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Research paper

Guanidinium-grafted polyethylenimine: An efficient transfecting agent for mammalian cells

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Abstract

Polyethylenimine (PEI) is one of the most efficient polycationic non-viral gene delivery vectors. Its efficiency and cytotoxicity depends on molecular weight, with the 25-kDa PEI being most efficient but accompanied with cytotoxicity. In the present study, enhancement in gene delivery efficiency along with reduction in cytotoxicity by attachment of guanidinium side group was explored. The hypothesis was that the guanidination would lead to the delocalization of charge present on primary amines of the polymer thereby leading to enhancement in gene delivery efficiency along with reduction in cytotoxicity. The polymer was guanidinated using *O*-methylisourea hemisulfate and the chemical linkage characterized by FTIR spectroscopy. The hydrodynamic diameter of guanidinated PEI–DNA complexes was determined using DLS. Subsequently, these complexes were used for DNA binding assay and zeta-potential measurements, taking native PEI as reference. Further, guanidinated PEI–DNA complexes were investigated for their gene delivery efficacy on HEK 293 cells. The hydrodynamic diameter of guanidinated PEI–DNA complexes was found to be in the range of 176–548 nm. As expected, the zeta potential values increased, on increasing the N/P ratios. It was found that guanidinated PEI had higher transfection efficiency at the majority of the N/P ratios tested as compared to commercially available transfecting agent lipofectin and native PEI itself. The toxicity of guanidinated PEI–DNA complexes was also reduced considerably in comparison to PEI polymer, as determined by MTT colorimetric assay. Out of the various derivatives prepared, gPEI 56% was found to be the most efficient in in vitro transfection.

Keywords: Polyethylenimine; Guanidination; Polyplexes; Transfection; Cytotoxicity

1. Introduction

Gene therapy, defined as a method of efficiently transferring the genetic material into somatic cells of individuals to produce-specific therapeutic proteins to prevent, diagnose, correct, or modulate disease, has been increasingly clinically applied in the field of severe diseases such as cancer [1,2]. One of the major factors for efficient and targeted gene delivery is the vector that delivers genes into cells for the production of therapeutic proteins. Two types of vectors, i.e. viral and non-viral have been rapidly developed in the recent years. Viral vectors have been widely investigated due to their high transfection efficiency [1–3]. However, viral vectors also bear certain disadvantages such as issues of safety, immunogenicity, mutagenesis and low gene carrying capacity.

Polycationic polymers have emerged as a promising vector amongst the proposed non-viral vectors as modifications can be easily incorporated into these polymers. These polymers also carry several advantages such as stability, safety, low cost and high gene carrying capacity [4]. Commonly used polymers consist of polyethylenimine [5,6], chitosan [7], dendrimers [8], and polylysine [9], of which the positive charges come from the amino groups.

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Among the cationic vectors, polyethylenimine (PEI) has received considerable attention due to its high transfection efficiency [10].

The amino groups in the cationic polymers play the major role in determining the transfection efficiency of the vector. The cationic polymers spontaneously form condensed particles with DNA due to electrostatic interactions between the positively charged amino groups of the polycation and the negatively charged phosphate groups of DNA [11]. Transfection begins with the entry of plasmid across the cell membrane after the exposure of the complexes to the cultured cells. These complexes carry extra positive charge on their surface, due to which the complexes tend to adhere onto the negatively charged proteoglycans of the cell membranes, thereby favoring endocytosis leading to enhancement in the transfection efficiency [12]. The complex then translocates from endosomes to the cytoplasm wherein the amino groups can facilitate the release, to avoid the degradation by the endocytic DNase [13]. The amino groups of PEI act as proton sponges, which increase the amounts of chloride ion inside the endosomes to induce osmotic swelling, and so disrupt the endosomes [5,14,15]. Once the released DNA or complexes enter the nucleus during mitosis, transcription begins [13,16–18].

