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The Synthesis and characteristic study of transferrin-conjugated liposomes carrying brain-derived neurotrophic factor

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Abstract. This study aimed to establish a novel non-viral liposome vector delivering brain derived neurotrophic factor (BDNF) through the blood brain barrier. For this purpose, different water-oil ratios were tested to create liposomes for packaging the prophase synthesized plasmids encoding the BDNF proteins. In order to increase the targeted and peripheral circulation time, we connected the liposomes with transferrin (Tf) and a polyethylene glycol (PEG) long chain. The non-isotope method was used to measure the liposome envelopment ratio and ligand-binding ratio, and also to detect molecular biological features, such as particle size and stability. Tf-conjugated liposomes could be synthesized satisfactorily under the following conditions: the ratio of phospholipid to cholesterol was 1:1; the ratio of enter to plasmid was 100:1; oil phase was dichloromethane; the oil to water ratio was 4:1; the rotary evaporation temperature was 30°C; the ultrasonic temperature was 10°C; the ultrasonic time was 10min; and 10% trehalose was in the presence. Generated liposomes had a uniform circular shape and particle size distribution. In this experiment, we successfully established a new type of Tf-conjugated liposomes carrying the gene of BDNF and the study provides an experimental basis for the future.

Keywords: Brain derived neurotrophic factor, Tf-conjuaged, non-viral vector, liposome, transferrin

1. Introduction

Brain derived neurotrophic factor (BDNF) is a member of the family of neurotrophic factors. In 1982, BDNF was found in the brain of pigs for the first time. BDNF and its receptor are widely distributed in the central nervous system [1]. It is one of the most important neurotrophic factors in the brain, which maintains and promotes the growth and differentiation of neurons. It also plays an important role in the process of repair [2,3]. However, the expression of endogenous BDNF is low, whereas exogenous BDNF cannot go through the blood brain barrier due to its large molecular weight. How to carry exogenous BDNF into the intracranial area is a current research hotspot. Carrying by a viral vector is one of the means for gene therapy and has been studied by many scholars. However, its side effects are obvious, including increasing the risk of infection and leading to demyelination and

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immunogenicity in the central nervous system [4,5]. Hence, it is crucial to develop safe and efficient non-viral vectors. Liposome is a kind of non-viral vector that can deliver molecules such as DNA and drugs to the action sites, and it has been used to carry exogenous gene productions [6]. However, traditional liposomes are limited by low coating rates which hamper their efficiency of transfection into cells. Moreover, the liposome envelopment rate and drug loadings are usually obtained by the means of isotope labeling, which requires specific equipment to prevent radiation. It limits liposome generation in places where radioisotopes cannot be used. Researchers have explored the method of assembling liposomes [7,8], such as liposomes self-assembled from electrosprayed composite microparticles and from amphiphilic electrospun nanofibers. In this study, we tested different water-oil ratios to build liposomes, which packages the prophase synthesized *BDNF* gene fused with a GFAP promoter. The liposomes were linked with transferrin and a PEG long-chain. We also created a new type of non-viral vector without isotope labeling, which could successfully carry exogenous *BDNF* gene. It provides an experimental basis for *in vivo* targeted gene therapy in the future.

2. Materials and Methods

2.1. Materials

Two efficient transfected plasmids, pGFAP-BDNF and pCMV- BDNF, were constructed in our laboratory. Cholesterol formic acid ester chloride, chloroform, N, N - dimethyl ethylenediamine, phospholipids, DNaseI, Exonuclease III, and trehalose were all purchased from Sigma, USA. Methylene chloride, EDTA, POPC, DSPE-PEG-MAL and DSPE-PEG were all purchased from Avanti, USA. AgaroseCL-4B was from GE, USA. Tf, anhydrous ethanol, dimethyl sulfoxide, SDS, PicoGreen nucleic acid quantitative kit and BCA protein kit were all purchased from Invitrogen, USA.

