A leptin derived 30-amino-acid peptide modified pegylated poly-l-lysine dendrigraft for brain targeted gene delivery

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ABSTRACT

The blood–brain barrier is the major obstacle that prevents diagnostic and therapeutic drugs being delivered to the central nervous systems in order to exert their effects. Specific ligand–receptor binding mediated endocytosis is one of the possible strategies to cross this barrier. A 30-amino-acid peptide (leptin30) derived from an endogenic hormone—leptin is exploited as brain-targeting ligand as it is reported to possess the same brain accumulation efficiency after intravenous injection. Dendrigraft poly-l-lysine (DGL) is used as non-viral gene vector in this study. DGL–PEG–Leptin30 was complexed with plasmid DNA yielding nanoparticles (NPs). The cellular uptake characteristic and mechanism were explored in brain capillary endothelial cells (BCECs) which express leptin receptors. Furthermore, brain parenchyma microglia cells such as BV-2 cells expressing leptin receptors could promote ligand-receptor mediated endocytosis leading to enhanced gene transfection ability of DGL–PEG–Leptin30/DNA NPs. The targeted NPs were proved to be transported across in vitro BBB model effectively and accumulate more in brains after i.v. resulting in a relatively high gene transfection efficiency both in vitro and in vivo. Besides, the NPs showed low cytotoxicity after in vitro transfection. Thus, DGL–PEG–Leptin30 provides a safe and noninvasive approach for the delivery of gene across the blood–brain barrier.

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1. Introduction

Central nervous system diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD) and brain tumors have been major threats to human health [1–3]. Though great efforts have been made during the last thirty years, there is still much to be done to develop efficient and safe therapies [4,5]. Comparing with the traditional chemical drugs, the gene therapy holds great promise in curing brain diseases in both laboratory and clinical applications [6,7].

One of the principal concerns about the gene therapy is the gene vectors. The ideal gene vector should possess such characteristics as high gene loading dose, low immunogenicity, better shelf-life, the possibility of repeated administration and being apt to further modification. Despite viral gene vectors have been proved to be efficient enough, the safety consideration has somehow prevented their further applications [8]. Among the non-viral approaches, synthetic polymers such as PEI, PLGA, PAMAM have been intensively investigated in gene delivery system [9–11]. PAMAM have been successfully applied to non-viral gene delivery systems in our previous studies [12–14]. The critical concerns about the synthetic polymers include safety and efficiency. Dendrigraft poly-l-lysine (DGL) have emerged as a new kind of synthetic polymers consisted of lysine [15–17] and herein been employed as gene vector due to its degradability and rich external amino groups. The dendrimers used in this study were DGL Generation 3 with 123 amino groups per molecular. Besides their biodegradability [17], the external amino groups could encapsulate DNA through electric interactions. Furthermore, they could be modified with PEG and targeting ligand rendering vectors long circulation and targeting properties.

Another major obstruction of brain gene therapy is the restrict conjunction of the blood–brain barrier (BBB). Several strategies have been developed to overcome the BBB [12–14,18–20]. Among these, cell-penetrating peptides (CPPs) are a group of short peptides with the potent ability to translocate across the BBB [18] and have been studied intensively during the last decades as materials well-suitable for the development of drug delivery vehicles [21]. However, because of lack of selectivity, the cell-penetrating peptide modified drug delivery system would cause unnecessary uptake by disease unrelated cells or tissues. Receptor-mediated endocytosis is one of the major mechanisms by which various agents can cross the BBB. The drug or gene delivery systems modified with ligands such as transferrin and lactoferricin, could be uptaken by brain capillary
endothelial cells (BCECs) and cross BBB via endocytosis pathway mediated by the specific ligand-receptor binding [12,22]. Compared with the above large molecule protein ligands, short peptide ligands have the advantages of a relatively small molecular weights, being easily synthesized, relatively low cytotoxicity and immunogenicity, and degradability [18]. There have been quite a few research papers about the short peptide ligands modified drug delivery systems recently. And we have previously reported a 29 amino acids peptide derived from rabies virus glycoprotein could serve as brain-targeting ligand inducing efficient brain-targeting gene delivery [13]. On the basis of the above evidence, a 30 amino acids peptide derived from leptin was investigated in this research.

Leptin, a 146 amino acid polypeptide secreted into the bloodstream by adipocytes, acts centrally on leptin receptor-expressing cells located in the hypothalamus and other parts of the brain, leading to decreased food intake and retarding weight gain [23,24]. It has been widely reported that leptin could be taken up by the brain [25,26]. Considering the 16 kDa molecular weight of leptin preclude the possibility of passive diffusion across the blood–brain barrier, a process that provides limited entry for some relatively small molecules, the leptin receptor mediated transport is believed to be one of the possible mechanisms besides leptin receptor independent pathways. Barrett’s group has identified several leptin derived peptides that are taken up by the brain [27]. Among these, peptide corresponded to position 61–90 (leptin30, 30 amino acids) was observed to possess the highest brain: plasma ratio which was equivalent to that of leptin. For leptin30, the majority of the radioactivity was localized more to parenchyma than capillaries. As the regions conferring brain uptake correspond closely to the sequences that are necessary for receptor binding, the results reinforce the view that the receptor pathway plays a large part in leptin uptake [27].

