Archive of SID

Iranian Journal of Basic Medical Sciences
Vol. 11, No. 1, Spring 2008, 33 - 40
Received: Jan 14, 2008; Accepted: Apr 28, 2008

IJBMS

Modified Polyethylenimine: Self Assemble Nanoparticle Forming Polymer for pDNA Delivery

[1]Reza K. Oskuee, [1]Ali Dehshahri, [2]Wayne. T. Shier, *[1]Mohammad Ramezani

Abstract

Objective
Polyethylenimine (PEI), a readily available synthetic polycation which has high transfection efficiency owing to its buffering capacity was introduced for transfection a few years ago. But it has been reported that PEI is cytotoxic in many cell lines. In this study, in order to enhance the transfection efficiency of 10 kDa PEI and reduce its toxicity, hydrophobic residues were grafted on PEI.
Materials and Methods
PEI polymers were modified by adding hydrophobic chains to the primary amines of PEI in different degrees of grafting using bromoacetic acid derivatives with different lengths. These polymers were complexed with plasmid DNA at different C/P ratios and the resulting nanoparticles were characterized by dynamic light scattering and EtBr-DNA binding assay to determine particle sizes and complex formation, respectively. Cytotoxicity and transfection efficiency of the polymers were also tested in cultured Neuro2a cell line.
Results
DNA condensation measurement revealed that the resulted polymers could form polyplexes with plasmid DNA and they have the ability to condense DNA in relatively low amounts of polymers. Particle size measurement of polyplexes showed that they form particles in the size range of below 190 nm. Transfection experiments showed that polymers which have been modified with hexanoic derivative could transfect pDNA as good as 25 kDa PEI with the advantage of being much less toxic.
Conclusion
Results indicate that the structure modifications of PEI accomplished in this study play a significant role in increasing the transfection efficiency and without inducing the cytotoxicity compared to PEI itself.

Keywords: Gene delivery, Nanoparticle, Polyethylenimine, Transfection

1- Department of Biotechnology, Pharmaceutical and Biotechnology Research Centers, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
*Corresponding author: Tel: +98 - 511- 8823255; Fax: +98- 511- 8823251; email: ramezanim@mums.ac.ir; P.O. Box: 91775-1365, Mashhad, Iran.
2- Department of Medicinal Chemistry, University of Minnesota-Twin Cities, Minneapolis, MN 55455, USA

www.SID.ir

Archive of SID

Introduction

Human gene therapy promises to prevent, treat, or cure a variety of conditions including several inheritable or acquired diseases. However, gene delivery remains a limiting challenge (1, 2). Generally, two different approaches have been utilized for the delivery of nucleic acids in gene therapy; viral vectors and non-viral delivery systems. Cationic polymers and cationic lipids are by far the most widely used vectors in non-viral gene and oligonucleotide delivery (3).

Viral vectors have been applied to deliver therapeutic genes into living cells, but their broad use is affected by the limited size of the genetic material that can be delivered with severe safety risks (4). Viral vectors including retroviruses, adenoviruses and adeno-associated viruses impress by their high efficiency in introducing their genetic material. Viral vectors show excellent transfection efficiencies. However, they develop a high immunogenicity after repeated administration, since the mammalian immune system has developed strategies to eliminate viral invaders as well. Other problems associated with viral vectors are their potential oncogenicity due to insertional mutagenesis and the limited size of DNA that can be carried (5-8).

