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RESEARCH PAPER

Angiopep-Conjugated Nanoparticles for Targeted Long-Term Gene Therapy of Parkinson's Disease

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ABSTRACT

Purpose To prepare an angiopep-conjugated dendrigraft poly-L-lysine (DGL)-based gene delivery system and evaluate the neuroprotective effects in the rotenone-induced chronic model of Parkinson's disease (PD).

Methods Angiopep was applied as a ligand specifically binding to low-density lipoprotein receptor-related protein (LRP) which is overexpressed on blood-brain barrier (BBB), and conjugated to biodegradable DGL via hydrophilic polyethyleneglycol (PEG), yielding DGL-PEG-angiopep (DPA). *In vitro* characterization was carried out. The neuroprotective effects were evaluated in a chronic parkinsonian model induced by rotenone using a regimen of multiple dosing intravenous administrations.

Results The successful synthesis of DPA was demonstrated via ^{1}H-NMR. After encapsulating the therapeutic gene encoding human glial cell line-derived neurotrophic factor (*hGDNF*), DPA/*hGDNF* NPs showed a sphere-like shape with the size of 119 ± 12 nm and zeta potential of 8.2 ± 0.7 mV. Angiopep-conjugated NPs exhibited higher cellular uptake and gene expression in brain cells compared to unmodified counterpart. The pharmacodynamic results showed that rats in the group with five injections of DPA/*hGDNF* NPs obtained best improved locomotor activity and apparent recovery of dopaminergic neurons compared to those in other groups.

Conclusion This work provides a practical non-viral gene vector for long-term gene therapy of chronic neurodegenerative disorders.

KEY WORDS angiopep-conjugated nanoparticles · dendrigraft poly-L-lysine · gene therapy · multiple dosing administrations · Parkinson's disease

ABBREVIATIONS

BBB	blood-brain barrier
BCECs	brain capillary endothelial cells
DGL	dendrigraft poly-L-lysine
DPA	DGL-PEG-angiopep
GFP	green fluorenscent protein
hGDNF	the gene encoding human glial cell line-derived neurotrophic factor
LRP	low-density lipoprotein receptor-related protein
NMR	nuclear magnetic resonance
NPs	nanoparticles
PAMAM	polyamidoamine
PBS	phosphate-buffered solution
PD	Parkinson's disease
PEG	polyethylene glycol
PPA	PAMAM-PEG-angiopep
TH	tyrosine hydroxylase

INTRODUCTION

Parkinson's disease (PD) is considered as a chronic and progressive neurodegenerative disorder, the major cause of

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which is addressed as selective loss of dopamine in nitrostriatal system. Because of the long course, PD requires long-term therapy. However, currently existing therapeutic options in clinic such as oral L-dopa are limited to compensating for the loss of dopamine and recovering only the symptoms (1). Furthermore, long-term therapy of L-dopa gradually loses its effectiveness as dopaminergic neurons are lost during PD progression, and it is associated with a series of side effects, including the on/off motor effect (1,2). Therefore, it is of great need to develop advanced therapy not only redress the fundamental causes of PD but also reduce the adverse effects of current treatments.

Gene therapy is now a promising approach for PD treatment (3). Gene therapy has the potential to transport therapeutic genes to the diseased region, and then the expressed products could maintain the healthy neurons or repair degenerated ones (4). This might slow or reverse the progressive course of PD fundamentally. Non-viral gene therapy now attracts researchers' attention due to some advantages compared to viral gene therapy. For example, it is less invasive and practical for repetitive or continuous treatments. Most importantly, non-viral gene vectors are able to cross the blood-brain barrier (BBB) via transvascular administration, which significantly limits the application of viral vectors (5). However, how to effectively cross the BBB and mediate gene expression in the target region still remains a tough problem for non-viral gene therapy of PD.

Receptor-mediated gene delivery has now a research focus for PD treatment. It has been reported that low-density lipoprotein receptor-related protein (LRP) is present on the luminal endothelial plasma membranes and could mediate the transport of its ligands across the BBB (6). In previous work, angiopep has been demonstrated as an efficient ligand to LRP, which possesses significantly higher brain penetration capability than other brain-targeting ligands including transferrin (7,8). Angiopep is a small peptide with 20 amino acids, and could be chemically synthesized (8). It is more stable than protein ligands for *in vivo* application. Thus in this study, angiopep was applied as the targeting ligand to mediate the BBB transport and cellular internalization of non-viral gene vectors.

On the other hand, dendrigraft poly-L-lysine (DGL) was applied as the main vector in this work. DGL is a kind of cationic, biodegradable, monodispersed, and well-defined poly-L-lysine-based dendrimer, which was induced to the gene delivery field for the first time in our lab (9). DGL-based gene vectors showed higher gene transfection efficiency and lower cytotoxicity compared to currently widely used polyamidoamine (PAMAM)-based counterparts (9). Thus DGL-based polymers have been exploited as non-viral vectors in gene therapy of tumors such as glioma (10).

In this paper, angiopep was conjugated to DGL via hydrophilic polyethylene glycol (PEG), constructing DGL-PEG-angiopep (DPA). And, the gene encoding human glial cell line-derived neurotrophic factor (*hGDNF*) was exploited as the therapeutic gene to be encapsulated in DPA. A regimen of multiple dosing administrations of DPA/*hGDNF* NPs was carefully designed, and the neuroprotective effects were evaluated in the rotenone-induced chronic parkinsonian model.

