One-Pot Construction of a Twice-Condensed pDNA Polyplex System for Peripheral Nerve Crush Injury Therapy

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Materials and methods

VEGF-A pDNA VEGF-A (1239 bp) pDNA encoding GFP was constructed by Bioroot Biology (Shanghai, China).

Cell Culture Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from ALLCELLS (Shanghai, China) and cultured in RPMI-1640 (GIBCO BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY, USA) and 1% antibiotics (GIBCO, Grand Island, NY, USA) at 37 ℃ in the cell incubator with 5% CO_2. Rat RSC96 Schwann cells (SCs) were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA)containing 10% FBS and 1% antibiotics at 37 ℃ in the cell incubator with 5% CO_2.

Preparation and Characterization of GPEI Polyplexes VEGF-A pDNA solution (20 ng/μl) was mixed with PEI1.8 kDa (Sigma-Aldrich, St. Louis, MO, USA) solution (2 mg/ml, in H_2O) and 0.125% glutaraldehyde (GA) (Sigma-Aldrich, St. Louis, MO, USA) water solution at different ratios, which were shown in Table 1. After the first 30-minute condensation, the mixture was heated in a water bath at 60℃ with stirring for 30 minutes (second condensation). When the solution cooled down to room temperature, extra GA and small fragments (<7 kDa) were removed by dialysis (Thermo, Rockford, IL, USA) for 4 hours. Finally, the brownish red GPEI polyplexes solution was stored at 4 ℃ for further use. Particle sizes, polydispersity index (PDI) and Zeta potential were measured using particle size analyzer (Brookhaven Particle Size Analyzer 90 Plus, USA). The morphology of the polyplexes was observed by transmission electron microscopic (TEM) (JEM 2010 system JEOL, Japan). The condensation ability of GPEI polyplexes was detected by agarose gel electrophoresis (AGE) stained with GEL RED (Biotium, Fremont, CA, USA).

Table 1 Preparation of GPEI polyplexes.

w/w Ratio	Approximate N/P Ratio	pDNA (μl)	PEI1.8k (μl)	GA (μl)	H_2O (μl)
1	7.53	1000	10	7	983
3	22.59	1000	30	21	949
5	37.65	1000	50	35	925
10	75.3	1000	100	70	830
20	150.6	1000	200	140	660
30	225.9	1000	300	210	490
40	301.2	1000	400	280	320

Preparation and Characterization of GPEI Polycation Analogue PEI1.8kDa solution (2mg/ml, in H_2O) was mixed with GA solution (0.125%, in H_2O) at the w/w ratio=5, and the volume of VEGF-A pDNA solution was replaced by ultrapure water. (PEI 1.8 kDa solution 5 ml, GA solution 3.5 ml, ultrapure water 191.5 ml) Then, the mixture were heated in a water bath at 60 ℃ with stirring for 30 minutes. When the solution cooled down to room temperature, extra GA and small fragments (<7 kDa) were removed by dialysis for 4 hours. Then, solution was freeze-dried for 48 hours. Finally, the faint yellow GPEI polycation analogue were stored at -20 ℃ for further use. The structure of GPEI polycation analogues was confirmed by Fourier transform infrared spectroscopy (FT-IR) (Thermo IR/Nicolet 6700, USA) and Proton nuclear magnetic resonance (^{1}H NMR) (Agilent NMR Systems 400MHz NMR Spectrometer, USA). Besides, the molecular weight of GPEI was tested by Gel Permeation Chromatography (GPC) (Agilent 1260 Infinity LC, PL aquagel-OH 30 8μm, Agilent GPC-Addon, UK).

Nucleic Acid Unpackaging and Degradation of pH-responsive Linkages After GPEI polyplexes had been prepared, hydrochloric acid (HCl) (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) 0.001M was used to adjust pH value to be 6.0. 4 hours later, Particle sizes and polydispersity index (PDI) a were measured using particle size analyzer (Brookhaven Particle Size Analyzer 90 Plus, USA). GPEI polycation analogue was dissolved in Formic buffer (pH =6.0) and PBS buffer (pH =7.4), kept in 37 ℃. The molecular weight of degradation products was tested by Gel Permeation Chromatography (GPC) (Agilent 1260 Infinity LC, PL aquagel-OH 30 8μm, Agilent GPC-Addon, UK).

