

Research Article

Correlation of Copper Interaction, Copper-Driven Aggregation, and Copper-Driven H₂O₂ Formation with A β 40 Conformation

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The neurotoxicity of A β is associated with the formation of free radical by interacting with redox active metals such as Cu²⁺. However, the relationship between ion-interaction, ion-driven free radical formation, and A β conformation remains to be further elucidated. In the present study, we investigated the correlation of Cu²⁺ interaction and Cu²⁺-driven free radical formation with A β 40 conformation. The Cu²⁺-binding affinity for A β 40 in random coiled form is 3-fold higher than that in stable helical form. Unexpectedly but interestingly, we demonstrate in the first time that the stable helical form of A β 40 can induce the formation of H₂O₂ by interacting with Cu²⁺. On the other hand, the H₂O₂ generation is repressed at A β /Cu²⁺ molar ratio ≥ 1 when A β 40 adopts random coiled structure. Taken together, our result demonstrates that A β 40 adopted a helical structure that may play a key factor for the formation of free radical with Cu²⁺ ions.

1. Introduction

β -Amyloid (A β) peptide is the main cause of Alzheimer's disease (AD) [1, 2]. A β has two major types, A β 40 and A β 42, which are derived from a ubiquitous type I transmembrane protein—amyloid precursor protein (APP) by a two-step secretase (β - and γ -secretase) pathway [3–5]. The amyloid cascade hypothesis predicts that the aggregated A β peptides in the brain have an early and essential role in the neuronal degeneration that leads to dementia [1, 2, 6]. The aggregation of A β occurs following a conformational conversion from either α -helix or random coil to β -sheet in a time-dependent manner, indicating that the formation of β -sheet structure is the key step for the peptide aggregation [7, 8].

The formation of A β aggregates is modulated by several factors, including metal ions [9, 10]. In amyloid plaques of AD-affected brain, remarkably high concentration of metals,

such as Cu (400 μ M), Zn (1 mM) and Fe (1 mM), has been found [11, 12]. *In vitro* studies, micromolar levels of Cu²⁺, Zn²⁺ and Fe³⁺ have been shown to sufficiently induce protease-resistant aggregation and precipitation of A β [13]. Copper ions are bound to the three His residues of A β located at the N-terminus, which forms a 3N1O square-planar motif as verified by EPR spectroscopy [14]. A β /Cu²⁺ complexes have been demonstrated to generate neurotoxic H₂O₂ from O₂^{•-} through Cu²⁺ reduction [15]. Copper-selective chelators can dissolve A β deposits extracted from AD postmortem brain specimens and reduce the level of free radical [16].

Curtain and his colleagues previously reported that the addition of Cu²⁺ to oligomeric A β 40 in a negatively charged lipid membrane can induce A β peptides reinserted into lipid membranes, convert A β conformation from β -strand into α -helix, and cause the lipid peroxidation [17]. Their result is the first study to show that instead of β -sheet, A β in α -helical

form can also generate free radicals in the presence of Cu ions. Recently, our group has characterized the conformation of A β in the presence of Cu ions into three structural states, stable helix, unstable helix, and random coil, in 40%, 25%, and 5% TFE, respectively, [18]. Therefore, this provides us a possibility to re-examine the relationship between A β conformation, and Cu-driven free radical generation.

In the present study, we examined the correlation between Cu²⁺ binding affinity, H₂O₂ formation, and A β 40 conformation. It was found that both Cu²⁺ binding affinity and H₂O₂ formation are well correlated with the conformation of A β 40. In general, Cu²⁺ ions can be bound to the different structural forms of A β 40. The copper-binding affinity of A β 40 in random coiled form is about 3-fold higher than that of A β 40 in stable helical form. For H₂O₂ formation, our result shows in the first time that the generation of H₂O₂ can also be induced by Cu²⁺ and the stable helical form of A β 40. In contrast, in random coiled form, the formation of H₂O₂ is inhibited by A β 40.

