Gene delivery to neuroblastoma cells by poly (L-lysine)-grafted low molecular weight polyethylenimine copolymers

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Abstract: Polyethylenimine (PEI) and poly (L-lysine) (PLL) are among the most investigated non-viral gene carriers. However, both polymers contain deficiencies that restrict their applications. In the present study, we synthesized PLL-alkyl-PEI conjugates via 6-carbon alkyl linker and investigated their possible advantages in gene delivery. Four PLL copolymers were synthesized with different molecular weights and ratios of PEI. The physiochemical properties of synthesized conjugates such as size, zeta potential, DNA condensation ability, buffering capacity and cytotoxicity were investigated. Renilla luciferase assay was employed to evaluate the gene transfection efficiency of pDNA-polymer to Neuro2A cell line. DNA condensation and particle size measurements showed that new PLL-PEI conjugates could form polyplexes in nano-scale size in the range of 99-122 nm and were able to condense DNA at low concentration. While cytotoxicity reduced in some groups, the transfection efficiency increased about 2.8 and 4 fold as compared to the unmodified PEI 1.8 kDa and 10 kDa, respectively. The results of the present study showed that the chemical modifications of PEI with PLL could significantly improve transfection efficiency and PLL10-10% shows the most promise as a new gene carrier.

Keywords: Cytotoxicity, Gene delivery, Nanoparticles, Non-viral vector, Poly (L-lysine), Polyethylenimine.

1. Introduction

Most of the gene therapy clinical trials have been performed in cancers, inherited monogenic and cardiovascular diseases. Two main challenges in gene therapy include the lack of efficient gene delivery system or/and appropriate *in vivo* selection guidelines [1]. Various delivery strategies and gene carriers have been employed in gene therapy studies which are categorized into viral and non-viral systems. Viral vectors have been utilized in a variety of clinical trials such as retrovirals in severe combined immunodeficiency (SCID) [2], lentivirals in X-linked adrenoleukodystrophy (X-ALD) [3] or recombinant adeno-associated viruses (rAAV) in Leber's congenital amaurosis (LCA) diseases [4]. Although viral vectors exhibit high and rapid gene delivery efficiency, their applications have declined in recent years because (1) they have small capacity and are unable to carry large genes which are necessary for clinical applications, (2) they may activate proto-oncogenes due to random insertion of their genome into chromosomes [5], (3) they induce strong immune responses limiting multiple injections and (4) there are difficulties in large scale production. Safety-concerns about the viral vectors have promoted the development of non-viral carriers. Although the transfection efficiencies of non-viral vectors are low in short-term delivery as compared with viral vectors, they have advantages including less infection risk, low cost of production, unlimited plasmid size and low immunogenicity [6]. Because of the flexibility in structures and designs of non-viral carriers, there have been many attempts to improve transfection efficiency and overcome their deficiencies. Liposomes and polymers are the most investigated non-viral vectors [7]. Enormous libraries of polymeric nanoparticles have been examined for gene delivery such as polyethylenimine (PEI) [8], dendritic poly(amidoamine) (PAMAM) [9], poly(β -amino ester) [10], polypropylenimine (PPI) [11] and poly(L-lysine) (PLL) [12]. Cationic polymers especially polyethylenimine and poly(L-lysine) are popular because they are positively charged and contain intrinsic affinity for negatively charged oligonucleotides and cell surfaces [13]. Both polymers can effectively condense pDNA and protect it from enzymatic degradation. PEI reveals excellent buffering capacity which can facilitate rupture of lysosomes and release their cargo into the cytoplasm, whereas PLL exhibits weak transfection efficiency due to slow release of PLL/DNA polyplexes from endosomes [14]. Unlike PLL, PEI is not biodegradable and its cytotoxicity depends on its molecular weight and the type of cell tested.

