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

Chi Zhang, Xu Wan, Xiaoyao Zheng, Xiayan Shao, Qingfeng Liu, Qizhi Zhang, Yong Qian

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 Aβ1-42, 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 (T3Q3-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 T3Q3-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 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
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, TGNYKALPHNG (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. Aβ, the predominant species of Aβ peptide (approximately 96% of the total) [11] and its monomer is the most toxic isoform because it has a strong tendency toward aggregation [12], although its protoflbril and fibril aggregation are also toxic [13,14]. Although the precise mechanism of Aβ precipitation is not clearly understood, the Aβ peptide might play a crucial role. Therefore, Aβ, in the amyloid plaques is a target for AD therapy [15]. A D-enantiomeric peptide, QSHYRHISPQV (denoted as QSH), was recently screened using a mirror-image phage display selection using Aβ as the target. QSH binds Aβ, in the sub-micromolar range and stains Aβ 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 Aβ 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)1000-poly (lactic acid)3000 (Male-PEG-PLA) and methoxy poly(ethylene glycol)3000-poly(lactic acid)1000 (Me-PEG-PLA) were synthesized by the East China University of Science. TGN (TGNYKALPHNG), QSH (QSHYRHISPQV) and Aβ, were obtained from the Chinese Peptide Company (Hangzhou, China). Comuramin-6, comuramin-7, 11'-diodotyrosyl-3,3',3''-tetramethylindotricarbocyanine iodide (DiR), 3(4, 5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazo-psilopium bromide (MTT) and cy5-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 Animal 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 Aβ1-42 samples

Monomer Aβ1-42 was prepared using the method reported by Zagorski [21]. Aβ1-42 peptide was dissolved in trifluoroacetic acid (TFA) and sonicated for 15 min. Subsequently, the TFA was removed using dry N2 gas. The remaining peptide was redissolved in 1, 1, 1, 3, 3, 3-hexafluoropropanol (HFIP) and sonicated for 15 min. After the HFIP was removed with dry N2 gas, the Aβ1-42 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 molar ratio. The resultant TGN- and QSH- and dual-modified nanoparticles were termed T5-NP, T3-NP, Q7-NP, Q5-NP, Q3-NP, Q2-NP, Q1-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 (TEDL2010_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% CO2.

For the qualitative studies, the cells were seeded at a density of 1.5 × 104 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 T5-NP, T3-NP, and T2-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 × 103 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, T5-NP, T3-NP, and T2-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

Monomer Aβ1-42 was dissolved in PBS and sonicated for 15 min. NP and Q7-NP, Q5-NP, Q3-NP, Q2-NP, Q1-NP and TQ-NP (5 mg/mL) were added to the Aβ, solution; the final concentration of Aβ was 100 μM. The samples were aggregated for 24 h at 37°C. After incubation, 10 μL of each Aβ1-42/nanoparticle mixture was added to 190 μL of 5% ThT in 50 mM Glycin-NaOH (pH 8.5). The fluorescence was measured in 96-well nonbinding plates (Greneir Bio One, Germany) using a microplate reader.
at excitation and emission wavelengths of 440 and 490 nm, respectively. NP fluorescence in the ThT solution without Ab1-42 was subtracted from each value to correct for the background fluorescence.

2.5.2. Surface plasmon resonance (SPR) experiments

The interaction between the nanoparticles and Ab1-42 was evaluated with a Biacore 3000 instrument (GE Health, Piscataway, NJ). The monomeric Ab1-42 was immobilized on CM5 sensor chips using amine-coupling chemistry. Briefly, the Ab1-42 solution (10 μM in acetate buffer pH 4.0) was injected for 5 min at 30 μL/min after surface activation, resulting in 6000 resonance units (1RU = 1 pg protein/mm²). The remaining activated groups were blocked with ethanolamine at pH 8.0. The naked surface (without peptide) was used as a reference. The anti-Ab antibody 6E10 was used to assess the activity of the immobilized Ab1-42. NP, as well as Q7-NP, Q5-NP, Q3-NP, Q2-NP and Q1-NP (5 mg/mL) were injected and flowed across the chip for 180 s at 5 μL/min and 25°C. After each injection, the chip surface was regenerated with running buffer (PBS). The data were evaluated using BioEvaluation 4.1.

