Nanomedicine Journal

Received: Feb. 25, 2013; Accepted: Apr. 12, 2013
Vol. 1, No. 1, Autumn 2013, page 55-62

Online ISSN 2322-5904
http://nmj.mums.ac.ir

Original Research

Gene transfer enhancement by alkylcarboxylation of poly(propylenimine)

Maryam Hashemi[1], Hamideh Sahraie Fard[2], Sara Amel Farzad[3], Hamideh Parhiz[3], Mohammad Ramezani[3*]

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

Abstract

Objective(s): Among synthetic carriers, dendrimers with the more flexible structure have attracted a great deal of researchers' attention in the field of gene delivery.
Materials and Methods: Followed by the promising results upon hydrophobic modification on polymeric structures in our laboratory, alkylated poly (propylenimine)-based carriers were synthesized by nucleophilic substitution of amines with alkyl moieties and were further characterized for their physicochemical and biological characteristics for plasmid DNA delivery.
Results: Although not noticeably effective gene transfer activity for hexanoate- and hexadecanoate- modified series was observed, but alkylation by decanoic acid significantly improved the transfection efficiency of the final constructs up to 60 fold in comparison with unmodified poly(propylenimine) (PPI). PPI modified by 10-bromodecanoic acid at 50% grafting, showed significantly higher gene expression at c/p ratio of 2 compared to Superfect as positive control. Overall, modification of PPI with 50% primary amines grafting with 10-bromodecanoic acid could increase the transfection efficiency which is occurred at lower c/p ratio when compared to Superfect, i.e. less amount of modified vector is required to exhibit the same efficiency as Superfect.
Conclusion: Therefore, the obtained constructs seem to be safer carriers for long-term gene therapy applications.

Keywords: Alkylation, Gene delivery, Hydrophobic modification, Poly (propylenimine)

***Corresponding author:** *Mohammad Ramezani, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.*
Tel: +985118823255, Email: Ramezanim@mums.ac.ir

Introduction

Synthetic carriers have been proposed as promising and safer alternatives to viral systems for gene delivery purposes (1). However, they are still suffering from the lower transfection efficiency compared to viral counterparts. Therefore, various studies have focused on the synthesis of efficient agents with gene delivery capability compared to viral ones. Among synthetic carriers, dendrimers with the more flexible structure have gained a great deal of researchers' attention in the field of gene delivery (2, 3). Dendrimers consist of three characteristic components: (a) an initiator core, (b) an interior layer (generations), com-posed of repeating units, radially expanded from the initial core and (c) exterior (terminal functionality) attached to the outermost interior generation (4, 5). Their unique structure confer them a superior multivalent surface, exposing high number of functional groups on the surface to do various modifications on (4). Poly (amidoamine) (PAMAM) and poly (propylenimine) (PPI) can be mentioned as the two most investigated dendrimers for both drug and gene delivery purposes (6). The differences between the two polymers are the hydrodynamic diameter and internal micro-environments. PPI dendrimers are smaller, more stable in higher temperature and less polar in internal microenvironment compared to PAMAM (7). A wide range of modifications from PEGylation to more sophisticated surface engineering have been studied on these structures to improve their gene transfer efficacy while reducing toxicity (8-12). However, fewer studies have been conducted on gene delivery efficiency of PPI dendrimers in comparison to PAMAM. The aim of this research was to evaluate the effect of alkylation on the transfection efficiency of PPI G4. Our group recently reported successful gene delivery by grafting different length alkyl moieties onto branched polyethylenimine-based structures (13-16).

Employing the hydrophilic parts in the structure of various multi-functional vectors has been investigated by other groups as well which could significantly improve their gene transfer properties (17, 18). In the current study, we synthesized the PPI-based carriers by nucleophilic substitution reaction of a series of bromoalkylcarboxylates with different alkyl chains with surface amine groups on PPI followed by their physicochemical and biological characterization for plasmid DNA delivery.

