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Self-triggered click reaction in an Alzheimer's disease model: *in situ* bifunctional drug synthesis catalyzed by neurotoxic copper accumulated in amyloid- β plaques†

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Cu is one of the essential elements for life. Its dyshomeostasis has been demonstrated to be closely related to neurodegenerative disorders, such as Alzheimer's disease (AD), which is characterized by amyloid- β (A β) aggregation and Cu accumulation. It is a great challenge as to how to take advantage of neurotoxic Cu to fight disease and make it helpful. Herein, we report that the accumulated Cu in A β plaques can effectively catalyze an azide-alkyne bioorthogonal cycloaddition reaction for fluorophore activation and drug synthesis in living cells, a transgenic AD model of *Caenorhabditis elegans* CL2006, and brain slices of triple transgenic AD mice. More importantly, the *in situ* synthesized bifunctional drug **6** can disassemble A β -Cu aggregates by extracting Cu and photo-oxygenating A β synergistically, suppressing A β -mediated paralysis and diminishing the locomotion defects of the AD model CL2006 strain. Our results demonstrate that taking the accumulated Cu ions in the A β plaque for an *in situ* click reaction can achieve both a self-triggered and self-regulated drug synthesis for AD therapy. To the best of our knowledge, a click reaction catalyzed by local Cu in a physiological environment has not been reported. This work may open up a new avenue for *in situ* multifunctional drug synthesis by using endogenous neurotoxic metal ions for the treatment of neurodegenerative diseases.

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Introduction

Cu is indispensable to life and plays an important role in energy generation, signal transduction, cellular respiration and antioxidant defense.¹ The fluctuation of Cu is associated with a wide range of diseases, including genetic diseases like Menkes and Wilson's diseases; neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, and prion diseases; metabolic disorders and even cancer.^{1,2} For diseases related to Cu accumulation, the most common treatment strategy is the direct use of Cu-chelators. However, systemic administration of chelating agents may cause adverse side effects because of indiscriminate metal chelation.³ It is a promising strategy to

harness toxic Cu under pathological conditions for defense against disease rather than just to chelate Cu.^{4–6}

Due to its high efficiency, fast kinetics, and mild reaction conditions, the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction has received special attention in many biochemical applications such as bioconjugation,^{7–11} biosensing,^{12–14} and drug synthesis.^{15,16} Many kinds of Cu sources are efficient for CuAAC bioorthogonal reactions. Besides typical Cu(I) complexes and the reduction of Cu(II) complexes,¹⁷ various Cu-containing nanoparticles (NPs) are increasingly used for CuAAC reactions, including Cu, Cu₂O and CuO NPs,^{18,19} Cu-immobilized inorganic and polymer NPs,^{20–22} as well as Cu-doped semiconductor NPs.^{23,24}

A major drawback of the CuAAC reaction in biological systems is Cu-triggered reactive oxygen species (ROS) from Fenton's reaction.²⁵ An additional but usually neglected side effect is that the addition of exogenous Cu-catalysts might disturb Cu homeostasis in physiological milieu. In terms of intracellular chemistry, a click reaction using endogenous Cu as the catalyst holds a special attraction.

Alzheimer's disease (AD), affecting about fifty million people worldwide, is suggested to be strongly correlated to amyloid- β (A β) aggregation and metal dysregulation.^{26–28} The typical concentration of Cu in A β plaques reaches up to 0.4 mM, while

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Organotypic brain slice cultures largely preserve brain architecture and possess major disease features, providing a practical platform for direct medication and real-time monitoring therapeutic effects.^{38,39} Therefore, this technology is widely used for studying neurodegenerative diseases. In addition, it has been observed that copper levels are elevated in senile plaques, the hippocampus, and cortex of AD model mice.^{40–42} Herein, we also investigated whether the click reaction can be catalyzed by Cu-accumulated plaques in the brain slices of triple transgenic AD ($3 \times \text{Tg-AD}$) model mice.⁴³ After incubation for 12 h, the fluorescence of the brain slices was much more intense for $3 \times \text{Tg-AD}$ mice than for normal mice, which demonstrated that the local Cu in brain slices from $3 \times \text{Tg-AD}$ mice can catalyze the CuAAC click reaction (Fig. S11 and S12†).