Cationic lipids such as Transfectam® are dioctadecylamidoglycyl spermine (DOGS), a synthetic, cationic lipopolyamine molecule. The strong positive charge contributed by the spermine headgroup gives the molecule a high affinity for DNA, coating the DNA with a cationic lipid layer, which facilitates binding to the cell membrane (9). Polyethylenimine (PEI), a readily available synthetic polycation was introduced for transfection a few years ago (10). PEI has a high density of cationic groups such as secondary and tertiary amines whose charge is pH-dependent so that they can act as endosomal buffering systems. Therefore, the nucleic acid can be released from the lysosome without degradation (endosomal escape) (10, 11). But it has been reported that PEI is cytotoxic in many cell lines (3). The effect of various chemical modifications of nitrogen atoms on the efficiency of polyethylenimines (PEIs) as synthetic vectors for the delivery of plasmid DNA into cells has been investigated including the conjugation of different residues to its amines such as surface coating with hydrophilic poly(ethylene glycole) (11, 12), ligand tagging (13,14), dextran grafting (15) and hydrophobic modifications (16, 17). There are also, reports that hydrophobic modification of hydrophilic PEI changed its characteristics such as method of interaction with cell membranes or channels as well as nuclear pores and could result in higher transfection efficiency (16, 18) or lower transfection efficiency (19).

In this study we developed a novel nonviral gene transfer system based on the advantages of both liposomes and polycations in order to combine the osmotic burst mechanism with the lipid depletion mechanism for lysing endocytotic vesicles after uptake of the DNA-containing complex by endocytosis. Therefore, PEI 10 kDa was modified by grafting of a short and medium length alkyl chains in two different percentages of grafting and tested for their transfection efficiency.

Materials and Methods

Materials

Branched polyethylenimine with an average molecular weight of about 10000 (PEI 10 kDa), were purchased from Polyscience Company, Inc. Branched polyethylenimine (PEI; average MW= 25 kDa), Bromoacetic acid, 6-Bromohexanoic acid, N-[2-hydroxythyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), 3-[4,5-imethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Munich, Germany). Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from GIBCO (Gaithersburg, USA). All solvents used, purchased from Aldrich, were of the highest purity available. Dialyses were carried out by using Spectra/Por dialysis (Spectrum Laboratories, USA).

www.SID.ir

Archive of SID

Methods

Preparation of PEI-carboxylic chain

For the preparation of PEI-carboxylic chain, 1-bromoacetic acid and 6-bromohexanoic acid were dissolved in dimethylformamide (DMF). PEI 10 kDa was also dissolved in the same solvent. The former was added dropwise to the latter for 2 hrs. The reaction was allowed to proceed at room temperature for 24 hrs. The final product was dissolved in water. The resulting solution was poured into a dialysis membrane (8000 cutoff, Spectra/Por membrane) and dialyzed against double-distilled water (DDW) for 3 days to exclude unreacted materials. After the dialysis, the aqueous solution was lyophilized.

Determination of the degree of grafting by TNBS

The branched PEI has an average of 25% primary, 50% secondary, and 25% tertiary amines according to its structure. The amounts of primary amines on unmodified PEI and those on modified-PEI were determined using 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) according to the standard protocol (20). The degrees of grafting were calculated by the differences in the amounts of primary amines on the unmodified PEI and those on the modified PEI.

Measurements of the size and zeta-potential of the DNA-polymer complexes

Unmodified and grafted PEIs were dissolved in water. The size and zeta-potential of the DNA-polymer complexes were examined using a salt-free buffer (20 mM Hepes, 5.2% glucose, pH 7.0) (HBG). The desired amounts of cationic polymers were diluted in 125 µl of each buffer and mixed with an equal volume of the same buffer containing DNA. The surface charge and particle size of the DNA/polymer complexes were analyzed using a Malvern Zetasizer nano ZS (Malvern, UK).

Measurement of the interactions between DNA and cationic polymer

Ethidium bromide; a DNA-intercalating dye, was used to examine the association of DNA with the cationic polymers. A solution of 400 ng/mL EtBr in HBG was prepared with further addition of 10 µg/mL of pDNA. The fluorescence intensity of EtBr was measured at an excitation wavelength 510 nm and emission wavelength 590 nm with a 5-nm slit using an spectrofluorimeter (Jasco; FP-6200, Japan) and fluorescence was set to 100%. Equal amoumts of modified polymers were added stepwise to the pDNA EtBr solution and the fluorescence intensity was recorded. All measurments were done in triplicate and a graph was constructed by plotting the relative fluorescence intensity (%) against the polymer concentration/plasmid DNA phosphate ratio.

