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# The Effect of Ultrasonication on the Fibrillar/ Oligomeric Structures of $A\beta_{1-42}$ at Different Concentrations

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### Abstract

The number of disease states linked the aberrant regular protein conformations to oligomers and amyloid fibrils. Amyloid beta 1–42 ( $A\beta_{1-42}$ ) peptide is very hydrophobic and quickly forms the  $\beta$ -rich structure and fibrillar protein aggregates in some solutions and buffer conditions. Ultrasonication pulses can disrupt amyloid fibrils to smaller fragments and produce  $A\beta_{1-42}$  peptides of different sizes and oligomers. Herein, we investigated the effects of buffer and ultrasonication on  $A\beta_{1-42}$  structure at low and high concentrations. After ultrasonication, the Western blot results showed that  $A\beta_{1-42}$  fibrils were disaggregated into different sizes. The transmission electron microscopy results indicated  $A\beta_{1-42}$  at low concentration (25  $\mu$ M) in Ham's/F12 phenol red-free culture medium formed short-size fragments and oligomers. In comparison,  $A\beta_{1-42}$  at higher concentration (100  $\mu$ M) formed fibrils that break down into smaller fragments after ultrasonication. However, after regrowth, it formed mature fibrils again. Cell viability assay indicated that  $A\beta_{1-42}$  oligomers formed at a low concentration (25  $\mu$ M) were more toxic to PC12 cells than other forms. In conclusion, by applying ultrasonication pulses and controlling peptide concentration and buffer condition, we can rich  $A\beta_{1-42}$  aggregates with a particular size and molecular structure.

Keywords Amyloid Fibrils · Protein Aggregation · Amyloid  $\beta$  Oligomers · Ultrasonic Pulses · Cell Toxicity

### 1 Introduction

Protein misfolding and aggregation have been introduced as the molecular mechanism of some neurodegenerative diseases. Aggregated conformers are classified based on their size, structure, and solubility. Recently, highly toxic Amyloid beta (A $\beta$ ) oligomers have been introduced as critical determinants in aggregate-induced toxicity [1–3]. A $\beta$  is one of the most critical extracellular aggregates responsible for

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Alzheimer's disease (AD). Therefore,  $A\beta$  peptides with different lengths,  $A\beta_{1-40}$  or  $A\beta_{1-42}$ , have been used in different in vitro studies. They could exist as a monomer or larger soluble entities called  $A\beta$  oligomers with non-fibrillar structures, and eventually insoluble fibrils. The soluble oligomers of  $A\beta_{1-42}$  contain mixed intermolecular parallel and intramolecular antiparallel  $\beta$ -sheets.  $\beta$ -amyloid species have different types of assemblies: dimers, trimers, protofibrils, annular or pore-like oligomers, and spherical (globulomers) [4–6].  $A\beta$  fibrils are unbranched, long supramolecular assemblies containing in-registered parallel  $\beta$ -sheets.

The cytotoxicity of  $A\beta_{1-42}$  is related to the soluble oligomers that, due to their interaction with the lipid bilayer of the cell membrane and pore formation, cause an uncontrolled ion flux [5, 7, 8]. It is because oligomeric  $A\beta_{1-42}$  can form the  $\beta$ -barrel in the cell membrane and permeabilize cells [9–11]. As mentioned above, different aggregated morphologies in neuronal cells exhibit different degrees of toxicity, and soluble oligomers are generally more toxic than amyloid fibrils [2, 12, 13]. Deposition of these aggregates is associated with various degenerative diseases, including AD, prion disease, and dialysis-related amyloidosis [14]. The in vitro protein aggregation mechanism is sensitive to

subtle differences in environmental conditions such as buffer composition, agitation, and protein concentrations. Even tiny changes in protein/ peptide concentration can lead to the formation of specific types of oligomers and intermediates [15–17]. In vitro studies indicated that a low concentration of A $\beta_{1-42}$  controls oligomers' formation and favors a modest conformational conversion into fibrils. In contrast, its higher concentration would promote heterogeneous nucleation and aggregation to form fibrils of different sizes [18, 19].

