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Influence of acyl chain length on transfection mediated by acylated PEI nanoparticles

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Abstract

Polyethylenimine (750 kDa) has been derivatized to influence the proton sponge mechanism and hydrophobic–hydrophilic balance. The polymer was acylated using acid anhydrides of varying carbon chain length, followed by cross-linking with PEG-bis-P to form compact nanoparticles. The chemical linkages in the particles were characterized by FTIR and NMR spectroscopy. The hydrodynamic diameter of nanoparticles was found to be in the range of 83.5–124 nm. AFM imaging of native and DNA-loaded nanoparticles revealed highly compact and spherical shape. The positive surface charge on particles decreased with the increase in percentage of acylation and also on complexing with DNA. The buffering capacity of PEI was reduced considerably on preparing acylated nanoparticles. The nanoparticles formed stable complexes with DNA and higher weight ratios were required for formation of electro-neutral complexes. Further, these nanoparticles were investigated for their gene delivery efficacy on COS-1 cells. It was found that acylated PEI nanoparticles were 5–12-fold more efficient transfecting agents as compared to native PEI (750 kDa) and commercially available transfecting agent lipofectin. The MTT colorimetric assay revealed of considerable reduction in toxicity of acylated PEI nanoparticles as compared PEI. Of all the systems prepared, nanoparticles with 30% acylation using propionic anhydride were found to be the most efficient in *in vitro* transfection.

Keywords: Polyethylenimine; Acylation; Nanoparticles; Hydrophobic; Hydrophilic

1. Introduction

Gene therapy has emerged as a promising strategy for the treatment of several inheritable or acquired diseases (El Aneed, 2004; Olefsky, 2000; Fajac et al., 1999). In the earlier stages, viral vectors have been used to deliver therapeutic genes into living cells, but later non-viral vectors have become an alternate area of interest, as they offer several advantages, such as safety, easy manipulability, high stability, low cost and flexibility regarding the size of gene to be delivered (Lehrman, 1999; Liu and Muruve, 2003; Felgner, 1997).

Polycationic polymers have been preferred over other systems due to their ease of preparation, purification and chemical modification along with the long shelf life (Zhao et al., 2003; Davis, 2002). Polyethylenimine (PEI), a polycationic polymer has emerged as a potential candidate for developing effective gene delivery vehicles (Boussif et al., 1995; Abdallah et al., 1996; Hong et al., 2004; Erbacher et al., 2004). The high molecular weight PEI (800 kDa) has been reported to exhibit higher transfection efficiency compared to low molecular weight PEIs, but accompanied with high cytotoxicity (Godbey et al., 1999; Kunath et al., 2003; Fischer et al., 1999). Of the various mechanisms of gene delivery suggested "proton sponge hypothesis" is the most accepted one (Boussif et al., 1995). However, this theory has recently been challenged (Godbey et al., 2000).

PEGylation of PEI carries advantages such as introduction of biodegradable linkages, reduction in toxicity and decrease in non-specific interactions with the serum proteins, increases polymer solubility, improves blood circulation time, which finally leads to better transfection efficiency (Sung et al., 2003; Tang et al., 2003; Bronich et al., 1998). Nanoparticle-based

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delivery vehicles have been developed during the past few decades to achieve better drug and gene delivery (Rhaese et al., 2003). Our earlier studies have demonstrated the use of PEG as a cross-linker to prepare nanoparticles with improved transfection efficiency (Nimesh et al., 2006).

Partial acetylation of PEI has been reported to reduce the physiological buffering capacity of the polymer (Forrest et al., 2004). The cytotoxicity of the acetylated polymer was unaltered, but the transfection efficiency was found to increase considerably as compared to unmodified PEI.

In the present study, we have systematically modified commercially available high molecular weight PEI (750 kDa) with the aim of improving PEI-mediated transfection. The chemical modifications were designed to influence the proton sponge mechanism and hydrophobic–hydrophilic balance. As the presence of a large number of amino groups have been found to be responsible for the proton sponge mechanism and the hydrophilicity. The amino groups present in the polymer were partially derivatized using three different acylating agents varying in carbon chain length from C-2 to C-4. Subsequently, the cross-linking of acylated PEI with PEG was achieved with a homobifunctional PEG derivative (PEG-bis-P). It is expected that the positively charged nanoparticles will efficiently interact with the negatively charged DNA and thus increase the physical concentration of DNA on the cell monolayers to enhance transfection. The nanoparticles prepared were characterized by measuring their size and surface charge by DLS, AFM and zeta potential, respectively. The transfection efficiency of the nanoparticles prepared by cross-linking acylated PEI with PEG was found to increase significantly along with reduction in cytotoxicity. PEI acylated with propionic anhydride was found to be the most efficient transfecting agent compared to acetic anhydride and butyric anhydrides.

2. Experimental procedures

2.1. General methods

Polyethylenimine (PEI, MW 750 kDa), high retention dialysis tubing (cut-off = 12 kDa), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), agarose, HEPES, Tris, EDTA, ethidium bromide (EtBr), bromophenol blue (BPB) and xylene cyanol (XC) were procured from Sigma Chemical Co., St. Louis, MO, USA. Polyethylene glycol 8000, BioChemika Ultra (PEG_{8000} MW range 7000–9000) was purchased from Fluka Chemie GmbH. All other chemicals and reagents were procured locally. Nanoparticles were sonicated using Misonix 3000 sonicator, West Chester PA, USA, with total sonication time of 3 min (6× 30 s pulse each followed by a 30 s stop time) at 4 °C (ice-bath) with power set at 3 W using micro tip probe. FTIR spectra were recorded on a single beam Perkin-Elmer (Spectrum BX Series) spectrophotometer, USA with the following scan parameters: scan range 4400–400 cm^{-1}: number of scan 16: resolution 4.0 cm^{-1}: interval 1.0 cm^{-1}: units %T. GFP protein expression was observed under Nikon Eclipse TE 2000-U inverted microscope, Kanagawa, Japan, fitted with C-Fl epifluorescence filter block B-2A consisting of excitation filter Ex 450–490 nm, Dichroic mirror DM 505 and barrier filter BA 520. Qiagen kit for plasmid isolation was purchased from Qiagen Inc., CA, USA. Cell culture media, Dulbecco's modified eagle's medium (DMEM), fetal calf serum (FCS) were from GIBCO-BRL-Life Technologies, Web Scientific Ltd., UK, EGFP plasmid from BD Biosciences, USA.

