Journal of Inorganic Biochemistry 190 (2019) 15–23

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Nitration of amyloid-β peptide (1–42) as a protective mechanism for the amyloid-β peptide (1–42) against copper ion toxicity

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ARTICLE INFO

Keywords:
Alzheimer's disease
Amyloid-β peptide
Copper ion
Tyrosine nitration

ABSTRACT

It is known that copper ion (Cu(II)) binds to amyloid-β peptide (Aβ), induces Aβ oligomer formation and ultimately exacerbates Aβ-aggregation neurotoxicity in Alzheimer's disease (AD). It becomes interesting to know that how this chemical modification of Aβ would affect interaction of Aβ and Cu(II) and their roles in the development of AD. In this work, we investigated the interaction of $Aβ_{1–42}$ nitration with the toxic Cu(II). It showed that Cu(II)induced $Aβ_{1–42}$ nitration in the presence of nitrite and hydrogen peroxide. Circular dichroism studies also revealed significant conformational change of $Aβ_{1–42}$ and Tyr10 nitrated amyloid-β peptide(1–42) ($Aβ_{1–42}$NT) when interacting with Cu(II). Even though nitration did not alter the binding of $Aβ_{1–42}$ to Cu(II) or the peroxidative activity of $Aβ_{1–42}$-Cu(II) complex, nitration ameliorated the aggregation and neurotoxicity of $Aβ_{1–42}$ induced by Cu(II), which was also further confirmed by the cell study. Given our previous findings that Aβ nitration dramatically inhibited its aggregation and thus reduced its toxicity, we speculated that nitration of $Aβ_{1–42}$ altered its intermolecular interaction, which protected itself against the toxicity of Cu(II). Based on this hypothesis, we propose that nitration of $Aβ_{1–42}$ may be an important protective mechanism for normal function of $Aβ_{1–42}$ and deserves more attention in AD drug development.

1. Introduction

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases [1]. Its pathophysiology involves oxidative stress and extensive deposition of extracellular amyloid plaques that consist of predominantly aggregated amyloid-β peptide (Aβ) [2,3]. Aβ, a peptide of 39 to 43 amino acids, is the product of the sequential cleavage of amyloid precursor protein (APP) by α- and β-secretases, and its most abundant forms are $Aβ_{1–40}$ and $Aβ_{1–42}$ [4–6]. Although $Aβ_{1–42}$ only differs two residues with $Aβ_{1–40}$, it is more inclined to aggregate and thus more pathogenic compared to $Aβ_{1–40}$ [7–9]. Soluble Aβ might aggregate into oligomers, protofibrils and mature fibrils, and even into senile plaques under pathological condition [10]. However, there is a poor correlation between the amount of senile plaques and cognitive deficits in the AD brain [11,12]. Instead, researches tend to suggest that Aβ oligomers may be the most neurotoxic to neurons and responsible for synaptic dysfunction and memory loss in AD [13,14]. It has been reported that copper ion (Cu(II)) binds strongly to Aβ, and the complex promotes the formation of Aβ oligomers and consequently elevates Aβ neurotoxicity in vitro [15–17]. Moreover, Cu(II) has been found abnormally high in senile plaques. Increasing evidence indicates that Cu (II) plays an important role in the metabolism and aggregation of Aβ in the development of AD [18–20]. Hence, it suggests a new strategy by disrupting the interaction of Aβ, which alleviates the Aβ oligomerization, for the prevention of AD development. Unfortunately, drugs designed on this assumption, as well as the aggregated-Aβ-targeted drugs, failed at clinical trials [21,22]. It generates skepticism that the view of considering Aβ, Cu(II) and the Aβ-Cu(II) complexes as the enemies tout court could be wrong [23]. In fact, synthetic monomer of $Aβ_{1–42}$ has been found to have a rescuing effect in both trophically deprived neurons and neurons undergoing excitotoxic death [24]. Accumulated evidence also suggests that Aβ may possess some important physiologic functions [25]. Therefore, there must be an unveiled mechanism to prevent Aβ from aggregating into toxic oligomers that impairs neurons.

Interestingly, our recent studies found that nitration of tyrosine (Y10) in Aβ could significantly inhibit the aggregation of Aβ and reduce its neurotoxicity by altering its intermolecular interaction. The nitration of Y10 in Aβ seemed a compensatory reaction against oxidative/

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https://doi.org/10.1016/j.jinorgbio.2018.10.005
Received 25 July 2018; Received in revised form 12 October 2018; Accepted 13 October 2018
Available online 16 October 2018

nitrative stress and Aβ aggregation [26,27]. Moreover, Kummer et al. found that nitrated Aβ was detected in the core of Aβ plaques of APP/PS1 mice and AD brains [28]. It indicated the existence of Aβ nitration in vivo. It is already known that Aβ-Cu(II) complex is capable to catalyze peroxynitrite production in the presence of hydrogen peroxide and nitrite [29]. The production of peroxynitrite may induce nitration of tyrosine (Tyr 10) in in Aβ that is close to the binding site of Cu(II) [30–32].It explains the toxicity of Cu(II) in the Aβ-associated development of AD. As aforementioned that nitration of Y10 in Aβ exhibits significant effect on its aggregation and neurotoxicity, it becomes interesting to know that how the Aβ nitration would affect the Cu(II)-induced Aβ toxicity. In this study, we investigated the differences between synthetic $Aβ_{1–42}$NT and $Aβ_{1–42}$ in the Cu(II)-mediated neurotoxicity. Our findings provide novel insights into the relationship between Cu(II)and $Aβ_{1–42}$ in the development of AD.

2. Methods

2.1. Materials

$Aβ_{1–42}$, Tyr10 nitrated amyloid-β peptide (1–42) ($Aβ_{1–42}$NT), $Aβ_{1–16}$ and Tyr10 nitrated amyloid-β peptide (1–16) ($Aβ_{1–16}$NT) (~95%, lyophilized powder) were purchased from China Peptides (Shanghai, China). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bicinchoninic acid (BCA), N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonicacid) (HEPES), desferrioxamine and coumarin-3-carboxylic acid (3-CCA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). All other reagents were analytical grade.

2.2. Preparation of Aβ monomer solution

$Aβ_{1–42}$ and $Aβ_{1–42}$NT were solubilized in HFIP overnight to remove pre-existing aggregates. Then HFIP was evaporated in vacuum, and the obtained peptide film was stored at −20 °C. The peptide film was dissolved to 5 mM in 10 mM NaOH solution and further diluted to desired concentration with 20 mM phosphate buffer solution (PBS), pH 7.4, prior to use. $Aβ_{1–16}$ and $Aβ_{1–16}$NT were dissolved to 1 mM in deionized water without pretreatment. Then the solution was diluted to required concentration with 20 mM PBS, pH 7.4.

2.3. Dot blot immunoassay

To study the nitration of $Aβ_{1–42}$ by Cu(II), 50 μM $Aβ_{1–42}$ was incubated with 50 μM Cu(II), 1 mM nitrite and 1 mM hydrogen peroxide in 20 mM PBS, pH 7.4, at 37 °C for 6 h. After 6 h incubation, 3 μl sample was transferred to nitrocellulose membrane. A rabbit polyclonal antibody against 3-nitrotyrosine was used to detect the nitrated Aβ.

