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Alkyl cross-linked low molecular weight polypropyleneimine dendrimers as efficient gene delivery vectors

Faezeh Moghadam Ariaee [1], Maryam Hashemi [2], Sara Amel Farzad [1], Khalil Abnous [1], Mohammad Ramezani [2]*

[1] Pharmaceutical Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[2] Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

Objective(s): In recent years, polypropyleneimine (PPI) dendrimers have attracted great interest as non-viral gene delivery systems because of their attractive features including highly branched architecture with number of reactive end groups. However, without being structurally modified, they are not efficient gene carriers. In the present study, generation 2 and 3 (G2 and G3) of PPI dendrimers were conjugated with alkylcarboxylate groups as linker to enhance the transfection efficiency while maintaining their low cell toxicity.
Materials and Methods: First, 10-bromodecanoic acid was covalently attached to all available surface primary amines of PPI G2 and G3 to increase their lipophilicity. In the subsequent step, PPIs were conjugated to the alkylcarboxylate groups of alkylcarboxylate-PPI derivatives to increase the number of surface primary amines. Physicochemical properties of modified PPIs were determined. Transfection experiments (using both luciferase and green fluorescent protein (GFP)- expressing plasmids) and cytotoxicity assay were performed to evaluate the efficiency of the final derivatives.
Results: Fabricated vectors condensed DNA effectively so that polyplexes with appropriate size (below 155 nm) and positive surface charge were constructed. Cross-linked low molecular weight PPIs (G2 or G3) with decanoate linkage increased transfection efficiency significantly while maintaining the low cytotoxicity. PPI G2 derivative exhibited increased buffering capacity which is believed to be responsible for better proton sponge mechanism leading to higher transfection efficiency.
Conclusion: Our results indicated that oligomerization of low molecular weight PPI (PPI G2-alkyl-PPI G2 conjugate) could be an approach to increase the transfection efficiency and to lower the cytotoxicity of low molecular weight polycations.

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Introduction

One of the main problems in gene therapy is the efficient transfer of genetic material into cells of various tissues without inducing a toxic response. Most of the current research efforts are focused on designing more effective non-viral vectors because of their ease of preparation, lower immunogenicity and well-defined structures (1, 2).

Among non-viral vectors, dendrimers are new class of polymers which have three dimensional structures, with high number of functional groups on their surfaces (3, 4). One of the most widely investigated dendrimers is polypropyleneimine (PPI). Different generations of PPI dendrimers are commercially available, however, without being structurally modified, they are not efficient gene carriers. There is a correlation between molecular weight of polymers and their tansfection efficiency and cytotoxicity. Polymers with higher molecular weight (HMW) are highly effective in gene transfection but are highly cytotoxic (5, 6). Thus, one strategy for obtaining low cytotoxicity is to structurally modify low molecular weight polymers so that their transfection efficiency enhances while maintaining low cytotoxicity.

In many studies, low molecular weight PEI derivatives were prepared by cross-linking them with various linkers to form ester (7, 8), disulfide, amide (9, 10), imine, carbamate (11, 12), and ketal linkages (13-15). In another study, high efficient gene carriers were prepared using disulfide cross-linked low generation PAMAM dendrimers (generation 2, G2) at a linker/dendrimer molar ratio of 1:1 (16).

Here, we first evaluated the use of alkylcarboxylate chains to increase lipophilicity of the PPI G2 and G3 structure based on the results of previous studies (17, 18). Then, PPIs were conjugated to the alkylcarboxylate groups of alkylcarboxylate-PPI to increase the surface primary amines. It was hypothesized that PPI G2 and G3 with lower cell toxicity *in vitro* and *in vivo* could be cross-linked with alkyl groups to enhance the transfection efficiency while maintaining their low cell toxicity.

**Corresponding author:* Mohammad Ramezani. Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. Tel: +98-51-38823255; Fax: +98- 51-3823251; *email:* ramezanim@mums.ac.ir

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Materials and Methods

Materials

Generation 2 and 3 of PPI preparations were obtained from Symochem (BV, Netherlands). 10-Bromohexanoic acid, N-hydroxybenzotriazole (HOBt), 1-ethyl-3-[3-dimethylaminopropyl] carbod - iimide hydrochloride (EDC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Munich, Germany). Chloroform was obtained from Merck (Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied by GIBCO (Gaithersburg, USA). Ethidium bromide was purchased from Cinnagen (Tehran, Iran).

Synthesis of alkylcarboxylate derivatives

In brief, 50 mg PPI G2 or G3 were dissolved in 5 ml chloroform separately. The degree of grafting was adjusted to 100% of the calculated amount of primary amines in PPIs. Therefore, 10- bromodecanoic acid was dissolved separately in 5 ml chloroform in the amount of 142.8 and 130.9 mg according to calculated primary amines of PPI G2 and PPI G3, respectively. 10-Bromodecanoic acid solution was added drop-wise to the vigorously stirred PPI solutions. After 24 h of incubation at room temperature, chloroform was removed using rotary evaporator (Heidolph, Germany). The product was dissolved in water and then lyophilized. The degree of substitution of PPI primary amines with alkylcarboxylate was determined by estimation of free primary amine groups through reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (19).