Ultrasonication as an environmental factor has opposite effects on the formation and breakdown of fibrils [20]. Ultrasonic-dependent fragmentation is a fundamental approach to breaking  $A\beta_{1-42}$  fibrils into smaller aggregates [20, 21]. The main reason is the repeated growth and collapse of bubbles, which are under the control of negative or positive pressures [22–24].

Because of the tendency to aggregation and fibril formation of  $A\beta_{1-42}$  peptide in some conditions and the nature of insoluble particles formed, in the present study, we tried to find a method and situation to dissolve the insoluble aggregates and then reform the monomeric and oligomeric structures for in vitro studies. Thus, we decomposed  $A\beta_{1-42}$  large fibrils in phosphate-NaCl buffer using ultrasonication. After recovering the monomers, oligomeric structures formed through ultrasonication. For this purpose, we modeled low and high concentrations of  $A\beta_{1-42}$  peptide in different buffers using different techniques.

### 2 Materials & Methods

#### 2.1 Materials

 $A\beta_{1-42}$  was bought from Sigma-Aldrich (St. Louis, USA) and GL Biochem. (Beijing, China) as the lyophilized powder. Hexafluoroisopropanol (HFIP) was purchased from Merck Co. (Darmstadt, Germany). Thioflavin T (ThT), thiazolyl blue tetrazolium bromide (MTT), 8-Anilinonaphthalene-1-sulfonate (ANS), and Hoechst were purchased from Sigma-Aldrich Chem. Co. (St. Louis, USA). The PC12 rat pheochromocytoma cell line was purchased from Pasture Institute (Tehran, Iran). Cell culture plates were acquired from SPL (Beijing, China). Primary antibodies (ab201060 and ab224275) and the secondary antibody (ab6721) were bought from Abcam (Abcam Inc., Cambridge, MA). ECL Plus Kit was purchased from Bio-Rad (Bio-Rad Laboratories Inc., Hercules, CA, USA). Alexa Fluor 594 (clone Poly4064) was purchased from BioLegend (San Diego, CA, USA). PVDF was purchased from GE Healthcare (Biosciences, Stockholm, Sweden). All other materials were of analytical grade.

### 2.2 Methods

**Preparation of Aβ Conformers.** Aβ fibrils were formed according to the method described previously by us. Briefly,  $A\beta_{1-42}$  (220 µM) was dissolved in 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM NaCl, pH 7.4, and incubated at 37 °C up to 5 h [25].

Other  $A\beta_{1-42}$  peptide conformers were prepared by dissolving its powder in cold HFIP at a 2 mg/ml concentration and incubated at room temperature (RT) for 1 h. Then, the solution was divided into 25 µl aliquots, and HFIP was evaporated. The resulting  $A\beta_{1-42}$  films were stored at -20 °C until further experiments. Before any experiment, the peptide was dissolved in DMSO at a final concentration of 5 mM. Then resuspended in the suitable buffer as follows and treated with ultrasonication.

The high fibril concentration was formed by resuspension of this peptide in 10 mM HCl at a final concentration of 100  $\mu$ M and incubating at 37 °C for 24 h.

The lower fibril concentration was prepared by diluting this solution to 25  $\mu$ M in Ham's/F12 phenol red-free culture medium.

Finally, preparation and propagation of oligomers were done by resuspension of  $A\beta_{1-42}$  solution in DMSO into Ham's/F12 (phenol red-free) at a final concentration of 100  $\mu$ M incubated at 4 °C for 24 h [26].

Ultrasonication Treatments.  $A\beta_{1-42}$  fibrils were placed on a water bath-type Ultrasonicator (EngoTech, Zurich, Switzerland). Ultrasonication pulses were applied to both low (25 µM) and high (100 µM) concentrations of  $A\beta_{1-42}$ solution for 120 min. The sonication output was set to 60 Hz and 280 watts, and the temperature was maintained at 4 °C throughout the treatment. The  $A\beta_{1-42}$  solution samples were named ultrasonicated high concentration (USH) and ultrasonicated low concentration of  $A\beta$  was also prepared and named as a non-ultrasonicated high concentration (NUSH).

