**Introduction**

Alzheimer’s disease (AD) is a stubborn neurodegenerative disorder that plagues millions of people worldwide. The accumulation and aggregation of amyloid-β (Aβ) peptides are implicated as the initial and prominent pathophysiology features of AD and further trigger a series of cascade reactions, such as oxidative stress, neurotransmitter deficiencies, and neuronal injury. Al-binding agents have been actively developed to modulate the amyloidogenic behavior and alleviate concomitant cytotoxicity. Tracking Aβ ligands in real-time is of great significance for precision medicine, which conduce to identify the distribution of the ligands and evaluate treatment effect. To tackle poly-pathological features in AD, the rational design of multifunctional agents by integrating two or more target-interacting moieties into one single molecule has gained particular attention.

As a quintessential bioorthogonal reaction, Cu(i)-mediated azide/alkyne cycloaddition (CuAAC) is characterized by high selectivity, mild conditions, and fast kinetics. Thus, it has been extensively investigated in the biochemical research, such as bioconjugation, biolabeling, and prodrug activation. From this point of view, bioorthogonal catalysis has a great potential to endow Aβ binders with additional capabilities for their localization and versatile modification to address the multifactorial nature of AD.

Owing to the distinctive physicochemical properties, various metal complexes display a fascinating affinity with Aβ and thus are exploited for the theranostics of AD. Given that Aβ aggregates carry the chirality at different-length scales, the incorporation of enantioselective recognition is a bonus for AD therapeutics. Chiral metallohelices, a type of three-dimensional metal complexes assembled by multidente organic ligands wrapping around two or more metal centers, are analogous to α-helical peptides in terms of size, amphiphilicity, and stereochemistry. As potential non-peptide mimetics, a pair of alkyl-bearing metallohelices enantiomers (AA and ΔA, Scheme 1) are chosen, as an example to employ bioorthogonal activity for enantioselective targeting and visualization of amyloid without the need of de novo synthesis.

**Results and discussion**

Enantioselective modulation of Aβ aggregation by AA

The influence of the chiral metallohelices AA and ΔA on the course of Aβ40 fibril formation was investigated using thioflavin T (ThT) assays, circular dichroism (CD), and transmission
The structures of Δ (left-handed twist) and Λ (right-handed twist) enantiomers of the chiral metallohelices.

Fig. 1 Enantioselective regulation on Aβ40 fibrillation with the chiral metallohelices. (a) Overview of interrupting Aβ40 aggregation with ΔA and ΛA. (b) ThT assays for monitoring Aβ40 fibrillogenesis. Error bars represented ±standard deviation (s.d.) of three independent experiments. (c) CD spectra of Aβ40. For the spectra of Aβ in the presence of metallohelices, the CD spectra of the metallohelices were subtracted to reduce interference. (d) Secondary structure content estimations. (e) TEM images of Aβ40 aggregates. Scale bars are 50 nm.

displayed a weak effect (Fig. 1b). The control experiment indicated the chiral metallohelices had a small impact on the binding of ThT to Aβ fibrils (Fig. S1b†). In addition, the free chiral ligands of ΔA and ΛA had very feeble regulation ability on Aβ40 aggregation (Fig. S1d†), indicating that stereoscopic structures of the chiral metallohelices played a vital role on Aβ discrimination.

CD spectra, secondary structure analysis, and TEM images further verified that ΔA prohibited Aβ40 from aggregating into β-sheet-rich fibrils more powerfully than its enantiomer (Fig. 1c–e). Besides, ΔA could also block Aβ42 fibrillation, as depicted in Fig. S2.† The performance of the chiral metallohelices on Aβ42 oligomerization was evaluated with a dot blotting immunoassay. As shown in Fig. S2d, the sample treated with ΔA displayed a weaker reactivity to Aβ oligomer-specific antibody A11, indicating that ΔA decreased the formation of toxic oligomeric intermediates. UV-Vis absorption spectra and high-resolution mass spectra (HRMS) demonstrated the chiral metallohelices were stable in the phosphate-buffered saline (PBS) (Fig. S3†).

The complexation between ΔA and Aβ

Isothermal titration calorimetry (ITC), a distinguished method for evaluating molecular interactions, was employed to estimate the apparent binding constant ($K_a$), enthalpy change ($\Delta H_f$), and binding stoichiometry ($n$) of Aβ40 with chiral metallohelices. According to Fig. 2a and b, an exothermic ITC isotherm was recorded and the binding stoichiometry was close to 1 : 1. ΔA showed a higher binding affinity to Aβ40 ($K_a = 8.05 \times 10^6 \text{ M}^{-1}$) than the Δ enantiomer ($K_a = 9.38 \times 10^5 \text{ M}^{-1}$), and the corresponding Gibbs free energy changes ($\Delta G$) were $−39.4$ and $−34.1 \text{ kJ mol}^{-1}$, respectively. Competition dialysis experiment (Fig. S4†) further confirmed the higher affinity between Aβ40 and ΔA.