Since leptin is known to be taken up into all regions of the brain [28,29], leptin30 could be exploited as the brain-targeting ligand modified on the surface of gene vectors. Furthermore, it could be deduced that after transcytosis across the BBB, leptin30 could enhance internalization to the brain parenchyma cells by the specific ligand–receptor mediated endocytotic pathway. Therefore, leptin30 modified gene delivery system may have significant repercussions for brain diseases therapeutics.

In this study, leptin30 was modified by covalent linkage bond on the surface of DGL through bifunctional polyethyleneglycol (PEG), yielding DGL–PEG–leptin30. The brain-targeting efficiency of DGL–PEG–leptin30 as gene delivery vectors was evaluated in vitro and in vivo. Furthermore, the uptake mechanism of DGL–PEG–Leptin30/DNA NPs was explored.

2. Materials and methods

2.1. Materials

DGL were purchased from Colcom, France. α-Maleimidyl-ω-N-hydroxy-tyrosinamidid polyethylene glycol (NHS-PEG–MAL, MW 3400) was obtained from GL biochem (Shanghai) Ltd. The red fluorescent protein (RFP) plasmid (Shanghai GeneChem Co., Ltd, China) and pGL2-control vector (Promega, Madison, WI, USA) were purified using QIAGEN Plasmid Mega Kit (QIAGEN GmbH, Hilden, Germany), Filipin, colchicine and were purchased from Sigma–Aldrich (St. Louis, MO, USA). EMA and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes (Eugene, OR, USA). Lipofectamine2000 was bought from Invitrogen. Male ICR mice (5 wk old) were kindly provided by Prof. J. N. Lou (the Clinical Medicine Research Institute of the Chinese–Japanese Friendship Hospital). Primary BCECs were cultured as described previously [30]. Briefly, BCECs were expanded and maintained in special Dulbecco’s modified Eagle medium (DMEM) (Sigma–Aldrich) supplemented with 2% heat-inactivated fetal calf serum (FCS), 100 μg/ml epidermal cell growth factor, 2 mMol/L-glutamine, 20 μg/ml heparin, 40 μU/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin and cultured at 37 °C under a humidified atmosphere containing 5% CO2. All cells used in this study were between passage 15 and passage 30.

2.2. Cell culture

The primary cultured brain capillary endothelial cells (BCECs) isolated from BALB/C mouse were kindly provided by Prof. J. N. Lou (the Clinical Medicine Research Institute of the Chinese–Japanese Friendship Hospital). Primary BCECs were cultured as described previously [30]. Briefly, BCECs were expanded and maintained in special Dulbecco’s modified Eagle medium (DMEM) (Sigma–Aldrich) supplemented with 2% heat-inactivated fetal calf serum (FCS), 100 μg/ml epidermal cell growth factor, 2 mMol/L-glutamine, 20 μg/ml heparin, 40 μU/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin and cultured at 37 °C under a humidified atmosphere containing 5% CO2. All cells used in this study were between passage 15 and passage 30.

2.3. Synthesis of DGL derivatives

DGL was reacted with NHS-PEG3400-MAL at different molar ratio in PBS (pH 8.0) for 2 h at room temperature. The primary amino groups on the surface of DGL were specifically reacted with the NHS groups of the bifunctional PEG derivative. The resulting conjugate, DGL–PEG, was purified by ultrafiltration through a membrane (cutoff = 5 kDa) and the buffer was changed to PBS (pH 7.0). Then DGL–PEG was reacted with peptide leptin30 in PBS (pH 7.0) for 24 h at room temperature. The MAL groups of DGL–PEG were specifically reacted with the thiol groups of Leptin30. Ethidium monoxide (EMA) is a fluorescent photoaffinity label that, after photolysis, binds covalently to plasmid DNA. Thus, after complexing with the EMA-labeled plasmid DNA, the DGL series NPs was fluorescent. For synthesis of EMA-labeled DNA, EMA was labeled on DNA through UV exposure 1 h and series purification.

2.4. Characterization of DGL–PEG–Leptin30

The characteristics of DGL–PEG–Leptin30 were analyzed by nuclear magnetic resonance (NMR) spectroscopy.Basically, DGL–PEG–Leptin30 was freeze-dried, solubilized in D2O and analyzed in a 400 MHz spectrometer (Varian, Palo Alto, CA, USA).