2.2. Preparation of C-cholesterol

2.25g (5mmol) of Cholesterol chloroformate was weighed and dissolved in 5ml of chloroform. It was then dried at 0°C in an ice bath. The excess N, N-dimethyl ethylenediamine chloroform solution was added in drops with stirring. The liquid was dried (18mmol of N, N-dimethylethylenediamine was dissolved in 3ml chloroform and dried). A Rotary evaporator was used until the reaction was complete. The reaction was terminated with cholesterol chloroformate. The generated powder was recrystallized twice with absolute ethanol, and then purified and dried in vacuo.

2.3. The synthetic and screening process of liposomes and determination of the liposome envelopment rate, oil type and oil phase ratio

0.6182g of phospholipids and 0.4149g of DC-Chol were weighed and dissolved with chloroform into a 25ml volume system. 1ml of phospholipid stock solution and 1ml of DC-chol stock solution were added into a round bottom tube and placed at in a dry rotary evaporator with the temperature set to 30°C. Diethyl ether, chloroform and methylene chloride were selected as the organic phase, respectively. Appropriate water was added according to the water-oil ratio of 1:1, 1:2, 1:3, 1:4 and 1:5. 4ml of the organic phase solvent was added into the stock solution while rotary evaporation was kept operating. The products were vortexed for 5 min. The compatibility of the two phases was monitored till a homogeneous opalescence phase was formed. A group of water-compatible phases were selected

and placed in an ultrasonic bath for 30min. The oil and water phases were not stratified in order to determine the appropriate water-oil ratio.

2.4. The proportion of phospholipids and cholesterol screening

Chloroform was used as the organic phase to investigate the encapsulation efficiency of liposomes under different phospholipid /cholesterol ratios (1:0.5, 1:1, 1:2 and 1:3). Phospholipid and DC-Chol were added into a round bottom test tube or beaker according to the above ratios and then dried by a rotary evaporator. 4ml of chloroform and 1ml of plasmid solution were added and vortexed for 5min, followed by 5 min-ultrasound at different temperature. The organic solvent was dried in a rotary evaporator at room temperature. The aqueous phase was collected. 200ul of sample was added into 1ml water and then filtered using a 0.45um filter. 450ul of solution was added into a 10kDa MWCO ultrafiltration tube. Centrifugal ultrafiltration was performed at the speed of 3000rpm for 30min. The lower supernatant was collected to determine the concentration, and the encapsulation efficiency was calculated. The density of the purified plasmid (pCMV-BDNF or pGFAP-BDN) DNA with the high transformation levels was 1mg/ml in a total volume of 4ml. According to different F values of plasmid concentrations, the standard curve was calculated and the concentrations of free DNA, entrapment efficiency and particle size were measured.

2.5. Synthesis conditions for liposomes screening and determination of liposomes

1ml of phospholipid stock solution and 1ml of DC-Chol stock solution were added into a test tube or beaker. The temperature of the rotary evaporator was set up at 20°C, 30°C and 40°C to dry samples, respectively. 4ml of chloroform was added to form 1ml plasmid aqueous phase. Liposomes were prepared under different conditions, including 4°C, 10°C and 20°C of ultrasonic temperature and 2min, 5min and 10min of ultrasonic time. According to the instructions of PicoGreen nucleic acid quantitative kit, the encapsulation efficiency of liposomes was determined.

2.6. Synthesis of Tf-conjugated liposomes

2.6.1. Synthesis of PEG-liposomes

0.4149g of DC-Chol was weighted and dissolved in chloroform to a 25ml system. 33mmol / L of DC-Chol was prepared. 259.71mg of POPC, 56.1mg of DSPE-PEG and 11.22mg of DSPE-PEG-MAL were accurately weighed and dissolved with chloroform to a final volume of 10ml. 33mmol / L of POPC, 1.65mmol / L of DSPE-PEG and 0.33mmol / L of DSPE-PEG-MAL were prepared. POPC chloroform solution was drawn. The DSPE-PEG chloroform, DSPE-PEG-MAL and DC-Chol chloroform solutions were mixed together to form a chloroform solution (385ul) and added into a beaker at room temperature. The solvent was dried in a rotary evaporator. 4ml of dichloromethane was added into 1ml of plasmid reconstitution solution (1mg/ml), followed by 5 min-vortex and 5 min-ultrasound. Chloroform was evaporated at 30°C to obtain plasmid liposome solution. Similarly, the plasmid solution with an aqueous solution was used to generate liposome solution. The obtained plasmid liposomes were filtered through a 100nm microporous membrane. The plasmid liposomes recovered were treated with Dnasei and Exonuclease III at 37°C for 1h. In the end, the reaction was stopped by EDTA.