2.2. Cell culture

The mammalian cell line, COS-1 cells (Simian virus 40 transformed kidney cells of an African green monkey), were maintained as monolayer cultures in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 50 μg/ml gentamicin.

2.3. Plasmid purification

All the transfection experiments were carried out using the plasmid encoding enhanced green fluorescent protein gene (EGFP) under the control of cytomegalovirus (CMV) immediate early promoter. The plasmid was transformed into *E. coli* bacterial strain DH5α and extracted from the culture pellets using the Qiagen Endofree Maxi-Prep kit (Qiagen S.A., Courtaboeuf, France) as per manufacturer's instructions.

2.4. Polyethylene glycol-bis (phosphate) (PEG-bis-P)

Polyethylene glycol (MW 8 kDa) was derivatized to obtain polyethylene glycol-bis (phosphate) (PEG-bis-P) following the procedure already reported from author's laboratory (Nimesh et al., 2006).

2.5. Acylation of polyethylenimine (PEI)

Polyethylenimine 750 kDa (2 g) was dried twice by co-evaporation with dry toluene (2× 40 ml) on a rotary evaporator and finally suspended in dry methanol (25 ml) followed by drop-wise addition of acetic anhydride (355.76 μl) with continuous stirring at 60 °C as reported earlier (Forrest et al., 2004). Subsequently, the reaction was stirred at 60 °C for 4.5 h and quenched by the addition of double distilled H_2O (1 ml). The reaction mixture was concentrated, re-dissolved in double distilled H_2O (25 ml) and dialyzed (12 kDa cut-off) against 5% aqueous sodium bicarbonate solution for 3 days with intermittent change of bicarbonate solution followed by distilled water (1 day). The solution was lyophilized in a speed vac to obtain acylated PEI and characterized by ^1H NMR and FTIR. IR (KBr) ν (cm^{-1}): 3436 (amino stretching), 2932, 1638 (carbonyl stretching), 1423.

Similarly, 10–50% acyl substituted PEI employing acetic, propionic and butyric anhydride were synthesized and characterized spectroscopically.

2.6. Determination of extent of acylation

The extent of acylation of each PEI derivatives was determined through quantification of the free amino groups remaining

on the polymer following 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (Tseng et al., 2004). The extent of derivatization of PEI was also confirmed by ^{1}H NMR (Forrest et al., 2004).

2.7. Preparation of nanoparticles of acylated polyethylenimines

An aqueous solution of acylated PEI (5 mg/5 ml) was taken in a 50 ml round bottom flask and a solution of PEG-bis-P dissolved in distilled water (17.9 mg for 5% cross-linking, 17.9 ml) was added drop-wise over a period of 30 min through a 5 ml syringe with continuous stirring at room temperature. The solution was allowed to stir at room temperature for 2 h and then dialyzed (using 12 kDa cut-off membrane) against distilled water for 2 days with intermittent change of water and finally lyophilized in a speed vac to obtain cross-linked nanoparticles of acylated polyethylenimine (yield = 76.4%).

Similarly, nanoparticles of other acylated polyethylenimines cross-linked with PEG-bis-P were prepared.

2.8. Characterization of nanoparticles

The nanoparticles prepared for the present study were characterized by the following procedures.

(i) *Atomic force microscopy*. The size and surface morphology of the nanoparticles was determined by atomic force microscopy using NANO-R AFM System (Pacific Nanotechnology, USA) operating in Close Contact Mode. A Silicon Close Contact Mode Probe (Model: P-MAN-SICC-O, Pacific Nanotechnology, USA) with substrate force constant of 40 N/m (nominal) and resonance frequency of 300 kHz (nominal) and tip radii <10 nm was used. Lyophilized powder (~0.5 mg) of nanoparticles was dispersed by sonication in double distilled water (1 ml) to obtain a suspension, 2–3 μl of this suspension was deposited on a "Piranha" cleaned glass slide and allowed to dry for overnight at room temperature. Subsequently, the glass surface containing the nanoparticles was imaged. Particle size was obtained using Nano Rule software. The height differences on the surface are indicated by the color code, lighter regions indicate higher heights.

(ii) *Dynamic light scattering* (DLS). The hydrodynamic diameter of the acylated PEI–PEG nanoparticles was determined by dynamic light scattering (DLS) measurements. Nanoparticles were suspended in 1 ml of double distilled water and sonicated prior to measurements. Nanoparticles size was determined using Zetasizer, Nano ZS (Malvern instruments, UK) employing a nominal 5 mW HeNe laser operating at 633 nm wavelength. The scattered light was detected at 135° angle. The refractive index (1.33) and the viscosity (0.89) of ultrapure water were used at 25 °C for measurements. All the data analysis was performed in automatic mode. Measured sizes were presented as the average value of 20 runs.

(iii) *Zeta potential measurements*. The acylated PEI–PEG nanoparticles were suspended in 1 ml 10 mM Tris, pH 7.4 followed by sonication and subjected to zeta potential measurements on a Zetasizer Nano ZS. Zeta potential measurements were carried out in automatic mode and the values were presented as the average value of 30 runs. The Smoluchowski approximation was used to calculate zeta potential from the electrophoretic mobility.