Conjugation of PPI to PPI-alkylcarboxylate

PPI G2 or G3 was covalently coupled to alkylcarboxylate derivatives of PPI using HOBt and EDC as coupling agents. Briefly, 50 mg alkylcarboxylate derivative of either PPI G2 or G3 was dissolved in 1 ml distilled water and stirred with 1 ml EDC solution for 30 min. Then, 2 ml aqueous solution containing either 217.5 mg PPI G2 or 306.2 mg PPI G3 and HOBt was added drop-wise to the vigorously stirred solutions of either PPI G2 or G3 and the mixtures were incubated for 24 h at room temperature. The reaction mixture was dialyzed against distilled water using dialysis membrane (3.5 and 12-14 kDa cut-off for PPI G2 and PPI G3 products, respectively, Spectra/Por membrane) to remove the unreacted materials. The final products were lyophilized. Amide bond formation was confirmed by Fourier transform infrared spectroscopy (FTIR). The ^{1}HNMR spectra of final products in D_2O were recorded at room temperature using a Bruker Avance-III 300.

Preparation of plasmid DNA

Renilla luciferase (pRL-CMV) plasmid (Promega, Madison, WI) was transformed into *E. coli* bacterial strain DH5α. The plasmid was extracted from the culture pellets using a Qiagen endotoxin free mega plasmid kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Ethidium bromide (EtBr) exclusion assay

The ability of PPI conjugates to condense pDNA was measured by the ethidium bromide (a DNA-intercalating dye) exclusion assay (20). Solutions of either PPI or PPI derivative in HBG buffer (HEPES-buffered glucose, 20 mM HEPES, 5%glucose, pH 7.4) were added to a solution of 5 μg/ml pRL-CMV and 400 ng/ml EtBr in HBG buffer at different range of carrier to plasmid weight ratios (C/P). Fluorescence intensities were recorded at excitation and emission wavelengths of 510 and 590 nm, respectively on a Jasco FP-6200 spectrofluorimeter (Tokyo, Japan). The 0% condensation standard was prepared by mixing plasmid with EtBr and measuring the resultant fluorescence intensity whereas the fluorescence intensity of EtBr without plasmid was used as the 100% condensation standard. Results are reported as mean±SD of samples in triplicate.

Particle size and zeta potential measurements

The particle size and zeta potential of transfection complexes were measured using Dynamic Light Scattering (DLS) and Laser Doppler Velocimetry (LDV), respectively by a Malvern NanoZS instrument and DTS software (Malvern Instruments, UK). Various amounts of PPI or modified PPIs in HBG buffer were added to a final DNA concentration of 5 μg/ml. The mixture was incubated for 20 min at room temperature. The results are reported as mean±SD. Each mean represents the average value of 30 measurements from each independent sample.

Buffering capacity determination

The buffering capacity of PPI derivatives over a pH range of 12 to 2.5 was determined by acid–base titration. Briefly, each PPI derivative (0.4 mg) was dissolved in 1 ml of double-distilled water. The pH was adjusted to 12 with NaOH solution. The pH of the solution was measured after each addition of 0.1M HCl in subsequent 5 μl increasments until the pH was reduced to 2.5.

Luciferase reporter gene expression

To study the transfection activity of the vectors, Neuro-2A murine neuroblastoma cells were seeded at a density of 1×10^4 cells/well in 96-well plates in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin, 24 h before transfection. Polyplexes at various C/P ratios [carrier / *p*DNA, (wt/wt)] containing 200 ng pDNA were added into each well and incubated at 37 ˚C for 4 hr. Subsequently, the media were replaced with fresh complete media and incubated for an additional 24 h at 37 ˚C. The luciferase activity assay in cell lysates was measured using a Promega luciferase assay kit on a luminometer (Berthold Detection System, Pforzheim,

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Germany). The results are reported as mean±sd, for n=3.

Cytotoxicity assay

The cytotoxicity of the various polyplexes prepared with PPI derivatives were assessed using MTT assay. In brief, cells were seeded in 96-well plates at an initial density of 1×10⁴ cells/well and then incubated for 24 h. Cells were then treated with the same amount of polyplex used for transfection experiments as described before. After 4 hr, the medium was replaced with fresh complete medium. At the end of the experiments, 20 µl of sterile filtered MTT stock solution in PBS (5 mg/ml) was added to each well. After 4 hr, the MTT solution was carefully removed from each well, and 100 µl DMSO was added to dissolve the MTT formazan crystals. The absorbance was read at 575/630 nm. The cell viability (%) relative to control wells containing cell culture medium without polyplex was calculated as [A] test/ [A] control × 100%.

