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Original Research

Preparation, characterization and transfection efficiency of nanoparticles composed of alkane-modified polyallylamine

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Abstract

Objective(s): Although viral vectors are considered efficient gene transfer agents, their board application has been limited by toxicity, immunogenicity, mutagenicity and small gene carrying capacity. Non-viral vectors are safe but they suffer from low transfection efficiency. In the present study, polyallylamine (PAA) in two molecular weights (15 and 65 kDa) was modified by alkane derivatives in order to increase transfection activity and to decrease cytotoxicity.

Materials and Methods: Modified PAA was synthesized using three alkane derivatives (1-bromobutane, 1-bromohexane and 1-bromodecane) in different grafting percentages (10, 30 and 50). The condensation ability of modified PAA was determined by ethidium bromide test. The prepared polyplexes, complexes of modified PAA and DNA, were characterized by size and zeta potential. Transfection activity of polyplexes was checked in Neuro2A cells. The cytotoxicity of vector was examined in the same cell line.

Results: DNA condensation ability of PAA was decreased after modification but modified polymer could still condense DNA at moderate and high carrier to plasmid (C/P) ratios. Most of polyplexes composed of modified polymer had mean size less than 350 nm. They showed a positive zeta potential, but some vectors with high percentage of grafting had negative surface charge. Transfection efficiency was increased by modification of PAA by 1-bromodecane in grafting percentages of 30 and 50%. Modification of polymer reduced polymer cytotoxicity especially in C/P ratio of 2.

Conclusion: Results of the present study indicated that modification of PAA with alkane derivatives can help to prepare gene carriers with better transfection activity and less cytotoxicity.

Keywords: Alkane derivatives, Cytotoxicity, Gene delivery, Polyallylamine

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Introduction

Gene transfer systems are classified into two categories, viral and non-viral vectors. Despite recent advances, the major problem in the application of gene transfer systems is lack of carrier with high efficiency and low toxicity [1, 2]. Viral vectors exhibit high transfection efficiency but application of these vectors has been limited due to induction of immune response, low gene transfer capacity, high cost of production and insertional mutagenesis [3, 4]. Therefore, non-viral systems have been considered more in recent decades. These vectors are less toxic, able to carry large DNA molecules, producible with low-cost in large-scale. However, their low transfection efficiency compared to viral carriers is fundamental problem in implementing of these vectors [5, 6].

Among non-viral systems, cationic polymers show special advantages as carriers for gene transfer. Among the cationic polymers, polyallylamine (PAA) can be noted. PAA is a synthetic polymer with high primary amine groups. Interaction of this cationic polymer is provided with the negatively charged DNA due to its high positive charge. Toxicity due to the high positive charge of the polymer and low buffering capacity has limited the administration of PAA as a gene transfer carrier [7].

PAA structure can be modified to create new derivatives with different structures in order to improve transfection activity and reduce the cytotoxicity of polymer. In the present study, PAA was modified by alkane derivatives with different chain lengths to achieve a library of compound with different physicochemical properties. The aim of the present study was to obtain a vector capable to enter into the cell to the greater extent and act efficiently in escaping from endosomes. Also, we expect reduction in cytotoxicity of polymer due to covering of amine groups. The mentioned modifications have not been conducted on PAA in previous studies. Nanocomplexes composed of PAA-dextran-DNA with an average size of about 150 nm were prepared in the study of Nimesh et al [8]. The results of this study showed that transfection efficiency of these nano-particles was higher and cytotoxicity as well as significantly reduced compared to the PAA-DNA nanoparticles. Also, conjugation of mildly basic groups such as imidazole to PAA was also investigated to increase the proton sponge effect [9]. By this modification, in vitro transfection efficiency of synthesized nanoparticles was increased up to several folds compared to native PAA.

Materials and Methods

Materials

PAA with different molecular weights (15 and 65 kDa) were purchased from Polyscience Inc (USA). Bromoalkanes (1-bromobutane, 1-bromohexane and 1-bromodecane) were ordered from Aldrich (USA). Ethidium bromide (EtBr) was from Cinnagen (Iran).

Cell culture

Neuro2A (murine neuroblastoma) cells (ATCC CCL-131) were cultured in DMEM supplemented with fetal bovine serum (10%), streptomycin (100 μg/ml), and penicillin (100 U/ml). The cultured cells were incubated at 37 °C under 5% CO_2.

