

Research Article



Cationic Liposomes Modified with Polyallylamine as a Gene Carrier: Preparation, Characterization and Transfection Efficiency Evaluation

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Abstract

Purpose: Cationic polymers and cationic liposomes have shown to be effective non-viral gene delivery vectors. In this study, we tried to improve the transfection efficiency by employing the advantages of both.

Methods: For this purpose, modified polyallylamines (PAAs) were synthesized. These modifications were done through the reaction of PAA (15 KDa) with acrylate and 6-bromoalkanoic acid derivatives. Liposomes comprising of these cationic polymers and cationic lipid were prepared and extruded through polycarbonate filters to obtain desired size. Liposome-DNA nanocomplexes were prepared in three carrier to plasmid (C/P) ratios. Size, zeta potential and DNA condensation ability of each complex were characterized separately and finally transfection efficiency and cytotoxicity of prepared vectors were evaluated in Neuro2A cell line.

Results: The results showed that mean particle size of all these nanocomplexes was lower than 266 nm with surface charge of 22.0 to 33.9 mV. Almost the same condensation pattern was observed in all vectors and complete condensation was occurred at C/P ratio of 1.5. The lipoplexes containing modified PAA 15 kDa with 10% hexyl acrylate showed the highest transfection efficacy and lowest cytotoxicity in C/P ratio of 0.5.

Conclusion: In some cases nanocomplexes consisting of cationic liposome and modified PAA showed better transfection activity and lower cytotoxicity compared to PAA.

Introduction

Disadvantages of viral vectors are serious concerns that restricted their application. Although they are efficient systems in gene delivery, their safety is not reliable.¹ These considerations favor the application of non-viral vectors over viral systems for gene delivery of genetic or acquired diseases. Regarding this fact, synthetic vectors play an important role in gene therapy. Non-viral vectors showed limited transfection efficiency in clinical applications. Most widely used synthetic DNA delivery systems generally consist of three categories: cationic polymer (polyplex), cationic lipid (lipoplex) or a mixture of these (lipopolyplex).^{2,3} Among these carriers, cationic polymers (polycations) are the most widely used. Polycations and DNA form compact complexes by electrostatic bonds between negatively charged DNA and positively charged polymers.^{4,5} The whole system carries net positive charge which facilitates the interaction with negatively charged cell surface, leading to better endocytosis.⁶ After endocytosis, nanoparticles undergo acidic pH of lysosomes. Endosomal escape is

considered to be one of the most important steps of gene delivery.³

Polyallylamine (PAA) is one of less investigated cationic polymer which has high density of primary amino groups (as free amine or as cationic ammonium salt). High positive charge density of PAA and the other polycationic polymers is the main reason for their cytotoxicity.⁶ Another limitation for application of PAA in gene delivery is low buffering capacity.³ The efficiency of gene delivery can be increased by modification of the polycation structure to reach an optimized vector. Different chemical modifications could decrease cytotoxicity and improve transfection efficiency of PAA. Boussif et al. used glycolylated derivatives of PAA.⁷ Their efforts decreased the cytotoxicity of PAA-DNA complex and also increased the transfection efficiency of this complex. Nimesh et al. prepared the nanocomplexes composed of PAA-dextran-DNA.⁶ They demonstrated transfection efficiency of these nanoparticles in HEK 293 cells increased and cytotoxicity reduced significantly compared to PAA-DNA nanoparticles. Conjugation of

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imidazole (as a mildly basic group) to PAA was also performed to increase the proton sponge effect and enhance endosomal escape.⁸

In the previous studies, we tried to modify PAA by acrylate³ and 6-bromoalkanoic acid⁹ derivatives with different chain lengths to achieve a library of compounds with more hydrophobic characteristics and we used them in polyplex structure in order to reduce toxicity and to improve the interaction with cell surface as well as maintaining the buffering capacity. In the present study, we selected most successful derivatives of our previous studies in gene delivery and used them in lipoplex structure in order to evaluate if the transfection properties could improve or not. We expected to achieve facilitated passage through cell membrane, better endosomal release (through flip-flop effect) and decreased cytotoxicity.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), butyl acrylate and hexyl acrylate were purchased from Sigma (USA). 6-bromohexanoic acid and 6-bromodecanoic acid were obtained from Sigma-Aldrich (Munich, Germany). Polyallylamine (PAA; average MW 15 kDa) was ordered from Polyscience, Inc (Warrington, USA). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesterol were from Avanti Polar Lipids (USA). Ethidium bromide, protamine, methanol and chloroform were ordered from Merck (Germany). Cyclopore polycarbonate membranes (0.1, 0.4 and 0.8 μm) were obtained from Wathman (Belgium). Dialyses were carried out using Spectra/Por dialysis membranes (Spectrum Laboratories, Houston, USA).