Cytotoxicity of GPEI Polyplexes In Vitro Cytotoxicity of GPEI polyplexes *in vitro* was determined by using cell counting Kit-8 (CCK-8) (DOJINDO LABORATORISE, Shanghai, China) assay. HUVECs and RSCs were seeded in 96-well plates (1×10^4 cells/well) and cultivated at 37 ℃ for 24 hours. Then 10 μl GPEI polyplex solutions at different w/w ratios were added into wells, and each ratio was repeated 6 times (PEI25kDa complexes as positive control, PBS as blank control). At the same time, GPEI polycation analogue was tested using the same method. After 4 hours and 24 hours, former culture medium was replaced by 60μl new culture medium containing 10 μl CCK-8. 2 hours later, 96-well plates were read by multifunctional microplate reader (SpectraMax M3 Multi-Mode Microplate Reader, Sunnyvale, CA, USA).

Annexin V-PI Detection Cell apoptosis was detected by Annexin V Apoptosis Detection Kit FITC (Sangon Biotech CO.,Ltd. Shanghai, China). HUVECs and RSCs were seeded in 6-well plates ($3\sim4\times10^5$ cells/well) and cultivated at 37 ℃ for around 24 hours. When cells occupied 80% of each well, culture medium in wells was replaced by 2000 μl RPMI-1640 or DMEM without FBS. Then, 400μl GPEI polyplex solutions at different w/w ratios were added and mixed completely (PEI25kDa polyplexes as positive control, PBS as blank control). After 4-hour incubation, cells were harvested and washed by PBS for three times. In the next step, cells were evaluated by Annexin V Apoptosis Detection Kit FITC according to its protocol, and flow cytometry (FCM) (BD LSRFortessa, USA) was used to analyze these cell samples.

Intracellular Uptake pDNA was labeled with the fluorophore Cy3 using the Label IT kit (Mirus, Madison, WI) according to manufacturer's protocol, and polyplexes (w/w Ratio =5) were prepared as regular methods. Then, culture medium of HUVECs and RSCs that had been seeded ($5\sim10\times10^4$ cells/well) before was replaced by 500μl RPMI-1640 or DMEM without FBS. Subsequently, 100μl GPEI polyplex solution was added into each well and mixed completely. After 2-hour transfection, cells were washed three times with PBS and LysoTracker Green (Beyotime, Shanghai, China) (50 nM) was used to mark the lysosome at 37℃, 30mins. Next, cells were washed three times with PBS and cells were fixed with 4% paraformaldehyde. Finally, DAPI (Roche Diagnostics, Mannheim, Germany) (1 μg/ml) was used to mark the cell nucleus at 37℃, 5 mins. The results were investigated using Confocal Laser Scanning Microscope (CLSM) (Leica Las AF lite, Germany). In addition, 4-hour intracellular uptake researches were conducted by the same method.

Gene Transfection Efficiency and VEGF Expression In Vitro HUVECs and RSCs were seeded in 24-well plates ($5\sim10\times10^4$ cells/well)

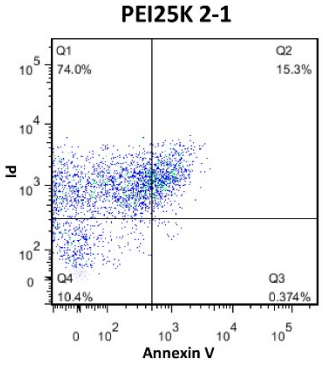
and cultivated at 37℃ for around 24 hours. When cells occupied 80% of each well, culture medium in wells was replaced by 500μl RPMI-1640 or DMEM without FBS. Then, 100μl GPEI polyplex solutions at different w/w ratios were added and mixed completely (PEI25kDa polyplexes as positive control, naked pDNA as negative control, PBS as blank control). After 4-hour incubation, cells were washed three times with PBS, and new culture medium was added. 72 hours later, flow cytometry (FCM) (BD LSRFortessa, USA) was used to analyze these two kinds of treated cells. FSC as well as SSC signals represented the cell status, and transfection efficiency could be calculated from Alexa Fluor 488 signals. VEGF concentration was detected by Elisa (Multi Sciences Biotech, Hangzhou, Zhejiang, China) according to manufacturer's protocol. In addition, total protein concentration was tested by micro bicinchoninic acid method (Micro BCA) (Micro BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA).