**Transmission Electron Microscopy (TEM).** Amyloid conformers (NUSH, USH, and USL) were immediately placed on the 400-mesh carbon-coated copper grids. After 1 min incubation for adsorption of bio-macromolecules on the grid surface, the excess solution was removed with filter paper. Next, the grid's surface was washed with deionized water. Then, the grids were negatively stained using 2% (w/v) uranyl acetate. After 15 min, the dried grids were used for TEM analysis. The electron micrographs were acquired using a transmission electron microscope (Model-EM208S) at 100 kV with a magnification of 200 K×. The images were prepared using a Digital camera. The electron micrographs were taken at Partow Rayan Rastak, Tehran, Iran.

Western Blotting. For Western blot,  $A\beta_{1-42}$  conformers were diluted to 2.5 mM in sample buffer. Then, the diluted samples were loaded onto the 12.5% SDS-PAGE. After electrophoretic separation, they were transferred to a polyvinylidene difluoride membrane (PVDF). The membranes were blocked with 3% BSA in Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.4, 0.9% NaCl) containing 0.1% Tween 20 (TBS-T), washed for 10 min with TBS-T, and incubated with specific primary  $A\beta_{1-42}$  antibody (Ab11132) overnight at 4 °C. The day after, the membrane was washed three times, 10 min each, with TBS-T. Then, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG Goat antibody in TBS-T containing 0.5% BSA for 1 h at RT. Next, the membrane was rewashed with TBS-T three times, 10 min each, and then with TBS. Immunereactive bands were visualized by the enhanced chemiluminescence (ECL) method on X-ray films. Subsequently, intensities of bands on the SAS-PAGE and WB membrane were quantified by using ImageJ software version 1.49t (NIH approved). So, the bands with less than 10 kDa, in the range of 10–50 kDa, and more than 50 kDa were considered the monomer, oligomer and fibril, respectively.

**Dot Blot.** Each  $A\beta_{1-42}$  conformer was transferred and spotted (1 µg) on PVDF. Afterward, the membrane was blocked with 10% BSA in TBS-T for 1 h at 37 °C. Next, it was incubated with rabbit polyclonal anti- $A\beta_{1-42}$  antibody for 2 h, at RT. Then the blots were washed in washing buffer, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at RT for 1 h, and developed with ECL.

**ThT Assay.**  $A\beta_{1-42}$  samples were diluted with ThT (0.4  $\mu$ M ThT in 50 mM Na<sub>2</sub>HPO<sub>4</sub> and 0.05% (w/v) NaN<sub>3</sub>, pH 7.4). The fluorescence intensity (FI) was measured using a Cytation 3 microplate reader (BioTek Instruments, Inc. Winooski, VT, USA). The FI of ultrasonicated and nonultrasonicated  $A\beta_{1-42}$  at different time intervals of protein incubation at 37 °C was recorded at excitation and emission wavelengths of 450 and 482 nm, respectively.

ANS Fluorescence Assay. Diluted solutions (10  $\mu$ M) of A $\beta_{1-42}$  in all forms of NUSH, USH, and USL were exposed to 20  $\mu$ M of ANS at different incubation time points. The ANS fluorescence intensity at 500 nm was read after the excitation of the samples at 380 nm at different time intervals. The data was obtained using a Cytation 3 microplate reader.

**Circular Dichroism (CD).** Far-UV (190–260 nm) CD spectra explored changes in the secondary structure of the diluted conformers of oligomers, NUSH, USH, and USL solutions of  $A\beta_{1-42}$  in 10 mM PBS, pH 7.4. These conformers were evaluated using a JASCO-J-810 Spectropolarimeter (JASCO Corporation, Tokyo, Japan), using a 5-mm path length cuvette at 25 °C. Spectra were recorded with wavelength intervals of 1 nm, a response time of 4 s, and a scan rate of 100 nm/min. Each spectrum is the average of 4 scans of samples after subtraction of the related baseline. The

noise component in data was smoothed using the JASCO J-810 software, including the fast Fourier-transform noise reduction method, which allows enhancement of most noisy spectra without distorting their peak shapes. The amounts of the secondary structures of the peptides were estimated using the Protein Secondary Structure Estimation Program (JWSSE-480), J-800 for windows. Yong plots were used as references for  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turn, and random coil.