Materials and Methods

Materials

Generation 4 Poly(propyleneimine) was obtained from Symo-chem. BV, Netherlands. 6-bromohexanoic acid, 10-bromodecanoic acid, 16-bromohexadecanoic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra zolium bromide (MTT) and N-[2-hydroxythyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) were purch-ased from Sigma-Aldrich (Munich, Germ-any). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from GIBCO (Gaith-ersburg, USA). Ethidium bromide was supplied by Cinnagen (Tehran, Iran).

Preparation of plasmid DNA

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

Conjugation of alkylcarboxylate to polypropyleneimine

The synthesis of PPI-alkylcarboxylates was carried out according to the previously reported method with minor modifications (13). In Brief, PPI G4 was dissolved in chloroform. 6-bromohexanoic acid, 10-bromodecanoic acid or 16-bromohexa-decanoic acid was dissolved in chloroform and added drop-wise to the vigorously stirred PPI solution. After 24 h stirring at room temperature, the solvent was removed under reduced pressure and the residue was

dissolved in DDW. The final product was dialyzed using dialysis Spectra/Por membrane with the range of 1000-3000 Da molecular weight cut-off (Spectrum Laboratories, Houston, USA) once against ethanol-water, then twice against water.
This was to remove the unreacted alkylating agents and other reagents used for facilitating the reaction. The final purified product was lyophilized for further use. The degree of grafting was measured by TNBS assay at 405 nm.

Ethidium bromide (EtBr) exclusion assay

PPI derivatives were prepared in HBG buffer (1 ml, 20 mM, pH 7.4 containing 5% glucose) and added at different weight polymer/weight plasmid ratios (c/p ratios) to a solution containing pRL-CMV (5 µg/ml) and EtBr (400 ng/ml). Fluorescence intensity was measured at 510 nm excitation and 590 nm emission wavelengths using a Jasco FP-6200 spectrofluorimeter (Tokyo, Japan). The fluorescence intensity of DNA with EtBr corresponds to 0% condensation, whereas the fluorescence intensity of EtBr without plasmid corresponds to 100% condensation. Results are reported as mean ±SD, n=3.

Particle size and zeta potential measurements

The particle size and zeta potential of PPI or the optimal modified PPIs were measured using Dynamic Light Scattering (DLS) and Laser Doppler Velocimetry (LDV) respectively by a Malvern Nano ZS instrument and DTS software (Malvern Instruments, UK). Three independent samples (n=3) were prepared and the results are reported as mean±SEM.
Each mean represents the average value of 30 measurements from each independent sample.

Cell transfection with plasmid RL-CMV

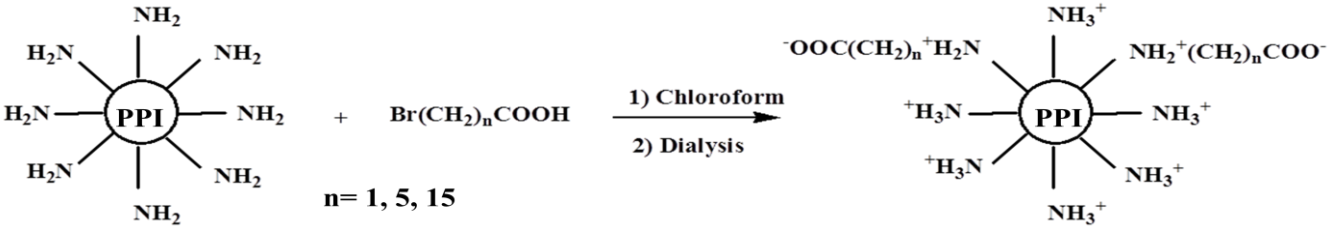
For cell transfection, murine neuroblastoma (Neuro-2A) cells (ATCC, CCL-131) were seeded at a density of 1×10^4 cells/well in 96-well plates and incubated in DMEM supplemented with 10% fetal bovine serum for 24 h. PPI G4, modified-PPI and Superfect were complexed at different weight/weight ratios ranging from 1:1, 2:1 and 4:1 with 200 ng pRL-CMV added into each well.
After 4 h, the media was removed and replaced with fresh complete media.
The cells were further incubated for 24 h at 37^{o}C and the luciferase activity in cell lysate was measured by using promega luciferase assay kit on Luminometer (Berthlod Detection System, Pforzheim, Germany).
The results are reported as mean±SD, n=3.