In order to increase the transfection efficiency of PLL and to reduce the cytotoxicity of PEI, we synthesized copolymers by conjugating low molecular weight PEI (1.8 and 10 kDa) to a PLL core to take advantage of the properties of both PLL and PEI.

2. Materials and methods

2.1. Materials

Branched polyethylenimine with an average molecular weight of 10,000 Da (PEI 10 kDa) and 1800 Da (PEI 1.8 kDa), poly(L-lysine) (PLL; Mw ~15–30 kDa) were purchased from Polysciences, Inc. (Warrington, PA, USA). The pRL-CMV and pEGFPN1 plasmids were obtained from Promega (Madison, WI, USA). Fetal bovine serum (FBS) and Roswell Park Memorial Institute medium (RPMI) were purchased from Gibco (Gaithersburg, MD). Methylthiazolotetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; tissue culture grade), 6-bromohexanoic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (Munich, Germany). Ethidium bromide was purchased from Cinnagen (Tehran, Iran). Spectra/Por dialysis membranes (Spectrum Laboratories, Houston, TX, USA) were utilized for dialyses.

2.2. Synthesis of nanoparticles

The branched polyethylenimine (1.8 kDa and 10 kDa) were dissolved in double distilled water (ddH₂O), then 14 mg (excess amount) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was separately dissolved in ddH₂O and added to PEI while the mixture was stirred vigorously. Poly(L-lysine) was dissolved in dimethylsulphoxide (DMSO) and 6-bromohexanoic acid was added dropwise over a period of 2 h to a vigorously stirred solution. The reaction was allowed to proceed at room temperature for another 24 h and then diluted with

ddH₂O. The resulted solutions were dialyzed using 8000 cut-off membrane (Spectra/Pormembrane) against 150 mM NaCl for 1 day and then ddH₂O for 2 days to remove unreacted alkylcarboxylates. The PLL-alkylcarboxylate solution was added dropwise over a period of 2 h to vigorously stirred PEI solution and the reaction was proceeded for overnight. For purification, the mixture containing PLL-PEI conjugates was transferred to a dialysis membrane with 10000 and 25000cut-off for 1.8 kDa and 10 kDa PEI solutions, respectively (Spectra/Por membrane). Dialysis was performed against 150 mM NaCl saline solution for 1 day and then ddH₂O for 2 days to remove unreacted polymers. After the dialysis, the remained solution was lyophilized to yield a dry powder. Stock solutions (1 mg/ml) were prepared for further application.

2.3. Size distribution and zeta-potential measurements

The particle size and surface charge were measured for vector/pDNA polyplexes by dynamic light scattering (DLS) and laser dopplervelocimeter (LDV) using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The desired amount of conjugated polymers was diluted in 150 μ l filtered doubly-distilled water in physiological pH (pH = 7.4) and mixed with 150 μ l pDNA solution (containing desired amount of plasmid) and left for 20 min at room temperature to form the polyplexes. Then, 700 μ l water was added and the solution was utilized for size and zeta potential measurements. The final polymers concentration was at cationic/plasmid (w/w) ratio at C/P=4. Values were presented as mean $\pm$ SDfor three measurements.

2.4. Determination of buffering capacity

Synthesized conjugates with concentration of 0.4 mg/ml were dissolved in 1 ml of ddH₂O and the pH was adjusted to 12 using 1 M NaOH. The pH was measured using a pH meter (Mettler Todelo, Greifensee, Switzerland) at room temperature (about 25 $^{\circ}$ C) after addition of each five microliter of 0.1 M HCl until pH of 2.5 was obtained. The buffering capacity of polymers was estimated from the slope of pH plot versus the amount of HCl added.