The fragments 12–28 and 25–35, which are the critical regions responsible for Aβ aggregation, were utilized to explore the binding sites of Aβ to the chiral metallohelices. As shown in Fig. S5†, ΔA efficiently blocked the aggregation of Aβ12–28 while it displayed modest modulation ability to Aβ25–35, implying that ΔA preferentially binds to Aβ12–28 rather than Aβ25–35. Remarkably, Aβ15–20 is considered the self-recognition region and often exploited as an Aβ-target peptide. Table S1† revealed that ΔA possessed comparative regulation capability towards the fibrillation of Aβ15–20 and Aβ1–40. It is inferred from the above results that ΔA might bind to Aβ15–20 as the mechanism of altering Aβ aggregation.

To further dissect the structural features of the chiral metallohelices and Aβ40, we performed docking studies with AutoDock Vina. Aβ1–40 monomer (PDB ID 2LFM), in which the core hydrophobic region adopts a helical conformation, was used as a working model for molecular docking. Interestingly, ΔA was nearly parallel to the α-helix of the Aβ monomer while ΔA was sloping (Fig. 2c–f). As a result, there were four sets of cation–π and σ–π interactions together between the aromatic nuclei of ΔA and the K16 residue of Aβ (Fig. 2d); this is in accord with aforementioned observation from inhibiting aggregation.
assays of Aβ fragments. In contrast, only one set of cation–π interaction was found between ΔA and Aβ (Fig. 2f). This structural analysis provides a rationale for the stronger binding between ΔA and Aβ and more powerful modulation activity towards amyloid aggregation of ΔA.

**Alleviation of Aβ-induced toxicity by ΔA in cells and the nematode model**

Aβ-triggered oxidative stress is another feature in AD pathogenesis. It has been proven that quenching overproduced reactive oxygen species (ROS) plays an active role in delaying AD progression. A series of Fe complexes have been reported to mimic catalase (CAT) or superoxide dismutase (SOD) and display promising anti-oxidation features. Hence we speculated that the chiral metallohelices might also scavenge deleterious ROS. As expected, ΔA and ΔA efficiently decomposed H$_2$O$_2$ (Fig. 3a and b) and slowed down pyrogallol auto-oxidation (Fig. 3c and d), confirming their CAT-mimic and SOD-mimic activities.

Based on the above encouraging results, we further investigated whether the metallohelices were able to protect PC12 cells from Aβ-induced toxicity. Intracellular ROS was monitored by the probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which can be oxidized to fluorescent dichlorofluorescin (DCF). Flow cytometric analysis revealed that ΔA-treatment significantly decreased Aβ-mediated oxidative stress, owing to the synergistic effect of regulating Aβ aggregation as well as CAT-mimic and SOD-mimic activities (Fig. 3e). Moreover, microscopic images presented the shrunken, rounded, and aggregated morphologies of Aβ-treated cells, while ΔA substantially restored these abnormalities (Fig. S6a†). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays further verified ΔA exhibited noticeable neuroprotection against Aβ-induced cytotoxicity (Fig. S6c†).

With wild-type N2 strain as the control, we further evaluated the therapeutic potential of chiral metallohelices in the transgenic AD model Caenorhabditis elegans (C. elegans) CL2006, which constitutively expressed Aβ. Thioflavin S (ThS) staining, ROS measurement, motility quantification, and survival curves demonstrated that Aβ aggregates led to paralysis phenotype, oxidative stress, behaviour defects, and shortened lifespan in the CL2006 model (Fig. 4 and S7†). Whereas the presence of ΔA reduced Aβ plaques, rescued Aβ-triggered paralysis, decreased the ROS level, and improved the motility of the CL2006 strain.
Likewise, ΔA performed better than its enantiomer ΔA in relieving these Aβ-induced toxicities.

**Improvement of cognitive function in AD model mice by ΔA**

We next evaluated the performances of the metallohelices in the triple-transgenic model mice of AD (3×Tg-AD mice). The potential blood–brain barrier (BBB) permeability of the metallohelices was evaluated after the attachment of fluorescent Cyanine5 (Cy5). Fig. S8† indicated that ΔA successfully crossed the BBB and accumulated in the brain parenchyma. Besides, their potential toxicity was investigated through hematoxylin and eosin (H&E) staining and biochemistry analysis (Fig. S9 and S10†). Neither obvious tissue harm nor significant change in histopathology analysis was found, suggesting favorable biocompatibility of the metallohelices.