2.4. Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) spectra of the Aβ and Aβ-Cu(II) complex were recorded on a time-of-flight high-resolution mass spectrometer (micrOTOF-II BRUKER), equipped with a conventional ESI source. The instrumental parameters were as follows: dry gas, nitrogen; temperature, 180 °C; scan range, *m/z* 400–3000, and capillary voltage, 4500 V. The scan range was m/z 400–3000. For ESI-MS experiment, stock solution of Aβ peptide was diluted to 100 μM in deionized water with 80 μM Cu(II) added.

2.5. Electron paramagnetic resonance spectroscopy

The X-band electron paramagnetic resonance (EPR) spectrum was recorded on a JEOL JES-FA200 X-band ESR spectrometer (JEOL, Tokyo, Japan). Experimental conditions were follows: microwave frequency, 9.15 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; temperature, 120 K; sweep time, 32 s; time constant, 100 ms; 3 averages. For EPR experiment, stock solution of Aβ peptide was diluted to 1 mM in deionized water with 0.8 mM Cu(II) added. The samples were rapidly transferred to EPR tubes and frozen immediately in liquid nitrogen. EPR data was processed and analyzed using the method described by Peisach and Blumberg [33].

2.6. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was carried out on a MicorCal iTC-200 microcalorimeter (Northampton, MA) at 25 °C. The reaction cell contained 60 μM peptide in 20 mM HEPES buffer at pH 7.4 with the ionic strength adjusted to 160 mM with NaCl. The buffer was chosen because of its negligible interference with Cu(II). The injection syringe contained 700 μM Cu(II) with 4 equivalent glycine in a matching buffer solution same to the reaction cell. All solutions were degassed under vacuum for 15 min prior to use. For the experiment, 3 μl 700 μM Cu(II) were titrated into 200 μl 60 μM Aβ peptide over 4 s with a 150 s interval between each injection. Thirteen injections were made in total. In addition, the stir speed was kept constant at 1000 rpm to achieve homogeneous mixture in the cell. ITC data analysis was performed by using one-site binding model in Origin 7.0 (Microcal). A non-linear least squares method was used to obtain the apparent association constant K, the number of binding sites n, and the change of enthalpy ΔH.

2.7. Gel electrophoresis analysis of Aβ

Aβ peptide (50 μM) were incubated in the presence or absence of Cu (II) (50 μM) at 37 °C for 3 days. Then the samples were mixed with loading buffer and resolved in Nu-PAGE 4%–12% Bis-Tris Protein Gels (invitrogen) with 2-(Nmorpholino) ethanesulfonic acid running buffer. Finally, the peptide was visualized by silver staining [34].

2.8. Circular Dichroism spectroscopy (CD)

Aβ samples were prepared by incubating with or without equimolar Cu(II) in 5 mM PBS, pH 7.4, at 37 °C for 12 h. The spectral region was recorded from 200 to 260 nm, with a 1 nm bandwidth, on a JASCO circular dichroism spectrometer at room temperature. A cuvette cell with 1 mm path length was used. The scanning speed was 100 nm/min and the spectra represented the average of three scans. The relevant baseline was subtracted by running PBS alone or PBS containing Cu(II) as a blank.

2.9. Measurements of hydroxyl radical

Hydroxyl radical generation was detected by 3-CCA assay. Nonfluorescent 3-CCA can be converted to highly fluorescent 7-OH-CCA by hydroxyl radical [35,36]. The reaction was started by the addition of ascorbate (300 μM) to the samples containing Cu(II) (10 μM) and peptide (10 μM). All measurements were performed in 20 mM PBS, pH 7.4, containing 1 mM 3-CCA. In this experiment, desferrioxamine was supplemented to remove trace metals in PBS. After 1 h incubation at 37 °C, samples were measured using a fluorescence spectrometer with excitation at 395 nm and emission at 450 nm.

2.10. Cell culture

SH-SY5Y cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicilin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.11. MTT assay

Cell viability was determined by MTT assay, which measured the ability of metabolically active cells to form a formazan through cleavage of the tetrazolium ring of MTT [37,38]. The cells were seeded in 24-well plate and cultured in DMEM with 10% FBS for 24 h. Then culture medium was replaced with fresh medium without FBS, and cells were treated with 10 μM Aβ peptide or 10 μM Aβ-Cu(II) complex at 37 °C for 48 h. Prior to use, Aβ peptide and Aβ-Cu(II) complex were incubated at 37 °C for 24 h to form oligomers. Finally, cells were treated with MTT solution for 4 h. Then DMSO was added to dissolve the intracellular formazan products. Absorbance values of formazan were determined at 570 nm with a microplate reader. Results were normalized with the control.

2.12. Data analysis

All of the experiments were repeated at least three times. The results were expressed as the means ± SD. Student's *t*-test was used for statistical analyses and $p < 0.05$ was considered significant.

3. Results

3.1. Nitration of $A\beta_{1-42}$ by Cu(II)

It has been reported that $A\beta_{1-42}$ could bind to Cu(II) through residues of His6, His13 and His14 [29]. The Aβ-Cu(II) complex would catalyze peroxynitrite production in the presence of hydrogen peroxide and nitrite [31,32]. Consequently, Y10 in $A\beta_{1-42}$ may subject to the Cu(II)-catalyzed nitration as it is close to binding site. In Fig. 1, we saw that $A\beta_{1-42}$ was significantly nitrated after treated with hydrogen peroxide, nitrite and Cu(II). In contrast, no nitration was detected in the control group that had no Cu(II). The result indicated that Cu(II) catalyzed the nitration of $A\beta_{1-42}$ in the presence of hydrogen peroxide and nitrite. It has been reported that microglia and astrocytes can produce reactive oxygen species in brain tissue [39]. And the appearance of nitrite is supported by the fact that it is the stable downstream product of nitric oxide, which is a key signaling molecule in the regulation of cerebral blood flow [40]. Thus, the catalytic reaction of Cu(II) with hydrogen peroxide and nitrite in vitro supported the notion of Aβ nitration by Cu(II) in vivo.

3.2. Effect of Aβ nitration on its binding to Cu(II)

Evidences have shown that His6, His13 and His14 play important roles in the interaction between Cu(II) and Aβ [30]. To investigate the effect of $A\beta_{1-42}$ nitration on its binding to Cu(II), three independent methods, e.g. ESI-MS, EPR and ITC, were used. In this experiment, $A\beta_{1-16}$ peptide was used instead of $A\beta_{1-42}$ for the following reasons: 1) $A\beta_{1-16}$ was more soluble even in moderate concentration; and 2) $A\beta_{1-16}$

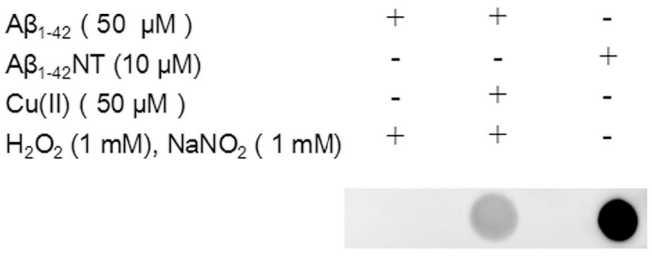

Fig. 1. Nitration of $A\beta_{1-42}$ by Cu(II). $A\beta_{1-42}$ was incubated with or without hydrogen peroxide, nitrite and Cu(II) at 37 °C for 6 h. Then, the samples were transferred to nitrocellulose membrane, and the nitrated $A\beta_{1-42}$ was detected using a rabbit polyclonal antibody against 3-nitrotyrosine. Synthetic nitrated $A\beta_{1-42}$ was used as a control.

maintained the structure integrity in the interaction with Cu(II) [41]. Worth noting was that deionized water instead of buffer solution was used in the ESI-MS and EPR measurements to diminish interference of buffer salts to Cu(II) as well as instruments [42].