2. Materials and Methods

2.1. Peptide Synthesis, Purification, and Sample Preparation.

The synthesis of full-length A β 40 peptide was performed in an ABI-433A solid-phase peptide synthesizer using the Fmoc protocol. Cleavage and deprotection of the synthesized peptide were performed by treatment with a mixture of trifluoroacetic acid/distilled water/phenol/thioanisole/ethanedithiol. Then the peptide was extracted with 1:1 (v:v) ether:H₂O containing 0.1% 2-mercaptoethanol. The synthesized A β peptides were purified on a reverse-phase C-18 HPLC with a linear gradient from 0% to 100% acetonitrile, and the molecular weight of A β 40 peptides and purity was verified by an MALDI-TOF mass spectroscopy. One mg of purified monomeric A β 40 peptide was dissolved in 1 mL 100% trifluoroethanol (TFE) as stock solution.

2.2. Circular Dichroism (CD) Spectroscopy.

A final A β peptide concentration of 30 μ M in either 5% or 40% TFE was prepared from fresh peptide stock solution diluted in phosphate buffer, pH 7.4, with or without the same concentration of Cu²⁺. A thousand μ M of Cu²⁺ stock solution was prepared by dissolved CuCl₂ salt in distilled H₂O purged by N₂ in a sealed flask and freshly diluted into the designed concentration before it was used. CD spectra were recorded using an Aviv spectropolarimeter equipped with a thermal circulator accessory. All measurements were performed in quartz cells with a path length of 0.1 cm. Data were collected at wavelengths from 190 to 260 nm in 0.2 nm increments. Every CD spectrum is reported as the average from at least three individual samples. The reported CD spectra were corrected using phosphate buffer, pH 7.4 for the baseline. All measurements were carried out at 25.0 \pm 0.2°C.

2.3. Electron Paramagnetic Resonance (EPR) Spectroscopy.

An equal molar 300 μ M of A β and Cu²⁺ in 30% glycerol phosphate buffer, pH 7.4, and 5% and 40% of TFE was employed for spin counting purposes. EPR spectra were obtained at

X-band using a Bruker EMX ER073 spectrometer equipped with a Bruker TE102 cavity and an advanced research system continuous-flow cryostat (4.2–300 K). During EPR experiments, the sample temperature was maintained at 10 K. The microwave frequency was measured with a Hewlett-Packard 5246L electronic counter.

2.4. Tyrosine Fluorescence Spectroscopy.

Tyrosine fluorescence spectroscopy was used to investigate the binding affinity for copper bound to A β . A β peptide stock solution was diluted in phosphate buffer, pH 7.4 to a final peptide concentration of 10 μ M with designed concentration of Cu²⁺. All measurements were performed in a 96-well plate using a microplate reader (FlexStation 3, MD). The excitation and emission wavelengths were selected at 278 and 305 nm, respectively. Reported data were the average obtained from three individual samples and three repeated measurements of each sample.

The binding affinity was calculated using the following equation:

$$\frac{F_x}{F_0} = \frac{I_x - I_\infty}{I_0 - I_\infty} = \frac{1}{1 + K_a [\text{Cu}^{2+}]^n}, \quad (1)$$

where I_0 and I_x are the fluorescence intensity for free and A β /Cu²⁺ complex, respectively, I_∞ is the fluorescence intensity at saturation state, $[\text{Cu}^{2+}]$ is the copper concentration, and n is the copper binding number. The related parameters were fitted using nonlinear curve fitting program Micro-Origin v6.0 (Microcal Software, Inc., Northampton, MA). In the initial fitting stage, the simplex method, which was set to 100 cycle runs, was used to calculate the initial parameter for further nonlinear curve fitting. A 0.95 confidence level was set to constrain the quality of curve fitting. The final fitting parameters were obtained when the value of χ^2 was less than 0.05 and the parameters and errors for the parameters reached the convergent and steady state.

2.5. Hydrogen Peroxide Assay.

The production of H₂O₂ was analyzed using the dichlorofluorescein diacetate (DCFH-DA) assay [19]. Dichlorofluorescein diacetate was dissolved in 100% dimethyl sulfoxide, deacetylated with 50% v/v 0.05 M NaOH for 30 min, and then neutralized (pH 7.4) to a final concentration of 200 μ M as stock solution. This stock solution was kept on ice and in dark until use. The reactions were carried out in Dulbecco's PBS, pH 7.4, in a 96-well plate (100 μ L/well) containing different concentration of A β 40, 30 μ M CuCl₂, TFE (5% and 40%), deacylated DCF (20 μ M), and horseradish peroxidase (5 μ M) at 37°C. For negative control, an extra of 1.0 mM catalase (*Aspergillus niger*) was used to quench H₂O₂. Measurements were performed on the day of sample preparation. Fluorescence readings were recorded on a microplate reader (MD, FlexStation 3), with the excitation and emission wavelengths selected at 485 and 530 nm, respectively.