To further enhance the transfection efficiency of PEI-mediated DNA–polymer complexes the amine structures are modified [19]. One of the most common approaches is the attachment of a pendant group onto the primary amines of the cationic polymer. Various other modifications incorporated into PEI include coating with human serum albumin [20–23], dextran [24–26], PEGylation [27–32] and acylation [33]. Another strategy is to conjugate a ligand onto the primary amines of the cationic polymers to enhance the transgene expression by targeted delivery [34–36], such as the use of RGD peptide [37], mannose [38], and transferrin [39] for targeting endothelial cells, dendritic cells, and hepatocytes, respectively. Micelles of polyethylenimine coupled to poly(ε-caprolactone) of differing molecular weights (MW) via an amide group complexed with DNA were found to be significantly less toxic to HepG2 cells than blank PEC micelles, and showed improved gene transfection efficiency both in presence and absence of DOX [40]. Imidazolyl substitution into PEI improves the gene delivery efficiency of PEI (750 kDa) by nine to tenfold and PEI (25 kDa) by three- to fourfold [41]. At high N/P ratios, the chitosan-graft-polyethylenimine (CHI-g-PEI)/DNA complex showed higher transfection efficiency than PEI 25 K in HeLa, 293 T and HepG2 cell lines [42]. PEI nanoparticles prepared with polyethylene glycol-bis-phosphate with 30% acylation using propionic anhydride were found to be the most efficient in *in vitro* transfection [43]. The distribution of PEI coupled to a specific targeting moiety was significant in target cells in animal models. Further studies indicated that PEI is rapidly cleared from the blood stream, primarily through the kidneys. However, at cellular level, a considerable toxicity associated with PEI is widely demonstrated [44,45].

In the present study, we have carried out proportionate guanidination of branched PEI 25 kDa. Guanidinium group being highly basic ($pK_a = 12.5$) is fully protonated at physiological pH. As reported earlier, the guanidine head group of arginine has been predicted to be a critical structural component responsible for the biological activity of cell penetrating peptides (CPPs) including protein transduction domain (PTD); further hydrogen bonding between the highly basic arginine guanidine groups and the phospholipids in the membrane lipid bilayer may be involved in protein transduction [46,47]. Hence, it is expected of synthetic polymer bearing guanidinium side chains to complex and condense DNA into small particles and to provide a better cell penetrating system. These particles were found to be in nanometer range on quantitation using DLS and AFM. The cytotoxicity of the branched PEI (25 kDa) was significantly reduced along with remarkable improvement in transfection efficiency compared to native PEI. Amongst the various degree of guanidination, 56% was found to be the most efficient transfecting system.

2. Materials and methods

2.1. General methods

Polyethylenimine (PEI, MW 25 kDa), high retention dialysis tubing (cut off, 12 kDa), *O*-methylisourea hemisulfate, 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ninhydrin, agarose, HEPES, tris, EDTA, ethidium bromide (EtBr), bromophenol blue (BPB), and xylene cyanol (XC) were procured from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals and reagents were procured locally. Characterization was carried out on Fei-Philips Morgagni 268D Transmission Electron Microscope (TEM) from FEI Inc., Hillsboro, Oregon, USA, and particle size analyzer NANO ZS from Malvern Instruments, Worcestershire, UK. FTIR spectra were recorded on a single beam Perkin-Elmer (Spectrum BX Series), 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. All the short spins were performed on Eppendorf Minispin, USA. 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., California, 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.

2.2. Cell culture

The mammalian cell line, HEK 293 cells (Human embryonic kidney 293), 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 *Escherichia coli* bacterial strain DH5α and extracted from the culture pellets using the Qiagen Endo-free Maxi-Prep kit (Qiagen S.A., Courtaboeuf, France) as per manufacturer's instructions.

2.4. Polymer conjugate synthesis

Polyethylenimine (20 mg) taken in 1.5 ml centrifuge tube was diluted to 1 ml using double distilled water. To this tube a known amount of *O*-methylisourea hemisulfate (2.85 mg for 20% guanidination) was added and kept on a thermo-mixture at 60 °C for 90 min. After 90 min the reaction mixer was transferred to 12 kDa dialysis tubing and dialysed against double distilled water for 2d with intermittent change of water. Afterwards, the dialysed solution was poured into 1.5 ml centrifuge tube and concentrated in a speed vac to obtain dry solid mass containing guanidinated-PEI (gPEI) in 76% yield. FTIR (KBr) ν (cm^{-1}): 3400 (amino stretching), 2922 (methylene, C—H asymmetric stretching), 1636 (imino, C=N stretching), 1048 (primary amine, C—N stretching).

Higher percentage of guanidinated PEIs were also prepared using above methodology, but with increased amount of *O*-methylisourea hemisulfate.

