.com/products/labeled-peptides-and-proteins/beta-amyloid-labeled-peptides-recombinant/). The SUMO protease used in this study was prepared in-house, the methodology is very simple and can be performed by any lab wishing to produce large quantities of the enzyme to reduce costs [43]. SUMO protease is also available commercially as 100,000 U for €600 (less than one cent per unit) and, as we estimate that 5,000-10,000 U should be sufficient to cut 25mg of the SUMO-Aß42 fusion overnight, this digestion step should not therefore be seen as prohibitively expensive. Additionally, even in the case that a laboratory would need to invest in the required FPLC columns—we assume possession of an FPLC system—the cost of the columns would be recovered in the first Aβ42 purification, and the columns themselves, especially Superdex 30, can be used for many other purifications. Therefore, the expression and purification protocol described herein offers an alternative inexpensive approach to previously described methods to obtain [U-^{15}N] Aβ42 [29,30] and the first method to achieve [U-^{2}H,^{13}C,^{15}N] Aβ42.

Acknowledgements

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Abbreviations

Alzheimer's disease (AD); amyloid-beta peptide (Aβ); amyloid-beta peptide comprising 42 residues (Aβ42); amyloid precursor protein (APP); dimethylsulfoxide-d_6 (DMSO-d_6); ethylenediamnetetraacetic acid (EDTA); familial Alzheimer's disease

(fAD); formic acid (FA); fast protein liquid chromatography system (FPLC); guanidinium thiocyanate (GdnSCN), heteronuclear single quantum coherence (HSQC); high-resolution mass spectrometry (HRMS); hydrogen deuterium exchange (HDX); immobilized metal affinity chromatography (IMAC); liquid chromatography coupled to MS (LC-MS); Luria Bertani (LB); nano electrospray (nanoESI); nuclear magnetic resonance spectroscopy (NMR); oxidation of the Met-35 side chain of Aβ42 to methionine sulfoxide (Met-35(ox)Aβ42); reversed phase high performance liquid chromatography (RP-HPLC); sample buffer (SB); size exclusion chromatography (SEC); small Ubiquitin-like Modifier (SUMO); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); trifluoroacetic acid (TFA); tris-buffered saline, 0.1% tween 20 (TBST); tris(2-carboxyethyl)phosphine (TCEP); Western blot (WB).

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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Tables:

Medium	Theoretical Mass (mono-isotopic)	Observed Mass	Theoretical number of isotopic substitutions	Observed number of isotopic substitutions	Isotopic incorporation (%)
P-5052 (^{15}N)	4566.1	4566.1	54.8	54.8	100.0
$M9_{D_2O}$ (^{2}H,^{13}C,^{15}N)	5014.3	5004.6 5002.9 4996.7	503.043	493.3 491.6 485.4	98.1 97.7 96.5

Table 1. Isotopic incorporation percentages. The theoretical monoisotopic masses were calculated using the Molecular Mass Calculator tool from the Biological Magnetic Resonance Data Bank website (http://www.bmrb.wisc.edu/metabolomics/mol_mass.php?formula=C210H321N56O61S2&subaction=Natural+Composition&updateIsoComp=1). In the case of [U-^{15}N] Aβ42, they were obtained assuming 100% substitution of ^{14}N isotopes for ^{15}N and in the case of [U-^{2}H,^{13}C,^{15}N] Aβ42 assuming 100% substitution of ^{12}C, ^{14}N and non-labile ^{1}H isotopes for ^{13}C, ^{15}N and ^{2}H. The theoretical and observed number of isotopic substitutions account for the difference between the theoretical mono-isotopic mass and the theoretical or observed mass of the different isotopically labeled Aβ42 peptides. Therefore, the theoretical and observed number of isotopic substitutions indicate the increase in mass due to the theoretical and experimental incorporation of ^{15}N and ^{2}H, ^{13}C or ^{15}N isotopes in [U-^{15}N] Aβ42 and [U-^{2}H,^{13}C,^{15}N] Aβ42, respectively.

