

The effect of Cu²⁺ and Zn²⁺ on the Aβ₄₂ peptide aggregation and cellular toxicity

Cite this: *Metallomics*, 2013, 5, 1529

Anuj K. Sharma,^a Stephanie T. Pavlova,^a Jaekwang Kim,^b Jungsu Kim^b and Liviu M. Mirica^{*a}

The coordination chemistry of Cu and Zn metal ions with the amyloid β (Aβ) peptides has attracted a lot of attention in recent years due to its implications in Alzheimer's disease. A number of reports indicate that Cu and Zn have profound effects on Aβ aggregation. However, the impact of these metal ions on Aβ oligomerization and fibrillization is still not well understood, especially for the more rapidly aggregating and more neurotoxic Aβ₄₂ peptide. Here we report the effect of Cu²⁺ and Zn²⁺ on Aβ₄₂ oligomerization and aggregation using a series of methods such as Thioflavin T (ThT) fluorescence, native gel and Western blotting, transmission electron microscopy (TEM), and cellular toxicity studies. Our studies suggest that both Cu²⁺ and Zn²⁺ ions inhibit Aβ₄₂ fibrillization. While presence of Cu²⁺ stabilizes Aβ₄₂ oligomers, Zn²⁺ leads to formation of amorphous, non-fibrillar aggregates. The effects of temperature, buffer, and metal ion concentration and stoichiometry were also studied. Interestingly, while Cu²⁺ increases the Aβ₄₂-induced cell toxicity, Zn²⁺ causes a significant decrease in Aβ₄₂ neurotoxicity. While previous reports have indicated that Cu²⁺ can disrupt β-sheets and lead to non-fibrillar Aβ aggregates, the neurotoxic consequences were not investigated in detail. The data presented herein including cellular toxicity studies strongly suggest that Cu²⁺ increases the neurotoxicity of Aβ₄₂ due to stabilization of soluble Aβ₄₂ oligomers.

Received 30th May 2013,
Accepted 23rd August 2013

DOI: 10.1039/c3mt00161j

www.rsc.org/metallomics

Introduction

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease that slowly destroys memory and thinking skills, and eventually causes senile dementia. More than 5 million in the US and 24 million people worldwide suffer from this disease.^{1,2} The pathogenesis of AD is far from being understood, and 42- and 40-amino acids long amyloid β peptides (Aβ₄₂ and Aβ₄₀, respectively) are proposed to play a central role in the onset of AD.³ Although Aβ₄₀ is present in larger amounts in the brain, Aβ₄₂ was found to be more neurotoxic and have a higher tendency to aggregate.^{4,5} The amyloid cascade hypothesis suggests the ultimate products of Aβ aggregation, the amyloid plaques, are responsible for neurodegeneration.⁶ However, recent *in vivo* studies have shown that soluble Aβ oligomers are more neurotoxic than amyloid plaques and most likely responsible for synaptic dysfunction and memory loss in AD.^{7–11}

Post-mortem examination of the brain suggested that bioavailable metals (*i.e.*, copper, zinc, and iron) are found in high

concentration in the amyloid plaques. These metal ions are believed to play a key role in the peptide aggregation processes.^{12–15} For example, it was shown that that metal ions promote Aβ aggregation,^{16,17} as well as formation of reactive oxygen species (ROS) and lead to oxidative stress.^{18–20} While several studies have investigated the metal coordination environment of Aβ-M²⁺ complexes,^{14,21–24} only few have employed the full-length Aβ peptides, shorter non-aggregating forms like Aβ_{1–16} or Aβ_{1–28} being used instead.^{14,22–25}

Although these studies suggest that metal ions alter the Aβ aggregation pathways and toxicity,²⁶ the molecular mechanisms of metal-Aβ species interactions are not completely understood, especially for the more neurotoxic Aβ₄₂ peptide.^{13,15,27,28} Several studies showed that Zn²⁺ promotes formation of nonfibrillar aggregates,^{16,29–31} yet conflicting results have been reported for the Cu²⁺-mediated Aβ aggregation.^{21,28,32,33} In some studies Cu²⁺ appears to be involved in disrupting or reducing Aβ₄₂ aggregation,^{28,32,34–37} while other studies report that Cu²⁺ enhances aggregation.^{17,33,38} Moreover, in the cell toxicity studies the presence of transition metal ions showed both increased^{39–42} or decreased^{34,43} toxicity of Aβ. The likely reason for these conflicting results is the use of different experimental conditions and methods to measure Aβ aggregation. There are reports using Thioflavin T (ThT) fluorescence,^{28,32,33} atomic force microscopy (AFM)^{32,35} or TEM,^{28,36,44} and

^a Department of Chemistry, Washington University, One Brookings Drive, St. Louis, Missouri 63130-4899, USA. E-mail: mirica@wustl.edu

^b Department of Neurology, Washington University School of Medicine, St. Louis, Missouri 63108, USA

various cellular toxicity³³ assays to evaluate the Cu²⁺-mediated A β aggregation and neurotoxicity. However, no report has employed all these techniques under similar experimental conditions to provide a detailed picture of the neurotoxicity implications of the Cu²⁺- and Zn²⁺-mediated formation of soluble and/or insoluble A β ₄₂ aggregates. Hence, more comprehensive studies on the effect of metal ions on A β ₄₂ aggregation and neurotoxicity are needed to better understand their role in AD progression.