2.6.2. Thiolated Tf

25mg of Tf was weighted and added into 8ml of 1mol / L hydrochloric solution, mixing. Deionized water was added to a final volume of 50ml. 1g of catalyst was added into EDAC solution with stirring until the catalyst was completely dissolved. 0.5ml of pure mercaptoacetic acid was added into the obtained solution. 2mol / L of NaOH was used to adjust pH till pH was 5. The reaction was performed in dark at room temperature for 5h. Samples are dialyzed at 10°C and at dark for once against 5mmol/L hydrochloric acid, twice against 5 mmol/L hydrochloric acid and 1% NaCl, and then three times against 1 mmol/L hydrochloric acid. Dialysis buffer was changed every 2 hours. The samples were further concentrated to 3-4 ml by solid dispersion with PEG20000 around the dialysis bag, and then lyophilized at -30°C and stored at 4°C.

2.6.3. Conjugation of Thiolated Tf and PEG- liposomes

The PEG-liposomes was diluted to 80 ug/ml with sterile water, and then 250 ul of this solution was mixed with 250 ul of 18 mmol/L sodium sulfate. The PEG-liposomes solution and 2ml of thiolated Tf were pre-warmed at 55C for 15 min. The Thiolated Tf solution was then added into the DNA solution at 100 ul/s with vortexing. The mixture was vortexed for another 30 seconds and incubated at room temperature for 30 minutes. The products were named as the Tf-DNA-PEG Nano-gold suspension.

2.6.4. Determination of the Tf coupling ratio and analysis of the physical characteristics of Tf-liposomes

The concentration of Tf - pCMV - BDNF - PEG, Tf - pGFAP - BDNF – PEG and Tf - H_2O - PEG proteins were measured using a BCA protein quantitative kit and a spectrophotometer at OD562. The coupling rate of liposomes was then determined. In order to confirm the plasmid DNA had been wrapped into the interior of liposomes, 10ul of plasmid-carrying liposomes was mixed with 20ul of 0.5% SDS for cracking. The plasmid, the plasmid-carrying liposome and liposome lysis plasmid were detected by electrophoresis using a 0.8% agarose gel. The electrophoresis result was observed using ultraviolet transillumination.

2.6.5. The electron microscope of Tf-PLs

The Tf-pCMV-BDNF-PEG, Tf-pGFAP-BDNF-PEG or Tf-H_2O-PEG suspension was added into 50ul vinyl alcohol formal copper network and fixed about 3min. Excess liquid was absorbed with absorbent paper. 1% uranyl acetate of copper was added to the copper grid, standing for 2min. Excess liquid was absorbed again. The samples were air dried for 1h, and then placed under an electron microscope to observe the shape and size distribution of liposomes.

3. Results

3.1. Oil type and oil phase ratio selection

The experimental results showed that a homogeneous phase was formed when the ratio of water to chloroform or methylene chloride was 1:4. Thus, chloroform or methylene chloride is a preferable organic solvent.