Figure Legends:

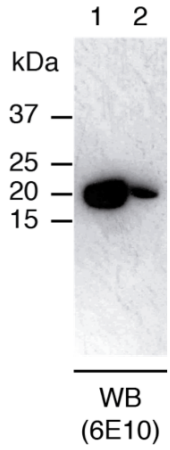

Fig. 1. SUMO-Aβ42 is predominantly expressed in the soluble fraction. WB analysis of the supernatants corresponding to the soluble (lane 1) and insoluble (lane 2) fraction of a crude lysate. Samples were blotted with 6E10 monoclonal antibody, which recognizes residues 3-8 of Aβ.

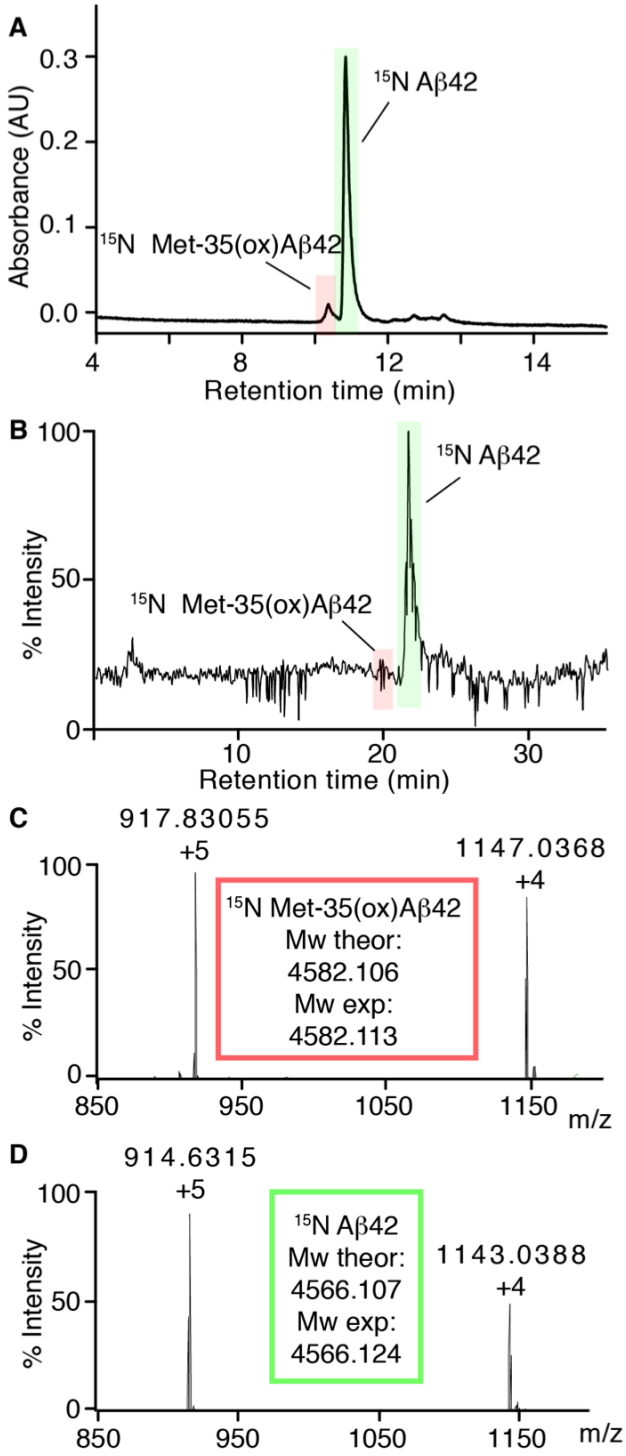

Fig. 2. Characterization of the retention time of Met-35(ox)Aβ42 and Aβ42 on RP-HPLC. (A) RP-HPLC chromatogram obtained for a sample containing Met-35(ox)Aβ42 (highlighted in red) and Aβ42 (highlighted in green). (B) LC-MS chromatogram obtained for the same sample containing Met-35(ox)Aβ42 (highlighted in red) and Aβ42 (highlighted in green). ESI-mass spectrum corresponding to the (C) first and the

(D) second peaks detected by LC-MS, assigned to Met-35(ox)Aβ42 and Aβ42, respectively. Mw theor refers to the theoretical monoisotopic mass and Mw exp to the experimental monoisotopic mass.