2.5. Preparation and characterization of dendrimers/DNA nanoparticles(NPs)

Dendrimers (DGL, DGL–PEG, and DGL–PEG–Leptin30) were freshly prepared and diluted to appropriate concentrations in PBS (pH 7.4). DNA solution (100 μg DNA/ml 50 mM sodium sulfate solution) was added to obtain specified weight ratios (6:1, DGL to DNA, w/w) and immediately vortexed for 30 s at room temperature. Freshly prepared NPs were used in the experiments that follow. The mean diameter and zeta-potential of DGL series vectors (DGL, DGL–PEG, DGL–PEG–Leptin30) were determined by dynamic light scattering using a Zeta-Potential/Particle Sizer Nicomp® 380 ZLS (PSS Nicomp Particle Size System, U.S.A.).

2.6. Electrophoresis of dendrimers/DNA NPs and their DNA protection assay

The three NPs including DGL/DNA, DGL–PEG/DNA and DGL–PEG–Leptin30/DNA were freshly prepared with DGL to DNA at weight ratio of 6:1:0.7% agarose gel electrophoresis was performed to evaluate the DNA encapsulation effect caused by DGL in the NPs compared with naked DNA. To determine the DNA integrity and morphology. After that, 15 mg/ml sodium heparin was added and incubated at room temperature for 2 h to release the DNA from the NPs. All the samples were analyzed by 0.7% agarose gel electrophoresis. The integrity of the plasmid in each sample was compared with untreated naked DNA and DNase I treated naked DNA.

2.7. Cellular uptake of dendrimers/DNA NPs and with different endocytosis inhibitors

BCECs were seeded at a density of 2 × 104 cells/well in 24-well plates (Corning-Coaster, Tokyo, Japan), incubated for 72 h, and checked under the microscope for similar confluency and morphology. After this, BCECs were incubated with EMA-labeled DGL–PEG/DNA or DGL–PEG–Leptin30/DNA NPs at the concentration of 20 μg/well measured by DGL in the present of serum-free medium for 1 h at 37 °C. In case of inhibitors groups, different inhibitors were pre-incubated with cells 10 min, following the addition of the DGL–PEG–Leptin30/DNA NPs treated at the condition mentioned above.

2.8. Scatchard analysis of leptin receptor-binding assay

BCECs were seeded at a density of 2 × 104 cells/well in 24-well plates, incubated for 72 h, and checked under the microscope for similar confluency and morphology. DGL was iodinated as described previously with radioactivity of 1 μCi/μg 125I-DGL [31]. Radiolabeled DGL was applied to synthesize DGL–PEG–Leptin30. BCECs were
incubated with 125I-DGL-PEG-Leptin30 at concentrations from 0.2 to 2500 nM (leptin30) at 4 °C for 2 h. After that, the contents of each incubation well were removed and washed with cold PBS 7.4 for three times. 200 μl 1 % NaOH were added to lysis cells for 3 h. 100 μl 2 % HCl was used to neutralize NaOH. The radioactivity of the final well contents was analyzed by a γ-counter (SN-695, China). The above experiments were performed to determine the total binding of vector with leptin receptors. Nonspecific binding was determined by adding 5 μM nonradioactive DGL-PEG-Leptin30 to each reaction mixture in a parallel assay. The amount of cellular protein was determined by a BCA Protein Assay. For each concentration, experiments were repeated for 4 times.

2.9. Transport studies of NPs across BCECs monolayer

BCECs were seeded at a density of 6 × 10^4 cells/cm² onto polycarbonate 24-well Transwell filters of 1.0 μm mean pore size, 0.33 cm² surface area (FALCON Cell Culture Insert, Becton Dickinson Labware, U.S.A.). After about 3 days' culture, the cells were checked under the microscope for complete confluence. The cell monolayer integrity was monitored using an epithelial voltohmmeter (MILLICELL Culture Insert, Becton Dickinson Labware, U.S.A.). After about 3 days, BCECs monolayers were treated with DGL/DNA and DGL-PEG-Leptin30/DNA NPs, with a final amount of 20 μg DGL/well, separately. The incubation was performed at 37 °C on a rocking platform at 50 rpm. The radioactivity of 125I in each aliquot was assessed using a γ-counter (SN-695, China). After that, 10 μl from the 20 μl aliquot was dissolved in 1 ml of scintillation cocktail, and analyzed in a liquid scintillation counter (Packard Tri-Carb, Chicago, IL, USA). The correction factor of 14C-sucrose was 25% for the equipment. The apparent permeability (Papp) was calculated according to Irvine et al. [33] as follows:

\[ \text{Papp} = \frac{(dQ/dt) \times (1/C_0)}{(A \times t)} \times \frac{1}{14C} \]

where dQ/dt is the permeability rate (nmol/s), C₀ is the initial concentration (nmol/ml) in the donor chamber, and A is the surface area (cm²) of the membrane filter. Both TEER and permeability of 14C-sucrose of BCECs monolayers were measured from time 0–60 min to monitor the integrity of monolayers [35]. Basically, the cleared volume of 14C-sucrose was plotted vs. time, and the slope was estimated by linear regression analysis to give the mean and standard error of the estimate. The PS (permeability surface area product) value for the BCECs monolayer, called PSc (cm²/min), was computed as follows:

\[ \text{PSc} = \frac{1}{P_S} - 1 = \frac{1}{P_F} - 1 = \frac{1}{1/PS_F} \]

where PS is the PS value for the BCECs monolayer and the filter, PSc is the PS value for the filter only.