Modification of PAA

PAA was modified by the reaction between PAA and a series of bromoalkanes (Figure

1). Briefly, a solution of bromoalkane in DMF was added dropwise to the stirring solution of PAA (0.1 g in 5 mL DMF) over 3 hours. After 24 hour additional stirring at room temperature, the reactions mixture was dialyzed once against 0.25 M NaCl and twice against water (10000 Da cut-off dialysis tubing) to remove unreacted compounds. Finally after freeze-drying of final solution, the achieved powder was kept at 2-8 °C. The modified polymers were labeled as PAAX-YC AlkZ%, in which X is molecular weight of PAA, Y is number of carbons in alkyl chain of bromoalkane derivative and Z% is the percentage of PAA primary amines substituted with alkyl chains. The primary amine content of modified polymer was evaluated using quantification of accessible primary amines by coupling with 2, 4, 6 trinitrobenzene sulfonic acid (TNBS). Briefly, freshly aqueous TNBS solution (15 mg/ml) was added to various amounts of PAA dissolved in double distilled water. The mixture was diluted by sodium bicarbonate buffer solution (0.8 M, pH 8.5) and the UV absorbance of this solution was recorded at 410 nm.
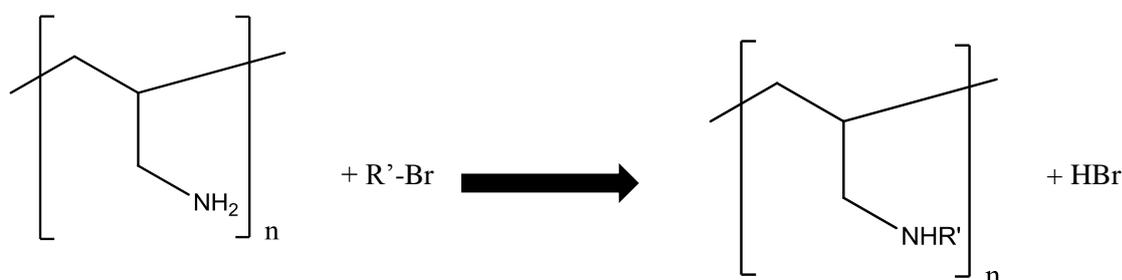
Ŕ =Butyl, Hexyl, Decyl

Figure 1. Schematic illustration of synthesis.

Preparation of polyplexes

For preparation of polyplexes, different amounts of modified polymer were mixed with DNA at different polycation to DNA weight ratios (C/P ratio) ranging from 2:1 to 6:1. The mixture was incubated at room temperature for 15 min before use.

Characterization of polyplexes

The mean size and zeta potential of nanoparticles were measured with Zetasizer Nano ZS (Malvern Instruments, UK). EtBr, a DNA-intercalating dye, was used to examine the association of DNA with the polymer solution to evaluate the condensation ability of modified polymer.

For this purpose, a solution of 400 ng/mL EtBr glucose was prepared with further addition of 10 μg/ml of DNA.

The fluorescence intensity of this mixture was measured at 510 (excitation wavelength) and 590 nm (emission wavelength) using a spectrofluorimeter (FP-6200; Jasco, Japan) and set to 100%. Next, equal amounts of polymer solutions were added stepwise to this solution, and the fluorescence intensity was recorded.

In vitro transfection activity

Twenty four hours before transfection, cells were seeded at a density of 1×10^4 cells per well in 96-well plates. Then

polyplexes were added to each well in different C/P ratios (2, 4 and 6) and incubated with cell for 4 hours, followed by replacement of the medium with fresh medium. After another 24 h, the medium was removed and lysis buffer were added to each well. The percentage of transfected cells was determined by measuring the fluorescence intensity of green fluorescent protein (GFP) at 498 nm (excitation wavelength) and 535 nm (emission wavelength) using fluorescent plate reader (Victor X5, Perkin-Elmer, USA).

Cytotoxicity of polyplexes

The metabolic activity of Neuro2A cells in the presence of polyplexes was determined using a thiazolyl blue tetrazolium bromide (MTT) assay.