Analysis of GFP reporter gene expression by flow cytometry

Neuro 2A cells were seeded in 12-well plates at a density of 8×10^4 cells per well 24 hr prior to transfection. Polyplexes in free-serum medium containing 3 μg DNA (pEGFP) and different concentration of carriers were added to each well. Medium was replaced after 4 h of incubation. Positive GFP-expressing cells transfected with modified PPIs was observed under a JuLi Smart Fluorescence Cell Analyzer (Ruskinn Technology Ltd, Bridgend,UK) 24 h after treatment. Then, cells were harvested by treatment with trypsin/EDTA solution and kept on ice until analysis. The percentage of transfected cells was determined using Partec flow cytometer (Partec, Germany). GFP fluorescence was excited at 488 nm and emission was detected using a 530/40 nm bandpass filter and a 575/25 nm bandpass filter to analyze GFP-positive cells by diagonal gating.

Transfection with bafilomycin A

The possibility of proton sponge mechanism was determined using bafimycin A1 (a vacuolar proton pump inhibitor). Luciferase gene transfer experiments were conducted either in the absence or presence of bafilomycin A1 in Neuro2a cells. Before addition of polyplexes, cells were incubated for 30 min at 37 °C with bafilomycin A1 (200 nM per well). Luciferase transfection assay was done as described above.

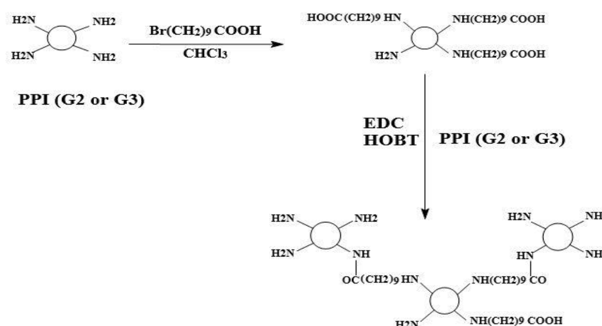
Statistical analysis

To analyse data, Student's t-test was done using GraphPad PRISM® 5 software. Data are presented as the mean±standard deviation (sd.). The statistical significance was determined using Student's *t*-test. *P* values of ≤0.05 were considered significant.

Results

Synthesis of modified PPI

The synthesis approach for chemical modification of either PPI G2 or G3 are presented in Scheme 1.



Scheme 1. Synthesis scheme for the preparation of PPI-PPI conjugates using 10-bromodecanoic acid as the linker.

Both PPI G2 and G3 were modified by alkylcarboxylation of their primary amines with 10-bromodecanoic acid to increase their lipophilicity. Then, either unmodified PPI G2 or G3 was conjugated to carboxylate groups of decanoate derivative of PPI prepared in the first reaction to increase the number of surface primary amines. The degrees of grafting were 77.9 and 76.3 for PPI G2 and PPI G3, respectively which were less than those calculated for 100% substitution of primary amines. The conjugation of the PPIs to alkycarboxylate chain was confirmed using FTIR spectra (Figure 1). The presence of signal corresponding to C=O stretching (carboxylic acid) was demonstrated by a strong peak at 1733.93 cm^{-1} for alkylcarboxylate PPI G2 which was weakened

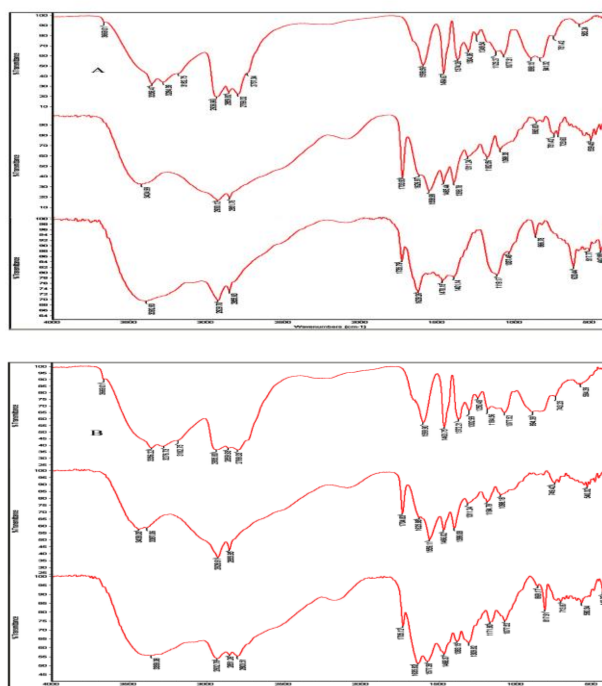


Figure 1. Modification of PPIs was demonstrated by comparing Fourier transform infrared (FTIR) spectra of unmodified PPIs, alkylcarboxylate PPIs and corresponding PPI-alkyl-PPI conjugates. Spectra A: upper spectrum: unmodified PPI G2, middle spectrum: alkylcarboxylate PPI G2, lower spectrum: PPI G2-alkyl-PPI G2 conjugate. Spectra B: upper spectrum: unmodified PPI G3, middle spectrum: alkylcarboxylate PPI G3, lower spectrum: PPI G3-alkyl-PPI G3 conjugate