2.10. In vitro transfection experiment

BV-2 cells were seeded at a density of 5 × 10⁵ cells/well in a 24-well plate in a DMEM medium containing 10% FBS, and grown to reach 70–80% confluence prior to transfection. Before transfection, the medium was exchanged with fresh serum-free medium. The cells were treated with different NPs solutions containing 5 μg of plasmid DNA for 4 h at 37 °C. After exchange with a fresh serum-containing medium, cells were further incubated for 2 days after transfection. 5 μg plasmid DNA mixed with Lipofectamine2000 according to the standard protocol as described in instruction served as positive control. In the case of qualitative evaluation, the red fluorescence images were taken using a fluorescence microscope. For luciferase activity assay, medium was removed and the cells were rinsed with DPBS and shaken for 30 min at room temperature in 150 μl of luciferase cell culture lysis reagent supplied by the Promega Luciferase Assay Kit. The lysis solution was centrifuged at 14,000g for 2 min at 4 °C. Luciferase activity was measured by BPCI-1 Ultra-Weak CL and BL Analyzer and total amount of cellular protein was determined by a BCA Protein Assay. The final results were reported in terms of relative light units (RLU/mg protein).

2.11. Cytotoxicity assay

The cytotoxicity of the polymers was measured by MTT assay. BCECs were seeded in a 96-well tissue culture plate at 5000 cells per well in 90 μl DMEM medium containing 10% FBS. Cells achieving 70–80% confluence after 24 h were exposed to 100 μl of different NPs solutions with various concentrations and Lipofectamine200 at equivalent to 0.2 μg DNA per well for 2 h. Then, 100 μl of stock solution of MTT (1 mg/ml in Hank’s) was added to each well. After 3 h of incubation at 37 °C, each medium was removed and 100 μl of DMSO was added to each well to dissolve the formazan crystals formed by proliferating cells. Cells without treatment were used as a control. Absorbance was measured at 570 nm using a microplate reader (BIO-TEK, MQX200R PowerWave™ XS). Cell viability of each group was expressed as a percentage relative to that of control cells.

The same experiments were also performed on BV-2 cells. For 48 h assay, DMEM was added after 2 h incubation with NPs.

2.12. In vivo imaging analysis

The DGL-PEG-Leptin30/DNA NPs labeled by EMA were injected into the tail vein of nude mice at dose of 50 μg DNA/mouse. Then, the mice were anesthetized. Images were taken by CRi in vivo imaging system 60 min after injection. Brains were excised 2 h after injection for comparing the relative accumulation. EMA-labeled DGL/DNA NPs served as negative control.

2.13. Distribution of gene expression qualitatively in mouse brain

The DGL-PEG-Leptin30/pRFP NPs (6:1, DGL to DNA, w/w) as well as DGL/prFP and DGL-PEG/prFP NPs were injected into the tail vein of mice at a dose of 50 μg DNA/mouse. About 48 h later, animals were anesthetized with diethyl ether and killed by decapitation. The brains were removed, fixed in 4% paraformaldehyde for 48 h, placed in 15% sucrose solution until subsidence (6 h), then in 30% sucrose until subsidence (24 h). After this, brains were frozen in OCT embedding medium (Sakura, Tokyo, CA, USA) at 80 °C. Frozen sections of 20 μm thickness were prepared with a cryotome Cryostat (Leica, CM 1900, Wetzlar, Germany) and stained with 300 nm DAPI for 10 min at room temperature. After washing twice with PBS (pH 7.4), the sections were immediately examined under the fluorescence microscope.


The DGL-PEG-Leptin30/pGL2 NPs (6:1, DGL to DNA, w/w) were injected into the tail vein of mice at a dose of 50 μg DNA/mouse. At 48 h after injection, the mice were humanely decapitated and the principal organs (including brain, heart, liver, spleen, lung, and kidney) were excised. The organs were carefully washed with distilled water and homogenized in 1 ml lysis reagent (Promega, Madison, WI, USA) using a JY92-11N tissue homogenizer. The homogenate was centrifuged at 14,000g for 20 min at 4 °C. Luciferase activity and cellular proteins in the supernatant were quantified by a Luciferase Assay System and total amount of cellular protein was determined by a BCA Protein Assay, respectively. The results were expressed as light units/mg protein. Meanwhile DGL/gl2 and DGL–PEG/gl2 NPs were also injected for comparison.