The effects of the metallohelices on amyloid deposition were determined via immunoassay. As depicted in Fig. 5a, ΔA significantly decreased the levels of Aβ species in the brain compared to the treatment with ΔA, further confirming the enantioselectivity towards Aβ. Morris water maze (MWM) assay was conducted to assess the spatial learning behavior of 3×Tg-AD mice after administration. Four evaluation indicators were analyzed, including the escape latency, crossing frequency, time spent in the target quadrant, and swimming path of the mice (Fig. 5b–e). After the treatment of ΔA, 3×Tg-AD mice took shorter paths and less time to reach the target platform and displayed a spatially oriented swimming behavior. Taken together, these results indicate that ΔA could recover impaired learning and memory in 3×Tg-AD mice.

**Bioorthogonal PAM of ΔA to extend its biochemical applications**

Inspired by protein post-translational modification (PTM), post-assembly modification (PAM) has been developed as a powerful tool to optimize metallosupramolecular performance by covalently introducing new functional groups, in which case the cautious pre-assembly ligand design is avoided while retaining the desired framework and properties. In this context, we investigated the chemoselective conjugation of bioactive moieties to the alkyne-bearing metallohelix ΔA via CuAAC reaction to broaden its biochemical applications. A commercial fluorescent dye Cyanine5–azide (Cy5–N3) was used for labelling the metallohelices in both PC12 cells and C. elegans CL2006 strain (Fig. 6). Colocalization quantitative analysis was evaluated via Manders’ colocalization coefficients (MCC), conducted using ImageJ software with the JACoP plugin. Encouragingly, most of ΔA (about 90%) was found to be co-localized with Aβ antibodies in PC12 cells (Fig. 6a), and the MCC value of ΔA with Aβ was more significant than that of ΔA (Fig. 6c and d). This is well consistent with the above observation that ΔA dramatically outperforms ΔA in terms of specificity on targeting Aβ, inhibition of amyloid aggregation, and attenuation of Aβ-mediated toxicity. Fig. 6b and e further displayed the potential of click reaction for metallohelix visualization in the worms.

Next, we integrated various functional moieties with ΔA via bioorthogonal PAM to modulate multiple facets of AD, as illustrated in Fig. 7a. Previous studies have demonstrated that photoactivated ThT and its analogs can oxygenate Aβ and thereby decrease the aggregation property and pathogenicity of Aβ. The azide derivative of ThT (ThT–N3) was grafted onto ΔA employing the CuAAC reaction. The obtained ΔA–ThT hybrid thus bore three copies of ThT moity (Fig. S11a†). UV-Vis absorption and CD spectra indicated that PAM did not disrupt the metallohelix architecture (Fig. S11b and c†).
Interestingly, photoactivated $\text{AA}$–$\text{ThT}$ could selectively oxygenate $\alpha\beta$ (Fig. 7b, c and S11f†), presumably because of the transient lifetime and restricted diffusion range of highly reactive singlet oxygen (1$\text{O}_2$) in biological systems. 47,48 Anilinonaphthalene-1-sulfonyl (ANS), which displays enhanced fluorescence upon binding to protein hydrophobic residues, was used for monitoring $\alpha\beta$ fibrillation. Our observations manifested that photoactivated $\text{AA}$–$\text{ThT}$ reduced $\alpha\beta$ hydrophobicity (Fig. S11f†) and prohibited $\alpha\beta$ aggregation (Fig. S11g†) through a synergistic mechanism.

Tacrine (1,2,3,4-tetrahydroacridine-9-amine, THA), the first clinically-used acetylcholinesterase (AChE) inhibitor for AD treatment, is hindered by severe adverse reactions, especially hepatic toxicity, which perhaps results from oxidative stress. 49-50 Due to reliable anti-AChE potency, classical pharmacophore, and synthetic accessibility, THA-based scaffolds are still at the forefront of developing safer AChE inhibitors. 51-53 Recent reports revealed that antioxidants could repress THA-triggered hepatotoxicity by scavenging overproduced ROS during administration. 54,55 In these contexts, the azide derivative of THA (THA–N$_3$) was also integrated with $\text{AA}$ through click reaction to obtain $\text{AA}$–THA (Fig. S12†). The AChE inhibitory activity of $\text{AA}$–THA was evaluated by modifying Ellman’s method. 56,57 As shown in Fig. 7d and e and Table S2,† the hybrid metallohelix $\text{AA}$–THA exhibited a modest anti-AChE activity and diminished hepatotoxicity, which might be ascribed to a ROS-quenching feature inherited from $\text{AA}$ (Fig. S12d–f†). In addition, $\text{AA}$–THA retained the capacity to intervene $\alpha\beta$ aggregation (Fig. S12g†).