(a) Synthesis of Aβ40-Cu. Compound 1 (a naphthyl-substituted coumarin derivative) reacts with compound 2 (4-ethynylphenyl) in the presence of Aβ40-Cu to form compound 3. The reaction is inhibited by Cu²⁺ ions, as indicated by the red 'X' over the ICT arrow.

(b) Time-dependent fluorescence increase of Aβ40-Cu. The graph shows fluorescence (a.u.) versus wavelength (nm) from 450 to 600 nm. The fluorescence intensity increases over time (0, 10, 20, 40, 60, 80, 120 min) upon addition of Aβ40.

(c) Binding of Aβ40-Cu to Aβ40. The graph shows normalized fluorescence versus time (min) from 0 to 120 min. The fluorescence increases rapidly and then plateaus, indicating binding.

(d) Fluorescence spectra of Aβ40-Cu with varying Aβ40 concentrations. The graph shows count versus wavelength (nm) from 450 to 600 nm. The spectra are shifted to the right (red-shifted) as the concentration of Aβ40 increases, indicating binding.

(e) Bar graph of mean fluorescence for different Aβ40 concentrations. The concentrations are +3, +1+2+AA+Aβ40-Cu, +1+2+AA, +1+2, and +1. The mean fluorescence increases significantly with the addition of Aβ40.

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Repeated systemic administration is often hindered by absent target specificity and unpredictable toxicity in non-targeted tissue.^{44,45} Drugs may also suffer from rapid deactivation, poor pharmacokinetics, and low tissue absorption.⁴⁶ *In situ* drug synthesis within living cells or organisms through targeted bioorthogonal catalysis may overcome the above mentioned drawbacks because it can greatly improve drug concentration at desired sites and reduce off-target effects.^{47–52} We speculated that *in situ* drug synthesis through CuAAC reaction would probably mitigate the cytotoxicity caused by the A β -Cu aggregates. Recent studies have indicated that the aggregation properties and cytotoxicity of A β are closely related to the hydrophobicity of the protein.^{53,54} Photo-oxygenating A β is capable of disintegrating A β aggregates through increasing the protein polarity, which is an attractive strategy for AD therapy.^{55–58} However, in the presence of Cu, just oxygenating A β may not entirely block its fibrillation because Cu can still promote protein aggregation by coordinating to A β . Considering that Cu and A β are two critical pathogenic factors of AD,^{59,60} we envisioned that *in situ* synthesized bifunctional drug agent with A β -oxygenating and Cu-chelating properties could effectively disassemble A β -Cu aggregates *in vivo*.

To this end, compound **6** was rationally designed based on the following considerations: (1) thioflavin T (ThT) and its analogues can selectively photo-oxygenate amyloid aggregates.^{61,62} Without binding to β -sheet aggregates, photo-excited ThT undergoes twisted intramolecular charge transfer and transforms light energy to heat; (2) propargylglycine **5** has often been used for synthesizing transition metal chelators through a “click-to-chelate” approach.^{63,64} Therefore, the bifunctional compound **6** was constructed by integrating an A β -specific photosensitizer with a Cu prochelator (Fig. 2a).

Spectral titration⁶ and isothermal titration calorimetry (ITC) experiments^{65–67} were carried out to measure the binding stoichiometry (n) and binding affinity of compound **6** with Cu. After adding CuCl₂, the absorbance of compound **6** at 321 nm was slightly decreased (Fig. S13†). A breakpoint was observed at a ratio of 0.5 : 1, illustrating the binding stoichiometry of Cu and **6** was 1 : 2. For ITC assays, a weaker competitor glycine was added to prevent the precipitation of Cu. As shown in Fig. 2b and c, without considering the competing chelation of glycine,⁶⁶ apparent binding constants (K_a) of Cu to A β 40 and compound **6** were $9.64 \times 10^5 \text{ M}^{-1}$ and $7.21 \times 10^6 \text{ M}^{-1}$, respectively. The affinity of compound **6** to Cu was appropriate to disrupt A β -Cu interaction (Table S1†), without affecting the normal function of the metal enzymes.⁶⁷ The binding affinity between compound **5** and Cu was too low to detect by ITC, showing that propargylglycine and glycine have equivalent chelating ability to Cu. Fig. S14† indicates that chelating Cu slightly decreases the fluorescence of compound **6**.

