

Attenuated cytotoxicity but enhanced β -fibril of a mutant amyloid β -peptide with a methionine to cysteine substitution

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Abstract Amyloid- β peptide (A β), the major constituent of senile plaques in the Alzheimer's disease (AD) brain, is the main source of oxidative stress leading to neurodegeneration. The methionine residue in this peptide is reported to be responsible for neurotoxicity. Structurally similar substitution with methionine 35 replaced by cysteine in A β ₄₀ was synthesized, and this result in enhanced β -sheet structures according to both circular dichroism (CD) spectra and β -fibril specific fluorescence assay but attenuated cytotoxicity whether in the presence of copper or not. These findings may provide further evidence on disclosing the connection between amyloid β -aggregation and A β -induced neurotoxicity.

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Keywords: Amyloid- β peptide; Circular dichroism spectra; Thioflavin T; Neuronal cell viability

1. Introduction

A great deal of evidence suggests that amyloid- β peptide (A β) plays a causal role in the development of Alzheimer's disease (AD) pathology, emerging from a variety of genetic, pathological, and biochemical studies [1,2]. A β is a proteolytic product derived from the larger amyloid precursor protein (APP), resulting in the production of A β peptides of varying length: a 40-amino acid A β peptide (A β ₄₀) and a 42-amino acid A β peptide (A β ₄₂) are the two species of most interest, of which A β ₄₀ is the major species [3]. A β has been shown to induce protein oxidation, lipid peroxidation, and reactive oxygen species (ROS) formation in AD brain [4,5]. The binding between A β and metals such as copper and zinc ions accelerates β -aggregation and generates reactive oxygen species (ROS), which in turn accelerates oxidative damage to other proteins [6–8].

Among the proposed mechanisms involving A β -induced oxidative stress, Methionine 35 is thought to be a critical residue

[5,9,10], since it is the most susceptible residue in A β peptide to oxidation, especially under conditions of oxidative stress [11]. A β peptides with Met 35 substituted by either norleucine or cysteine are reported to be non-toxic and unable to induce oxidative stress responses in cells [5]. The substitution of Met 35 in A β (25–35) with Nle, Lys, Leu, and Tyr inhibits aggregation and neurotoxicity of the peptide [7]. It is also reported that A β (1–28) does not induce oxidative stress or neurotoxicity in the absence of methionine [12]. Oxidation of Met 35 in A β (1–40) hinders a conformational transition from random coil to β -sheet and reduces the propensity of the peptide to aggregate to form fibrils [13]. The chemistry of Met in A β (25–35) is very different than that of A β (1–42) for the C-terminal nature of Met in A β (25–35), though both species end up with a sulfuranyl free radical, but by different mechanisms [14]. A β (25–34) that lacks terminal methionine is nontoxic, suggesting a key role of methionine leads to oxidative stress and neurotoxicity [7].

Numerous studies have focused on the structure, aggregate properties [15,16] and fibril formation [17,18], and neurotoxicity [19] of A β peptides and their roles in AD. However, the link between β -aggregation and neurotoxicity is still in dispute, since it is difficult to define whether these fibrillar deposits are the byproducts of neurodegeneration or precede the degenerative process [5]. The β -amyloid cascade hypothesis states that excessive aggregation of A β is of importance in the pathogenesis of AD [2]. Mclean et al. [20] reported a negative correlation between the density of deposits and the severity of the disease, while soluble A β and synaptic damage and cognitive impairment were in good correlativity. Due to the importance of Met 35 in oxidative stress and its possible role as an electron donor for the reduction of A β -bound transitional metals [21], we use a synthetic A β (1–40)M35C to test its aggregate and neurotoxic properties in the presence of copper or not. Our results show that mutation of methionine to cysteine significantly enhances the β -fibril components but impedes the cytotoxicity with practically unchanged total aggregation. These data support for a negative correlation between the density of β -fibril and the severity of the disease.