2.9. Measurement of buffering capacity of acylated PEI–PEG nanoparticles

To evaluate the buffering capacity of PEI and acylated PEI nanoparticles, acid base titration was carried out as reported (Tang and Szoka, 1997). The samples were prepared by suspending nanoparticles at a concentration of 1 mg/10 ml in 150 mM NaCl. Initially, the pH was adjusted to nearly 11.5 and then PEI, acylated PEI nanoparticles were titrated with small increments of 0.1N HCl (50 μl) until a pH of 3.0 was reached. The slope of the graph plotted between pH and the amount of HCl added provides an indication of the intrinsic buffering capability of the delivery vehicle.

2.10. DNA retardation assays

The DNA/nanoparticle complexes were prepared by taking a known amount of plasmid DNA (0.5 μg) with varying amount of acylated PEI–PEG nanoparticles in 20 mM HEPES buffer, pH 7.2, containing 150 mM NaCl and by incubating for 30 min at room temperature. The resultant complexes were loaded onto a 0.8% agarose gel (Tris–acetic acid buffer, TAE) after mixing with loading buffer containing a tracking dye (xylene cyanol) and electrophoresed at 100 V for 45 min in the same buffer. The bands corresponding to plasmid DNA and DNA/nanoparticle complexes were visualized under ultra violet light after staining the gels with ethidium bromide.

2.11. In vitro cell transfection studies

COS-1 cells (Simian virus 40 transformed kidney cells of an African green monkey) were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 50 μg/ml gentamicin. The cells were grown at 37 °C in humidified 5% CO_2 atmosphere. COS-1 cells were seeded prior to transfection into 96-well plates at a density of 3.5×10^3 cells/well and incubated for 16 h for adherence. After stipulated time the cells were washed once with serum free DMEM. Reporter gene encoding for green fluorescent protein (GFP) was used to assess the efficiency of acylated PEI–PEG nanoparticles-mediated transfection. DNA–nanoparticles complexes were prepared with varying concentration of nanoparticles and 0.5 μg of plasmid as described in DNA retardation assay. Subsequently, the DNA–nanoparticles complexes were diluted with serum free DMEM to a final volume of 80 μl and this transfection media was added to each well, followed by incubation at 37 °C in humidified 5% CO_2 atmosphere for 4 h. The transfection media

was replaced with 150 μl of serum supplemented DMEM and cells were further incubated for 36 h under same conditions. Thereafter, the cells, transfected with GFP reporter gene, were observed under bright field and UV using GFP filter, at 10× magnification, under an inverted fluorescent microscope to observe the expression of green fluorescent protein.

2.12. Analysis of EGFP expression

The fluorescence intensity was measured to quantitate the GFP expression in the mammalian cells. The cells were washed twice with PBS and lysed by incubating for 30 min with 100 μl buffer containing 10 mM Tris, 1 mM EDTA and 0.5% SDS pH 7.4. The lysed cells were centrifuged at 4000 rpm for 30 min and the supernatant aspirated. Ten microliters of lysate was used to estimate the expressed reporter gene product, green fluorescent protein (GFP), spectroflurometrically at an excitation wavelength 488 nm and emission at 509 nm. Background fluorescence and auto-fluorescence were determined using mock-treated cells. The total protein content in cell lysate from each well was estimated using Brad Ford's reagent (Bio Rad) taking BSA as a standard. The level of fluorescence intensity of GFP was calculated by subtracting the background values and normalized against protein concentration in cell extract. The data is reported as arbitrary unit (AU)/mg of cellular protein and represent mean ± standard deviation for triplicate samples.

2.13. Cytotoxicity

The toxicity of DNA-loaded acylated PEI–PEG nanoparticles was evaluated by MTT colorimetric assay (Mosmann, 1983). COS-1 cells were seeded onto 96-well plates at a density of 8×10^3 cells/well and incubated for 16 h for adherence. The cells were incubated with DNA-loaded acylated PEI–PEG nanoparticles as described above in the transfection experiment. After 36 h, 50 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (2 mg/ml in DMEM) was added to the cells and incubated for another 2 h. The MTT containing medium was aspirated, and the formazan crystals formed by the living cells were dissolved in 100 μl isopropanol containing 0.06 M HCl and 0.5% SDS. Aliquots were drawn from each well after 1 h of incubation and the absorbance measured spectrophotometrically in an ELISA plate reader at 540 nm. Untreated cells were taken as control with 100% viability and cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. The relative cell viability (%) compared to control cells was calculated by $[abs]_{sample}/[abs]_{control} \times 100$.

3. Results

3.1. Acylation of PEI

To investigate the effects of alkyl chain (charge masking and hydrophobicity) on the polymer buffering capacity and gene delivery efficiency, commercially available high molecular weight branched 750 kDa PEI was systematically modified with various amounts of acetic anhydride, propionic anhydride and butyric anhydride to acylate the primary and secondary amines. Increasing amounts of the acylating agents were mixed with PEI and the reaction was incubated at 65 °C for 4.5 h. The concentration or number of amine groups blocked was determined using TNBS method (Table 1). The extent of primary and secondary amine acylation was determined from the relative methyl and methylene signals in ^{1}H NMR spectra. The extent of actual acylation was found to be more than 60% of the theoretical values in most of the acylated PEI preparations. The reproducibility of synthesis was ascertained by carrying out several independent preparations.