Preparation of plasmid DNA

Plasmid DNA encoding *Renilla* luciferase (pRL-CMV) (Promega, Madison, WI), was transformed into *E. coli* bacterial strain DH5α, incubated in selective Luria-Bertani (LB) medium and extracted from the culture pellets using a QIAGEN endotoxin free Mega Plasmid kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The purity and identity of the plasmid was confirmed by agarose gel electrophoresis.

Cell culture

Neuro2a murine neuroblastoma cells (ATCC CCL-131), were grown in DMEM supplemented with 10% FBS, streptomycin at 100 µg/ml and penicillin at 100 U/ml. All cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

Transfection Procedures

Cells were seeded at a density of 1×10^4cells/well in 96 well plates one day prior to transfection experiments, and grown in the appropriate medium with 10% fetal bovine serum. The cell lines were 60-90% confluent at the time of transfection. Polymer/ pRL-CMV complexes were prepared in HBG. Each complex solution was further incubated for 20 mins at room temperature and added to the

www.SID.ir

Archive of SID

cells. Transfection was performed in complete medium for 4 hrs. The medium was replaced with a fresh complete medium and gene expression was assayed 24 hrs post-transfection. Control transfections were performed by using branched PEI 25 kDa.

Cytotoxicity assay by MTT

Cells were seeded in 96-well plates and treated after 24 hrs with the same amounts of polyplex used for transfection experiment. After 3 hrs medium was replaced by 100 µl fresh culture medium. Metabolic activity of each well was determined using a methylthiazoletetrazolium (MTT)/thiazolyl blue assay after 24 hrs as follows: 10 µL of a 5 mg/mL solution of MTT in sterile PBS buffer was added to each well. After incubation for 2 hrs at 37 °C, the medium was removed, 100 µl of dimethylsulfoxide (DMSO) added and samples were further incubated at 37 °C for 30 mins under constant shaking. Optical absorbance was measured at 590 nm (reference wavelength 630 nm) using a microplate reader and cell viability was expressed as a percent relative to untreated control cells. Values of metabolic activity are presented as means +/- SD of triplicates.

Statistical Analysis

Multiple measurement, comparisons were performed using student t-test. A *P* value of 0.05 or less was considered significant.

Results

Synthesis and characterization of the modified polymer

In the present investigation, alkyl carboxylic acids (acetic acid and hexanoic acid) were grafted on PEI primary amines in low and high degrees of substitutions with feed mole percents of 10 mol% and 50 mol% with respect to PEI's primary amine content. However, as shown in Table 1 and by determining the amount of primary amines of the modified polymers by TNBS test, the degree of substitution was lower than the feeds (Table 1). No significant effect of alkyl length upon conjugation efficiency was observed. The nomenclature of the polymers is expressed as: 10-X, Y, where 10 represents the PEI 10 kDa, X and Y represent reagent by which PEI was modified and percent of grafting, respectively.

Characterization of Polymer/DNA complexes

The formation of polyplexes was confirmed by ethidium bromide exclusion assay of complexes prepared from pRL-CMV and modfied-PEI. The interaction between the polycationic polymer and plasmid DNA results in neutralization of negative charges in the phosphate backbone of DNA, which results in the decrease in the fluorescence intensity of EtBr. Keeping the amount of DNA constant, different amonts of conjugates was added to form conjugate/DNA complexes.

Figure 1 shows that in C/P ratio of 2, almost all of vectors were able to condense the plasmid DNA. It was also obvious that by adding hydrophobic chains to PEI the condensation ability of polymer slightly decreases in comparison to the unmodified PEI (Modified polymers form loose complexes with DNA and therefore, more quantity of free DNA is available for interaction with EtBr to produce enhanced fluorescence).