2. Materials and methods

2.1. Chemical reagents and materials

All cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), thioflavin T (ThT), dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and

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Abbreviations: AD, Alzheimer's disease; A β , amyloid- β peptide; APP, amyloid precursor protein; ROS, reactive oxygen species; CD, circular dichroism; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; WT, wide type; ThT, thioflavin T; OD, optical density; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide

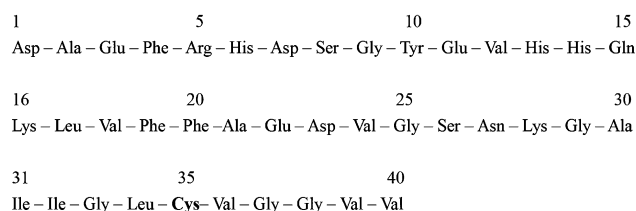


Fig. 1. Synthesized A β (1–40)M35C with a cysteine substitution at position 35.

wide type A β _{1–40} (WT A β ₄₀) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other chemical reagents including CuCl₂ · 2H₂O, L-glycine were obtained from Beijing Chemical Industry (Beijing, China). Prior to use all buffers and stock solutions of copper ions were filtered through a 0.22- μ m filter (Millipore, Cork, Ireland) to remove any particulate matter.

2.2. Peptide preparation

A β (1–40)M35C (³⁵A β ₄₀) (Fig. 1) was synthesized by employing solid phase Fmoc chemistry and produced by Biosynthesis Biotech Co. Ltd. (Beijing, China). After removal from the resin and de-protection, the sample was purified and characterized by reverse phase HPLC using UV/Vis detection.

The lyophilized peptide was initially dissolved in HFIP to a final concentration of 1 mg/ml, shaken for 2 h at 4 °C to entirely dissolve the peptide, then sonicated for 1 min to remove any preformed structures [22]. The stock solution was aliquoted into siliconized tubes and stored at –20 °C. Prior to each experiment, the stock solution was spin-vacuumed using Integrated Speed-Vac System (Thermo Savant) then further diluted either with serum free neurobasal medium or Tris-buffered solutions to a desired concentration. For cortical cultures treated with A β –Cu²⁺ mixtures, Cu²⁺ was present as a solution of one part CuCl₂ to six parts glycine [26].

2.3. Circular dichroism measurements

Circular dichroism (CD) spectra were recorded at 25 °C using a Jasco (Tokyo, Japan) 810 spectrometer. Briefly, the peptide was dissolved in HFIP and spin-vacuumed, then dissolved in 20 mM Tris–HCl (pH 7.4) with or without different concentration of Cu²⁺, and the final concentration of A β in each sample was 50 μ M. Next, each test sample was added into a 1-mm path length cell (Hellma) and scanned. All CD measurements were carried out using the following parameters: 2-nm bandwidth, 20 nm/min run speed, 0.5-nm step size, and 2-s response time. Background values for each test were subtracted from the corresponding CDs of each sample prior to the calculation of CD values. Direct CD measurements (θ , in mdeg) were converted to molar ellipticity, $[\theta]$ (deg cm² mol^{–1}) using the formula $[\theta] = \theta / 10 \times c \times l$, where c represent the molar concentration and l the path length (cm).

2.4. Fluorescence spectroscopy (ThT assay)

The degree of β -aggregation is determined by thioflavin T (ThT) that specifically binds to fibrous structures [8,23]. First, WT A β or ³⁵A β ₄₀ stock solution was diluted with 20 mM Tris–HCl in the presence of copper or not, to a concentration of peptide at 10 μ M. ThT was then added to each test sample to a final concentration of 10 μ M. Each sample was prepared in 96-well Black Cliniplate (Corning) and shaken for 10 s prior to each measurement. Measurements were carried out every 20 min.

The relative degree of β -aggregation was determined in terms of fluorescence intensity, which was measured at 37 °C using a Varioskan multimode microplate spectrophotometer (Thermo) under kinetic fluorometric measurement mode. Measurements were performed at the excitation and emission wavelength of 450 and 485 nm respectively, which resulted in the optimum detection of amyloid fibrils [24]. To account for background fluorescence, the fluorescence intensity measured from each control solution without A β peptides was subtracted from that of each solution containing A β .