Table 1
Percentage acylation of PEI 750 kDa

Sample no.	Partial acylated PEI	% Acylation	
		Theoretical	Actual estimated by TNBS (NMR values)
1.	PA 1	10	8.1
2.	PA 2	20	16.1 (15.7)
3.	PA 3	30	24.3
4.	PA 4	40	33.2
5.	PA 5	50	41.7
6.	PP 1	10	7.9
7.	PP 2	20	14.3
8.	PP 3	30	22.4 (21.1)
9.	PP 4	40	28.7
10.	PP 5	50	36.1
11.	PB 1	10	7.2 (6.5)
12.	PB 2	20	13.4
13.	PB 3	30	18.0
14.	PB 4	40	25.4
15.	PB 5	50	31.8

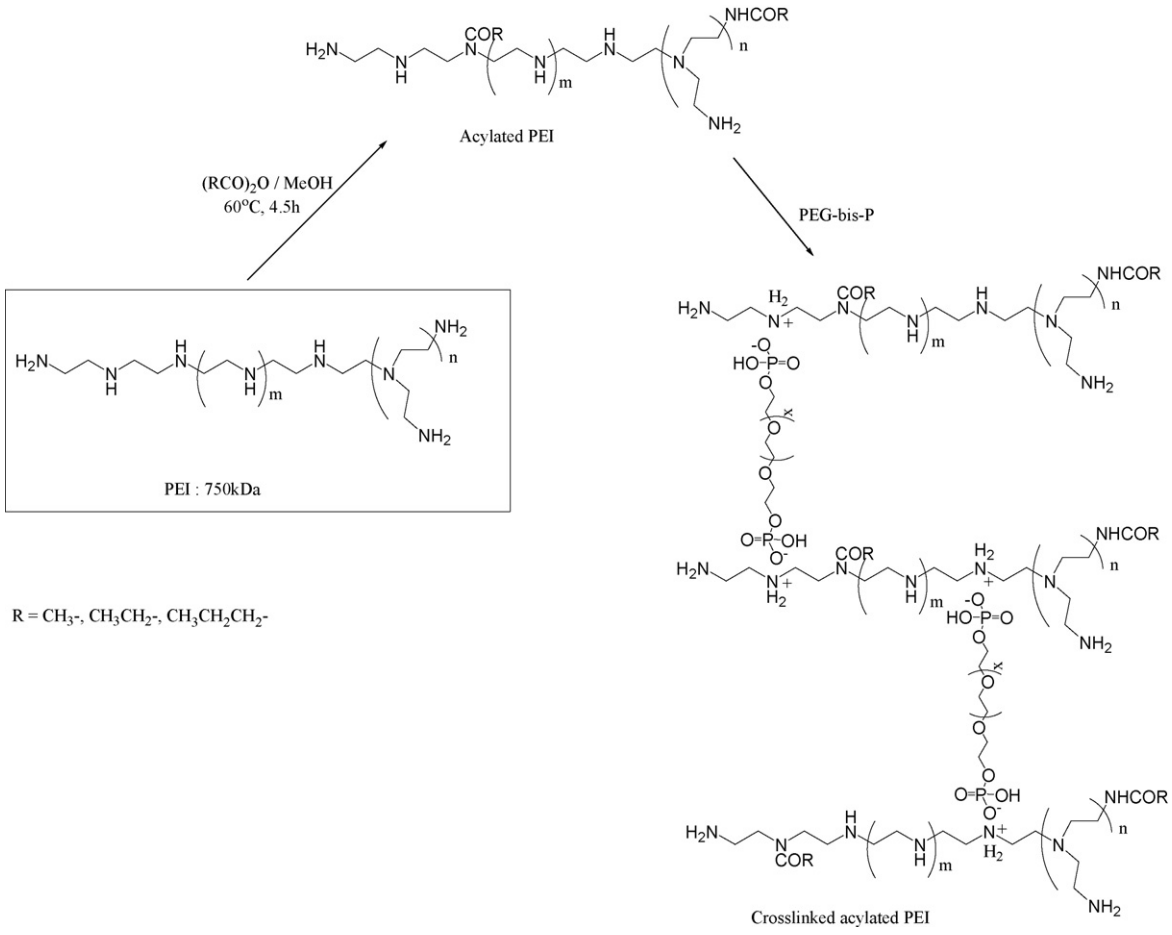
PA: PEI 750 kDa acylated using acetic anhydride; PP: PEI 750 kDa acylated using propionic anhydride; PB: PEI 750 kDa acylated using butyric anhydride.

3.2. Preparation and characterization of nanoparticles of acylated polyethylenimine

Ionic cross-linked nanoparticles of acylated PEI–PEG-bis-P with 5% cross-linking were prepared by reaction of acylated PEI with PEG-bis-P, as mentioned in Section 2. The nanoparticle formation occurred due to ionic interaction between the positively charged amino groups of PEI and the negatively charged phosphate groups of PEG-bis-P, as shown in Scheme 1. The formation of nanoparticles was confirmed by AFM imaging of virgin and DNA-loaded nanoparticles. AFM observations of native and DNA-loaded acylated PEI–PEG nanoparticles showed spherical and compact complexes (Fig. 1). The morphology and shape of the nanoparticles was maintained even after binding to DNA. The discrete particles were evident and the three-dimensional image reveals homogeneous population with a clear absence of aggregates even after 2 h.

3.3. Surface charge, size and morphology of acylated PEI–PEG nanoparticles

To determine the effect of degree and type of acylating agent on the surface charge of PEI nanoparticles, zeta potential was



Scheme 1. Preparation of PEG cross-linked nanoparticles of acylated PEI.

measured in the presence and absence of DNA at physiological pH. The zeta potential values were found to decrease with an increase in the degree of acylation in all the nanoparticles, which is consistent with the decreased percentage of available amino functions in the nanoparticles (Table 2). As expected, the zeta potential values of nanoparticles were also found to decrease significantly on complexing with DNA (Table 2). The significant amount of positive charge was observed on all the nanoparticles even after DNA complexation, suggesting prevention of aggregation due to electrostatic repulsion between the cationic complexes.

The DLS measurements revealed that the average diameter of nanoparticles was in the range of 83.5–124 nm. The size of the nanoparticles thus formed was determined by DLS (Fig. 2). As evident from size determination studies, the degree of acylation as well as the type of acylating agent used has small but significant effect on the size of the nanoparticles formed (Table 2). Further, to assess the stability of these particles in buffer and serum dilutions, DLS measurements were carried out in 0.01 M phosphate buffered saline, pH 7.4 and 10% FCS. The size of the particles was found to be essentially the same as reported in distilled water.