We then assessed the ability of compound **6** to photo-oxygenate A β and inhibit its aggregation. 1,3-Diphenylisobenzofuran (DPBF)⁶⁸ was used to detect singlet oxygen (¹O₂) generated by photo-excited compound **6**. Glycerol, which can increase the solution viscosity, was used to restrict the bond rotation of the ThT analogue **6**. In contrast to 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, compound **6**

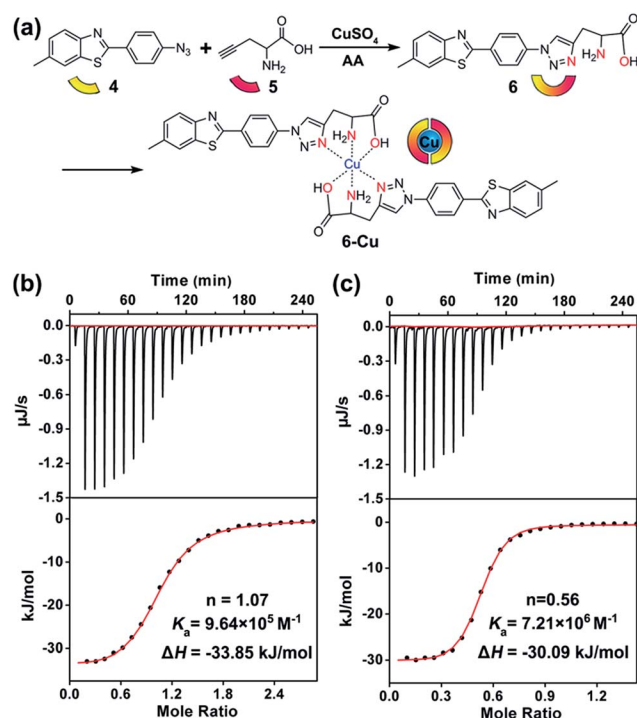


Fig. 2 Cu-chelating properties of **6**. (a) Scheme of the synthesis of the bifunctional compound **6**. (b and c), ITC data of CuCl₂ binding to A β 40 and compound **6**, respectively. The bottom panels show the fitting results. Details are described in the Experimental section.

produced more ¹O₂ in a glycerol/HEPES mixed solution (Fig. S15†).^{55,61} A β 40 (10 μM) and compound **6** (20 μM) were irradiated with UV light (1 W cm⁻²) for 1 h and then photo-oxygenated A β was detected by MS. Fig. S16† illustrates that both compounds **4** and **6** can effectively oxygenate A β 40.

A β aggregation kinetics were measured with Nile Red (NR), a widely used dye for studying fibril formation.⁶⁹ As shown in Fig. S17,† the effect of ThT analogues **4** and **6** on NR fluorescence is negligible. Fig. S18† demonstrates that **4** and **6** can inhibit A β 40 aggregation under light illumination. 8-Anilidonaphthalene-1-sulfonate (ANS), the fluorescence of which is strongly enhanced in the presence of the hydrophobic protein, was also applied to monitor A β fibrillation (Fig. S19†). ANS fluorescence was greatly enhanced for native A β 40 after incubation for 7 days, in contrast to the slight fluorescence change for photo-oxygenated A β 40. This implies that photo-oxygenation decreased A β 40 fibrillation and, possibly, toxicity, through increasing protein polarity.⁵⁶

The inhibition effect of compound **6** on Cu-accelerated A β 40 aggregation was then evaluated. After UV light (1 W cm⁻²) irradiation for 1 h, A β 40 monomer (10 μM) and Cu (10 μM) were incubated with 2 equiv. of **4** or **6** for 24 h. Then, NR assays, circular dichroism (CD), and AFM were conducted to monitor A β 40 aggregation. As the incubation period progressed, Cu induced A β 40 to form β -sheet-rich fibrils, while compound **6** blocked this process (Fig. 3). However, due to the lack of a Cu-chelating moiety, compound **4** could not inhibit Cu-induced A β 40 aggregation.