MATERIALS AND METHODS

Materials

Dendrigraft poly-L-lysine (DGL) [generation = 3, containing 123 primary amino groups] was purchased from COLCOM (Montpellier Cedex, France). Polyamidoamine (PAMAM) dendrimer [generation = 5, containing 128 primary amino groups] was purchased from DENDRITECH (Midland, MI, U.S.A.). α-Malemidyl-ω-*N*-hydroxysuccinimidyl polyethyleneglycol (MAL-PEG-NHS, MW 3500) was obtained from JenKem Technology Co., Ltd (Beijing, China). Angiopep with a sequence of TFFYGGSRGKRNNFKTEEYC was synthesized by Chines Peptide Company (Hangzhou, China). The fluorescent dyes, green BODIPY (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-*s*-Indacene-3-Pentanoic Acid, Succinimidyl Ester), and red BODIPY (6-(((4,4-Difluoro-5-(2-Pyrrolyl)-4-Bora-3a,4a-Diaza-*s*-Indacene-3-yl)Styryloxy) Acetyl)Aminohexanoic Acid, Succinimidyl Ester), were purchased from Molecular Probes (Eugene, OR, U.S.A). Anti-tyrosine hydroxylase (TH), clone LNC1, was purchased from Chemicon (Temecula, CA, U.S.A.). The therapeutic plasmid DNA (pDNA), pEF-Bos-*hGDNF* (5.9 kb) coding for hGDNF, was a gift from Prof. Saarma Mart (University of Helsinki). The plasmid pEGFP-N2 coding green fluorenscent protein (GFP) (Clontech, U.S.A) and pEF-Bos-*hGDNF* were purified by using QIAGEN Plasmid Mega Kit (Qiagen GmbH, Germany). Rotenone, and other relating reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Animals

Sprague-Dawley rats, male, weighing 250-300 g (purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences) were housed under standard laboratory conditions. They were allowed free access to food and water during the experiments. The rats were kept in a 12-h dark/light environment. All animal experiments were carried out in accordance with the guidelines evaluated and approved by the ethics committee of Fudan University.

Synthesis of DGL-based Vectors

First, DGL was dried under a nitrogen atmosphere to remove methanol. At the same time, PEG was dissolved in phosphate-buffered solution (PBS, pH 8.0). The specific

reaction between the primary amino groups on the surface of DGL and the NHS groups of the bifunctional PEG was performed at room temperature for 2 h. The resulting conjugate (DGL-PEG, DP) was purified by ultrafiltration using a 10-kDa molecular weight cutoff membrane to remove unreacted PEG and the buffer was exchanged into PBS (pH 7.0). Then the MAL groups on the terminal of DP were reacted with the thiol groups of angiopep for 24 h at room temperature, yielding DGL-PEG-angiopep (DPA). For the synthesis of BODIPY-labeled vectors, DGL was first reacted with BODIPY in 100 mM $NaHCO_3$ for 12 h at 4°C, and purified by ultrafiltration using a 5-kDa molecular weight cutoff membrane to remove unreacted BODIPY. PAMAM possesses similar features with DGL, thus PAMAM-PEG-angiopep (PPA) was synthesized in similar process with DPA.

^{1}H-NMR Analysis of DPA

The characteristic of DPA was analyzed by nuclear magnetic resonance (NMR) spectroscopy. DPA was dissolved in D_2O and analyzed in a 400 MHz spectrometer (Varian, U.S.A.).

Preparation of Different Nanoparticles

Vectors (DP, DPA and PPA) were freshly prepared and diluted to appropriate concentrations in distilled water. pDNA solution (100 μg pDNA/mL 50 mM sodium sulfate solution) was added to obtain specified weight ratios (DGL to DNA or PAMAM to DNA, *w*/*w*) and immediately vortexed for 30 s at room temperature. Reporter gene (*GFP*) and the therapeutic gene (*hGDNF*) were used to prepare different nanoparticles (NPs), according to different purposes. PPA/DNA NPs were used as positive control in this work. All used NPs were freshly prepared in the following experiments.

Gel Retardation Assay

Agarose gel retardation assay was carried out to determine the *hGDNF* binding ability of DPA. DPA/*hGDNF* NPs were prepared at various weight ratios (DGL to pDNA, w/w) ranging from 0.05:1 to 10:1. NPs were mixed with appropriate amounts of 6×loading buffer and then electrophoresced on a 0.7% (*w*/*v*) agarose gel containing ethidium bromide (0.25 μg/ml of the gel). The location of *hGDNF* in the gel was analyzed on a UV illuminator and photographed using a Canon IXUS 950IS camera.

Characterization of DPA/hGDNF NPs

The mean diameter and zeta potential of DPA/*hGDNF* NPs with a DGL to *hGDNF* weight ratio at 3:1 were determined by dynamic light scattering by Zeta Sizer Nano (Malvern, UK). And also, the size and morphology of DPA/*hGDNF* NPs were examined under a high-resolution transmission electron microscope (JEM-2010, JEOL, Japan).

Cell Culture

Brain capillary endothelial cells (BCECs) were cultured as described previously (11). Briefly, BCECs were maintained in special Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, U.S.A.) supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 μg/ml epidermal cell growth factor (ECGF), 2 mM L-glutamine, 20 μg/ml heparin, 40 μU/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under a humidified atmosphere containing 5% CO_2. Human neuroblastoma cells (SH-SY5Y) were purchased from Shanghai Cell Bank, Chinese Academy of Medical Sciences. SH-SY5Y cells were cultured in RPMI 1640 medium (Gibco, Tulsa, OK) containing with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (12). Cells were grown at 37°C under a humidified atmosphere containing 5% CO_2.

Cellular Uptake at Different Concentrations

BCECs were seeded at a density of 2×10^4 cells/well in 24-well plates (Corning-Coaster, Tokyo, Japan), incubated for 72 h, and checked under the microscope for confluency and morphology. Following this, BCECs were incubated with green BODIPY-labeled DPA/*GFP* in a vector concentration range of 0.5 to 20 μM for 30 min. The cells were washed 3 times with PBS (pH 7.4), visualized and photographed under a DMI4000B fluorescent microscope (Leica, Germany).