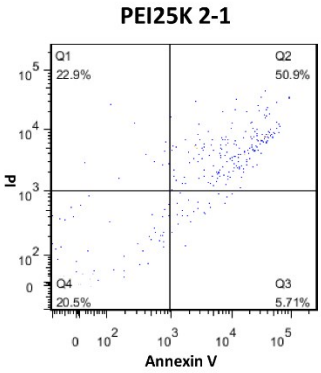
Sciatic Nerve Crush Injury Model Study 16 Sprague-Dawley rats (male, weighing 150-200 g) were housed under standard laboratory conditions for one week before the surgery. After one week, rats weighting around 250 g were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium, and the sciatic nerve was exposed through a 2-cm-long incision on the left thigh under sterile conditions. Then the sciatic nerve was crushed with a vascular clamp for 30 seconds to ensure a successful SNCI model. Finally, the incision was closed with a suture. After the operation, these rats were randomly divided into four groups (n=4): the blank control group, the negative control group, the positive control group and the GPEI group. When the wound had healed completely, drugs (pDNA concentration = 100 ng/μl) were injected intramuscularly (0.1 ml/ 100 g) at the model position once every three days. The blank control group was injected with normal saline (NS). The negative control group was injected with VEGF pDNA (100 ng/μl). The positive control group was injected with PEI25 kDa 2/1 polyplexes which contained VEGF pDNA (100 ng/μl). And the GPEI group was injected with GPEI 5/1 polyplexes which contained VEGF pDNA (100 ng/μl). Besides, each rat was injected with 5-Bromodeoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, MO, USA) according to the protocol during the experiment. 4 weeks after the surgery, rats were sacrificed for further investigation.

Blood sample were collected before rats being sacrificed, and several cytokines and hepatic enzymes from blood were detected by Elisa (Rat IL-6 EK306, Rat IFN-γ EK380V2 Multi Sciences Biotech, Hangzhou, Zhejiang, China; Rat TNF alpha Uncoated ELISA, Thermo Scientific, Rockford, IL, USA; C003, C018, C052, C072, CHANGCHUN HUILI BIOTECH CO.,LTD., Changchun, Jilin, China). Sciatic nerves and gastrocnemii were fixed in 4% paraformaldehyde to determine the angiogenesis through CD34 and CD31 staining. BrdU antibodies were used to mark the renewing cell population. To observe the ultra-structure of myelin sheaths and prove the nerve remyelination after the crush injury, TEM was used to take photos of those sciatic nerves which were cut around 2-mm long and fixed in 2.5% GA for 4hours. Toluidine blue (TB) staining was also applied to analyze the treatment effects of each group. In addition, MBP & S100 as well as β-tubulin & NF200 immunofluorescence (IF) images of sciatic nerves were captured to investigate the recovery level of sciatic nerves. Investigators were blinded for image analyses. Besides, West Bolt (WB) results of VEGF level in gastrocnemii, myelin basic protein (MBP) level in sciatic nerves could show therapeutic effects quantitatively (antibody: Wuhan Servicebio technology CO.,LTD, Wuhan, Hubei, China) . When the animal study finished, main organs were harvested to determine the toxicity *in vivo* by hematoxylin-eosin (HE) staining. Investigators were blinded for image analyses.

Sciatic function index (SFI) and electrophysiological parameters were used to evaluate the functional recovery in these four groups. After feet of rats were covered by inkpad, rats were allowed to walk freely on white paper, and SFI values were calculated by the walking track and footprints. Then sciatic nerves were exposed when rats were anesthetized. A digital MYTO electromyograph machine (Esaote, Genoa, Italy) was used to evaluate the nerve conduction velocity (NCV) and distal compound motor action potential (DCMAP).

Results





HUVEC RSC

Fig.S1 Annexin V-PI Detection of HUVECs and RSCs was analyzed by FCM, compared with controlled group treated by PEI25kDa 2/1 Cell numbers of HUVECs (5000 events) and RSCs (1000 events) controlled group were too small to finish 10000-event analysis, and most of those cells were not in normal conditions.