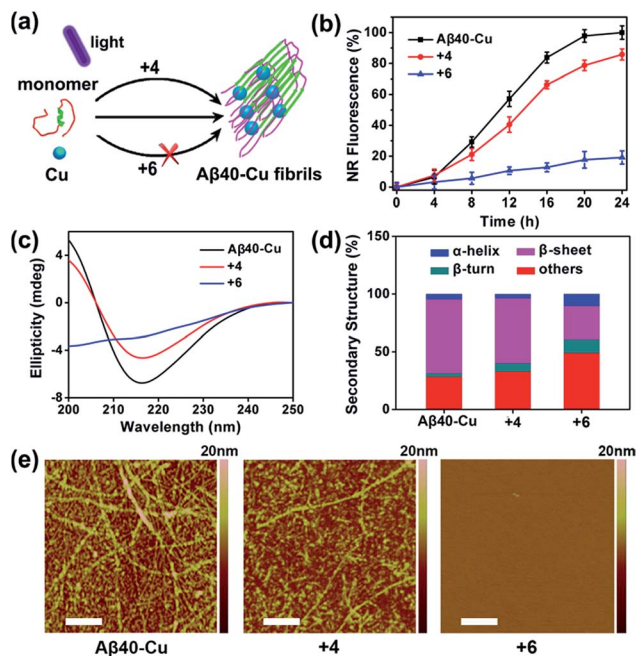


Fig. 3 Inhibition of Cu-induced A β 40 aggregation. (a) Scheme of the interruption of Cu-induced A β 40 fibrillation with compound 6 under light illumination. (b) Fibrillation kinetics of A β -Cu alone or in the presence of compounds 4 and 6, monitored by NR. Each experiment was repeated three times. Error bars indicate $\pm$ s.d. (c) CD spectra of A β 40-Cu alone or in the presence of compounds 4 and 6. (d) Secondary structure analysis of A β 40-Cu alone or in the presence of compounds 4 and 6. (e) AFM images of A β 40-Cu alone or in the presence of compounds 4 and 6. Scale bars: 500 nm.

Furthermore, photo-activated compound 6 could also dissociate A β 40-Cu aggregates. As shown in Fig. S20,† A β 40-Cu aggregates further assembled into long fibrils after incubation for 24 h. However, NR fluorescence gradually decreased for A β 40-Cu aggregates when incubated with compound 6, indicating that the A β 40-Cu aggregates were dissociated over the course of time in the presence of compound 6. AFM and ANS assays further supported that compound 6 dissociates A β 40-Cu aggregates (Fig. S20†). Compound 6 also inhibits and disassembles Cu-induced A β 42 aggregates (Fig. S21 and S22†).

Given the above results, we assessed the neuroprotective effect of compound 6 on the cytotoxicity triggered by A β 40-Cu aggregates. Immunofluorescence analysis showed the co-localization of compound 6 and anti-A β antibodies in PC12 cells (Fig. S23†). This implied that photo-activated compound 6 reduces non-specific oxygenation to lipid, DNA or other proteins in the cells.⁶¹ Methyl thiazolyl tetrazolium (MTT) assays revealed that 6 protects against A β -Cu-mediated cellular toxicity under light illumination (Fig. S24†).

We further evaluated the effectiveness of disassembling A β -Cu aggregates by 6 in brain slices of 3 $\times$ Tg-AD mice. A β plaques were labeled with NR to monitor the therapeutic effects. As shown in Fig. S25,† many A β aggregates could be observed in the hippocampus and cortex. For brain slices treated with compound 6 and UV light, the fluorescence of NR became weak after incubating for 12 h (Fig. S25, Table S2†), indicating that

photo-activated 6 was able to reduce Cu-A β aggregates in brain slices. The immunofluorescence study further confirmed the effectiveness of clearing A β plaques using photo-excited compound 6 (Fig. S26†).³⁹

We next investigated whether A β 40-Cu aggregates could act as a catalyst for the *in situ* synthesis of the bifunctional drug 6 in cells. It has been proven that silica nanoparticles can penetrate the blood-brain barrier (BBB) by inducing tight junction loss and cytoskeleton arrangement.^{70,71} Moreover, certain types of silica-based nanoparticles have been approved by the Food and Drug Administration (FDA) for clinical use.⁷² Herein, H₂O₂-responsive mesoporous silica nanoparticles (MSN) were used as a controlled-release nanocarrier to deliver the prodrugs to desired sites.^{73,74} Firstly, phenylboronic acid (BA) was modified on MSN and then MSN-BA was loaded with the prodrugs (4, 5, and AA). Immunoglobulin G (IgG) acted as a capping agent and the formed boronates were sensitive to H₂O₂.⁷⁵ The successful fabrication of MSN-IgG was validated by transmission electron microscopy (TEM, Fig. S27†), nitrogen adsorption-desorption isotherms (Fig. S28, Table S3†), Fourier transform infrared spectroscopy (FTIR, Fig. S29†), and thermogravimetric analysis (Fig. S30†). As shown in Fig. S31,† MSN-IgG efficiently released the loaded prodrug 4 when treated with 0.5 mM H₂O₂. It has been proved that H₂O₂ level is remarkably elevated in AD because of A β -Cu⁷⁶⁻⁷⁸ and H₂O₂ levels in partial area of cells can be 1 mM.⁷⁹ Table S4† shows that MSN can enter the cells.