3.2. The synthetic process and prescription screening of liposome and determination of the liposome envelopment rate

Chloroform was used as the organic phase. Different phospholipid and cholesterol ratios (1:0.5, 1:1, 1:2 and 1:3) were investigated. The encapsulation efficiency of liposomes was calculated and the phospholipid and cholesterol feed ratio was determined. different F values of plasmid concentrations. The standard curve was calculated with the equation: Y = 15.024X +140.3931. The concentration of free DNA and entrapment efficiency as well as the particle size were measured . According to the kit instructions, PicoGreen nucleic acid was used to quantify the encapsulation efficiency of liposome. When the rotary evaporator temperature was at 20°C and 30°C, the liposome encapsulation efficiency was 55.32% and 87.45%, respectively. When the temperature was 40°C, the evaporated solution had a large number of bubbles. Hence, liposomes were prepared at 30°C. When the ultrasonic temperature was 4°C, water couldn't be fully mixed with oil. In contrast, water could be fully mixed at 10°C, and the particle size was 250nm. At 20°C, the particle size was 450nm. Different ultrasonic time was tested. Oil failed to be thoroughly mixed with water after 2 min-ultrasound. The particle size was 248nm and 452nm after 5 min- and 10 min-ultrasound, respectively. By comparison, 30 °C was selected as the liposomes rotational temperature and 10°C and 5min were chosen as the appropriate ultrasound temperature and time. Given the above conditions, the encapsulation efficiency was high and smaller particle size and uniform liposomes could be obtained. Different concentrations of trehalose were tested in the liposome size distribution analysis under circumstances described above. The results showed that only 10% of trehalose was suitable to be used as stabilizer, with which the particle sizes of liposomes render a standard distribution. Thus, Tf-conjugated liposomes could be synthesized satisfactorily under the following conditions: the phospholipid/cholesterol ratio was 1:1; the enter/plasmid ratio was 100:1; dichloromethane was used as the oil phase; the oil/water ratio was 4:1; the rotary evaporation temperature was 30°C; the ultrasonic temperature was 10°C; the ultrasonic time was 10min; and 10% trehalose was in the presence.

3.3. Synthesis of Tf-conjugated liposomes

3.3.1. Synthesis of PEG-liposomes

Agarose gel electrophoresis was performed to detect plasmid DNA wrapped by liposomes and unwrapped DNA which was degraded by nucleases (Fig. 1). The figure showed that the plasmid DNA wrapped by liposomes moved slower in the agarose gel than the unwrapped DNA.

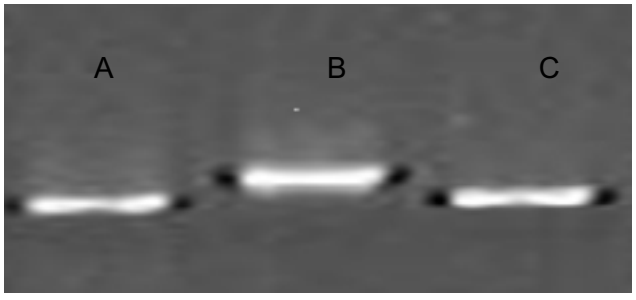

Fig.1 The electrophoresis of liposomes From L-R: A: unwrapped plasmid DNA; B : wrapped plasmid DNA; C : unwrapped plasmid DNA

3.3.2. Determination of the Tf coupling ratio

The values of the standard proteins were 1:0, 2:0.137, 3:0.270, 4:0.432, 5:0.554 and 6:0.662. The measured values of the Tf-pCMV-BDNF-PEG eluent, which went through the column at different stages, were 0, 0.081, 0.112, 0.131, 0.132, 0.120, 0.334, 0.530, 0.553, 0.542, 0.131, 0.221, 0.232, 0.122 and 0.02. The measured values of the Tf-pGFAP-BDNF-PEG eluent, which went through the column at different stages, were 0, 0.02, 0.122, 0.344, 0.522, 0.564, 0.532, 0.191, 0.201, 0.232, 0.222, 0.112, 0.101, 0.08 and 0.04. The measured values of the Tf-H_2O-PEG eluent, which went through the column at different stages, were 0, 0.01, 0.121, 0.155, 0.321, 0.532, 0.576, 0.542, 0.111, 0.321, 0.232, 0.121, 0.110, 0.09 and 0.01.The standard curve was calculated according to Y=6.7471X+0.0051. The coupling rates of Tf thiolated with PEG-liposomes were 51.75% for Tf-pCMV-BDNF-PEG, 52.10% for Tf-pGFAP-BDNF-PEG and 51.56% for Tf-H_2O-PEG, respectively. The three liposome elution curves after coupling were showed in Fig.2-4. According to the figure, the first peak should be Tf connected with liposomes and the second peak might be free Tf. The peaks showed particle size. The first peak detected at 400nm indicated Tf-pCMV-BDNF-PEG (76nm), Tf-pGFAP-BDNF-PEG(74nm) and Tf-H_2O-PEG(69nm). The values of the three PDI were 0.221 for Tf-pCMV-BDNF-PEG, 0.219 for Tf-pGFAP-BDNF-PEG and 0.198 for Tf-H_2O-PEG, respectively (Fig. 5-7). The second peak(200nm) showed not-uniformly distributed liposome size, and PDI> 0.8. Therefore, the first peak suggests Tf-conjugated liposomes.