Herein we have employed for the first time a wide range of techniques to study the effect of metal ions on the oligomerization and aggregation of the more neurotoxic A β ₄₂ peptide. ThT fluorescence, native gel electrophoresis and Western blotting, transmission electron microscopy (TEM), and cellular toxicity studies were used to determine the formation in presence and absence of Cu²⁺ and Zn²⁺ of both soluble and insoluble A β ₄₂ aggregates. Most importantly, we have correlated these *in vitro* results with neurotoxicity studies, in order to address to potential *in vivo* role of metal–A β ₄₂ interactions in AD. We observed a significantly low ThT fluorescence for Cu²⁺- and Zn²⁺-containing A β aggregates, and TEM and native gel/Western blotting studies suggest that Cu²⁺ and Zn²⁺ both inhibit fibrillization. While Zn²⁺ leads to formation of amorphous aggregates, Cu²⁺ is shown to stabilize soluble A β species. Cellular toxicity studies suggest that while Zn²⁺ reduces the A β ₄₂ toxicity, Cu²⁺ significantly increases A β ₄₂ neurotoxicity. Based on these results, we propose that Cu²⁺ stabilizes the neurotoxic soluble A β ₄₂ oligomers, with direct implications into the physiological role of Cu²⁺ in increasing A β ₄₂ neurotoxicity in AD.

Experimental details

Materials

All reagents were purchased from commercial sources and used as received unless stated otherwise. All solutions and buffers were prepared using metal-free Millipore water that was treated with Chelex overnight and filtered through a 0.22 μ m nylon filter.

A β samples preparation

A β monomeric films were prepared by dissolving synthetic A β ₄₂ or A β ₄₀ peptide (Keck Biotechnology Resource Laboratory, Yale University) in hexafluoroisopropanol (HFIP) (1 mM) and incubating for 1 h at room temperature. The solution was then aliquoted out and evaporated overnight. The aliquots were vacuum centrifuged and the resulting monomeric films stored at –80 °C. A β fibrils were generated by dissolving monomeric A β films in DMSO, diluting into the appropriate buffer, and incubating for 24 h at 37 °C with continuous agitation at 150 rpm (final DMSO concentration was <2%). For metal-containing fibrils, the corresponding metal ions were added in various stoichiometric ratios (0.25 to 2 equiv. vs. A β) before initiation of fibrillization. For preparation of soluble A β ₄₂ oligomers, a literature protocol was followed:⁴⁵ a monomeric film of A β ₄₂ was dissolved in anhydrous DMSO and diluted in DMEM:F12 media (1:1 v:v, without phenol red, Invitrogen).

The A β ₄₂ solution (50–100 μ M) was incubated at 4 °C for 24 h and then centrifuged at 10 000g for 10 min. The supernatant was used as a solution of soluble A β ₄₂ oligomers.

Native gel electrophoresis and Western blotting

All gels, buffers, membranes, and other reagents were purchased from Invitrogen and used as directed except where otherwise noted. Samples were separated on 10–20% gradient tris-tricine mini gels. The gels were transferred to a nitrocellulose membrane on an ice bath and the protocol was followed as directed except that the membrane was blocked overnight at 4 °C. After blocking, the membrane was incubated in a solution (1:2000 dilution) of 6E10 anti-A β primary antibody (Covance) for 3 h. Invitrogen's Western Breeze Chemiluminescent kit was used to visualize the A β species. An alkaline phosphatase antimouse secondary antibody was used, and the protein bands were imaged using a FUJIFILM Luminescent Image Analyzer LAS-1000CH.

Cytotoxicity studies

Cell viability studies were performed using the Alamar Blue assay (Invitrogen). Mouse neuroblastoma Neuro2A (N2A) cell lines were purchased from the American Type Culture Collection (ATCC). Cells were grown in DMEM/10% FBS, which is the regular growth media for N2A cells. N2A cells were plated in each well of a 96 well plate (2.5 \times 10⁴/well) with DMEM/10% FBS. The media was changed to DMEM/N2 after 24 h. After 1 h, the reagents (20 μ M A β ₄₂ species and metal ions) were added. After an additional incubation of 40 h, the Alamar blue solution was added in each well, the cells were incubated for 90 min at 37 °C, and the absorbance was measured at 570 nm (control OD = 600 nm).

Fluorescence measurements

All fluorescence measurements were performed using a SpectraMax M2e plate reader (Molecular Devices). For ThT fluorescence studies, samples were diluted to a final concentration of 2.5 μ M A β in PBS containing 10 μ M ThT and the emission measured at 485 nm (λ_{ex} = 435 nm).

Transmission electron microscopy (TEM)

Glow-discharged grids (Formvar/Carbon 300-mesh, Electron Microscopy Sciences) were treated with A β samples (25 μ M, 5 μ L) for 2–3 min at room temperature. The excess solution was removed using filter paper and the grids were rinsed twice with H₂O (5 μ L). Grids were stained with uranyl acetate (1% w/v, H₂O, 5 μ L) for 1 min, blotted with filter paper, and dried for 15 min at room temperature. Images were captured using a FEI G2 Spirit Twin microscope (60–80 kV, 6500–97 000 magnification).