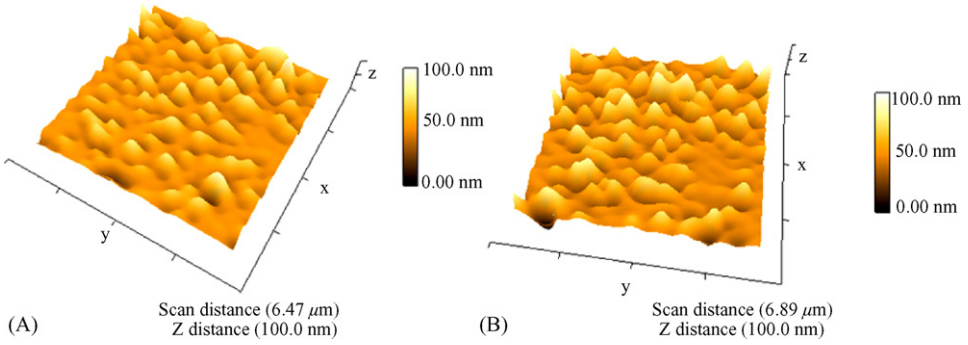


Fig. 1. Atomic force microscope image of (A) PPA 3 alone and (B) PPA 3–DNA complexes. Average size in each case is 100 nm.

Table 2
Size and zeta potential of PEI 750 kD nanoparticles

Sample no.	PEI nanoparticles	Average particle size (nm) by DLS in dH_2O	Polydispersity index	Zeta potential (S.D.)				Ratio of nanoparticle: DNA (w/w)
				Nanoparticles alone in dH_2O	DNA-loaded nanoparticles			
					dH_2O	PBS	FCS	
1.	PAA 1	83.5	0.433	30.2 (1.6)	16.2 (0.9)	17.9 (1.1)	−18.7 (1.1)	15:1
2.	PAA 2	87.4	0.391	27.2 (1.4)	9.12 (0.5)	10.2 (0.7)	−20.9 (1.4)	15:1
3.	PAA 3	89.6	0.455	26.2 (1.5)	15.7 (1.1)	17.5 (1.3)	−20.6 (0.9)	30:1
4.	PAA 4	93.0	0.476	23.5 (2.1)	8.80 (0.4)	9.79 (0.7)	−21.6 (1.2)	30:1
5.	PAA 5	98.2	0.434	19.6 (1.1)	9.72 (0.5)	10.8 (0.5)	−19.6 (0.8)	45:1
6.	PPA 1	102	0.403	32.5 (1.9)	8.21 (0.6)	9.13 (0.6)	−15.5 (0.6)	15:1
7.	PPA 2	106	0.560	30.1 (2.3)	17.4 (1.1)	19.3 (1.4)	−15.4 (1.2)	15:1
8.	PPA 3	108	0.598	28.8 (1.7)	19.1 (1.2)	21.2 (1.3)	−16.3 (1.1)	30:1
9.	PPA 4	110	0.681	25.0 (1.9)	17.9 (1.1)	17.3 (1.1)	−16.0 (1.4)	45:1
10.	PPA 5	113	0.631	22.4 (1.3)	14.4 (0.8)	15.6 (1.4)	−15.6 (1.3)	45:1
11.	PBA 1	105	0.467	21.7 (1.1)	5.85 (0.3)	6.51 (0.8)	−19.4 (1.4)	30:1
12.	PBA 2	108	0.607	21.0 (1.4)	7.24 (0.6)	8.05 (1.1)	−22.2 (1.8)	30:1
13.	PBA 3	111	0.602	20.8 (1.3)	9.46 (0.5)	10.5 (1.2)	−21.9 (1.6)	45:1
14.	PBA 4	115	0.481	20.1 (1.2)	14.6 (1.1)	16.2 (1.3)	−22.3 (1.1)	45:1
15.	PBA 5	124	0.735	18.2 (1.1)	11.5 (0.5)	12.8 (0.9)	−15.6 (0.8)	45:1

PAA: nanoparticles of PEI 750 kDa acylated using acetic anhydride; PPA: nanoparticles of PEI 750 kDa acylated using propionic anhydride; PBA: nanoparticles of PEI 750 kDa acylated using butyric anhydride; dH_2O: double distilled water at pH 7.00; PBS: phosphate buffered saline at pH 7.4; w/w: weight by weight ratio of nanoparticles to DNA.

3.4. Buffering capacity

The buffering capacity is one of the major driving forces behind the release of PEI–DNA complexes from the endosomes. The effect of degree of acylation and acylating agent used on the buffering capacity of PEI was estimated by measuring the change in pH of the suspension (1 mg/10 ml) upon titration with 0.1N HCl. A polymer with high buffering capacity undergoes a small change in pH when titrated with the same amount of HCl. As evident from Fig. 3 the PEI 750 kDa has considerable buffering capacity over almost entire range of pH. However, the modified PEI nanoparticles showed high extent of change in pH on titration with HCl as compared to unmodified PEI, indicating a reduction in the buffering capacity on substitution with acyl chain. The buffering capacity was found to decrease with the increase in degree of substitution of acyl chains. The protonation tendency of PEI nanoparticles reduced significantly on acylation, as the total number of amino groups available for protonation has decreased.