Cellular Uptake with Different NPs

BCECs were seeded at a density of 2×10^4 cells/well in 24-well plates, cultured for 72 h, and checked under the microscope for confluency and morphology. After that, BCECs were incubated with red BODIPY-labeled DP/*GFP*, DPA/*GFP* and PPA/*GFP* in a vector concentration at 5 μM for 30 min. The cells were washed 3 times with PBS (pH 7.4), visualized and photographed under a DMI4000B fluorescent microscope (Leica, Germany).

Gene Expression with Different NPs

SH-SY5Y cells were seeded in 24-well plates at a density of 1×10^4 cells/well. Cultured for 48 h, the cells reached 70% confluence. After that, different NPs (DP/*GFP*, DPA/*GFP* and PPA/*GFP*), with DGL to DNA or PAMAM to DNA weight ratio at 3:1, were added to the cells in FCS-free

medium and the mixture was incubated at 37°C for 30 min. Then the cells were washed 3 times with PBS (pH 7.4). After 2 days, GFP expression was visualized and photographed under a DMI4000B fluorescent microscope (Leica, Germany).

Treatments and Experimental Groups

Rotenone was emulsified in corn oil with a concentration of 1.25 mg/ml and then was intraperitoneally injected to rats once a day at 2.5 mg/kg for 45 days. The therapeutic effect of NPs loading *hGDNF* was investigated focusing on the lesion of rotenone on nigrostriatal system. And animals were monitored for weight every 2 days and signs of motor impairments throughout the study. Six groups of rats (n= 15 in each group) were categorized as follows. For group 1, rats were injected with corn oil only every day without any therapy, designed as the blank control. For group 2, rats were injected with rotenone every day and treated with five injections of DPA/*GFP* every other day from 35th day, designed as the negative control. For group 3, rats were injected with rotenone every day and treated with one injection of DP/*hGDNF* at 43th day. For group 4, rats were injected with rotenone every day and treated with one injection of DPA/*hGDNF* at 43th day. For group 5, rats were injected with rotenone every day and treated with triple injections of DPA/*hGDNF* every other day from 39th day. And for group 6, rats were injected with rotenone every day and treated with five injections of DPA/*hGDNF* every other day from 35th day. The dose for one injection was 200 μg pDNA per rat in a total volume of 500 μl solution at a DGL to pDNA weight ratio at 3:1. The design was exhibited in Table I in detail.

Behavioral Tests (Open-Field Test)

Each rat was placed in an open field (80 cm×48 cm, with a 50 cm wall around), the floor of which was divided into 15 equal-sized squares (16 cm×16 cm). The time period to observe the movement and behavior of rats were 5 min. Five parameters were recorded including line crossing (number of lines crossed), rearing (number of rears), head dipping (number of dips), defecating (number of faces) and inactive sitting (time in seconds). Locomotor activity was recorded on the 15th, 25th, 35th and 45th day from the beginning of rotenone injection. Evaluation was done by an investigator unaware of the treatments.

TH Immunohistochemistry

The detailed process for TH immunohistochemistry was shown previously (4). Briefly, on specified days, rats were anaesthetized and perfused transcardially. The brains were rapidly removed and postfixed for 24 h, then transferred to PBS containing 30% sucrose at 4°C until subsidence. Coronal brain sections were made at a thickness of 30 μm and processed for TH immunohistochemical staining to examine the extent of dopaminergic neuronal loss. Finally, sections were visualized under a Canon IXUS 950IS camera (striatum) and a LEICA DMI4000B microscope (substantia nigra, SN). For quantitative evaluation, TH-positive cells were counted on both lesioned side and intact side of SN to determine the extent of neuron recovery as described previously (13).

Statistical Analysis

All data were expressed as mean±standard error of the mean (S.E.M.). Statistical analysis was performed by one-

Table I The Regimen of Multiple Dosing Intravenous Administrations of Rats

Days	0 •••	15 •••	25 •••	35	37	39	41	43	45
Group I (oil)		BT	BT	BT					BT,IH
Group 2 (DPA/*GFP*)		BT	BT	BT	💉	💉	💉	💉	BT,IH
Group 3 (DP/*hGDNF*)		BT	BT	BT				💉	BT,IH
Group 4 (DPA/*hGDNF*)		BT	BT	BT				💉	BT,IH
Group 5 (DPA/*hGDNF*)		BT	BT	BT		💉	💉	💉	BT,IH
Group 6 (DPA/*hGDNF*)		BT	BT	BT	💉	💉	💉	💉	BT,IH

💉 represents one injection of corresponding NPs; BT, behavioral test; IH, immunohistochemistry

way ANOVA followed by Student-Newman-Keuls post hoc test, using GraphPad InStat software. Statistical significance was defined as $p<0.05$.

RESULTS

Characterization of DPA and DPA/hGDNF NPs

The result of NMR spectra was shown in Fig. 1a. Around 4.65 ppm appeared the solvent peak of D_2O. Peaks of H protons of $3CH_2$ in lysine units were observed between 1 and 1.8 ppm. The peak around 2.8 ppm represented H protons of $1CH_2$ epsilon, while that around 4.2 ppm was representative of H protons on the chiral C in lysine units. The sharp peak at 3.5 ppm represented the repeat units of PEG, indicating the existence of PEG (9). The disappearance of the characteristic peak of the MAL group at 6.7 ppm demonstrated the reaction between MAL group in PEG and thiol group in angiopep (14). Also, the short peaks between 2.9 and 3.5 ppm indicated the existence of angiopep. The NMR result proved the successful synthesis of DPA.

