RESEARCH ARTICLE

Construction of Brain-Targeted Nanoreactor System and Intracerebral Drug Delivery

ABSTRACT

Poly(lactic-co-glycolic acid (PLGA) copolymer) nanoparticles with an average particle diameter of 142.8 nm and a polydispersity of 0.161 were prepared and optimized by an emulsion solvent evaporation method. Blank nanoparticles were modified with the transmembrane peptide (trans-activator of transcription, TAT) and the brain-targeting peptide (Angiopep-2), and the modification rate was determined. It is obvious that the modification rates of TAT and Angiopep-2 were 65.4% and 71.1%. After the preparation of coumarin-6-loaded nanoparticles, high-performance liquid chromatography analysis method was established, the entrapment efficiency, drug loading capacity (DLC) and drug release properties of coumarin-6 nanoparticles were determined. It was indicated that the entrapment efficiency of coumarin-6 nanoparticles was 38.21%, DLC was 0.229%, with a sustained release effect within 48 h. Fluorescence imaging technology was used to explore the targeting of nanoparticles in mice. TAT and Angiopep-2 modified nanoparticles exhibited strong brain targeting effects.

KEYWORDS: nano-release system, polyactic acid-glycolic acid copolymer, brain-targeting peptides, cell-penetrating peptides

INTRODUCTION

Currently, central nervous system diseases have become one of the major diseases affecting public health. However, conventional drugs are not effective in treating such diseases due to the fact that the body’s blood–brain barrier (BBB) limits the entry of many drugs into the central nervous system. Poly(lactic-co-glycolic acid (PLGA) is a block copolymer of lactic acid (LA) and glycolic acid (GA) with high biocompatibility and the product obtained by Krebs cycle after elimination of water and carbon dioxide in vivo. Therefore, PLGA as a material for preparing nanoparticles, its irritation and the side effects are small. PLGA nanoparticles are typically nanoscale in size with good tissue permeability and targeting, in addition to protecting encapsulated macromolecular drugs from enzymatic degradation, and change the dynamic behavior of the drug and tissue distribution in vivo. It is a widely used material in the preparation of brain-targeting nanoparticles.

Polyethylene glycol (PEG) is the most commonly used polymer in drug delivery. The use of PEGylated PLGA material (PLGA-MePEG and PLGA-PEG-Mal) can increase the solubility and stability of the drug in the aqueous phase, reduce the aggregation between molecules, reduce the immunogenicity and prolong the circulation time of the drug. Moreover, PEG acts as a spacer between the drug-loading system and Mal, which enables the target molecules on the subsequent attachment to be exposed on the surface of the drug-carrying system to freely target, bind and mediate the cell internalization function.

Cell-penetrating peptides (CPPs) are a series of short, positively charged peptides consisting of not more than 30 amino acid residues that have a specific conserved amino acid sequence. CPPs are derived from the transversion of the HIV viral gene trans-activator of transcription (TAT), TAT is composed of 86 amino acid residues, of which the amino acid sequence from 48th to 60th (GRKRRQRRPQPQ) is arginine-rich, which is a key structural region with translocation function and contains two lysine residues and six arginine residues determine the hydrophilicity of the whole TAT fragment. Not only small molecules but also macromolecules that are 100 times large can also be carried into cells by membrane-penetrating peptides.

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Angiopeps, a family of peptides that target the receptor-related protein (LRP) domain, have been shown to have better ability to assist the carrier in penetrating the BBB. Wherein Angiopep-2 (TFFYGGSRGKRNNFKTEEYC, MW = 2.4 kDa) was indicated stronger brain penetration than other LRP-related proteins and most BBB-capable proteins (such as aprotinin and transferrin)\(^5\).

In this paper, PLGA-PEG-Mal and PLGA-MePEG were used as materials to prepare nanoparticles by emulsion solvent evaporation, optimize the preparation process of nanoparticles, modified the nanoparticles with the transmembrane peptide TAT and the brain-targeting peptide Angiopep-2 and characterized them. In vivo fluorescence imaging was used to observe the brain involvement of different types of nanoparticles after intravenous administration.