3.5. DNA retardation assay

The interaction between the polycationic nanoparticles and plasmid DNA results in neutralization of negative charge on the phosphate backbone of DNA, which results in the formation of neutral complexes, unable to migrate under the influence of electric field during gel electrophoresis. To estimate the optimal concentration of nanoparticles required for complete electro-neutralization of DNA, the complexes were prepared with plasmid DNA at different weight ratios, keeping the amount of DNA constant and retardation was analyzed on 0.8% agarose gel. Indeed, acylated nanoparticles were compe-

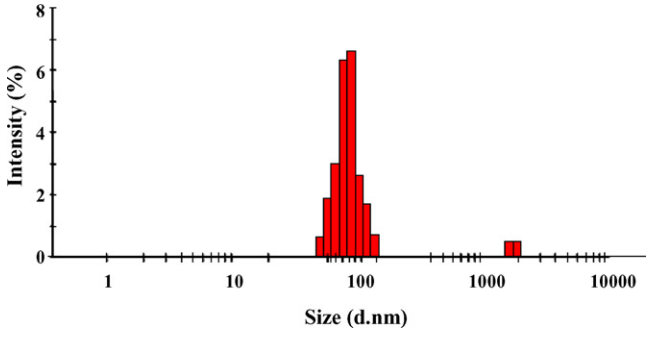


Fig. 2. Representative dynamic light scattering spectrum of PPA 3 nanoparticles in double distilled water. The average hydrodynamic diameter in this case is 108 nm.

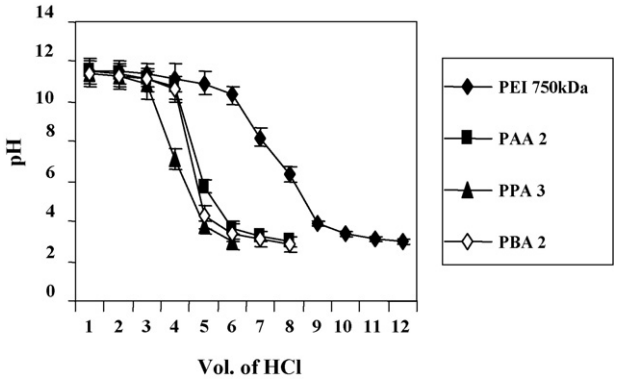


Fig. 3. Buffering capacity of acylated PEI nanoparticles and native PEI. Acid titration curves profile of aqueous solution of nanoparticles and native PEI was obtained by adding 50 μl aliquots of 0.1N HCl to 1 mg/10 ml concentration of samples dissolved in 150 mM NaCl.

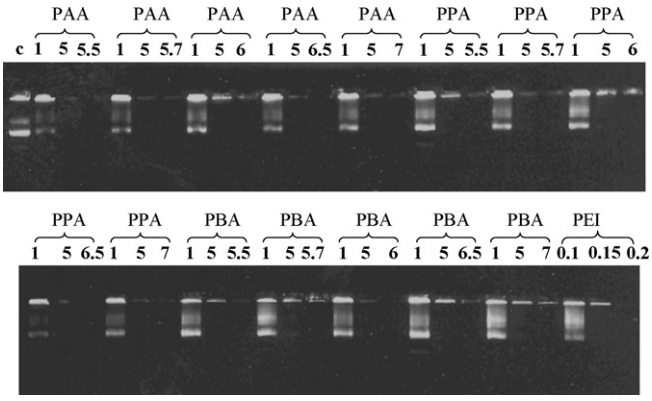


Fig. 4. DNA binding and retardation assay. Plasmid DNA, 0.5 μg was incubated with increasing amounts of acylated PEI nanoparticles and in presence of salt (150 mM NaCl) for 30 min. The samples were analyzed on a 0.8% agarose gel. Lane C represents DNA without nanoparticles, the values mentioned correspond to the amount of nanoparticles (μg) used in a 20 μl reaction to condense the DNA by charge neutralization.

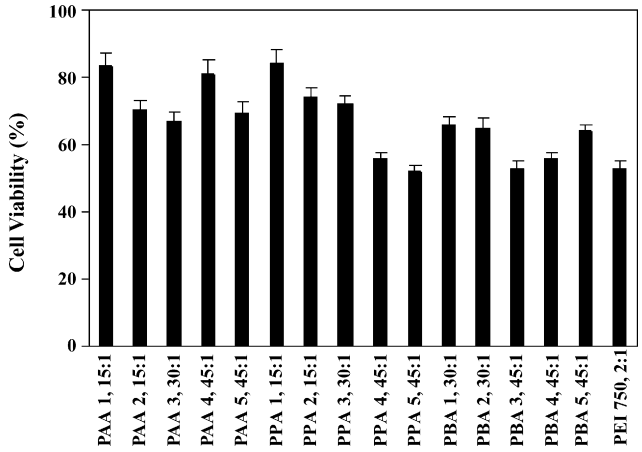


Fig. 5. Cell viability assay. COS-1 cells were treated with DNA-loaded acylated PEI nanoparticles under the transfection conditions. The ratios corresponding to the optimal transfection efficiency by PEI–PEG nanoparticle:DNA ratios are presented here. The assays were done in triplicate and the standard error is shown.

tent in interacting with DNA and neutralize the charge. Complete retardation for native PEI was observed at 0.4 μg/μg plasmid DNA, whereas acylated PEI–PEG nanoparticles were required in higher amounts. In case of nanoparticles of PEI acylated with acetic anhydride, propionic anhydride and butyric anhydride, the DNA mobility gradually decreased with increase in concentration of nanoparticles. The maximum retardation was observed within 5–7 μg/μg of nanoparticles:DNA weight ratio for propionated nanoparticles (Fig. 4).

3.6. Cytotoxicity

PEGylation as well as acylation reduce the overall surface charge and are thus expected to reduce the charge-associated toxicity. The cytotoxicity of the nanoparticles was estimated using MTT colorimetric assay.