To further assess the universality of bioorthogonal chemistry, $\text{AA}$ was also conjugated with azide functionalized nanoparticles. Magnetic nanoparticles (MNPs) have been widely exploited as versatile nano-platforms for the theranostics of neurodegenerative diseases, because of their excellent biocompatibility, BBB permeability, and attractive magnetic properties. 58-59 Recently, Hyeon’s group constructed multifunctional nano-assemblies (magnetite and ceria nano-composites) for $\alpha\beta$ clearance through selective binding and magnetic separation. 60 Inspired by that, $\text{AA}$ was attached to the MNPs to form MNP–$\text{AA}$ through the same click reaction (Fig. S13 and S14†). As a proof of concept, MNP–$\text{AA}$ was harnessed for $\alpha\beta$40 capture, and the adsorbed $\alpha\beta$40 was measured by MS quantitative analysis with $\alpha\beta$42 as the internal standard. The amino acid sequences of $\alpha\beta$1–40 and $\alpha\beta$1–42 differ by only two amino acids, so $\alpha\beta$42 is an ideal internal standard for $\alpha\beta$40 in MS analysis (Fig. 7f). Fig. 7g showed that MNP–$\text{AA}$ was able to remove $\alpha\beta$40 from the mice serum in a concentration-dependent manner. Taken together, these results indicate that, through bioorthogonal PAM, $\text{AA}$ can act as a universal scaffold to integrate various bioactive moieties, thus conferring the superiority of multidirectional treatment for AD.

Fig. 6 Co-localization of $\text{AA}$ and $\alpha\beta$ in the PC12 cells and CL2006 strain. (a) Immunofluorescence staining of PC12 cells treated with $\alpha\beta$ and the chiral metallohelices. $\alpha\beta$ was labeled with $\alpha\beta$–specific antibody B4 (green channel), and the alkyne-bearing metallohelices $\text{AA}$ and $\text{AA}$ were visualized by Cy5–N$_3$ (red channel) via CuAAC reaction. The nucleus was dyed with DAPI. Scale bars: 10 μm. (b) Fluorescence images of the CL2006 strain, which were treated with the chiral metallohelices and then labeled with Cy5–N$_3$ and $\alpha\beta$ antibody. Scale bars: 100 μm. (c) and (d) were the MCC values of $\alpha\beta$ with Cy5-labelled metallohelices in the PC12 cells, respectively. (e) and (f) were the MCC values of $\alpha\beta$ with Cy5-labelled metallohelices in the CL2006 strain, respectively.
Conclusion

In summary, chiral metallohelix AA displayed enantioselectivity on targeting and regulating Aβ aggregation. Meanwhile, it mimicked CAT and SOD to quench overproduced ROS and maintain redox homeostasis. Such synergism enabled the impressive ability of AA to attenuate Aβ-mediated toxicity in cells and the C. elegans CL2006 strain, while the Δ enantiomer was far less efficient. Moreover, AA efficiently reduced the Aβ burden in the brain of 3×Tg-AD mice and recovered their impaired learning and memory. Beyond the inherent bioactivity, the alkyne-bearing AA served as a building block that allowed subsequent chemoselective conjugation via click reaction for extended functionalities, including biolabeling, bioconjugation, and nanoparticle construction. Through fluorescent tagging, clickable AA was found to be well colocalized with Aβ in vivo. As representatives, several types of multi-target-directed hybrid agents against the interrelated pathological features in AD were constructed, where AA-ThT reduced Aβ hydrophobicity and aggregation tendency; AA-THA showed modest anti-AChE potential and attenuated hepatotoxicity; and MNP-AA conveniently captured and cleared Aβ. More importantly, incorporating new functional units did not interfere with the core metallohelix framework, and the final hybrid simultaneously inherited the biological activities from diverse parent frameworks. Hence, bioorthogonal chemistry promises a fresh and unique perspective for customized supramolecules with desirable functionality against multifaceted AD.

Data availability

We have included all data in the ESI Section.

Author contributions

X. Q. and J. R. conceived the project. Z. D. performed the experimental study and drafted the manuscript. H. S. and P. S. synthesized the chiral triplex metallohelices, and P. S. assisted in revising the manuscript. C. L., and Z. L. carried out the experiments on the AD mice and analyzed the data. X. D. helped to analyze the data. All authors approved the final version. Z. D., C. L., and Z. L. contributed equally to this work.

Conflicts of interest

There are no conflicts to declare.

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