Title

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Effects of docosahexaenoic acid on *in vitro* amyloid beta peptide 25–35 fibrillation

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ABSTRACT

Amyloid β peptide$_{25-35}$ ($A\beta_{25-35}$) encompasses one of the neurotoxic domains of full length $A\beta_{1-40/42}$, the major proteinaceous component of amyloid deposits in Alzheimer's disease (AD). We investigated the effect of docosahexaenoic acid (DHA, 22:6, n-3), an essential brain polyunsaturated fatty acid, on the *in vitro* fibrillation of $A\beta_{25-35}$ and found that it significantly reduced the degree of fibrillation, as shown by a decrease in the intensity of both the thioflavin T and green fluorescence in confocal microscopy. Transmission electron microscopy revealed that DHA-incubated samples were virtually devoid of structured fibrils but had an amorphous-like consistency, whereas the controls contained structured fibers of various widths and lengths. The *in vitro* fibrillation of $A\beta_{25-35}$ appeared to be pH-dependent, with the strongest effect seen at pH 5.0. DHA inhibited fibrillation at all pHs, with the strongest effect at pH 7.4. It also significantly decreased the levels of $A\beta_{25-35}$ oligomers. Nonreductive gradient gel electrophoresis revealed that the molecular size of the oligomers of $A\beta_{25-35}$ was 10 kDa (equivalent to decamers of $A\beta_{25-35}$) and that DHA dose-dependently reduced these decamers. These results suggest that DHA decreases the *in vitro* fibrillation of $A\beta_{25-35}$ by inhibiting the oligomeric amyloid species and, therefore, $A\beta_{25-35}$-related neurotoxicity or behavioral impairment could be restrained by DHA.

1. Introduction

Deposition of insoluble neuritic plaques and neurofibrillar tangles of amyloid β peptides (Aβ) in the brain is the neuropathological stamp of Alzheimer's disease (AD), characterized by progressive loss of neurons and the deterioration of memory-related learning ability [1]. The major components of neuritic plaques are the 40–42-amino acid-residue-long Aβs that are proteolytically released from membrane-bound amyloid precursor proteins (APP) [2]. After intersecting with the environment, these Aβs self-transform from their native coiled α-helical structures into insoluble fibrillar forms [3]. Although $A\beta_{1-40}$ and $A\beta_{1-42}$ are the predominant forms, other fragments can be present as well: in the brains of aged patients, $A\beta_{25-35}$, a stretch of 11 amino acid-long residues of the full length from position 25 to 35, is produced by the proteolysis of full-length $A\beta_{1-40}$ [4]. The $A\beta_{25-35}$ fragment is biologically active and analogous to its full length Aβs [5]; it is thus conceivable that learning-related memory impairment could also be induced by the infusion of this short amyloid into the cerebral ventricle of rats. The hypothesis that $A\beta_{25-35}$ forms fibrils and confers toxicity analogous to that of the full-length peptide has been proved by *in vitro* studies with neurons [6]. These findings are further supported by *in vivo* studies demonstrating that direct cerebral infusion of $A\beta_{25-35}$ into the ventricle impairs memory in mice [7,8]. We have previously reported that the memory impairment of $A\beta_{1-40}$-infused AD model rats is ameliorated and/or prevented by the dietary administration of docosahexaenoic acid (DHA, C22:6, n-3) [9,10]. The brain utilizes large amounts of DHA before birth, during growth spurts and throughout the growing periods [11–12]. The level of DHA decreases in the hippocampus of AD patients [13]. Accordingly, dietary intervention with DHA has been shown to improve AD-related symptoms [14], and supplementation with DHA has been associated with the improvement of neurobehavioral complications in AD model animals [15]. Recently, it has been shown that DHA significantly decreases the cerebral cortical levels of Aβ peptides with concomitant ameliorative effects on memory-related learning ability [9,10,15]. Though the amyloid deposition is considered as one of the key steps in the pathogenesis of AD and DHA could be targeted as one of the potential therapies to clear up the amyloid deposits [9,10,15], the mechanism of the action of DHA on the *in vitro* fibrillation is scarcely reported. *In vitro* polymerization is postulated to be a model that explains well the mechanistic milieu of fibrillation of Aβ proteins in AD. Thus, the direct interruption of amyloid fibril formation by DHA would be predicted to relate its Aβ-clearance from the *in vivo* scenario of AD brain.

The effect of DHA on neurodegenerative diseases is very significant in that DHA benefits brain functions. Numerous structural and functional studies on full-length $A\beta_s$ have also stressed the role of $A\beta_{25-35}$ in neurotoxicity: $A\beta_{25-35}$ induces neuronal cell death [16],

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synaptic loss [17] and inhibits neurogenesis [18]. The effects of DHA on *in vitro* fibrillogenesis of $A\beta_{25\text{-}35}$ sequences have not been reported. Therefore, in this study we investigated whether DHA inhibits *in vitro* fibrillation of $A\beta_{25\text{-}35}$ and what the relevant mechanism(s) is.

2. Materials and methods

2.1. Materials

The trifluoroacetate form of amyloid β-peptide ($A\beta_{25\text{-}35}$) was purchased from the Peptide Institute Inc. (Osaka, Japan); Docosahexaenoic Acid (DHA (C22:6, n-3)) (formal name: 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid, purity ≥98%) was purchased from Cayman Chemical Company, MI, USA; Thioflavin T (ThT) was purchased from Sigma-Aldrich (St. Louis, Mo.); and Rabbit polyclonal anti-oligomer antibody (A11) was purchased from Invitrogen (CA). All other chemicals were of analytical grade.

2.2. Preparation of $A\beta_{25\text{-}35}$ peptide for analysis of aggregation

$A\beta_{25\text{-}35}$ peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Wako, Japan) at a concentration of 500 μM to produce uniform, non-aggregated Aβ [19]. Aliquots of the soluble $A\beta_{25\text{-}35}$ were then stored at −80 °C until use. HFIP was extensively blown with N_2 gas at ice-cold temperatures and re-dissolved in the assembly buffer as required for aggregation studies.