MATERIALS

PLGA-PEG3500-Mal (LA/GA = 50:50, MW45000) and PLGA-MePEG (LA/GA = 50:50, MW45000) were obtained from Polymtek Biomaterial Co., Ltd. (Shenzhen, China). Sodium cholate and coumarin-6 were purchased from Sigma-Aldrich (St. Louis, MO, USA). RAGE antagonist (RP-1, APDTKTQ, MW759.82), Angiopep-2 (TFFYGGSRGKRNNFKTEEY, MW2404.66) and TAT (CGRKKRRQRRRPPQ, MW1822.18) were synthesized by Shanghai China Peptides Co., Ltd. Company (Shanghai, China). Dichloromethane, methanol and acetonitrile were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Trifluoroacetate was purchased from Shanghai ShiFeng Co., Ltd. Company (Shanghai, China), Total thiol detection kit was purchased from Shanghai Best Bio Co., Ltd. (Shanghai, China), DiR iodide (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine iodide) was purchased from AAT Bioquest Co., Ltd. (USA).

METHODS

Preparation of blank PLGA nanoparticles

Weigh a certain amount of PLGA-PEG-Mal and PLGA-MePEG, dissolved in 1 mL dichloromethane; add 2 mL of sodium cholate solution, 200 W power ice bath intermittent ultrasound 90 s, the resulting colostrum was added dropwise to a certain concentration of sodium cholate aqueous solution under magnetic stirring for 5 min; solution was evaporated using a rotary evaporator for 45 min and removed dichloromethane; 12,000 rpm at 4°C centrifuged for 45 min, the precipitate was collected to obtain nanoparticles.

Optimization of the preparation process

According to the single-factor experimental conditions in the previous method, the results of the study to determine the ultrasonic time of 90 s, the ultrasonic power of 200 W. Selected the concentration of sodium cholate (A), the total amount of PLGA-MePEG and PLGA-PEG-Mal (B), the concentration of dispersed sodium cholate (C) and the ratio of PLGA-MePEG to PLGA-PEG-Mal (D) as the test factors. Choose three levels for each factor. According to four factors and three levels, orthogonal experimental designs are shown in Table 1. Each factor and each level was repeated three times. The particle size of the nanoparticles was used as an index for evaluation. Orthogonal design factors and experimental arrangements are as follows:

Verify the best process

According to the optimal process conditions, nanoparticles are prepared and their particle size, potential and polydispersity (PdI) are measured.

Morphological observation of nanoparticles

Take the nanoparticles placed on the copper net, the copper mesh immersed in tungstophosphoric acid staining for 2 min, the morphology of nanoparticles observed using transmission electron microscopy (TEM).

Surface modification of nanoparticles

Take the nanoparticles, add 1 mL HEPES (pH 7.0) solution mixed and dispersed, transferred to a vial. TAT and Angiopep-2 were added at the ratio of Thiol:Mal = 2:1.

Table 1

<table>
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<th>C (%)</th>
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Table 2

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<td>R</td>
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<td>72.2</td>
<td>118.5</td>
<td>11.9</td>
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Magnetic stirring at room temperature for 6 h, then centrifuged at 12,000 rpm for 45 min at 4°C, washed twice by ddH₂O and kept at 4°C for further use.

**High-performance liquid chromatography (HPLC) analysis**

1) Chromatographic conditions
The reversed-phase column (diamonsil C18 5 um, 200 × 4.6 mm) was used at 25°C. Mobile phase: Methanol-ddH₂O (95:5). Injection volume: 20 μL. The flow rate was set at 1.0 mL/min and the detection wavelength was 448 nm.

2) Exclusive
Dissolve the blank nanoparticles with acetonitrile, dilute with methanol and serve as a blank solution. Weigh a certain amount of coumarin-6, add methanol volume, formulated as a reference solution. Dissolve appropriate amount of coumarin-6 nanoparticles with acetonitrile, add appropriate amount of methanol to dilute the volume, and prepare the test solution. Inject 20 μL and record the chromatograms.

3) Standard curve
Precision drawing coumarin-6 solution amount, diluted with methanol 10, 5, 2.5, 1.25, 0.625, 0.03125, 0 μg/mL series of solution, take 20 μL injection analysis, the concentration of each sample three times the average peak area A. Linear regression of the average peak area A (mAU) for each, respectively, preparation of 0.625, 2.5 and 10 μg/mL of concentration at coumarin-6 concentration C (μg/mL).

1) Precision
Coumarin-6 methanol solution, injection of 20 μL each time, each concentration were injected six times, calculate the concentration of peak area RSD.

2) Method recovery
Take 0.1 mL of blank nanoparticle colloidal solution, by adding appropriate amount of acetonitrile dissolved material, coumarin-6 mother liquor amount, and methanol dilution, formulated the solution into 0.625, 2.5, 10 μg/mL, respectively. Three concentrations of the solutions were injected 20 μL determination, into the standard curve, calculate the recovery rate (n = 3).

