Characterization of Polyethylene Glycol-Polyethyleneimine as a Vector for Alpha-Synuclein siRNA Delivery to PC12 Cells for Parkinson's Disease

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Keywords

Alpha-synuclein; Gene therapy; Nanomedicine; Parkinson's disease; Polyethylene glycol-polyethyleneimine; RNA interference.

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Received 3 May 2013; revision 15 July 2013; accepted 5 August 2013

SUMMARY

Aims: Gene therapy targeting the SNCA gene yields promising results in the treatment of Parkinson's disease (PD). The most challenging issue of the RNAi gene therapy strategy is maintaining efficient delivery without inducing significant toxicity and other adverse effects. This study aimed to characterize polyethylene glycol-polyethyleneimine as a vector for alpha-synuclein siRNA delivery to PC12 cells for Parkinson's disease. **Methods:** The characteristics of PEG-PEI/siSNCA were analyzed via gel retardation assay and assessments of particle size and zeta potential. MTT cytotoxicity assay and flow cytometry were used to detect cytotoxicity and transfection efficiency in PC12 cells. Confocal laser scanning microscopy was employed to examine the intracellular distribution of PEG-PEI/FITC-siSNCA after cellular uptake. RT-PCR and western blotting were used to measure SNCA expression. The MTT cytotoxicity assay was used to study the effect of PEG-PEI/siSNCA on cell viability. The protective effect of PEG-PEI/siSNCA on MPP⁺-induced apoptosis in PC12 cells was examined via flow cytometry and Hoechst staining. **Results:** PEG-PEI/siSNCA complexes were well-developed; they exhibited appropriate particle sizes and zeta potentials at a mass ratio of 5:1. *In vitro*, PEG-PEI/siSNCA was associated with low cytotoxicity and high transfection efficiency. Complexes were capable of successfully delivering siSNCA into PC12 cells and releasing it from the endosome. Furthermore, PEG-PEI/siSNCA could effectively suppress SNCA mRNA expression and protected cells from death via apoptosis induced by MPP⁺. **Conclusions:** Our results demonstrate that PEG-PEI performs well as a vector for alpha-synuclein siRNA delivery into PC12 cells. Additionally, PEG-PEI/siSNCA complexes were suggested to be able to protect cells from death via apoptosis induced by MPP⁺. These findings suggest that PEG-PEI/siSNCA nanoparticles exhibit remarkable potential as a gene delivery system for Parkinson's disease.

doi: 10.1111/cns.12176

The first two authors contributed equally to this work.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by two major progressive processes: (1) accumulation of intraneuronal Lewy bodies/Lewy neurites (LBs/LNs) that are composed of filamentous α -synuclein (SNCA) aggregates and (2) selective reduction of dopamine (DA) neurons of the nigrostri-

atal system, which leads to tremor, rigidity, and bradykinesia [1]. The incidence of PD has increased dramatically over the last two decades; however, the available therapeutics for treating PD remain limited. The effectiveness of therapies that are directed toward PD-related motor dysfunction decline as the disease progresses [2]. Currently, there are no therapeutic agents available to arrest or decelerate the progression of PD [3]. Recent studies have

shown treatments targeting the SNCA gene may represent a new frontier in the treatment of PD [4].

SNCA is a small (140 amino acid) peripheral membrane protein that specifically localizes to axon terminals and is the main component of LB inclusions [5]. Numerous studies have indicated that a key step in PD-related LB formation and neuronal loss associated involves the aggregation and modification of SNCA [6]. For its central role in PD, SNCA has been suggested to be a novel therapeutic target for new PD treatments. Patients with either familial or idiopathic PD may benefit from therapies that reducing SNCA expression [7].

Gene therapy is just one of the promising therapeutic techniques being developed for future use to ameliorate PD motor symptoms and motor complications that result from the current standard of clinical care [8,9]. RNA interference (RNAi) represents an endogenous gene-silencing mechanism that involves double-stranded RNA-mediated sequence-specific mRNA degradation; this technique is widely used in gene therapy [10]. The most challenging issue associated with successful RNAi gene therapy involves ways to efficiently deliver genetic material to target cells without inducing significant toxicity or other adverse effects [11]. Viral vectors are currently the most efficient systems used in gene therapy; however, viral vectors are associated with potent immunogenicity, mutagenesis, and other the biohazards, which weaken their application [12]. Non-viral gene vectors demonstrate advantages over viral vectors; they are able to target specific cells, do not integrating into the host genome and are less immunogenic. However, many studies have associated less sustained gene expression with non-viral vectors [13].