The result of gel retardation assay was shown in Fig. 1b, demonstrating that the *hGDNF* could be completely packed by DPA at a DGL to *hGDNF* weight ratio greater than 1, since the *hGDNF* could not be intercalated by ethidium bromide anymore. When the DGL to *hGDNF* weight ratio was fixed at 3:1 in this work, *hGDNF* could be effectively incorporated in DPA.

The mean diameter of DPA/*hGDNF* NPs was 119± 12 nm. And the zeta potential was 8.2±0.7 mV. The TEM image showed that DPA/*hGDNF* NPs had compact structure with a sphere-like shape (Fig. 1c).

Cellular Uptake Studies

The uptake of DPA/*GFP* NPs in BCECs exhibited a concentration-dependent manner (Fig. 2). Images were taken under a fluorescent microscope with similar cell density. When the concentration reached 5 μM, the cellular uptake was sufficient for observation and cells maintained good morphology. The fluorescence increased with the concentration of NPs, and at the same time, cells gradually turned round (data not shown). Thus 5 μM was used in the targeting ability evaluation study. As shown in Fig. 3, the angiopep-modified NPs significantly enhanced the cellular uptake compared to unmodified counterpart, indicating the targeting effect of angiopep. The cellular uptake of DPA/*GFP* NPs was higher

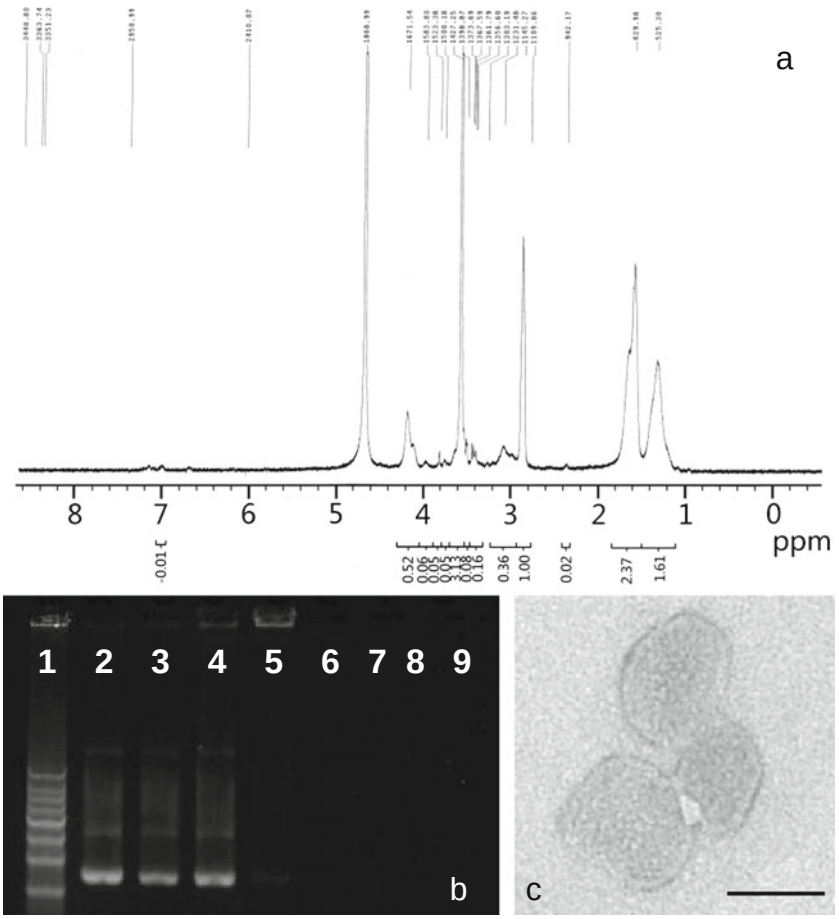

Fig. 1 Characterization of DPA vector and DPA/DNA NPs. (**a**) H^1-NMR spectra of DPA in D_2O at 400 MHz. (**b**) Agarose gel retardation assay of DPA/DNA NPs with different DGL to pDNA weigh ratios. Lane 1, DNA Marker, Hind III digested; lane 2, pDNA alone; lane 3, 0.05:1 (*w/w*); lane 4, 0.1:1 (*w/w*); lane 5, 0.5:1 (*w/w*); lane 6, 1:1 (*w/w*); lane 7, 2:1 (*w/w*); lane 8, 5:1 (*w/w*), and lane 9, 10:1 (*w/w*). **c**. TEM image of DPA/DNA NPs. Bar = 100 nm.