2.6. Turbidity Assay. UV/Vis turbidity assay was used to detect the aggregation process of A β . 200 μ L of 30 μ M A β 40

in phosphate buffer, pH 7.4 containing either 5% or 40% TFE was freshly prepared from stock solution. Fresh prepared samples with or without 30 μM Cu^{2+} placed in a 96-well plate were incubated at 37°C. Turbidity was measured using a microplate reader (MD, FlexStation 3) at a wavelength of 450 nm.

3. Results

The aim of this study is to examine the relationship between $\text{A}\beta_{40}$ conformation and other physical and chemical properties such as aggregation, Cu-binding affinity, and free radical formation. In the present study, we applied 5% and 40% TFE to mimic the conformation of $\text{A}\beta_{40}$ in random coil and stable helix, respectively, [18]. Therefore, the effect of TFE on Cu^{2+} binding affinity and free radical generation has to be evaluated. As depicted in supplemental data (see Supplementary Material available online at doi:10.4061/2011/607861), the main effect of TFE on the system is only to quench the DCF fluorescence intensity. Neither was the solubility of Cu^{2+} ions nor the formation of H_2O_2 affected by TFE.

3.1. Secondary Structure of $\text{A}\beta_{40}$ and $\text{A}\beta_{40}/\text{Cu}^{2+}$. First of all, we characterized the structural state of $\text{A}\beta_{40}$ with and without Cu^{2+} in either 5% or 40% TFE using circular dichroism (CD) spectroscopy. Figures 1(a) and 1(b) show the CD spectra for $\text{A}\beta_{40}$ and $\text{A}\beta_{40}/\text{Cu}^{2+}$ in 5% and 40% TFE, respectively. It can be seen that, in 5% TFE, the pattern of CD spectrum for $\text{A}\beta_{40}$ in the presence of Cu^{2+} showed a dramatic change compared to that for $\text{A}\beta_{40}$ only, while the CD spectra of $\text{A}\beta_{40}$ with or without Cu^{2+} in 40% TFE did not show any significant change. The secondary structure of $\text{A}\beta_{40}$ in 5% TFE changes from 63% random coil, 34% β -sheet and 3% helix in Cu^{2+} -free state to 45% random coil, 50% β -sheet and 5% helix in Cu^{2+} -bound state, representing that there is a structural conversion from random coil into β -sheet. On the other hand, the conformation of $\text{A}\beta_{40}$ with or without Cu^{2+} in 40% TFE remains stable in which the secondary structure is 74% helix, 14% β -sheet, and 12% random coil in Cu^{2+} -free state and 73% helix, 15% β -sheet, and 12% random coil in Cu^{2+} -bound state, indicating that there is no conformational conversion of $\text{A}\beta_{40}$ in 40% TFE while adding Cu^{2+} ions.

3.2. Correlation of Aggregation and $\text{A}\beta_{40}$ Structure. It has been shown that Cu^{2+} ions are able to accelerate the aggregation of $\text{A}\beta_{40}$ [9, 15, 18, 20]. Hence, we characterized the aggregation process of $\text{A}\beta_{40}/\text{Cu}^{2+}$ and $\text{A}\beta_{40}$ in 5% and 40% TFE. The aggregation process of $\text{A}\beta_{40}/\text{Cu}^{2+}$ and $\text{A}\beta_{40}$ in 5% and 40% TFE was analyzed by turbidity assay as shown in Figure 2. In general, in both 5% and 40% TFE, $\text{A}\beta_{40}$ in the presence of Cu^{2+} was aggregated much faster than that in the absence of Cu^{2+} . Furthermore, the aggregation rate in 5% TFE for both $\text{A}\beta_{40}/\text{Cu}^{2+}$ and $\text{A}\beta_{40}$ was much faster than that in 40% TFE. The aggregation process in 5% TFE for $\text{A}\beta_{40}/\text{Cu}^{2+}$ and $\text{A}\beta_{40}$ showed a typical sigmoidal profile. On the other hand, the aggregation process in 40% TFE for $\text{A}\beta_{40}$ with and without Cu^{2+} mostly stayed in the nucleation or the

early elongating stage within 24 hrs. The aggregation process for $\text{A}\beta_{40}$ and $\text{A}\beta_{40}/\text{Cu}^{2+}$ in 5% and 40% TFE echoes with the result obtained in the analyses of secondary structure in which the aggregation ability is correlated with the ability of conformational formation of β -sheet [8].