Cells (1×10^4) were seeded and treated with the same amounts of polyplexes used for transfection experiment. After 4 hours

incubation, the medium was replaced by fresh culture medium. After 18 hours, 10 μl of MTT solution was added to each well. Then the medium was removed after incubation for 2 hours at 37°C, 100 μl of dimethyl sulfoxide added, and the samples further incubated at 37 °C for 30 minutes under constant shaking. Optical absorbance was measured at 590 nm (reference wavelength 630 nm) using a microplate reader (M200, Tecan, Switzerland), and cell viability was expressed as a percent relative to untreated control cells. Values of metabolic activity are presented as mean±SD for three samples.

Statistical analysis

One-way ANOVA test was used to analyze our data. Differences between means were statistically significant if the p-value was less than 0.05.
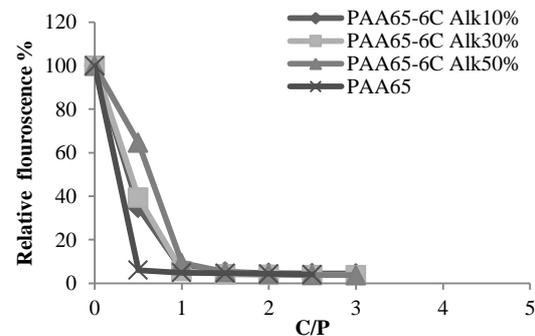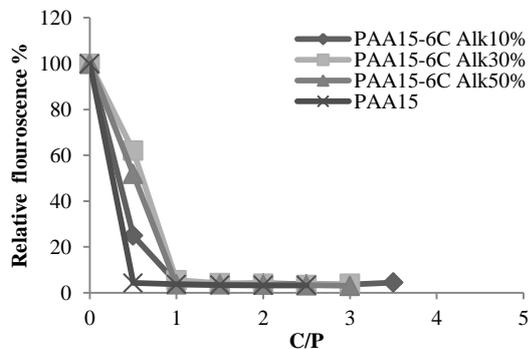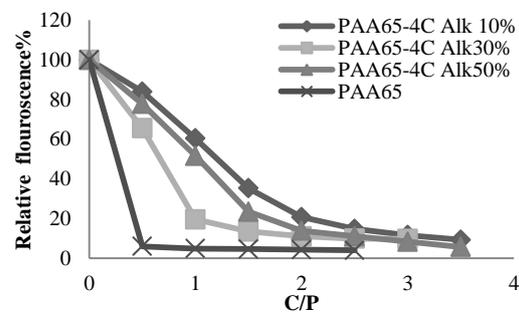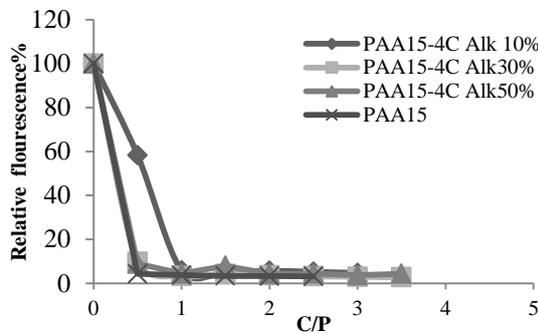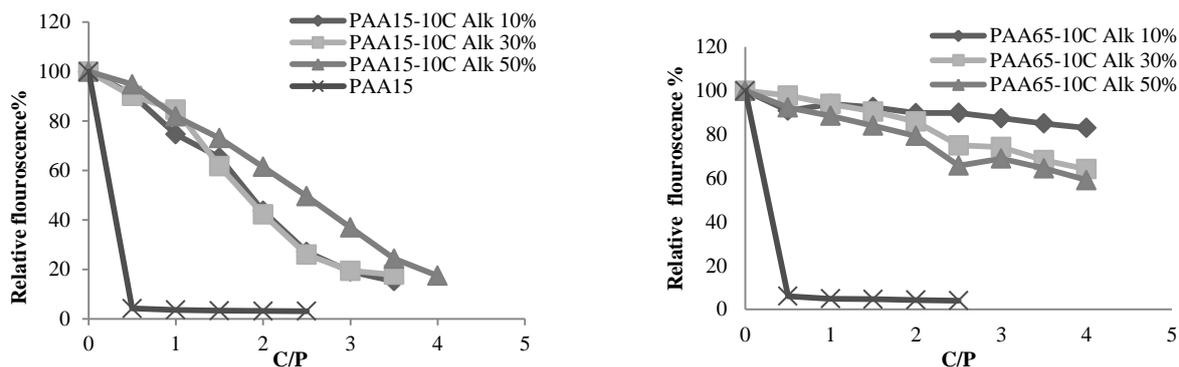
Figure 2. The DNA condensation ability of polyallylamines (15 and 65 KDa) and modified- polyallylamines by bromoalkane derivatives. DNA condensation measured as a decrease in fluorescence of EtBr.