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to 1735.79 cm^{-1} for PPI G2-alkyl-PPI G2 conjugate due to amidation of carboxylate in final product. Amide bond formation in final product is shown by a distinct peak at 1629.26 cm^{-1} for PPI G2-alkyl-PPI G2 conjugate but either it is either weakened or absent for alkylcarboxylate PPI G2 and unmodified PPI G2, respectively (Figure 1.A). Absorption band at 1734.83 cm^{-1} for alkylcarboxylate PPI G3 at 1735.12 cm^{-1} which was weakened in PPI G3-alkyl-PPI G3 conjugate is attributed to stretching of C=O bond (carboxylic acid). The carbonyl amide stretch was indicated by a peak at 1635.82 cm^{-1} for PPI G3-alkyl-PPI G3 conjugate but it is either weakened or absent for alkylcarboxylate PPI G3 and unmodified PPI G3, respectively (Figure 1.B). The structure and purity of final products were also confirmed by ^{1}HNMR (D_2O) (Figure 2). For PPI G2-alkyl-PPI G2 conjugate and PPI G3-alkyl-PPI G3 conjugate, peak "a" corresponded to $-CH_2CO-$ in alkylcarboxylate chain, peak "b" was assigned to $-CH_2-$ adjacent to 1 º, 2 º, and 3 º amines in modified PPI and peak "c" was attributed to –CHN- and also $-CH_2NHCO-$ resulting from amide formation (upper spectrum in Figure 2 A,B). Unmodified PPI G2 and PPI G3 indicated peak "b" corresponding to $-CH_2-$ adjacent to 1º and 3º amines in PPIs. Neither peak "a" nor peak "c" appeared for unmodified PPIs (lower spectrum in Figure 2 A, B).

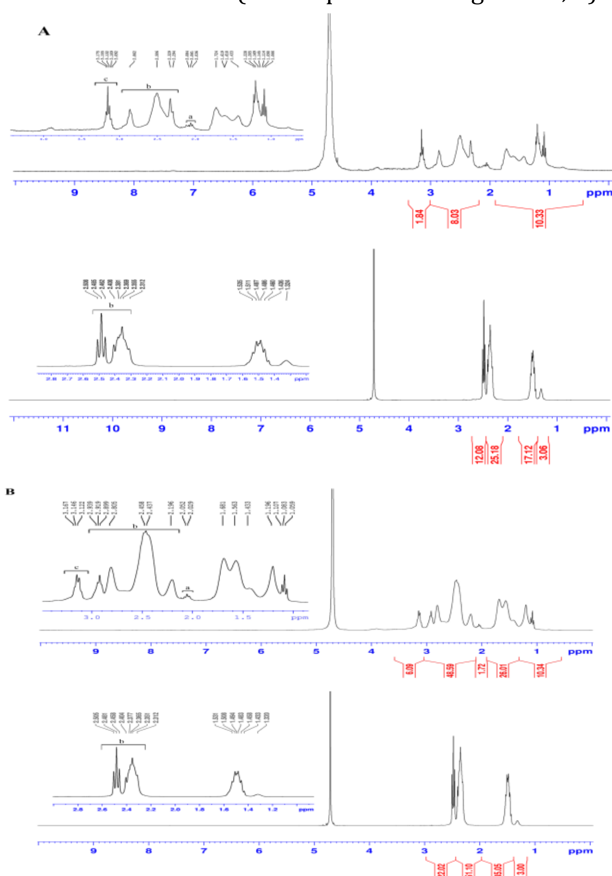

Figure 2. Characterization of the final PPI derivatives by ^{1}HNMR. A: upper spectrum: PPI G2-alkyl-PPI G2 conjugate, lower spectrum: unmodified PPI G2. B: upper spectrum: PPI G3-alkyl-PPI G3 conjugate, lower spectrum: unmodified PPI G3

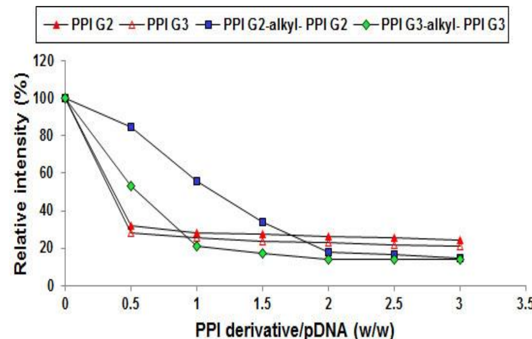

Figure 3. The EtBr exclusion assay for pDNA complexed with PPI G2 or G3 derivatives

DNA condensation

Binding affinity of the PPI derivatives for plasmid DNA was evaluated by the ethidium bromide (EtBr) exclusion assay. The results of EtBr exclusion analysis indicated that all PPIs derivatives could reduce the relative fluorescence by more than 80% at C/P ratio 2, whereas PPI G2-alkyl-PPI G2 derivatives exhibited lower fluorescence intensity than all other polymers tested at C/P ratio 1 indicating that the derivatization of PPI G2 has lower binding affinity for DNA (Figure 3).

Size and surface charge of PPI derivatives complexed with plasmid DNA

Particle size and zeta potential measurements are shown in Table 1. All polyplexes were prepared in distilled water with 5 μg plasmid DNA. The polyplexes of PPI derivatives showed smaller size than unmodified PPIs. The PPI-alkyl-PPI conjugates exhibited a typical particle size of < 155 nm.