2.6. Evaluation of dual-targeting effect of TQ-NP

2.6.1. In vivo model of Alzheimer’s disease

Animals subjected to intracerebroventricular injection of Ab1-42 suffer from progressive memory deficits, neuronal dysfunction and abnormal cholinergic function and therefore might be used to model AD [22–24]. Briefly, Ab1-42 was dissolved in saline (1 mg/mL) and incubated at 37°C for 7 days. ICR mice were anesthetized using i.p. of 5% chloral hydrate and fixed in a stereotaxic apparatus. Each mouse was injected bilaterally into the hippocampus (±1.8 mm lateral to the midline, 2.3 mm posterior to the bregma and 2.0 mm ventral to the skull surface) with 5 μL of Ab1-42 within 5 min. The needle was kept still for another 3 min and then slowly withdrawn. Animals injected with saline were used as the control group.

2.6.2. Ex vivo imaging

One week after the operation, the AD model mice were injected with DiR-loaded NP (0.5 mg DiR/kg), T3-NP (evaluated molar ratio of TGN was 3), and T3Q3-NP (evaluated molar ratio of QSH was 3) via the tail vein. One hour after administration, the mice were killed, and their brains were divided into the cerebrums, cerebellums and hippocampi. The images were taken using the imaging system.

2.6.3. In vivo distribution of coumarin-6-loaded nanoparticles in the brain tissues of AD model mice

For this study, 108 ICR mice had AD induced as described in 2.6.1 and were randomly divided into three groups to receive coumarin-6-loaded NP, T3-NP or T3Q3-NP (300 μg/kg). Subsequently, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after intravenous injection, the blood samples and brain tissues were collected. Each brain was subdivided into its cerebrum, cerebellum and hippocampus. The coumarin-6 concentrations in the blood and brain tissues were measured using HPLC with coumarin-7 as the internal standard, as previously described [25].

All of the concentration data were normalized based on dose and weight. The area under the blood or brain tissue concentration of coumarin-6 versus time curve (AUC(0-t)) was calculated using the trapezoidal method. The variance for the AUC(0-t) was normalized to the AUC(0-t) of coumarin-7.

Table 1

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>96.03 ± 3.64</td>
<td>−25.07 ± 0.32</td>
</tr>
<tr>
<td>T2-NP</td>
<td>100.32 ± 3.11</td>
<td>−24.12 ± 0.21</td>
</tr>
<tr>
<td>T2-NP</td>
<td>102.18 ± 2.06</td>
<td>−23.16 ± 0.18</td>
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<tr>
<td>T2-NP</td>
<td>108.21 ± 4.13</td>
<td>−22.38 ± 0.43</td>
</tr>
<tr>
<td>Q3-NP</td>
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<td>−23.69 ± 0.45</td>
</tr>
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<td>101.25 ± 3.38</td>
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</tr>
<tr>
<td>Q3-NP</td>
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<td>−22.76 ± 0.31</td>
</tr>
<tr>
<td>Q3-NP</td>
<td>107.34 ± 1.23</td>
<td>−21.05 ± 0.24</td>
</tr>
<tr>
<td>Q3-NP</td>
<td>111.76 ± 5.65</td>
<td>−19.87 ± 0.46</td>
</tr>
<tr>
<td>T3Q3-NP</td>
<td>107.45 ± 2.77</td>
<td>−21.33 ± 0.16</td>
</tr>
</tbody>
</table>

Fig. 1. Transmission electron micrographs of NP (A), T2-NP (B), Q2-NP (C) and T3Q2-NP (D), respectively. The bar represents 200 nm.
was estimated using the method described by Yuan [26]. The amount of TGN-modified nanoparticles targeting the brain after i.v. administration was evaluated using the drug targeting index (DTI) [27,28] and calculated using the equation below:

\[
\text{DTI} = \frac{\text{AUC}_{\text{brain}}}{\text{AUC}_{\text{blood}}} \equiv \frac{\text{modified NP}}{\text{NP}} = \frac{\text{AUC}_{\text{brain}}}{\text{AUC}_{\text{blood}}}
\]

2.7. Cytotoxicity assay

The cytotoxicity was assessed using a MTT assay based on the reduction of the MTT formazan crystals by living cells [29]. Both PC12 and bEnd.3 cells were seeded on 96-well plates at a density of 2 x 10^4 cells/well, and cultured for 24 h. Subsequently, the cells were exposed to nanoparticles (NP, T3-NP, T2-NP and T3Q3-NP) dispersed in DMEM at different concentrations (0.1, 0.5, 1, 5 and 10 mg/mL). After 24 h of incubation, the MTT method was used to determine the cell viability. Untreated cells were used as the control, and the viability was expressed as the percentage of the absorbance of the control.

