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Dual-functional nanoparticles targeting amyloid plaques in the brains of Alzheimer's disease mice

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ABSTRACT

Alzheimer's disease (AD) is a common neurodegenerative disorder with few treatments. The limitations imposed by the blood–brain barrier (BBB) and the non-selective distribution of drugs in the brain have hindered the effective treatment of AD and may result in severe side effects on the normal brains. We developed a dual-functional nanoparticle drug delivery system based on a PEGylated poly (lactic acid) (PLA) polymer. Two targeting peptides that were screened by phage display, TGN and QSH, were conjugated to the surface of the nanoparticles. TGN specifically targets ligands at the BBB, while QSH has good affinity with $A\beta_{1-42}$, which is the main component of amyloid plaque. Tests probing the bEnd.3 cell uptake and *in vivo* imaging were conducted to determine the best density of TGN on the nanoparticles' surfaces. The optimal amount of QSH was studied using a Thioflavin T (ThT) binding assay and surface plasmon resonance (SPR) experiments. The optimal maleimide/peptide molar ratio was 3 for both TGN and QSH on the surface of the nanoparticles (T_3Q_3-NP), and these nanoparticles achieved enhanced and precise targeted delivery to amyloid plaque in the brains of AD model mice. A MTT assay also validated the safety of this dual-targeted delivery system; little cytotoxicity was demonstrated with both bEnd.3 and PC 12 cells. In conclusion, the T_3Q_3-NP might be a valuable targeting system for AD diagnosis and therapy.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly, affecting more than 35 million people worldwide [1]. The number of people with AD increases annually and will most likely triple over the next 40 years [2]. AD is a progressive neurodegenerative disorder characterized by memory loss, confusion and cognitive disabilities [3]. Currently, therapeutic drugs that treat AD, such as acetylcholinesterase inhibitors and N-methyl-D-aspartate receptor antagonists, are only able to alleviate the symptoms. Although numerous biotech drugs have been developed from studies of the molecular pathogeneses of AD, few can be used in clinical treatment [1].

The blood–brain barrier (BBB) is a formidable obstacle for biotech drugs targeting the brain. Though some aspects of the BBB of AD patients may differ from the normal BBB, such as P-gp function, cerebral blood flow and cerebrospinal fluid reabsorption, it retains its integrity [4]. Therefore, negotiating the BBB is essential for successful AD treatment using biotech drugs. Due to the development of brain targeting delivery systems, some drugs encapsulated by nanoscale particles that were conjugated with BBB targeting ligands can be transported directly into the brain. Unfortunately, few systems have been able to deliver these drugs to the diseased region after passage through the BBB. The drug distribution in normal brain tissues might cause serious central nervous system (CNS) side effects. For example, nerve growth factor (NGF) is a potential agent for treating AD. However, it may induce abnormal Schwann cell, sensory and sympathetic neuron hyperplasia in normal brains [5]. However, the drug distribution spread across the whole brain also decreases the amount of drug reaching the target [6], reducing the therapeutic effects. Therefore, a delivery system must be developed that precisely targets the lesions.

In our previous studies, a "dual targeting" strategy was proposed and achieved some success when treating brain glioma [6,7]. However, whether this strategy is also a promising solution for AD diagnosis and treatment remains to be confirmed. Therefore, a

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cascade targeting delivery system for AD was developed in this study.

Dual functional ligands are a crucial component of the dual-targeted delivery system. To overcome the first barrier of AD treatment, targeting ligands must be chosen to overcome the BBB. A new ligand composed of 12-amino acids, TGNYKALHPHNG (denoted as TGN), was obtained during our previous study using the *in vivo* selection of a phage displayed peptide library and has great potential for brain transport [8]. The nanoparticles modified with TGN were able to achieve 3.6 times more accumulation in the brain than unmodified nanoparticles. Accordingly, TGN was employed as the first-order ligand for targeting and penetrating the BBB.

