Near-infrared Two-region AIE Nanoprobe Study for AD Diagnosis and Treatment Integration

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Abstract. In this paper, we address the lack of specific targeting of β-amyloid β-protein (Aβ) in the current Alzheimer's disease (AD) diagnosis and treatment, and build a novel AIE nanoprobe molecule based on molecular design and nano-self-assembly technology. Based on the molecular design and nano-self-assembly technology, a new two-component AIE nanoprobe molecule with integrated AD diagnosis and treatment was constructed by co-assembling two-component AIE components. The NIR AIE nanomolecule can effectively penetrate the blood brain barrier (BBB) and depolymerize Aβ fibers, alleviate reactive oxygen species (ROS) in the focal area, and achieve highly sensitive imaging and specific depolymerization.

Keywords: AIE probe, Alzheimer's disease, nanocomplex, blood brain barrier, β-amyloid

1. Introduction

Firstly, in this paper, AIE properties and solubilization were achieved by introducing alkylthiophene as the electron donor and combining with multi-rotor design, while the strong electron-absorbing effect of benzobisthiadiazole as the molecular backbone (electron acceptor) interacted with the hydrophobic end of Aβ and dihydroxyethylamine formed hydrogen bonds with Aβ hydrophilic amino acids, thus achieving effective depolymerization of Aβ fibril structure. In addition, the reduction of the probe molecule was significantly enhanced by complexing Ce(III) in the second motif TpPTB-Ce, and the oxidative stress in the AD focal area was effectively reduced. Finally, the two-component AIE nanoprobe molecule was modified with a targeted peptide (angiopoietin-2, Ang-2), which enabled the probe molecule to possess an efficient BBB penetration ability. The two-component AIE nanoprobe molecules exhibited excellent NIR two-zone emission and NIR absorption properties by UV and fluorescence spectroscopy. The cytotoxicity assay showed that the nanoprobe molecules were biocompatible and could effectively reverse the cytotoxicity of neuroendocrine cells (PC12) for neuroprotection. The results of in vivo and in vitro experiments showed that the two-component AIE nanoprobe molecules have good biosafety. This work provides a new idea of clinical prevention and treatment for the visualization and precision treatment of AD by realizing the brain-targeted ROS response combined with NIR AIE through structural design and nano self-assembly method.

2. Innovative ideas

In this paper, PTB, which has the function of depolymerizing Aβ fibrils and inhibiting Aβ monomer aggregation, and TpPTB-Ce, which has the function of reducing ROS, were co-assembled by microemulsion-assisted mesophase transfer co-assembly to construct AIE nanocomplexes with dual functions of depolymerizing Aβ and reducing ROS for AD treatment and research. When the nanocomplexes reach the high ROS environment in the AD region, the ketone condensation thiol structure (TK) in the emulsifier is disrupted, which releases both PTB and TpPTB-Ce components inside. When bound to Aβ in the brain, AIE emits intense near-infrared second-zone fluorescence, enabling in vivo high-sensitivity imaging. To confer specific targeting function to the nanocomplex, we modified the targeting peptide Ang-2 on its surface that specifically binds to low-density lipoprotein receptor-related protein 1 (LRP-1) overexpressed on BBB endothelial cells. we demonstrated that Ang-2-modified brain-targeted ROS-responsive NIR AIE nanocomplex (Ang-PTB/TpPTB-Ce) can effectively traverse the BBB and accumulate in the brain, ultimately effectively inhibiting Aβ self-assembly, depolymerizing Aβ, as well as remodeling the ROS environment in the AD focal area, enabling the integration of Alzheimer's disease diagnosis and treatment.

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3. Innovation points

3.1 Molecular Design and Self-assembly of Two-component NIR AIE Nanoprobes

Firstly, the AIE properties and solubilization were achieved by introducing alkylthiophene as the electron donor and combining with multi-rotor design, while the strong electron-absorbing effect of benzobisthiadiazole as the molecular backbone (electron acceptor) interacted with the hydrophobic end of Aβ and dihydroxyethylamine formed hydrogen bond with Aβ hydrophilic amino acid, so as to achieve effective depolymerization of Aβ fiber structure. In addition, the reduction of the probe molecule was significantly enhanced by complexing Ce(III) in the second motif TpPTB-Ce, and the oxidative stress in the AD focal area was effectively reduced. Finally, the two-component AIE nanoprobe molecule was modified with a targeted peptide (angiopoietin-2, Ang-2), which enabled the probe molecule to possess efficient BBB penetration ability.