2.8. Statistical analysis

All of the data were expressed as the mean ± SD. Significant differences were determined using a one-way analysis of variance (ANOVA), followed by a Dunnett’s post hoc analysis for multi-group comparison. Student’s t-test was used when comparing two groups, and statistical significance was when \( p < 0.05 \).

3. Results

3.1. Characterization of nanoparticles

The transmission electronic microscope images revealed that the nanoparticles had a round shape (Fig. 1). The TGN- and QSH-modified nanoparticles were approximately 100 nm, and their zeta potentials were approximately −20 mV (Table 1). The particle...
size was slightly increased and zeta potential was slightly decreased when the number of surface peptides increased.

3.2. Density optimization of TGN on NP

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

bEnd.3 cells are from an immortalized mouse brain endothelial cell line and are reported to be an attractive candidate for modeling the BBB [30,31]. Therefore, these cells were chosen to evaluate the cellular uptake of the nanoparticles. For the qualitative study, the fluorescence signal was correlated with the incubation time (Fig. 2). Similar intensity was observed in cells treated with T3-NP and T2-NP at each time point. The quantitative analysis of the nanoparticles' cellular uptake was studied from 15 min to 2 h and at different concentrations. The fluorescence intensity displayed both time- and concentration-dependent behavior (Fig. 3B). At every time point and each concentration studied, the uptake of the TGN-modified nanoparticles was significantly higher than the uptake of the unmodified nanoparticles, particularly T3-NP and T2-NP: T3-NP and T2-NP demonstrated 3.87–3.93 times more fluorescent intensity than the NP at 0.5 h (Fig. 3A). There was no significant difference between the cellular uptake of these two nanoparticles, indicating that the molar ratio between maleimide on the nanoparticles and thiol on the TGN (3) was sufficient for attaining best cellular uptake.

3.2.2. In vivo imaging

The in vivo images were taken 1 h after injection into the vein (Fig. 4A). Relative to the NP-treated mice treated, a slight increase in the nanoparticle accumulation in brain was observed for T3-NP, and the signal strengthened until the molar ratio between the maleimide of the nanoparticles and the thiol from TGN decreased to 3 for both TGN and QSH. After incubation with bEnd.3 cells, similar fluorescence was observed in cells treated with T3TQ3–NP and T3-NP at each time point, achieving 3.64 and 3.87 times more fluorescent intensity than NP at 0.5 h, respectively (Fig. 3A). A similar phenomenon was also observed in the in vivo images: little difference was found in T3-NP and T3TQ3–NP accumulation in brain (Fig. 4A), indicating that the modification of QSH did not influence the brain-targeting effect of the TGN modified nanoparticles.

3.3. Density optimization of QSH on NP

3.3.1. Thioflavin T binding assay

An established ThT assay was used to monitor the NP-induced Aβ1-42 peptide aggregation through the β-sheet formation pathway [1] and was therefore chosen to evaluate the interaction between the nanoparticles and Aβ1-42. The NP had no effect on the Aβ1-42 aggregation, and the fluorescent intensity had no significant correlation with Aβ1-42 alone (Fig. 5). After the modification using QSH, the fluorescent intensity decreased with the QSH density. The lowest values were observed for Q3-NP, Q2-NP and Q1-NP, suggesting that the maleimide/QSH molar ratios from 3 to 1 had a similar effect on the Aβ1-42 aggregation. Therefore, a maleimide/QSH molar ratio of 3 might be optimal for the dual-functional nanoparticles.