Results

The extent of the modification of primary amines in the structure of PAA after conjugation by different boromoalkane derivatives was estimated by the TNBS method. As it was presented in Table 1, the degree of conjugation was in the range of 8–30 mol% depending on the molar ratios in the feed.

Table 2 shows the mean size and zeta potential of the prepared polyplexes. Size of polyplexes ranged from 117 nm to several microns.

For both molecular weights of PAA, nanoparticles composed of 1-bromodecane had the highest particle size. Type of bromoalkane derivative used and grafting percent had effect on mean size and zeta potential of final vectors. Zeta potential of nanoparticles changed from positive to negative in some cases.

PAA in both molecular weights condensed the plasmid at C/P ratio of 0.5 (Figure 2). The complete condensation was occurred at higher C/P ratio after modification of PAA. In case of modified PAA 65 KDa with 1-bromodecane, complete condensation was not occurred even in high C/P ratio.

For PAA 15 KDa, the transfection activity of polyplexes was increased by increasing in C/P ratio (Figure 3).

Modification of PAA 15 KDa with 1-bromohexane and 1-bromodecane at 30 and 50% in selected C/P ratios increased the transfection efficiency significantly ($P<0.05$). 15 KDa modified with 1-bromodecane (grafting of 50%) at C/P ratio of 4 showed the highest gene transfer ability. In case of vectors prepared with PAA 65 KDa, modification of polymer with bromoalkane derivatives did not affect the transfection activity of vector ($P>0.05$).

The cytotoxicity of PAA decreased remarkably by covering of the amine groups of polymer especially in the grafting percent of 10% (Figure 4).

The cytotoxicity of the modified polymer was increased by increasing in C/P ratio in both molecular weight of PAA. The vectors with the highest transfection activity had reasonable cytotoxicity.

Alkane-modified polyallylamine as gene delivery vector

Table 1. Composition of PAA polymers modified with acrylate derivatives.

Samples	Initial reaction feed (mol%)	Substitution of amines by acrylate (Mol%)
PAA15-4C Alk 10%	10	7.0
PAA15-4C Alk 30%	30	23.7
PAA15-4C Alk 50%	50	41.2
PAA15-6C Alk10%	10	3.2
PAA15-6C Alk30%	30	18.1
PAA15-6C Alk50%	50	39.7
PAA15-10CAlk10%	10	4.2
PAA15-10C Alk30%	30	22.4
PAA15-10C Alk50%	50	36.1
PAA65-4C Alk 10%	10	5.6
PAA65-4CAlk30%	30	23.4
PAA65-4C Alk 50%	50	35.4
PAA65-6C Alk10%	10	3.8
PAA65-6C Alk30%	30	17.9
PAA65-6C Alk50%	50	32.5
PAA65-10C Alk10%	10	4.8
PAA65-10C Alk30%	30	25.3

Table 2. Mean size and zeta potential of polyplexes of modified polyallylamine/DNA weight ratio of 4 (mean±SD, n=3).