2.5. Characterization of polymer conjugate

The extent of guanidination of each polymer synthesized was determined through quantitation of the free primary amines remaining on the polymer by using the well-characterized ninhydrin method [48,49].

2.6. Preparation of gPEI plasmid DNA complexes

Complexes of plasmid EGFP and gPEI were prepared by adding EGFP to gPEI taken in a 1.5 ml centrifuge tube and then diluting it with HBS (20 mM HEPES buffer, pH 7.4, containing 150 mM NaCl) to 20 μl. The resultant mixture was vortexed for 20 s to mix all the constituents, centrifuged briefly to settle down all the constituents at the bottom of the tube and left at room temperature for 30 min. The amount of DNA (0.5 μg) was kept constant and that of gPEI varied depending on the amount required according to N/P ratios. These complexes were further diluted to 1 ml using physiological buffer and used for size and zeta potential measurement studies. However, complexes of EGFP and gPEI were prepared fresh each time prior to *in vitro* cytotoxicity studies and *in vitro* transfection evaluation.

2.7. Characterization of complexes

The DNA–gPEI complexes prepared in the present study were characterized by the following procedures.

2.7.1. 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 NANO-TECHNOLOGY, USA) with substrate force constant of 40 N/m (nominal)and resonance frequency of 300 kHz (nominal) and tip radii <10 nm was used. Complexes containing 0.5 μg DNA were prepared at various N/P ratios. After 30 min of incubation, complex solutions were diluted to a final volume of 1 ml using physiological buffer. 2–3 μl of this solution 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 indicating higher heights.

2.7.2. Dynamic light scattering (DLS)

The hydrodynamic diameter of the DNA–gPEI complexes was determined by dynamic light scattering (DLS) measurements. Complexes containing 0.5 μg DNA were prepared at various N/P ratios. After 30 min of incubation, complex solutions were diluted to a final volume of 1 ml using physiological buffer prior to measurements. Complex sizes were determined by dynamic light scattering (DLS) employing Photon Correlation Spectrometer, PHOTOCOR FC fitted with argon ion laser operating at 632.8 nm as the light source using a digital correlator. Measurements were carried out at an angle perpendicular to the incident light and the data were collected over a period of 3 min. The mean particle size was obtained from the method of cumulants. Instrument was calibrated with the monodisperse particle standards (silica, diameter 200 nm and latex beads, diameter 50 nm) supplied with the equipment before measuring particle size in the present investigation.

2.7.3. Zeta potential measurements

The DNA–gPEI complexes prepared for size determination were further used for zeta potential measurements. Zetasizer Nano ZS (Malvern instruments, UK) employing a nominal 5 mW HeNe laser operating at 633 nm wavelength was used for carrying out zeta potential studies. 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.

2.8. DNA retardation assays

The DNA–gPEI complexes were prepared by taking a known amount of plasmid DNA (0.5 μg) with varying amount of guanidinated PEI in a 20 mM HEPES buffer, pH 7.2, composed of 150 mM NaCl and by incubating for 30 min at room temperature. The resulted 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–gPEI complexes were visualized under ultra violet light after staining the gels with ethidium bromide.

2.9. In vitro cell transfection studies

Human embryonic kidney 293 (HEK 293) cells 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. HEK 293 cells were seeded prior to transfection into 24-well plates at a density of 5×10^4 cells/ well and incubated for 16 h for adherence. After stipulated time the cells were washed once with serum-free DMEM. Reporter gene CMV-GFP encoding for green fluorescent protein was used to assess the efficiency of guanidinated PEI-mediated transfection. DNA–gPEI complexes were prepared with varying concentration of gPEI and 0.5 μg of plasmid as described in DNA retardation assay. Subsequently, the complexes were diluted with serum free DMEM to a final volume of 400 μl and this transfection medium was added to each well, followed by incubation at 37 °C in humidified 5% CO_2 atmosphere for 4 h. The transfection media were replaced with 400 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.

2.10. Transfection efficiency measurements

To determine the nature and extent of transfection, fluorescence activated cell sorter (FACS) analysis was performed. After 36 h of transfection the serum supplemented DMEM was aspirated from the 24-well plate and cells were washed twice with 0.5% bovine serum albumin/phosphate-buffered saline (BSA/PBS). Cells were resuspended in 200 μl of 0.5% BSA/PBS and subjected to FACS analysis. Data were obtained on BD-LSR (Becton–Dickinson) and analyzed by Cell Quest and Win MDI softwares.