Non-viral vectors that are based on nanotechnology have generated much interest in the last few years. Nanotechnology has played an important role in the development of delivery vectors, and numerous research teams have combined these technologies to create nanosystems for small interference RNA (siRNA) delivery [14,15]. The design and synthesis of nanocarriers that are able to efficiently and safely deliver siRNA are challenging and rapidly developing; these processes involve targeting potential application sites and overcoming ubiquitous biological barriers [16]. We previously reported that polyethylene glycol-polyethyleneimine (PEG-PEI) may be used as a nanocarrier system to apply siRNA delivery into cells with low cytotoxicity and high transfection efficiency [17].

In the present study, we proposed to use PEG-PEI as the siRNA delivery system to reduce SNCA expression in PC12 cells in Parkinson's disease. We assessed the characteristics of PEG-PEI/siSNCA and evaluated the potential of PEG-PEI/siSNCA for use as effective gene therapy systems for PD. In addition, we examined the effect of PEG-PEI/siSNCA pretreatment in an N-methyl-4-phenylpyridinium (MPP⁺)-induced cellular model of PD.

Cell culture and reagents

Rat pheochromocytoma (PC12) cell lines were obtained from ATCC and cultured in RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cells were differentiated via treatment with

100 ng/mL nerve growth factor-7S for 9 days; they were then washed with RPMI 1640 medium containing 1% fetal bovine serum 24 h prior to being used in experiments.

PEG-PEI was synthesized in-house using previously reported techniques [17]. Lipofectamine 2000 (Lipo) was purchased from Invitrogen (Carlsbad, CA, USA). The siSNCA, which targeted SNCA in the rat, negative control siRNA (siNC) and FITC-labeled siSNCA (FITC-siSNCA) were purchased from GenePharma (Shanghai, China). Nerve Growth Factor-7S and MPP⁺ iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Z-VAD was purchased from the Sigma Chemical Co (St. Louis, MO, USA). SNCA rabbit mAb and Hoechst 33,342 (4082S) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Analysis of the characteristics of PEG-PEI/siSNCA by gel retardation assay and measurements of particle size and zeta potential

Gel retardation assay was used to evaluate the complexation between PEG-PEI and siSNCA. PEG-PEI and siSNCA were mixed at various mass ratios of 0.5:1, 1:1, 1.5:1, 3:1, and 5:1 to yield different formulation compositions. The resulting PEG-PEI/siSNCA complexes were incubated at room temperature for 30 min to facilitate complexation and then mixed with 10× loading buffer. Complexes were then loaded into 1% agarose gels that were pre-stained with ethidiumbromide (EtBr, 0.1 mg/mL). The samples were electrophoresed at 90 V for 30 min in Trisacetate (TAE) buffer (0.045 M TAE; 0.001 M EDTA), and bands were visualized using a UV imaging system (UVIdoc, UVItect Ltd., Cambridge, UK).

To measure particle size and zeta potential, PEG-PEI/siSNCA complexes were prepared at mass ratios of 1.5:1, 3:1, and 5:1 and then diluted to a volume of 1.5 mL with DPEC water. Particle size and zeta potential were analyzed using the Zeta-Plus instrument (Brookhaven, NY, USA) with a detection angle of scattered light at 90 and 15°C, separately.

MTT cytotoxicity assay and flow cytometry

For MTT cytotoxicity assay, PC12 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and cultured for 12 h at 37°C with 5% CO₂. Cells were then transfected for 48 h with different concentrations of PEG-PEI (5, 10 and 30 µg/mL) delivering either 100 nM siSNCA, as either Lipo conjugated with siSNCA, or the siSNCA only, or phosphate-buffered saline (PBS) control. The medium was then removed and replaced with 100 µL of fresh medium and 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After 4 h of incubation, 150 µL of dimethyl sulfoxide (DMSO) was added to replace the MTT-containing medium and gentle agitation was applied for 10 min. The absorbance at 490 nm was measured on a TecanInfinite F200 Multimode plate reader (Tecan Trading AG, Männedorf, Switzerland).