Results and discussion

A β ₄₂ aggregation monitored by ThT fluorescence

Thioflavin T (ThT) is a fluorescent dye widely used for detection of amyloid peptide aggregation.⁴⁶ However, ThT does not interact with unstructured A β monomer or oligomers, nor with

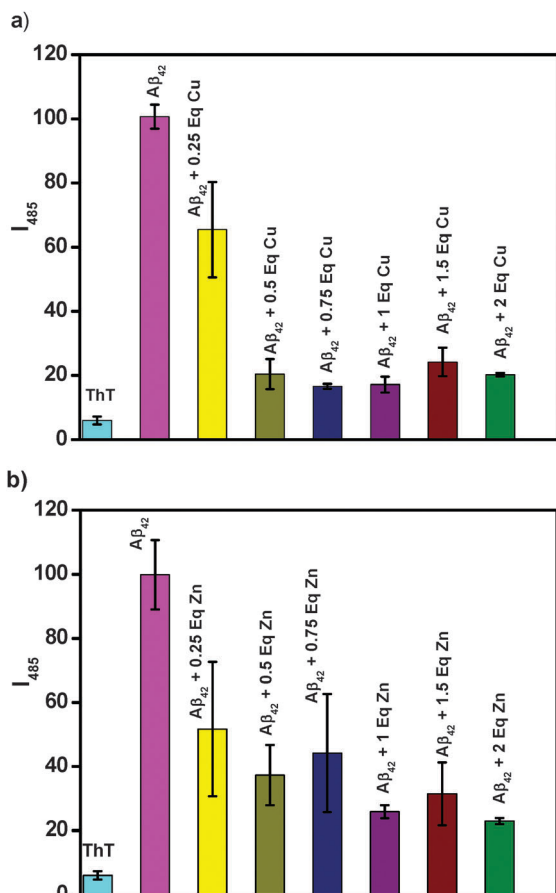


Fig. 1 ThT fluorescence for Aβ₄₂ incubated with various stoichiometric ratios of (a) Cu²⁺ and (b) Zn²⁺ for 24 h at 25 °C in PBS. Conditions: [Aβ₄₂] = 25 μM, [M²⁺] = 0–2 equiv., [ThT] = 10 μM.

amorphous aggregates.⁴⁶ The fluorescence intensity of ThT increases by 20 fold in presence of pre-formed Aβ fibrils (Fig. 1), as observed previously for similar Aβ₄₂ peptide concentrations.³² Interestingly, we observe a significantly low ThT fluorescence with the Aβ₄₂ aggregates prepared in presence of various amounts of Cu²⁺ and Zn²⁺ (Fig. 1). A decreased ThT fluorescence^{32,36} in presence of Cu²⁺ ions can be accounted for by less fibril formation, although a quenching of fluorescence by the paramagnetic Cu²⁺ ions could also be involved.⁴⁷ Previous conflicting results suggested an increased or decreased ThT fluorescence intensity for Cu-Aβ aggregates. Exley *et al.*^{28,36} and Sugimoto *et al.*³² reported a decreased ThT fluorescence and suggested that Cu²⁺ prevents amyloid fibril formation, while Viles *et al.*³³ suggested an increased ThT fluorescence and thus an increased extent of fibrillization for the Aβ-Cu²⁺ species. Overall, our results are in line with the former reports, with a decreased ThT fluorescence observed for the Cu-Aβ₄₂ aggregates. In the presence of diamagnetic Zn²⁺ ions, although the effect is slightly lower than for Cu²⁺, the decrease in ThT fluorescence suggests the formation of non-fibrillar aggregates (see below),⁴⁸ suggesting that the nature of Aβ aggregates determines the intensity of ThT fluorescence.

As described above, the use of ThT fluorescence to probe the aggregation of Aβ peptide has limitations as the results can be

affected by metal complex formation, paramagnetic quenching effect, pH of media, and formation of amorphous aggregates. Thus, other methods of analysis such as TEM and native gel electrophoresis/Western blotting are needed to accurately evaluate the effect of metal ions on Aβ₄₂ aggregation.

Aβ aggregation monitored by TEM and native gel/Western blotting

Using both native gel electrophoresis/Western blotting and TEM techniques, a more detailed picture of the extent and pathways of Aβ aggregation can be obtained. While the former type of analysis reveals the presence of smaller, soluble Aβ species, the latter method reveals the morphology of the larger, insoluble Aβ aggregates that cannot be characterized by gel electrophoresis.

We have focused mainly on studying the effect of Cu²⁺ and Zn²⁺ on the Aβ₄₂ peptide aggregation, since Aβ₄₂ was shown to be more neurotoxic and form soluble Aβ₄₂ oligomers.^{7,8,11} To confirm this observation, we studied the aggregation of Aβ₄₀