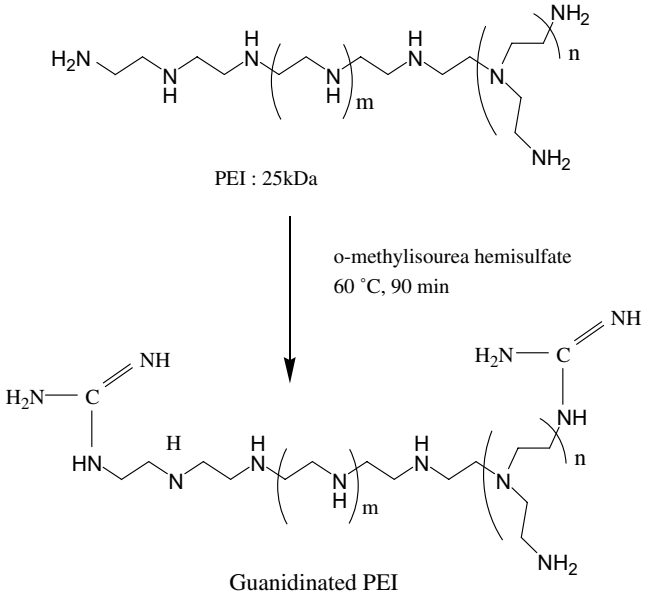
2.11. Cytotoxicity

The toxicity of DNA loaded guanidinated PEI was evaluated by MTT colorimetric assay [46]. HEK 293 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–gPEI complexes 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. Polymer synthesis

The amino groups of PEI substituted with guanidino groups in four different percentages (20%, 40%, 60%, 80%) were synthesized to investigate the possible effect of the amino/guanidino ratio on DNA condensation, cytotoxicity and transfection efficiency. The conjugates were



Scheme 1. Preparation of guanidinated PEI derivative.

made in a single-step reaction as shown in Scheme 1. On quantification of the guanidination extent by ninhydrin the % guanidino substitution was found to be 18, 37, 56 and 74 and designated as "gPEI 18", "gPEI 37", "gPEI 56" and "gPEI 74". Each guanidination effectively attaches a guanidino moiety to the primary amino group of PEI, resulting in the randomization of cationic charges along the polymer chain.

3.2. Preparation and characterization of g-PEI plasmid DNA complexes

Complexes of guanidinated-PEI plasmid DNA were prepared at different N/P ratios out of which the ratios showing optimum transfection, i.e. 15, 30, 74 have been reported in this study. The polyplex formation occurred due to ionic interaction between the positively charged amino groups of PEI and the negatively charged phosphate groups of plasmid DNA. The formation of polyplexes was confirmed by AFM imaging of complexes prepared from pEGFP and gPEI. AFM observations of guanidinated PEI-plasmid DNA showed spherical and compact complexes (Fig. 1). The discreet particles were evident and the three-dimensional image reveals homogeneous population with a clear absence of aggregates.

The size of the polyplexes was further determined by DLS (Fig. 2) and the average hydrodynamic diameter was from 176 to 548 nm (Table 1). The DLS spectrum provides a plot of population distribution in percentile vs size distribution in nm of the polyplexes. As evident from size determination studies the size of polyplexes was considerably decreased with the increase in the N/P ratio from 15 to 74. Further, to determine the surface charge of the various DNA–gPEI complexes zeta potential measurements were carried out at the three different N/P ratios in physiological buffer. As expected, the zeta potential values increased with the increase in the N/P ratio. However in the presence of serum the zeta potential values were found

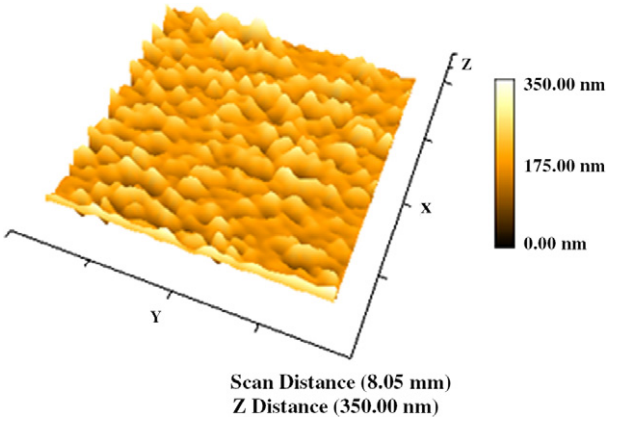


Fig. 1. Atomic force microscope image of gPEI 56% – DNA complexes at N/P ratio of 30 in PBS (phosphate-buffered saline at pH 7.4). The average particle size in this case is 350 nm.