2.5. Ethidium bromide (EtBr) exclusion assay

DNA condensation ability of nanopolymerswas evaluated by ethidium bromide (EtBr) exclusion assay. The fluorescence intensity of 400 ng/ml ethidium bromide solutionwas measured at λ_{ex} 510 nm and λ_{em} 590 nm using a Jasco FP-6200 spectrofluorimeter (Jasco Global, FP-6200, Japan). Upon addition of pDNA(10 μ g/ml) to ethidium bromide solution, the fluorescence intensity increased which was regarded as 100%. The decrease of EtBr fluorescence intensity was measured following addition of pre-determined amounts (2.5 μ l of 1mg/ml solution) of PLL-PEI conjugates to the pDNA-ethidium bromide solution. The experiments were performed in triplicate and results were reported by plotting the relative fluorescence intensity (%) against the carrier/plasmid ratio (w/w).

2.6. Measurement of primary amine content

The primary amines of PLL after grafting with 6-bromohexanoic acid was determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay using the standard method [15]. The difference in the amounts of primary amines on unmodified PLL and conjugated PLL, revealed the degrees of alkylcarboxylate grafting to PEI.

2.7. Preparation of plasmid DNA

The pRL-CMV plasmid (Promega, Madison, WI) coding Renilla luciferase reporter gene was transformed into *Escherichia coli* bacterial strain DH5 α . The transformed bacteria was cultured in selective Luria–Bertani (LB) medium and plasmid was extracted from the cell pellets using Qiagen endotoxin-free Mega Plasmid kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The concentration and purity of the plasmid were estimated using the A260 and A260/A280, respectively, using a UV-Visible spectrophotometer (Pharmaspec, UV-1700; Specto, Shimadzu, Japan). The highest purity and mostly supercoiled plasmids were selected after agarose gel electrophoresis.

2.8. Cell culture and cytotoxicity assay

Neuro2A (murine neuroblastoma) cells (ATCC CCL-131) were cultured in DMEM (1 g/l glucose, 2 mM glutamine) supplemented with 10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin. All cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured for 24 h before transfection and toxicity assay. Polyplexes containing 2µg plasmid/well were prepared at C/P ratios of 2:1, 4:1 and 6:1 (w/w). Polyplexes were mixed in aqueous solution (20 µl) without serum and incubated for 30 min at room temperature then added to each well. After 4 h, the medium was removed and replaced with 100 µl fresh complete growth media and incubated for additional 24 h. The metabolic activity assay (MTT) was performed by adding 20 µl of MTT solution (5 mg/ml) to each well and incubating the plates for 2 h at 37 °C. The absorbance was measured at 550 nm (reference wavelength 630 nm) using a microplate reader (ELx800; BioTekInc, Winooski, VT, USA). The relative metabolic activities were expressed as absorbance percentage of treated cells related to control cells ($\text{Cell viability} = A_{\text{treated}}/A_{\text{control}} \times 100$) and presented as the mean $\pm$ SD of triplicate.

2.9. Transfection procedure

Transfection experiments were performed in similar conditions with the same amount of polyplexes used for cytotoxicity experiment. The polyplexes were prepared in different C/P ranging from 2:1 to 6:1 and were added to cells. The medium was removed after 4 h and replaced with 100 µl of serum supplemented DMEM. The cells were harvested 24 h post-transfection and resuspended in lysis buffer. The luciferase activity was measured using PromegaRenilla Luciferase assay kit and a luminometer (Berthold Detection Systems, Pforzheim, Germany). The results were presented as relative light units (RLU) per number of seeded cells, mean $\pm$ SD, n =3. Branched 25 kDa PEI in C/P 0.8 was used as a positive control for transfection experiments.