3.3. EPR Spectroscopy of $\text{A}\beta_{40}/\text{Cu}^{2+}$ in 5% and 40%. In the present study, we showed that the conformational states for $\text{A}\beta_{40}/\text{Cu}^{2+}$ and $\text{A}\beta_{40}$ in 5% are dramatically different, while no such conformational change can be observed between $\text{A}\beta_{40}/\text{Cu}^{2+}$ and $\text{A}\beta_{40}$ in 40% TFE. Although Cu^{2+} ions have been shown to be bound to $\text{A}\beta$ in aqueous solution [14], then, whether Cu^{2+} ions are bound to $\text{A}\beta_{40}$ in 40% TFE is needed to be investigated since the structural state for $\text{A}\beta_{40}/\text{Cu}^{2+}$ and $\text{A}\beta_{40}$ in 40% TFE shows no difference. To address this issue, EPR spectroscopy was applied to explore the interaction between Cu^{2+} and $\text{A}\beta_{40}$ in 40% TFE. As shown in Figure 3, the EPR spectra of $\text{A}\beta/\text{Cu}^{2+}$ both in 5% and 40% TFE showed two major groups of hyperfine peaks, while no hyperfine peak could be observed in the EPR spectrum for Cu^{2+} alone, indicating that Cu^{2+} ions were bound to $\text{A}\beta_{40}$ in both 5% and 40% TFE. The pattern of hyperfine peaks for $\text{A}\beta_{40}/\text{Cu}^{2+}$ in 5% and 40% TFE is similar, but the position of hyperfine peaks in 40% TFE shifts to low magnetic field. The related EPR parameters of g_{\parallel} , A , and g_{\perp} are 2.28, 156.8, and 2.06 for $\text{A}\beta_{40}/\text{Cu}^{2+}$ in 5% TFE, respectively. The g_{\parallel} and g_{\perp} values in 5% TFE are very close to those in the literature measured in aqueous condition [14], representing that the binding geometry of $\text{A}\beta_{40}/\text{Cu}^{2+}$ in 5% TFE should adopt a similar 3N1O coordination. On the other hand, the related EPR parameters of g_{\parallel} , A , and g_{\perp} are 2.40, 119.6, and 2.09 for $\text{A}\beta_{40}/\text{Cu}^{2+}$ in 40% TFE, indicating that the copper-binding geometry is different from the 3N1O mode and may be locally distorted in 40% TFE. Meanwhile, the A value in 5% TFE is larger than that in 40% TFE. This further indicates that the binding affinity of Cu^{2+} to $\text{A}\beta_{40}$ in random coiled form may be stronger than that to $\text{A}\beta_{40}$ in stable helical form.

3.4. Cu^{2+} -Binding Affinity in 5% and 40% TFE. As shown in EPR studies, Cu^{2+} can be bound to different structural forms of $\text{A}\beta_{40}$ with a different binding affinity. In order to further elucidate the binding property of Cu^{2+} , we applied the tyrosine fluorescence spectroscopy to determine the Cu^{2+} -binding constant, since tyrosine 10 is the only fluorophore in $\text{A}\beta$ amino acid sequence and locates at the binding pocket, in which the binding of Cu^{2+} to $\text{A}\beta_{40}$ may cause the change of tyrosine fluorescence.

The titration curves of Cu^{2+} concentration versus tyrosine fluorescence intensity in 5% and 40% TFE are shown in Figures 4(a) and 4(b), respectively. The binding constants, K_a , were estimated using the equation as described in the experimental section with molar ratio of $\text{A}\beta:\text{Cu}^{2+} = 1:1$, that is, $n = 1$. The calculated K_a values are 0.14 μM^{-1} ($R^2 > 0.97$) and 0.05 μM^{-1} ($R^2 > 0.96$) for $\text{A}\beta/\text{Cu}^{2+}$ in 5% and 40% TFE, respectively. The K_a of $\text{A}\beta_{40}/\text{Cu}^{2+}$ in 5% TFE is around 3-fold higher than that in 40% TFE, suggesting that Cu^{2+} is bound to random coiled form of $\text{A}\beta_{40}$ more strongly than to