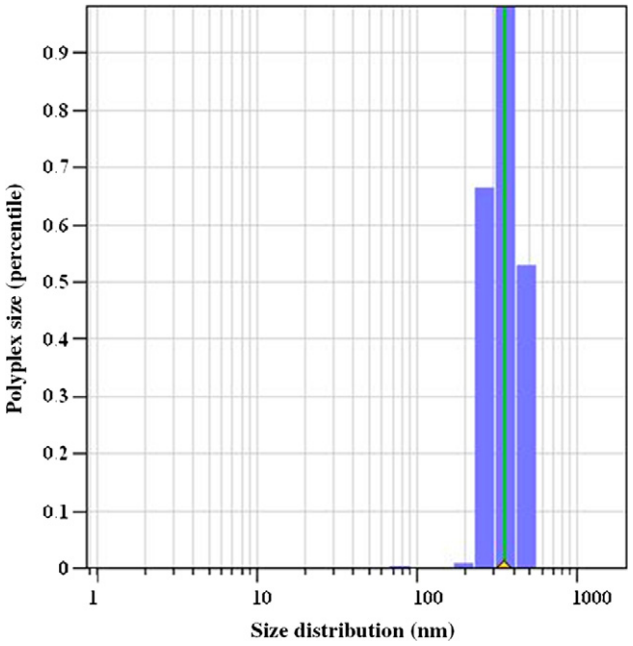


Fig. 2. Representative dynamic light scattering spectrum of gPEI 56% – DNA complexes at N/P ratio of 30 in PBS (phosphate-buffered saline at pH 7.4). The average hydrodynamic diameter in this case is 355 nm.

to be negative which could be due to the obstructive effect of serum which may be partly attributed to the non-specific interaction of the vector complexes with proteinous components.

3.3. DNA retardation assay

The interaction between the polycationic polymer and plasmid DNA results in neutralization of negative charges in the phosphate backbone of DNA, which results in the formation of neutral complexes unable to migrate under the influence of electric field in agarose gel. To estimate the optimal concentration for complete electro-neutralization of DNA, guanidinated PEI complexes were prepared with plasmid DNA at different N/P ratios, keeping the amount of DNA constant and retardation analyzed on 0.8% agarose. Native PEI was electro-neutralized at 0.4 μg/μg of plasmid DNA whereas guanidinated PEI was used in higher amount. In case of gPEI 18%, the DNA movement was slowly hampered with the increase in the concentration of gPEI and maximum retardation at 0.6 μg/μg of DNA (Fig. 3). DNA–gPEI 74% complexes showed complete retardation at 0.5 μg/μg of DNA.

3.4. Cytotoxicity

The positive charge of the polycationic polymers has been found to be the main reason of cellular toxicity exhibited. As revealed by a comparative study between polycationic, neutral and polyanionic polymers, the polycationic polymers due to strong electrostatic interaction with plasma membrane proteins lead to destabilization and ulti-

Table 1
Size and zeta potential of gPEI nanoparticles

S. No.	Nanoparticles	Average particle size in nm by DLS at different N/P in PBS (S.D.), PDI			Zeta potential at different N/P ratios (S.D.)					
		15	30	74	PBS			FCS		
					15	30	74	15	30	74
1	gPEI 18	548(26) 0.33	325(12) 0.42	257(13) 0.22	9.27(0.45)	12.7(0.75)	18.9(1.1)	−22.1(1.3)	−21.9(1.2)	−21.6(1.3)
2	gPEI 37	430(18) 0.27	360(16) 0.41	288(11) 0.32	10.49(0.5)	15.4(0.72)	18.8(0.91)	−17.1(1.1)	−14.9(0.9)	−20.6(0.9)
3	gPEI 56	409(20) 0.18	355(15) 0.21	284(9) 0.2	8.03(0.41)	12.3(0.62)	17.8(0.9)	−19.4(1.4)	−19.3(0.8)	−14.4(0.82)
4	gPEI 74	297(12) 0.15	206(14) 0.31	176(8) 0.13	10.1(0.43)	16.1(0.45)	20.5(1.3)	−17.2(0.9)	−13.8(0.56)	−20.9(0.94)

PBS, phosphate-buffered saline at pH 7.4; FCS, 10% fetal calf serum pH 7.2; N/P, ratio of nitrogen of PEI nanoparticles to phosphate of DNA; S.D., standadrd deviation; PDI, polydisersity index.