Samples	Z-average (nm)	Zeta Potential (mV)
PAA15	189.7±5.4	19.2±3.0
PAA15-4C Alk10%	172.0±10.3	20.9±8.2
PAA15-4C Alk30%	152.2±36.3	13.3±0.9
PAA15-4C Alk50%	789.2±71.5	3.5±2.4
PAA15-6C Alk10%	345.6±45.8	10.3±2.4
PAA15-6C Alk30%	365.8±30.5	15.8±8.8
PAA15-6C Alk50%	307.1±97.9	21.3±3.4
PAA15-10CAlk10%	5057.7±277.5	16.0±1.8
PAA15-10C Alk30%	524.1±16.7	26.1±2.7
PAA15-10C Alk50%	872.3±146.5	-15.5±6.2
PAA65	194.7 ±44.2	21.5±1.4
PAA65-4C Alk 10%	206.2±9.8	22.2±3.3
PAA65-4CAlk30%	291.1±128.6	21.7±0.5
PAA65-4C Alk 50%	311.5±80.3	19.3±3.2
PAA65-6C Alk10%	194.4±4.6	14.4±1.7
PAA65-6C Alk30%	117.2±0.1	23.4±3.1
PAA65-6C Alk50%	198.6±15.7	28.4±2.5
PAA65-10C Alk10%	1207.8±102.6	-20.9±5.0
PAA65-10C Alk30%	507.9±158.5	-24.7±1.0
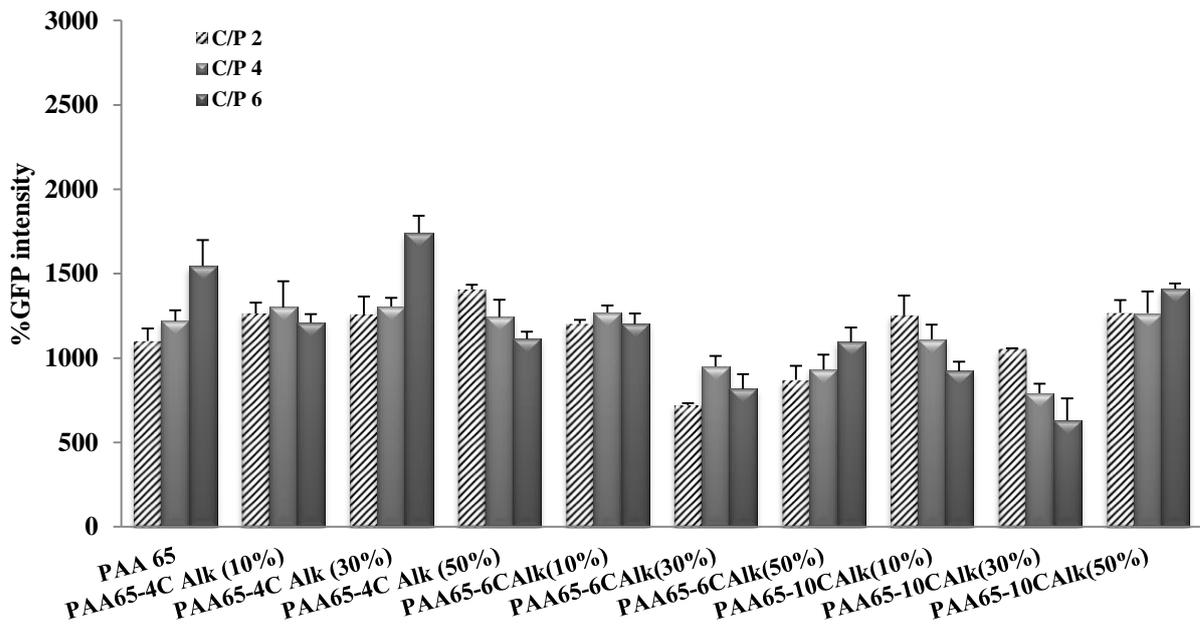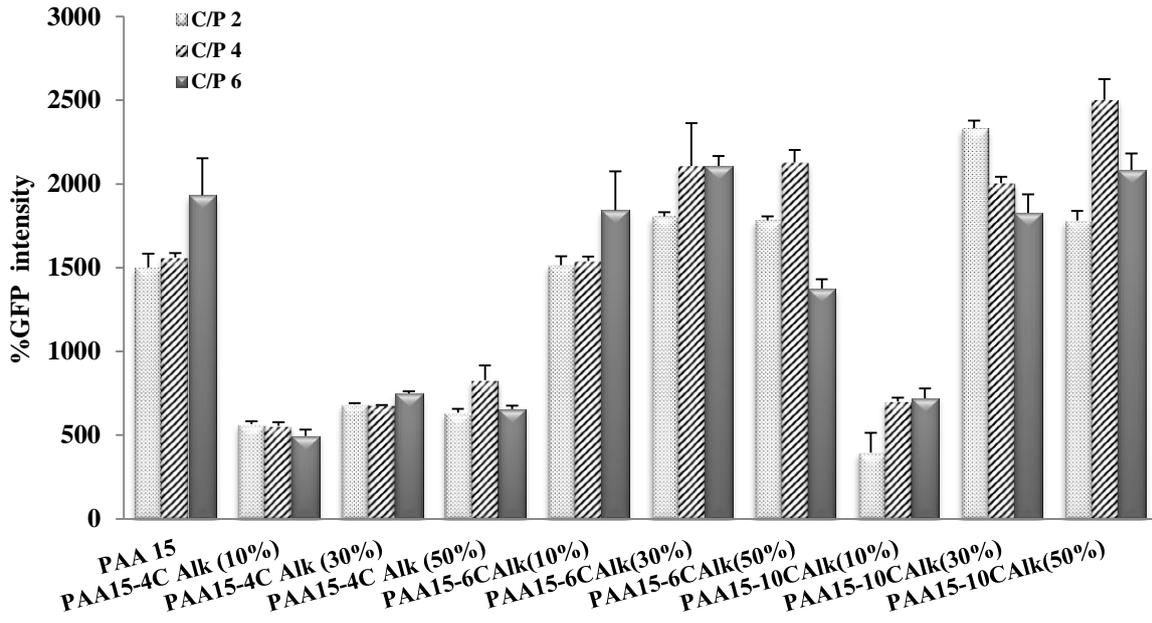
Figure 3. Transfection activity of polyplexes prepared by polyallylamine (15 and 65 kDa) or modified polyallylamine in Neuro2A cells. Complexes were prepared at three different C/P ratios (mean±SD, n=3).