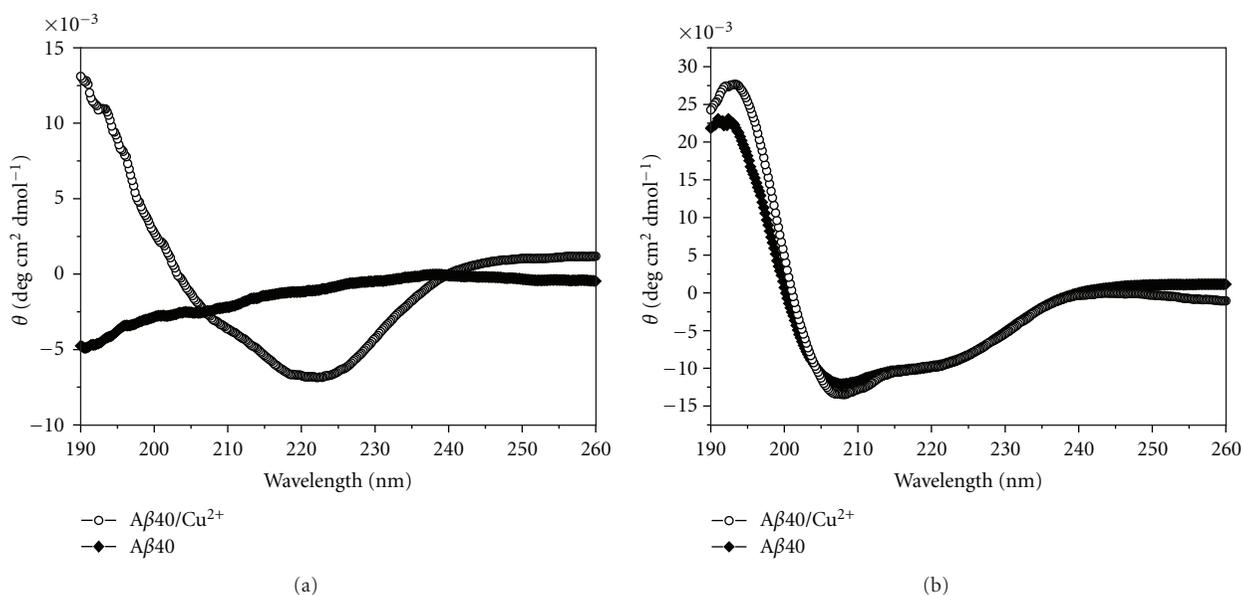


FIGURE 1: Circular dichroism spectra of A β 40 peptide with or without Cu $^{2+}$ in (a) 5% TFE and (b) 40% TFE. The concentration for both A β 40 and Cu $^{2+}$ used in measurements was 10 μ M. It can be seen that the conformation of A β 40 in 5% TFE shows a dramatic change in the presence of Cu $^{2+}$, while the conformation of A β 40 in 40% TFE remains unchanged in the presence or absence of Cu $^{2+}$.

