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Research Article

Preparation of Effective and Safe Gene Carriers by Grafting Alkyl Chains to Generation 5 Polypropyleneimine

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Abstract. Gene therapy is a novel method to treat a variety of diseases including genetic disorders and cancer. Nonviral gene carriers have now gained considerable attention as gene carrier systems. Polyamidoamine (PAMAM) and polypropyleneimine (PPI) are the two most widely used denderimers in gene delivery studies. The aim of the current study was to investigate the effects of modification of generation 5 polypropyleneimine (G5 PPI) dendrimers with alkanoate groups as hydrophobic moieties on DNA transfection and cytotoxicity. Six, 10, and 16 carbon derivatives of bromoalkanoic acids were conjugated onto PPI with 10%, 30%, and 50% of surface amine grafting. Ethidium bromide exclusion assay results proved the ability of modified carriers to condense DNA. Transfection assay showed higher DNA delivery potential for 30% and 50% grafting with decanoate moieties compared to native G5 PPI and Superfect™. 3-(4,5-Dimethylthiazol-2-yl)-2,5-di phenyltetrazolium bromide (MTT) and apoptosis experiments showed lower toxicity for modified carriers compared to unmodified PPI. The hemolytic effect of grafted carriers was not significantly different from G5 PPI. Size and zeta potential measurements revealed that polyplex size was less than 200 nm and electrical charges were in the range 14–25 mV. The hydrophobic modifications improved transfection activity and toxicity of G5 PPI without negatively affecting hemocompatibility. These modified carriers are therefore promising candidates for further *in vivo* investigations.

KEY WORDS: gene delivery; hydrophobic modification; nonviral vector; polypropyleneimine; transfection.

INTRODUCTION

The application of nucleic acids with different structures and functions as therapeutic agents has long been an intriguing challenge in molecular medicine. All types of nucleic acids need to be delivered into specific cells by a carrier (1). Nonviral gene delivery systems offer many advantages over their viral counterparts such as safety, high loading capacity, potentials for targeted delivery, low cost, and ease of preparation (2).

Cationic polymers are one of the main categories of nonviral gene carriers (3). Dendrimers are cascade polymers which consist of three main regions: an initial core, repetitive units expand toward the outermost layer (generations), and the terminal layer which, in case of cationic dendrimers, usually provides surface primary amines (4). Dendrimers have high potentials in nano-medicine, because of their uniform structure and monodispersity, high loading capacity, and relatively low toxicity (5). Polyamidoamine (PAMAM) and polypropyleneimine (PPI) are the two most widely investigated dendrimers (6). However, fewer studies have been done on PPI compared to PAMAM in the field of gene delivery (7). The greatest problem with dendrimers and PPI, in particular, is low transfection efficacy (6). Therefore, several modifications have been made on this dendrimer which have resulted in improved transfection and reduction in toxicity. Some of these modifications include addition of arginine, guanidyl groups, oligoethyleneimine; quarternerization of primary amines by methylation; and targeting of PPI dendrimers by transferrin, folate galactose, and dextran (8–13).

Hydrophobic/hydrophilic balance is one of the major factors involved in the success of a chemical vector (14). The addition of hydrophobic moieties to polycations has led to enhanced gene transfection in a variety of constructs such as polyethyleneimine (PEI), poly-l-lysine (PLL), PAMAM dendrimers, dendrons, and other similar structures (14,15). These modifications are thought to show their effects by a variety of mechanisms such as improved physical

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encapsulation of nucleic acid in polyplexes due to flexible three-dimensional conformation of the conjugated hydrophobic chains, enhanced cell adsorption, induction of endocytosis, and thus increased cell uptake (14). Alkylation or acylation of primary amines in PEI decreases the buffering capacity of PEI in the endosomal pH range (16,17). Substitution of primary amines with hydrophobic groups may also weaken the DNA condensation ability of polycations. However, this effect has been shown to be beneficial in some cases in terms of efficient release of DNA from polymer in the cytosol (18). In general, a balance between hydrophobicity and hydrophilicity seems to be essential for optimal delivery of genetic material.

Previously, we have shown that hydrophobic substitution of PEI (19,20) and generation 4 PPI (G4 PPI) (21) enhances gene transduction efficacy without concerns about cytotoxicity. Generation 5 polypropyleneimine (G5 PPI) has more surface amine groups compared to G4 PPI, which may provide better transfection efficacy. We therefore modified G5 PPI dendrimers with similar hydrophobic groups in order to achieve an optimal nonviral vector with higher transfection and desired biocompatibility. Linear bromoalkylcarboxylate groups with three different side-chain lengths (6, 10, and 16 carbons) and three different degrees of substitution (10%, 30%, and 50% of surface primary amines) were added onto G5 PPI dendrimers. The effects of these modifications on transfection, toxicity, apoptosis, and hemocompatibility were then evaluated.

MATERIALS AND METHODS

Materials

G5 PPI was purchased from SyMO-Chem BV (Eindhoven, The Netherlands). 6-Bromohexanoic acid, 10-bromodecanoic acid, 16-bromohexadecanoic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-di phenyltetrazolium bromide (MTT), N-[2-hydroxythyl] piperazine-N′-[2-ethane sulfonic acid] (HEPES), and propidium iodide (PI) were obtained from Sigma-Aldrich (Munich, Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were bought from GIBCO (Gaithersburg, USA). Ethidium bromide was supplied by CinnaGen (Tehran, Iran).

Synthesis of Bromoalkylcarboxylate Derivatives of G5 PPI Dendrimers

Bromoalkyl carboxylation was done by nucleophilic substitution as previously described (19). Briefly, 20 mg of G5 PPI was dissolved in 5 ml of chloroform. The substituents were also dissolved in 5 ml of chloroform and added dropwise to the PPI chloroform solution while being rigorously stirred. The reaction was allowed to proceed overnight. Chloroform was then removed by a rotary evaporator and the precipitate was dissolved in 5–10 ml of double-distilled water (DDW). The aqueous solution was freeze-dried. The resulting milky to brownish lyophilized powder was stored for further investigations.