The locations of AD lesions in the brain are unquestionably within the region for second-stage targeting. One of the primary histopathological characteristics of AD is the extracellular aggregation of amyloid plaque. The formation of amyloid plaque is caused by the increasing production, accumulation and aggregation of the amyloid-β (Aβ) peptide [9,10]. $A\beta_{1\text{-}42}$ is the predominant species of Aβ peptide (approximately 96% the total) [11] and its monomer is the most toxic isoform because it has a strong tendency toward aggregation [12], although its protofibrillar and fibrillar aggregates are also toxic [13,14]. Although the precise mechanism of AD progression is not clearly understood, the Aβ peptide might play a crucial role. Therefore, $A\beta_{1\text{-}42}$ in the amyloid plaques is a target for AD therapy [15]. A D-enantiomeric peptide, QSHYRHISPAQV (denoted as QSH), was recently screened using a mirror-image phage display selection using $A\beta_{1\text{-}42}$ as the target. QSH binds $A\beta_{1\text{-}42}$ in the sub-micromolar range and stains $A\beta_{1\text{-}42}$ deposits in the brains of both AD model mice and humans [16–18]. Moreover, this D-peptide is also protease-resistant and non-immunogenic [16], making it suitable as a targeting moiety. Therefore, QSH is an excellent second-stage targeting ligand for the $A\beta_{1\text{-}42}$ deposits delivery in brain.

Poly(ethylene glycol)-Poly (lactic acid) (PEG-PLA) is an ideal candidate among biodegradable polymers for nanoparticle formulation because it is a safe material with low immunogenicity. Copolymer nanoparticles are characterized by their core–shell architecture that features a segregated hydrophobic core (PLA) surrounded by a hydrophilic and sterically stabilized shell (PEG) [19]. PEG-PLA nanoparticles have been extensively studied for improving the bioavailability, solubility and retention time of drugs and bioactive molecules [20].

In this study, we constructed a dual-functional targeted PEG-PLA nanoparticle system modified with both TGN and QSH for delivering nanoparticles to AD brain lesions. The density optimization of the two ligands was conducted using cellular uptake, a Thioflavin T (ThT) binding assay, surface plasmon resonance (SPR) experiments and *in vivo* imaging. The dual-targeting effect was confirmed by brain distribution studies of nanoparticles and *ex vivo* imaging; the cytotoxicity was evaluated using a MTT assay.

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 $A\beta_{1\text{-}42}$ were obtained from the Chinese Peptide Company (Hangzhou, China). Coumarin-6, coumarin-7, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine Iodide (DiR), 3-(4, 5-Dimethyl-2-thiazolyl)-2,5-diphenyltetra-zoplium bromide (MTT) and cy3-labeled sheep antimouse IgG were purchased from Sigma (USA). The brain capillary endothelial cells (bEnd.3) and PC 12 cells were obtained from the Chinese Academy of Sciences Cell Bank. Dulbecco's Modified Eagle 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.

Adult male nude mice (16–20 g) and ICR mice (18–22 g) were obtained from Shanghai Sino-British Sippr/BK Lab Animial Ltd. (Shanghai, China) and maintained at a constant temperature (25 ± 1 °C). The animal studies were carried out according to the protocols approved by the ethical committee of Fudan University.

2.2. Preparation of $A\beta_{1\text{-}42}$ samples

Monomeric $A\beta_{1\text{-}42}$ was prepared using the method reported by Zagorski [21]. $A\beta_{1\text{-}42}$ peptide was dissolved in trifluoroacetic acid (TFA) and sonicated for 15 min. Subsequently, the TFA was removed using dry N_2 gas. The remaining peptide was redissolved in 1, 1, 1, 3, 3, 3-hexafluroisopropanol (HFIP) and sonicated for 15 min. After the HFIP was removed with dry N_2 gas, the $A\beta_{1\text{-}42}$ peptide was dried in a vacuum oven overnight at room temperature and stored at −20 °C until used.