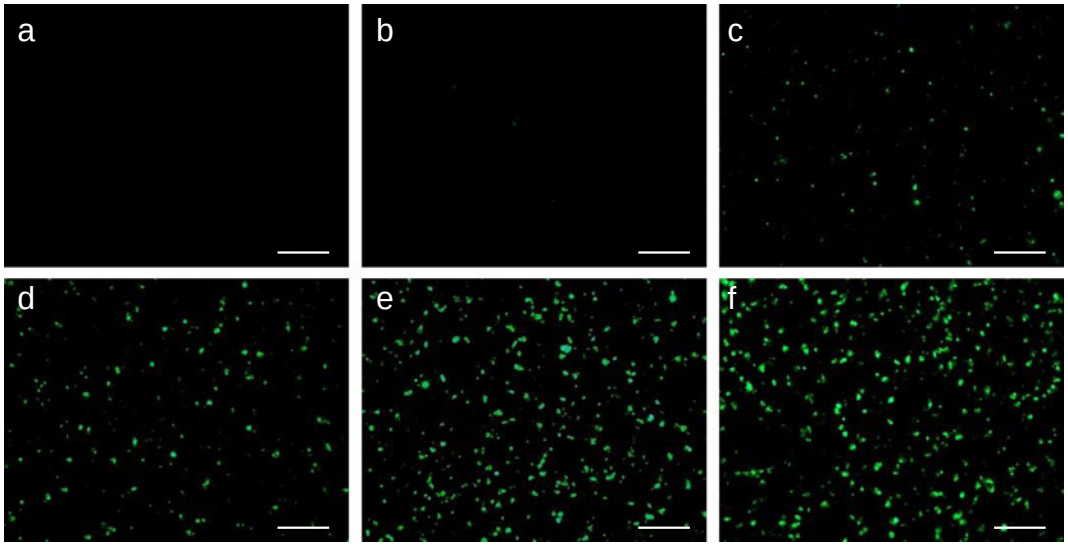

Fig. 2 Cellular uptake of green BODIPY-labeled DPA/*GFP* in the vector concentration of 0.5 μM (**a**), 1 μM (**b**), 2 μM (**c**), 5 μM (**d**), 10 μM (**e**) and 20 μM (**f**), on BCECs after a 30-min incubation. Results were performed as fluorescent microscopy images. Bar = 100 μm.

than PPA/*GFP* positive control, showing the advantage of DGL.

Gene Expression

The gene expression result shown in Fig. 4 was consistent with cellular uptake result (Fig. 3). The highest GFP expression was obtained when SH-SY5Y cells were transfected with DPA/*GFP* NPs, further demonstrating the positive effects of both angiopep and DGL.

Body Weight Measurement

Rats in model groups maintained the body weight when injected with rotenone daily and before any therapeutic treatment (Fig. 5). Rats in therapeutic groups via multiple dosing of DPA/*hGDNF* NPs (group 5 and group 6) exhibited gradually increased body weight, showing the improvement of rats' situation. One dosing of either DP/*hGDNF* or DPA/*hGDNF* NPs could not apparently change the body weight loss. Rats in blank group without rotenone increased body weight in a normal speed, markedly heavier than those in model groups. Monitoring the body weight could reflect the physical conditions of rats in some extent.

Behavioral Observation

Neuroprotective effects were evaluated via the five parameters of behavioral observation. The closer the parameters were observed to rats in blank group, the better locomotor activity was achieved. Regarding the number of lines crossed, there was no apparent difference in the former 35 days between model groups exposure to rotenone, while rats in blank group could cross apparently more lines (Fig. 6a). Similar result could be obtained from inactive sitting evaluation, where rats in model groups exhibited much longer inactive time than those in blank group without rotenone exposure (Fig. 6e), indicating the successful construction of rotenone-induced PD models. After different therapeutic treatments, behavioral data on the 45th day were statistically analyzed. The result showed that treatments of NPs loading *hGDNF* especially multiple dosing of DPA/*hGDNF* NPs (group 5 and group 6) could markedly increase the number of line crossings and decrease the

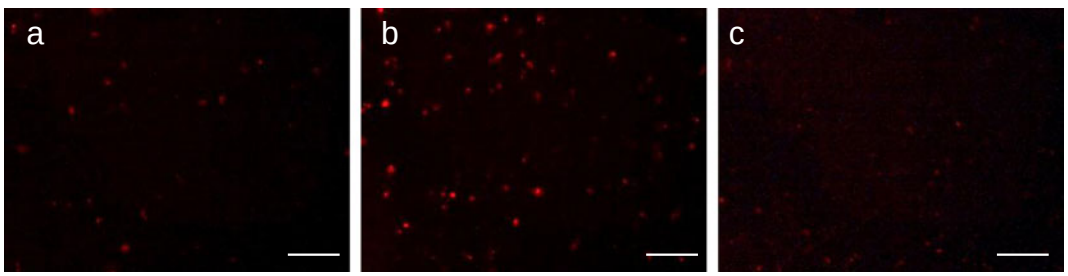

Fig. 3 Cellular uptake of red BODIPY-labeled DP/*GFP* (**a**), DPA/*GFP* (**b**) and PPA/*GFP* (**c**) in the vector concentration of 5 μM on BCECs after a 30-min incubation. Results were performed as fluorescent microscopy images. Bar = 100 μm.

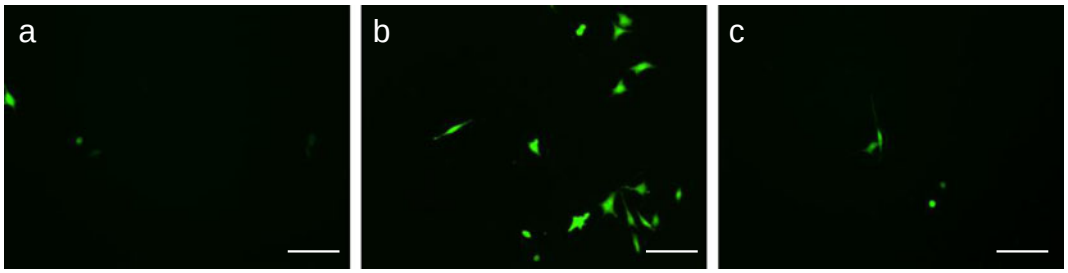

Fig. 4 GFP expression of DP/*GFP* (**a**), DPA/*GFP* (**b**) and PPA/*GFP* (**c**) in the vector concentration of 5 μM on SH-SY5Y cells after a 30-min incubation. Results after 2 days were performed as fluorescent microscopy images. Bar = 100 μm.

inactive time, compared to treatment of NPs loading reporter gene *GFP* (the negative group 2). This suggested the improved locomotor activity of rats after positive therapy. Five injections of DPA/*hGDNF* NPs could achieve best locomotor activity improvement of rats. Moreover, no significant difference was observed in the records of rearing, head dipping and defecating between different groups (Fig. 6b, c and d).

