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Delivery of cationic polymer-siRNA nanoparticles for gene therapies in neural regeneration

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ABSTRACT

The therapeutic applications of neural stem cells (NSCs) have potential to promote recovery in many obstinate diseases in central nervous system. Regulation of certain gene expressions using siRNA may have significant influence on the fate of NSC. To achieve the optimum gene silencing effect of siRNA, non-viral vector polyethylene glycol-polyethyleneimine (PEG-PEI) was investigated in the delivery of siRNA to NSCs. The characteristics of PEG-PEI/siRNA polyplexes were detected by scanning electron microscopy (SEM). The effects of nanoparticles on cell viability were measured via CCK-8 assay. In addition, the transfection efficiency was evaluated by fluorescence microscope and flow cytometry, and real-time PCR and Western Blot were employed to detect the gene inhibition effect of siRNA delivered by PEG-PEI. The SEM micrographs showed that PEG-PEI could condense siRNA to form diffuse and spherical nanoparticles. The cytotoxicity of PEG-PEI/siRNA nanocomplexes (N/P = 15) was significantly lower when compared with that of Lipofectamine 2000/siRNA ($P < 0.05$). Moreover, the highest transfection efficiency of PEG-PEI/siRNA nanoparticles was obtained at an N/P ratio of 15, which was better than that achieved in the transfection using Lipofectamine 2000 ($P < 0.05$). Finally, the gene knockdown effect of PEG-PEI/siRNA nanoparticles was verified at the levels of mRNA and protein. These results suggest that PEG-PEI may potentially be used as a siRNA delivery vector for neural regeneration therapy.

1. Introduction

Therapies based on neural stem cells (NSCs) bring hopes to many neurological diseases, ranging from acute central nervous system (CNS) injuries, such as spinal cord trauma and stroke, to neural degeneration diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD). However, it is unexpected that the majority of exogenous NSCs transplanted in vivo differentiate into astrocytes rather than neurons [1], let alone establish functional neural circuits, thus limiting their clinical applications in neural regeneration [2,3]. In order to induce NSCs to generate desired cells or tissues to replace damaged parts in CNS, mediating the expression of certain genes in NSCs would be a potent strategy.

Nogo receptor (NgR), a common receptor for three myelin-related axon growth inhibitory proteins: Nogo-A, oligodendrocyte-myelin glycoprotein (OMgp) [4] and myelin associated glycoprotein (MAG) [5], has been described as a therapeutic target for CNS regeneration [6]. NgR has a widespread influence on CNS, including regulation of neural precursor migration during cortical development [7], mediating neural inflammation [8] and participating in the pathological processes of AD [9] and stroke [10]. In the field of neural regeneration, NgR not only impedes the neurite growths of injured neurons [11], but also affects the differentiation of NSC [12]. A recent study has shown that Nogo-66 promotes astroglial differentiation of neural stem cells via triggering of NgR [13]. Therefore, suppressing the expression of NgR in NSC might be a potential strategy of CNS regeneration.

RNA interference (RNAi) is regarded as a promising technique for gene silencing because the small interfering RNA (siRNA) can recognize its complementary mRNA with high specificity and degrade it in a short time [14,15]. However, low cellular uptake and instability of the naked siRNA in physiological conditions obviously affect its clinical application. Thus, it is crucial to employ a

Abbreviations: PEG-PEI, polyethylene glycol-polyethyleneimine; NSCs, neural stem cells; NgR, Nogo receptor; siRNA, small interfering RNA; scr, scrambled; Lipo 2000, Lipofectamine 2000; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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proper vector system to keep integrity of siRNA in circulation and assist them to transfer into cells.

Although viral vectors have the advantage of high transfection efficiency, their in vivo uses have been seriously challenged by some safety concerns, such as biological immunogenicity and probable carcinogenicity resulting from insertion mutation [16]. Hence, non-viral vectors have emerged as effective and promising alternatives due to their own merits: ease of production in lower price, having the capability to carry larger pieces of genetic material and inducing less immune response than viral vectors.