Buffering capacity of PPI conjugates

Acid–base titration experiments were carried out to evaluate the buffering capacity of PPI G2 and its conjugate. The buffering capacity in the endosomal pH range from 4.5 to 7.5 is responsible for driving the osmotic burst mechanism for release of polyplexes from the endosome, a key step in the transfection process. The higher buffering capacity of polymer is known as a higher resistance to pH variations during titration with the same amount of HCl. As seen in Figure 4, PPI G2-alkyl-PPI G2 conjugate had higher buffering capacity than PPI G2 itself.

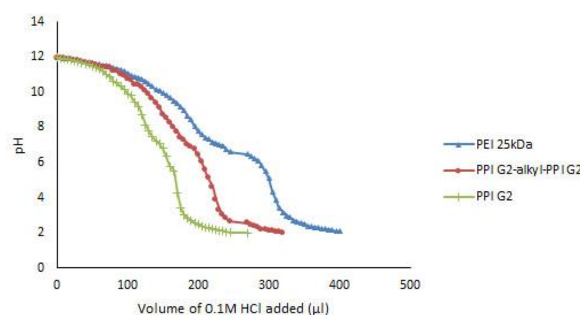

Figure 4. Buffering capacity assay for PEI 25, PPI G2 and its derivative by titration of aqueous polymer solutions (0.4 mg/ml) with 0.1 M HCl

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Table 1. The particle size and zeta potential of PPI-alkyl-PPI conjugates at different C/P ratios

Polymer	Carrier to plasmid ratio (C/P, wt/wt)	Particle size (nm) ± SD	Potential zeta (mV) ± SD
PPI G2	2	244.5 ± 14.4	9.75 ±0.5
PPI G2	4	238.7 ± 9.3	10.11 ± 0.8
PPI G2	6	231.2 ± 11.6	10.26 ± 2
PPI G2-alkyl-PPI G2	2	153.7 ± 6.3	5.46 ± 1.5
PPI G2-alkyl-PPI G2	4	147.9 ± 10.2	6.32 ± 0.2
PPI G2-alkyl-PPI G2	6	142.4 ± 13.5	8.85 ± 0.6
PPI G3	2	263.3 ± 19.6	8.02 ± 0.9
PPI G3	4	255.2 ± 12	9.91 ± 2.3
PPI G3	6	238.7 ± 9.6	9.45 ± 1.4
PPI G3-alkyl-PPI G3	2	150.8 ± 10.8	8.08 ± 1.5
PPI G3-alkyl-PPI G3	4	149.5 ± 4.9	8.23 ± 1.9
PPI G3-alkyl-PPI G3	6	143.1 ± 5.7	12.51 ± 0.7

Transfection efficiency experiment

Neuro2A cells were transfected with 200 ng of plasmid DNA per 10,000 cells, complexed with PPI derivatives at C/P ratios of 2, 4 and 6 based on the concentration of PPI. The transfection data indicated that the crosslinking of PPI G2 molecules through an alkylcarboxylate chain resulted in the enhancement of the ability of PPI to transfer a reporter gene into the cell line. As shown in Figure 5, there was a significant increase in transfection efficiency with PPI G2-alkyl-PPI G2 conjugate compared to PPI G2 itself at C/P ratio of 2 (P<0.05), 4 (P<0.001) and 6 (P <0.05). This enhancement was observed with PPI G3-alkyl-PPI G3 conjugate as well compared to PPI G3 itself at C/P ratio of 2 (P<0.001), 4 (P<0.01) and 6 (P<0.01). PPI G2-alkyl-PPI G2 conjugate had significantly higher gene transfection efficiency when tested at C/P 4 , 6 in comparison to PEI 25 kDa, as the positive control (P <0.01). The highest gene transfection efficiency was obtained at C/P 4 with PPI G2-alkyl-PPI G2 conjugate which exhibited a 1.7 fold increase over that of PEI 25 kDa.

Analysis of GFP reporter gene expression

To confirm the result of transfection study, positive EGFP-expressing cells were observed using fluorescent microscopy. As shown in Figure 6, PPI G2-alkyl-PPI G2 conjugate was more efficient at delivering reporter genes into Neuro-2A cells compared to other vectors.