For *in vitro* transfection, PC12 cells were seeded in a 6-well plate at a density of 2×10^5 cells/well and allowed to sit overnight to achieve 60–80% confluence prior to the transfection reaction. Cells were then transfected with FITC-siSNCA, Lipo/FITC-siSNCA, or PEG-PEI/FITC-siSNCA at the mass ratio of 5:1 with different dosages of FITC-siSNCA (50, 100, and 200 nM). After 6 h of

incubation at 37°C with 5% CO₂, the medium was replaced with the same volume of fresh medium. The cells were inspected under a fluorescence microscope (Nikon, Tokyo, Japan). Cells were also collected and analyzed by flow cytometry (Beckman-Coulter Inc, Fullerton, CA, USA).

Intracellular distribution of PEG-PEI/FITC-siSNCA after cell uptake

PC12 cells were seeded at a density of 5×10^3 cells per dish into a confocal laser dish and incubated for 24 h at 37°C with 5%CO₂ for detection by confocal laser scanning microscopy (CLSM). PEG-PEI/FITC-siSNCA(100 nM)complexes at a 5:1 mass ration were added into each dish and incubated for predetermined amounts of time (1, 4, and 8 h). At the predetermined time intervals, cells were washed three times with phosphate-buffered saline(PBS), and the nuclei were stained with DAPI (1 mg/mL) for 10 min and incubated with LysoTracker Red (50 nM) for 1 min. Finally, the cells were washed 3 times with PBS and prepared for observation under a confocal Zeiss LSM 510 META microscope (Carl Zeiss Co., Ltd., Gottingen, Germany).

Analysis of SNCA expression by RT-PCR and western blotting

PC12 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated at 37°C with 5% CO₂ overnight to reach approximately 60% confluence. PEG-PEI/siSNCA (100 nM, 5:1 mass ratio), PEG-PEI/siNC (100 nM, 5:1 mass ratio), PEG-PEI/siSNCA (100 nM), or the same volumes of PBS were added to the plates which were then incubated for 48 h (for mRNA isolation) or 72 h (for protein extraction).

SNCA mRNA expression was analyzed via real-time PCR assay. The total amount of RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using PrimeScript RT reagent Kit according to the manufacturer's instructions (Promega, Madison, WI, USA). Real-time PCR was conducted using an ABI 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The SNCA primer sequences used were as follows: forward, 5'-CCAGAGCCTTTCACCCCTCTT-3' reverse, 5'-TCTTTGCTCCACA CGGTCT-3'. The GAPDH primer sequences were as follows: forward, 5'-GGGAAGCCCATCACCATCT-3', reverse, 5'-CGGCCTCAC CCCATTG-3'. PCR was conducted at 95°C for 10 min, 40 cycles at 95°C for 5 s and 40 cycles at 60°C for 34 s.

SNCA protein expression was analyzed via real-time PCR assay. The total amount of protein was extracted using Proteo JETTM mammalian cell lysis reagent (Formentas Life Science) with phenylmethanesulfonyl fluoride (PMSF). The concentrations of these reagents were determined using a BCA-100 Protein Quantitative Analysis Kit (Biocolors, Shanghai, China). Different protein samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked using 5% nonfat dry milk in 1 × Tris buffered saline containing 0.1% Tween-20 for 3 h, and then incubated with anti- α -Synuclein monoclonal antibody (1:500) at 4°C overnight. After being washed three times with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:3000) for 2 h at

room temperature. After exposure to X-ray film, protein bands were imaged using a UV imaging system. Expression of the housekeeping gene, beta-actin, was used as the control.

The effect of PEG-PEI/siSNCA on cell viability induced by MPP⁺ *in vitro*

PC12 cells were seeded onto 96-well plates at a density of 1×10^4 cells per well. The cells were transfected with different concentrations of MPP⁺ (0, 5, 10, 50, 100, 200 μ M) for 48 h; cell viability was then determined via MTT assay as previously described.

The 100 μ M concentration of MPP⁺ was chosen for subsequent experiments. PC12 cells were seeded and transfected with PEG-PEI/siSNCA, PEG-PEI/siNC, or the same volumes of PBS for 24 h. Cells were washed three times with PBS, and fresh medium containing MPP⁺ was then added to yield final concentration of 1 mM. The plates were incubated for 48 h. Identical volumes of PBS were added to the control plate instead of MPP⁺. The cell viability of each well was then measured via MTT assay.