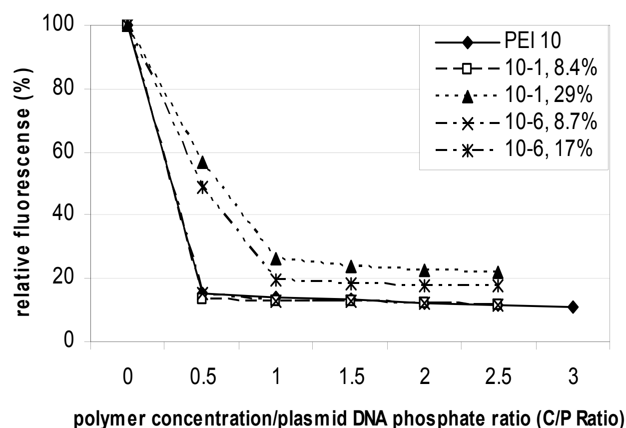

Figure 1. DNA binding of modified polymers measured by EtBr exclusion assay in HBG.

For efficient transfection, the particles should be compact and positively charged to interact with lipid bilayer membranes. Particle size and zeta potential measurement results are

www.SID.ir

Archive of SID

shown in Table 1. These results demonstrated that modified PEI polymers were able to form nanoparticles in the range of 70-200 nm. As shown in the Table 1 addition of alkyl chains causes significant reduction in zeta potential from 32.1 mv in the case of 10 kDa PEI to 9.8 and 5.6 for acetic acid and hexanoic acid grafted PEI's, respectively.

Table 1. Various degrees of alkyl chain grafted on PEI, and the biophysical properties of the DNA-polymer complexes prepared with either the modified or unmodified PEI.

Polymers name	Degree of alkyl chain grafting (%)	Size of DNA/polymer complex (nm)	Zeta potential of DNA/ polymer complex (mV)
PEI 10kD	0	75.9	35.1
PEI 10-1, 8.4%	8.4	95	9.25
PEI 10-1, 29%	29	150	5.6
PEI 10-6, 8.7%	8.7	191	16
PEI 10-6, 17%	17	205	8

Cell viability assay and transfection efficiency

Cytotoxicity of polycation/DNA nanoparticles was evaluated with regard to their effects on *in vitro* viability of Neuro2a cells by MTT assay. MTT experiments revealed that most of the modified PEIs induced less cytotoxicity than PEI 25 kDa when the cells were incubated with the DNA-polymer nanoparticles (Figure 2). The level of cytotoxicity did not show a significant difference with 10 kDa PEI itself.

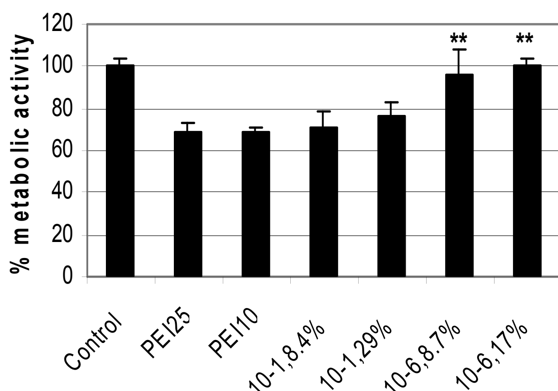

Figure 2. Cellular cytotoxicity induced by unmodified and modified PEIs. Neuro2a cells were transfected with polyplexes consisting of pRL-CMV complexed with modified PEI series at a C/P ratio of 4. Cytotoxicity was assayed by the MTT method, and expressed as the percentages of cell viability by setting the untransfected cells as 100%. Metabolic activity (MTT assay) is presented as mean values ± SD of triplicates. ***P*<0.05 compared with PEI 25 kDa.

The transfection efficiency of the polyplexes was evaluated in Neuro2A cells by using the plasmid pRL-CMV encoding *Renilla* luciferase. Transfection experiment was performed in serum containing media for four derivatives of PEI at C/P ratio of 4. As shown in Figure 3, the transfection efficiency was dependent on alkyl chain length. Moreover, transfection efficiency of 10-6 conjugates was much higher than 10-1 conjugates. As demonstrated in Figure 3 in most cases the transfection efficiency of these new conjugates was similar to PEI 25 kDa with the advantage of less toxicity (Figure 2 and Figure 3).