2.10. Statistical analysis

Results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $p \leq 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Preparation and characterization of polyplexes

In the present study, 1.8 kDa and 10 kDa PEIs were grafted to PLL via an alkylcarboxylate (6-bromohexanoic acid) as the linker. Thus, alkylcarboxylate moiety was reacted with 10% and 50% of the PLL primary amine groups. The TNBS assay was performed to estimate the degree of PLL primary amine substitutions by alkylcarboxylate groups. As shown in Table 1, the percentages of substitutions were 8.2% and 35.8% for 10% and 50% feed mol ratio, respectively. In the next step, solution of alkylcarboxylate derivative of PLL was added dropwise to the activated PEI solution, to yield PLL-PEI conjugates (Fig. 1). New PLL-PEI conjugates with structure of PLL-hexanoate-10%-PEI 1.8 kDa and PLL-hexanoate-50%-PEI 1.8 kDa were abbreviated as PLP1.8-10% and PLP1.8-50%, respectively. Similarly, conjugates having PEI 10 kDa were abbreviated as PLP10-10% and PLP10-50%, respectively.

The effective diameters of polyplexes had been reported in the range of 30 to 500 nm [16]. The results showed that the new PLL-PEI conjugates were able to form nanoparticles in the range of 99-122 nm. However, their zeta potentials were lower than unmodified PEIs, the zeta potentials of these polyplexes were between 15.2 and 23.5 mV which increased with increasing the feed mole percentages of coupled PEI.

Table 1

The particle size and surface charge of nanoplexes. Data are expressed as mean values $\pm$ SD. PLL-hexanoic-10%-PEI and PLL-hexanoic-50%-PEI abbreviated to PLPX10% and PLPX50%, respectively. X represents the PEI molecular weight 1.8 or 10 for PEI 1.8kDa and PEI 10kDa.

NPs	Size of DNA/polymer complex (nm±SD)	Zeta potential of DNA/polymer complex (mV±SD)
PEI 1.8kDa	61.3±4.0	23±2
PLP1.8-10%	122.3±3.4	15.2±0.9
PLP1.8-50%	109.3±9.4	20.6±2.8
PEI 10kDa	87.6±2.5	24.2±3.7
PLP10-10%	101.8±15.7	21.2±2.6
PLP10-50%	99±7	23.5±2
PLL	73.6±1.06	4.9±2

Fig. 1. Synthesis of alkyl carboxylate PLL-grafted PEI derivatives.

3.2. DNA condensation and buffering capacity of polymers

The ability of PLL-PEI conjugates to condense pDNA and to form polyplexes is critical for efficient gene delivery into cells. The polyplex formation was evaluated by ethidium bromide (EtBr) exclusion assay. Ethidium bromide is an intercalating dye and could associate with free dsDNA. Fluorescence intensity decreases as polymer condense DNA and excludes EtBr from dsDNA. All conjugates were able to condense pRL-CMV plasmid

DNA at C/P ratios more than 2 whereas PLL showed a weak positive charge density and could not tightly bind to DNA and therefore, condensed pDNA at C/P more than 4 (Fig.2). Appropriate DNA condensation ability is one of the necessary features of polymers for gene delivery. Because of few positive charge of PLL, it condensed DNA at higher C/P ratio, whereas all modified polymers were able to condense pDNA in the presence of low polymer concentrations ($C/P < 1$). Sizes of prepared polyplexes were less than 200nm which were suitable for being taken up by the cells via clathrin-mediated endocytosis [17].

Fig.2. Condensation profile of pDNA, measured by ethidium bromide (EtBr) exclusion assay in HBG buffer. PLL-PEI conjugates with (a) 1.8 kDa and (b) 10 kDa PEI.

The PEI can easily induce proton sponge effect which is the main mechanism leading to osmotic swelling and rupturing, due to excellent buffering capacity over the pH range (5.5-7) of endosome [18, 19]. However, PLL exhibits weaker buffering capacity at this pH range which is attributed to the lack of secondary and tertiary amines [20]. Therefore it is expected that PLL alone could not facilitate endosomal release of polyplexes through osmotic burst mechanism. According to the results, all synthesized conjugates exhibited buffering capacity over the endosomal pH ranges similar to unmodified PEI 1.8 kDa and PEI 10 kDa, whereas the PLL showed weak buffering capacity at this critical pH range. These results confirmed the weak buffering capacity of PLL and indicated that PLL-PEI conjugates had the appropriate buffering capacity at the endosomal pH range.