Cell Toxicity Assay.  $A\beta_{1-42}$  toxicity was evaluated by cell viability assay using MTT. Therefore, we firstly grew rat adrenal pheochromocytoma cells (PC12) in RPMI-1640 medium supplemented with 5% (v/v) fetal bovine serum (FBS), 10% (v/v) horse serum, and 1% (v/v) penicillin/ streptomycin. Then, PC12 cells were trypsinized and, after washing, were seeded in 96-well plates. After 24 h, different types of  $A\beta_{1-42}$  peptides (NUSH, USH, and USL) were separately added to the PC12 cells. The cells were incubated for an additional 24 h at 37 °C, and then the MTT solution (20 µl of 5 mg/ml) was added to the wells. After 4 h of incubation with MTT, the media were removed and replaced with 200 µl of DMSO. The color intensity of the formazan solution was quantified using an ELISA plate reader (Tecan, Zurich, Switzerland) at 570 nm. The control (untreated) cells were assumed 100% viable, and the viability of the  $A\beta_{1-42}$  treated cells was calculated relative to the control and expressed as the percentages (%) of viability.

Immunofluorescence Assay of  $A\beta_{1-42}$  Cellular Uptake. PC12 cells were grown in RPMI-1640 medium supplemented with 5% fetal bovine serum, 10% horse serum, and 1% penicillin/ streptomycin. Then, cells were exposed to the IC<sub>50</sub> value of  $A\beta_{1-42}$  conformers. After 24 h, treated cells were washed with PBS and fixed in 70% chilled methanol for 10 m at RT. After PBS washing, nuclei were permeabilized in 0.3% Triton-X for 10 m at RT. Next, PC12 Cells were washed with PBS, and unspecific binding sites were blocked with 1% BSA for 30 m at RT. Then, cells were subjected to primary  $A\beta_{1-42}$  antibody at RT overnight. After that, cells were incubated with Alexa Fluor 594, as a fluorescent secondary antibody, for 1 h at RT, followed by counterstaining with Hoechst 33,258 solution. Finally, cells were scanned and imaged with a Cytation<sup>™</sup> 3 microscope (BioTek Instruments, Inc. Winooski, VT, USA).

### **3 Results**

### 3.1 The TEM Analyzes

TEM analysis was performed to obtain information about the shape and size of ultrasonicated  $A\beta_{1-42}$  aggregates. Figure 1a shows long fibrillar aggregates of NUSH, while Fig. 1d shows  $A\beta_{1-42}$  oligomers with spherical structures. Fig. 1 Transmission electron microscopy (TEM) images of different forms of  $A\beta_{1-42}$ . (a) The non-ultrasonicated 100  $\mu$ M (NUSH), (b) The ultrasonicated 100  $\mu$ M  $A\beta_{1-42}$  (USH), (c) The ultrasonicated 25  $\mu$ M  $A\beta_{1-42}$ concentration (USL), and (d) The oligomer conformer. TEM analysis showed that  $A\beta$  oligomeric species present more in the 25  $\mu$ M concentration than 100  $\mu$ M concentration of  $A\beta_{1-42}$ . The yellow arrows show fibrils and red arrows show globular oligomers



Figure 1b and c show TEM images of USH and USL of  $A\beta_{1-42}$ . Comparing these two figures indicate a marked decrease in fibrillar size and the effective fragmentation of amyloid fibrils to smaller species and oligomers at low concentrations of  $A\beta_{1-42}$  solution.