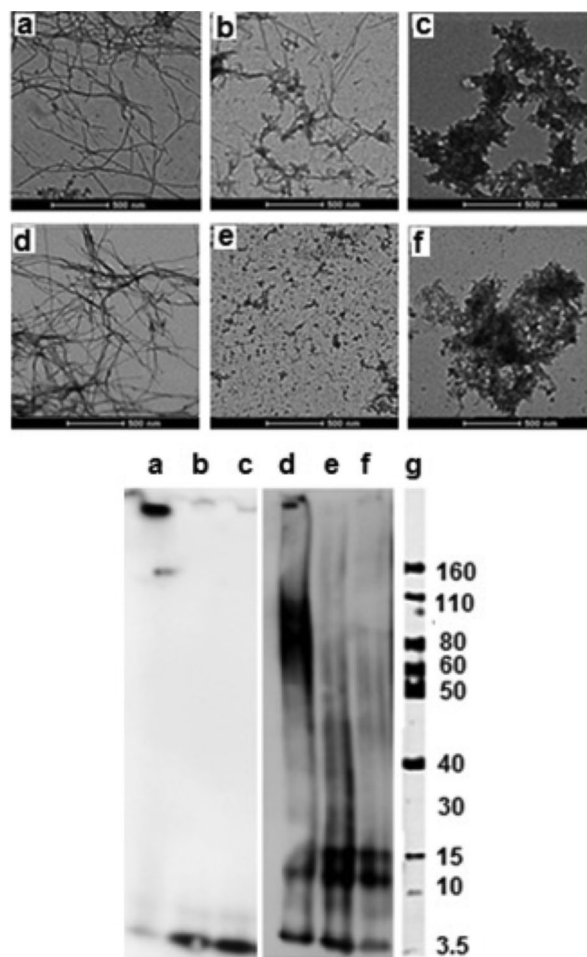


Fig. 2 TEM images and native gel/Western blots of Aβ₄₀ and Aβ₄₂ fibrils in absence and presence of Cu and Zn. Top: (a) Aβ₄₀; (b) Aβ₄₀ + Cu²⁺; (c) Aβ₄₀ + Zn²⁺; (d) Aβ₄₂; (e) Aβ₄₂ + Cu²⁺; (f) Aβ₄₂ + Zn²⁺; bottom: Western analysis of same samples. Conditions: 24 hours with agitation in PBS at 37 °C, [Aβ] = [M] = 25 μM. The scale bar represents 500 nm.

and $A\beta_{42}$ in presence and absence of Cu^{2+} and Zn^{2+} . TEM images and the corresponding native gel/Western blots are shown in Fig. 2. Panels a–c show the TEM images of $A\beta_{40}$ aggregation in absence and presence of Cu^{2+} and Zn^{2+} , respectively. $A\beta_{40}$ and $A\beta_{42}$ both form fibrils after incubation for 24 h at 37 °C (Fig. 2a and d). In the Western blot, although $A\beta_{40}$ does not show formation of any oligomeric species, it shows a dark band at the top of gel (lane a). This band likely corresponds to $A\beta$ fibrils or large aggregates which cannot enter the gel. The Western blot of $A\beta_{42}$ shows formation of various size oligomers (trimer, tetramer, and higher MW oligomers) as well as a small band at gel entrance (lane d). These results strongly support the fact that $A\beta_{42}$ is the oligomer-forming peptide rather than $A\beta_{40}$.

In presence of Cu^{2+} , $A\beta_{40}$ forms fibrils although in a lower amount as observed by TEM, yet the nature of aggregates formed remains fibrillar (Fig. 2, panel b). Notably, the $A\beta_{40}$ peptide does not form oligomers either in presence or in absence of metal ions (Fig. 2, lanes a–c), a similar behavior being observed for $A\beta_{40}$ by SDS-PAGE.⁴⁹ In contrast to $A\beta_{40}$, $A\beta_{42}$ did not form amyloid fibrils in presence of Cu^{2+} , small $A\beta_{42}$ aggregates being formed instead as seen by TEM (panel e). Western blot confirms the formation of small soluble $A\beta_{42}$ oligomers (lane e), the formation of high MW oligomers being inhibited. Thus, Cu^{2+} seems to reduce the aggregation of $A\beta_{42}$ quite significantly. In presence of Zn^{2+} , both $A\beta_{40}$ and $A\beta_{42}$ form amorphous aggregates, as observed by TEM (panels c and f). Western blotting of Zn^{2+} - $A\beta_{42}$ aggregates shows formation of small soluble $A\beta_{42}$ oligomers, while high MW oligomers were not observed. Overall, the TEM and Western blotting results suggest that metal ions inhibit the $A\beta_{42}$ fibrillization process by stabilizing soluble $A\beta_{42}$ aggregates or formation of amorphous aggregates. Since the $A\beta_{42}$ aggregation was significantly inhibited by Cu^{2+} and thus could influence the etiology of AD, we have probed the effect of buffer, temperature, incubation time, and Cu^{2+} (and Zn^{2+}) stoichiometry on the observed metal-mediated formation of various $A\beta_{42}$ species and their neurotoxicity.

Effect of buffer on Cu^{2+} -mediated $A\beta_{42}$ aggregation

The commonly used buffers for $A\beta$ aggregation studies are HEPES and PBS. In addition, we tested two concentrations of HEPES, 20 μ M (pH 6.6) and 20 mM (pH 6.6 and pH 7.4). The former concentration has been used in previous reports for studying $A\beta$ aggregation in presence of Cu^{2+} ,^{50–52} although we believe this concentration may not be enough to maintain a buffered solution of 25 μ M $A\beta$. We did not observe any significant change between the two different pHs of HEPES. Under all conditions tested, TEM analysis shows a drastically reduced amount of $A\beta_{42}$ fibrils formed in presence of Cu^{2+} (Fig. 3). While some large $A\beta_{42}$ aggregates were observed in 20 μ M HEPES, overall the inhibition of $A\beta_{42}$ aggregation by Cu^{2+} does not seem to be dependent on the buffer used.

Effect of temperature on Cu^{2+} -mediated $A\beta_{42}$ aggregation

As temperature is expected to dramatically affect the aggregation process, we performed experiments at both 25 °C^{30,53} and 37 °C (physiological temperature),^{32,50–52} the most commonly