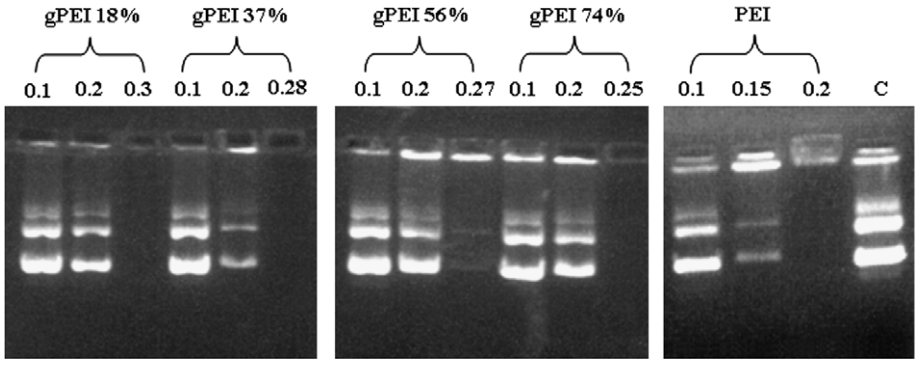


Fig. 3. DNA binding and retardation assay. Plasmid DNA, 0.5 μg, was incubated with increasing amounts of guanidinated PEI in presence of salt (150 mM NaCl) for 30 min. The samples were analyzed on a 0.8% agarose gel. Lane C represents DNA without nanoparticles, the values mentioned correspond to the amount of guanidinated PEI (μg) used in a 20 μl reaction to condense the DNA by charge neutralization.

mately rupture of the cell membrane. Guanidination results in delocalization of the polymer charge which leads to reduction in toxicity of the polymer–DNA complexes. The cytotoxicity of the DNA–gPEI complexes was evaluated using MTT colorimetric assay. HEK 293 cells were incubated with DNA–gPEI complexes prepared at various N/P ratios initially for 4 h in the absence of serum and extending upto 48 h in the presence of serum. A N/P ratio dependent cytotoxicity was observed with the complexes (Fig. 4).

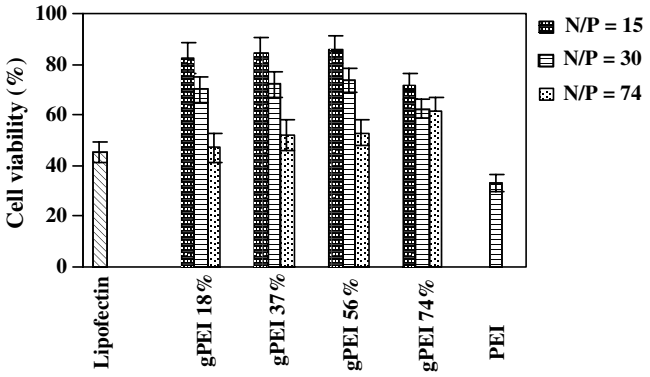


Fig. 4. Cell viability assay. HEK 293 cells were treated with guanidinated PEI–DNA under the transfection conditions. The ratios corresponding to the optimal transfection efficiency by guanidinated PEI–DNA ratios are presented here. The assays were done in triplicate and the standard error is shown.

The native polymer was found to exhibit high cell morbidity rate as compared with the gaunidinated polymer. At lower N/P ratio (i.e. 15) the cell viability percentage was found to be above 80% in case of 18–56% guanidinated polymer. The cell viability was however found to decrease with the increase in N/P ratio. The DNA–gPEI complexes with 56% gaunidination showed highest cell viability of 86% whereas in case of PEI polymer only 40% cells were viable. Thus, the guanidination has been found to decrease the cytotoxicity of PEI.