3.2 Good Biocompatibility and in Vivo Biosafety

The cytotoxicity assay showed that the targeted nanocomplex has good biocompatibility, and also effectively reverses the cytotoxicity of neuroendocrine cells (PC12) by depolymerizing Aβ fibers to achieve the neuroprotective function. The nanocomplexes were tested to have good in vivo biosafety using hemolysis, routine blood and blood biochemistry assays. The pharmacokinetic data showed that the targeted modification effectively prolonged the in vivo circulation time (elimination half-life of 3.22 hours) of the nanocomplex to 5.82 hours.

3.3 Integration of AD Therapy

Through a rational and ingenious structural design, the purposeful introduction of multiple aromatic rings and heteroatoms, which produce multi-location depolymerization with Aβ, realizes an innovative expansion of the single imaging function of traditional Aβ probes, and realizes the visual monitoring of the occurrence, development and treatment of AD by screening and studying the conformational relationship and performance modulation elements of the probes.

4. Experimental methods

4.1 Preparation of Non-targeted Nanocomplexes

The desired nanocomplexes were prepared by co-assembly method, firstly, 1 mg of PTB and 1 mg of TpPTB-Ce were accurately weighed and dissolved in 100 μL of N,N-dimethylformamide, and then rapidly injected into deionized water (10 mL) containing 4 mg of DSPE-TK-PEG2000 at a time. Finally, sonication was performed for 1 min with the assistance of ultrasound (power: 80 W, on/off cycle: 5s/5s) and stirring. After sonication, the resulting solution was centrifuged at 15000 rpm for 15 min, washed once with water and dispersed in deionized water to obtain AIE nanocomplexes (PTB/TpPTB-Ce).

4.2 Preparation of Targeting Nanocomplexes

In order to prepare nanocomplexes with brain targeting function, we changed the above nanocomplex preparation process of DSPE-TK-PEG2000 into DSPE-TK-PEG2000 and DSPE-TK-PEG3400-Ang for co-assembly. Firstly, 1 mg of PTB and 1 mg of TpPTB-Ce were dissolved in 100 μL of N,N-dimethylformamide and then injected into 10 mL of deionized water containing 1 mg of DSPE-TK-PEG3400-Ang and 3 mg of DSPE-TK-PEG2000 using the same sonication, stirring and centrifugation as the above nanocomplex treatment steps to obtain targeted AIE nanocomplexes (Ang-PTB/TpPTB-Ce NPs). Here DSPE-TK-PEG3400-Ang needs to be preformed in advance: using Michael addition reaction, firstly, 50 mg of Mal-PEG3400-TK-DSPE was dissolved in 3 mL of PBS solution, 42 mg of Angiopep-2-SH peptide (1.2 eq) was added and stirred at room temperature for 24 h. Then the obtained product system was transferred to A dialysis bag with a cut-off molecular weight of 3500 was placed in deionized water for dialysis purification for 24 h. The obtained dialysate was freeze-dried to obtain Ang-PEG-TK-DSPE.

5. Results

5.1 Evaluation of Two-component Near-infrared AIE Nanoprobe Molecules for in Vitro Level Diagnosis and Treatment

5.1.1 Binding and Depolymerization of Aβ by Two-component Near-infrared AIE Nanoprobe Molecules

A clear linear concentration-dependent increase in fluorescence intensity was observed by incubating a series of different concentrations of PTB monomers with the same concentration of Aβ fibers using near-infrared fluorescence imaging (at 1350 nm) and intensity statistics. A series of competing species were then set up and the results showed a stronger specific binding ability of PTB to Aβ fibers compared to other competing species by comparing the PTB molecules with their binding fluorescence. The detailed binding process was further examined using fluorescence spectroscopy, and the results showed that PTB molecules could bind to Aβ fibers rapidly within 5 min. Transmission electron microscopy observations showed that PTB molecules could effectively depolymerize long fibrillar Aβ fibers into shorter, amorphous Aβ fibers. The change of its secondary structure was further monitored using circular dichroism (CD) spectroscopy, as shown in Figure 5F. Compared with the Aβ monomer, which had no obvious signal peak at 218 nm, the Aβ fiber showed a distinct chiral signal peak at 218 nm, attributed to its β-folded sheet-like structure characteristic peak. After the addition of PTB molecules, the signal peak was significantly weakened, indicating the reduction of the β-folded

lamellar structure. In summary, PTB molecules have excellent specific binding and depolymerization ability to Aβ fibers.