Polyethylenimine (PEI) contains a number of amino groups that can be protonated at physiological pH, thus possessing the potential to combine with negatively charged nucleic acids effectively and prevent them from biodegradation before they reach the target cells. In addition, 'proton sponge effect' hypothesis [17] suggests that PEI can assist its cargo to escape from endosome to cytoplasm. Therefore, PEI-based cationic polymers have been widely explored as non-viral gene vectors in the introduction of DNA and RNA molecules into target cells [18].

PEIs are classified by the molecular weight and structure (linear or branched polymer). A growing body of study demonstrates that the biological effects of PEIs on mammalian cells, such as transfection efficiency and cytotoxicity, vitally rely on the material properties and dosages [19]. High molecular weight (HMW) PEIs tend to yield higher transfection efficiency than low molecular weight (LMW) PEIs at the same concentration [20]. However, HMW PEIs often display high cytotoxicity, which impedes them to be considered as ideal transfection reagents [21]. Another problem facing the use of PEI is the non-specific interactions of blood components with PEI-based complexes. These combinations can be easily trapped and removed by reticuloendothelial system (RES). Therefore, in order to improve gene-delivery properties of PEI and reduce its detrimental effects on target cells, some chemical modifications of this material are necessary.

Among the various attempts, PEGylation is one of the most effective approaches. After coupling PEI with PEG, the surrounded layer of nonionic PEG polymers can provide dimensional shielding to minimize PEI's cationic toxicity. Moreover, the hydrophilic property of PEG can improve biological stability of the transfection complexes and prolong their circulation times via reducing non-specific protein binding in the serum [22].

In order to effectively transfer siRNA targeting NgR into neural stem cells, polyethylene glycol-polyethyleneimine (PEG-PEI) was synthesized with PEI (25 kDa) and PEG (2 kDa). We tested the biochemistry properties of PEG-PEI/siRNA nanoparticles at different N/P ratios (The molar ratio of nitrogen atoms in PEG-PEI to phosphorus atoms in siRNA) to select the most suitable one to achieve the optimum gene silencing effect of siRNA.

2. Materials and methods

2.1. Materials and reagents

Branched polyethyleneimine (PEI, MW 25 kDa) was purchased from Sigma Aldrich Co., Ltd., (St. Louis, MO, USA). Rabbit anti-mouse Nogo receptor antibody was purchased from Millipore Corporation (Billerica, MA, USA); Lipofectamine 2000 (Lipo2000) was obtained from Invitrogen Corporation (Carlsbad, CA, USA); FAM-(carboxyfluorescein)-labeled siRNA, siRNA targeting NgR, and negative control siRNA were designed and synthesized by GenePharma (Shanghai, China). Becton–Dickinson FACSCalibur flow cytometer (San Joes, CA) was used to detect the transfection efficacy; Nikon Eclipse Ti-S fluorescence microscope (Nikon, Tokyo, Japan) was employed to observe the cells.

2.2. Cell culture

C17.2 neural stem cells were preserved in our laboratory. The NSCs were cultured with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 5% horse serum (Sigma Aldrich, St. Louis, MO, USA) and 2 mM glutamine in a humidified incubator (5% CO2, 95% air) at 37 °C. When the cell monolayers were grown to approximately 90% confluence, they were detached by trysinization for reseeding.

2.3. Synthesis of PEGylated poly (ethylene imine) copolymer

PEG 2k was prepared by School of Chemistry and Chemical Engineering of Sun Yat-sen University. PEG20k-PEI25k was synthesized according to our previous study [23]. Nuclear magnetic resonance spectroscopy (^{1}H-NMR, Mountain View, CA) was employed to characterize the structure of PEG-PEI.

2.4. Preparation of PEG-PEI/siRNA complexes

PEG-PEI copolymers were dissolved in deionized water (1–3 mg/mL) and stored at 4 °C. Dilution of siRNA (20 μM) was stored at −20 °C. To prepare PEG-PEI/siRNA complexes, appropriate amounts of PEG-PEI copolymers were diluted with deionized water according to different N/P ratios; meanwhile, siRNA stock solution was also diluted with deionized water to the same volume as that of PEG-PEI. Finally, both the dilutions were gentle mixed and incubated for 30 min at room temperature.