2.3. Preparation of DHA

DHA (50 mg) dissolved in 200 μl ethanol, bathed in N_2, was stored (in 5.0-μl aliquots) at −80 °C until use. It was then directly suspended in ultrapure water and used at the desired concentration containing 0.002% ethanol. Only freshly prepared DHA was used in all the experiments.

2.4. Aβ fibrillation in vitro

The HFIP was dried-off from the $A\beta_{25\text{-}30}$ aliquot and the peptide film was suspended in assembly buffer to initiate fibrillation. The fibrillation experiment was conducted according to the method of Ono et al. [20] with a slight modification. In brief, 200 μl of 50 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl and 0.01% sodium azide, as assembly buffer, was added to the dried peptide film of 50 μM $A\beta_{25\text{-}35}$ (final concentration). Immediately after a brief vortex, the mixture was incubated at 37 °C for 24 h. For the $A\beta_{25\text{-}35}$+DHA samples, the assembly buffer contained 20 μM of DHA (or 5, 10 and 20 μM of DHA for the dose-dependent study) plus 50 μM $A\beta_{25\text{-}35}$. The reaction mixture was taken into oil-free PCR tubes (Takara Shuzo, Otsu, Japan) which were then flushed with nitrogen gas, sealed with paraffin film and placed into a DNA thermal cycler (PJ480; Perkin Elmer Cetus, Emeryville, CA). Starting at 4 °C, the temperature was elevated at maximal speed to 37 °C for 24 h, and the incubation was stopped by placing the tubes on ice.

2.5. ThT fluorescence spectroscopy

After 24 h of incubation at 37 °C, 40-μl aliquots from each tube were mixed with 210 μl of 5 μM ThT in 50 mM glycine-NaOH buffer (pH 8.5) and subjected to fluorescence spectroscopy (Hitachi F-2500 fluorescence spectrophotometer) at excitation and emission wavelengths of 448 and 487 nm, respectively. The scan was recorded at the speed of 1500 nm/min at every 0.5 nm in 0.5 ml four-side quartz cuvette.

2.6. Microfluorescence study

A 2.5-μL aliquot of the fibrillated $A\beta_{25\text{-}35}$ peptide (50 μM) sample was diluted ×2 with 5 μM thioflavin T in 50 mM glycine-NaOH buffer (pH 8.5) and transferred onto a slide. Fluorescent signals (488 nm) were then visualized by the confocal laser microscope system (CLSM FV300, Olympus, Tokyo, Japan) and processed by Adobe Photoshop (Adobe Systems, Mountain View, CA, USA) or NIH image version 1.63. The fluorescence intensity was visualized in each of three random fields of the sample. As an alternative, the aggregates were allowed to air-dry on the slide, and 2.5 μl thioflavin T in 50 mM glycine-NaOH buffer (pH 8.5) was added to the air-dried film; the slides were then viewed under the microscope; no difference in fluorescence intensity between the two assays was observed, however.

2.7. Transmission electron microscopy

After completion of $A\beta_{25\text{-}35}$ fibrillation for 24 h at 37 °C with or without DHA, an aliquot was used for electron microscopy. In brief, a 5-μl droplet of the sample was placed on a carbon-coated copper grid, stained with 1% uranyl acetate, and excess uranyl acetate was then removed from the grid with distilled water. The grid was then dried, and the sample was examined under a Hitachi H-7000 transmission electron microscope with an operating voltage of 75 kV.

2.8. pH dependent assay

After drying the HFIP from the $A\beta_{25\text{-}30}$ stock-aliquot, the peptide film was immediately suspended in assembly buffer (pH 3.0, 4.0, 5.0, 6.0, 7.4 and 8.0) to initiate *in vitro* fibrillation. Briefly, 200 μl of 50 mM Tris–HCl buffer (pH 3.0 –8.0) containing 100 mM NaCl and 0.01% sodium azide was added to the dried peptide film. Immediately after a brief vortex, the mixture was incubated at 37 °C for 24 h. The stock solution of DHA in ultrapure water was diluted with the respective pH buffers and added to the assembly mixture at a final concentration of 20 μM DHA. The reaction mixture was taken into oil-free PCR tubes (Takara Shuzo, Otsu, Japan) which were then flushed with nitrogen gas, sealed with paraffin film and placed into a DNA thermal cycler (PJ480; Perkin Elmer Cetus, Emeryville, CA). Starting at 4 °C, the temperature was elevated at maximal speed to 37 °C for 24 h, and the incubation was stopped by placing the tubes on ice.

2.9. Dose dependent effect of DHA on the in vitro Aβ fibrillation

Each 200 μL of assembly buffer (50 mM Tris–HCl buffer (pH 7.4), 100 mM NaCl, 0.01% sodium azide) contained 50 μM $A\beta_{25\text{-}35}$, (as dried film) with (final concentration of 5, 10 and 20 μM) or without (0 μM)

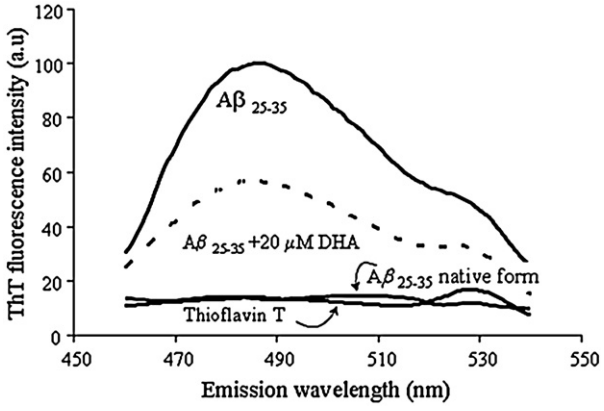

Fig. 1. Representative emission spectra of the thioflavin T (ThT) for the effect of DHA on the *in vitro* fibrillation of $A\beta_{25\text{-}35}$ (50 μM). $A\beta_{25\text{-}30}$ was initially incubated in the presence and/or absence of 20 μM of DHA at 37 °C for 24 h. After the completion of fibrillation, an aliquot of 40 μl of the sample was added to 210 μl of 5 μM ThT in glycine buffer (pH 8.5) and analyzed for emission spectra at the excitation wavelength of 448 nm. ThT bound only to the amyloid β-sheet and displayed a characteristic emission peak at 488 nm.