Estimation of the Degree of Substitution of Bomoalkylcarboxylate Groups on PPI Primary Amines by TNBS Colorimetric Assay

The degree of substitution of PPI primary amines with bromoalkylcarboxylate was determined by estimation of free primary amine groups through reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS), which selectively binds primary amino groups and forms a yellow-colored conjugate (22). Freshly prepared aqueous TNBS solution (2.5 μl of 8.75 mg/ml solution) was simultaneously added to the wells of a 96-well plate containing different amounts of PPI (to obtain a standard curve for absorbance against unmodified PPI concentration) or PPI derivatives dissolved in 100 μl of 0.1 μM borate buffer solution. The UV absorbance of the samples was measured by an ELISA plate reader at 410 nm. Wells without PPI were used as blank to measure the background unreacted TNBS absorbance.

Equivalent PPI concentration for each carrier in the well (μg/100 μL) was obtained by interpolation of the standard curve. Since the number of primary amine groups per G5 PPI molecule is 64, then the number of primary amine groups in each carrier per well (n) could calculated. The percentage of grafting (X) in the modified PPI carriers was estimated using the following formula:

$$C \times (64-X) = n \times [7168.1 + (X \times S)]$$

where C is the amount of each carrier in the well (μg/100 μL), n is the number of primary amine groups per G5 PPI molecule in each carrier, S is the molecular weight of the hydrophobic substituents, 64 is the number of primary amine groups per G5 PPI molecule, and 7168.1 is the molecular weight of G5 PPI.

Determination of pDNA Condensation Properties of Modified PPI by the Ethidium Bromide Exclusion Assay

Ethidium bromide (EtBr), a DNA intercalating agent, was used to measure the ability of polymers to condense DNA. HBG buffer containing EtBr (400 ng/ml) was freshly prepared. To obtain peak fluorescence, 5 μg plasmid was added to the 1 ml of the buffer solution. PPI or PPI derivatives were added stepwise (2.5 μg each time) and gently stirred, and the reduction of fluorescence was measured after each addition at 510 nm excitation and 590 nm emission wavelengths using a Jasco FP-6200 spectrofluorometer (Tokyo, Japan). The addition was repeated until plateau fluorescence was reached. All measurements were repeated three times and relative fluorescence (%) was plotted against carrier weight/plasmid weight (c/p) ratio.

Plasmid DNA Preparation

pRL-CMV-luc was transferred into DH5a *Escherichia coli* bacterial strain, proliferated in selective Luria–Bertani (LB) medium and centrifuged. The plasmid DNA was then extracted using the Qiagen Endofree Mega Plasmid Kit (QIAGEN, Hilden, Germany) according to the manufacturer's manual. Green Fluorescent Protein (GFP) plasmid was also prepared the same way as pRL-CMV-luc.

Cell Culture and Luciferase Transfection Assay

In order to compare DNA transfection efficiency of the carriers, murine neuroblastoma (neuro-2a) cells (ATCC, CCL-131) were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h in DMEM medium containing 10% FBS. G5 PPI and bromoalkylcarboxylate derivatives were complexed with pRL-CMV plasmid at *c*/*p* ratios of 2, 4, and 6 and added to each well (200 ng plasmid was used per well). Superfect™ was used at the same ratios as positive control. The media was replaced with fresh media after 4 h. Cells were further incubated overnight at 37°C and luciferase activity in cell lysate was measured using Promega luciferase assay kit on Luminometer (Berthold Detection System, Pforzheim, Germany). The results were reported in relative luminescence units (RLU)/4000 cells as mean±SD of at least three independent experiments.

Cell Transfection with GFP Reporter Gene Expression

Neuro-2a cells were seeded in 24-well plates (4×10^4 cells per well). Polyplexes made with Superfect™, G5 PPI, or the vectors with highest luciferase transfection efficiency were added to the cells at different *c*/*p* ratios (3 μg pEGFP plasmid per well). Culture medium was refreshed after 4 h. Cells were harvested 18 h after transfection and kept on ice until analysis. Live transfected cells were observed using a JuLI™ digital fluorescence microscope (NanoEnTek, Korea Republic).

MTT Cytotoxicity Assay

In order to assess cytotoxicity caused by G5 PPI and its hydrophobic derivatives, 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 as for the transfection experiment. The medium was replaced with fresh complete medium after 4 h of exposure to dendriplexes. After 18 h, 20 μl sterile filtered MTT stock solution in PBS (final MTT concentration 5 mg/ml) was added to each well, and after 4 h, the MTT-containing medium was withdrawn and the remaining formazan precipitate was reconstituted with 100 μl DMSO. The absorbance (*A*) was measured at 570 nm. Cell viability (%) relative to control wells containing cell culture medium without the addition of polyplexes was calculated.

Flow Cytometric Analysis of Apoptosis Caused by the Modified Vectors

In order to investigate the effect of the substituted groups on induction of apoptosis, PI staining method was used. In brief, neuro-2a cells were cultured overnight in a 48-well plate at the initial density of 2×10^4 cells/well and then treated with decanoate-G5 PPI derivatives at a *c*/*p* ratio of 4. After 4 and 24 h, cells were then harvested and incubated 2 h at 4°C in the dark with 150 μl of a hypotonic buffer (50 μg/ml PI in 0.1% sodium citrate, 0.1% Triton X-100, and 100 μg/ml RNAase A). The resulting cells were analyzed by flow cytometry (Partec, Germany) to detect sub G1 peak (apoptotic cells) which showed reduced DNA stainability following staining with a PI as high-affinity DNA binding fluorochrome.

Hemolysis Assay

RBC hemolysis assay was tested as previously described (10). Human red blood cells (RBCs) were freshly isolated and washed four times in phosphate-buffered saline by centrifugation at 800*g* for 10 min at 4°C. The erythrocyte pellet was suspended in 150 mM NaCl at a concentration of 10% (V/V). Serially diluted concentrations of decanoate-PPI derivatives (ranging from 2.5 to 80 μg/ml) were prepared in 150 μl of the HEPES-buffered saline per well in a V-bottom 96-well plate. Control wells contained 100 μl of buffer with 1% Triton-X-100 (corresponding to 100% hemolysis). Added to each well was 10 μl of erythrocyte suspension, and the plates were incubated at 37°C for 30 min under constant shaking. After centrifugation at 2200 rpm for 10 min, 70 μl of supernatant was analyzed for hemoglobin release at 405 nm using an ELISA microplate reader (n=3 for each sample). The results were represented as % hemolysis (compared to Triton-X-100 1%) against carrier concentration (μg/ml).