2.5. Particle size and Zeta potential analysis

PEG-PEI/siRNA complexes were prepared according to various N/P ratios, and then diluted in 1.5 mL deionized water. ZETA-Plus instrument (Brookhaven, NY, USA) was employed to detect their sizes and Zeta potentials. The data were collected from three independent experiments.

2.6. Morphological analysis – scanning electron microscopy (SEM)

Scanning electron microscopy (Quanta 400 FEG) was applied in morphological analysis of nanoparticles. PEG-PEI/siRNA complexes were prepared as mentioned above. The suspension of nanoparticles was dropped on cover slips and dried at room temperature. The SEM samples were gilded and then observed under the scanning electron microscope. Finally, the images of nanocomplexes were captured and recorded.

2.7. Cytotoxicity assay in vitro

The WST-8 Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was employed to measure the cytotoxicity of PEG-PEI/siRNA nanoparticles at different N/P ratios. The C17.2 NSCs were seeded in 96-well plates at a density of 5000 cells/ well 24 h prior to transfection. To deliver 10 pmol siRNA per well, PEG-PEI/siRNA polyplexes were prepared according to N/P ratios of 0, 5, 10, 15, 30, and 50, respectively. Then the original medium was replaced with serum-free DMEM containing nanoparticles. Each concentration was replicated with 6 wells. After incubation for 6 h, the media were removed again and the cells were cultured with normal medium for another 48 h. In addition, the viabilities of NSCs treated with PEG-PEI/siRNA nanoparticles (N/P = 15) at different time points were evaluated. The transfection using Lipofectamine 2000 was employed as a control. After exposure to PEG-PEI/siRNA complexes for 4, 6, 8 and 12 h, the NSCs were incubated for another 48 h with fresh complete DMEM. Then 10μL WST-8 reagent was added per well. After 4 h, the absorbance was recorded at 450 nm on a

microplate reader (Labsystem Dragon, Oy, Finland). All experiments were conducted in triplicate.

2.8. Transfection efficiency assay

C17.2 neural stem cells were seeded in 12-well plates at a density of 2×10^4 cells / well 24 h before transfection. A series of polyplexes (N/P = 5, 10, 15 and 30) were formed by mixing 80 pmol FAM-labeled siRNA molecules and the corresponding amounts of PEG-PEI. The Lipo2000/siRNA liposome complex (2 μL Lipo2000 and 80 pmol siRNA) served as a positive control. Immediately before transfection, the original culture medium was replaced with DMEM (1 mL/well) containing transfection complexes. After incubation for 6 h at 37 °C, the medium was removed again and normal culture medium was added to NSCs for an additional 18-h incubation period. Then the cells were observed under the fluorescence microscope (Nikon, Tokyo, Japan) and the results were recorded as fluorescent images. Moreover, the transfection efficiency was assessed by flow cytometry via calculating the percentage of cells with green fluorescence 24 h after transfection. The experiments were done in triplicate.

2.9. Quantitative real-time PCR

After transfection for 24 h, total RNA extraction was carried out using TRIzol reagent (Takara shuzo, shiga, Japan) according to the manufacturer's instructions. The sequences of primers were as follows: Nogo receptor (forward primer: 5′- GGCACT TCCCGACAAC AC- 3′and reverse primer: 3′- TGCAAGAGGAGACGGTCAA-5′); GAPDH (forward primer: 5′-GCACAGTCAAGGCCGAGAAT-3′ and reverse primer: 5′-GCCTTCTCCATGGTGGTGAA-3′). Reverse transcription and real-time PCR were performed in accordance with the protocol of One Step SYBR® RT-PCR Kit (Takara shuzo, shiga, Japan). Briefly, the reaction mixture that contained 2ul cDNA, 0.5uL forward primer (10 μM), 0.5uL reverse primer (10 μM), 10uL SYBR Premix Ex Taq (2×), and 7 μL Rnase-free water was prepared. Then the thermal cycling process was performed using LightCycler instrument (Roche Diagnostics, USA). The two-step cycling program was set as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles for amplification and quantification (each cycle is 5 s at 95 °C followed by 30 s at 60 °C). Finally, the melting curve program was performed at the end of each reaction. The relative expression of mRNA was assessed by the comparative Ct Method (ΔΔCT Method), which normalize the mRNA level of NgR to that of housekeeping gene GAPDH.

