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Dual-functional nanoparticles for precise drug delivery to Alzheimer's disease lesions: Targeting mechanisms, pharmacodynamics and safety

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia and is characterized by the cerebral accumulation of extracellular amyloid plaques. In a previous study, this histopathological hallmark was used as a target on a dual-functional nanoparticle (TQNP) to deliver biotechnological drugs, such as the H102 peptide, a β-sheet breaker, to AD lesions precisely. This delivery system could reduce the amyloid-β (Aβ) burden in the brains of AD model mice, as well as ameliorated the memory impairment of the mice. Regretfully, the mechanism how nanoparticles penetrated the BBB and subsequently targeted to the plaques is still unclear. In this study, the internalization, subcellular fate and transportation of the nanoparticles on bEnd.3 cells and an *in vitro* BBB model, demonstrated that TQNP could be taken up through various routes, including caveolae-mediated endocytosis, suggesting that some of TQNP were able to cross the BBB intact. Then, the TQNP were visualized to specifically bind to the Aβ plaques. TQNP targeting to amyloid plaques might lead to enhanced therapeutic efficacy, which was further evaluated in APP/PS1 transgenic mice. The TQNP/H102 obtained better ability in decreasing amyloid plaques, increasing Aβ-degrading enzymes, reducing tau protein phosphorylation, protecting synapses and improving the spatial learning and memory of transgenic mice than nanoparticles modified with a single ligand. And good biocompatibility of TQNP was indicated with subacute toxicity assays. In conclusion, TQNP was a valuable nanodevice for the precise delivery for biotechnological drugs to treat AD.
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1. Introduction

Alzheimer's disease (AD) is defined as an irreversible progressive neurodegenerative disease that gradually and intensively impairs cognition and memory, mainly in the geriatric population (Ferri et al., 2005). AD currently affects approximately 36 million people worldwide, with the estimated prevalence expected to be over 115 million by 2050 (Gregori et al., 2015). AD is now quickly becoming one of the most pressing universal healthcare problems. Several distinct neuropathological features have been identified in the brains of AD patients, including extracellular amyloid-β (Aβ) deposition (plaques), intracellular neurofibrillary tangles composed of abnormally phosphorylated tau protein, and degeneration of cholinergic neurons of the basal forebrain (Popovic and Brundin, 2006). Aβ peptide (39–42 amino acids in length) spontaneously and progressively undergoes aggregation, forming toxic oligomers and fibrils, which are now considered to be the main factor responsible for the synaptic damage and memory deficits in AD (Gilbert, 2013). Therefore, in the last years, many efforts toward AD therapy have focused on the "amyloid hypothesis", which aims at preventing Aβ formation, blocking Aβ aggregation into plaques, or lowering the levels of soluble Aβ in the brain and, perhaps, also disassembling existing aggregates (Taylor et al., 2011; Balducci et al., 2014; Song et al., 2014).

β-Sheet breaker peptides are a new class of oligopeptide drugs designed to specifically interfere with β-sheets within Aβ, preventing the misfolding and deposition of Aβ and decreasing its neurotoxicity (Soto and Estrada, 2005; Bolukbasi and Hatip-Al-Khatib, 2013). Regretfully, these peptides are poorly transported across the blood-brain barrier (BBB) and can be rapidly cleared after intravenous injection, which limits their clinical application. To overcome these problems, we previously developed a multi-

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functionalized nanoparticle system based on poly(ethylene glycol)-poly(lactic acid) (PEG-PLA), modified with TGN peptides as the BBB ligand and QSH peptides for the Aβ42-binding (TQNP) (Zhang et al., 2014a), to target amyloid plaques in the brain. TQNP can enhance the drug delivery to the hippocampus in a mouse model of AD (3.41 and 1.84 times the levels of non-modified nanoparticles (NP) and TGN-modified nanoparticles (TNP), respectively) and showed better AD therapeutic effects after encapsulation a β-sheet breaker, H102 (HKQLPFFEED), than free drug or TNP/H102 (Zhang et al., 2014b).

These achievements might have resulted from a dual-targeting effect that allowed the nanoparticles to cross the BBB and subsequently concentrate at the plaques. However, there was limited investigation into the subcellular fate and transport of the nanoparticles, which is important for understanding the mechanism of the dual-targeting effect. Moreover, no direct evidence has been obtained showing that TQNP can penetrate the BBB in an intact form and accumulate around the amyloid plaques. Additionally, a non-transgenic AD mice model (created by injecting Aβ42 aggregates into the bilateral hippocampus of mice) was used in our previous study and could not fully mimic the pathological changes of AD, resulting in some limitations. In the present study, the cellular uptake mechanism of the TQNP and their ability to cross an *in vitro* BBB model was first investigated, then, an *in vivo* co-localization experiment was conducted to demonstrate the lesion-specific delivery by the TQNP. Finally, the neuroprotective effects of the H102 preparations on APP/PS1 transgenic mice were systematically evaluated, and the *in vivo* toxicity of the TQNP was also investigated.

