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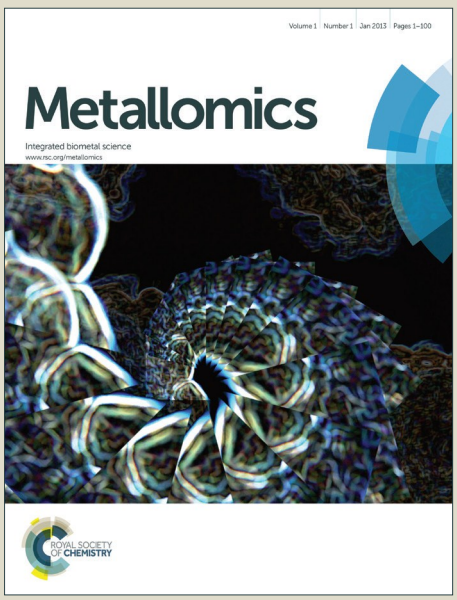

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$[Ru(phen)_2dppz]^{2+}$ (phen=1,10-phenanthroline, dppz= dipyrido [3,2-a:2'.3'-c] phenazine) holds red-shifted emission, large stokes shifts about 190 nm and high photo-stability, which has been widely used to monitor protein aggregation and used to monitor in real-time the transition of $A\beta_{1-40}$ monomers into fibrils.[16,17] As shown in Figure 1, Compound $[Ru(phen)_2dppz]^{2+}$ itself showed very weak fluorescence emission (Fig.1a), which was significantly increased after mixed with $A\beta_{1-42}$ with Cu^{2+} ions (Fig.1b), suggesting high affinity of this compound for amyloidogenic $A\beta_{1-42}$ with Cu^{2+} aggregates. After addition of CeONP, the fluorescence intensity of $[Ru(phen)_2dppz]^{2+}$ with $A\beta_{1-42}$ with Cu^{2+} ions was decreased 62% (Fig. 1c). These results further confirmed that CeONP can effectively reduce the amyloid peptide aggregation in vitro.

The effect of CeONP in inhibiting amyloid peptide from aggregation by photoluminescence of $[Ru(phen)_2dppz]^{2+}$ was also detected in SH-SY5Y cells. External $A\beta_{1-42}$ could enter SH-SY5Y cells and aggregated in cytoplasm, which were confirmed by fluorescence microscopy images of SH-SY5Y cells stained with FITC-labeled $A\beta_{1-42}$(50μM, green) (Fig. S1) (FITC means fluorescein isothiocyanate isomer). We observed that FITC-labeled $A\beta_{1-42}$ aggregated around SH-SY5Y nuclei, which was stained with DAPI (blue). $A\beta_{1-42}$ or $A\beta_{1-42}$ plus Cu^{2+} was incubated with SH-SY5Y cells in the presence of $[Ru(phen)_2dppz]^{2+}$. Photoluminescence properties of $A\beta_{1-42}$-treated SH-SY5Y cells were examined by confocal scanning laser microscopy. SH-SY5Y cells, after incubated with $A\beta_{1-42}$, displayed some red photoluminescence intensity of $[Ru(phen)_2dppz]^{2+}$ (Fig. 2B), comparing with the control of SH-SY5Y cells alone (Fig. 2A). The treatment of SH-SY5Y cells with $A\beta_{1-42}$ plus Cu^{2+} resulted in dramatic increase in $[Ru(phen)_2dppz]^{2+}$ photoluminescence intensity, suggesting the binding of $[Ru(phen)_2dppz]^{2+}$ with aggregated amyloid peptide (Fig. 2C). These results indicated that Cu^{2+} ions induced tremendous $A\beta_{1-42}$ aggregation, and $[Ru(phen)_2(dppz)]^{2+}$ interacted strongly with the fibril framework of the amyloid peptide, which changed the polarity of the microenvironment felt by the dppz ligand, favoring the luminescent state. However, in the presence of CeONP, the red $[Ru(phen)_2dppz]^{2+}$ photoluminescence intensity of SH-SY5Y cells treated with $A\beta_{1-42}$ plus Cu^{2+} dramatically decreased (Fig. 2D). This study revealed that CeONP played a pivotal role in suppression of $A\beta_{1-42}$ aggregation.