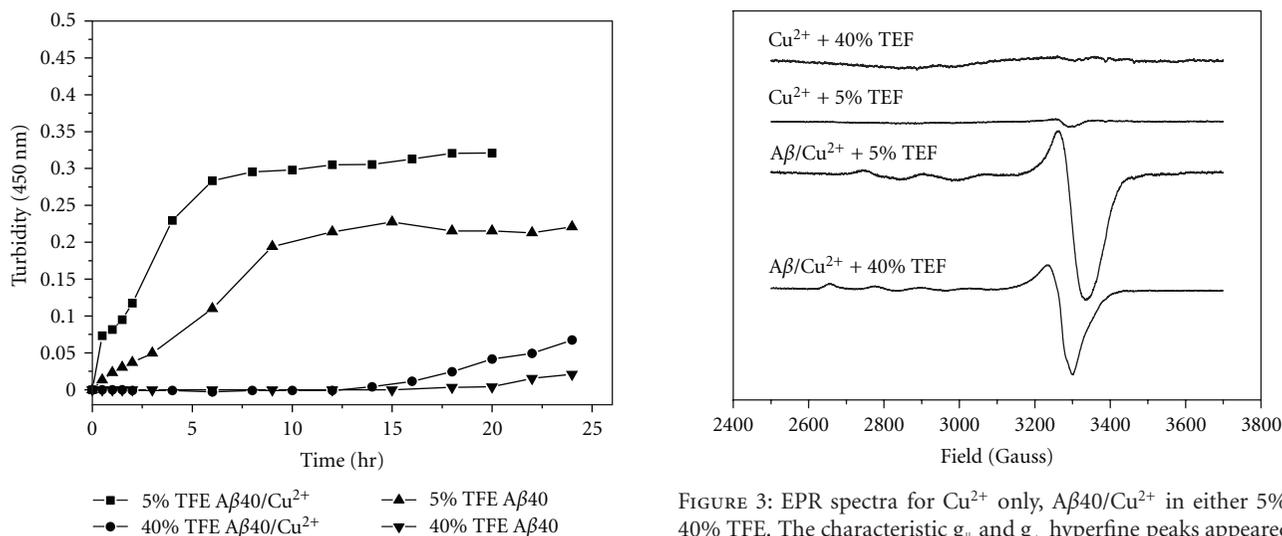


FIGURE 2: The aggregation process of A β 40 and A β 40/Cu $^{2+}$ complex in 5% and 40% TFE measured by turbidity assay. (■) A β 40/Cu $^{2+}$ in 5% TFE, (▲) A β 40 in 5% TFE, (●) A β 40/Cu $^{2+}$ in 40% TFE, and (▼) A β 40 in 40% TFE.

stable helical form of A β 40 which is consistent with the EPR studies.

3.5. Hydrogen Peroxide Formation in 5% and 40% TFE. It has been demonstrated that A β 40 coordinated with Cu $^{2+}$ can cause the reduction of Cu $^{2+}$ and then induce the formation of H $_2$ O $_2$ from O $_2$ in a catalytic manner [15, 20]. To examine the relationship between free radical formation and A β 40

FIGURE 3: EPR spectra for Cu $^{2+}$ only, A β 40/Cu $^{2+}$ in either 5% or 40% TFE. The characteristic g_{\parallel} and g_{\perp} hyperfine peaks appeared in 5% TFE represent that copper ion coordinates with A β 40 in a 3N1O mode. The characteristic bands in 40% TFE are slightly shift downfield compared with the hyperfine peaks appeared in 5% TFE, suggesting that A β 40 adopts different conformation in 5% and 40% TFE.

conformation, DCF assay was used to detect the formation of H $_2$ O $_2$. Figures 5(a) and 5(b) show the plots of DCF fluorescence intensity versus A β concentration in 5% and 40% TFE as incubated at 37°C for 1 hr.

In general, results clearly show that the formation of H $_2$ O $_2$ is also correlated with A β 40 conformation. In 5% TFE, the H $_2$ O $_2$ level was decreased with an increase of A β 40 concentration. The level of H $_2$ O $_2$ was reduced to zero when