2. Materials and methods

2.1. Materials and animals

Maleimide-poly(ethylene glycol)$_{3000}$-poly(lactic acid)$_{70,000}$ (Mal-PEG-PLA) and methoxy-poly(ethylene glycol)$_{3000}$-poly(lactic acid)$_{50,000}$ (MePEG-PLA) were synthesized by the East China University of Science. TGN (TGNYKALHPHNGC), QSH (QSHYRHISPAQVC) and Aβ42 were obtained from the Chinese Peptide Company (Hangzhou, China). H102 peptide (HKQLPFFEED) was purchased from GL Biochem Ltd. (Shanghai) (purity 97%). Coumarin-6, chlorpromazine hydrochloride, sodium azide, filipin, monensin and cytochalasin B were purchased from Sigma (USA). Anti-neprilysin antibody (NEP), anti-Drp1 antibody, anti-Aβ16 antibody and anti-phospho-PHF-tau mouse monoclonal antibody were obtained from Abcam, Millipore, Covance and Pierce (USA), respectively. Anti-synaptophysin1 antibody was obtained from the Bioss Company (Beijing, China). LysoTracker^{TM} Red DND-99 and Hoechst 33342 were purchased from Molecular Probes, Invitrogen (USA). Brain capillary endothelial cells (bEnd.3) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA solutions were purchased from Gibco (CA). All of the other chemicals were analytical or reagent grade.

5XFAD (males, N = 12) and wild-type littermates (males, N = 9) that were 6–8 months of age were kindly donated by Professor Zheng Yan (Capital Medical University, China). APP/PS1 double transgenic mice (male, B6C3-Tg (APPswe, PSEN1dE9)85Dbo/J strain) were obtained from the Model Animal Research Center of Nanjing University (China). Balb/c mice were obtained from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China). Mice were raised in separate cages in a 12-h light-dark cycle at a constant temperature with free access to food and water. The animal studies were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC), School of Pharmacy, Fudan University. All efforts were made to minimize the number of animals and their suffering.

2.2. Preparation of nanoparticles

H102-loaded PEG-PLA nanoparticles (NP/H102) were prepared using the emulsion/solvent evaporation method as previously described (Zhang et al., 2014b). Briefly, 17.5 mg Mal-PEG-PLA and 7.5 mg MePEG-PLA were dissolved in 1 mL of dichloromethane, then 100 μL of H102 solution (10 mg/mL) was added. The water-in-oil (w/o) emulsion was obtained by tip sonication in an ice-water bath and was then sonicated in a 1% sodium cholate solution (2 mL) to obtain the water-oil-water (w/o/w) emulsion. This double emulsion was diluted in a 0.5% sodium cholate solution (20 mL) with continuous stirring, followed by evaporation with a rotary vacuum. NP were collected by centrifugation and washed in deionized water three times to remove free H102. The coumarin-6-loaded nanoparticles (NP/Cou-6) were prepared using the same method, except that coumarin-6 was added to the dichloromethane copolymer solution before emulsification, and the nanoparticles were subjected to a sepharose CL-4B column to remove the free coumarin-6.

To obtain the TGN-, QSH- and dual-modified nanoparticles (TNP, QNP, TQNP), a maleimide-thiol coupling reaction was conducted at room temperature under nitrogen for 4 h, with a molar ratio of 3 for both maleimide/TGN and maleimide/QSH (Zhang et al., 2014a). The nanoparticles were collected by centrifugation and washed three times with deionized water.

2.3. Characterization of nanoparticles

The particle size, polydispersity index (PDI) and zeta potential of the nanoparticles were determined using a Malvern Zetasizer (Malvern, nanoZS, UK). The drug-loading capacity (DLC) and the encapsulation efficiency (EE) of the H102/Coumarin-6-loaded nanoparticles were measured using HPLC as previously reported (Zhang et al., 2014a,b).

The surface composition of the four preparations (NP, TNP, QNP and TQNP) was analyzed using X-ray photoelectron spectra (XPS) on an RBD-upgraded PHI-5000C ESCA system (Perkin Elmer).

The conjugation efficiency and density of TGN and QSH on the NP, measured by HPLC, were calculated as indicated below:

$$\text{Conjugation efficiency}(\%) = \frac{\text{Total amount of TGN/QSH added initially} - \text{amount of TGN/QSH in supernatant}}{\text{Total amount of TGN/QSH added initially}} \times 100$$

$$\text{Density} = \frac{\text{Total amount of TGN/QSH added initially} - \text{amount of TGN/QSH in supernatant}}{\text{Weight of nanoparticles}}$$

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