Modification of PAA

The strategy of PAA modification was to convert the primary amines of PAA to the secondary amines using acrylate or 6-bromoalkanoic acid derivatives. Based on the previous studies, the reaction can take place in water free solution without the use of conjunctive reagents.³ Modification was performed with hexyl acrylate in substitution percent of 10, 30 and 50; butyl acrylate in substitution percent of 50 and also with 6-bromohexanoic acid and 6-bromodecanoic acid in substitution percent of 30 and 50 of each. Chain length and substitution percent of each derivative was selected based on the results of our previous studies.^{3,9} The modified polymers were labeled as HAX or BAX or BHX or BDX in which X is percentage of PAA primary amines substituted with acrylate or bromoalkane derivatives, HA is hexyl acrylate, BA is butyl acrylate, BH is 6-bromohexanoic acid and BD is 6-bromodecanoic acid.

Briefly, various amounts of acrylate or 6-bromoalkanoic acid derivatives were dissolved in dimethylformamide (DMF). This solution was added

dropwise to the stirring solution of PAA 15 kDa (0.1 g in 5 ml DMF) and the reaction was stirred for 24 overnight at room temperature. After 24 hours, the reaction mixture was dialyzed once against 0.25 M NaCl and twice against water (10,000 Da cut-off dialysis tubes) in order to remove unreacted agents. The solution of final product was freeze dried.^{3,9}

Preparation of liposomes

For preparation of DOTAP:modified PAA liposomes (10 mg/ml based on DOTAP), 10:1 mole ratio of DOTAP and lipopolymer were dissolved in methanol-chloroform (1:1 v/v) solvent. After complete dissolution, the organic solvent was evaporated by rotary evaporator (Heidolph, Germany) in order to form a thin lipid film. The thin film was then hydrated by deionized water at 50 °C and the container was placed in bath sonicator (40 °C) (Branson, USA) to form the liposomal vesicles. To prepare DOTAP: cholesterol liposome (10 mg/ml based on DOTAP), 1:1 molar ratio of DOTAP and cholesterol were dissolved in the same organic solvent and liposomes were prepared in the same procedure described above. In order to reduce the size, liposomal formulations were extruded through 800, 400 and 100 nm polycarbonate membranes repeatedly at 50 °C using Thermobarrel extruder (Northern Lipid, Canada).

Preparation of lipoplexes and polyplexes

Three carrier to plasmid (C/P) mass ratios (0.5, 1.5 and 3) of lipoplexes (sample and control) and corresponded polyplexes (PAA or modified PAA) were premixed and left for 20 min at room temperature to form structure. Liposome-protamine-DNA (LPD) complexes were prepared as a control. DOTAP: cholesterol liposomes were used in these structures. Particle size, polydispersity index (PDI) and zeta potential of nanocomplexes were analyzed with Zetasizer Nano ZS (Malvern Instruments, UK) after suitable dilution.

Ethidium bromide test

In order to evaluate the pDNA condensation ability of prepared vectors, ethidium bromide (EtBr) test was performed. Ethidium bromide in HBG buffer (HEPES buffer + glucose 5%) was used in this test. After adding sequential 2.5 μl of vector to the mixture of 0.5 μl pDNA solution (1 mg/ml) and 1 ml ethidium bromide (0.4 $\mu\text{l/ml}$), the *spectrofluorometer* (Jasco, Japan) read the light emission. The fluorescence intensity was measured at an excitation and emission wavelength of 510 and 590 nm. The lowest light emission showed the best condensation ability.