To evaluate the therapeutic potential of *in situ* synthesized drug 6 in cells, PC12 cells were incubated with A β 40-Cu aggregates and prodrug-loaded MSN-IgG. As shown in Fig. 4a,

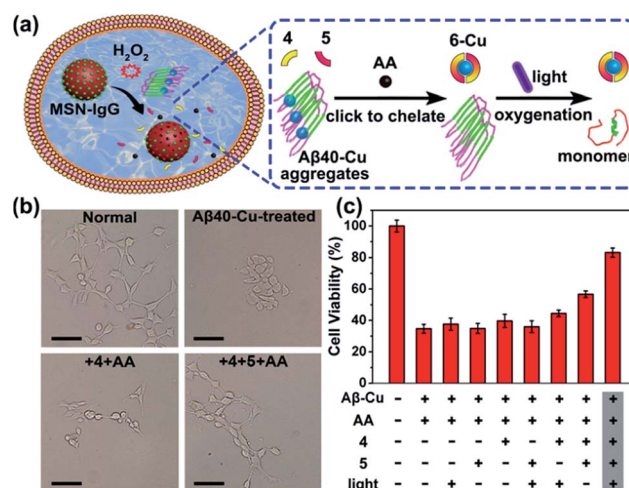


Fig. 4 *In situ* synthesis of bifunctional compound 6 for mitigating A β 40-Cu-induced cytotoxicity. (a) Overview of the *in situ* synthesis of compound 6 for disassembling A β 40-Cu aggregates. (b) Microscope images of the normal PC12 cells and A β 40-Cu-treated PC12 cells in the presence or absence of prodrug-loaded MSN. Scale bars: 20 μ m. (c) MTT assays. Each experiment was repeated three times. Error bars indicate $\pm$ s.d. PC12 cells were incubated with A β 40-Cu aggregates (10 μ M) and prodrug-loaded MSN-IgG (0.1 mg mL⁻¹) for 24 h and then irradiated with UV light (1 W cm⁻²) for 10 min in 20 min intervals 6 times. Cells were incubated for 24 h after the light irradiation and then MTT assays were carried out.



prodrug-loaded MSN-IgG was responsive to H_2O_2 induced by A β 40-Cu aggregates,⁷⁵ and the prodrugs (**4**, **5**, and AA) were released in the cells. Then A β 40-Cu aggregates catalyzed the click reaction and compound **6** was *in situ* synthesized, as verified by MS (Fig. S32†). Moreover, the yield of compound **6** increased upon an increase in the concentration of A β -Cu (Fig. S33†). In this regard, *in situ* drug synthesis without the use of the exogenous Cu-catalyst could be self-triggered and self-regulated by the level of A β -Cu aggregates in cells.

As shown in Fig. 4b and c, A β 40-Cu aggregates destroyed the cell morphology and caused cell death. In contrast, the *in situ* synthetic drug **6** restored PC12 cells to their normal morphologies and improved the cell viability from 37% to 82%. However, compound **4** did not display similar neuroprotective activity. Collectively, our results show that compound **6** mitigates the cytotoxicity induced by A β -Cu aggregates as a consequence of chelating Cu and photo-oxygenating A β .

The AD model *C. elegans* has been widely used to enhance the understanding of the molecular pathogenesis and identify promising therapeutic strategies.^{80,81} It has been reported that ThT rescues A β -mediated paralysis and increases the longevity of transgenic worms,⁸² and Cu-chelator clioquinol (CQ) ameliorates A β -Cu toxicity to *C. elegans*.⁸³ As shown in Fig. S34,† photo-excited compound **6** can prolong the lifespan of *C. elegans* CL2006. We then explored whether the *in situ* synthesis of bifunctional compound **6** can suppress worm paralysis triggered by A β -Cu aggregates. CL2006 worms were cultured in NGM containing 1 mM $CuCl_2$ for 24 h and then Cu-treated worms were transferred to NGM containing prodrug-loaded MSN-IgG (1 mg mL⁻¹) for a further 24 h.