Particle Size and Zeta Potential Measurements

Various amounts of PPI derivatives, unmodified PPI, and Superfect™ (calculated to prepare the *c*/*p* ratios of 2 and 4) were prepared in 125 μl filtered DW and added to equal volume of solution containing 5 μg pDNA plasmid, gently stirred, and left for 20 min to allow polyplexes to form. The final volume was adjusted to 1 ml by filtered DW. Particle size and zeta potential of PPI or the optimal modified vectors were measured using dynamic light scattering (DLS) and laser Doppler velocimetry (LDV), respectively, by a Malvern Nano ZS instrument (Malvern Instruments, UK). The results are reported as mean±SD (N=3). Each mean represents the average value of 30 measurements from each sample.

Statistical Analysis

Data was analyzed by the one-way ANOVA test and Tukey–Kramer posttest using GraphPad PRISM® 5 software.

RESULTS AND DISCUSSION

Structure Enhancement of G5 PPI with Hydrophobic Bromoalkylcarboxylate Residues

Hydrophobic modification has been shown to improve gene delivery properties of polycations in several studies (14). To investigate the effects of these modifications in G5 PPI, bromoalkanoic acids containing 6, 10, and 16 carbon side chains were added to PPI by nucleophilic substitution reaction at substitution rates of 10%, 30%, and 50%, of surface primary amines (Fig. 1). These carriers were designated by the origin, length of hydrophobic substitution, and supposed degree of substitution (*e.g.*, PPI G5-6Br-10% for PPI substituted with 6-bromo hexanoic acid at 10% degree of substitution). TNBS assay results showed that substitution was done on surface primary amines close to expected grafting percentages

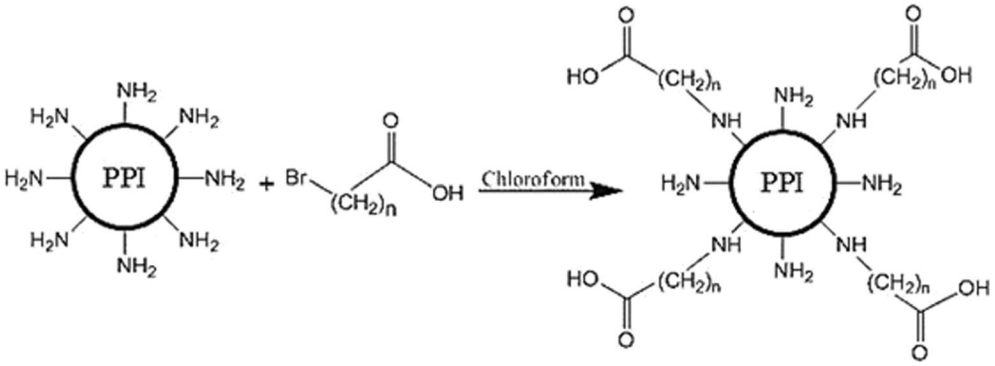

Fig. 1. Schematic synthesis of alkylcarboxylated derivatives of G5 PPI by nucleophilic substitution reaction between 6-bromohexanoic acid (n=5), 10-bromodecanoic acid (n=9), and 16-bromohexadecanoic acid (n=15) and primary amines of PPI

(Table I). However, at higher degrees of substitution, the estimated grafting was lower than expected.

Hydrophobic Moieties Mildly Weaken PPI-pDNA Interactions

Successful packaging of pDNA is an important step in DNA delivery (19). Polymers must pack DNA into condensed form to allow protection from different nucleases and provide efficient cell penetration (19). Ethidium bromide exclusion assay was carried out to evaluate the effect of our hydrophobic modifications on these dendrimer–DNA interactions. The results show that all the carriers condensed pDNA at *c*/*p* ratios between 1.5 and 2.5, and substitution with hydrophobic alkylcarboxylate groups shifted the maximum condensation *c*/*p* ratio by 0.5 to 1.0 units higher (Fig. 2). These results indicate that although hydrophobic modifications lowered DNA condensation properties of PPI, the reduction was not too profound to hinder the formation of polyplexes at suitable *c*/*p* ratios. Nevertheless, PPI G5-16Br-50% did not reach a fluorescence plateau until the *c*/*p* ratio of 3.5. This carrier was excluded from further test due to poor condensation properties and low water solubility.

Higher degrees of grafting with alkyl chains results in the reduction of the number of amine groups per mole of the dendrimer, which may explain the reason our substituted vector were needed at higher *c*/*p* ratios to do the condensation. This effect, however, did not interrupt packing of DNA, probably due to the presence of abundant unsubstituted primary amine groups. Nonetheless, this phenomenon did not occur for one of the polymers with the longest side chain at the highest degree of grafting. Insufficient DNA condensation may lead to failure in formation of complexes and total loss of transfection activity such as observed by Okuda *et al.* with histinylated PLL (23). On the other hand, strong binding may result in unsuccessful release of DNA inside the cell and thus low transfection, as it was concluded by Gabrielson *et al.*, that enhancement of transfection activity of acetylated PEI compared to unmodified PEI was due at least in part to facilitated dislocation of condensed DNA from the polyplex inside the cell (18). A balanced polymer/DNA interaction is thus needed in order to achieve high transfection. The observed mild reduction in the DNA condensation ability of these polymers may have contributed to the observed higher transfection by maintaining such a balance.

Table I. Calculated Degree of Substitution of Alkylcarboxylate Groups on Primary Amines of G5 PPI by Derivatization with 2,4,6-Trinitrobenzenesulfonic Acid (TNBS)

Carrier	Calculated % of primary amines substitution	% of primary amines substitution measured by TNBS assay
PPI5-6Br-10	10	9.1
PPI5-6Br-30	30	23.9
PPI5-6Br-50	50	40.4
PPI5-10Br-10	10	9.5
PPI5-10Br-30	30	14.0
PPI5-10Br-50	50	31.5
PPI5-16Br-10	10	8.3
PPI5-16Br-30	30	26.2
PPI5-16Br-50	50	34.3

Alkylcarboxylation of G5 PPI Significantly Enhances pDNA Transfection

Transfection is one of the key properties of a gene delivery system (2). Neuro-2a cells were exposed to 200 ng plasmid DNA complexed dendrimers at different *c*/*p* ratios from 2:1 to 6:1, and Renilla luciferase assay was then done to determine the ability of our vectors to introduce genetic material into cells as measured by the expression of luciferase. Superfect™, another dendrimer-based commercial transfection agent was used as positive control at the same *c*/*p* ratios.