3.3.2. SPR experiments

SPR is a surface-sensitive optical technique based on the changes in the refractive index near a metal surface used to investigate various dynamic biological processes, such as protein–protein and protein-DNA interactions [32]. In the present study, a SPR experiment was conducted to determine whether the QSH on the surface of the nanoparticles was able to bind Aβ1-42 and find out the optimal density of QSH. The rise of the response signal for the QSH modified nanoparticles indicated an increased binding ability toward chip-immobilized Aβ1-42. The response signal of nanoparticles increased with the maleimide/QSH molar ratio from 7 to 3, and decreased slightly when the ratio was reduced to 2 (Fig. 6A). Because the response signal is directly proportional to the average mass of the nanoparticles per Aβ1-42 [33], the QSH density on the Q2–NP surface was higher than on the Q3–NP, possibly causing one QSH modified nanoparticle to bind with more than one Aβ1-42 molecule on the chip and inducing a decreased signal for Q2–NP. The interaction was specific for Aβ1-42 given that no binding was observed on the naked surface (Fig. 6B). The results of the SPR experiments were consistent with the observation of the ThT assay; therefore, the optimal maleimide/QSH molar ratio for dual-functional nanoparticles was 3.

3.4. Confirmation of TGN and QSH densities on NP

After the density optimization of both TGN and QSH on nanoparticles was conducted, it was necessary to investigate the targeting effect when these two peptides were introduced by nanoparticles simultaneously and to determine whether they influence one another. The previous density optimization achieved satisfactory targeting effects when the molar ratio of the maleimide on the nanoparticles and the thiol on peptide decreased to 3 for both TGN and QSH. After incubation with bEnd.3 cells, similar fluorescence was observed in cells treated with T3TQ3–NP and T3-NP at each time point, achieving 3.64 and 3.87 times more fluorescent intensity than NP at 0.5 h, respectively (Fig. 3A). A similar phenomenon was also observed in the in vivo images: little difference was found in T3-NP and T3TQ3–NP accumulation in brain (Fig. 4A), indicating that the modification of QSH did not influence the brain-targeting effect of the TGN modified nanoparticles.

In Th-T assay, T3TQ3–NP caused an obvious decrease in the aggregation compared to the NP, and the fluorescence value was near the value for Q3–NP (Fig. 5). Interestingly, the affinity of the T3TQ3–NP for Aβ1-42 during the SPR experiments was slightly higher than...
was observed for the Q3-NP (Fig. 6I), possibly due to the uniform spatial distribution of QSH caused by the TGN modification. Therefore, T3Q3-NP was the optimal species to use as the dual-targeted drug delivery system in the present study.

3.5. Evaluation of dual-targeting effect of T3Q3-NP

3.5.1. Characterization of T3Q3-NP

According to the above screening results, T3Q3-NP was chosen for further investigation. T3Q3-NP was spherical and uniform when observed using transmission electron microscopy (Fig. 1D). The particle sizes of the T3Q3-NP were 107.45±2.77 nm, and the zeta potential was −21.33±0.16 mV.

3.5.2. Ex vivo imaging

To determine the dual-targeting effect of nanoparticles in vivo (the highest capacity to concentrate on the amyloid plaques), DiR-loaded NP, T3-NP and T3Q3-NP were administered to AD model mice via the tail vein. The brains were harvested 1 h after injection and divided into their cerebrums, cerebellums and hippocampi (Fig. 4D and E). Similar fluorescence was detected in cerebrums of mice treated with T3-NP and T3Q3-NP, while signal in the cerebellums was slightly stronger for T3-NP than T3Q3-NP. For the hippocampi, which are the locations of the amyloid plaques, the fluorescence attributed to the T3Q3-NP was significantly stronger than for T3-NP. These results indicated that T3Q3-NP was transported into the brain and realized a good amyloid plaques targeting effect.

3.5.3. In vivo distribution of coumarin-6-loaded nanoparticles in the brain tissues of AD model mice

Coumarin-6 was incorporated into nanoparticles and used as a fluorescent marker to quantitatively evaluate the distribution of the NP, T3-NP and T3Q3-NP in the brain. Similar concentration–time profiles in the blood samples were found among these three nanoparticles (Fig. 7D). After injection, the concentration of coumarin-6 in the blood decreased rapidly within 1 h before generally declining. In the brain tissues, the peak exposure of coumarin-6 occurred at 1 h (Fig. 7A–C). The maximum
Fig. 6. (I) Surface plasmon resonance (SPR) sensorgram of chip-immobilized Ab1-42 interacting with nanoparticles modified with different ratios of QSH. NP (A), Q2-NP (B), Q3-NP (C), Q5-NP (D), Q7-NP (E) and T3Q3-NP (F) were injected and flowed across the chip for 180 s at 5 μL/min at 25 °C, respectively. (II) SPR sensorgram of the naked chip surface interacting with NP.