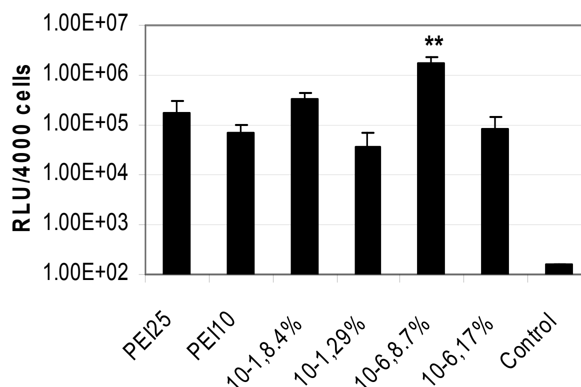

Figure 3. Transfection efficiency after incubation with the modified polymers. The Neuro2a cells were transfected with polyplexes consisting of pRL-CMV complexed with the modified PEI series at a C/P ratio of 4. Luciferase activity is presented as mean values ± SD of triplicates. ***P*<0.001 compared with PEI 25 kDa.

Discussion

Polyethylenimine (PEI) has been used for the gene delivery system *in vitro* and *in vivo*. Since it has a high density of cationic groups such as secondary and tertiary amines whose charge is pH-dependent, they can act as endosomal buffering systems (10). Therefore, the nucleic acid can be released from the endosome without degradation (endosomal escape) (11). However, the use of PEI for gene delivery is limited due to cytotoxicity,

www.SID.ir

Archive of SID

nonspecificity and unnecessary interaction with serum components (21).

Cationic liposomes form a complex with polyanionic DNA molecules and are thought to deliver DNA through endosomes, after endocytosis of the complex. The complexes of DNA and lipid form aggregates, due to ionic interactions between the positively charged cationic lipid and the negatively charged phosphate groups of the DNA (22). In order to achieve more efficient and less toxic non-viral vectors for gene delivery, the structure of PEI has been widely modified. It has been reported that the reduction of buffering capacity of polymer can greatly enhances gene delivery activity of the polymer (2). However, there are some reports that decreasing the amount of surface amines decreases the surface charge and overall buffering capacity; one of the primary mechanisms for endosomal escapes (23).

We developed a novel non-viral gene transfer system by combining the advantages of both liposomes and polycations in order to combine the osmotic burst mechanism with the lipid depletion mechanism for lysing endocyotic vesicles after uptake of the DNA-containing complex by endocytosis.

In this study, we synthesized modified PEI denderimers by adding two different lengths of carboxylic chains in two different percentages to the primary amines of PEI and used them as new gene carriers.

Hydrophobic modification of PEI was achieved by different approaches like conjugation of cholesterol and myristate (16), acetylation of PEI (2), addition of saturated hydrocarbons (24) and palmitic acid (19). Each of these modified polymers has their own conjugation chemistry along with some drawbacks like labor methods, solubility problems, and low efficiency. We have succeeded to modify PEI polymer with a simple chemistry and relatively high efficiency.

One prerequisite of a polymeric gene carrier is DNA condensation. The extent of DNA association with the polymer was measured by the changes in the fluorescence intensity of a DNA-intercalating dye, EtBr. The dye strongly binds to free DNA (high fluorescence intensity), whereas, PEI particles form complexes with DNA and therefore, free DNA is relatively less available to intercalating dye, EtBr (low fluorescence intensity. The results showed that modified-PEI polymers were able to condense DNA. The condensation ability was decreased by addition of hydrocarbon chains. Some recent reports also support this observation that substitution of primary amines of PEI would lead to looser polymer/DNA complexes (15, 25, 26) and it could have a positive effect on the overall transfection efficiency of polymers because a cationic polymer's high affinity for DNA may be a limiting step for successful transfection, due to the difficulty in release of DNA from the gene carrier (25).