Figure 4. Cytotoxicity of polyplexes prepared by polyallylamine (15 and 65 kDa) or modified polyallylamine at different C/P ratios (mean±SD, n=3).

Discussion

Gene delivery approaches have been developed for the treatment of many diseases [10]. Transfection efficiency of non-viral systems such as cationic polymer is lower than with viral vectors but because of several advantages including ease of synthesis, low cost and safety, these gene delivery systems have been considered more [11].

PAA is a synthetic polymer with a high density of primary amines. This polymer has been used as non-viral gene transfer system [12] but application of this polymer has been limited because of cell toxicity. Also, one of drawbacks of this polymer is

low buffering capacity [8, 9]. In this study, in order to increase the hydrophobicity and to reduce toxicity of polymer, PAA was modified with bromoalkane derivatives in three grafting percentages (10, 30 and 50%). Hydrophobic groups in the structure of polymer can increase the interactions of carrier with both cell and endosome membranes. This modification may increase the possibility of vector crossing from cell membrane and improve the efficiency of the polyplex escape from endosomes [13]. Consequently, variation in transfection activity among different modified PAAs can be related to favorable hydrophobic-hydrophilic balance of polymer [14].

Covering the amine groups of PAA decreased the ability of polymer to condense DNA. This speculated result is due to the substitution of positively-charged groups by hydrophobic groups [15]. Since the condensation is resulting from the interaction of positively charged groups of polymer and negatively charged phosphate groups of DNA, full condens-ation occurred for modified polymer in higher C/P ratio. On the other hand, the ability to condense DNA is an advantage for a designed vector but DNA release from vector after cell uptake should be considered. Consequently, considering the optimal balance between condensation and separation of DNA from vector is necessary [14]. Size and surface charge are two important parameters in controlling uptake by cells [16]. Size of the carriers has an important role in carrier entrance into the cell via endocytosis. Having an appropriate surface charge is necessary for the interaction of vector with cell surface [17]. Previous studies related to this field have shown that the hydrophobic

modification of polymer can increase size of resultant polyplexes compared to unmodified polymer. This increase in size may be cut back due to poor interactions of modified polymer and DNA. In this study, almost with increasing grafting percent and also increase the number of carbon substituent In some vectors, the zeta potential was unusual and final polyplexes showed negative surface charge. As it was modeled in the study of Kuhn et al., the charge inversion in case of polyplexes or lipoplexes may happen with even small concentration of cationic amphiphile (polymer or lipid), if it is sufficiently hydrophobic [18].

Different studies show gene carriers enter cells according to their size. For example, particles with a diameter less than 200 nm are endocytosed by clathrin-mediated pathway. Polyplexes formed from modified polymer with 10% grafting had the mean size around 200 nm. Probably these carriers are entering into the cells by clathrin mediated- endocytosis. Better transfection achieved with this modified polymer may be related to the difference in uptake pathway. Although the transfection ability of PAA after modification was improved, it may not consider being a great achievement but from our point of view the importance of it will be clarified when the results of toxicity are considered as well. In the other word, modification of PAA has resulted to some vectors with better transfection ability with reduced toxicity. Several studies have reported the reduction of toxicity after conjugation of hydrophobic moieties to polycations [19, 20].

Conclusion
The results of study showed that

modification of PAA with bromoalkane derivatives increased transfection activity and reduced the vector cytotoxicity.

Acknowledgments

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