Results of ESI-MS directly supported the binding of $A\beta_{1-16}$ to Cu(II), as shown in Fig. 2. The molecular ion peak of $A\beta_{1-16}$ was evidenced by the *m/z* peak at 1956.8, while m/z peaks at 978.9, 652.9 and 489.9 corresponded its doublet, triplet and quartet protonated peaks. When the $A\beta_{1-16}$ was incubated with Cu(II), the appearance of the peak at *m/z* 1010.6 showed the doublet protonated peak of $A\beta_{1-16}$-Cu(II) complex, which corresponded to the triplet and quartet protonated peak of the complex. Similarly, $A\beta_{1-16}$NT was evidenced by its molecular ion peak at m/z of 1999.8, with its doublet, triplet and quartet protonated peak at m/z of 1000.9, 667.2 and 500.7. After the addition of Cu(II), the doublet, triplet and quartet protonated peaks of the $A\beta_{1-16}$NT-Cu(II) were observed at 1031.5, 688.25 and 516.2. These results indicated the interaction of $A\beta_{1-16}$NT with Cu(II) even after the nitration.

The ligation structure of Aβ-Cu(II) complex was further studied by EPR. EPR has been widely used to gain insight into Cu(II) coordination, as the characteristics of EPR parameters are correlated to the number, chemical nature, and charge of the equatorial ligands [43]. When peptide binds to Cu(II), low absorption at $g_{//}$ can be observed in the low field. In addition, $A_{//}$ is the nuclear hyperfine splitting at $g_{//}$ [33]. Values of $g_{//}$ and $A_{//}$ can be used to decipher the ligand environment around the Cu(II). As shown in Fig. 3, the EPR spectrum of $A\beta_{1-16}$-Cu(II) complex was consistent with previous reports at low pH, with $g_{//}$ at ~ 2.283 and $A_{//}$ at $\sim 165 \times 10^{-4}\,cm^{-1}$, which were typically three nitrogen and one oxygen ligands (3N1O) or two nitrogen and two oxygen ligands (2N2O) [44]. For 3N1O ligation, it could be originated from the NH_2 group of Asp 1, two of the imidazole rings of His6 and His13 or His14, and a CO group of Asp 1 [30]. In addition, there seemed no significant differences between the EPR spectrum of $A\beta_{1-16}$-Cu(II) and $A\beta_{1-16}$NT-Cu(II) complex. The values of $g_{//}$ and $A_{//}$ obtained from the EPR spectrum of $A\beta_{1-16}$NT-Cu(II) complex were similar to the $A\beta_{1-16}$-Cu(II) complex. This result suggested that Aβ nitration had no effect on the Cu(II) binding with Aβ, which was consistent with previous report that the tyrosine in Aβ was not involved in its interaction with Cu(II) [45].

Finally, we used ITC to characterize the thermodynamics of Aβ binding with Cu(II). As the affinity of Aβ to Cu(II) was too high to be measured directly by ITC, glycine was supplemented as a competitor [46,47]. Fig. 4 showed the isotherms of the titration of Aβ by Cu(II) (stabilized by glycine) in 20 mM HEPES buffer at pH 7.4, 25 °C with 0.16 M ionic strength. The binding constants and enthalpies determined from these data were listed in Table 1. By ignoring the competing equilibrium between the metal and glycine, the binding constant of $A\beta_{1-16}$ to Cu(II) was revealed as $2.78 \times 10^5\,M^{-1}$ in the ITC assay, which was similar to the report by Hatcher and colleagues [46]. According to previous studies, the experimental binding constant was associated with the Cu(II)-$A\beta_{1-16}$ binding constant. Our experimental conditions were the same as in the work by Hatcher et al. [39]. Thus, according to the data reported by Hatcher et al., the K value of $A\beta_{1-16}$ to Cu(II) was $6.4 \pm 0.14 \times 10^8\,M^{-1}$, and the K value of $A\beta_{1-16}$NT to Cu(II) should be $4.7 \pm 0.11 \times 10^8\,M^{-1}$. We also found that there were no significant differences between $A\beta_{1-16}$ and $A\beta_{1-16}$NT in terms of the binding constants to Cu(II). These results further suggested that nitration of Aβ had little impact on the interaction between Aβ and Cu(II).

3.3. Effect of Cu(II) on Aβ aggregation

Substantial evidence has showed that physiological Cu(II) is able to accelerate $A\beta_{1-42}$ aggregation that results in the formation of amorphous aggregates [48]. The aggregation of Aβ is tightly associated with its neurotoxicity. Inhibition of Cu(II)-induced $A\beta_{1-42}$ aggregation may be an attractive strategy to ameliorate its neurotoxicity. To investigate the effect of $A\beta_{1-42}$ nitration to the Cu(II)-induced $A\beta_{1-42}$ aggregation,