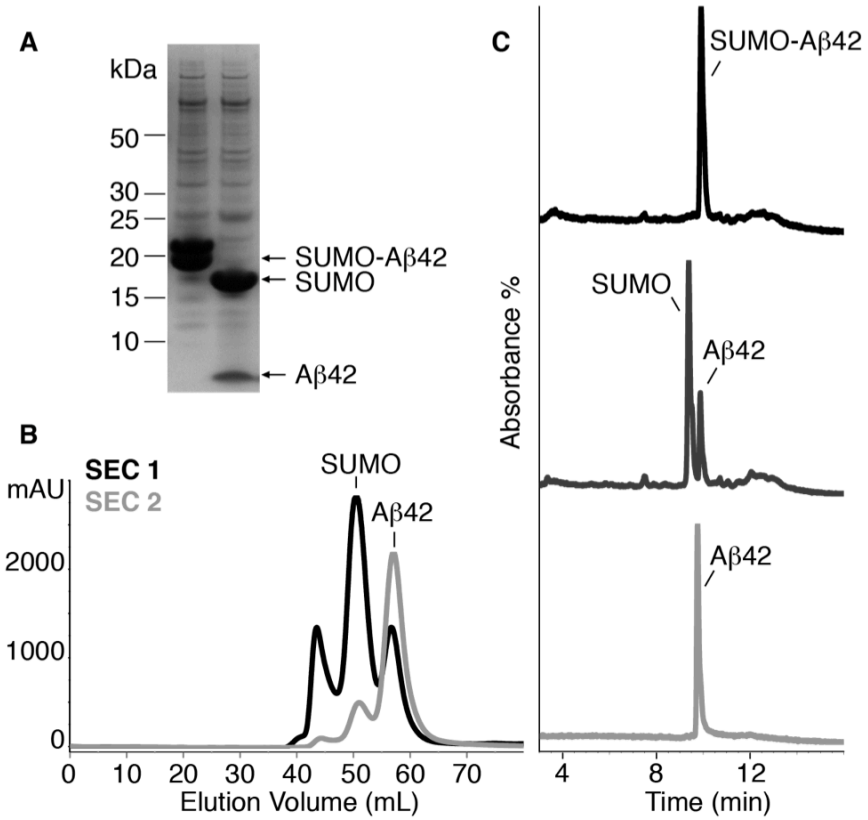

Fig. 3. Aβ42 purification. (A) SDS-PAGE analysis of SUMO-Aβ42 fusion protein before (lane 1) and after (lane 2) cleavage with SUMO protease (Ulp1). (B) SEC chromatograms corresponding to the first (black) and second (light gray) purification step of the SUMO and Aβ42 sample obtained after cleavage. SUMO and Aβ42 eluted at 50.5 mL and 57.1 mL, respectively. (C) RP-HPLC chromatograms at different stages of the Aβ42 purification: SUMO-Aβ42 before (black) and SUMO Aβ42 after cleavage (dark gray), and pure Aβ42 (light gray) obtained after the second SEC. SUMO-Aβ42, SUMO and Aβ42 eluted at 9.9 min, 9.3 min and 9.8 min, respectively.