Evaluation of transfection efficiency and cytotoxicity of vectors

Neuro2A murine neuroblastoma cells (ATCC CCL-131), were cultured in DMEM containing 10% fetal bovine serum, streptomycin at 100 $\mu\text{g/ml}$ and

penicillin at 100 U/ml. These cells were incubated at 37 °C under an atmosphere containing 5% CO₂. Cells were seeded in 96-well plates in a density of 1 × 10⁴ cells per well. Lipoplexes were prepared in C/P ratios of 0.5, 1.5 and 3 and added to the cells in 5 repetitions. After 3-4 hours in 37 °C incubator, medium was replaced and further incubation in 37 °C was done for 24 hours. Transfection and lysis buffer were added to each well. The percentage of transfected cells was determined reading Green fluorescent protein (GFP) fluorescence by fluorescent plate reader (Victor X5, Perkin-Elmer, USA). Excitation and emission wavelength was adjusted on 498 and 535 nm, respectively.

For cytotoxicity evaluation, metabolic activity was measured using MTT assay. After 24 hours incubation, seeded cells were treated with the same amounts of lipoplexes used for transfection experiment. After 4 hours of incubation, the medium replacement was done. 24 hours later, 10 µl of MTT solution (5 mg/ml in sterile PBS) was added to each well. After 2h hour incubation in 37 °C, the medium was removed and 100 µl of dimethyl sulfoxide added and the plates were put on shaker incubator for 30 min (5000 rpm and 37 °C). Results were read in ELISA reader apparatus (Statfax-2100, Awareness Technology, USA) at 590 nm (reference wavelength 630 nm). Cell viability was expressed as a percent relative to untreated cells.

Statistical analysis

One-way ANOVA and Tukey–Kramer test was used to analyze the obtained data. Differences were statistically significant if the P-value was less than 0.05.

Results

Mean size, polydispersity index (PDI) and zeta potential of lipoplexes were summarized in Table 1. All the complexes showed positive surface charge and a mean size between 136 to 266 nm with PDI bellow 0.5. Increasing in grafting percent did not make a significant difference in surface charges but increased mean size in some cases.

Table 1. Mean size, polydispersity index (PDI) and zeta potential of lipoplexes (composing of DOTAP: modified PAA: DNA) and LPD (liposome:protamine:DNA) (mean±SD, n=3).

Sample Name	Z-Average (nm)	PDI	Zeta Potential (mV)
LPD*	183.9±3.3	0.279	33.9±0.5
Lipo-BD 30	138.2±6.8	0.195	30.0±0.1
Lipo-BD 50	204.2±5.2	0.369	31.9±1.8
Lipo-BH 30	136.9±4.7	0.237	28.0±1.8
Lipo-BH 50	177.0±27.8	0.369	28.0±1.7
Lipo-HA 10	266.3±10.4	0.377	30.6±1.5
Lipo-HA 30	189.7±12.1	0.334	26.4±1.3
Lipo-HA 50	265.7±14.5	0.461	27.8±1.5
Lipo-BA 50	202.2±8.7	0.324	28.9±2.6

*LPD = Liposome : Protamine : DNA

All vectors condensed the pDNA at C/P ratio of 1.5 (Figure 1). Increasing in the grafting percent of polymer had no impressive effect on condensation ability of vector. Almost the same pattern was observed in all cases.

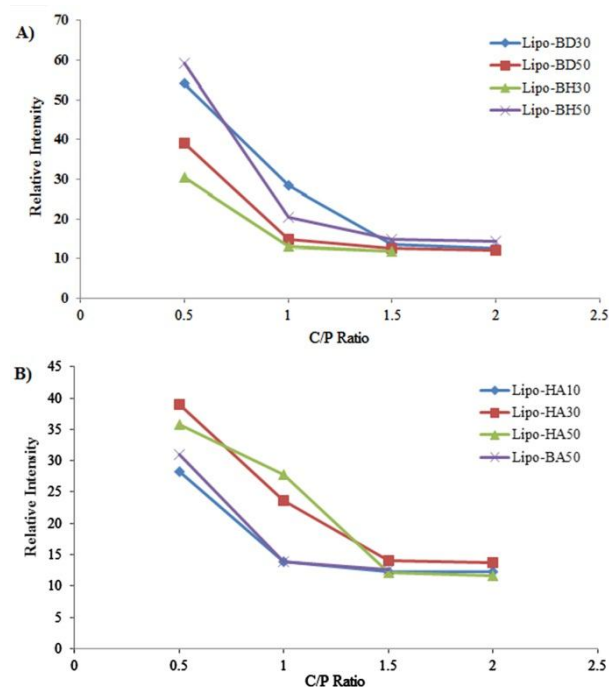


Figure 1. Ethidium bromide test in order to evaluate the DNA condensation ability of A) vectors prepared by DOTAP:PAA modified by bromoalkane derivatives, B) vectors prepared by DOTAP:PAA modified by acrylate derivatives