Luciferase transfection results (Fig. 3) showed that almost all polyplexes had higher luciferase activity than unmodified carriers at the same *c*/*p* ratios. The highest transfection activity was observed with 10-Br modified PPI with 50% primary amine substitution and the optimal *c*/*p* ratio for this carrier was found to be 4:1. This vector increased transfection 1642-fold compared to native G5 PPI and 3.5-fold compared to Superfect™ at the same *c*/*p* ratio (p<0.001). The transfection for this vector was significantly higher (1.2-fold) than the highest transfection obtained by Superfect™, which occurred at the *c*/*p* of 6 (p<0.05).

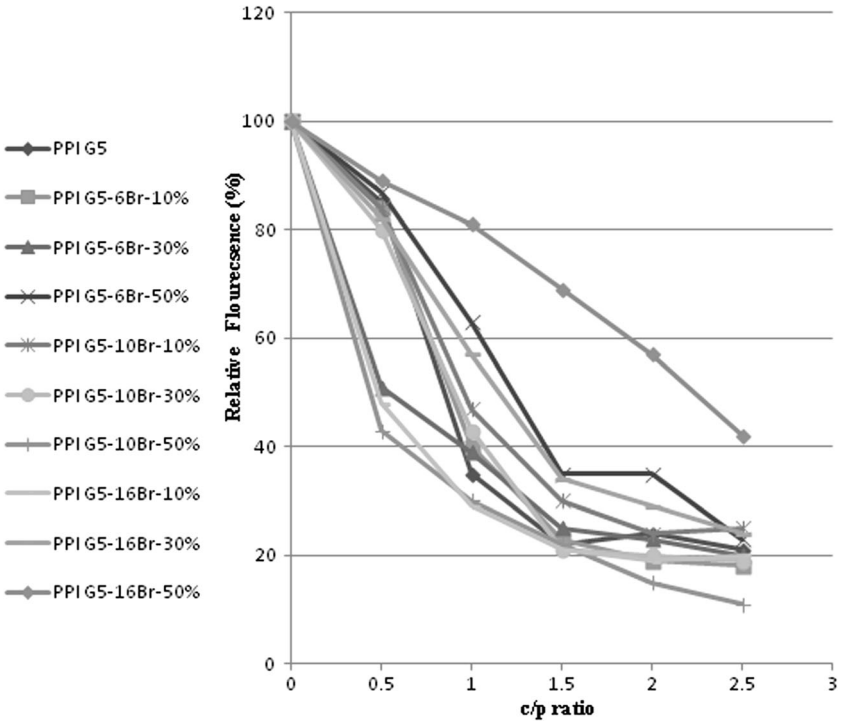

Fig. 2. Plasmid DNA condensation by G5 PPI and its alkylcarboxylated derivatives as measured by their ability to displace DNA from its fluorescent complex with ethidium bromide in HBG buffer medium. The *c*/*p* ratio with highest condensation was 1.5 for unmodified PPI; 1.5, 1.5, and 2.5 for 6Br-modified dendrimers; 1.5, 1.5, and 2 for 10-Br-modified carriers; and 1.5, 2, and 3.5 for 10%, 30%, and 50% substitution levels, respectively

Thirty percent substitution of primary amines by 10-Br groups also resulted in higher transfection than Superfect™ at c/p ratios of 2 and 4, respectively ($p<0.001$).

To confirm luciferase assay results, polyplexes with the highest luciferase activity were used to transfect cells with GFP plasmid. Viable GFP-expressing cells were observed under a fluorescence microscope and a representative field of view is shown in Fig. 4.

Hydrophobic modifications have been shown to enhance transfection in several studies and various mechanisms have been proposed for this observed effect. It has been shown that hydrophobic moieties may interact with membrane phospholipids, which enhances internalization of polyplexes (24,25).

The effect of hydrophobic side chain length on transfection seems to be highly dependent on the structure and architecture of the native polymer. For example, upon acetylation,

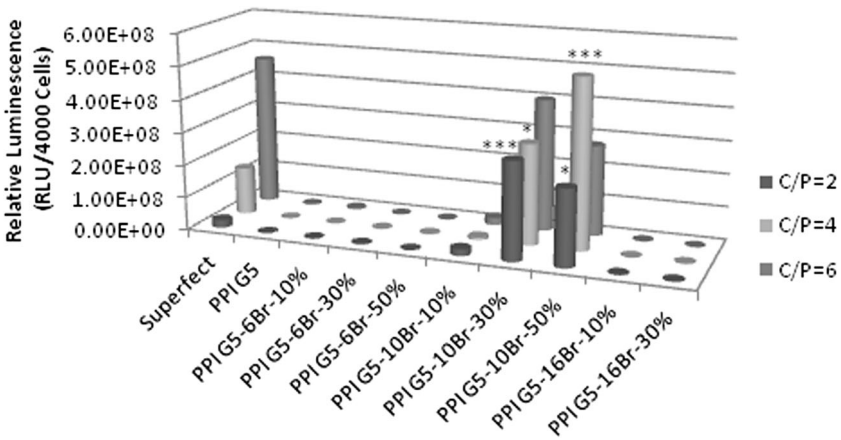

Fig. 3. Luciferase transfection assay in neuro-2a cell line. G5 PPI dendrimers and their alkylcarboxylate with 6, 10, and 16 carbon atom length derivatives were complexed with pDNA and tested for their transfection activity. Results are presented in relative luciferase units (RLU)/4000 cells of at least three repeats and compared to Superfect at the same *c*/*p* ratios