2.5. Primary neuronal cultures

Cortical neuronal cultures were performed as described previously [25–27] with some modifications. Briefly, embryonic day 17 Sprague–Dawley rat cortices were dissected free of meninges and cut into 1–2 mm³ tissues, then dissociated in 0.125% (w/v) trypsin in Krebs buffer. Cells were triturated using a filter-plugged fine pipette tip, pelleted, resuspended in plating medium (MEM with 10% FBS and 10% HS). After 2 h the plating medium was replaced with fresh neurobasal medium plus 2% B27 supplements, 1% penicillin–streptomycin, and 0.5 mM L-glutamine. This method resulted in cultures highly enriched for neurons (>95% purity) as determined by immunostaining (data not shown).

The neuronal cells were allowed to mature for 5 d in culture before commencing treatment using freshly prepared serum free neurobasal medium plus B27 supplements minus antioxidants [25,26]. Samples were freshly prepared at Cu²⁺:peptide molar ratios of 0:1 and 1:1 respectively, and coincubated at 25 °C for 15 min, then the mixtures were added to neurons for 96 h [26].

2.6. Cell viability assay

The toxicity of variant A β peptides with or without Cu²⁺ on cell cultures was assessed quantitatively using MTT reduction assay [28,29]. Solutions containing A β alone or A β together with Cu²⁺ were prepared as above. Briefly, the medium was replaced with neurobasal medium plus B27 lacking antioxidants (250 μ l), and 10% MTT (v/v) was added to each well in a 48-well plate then incubated for 4 h at 37 °C with 5% CO₂. Upon removal of the medium, DMSO was added to solubilize the MTT formazan. Absorbance was determined using a μ Quant MQX200 reader (Bio-Tek) at a wavelength of 570 nm and a reference wavelength of 630 nm, and background readings of MTT incubated in cell-free medium were subtracted from each value before calculations. The data were normalized and calculated as a percentage of untreated control values prior to analysis.

3. Results

AD is characterized by a change in the main chain conformation of A β . Therefore, to determine the structure change of peptides induced upon copper binding is of special interest. CD spectra in the far UV region (190–240 nm) were recorded to investigate the structuring of peptides upon Cu²⁺ addition. The spectral curve of WT A β ₄₀ in the absence of Cu²⁺ exhibited a minimum ellipticity at around 200 nm (Fig. 2a), suggesting the secondary structure of A β under these conditions is mainly random coil structure [30]. As Cu²⁺ was titrated in, the spectral curve slightly shifted to 198 nm, and the negative signal reduced in intensity, indicating either the peptide is becoming slighter more orderly, or that the change observed could be due to sample precipitation. To investigate if the effect was resulted from precipitation of A β and thus loss of CD signal, varying concentration of peptide were used. The loss of signal was definitely dependent on the concentration of peptide (data not shown), suggesting the effect is due to precipitation upon addition of copper. Of all the spectra detected, hardly any significant change in structuring of A β ₄₀ could be observed after the addition of 1 mol equivalent of Cu²⁺.

In the contrast, the spectral curve of ³⁵A β ₄₀ alone displayed a minimum at around 213 nm (Fig. 2b), which is a characteristic of β -sheet structure. With the addition of gradient concentration of Cu²⁺, these curves had slightly negative ellipticities around 220 nm and positive ellipticities around 203 nm. This type of curve was especially conspicuous when one mol equivalent Cu²⁺ was contained in the sample. These curves most resemble the type II β -turn conformation, although it is insufficient to make such a conclusion. However, the ³⁵A β ₄₀ spectral curve with addition of copper suggests a non- β -sheet conformation.