3. Results

3.1. Characterization of DGL–PEG–Leptin30 and DGL–PEG–Leptin30/DNA NPs

In NMR spectra, the solvent peak of D₂O was found at 4.65 ppm. The methylene protons of branching units of DGL and a series of protons of leptin30 peptide have multiple peaks between 4.3 and 11 ppm. The NMR spectrum of DGL–PEG had a characteristic peak of the MAL group in PEG at 6.7 ppm (data not shown). The MAL peak disappeared in the NMR spectrum of DGL–PEG–leptin30, whereas the repeat units of PEG still presented a sharp peak at 3.6 ppm (Fig. 1) showing that the MAL group had reacted with the thiol group of peptide leptin30. The NMR spectra result proved the existence of the conjugate structure of DGL–PEG–leptin30. Meanwhile Eilman’s reagent was used to determine the percentage of unreacted thiol. And little thiol was detected (data not shown) which could be explained as the thiol at the end of leptin30 reacted with the MAL at one end of PEG specifically. The particle size and zeta-potential were measured. The results showed the vector/pDNA complex was nano-sized (118 ± 69 nm, 114 ± 49 nm, 141 ± 33 nm for DGL/DNA, DGL-PEG/DNA and DGL–PEG–Leptin30/DNA NPs, respectively) and positively charged (+1.30 ± 0.56 mV, +0.87 ± 0.43 mV, +1.15 ± 0.47 mV for DGL/DNA, DGL–PEG/DNA and DGL–PEG–Leptin30/DNA NPs, respectively).

3.2. Electrophoresis results

Fig. 2A showed the electrophoresis results of DGL series vector/ DNA NPs. All three NPs (DGL/DNA, DGL–PEG/DNA and DGL–PEG–Leptin30/DNA) with DGL to DNA at weight ratio of 6:1
could encapsulate DNA completely with no electrophoresis shift (Fig. 2A lane 3–5) compared with naked DNA (Fig. 2A lane 2). To investigate whether DGL series vectors could protect DNA against enzymatic degradation, naked DNA and the three DGL series NPs were incubated with DNase I. For all the three NPs, no electrophoresis shifts were observed after that (data not shown). To check the integrity of plasmid DNA in NPs after DNase I incubation, sodium heparin was added to release DNA. Fig. 2B shows that naked plasmid DNA (lane 2) was completely digested by DNase I, whereas all the three NPs (lane 3–5) could protect DNA from digestion.

3.3. Uptake comparison of DGL series vector/DNA NPs by BCECs in vitro

The EMA-labeled DGL series vector/DNA NPs were used to investigate cellular uptake characteristics. The results were shown qualitatively using fluorescent images. DGL with different PEGylation and leptin30 brain-targeting modification were synthesized as the gene vectors. The uptake efficiency was investigated in BCECs to determine an appropriate proportion for the subsequent experiments. The fluorescence intensity exhibited by BCECs had slightly increased when cells were treated with the EMA-labeled DGL–PEG–leptin30/DNA NPs at 1:5:2 (DGL:PEG:Leptin30, weight ratio) and 1:10:3 (Fig. 3C and D) compared with DGL/DNA NPs and DGL–PEG–leptin30/DNA (1:2:1) (Fig. 3A and B). As the ratio 1:5:2 and 1:10:3 showed no significant difference in uptake, DGL:PEG:Leptin30 at the weight ratio of 1:5:2 was selected and studied in further experiments from the economic consideration.

3.4. The exploration of NPs’ cell uptake mechanism

As shown in Fig. 4A–C, the BCECs uptake of DGL–PEG–leptin30/DNA NPs was higher than that of DGL/DNA and DGL–PEG/DNA NPs. The fluorescence intensity of all the three DGL series vector/DNA NPs declined dramatically at 4°C (Fig. 4D–F) compared with treated at 37°C (Fig. 4A–C). Excessive free leptin30 could inhibit the uptake of DGL–PEG–leptin30/DNA NPs (Fig. 4G), but had little influence on that of non-leptin30 modified NPs (Fig. 4H and I). Different endocytotic pathway inhibitors were used to clarify the endocytosis process. Treatment with transferrin resulted in a decrease of fluorescent intensity (Fig. 4K and L) of the DGL–PEG/DNA and DGL–PEG–Leptin30/DNA NPs uptake while showed no
effect on the uptake of DGL/DNA NPs (Fig. 4J). And the fluorescent intensity of DGL/DNA and DGL-PEG-Leptin30/DNA NPs declined to some extent (Fig. 4M and O) by filipin. The treatment of colchicine seemed to have little influence on the DGL-PEG/DNA NPs uptake (Fig. 4Q). However, the fluorescent intensity of DGL/DNA and DGL-PEG-Leptin30/DNA NPs decreased with the pre-incubation of colchicine (Fig. 4P and R).