To verify whether PTB molecules can continue to perform the dual functions of NIR imaging and depolymerization of Aβ fibers after assembling into nanocomplexes. We first observed different concentrations of aqueous nanocomplexes by NIR imaging, and the results showed that the nanocomplexes exhibited excellent concentration-dependent NIR fluorescence at 1150 nm, 1350 nm and 1550 nm filters. We selected 1350 nm, which has high luminescence and large penetration depth, as the target wavelength for in vivo monitoring. We then tested the ROS-stimulated release performance of the nanocomplexes based on the particle size variation of the nanoparticles, using H2O2 to simulate the high ROS environment in the AD region of the brain, and the results showed that H2O2 could effectively stimulate a significant increase in the particle size of the nanocomplexes and achieve controlled release. For Aβ monomer, it has a good effect of inhibiting the aggregation of Aβ monomer. In summary, the obtained nanoparticles have good ROS-responsive release performance and inherit the dual functions of NIR two-region luminescence and depolymerization/inhibition of Aβ of PTB molecules.

5.1.2 In vitro experiments of two-component near-infrared AIE nanoprobe molecules

In order to evaluate the brain-targeting function, biosafety and the protective effect of nanoparticles on neurons after depolymerization of Aβ fibers. We first used the Transwell model that simulates the in vitro BBB, added the material to the upper chamber, and examined the transmission rate of the nanocomplexes in the lower chamber to assess the efficiency of the nanocomplexes to cross the BBB. The penetration efficiency of the targeted nanocomplexes was 1.5 times higher than that of the non-targeted nanocomplexes across the BBB. Also the nanocomplexes have good biosafety for a variety of cell lines (SH-SY5Y, BV2, Neuro-2a, PC12). Therefore, we selected PC12 cells as the target cell line for the assay of antioxidant neuroprotection. By confocal imaging characterization of PC12 cell endocytosis nanocomplexes, the results showed that the targeted nanocomplexes had higher cellular endocytosis uptake compared to the non-targeted modified nanocomplexes. The results of fluorescence intensity based on the green fluorescent 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), a reactive oxygen probe, after uptake showed that it could effectively reduce intracellular ROS levels combined with the depolymerization of Aβ fibers and effectively reduce the damage of Aβ plaques and oxidative stress on PC12 cells.CCK-8 cytotoxicity results showed that at a concentration of 200 μM, the targeted nanocomplex complexes were able to reverse PC12 cell activity to a level close to 100%. In summary, the targeted nanomaterials have excellent BBB penetration, enhanced cellular endocytosis, high safety, effective antioxidant and multifunctional in enhancing neuronal activity, which are beneficial for use in the next in vivo therapeutic studies.

5.2 Evaluation of Two-component NIR AIE Nanoprobe Molecules for in vivo Level Therapy

5.2.1 Blood safety results

The safety of nanomedicines is the basis of treatment, therefore, we firstly used hemolysis experiments to conduct a preliminary investigation of the safety of nanomedicines by co-incubating nanocomplexes with red blood cells, and the results of absorbance values of supernatants after centrifugation are shown in Figure 8A. The results showed that compared with the deionized water control group, different concentrations of targeted nanomaterials showed higher biosafety (hemolysis <5%), and their The optical photographs showed that the supernatant of the targeted nanomaterials group was colorless and transparent compared with the red supernatant of the deionized water-treated erythrocyte blood samples after centrifugation, indicating that it did not cause obvious cytolytic phenomenon. The in vivo safety of the targeted nanocomplex was further verified by routine blood and blood biochemistry experiments. After the tail vein injection, blood was collected and sent for examination on the seventh day, and several routine blood and blood biochemistry blood indices including red blood cells (RBC), hemoglobin (HGB), and liver function albumin (ALB) of the nanocomplex-treated group were not significantly different from those of the PBS group, showing a high safety level (Figure 8C). In summary, the above experiments showed that the resulting targeted nanocomplexes have high biosafety and can be used for the next in vivo real-time NIR imaging tracing, therapeutic and behavioral assessment experiments.