Cell transfection with GFP reporter gene expression

Neuro 2A cells were seeded at a density of 8×10^4 cells per well in 24-well plates. Polyplexes with 3 μg of DNA (pEGFP) at different c/p ratio of Superfect, PPI G4 and the vector with the highest luciferase transfection efficiency were added to the cells. Medium was refreshed after 4 h.
Cells were harvested 24 h post-transfection and kept on ice until analysis. The transfected cells were observed using a JuLI™ digital fluorescence microscope **(**NanoEnTek).

Cytotoxicity assay

Neuro 2A cells were seeded in 96-well plates at an initial density of 1×10^4 cells/well and incubated for 24 h. Cells were then treated with the same amount of polyplexes used for transfection experiment.
After 3 h, the med-ium was replaced with fresh complete medium. After 24 h, 10 µL sterile filtered MTT stock solution in PBS (5 mg/ml) was added to each well and the absorbance was read at 575/630 nm.
The cell viability (%) relative to control wells containing cell culture medium without polymer was calculated by [A] test/ [A] control × 100.



Scheme 1. Representative pathway for the synthesis of alkylcarboxylate PPI G4.

Results and Discussion

Synthesis and physicochemical characterization of alkyl-PPI G4

As recently discussed by several groups, alkylation of cationic polymers might be advantageous due to enhancement in both lipophilicity and interaction with biological membranes (13, 15-21).

In order to investigate the influence of the alkyl chains on the transfection and cytotoxicity of PPI G4 dendrimer, different percentages of primary amines of PPI were reacted with 6-bromohexanoic, 10- bromodecanoic or 16-bromohexadecanoic acids separately.

The degrees of substitution, determined by TNBS assay, were in the range of 9-13%, 19-21% and 35-42% for supposed 10, 30 and 50 % alkylation of primary amines, respectively. Synthesis route is presented in Scheme 1. The obtained vectors were presented as PPI–X–Y%, in which X is the number of carbons in the alkyl chain and Y% is the percentage of primary amines supposed to be substituted with alkyl chains.

The first requirement for cationic polymers in gene delivery systems is the ability to condense DNA into polyplexes with optimal size ranges (22). Ethidium bromide (EtBr) exclusion assay can be used to measure the extent of association of DNA with cationic polymers (23). At the c/p ratio of 1 and 2, almost all of vectors were able to condense the plasmid DNA (Figure 1). As expected, with increasing the percentage of substitution, more polycation required to condense DNA. It could be due to negatively charged carboxylate anions on the surface of the polycation which reduce the electrostatic binding affinity of the polymer to DNA. In other studies with hydrophobic moieties grafted onto cationic polymers, more vectors were required to highly condense DNA as the percentage of grafting increased (24-26).

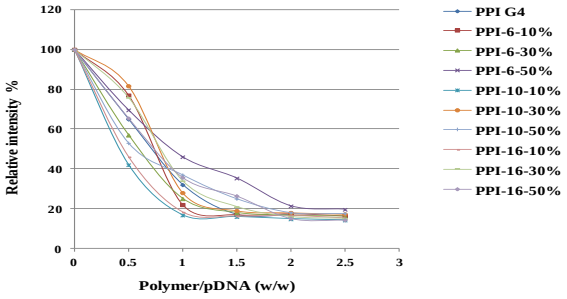


Figure 1. DNA binding affinity of alkylcarboxylate derivatives of PPI measured by ethidium bromide exclusion assay.