Transfection efficiency was significantly decreased by using of lipoplexes containing PAA 15 KDa modified with different 6-bromoalkanoic acid compared to the same polyplexes in selected C/P ratios (P<0.05). The difference in transfection ability of all lipoplexes in all C/P ratios was not significant compared to LPD (Figure 2). Transfection efficiency of lipoplexes in C/P 3 was significantly higher than unmodified PAA (P<0.05) but they could not make a significant difference in lower C/P ratios (Figure 2). These results showed that lipoplexes have better gene transfer ability compared to unmodified PAA in higher C/P ratios.

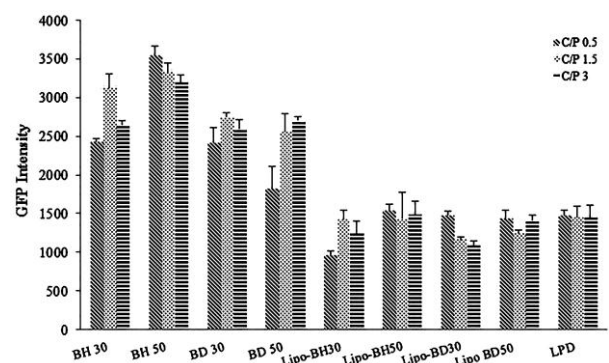


Figure 2. Transfection efficiency of lipoplexes (composing of DOTAP: PAA modified by bromoalkane derivatives: DNA), LPD (liposome:protamine:DNA), PAA and PAA modified by bromoalkane derivatives in Neuro2A cell line (Mean±SD, n=5).

Lipoplexes containing modified PAA 15 kDa with acrylate derivatives (in different grafting degrees) significantly increased the transfection efficiency compared to analogous polyplexes in selected C/P ratios ($P < 0.05$) and most of them showed higher transfection activity than LPD (Figure 3). All new lipoplexes in all C/P ratios made remarkable higher gene transfer in comparison with unmodified PAA. Among lipoplexes, the highest gene transfer ability belonged to lipoplex composed of PAA 15 kDa modified with 10% hexyl acrylate at C/P ratio of 3 (Figure 3).

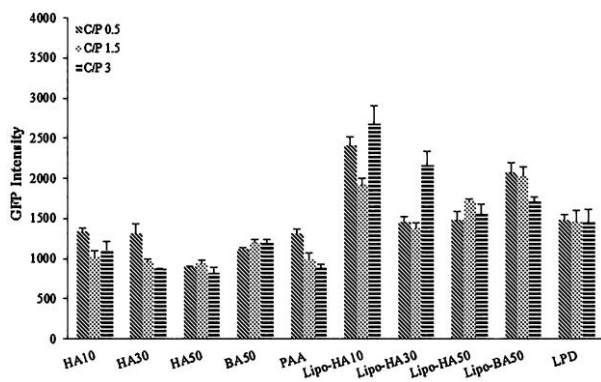


Figure 3. Transfection efficiency of lipoplexes (composing of DOTAP: PAA modified by acrylate derivatives: DNA), LPD (liposome:protamine:DNA), PAA and PAA modified by acrylate derivatives in Neuro2A cell line (Mean±SD, n=5).

As shown in Figures 4 and 5, most vectors showed noticeable cell cytotoxicity. The majority of new nanocomplexes showed increasing cytotoxicity by increasing in C/P ratio. The differences in cytotoxicity of lipoplexes and their analogous polyplexes were not significant in C/P 0.5 and 1.5. Lipoplex composed of modified PAA with 10% hexyl acrylate (lipoplex HA10) showed significant lower cell toxicity compared to analogous polyplex in C/P 3 ($P < 0.05$), but lipoplex containing modified PAA with 30% hexyl acrylate (lipoplex HA 30) showed higher cytotoxicity than analogous polyplex ($P < 0.05$). Modification with acrylate derivatives could decrease cytotoxicity compared to unmodified PAA, this difference became remarkable by increasing in C/P ratio.