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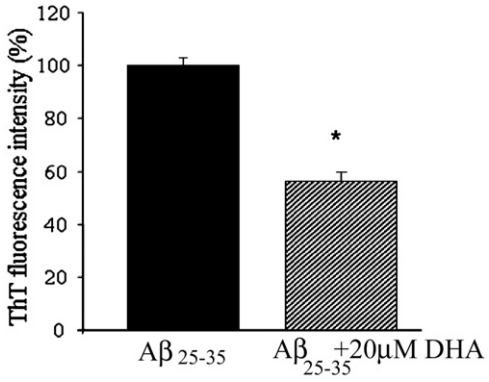

Fig. 2. The effect of DHA on *in vitro* fibrillation of $A\beta_{25-35}$. For fibril formation, $A\beta_{25-30}$ peptides (50 μM) were incubated at 37 °C for 24 h in the presence or absence of 20 μM of DHA. After completion of fibrillation, 40 μl of the sample was added to 210 μl of 5 μM ThT in glycine buffer (pH 8.5), and fluorescence intensity was measured at excitation and emission wavelengths of 448 and 488 nm, respectively. Results are means ± SE ($n=5$). Significant difference at $^*P<0.05$ (unpaired Student's *t*-test).

DHA. The mixture was taken into oil-free PCR tubes, which were then air-evacuated with N_2 gas, sealed with paraffin film and incubated at 37 °C for 24 h. The reaction was stopped by placing the tubes on ice. An aliquot of 40 μL from each tube was mixed with 210 μl of 5 μM thioflavin T in 50 mM glycine-NaOH buffer (pH 8.5) and immediately subjected to fluorescence assay at excitation and emission wavelengths of 448 and 488 nm, respectively.

2.10. $A\beta_{25-35}$ oligomer preparation and assay by oligomer-specific antibody (A11)

The fibrillated samples (50 μM $A\beta_{25-35}$) with or without DHA were centrifuged at 10,000 ×*g* for 5 min at 4 °C to pellet down the insoluble fibers, and the supernatant containing soluble oligomers were subjected to ELISA. The soluble oligomers were diluted in coating buffer (0.1 M sodium bicarbonate, pH 9.6), placed in a 96-well plate and incubated at 4 °C for 24 h. The wells were then washed three times with PBS and again supplemented with 200 μL of blocking buffer (10% ELISA grade BSA in TBST), according to the manufacturers' instructions, and incubated at 37 °C for 1 h. After washing with PBS, the primary antibody (A11) [21] was added to each well at a ratio of 1:1000 and incubated for an additional 1 h at 37 °C. The plates were again washed and incubated with the secondary antibody (goat F(ab')2 anti-rabbit IgG horseradish peroxidase conjugate (BioSource Cat.# AL14404)). After incubation for 1 h at 37 °C, the plates were washed and 100 μl of chromogen (BioSource lot # SB01/N021302) was added to develop color; the reaction was stopped after 30 min with stop solution (BioSource Cat. # SS01/M091003) and the absorbance was measured at 450 nm in the multiwall plate reader.

2.11. Gel electrophoresis of $A\beta_{25-35}$ oligomer

In a parallel set of experiments, the supernatant fractions containing the soluble oligomers were subjected to nonreducing Tris/Glycine 4–20% gradient gel electrophoresis (Novex gel; Invitrogen, Carlsbad, CA) under native conditions at 60 V for 4 h. Proteins of known MW were used as size standards (Bio-Rad, Hercules, CA, USA). The bands were stained with Coomassie brilliant blue (Fig. 6) and visualized with Molecular Imager FX (Bio-Rad, Hercules, CA, USA).

2.12. Statistical analysis

Results are expressed as means ± S.E. The data were analyzed by unpaired Student's *t*-test and one-way ANOVA. ANOVA followed by Fisher's PLSD was used for post hoc comparisons. The statistical program used was StatView® 4.01 (MindVision Software, Abacus Concepts, Inc., Berkeley, CA, USA). A level of $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of DHA on Aβ fibrillation

Examination of emission peak characteristics of the short stretch of $A\beta_{25-35}$ after its binding to thioflavin T (ThT) (Fig. 1) revealed that the fluorescent enhancement of ThT at 488 nm was proportional to the amount of amyloid formed [22,23]. The amplitude of the emission peak of the polymerized $A\beta_{25-35}$ increased about 10–15 times as compared with that of the ThT-blank alone, thus confirming the formation of $A\beta_{25-30}$ fibers from the precursor monomeric $A\beta_{25-30}$. The results demonstrated that the short sequence formed amyloid β sheets while retaining full-length $A\beta_{1-40/42}$. After confirming that $A\beta_{25-35}$ was