The formation of polymer/DNA complexes was also monitored by observation of changes in particle size and zeta potential of the complex at their optimum transfection weight ratios. Zeta potential also supports the effect of alkyl chain addition on decreasing the positive surface charge of polyplexes.

Previous studies on the modification of surface positive charge of nanoparticles reported the same trend of decrease in charge with increase in substitution degree (26).

Many factors affect the cytotoxicity profile of PEI polyplexes, such as molecular weight and degree of branching (27). Mostly, PEIs larger than 10 kDa have been used successfully in the transfection studies (18, 27). Lower molecular weight PEIs are less toxic, but ineffective in gene transfer (28). On the other hand, the effectiveness of large PEI, such as 25 kDa, is affected by its cytotoxicity (29). The toxicity is related to strong positive charge of the polymer, which leads to electrostatic interaction of this polycation to the cell membrane and causes damage (30). Our data shows that the charge, responsible for the toxicity (primary amines), gets partially converted to the charge, which is helpful in improving the transfection efficiency. Therefore, among different approaches of decreasing PEI cytotoxicity, reducing the surface charge of PEI might be useful.

www.SID.ir

Archive of SID

Transfection efficiency of the modified PEI/DNA complexes increased with increasing the alkyl chain length, which can be explained by the endosomolytic effect of longer alkyl chains.

Increased transfection efficiency through the acetylation of PEI has been reported previously (2, 26). Enhanced unpacking of the modified polymer/DNA complexes along with reduced positive surface charge of polymer was found to be the reason. High reduction of positive charge, however, might also reduce the transfection ability of polycation, as reported by Thomas *et al*. (31). In another study, the effect of lipid length on transfection efficiency was reported. They found that there are optima for all the lipids, and that these optima appear at different degrees of hydrophobicity (24). Our data fits well with the mentioned studies, where modifications of branched PEI with acetic and hexanoic led to increase in the transfection efficiency (at low degrees of substitutions).

The beauty of the system lies in the fact that it significantly reduces the charge dependent toxicity of native PEI and hence improves the transfection efficiency.

Conclusion

The structure modifications of PEI accomplished in this study play a significant role in increasing the transfection efficiency without inducing the cytotoxicity compared to PEI itself. They showed the great ability to form complexes with DNA and showed suitable physicochemical properties for the gene delivery system. These polymers had low cytotoxicity and exhibited much enhanced gene transfer efficiency than PEI itself. Additional investigation is necessary to understand the effect of higher alkyl chain lengths and the mechanism through which alkylation of branched polyethylenimine influences the transfection efficiency *in vitro*.

Acknowledgements

This work was supported by the grants from Research Committee, Mashhad University of Medical Sciences, Mashhad, Iran. The financial support by Iranian Nanotechnology Initiative is gratefully acknowledged. We also, would like to acknowledge Prof. Dr. Ernst Wagner (Pharmaceutical Biology-Biotechnology, Ludwig Maximilians University, München, Germany) for providing us the Neuro2a cell line.

References

1. Cavazzano-Calvo M, Thrasher A, Mavilio F. The future of gene therapy. Nature 2004; 427: 779-781.
2. Forrest LM, Meister EG, Koerber J, Pack D. Partial acetylation of polyethylenimine enhances *in vitro* gene delivery. Pharm Res 2004; 21: 365-371.
3. Lungwitz U, Breunig MT, Blunk Gopferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm 2005; 247-266.
4. El-Aneed A. An overview of current delivery systems in cancer gene therapy. J Control Release 2004; 94: 1-14.
5. Lehrman S. Virus treatment questioned after gene therapy death. Nature 1999; 401:517-518.
6. Liu Q, Muruve DA. Molecular basis of the inflammatory response to adenovirus vectors. Gene Ther 2003; 10: 935-940.
7. Sun JY, Anand-Jawa V, Chatterjee S, Wong K K. Immune responses to adeno-associated virus and its recombinant vectors. Gene Ther 2003; 10:964-976.
8. Merdan T, Kopecek J, Kissel T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. Adv Drug Deliver Rev 2002; 715-758.
9. Zhang S, Xu Y, Wang B, Qiao W, Liu D, Li Z. Cationic compounds used in lipoplexes and polyplexes for gene delivery. J Control Release 2004; 100:165-180.
10. Boussif O, Lezoualc'h F, Zanta MA. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA 1995; 92:7297–7301.
11. Cho YW, Kim J, Park K. Polycation gene delivery systems: escape from endosomes to cytosol. J Pharm Pharmacol 2003; 55:721-734.
11. Kursa M, Walker GF, Roessler V, Ogris M, Roedl W, Kircheis R, *et al*. Novel shielded transferrin–polyethylene glycol–polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer. Bioconjugate Chem 2003; 14: 222-231.
12. Kircheis R, Schuller S, Brunner S, Ogris M, Heide KH, Zauner W, *et al.* Polycation-based DNA complexes for tumor-targeted gene delivery *in vivo*. J Gene Med 1999; 1:111-120.