Transfection with luciferase and GFP reporter genes

The ability of the alkylcarboxylated PPI G4 to transfect mammalian cells was evaluated in murine neuroblastoma cells. Cells were transfected with polyplexes containing 200 ng of pRL-CMV at various c/p ratios. As shown in Figure 2, the optimum alkylcarboxylate chain length was found to be ten carbons. Although not noticeably effective for hexanoate- and hexadecanoate- modified series, but alkylcarboxylation with 10-bromodecanoic acid could significantly improve the transfection efficiency of the final constructs up to 60 fold in comparison with unmodified PPI G4 (Figure 2B). For example, PPI modified by 10-bromodecanoic acid at 50% grafting, showed significantly higher gene expression at c/p ratio of 2 compared to Superfect as positive control.

Hydrophobic modification has been investigated as an efficient strategy in improving the gene transfection efficiency of cationic polymers (19-21). Our group has recently reported the structure of a series of alkyl-oligoamine derivatives of PEI 10 kDa with enhanced efficiency due to increase in lipophilicity while maintaining low toxicity (13, 27). These derivatives of PEI 10 kDa showed comparable transfection efficiency to PEI 25 kDa with significantly lower cell toxicity (13). In another study, gene expression in MSCs was enhanced using PAMAM dendrimer G5 linked to hydrophobic chains. However in this case, higher transfection efficiency wasobserved in vectors containing the shortest hydrophobic chains (19).

In our study, the results obtained revealed a significant improvement by alkylation of PPI G4 as PPI itself has low transfection efficiency. The alkylcarboxylate chains with intermediate number of carbons, i.e. 10 carbons seem to be the optimal alkyl length for the alkylcarboxylate-modified PPI G4 series compared to Superfect. In a series of alkylcarboxylated PEI 10 kDa which were synthesized by Dehshahri *et al* (13), the authors suggested the more favorable hydrophobic-hydrophilic balance on the surface of polymer as a probable reason of the observed higher gene transfection efficiency. However, they demonstrated the 6 carbon alkyl chain as the optimal number of carbons with 10 and 30% substitution in their study. The longer optimal alkyl length in our study compared to the previous PEI 10 kDa derivatives may be due to the fact that PPI G4 has lower amine content than PEI 10 kDa. Therefore, it may need stronger synergistic hydrophobic content to exhibit an optimal gene transfection effect. The longer alkyl chains would probably be more effective at disturbing endosomal membrane and facilitating endosomal release. That may be the reason for the observed higher transfection efficiency by PPIs with ten carbon atoms alkyl chains compared to the ones with 6 carbon atoms.

The most efficient vector in luciferase gene experiments was further used to transfect the Neuro2A cells by the GFP plasmid. Figure 3 shows positive GFP-expressing cells transfected with PPI-10-50%, PPI and Superfect at c/p ratios of 2 and 4.

Cytotoxicity assay

The cytotoxicity of polyplexes prepared with alkylcarboxylation of PPI G4 was evaluated by MTT assay in Neuro2A cell culture. Although not significant, but in most modified PPIs, a slightly decrease in cellular viability was observed compared to unmodified PPI at almost all c/p ratios. The results also showed that cytotoxicity was dependent on degree of substitution of primary amines of PPI with alkylcarboxylate residues especially at higher c/p ratio, i.e generally higher substitution resulted in higher toxicity.

In a study done by Santos *et al* (19) in which PAMAM dendrimer G5 was conjugated to hydrophobic chains, the less lipophilic functionalized dendrimers were as cytotoxic as G5 dendrimers whereas the more lipophilic functionalized dendrimers exhibited a cytotoxicity profile similar to Superfect. However, cytotoxicity results obtained in this study is in correlation with our previous studies in which hydrophobic modification did not significantly increase the toxicity (15, 16), although it was also reported that alkylcarboxylation improved the cellular viability in their experiments (13).

Particle size and surface charge of optimal PPI derivatives

Particle size and zeta potential measurements of the polyplexes from the most efficient vectors are shown in Figure 5. The PPI-10-50% derivative exhibited a typical particle size of < 200 nm and surface charges about 20-24 mV, both suitable for a nanoparticle to be used for gene delivery purposes.