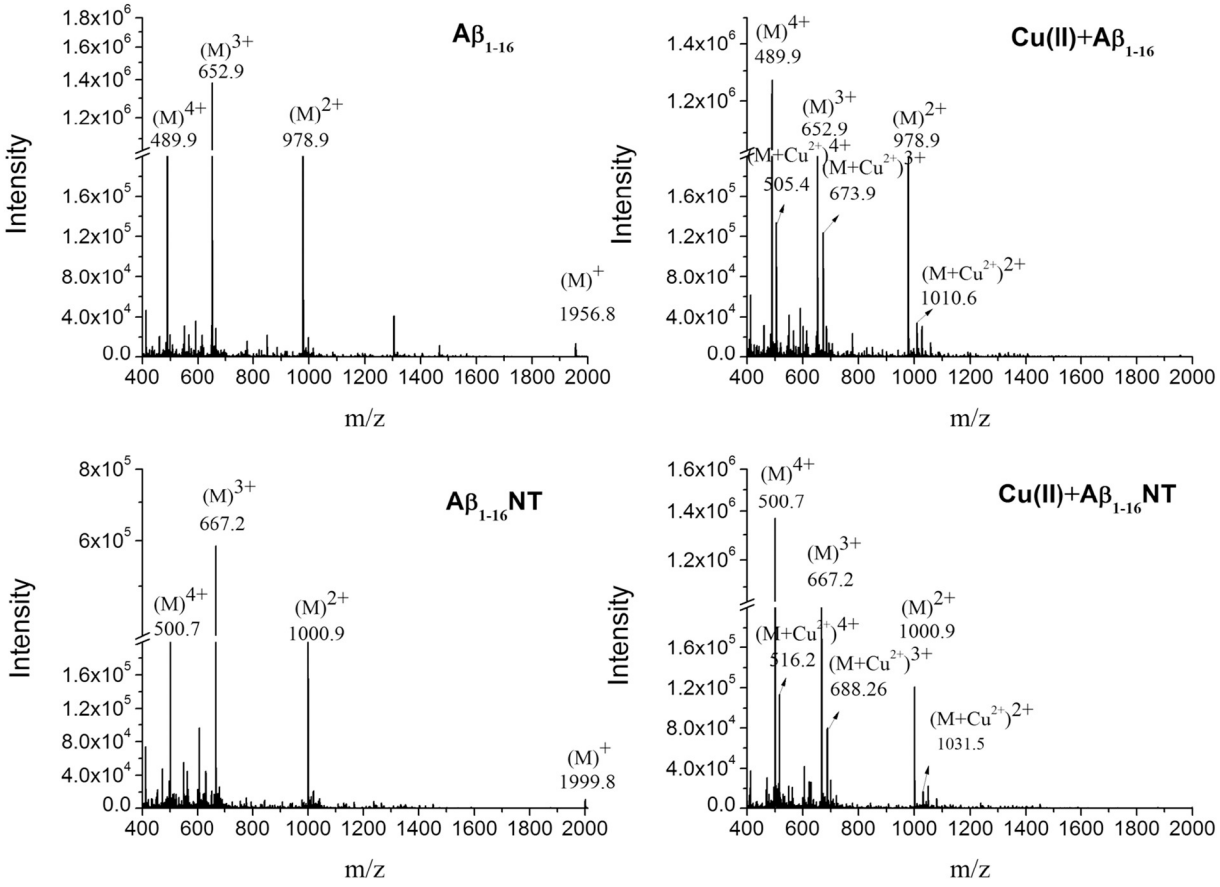

Fig. 2. ESI-MS spectra of the Aβ-Cu(II) complex. $Aβ_{1–16}$ or $Aβ_{1–16}$NT (100 μM) was incubated with or without 0.8 equimolar Cu(II) in water. The instrumental parameters were as follows: dry gas, nitrogen; temperature, 180 °C; scan range, *m/z* 400–3000, and capillary voltage, 4500 V. The scan range was m/z 400–3000.

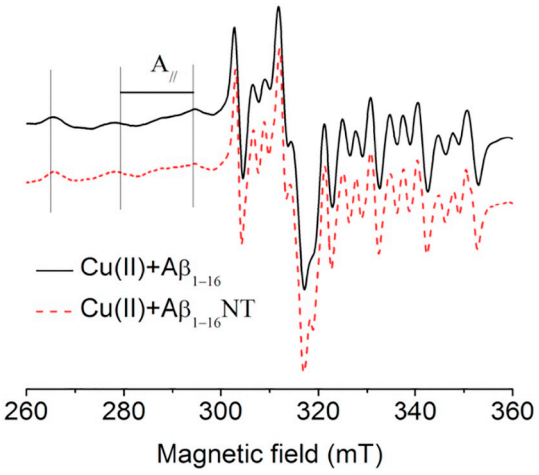

Fig. 3. EPR spectrum of the Aβ-Cu(II) complex. The Aβ peptide (1 mM) were incubated with Cu(II) (0.8 mM) in deionized water at 37 °C for 30 min. Then the samples were flash frozen in liquid nitrogen. Experimental conditions were as follows: microwave frequency 9.15 GHz, microwave power 20 mW, modulation frequency 100 kHz, temperature 120 K.

gel electrophoresis assay was employed to analyze the aggregation of Aβ peptide. After 72 h incubation with Cu(II), little $Aβ_{1–42}$ at low molecular weight was detected, while the amount of $Aβ_{1–42}$NT at low molecular weight remained appreciable (Fig. 5). As expected, Cu(II) exacerbated the $Aβ_{1–42}$ aggregation. In contrast, the nitration of $Aβ_{1–42}$ significantly diminished the Cu(II)-induced aggregation. These results indicated that nitration of $Aβ_{1–42}$ could significantly inhibit Cu(II)-induced $Aβ_{1–42}$ aggregation. As aforementioned, Aβ nitration seemed little influence on the interaction between Cu(II) and Aβ (Figs. 2, 3, 4). We speculated that the observed inhibitive effect of the nitration on the Aβ aggregation was solely attributed to the interfered Aβ intermolecular interaction by the nitration, which was consistent with our previous report [26,27].

3.4. Effect of Cu(II) binding on conformation of $Aβ_{1–42}$ and $Aβ_{1–42}$NT

It is commonly accepted that copper ion has remarkable impact on secondary structure of Aβ, which is thought to be critical to the Aβ aggregation [49]. Herein, the effect of Cu(II) binding on the conformational changes of $Aβ_{1–42}$ and $Aβ_{1–42}$NT were studied by CD spectroscopy. As shown in Fig. 6, $Aβ_{1–42}$ and $Aβ_{1–42}$NT showed an obvious negative peak at 217 nm, which indicated β conformation for Aβ [50]. But a reduction in the negative band at 217 nm was observed in the CD spectrum upon the incubation with Cu(II). This observation was similar to results reported by Hernández-Rodríguez [51]. It suggested that Cu(II) could significantly affect the conformational changes of $Aβ_{1–42}$. It changed the aggregation of $Aβ_{1–42}$ from fibrils to nanoscale oligomers and amorphous aggregates instead [52]. Nevertheless, the result indirectly indicated the interaction of Cu(II) with $Aβ_{1–42}$NT, and interaction induced conformational change of $Aβ_{1–42}$NT.

3.5. Influence of Aβ nitration on hydroxyl radical production by $Aβ_{1–42}$-Cu (II) complex

$Aβ_{1–42}$-Cu(II) complex could be involved in the production of hydroxyl radical, which is highly toxic to cells [53,54]. Previous results indicated that nitration of Tyr10 in Aβ had little effect on the binding of

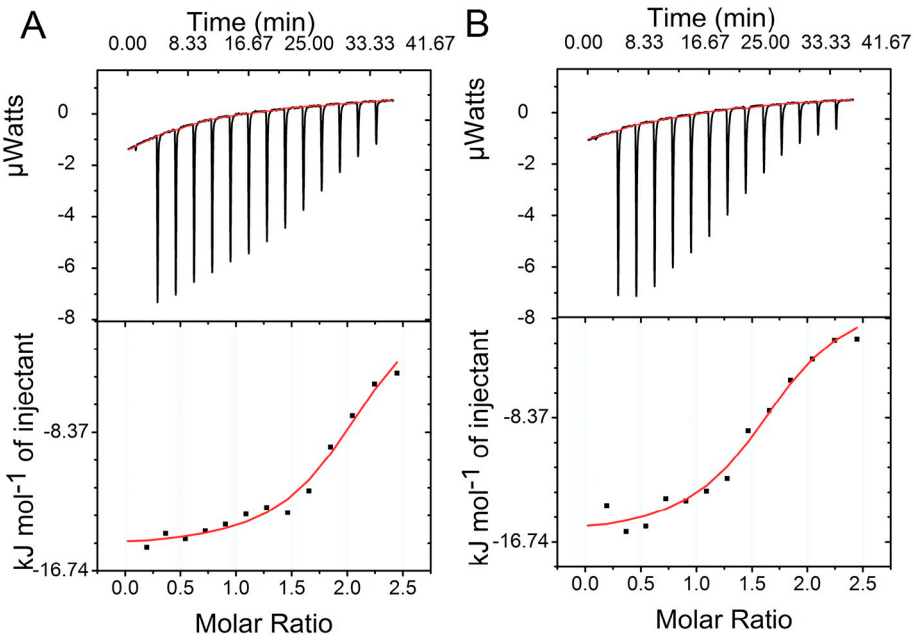

Fig. 4. ITC binding isotherms for Cu(II) binding to Aβ and mutant. Titration of 700 μM Cu(II) with 4 equivalent glycine and $Aβ_{1–16}$ (A) and $Aβ_{1–16}$NT (B) (60 μM) in 20 mM HEPES buffer, pH 7.2 at 25 °C. Ionic strength was 0.16 M. The top panels show the differential power signal measured for each injection throughout the experiment, and the bottom panels show the integrated peak areas corresponding to the measured heat released per injection. The solid line represents the best fit for single binding site model, and the resulting parameters are listed in Table 1.