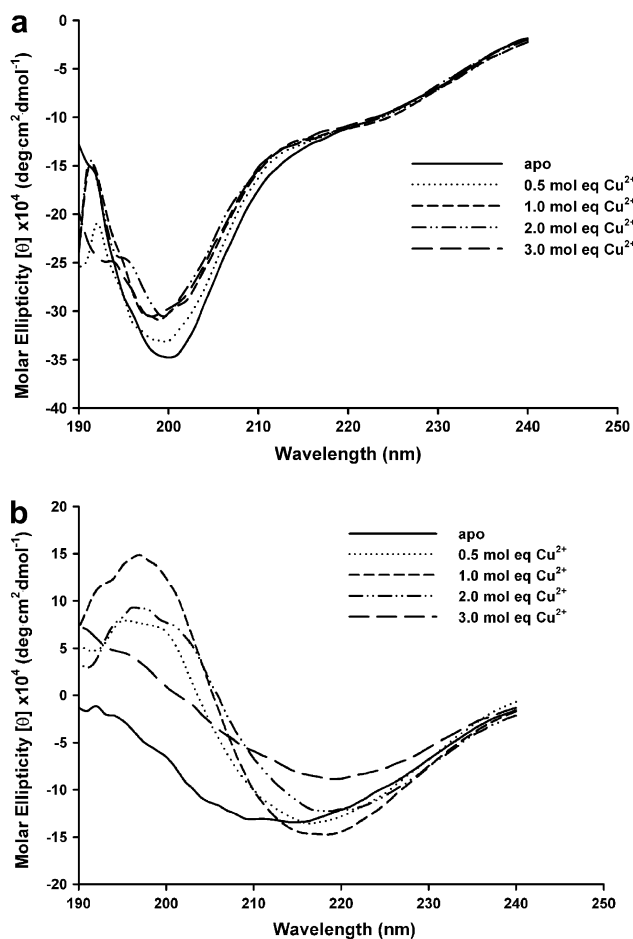


Fig. 2. UV region CD spectra of various A β peptides titrated with Cu $^{2+}$. Molar ellipticity of WT A β_{40} (a) and 35 A β_{40} (b) was recorded in the UV region (190–240 nm) with increasing mol equivalent of Cu $^{2+}$. Variant A β peptide was diluted using 20 mM Tris–HCl (pH 7.4) to a final concentration of 50 μ M in each sample. The data obtained represent for the average of 10 runs.

To further validate the results obtained from CD spectra, we determined the influence of Cu $^{2+}$ on β -sheet structure and total aggregation in A β using the same peptide solutions. By using ThT that specially binds to fibrous structures, we assessed the time course of β -aggregation formed by WT A β_{40} and 35 A β_{40} in the absence or presence of Cu $^{2+}$. Since CD spectra of various peptides exhibited definite changes when one mol equivalent Cu $^{2+}$ was present, we adopted a Cu $^{2+}$:peptide molar ratio of 1:1 here and in the following experiments unless otherwise indicated.

It is interesting to note that 35 A β_{40} produced much higher fluorescence signal than WT A β_{40} , this increase in signal may be due to secondary structure changes of modified A β (35 A β_{40}) leading to a greater binding capacity for ThT or an increase in the total amount of aggregation. In the absence of copper, WT A β_{40} showed higher fluorescence signal suggesting much content of β -aggregation (Fig. 3a), while it is the contrary case to 35 A β_{40} . A decrease in ThT intensity was detected during the whole incubation course, and additional ThT was added to determine if the decreased ThT signal was due to degradation of fluorescent dye or structural alteration. As expected, no markedly increased signal (data not shown) could