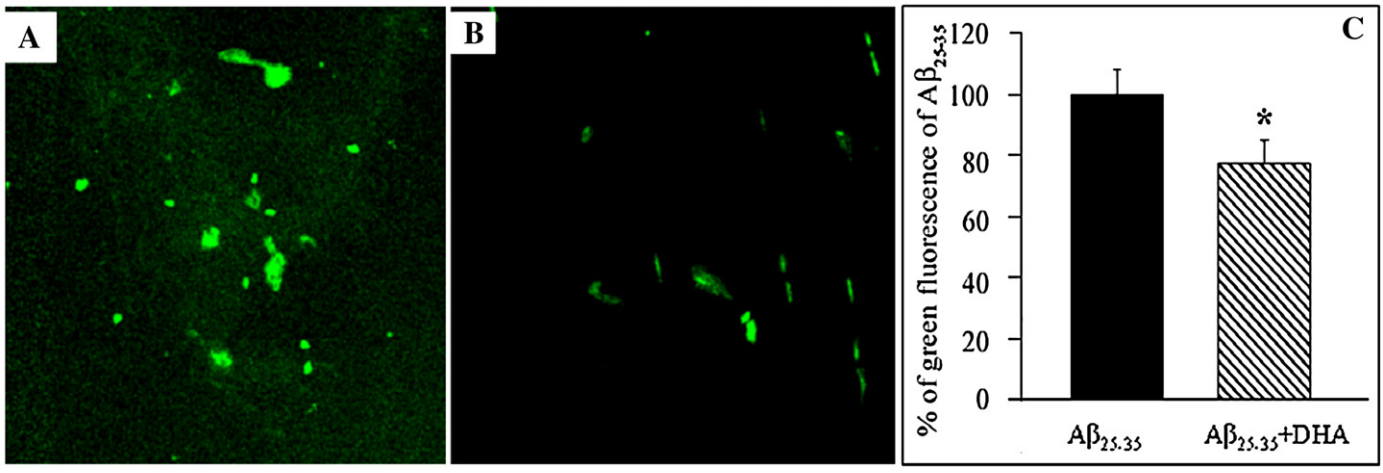

Fig. 3. Thioflavin T staining of polymerized $A\beta_{25-35}$ (50 μM) aggregating materials in the absence (Control: A) and presence (B) of 20 μM of DHA. Under fluorescence illumination with thioflavin T staining, amyloid aggregates appear green. DHA shows a clear inhibitory effect on $A\beta_{25-35}$ fibril formation, as evidenced by the smaller number of illuminated areas of $A\beta_{25-35}$ deposits. The microfluorescence signals were digitized to histogram data by using NIH Image analyzer and calculated each of three random fields per slide (C). Data are indicated as the means ± S.E. Statistical significance was evaluated by Student's *t*-test; a level of $^*P<0.05$ was considered significant.

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polymerized and assembled into aggregated fibrous forms analogous to full-length $A\beta_{1-40/42}$, $A\beta_{25-35}$ fragments were incubated with or without DHA (20 μM) for 24 h to complete the fibrillation process, and the intensities of other samples was routinely measured at the emission wavelength of 488 nm. The mean level of fluorescence intensity decreased significantly (by about 50%) in the DHA-incubated samples (Fig. 2).

3.2. Effect of DHA on the microfluorescence of aggregated $A\beta_{25-35}$

The effect of DHA on *in vitro* fibrillogenesis was evaluated by microfluorescence assay based on fluorescence emission by ThT. ThT binds specifically to amyloid fibrils with a concurrent display of green emission fluorescence. The fluorescence intensity of the green illuminated areas was significantly lower in DHA-treated samples (Fig. 3) than in the controls.

3.3. Effect of DHA on $A\beta_{25-35}$ fiber morphology

The Aβs were viewed under a transmission electron microscope using 50 μM peptide assembly buffer (pH 7.4) with (20 μM) or without DHA. After incubation for 24 h, the control samples had clear-cut fibrils with a flat and ribbon-like morphology, averaging 100–200 nm long and 9–14 nm wide (Fig. 4A,C). On the other hand, the DHA-incubated samples had very few, poorly defined fibril-like structures, if at all. In the presence of DHA, the fibers were practically abolished with little staining on the grids and appeared as densely amorphous-like and occasionally granular structures (Fig. 4B, D).