The difference in cytotoxicity of lipoplexes and polyplexes was not significant in C/P ratio of 0.5 and 1.5, but in C/P 3 lipoplexes showed increased cell toxicity compared to their corresponding polyplexes. Metabolic activity in C/P ratios of 1.5 and 3 in all grafting percent was higher than unmodified PAA. About the differences between these nanolipoplexes and LPD, in most cases there were no significant differences.

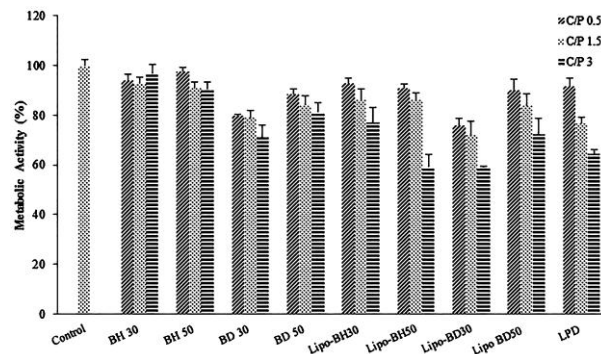


Figure 4. Cell cytotoxicity of lipoplexes (composing of DOTAP: PAA modified by bromoalkane derivatives: DNA), LPD (liposome:protamine:DNA), PAA and PAA modified by bromoalkane derivatives in Neuro2A cell line (Mean±SD, n=5).

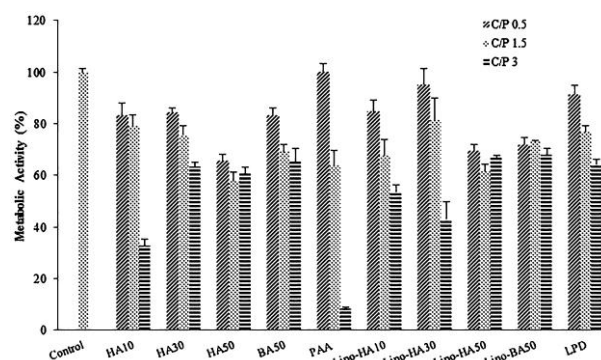


Figure 5. Cell cytotoxicity of lipoplexes (composing of DOTAP: PAA modified by acrylate derivatives: DNA), LPD (liposome:protamine:DNA), PAA and PAA modified by acrylate derivatives in Neuro2A cell line (Mean±SD, n=5).

Discussion

Cationic polymers and lipids can cause transient destabilization of endosomal membrane, but their mechanisms of action are different. For cationic polymers some amine groups are involved in electrostatic interaction with negatively charged pDNA, and other amine groups contributed in endosomal disruption at acidic pH (proton sponge effect). Cationic lipids have fusogenic features. They help endosomal escape via bilayer-to-micelle conversion and lamellar-to-inverted hexagonal transition.¹ Regarding the fact that osmotic endosomal swelling is not induced by PAA, so considering of another strategy for endosomal escape can improve the efficiency of transfection. For this purpose, using cationic lipid beside PAA (in the structure of lipoplex) can be effective.¹⁰

In the present study, we tried to improve transfection efficiency of modified PAA 15 kDa. A high density of primary amine groups is covering the structure of PAA. We investigated the effect of substitution of primary amines with hydrophobic moieties in gene transfection ability and cell toxicity of this polymer in our previous study.¹⁰ The synthesized polymer beside DOTAP was used in liposome preparation in the present study. This modification could successfully improve gene

transfection ability of some new vectors compared to polyplexes of analogous modified PAA.

PAA is a cationic polymer that has been less investigated. Cell toxicity of this polymer is not acceptable and low buffering capacity can't induce enough endosomal disruption so transfection efficiency is low.³ To overcome these limitations, some modifications have been done on this polymer. The molecular weight of 15 kDa for PAA was selected according to our previous studies results.^{3,9} Cationic nature of this polymer appears to be the main reason of cytotoxicity.⁶ We tried to increase cell viability by covering different percentages of amine groups with acrylate and 6-bromodekanoic acid derivatives. These modified polymers were used in the structure of a new nanoplex gene delivery system composing of modified PAA along with DOTAP (1:10 molar ratio) in liposomal form to evaluate transgenic characteristics and cell viability.