5.2.2 Pharmacokinetic results

Based on the safety validation, we further investigated the in vivo pharmacokinetic performance of the nanodrugs. On healthy mice, non-targeted and targeted nanocomplexes were injected via tail vein, and blood samples were taken and the absorbance of nanomaterials at 760 nm in blood samples was measured at different time points after injection. The results were obtained by Origin9 software through quadratic exponential decay fitting, and the Angiopep-2 modification effectively prolonged the in vivo blood circulation time of the nanocomplex to 5.82 h compared to the elimination half-life (t1/2,β) of the non-targeted nanocomplex of 3.22 h, thus contributing to the increase of the targeted accumulation in the brain and the ultimate therapeutic efficiency.

5.2.3 In vivo crossing BBB ability and behavioral results analysis

At different wavelengths, the nanocomplexes could clearly show the clear detailed outline of brain blood vessels, especially at 1350 nm, which indicates that the nanocomplexes have better NIR imaging tracer ability. Then we injected targeted and non-targeted nanocomplexes into AD mice by tail vein and examined their brain imaging efficiency at different time points on AD mice, and the results showed that the targeting agent could improve the ability of nanocomplexes to cross the BBB and reach the maximum accumulation in the brain at 12~24 hours.

After crossing the BBB, the nanocomplexes released AIE molecules and bound to Aβ fibers in the lesions under high level of ROS stimulation in the AD lesion area, deconjugating Aβ fibers and antioxidant to improve the cognitive function of AD mice. Wild-type mice and AD mice were selected and injected with the nanocomplex through the tail vein every 2 days, and after 6 consecutive injections, each group of mice was subjected to nesting and water maze behavioral tests. Nesting tests were used to assess rodent health status and hippocampal function. The results of the nesting test showed that the behavioral improvement was more pronounced after the targeted nanocomplex treatment, and the paper clusters were more aggregated in the targeted group, with the highest behavioral scores and closest to the wild group, indicating that the targeted nanocomplex treatment could restore the cognitive impairment in AD mice. Next, the spatial learning and memory abilities of the mice were assessed by water maze test. Specifically, by recording the time mice spent searching for hidden platforms in the pool (latency period) every day for 5 consecutive days. As the number of training sessions increased, the latency periods of all mice were gradually reduced. The targeted group also showed superior behavioral improvement efficiency, obtaining the closest latency to that of wild-type mice. Brain-targeted nanocomplexes have excellent therapeutic functions in improving behavioral cognition and memory in AD mice, showing great potential for AD treatment.

After behavioral testing, one mouse was taken in each group, and the brains were executed and collected using the euthanasia method for brain section processing and immunofluorescence staining analysis and neuronal morphology analysis. After Aβ antibody staining, it was observed that no significant Aβ plaques appeared in the brains of wild group mice and a large number of Aβ plaques appeared in the brains of AD mice. Compared with the large number of Aβ plaques in the non-targeted group, the Aβ plaques in the hippocampal region of the targeted nanocomplex group were significantly reduced, and their fluorescence quantification results also showed a significant reduction of Aβ plaques in both the hippocampal and cortical regions. The reduction of plaques effectively protected the neuronal activity, and the results of nisin staining indicated that the nuclei in the cortical and hippocampal regions of AD mice were shrunken, representing impaired neuronal structure, while the mice in the targeted nanocomplex group had full morphology and high activity, which were close to the neuronal state of wild mice.

5.2.4 Analysis of tissue safety results

To further test the biosafety of the nanocomplexes, the major organs (heart, liver, spleen, lung and kidney) of the mice were collected at the end of the treatment and the tissue sections were stained with H&E for observation, and the results indicated that there was no significant visceral damage in each visceral organ of the mice treated with non-targeted and targeted nanocomplexes compared with the PBS group, indicating that the prepared nanocomplexes have high biosafety. In summary, our prepared brain-targeted Ang-PTB/TpPTB-Ce nanocomplexes have good biocompatibility and excellent NIR imaging, as well as strong binding/depolymerization and antioxidant ability with Aβ fibers, effectively disintegrating neurotoxic Aβ plaques and reshaping redox homeostasis in the brain, effectively improving behavioral cognition and memory functions in mice. It not only confirms the rationality of our design, but also demonstrates the great potential of this brain-targeted NIR neuroprotective function of Ang-PTB/TpPTB-Ce nanocomplex in the field of neurodegenerative diseases.

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