Conclusion

In conclusion, modification of PPI G4 with substitution of 50% of its primary amines by

10-bromodecanoic acid increased the transfection efficiency which was occurred at lower c/p ratio compared to Superfect making it a safer vector by using lesser amount for long-term gene therapy applications.

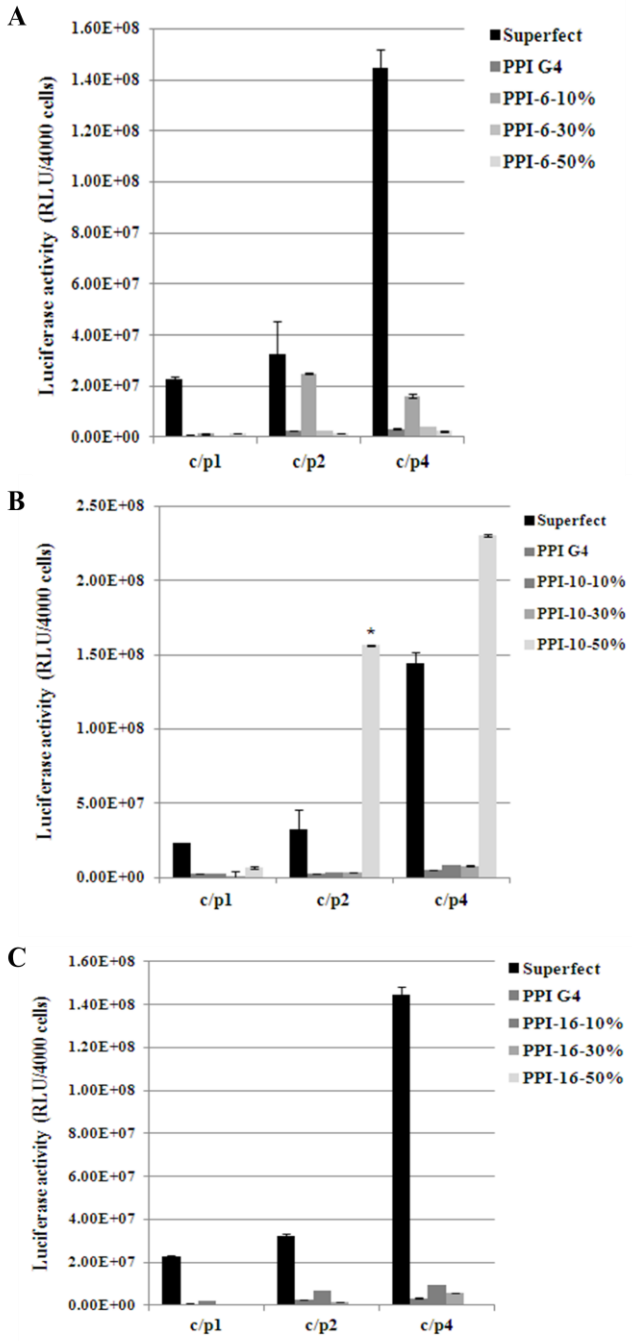


Figure 2. Transfection efficiency of nanoparticles formed with alkylcarboxylate-grafted PPI G4, PPI G4 and Superfect as positive control.