2.10. Western blotting

The NSCs were seeded at a density of 2×10^5 cells in 6-cm culture dishes 24 h prior to transfection. PEG-PEI/siRNA-NgR, PEG-PEI/siRNA-scr complexes at N/P = 15 and Lipo2000/siRNA-NgR were prepared to transfect cells respectively. 48 h later, the cells were lysed in ice-cold RIPA buffer, and then centrifuged for 30 min (12000 g, 4 °C). Equal amounts (25 μg) of the proteins from different samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Then the gels were transferred to PVDF membranes, which were then blocked in Tris-buffered saline Tween-20 (TBST) containing 5% fat-free dry milk at room temperature for two hours. Immunoblotting was performed using anti-Nogo receptor primary antibody (1:1000), followed by incubation with HRP-conjugated secondary antibody. Finally, the extended signal of protein-antibody complexes was detected through exposure to X-ray film. To standardize the protein expression, immunoblotting of β-Actin was conducted as a loading control.

2.11. Statistical analysis

The software SPSS16.0 was applied in statistical analysis. Experiments were replicated at least thrice and the results were presented as mean ± standard deviation. The differences in means between the groups were statistically analyzed using ANOVA. P values < 0.05 indicated significant differences.

3. Results

3.1. Biophysical characteristics of PEG-PEI/siRNA complexes

3.1.1. Size and Zeta-potential measurements

The size and Zeta-potential of PEG-PEI/siRNA complexes depended on N/P ratios. As shown in Fig. 1A, a decreasing trend of sizes was found as the N/P ratio gradually enhanced. At N/P = 5, the size of composites was 398.9 ± 82.5 nm, then reduced to 302.8 ± 6.7 nm at N/P = 10. Finally the average diameter of the nanoparticles was just 131.7 ± 2.8 nm at N/P = 30. In addition, Zeta-potential of PEG-PEI/siRNA polyplexes was positive at N/P = 5 and gradually enhanced with increasing N/P ratios. At N/P = 5, Zeta potential of the nanoparticles was just 3.4 ± 2.6 mV, and increased to 25.8 ± 1.3 mV at N/P = 30.

3.1.2. Morphology analysis – scanning electron microscopy (SEM)

Scanning electron microscopy was used to image the formation of Polyplexes prepared at N/P = 15. As shown in Fig. 1B, the siRNA molecules were condensed well by PEG-PEI and formed dispersed and spherical nanoparticles with the particle size of approximately 250 nm.

3.2. CCK-8 cytotoxicity assays

The cytotoxicities of PEG-PEI at different concentrations were determined. The cell viability of the NSCs without treatment (blank control) was set as 100%. As showed in Fig. 2A, NSCs maintained high survival rate (85.3%) after exposure to nanocomplexes at N/P = 15 for 6 h. The cell viability dropped remarkably when the N/P ratios were above 15. Fig. 2B demonstrated that the cytotoxicity of PEG-PEI /siRNA was significantly lower than that of Lipo2000/siRNA at the same incubation time. ($P < 0.05$).

3.3. Transfection efficiency assay

The images of NSCs taken with fluorescence microscope directly demonstrated the cellular uptake of FAM-(carboxyfluorescein)-labeled siRNA after transfection. Fig. 3A showed that the fluorescent siRNA molecules were distributed within the cytoplasm, especially in the perinuclear area. In addition, data from flow cytometry indicated that transfection efficiency relied on the N/P ratios. As shown in Fig. 3B, transfection efficiency at N/P = 5 was lowest (5.37 ± 1.48%), consistent with the weakest fluorescence in the image of NSCs treated with PEG-PEI/siRNA nanocomplexes at the same N/P ratio. The highest percentage of neural stem cells containing fluorescent siRNA was obtained after transfection using PEG-PEI at N/P = 15 (86.05 ± 5.22%), which was better than that using the positive control Lipofectamine 2000 delivery system (70.66 ± 5.96%, $P < 0.05$). But the transfection efficiency of PEG-PEI/siRNA polyplexes slightly decreased at N/P = 30 (70.52 ± 6.92%), suggesting that the preparation of PEG-PEI/siRNA at a proper N/P ratio was the determinant of optimal transfection efficiency.