Table 1
Binding constants for the addition of Cu(II) to $Aβ_{1–16}$ and $Aβ_{1–16}$NT in HEPES buffer at 25 °C, pH 7.4 with 0.16 M ionic strength.

	$Aβ_{1–16}$	$Aβ_{1–16}$NT
K ($\times 10^5\ M^{-1}$)	2.78 ± 0.61	2.03 ± 0.5
ΔH ($\times 10^{-4}$ cal/mol)	−1.539 ± 0.03	−1.643 ± 0.05
n	2.05	1.66

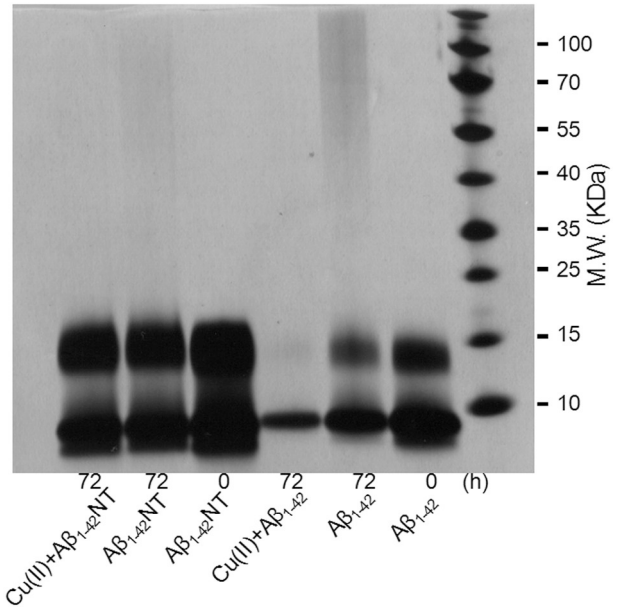

Fig. 5. Effect of Cu(II) on the aggregation of $Aβ_{1–42}$. $Aβ_{1–42}$ or $Aβ_{1–42}$NT (50 μM) was incubated in the absence or presence of equimolar Cu(II) in 50 mM PBS, pH 7.4, at 37 °C for 0 or 72 h. Then the aggregation of samples were detected by Nu-PAGE and visualized by silver staining.

Cu(II) to Aβ (Figs. 2, 3, 4). However, it remains a question whether tyrosine nitration affect the redox property of $Aβ_{1–42}$-Cu(II) complex. Thus, we used 3-CCA to quantitatively detect the production of hydroxyl radical [37,43]. It can react with hydroxyl radical to produce a fluorescence emitted at 452 nm. As ascorbate was reported found at high concentration in brain cells, it was chosen as a reducing agent. Fig. 7 showed that Cu(II) reacted with ascorbate that generated hydroxyl radical [55]. When Cu(II) was incubated with Aβ peptide, the production of hydroxyl radicals significantly reduced. Among the four peptides, $Aβ_{1–42}$ was more effective at reducing hydroxyl radical formation, which showed about 60% reduction compared to the positive control. The other peptide including $Aβ_{1–42}$NT, $Aβ_{1–16}$ and $Aβ_{1–16}$NT exhibited similar reduction, i.e. around 40%. This phenomenon may be mainly due to Cu(II) binding to $Aβ_{1–42}$ and promoting its aggregation. As a result, a part of Cu(II) will be buried in the aggregated $Aβ_{1–42}$ and prevent it from free access to ascorbate. In contrast, although Cu(II) can still bind to $Aβ_{1–42}$NT, $Aβ_{1–16}$ or $Aβ_{1–16}$NT, it does not interfere with the aggregation of these peptides, making it react with ascorbate freely. Thus, $Aβ_{1–42}$ was more effective on reducing hydroxyl radical among the four peptides. It has been reported that Aβ did not silence redox cycling of Cu(II) in the presence of ascorbate but quenched hydroxyl radical, and histidine and methionine in Aβ were identified as specific oxidation sites by ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy [56]. Similar reaction of Aβ with Cu(II)-induced hydroxyl radicals was also reported by Atwood et al.) [57]. So the reduction of hydroxyl radicals, which was produced in the reaction of Cu(II) with ascorbate, might be attributed by Aβ reacting with hydroxyl radical. By the reaction Aβ acted as a sacrificial antioxidant by quenching hydroxyl radical produced by Cu(II) in vivo.

3.6. Effect of Aβ nitration on Cu(II)-induced $Aβ_{1–42}$ neurotoxicity

To examine the effect of Aβ nitration on Cu(II)-induced $Aβ_{1–42}$ neurotoxicity, we incubated Aβ peptide or Aβ-Cu(II) complex with SH-SY5Y cells for 48 h. Then the cell viability was assessed by MTT assay. As shown in Fig. 8A, Cu(II) revealed negligible toxicity to SH-SY5Y cells with cell viability maintained 92.4 ± 5.7%. As expected, the cell viability of the $Aβ_{1–42}$-Cu(II) complex group was 52.2 ± 2%, which was lower than that of $Aβ_{1–42}$ group as60.6 ± 1.5%. It supported the notion of the more neurotoxic nature of $Aβ_{1–42}$-Cu(II) complex. This result was in line with previous studies [58,59]. As to $Aβ_{1–42}$NT, the toxicity to cells was mild as cell viability measured as 88.5 ± 1.2%. Interestingly, binding of Cu(II) with $Aβ_{1–42}$NT did not alter its toxicity to cells with cell viability measured as 87.5 ± 2.9%, which was similar to the measurement of $Aβ_{1–42}$NT. It suggested that nitration of $Aβ_{1–42}$ not only reduced neurotoxicity of $Aβ_{1–42}$, but also

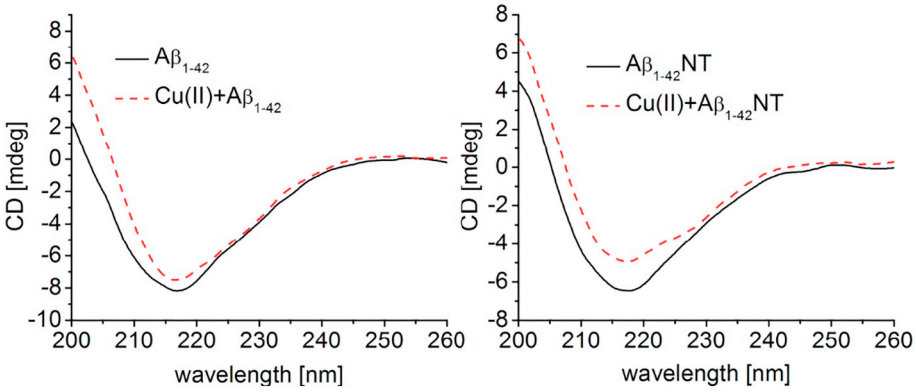

Fig. 6. CD spectra of Cu(II)-induced Aβ conformational changes. (A) $Aβ_{1–42}$ + Cu(II); (B) $Aβ_{1–42}$NT + Cu(II). $Aβ_{1–42}$ or $Aβ_{1–42}$NT (30 μM) was incubated with equimolar Cu(II) in 5 mM PBS, pH 7.4, at 37 °C for 12 h.