TH Immunohistochemical Staining

The results of TH immunohistochemistry revealed severe loss of dopaminergic neurons in nigrostriatal system in the negative group treated with NPs loading reporter gene *GFP* (Figs. 7b and 8b). Apparent recovery was found in therapeutic groups treated with NPs loading *hGDNF* (Figs. 7 and 8). Increasing the injection number of NPs could decrease the loss of dopaminergic neurons. Five injections of DPA/*hGDNF* NPs (group 6) showed no apparent difference with the oil-treated group (group 1) (Figs. 7f and 8f and g).

DISCUSSION

In this work, angiopep-conjugated DGL-based NPs were successfully synthesized and the neuroprotective effects in the rotenone-induced chronic PD model via multiple dosing intravenous administrations were systematically evaluated.

PD is a chronic neurodegenerative brain disorder which requires long-term therapy. Non-invasive and effective modalities are preferred. One of the tough problems is the existence of BBB, which significantly limits the transport of drugs including gene medicine to diseased areas (15). Effective therapy of PD via non-invasive intravenous administration needs the efficient transport of genes or gene-loaded vectors across the BBB and the following expression of gene products in focus. It has been reported that the presence of LRP on the

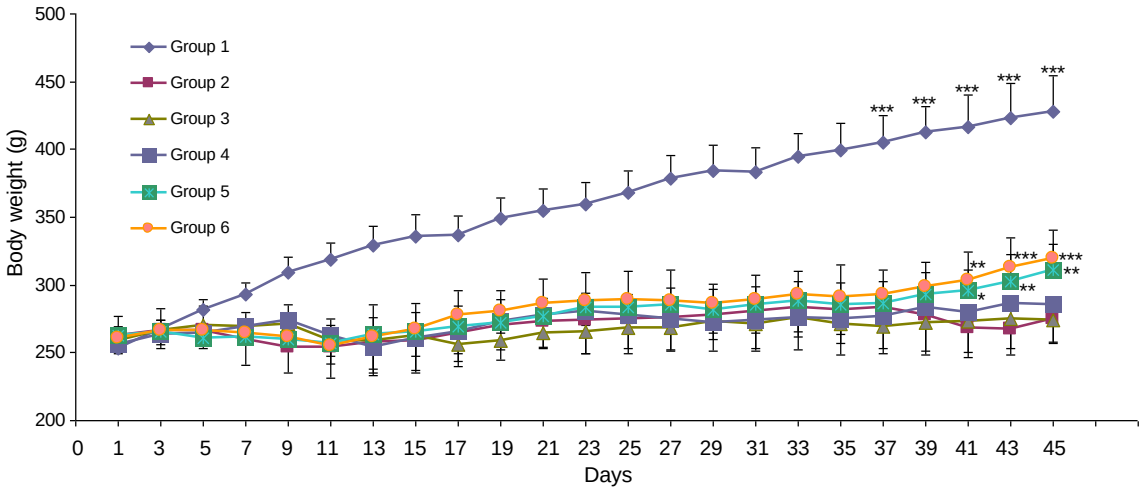

Fig. 5 Body weight monitoring of rats in different groups every 2 days during the 45 days of experiments. Group 1, blank control, rats were treated with corn oil only every day; group 2, negative control, rats were injected with rotenone every day and treated with five injections of DPA/*GFP* every other day from 35th day; group 3, rats were injected with rotenone every day and treated with one injection of DP/*hGDNF* at 43th day; group 4, rats were injected with rotenone every day and treated with one injection of DPA/*hGDNF* at 43th day; group 5, rats were injected with rotenone every day and treated with triple injections of DPA/*hGDNF* every other day from 39th day; group 6, rats were treated with rotenone every day and treated with five injections of DPA/*hGDNF* every other day from 35th day. The dose for one injection was 200 μg pDNA per rat in a total volume of 500 μl solution at a DGL to pDNA weight ratio at 3:1. Significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, significantly different as compared to group 2 after 35th day.