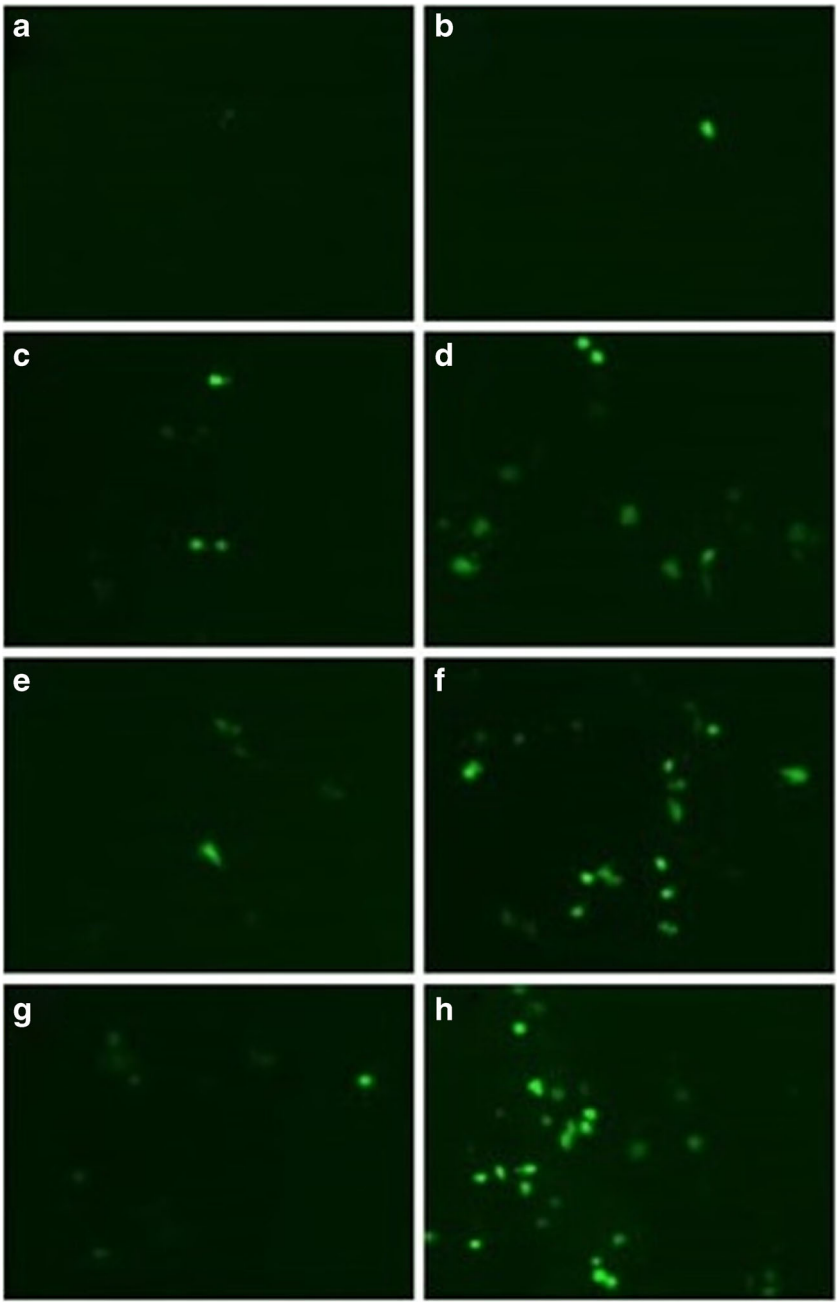

Fig. 4. GFP expression mediated by Superfect (positive control) (**a** and **b**), G5 PPI (**c** and **d**) and its decanoic acid derivatives with 30% (**e** and **f**) and 50% (**g** and **h**) grafting of primary amines at *c*/*p* ratios of 2 (**a**, **c**, **e**, **g**) and 4 (**b**, **d**, **f**, **h**)

propionylation, and butyration of 750 kDa PEI, Nimesh *et al.* found that optimal transfection was observed with propionylated 750 kDa *PEI* (17). In another study, Doody *et al.* conjugated branched PEI with acetate, butanoate, and hexanoate and found no special trend in transfection activity based on side chain lengths (16). Also, G5 PAMAM dendrimers modified with lauric (12 carbons), myristic (14 carbons), and palmitic (16 carbons) acids showed the best results with lauric acid (15).

In the present study, the optimal side chain length was found to be 10 carbons for G5 PPI dendrimers. This is in line with the results obtained from a recent study in our lab on G4 PPI denrimers, where ~40% grafting with decanoic acid resulted in higher transfection compared to Superfect at *c*/*p* ratios of 2 and 4. Our previous studies show the optimal side chain length to be six carbons for 10 kDa PEI, as well as high molecular weight PEI (20,26). The different optimal side chain length for PPI as compared to PEI may be attributable to the highly hydrophilic shell of PPI, which requires more hydrophobic groups to reach a hydrophobic/hydrophilic equilibrium. Substitution of 50% primary amines of G5 PPI with 16-bromohexadecanoate produced a vector which was unable to condense DNA effectively. This carrier did not show any transfection activity and was excluded from further

experiments. In other 16 carbon-G5 PPI derivatives, the transfection activity was not comparable to their decanoate-modified counterparts. These results also suggest that an appropriate balance between cationic and hydrophobic regions of alkylated G5 PPI is required for obtaining an efficient vector. In another study, although quaternization of tertiary amines of 25 kDa PEI with dodecyl and hexadecyl side chains led to a fivefold increase in transfection efficiency compared to native PEI, dodecylation and hexadecylation of primary amines of 25 kDa PEI resulted in low or no transfection activity of the polymer (27). The effects of the degree of substitution of primary amines with hydrophobic groups have been investigated in many studies. Nevertheless, on the study done on G5 PAMAM (128 surface amines), 14- and 16-carbon long side chains could produce transfection activity comparable to Superfect™ at substitution degrees as low as about 5% of primary amines. It is interesting that increasing the grafting by another 5% resulted in very low transfection and higher toxicity. It may therefore be worth trying lower percentages of grafting for the hexadecanoic acid in future studies.