www.SID.ir

Archive of SID

13. Ogris M, Walker G, Blessing T, Kircheis R, Wolschek M, Wagner E. Tumor-targeted gene therapy: strategies for the preparation of ligand–polyethylene glycol–polyethylenimine/DNA complexes. J Control Release 2003; 91:173-181.
14. Kircheis R, Wightman L, Schreiber A, Robitza B, Rossler V, Kursa M, *et al*. Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. Gene Ther 2001; 8:28-40.
15. Tseng WC, Jong CM. Improved stability of polycationic vector by dextran-grafted branched polyethylenimine. Biomacromolecules 2003; 4:1277-1284.
16. Kim S, Choi JS, Jang HS, Suh H, Park J. Hydrophobic modification of polyethylenimine for gene transfectants. Bull Korean Chem Soc 201; 22:1069-1075.
17. Thomas M, Klibanov AM. Non-viral gene therapy: polycation mediated DNA delivery. Appl Microbiol Biotechnol 2003; 62:27-3.
18. Thomas M, Klibanov AM. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. Proc Natl Acad Sci USA 2002; 99:14640–14645.
19. Brownlie A, Uchegbu A, Schatzlein AG. PEI-based vesicle-polymer hybrid gene delivery system with improved biocompatibility. Int J Pharm 2004; 274:41–52.
20. Snyder SL, Sobocinski PZ. An improved 2, 4, 6-trinitrobenzenesulfonic acid method for the determination of amines. Anal Biochem 1975;64: 284-288.
21. Kichler A, Leborgne Ch, Coeytaux E, Danos O. Polyethylenimine-mediated gene delivery: a mechanistic study. J Gene Med 2001; 3:135-144.
22. Liu D, Ren T, Gao X. Cationic transfection lipids. Curr Med Chem 2003; 10: 1307-1315.
23. Neu M, Fischer D, Kissel T. Recent advances in rational gene transfer vector design based on poly (ethylene imine) and its derivatives. J Gene Med 2005; 7:992–1009.
24. 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; 116:227-237.
25. Chen DJ, Majors BS, Zelikin A, Putnam D. Structure–function relationships of gene delivery vectors in a limited polycation library. J Control Release 2005; 103:273-283.
26. Gabrielson NP, Pack DW. Acetylation of polyethylenimine enhances gene delivery via weakened polymer/DNA interactions. Biomacromolecules 2006; 7:2427-2435.
27. Fischer D, Bieber T, Li Y, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm Res 1999; 16:1273-1279.
28. Godbey WT, Wu KK, Mikos AG. Size matters: molecular weight affects the efficiency of poly (ethylenimine) as a gene delivery vehicle. J Biomed Mater Res 1999; 45: 268-275.
29. Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk A. A two-stage poly (ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. Mol Ther 2005; 11:990-995.
30. Hunter AC. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. Adv Drug Deliver Rev 2006; 58:1523-1531.
31. Thomas M, Lu JJ, Ge O, Zhang C, Chen J, Klibanov AM. Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. Proc Natl Acad Sci USA 2005; 102:5679-5684.

www.SID.ir