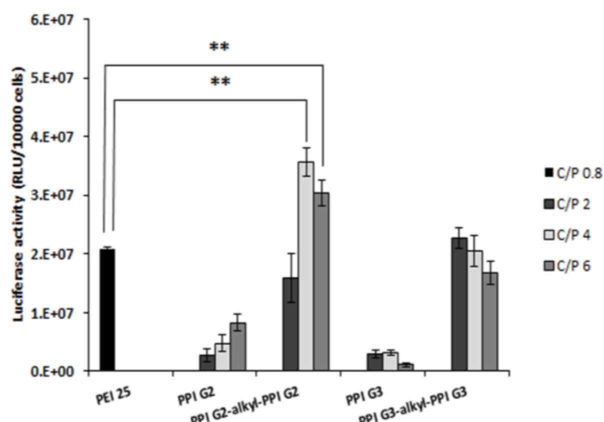

Figure 5. The transfection efficiency of unmodified and modified PPIs after complexation with pDNA encoding the luciferase gene. PEI 25 kDa was used as control. * for P<0.05, ** for P<0.01, or *** for P<0.001 (t-test)

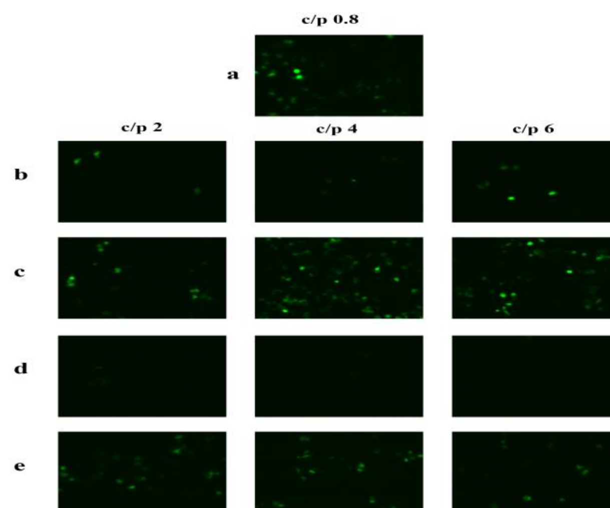

Figure 6. The transfection efficiency of green fluorescent protein in Neuro 2A cells expressed at C/P ratio 2, 4, and 6 for PPIs and PPI derivatives by fluorescent microscopy. a) PEI 25 kDa as the control, b) PPI G2, c) PPI G2-alkyl-PPI G2 conjugate, d) PPI G3, e) PPI G3-alkyl-PPI G3 conjugate

To further study the transfection efficiency, PPIs-alkyl-PPIs were evaluated for the EGFP positive cells by flow cytometry. It was found (Figure 7, Figure 8) that PPI G2-alkyl-PPI G2 conjugate caused significantly higher transfection efficiency in Neuro-2a cells line at C/P ratio 4 in comparison with intact PPI G2 and PEI 25 kDa as positive control.

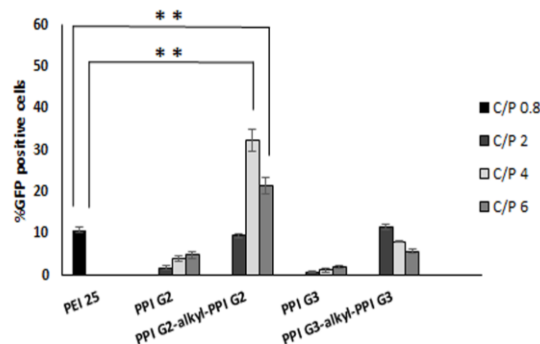

Figure 7. Expression of green fluorescent protein in Neuro-2a cells transfected with polyplexes prepared from an EGFP expressing plasmid DNA and PPIs or the PPIs modified with alkylcarboxylate chains. * for P<0.05, ** for P<0.01, or *** for P<0.001 (t-test)

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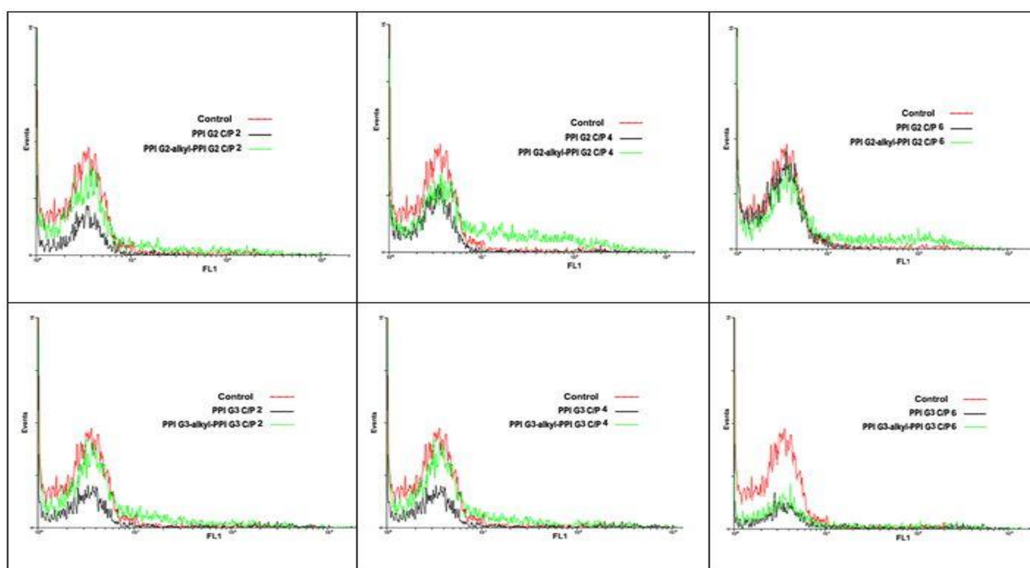