Fig. 7. Concentration vs. time curves of coumarin-6 in the cerebrum (A), cerebellum (B), hippocampus (C) and blood (D), respectively. AD model mice were treated with 300 μg/kg NP, T3-NP and T3Q3-NP, respectively. The data are represented by the mean ± S.D., n = 4.

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concentrations of T3-NP and T3Q3-NP in cerebrum and cerebellum were 1.82–2.25 times higher than the NP concentration; the AUC values were 0.87–1.23 times higher (Table 2), suggesting that the TGN modification might improve the brain penetration of nanoparticles. The highest uptake of coumarin-6 in the hippocampus by T3Q3-NP was 103.52 ng/g, which was 1.79 and 2.81 times the uptake of T3-NP (57.91 ng/g) and NP (36.84 ng/g) (Fig. 7C), respectively. These results support the conclusions from the in vivo and ex vivo images (Fig. 4). The drug targeting index (DTI) for hippocampus by T3Q3-NP was 3.77 ± 0.008, while it was 2.33 ± 0.008 for T3-NP (Table 2). Accordingly, the nanoparticles modified with both TGN and QSH had an excellent hippocampus-targeting effect relative to the NP and TGN-NP.

### 3.6. Cytotoxicity assay

The in vitro cytotoxicity of the NP, T3-NP, Q3-NP and T3Q3-NP were detected using bEnd.3 and PC12 cells after 24 h incubation using MTT (Fig. 8). At every studied concentration, the viability of both bEnd.3 and PC12 cells was above 90% for all four nanoparticles, and no significant difference was found between the formulations, indicating they had uniformly low cytotoxicity. Because PLA and PEG polymers are safe materials with FDA approval, the PEG-PLA nanoparticles modified with TGN and QSH might be promising drug carriers with little cytotoxicity.

### 4. Discussion

The BBB remains the primary challenge for developing AD therapeutics because the vast majority of the brain microvessels in demented individuals displayed normal BBB features [34]. However, the intra-cerebral injection of a drug or polymeric implant is ineffective due to the diffusion limitations drugs within the brain [35]. In addition, the drug distribution in normal brain tissues

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**Table 2**

Comparing the AUC(0,4) and DTI of NP, T3-NP and T3Q3-NP in brain versus blood in AD model mice. (mean ± S.D., n = 4).

<table>
<thead>
<tr>
<th></th>
<th>AUC(0,4)</th>
<th>Ratiobrain/blood</th>
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<tr>
<td>Blood</td>
<td>NP</td>
<td>T3-NP</td>
<td>T3Q3-NP</td>
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<tr>
<td></td>
<td>402.66 ± 15.73</td>
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<td></td>
<td>0.41</td>
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</tr>
<tr>
<td>Cerebrum</td>
<td>NP</td>
<td>T3-NP</td>
<td>T3Q3-NP</td>
</tr>
<tr>
<td></td>
<td>163.12 ± 7.70</td>
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<td>319.92 ± 13.00^a</td>
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<td>0.83</td>
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<td>T3-NP</td>
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<td>653.59 ± 32.14</td>
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<td>Cerebellum</td>
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<td>T3Q3-NP</td>
</tr>
<tr>
<td></td>
<td>157.59 ± 11.81</td>
<td>355.68 ± 15.40^c</td>
<td>319.92 ± 13.00^a</td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>0.91</td>
<td>2.33 ± 0.008</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>NP</td>
<td>T3-NP</td>
<td>T3Q3-NP</td>
</tr>
<tr>
<td></td>
<td>542.00 ± 24.06^c</td>
<td>542.00 ± 24.06^c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.47</td>
<td>1.47</td>
<td>3.77 ± 0.008</td>
</tr>
</tbody>
</table>

\[ \text{DTI} = \frac{\text{AUC}_{\text{brain}}}{\text{AUC}_{\text{blood}}} \]  
\[ \text{modified NP} = \frac{\text{NP}}{\text{NP}}. \]

\(^a\) Significantly different from NP, p < 0.05.  
\(^b\) Significantly different from T3-NP, p < 0.05.  
\(^c\) Significantly different from T1-NP, p < 0.05.