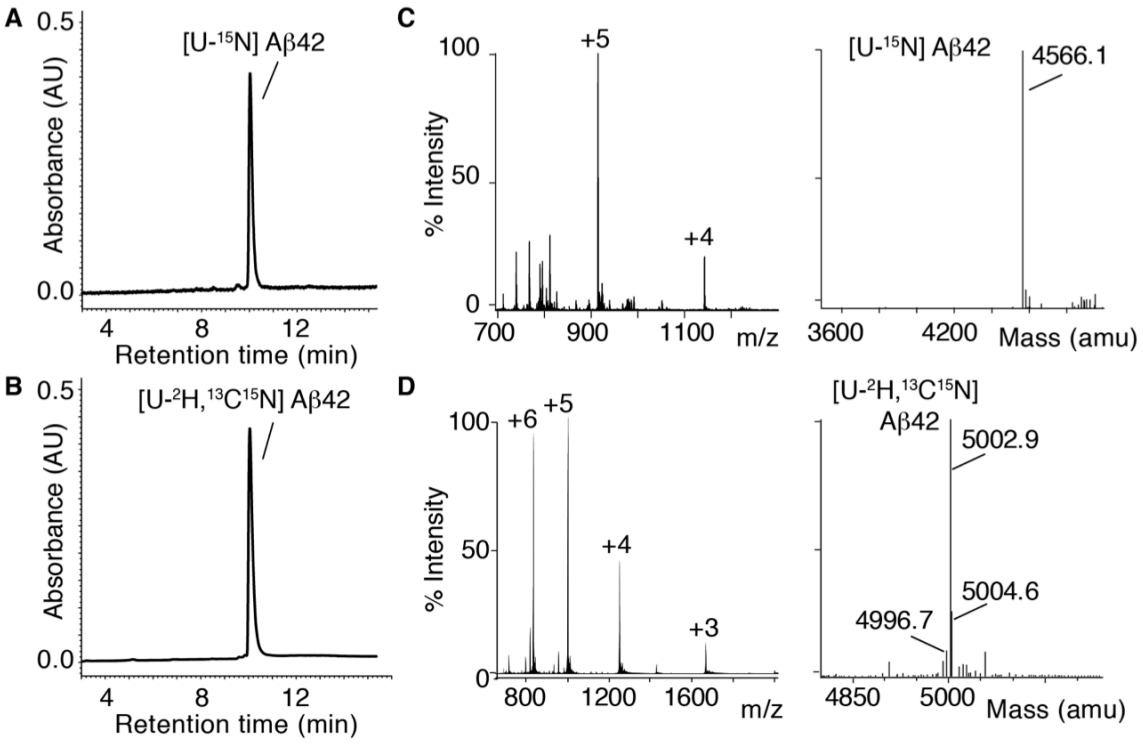

Fig. 4. Purity and identity of [U-^{15}N] Aβ42 and [U-^{2}H,^{13}C,^{15}N] Aβ42. RP-HPLC analysis of (A) [U-^{15}N] Aβ42 and (B) [U-^{2}H,^{13}C,^{15}N] Aβ42. HRMS analysis of (C) [U-^{15}N] Aβ42 and (D) [U-^{2}H,^{13}C,^{15}N] Aβ42. ESI-mass spectrum (right) and deconvoluted mass spectrum showing the mass of the peptides (left).