Fig.3. The titration curves of PLL-PEI conjugates. At first, pH of all samples were adjusted to 12 with NaOH (1M) and then titration curves were plotted by subsequent addition of 5 μ l 0.1 M HCl until the pH reached 2. Deionized water and PEI 25 kDa were included as the controls.

3.3. Cytotoxicity and transfection efficiency of polyplexes

Although both polyethylenimine (PEI) and poly (L-lysine) (PLL) are the most investigated polymers as non-viral gene carriers, but there are drawbacks in their applications. PLL reveals poor transfection efficiency and low stability [14], while, PEI with its excellent “proton sponge” effect is not biocompatible and shows cytotoxicity [21]. Many attempts have been made to modify PLL structure and make a suitable gene delivery system such as pullulan-g-poly(L-lysine) [22] and PLL-PEG-FOL conjugate [23].

Contrary to PEIs, PLL is a biodegradable molecule and seems to be more suitable for in vivo applications. There are different PEIs with molecular weight ranging from few hundred to 1500 kDa [24] and cytotoxicity of PEIs depend on their molecular weight (MW), structure and the cell type used [21, 25]. It was reported that in vitro transfection efficiency increased with higher MW branched PEIs, ranging from 1.8 to 70 kDa [26]. In contrast, another study found that in vitro transfection activity decreased as MW increased from 1.8 to 70 kDa [27]. In our study, we synthesized PLL-PEI conjugates to take advantages of both PLL and PEI properties. Thus, 1.8 kDa and 10 kDa PEIs were added to primary amines of PLL via hexanoate as the linker.

The cytotoxicity of polyplexes was determined using MTT assay at C/P ratios of 2:1, 4:1 and 6:1 in Neuro2a cells. The cytotoxicity of the produced copolymers was similar to that of unmodified PEI. The cell viabilities after transfection with low molecular weight PEI derivatives were not significantly different from those of PEI 1.8 kDa itself (Fig. 4c). The cell viability of PLP10-10% enhanced at C/P=2 compared to unmodified PEI 10 kDa. Cytotoxicity increased at higher C/P ratios, though at lower grafting conjugate (i.e. PLP10-10%), it was not significantly different from those unmodified observed with PEI 10 kDa (Fig. 4d). However, PLP10-50% showed cytotoxicity at higher C/P ratios of 4:1 and 6:1. As expected, PLL showed no cytotoxicity at all C/P ratios tested.

1 The gene transfection efficiency of PLL-PEI conjugates was evaluated in Nero2A cells using Renilla luciferase
2 assay at C/P ratios of 2:1, 4:1 and 6:1. The results were compared to those of unmodified PEIs (1.8 kDa and 10
3 kDa) and PLL. In overall, the results showed the transfection efficiency in PLPX-10% series were enhanced
4 regardless of the molecular weight of used PEI. Higher degree of PEI substitution (PLPX-50%) resulted in
5 lower or no significant changes in gene transferring ability to cells. It was assumed that increase in grafting
6 percentage of PEIs in the PLL-PEI copolymers had an impact on the cytotoxicity of polyplexes thus reducing
7 the transfection activity. Although cell viability showed no differences in PLP1.8-10% or PLP1.8-50%
8 treatments, but PLP10-50% displayed higher cytotoxicity even more than that of unmodified PEI 10kDa at
9 higher C/P ratios which might resulted in lower transfection efficiency.

10 It was observed that both PLP1.8-10% and PLP10-10% were able to transfer plasmid DNA more efficiently
11 than their unmodified PEIs, while PLL itself showed the lowest transfection efficiency at all tested C/P ratios.
12 PLP1.8-10% enhanced gene transfection ability up to 2 and 2.8 folds at C/P 4 and 6, respectively. Transfection
13 efficiency of PLP10-10% increased about 4 and 3 folds at C/P 2 and 4, respectively (Fig. 4a-b).