3.5. Scatchard analysis of leptin receptor-binding assay

Binding of 125I-DGL-PEG-Leptin30 to BCECs was saturable in the presence of excess of nonradiolabeled DGL-PEG-Leptin30 in both low (Fig. 5, main graph A) and high (Fig. 5, main graph B) concentration ranges. Scatchard analysis of the binding data (Fig. 5, inset) indicated the presence of two independent leptin binding sites. The parameters governing the binding of DGL-PEG-Leptin30 to the BCECs, estimated by nonlinear regression analysis, indicated a high-affinity binding site (Fig. 5, inset A) with a dissociation constant $K_d$ of $1.737 \pm 0.21$ nM (estimate $\pm$ SD) compared with that of leptin ($5.1 \pm 2.8$ nM reported by Pardridge [25]) and binding maximum ($B_{\text{max}}$) of $0.6258 \pm 0.02$ pmol/mg pro. (compared with leptin, $0.34 \pm 0.16$ pmol/mg pro. reported). While, low-affinity and high-capacity class of binding sites (Fig. 5, inset B) had an estimated dissociation constant ($K_d$) of $421.8 \pm 66.13$ nM (leptin, $743 \pm 226$ nM reported) and binding maximum ($B_{\text{max}}$) of $372.1 \pm 18.24$ pmol/mg pro. (leptin, $26 \pm 5$ pmol/mg pro. reported).

3.6. Transport studies of NPs across BCECs monolayer

The results of transport studies of DGL/DNA and DGL-PEG-Leptin30/DNA NPs were shown in Fig. 6A. $P_{\text{app}}$ of DGL-PEG-Leptin30/DNA NPs was significantly higher than that of DGL/DNA and DGL-PEG/DNA NPs after 10 min. The transendothelial electrical resistance (TEER) showed no significant difference from that of controls (data not shown). The permeability of 14C-sucrose of BCECs monolayers (Fig. 6B) measured by $PS_{\text{app}}$ was $7.02 \times 10^{-3}$ cm/min, $5.84 \times 10^{-3}$ cm/min and $5.35 \times 10^{-3}$ cm/min for DGL/DNA, DGL-PEG/DNA and DGL-PEG-Leptin30/DNA NPs respectively, which was close to that reported by Pardridge [35]. The above results verified the integrity of BCECs monolayer during the experiments.

3.7. In vitro gene transfection

The transfection efficiency mediated by DGL series vector/DNA NPs was assessed in BV-2 cells. Fig. 7 gave a qualitative comparison of the DGL series vector/DNA NPs as well as positive control lipofectamine2000. The RFP expression of DGL-PEG-Leptin30/DNA and lipofectamine2000 was much higher that of DGL/DNA and DGL-PEG/DNA NPs. The luciferase activity of DGL-PEG-Leptin30/DNA and lipofectamine2000 was 4-fold higher than that of DGL/DNA and DGL-PEG/DNA NPs.

3.8. In vitro cytotoxicity

The cytotoxicity of the three DGL series vector/DNA NPs at different concentrations was evaluated in BCECs and BV-2 cells by MTT assay. Lipofectamine2000 was used as positive control. As shown in Fig. 9A, the cytotoxicity of the three NPs at low, medium, high concentration in BCECs was similar to that of lipofectamine2000 which was thought to be safe enough in cells. Meanwhile the cell viability in BV-2 cells at both 2 h and 48 h showed no significant difference from that of lipofectamine2000 (Fig. 9B and C). Fig. 9B revealed that the PEGylation and/or brain-targeting ligand modification had improved the cell viability slightly.

3.9. In vivo imaging of mice administrated NPs

Nude mice were injected with the EMA-labeled DGL-PEG-Leptin30/DNA NPs, and DGL/DNA NPs as control. In vivo fluorescent images were taken at 80 min after injection. As shown
Fig. 4. Cellular uptake of DGL/DNA (A, D, G, J, M, P), DGL-PEG/DNA (B, E, H, K, N, Q) and DGL-PEG-Leptin30/DNA (C, F, I, L, O, R) NPs at 4°C (D–F) and with different inhibitors (G–R) was examined by fluorescent microscopy after a 60-min incubation. BCECs were treated with different inhibitors including free leptin30 peptide (G–I), transferrin (J–L), filipin (M–O) and colchicine (P–R). A–C was control without any inhibition. Red: EMA-labeled DNA. Original magnification: ×200.
in Fig. 10, EMA-labeled DNA was obviously accumulated in brain in the mice treated with the DGL-PEG-Leptin30/DNA NPs (Fig. 10B). While, the fluorescence in the brain of the DGL/DNA NPs treated mice was not so significant (Fig. 10A). Fig. 10 inset shows that the brain uptake of DGL-PEG-leptin30/DNA NPs (right) was relatively higher than that of DGL/DNA NPs (left).

3.10. Qualitative analysis of distribution of gene expression in the mouse brain

RFP expression in the cortical layer, hippocampus, caudate putamen and substantia nigra at 48 h after administrated DGL/DNA, DGL-PEG/DNA, or DGL-PEG-leptin30/DNA NPs were shown in Fig. 11A–L. The RFP expression in the four regions had nearly no difference between DGL/DNA and DGL-PEG/DNA NPs (Fig. 11A–H). For the DGL-PEG-leptin30/DNA NPs, gene expression was observed in all the four regions (Fig. 11I–L) and was much higher than that of the DGL/DNA and DGL-PEG/DNA NPs, especially in hippocampus and substantia nigra.