COS-1 cells were incubated with DNA-loaded acylated PEI–PEG nanoparticles at various weight ratio of nanoparticles and DNA for 4 h (absence of serum), followed by further incubation for 36 h (presence of serum). A dose-dependent cyto-

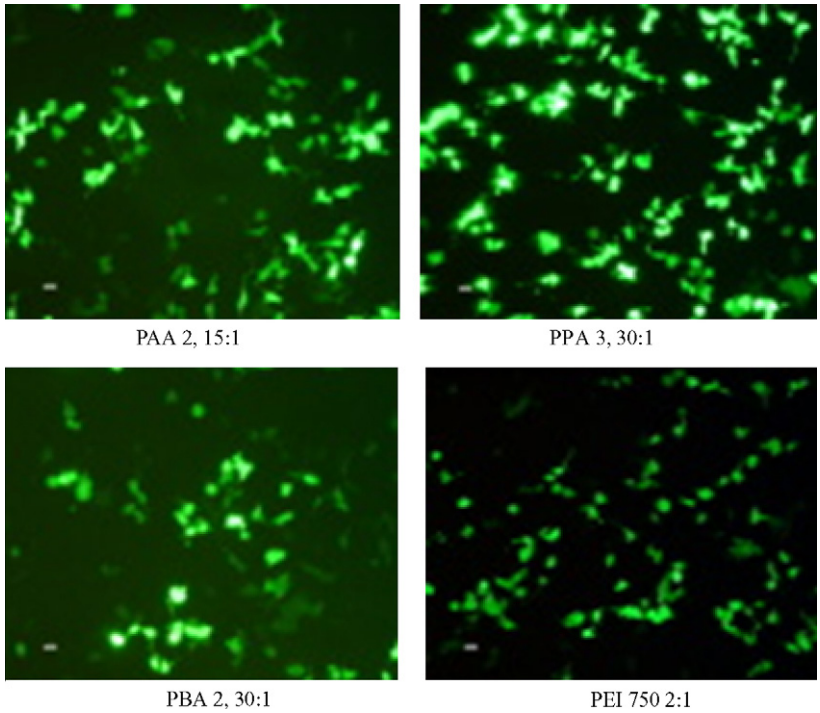


Fig. 6. Comparison of transfection efficiency of various acylated PEI nanoparticle complexes. COS-1 cells were incubated with DNA-loaded acylated PEI nanoparticle complexes at various weight ratios and incubated for 4 days. The green fluorescent protein (GFP) expression was observed under fluorescent microscope at 10× magnification. The data was recorded at optimal transfection efficiency at the mentioned acylated PEI nanoparticle:DNA ratio.

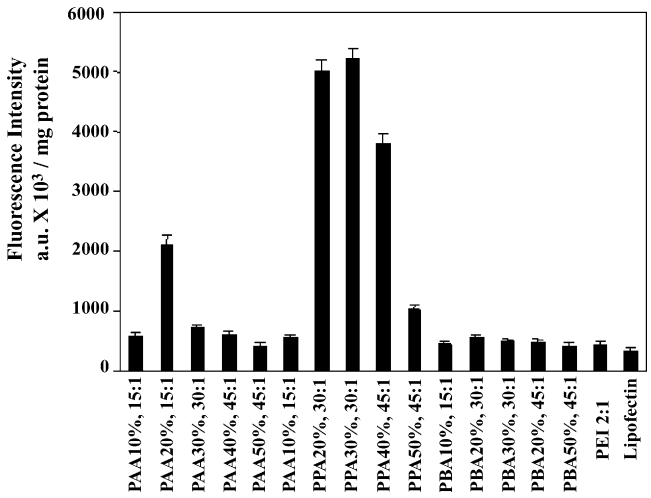


Fig. 7. Comparison of transfection efficiency of various acylated PEI nanoparticle complexes. COS-1 cells were incubated with DNA-loaded acylated PEI nanoparticle complexes at various weight ratios for 4 h and the expression of GFP was monitored after 36 h. The fluorescent intensity of GFP fluorophore in the cell lysate was measured on spectroflurometer and the results are expressed in terms of arbitrary units/mg total cellular protein. The results represent the mean of two independent experiments performed in triplicates. The data was recorded at optimal transfection efficiency at the mentioned acylated PEI nanoparticle:DNA ratio transfection efficiency with acylated PEI nanoparticles, DNA complexes.

toxicity was observed on increasing the weight ratio (data not shown) in DNA–nanoparticle complexes.

Microscopic examination revealed considerable toxicity and cell morbidity, induced by PEI polymer. However, the DNA-loaded acylated PEI nanoparticles were slightly toxic after 48 h nearly 72–84% cell viability was observed in the case of PPA samples, exhibiting maximal transfection (Fig. 5). When the cells were incubated with DNA-loaded PBA nanoparticles, around 60% viability was observed whereas in case of PAA nanoparticles cell viability scored above 70%. However, the treatment of cells with native PEI resulted in 53% cell viability.

3.7. In vitro cell transfection

To determine the transfection efficiency of acylated PEI–PEG nanoparticles, transfection studies were carried out on COS-1 cells and compared to commercially available transfection reagent, lipofectin (Invitrogen) and native PEI polymer (750 kDa). The efficacy of nanoparticles for gene delivery was determined by using a plasmid carrying reporter gene, Green fluorescent protein (GFP). Nanoparticle–DNA complexes were prepared at various weight ratios under physiological salt concentrations (150 mM) and used to transfect COS-1 cells. The gene expression of GFP carrying plasmid was observed and quantified after 48 h (Fig. 6). Sufficient amount of GFP expression level was observed in all cells which sustained even after 3–4 days (post-transfection). Amongst all the samples studied on COS-1 cell line, the highest expression levels was found with 30% propionoylated derivative, i.e. 12-fold for nanoparticles prepared from PEI 750 kDa (Fig. 7). The transfection efficiency was found to be dependent on the acyl chain length and the degree of acylation.

4. Discussion

In this study, we have first partially acylated PEI's with acetic, propionic, and butyric anhydrides to prepare various derivatives which were later cross-linked with PEG-bis-P to obtain nanoparticles. We presume that nanoparticles of PEI, thus prepared, will carry the advantages of acylation, as well as PEGylation and will emerge as better transfecting agents.