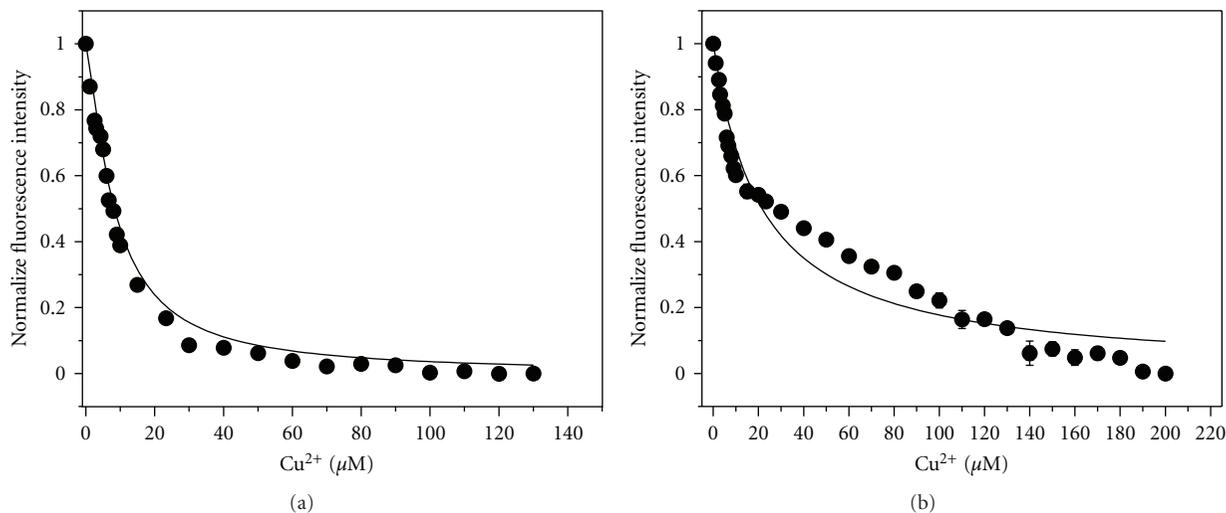


FIGURE 4: The binding affinity of Cu^{2+} to $\text{A}\beta_{40}$ peptide in (a) 5% TFE and (b) 40% TFE measured by tyrosine fluorescence spectroscopy. Insets in (a) and (b) show the Jot plot. The concentration of $\text{A}\beta_{40}$ was $10 \mu\text{M}$. The solid lines represent the fitting curve using the equation as depicted in Section 2 with $n = 1$. The R^2 values in (a) and (b) are all >0.96 , and the χ^2 values are <0.05 .

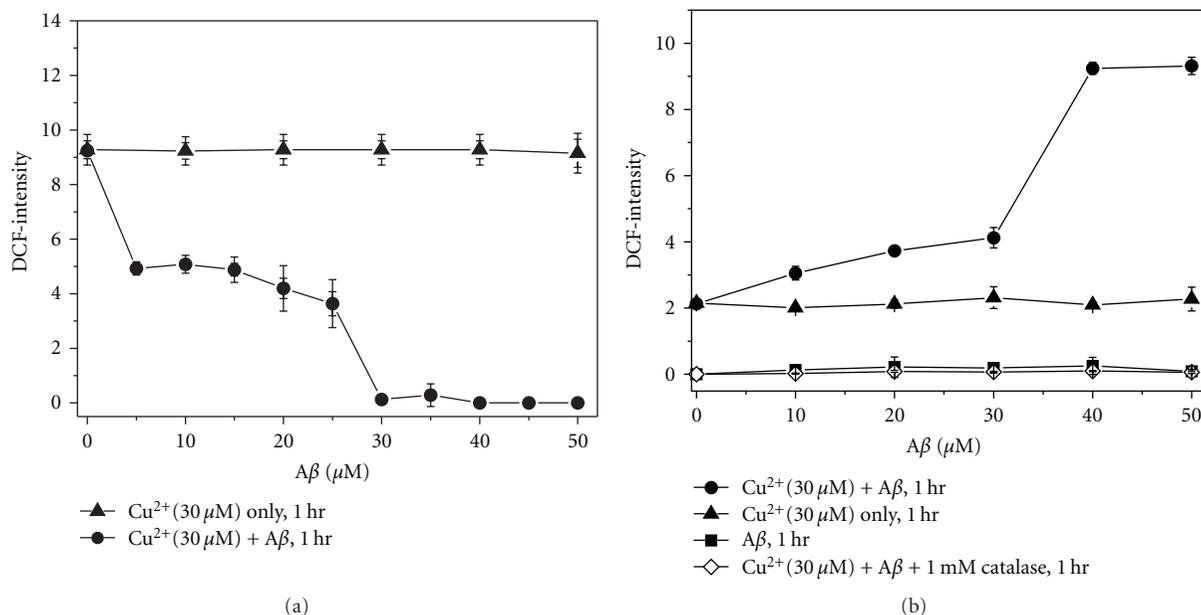


FIGURE 5: H_2O_2 generation assay for $\text{A}\beta_{40}$ in (a) random coiled state (5% TFE) and (b) stable helical state (40% TFE). In (a), instead of generating free radicals, $\text{A}\beta_{40}$ shows to inhibit the generation of free radical. (\blacktriangle) $30 \mu\text{M}$ Cu^{2+} alone and (\bullet) titration of $\text{A}\beta_{40}$ with $30 \mu\text{M}$ Cu^{2+} . On the other hand, in stable helical structure (b), $\text{A}\beta_{40}$ produces free radical with copper, in (\blacktriangle) $30 \mu\text{M}$ Cu^{2+} alone, (\blacksquare) $\text{A}\beta_{40}$ alone, (\bullet) titration of $\text{A}\beta_{40}$ with $30 \mu\text{M}$ Cu^{2+} , and (\diamond) $\text{A}\beta_{40}$ with $30 \mu\text{M}$ Cu^{2+} and 1 mM Catalase. All measurements were incubated at 37°C for 1 hour.