Figure 8. Flow cytometry histogram of cells transfected with polyplexes prepared from an EGFP expressing plasmid DNA and PPIs or the modified PPIs with alkylcarboxylate chains

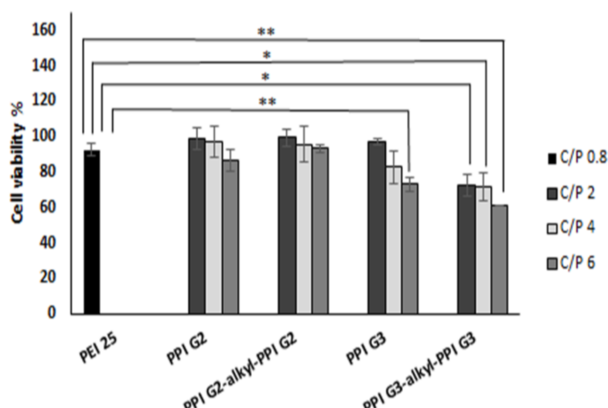

Figure 9. Cellular toxicity of PPI-alkyl-PPI conjugates at different C/P ratios. * for P <0.05, ** for P <0.01 or *** for P <0.001 (t-test)

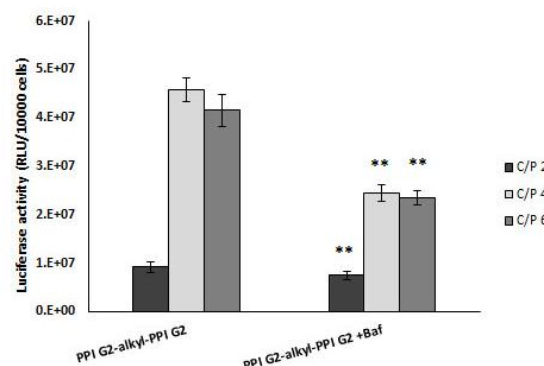

Figure 10. Transfection efficiency of PPI G2-alkyl-PPI G2 and unmodified PPI G2 in bafilomycin-treated cells

Cytotoxicity assay

The cytotoxicity of PPI-alkyl-PPI conjugates was evaluated by the MTT assay in the neuro-2A cell lines. PEI 25 kDa was used as positive control. As shown in Figure 9, cells treated with PPI G2-alkyl-PPI G2 conjugate was more viable than those with unmodified PPI G2 at C/P ratio 6. However, PPI G3-alkyl-PPI G3 conjugate showed more cytotoxicity than PEI 25 kDa at all C/P ratios.

Bafilomycin A1 experiment

Bafilomycin A1 as a proton pump inhibitor could inhibit endosomal acidification following cellular uptake of particles and thus inhibits the proton sponge effect. Transfection activity of PPI G2-alkyl-PPI G2 conjugate in the presence of bafilomycin A1 decreased by 1.2, 1.9 and 1.8 fold at C/P ratios 2, 4 and 6, respectively (Figure 10).

Discussion

In the present study, we synthetized a novel cross-linked low molecular wight derivative of PPI (G2 or G3) with 10-bromodecanoic acid linkage. In recent years, dendrimers have gained a great attention because of highly branched with well-defined and three-dimensional structures (21, 22). Polypropyleneimine (PPI) is one of the most investigated dendrimers for gene delivery purposes. Increasing the molecular weight of this polymer often enhances gene expression but poses a risk of increased cytotoxicity (23).

It was reported that when low-molecular-weight polycationic vectors were crosslinked with biodegradable bonds, the transfection efficiency can increase while maintaining low cytotoxicity. Various degradable linkages were potentially used to synthesize degradable PEIs through crosslinking between PEIs and grafting agents (24). Yu-Qiang Wang *et al* synthesized a small-molecular-weight PEI derivative (PEI 800 Da) with ethylene biscarbamate linkages (15). This polymer exhibited significantly higher transfection efficiency and lower cytotoxicity compared to PEI 25 kDa at the same concentration. In another study, branched PEI (2 kDa) was crosslinked with PEI 2 kDa

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or linear PEI (423 Da) via ester and/or amid linkage (25). The results showed that these derivatives could improve gene delivery efficiency of small PEIs both *in vitro* and *in vivo* significantly without increasing the cytotoxicity. In recent study, crosslinked bioreducible polypropyleneimine-cystamine bisacrylamides (PPI-CBAs) were synthesized as gene delivery systems. PPI-CBAs showed high transfection efficiency comparable to PEI 25 KDa. However, bioreducible PPI–CBAs displayed considerable cytotoxicity (26).

In the current study, PPI (G2 and G3) were first modified with 10-bromodecanoic acid to provide the carboxylate functional group which provided the possibility of further functionalization and also increase of lipophilicity. TNBS assay revealed the degrees of alkylcarboxylate grafting as 77.9% and 76.3% for PPI G2 and PPI G3, respectively which were less than those calculated for 100% substitution of surface primary amines. In the next step, the carboxylic end group of the decanoate derivative was further conjugated to PPIs with the same generation through amide linkages.