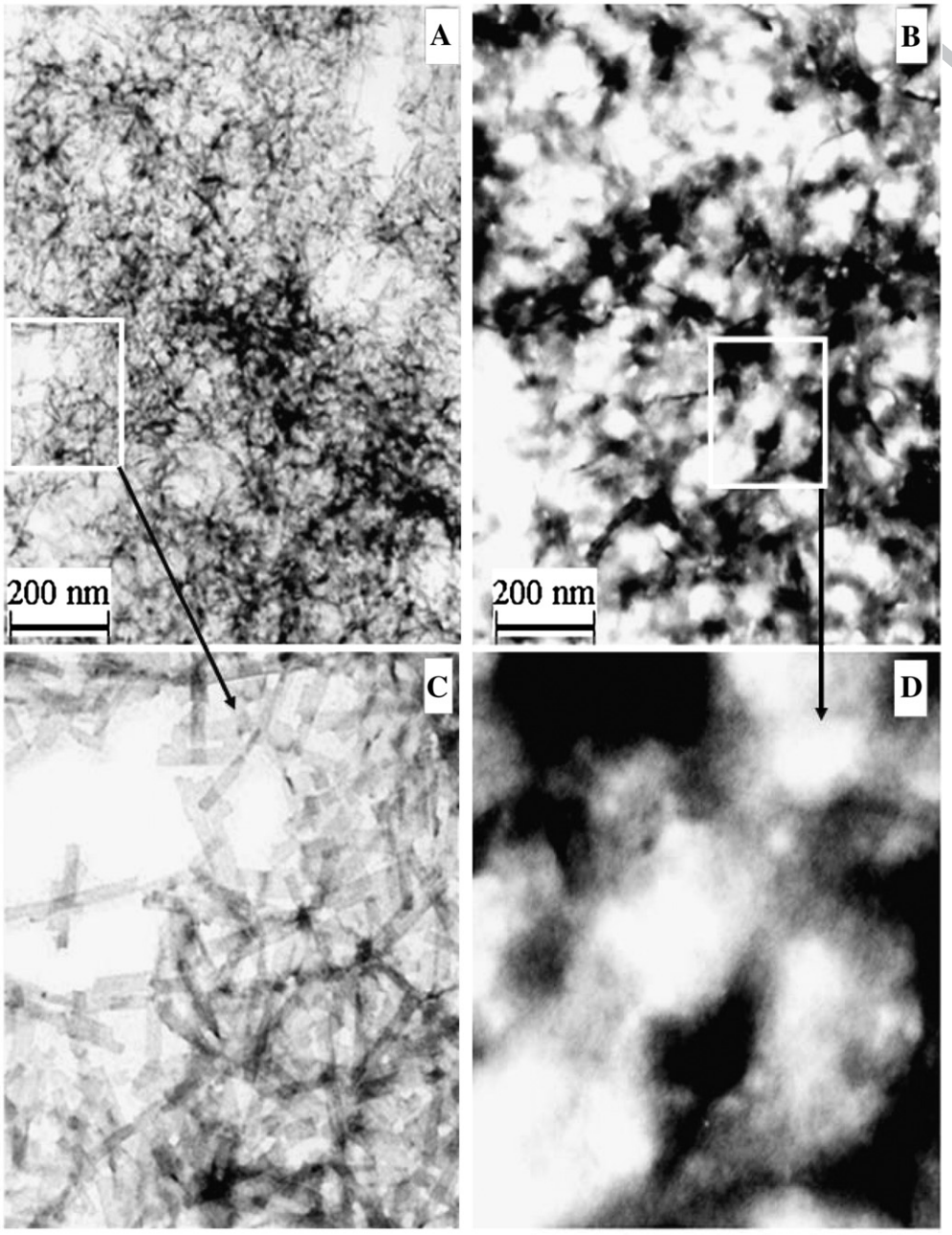

Fig. 4. Representative transmission electron micrographs of the effect of DHA on $A\beta_{25-35}$ fibril morphology. Aβ peptide$_{25-35}$ (5[illegible]was incubated for 24 h at 37 °C in the absence or presence of 20 μM of DHA. Six microliters of each sample was applied to a 400-mesh grid, stained with 1% uranyl acetate, ~~access~~ stain was removed, dried for 1 min, and visualized under transmission electron microscope. The morphology of the control fibrils displayed ribbon-like structures (A; C, boxed area in A), while that of the DHA-incubated samples was highly unstructured (B; D boxed area in B) and mostly of amorphous consistency.

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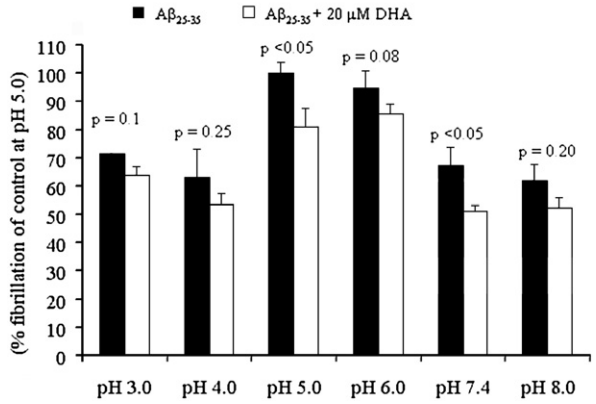

Fig. 5. The effect of DHA on pH-dependent $A\beta_{25-35}$ fibril formation. Aβ peptide$_{25-35}$ (50 μM) was incubated in the presence or absence of DHA (20 μM). An aliquot (40 μl) of the samples of each pH was mixed with 210 μl of 5.0 μM of THT in glycine buffer (pH 8.5) and immediately subjected to fluorescence assay at excitation and emission wavelengths of 448 and 488 nm, respectively. Results are means±SE of 4 determinations, as a percent of fibrillation occurring at pH 5.0. Significant effect of DHA is indicated by $P<0.05$ (unpaired Student's *t*-test) at the given pH.

3.4. Effect of DHA on pH-dependent fibrillation

In the absence of DHA, the highest aggregation of $A\beta_{25-35}$ fibrils was found in the assembly solution at pH 5.0 and, in descending order, lower at pH 6.0, 3.0, 7.4, 4.0 and 8.0. Fibril formation at pH 5.0 was 26–36% higher than at pH 3.0, 4.0, 7.4, 8.0 and only 5% higher than at pH 6.0, demonstrating a bell-shaped pH profile of fibrillation. In the presence of DHA, the degree of fibrillation decreased at all pHs tested; however, the inhibitory effect was significant ($P<0.05$) only at pH 5.0 and 7.4 (Fig. 5). At pH 6.0, only the tendency ($P=0.08$) of inhibition was observed.

3.5. Dose dependent Effect of DHA on the $A\beta_{25-35}$ fibrillation

Evaluation of $A\beta_{25-35}$ fibrillation revealed that DHA (5, 10 and 20 μM) dose-dependently inhibited *in vitro* $A\beta_{25-35}$ fibrillogenesis, with the strongest inhibitory effect at 20 μM (Fig. 6).

3.6. Effect of DHA on $A\beta_{25-35}$ oligomer levels

DHA dose-dependently decreased the levels of soluble $A\beta_{25-35}$ oligomers (Fig. 7A). The strongest effect was observed at 20 μM DHA

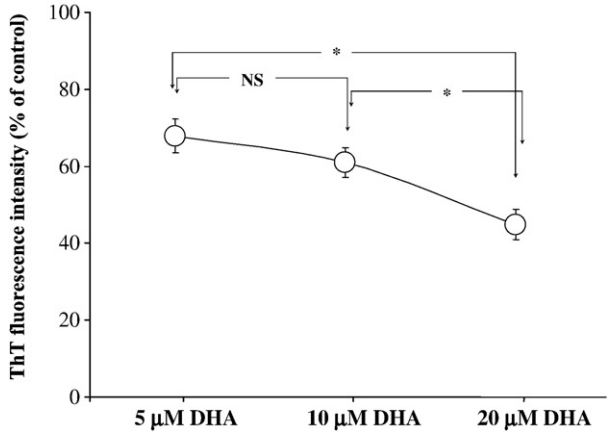

Fig. 6. The dose-dependent effect of DHA on the formation of $A\beta_{25-35}$ fibrils (50 μM). $A\beta_{25-35}$ fibril formation was initiated from fresh $A\beta_{25-35}$ monomers incubated with 5, 10 and 20 μM of DHA. The extent of fibril formation was evaluated by thioflavin T fluorescence measurements (see Materials and methods). The data were analyzed by one-way ANOVA followed by Fisher's PLSD (Protected Least Square Difference) test for post hoc comparisons. Each symbol represents the mean±S.E ($n=4$). *$P<0.05$.