3.5. In vitro cell transfection

To determine the transfection efficiency of guanidinated PEI–DNA complexes, transfection experiment was carried out on HEK 293 cells and compared with native PEI 25 kDa and commercially available transfecting agent Lipofectin. The efficacy of complexes was determined by using plasmid carrying reporter gene CMV-Green fluorescent protein (GFP) for gene delivery. DNA–gPEI complexes were prepared at various N/P ratios under physiological salt concentrations (150 mM) and used to transfect HEK 293 cell lines. The gene expression of GFP carrying plasmid was observed under an inverted fluorescent microscope after

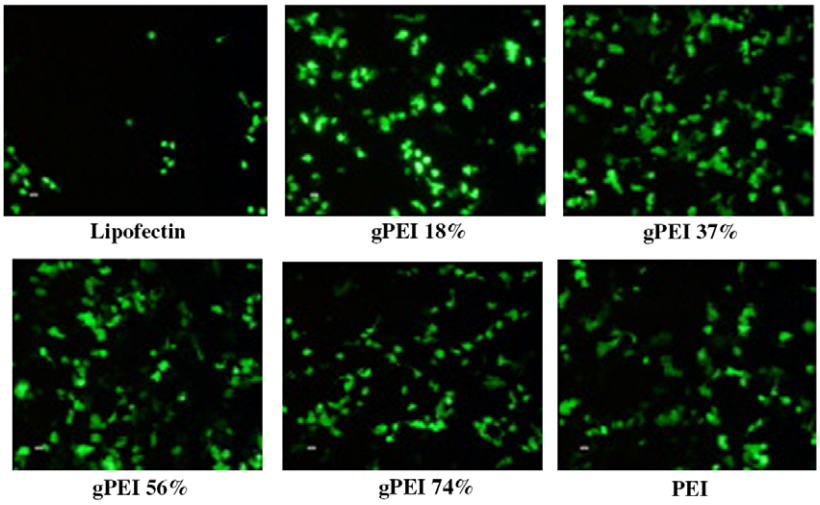


Fig. 5. Comparison of transfection efficiency of various guanidinated PEI–DNA complexes. HEK 293 cells were incubated with guanidinated PEI–DNA complexes at various N/P ratios and incubated for 48 h. The Green Fluorescent Protein (GFP) expression was observed under fluorescent microscope at 10× magnification. The data were recorded at optimal transfection efficiency at the mentioned guanidinated PEI: DNA ratio.

36 h (Fig. 5). Sufficient amount of GFP expression level was observed which sustained even after 3–4 days post-transfection.

3.6. Transfection efficiency measurements

The transfection efficiency of HEK293 cell line with gaunidinated PEI was studied by the calculation of green cell percentage with flow cytometry. As shown in Fig. 6 gPEI 56% had the highest transfection efficiency at the majority of the N/P ratios tested. With the increase in N/P ratio, gPEI 56% transfection efficiency increased and reached the highest at the N/P ratio of 30. All the other derivatives of PEI also showed increase in transfection efficiency with the increase in N/P ratio. The commercially available transfecting agent lipofectin showed only 15% of green color expressing cells, whereas as native PEI showed 34% cells. With the increase of the N/P ratio, more cells were dead, indicated by detachment from the bottom of plate, floating and aggregation.

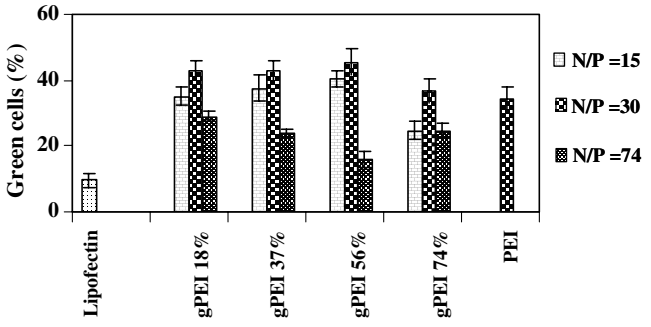


Fig. 6. Comparison of transfection efficiency of various guanidinated PEI–DNA complexes. HEK 293 cells were incubated with guanidinated PEI–DNA complexes at various N/P ratios for 4 h and the expression of GFP was monitored after 48 h. The green cell percentage was calculated by FACS. The results represent means of two independent experiments performed in triplicate.