In the presence or absence of CeONP, the aggregation extents and morphology of $A\beta_{1-42}$ plus Cu^{2+} were monitored by transmission electron microscopy (TEM) (Fig. S2).[14] After 72 h of quiescent incubation at 37ºC, $A\beta_{1-42}$ plus Cu^{2+} displayed abundance of cross-linked fibril aggregations (Fig. S2A). The aggregation fibrils of $A\beta_{1-42}$ induced by Cu^{2+} was obviously reduced after CeONP-treatment (Fig. S2B). The results, consistent with photoluminescence of $Ru(phen)_2dppz]^{2+}$ study, suggested that CeONP decreased the aggregation tendency and intensity of $A\beta_{1-42}$.

The study by mass spectroscopy (Fig. S3) demonstrated that CeONP can effectively decrease Cu^{2+}-induced $A\beta_{1-42}$ aggregation intensity. The mass spectrometry analysis indicated that $A\beta_{1-42}$ appeared at m/z = 1083. After addition of Cu^{2+} ions, peaks of amyloid peptides shifted to m/z = 1099, corresponding to more aggregated oligomer forms. Cu^{2+} ions promoted the processes of $A\beta_{1-42}$ aggregation. In the presence of CeONP, the oligomers of $A\beta_{1-42}$ with Cu^{2+} at m/z = 1099 shifted back to m/z = 1083. These peaks shift of $A\beta_{1-42}$ indicated that CeONP inhibits the aggregation of $A\beta_{1-42}$, despite it is difficult to quantitatively identify what kinds of $A\beta_{1-42}$ oligomers.

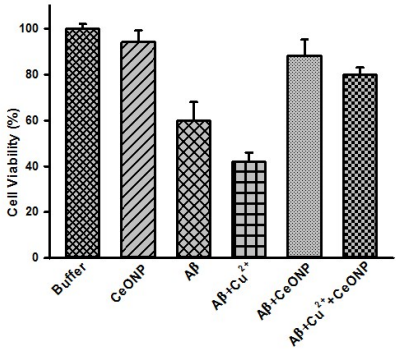

Figure 3. Cell viability of SH-SY5Y cells in different conditions. The cells were treated with 50 μM $A\beta_{1-42}$ or 50 μM Cu^{2+}-$A\beta_{1-42}$ in the presence or absence of 50 μM CeONP.

To evaluate the role of CeONP on neuronal cytotoxity of $A\beta_{1-42}$ plus Cu^{2+} ions, cell viability was determined quantitatively by MTT assay using SH-SY5Y human neuroblastoma cells. $A\beta_{1-42}$ in the oligomeric state is cytotoxic to neuronal cells.[18] As Figure 3 shown, the cell viability was about 60% and 42%, when incubated the cells with $A\beta_{1-42}$ and Cu^{2+}+$A\beta_{1-42}$, respectively. Upon treatment of the cells with CeONP, the cell viability was increased to 90% and 81%, with $A\beta_{1-42}$ and Cu^{2+}+$A\beta_{1-42}$, respectively. The results indicated that CeONP can significantly protect against neuronal cytotoxicity from amyloid peptide and copper ions.

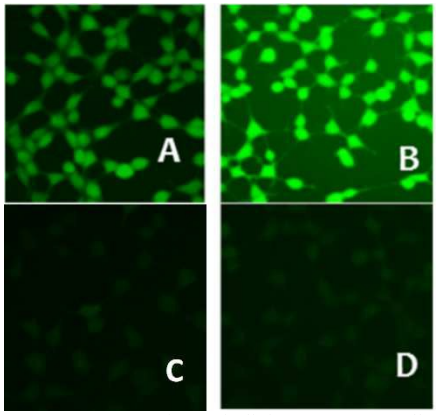

Figure 4. Fluorescence assays of ROS in living SH-SY5Y cells. The ROS was monitored by a fluorescent assay kit of DCFH. (A) $A\beta_{1-42}$; (B) Cu^{2+} + $A\beta_{1-42}$; (C) $A\beta_{1-42}$ + CeONP; (D) Cu^{2+} +$A\beta_{1-42}$ + CeONP, respectively. Concentrations of both Aβ and CeONP are 50 μM.

Reactive oxygen species (ROS) is the critical factor responsible for neuronal cytotoxicity induced by $A\beta_{1-42}$ and redox metal ions. The concentration of ROS in living cells was sensitively detected by the fluorescent probe with an ROS assay.[15] Green fluorescence intensity is in proportion to the ROS concentration in the cells. The reduction of ROS by the CeONP was detected using the fluorescent probe DCFH in SH-SY5Y cells in the presence of $A\beta_{1-42}$ or Cu^{2+}+$A\beta_{1-42}$. As shown in