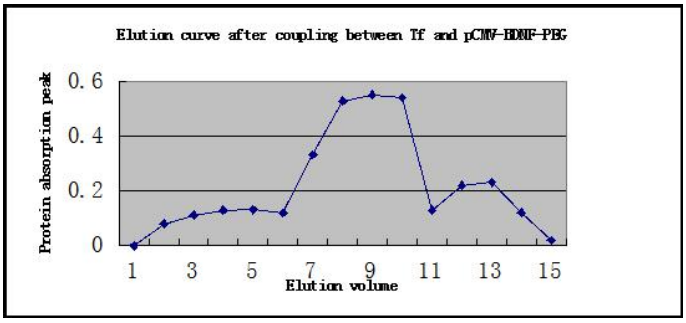

Fig.2 Elution curve after coupling of Tf and pCMV-BDNF-PEG X -axis means elution volume, Y-axis shows the protein absorption peak

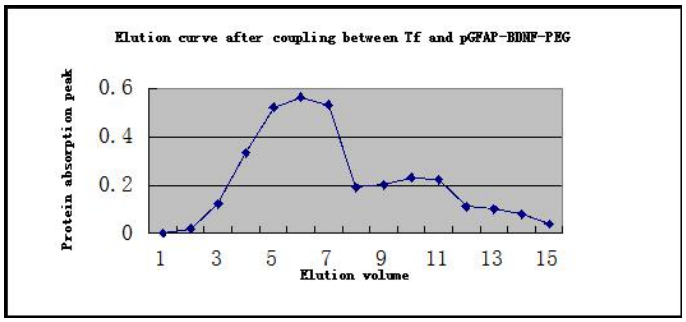

Fig.3 Elution curve after coupling of Tf and pGFAP-BDNF-PEG X -axis means elution volume, Y-axis shows the protein absorption peak

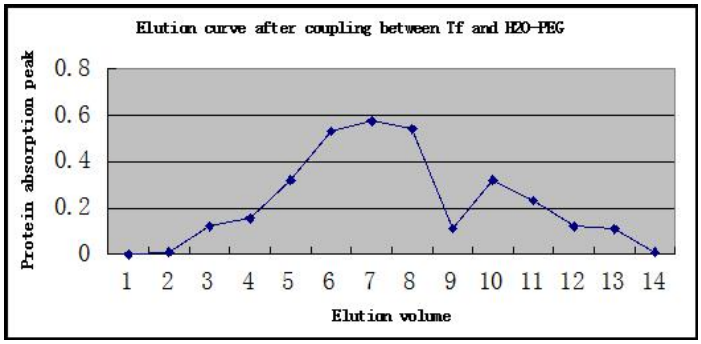

Fig.4 Elution curve after coupling of Tf and H_2O-PEG,X -axis means elution volume, Y-axis shows the protein absorption peak

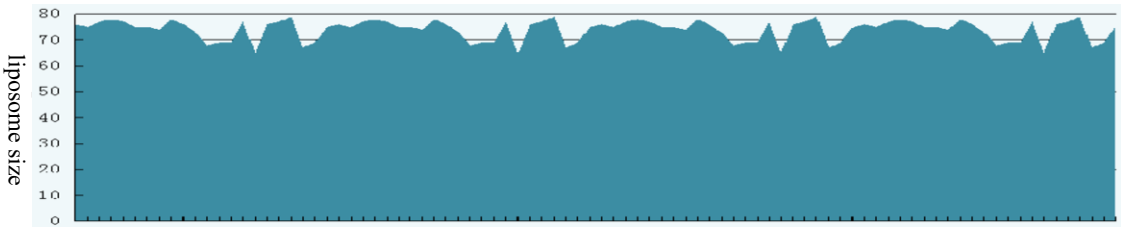

Fig.5 Tf-pCMV-BDNF-PEG size distribution, X -axis means Tf-pCMV-BDNF-PEG liposome, Y-axis means liposome size