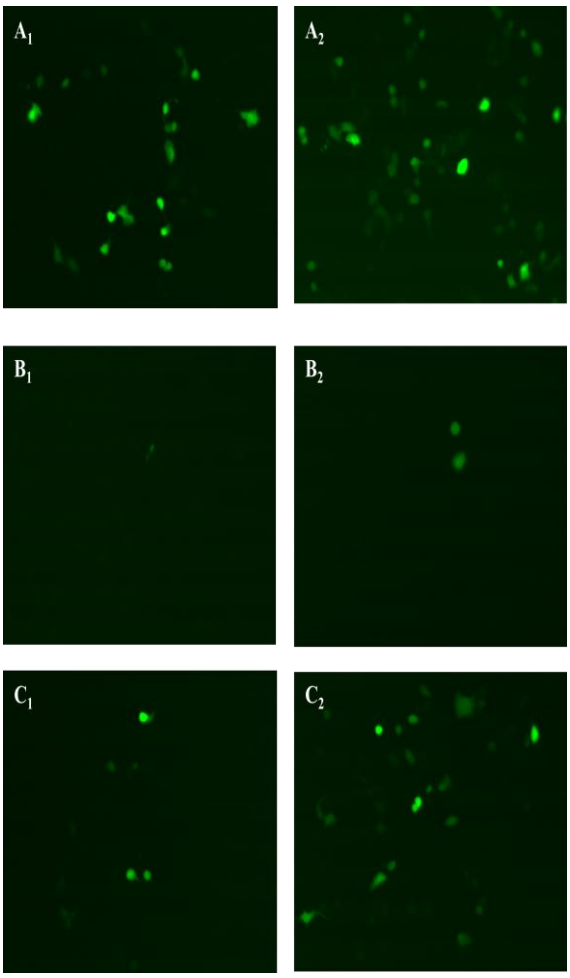


Figure 3. The transfected cells with GFP-expressing plasmid in Neuro 2A cells: (A_1) PPI-10-50% c/p2, (A_2) PPi-10-50% cp4, (B_1) PPI G4 c/p2, (B_2) PPI G4 c/p4, (C_1) Superfect c/p2, (C_2) Superfect c/p4.

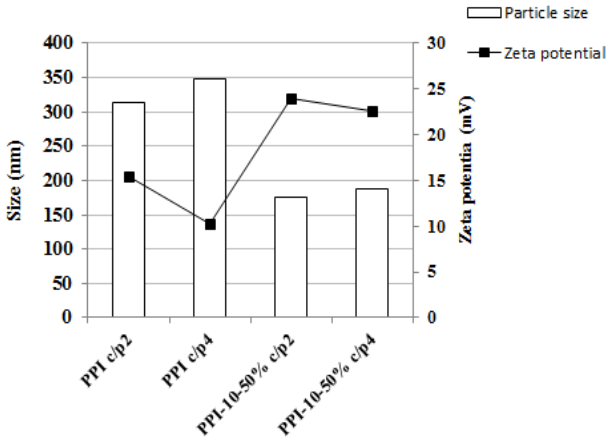


Figure 5. Size and zeta potential of the polymer/DNA nanoparticles prepared the best modified-PPI vector and unmodified PPI G4.