## 3.2 Western blot proProfiles $A\beta_{1-42}$ pepPeptides difDifferentnditions

Figure 2a shows the Western blot bands. From left to right, it shows the oligomeric form of protein in lane 1, and the fibril with no ultrasonication in lane 2. Lanes 3 and 4 show the ultrasonicated high and low concentrations  $A\beta_{1-42}$  peptides, respectively. As can be seen, ultrasonication breaks proteins and smears appear. However, two 45 and 25 kDa bands at lower concentrations (USL) indicate oligomeric structures. Figure 2b is the histogram of Western Blot data, indicating the portion of monomer + oligomer vs. fibrils in each sample.

Figure 2c shows Dot blot spots. It indicates the smaller species content at low and high concentrations of  $A\beta_{1-42}$  peptides after ultrasonication. Figure 2d, the histogram of Dot blot data, indicates that the oligometric form was considerably higher at USL ( $A\beta_{1-42}25 \mu M$ , after ultrasonication) than in others.

### 3.3 ThT Fluorescence Intensity and ANS Binding Assay

The interaction of  $A\beta_{1-42}$  peptides with ThT, a dye reactive with  $\beta$ -sheet-rich conformers, and ANS, a hydrophobic dye, were studied. Figure 3a shows the enhancement of

ThT fluorescence emission following its incubation with different forms of  $A\beta_{1-42}$  peptides. NUSH showed higher fluorescence intensity after interaction with ThT, which was increased up to 3 h. However, the ultrasonicated forms showed lower fluorescence intensity changes. Figure 3a shows that the lowest ThT fluorescence intensity belongs to oligomer and USL of  $A\beta_{1-42}$ , indicating the lowest fibrillar form.

Figure 3b shows the results of the ANS binding assay. The highest ANS fluorescence intensity was observed in the solutions containing oligomers and USL of A $\beta$  after 150 min incubation. In contrast, the lowest fluorescence intensity was observed in the NUSH solution. It indicates the formation of A $\beta_{1-42}$  species with higher surface hydrophobicity, such as those observed in A $\beta$  oligomers in the USL. These results suggest that ultrasonication shifts the equilibrium toward A $\beta_{1-42}$  fibrils depolymerization, monomer, and oligomer formation. Furthermore, these changes were more at lower A $\beta_{1-42}$  concentrations.

### 3.3.1 Secondary Structure of Different Forms of $A\beta_{1-42}$

Figure 4a and c indicate oligomers' higher percentage of  $\beta$ -turn and random coil (40.0% and 46.9%, respectively). The NUSH solution showed a  $\beta$ -sheet-rich structure for fibrils (Fig. 4b and c), while after ultrasonication, at higher concentration, the percentages of  $\beta$ -sheet decreased (from ~72.2% to ~37.6%) at the expense of  $\beta$ -turn (from 0.8 to 28.0%) and random coil (from 18.3 to 20.2%) conformations. At lower concentrations, the  $\beta$ -turn was (45.2%) higher than in other structures (Fig. 4c).

Fig. 2 Western blot and Dot blot data of different forms of  $A\beta_{1-42}$ . (a) Western blot analysis of different forms of  $A\beta_{1-42}$ . Lanes 1 to 4 show oligomers, non-ultrasonicated 100 µM (NUSH), ultrasonicated 100 and 25 µM (USH and USL, respectively).  $A\beta_{1-42}$ disrupted due to ultrasonication to fragments with different sizes. (b) The related histogram of semi quantitative analysis of the bands in Western blot using ImageJ software. (c) Dot-blot analysis of the mentioned samples. (d) The histogram shows semi quantitative analysis of the dot blot spots of NUSH, USH and USL. The results are expressed as mean density of indicated bands  $\pm$  SD based on three independent experiments



## 3.4 Cell Toxicity Assay of $A\beta_{1-42}$ at Different Conditions

The toxicity of different forms of A $\beta$  peptide against PC12 cells was investigated using MTT assay and expressed as percentages of cell viability. The results in Fig. 5 indicated more toxicity of ultrasonicated A $\beta_{1-42}$  peptide (USL) than non-ultrasonicated one (NUSH) against these cells. The IC50 of oligomers was about 5  $\mu$ M, and the IC50 of the USL was 10  $\mu$ M, which is the highest cytotoxicity compared to other forms.