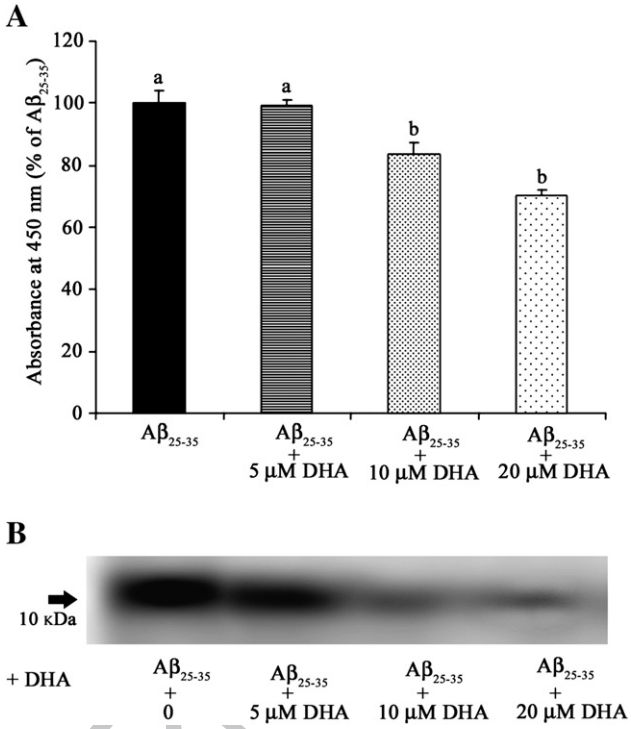

Fig. 7. (A) Inhibitory effect of DHA on the levels of soluble oligomeric amyloid $A\beta_{25-35}$. Oligomer levels were determined by conformation-specific antibody (A11). Results are means±SE of 3 samples each for duplicate determinations. Significantly different [a]$P<0.05$. (B) Tris-glycine (4–20%) gradient gel electrophoresis of $A\beta_{25-35}$ oligomers. After fibrillation for 24 h, the oligomers were subjected to electrophoresis, stained with Coomassie brilliant blue and visualized with Molecular Imager FX (Bio-Rad, Hercules, CA, USA). The data were analyzed by one-way ANOVA followed by Fisher's PLSD (Protected Least Square Difference) test for post hoc comparisons. Each symbol represents the mean±S.E ($n=4$). *$P<0.05$.

when the level decreased by 30%; at 10 μM, however, the level decreased by ~15%. The effect of DHA on the size of oligomers is shown in Fig. 7B. The band intensity at molecular mass 10 kDa was lower in the DHA-incubated samples, with the strongest effect observed at 20 μM of DHA.

4. Discussion

$A\beta_{25-35}$ is considered one of the neurotoxic domains of full-length $A\beta_{1-40/42}$. Our examination of whether DHA inhibits *in vitro* fibrillation of this short stretch Aβ revealed that DHA inhibits *in vitro* $A\beta_{25-35}$ fibrillation and that the inhibitory effect depends on DHA at a dose below its critical micellar concentration. The present study reaffirms that $A\beta_{25-35}$, analogous to its counterpart full-length $A\beta_{1-40/42}$, also forms fibrils through an intermediate species of oligomers, including 10-mers, and that DHA inhibits the maturation of fibrils by reducing the levels of these 10-mer $A\beta_{25-35}$ species. That $A\beta_{25-35}$ was present as a native conformation before incubation was confirmed by the absence of the characteristic ThT peak at the emission wavelength of 486 nm (Fig. 1). $A\beta_{25-35}$ displayed a higher ThT-fluorescence intensity peak than that of the un-incubated (zero time) sample and the ThT-blank, indicating that $A\beta_{25-35}$ forms clear-cut Aβ fibers corresponding to those of its counterpart full-length $A\beta_{1-40/42}$. Conversely, the DHA-incubated samples had a lower ThT fluorescence intensity than the controls, demonstrating the inhibitory effect of DHA on fibril formation.

The DHA-incubated samples also displayed lower green-fluorescence intensity, as determined by confocal laser microfluorescence spectroscopy [24], again suggesting that DHA had interrupted *in vitro* $A\beta_{25-35}$ fibrillation. Moreover, DHA dose-dependently inhibited $A\beta_{25-35}$ fibrillation with the strongest effect observed at 20 μM. The inhibitory effect of DHA on the $A\beta_{25-35}$ fibrillation was thus confirmed

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by the significant decrease in the intensity of ThT- and green-fluorescence. With this evidence, we examined the effect of DHA on the morphology of $A\beta_{25-35}$ fibers in the course of their forming amyloid fibrils. Transmission electron microscopy revealed that DHA-incubated samples did not have defined and structured fibrils, but had some typical morphology, including amorphous and granular aggregates (Fig. 4). The control fibers were ribbon-like and of various lengths. Thus, the transformation of the native forms into fibrils could be attributed to morphological changes in the assembly buffer.