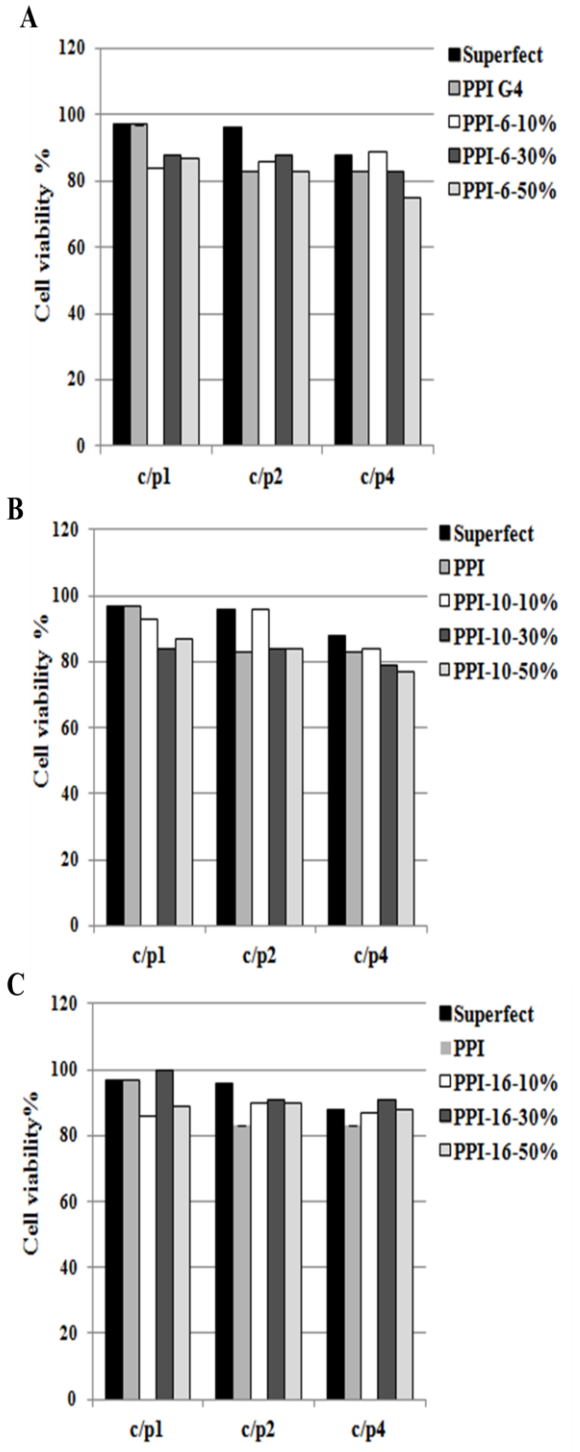


Figure 4. Viability profile of Neuro2A treated with alkyl-modified PPI G4, unmodified PPI G4 and Superfect.

Acknowledgments

This work was supported financially by a research grant from the Pharmaceutical Research Center for Research of Mashhad University of Medical Sciences, Mashhad, Iran.

References

1. Li SD, Huang L. Non-viral is superior to viral gene delivery. J Control release. 2007; 123(3): 181-183.
2. Dufes C, Uchegbu IF, Schatzlein AG. Dendrimers in gene delivery. Adv Drug Deliv Rev. 2005; 57(15): 2177-2202.
3. Parekh HS. The advance of dendrimers--a versatile targeting platform for gene/drug delivery. Curr Pharm Des. 2007; 13(27): 2837-2850.
4. Nanjwade BK, Bechra HM, Derkar GK, Manvi FV, Nanjwade VK. Dendrimers: emerging polymers for drug-delivery systems. Eur J Pharm Sci. 2009; 38(3): 185-196.
5. Tomalia DA, Baker H, Dewald J, Hall M, Kallos G, Martin S, et al. A new class of polymers: starburst-dendritic macro molecules. Polym J. 1985; 17: 117-32.
6. Shao N, Su Y, Hu J, Zhang J, Zhang H, Cheng Y. Comparison of generation 3 polyamidoamine dendrimer and generation 4 polypropylenimine dendrimer on drug loading, complex structure, release behavior, and cytotoxicity. Int J Nanomedicine. 2011; 6: 3361-3372.
7. Shishu, Maheshwari M. Dendrimers: The Novel Pharmaceutical Drug Carriers. IJPSR. 2009; 2(2): 493-502.
8. Takahashi T, Hirose J, Kojima C, Harada A, Kono K. Synthesis of poly(amidoamine) dendron-bearing lipids with poly(ethylene glycol) grafts and their use for stabilization of nonviral gene vectors. Bioconjug Chem. 2007; 18(4): 1163-1169.
9. Wen Y, Guo Z, Du Z, Fang R, Wu H, Zeng X, et al. Serum tolerance and endosomal escape capacity of histidine-modified pDNA-loaded complexes based on polyamidoamine dendrimer derivatives. Biomaterials. 2012; 33(32): 8111-8121.
10. Biswas S, Dodwadkar NS, Piroyan A, Torchilin VP. Surface conjugation of triphenylphosphonium to target poly(amidoamine) dendrimers to mitochondria. Biomaterials. 2012; 33(18): 4773-4782.
11. Russ V, Gunther M, Halama A, Ogris M, Wagner E. Oligoethylenimine-grafted poly propylenimine dendrimers as degradable and biocompatible synthetic vectors for gene delivery. J Control Release. 2008; 132(2): 131-140.
12. Aldawsari H, Edrada-Ebel R, Blatchford DR, Tate RJ, Tetley L, Dufes C. Enhanced gene expression in tumors after intravenous administration of arginine-, lysine- and