To examine whether MPP⁺ toxicity could be reduced by caspase inhibitors, Z-VADfmk was dissolved in culture media and added 2 h prior to the addition of MPP⁺ (100 μ M) to the cultures at concentrations of 0, 20, and 100 μ M. After 48 h of incubation, the culture medium was removed, PC12 cells were washed with PBS and the MTT experiments were conducted.

The effect of PEG-PEI/siSNCA on MPP⁺-induced cell apoptosis *in vivo*

To analyze apoptosis, PC12 cells were seeded in 6-well plates at a density of 2×10^5 cells per well. Cells were transfected with the formulations described above, and MPP⁺ was similarly added. Cells were then centrifuged to remove the medium, washed with PBS and stained with Annexin V-PE and propidium iodide (PI) using an Annexin V Apoptosis Detection Kit (BD biosciences) according to the manufacturer's instructions. The percentage of apoptotic cells was quantified via flow cytometry; viable cells were both Annexin V-PE and PI negative.

For immunocytochemical staining, PC12 cells were seeded on PLL-coated glass slides at a density of 2×10^5 cells per well. After various treatments, cells were fixed in 4% paraformaldehyde and stained with 10 mg/mL Hoechst 33,342 at 37° for 10 min. Cells were then washed three times with PBS and fluorescence was assessed via fluorescence microscopy (original magnification 600×). The number of Hoechst 33,342-positive cells was counted in five fields of each section. The results of the Hoechst 33,342-positive cell count are expressed as the percentage of the MPP⁺ control cells identified in each section.

Statistical analysis

All of the experiments were replicated at least three times and the results are expressed as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA), and significance differences were evaluated via one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. In all tests, a *P* value of <0.05 was considered statistically significant.

Characteristics of PEG-PEI/siSNCA

Condensation of siSNCA by PEG-PEI was determined via agarose gel electrophoresis. The ratio of PEG-PEI to siSNCA ranged from 0.5:1 to 5:1. As shown in Figure 1A, as electrophoresed mass ratios increased, the intensity of the siSNCA bands decreased. The siRNA band disappeared when the mass ratio of PEG-PEI/siSNCA was 1.5:1. These results suggest that siSNCA was fully condensed by PEG-PEI at a 1.5:1 mass ratio.

Based on the gel electrophoresis results, we chose mass ratios of PEG-PEI/siSNCA ranging from 1.5:1 to 5:1 to measure particle size and zeta potential. As shown in Figure 1B, as the mass ratio increased, the PEG-PEI/siSNCA particle size decreased. However, as the mass ratio increased, zeta potential increased. At the mass ratio of 5:1, the PEG-PEI/siSNCA particle size was 129.9 nm, and the zeta potential was 26.8 mV. We also analyzed the size distribution of PEG-PEI/siSNCA complexes at the 5:1 mass ratio; the extreme values of the distribution may represent particles that did not complex well (Figure 1C).

Cytotoxicity and transfection efficiency of PEG-PEI/siSNCA in PC12 cells

The cytotoxicity of PEG-PEI/siSNCA was evaluated via MTT assays. siSNCA was used as the negative control and Lipo/siSNCA was used as the positive control. Cells were treated with PEG-PEI/siSNCA complexes containing different concentrations of PEG-PEI.

As shown in Figure 2, even at high concentrations of PEG-PEI/siSNCA complexes (30 mg/mL), cell viability was 83.92%, indicating that these complexes performed well. In contrast, the cell viability after treatment with Lipo/siSNCA was 63.26%, which clearly indicated increased cytotoxicity compared to PEG-PEI/siSNCA ($P < 0.05$). All of these experiments clearly indicated that PEG-PEI could be used as a vector to deliver siSNCA; PEG-PEI/siSNCA performed well with lower cytotoxicity than Lipo/siSNCA.

To demonstrate the delivery efficiency of PEG-PEI/siSNCA into PC12 cells, we first analyzed the transfection of PEG-PEI/siSNCA complexes in PC12 cells via inverted fluorescence microscopy. siSNCA was labeled with FITC for this purpose. As shown in Figure 3A, FITC-siSNCA was observed in PC12 cells after 8 h of transfection by PEG-PEI/FITC-siSNCA; this indicated that cellular uptake of PEG-PEI/FITC-siSNCA occurred.