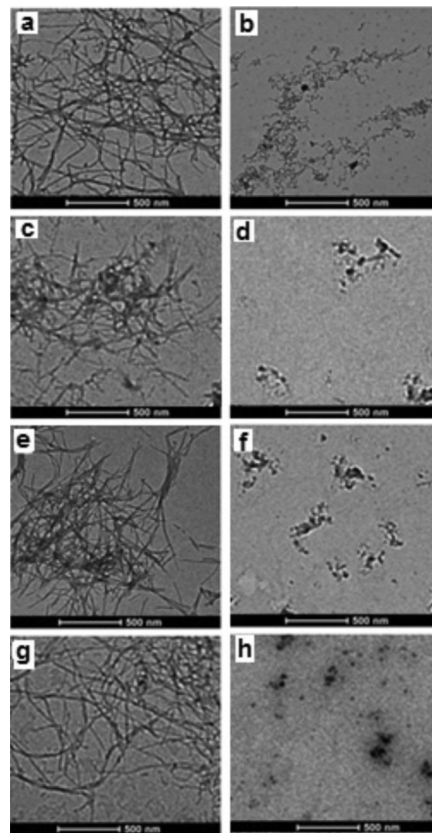


Fig. 3 TEM images of samples containing (a) $A\beta_{42}$ (20 μ M HEPES, 150 μ M NaCl, pH 6.6); (b) $A\beta_{42}$ + Cu^{2+} (20 μ M HEPES, 150 μ M NaCl, pH 6.6); (c) $A\beta_{42}$ (20 mM HEPES, pH 6.6); (d) $A\beta_{42}$ + Cu^{2+} (20 mM HEPES, pH 6.6); (e) $A\beta_{42}$ (20 mM HEPES, pH 7.4); (f) $A\beta_{42}$ + Cu^{2+} (20 mM HEPES, pH 7.4); (g) $A\beta_{42}$ (PBS, pH 7.4); (h) $A\beta_{42}$ + Cu^{2+} (PBS, pH 7.4). Conditions: 24 hours with agitation at 37 °C, $[A\beta] = [Cu^{2+}] = 25 \mu$ M. The scale bar represents 500 nm.

used temperatures to study $A\beta$ aggregation *in vitro*. $A\beta_{42}$ forms amorphous aggregates after incubation for 72 h at room temperature and forms a range of soluble oligomers (Fig. 4, panel a and lane a). However, the presence of Cu^{2+} limits the formation of insoluble aggregates and high MW oligomers and seems to stabilize small MW oligomers (Fig. 4, panel b and lane b). At 37 °C the $A\beta_{42}$ peptide forms amyloid fibrils within 24 h as observed in TEM, as well as large MW oligomers as seen in the Western blot (Fig. 4, panel c and lane c). By contrast, the presence of Cu^{2+} leads to formation of small MW oligomers, which suggests that Cu^{2+} stabilizes $A\beta_{42}$ oligomers even at 37 °C (Fig. 4, lane d). Overall, these experiments suggest that Cu^{2+} stabilizes soluble $A\beta_{42}$ species at both temperatures and use of RT allows for a detailed study of the Cu^{2+} -mediated oligomerization process.

Dose-dependent Cu^{2+} - $A\beta_{42}$ aggregation

The presence of a trace amount of Cu^{2+} was found to affect the $A\beta$ aggregation behavior in AFM studies.³⁵ Thus, various concentrations of Cu^{2+} were added to a fixed $A\beta_{42}$ concentration (25 μ M) and the aggregation behavior was investigated by TEM and Western blotting. Five different concentrations of

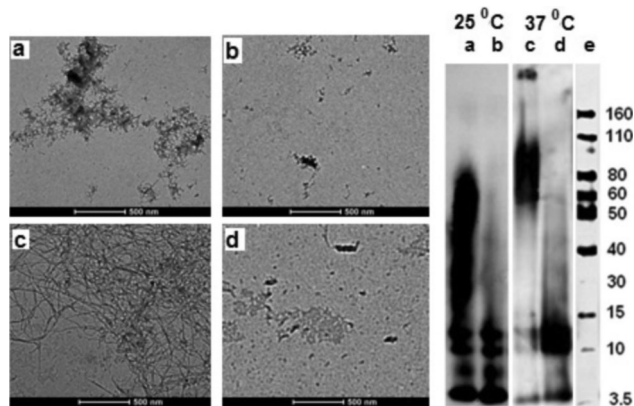


Fig. 4 TEM images and native gel/Western blot of $A\beta_{42}$ aggregation in absence and presence of Cu^{2+} . Left: TEM images for (a) $A\beta_{42}$ aggregation for 72 h at 25 °C; (b) $A\beta_{42} + Cu^{2+}$ aggregation for 72 h at 25 °C; (c) $A\beta_{42}$ aggregation for 24 h at 37 °C; (d) $A\beta_{42} + Cu^{2+}$ aggregation for 24 h at 37 °C. Right: the corresponding Western blots, (e) molecular weight ladder. Conditions: $[A\beta] = [Cu^{2+}] = 25 \mu M$, PBS. The scale bar represents 500 nm.

Cu^{2+} ranging from 0.25 to 2 equiv. relative to $A\beta_{42}$ were tested (Fig. 5). All concentrations of Cu^{2+} were effective in reducing $A\beta_{42}$ fibrillization, as monitored by ThT fluorescence (Fig. 1) and TEM (Fig. 5). While the presence of 0.25 equiv. Cu^{2+} (6.25 μM) does not completely inhibit $A\beta_{42}$ aggregation, Cu^{2+} amounts larger than 0.5 equiv. lead to formation of soluble $A\beta_{42}$ species. Importantly, the TEM images in Fig. 5 are representative of the entire surface of TEM grids that show a homogeneous distribution of the observed $A\beta$ species. In addition, the Western blots of these dose-dependent experiments suggest that all Cu^{2+} concentrations inhibit the $A\beta_{42}$ aggregation. The formation of insoluble aggregate and high MW oligomers (80–160 kDa) was inhibited by any amount of Cu^{2+} present (Fig. 5), and higher concentrations of Cu^{2+} lead to an increased formation of smaller soluble $A\beta_{42}$ oligomers (Fig. 5, lanes d–f).