As shown in Fig. S35 and S36,† compound **6** was synthesized in Cu-treated CL2006 worms and **6** was able to co-localize with

A β . Subsequently, it was confirmed that MSN can access worm tissue (Table S5†). As shown in Fig. 5, A β -Cu aggregates induce AD model CL2006 strain paralysis, reducing the lifespan, and behavioral defects,^{37,83} while the *in situ* synthesized drug **6** decreases A β deposits, rescues A β -associated paralysis and improves the motility of the CL2006 strain.

Conclusion

In summary, the CuAAC click reaction has been realized by endogenous Cu *in vivo*, instead of using exogenous Cu-catalysts. We have demonstrated that A β -Cu aggregates can catalyze a click reaction in cells, an AD model of *C. elegans* and brain slices of 3 × Tg-AD mice. The reaction is self-triggered and regulated by the level of A β -Cu aggregates. Harnessing the synergy of photo-oxidizing A β and chelating Cu, the *in situ* synthesized drug **6** can dissociate A β -Cu aggregates, reduce A β burden, suppress A β -mediated paralysis and diminish the locomotion defects of the AD model CL2006 strain. Our work provides new insights into *in situ* drug synthesis against AD using bioorthogonal chemistry.

Experimental section

Synthesis of compound 4

A mixture of 2-(4-aminophenyl)-6-methyl-1,3-benzothiazole (1 mmol) and $NaNO_2$ (1.2 mmol) in 5 M HCl (10 mL) was stirred at 4 °C for 30 min. Then, a NaN_3 (4 mmol) solution was added dropwise and the mixture was stirred for 2 h at 4 °C. After adjusting the pH to 7, the resulting yellow precipitate was filtered off and washed with water, affording compound **4** (189 mg, 71%). ¹H NMR (600 MHz, $CDCl_3$), δ (ppm): 8.08–8.03 (m, 2H), 7.93 (d, J = 8.3 Hz, 1H), 7.68 (s, 1H), 7.33–7.28 (m, 1H), 7.14–7.09 (m, 2H), 2.50 (s, 3H). MS (ESI) m/z : $[M + H]^+$ calcd for $C_{14}H_{10}N_4S$, 267.3; found, 267.2.

Synthesis of compound 6

Compound **4** (1 mmol), **5** (1.1 mmol), $CuSO_4$ (0.1 mmol) and AA (0.2 mmol) were added to a solution of H_2O and ethanol (1 : 1, 20 mL) and stirred in the dark at 37 °C for 24 h. Then, ethylenediaminetetraacetic acid disodium salt (EDTA, 0.3 mmol) was added and the mixture was stirred for 1 h to chelate Cu. The ethanol was then removed under reduced pressure and the pH of the solution was adjusted to 7. The resulting yellow precipitate was filtered off and washed with saturated salt water, affording compound **6** as a yellow solid (345 mg, 91%). ¹H NMR (600 MHz, D_2O/DCI), δ (ppm): 7.15 (s, 1H), 6.34 (d, J = 8.3 Hz, 2H), 6.19 (d, J = 8.3 Hz, 2H), 5.98 (d, J = 9.1 Hz, 2H), 5.60 (d, J = 8.5 Hz, 1H), 2.86 (t, J = 6.4 Hz, 1H), 1.91 (qd, J = 15.9 Hz, 2H), 0.46 (s, 3H). MS (ESI) m/z : $[M - H]^-$ calcd for $C_{19}H_{17}N_5O_2S$, 378.4; found, 378.4.