3) Extraction recovery
Precision drawing coumarin-6 mother liquor in blank nanoparticles solution, add acetonitrile to dissolve the nanoparticle material, then add methanol diluted into 0.625, 2.5, 10 μg/mL. coumarin-6 standard solution, take 20 μL injection, calculate the peak area A1. Then, take the same amount of coumarin-6 solution, add methanol diluted with the same volume of the control solution without the above treatment steps, take 20 μL injection, calculate the peak area A2. The recovery of coumarin-6 can be calculated by comparing A1 with A2.

**Drug-loading coefficient and encapsulation ratio**

Cou-6/NP, TAT-Cou-6/NP and ANG-Cou-6/NP were prepared at a coumarin-6 loading of 0.6%. After washing the precipitate, 200 μL of acetonitrile was added to dissolve the material, and add methanol to dilute, then centrifuged at 12,000 rpm for 5 min at 4°C. Take 20 μL of supernatant for injection analysis. According to the peak area and coumarin-6 standard curve, the concentration of coumarin-6 in the nanoparticles was calculated, and the entrapment efficiency and drug loading capacity (DLC) were calculated according to the dosage and the mass of the nanoparticles.

Encapsulation ratio (%) = mass of encapsulated drug/dosage × 100%

DLC (%) = mass of encapsulated drug/total mass of nanoparticle material × 100%

**Brain-targeting effects in vivo**

According to the previous procedure, DiR/NP, ANG-DiR/NP and TAT/ANG-DiR/NP nanoparticles are prepared. Take four C57BL/6 mice, tail intravenous injection of nanoparticles (a dose of 50 mgNP/kg) was given, respectively, the control group was given an equal volume of saline. After 1 h, mice were anesthetized with isoflurane. Cut off the skin and skull, exposing the brain. Fluorescent imaging of the brain to observe the brain into the brain.

**RESULT**

**Prescription screening**

In this experiment, the influence of each factor on the size of nanoparticles was C>, B>, A> and D>. The optimal prescription is C₃B₁A₂D₂. That is, the concentration of sodium cholate in the internal aqueous phase was 1.5%, the total amount of PLGA-MePEG and PLGA-PEG-Mal were 5 mg, the ratio of Mal-PEG-PLGA and MePBG-PLGA was 1:9 and disperse phase sodium cholate concentration of 0.1%.  

**Verify the best process**

According to the best process, three batches of nanoparticles were prepared, and their particle size, potential and PDI were measured.
It was indicated that the average particle size was 142.8 nm, the average Zeta potential was $-11.93 \text{ mV}$ and the average polydispersity (PdI) was 0.161.

**Morphological observation of nanoparticles**

The results shown in Figure 2, observed under electron microscopy in uniform size, appearance rounded.

**Surface modification of nanoparticles**

The modified rates of TAT-NP and ANG-NP were 65.4% and 71.1%, respectively, using the total thiol detection kit (Ellman’s method).

**HPLC analysis**

1) **Exclusive**

The peak time of coumarin-6 in vitro analysis of samples was about 4.8 min, the peak shape is good, the blank sample does not interfere with the determination, and this method has good specificity.

2) **Standard curve**

The HPLC analysis was calibrated with standard solutions of $0.03125–10 \mu \text{g/mL}$ of coumarin-6 dissolved in methanol (Equation: $A = 132.17C - 5.1781$; correlation coefficient of $R^2 = 0.9994$).

3) **Precision**

Preparation of 0.625, 2.5 and 10 μg/mL of coumarin-6 methanol solution, respectively, injection of 20 μL each time, each concentration were injected into six times, calculate the concentration of peak area RSD < 2%. It confirmed the instrument precision is good.

4) **Method recovery**

The recovery of coumarin-6 at different concentrations was between 97% and 101% with an RSD < 3%, indicating that the recovery of this method was good and in line with the requirements of in vitro sample determination.

5) **Extraction recovery**

High, medium and low concentrations of the coumarin-6 of extraction recovery between 96% and 100%, RSD < 2%. It is in line with the requirements of in vitro sample determination.

**Drug-loading coefficient and encapsulation ratio**

As shown in Figure 4, the DLC and entrapping efficiency (EE) of Cou-6/NP was about 0.229% and 38.21%. The DLC and EE of TAT-Cou-6/NP was about 0.164% and 27.311%. The DLC and EE of ANG-Cou-6/NP was about 0.23% and 38.377%.