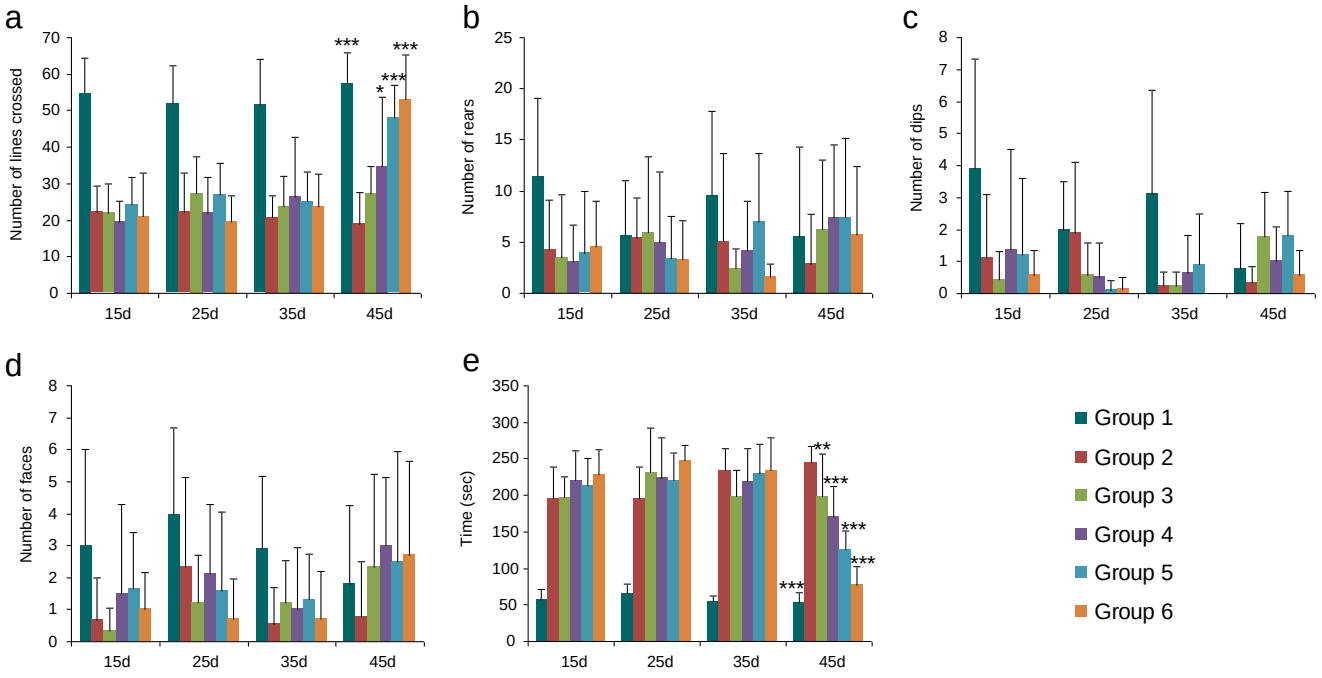

Fig. 6 Behavioral changes of rats in different groups in line crossing (**a**), rearing (**b**), head dipping (**c**), defecating (**d**), and inactive sitting (**e**) on 15th, 25th, 35th and 45th day from the beginning of rotenone injection. Data are expressed as mean ± S.E.M ($n = 8$). Statistical analysis was done with data on the 45th day. Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, significantly different as compared to group 2 (the negative control group).

luminal endothelial plasma membranes make it a potential target for brain targeting drug delivery (6,8). Angiopep, one of ligands to LRP, has been reported to exhibit higher transcytosis capacity and parenchymal accumulation than currently used ligands including transferrin and lactoferrin (7). Furthermore, angiopep has been exploited as an efficient brain targeting ligand in our previous work (8,16). Thus angiopep was used here to conjugate NPs for enhanced BBB-crossing delivery. The other problem is the essentiality of multiple dosing administrations. Till now, it is nearly impossible to treat PD via a single dose. Thus non-invasive modalities are of great demand, among which intravenous administration is an ideal manner. It has been reported that the expression of GDNF within brain via a single dosing of

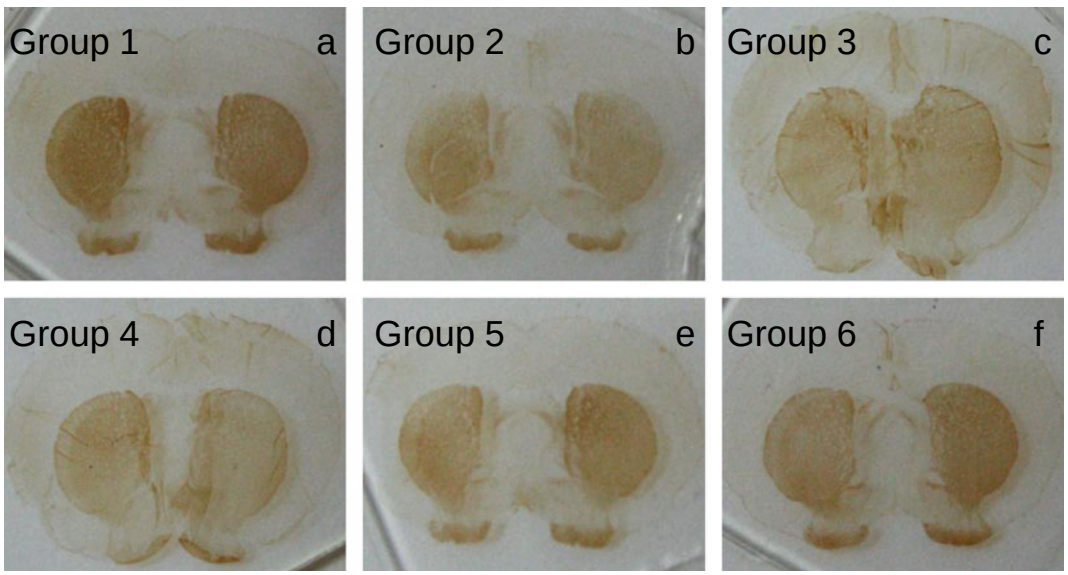

Fig. 7 TH-immunoreactivity in the striatum. Representative immuno-staining sections of rats selected from group 1 (**a**), group 2 (**b**), group 3 (**c**), group 4 (**d**), group 5 (**e**) and group 6 (**f**) were shown on the 45th day from the beginning of rotenone injection. The images showed the striatal dopaminergic terminals.