The size of nanoparticles prepared in the present investigation was found to be in the range of 83.5–124 nm (average hydrodynamic diameter) with almost uniform distribution (Table 2). Derivatization of primary and secondary amines to corresponding amides by acylation, resulted in the reduction of net positive charge on the polycationic polymer which might affect the ionic interaction with the negatively charged cross-linker, PEG-bis-P used for preparation of nanoparticles, as well as binding with DNA. This could affect the size and surface properties of the nanoparticles. Moreover, this weaker interaction might lead to quicker and easier release of DNA from the nanoparticle–DNA complex. DLS and AFM studies were used to differentiate between the sizes of the nanoparticles prepared from various acylated PEIs. It was concluded from DLS measurements that the average size of the nanoparticles varied from 83.5 to 124 nm on using different types of acylating agents and also on varying the degree of acylation. The increase in average particle size was also observed when the carbon chain length was increased. AFM technique was used to study the morphology of the native and DNA-loaded nanoparticles. The size in both the cases for PPA 3 was found to be in the range of 100 nm (Fig. 1). The physical attributes of nanoparticles, viz., size, surface charge, etc. may contribute significantly towards binding between PEI and DNA.

The specific conditions required to prepare small oligomolecular DNA complexes for efficient transfection have always been of interest to researchers (Choi et al., 2001). Here, we again prove the efficacy of nanoparticle approach to control the size of DNA–polymer complexes, avoiding formation of aggregates, even after modifying the PEI by acylation. Large polymer–DNA complexes are not good for many *in vivo* applications due to poor extravasation through capillary endothelia. PEI 25 kDa–pDNA complexes are known to be prone to aggregation (Sung et al., 2003), but the acylated nanoparticles–DNA complexes did not form aggregates even after 2 h of incubation (data not shown). Increased solubility, a consequence of PEG cross-linker may also help in reducing the H-bonding between PEI strands giving rise to discrete particles, thus preventing the aggregation.

The nanoparticles effectively interacted with DNA and neutralized the negative charge (Fig. 4). The protonated amines in nanoparticles required for DNA condensation are still sufficient. The weight ratio, nanoparticles:DNA was increased in complexes to attain a net positive surface charge for transfection studies. Though the buffering capacity of PEI reduced on acylation and cross-linking with PEG-bis-P to form nanoparticles, still sufficient to induce the endosomal buffering. The tertiary amines which are not participating in DNA interaction and acylation, mainly contribute towards buffering capacity. After establishing all the important parameters critical for efficient gene delivery, the cytotoxicity was evaluated on a model cell line.

Cell confluencies, a qualitative measure of cell viability based upon cell coverage on the well surface, as judged by microscopy, indicated little toxicity at the levels used for transfection. To get a more quantitative measure of cell viability, we assayed cell metabolism using MTT. Forrest et al. demonstrated that acetylation of PEI have no profound effect on the cytotoxicity of the polymer (Forrest et al., 2004). The cell line MDA-MB-231 used was equally resistant to the unmodified and acetylated PEI at concentrations up to 25 μg/ml. In one of our earlier studies on cytotoxicity, we have demonstrated that the cells were virtually resistant to DNA–PEI–PEG nanoparticles complexes even after 48 h (Nimesh et al., 2006). The cytotoxicity experiment performed on COS-1 cell line indicated that the cells were quite resistant to DNA–nanoparticles complexes even after 48 h. However, a little toxicity was observed at DNA–nanoparticles complexes concentration ranging from 187 to 750 μg/ml.

Herein, we report a 5–12-fold improvement in transfection efficiency of acylated PEI–PEG nanoparticles as compared to commercially available transfection reagent, lipofectin, native PEI polymer (750 kDa). The maximum transfection efficiency was obtained with 30% propionoylated nanoparticles in COS-1 cell line. The carbon chain in acylated PEI nanoparticles is presumed to facilitate the interaction with cell membrane owing to mild hydrophobicity, in addition to transfection properties of PEI backbone.

Acylation considerably decreases the pK_a and buffering capacity of PEI. The carbon chain length also appears to play an important role in endocytic trafficking of nanoparticle–DNA complexes and the final transport of DNA to nucleus. As a consequence of acylation and PEGylation, the number of positively charged amines in PEI at a given pH is also reduced. Hence, one may expect weaker binding between the modified polymer and DNA, which may lead to more efficient DNA release inside the cells, resulting in improved gene delivery. Erbacher et al. have reported increase in transfection efficiency of the polycation with decrease in the positive charge (Erbacher et al., 2004). An appropriate balance between the lipophilicity and hydrophilicity is necessary for the vector to cross the plasma membrane barrier of the cells. Acylation not only reduces the net positive surface charge of the polymer but may also increase the lipophilicity of the polymer, which could help in increasing transfection efficiency. On acylating with different acylating agents, viz. acetic, propionic and butyric anhydrides, there is an increase in the number of carbon atoms, thereby leading to increase in the lipophilicity of PEI. It could be possible that on acylation with propionic anhydride an appropriate amount of lipophilicity may have attained leading to maximum transfection efficiency. Size restriction of polycations to nanometer range by preparing nanoparticles before complexing with DNA is also an added advantage for improving transfection.

5. Conclusion

We have prepared various acylated PEI derivatives of 750 kDa by treating them with different acylating agents, i.e. acetic, propionic and butyric anhydrides, which were further non-covalently cross-linked with PEG-bis-P to obtain nanoparticles. These nanoparticles have been found to have improved transfection efficiency by 5–12-folds. The acylation improves transfection by neutralizing the positive charge of PEI and maintaining balance between lipophilicity and hydrophilicity. The PEG derivative used here for cross-linking improves the transfection by inhibiting aggregation of nanoparticles. The improved transfection efficiency data opens up new avenue for use of these nanoparticles in *in vivo* studies.

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