In several investigations, modification of cationic polymers with alkyl chains was used to improve the gene delivery efficiency. Conjugation of PEI with cholesterol and myristate (1), acetylation (27, 28) and amide linkage of saturated hydrocarbon chains (29) could increase gene delivery of PEI. Our group have recently reported that alkylcarboxylation of primary amines on PEI and PPI could significantly enhance transfection efficiencies while maintaining low cell toxicity (18, 30, 31).

One of the primary requirements of a cationic polymer to form nano-polyplexes is suitable DNA condensation activity. Ethidium bromide assay showed that crosslinking of LMW-PPIs decreased fluorescence intensity more than PPI alone at higher C/P ratios. In addition, particle size decrease was observed in comparison to PPIs when PPIs were conjugated together through alkylcarboxylate linkage. Since primary amine groups have a significant role in DNA condensation, therefore final products with higher density of amines on the surface could condense DNA more efficient than unmodified PPIs.

The size and surface charge of the polyplexes play a major role in the uptake efficiency and cytotoxicity (32). In the present study, the average size of PPI-alkyl-PPI conjugates were in the range of 142 to 154 nm. In this range of particle size, nanoparticles would enter the cells through endocytosis (33, 34). Furthermore, the surface charge of the modified conjugates remained enough positive to efficiently condense the plasmid DNA and to interact with the cell membrane.

In transfection study with Renilla and EGFP plasmids, there was a significant increase in transfection efficiency with both PPI G2-alkyl-PPI G2 and PPI G3-alkyl-PPI G3 conjugates compared with corresponding PPI G2 and PPI G3, respectively. The reason may be due to enhancement of lipophilicity, smaller size and increased number of amines on the surface. The highest transfection and lowest cytotoxicity was observed with PPI G2-alkyl-PPI G2 conjugate.

There was an enhancement in transfection efficiency of PPI G2-alkyl-PPI G2 at C/P 4 compared to C/P 2 due to the presence of further amounts of polyplex to deliver plasmid DNA efficiently. However, while there was no significant difference between cytotoxicity of polyplex at C/P ratios 4 and 6, the decrease in transfection efficiency at C/P ratio 6 could be attributed to the fact that at higher polymer ratio in polyplex (C/P 6 compared to 4), the unpackaging of the polyplex which is required for the release of the DNA inside either cytosol or nucleus is going to happen in lesser extent. On the other hand, by increasing C/P ratio, a plateau level was obtained in which the polyplex had the maximum ability to deliver the plasmid DNA. PPI G3-alkyl-PPI G3 conjugate was more cytotoxic than other derivaties at all C/P ratios tested. It may be due to higher amines on the surface.

It has been suggested that the amine groups in cationic polymers participate in DNA binding, promote cellular uptake of polyplexs, exert endosome buffering effect and enhance the release of DNA into the cytoplasm. On the other hand, these groups involved in cytotoxicity of polycations (35). Therefore, the number of amine groups on the surface of polymer has to be optimized so that while it provides the suitable effects as described above leading to significant transfection efficiency, it could also exert lower cytotoxicity.

We further examined the ability of modified vector to release from degrading environment of endosomes by evaluating the extent of protonation of the polymer at practical pH ranges. Endosomal escape is believed to be the major limiting step for polycation-mediated transfection, therefore, great attention has been paid to the modification of polymer structure to increase proton buffering ability at endosomal pH. Various buffering agents such as histidine (36, 37), was conjugated onto polymers to enhance the transfection efficiency.

In the present study, the results of buffering capacity experiment showed that crosslinking of low molecular wight PPI G2 *via* decanoate linkage could increase buffering capacity of unmodified PPI G2 at the endosomal pH range as compared with PEI 25 kDa.

The higher transfection efficiency of PPI-alkyl-PPI conjugates may be explained by enhanced endosomolytic activity based on the proton sponge effect (38, 39). To investigate this in more detail, luciferase gene transfer experiments were conducted in either absence or presence of bafilomycin A1 in Neuro2A cells. Bafilomycin A1 inhibits specifically the vacuolar type of H^+-ATPases by inhibiting the endo-/lysosomal proton pump. A 1.9 fold decrease in transfection efficiency at C/P ratio 4 in the presence of bafilomycin suggested the proton sponge effect as an

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underlying mechanism for enhanced transfection of PPI G2-alkyl-PPI G2 conjugate. Singh *et al* showed that when low molecular weight polyethylenimine (PEI) was crosslinked with glycerol molecules, the lower contant of glycerol (LG-PEI) with higher buffering capacity indicated more transfection efficiency than higher content of glycerol (HG-PEI0). Since these vectors showed apparently similar DNA binding affinity, DNA unpacking and cellular uptake abilities, therefore the buffering capacity of the polymers primarily effects on endosomal escape and subsequent transfection (40).

Conclusion

Our results indicated that oligomerization of low molecular weight PPI (PPI G2-alkyl-PPI G2 conjugate) could be an approach to increase the transfection efficiency and lower the cytotoxicity of low molecular weight polycations.

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