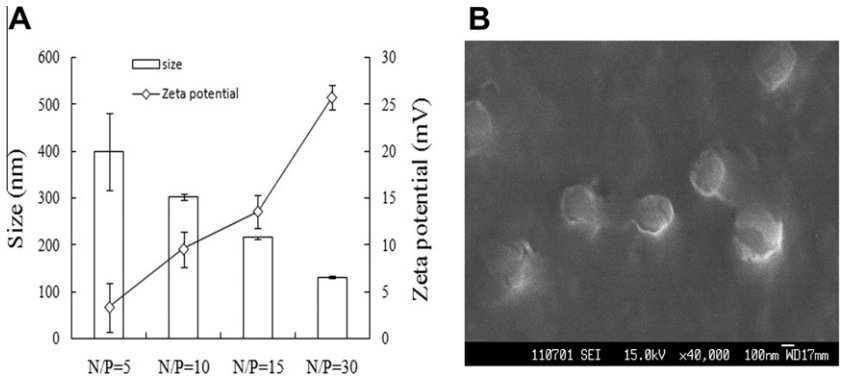

Fig. 1. Properties of PEG-PEI/siRNA nanoparticles: (A) hydrodynamic diameter and Zeta potential. The data were representative of three independent experiments and expressed as mean ± SD. (B) Morphology of PEG-PEI /siRNA polyplexes at N/P = 15 observed by scanning Electron microscopy. Scale bar is equivalent to 100 nm.

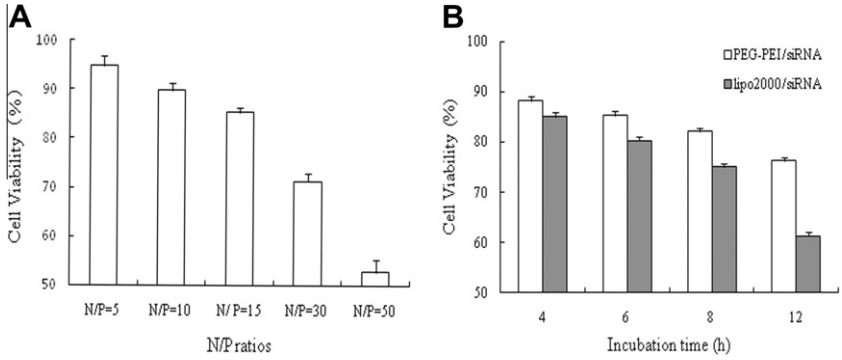

Fig. 2. Effect of the concentration and incubation time of PEG-PEI on C17.2 neural stem cells. (A) The cell viability of C17.2 NSCs after exposure to PEG-PEI/siRNA complexes at various N/P ratios (5, 10, 15, 30 and 50). (B) The cell viability of C17.2 NSCs treated with PEG-PEI/siRNA complexes at N/P = 15 for 4, 6, 8, and 12 h. Values are mean ± standard deviation (n = 3).

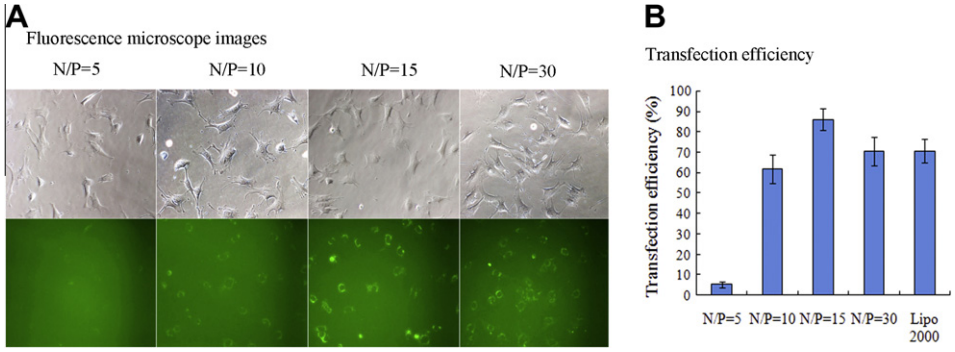

Fig. 3. Transfection efficacy of PEG-PEI/siRNA nanoparticles in C17.2 neural stem cells (NSCs) (A) fluorescence microscope images of C17.2 NSCs treated with PEG-PEI/siRNA nanoparticles at different N/P ratios (5, 10, 15, and 30). (B) Transfection efficiency of PEG-PEI/siRNA complexes detected by flow cytometry in comparison to Lipo2000/siRNA. ($\bar{x}$±SD, n = 3).