### 3.5 Uptake of $A\beta_{1-42}$ into the PC12 Cells

The A $\beta$  uptake by PC12 cells was studied by incubating the cells with a primary antibody against the A $\beta_{1-42}$  peptides. Then, cells were stained with a secondary antibody against A $\beta_{1-42}$  and Hoechst 33,258 for the nucleus. Figure 6a and b show the maximum and significant (p=0.000) cellular uptake in cells exposed to the ultrasonicated-25  $\mu$ M (USL) and A $\beta$ Os. Fig. 3 ThT and ANS fluorescence intensity of  $A\beta_{1-42}$  at different conditions. (a) Fluorescence intensity of the ThT-treated  $A\beta_{1-42}$  peptide at different conditions (ultrasonicated 25 and 100 µM as USL and USH, respectively; non-ultrasonicated 100 µM as NUSH and oligomer) and different time intervals; where  $\lambda_{ex}$  and  $\lambda_{em}$  was 450 and 482 nm, respectively. (b) ANS fluorescence intensity after 150 min incubation with different forms of  $A\beta_{1-42}$ , where  $\lambda_{ex}$  and  $\lambda_{em}$  was 380 and 500 nm, respectively. All results shown are representative of at least three independent experiments, and the results are shown as means  $\pm$  SD.



### **4** Discussion

In the present study, stable  $A\beta_{1-42}$  fibrils (erroneously formed at 220 µM in a phosphate buffer containing NaCl incubated at 37 °C) were disaggregated through ultrasonication and then, refolded to oligomers. To optimize the method, we first tried to form the A $\beta$  fibrils at high and low concentrations (100 and 25 µM) and oligomers by the standard methods of Stine et al. [25]. Then, we evaluated some properties of  $A\beta_{1-42}$  before and after ultrasonication. Our findings show that ultrasonicated-25 µM of  $A\beta_{1-42}$  was more toxic against PC12 cells than 100 µM before or after ultrasonication. The Western blot data showed  $A\beta_{1-42}$  fibrillation, mostly at 100 µM, before ultrasonication. Due to ultrasonication, the fibrils broke through into smaller species, resulting in oligomer formation. However, a low concentration of  $A\beta_{1-42}$  adopts a predominantly smaller size aggregate and oligomeric structure. The changes in the fluorescence intensities of different forms of  $A\beta_{1-42}$  in the presence of ThT and ANS were also other affirmative reasons for this finding. Dot blot analysis also indicated the presence of higher amounts of monomer and oligomer species after ultrasonication of 25  $\mu$ M preparations than in the 100  $\mu$ M preparations. We also showed the differences in the  $A\beta_{1-42}$  structures at high and low concentrations using TEM images. Given the toxicity assay, we showed that USL was more toxic than other forms for PC12 cells due to their uptake by the cells, confirming their oligomeric structure.

Fig. 4 Characterization of the  $A\beta_{1-42}$  secondary structure after ultrasonication. (a) The Secondary structures of oligomers, USH, and USL (100 and 25  $\mu$ M of ultrasonicated A $\beta_{1-42}$ , respectively); (b) The CD plot of NUSH (non-ultrasonicated, 100  $\mu$ M) A $\beta_{1-42}$ . It shows a characteristic negative peak around 220 nm of  $\beta$ -sheet structure for NUSH. After ultrasonication the Secondary structures for the peptides in solution were changed. (c) shows the percentages of the Secondary structures at different situations as estimated by the software



More than 80 functional or pathologic amyloid fibril proteins have been characterized [27]. However, forming  $A\beta_{1-42}$  fibers may happen in two different in vitro situations. Firstly, a controlled situation using a certain peptide concentration in the presence of heparin, dithiothreitol (DTT), and temperature [28]. These types of aggregates may be disaggregated in the presence of some peptides or small molecules as inhibitors [29, 30].

The second is an uncontrolled random situation due to inappropriate very high concentration, buffer, temperature, pH, some ions like Cu<sup>2+</sup>, agitation, and so on [31]. These types of fibrillization are not inhibited readily and cause

severe problems in experimental situations and use  $A\beta$  peptide in the experiments.