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**Fig. 8.** In vitro cytotoxicity. In vitro cytotoxicity of NP, T3-NP, Q3-NP and T3Q3-NP incubated with bEnd.cells (A) and PC12 cells (B) for 24 h at 37 °C.

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might cause unexpected side effects. Therefore, improving drugs’ brain penetration and targeting effects is crucial for AD treatment. Polymeric nanoparticles with appropriate surface modifications are promising candidates for AD treatment because they are capable of crossing the BBB, have high drug loading capacities and target AD-specific mutagenic proteins [36].

In recent years, several dual-targeted drug delivery systems [7,37] or nanoprobes [38,39] have been reported. In most cases, they were designed to detect or treat cancers, and the two ligands recognized the receptors or carriers of the tumor cells. Sanjib et al. [40] developed gold nanoparticles modified with epidermal receptor growth factor and folate receptor antibodies for selective delivery to cancer cells and subsequent use in cancer therapy. In this delivery system, both ligands targeted the cancer cells; in other systems, the two ligands utilize a sequential targeting effect [6,7]. Gao [6] et al. revealed nanoparticles conjugated with angiopep-2 peptide and EGFP-EGF1 protein. The two ligands selectively recognized different receptors for either the BBB or tumor cells.

In our research, the dual targeting strategy was sequential. TGN was used to enhance the BBB penetration of the PEG-PLA nanoparticles. Previous studies demonstrated that TGN might facilitate the delivery of nanoparticles to the brain [7,8]. Our research confirmed this effect; a significantly higher cellular uptake and brain distribution occurred with TGN modified nanoparticles compared to the naked nanoparticles. Subsequently, QSH targeted Aβ1-42 and guided the nanoparticles to the AD lesions. Because the AD mouse model was produced by bilaterally injecting Aβ1-42 into the hippocampus, it was a simple matter to evaluate the Aβ1-42 targeting effect by detecting the accumulation of nanoparticles in hippocampus. Our results in both ThT assays and SPR experiments suggested that QSH-modified nanoparticles might have good affinity with Aβ1-42 in vitro. The amount of nanoparticles in the hippocampi of the AD mouse models was significantly higher when administered using the T3Q3-NP compared to both NP and T3-NP. Therefore, these dual-functional nanoparticles achieved an excellent Aβ1-42 targeting effect in vitro and in vivo.

The density evaluation of the TGN and QSH on the nanoparticles was essential to obtain the optimal dual-targeting effect. Because the excessive modification of peptides on the surface might lead to nanoparticle aggregation, the best targeting effects were achieved with a minimal amount of peptides. Our study indicated that TGN and QSH did not influence one another’s targeting effects, allowing their density to be optimized, respectively. The evaluation of the TGN density was conducted using in vitro cellular uptake and in vivo imaging. The results for both experiments agreed, suggesting that a maleimide/TGN molar ratio of 3 might attain the best brain targeting effect. The optimization of QSH was studied using ThT assays and SPR experiments. A maleimide/QSH molar ratio of 3 may be optimal for Aβ1-42 targeting because increasing the modification did not improve the effects. Accordingly, the determined maleimide peptide molar ratio was 3 for both TGN and QSH on the surface of nanoparticles.

AD is a progressive neurodegenerative disorder, and some of its lesions begin to form 20–30 years before AD becomes clinically apparent [41]. Therefore, early diagnosis is crucial for therapeutically slowing the AD progression and long-term care. Most diagnoses of AD today are based on the NINCDS-ADRDA criteria and Diagnostic and Statistical Manual of Mental Disorders, 4th ed., Text Revision (DSM-IV-TR). However, the cognitive deficits during the pre-dementia stage of AD might not meet these criteria [42]. Therefore, using nanoprobes for AD that are sensitive to the earliest cognitive or biological changes might be a promising technique for early diagnosis. Because the generation and deposition of Aβ peptide are believed to be the key mechanisms for AD [43], our dual-targeted delivery system might be used in the treatment and early diagnosis of AD.

5. Conclusions

A dual-functional drug delivery system was developed to target AD lesions using TGN and QSH appended to the surface of PEG-PLA nanoparticles. The TGN/maleimide and QSH/maleimide molar ratios were 1:3 to achieve the optimal targeting effect. This cascade delivery system avoided cytotoxicity and could be used for early diagnosis or treatment of AD.

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