Figure 4, $A\beta_{1-42}$ and Cu^{2+}-$A\beta_{1-42}$ produced obvious amount of ROS. With the addition of CeONP, green fluorescence intensity of DCFH decreased significantly indicating that CeONP can reduce the amount of ROS in cells. The results are consistent with those found in cell viability experiments to ascertain that the cellular toxicity is in line with the generation of ROS induced by $A\beta_{1-42}$ or Cu^{2+}+$A\beta_{1-42}$.[7]

To further probe the molecular mechanism for CeONP to protect against neuronal cytotoxicity from amyloid peptide and copper ions, EPR was applied. The redox active Cu^{2+} ions bound with $A\beta_{1-42}$ produce reactive oxygen species (ROS) by Fenton-type and Harber–Weiss-type reactions, resulting in oxidative stress of the cells.[6] The generated ROS (especially hydroxyl radicals) can influence cell metabolism and also promote $A\beta_{1-42}$ aggregation. We now know that CeONP can protect from neurotoxicity of Cu^{2+}-$A\beta_{1-42}$, however, it is not clear whether CeONP reacts with redox copper ions directly, or eliminates ROS generated from $A\beta_{1-42}$ plus copper ions? In both cases, the neuronal cytotoxicity from amyloid peptide and copper ions could be reduced. As indicated in Figure 5A, treating Cu^{2+}-$A\beta_{1-42}$ with CeONP did not reduce or abolish Cu^{2+}-EPR signals, while the free radical signals induced by Cu^{2+}-$A\beta_{1-42}$ were reduced and then disappeared upon the addition of CeONP (Figure 5B). These results suggested that CeONP can be administered in an amount sufficient to abolish ROS from Cu^{2+}-$A\beta_{1-42}$ by Ce^{3+}/Ce^{4+} catalytic cycles.

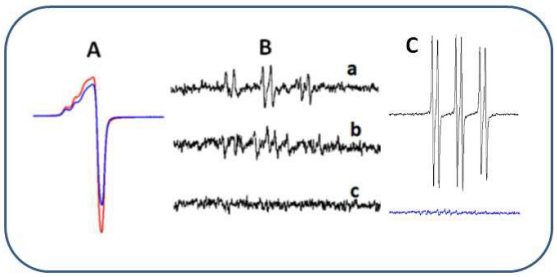

Figure 5. EPR of Cu^{2+} plus $A\beta_{1-42}$ in the presence and absence of CeONP. Panel A, Cu^{2+} ions EPR of Cu^{2+}-$A\beta_{1-42}$ (blue line) and Cu^{2+} plus $A\beta_{1-42}$ with CeONP (red line). Panel B, CeONP blocks Cu^{2+} and $A\beta_{1-42}$-induced free radical formation [a, Cu^{2+} plus $A\beta_{1-42}$ (50μM); b, Cu^{2+} plus $A\beta_{1-42}$ (50μM) and CeONP(50μM); c, Cu^{2+} plus $A\beta_{1-42}$ (50μM) and CeONP (100μM)]. Panel C, CeONP abolishes radicals generated from Cu^{2+} plus $A\beta_{1-42}$ and H_2O_2.

Cu^{2+}-$A\beta_{1-42}$ induced the production of free radicals, and this occasionally causes the formation of partially reduced oxygen forms, commonly known as ROS.[19] These can be highly reactive and potentially very dangerous to neural cells, and must be scavenged by exogenous or endogenous antioxidant systems to keep their level below a critical threshold. The Ce^{3+}/Ce^{4+} switch of CeONP resembles the mechanism of redox enzymes. Reactions involving redox cycles between the Ce^{3+} and Ce^{4+} oxidation states allow CeONP to react catalytically with superoxide and hydrogen peroxide, mimicking the behaviour of two key antioxidant enzymes, SOD and catalase, potentially abating all noxious intracellular ROS via a self-regenerating mechanism.

In conclusion, morphology of TEM, MS, and Fluorecent spectroscopy of $[Ru(phen)_2dppz]^{2+}$ revealed that CeONP reduces $A\beta_{1-42}$ aggregation. Cell viability assay and fluorescence assays of ROS in living SH-SY5Y cells indicated that CeONP protects neurotoxicity of $A\beta_{1-42}$ or Cu^{2+}-$A\beta_{1-42}$ by scavenging ROS radical production via Ce^{3+}/Ce^{4+} catalytic cycles. EPR studies demonstrated that CeONP doesn't react directly with Cu^{2+} ions, while it scavenges ROS generated from Cu^{2+}-$A\beta_{1-42}$. All these results provide valuable insights into the molecular mechanism for CeONP as a therapeutic intervention to reduce oxidative damage in Alzheimer's disease.

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Notes and references

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