the $\text{A}\beta_{40}/\text{Cu}^{2+}$ molar ratio ≥ 1 , indicating that the formation of H_2O_2 is inhibited when $\text{A}\beta_{40}$ adopts the random coiled form. Unexpectedly but interestingly, unlike the formation of H_2O_2 which was inhibited in 5% TFE, the level of H_2O_2 in 40% TFE was increased with an increase of $\text{A}\beta_{40}$ concentration and was much higher than those of either Cu^{2+} or $\text{A}\beta_{40}$ alone, suggesting that the formation of H_2O_2 was induced when $\text{A}\beta_{40}$ was in the stable helical form.

4. Discussion

According to amyloid cascade hypothesis, aggregated $\text{A}\beta$ is the main toxic species to cause the Alzheimer's disease [6]. The various forms of $\text{A}\beta$ aggregate have been shown to coordinate with redox active transition metals, such as Cu^{2+} and Fe^{3+} , and induce the generation of reactive oxygen species [15, 20]. However, the correlation of copper-binding

affinity, copper-driven aggregation, and free radical formation with A β 40 conformation still needs to be elucidated.

For ion interaction, our results demonstrate for the first time that Cu²⁺ ions can interact with both random coiled and stable helical forms of A β 40. As a larger A value and binding constant obtained for A β 40 in 5% TFE, the Cu²⁺-binding affinity for random coiled form of A β 40 is much stronger than that for stable helical form of A β 40. The possible explanation may be attributed to the different flexibility of A β 40 conformation in 5% and 40% TFE. As shown in CD spectra, the conformation of A β 40 in 5% TFE is relatively flexible and can easily convert structure from random coil into β -sheet while coordinating with Cu²⁺. On the other hand, the overall conformation of A β 40 with or without Cu²⁺ is relatively rigid and remains in stable helix in 40% TFE, and only the local geometry of Cu²⁺-binding site is distorted while interacting with Cu²⁺ ions as indicated by the shift of g_{||} value. Therefore, the interaction between Cu²⁺ and A β 40 is strong in 5% TFE and relatively weak in 40% TFE. The binding affinity for stable helical form of A β 40 is almost 3-fold lower than that for random coiled form of A β 40.

The Cu²⁺-binding affinity can further be used to account for the differences for Cu²⁺-driven aggregation between random coiled and stable helical forms of A β 40. It is well known that the formation of β -sheet plays a key step for the aggregation of A β 40 [8]. As shown in the present study, the conformation of A β 40 in 5% TFE is much easier than that in 40% TFE to convert into β -strand structure by Cu²⁺ ions. Thus, in the presence of Cu²⁺, the random coiled form of A β 40 (in 5% TFE) aggregates faster and more severe than the stable helical form of A β 40 (in 40% TFE).

Unlike the cascade hypothesis that only β -sheet form of aggregated A β can produce free radical, we demonstrate that helical form of A β 40 can also induce the formation of H₂O₂. From the aggregation assay, this helical form of A β 40 may possibly exist as a monomer during the early incubation period. Then, this further implies that monomeric A β 40 in stable helical form by coordinating with Cu²⁺ can induce the formation of H₂O₂. In contrast, A β 40 in random coiled form shows to inhibit the formation of H₂O₂ during the early incubation period. The inhibition of H₂O₂ formation becomes more significant with an increase of A β 40 concentration, and the formation of H₂O₂ further is completely inhibited when the molar ratio of A β /Cu²⁺ molar ratio ≥ 1 . Our observation is consistent with a recent study by Viles and his colleagues, in which they showed that, at A β /Cu²⁺ molar ratio = 1, the formation of H₂O₂ is inhibited by both monomeric and fibrillar A β peptides [21]. Taken together, it may suggest that helical structure may play a key factor for the generation of H₂O₂ by A β 40/Cu²⁺.

In summary, our present results demonstrate that both ion interaction and copper-driven free radical formation are well correlated with A β 40 conformation. When A β 40 adopts a stable helical structure, the Cu²⁺ binding affinity is weaker, and the free radical is produced. On the other hand, when A β 40 adopts a random coil in 5% TFE, the Cu²⁺ binding affinity is stronger, and the formation of H₂O₂ is inhibited.

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