In the present study, we tried to disaggregate the fibrils of the second category, which are very stable, using ultrasound and a suitable buffer, and then convert the monomers into oligomers. For this purpose, we used various methods to characterize the aggregates.

The ThT-binding assay is a method of choice for detecting the presence of amyloid fibrils [32–34]. It showed a higher fluorescence intensity of NUSH, indicating more amyloid fibril formation. In contrast, the USL displays the lowest ThT fluorescence intensity, indicating the lowest fibrillar content. These data are consistent with the data



**Fig. 5** The effect of different forms of  $A\beta_{1-42}$  on PC12 cell viability. Toxicity of different forms of  $A\beta_{1-42}$  NUSH (100  $\mu$ M) and USH (ultrasonicated 100  $\mu$ M) and USH (ultrasonicated 25 $\mu$ M), as well as  $A\beta_{1-42}$  oligomers was measured by MTT assay after 24 h. In this experiment, different concentrations of the ultrasonicated and non-ultrasonicated solutions were used. The order of toxicity of different structure was oligomers > USL > USL > NUSH. The graph represents the mean  $\pm$  SD from triplicate wells

obtained by TEM analysis. TEM examination of incubated NUSH also showed a tremendous amount of amyloid fibril content, while the lowest amount was observed at 25  $\mu$ M solution after ultrasonication.

In addition, the highest ANS binding to the USL is another reason for A $\beta$  oligomer formation. ANS is highly sensitive to the polarity of peptides and proteins. The fluorescence intensity increases upon interaction with the exposed hydrophobic regions in native or partially unfolded proteins [35]. Ultrasonic disruption of the fibrils increased to the extent of solvent exposure of hydrophobic A $\beta_{1-42}$  fragments and led to evaluate the hydrophobicity of A $\beta$  using the ANSbinding assay. A $\beta_{1-42}$  oligomers are more hydrophobic [18] and, thus, bind more ANS. While hydrophobicity of both NUSH and USH were lower than USL.

Aβ<sub>1-42</sub> fibrils are composed of two in-register interstrand parallel β-sheets connected by a bend between residues 25 and 30 [5, 36, 37]. Residues 10–16 are part of the first β-sheet in fibrils [11, 36, 37] and disordered in the preglobulomer [38]. Two hydrophobic peptide segments within Aβ<sub>1-42</sub>, residues 16–22 and 30–42, are solvent-exposed in the toxic Aβ<sub>1-42</sub> oligomers [18]. We also evaluated the secondary structure of the peptides by CD spectroscopy. The spectrum of NUSH would possess a sharp negative peak at about 220 nm, indicating that the peptide adopts mainly (~60%) the β-sheet conformation. In contrast, the spectra of other peptides show different characteristic peaks, indicating the decreased  $\beta$ -sheet percentage at the expense of  $\beta$ -Turn and random coil formation.

We also investigated the effect of ultrasonication on  $A\beta_{1-42}$  fragmentation. According to the Western blot analysis, ultrasonication of 25 µM of  $A\beta_{1-42}$  induced a significant increase in the densities of the bands around 20–60 kDa. This pattern is compatible with the formation of  $A\beta_{1-42}$  monomers (~10 kDa), oligomers (~25 kDa), and fibrils (~75 kDa) [39–41]. These results are in excellent agreement with those obtained from Dot blot in which the antibody recognized oligomers in USL more than those in USH solutions of  $A\beta_{1-42}$ . Furthermore, these data are compatible with the model that demonstrated two distinct pathways for the amyloid  $A\beta_{1-42}$  soluble (oligomer) and insoluble (fibril) peptides [6, 42]. Similar studies have been performed on 25 µM [43] and 8 µM [44] of the  $A\beta_{1-40}$  peptide, illustrating the role of environmental factors in the peptide structure.