Doody *et al.* observed that substitution of primary amines of branched PEI with saturated hydrocarbons enhances transfection only at low degrees of substitution (below 25%) (16). Acylation of 750 kDa PEI also enhanced transfection by grafting up to 30% propionylated 750 kDa PEI (17). In our study with alkylcarboxyl-grafted 10-kDa PEI, substitution of more than 35% of primary amines with hexanoate abated transfection activity due to high toxicity (20).

However, G4 PPI dendrimers showed better transfection at higher grafting percentages (21), which is in line with the findings of the present study. The different effects of the degree of substitution on transfection of PPI as compared to PEI may be explainable by relatively low toxicity of native PPI. The lower toxicity of native PPI allows for higher substitution while maintaining the biocompatibility of the polymer. Another explanation might be that the dendritic structure of PPI provides a hydrophilic shell which needs more hydrophobic groups to achieve a hydrophobic balance, while PEI-hydrophobic regions are more exposed and thus require fewer hydrophobic groups. This may also explain the reason why 6-carbon chain length is optimal in PEI while the appropriate side chain length is 10 carbons for PPI dendrimers.

Balanced Alkylation Reduces the Cytotoxicity of G5 PPI

Dendrimers have been shown to have lower toxicity than some conventional polycations (5). However, the toxicity associated with dendrimers, and PPI, in particular, is a basic hurdle in their biomedical application (6). The cytotoxicity of polyplexes used in the transfection study was tested by MTT assay, where cell viability is measured by the ability of the cells to reduce MTT to its colored derivative. Cell viability was tested for all polyplexes with 4 h

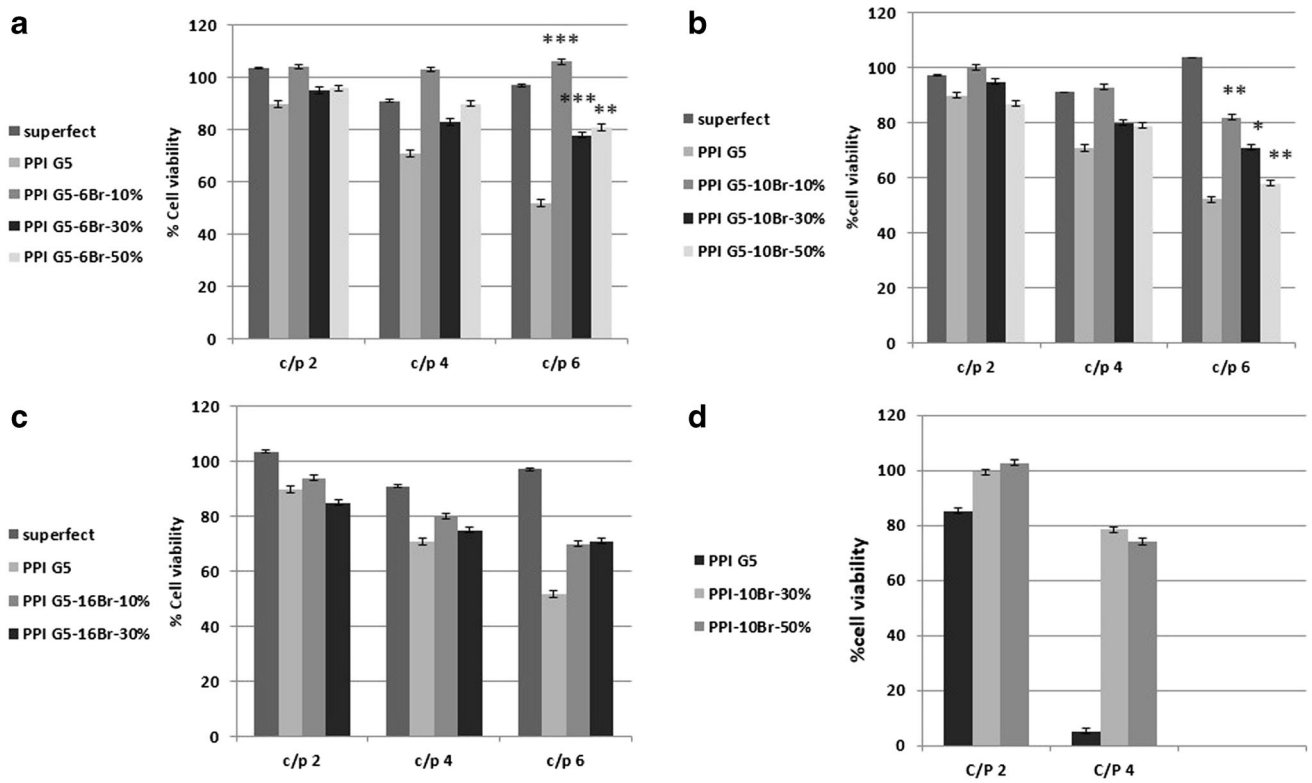

Fig. 5. Cell viability (%) of neuro-2a cells after 4 h exposure to Superfect, G5 PPI, and 6-Br (**a**), 10-Br (**b**), and 16-Br (**c**) derivatives by the MTT cytotoxicity assay. MTT test was also done with 24 h exposure for 10-Br-substituted dendrimers (**d**). Results are represented as mean±SD of relative viability as compared to untreated cells (n=3)

exposure time. Because high transfection results were observed with 10-Br substituted dendrimers, these polymers were also tested with 24 h exposure, among other further investigations. The results showed a *c*/*p* ratio dependent increase in toxicity of unmodified PPI, with relative viability reduction down to less than 60% at a *c*/*p* ratio of 6. Generally, modified dendrimers were less toxic than unmodified PPI (Fig. 5). The toxicity showed an increasing trend with increased *c*/*p* ratios in the modified polymers as well. Compared to unmodified PPI, however, the increase in toxicity with these vectors was less evident with increased *c*/*p* ratio. Superfect™ showed more than 90% viability at all *c*/*p* ratios, and cells treated with this polymer were more viable than G5 PPI and its hydrophobic derivatives.

Toxicity tended to increase with increased degree of substitution for the same side-chain length and *c*/*p* ratio. Cell viability was lower than 60% in PPI-10–50% at the *c*/*p* ratio of 6, which was significantly lower than PPI-10–30% at the same c/p ratio ($p<0.001$) (Fig. 5b). It can be concluded from this observation that toxicity *was a possible reason* for lower transfection of PPI-10–50% compared to PPI-10–30% at *c*/*p* 6, while the transfection for 50% substituted PPI was higher at *c*/*p* of 4. Increased side chain length also resulted in higher toxicity.