3.11. In vivo gene quantitative expression

The transfection efficiencies of DGL/pGL2, DGL-PEG/pGL2, and DGL-PEG-Leptin30/pGL2 NPs in the principal organs were measured after 48 h (Fig. 12). The luciferase expression of the DGL-PEG-Leptin30/pGL2 NPs in the brain was 1160 units/mg protein, about 1-fold higher than that of the DGL/pGL2 NPs (639 units/mg protein) and DGL-PEG/pGL2 NPs (715 units/mg protein) (Fig. 12A).

The luciferase expression of DGL/DNA NPs was higher in heart and lower in kidney than that of DGL-PEG/DNA, DGL-PEG-Leptin30/DNA NPs. In spleen, the luciferase expression is similar for DGL/DNA and DGL-PEG/DNA NPs, whereas the expression levels of liver and lung were not changed markedly (Fig. 12B).
4. Discussion

Synthetic dendrimers have been successfully applied as non-viral gene vectors. Meanwhile, PEGylation and targeting ligand modification render them better biocompatibility and tissue-selectivity. Diagnostic and therapeutic gene could be delivered into brain by the specific binding between brain-targeting ligands and receptors on BBB mediated transcytosis pathway. As a result, brain-targeting gene delivery systems based on dendrimers especially

Fig. 6. (A) The permeability of $^{125}$I-labeled NPs across BCECs monolayers at various incubation times. Results are reported as mean ± S.E.M (n = 4). *p < 0.05; **p < 0.01; ***p < 0.001, significance represents DGL-PEG-Leptin30/DNA NPs vs. other two groups. (B) $^{14}$C-sucrose volume cleared across BCECs monolayers in continuous culture plotted vs. time of transport at 37 °C with NPs. Results are reported as mean ± S.E.M (n = 4).

Fig. 7. The qualitative evaluation of gene expression in vitro. The fluorescence images of RFP expression in BV-2 cells were taken 48 h post-infection with DGL/DNA (A), DGL-PEG/DNA (B), DGL-PEG-Leptin30/DNA NPs (C) and Lipofectamine2000/DNA (D) NPs. Red: RFP. Original magnification: ×200.

Fig. 8. The quantitative evaluation of gene expression in vitro. Luciferase activity was measured 48 h post-transfection and expressed as light units per mg pro. ***p < 0.001, compared with the DGL/DNA and DGL-PEG/DNA NPs. Data represent the mean ± S.E.M (n = 6).
that could be degraded in vivo hold great promise for the further experimental or clinical applications.

The purpose of this work was to evaluate the brain-targeting efficiency of the leptin30 modified gene delivery system. The DGL−PEG−Leptin30 was proved to be synthesized successfully (Fig. 1) and complexed with plasmid DNA by electrostatic interactions (Fig. 2), yielding nano-scaled, positively charged nanoparticles (NPs).

**Fig. 9.** Cell viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay performed 2 h in BCECs (A) and BV-2 cells (B) or 48 h in BV-2 cells (C) after transfection of DGL/DNA, DGL-PEG/DNA, DGL-PEG-leptin30/DNA NPs at different concentrations compared with lipofectamine2000 (n = 3).
Three factors might influence the cellular uptake of DGL–PEG–Leptin30/DNA NPs in BCECs, namely surface charge, PEGylation and brain-targeting ligand leptin30 modification. The cellular uptake efficiency increased with the degree of PEGylation and brain-targeting ligand modification on the DGL–PEG–Leptin30/DNA NPs from none to median (Fig. 3A–C). However, the uptake efficiency changed slightly between the NPs with median and high (Fig. 3C and D) modification of the PEGylation and brain-targeting ligand. It was possible that no significant difference in uptake between the median and high brain-targeting modification NPs. The similar results were observed in a folate-decorated drug delivery system [34].

The cellular uptake of all the three NPs could be inhibited at 4 °C (Fig. 4D–F) indicating the endocytosis was an energy-dependent process. The endocytosis mechanism of NPs was preliminary investigated. The uptake of DGL–PEG–Leptin30/DNA NPs was dependent with leptin30 specifically and inhibited by the excess free leptin30, which was different from the other two NPs (Fig. 4). And the $K_d$ of DGL–PEG–Leptin30 binding to BCECs determined in this study (Fig. 5) was lower than of leptin reported by Pardridge [35]. In addition, the maximum binding of DGL–PEG–Leptin30 enhanced compared with that of leptin. This difference may attribute to the adsorptive effect between positively charged DGL and negatively charged cell membrane (Fig. 5). It may also be due to the
short peptide derived from longer peptide exhibits higher receptor affinity than the original peptides [36].