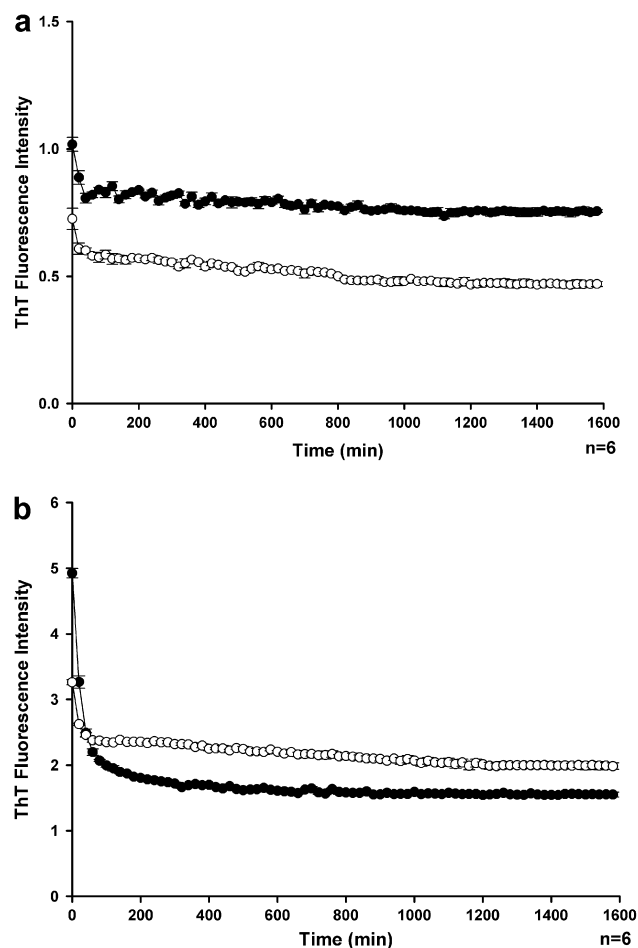


Fig. 3. Kinetic measurements of β -aggregation resulted from incubation of WT A β_{40} (a) and 35 A β_{40} (b) with or without addition of Cu $^{2+}$. Each sample containing 10 μ M A β was incubated with 10 μ M ThT in the absence (●) or presence (○) of 10 μ M copper. Experiments were carried out at pH 7.4. Each point represents the average of six independent experiments. Error bars represent S.E. values.

be observed in the presence of excessive ThT, suggesting a loss of β -aggregation during the time course. Copper induced shifts in the aggregation profile from β -aggregation to amorphous aggregates were evident for WT A β_{40} , whereas more β -aggregation reserved as for 35 A β_{40} in the presence of copper (Fig. 3b). Samples containing WT A β_{40} showed lower ThT fluorescence intensity compared with 35 A β_{40} , whether in the presence of copper or not.

OD $_{214}$ assay was adopted to assess total aggregation of WT A β_{40} and 35 A β_{40} under the same conditions with ThT assay, whether in the presence or absence of copper respectively. The results indicated that WT A β_{40} or 35 A β_{40} contained similar amount of aggregates (data not shown), while a comparatively higher proportion of deposits when copper was present.

In order to determine if there is a direct correlation between the cytotoxicity of A β peptides and the peptides' ability to form amyloid material, WT A β_{40} and 35 A β_{40} at Cu $^{2+}$:peptide molar ratios of 0:1 and 1:1 was added to cortical neuronal cultures to test for the neurotoxicity (Fig. 4). Only samples containing WT A β_{40} exhibited neurotoxicity at 5 μ M, with the Cu $^{2+}$:peptide molar ratio of 1:1 the most toxic. Toxicity was completely rescued with methionine substituted by cysteine

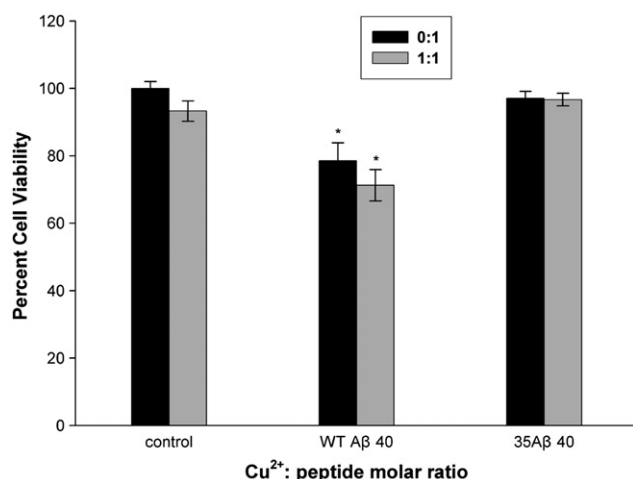


Fig. 4. Cytotoxicity of WT A β_{40} and 35 A β_{40} at variant Cu $^{2+}$:peptide molar ratios. Primary neuronal cells were grown at low density (1.25×10^5 cells/cm 2) for 5 d, then neuronal cells viability following different treatment was determined by measuring the inhibition of MTT reduction. Cortical neurons were exposed to WT A β_{40} or 35 A β_{40} at a final concentration of 5 μ M at Cu $^{2+}$:peptide molar ratios of 0:1 (black bars) and 1:1 (grey bars) for 96 h in serum free media. Statistical comparisons on groups were done using Student's *t*-test. Error bars represent S.E. values. * symbolize $P < 0.01$ versus control. At least three samples were done/group, each experiment was carried out in triplicate.

at position 35, whatever in the presence of Cu $^{2+}$ or not. It therefore appears that conditions leading to the cytotoxicity of A β could be attributed to Met 35. Viability of neurons treated with copper alone was in close similarity with that left untreated.