For better understanding of the effects of exposure time on toxicity, 10-Br-modified carriers were also evaluated with 24 h exposure time. Longer exposure of dendriplexes significantly reduced cell viability. In the 24 h test, the effects of substitution degree and *c*/*p* ratio were similar to those seen in the 4 h test, though in a more exaggerated manner. Cells exposed to 10-Br-modified dendriplexes with a *c*/*p* ratio of 6 showed no mitochondrial activity after 24 h of exposure (Fig. 5d). Similar to the 4 h test, in the 24 h test, the reduction of toxicity of modified polyplexes with increased *c*/*p* ratio was not as much as it was for unmodified PPI.

The effects of hydrophobic modifications on toxicity of cationic polymers are controversial. Our previous experiments with hydrophobic modifications such as alkyl carboxylation of G4 PPI did not show any marked toxicity (21).

Oskuee *et al.* (20) demonstrated that hydrophobized 10 kDa PEIs induced less cytotoxicity than unmodified 10 or 25 kDa PEI and that cytotoxicity was dependent on degree of

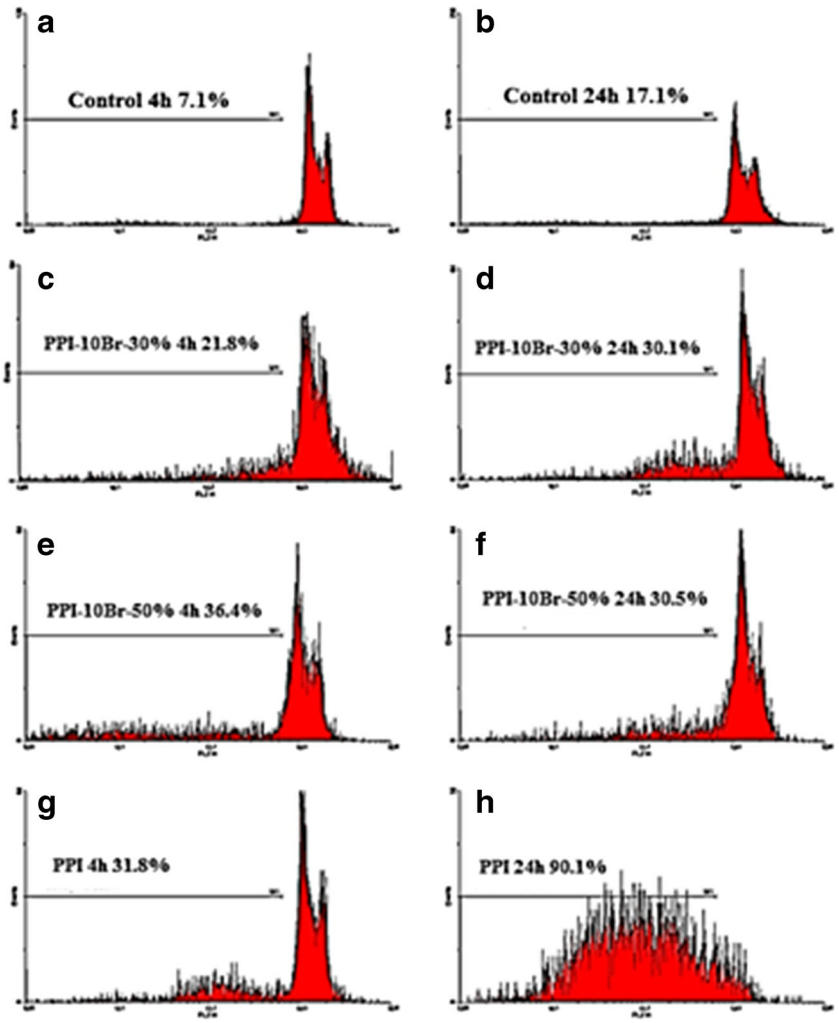

Fig. 6. Comparison of induction of apoptosis by treatment of neuro-2a cells with G5 PPI and its alkylcarboxylated derivatives complexed with pDNA. The results are shown for 4-h (**a**, **c**, **e**, **g**) and 24-h (**b**, **d**, **f**, **h**) treatment as measured by propidium iodide (PI) staining assay

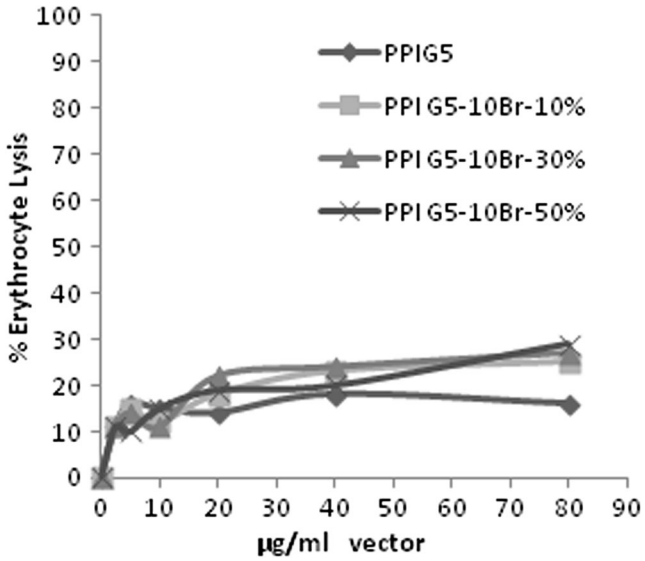

Fig. 7. Hemolytic activity of G5 PPI and alkylcarboxylated PPI. Human red blood cells were incubated for 45 min at 37°C with different concentrations of PPI G5 and its modified counterparts. Hemoglobin release was measured by absorption at 405 nm in the supernatant after centrifugation (n=3)

substitution of primary amines. In their investigation, cytotoxicity was observed only with 6-bromohexanic acid at the lowest percentage alkylation and with long alkyl chains (10 and 16 carbons), while hexanoate-derivatized PEIs showed no toxicity at all (20). This is in line with our study, as the lowest toxicity was observed with hexanoate-modified G5 PPI. In another study 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 higher toxicity (15).