2.3. Preparation and characterization of nanoparticles

The PEG-PLA nanoparticles (NP) were prepared using Mal-PEG-PLA and MePEG-PLA in a 3:7 ratio (weight) using the emulsion/solvent evaporation method. Briefly, 7.5 mg Mal-PEG-PLA and 17.5 mg MePEG-PLA were dissolved in 1 mL dichloromethane and added to a 3 mL 1% sodium cholate solution. The water-in-oil (w/o) emulsion was obtained using tip sonication (240 W, 30 s) in an ice-water bath. The emulsion was then diluted with 20 mL 0.5% sodium cholate solution with continuous stirring followed by rotary evaporation vacuum to remove the dichloromethane. The nanoparticles were collected by centrifugation at 14,000 rpm for 45 min at 4 °C. The coumarin-6-loaded or DiR-loaded nanoparticles were prepared using the same method, except that coumarin-6 or DiR 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 or DiR.

To conjugate the TGN and QSH, a maleimide-thiol coupling reaction was conducted at room temperature under nitrogen for 4 h. A maleimide on the nanoparticle was reacted with a thiol from TGN or QSH at a 7:1, 5:1, 3:1, 2:1 or 1:1 M ratio. The resultant TGN-, QSH- and dual-modified nanoparticles were termed T_5-NP, T_3-NP, and T_2-NP, Q_7-NP, Q_5-NP, Q_3-NP, Q_2-NP, Q_1-NP and TQ-NP (modified with different molar ratio of TGN and QSH), respectively. The nanoparticles were collected by centrifugation at 14,000 rpm for 45 min at 4 °C before being washed three times with deionized water.

The nanoparticles' morphology was examined using transmission electron microscopy (TEOL2010, JEM) and negative staining with 2% phosphotungstic acid solution. The mean diameter and Zeta Potential of the nanoparticles were measured using the light scattering method with a Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK).

2.4. Density optimization of TGN on NP

2.4.1. In vitro uptake of TGN-NP on bEnd.3 cells

The bEnd.3 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 μg/mL penicillin and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2.

For the qualitative studies, the cells were seeded at a density of 1.5×10^4 cells/well on 24-well plates and cultured for 24 h. After a 5 min equilibration with Hank's Balanced Salt Solution (HBSS), the cells were incubated with the coumarin-6-loaded NP, as well as T_5-NP, T_3-NP, and T_2-NP, at 0.1 mg/mL in HBSS for 0.5, 1 and 2 h, respectively, at 37 °C. Subsequently, the cells were washed with 0.01 M PBS three times and fixed with a 4% paraformaldehyde solution. The fluorescence intensity of the cells was examined under a fluorescent microscope (Leica DMI 4000B, Germany).

For the quantitative analysis, cells were seeded into 24-well plates at a density of 5×10^5 cells/well. After 24 h, the cells were treated as described above for 1 h. Subsequently, the cells were harvested and resuspended in 0.6 mL PBS. The fluorescence intensity was determined with a FACS Aria Cell Sorter (BD, USA).

2.4.2. In vivo imaging

The brain targeting effect was evaluated using an IVIS Spectrum Imaging System (Caliper) using a near infrared dye, DiR, as a probe.

The nude mice were injected with DiR-loaded NP, T_5-NP, T_3-NP, and T_2-NP (0.5 mg DiR/kg) via tail vein. One hour after administration, the mice were anesthetized with isoflurane and *in vivo* fluorescence images were taken. The mice were killed, and their brains were dissected to acquire images using the imaging system.

2.5. Density optimization of QSH on NP

2.5.1. Thioflavin T binding assay

Monomeric $A\beta_{1\text{-}42}$ was dissolved in PBS and sonicated for 15 min. NP and Q_7-NP, Q_5-NP, Q_3-NP, Q_2-NP and Q_1-NP (5 mg/mL) were added to the $A\beta_{1\text{-}42}$ solution; the final concentration of $A\beta_{1\text{-}42}$ was 100 μM. The samples were aggregated for 24 h at 37 °C. After incubation, 10 μL of each $A\beta_{1\text{-}42}$/nanoparticle mixture was added to 190 μL of 5 μM ThT in 50 mM Glycin-NaOH (pH 8.5). The fluorescence was measured in 96-well nonbinding plates (Greiner Bio One, Germany) using a microplate reader