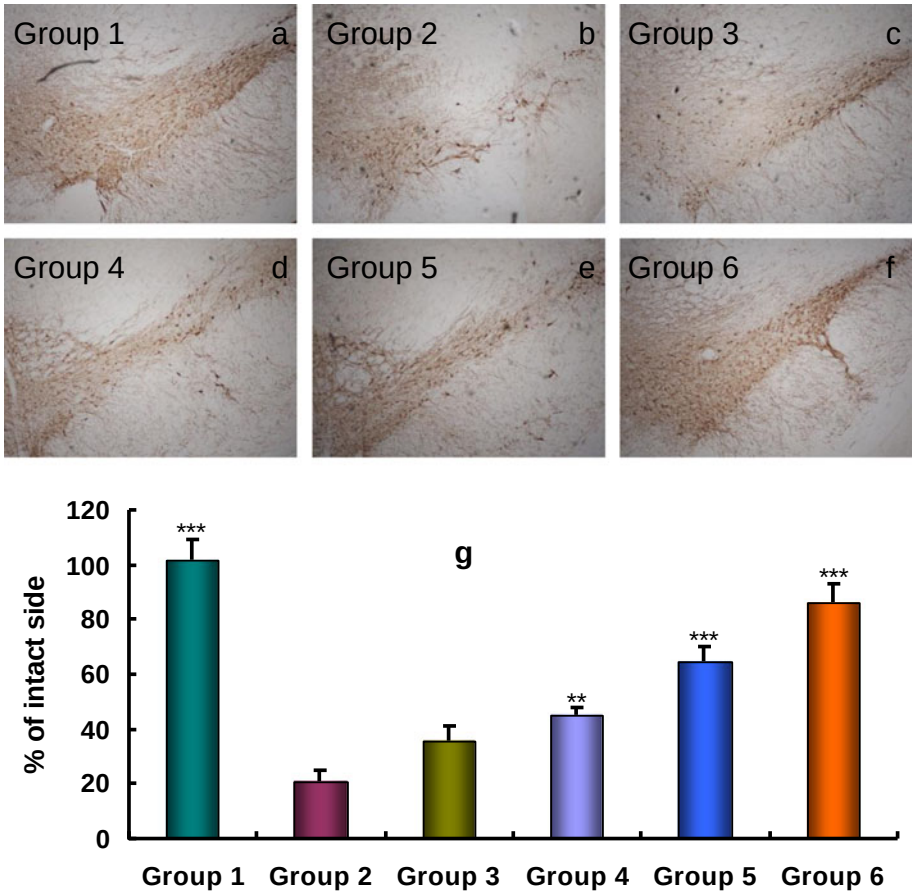

Fig. 8 TH-immunoreactivity in the SN. Representative immunostaining sections of rats selected from group 1 (**a**), group 2 (**b**), group 3 (**c**), group 4 (**d**), group 5 (**e**) and group 6 (**f**) were shown on the 45th day from the beginning of rotenone injection. The images showed the dopaminergic soma in the SN. Original magnification: 100×. The percentage of TH-positive neurons of lesioned side to intact side (**g**) was calculated. Data are expressed as mean ± S.E.M ($n = 6$). Statistical analysis was done with data on the 45th day. Significance: **, $p \leq 0.01$; ***, $p \leq 0.001$, significantly different as compared to group 2.

lactoferrin-modified NPs gradually decreased with time, and increasing the injection number of NPs could enhance GDNF amounts correspondingly (4). The positive effects of multiple dosing administrations were also verified in this work, where rats in group 6 with five injections of DPA/*hGDNF* NPs obtained best improved locomotor activity (Figs. 5 and 6) and apparent recovery of dopaminergic neurons (Figs. 7 and 8). Actually, the effectiveness of multiple dosing administrations has been demonstrated in our lab before (4). Thus multiple dosing administrations of gene-loaded NPs could be applied in long-term gene therapy of chronic PD.

Rotenone-induced parkinsonian rat model was successfully constructed. Rotenone is a widely used pesticide and considered as one important environmental factor in the etiology of PD (17,18). The rotenone-induced chronic model was reported to produce most of the movement disorder symptoms and the histopathological features of PD (19,20). And the lesion of rotenone was gradual and uniform in a global area in the nigrostriatal system, unlike unilateral 6-hydroxydopamine (6-OHDA) rat model which mainly caused local degeneration of neurons (4,21).

The main vector used here is lysine-based DGL, which has been a hot material in gene therapy field recently (22,23). DGL has similar features with commonly used dendrimers such as PAMAM, containing several primary amino groups (9). Thus PAMAM was applied as the positive control in this work. In the same concentration, DGL-based NPs showed higher cellular uptake and gene expression compared to PAMAM-based counterpart (Figs. 3 and 4). On the other hand, DGL possesses some apparent advantages, such as biodegradability and low cytotoxicity (9,24). DGL-based NPs in this study exhibited no apparent cytotoxicity against BCECs and SH-SY5Y cells (data not shown). These properties make DGL a potential dendrimer in the field of gene therapy.

The gene encoding GDNF was chosen as the therapeutic gene in this study. As known, GDNF was firstly identified as a survival factor for midbrain dopaminergic neurons (25). Further studies demonstrated that GDNF is a kind of trophic factor not only for neurons but also for peripheral organs (26). Thus, the expression of GDNF in peripheral organs including liver and spleen would not greatly affect the function of normal tissues. Moreover, GDNF is a secretive protein (27). That means GDNF could be secreted out of cells via autocrine or paracrine pattern, then bind specifically with its receptors and stimulate multiple signal pathways to promote the differentiation, growth and survival of central neurons. That further means, once NPs are taken up by brain cells including BCECs and other neuron cells, the expressed GDNF will be secreted and following play its

neurotrophic function. To avoid the similar experimental design, BCECs were used in the cellular uptake studies and SH-SY5Y cells were applied in the gene expression research. When genes encoding non-secretive proteins are chosen and these therapeutic genes are required to be expressed in specific brain cells for their function, further studies should be performed to evaluate BBB-crossing efficiency and final expression of proteins in focus.

CONCLUSION

In summary, angiopep-conjugated DGL-based NPs could mediate effective gene expression *in vitro* and *in vivo*. Multiple dosing intravenous administrations of angiopep-modified NPs could achieve apparently improved recovery of rotenone-induced parkinsonian rats. The findings may have implications for long-term non-invasive gene therapy for neurodegenerative diseases in general.

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