Overall, both TEM and native gel/Western blotting studies strongly suggest that Cu^{2+} has an inhibitory effect on $A\beta_{42}$ fibrillization and leads to the formation of soluble $A\beta_{42}$ oligomers. Our results are in line with the recent report by Exley *et al.*²⁸ that employs ThT fluorescence and TEM to study the role of Cu^{2+} in preventing fibril formation. However, we have also employed native gel/Western blotting to reveal the formation of soluble $A\beta_{42}$ species in presence of Cu^{2+} , strongly supporting our hypothesis that Cu^{2+} stabilizes such soluble $A\beta_{42}$ species. In addition, the Cu^{2+} -mediated stabilization of soluble $A\beta_{42}$ species also occurs in presence of Zn^{2+} ions – as observed by TEM, and thus is expected to be physiologically relevant. Importantly, these results have direct implications into the neurotoxicity of $A\beta_{42}$ (see below).

Dose-dependent Zn^{2+} - $A\beta_{42}$ aggregation

A similar experiment was carried out to study the effect of Zn^{2+} concentration on $A\beta_{42}$ aggregation, and five different concentrations of Zn^{2+} ranging from 0.25 to 2 equiv. relative to $A\beta_{42}$ were tested. Interestingly, while $A\beta_{42}$ fibrils were observed after incubation in absence of Zn^{2+} at room temperature for 1 day,

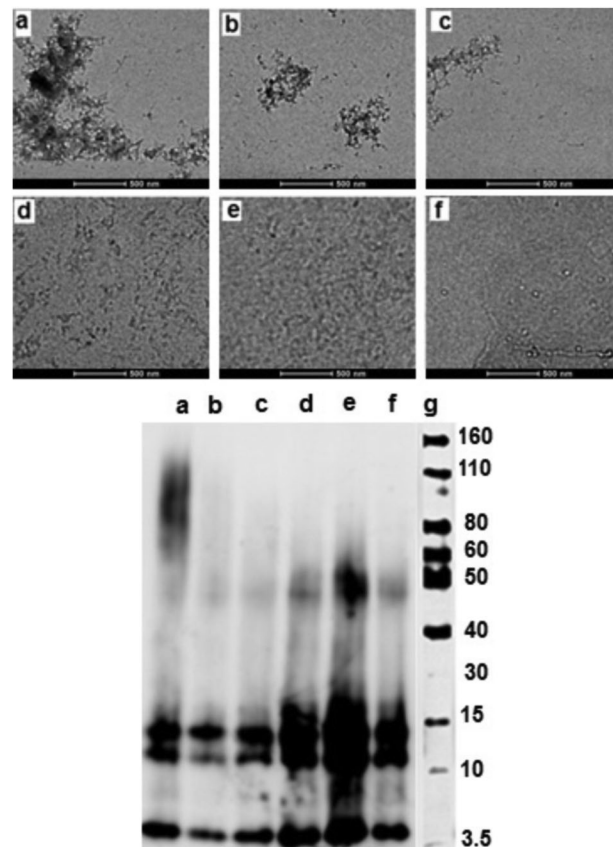


Fig. 5 Top: TEM images and for samples containing 25 μM $A\beta_{42}$ with: (a) 0 equiv. Cu^{2+} ; (b) 0.25 equiv. Cu^{2+} (6.25 μM); (c) 0.5 equiv. Cu^{2+} (12.5 μM); (d) 1.0 equiv. Cu^{2+} (25 μM); (e) 1.5 equiv. Cu^{2+} (37.5 μM); (f) 2 equiv. Cu^{2+} (50 μM). Bottom: the corresponding native gel/Western blots, (g) molecular weight ladder. Conditions: 24 h, 25 °C, PBS. The scale bar represents 500 nm.

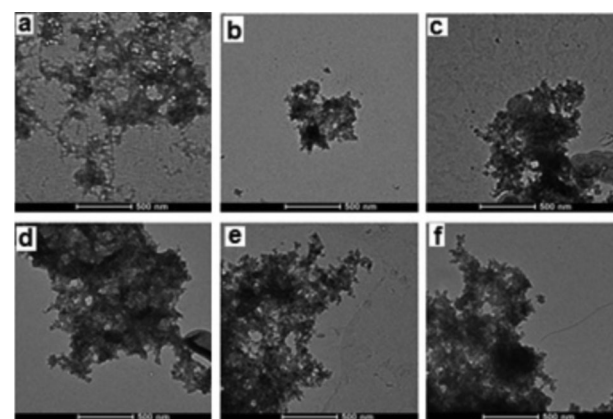


Fig. 6 TEM images of samples containing 25 μM $A\beta_{42}$ with: (a) 0 equiv. Zn^{2+} ; (b) 0.25 equiv. Zn^{2+} (6.25 μM); (c) 0.5 equiv. Zn^{2+} (12.5 μM); (d) 1 equiv. Zn^{2+} (25 μM); (e) 1.5 equiv. Zn^{2+} (37.5 μM); (f) 2 equiv. Zn^{2+} (50 μM). Conditions: 24 h, 25 °C, PBS. The scale bar represents 500 nm.

presence of as low as 0.25 equiv. Zn^{2+} causes formation of amorphous aggregates as observed by TEM (Fig. 6), a similar behavior being observed in the presence of higher concentrations of Zn^{2+} . The experiment suggests that even a substoichiometric