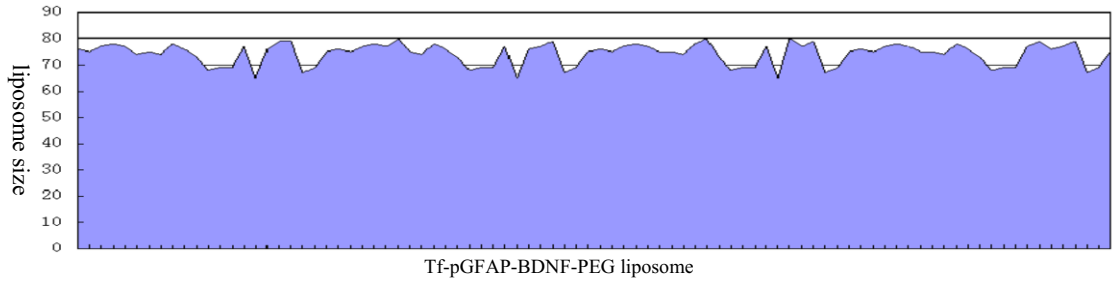

Fig.6 Tf-pGFAP-BDNF-PEG size distribution, X -axis means Tf-pGFAP-BDNF-PEG liposome, Y-axis means liposome size

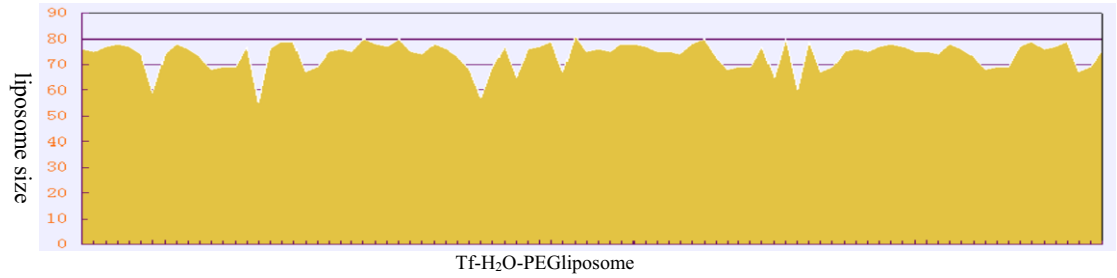

Fig.7 Tf-H_2O-PEG size distribution, X -axis means Tf-H_2O-PEG liposome, Y-axis means liposome size

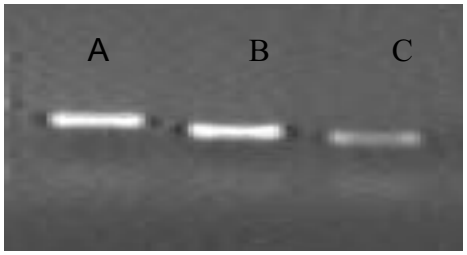

Fig.8 The agarose gel electrophoresis result of Tf-pCMV-BDNF-PEG （From L-R : Marker: D15000, wrapped DNA , plasmid DNA, released DNA）

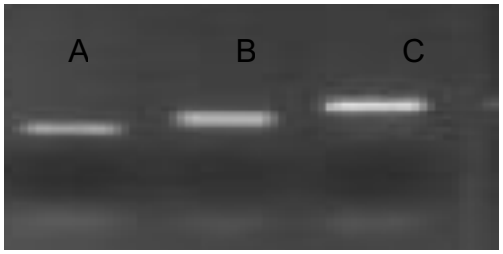

Fig.9 The agarose gel electrophoresis result of Tf-pGFAP-BDNF-PEG(From L-R : Marker: D15000, released DNA, plasmid DNA, wrapped DNA)