leucine-bearing polypropylenimine polyplex. Biomaterials. 2011; 32(25): 5889-5899.
13. Dehshahri A, Oskuee RK, Shier WT, Hatefi A, Ramezani M. Gene transfer efficiency of high primary amine content, hydrophobic, alkyl-oligoamine derivatives of poly ethylenimine. Biomaterials. 2009; 30(25): 4187-4194.
14. Oskuee RK, Dehshahri A, Shier WT, Ramezani M. Alkylcarboxylate grafting to polyethylenimine: a simple approach to producing a DNA nanocarrier with low toxicity. J Gene Med. 2009; 11(10): 921-932.
15. Parhiz H, Hashemi M, Hatefi A, Shier WT, Farzad SA, Ramezani M. Molecular weight-dependent genetic information transfer with disulfide-linked polyethylenimine-based nonviral vectors. J Biomater Appl. 2013; 28(1): 112-124.
16. Hashemi M, Parhiz BH, Hatefi A, Ramezani M. Modified polyethyleneimine with histidine-lysine short peptides as gene carrier. Cancer Gene Ther. 2011; 18(1): 12-19.
17. Forrest ML, Meister GE, Koerber JT, Pack DW. Partial acetylation of polyethylenimine enhances in vitro gene delivery. Pharm Res. 2004; 21(2): 365-71.
18. Thomas M, Klibanov AM. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. Proc Natl Acad Sci U S A. 2002; 99(23): 14640-14645.
19. Santos JL, Oliveira H, Pandita D, Rodrigues J, Pego AP, Granja PL, et al. Functionalization of poly(amidoamine) dendrimers with hydrophobic chains for improved gene delivery in mesenchymal stem cells. J Control Release. 2010; 144(1): 55-64.
20. Morales-Sanfrutos J, Megia-Fernandez A, Hernandez-Mateo F, Giron-Gonzalez MD, Salto-Gonzalez R, Santoyo-Gonzalez F. Alkyl sulfonyl derivatized PAMAM-G2 dendrimers as nonviral gene delivery vectors with improved transfection efficiencies. Org Biomol Chem. 2011; 9(3): 851-864.
21. Eltoukhy AA, Chen D, Alabi CA, Langer R, Anderson DG. Degradable terpolymers with alkyl side chains demonstrate enhanced gene delivery potency and nanoparticle stability. Adv Mater. 2013; 25(10): 1487-1493.
22. Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev. 2003; 55(3): 329-347.
23. Geall AJ, Blagbrough IS. Rapid and sensitive ethidium bromide fluorescence quenching assay of polyamine conjugate-DNA interactions for the analysis of lipoplex formation in gene therapy. J Pharm Biomed Anal. 2000; 22(5): 849-859.
24. Gabrielson NP, Pack DW. Acetylation of polyethylenimine enhances gene delivery via weakened polymer/DNA interactions. Biomacromolecules. 2006; 7(8): 2427-2435.
25. Doody AM, Korley JN, Dang KP, Zawaneh PN, Putnam D. Characterizing the structure/function parameter space of hydrocarbon-conjugated branched polyethylenimine for DNA delivery in vitro. J Control Release. 2006; 116(2): 227-237.
26. Tseng WC, Jong CM. Improved stability of polycationic vector by dextran-grafted branched polyethylenimine. Biomacromolecules. 2003; 4(5): 1277-1284.
27. Oskuee RK, Dehshahri A, Shier WT, Ramezani M. Alkylcarboxylate grafting to polyethylenimine: a simple approach to producing a DNA nanocarrier with low toxicity. J Gene Med. 2009; 11(10): 921-932.