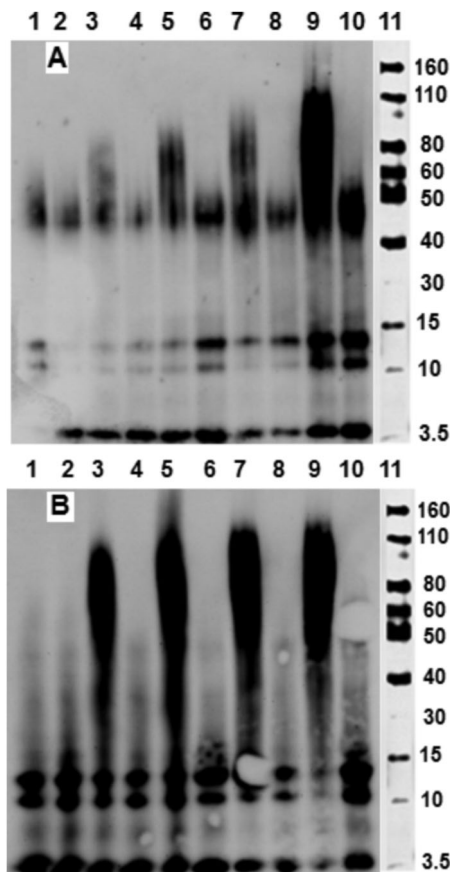


Fig. 7 Native gel/Western blots for time-dependent aggregation of Aβ₄₂ with and without Cu²⁺ or Zn²⁺. Panel A: lane 1, Aβ₄₂, day 0; lane 2, Aβ₄₂ + Cu²⁺, day 0; lane 3, Aβ₄₂, day 1; lane 4, Aβ₄₂ + Cu²⁺, day 1; lane 5, Aβ₄₂, day 2; lane 6, Aβ₄₂ + Cu²⁺, day 2; lane 7, Aβ₄₂, day 3; lane 8, Aβ₄₂ + Cu²⁺, day 3; lane 9, Aβ₄₂, day 4; lane 10, Aβ₄₂ + Cu²⁺, day 4. Panel B: lane 1, Aβ₄₂, day 0; lane 2, Aβ₄₂ + Zn²⁺, day 0; lane 3, Aβ₄₂, day 1; lane 4, Aβ₄₂ + Zn²⁺, day 1; lane 5, Aβ₄₂, day 2; lane 6, Aβ₄₂ + Zn²⁺, day 2; lane 7, Aβ₄₂, day 3; lane 8, Aβ₄₂ + Zn²⁺, day 3; lane 9, Aβ₄₂, day 4; lane 10, Aβ₄₂ + Zn²⁺, day 4; lanes 11, molecular weight ladder. Conditions: 24 h, 25 °C, PBS.

amount of Zn²⁺ dramatically affects Aβ₄₂ fibrillization and leads to formation of amorphous Aβ₄₂ aggregates instead of soluble oligomers.

Time-dependent Aβ₄₂ oligomerization–aggregation with Cu²⁺ and Zn²⁺

We then investigated the time dependency of the Aβ₄₂ oligomerization–aggregation process in absence and presence of metal ions. Equivalent amounts of Aβ₄₂ and M²⁺ were incubated at room temperature for four days and the aggregation was monitored by native gel/Western blotting. Aβ₄₂ by itself forms large MW oligomers at room temperature (Fig. 7); this is in contrast with the incubation at 37 °C in which Aβ₄₂ fibrils are formed instead (Fig. 4). Interestingly, the presence of Cu²⁺ seems to inhibit formation of the large MW oligomers and selectively stabilizes small MW oligomers, even after four days (Fig. 7A). On the other hand, Zn²⁺ leads to disappearance of high MW oligomers, only small Aβ₄₂ oligomers being observed

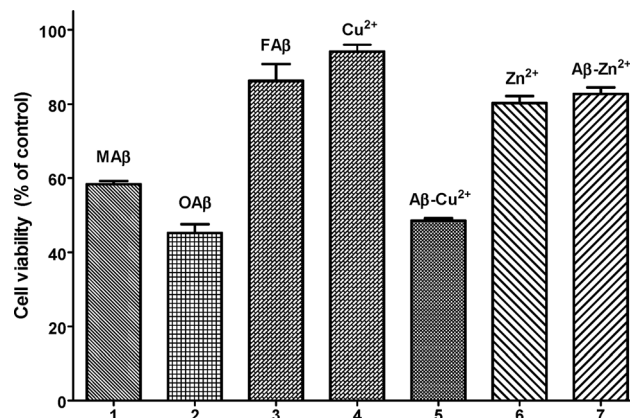


Fig. 8 Cell viability (% of control) upon incubation of Neuro-2A cells with (1) monomeric Aβ₄₂ (MAβ); (2) Aβ₄₂ oligomers (OAβ); (3) Aβ₄₂ fibrils (FAβ); (4) Cu²⁺; (5) MAβ₄₂ + Cu²⁺; (6) Zn²⁺; (7) MAβ₄₂ + Zn²⁺. Conditions: [Aβ₄₂] = [M²⁺] = 20 μM. The *t*-test analysis of experimental data reveals values of *p* < 0.001 for any two of the above lanes, except for lanes 2 and 5 that are not statistically different (*p* = 0.206).

(Fig. 7B) that form likely along with an appreciable amount of insoluble amorphous aggregates (Fig. 6).