3.3.3. Analyze the physical characteristics of Tf-liposomes

As shown in Figure 8-9, most of DNA encapsulated by liposomes remained inside of the vesicle and could not migrate in an agarose gel. After being treated with the SDS detergent, liposomes were broken-down and the DNA inside the plasmid was released into the solution without being impaired. Liposomes were placed at 4°C for 1h, 6h and 24h to detect the stability. Condensation conditions in PBS were then observed. No condensation phenomenon was detected, indicating that the stability of synthetized liposomes meets the requirement of the experiment.

3.3.4. Tf-PLs observed using an electron microscope

Tf-pCMV-BDNF-PEG, Tf-pGFAP-BDNF-PEG and Tf-H_2O-PEG were placed under an electron microscope to observe the form and liposome size distribution (80KV, 30000) (Fig.10-12). The figures showed that compared with Tf - H_2O - PEG, synthesized Tf - pCMV - BDNF - PEG and Tf - pGFAP - BDNF had a uniform circular shape and particle size distribution.

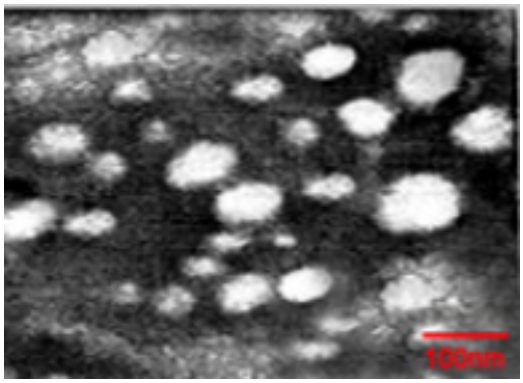

Fig.10 Tf-pCMV-BDNF-PEG observed using an electron microscope e,80KV,30000

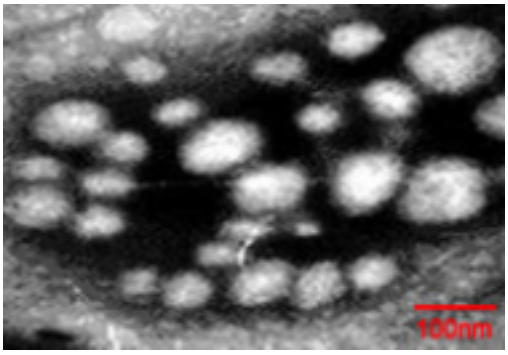

Fig.11 Tf-pGFAP-BDNF-PEG observed using an electron microscope,80KV,30000

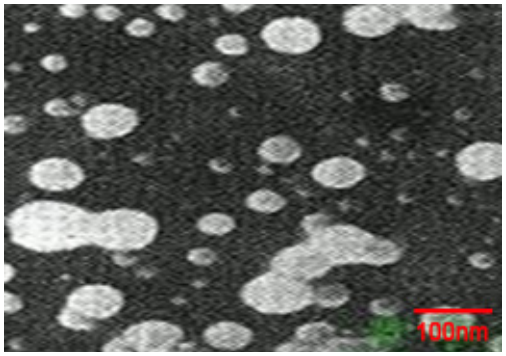

Fig.12 Tf-H_2O-PEG, observed using an electron microscope, 80KV,30000

4. Discussion

As a member of the family of neural nutrients, BDNF plays an important role in many aspects, such as promoting the growth and differentiation of neurons and damage repair in the central nervous system. Due to the presence of the blood-brain barrier, it is difficult to put exogenous BDNF into the brain, and the secretion of endogenous BDNF is not enough to play a significant biological role [9]. How to carry BDNF through the blood brain barrier becomes a research hotspot. Currently, the direct lateral ventricle injection and using a virus vector are main approaches for treatment [10]. However, the side effects such as the invasive or viruses immunogenicity limit its broad application [11]. Yeast two-hybrid bait vectors and pTAT prokaryotic expression vectors are better, but the expression of BDNF failed to meet research needs due to the low expression levels of these vectors and strict expression requirement [12]. In recent years, some scholars try to use liposomes as a kind of non-virus vector to carry target genes to penetrate the blood brain barrier [13]. Liposomes are made from natural biodegradable lipid bilayer which is similar to animal cell membrane structure. Due to its non-immunogenicity and low toxicity, it can avoid triggering the host immune response. Compared with direct DNA, nanoparticles of liposomes can provide an internal space to target genes and play a better effect *in vivo* [14]. The outer surface of liposomes can connect with targeting ligands to form targeted liposomes and complete the transfer function. Thus, molecular-weight polymer and cationic liposome has the potential to replace the viral vector. However, it has to overcome several common limitations such as low liposome package space, short life cycle, easily broken, etc.