Put together, It can be conferred that although cytotoxicity may interfere with efficient transfection at high grafting percentages and high c/p ratios (such as PPI-10–50% at c/p ratio of 6), a balanced side-chain length, degree of substitution, and c/p ratio may exert great transfection while decreasing the cytotoxicity of the vector (*e.g.*, PPI-10–50% at c/p 4).

Alkylcarboxylation Alleviates the Apoptotic Effects of G5 PPI

The effect of treatment with polyplexes on induction of apoptosis was examined after 4 or 24 h exposure by PI staining as hypodiploid peaks. In the 4 h group, the percent of apoptotic cells was 21.8% and 36.4% for 30% and 50% substitution with 10Br, respectively (Fig. 6c, e). The apoptosis caused by unmodified PPI was 31.8% (Fig. 6g).

In the 24 h exposure group, the apoptotic bodies made 30.1% and 30.5% of total cells for 30% and 50% substituted PPI, respectively (Fig. 6d, f). Unmodified PPI showed 90.1% apoptotic cells after 24 h of exposure (Fig. 6h).

In previous studies, it was suggested that polyethylenimine might induce two stages of toxicity in gene delivery process (28). First, in polyplex solution, where free PEI and PEI/DNA complexes are at equilibrium, interaction between aggregated free PEI and cell membrane induces immediate toxicity. In delayed toxicity, DNA release from polyplex produces free PEI in the cell which may interact with cellular components with negative charge and induce apoptosis (28).

Our results indicate that alkylcarboxylation of PPI dendrimers may have inhibitory effects on induction of apoptosis by this dendrimer, which is more obviously observed with longer exposure times.

Hemocompatibility of G5 PPI is not Markedly Affected by Alkylcarboxylation

Because one of the major problems with cationic polymers is induction of hemolysis, the effects of nonviral candidates of *in vivo* gene delivery on human erythrocytes (RBCs)

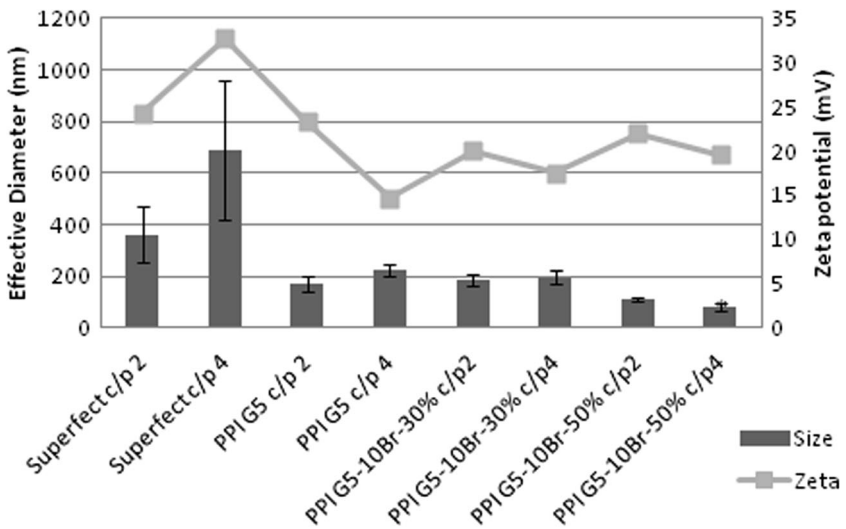

Fig. 8. Size (nm) and zeta potential (mV) of the polymer/DNA complexes of PPI and its derivatives with high transfection activity. Results are presented as mean±SD of three independent measurements

must be studied to ensure the hemocompatibility of these systems (28). We therefore tested the hemolytic effects of our dendrimers with high transfection activity on human erythrocytes with a variety of concentrations at pH 7 (close to physiologic pH). The polymers showed concentration-dependent hemolysis, in the range of 11% to 29% (Fig. 7). Unmodified G5 PPI exerted 16% hemolysis at the highest concentration tested. There was a trend for increased hemolytic effects with increased degree of substitution which was not statistically significant ($p>0.05$). These results indicate that the modifications done in this study do not result in marked increase in hemolytic effects of G5 PPI.

Fischer *et al.* (28) studied the cytotoxicity and hemolytic effects of a series of cationic polymers and found PEI and PLL to have the highest hemolytic activity, while PAMAM dendrimers showed negligible toxicity even at the highest concentrations tested (28). Alkylcarboxylation of PEI reduced hemolytic activity compared to 25 and 10 kDa PEIs for all alkyl chain lengths and all degrees of carboxyalkylation, which was attributed to reduction of positive charge of the polymers (19,20).

Particle Size and Zeta Potential Analysis

Particle size is an important property which affects cell uptake efficiency and pathways (29). Polyplexes of vectors with the most efficient transfection activities were formed at various *c/p* ratios and tested for their size and zeta potential. All PPI polyplexes except unmodified PPI at $c/p=4$ had effective diameters of less than 200 nm. Modified polymers formed smaller complexes than unmodified PPI and this reduction was statistically significant in PPI G5-10Br-50% at *c/p* 4 ($p<0.05$). This observation is in contrast with many other studies in which adding more hydrophobic groups resulted in larger particle size, possibly as a result of lower DNA binding affinity (15,17,26). One possible explanation for this observation is involvement of hydrophobic interactions in formation of polyplexes as well as conformational changes and the flexibility of the polymer, similar to the observations by Alshamsan *et al.* (30).

Zeta potential was in the range of 17–25 mV (Fig. 8). The zeta potentials for modified vectors were not significantly different from native PPI, which suggests that alkylcarboxylation does not affect the positive charge of the polyplexes. Similar results were obtained by Alshamsan *et al.* (30). They believed that variability in surface charge could attribute to the relatively flexible three-dimensional conformation of the grafted alkyl chains.

CONCLUSION

In this study, we developed a small library of modified G5 PPI dendrimers. It was shown that a balanced hydrophobic modification results in improved transfection higher than a commercially available dendrimer-based transfection agent (Superfect). The balanced polymers with optimal transfection activity showed low toxicity and hemolytic activity as well, which suggest some of these dendrimers as candidates for further *in vivo* experiments.

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