To analyze PEG-PEI/FITC-siSNCA transfection efficiency more closely, we incubated PEG-PEI/FITC-siSNCA with PC12 cells for 8 h and then measured the cells by flow cytometry. Lipo/FITC-siSNCA was used as a positive control. As shown in Figure 3B, in 50–100 nM doses of FITC-siSNCA, the percentages of fluorescent cells were greatly enhanced; however, only slight changes in transfection efficiency were observed when the dose of FITC-siSNCA was increased to 200 nM. In 150 nM doses of FITC-siSNCA, the PEG-PEI/FITC-siSNCA transfection efficiency was 78.22%; this indicates that PEG-PEI/FITC-siSNCA performed better than Lipo/FITC-siSNCA (transfection efficiency: 72.45%), although the increase in efficiency was small (Figure 3C).

Figure 1 Characteristics of PEG-PEI/siSNCA. (A) Gel retardation electrophoresis of PEG-PEI/siSNCA complexes at different mass ratios. (B) The particle size and zeta potential of PEG-PEI/siSNCA complexes at different mass ratios. (C) Size distribution of PEG-PEI/siSNCA complexes at a 5:1 mass ratio measured by Zeta-Plus instrument. Data are presented as the mean $\pm$ SD of experiments ($n = 3$).

Figure 2 Cytotoxicity of PEG-PEI/siSNCA in PC12 cells. Cell cytotoxicity of PEG-PEI/siSNCA was assessed via MTT assay at 48 h post-transfection and the percentage of viable cells was calculated relative to untreated control cells. Data are represented in the graph as the mean $\pm$ SD of three independent experiments (* $P < 0.05$ compared to PEG-PEI/siSNCA).

Intracellular distribution of PEG-PEI/FITC-siSNCA

To investigate whether PEG-PEI/FITC-siSNCA was taken up by PC12 cells and if FITC-siSNCA could escape lysosomes, the intracellular distribution of PEG-PEI/FITC-siSNCA complexes was evaluated via confocal laser scanning microscopy (CLSM). PC12 cells were treated for different periods of time prior to microscopy. Cell nuclei were stained blue with DAPI and lysosomes were stained red with Lyso-Tracker Red. As shown in Figure 4, the green fluorescence was weak and mainly distributed within areas of red fluorescence in shorter treatment periods, whereas the areas of green fluorescence increased as treatment periods increased. After 8 h of incubation, green fluorescence was distributed throughout the sample and exceeded that of the red fluorescence. This indicates that PEG-PEI/FITC-siSNCA was uptaken by PC12 cells and that FITC-siSNCA could successfully escape the lysosome.

Suppression of SNCA gene expression

Suppressed SNCA gene expression was observed in PC12 cells and evaluated at both the mRNA and protein level via real-time PCR and western blotting, respectively. As shown in Figure 5B, the PEG-PEI/siSNCA group exhibited levels of mRNA that were reduced to 78.64% and the Lipo/siSNCA group demonstrated mRNA levels that were reduced to 74.28%. Both treatment groups exhibited mRNA levels that were clearly less than those of the control groups ($P < 0.05$). As determined by western blotting, SNCA protein levels consistently changed in a similar manner (Figure 5A).

Cytoprotective effects of PEG-PEI/siSNCA on cell viability and MPP⁺-induced apoptosis

MTT assay was used to examine the effect of various concentrations of MPP⁺ (0–200 μ M) on the cell viability. As shown in Figure 6A, treatment with higher concentrations of MPP⁺ resulted

Figure 3 Transfection efficiency of PEG-PEI/siSNCA in PC12 cells. (A) Fluorescence images for FITC-siRNA fluorescence under an inverted fluorescence microscope 8 h after the final transfection. (B) The ratio of FITC-positive cells detected by flow cytometry. (C) Representative histograms and the mean $\pm$ SD of the percentage of fluorescent cells analyzed by flow cytometry. The means $\pm$ SD are cumulative results from three independent experiments. FITC-siSNCA refers to fluorescein isothiocyanate-labeled, small interfering RNA-targeted SNCA.

in significantly reduced cell viability in a concentration-dependent manner. Treatment with a MPP⁺ at a concentration of 100 μ M for 48 h was selected for subsequent experiments because it was previously found to reduce cell viability to approximately 43% of the control ($P < 0.001$).