A switch in mechanism with concentration has also been observed with  $A\beta_{1-42}$ . In a narrow concentration range  $(20-25 \ \mu\text{M})$ , spherical  $A\beta_{1-42}$  oligomers formed which were positive for the oligomer-specific A11 antibody and showed a high capacity to disrupt lipid bilayers [18]. By contrast, at higher concentrations, a different type of oligomer was formed that did not react with the A11 antibody that did not disrupt lipid bilayers, despite having a similar size and secondary structure at low concentrations (<20  $\mu$ M) [18]. Studies such as these show the importance of recognizing the influence of concentration-dependent mechanistic changes and the need for accurate concentration measurements before experiments start[17].

Previous studies have shown that the  $A\beta_{1-42}$  amyloid fibrils in the membrane consist of two intermolecular  $\beta$ -sheets:  $\beta$ 1, which is in the non-transmembrane (NTM) region (residues 17-28), and  $\beta 2$ , is in the transmembrane (TM) region (residues 29-42) [5]. Under ultrasonication, a bubble was usually created around hydrophobic residues in the TM region after pressure becomes negative. The assembly of hydrophobic residues in the TM region acts as a nucleus for bubble formation, and the bubble breaks down the fibrils. The amyloid fibrils keep their  $\beta$ -sheet structure even in the bubble. When the pressure becomes positive, the bubbles shrink and collapse, water molecules crash against the hydrophilic residues in the NTM region, and then amyloids disrupt. The deformation and disruption of the  $\beta$ -sheet structure at the NTM region are possible because of the presence of Glu22 and Asp23 with negative electric charges. The repulsion between these residues is shielded in water but not in the bubble, which is the main reason for  $\beta$ -sheet structure destruction in the NTM region in the bubble. In the case of short amyloid fibrils like trimer, hexamer, and dodecamer, the Aß peptides do not have

Fig. 6 PC12 Cell uptake of  $A\beta_{1-42}$ . (a) PC12 cells that were cultured in the presence USL and USH (ultrasonicated 25 and 100 µM), NUSH (nonultrasonicated 100  $\mu$ M) of A $\beta_{1-42}$ and oligomers for 24 h. Then, they were exposed to primary  $A\beta_{1-42}$  antibody, Alexa Fluor 594 and Hoechst 33,258 and images were prepared with fluorescent microscopy. The  $A\beta_{1-42}$  uptakes observed in USL (ultrasonicated low concentration (25 µM)) more than USH (higher concentration (100 µM)) before and after ultrasonication. (b) The histogram shows the ratio of red color intensity of  $A\beta_{1-42}$  to the cell nuclear number, in PC12 cells. All results shown are representative of at least three independent experiments, and the results are shown as means  $\pm$  SD.



Αβ1-42

enough hydrophobic residues to create a bubble. Thus, amyloid fibrils do not disrupt. Therefore, more time of negative pressure is required for bubble formation [22]. It appears that following ultrasonication, the potential for formation of antiparallel  $\beta$ -sheet at low A $\beta_{1-42}$  concentrations is greater than its high concentrations. It may be due to the repulsion between the negatively charged amino acids, Glu22 and Asp23, and the critical concentration of micelle-like formation. It has shown that after ultrasonication of both low and high concentrations,  $A\beta_{1-42}$  fibrils break down into monomers and smaller species of aggregates. However, the kinetic, thermodynamic stability, and energy landscape differences lead to possible conformations.

### **5** Conclusions

We examined the effect of the ultrasonic application on low and high concentrations of  $A\beta_{1-42}$  peptide to study the simultaneous effects of the concentration and ultrasonication on  $A\beta_{1-42}$  fibril disruption. The results indicated that due to ultrasonication of low concentration of  $A\beta_{1-42}$ , long amyloid fibrils disrupted and fragmented to smaller species of aggregates, including 2-12 mers. Furthermore, these small spherical  $A\beta_{1-42}$  aggregates were more toxic against PC12 cells than the fibrillar aggregates at higher concentrations of  $A\beta_{1-42}$ . In addition, their characteristics were very similar to  $A\beta$  oligomers. The ThT and CD studies indicated the following order for  $\beta$ -sheet content in the A $\beta_{1-42}$  peptides: NUSH>USH>USL>Oligomers.

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### Declarations

Competing Interests The authors declare no competing interests.

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