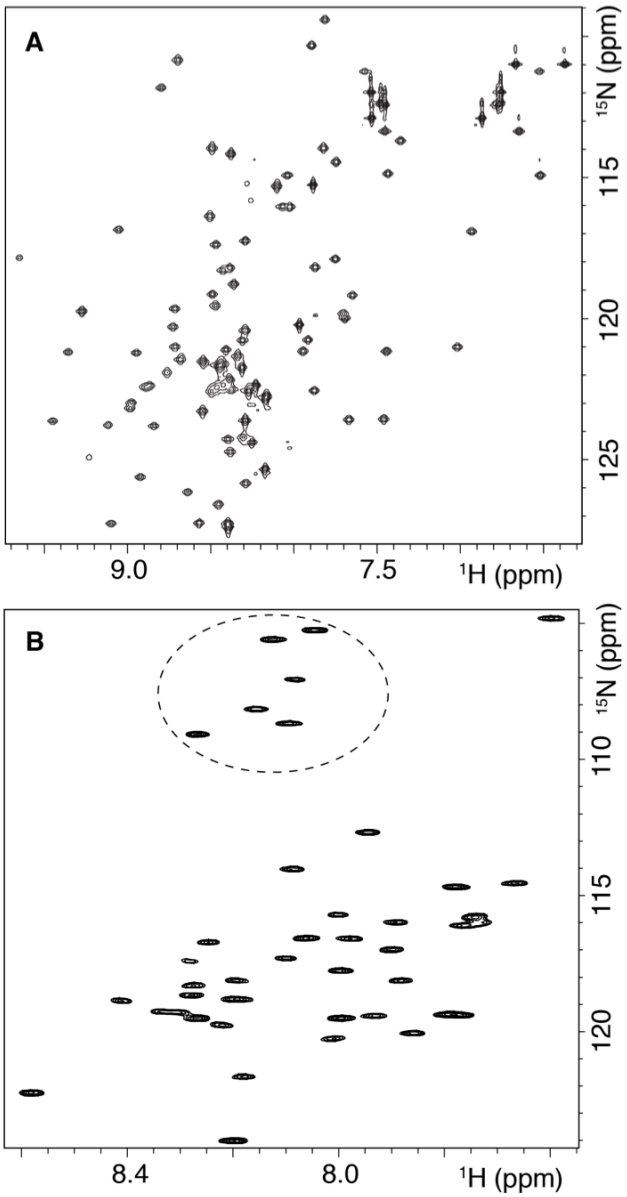

Fig. 5. Labeled SUMO and Aβ42 are suitable for NMR studies. (A) ^{1}H-^{15}N HSQC NMR spectra of 400 μM [U-^{15}N] SUMO in 50 mM sodium phosphate, pH 7.4, 90% H_2O/10% D_2O and (B) 150 μM [U-^{15}N] Aβ42 in 100% DMSO-d_6, 5% H_2O at pH* 4.6. The dotted circle indicates the position of the peaks appearing in the region characteristic of the glycine residues.

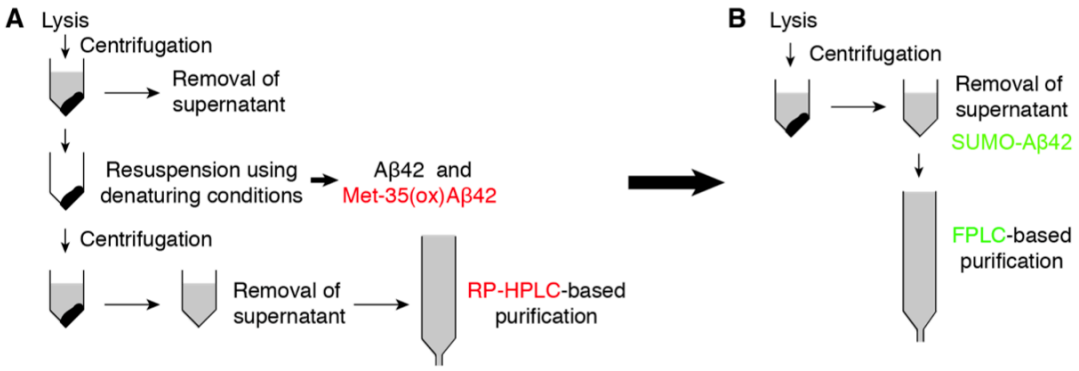

Fig. 6. Schematic diagram for purification of recombinant Aβ42. (A) Previous strategies report accumulation of the fusion-Aβ42 protein in inclusion bodies. This requires the use of denaturing conditions in the initial stages of purification, which leads to the formation of Met-35(ox)Aβ42. To separate Met-35(ox)Aβ42 from Aβ42, costly and time-consuming preparative RP-HPLC purification is required. (B) Proposed strategy based on the expression of Aβ42 fused to SUMO, which prevents Aβ42 aggregation. The solubility of SUMO-Aβ42 circumvents the requirement of denaturing conditions in the initial stages of purification, thereby preventing the formation of Met-35(ox)Aβ42. This protocol allows all the purification steps to be performed with the generally accessible fast protein liquid chromatography system (FPLC).