4. Discussion

Non-viral gene delivery vectors that can efficiently and specifically induce gene expression in targeted cells would be valuable tools in the treatment of diseases arising from genetic malfunction. Moreover, these vectors should be easy to prepare with diminished cytotoxicity. A major limiting factor in development of non-viral vectors is their poor efficiency of gene transfer with respect to their viral counterparts. Polyethylenimine, a polycationic polymers has emerged as one of the most promising non-viral vectors with efficient DNA condensation and lysosome rupture properties. Until date, a large no. of derivatives of PEI have been proposed, most of them focusing on modulation of the types of amino groups to enhance the transfection efficiency.

In PEI, every third atom is a nitrogen atom capable of undergoing protonation, thus conferring to the molecule a high DNA-condensing and a high buffering capacity [7]. Strong ionic interactions between positively charged primary amines and the negatively charged phosphate groups result in the DNA condensing property of the PEI. The mean size and the polydispersity of the particles are affected by the ratio of PEI nitrogens to DNA phosphates [3]. Thus, the determination of the relative concentrations of PEI and DNA are critical factors that influence the transfection efficacy of the complexes. Cell lines transfected with complexes bearing a net positive charge were found to have high expression levels, as positive charge facilitates their interaction with negatively charged cell surface proteoglycans [50–52].In one of the earlier studies poly(propylene imine) dendrimer has been completely or partially functionalized with guanidinium groups. The fully guanidinylated dendrimer has been found to exhibit the best transfection efficiency under all the conditions studied [53].

Our proposed study was carried out at several N/P ratios formed by complexation of positively charged poly-

mer conjugate and negatively charged DNA (data not shown). gPEI at N/P ratios of 15–74 corresponds to positively charged complexes as evident from high zeta potential values (Table 1). When using the EGFP expression analysis, the complexes formed in 150 mM NaCl achieved transfection levels of up to 45.6% transfected cells. The ionic strength of the PEI/DNA mixing solution influences the size and the morphology of the particles. We have observed that under physiological salt concentration, DNA–gPEI complexes rapidly aggregated into large particles (>200 nm). The size of the complex formed was found to depend on the N/P ratio, i.e. size decreased with the increase in N/P ratio, which is in accordance with the earlier studies. Aggregation of polyplexes was prevented to some extent due to strong electrostatic repulsion by the positive charge (the positive zeta potential values). The DNA complexing capacity of gPEI was further studied by DNA retardation assay. The PEI bearing maximum amount of guanidino groups was found to retard at 0.5 μg/μg of DNA as gaunidination leads to increase in the overall positive charge of the polymer by increasing the number of secondary amines.

The complexes of DNA prepared with gPEI were found to exhibit higher transfection efficiency as compared to native polymer. gPEI with 56% of amino groups grafted with guanidino groups exhibited highest transfection efficacy with 45.6% population of cells. The extensive grafting with guanidino residues leads to beneficial delocalization of charge present onto primary amines with increased hydrophilicity compared with native PEI/DNA complexes. Moreover, as reported earlier, the higher transfection efficacy exhibited by the large particles (>200 nm) results from the conjunction of several features (i) they sediment onto the cell surface more rapidly than the small particles, (ii) since they contain a large proportion of free cationic polymers in addition to those complexed with DNA, they destabilize the membrane favoring their entry into cells, and (iii) their endosomolytic activity is far higher than that of the small particles. PEI derivatives were significantly more efficient than the other commercially available vectors such as lipid Lipofectin for mammalian gene transfer. Indeed, the injection of cDNA complexed with cationic lipids or PEI into the cytoplasm or into the nuclei of cells showed that DNA complexed with PEI could produce gene expression without disruption of the transcription process while cationic lipids showed no expression of the transgene [18]. Also, the toxicity of the transfected cell decreased with degree of guanidination from 18% to 56%. The cell viability decreased with incubation time and was dependent on the N/P ratio. Thus, the comparison of cell viability after transfections of HEK 293 cells with PEI derivatives showed that guanidinated PEIs were less cytotoxic than unsubstituted PEIs.

5. Conclusion

We have prepared partially guanidinated derivatives of branched PEI 25 kDa. The preparation methodology involves mild processing conditions and completely aqueous nature with high yields. As expected, the incorporation of guanidino moiety into PEI led to the reduction in cytotoxicity along with moderate improvement in transfection efficiency. This study suggests that guanidinated PEI–DNA complexes lead to *in vitro* reduction in cytotoxicity along with remarkable improvement in transfection efficiency.

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