To evaluate the effects of PEG-PEI/siSNCA on MPP⁺-induced cytotoxicity, PC12 cells were pre-treated with PEG-PEI/siSNCA or PBS and incubated for 48 h. After exposure to MPP⁺ for 48 h,

Figure 4 The intracellular distribution of PEG-PEI/FITC-siSNCA in PC12 cells was analyzed by confocal laser scanning microscope (CLSM). Cell nuclei were stained with DAPI (blue). siSNCA were labeled with FITC (green). Lysosomes were stained with Lyso-Tracker Red (red).

Figure 5 PEG-PEI/siSNCA efficacy in suppressing SNCA gene expression in PC12 cells. (A) Protein expression suppression of the SNCA gene was evaluated by western blot analysis ($n = 3$). (B) Suppression of the SNCA mRNA levels was quantified by real-time PCR analysis ($n = 3$). Dose: 100 nM siRNA per well. *** $P < 0.001$ versus control.

cellular survival was measured via MTT assay. As shown in Figure 6B, the group receiving MPP⁺ treatment alone exhibited cell viability levels that were reduced to 43.62% of the control group. The group treated with PEG-PEI/siSNCA followed by MPP⁺ exhibited cell viability levels that were reduced to 70.59% of the control group. The PEG-PEI/siSNCA plus MPP⁺ group also demonstrated significantly enhanced cell viability than the MPP⁺ control or PEG-PEI/siNC plus MPP⁺ groups; this indicates that PEG-PEI/siSNCA exhibited cytoprotective effects on MPP⁺-induced cytotoxicity.

The addition of a caspase inhibitor (V-ZAD) had no effect on MPP⁺ toxicity at any concentration (Figure 6C). These data indicate that MPP⁺ elicits caspase-independent cell death in PC12 cells.

Cytoprotective effects of PEG-PEI/siSNCA on MPP⁺-induced cell apoptosis

We wondered whether SNCA gene knockdown mediated by PEG-PEI/siSNCA complexes would affect MPP⁺-induced PC12 cell apoptosis. Cells were pretreated with PEG-PEI/siSNCA or PBS and incubated for 48 h. After exposure to MPP⁺ for 48 h, cellular apoptosis was detected staining with Annexin V-FITC and propidium iodide followed by flow cytometry. As shown in Figure 7A, 52.3% of PC12 cells of the MPP⁺ treatment alone group experienced apoptosis in, while MPP⁺ treatment followed by PEG-PEI/siSNCA induced apoptosis in only 24.2% of PC12 cells. The PEG-PEI/siSNCA plus MPP⁺ group exhibited levels of cell apoptosis that were significantly lower than those of the MPP⁺ control and PEG-PEI/siNC plus MPP⁺ groups ($P < 0.05$, Figure 7B). This demonstrated that PEG-PEI/siSNCA could reduce MPP⁺-induced apoptosis in PC12 cells.

Hoechst 33,342 staining was performed to determine the level of MPP⁺-induced nuclear damage in PC12 cells (Figure 8A,B). In the MPP⁺ control group, MPP⁺ insult caused DNA fragmentation

Figure 6 Effects of PEG-PEI/siSNCA on cell viability induced by MPP⁺. (A) Effects of MPP⁺ on cell viability for 48 h at different concentrations (0–200 μM). (ANOVA *F*-test, **P* < 0.05 vs. control; ***P* < 0.01 vs. control; ****P* < 0.001 vs. control) (B) Effects of PEG-PEI/siSNCA on the viability of MPP⁺-treated PC12 cells was assessed via MTT analysis. Pretreatment consisted of PEG-PEI/siSNCA, PEG-PEI/siNC or PBS, as indicated. **P* < 0.05 vs. MPP⁺ control. (C) Cells were treated with MPP⁺ in the presence (20 or 100 μM) or absence of the caspase inhibitor z-VAD. Data are expressed as the percentage of cell survival compared to that of untreated cultures and the mean ± SD of the experiments (*n* = 3).

and condensation of the nuclear chromatin. Treatment with 100 mM PEG-PEI/siSNCA prevented these morphological changes in injured PC12 cells; this indicated a protective effect of PEG-PEI/siSNCA against nuclear damage.