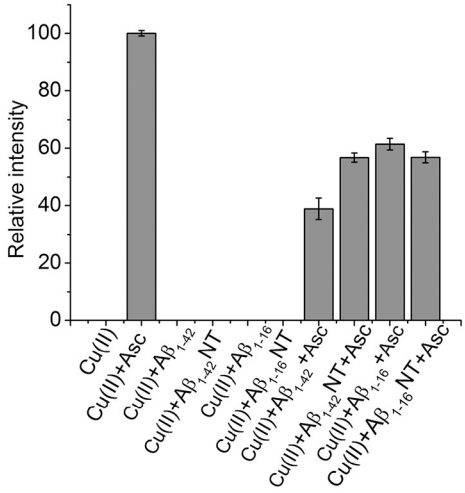

Fig. 7. Hydroxyl radical production by Cu(II) and ascorbate in the presence of Aβ. Aβ peptide (10 μM), Cu(II) (10 μM) and ascorbate (300 μM) were co-incubated in 20 mM PBS, pH 7.4, containing 3-CCA (1 mM). Cu(II) and ascorbate alone was used as a control. The fluorescence intensity at 460 nm was measured to evaluate the relative content of hydroxyl radical. All data points are means ± SD, $n = 3$.

ameliorated Cu(II) exacerbated neurotoxicity of $Aβ_{1–42}$ toward SH-SY5Y cells.

To confirm the cell viability upon various treatments, cell morphology was also thoroughly examined. The cells of the control group showed round cell bodies and clear neurite. When the cells were treated with toxic species, cells would change their neuron-like morphology, lose their neuritic processes, become rounded, and eventually compromise their viability. As shown in Fig. 8B, a lot of rounded cells but few normal cells were observed with the treatment of $Aβ_{1–42}$ and $Aβ_{1–42}$-Cu(II) complex. In contrast, few rounded cells were observed in the group of Cu(II), $Aβ_{1–42}$NT and $Aβ_{1–42}$NT-Cu(II) complex. This result further demonstrated that nitration of $Aβ_{1–42}$ significantly prevented neurons from Cu(II)-exacerbated $Aβ_{1–42}$ neurotoxicity.

4. Discussion

It's well established that interaction between Cu(II) and Aβ plays a critical role in the development of AD, as abnormally high concentration of Cu(II) has been found in senile plaques [18–20]. It has been reported that Cu(II)could bind strongly to Aβ. As a result, this interaction would promote toxic Aβ oligomers formation and ultimately elevate Aβ neurotoxicity in vitro [15–17]. Thus, many drugs were designed to disrupt the interaction of Aβ with Cu(II) to alleviate the Aβ oligomerization. Unfortunately, all the drugs have failed at clinical trials [21,22]. In addition, accumulated evidence suggested that Aβ may possess some important physiologic functions [24,25]. It indicated that there ought to be an unveiled mechanism to prevent Aβ from aggregating into toxic oligomers that impaired neurons. Interestingly, our previous research results showed that nitration of Aβ could significantly decrease its aggregation and reduce its neurotoxicity toward SH-SY5Y cells [26,27]. Note that our observations were different from the results reported by Kummer et al., in which they reported that nitration of Aβ critically increased its aggregation [28]. However, they used peroxynitrite to induce tyrosine nitration, which would introduce oxidation and dityrosine cross-link to Aβ. In order to ascertain the effect of tyrosine nitration on the structure and function of Aβ, we chose synthetic nitrated Aβ as the subject of our research to rule out the interference of oxidative byproducts in peroxynitrite caused Aβ nitration. We found that mutation of Tyr10 with glycine in $Aβ_{1–40}$ could inhibit its aggregation [60], which indicated that Tyr10 in $Aβ_{1–40}$ may play an important role in the aggregation of $Aβ_{1–40}$. Recently, Guivernau et al. also reported that nitration of $Aβ_{1–42}$ could significantly inhibit its aggregation [61]. All these results indicated the conclusion that nitration on Tyr10 might inhibited its aggregation.

It has been reported that Aβ-Cu(II) complex is capable of catalyzing peroxynitrite production in the presence of hydrogen peroxide and nitrite [29]. As the tyrosine residue in Aβ is close to the binding site with Cu(II), it would get nitrated due to the high chemical reactivity of peroxynitrite. Thus, it necessitated to detect the effect of nitration of $Aβ_{1–42}$ on its aggregation and toxicity induced by Cu(II). In this work, we firstly proved that Tyr10 in $Aβ_{1–42}$ could be nitrated by binding to Cu(II) in the presence of nitrite and hydrogen peroxide (Fig. 1). Then, we tested the effect of nitration of Tyr10 on the binding of $Aβ_{1–42}$ to Cu(II). The results of ESI-MS indicated that Cu(II) could bind to $Aβ_{1–42}$NT (Fig. 2), even though Tyr10 was close to the binding site. The EPR spectra revealed that nitration of Tyr10 had no effect on the binding model of $Aβ_{1–42}$-Cu(II) complex (Fig. 3). Similarly, the results of ITC suggested that nitration of Tyr10 had little impact on the interaction between $Aβ_{1–42}$ and Cu(II) (Fig. 4). Additionally, these results also clearly supported that Tyr10 in $Aβ_{1–42}$ was not involved in the binding of $Aβ_{1–42}$ to Cu(II), which were consistent with reported results [51].

Furthermore, we tested the effect of nitration of $Aβ_{1–42}$ on its aggregation induced by Cu(II). The observation from gel electrophoresis analysis indicated that Cu(II) had little effect on the aggregation of $Aβ_{1–42}$NT (Fig. 5). In addition, we also found that Cu(II) could promote the aggregation of $Aβ_{1–42}$, which was consistent with previous studies [56]. Accumulated evidence indicated that Cu(II) could strongly bind to Aβ and change its secondary structure. In this case, Aβ tended to aggregate into toxic oligomers [15–17]. We also observed from the CD spectra that Cu(II) had remarkable impact on secondary structure of Aβ (Fig. 6). Similarly, the secondary structure of $Aβ_{1–42}$NT was also