Previous studies have implicated lysosomes or early endosomes of pH ~6.0 as sites for the accumulation and processing of Aβ peptides [25,26], thus supporting the proposition that Aβ peptides preferentially fibrillate in an acidic environment. Full-length Aβ aggregates most rapidly at pH 4.0–5.7 [27]. Nonetheless, the pH-dependent behavior of $A\beta_{25-35}$, analogous to that of the full-length amyloid, is very difficult to understand, even if the relation between fibrillation and the ionization of the amino acid side chains is justified. The change in fibrillation occurring at pH values around 5.0 probably arises from the titration of an Asp or Glu acid residue. In both molecular dynamic simulations and *in vitro* studies, the pH-dependent electrostatic changes in aspartic/glutamic acid and histidine/lysine residues and the resulting salt-bridging between them (Asp/Glu vs His/Lys) have been described as crucial for amyloidogenesis of $A\beta_{1-40}$, $A\beta_{1-42}$ and other Aβs [27–29]. An intramolecular salt bridge between Lys28 and Asp23, proposed for the fibrils of $A\beta_{1-40}$ [30], is the same intermolecular one in the fibrils of $A\beta_{1-42}$ [31]. In the current study, the fibrillation of $A\beta_{25-35}$ must, therefore, be independent of salt bridges because of the lack of Asp/Glu in the $A\beta_{25-35}$ stretch, and other interactions might be involved in effecting its fibrillation. $A\beta_{25-35}$ contains only one ionizable residue, namely Lys28, and the p*K*a of Lys28 is ≥10.0; thus, the ε-amino group of Lys28 is positively charged within the pH range of 3.0–8.0. The assembly and aggregation of $A\beta_{1-40}$ at pH 10.0 (at which $A\beta_{1-40}$ contains a net negative charge) involves charge–charge repulsion that gives rise to monomers with a random coil together with oligomers and polymers in a β-sheet conformation [32]. It is thus not

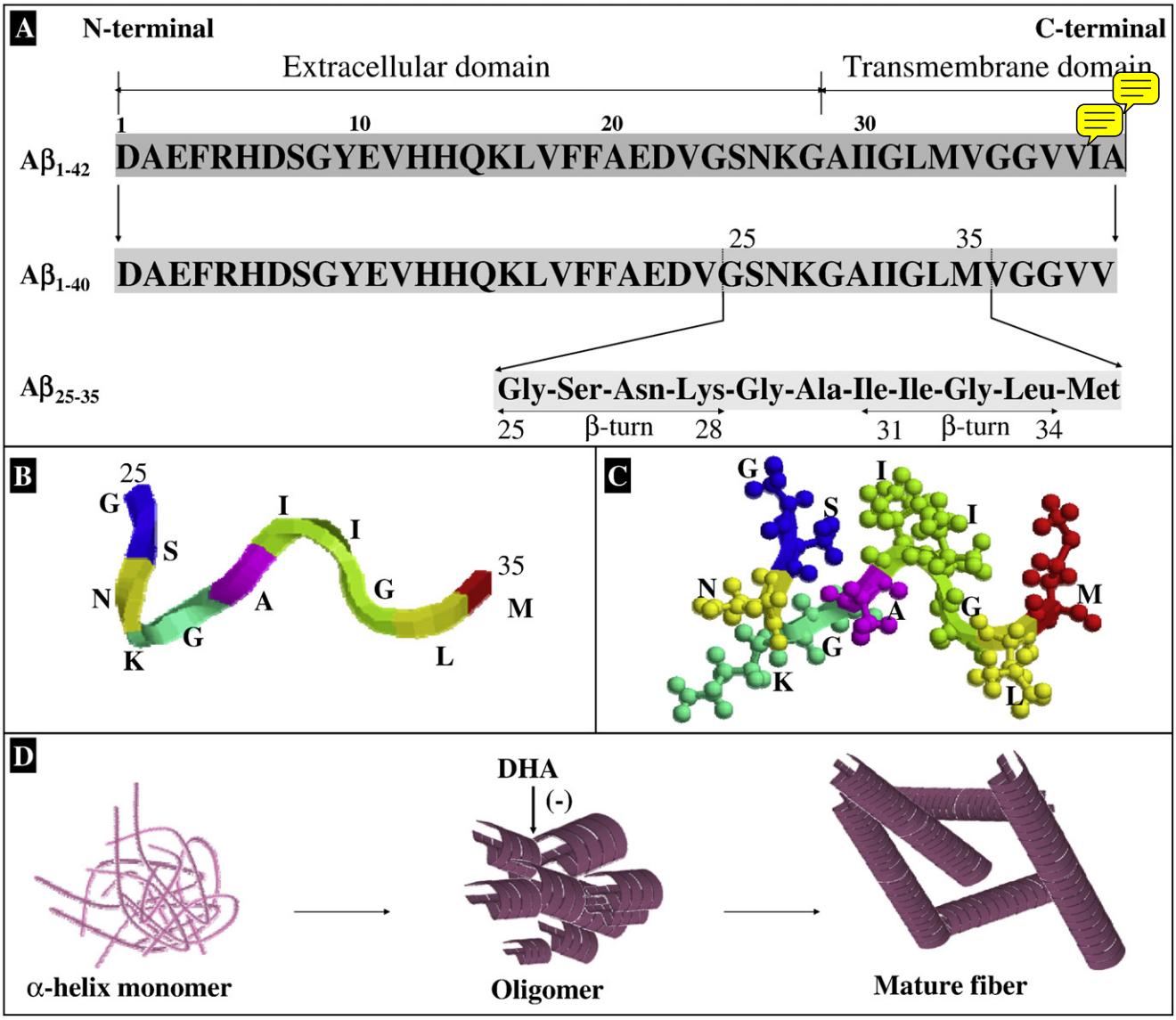

Fig. 8. (A) Short sequence of $A\beta_{25-35}$ (and its parent $A\beta_{1-42}$ or $A\beta_{1-40}$) with the probable localization of the β turn that facilitates its transformation from α-helix into Aβ fibrils. (B) The schema $A\beta_{25-35}$ with its β turn. (C) Ball-stick configuration of the peptide skeleton ((D'ursi et al., [41] pdb code 1qxc, figures were drawn by RasWin, v-3.7.2)). (D) Schematic diagram of fibrillation: the random coiled α-helix Aβ is transformed into β sheets that stack orthogonally by hydrogen bonding and hydrophobic association to form the oligomer, which ultimately extends to mature fibers. DHA, however, disrupts the maturation of the fibers by inhibiting the oligomeric $A\beta_{25-35}$ species.