The above evidence suggested the combined cellular internalization promoting effect by the DGL polymer and leptin30 ligand. The cellular uptake of DGL—PEG—Leptin30/DNA NPs could be inhibited by transferrin which was thought to be the typical marker binding cholesterol, an important component for caveolae formation [40]. The cellular internalization appears to be also mediated by surface charge, PEGylation and leptin30 modifications [17], hence possess better biocompatibility due to the peptide-like nature of the polymer [41]. The uptake of DGL/DNA NPs was mediated by caveolae mediated endocytosis and macropinocytosis. The similar concepts were mentioned as macropinocytosis inhibitor [42]. The uptake of DGL/DNA NPs was mediated by caveolae mediated endocytosis and macropinocytosis. The similar concepts were mentioned as macropinocytosis inhibitor [43].

DGL—PEG—Leptin30/DNA NPs endocytosis had relation to all the three endocytosis pathway. It confirmed that the ligand and the polymer both contribute to the cellular internalization of the NPs. In addition, the confocal analysis of BCECs incubated with DGL/DNA NPs indicated that EMA-labeled NPs could escape from endosomes effectively at 1 h after incubation (data not shown).

DGL—PEG—Leptin30/DNA NPs could be transported across in vitro BBB model consisted of BCECs monolayers more effectively than DGL—PEG/DNA and DGL/DNA NPs because the permeability of DGL—PEG—Leptin30/DNA NPs was significantly higher than that of DGL—PEG/DNA and DGL/DNA NPs from 10 min after incubation (Fig. 6A). While transendothelial electrical resistance (TEER) and permeability of 14C-sucrose (Fig. 6B) demonstrated that the integrity remained during the whole incubation process [35]. It could be deduced the specific binding between leptin30 and leptin receptors played an active role in the transcytosis of NPs.

In vitro transfection efficiency was evaluated in BV-2 microglia cells. BV-2 cells as well as many other brain cells were reported to expressed the long (OBRl) and short (OBRs) isoforms of the leptin receptors [29]. Both qualitative (Fig. 7) and quantitative (Fig. 8) results revealed the transfection ability of DGL—PEG—Leptin30/DNA NPs was higher than that of DGL/DNA and DGL—PEG/DNA NPs while equal to that of lipofectamine2000. It could be also explained that leptin30 could bind leptin receptors on BV-2 cells and be internalized by receptor mediated endocytosis. The DNA accumulation of DGL—PEG—Leptin30/DNA NPs was found higher than that of DGL/DNA NPs in brain after 80 min (Fig. 10). The report gene expression at different regions in brain (cortical layer, hippocampus, caudate putamen and substantia nigra) has clearly revealed the preferential localization of DGL—PEG—Leptin30/DNA NPs in the brain when compared with other two controls (Fig. 11). The transfection efficiency in vivo was in accordance to the results in BV-2 cells. A relative high report gene expression also showed qualitatively in brain (Fig. 12A). The in vivo transfection ability declined slightly compared with the in vitro results (Figs. 7 and 8).

The reasons may be the specific binding between targeting DGL—PEG—Leptin30/DNA NPs and leptin receptors on BBB was interfered by endogenic leptin. It is reported the plasma level of leptin in normal mice is about 280 nm [44] and doesn't change acutely with food administration [45], while the plasma level of leptin30 modified NPs was estimated no more than 5000 nm in this study. The targeting modification of NPs was sufficient enough to exert their targeting effects. This result was in agreement with the cellular uptake comparison result which demonstrated the uptake efficiency altered slightly as the targeting modification increasing from median to high. The accumulation in heart may be caused by the property of DGL but not relate to the modification of Leptin30 since the report gene expressions were higher in DGL/DNA NPs administration group than that in DGL—PEG/DNA and DGL—PEG—Leptin30/DNA NPs administration groups (Fig. 12B). The similar results were observed in gene delivery systems based on PAMAM with different modification [12,13]. It is worth optimizing the charge ratio N/P to achieve the best in vivo transfection efficiency further.

The cytotoxicity of the three series vector/DNA NPs at 2 h and 48 h in BCECs and BV-2 cells was relatively low at all the concentrations tested, while lipofectamine2000 didn't show much cytotoxicity in this experiment (Fig. 9). Compared with some synthetic polymers, DGL was thought to be less toxic and biodegradable [17], hence possess better biocompatibility due to the peptide-like linkage between the monomers. And the accumulative or long-term toxicity was relatively low. The above results further indicate the potent brain-targeting ability of leptin30 modified PEGylated DGL as the efficient gene vector.

5. Conclusions

DGL—PEG—Leptin30 was synthesized and complexed with plasmid DNA yielding nanoparticles. The mechanism of DGL—PEG—Leptin30/DNA NPs mediated brain-targeting is affected by surface charge, PEGylation and leptin30 modification and could be characterized as a clathrin and caveolae mediated energy-dependent endocytosis. Compared with in vitro transfection results...
in vivo transfection ability may be interfered by endogenic leptin slightly, the DGL—PEG—Leptin30/DNA NPs were demonstrated to be efficient enough for brain-targeting gene delivery. It could be believed that DGL—PEG—Leptin30 holds great promise as the non-viral vector for relatively safe and efficient brain-targeting gene delivery.

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Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figs. 3, 4, 7, 10 and 11 are difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2010.03.011.

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