3.4. Gene knockdown effect of siRNA on NgR in NSCs

24 h after transfection, the gene knockdown effect of nanocomplexes at N/P = 15 was measured by real-time PCR. Fig. 4A revealed that PEG-PEI/siRNA-NgR resulted in significant inhibition of NgR mRNA (18.22 ± 8%, $P < 0.05$) compared to NSCs without treatment (blank control). Meanwhile the positive control Lipofectamine2000/ siRNA-NgR suppressed NgR mRNA expression to 24.9 ± 11% of the blank control, indicating that the siRNA targeting NgR was highly specific for silencing NgR gene. In addition, the result of real-time PCR coincided with that of Western blot 48 h after transfection. As shown in Fig. 4B, the expression of NgR protein decreased to 30.69 ± 2.8% ($P < 0.05$) and 53.41 ± 6.6% ($P < 0.05$) of the blank control after treated with PEG-PEI/siRNA-NgR and Lipo2000/siRNA-NgR, respectively.

4. Discussion

Given that a proper gene modification can help NSCs differentiate into functional cells to realize the dreams of neuroprotection and neuroregeneration [24], the strategies based on RNA interference (RNAi) have opened up the therapeutic options of a wide range of obstinate neurological disorders [25]. To overcome the

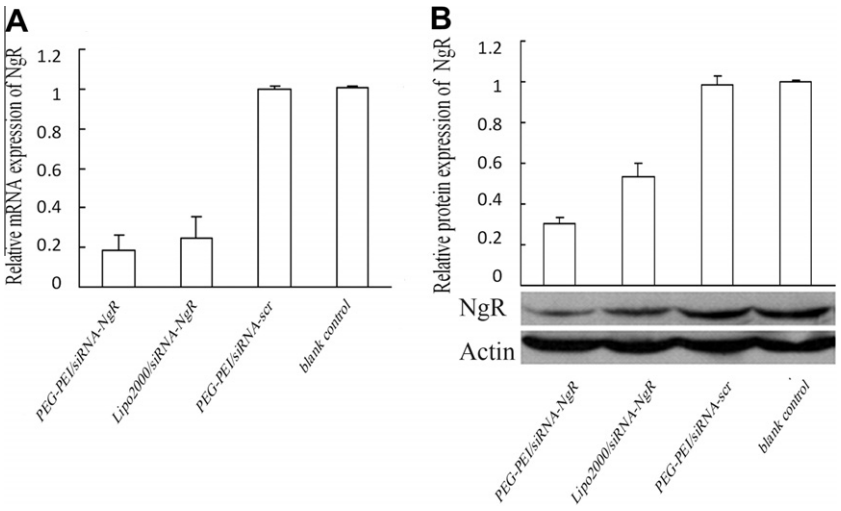

Fig. 4. Gene silencing effects of PEG-PEI/siRNA-NgR polyplexes (A) Relative mRNA expression of NgR in NSCs after transfection for 24 h. The gene expressions of Nogo receptor in neural stem cells exposed to PEG-PEI/siRNA-NgR at N/P = 15, lipo2000/siRNA-NgR and PEG-PEI/siRNA-scr were measured. The mRNA level of NgR was determined by real-time PCR and relative to that in the blank control. GAPDH was used as an internal standard to normalize the data. (B) Western blotting analysis of gene knockdown effect of siRNA targeting Nogo receptor in NSCs after transfection for 48 h. All values were mean ± SD of three experiments.

obstacles that prevent siRNA from reaching the desired site for target mRNA degradation, a non-viral vehicle PEG-PEI was applied in the siRNA delivery to NSCs.

Our study showed that this vector successfully condensed siRNA into compact and stable nanoparticles that were ready for transfection. Furthermore, PEG-PEI copolymers could retain the stability of siRNA in medium and strengthen its ability to penetrate through the living cell membranes. In addition, these nanocomplexes at an appropriate N/P ratio could achieve excellent cell transfection efficacy without affecting the cell viability obviously. Finally, these vectors could release siRNA into cytoplasm to mediate efficient inhibition of the expression of NgR at the gene and protein levels.