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unlikely that the presence of net ε-positive charges of Lys28 leads native $A\beta_{25-35}$, at least partially, to a putative orientation that facilitates the formation of a β-sheet and subsequent fibrillation. Lower forces are required to unzip β sheets formed of the Lys28-acetylated $A\beta_{25-35}$ peptide than those formed of the wild type, indicating that Lys28 plays a crucial role in the fibrillation of $A\beta_{22-35}$ [33]; there it is speculated that acetylation weakens the interactions of the β sheets by abolishing the positive charge of the Lys28 ε-amino groups. In this context, $A\beta_{25-35}$ fibrils also have been suggested to be stabilized by the electrostatic interactions between the Lys28 ε-amino group and the C terminus of a neighboring peptide [34]. We do not exclude either of the above two possibilities regarding $A\beta_{25-35}$ fibril formation.

DHA decreased $A\beta_{25-35}$ fibrillation in the current study. Fatty acids in water do not form a micellar phase below pH 9.0 [35]. On the other hand, the concentrations of DHA used (5, 10 and 20 μM) were lower than the critical micellar concentration of DHA (60–90 μM) [36,37]. Thus DHA might not have formed micelles at pH 3.0–8.0 in our assembly buffer. Consequently, the effects of DHA on $A\beta_{25-35}$ fibrillation could not be attributed to the detergent effect of DHA-micelles, which may solubilize the Aβ and disrupt fibrillation. DHA at micellar concentration stabilizes soluble $A\beta_{1-40}$ protofibrils and hinders fibrillation by directly interacting with the Aβ peptide [38]. When the p*K*a value of DHA is 8.5 [38], the free DHA putatively interacts with the positive charges of Lys28, and thereby inhibits fibrillation. The assumption would be true only if the positive charge of Lys28 is involved in $A\beta_{25-35}$ fibrillation and DHA remains negatively charged at the pHs used. Little is known about the influence of pH shifts on the folding of $A\beta_{25-35}$ and about amino acid residues that are directly affected by pH. Residues 31 to 35 (Ile31–Ile32–Gly33–Leu34–Met35) of $A\beta_{25-35}$ adopt a reverse turn conformation, inducing short anti-parallel strands in the surrounding residues, which in turn promote fibril formation [39]. The ILe31 and Leu34 residues of $A\beta_{25-35}$ both show elements of the β-sheet structure [40]. $A\beta_{25-35}$ contains type I β-turn motif located at residues 25–28, analogous to $A\beta_{1-42}$, indicating that $A\beta_{25-35}$ retains the structural features of its parent peptide in similar environments [41]. DHA itself has six double bonds with an ultimate formation of kinks along its axis with a large hydrophobic volume. The α-helix to β-sheet transformation involves interactions of the central hydrophobic regions and those of the C-terminal hydrophobic residues of the full length [28]. It is, thus, also speculated that DHA may intervene between monomers and constrain the planar orientation that leads to the formation of Aβ pleated sheets by amino acid side chains. In so doing, DHA might inhibit β-sheet formation, inter-β-sheet stacking and/or block molecular crowding that drives β-sheets together to form oligomers/protofibrils and, consequently, inhibits the formation of final fibrils. The effects of DHA on β-sheet formation at the amino acid levels of the $A\beta_{25-35}$ skeleton and/or on the staking of β sheets into full fibrils have remained without further predication (Fig. 8).

Mature fibrils pass through intermediate stages at which β units form oligomers or protofibrils. There is active debate about the relative toxicity between oligomers and mature fibrils with regard to AD pathology [21,42,43]. Mature insoluble fibrils being nontoxic, suggesting that toxicity is attributable more to the folded structure than to sequence. At this point we evaluated the effect of DHA on the oligomers prepared for this study. In our study, DHA significantly reduced the level of oligomers, suggesting that the reduction might suppress neurotoxicity and ameliorate its attendant impairment of learning-related ability. This speculation is consistent with studies demonstrating that pretreatment with DHA reduces neuronal apoptosis associated with soluble $A\beta_{1-40}$ peptide [44]. Further studies are under way to determine whether DHA-administration prevents pathological states concurrently with the amelioration of AD-related symptoms. Nonetheless, to obtain further information about oligomers, we determined the size of oligomers prepared from $A\beta_{25-35}$ in the presence or absence of DHA. Soluble oligomers were, however, not visible in reductive 12.5% SDS gel electrophoresis (data not shown), indicating that $A\beta_{25-35}$ oligomers are dissociated and solubilized by reductive conditions. This is qualitatively in agreement with previous studies [45,46], demonstrating that the population of $A\beta_{25-35}$ aggregates, are undetectable by gel electrophoresis because of the small size of $A\beta_{25-35}$; however, the oligomers of molecular size 10 kDa were visualized by nonreductive gradient gel. The band intensities were reduced when fibrillation occurred in the presence of DHA. These results suggest that DHA may inhibit the transformation of oligomers from the β units and the subsequent formation of fibrils, although the mechanisms remain to be further clarified. In addition, the effects of other polyunsaturated fatty acids (particularly arachidonic acid (AA) which comprises, second to DHA, a substantial amount of total polyunsaturated acids in the brain) on the fibrillation by $A\beta_{25-35}$ need to be evaluated as to whether the effects observed on the levels of $A\beta_{25-35}$ oligomer and the subsequent fibrillation are specific to only DHA.

In conclusion, our results suggest that DHA inhibits *in vitro* $A\beta_{25-35}$ fibrillation through the inhibition of oligomeric amyloid species of molecular size 10 kDa. Further studies are needed to determine the outcome of the inhibitory effect of DHA on $A\beta_{25-35}$ fibrillation, particularly under *in vivo* conditions.

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