Discussion

Parkinson disease is a progressive neurodegenerative disorder, and there is no current therapy that can reverse the progression of this disease. Alpha-synuclein is associated with Parkinson's disease and is a viable target for disease-modifying therapeutic interventions in PD [18]. Several new methods have been recently tested to suppress the SNCA gene in Parkinson disease, such as drugs [3,19], prolyl oligopeptidase inhibitors [20], peptides [21], shRNA [22], and siRNA [23]. However, of these therapies, none were based on nanomedicine, which represents remarkable advantages for gene therapy in nervous system diseases. In this study, we first applied the nanoparticles PEG-PEI as a vector of siSNCA to target SNCA mRNA.

Prior to conducting biological experiments, it was essential to evaluate the characteristics of the PEG-PEI/siSNCA nanoparticles because they were of great importance for the remainder of the study. We analyzed PEG-PEI/siSNCA characteristic via gel retardation assay, and measuring particle size and zeta potential. Agarose gel electrophoresis was used to evaluate the affinity between PEG-PEI and siSNCA. Our results revealed that siSNCA was fully condensed by PEG-PEI at a 1.5:1 ratio, thus, ErBt cannot intercalate between double-stranded siRNA and disable fluorescence. Although increased mass ratios led to better siRNA condensation and smaller particle sizes, they result in high toxicity and instability due to electrostatic interactions [24]. Based on our particle size and zeta potential measurements, we concluded that the optimal mass ratio of PEG-PEI/siSNCA is 5:1.

Nanoparticles were assessed as potential delivery vehicles because of their low toxicity and high transfection efficiency [25]. The cytotoxicity of PEG-PEI/siSNCA was evaluated via MTT assay and transfection efficiency was determined using flow cytometry. Our results indicated that PEG-PEI/siSNCA exhibited no obvious cytotoxicity, even at high concentrations. As a non-viral delivery system, Lipofectamine 2000 is widely used to deliver siRNA; however, our results indicated that Lipofectamine 2000 obviously exhibited high cytotoxicity, which was consistent with previous reports [26]. High transfection efficiency was observed in PEG-PEI/siSNCA when siRNA doses were 100 nM, which is even higher than Lipo/siSNCA doses. Using these data, we indicated that PEG-PEI/siSNCA complexes are promising siSNCA gene

Figure 7 Effects of PEG-PEI/siSNCA on MPP⁺-induced cell apoptosis. **(A)** PC12 cells that were treated with PEG-PEI/siSNCA complexes or other formulations were stained with Annexin V-PE and propidium iodide (PI). Apoptosis was analyzed via flow cytometry. **(B)** Quantification of the percentage of apoptotic PC12 cells after exposure to MPP⁺ in the absence or presence of PEG-PEI/siNC or PEG-PEI/siSNCA. The numbers of cells in: C1 (AV-/PI+) represent necrotic cells, C2 (AV+/PI+) represent late-phase apoptotic cells, C3 (AV-/PI-) represent normal cells and C4 (AV+/PI-) represent early-phase apoptotic cells. The data are presented as the means $\pm$ SD from three experiments ($n = 3$). ** $P < 0.01$ vs. MPP⁺ control.

Figure 8 Effect of PEG-PEI/siSNCA on MPP⁺-induced nuclear morphological alterations of PC12 cells. **(A)** Hoechst 33,342 staining was performed in PC12 cells that were treated with MPP⁺ in the absence or presence of PEG-PEI/siSNCA. Cells were stained with DAPI as a counterstain for all cell nuclei. **(B)** Quantitative analysis of the percentage of Hoechst-positive cells via standard cell counting methods ($n = 3$). ** $P < 0.01$ vs. MPP⁺ control.

delivery systems that are associated with high transfection efficiency and low cytotoxicity.

After cellular uptake, siRNA lysosomal escape is crucial for subsequent post-transcriptional cytoplasmic gene silencing [27].

CLSM was used to improve visualization PEG-PEI/siSNCA complex uptake. Our results revealed that as the treatment period increased in time, PEG-PEI/siSNCA complexes were released from the lysosome with high efficiency. Additionally, our RT-PCR and

western blotting experiments demonstrated that efficient SNCA gene knockdown was achieved in PC12 cells when PEG-PEI was used to deliver siSNCA.

PC12 cells, which are a clonal rat pheochromocytoma cell line, represent the site of dopamine synthesis, metabolism, and transporting systems. Therefore these cells have been used as a model for studies of MPP⁺ neurotoxicity and PD [28]. Although PC12 cells are not true brain dopaminergic neurons, these cells are able to synthesize and store the catecholamines dopamine and norepinephrine; in addition these cells express dopaminergic transporters [29]. Upon nerve growth factor (NGF) stimulation, PC12 cells not only display abundant neuritic growth, but they also adopt a neurochemical dopaminergic phenotype [30]. Both undifferentiated and NGF-treated PC12 cells synthesize, store, secrete and take up dopamine by processes that are similar to those present in true dopaminergic neurons [31,32].