14 The cytotoxicity of polyethylenimine is the most limiting aspect for utilizing them as a gene carrier. Different
15 modification of PEI were reported in attempts to reduce the cytotoxicity of PEI, such as PEGylation [28] or
16 acylation [29]. According to our results, PEI 10 kDa showed moderate cytotoxicity at low C/P, but cell viability
17 reduced as the amount of polymers increased. Former studies also revealed that PEI 1.8 kDa was less cytotoxic
18 and could be eliminated from body through kidneys.[30]. It was reported that low molecular weight PEIs were
19 less toxic but inefficient for gene delivery purposes [21]. Although several PEI 1.8 kDa molecules existed in
20 PLP1.8-10% structure but its cytotoxicity did not increase, while transfection efficiency increased about 2.8 fold
21 as compared to unmodified PEI 1.8 kDa. Interestingly, cell viability increased at low C/P ratio in PEI 10 kDa-
22 based polyplexes. Decrease in cytotoxicity may have resulted in enhancement of PLP10-10% transfection
23 efficiency by about 4 fold as compared to unmodified PEI 10kDa. These results indicated that our hypothesis for
24 achieving more efficient and less toxic non-viral vector was somewhat successful. Although the branched PEI
25 25 kDa with high cationic charge induced cytotoxicity due to that strong attachment and disruption of cell
26 membrane, but is still considered as golden standard for gene delivery studies [31].

27 Direct conjugation of PLL to linear PEI [32] was prepared and tested for gene delivery where amino groups of
28 PLL were partially grafted with low molecular weight linear PEI. The results showed reduction in cytotoxicity
29 and increase in transfection efficiency as compared to each of the unmodified polymers. Beside, conjugation of
30 biodegradable polycations such as polycarbonates and polyaspartamide to branched PEIs with low molecular
31 weights could reduce the safety concern of gene transfection [33, 34]. In a similar study, PLL was introduced
32 into branched PEI 25 kDa by ring-opening polymerization (ROP) of ϵ -benzyloxycarbonyl-L-lysine N-
33 carboxyanhydride [35]. In this study, different feed ratios of Lys(Z)-NCA were added to PEI which resulted in
34 new polymers for gene delivery to induce apoptosis.
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Fig.4. Transfection efficiency (a-b) and cell viability (c-d) of modified polymers at different polycation/plasmid ratios (w/w) of 2:1, 4:1 and 6:1. Neuro2A cells were transfected with polyplexes of PLP1.8 (a, c), PLP10 series (b, d). Polyplexes with PLL, PEI 1.8 kDa and 10 kDa utilized as controls at the indicated C/P ratios, as well as the polyplexes prepared with 25 kDa PEI in C /P 0.8. All experiments were performed in triplicate and data were presented as mean±SD. *p < 0.05, **p < 0.01 and ***p < 0.001 synthesized polymers as compared with unmodified PEIs.

4. Conclusion

In the present study, PLL-PEI conjugates with different molecular weight PEIs (1.8 kDa and 10 kDa) were synthesized. The physicochemical properties such as size, zeta potential and DNA condensation capability showed that these conjugates were suitable for gene delivery. The transfection efficiency of PLP10-10% was nearly four folds higher than that of PEI 10 kDa. The cell viability and transfection results exhibited that PLP1.8-10% and PLP10-10% were better gene transfer carriers for gene delivery as compared to unmodified PEI. Our results suggested the PLL-PEI conjugates as appropriate carriers for gene delivery purposes.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

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Figure captions:

- **Figure1.** Synthesis of alkylcarboxylate PLL-grafted PEI derivatives.
- **Figure2.** The ethidium bromide exclusion assay
- **Figure 3.** Buffering capacities of polymers.
- **Figure4.** Cellular toxicity and transfection efficiency of modified polymers.