Cell toxicity studies

The role of metal ions in the etiology of AD is still unclear, and conflicting results on the effect of metal ions on Aβ toxicity have been reported. An increased^{39–42} or decreased^{34,43} toxicity of Aβ has been observed in the presence of transition metal ions, and these *in vitro* studies have employed either Aβ₄₀ or Aβ₄₂ peptides. It was reported recently that an increased ratio of Aβ₄₂/Aβ₄₀ in amyloid plaques increases their toxicity,⁵ therefore we have investigated the effect of Cu²⁺ and Zn²⁺ on the cell toxicity of the more neurotoxic Aβ₄₂ peptide.^{54–56} The viability of Neuro-2A (N2A) cells⁵⁷ was probed using the Alamar Blue cell assay, which was shown to be more appropriate for Aβ toxicity studies *vs.* the MTT assay.^{58,59}

The cell toxicity results suggest that monomeric Aβ₄₂ leads to 58 ± 2% cell survival, likely due to formation of soluble Aβ₄₂ oligomers during the 40 h incubation with the cells (Fig. 8, lane 1). In addition, the cell survival with preformed Aβ₄₂ oligomers (OAβ) and Aβ₄₂ fibrils (FAβ) is 45 ± 6% and 86 ± 8%, respectively (Fig. 8, lanes 2 and 3), supporting the more neurotoxic nature of Aβ₄₂ oligomers.^{60,61} While Cu²⁺ was not toxic to cells in the absence of Aβ₄₂ – a 90 ± 5% cell survival being observed in presence of Cu²⁺ (Fig. 8, lane 4), a markedly reduced 48 ± 2% cell survival was observed in the presence of Aβ₄₂ and Cu²⁺ (Fig. 8, lane 5). Thus, the presence of Cu²⁺ leads to a statistically significant decrease in cell viability, which is similar to that observed for preformed Aβ₄₂ oligomers (Fig. 8, lane 5 *vs.* lane 2). It is important to mention here that earlier it was suggested that substoichiometric amounts of Cu²⁺ promote Aβ fibrillization and thus enhance Aβ-induced toxicity, yet an equimolar or excess amount of Cu²⁺ did not have a similar effect.³³ However, our results suggest that Cu²⁺ in an equimolar ratio to Aβ₄₂ also leads to an appreciable cell toxicity, strongly

supporting our hypothesis that Cu^{2+} stabilizes $\text{A}\beta_{42}$ oligomers and thus increases the neurotoxicity of $\text{A}\beta_{42}$ aggregates. By contrast, Zn^{2+} reduces the toxicity of monomeric $\text{A}\beta_{42}$, (Fig. 8, lane 6 vs. lane 1), while a similar cell survival ($80 \pm 5\%$) was observed for Zn^{2+} in both presence or absence of $\text{A}\beta_{42}$ (Fig. 8, lanes 6 and 7). Zn^{2+} reduces the toxicity of $\text{A}\beta_{42}$ most likely by formation of non-toxic amorphous aggregates. The results are in line with previous reports suggesting a neuroprotective role of Zn^{2+} .^{30,62} Overall, these cell toxicity studies are supported by ThT fluorescence, native gel/Western blotting, and TEM, showing the formation of neurotoxic soluble $\text{A}\beta_{42}$ species in presence of Cu^{2+} , while non-toxic amorphous aggregates were observed in presence of Zn^{2+} (see above).

Conclusion

In summary, we have studied herein the effect of Cu^{2+} and Zn^{2+} on $\text{A}\beta_{42}$ oligomerization and aggregation. ThT fluorescence, native gel electrophoresis/Western blotting, and TEM studies suggest that Cu^{2+} and Zn^{2+} inhibit $\text{A}\beta_{42}$ fibrillization: while Zn^{2+} forms insoluble amorphous aggregates, Cu^{2+} leads to formation of soluble $\text{A}\beta_{42}$ oligomers. Importantly, the cell toxicity assays show that Cu^{2+} leads to an enhanced toxicity due to formation of neurotoxic soluble $\text{A}\beta_{42}$ oligomers, while Zn^{2+} decreases $\text{A}\beta_{42}$ toxicity by formation of non-toxic insoluble amorphous aggregates. Previous studies have reported conflicting results, especially regarding the effect of Cu^{2+} on $\text{A}\beta_{42}$ aggregation. Our cellular toxicity studies confirm that $\text{A}\beta_{42}$ oligomers are more neurotoxic than insoluble $\text{A}\beta_{42}$ aggregates and the increased toxicity observed in presence of both $\text{A}\beta_{42}$ and Cu^{2+} strongly supports our hypothesis that Cu^{2+} stabilizes soluble $\text{A}\beta_{42}$ oligomers. These studies rule out a preventive role of Cu^{2+} and suggest that Cu^{2+} may lead to an increased $\text{A}\beta_{42}$ neurotoxicity *in vivo* due to formation of $\text{A}\beta_{42}$ oligomers. Thus, this Cu^{2+} -promoted increased neurotoxicity of soluble $\text{A}\beta_{42}$ species may play an important role in the etiology of AD.

It has been considered that modulating metal ion homeostasis *via* metal chelation therapy may be a valid method to control the onset of AD.⁶³ However, we have previously shown that for the $\text{A}\beta_{42}$ peptide, in contrast to the $\text{A}\beta_{40}$ peptide, the previously employed strategy of inhibiting $\text{A}\beta$ aggregation and promoting amyloid fibril disaggregation may not be optimal for the development of potential AD therapeutics, due to formation of neurotoxic soluble $\text{A}\beta_{42}$ oligomers.²⁹ Thus, we consider that the development of metal chelating compounds which do not lead to formation of toxic $\text{A}\beta_{42}$ oligomers should be promoted. Research efforts in this direction are currently ongoing in our laboratory.

Notes and references

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