<table>
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<tr>
<th>Formulation</th>
<th>DLC (%)</th>
<th>EE (%)</th>
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<tbody>
<tr>
<td>Cou-6/NP</td>
<td>0.229 ± 0.004</td>
<td>38.210 ± 0.626</td>
</tr>
<tr>
<td>TAT-Cou-6/NP</td>
<td>0.164 ± 0.001</td>
<td>27.311 ± 0.191</td>
</tr>
<tr>
<td>ANG-Cou-6/NP</td>
<td>0.230 ± 0.002</td>
<td>38.377 ± 0.230</td>
</tr>
</tbody>
</table>

**Table 3** The result of authenticate test.

<table>
<thead>
<tr>
<th>No.</th>
<th>Size/nm</th>
<th>PDI</th>
<th>Zeta (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>140.1</td>
<td>0.209</td>
<td>−11.7</td>
</tr>
<tr>
<td>2</td>
<td>146.6</td>
<td>0.128</td>
<td>−11.5</td>
</tr>
<tr>
<td>3</td>
<td>141.6</td>
<td>0.147</td>
<td>−12.6</td>
</tr>
<tr>
<td>X ± SD</td>
<td>142.8 ± 3.40</td>
<td>0.161 ± 0.042</td>
<td>−11.93 ± 0.59</td>
</tr>
</tbody>
</table>

**Fig. 1** Particle size distribution of nanoparticles.

**Fig. 2** TEM of nanoparticles 24,000×.

**Fig. 3** HPLC chromatograms of coumarin-6. (A) blank; (B) control solution; (C) sample solution.
In vitro release

The results showed that the cumulative release rate of coumarin-6 within 48 h does not exceed 8%, which is not easy to leak. It is a stable fluorescent probe.

Brain-targeting effects in vivo

It was indicated that the nanoparticles were mainly distributed in the main organs of the body at 1 h, which was due to the non-specific phagocytosis of the nanoparticles by the reticuloendothelial system in the body. However, all three kinds of nanoparticles had different degrees of brain penetration, TAT and Angiopep-2 modified nanoparticles significantly increased brain capacity. The brain-targeting of the three nanoparticles can be ranked in the following order from the live biofluorescence imaging of the brain in Figure 5: TAT/ANG-DiR/NP > ANG-Di/NP > DiR/NP.

DISCUSSION

The emulsification solvent evaporation method is the polymer material and the drug’s organic phase (such as methylene chloride, chloroform, etc.) and emulsifier-containing aqueous phase made of Oil/Water emulsion, diluted with a large amount of water and evaporated to remove the organic solvent, the original of the oil droplets solidified into drug-loaded polymer particles. According to the emulsification solvent evaporation, the concentration of external sodium cholate had the greatest effect on the particle size. The particle size of the nanoparticles decreased with the decrease of the concentration of sodium cholate, it is possible that the increase of the concentration of external aqueous solution hindered the diffusion of the organic solvent and the solidification rate of the emulsion slowed down, resulting in the accumulation of droplets and the increase of the particle size. The amount of nanoparticles also has a significant effect on the particle size of the nanoparticles. When the concentration of the material is decreases in the system, the particle size of the nanoparticles decreases. This may be due to the decrease in the mass of the nanoparticles. The probability of collision between the droplets is reduced, making the emulsion droplets easier to maintain the initial state and quickly solidified into a ball, to reduce the degree of agglomeration of nanoparticles into the ball, so that the particle size decreases.

Coumarin-6 is a fat-soluble dye with high laser conversion efficiency and stable performance. Coumarin-6 was detected by HPLC and its sensitivity was high. The coumarin-6 was encapsulated in the nanoparticles, the nanoparticles were characterized and investigated in vitro release characteristics of nanoparticles in 48 h, the cumulative release rate of nanoparticles in vitro is less than 8%, indicating that coumarin-6 nanoparticles with sustained-release function, better stability.

The near-infrared dye DiR has excitation and emission wavelengths of 748 and 780 nm, respectively, at which the absorption of fat, hemoglobin and water can be kept low, thus, maximizing the elimination of background when performing live fluorescent photography interference. To perform the fluorescence imaging of differently modified nanoparticles into the brain in vivo, it was found that the amount of brain-targeting nanoparticles and transmembrane peptide-modified nanoparticles increased compared with the blank nanoparticles after 1 h. Among them, the brain-targeting of the double-modified nanoparticle with penetrating peptide and the brain-targeting peptide was the best. This study shows that the nano-delivery system constructed with transmembrane peptides and brain-targeting peptides can be used for the delivery of essential therapeutic drugs in the central nervous system.

Fig. 4 Cumulation release of coumarin-6 from coumarin-6-loaded NP (n = 3).

Fig. 5 In vivo fluorescence imaging of C57BL/6 mice after intravenous injection of nanoparticles.
REFERENCES