The ethidium bromide dye exclusion assay was done for evaluation of DNA condensation ability of new vectors. Our previous studies showed that unmodified PAA could condense pDNA in C/P ratio of 0.5.³ These results confirmed that modification of PAA decreased surface positive charge and thereupon condensation occurred in higher C/P ratios (C/P 1.5). Polycationic polymers form nanocomplexes after interaction with negatively charged DNA. This compaction is necessary for efficient cell uptake.¹¹ Although DNA condensation ability of vector is an advantage, DNA release from vector after cell internalization also would be important for efficient gene delivery to the nucleus. Consequently, optimal balance between condensation and separation is needed.¹²

Previous researches revealed that particle size is an important parameter that can determine cell internalization mechanism. Rejman et al. showed that particles less than 200 nm are entering to the cells by clathrin-mediated endocytosis and particles with a size of 200-500 nm are entering through the caveolae-mediated endocytosis pathway.¹³ In first pathway, nanoparticles are faced with acidic pH of early and late endosome and in second one they are not faced lysosome.¹⁴

All nanoparticles showed positive surface charge. Positive zeta potential prevents aggregation by electrostatic repulsions and can help attachment to anionic cell surfaces.¹⁰ It expected that increasing in grafting percent decrease the positive charge of modified PAA. But the differences between surface charges of liposomal nanoparticles were not significant. This could be related to the presence of DOTAP (as a cationic lipid) along with these cationic polymers. On the other hand, the size of these structures is dependent to the level of compaction induced by their surface positivity. Consequently, the differences between their particle sizes were not significant too.

Hydrophobic-hydrophilic balance, buffering capacity of modified vectors and nuclear localization are three

important factors that affect their transfection efficiency. The variation was observed in transfection after modification can be related to these factors.^{3,10}

In this study, transfection efficacy of new nano-vectors did not follow up a regular pattern. This can be related to change in hydrophobic-hydrophilic balance induced by modification which can change interaction of nanoparticles with cell membrane and cell internalization. These hydrophobic interactions are known to play a key role in the binding of the preparations to the hydrophobic surface of cell membrane and endocytosis.¹⁰ Also using modified polymer in liposomal formulation can alter endosomal escape capability as an important part of successful gene delivery. The exact effect of modification type on internalization and endosomal release is unclear.

Previously, it has shown that modification of PEI 750 kDa with 6-bromoalkanoic acids was done and 6-bromohexanoic acid created the best transfection efficiency.¹⁵ Long alkyl chains have endosomolytic properties that can improve transfection activity.¹⁶ This improvement was optimum for the chain length of 6-bromohexanoic acid. The transfection data indicated that the alkylation enhanced the ability of unmodified PAA to transfer a reporter gene into Neuro2A cell line. But using DOTAP along with the same modified polymers decreased gene transfer efficiency compared to their analogous polyplexes. This can be related to impaired hydrophilic-hydrophobic balance or impaired endosomal release.

In one previous study, PAMAM modification with acrylate derivatives improved gene delivery ability.¹⁷ In our study, lipoplexes showed better transfection than their analogous polyplexes in some cases. Presumably using cationic lipid beside these polymers was improved cell internalization or endosomal escape. Among all vectors of this group lipoplex containing modified PAA with 10% hexyl acrylate (lipoplex HA10) showed the best transfection efficiency. In a previous study, lipopolyplex contained this modified PAA, enhanced gene transfer ability obviously.¹⁰ Our results in both kind of modification (acrylate and 6-bromoalkanoic acid derivatives) revealed better transfection activity for six-carbon chain components in lipoplex structures.

The cytotoxicity of vectors was increased by increasing in C/P ratio. Cytotoxicity was dependent to the surface zeta potential and increased by increasing in positive charge. Positively charged particles can damage cells following an electrostatic interaction with cell surface.¹⁸ Metabolic activity of synthesized vectors was ranged from 50%-95% of control group. The highest toxicity level was related to C/P 3 of unmodified PAA and modifications could decrease cell toxicity of vectors by decreasing positive zeta potential.

Conclusion

Among prepared vectors, lipoplex HA10 (C/P 0.5) was the best vectors for gene delivery due to their capability

in transfection and their low cytotoxicity (90% cell viability).

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Ethical Issues

Not applicable.

Conflict of Interest

The authors declare no conflict of interests.

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