In order to synthesize effective liposomes, we investigated different phospholipid and cholesterol ratios (1:0.5, 1:1, 1:2 and 1:3), calculated the encapsulation efficiency of the liposomes and determined the favorable phospholipid/cholesterol feed ratio. Through comparison, Tf-conjuated liposomes were found to achieve satisfactory synthesis under the following conditions: the phospholipid/cholesterol ratio was 1:1; the ester/plasmid ratio was 100:1; dichloromethane was used as oil phase; the oil/water ratio was 4:1; the rotary evaporation temperature was 30°C; the ultrasonic temperature and time were 10°C and 10min, respectively; and 10% trehalose was in the presence. GPAF promoter can lead to expression of specific target genes in neurons cells and astrocytes. In order to increase targeted combination with central neurons, in the previous experiments, we successfully constructed the pCMV - BDNF plasmid using genetic engineering technology, and further generated the pGPAF - BDNF expression vector, which can be transfected into cells with the recombinant plasmid. Using the above method and under experimental conditions, the coating rate of liposomes was higher (52%) and uniform nano liposomes with smaller particle size (< 100 nm) were generated. The liposomes were observed under an electron microscope. They had a uniform circular shape and particle size distribution. Specific proteins should be connected and the cycle time of peripheral blood circulation needs to be extended to enable the synthesized liposomes to effectively arrive the brain tissue from peripheral blood circulation. Transferrin (Tf) belongs to the class of iron-binding proteins. It has become a focus object in science since its serum transferring function was discovered 40 years ago [15]. Tf has a 670 to 700 amino acid polypeptide chain with a molecular weight of 80KDa. Its isoelectric point is about 5.6-5.8 [16]. Tf is used as a carrier or targeting ligand. It can be linked to liposomes through a combination with proteins and has been applied in the transport of anticancer drugs [17,18]. Tf-conjuaged liposome chemotherapy drugs have been widely used and they have characteristics like low toxicity, long cycle and cell-specific targeting. In a study, liposomes were conjugated with Tf and leukemia HL60 cells and the conjugated liposomes were cultured and investigated. The results showed that the liposomes binding to specific cells were delivered into cells *in vivo* by receptor-mediated endocytosis [19]. Organs such as liver, spleen, lung, and capillary endothelial cells of blood brain barrier are relatively rich of Tf receptor. Tf targeting PEG-2000 lipid surface exhibits a longer circulation time and lower absorption. It has been reported that the blood half-life of PEG-liposomes made by this method was extended up to 90h [20].

Liposome stability is the basis of guaranteeing the follow-up test. In the present study, liposomes were placed at 4°C for 1h, 6h and 24h and then condensation conditions in PBS were observed. No condensation phenomenon was detected, indicating that the stability of synthesized liposomes meets the needs of the experiment.

5. Conclusions

This experiment successfully built a new kind of non-virus gene carrier by measuring its biological characteristics. The carrier had a long chain of PEG liposomes whose size was only about 100 nm. The BDNF genes were packaged into the interior of the nanoparticles liposomes. The neurons cell-specific promoter GFAP replaced CMV. At the same time, Tf was coupled to the end of the long chain of PEG on the surface of liposomes. It has dual targets in the process of transportation and plays a role in the body by stimulating cell membrane receptors. The study provides an experimental basis for *in vivo* targeted therapy in the future.

Acknowledgments

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