4. Discussion

After being secreted from APP, A β peptide changes its soluble state to form aggregates, finally deposits as senile plaques, manifested as the main pathological hallmark of AD [1,2]. Senile plaque cores contain abnormally high level of copper and zinc ions [31], suggesting these metal ions may play a significant role in the formation and neurotoxicity of A β fibrils or oligomers. Recently, direct evidence (from EPR and raman microscopy spectroscopy) shows that Cu $^{2+}$ is bound to A β via the histidine bridge within senile plaque cores [26,32].

It is reported that a key stage in the pathogenesis of A β is a structural alteration of the main-chain from the predominantly random structure of soluble A β to a β -sheet rich conformation in senile plaques [33]. CD bands in the amide region (190–240 nm) give information about the peptide bonds and secondary structure of a protein [34]. For this reason, the effect of copper ions on the main-chain conformation is of significant interest. Our CD data from metal-binding of WT A β (1–40) suggest that copper chelation does not induce a profound structural transition or changes in the secondary structure (Fig. 2a). Reduction of CD values in the far UV region can be attributed to Cu $^{2+}$ -induce aggregation thus loss of signal. Taken together with comparisons of variant spectral curves of the peptide with Cu $^{2+}$ titration, our data suggest that binding of the first mol equivalent of Cu $^{2+}$ appears to be sufficient for the A β -Cu $^{2+}$ complex.

Met-35 in the pathogenic activity of A β has been suggested that this residue is necessary for promoting neurotoxicity, aggregation, and the generation of ROS [5,21]. Since metal binding occurs near the N-terminus of A β [26,32], methionine residue could not be expected to have large effects on the metal binding activity. This was confirmed by our CD spectra (Fig. 2b) showing a distinct spectral curve with addition of Cu $^{2+}$, suggesting copper binding activity was unaffected though methionine was replaced by cysteine. WT A β_{40} is predominately random coil [24], whereas the mutant 35 A β_{40} alters the secondary structure of this peptide resulting in a higher proportion of β -sheet structure under the same case. Moreover, the addition of Cu $^{2+}$ to 35 A β_{40} drives a structural transition to presumably β -turn structure, and the spectral curves seem to change all along the copper titration (Fig. 2b), implying a dimensional alteration may occur on 35 A β_{40} structures that affect the binding capability for Cu $^{2+}$. However, the addition of copper to WT A β_{40} does not result in such a structural transition, and β -sheet structures are not observed (Fig. 2a). Significantly, the lack of Met-35 does not change the metal binding properties of A β peptide, which is in agreement with previous finding shows coordination of Cu $^{2+}$ to A β_{1-28} [35].

One controversy in the amyloid hypothesis is whether or not A β plaques are required for toxicity [17,22]. At least two variant aggregation pathways formed for A β : one leads to β -structure aggregate and the other to unstructured aggregate [17]. The β -structured aggregate is mainly composed of A β peptides that have β -sheet secondary structure, but the unstructured aggregate lacks ordered structure, and its constituent peptides are not tightly associated [36]. Results from the ThT and OD assays lead us to hypothesize that, in addition to β -aggregate, another type of aggregate is formed. The idea logically origins from higher β -aggregation contents in A β M35C compared to WT A β_{40} , but almost unaltered overall aggregation. With or without addition of copper hardly exerts any influence on the total aggregate profile. Although CD spectroscopic pattern may suggest that Cu $^{2+}$ induce 35 A β_{40} to take β -turn conformation, more specific method such as NMR would be necessary for the precise identification [24]. However, the reduction of ThT fluorescence of WT A β_{40} with addition of copper or not during the time course (Fig. 3a), is contrary to the previous studies which suggest enhanced fluorescence intensity [24,37]. The reason for these discrepancies is not clear, although they could be the result of several factors such as differences with the handling of A β (lot-to-lot variability) [38] or differences in the solution conditions, such as pH, peptide concentrations, temperature, agitation, time course and the type of peptide employed (A β_{1-40} or A β_{1-42}). As for the final fibrous appearance, WT A β_{40} exhibits higher fluorescence than that observed in the presence of Cu $^{2+}$ (Fig. 3a), which is contrast to β -aggregates formed in sample containing 35 A β_{40} where Cu $^{2+}$ -binding resulted in more β -fibrils upon the overall time course (Fig. 3b). During the time course of β fibril degradation *in vitro*, it appears that steady fluorescence signal is displayed from the time point of 1000 min.