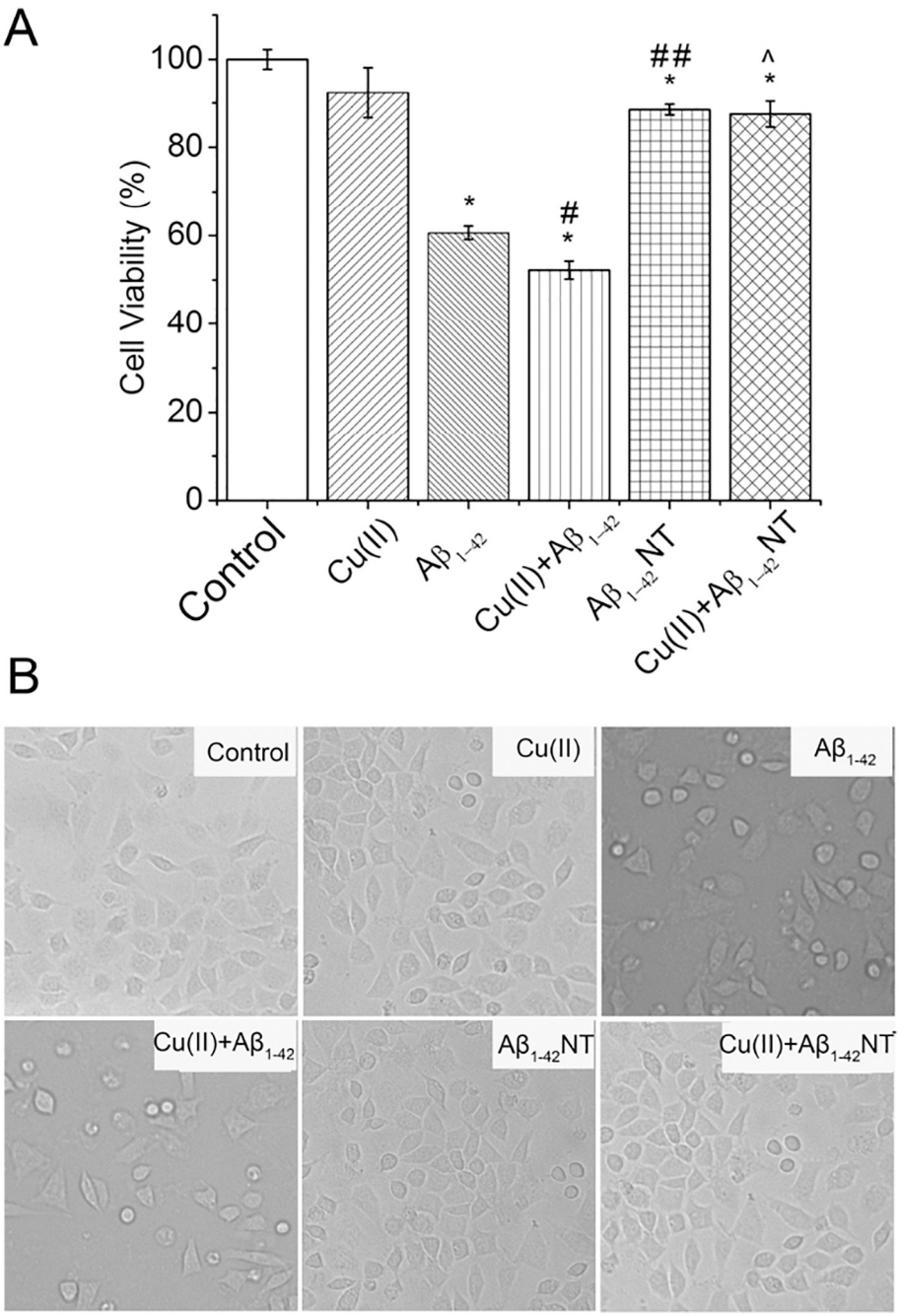

Fig. 8. Effect of nitration of Y10 in $A\beta_{1-42}$ on its neurotoxicity induced by Cu(II). (A) Cell viability assay of SH-SY5Y cells upon treatment with PBS; Cu(II); $A\beta_{1-42}$; $A\beta_{1-42}$-Cu(II); $A\beta_{1-42}$NT; $A\beta_{1-42}$NT-Cu(II). The cells were treated with different samples for 48 h. MTT assay was used to determine the cell viability. Data are means ± SD from independent experiments ($n = 3$). $^{**}p < 0.001$, compared with control. $\hat{}\hat{}p < 0.001$, compared with the group of $A\beta_{1-42}$-Cu(II). $\#p < 0.01$, $\#\#p < 0.001$, compared with the group of $A\beta_{1-42}$. (B) Representative photomicrographs of SH-SY5Y cells with different treatments for 48 h.

changed by Cu(II). The results of CD spectra further confirmed that nitration of $A\beta_{1-42}$ had little effect on the interaction between $A\beta_{1-42}$ and Cu(II). In our previous study, we speculated that Tyr10 played an important role in the self-assembly processes of Aβ through forming π-π stacking interactions, and tyrosine nitration may inhibit these interactions and decrease the aggregation of Aβ [26,27]. Herein, we showed that Cu(II) only induced conformational changes of Aβ, while it had little impact on the aggregation of $A\beta_{1-42}$NT. In addition, we noted that N-terminal residues of Aβ, particularly the stretch between Asp7 and Tyr10, played an important role in the aggregation of Aβ. Haupt et al. found that a stable N-terminal β strand controlled the partitioning between oligomer and protofibril formation, and disrupting the N-terminal β strand favored protofibrils relative to oligomers [62]. Rezaei-Ghaleh et al. found that phosphorylation of amyloid at Ser8 increased the stability of its pathogenic aggregates [63]. They attributed this observation to the enhancement of hydrogen bonds at the N terminus of Aβ. They also found that Aβ phosphorylation at Ser8 caused structural changes in the N-terminal region of Aβ aggregates in favor of less compact conformations [64]. It seems that the hydrogen bond interaction in the N-terminal region of Aβ may have effect on the aggregation of Aβ. More importantly, Tycko's group suggested that the N-terminal region of Aβ, particularly the stretch between Asp7 and Tyr10, was involved in a rich network of hydrogen bonds, both intra- and intermolecularly, sometimes with residues far away in the sequence like Ser26 and Asn27 [65]. These findings supported that posttranslational modifications played an important role in the structural polymorphism

of Aβ and may have modulatory effect on neurodegeneration. It also indicated that nitration of Aβ inhibiting its aggregation may due to the enhancement of hydrogen bonds induced by tyrosine nitration.

It is well known that the Aβ-Cu(II) complex could produce reactive oxygen species, which are harmful to neurons [29]. It is worth noting that Tyr10 is close to the active site of the Aβ-Cu(II) complex. Thus, we used 3-CCA assays to further detect the effect of nitration of $Aβ_{1–42}$ on the catalase activity of the $Aβ_{1–42}$-Cu(II) complex. We found that the production of reactive oxygen species were reduced, when Cu(II) bound to Aβ peptide (Fig. 7). Among the four peptides, $Aβ_{1–42}$ was more effective at reducing hydroxyl radical. We speculate that this observation may be due to Cu(II) promoting the aggregation of $Aβ_{1–42}$ so that part of Cu(II) is buried in the aggregated $Aβ_{1–42}$, preventing it from free accesses to ascorbate. Thus, $Aβ_{1–42}$ exhibited greater ability to reduce hydroxyl radical.