MPP⁺ has been widely used to induce acute cellular PD models [33]. MPP⁺ is a toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which produces clinical, biochemical, and neuropathological changes that are similar to those observed in PD [34]. MPP⁺ selectively induces dopaminergic neuron death and has thus been widely used as a dopaminergic neurotoxin [35]. MPP⁺ also renders dopaminergic cells more vulnerable to apoptosis, which has recently been recognized as an important cell death process in PD [36].

After PEG-PEI/siSNCA complexes were efficiently delivered into PC12 cells, they successfully suppressed SNCA gene expression. Therefore, we proposed that these complexes could be therapeutically applied in PD. As a result, we examined the effect of PEG-PEI/siSNCA pretreatment on MPP⁺ cytotoxicity in PC12 cells.

In the present study, we report evidence that pretreatment with PEG-PEI/siSNCA exhibited protective activity against MPP⁺ injury in PC12 cells. Many *in vitro* studies have shown that apoptosis and necrosis are involved in MPP⁺ toxicity-induced neuronal death pathways; this finding was also confirmed in our study. The flow cytometry and Hoechst 33,342 staining results revealed that PEG-PEI/siSNCA significantly inhibited apoptotic cell death in PC12 cells after MPP⁺ administration. The present findings reveal that apoptotic cell death was remarkably reduced in the presence of PEG-PEI/siSNCA; this is particularly important to their potential use in PD therapeutics.

It is well known that MPP⁺ causes dopaminergic cell death by impairing mitochondrial function in these cells. This depletes ATP levels and induces both ROS production and the activation of various apoptotic pathways [37]. Exposure to the neurotoxin MPP⁺ resulted in a concentration-dependent increase in cell death of

PC12 cells; approximately 40% cell death occurred with exposure to 1 mM MPP⁺ over a 48-h period. Several reports have suggested that MPP⁺ may induce apoptosis in PC12 cells at concentrations ranging from 10 μ M [38] to 1 mM [39]. It has also been reported that MPP⁺ is toxic to DA neurons of mesencephalic cultures at concentrations as low as 0.5–2.0 μ M; however, MPP⁺ is toxic to immortalized cell lines only at concentrations exceeding 100 μ M [40]. This is a relatively high dose of MPP⁺ and was required to achieve significant cell death in our cellular system as well as in that of other researchers [30,41].

Several studies have already noted a connection between SNCA and MPP⁺ [42]. MPP⁺ was found to accelerate the rate of SNCA aggregation [43]. Similarly, SNCA exhibited striking resistance to MPTP-induced apoptosis [44] and was highly expressed in the nigrostriatal pathway of normal mice following MPP⁺-induced injury [45]. SNCA knockdown mechanisms may offer significant neuroprotection against MPP⁺-induced injury via up-regulation of the Bcl-2/Bax ratio. Such an effect would attenuate mitochondrial membrane potential depression, inhibit cytochrome c release to the cytosol and prevent apoptosis [46].

In the current study, we constructed PEG-PEI/siSNCA complexes as non-viral siSNCA delivery systems for Parkinson disease. The complexes exhibited no obvious cytotoxicity and high transfection efficiency. The complexes were capable of delivering siSNCA into neural PC12 cells. These complexes also promoted the release of loaded siSNCA from the endosome into the cytoplasm and synapsis, which resulted in down-regulation of the target SNCA gene *in vitro*. In addition, PEG-PEI/siSNCA complexes were able to protect cells against cell death and MPP⁺-induced apoptosis. All of these findings revealed that PEG-PEI/siSNCA nanoparticles demonstrate remarkable potential as gene therapy agents for Parkinson disease.

Acknowledgments

This work was supported by the Natural Science Foundation of China (Grant number 81000466) and the Natural Science Foundation of Guangdong province (S2013010014550, S2013010014804). We would like to thank the Key Laboratory of Malignant Tumor Gene Regulation and Target Therapy of the Guangdong Higher Education Institutes of Sun Yat-Sen University.

Conflict of Interest

The authors declare no conflict of interest.

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