A β_{1-40} has been found in AD brains with high concentrations, and this peptide is highly soluble and toxic [2,3]. The synaptic damage and cognitive impairment associated with AD correlates with the soluble forms of A β , not the deposited forms [20]. Additionally, the *C. elegans* model expressing human A β (1–42) has been used to show that protein oxidation precedes the fibrillar aggregation [39], supporting the role of

small soluble aggregates as the toxic species of A β . The lack of oxidative stress and neurotoxic properties of the norleucine derivative of A β (1–42) but still produce fibrils [9,40,41], also foretell the results of our present study of A β M35C derivative. The deleterious effect of WT A β ₄₀ on neuronal cells is remarkable upon a time course of 96 h, even at a low concentration at 5 μ M (see Fig. 4), and the toxic effect is consistent with former studies [24,25]. Moreover, we do observe a decrease of Cu²⁺-induced β -aggregation in WT A β ₄₀ and thus fibrillogenesis, resulting in enhanced cytotoxicity in primary neuronal cultures quantitated by MTT assay compared to those containing WT A β ₄₀ alone. One explanation for this finding is that A β -Cu²⁺ compound is capable of promoting potentially toxic, pro-oxidative reactions, resulting in the generation of ROS [6,42], though the precise mechanism is unknown. As A β binds Cu²⁺ and reduces it to Cu⁺, methionine is oxidized to sulfoxide [43], thus when Cu²⁺ is present, such involvement is a consequence of the presence and properties of methionine. Since the Cu²⁺-induced toxicity was partly rescued by catalase or antioxidants [42,44], these studies provide further evidence indicating that the enhanced toxicity involving Cu²⁺ supplementation was mediated by ROS production.

The toxic species of A β involving Met is likely the sulfuranyl free radical on methionine, which initiates a chain reaction of propagating free radical reactions involving abstraction of hydrogen atoms from allylic carbons on acyl chains of the lipids, when A β as a small oligomer solubilizes in the lipid bilayer of neuronal membranes [45]. Computational studies showed that the sulfur-centered radical cation of methionine can lead to the generation of α -carbon-centered radical on glycine 33, thus producing hydrophobic species that can initiate peroxidation in the lipid bilayer [46,47]. The basis of how A β species exert their toxic effects *in vivo* is a complex and not yet fully understood subject, although it appears that some form of peptide assembly is an inevitable step [48,49]. Substitution of methionine with cysteine residue, which converts a hydrophobic residue into a more hydrophilic one, can markedly alter the properties of a protein. For example, conformational transition from α -helix to β -sheet structure can occur [50]. In the case of A β ₄₀, the replacement of Met-35 for cysteine has been shown to attenuate the neurotoxic property of the peptides [5]. Our results are consistent with A β M35C markedly altering the structural and neurotoxic properties of A β [5,40], definitively show decreased toxicity to neuronal cells in primary culture, whether in the presence of copper or not (Fig. 4). This finding coupled with former studies using mutant peptides has demonstrated a critical role of methionine in the neurotoxic properties of A β ₄₀ [5,7,10].

Due to the differences between the native peptide and the methionine mutant peptide, including the contents of β -aggregation, and the cytotoxicity of these peptides, it is possible that non- β fibril species are the most likely candidates responsible for the neuronal damage and cognitive impairment observed in the AD brain. Our findings are consistent with the lack of correlation between fibrillar aggregates and oxidative stress and neurotoxicity [39,41]. Furthermore, it has been suggested that toxicity induced by A β can be abolished by converting the oligomeric form to the fibrillar form [51]. In the present study, the mutant peptide A β M35C provides us with a new research tool with its abolished neurotoxicity compared with wide type A β ₄₀. Studies are currently in progress to further elucidate the mechanism by which amyloid plaques are associated with A β -induced cytotoxicity [27,39]. In conclusion, we believe

these results enlighten on mechanistic knowledge of fibrillation reactions [52] to provide a more profound understanding of both cause and effect of A β -mediated cytotoxicity.

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