Peptide preparation

A β 1–40 and A β 1–42 were purchased from ChinaPeptides Co., Ltd. The peptides were dissolved in hexafluoroisopropanol (HFIP) and stored at –20 °C as the stock solution. Before use,

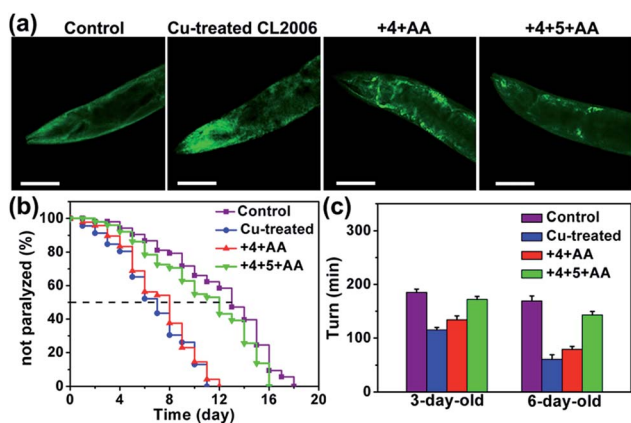


Fig. 5 The *in situ* synthesis of compound **6** for suppressing the toxicity induced by A β -Cu fibrils in worms. (a) Representative ThT-staining images of N2 and Cu-treated CL2006 in the presence or absence of prodrug-loaded MSN on the 6th day. Scale bars: 40 μ m. (b) Survival curves. (c) Quantification of the worm movement in M9 buffer (turns per minute). Each experiment was repeated three times. Error bars indicate $\pm$ s.d. Cu-treated worms were transferred to NGM containing prodrug-loaded MSN-IgG (1 mg mL⁻¹) for 24 h before worms were irradiated with UV light (1 W cm⁻²) for 10 min in 20 min intervals 6 times. A wild-type N2 strain was used as the control.



the solvent HFIP was evaporated under a gentle stream of nitrogen and A β was re-dissolved in HEPES buffer (20 mM, pH 7.4). For preparing A β 40–Cu aggregates, 100 μ M A β 40 was incubated with an equimolar amount of CuCl₂ at 37 °C for 8 h and then diluted to the corresponding concentration before use. For preparing A β 42–Cu aggregates, 100 μ M of A β 42 was incubated with an equimolar amount of CuCl₂ at 37 °C for 4 h and then diluted to the corresponding concentration before use. The A β –Cu species were dialyzed to remove any free Cu before the click reaction.

ITC measurements

ITC experiments were carried out on a NANO ITC System (TA Instruments Inc., USA) in HEPES buffer (20 mM, pH 7.4) with stirring at 300 rpm at 25 °C. A β or compound **6** was placed in the cell and the CuCl₂ solution was loaded *via* a syringe. To prevent the precipitation of Cu²⁺, 4 equiv. of glycine were added to the CuCl₂ solution. All solutions were degassed for 20 min before titration. The apparent binding constant (K_a), binding enthalpy (ΔH), and binding stoichiometry (n) were determined by fitting ITC data following information from the literature without considering the competing effects of glycine with Cu²⁺.^{66,67} When Cu²⁺–glycine was taken into consideration, the K_a values of Cu to A β and compound **6** were $1.63 \times 10^9 \text{ M}^{-1}$ and $1.04 \times 10^{10} \text{ M}^{-1}$, respectively.⁶⁵ For binding between A β and Cu²⁺, [A β] = 20 μ M, [Cu²⁺] = 200 μ M. For binding between **6** and Cu²⁺, [6] = 40 μ M, [Cu²⁺] = 200 μ M.

CD measurements

CD spectra were collected at 37 °C at a speed of 5 nm min^{−1} over the wavelength range from 200 to 250 nm. The secondary structure was analyzed using the BeStSel program (<http://bestsel.elte.hu/>).⁸⁴

C. elegans experiments

Transfected *C. elegans* strain CL2006 constitutively expresses human A β 1–42 in muscle tissues and thus displays an age-related progressive defect of muscle-specific motility. CL2006 and wild-type (N2) strain were fed with *E. coli* (OP50) and cultured at 20 °C in NGM. For worm paralysis assay, N2 and CL2006 worms were cultured at 20 °C on new NGM plates (about 50 worms per plate) after egg synchronization. Worms were classified as paralyzed if they failed to respond to a touch-provoked movement. For ThT-staining experiments, worms were fixed with 4% paraformaldehyde for 24 h at 4 °C and rendered permeable in permeabilization buffer (1% Triton X-100, 5% β -mercaptoethanol) for a further 24 h at 37 °C. After washing with PBS, the worms were immersed in ThT solution (2 mM) for 5 min. After washing with PBS, the worms were mounted on slides and observed using a fluorescence microscope.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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