Finally, the toxicity of $Aβ_{1–42}$-Cu(II) and $Aβ_{1–42}$NT-Cu(II) complex was tested by the MTT assay. It is established that cytotoxicity of Aβ is directly correlated with its aggregation level [66]. Interestingly, we found that nitration of $Aβ_{1–42}$ remarkably reduced the toxicity of $Aβ_{1–42}$ induced by Cu(II). This result was consistent with the result obtained from the aggregation experiment (Fig. 8). The results of the MTT assay may mainly because that nitration of Tyr10 in $Aβ_{1–42}$ can significantly inhibit Cu(II)-enhanced $Aβ_{1–42}$ aggregation. It also suggested that nitration of $Aβ_{1–42}$ might be an important protection for the normal functions of $Aβ_{1–42}$. More importantly, Lin et al. found that the level of Aβ secretion increased when PC12 cells were treated with high concentration Cu(II) [67]. Our results, together with the results reported by Lin et al., suggested that the production of Aβ may be a response for the Cu(II) toxicity and a protective mechanism for neurons from Cu(II) toxicity by chelating excess Cu(II) and reacting with hydroxyl radical (Fig. 9). Moreover, nitration of $Aβ_{1–42}$ is crucial for the normal function of $Aβ_{1–42}$. It is apparent that nitrite plays a crucial role in this protection mechanism. It has been reported that levels of nitrite and nitrate in the cerebrospinal fluid of AD patients notably decreases as compared with that of the controls [68]. Besides, it is demonstrated that NO synthase deletion promotes multiple AD pathologies in a mouse model of AD [69]. However, the mechanism underlined the correlation between decreased nitrite and nitrate levels and the pathogenesis of AD remains unclear. Our findings may propose a new explanation for the link between the decreased levels of nitrite plus nitrate and AD, since decreased nitrite and nitrate means the decrease of Aβ nitration, which leads to more Aβ aggregation and neuron damage. This study may open a new avenue for understanding the function of $Aβ_{1–42}$.

5. Conclusion

In conclusion, we systematically investigated the correlation between Aβ and Cu(II). We found that $Aβ_{1–42}$ bound with Cu(II) and was subjected to nitration afterwards in the presence of nitrite and hydrogen peroxide. But the nitration of $Aβ_{1–42}$ had little effect on the $Aβ_{1–42}$ interaction with Cu(II) or redox property of $Aβ_{1–42}$-Cu(II) complex. Even though the CD spectrum showed that Cu(II) induced a pronounced change in the conformation of $Aβ_{1–42}$NT as it did on $Aβ_{1–42}$, Cu (II) had negligible effect on the aggregation of $Aβ_{1–42}$NT. It indicated that the nitration of Aβ protected itself from Cu(II)-induced toxicity. The possible mechanism might be that nitration of $Aβ_{1–42}$ significantly disturbed its intermolecular interactions and thus protected itself against Cu(II)-induced aggregation. Our further study on cells also supported this hypothesis that nitration of Aβ ameliorated Cu(II)-induced neurotoxicity of Aβ. Based on these hypothesis, we proposed that the cell may generate Aβ together with nitric oxide to protect itself when neurons subjected Cu(II) toxicity and oxidative stress. Under the oxidative condition, Aβ acted as a sacrificial antioxidant and thus was nitrated. Then the nitration prevented itself from further aggregation induced by Cu(II). When the nitration of Tyr10 in $Aβ_{1–42}$ is lower in mild nitration condition, $Aβ_{1–42}$ is easy to form toxic oligomers and impairing neurons. Thus, nitration of Tyr10 in $Aβ_{1–42}$ is crucial to protect the normal functions of $Aβ_{1–42}$. Our findings may open a new avenue in deeply understanding the physiological function of $Aβ_{1–42}$, and the decrease of nitrite level deserves more attention in the development of AD.

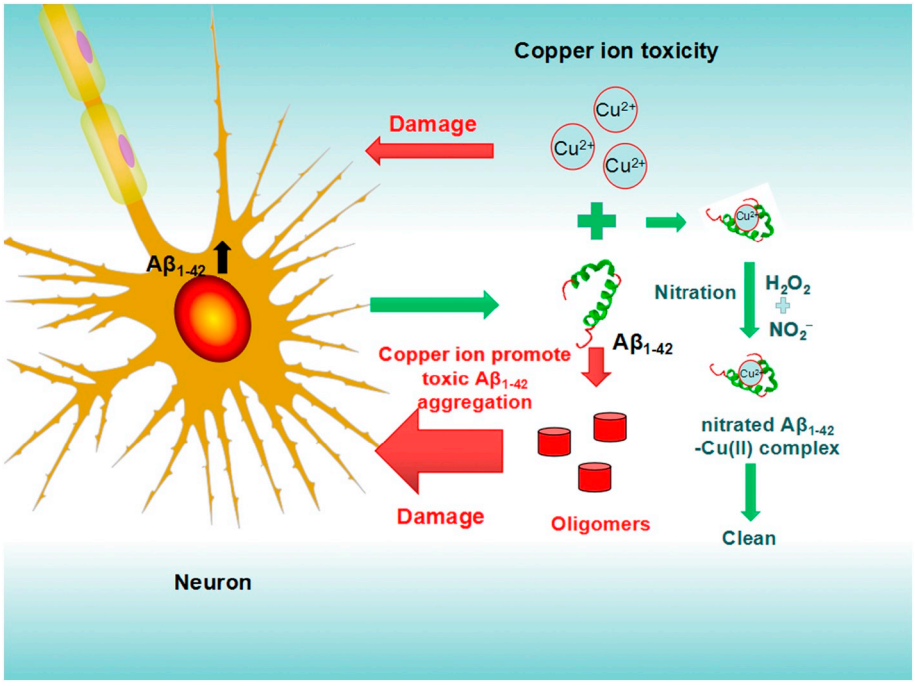

Fig. 9. Proposed effects of nitration of $Aβ_{1–42}$ on the neurotoxicity of $Aβ_{1–42}$ induced by Cu(II). Cu(II) can bind to $Aβ_{1–42}$ and affect its conformational changes. As a result, Cu(II) can enhance $Aβ_{1–42}$ neurotoxicity due to the formation of $Aβ_{1–42}$ oligomers. However, nitration of $Aβ_{1–42}$ can dramatically inhibit the Cu (II)-induced $Aβ_{1–42}$ oligomerization and neurotoxicity. These phenomena are mainly due to that nitration of Y10 can significantly disturb the intermolecular interactions of $Aβ_{1–42}$, while Cu(II) only affects conformational changes of $Aβ_{1–42}$. These results indicated nitration of $Aβ_{1–42}$ may be an important protective mechanism for $Aβ_{1–42}$'s function.

Abbreviations

Aβ	amyloid-β peptide
$Aβ_{1–42}$NT	Tyr10 nitrated amyloid-β peptide (1–42)
$Aβ_{1–16}$NT	Tyr10 nitrated amyloid-β peptide (1–16)
AD	Alzheimer's disease
APP	amyloid precursor protein
PBS	phosphate buffer solution
ESI-MS	Electrospray Ionization Mass Spectrometry
EPR	electron paramagnetic resonance
ITC	Isothermal titration calorimetry
CD	Circular Dichroism Spectroscopy

Conflict of interest/disclosure

Authors declare no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (31170808, 31570810, 31770866), Natural Science Foundation of Hubei Scientific Committee (2016CFA001) and the Fundamental Research Funds for the Central Universities of China (2017KFYXJJ167). The Analytical and Testing Center of Huazhong University of Science and Technology is thanked for its help in CD analysis.

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