The measurements of the characteristics of PEG-PEI/siRNA polyplexes suggested that the formation of the nanoparticles was dependent on the N/P ratio. The size and Zeta potential contribute to the stability of extracellular polyplexes in aqueous and the interactions of nanoparticles with membranes of cells. Therefore, they are important factors to evaluate which is the best N/P ratio of nanoparticles for transfection. At N/P = 5, the size and Zeta potential of polyplexes fluctuated obviously, demonstrating that nanoparticles with the N/P ratio below 10 were unstable, hence, they were ineligible for siRNA delivery. At N/P = 15, the size of nanoparticles reduced to around 216.2 ± 2.3 nm and the Zeta potential increased to 13.6 ± 1.7 mV. It can be partly explained by the fact that the electrostatic interaction of PEG-PEI with siRNA becomes stronger when the N/P ratio enhances, thus resulting in the formation of nanoparticles with smaller size and more stable structure [26]. Meanwhile, the results of dynamic light scattering measurement were confirmed by SEM images, because the sizes of PEG-PEI/siRNA nanocomplexes at N/P = 15 measured in the respective experiments were in agreement. Moreover, the images of dispersed and spheral nanoparticles (N/P = 15) revealed that the electrical repulsion between them was strong enough to prevent aggregation. Therefore, PEG-PEI/siRNA polyplexes with N/P ratio of 15 were suitable for transfection.

For the sake of safe utilization of the cationic polymer PEG-PEI, its cytotoxicity was detected using CCK8 assays. The results indicated that PEG-PEI could induce the impairment of cells in a concentration-dependent manner, which accorded with other literatures [27]. When the N/P values were above 15, the cytotoxicity of PEG-PEI increased greatly. Therefore, PEG-PEI/siRNA complexes at N/P = 15 were chosen to further detect their effects on cell viability compared with Lipofectamine 2000/siRNA at the different time points. The results revealed that PEG-PEI had weaker effect on cell survival rate than Lipofectamine. Thus, PEG-PEI would be served as a low-toxicity reagent to transfer siRNA into cells.

Transfection efficiency correlates to the nanocomplexes' stability and their capability to penetrate into cells [28]. The result of flow cytometry showed that cell transfection efficiency gradually enhanced when N/P ratios increased from 5 to 15. The most likely explanation is the higher N/P ratio leads to an enhanced positive Zeta potential, which not only stabilizes the PEG-PEI/siRNA complexes via preventing the formation of aggregates, but also facilitates the adhesion of nanoparticles to negatively charged cell membranes. However, the transfection efficiency declined slightly at N/P = 30. Considering the result of cytotoxicity assay, it might result from the deaths of NSCs induced by high cationic toxicity of an excess of PEG-PEI. Therefore, it is necessary to choose an appropriate N/P ratio to obtain higher transfection efficiency.

Apart from the effective penetration into cells, endosomal release is also an important step during the successful siRNA delivery. The results of real-time PCR demonstrated that PEG-PEI could rapidly release its cargo into cytoplasm and led to a significant decline in NgR expression, implying that PEG-PEI was a potent gene transfer vector. Western blot assay further confirmed that PEG-PEI mediated siRNA to downregulate the expression of NgR dramatically, even better than Lipofectamine 2000 delivery system. Several studies reported that Lipofectamine 2000 showed high cytotoxicity [29], which might induce the damage or even death of the cells when maintaining the high transfection efficiency, thus influencing siRNA molecules to exert their gene inhibition effect.

In this work, we verified that PEG-PEI was an attractive alternative as gene delivery system in the applications targeting NSCs. The nanoscale particles formed by PEG-PEI and siRNA were able to silence the expression of NgR with properties of low toxicity and high efficacy. Thus, PEG-PEI might be a gene ideal gene therapy tool for neural stem cell replacement treatments. Further studies

are required to test the biological functions of PEG-PEI/siRNA polyplexes in vivo, to evaluate their long-term use and the most suitable dose for mediating genes expression in CNS.

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