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ScienceDirect
International Journal of Pharmaceutics 335 (2007) 180–192
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Imidazolyl-PEI modified nanoparticles for enhanced gene delivery

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Received 24 August 2006; received in revised form 1 November 2006; accepted 3 November 2006
Available online 17 November 2006

Abstract

The derivatives of polyethylenimine (PEI 25 and 750 kDa) were synthesized by partially substituting their amino groups with imidazolyl moieties. The series of imidazolyl-PEIs thus obtained were cross-linked with polyethylene glycol (PEG) to get imidazolyl-PEI-PEG nanoparticles (IPP). The component of hydrophobicity was introduced by grafting the lauryl groups in the maximal substituted IPP nanoparticles (IPPL). The nanoparticles were characterized with respect to DNA interaction, hydrodynamic diameter, zeta potential, *in vitro* cytotoxicity and transfection efficiency on model cell lines. The IPP and IPPL nanoparticles formed a loose complex with DNA compared to the corresponding native PEI, leading to more efficient unpackaging of DNA. The DNA loading capacity of IPP and IPPL nanoparticles was also lower compared to PEI. The imidazolyl substitution improved the gene delivery efficiency of PEI (750 kDa) by nine- to ten-fold and PEI (25 kDa) by three- to four-fold. At maximum transfection efficiency, the zeta potential of nanoparticles was positive after forming a complex with DNA. The maximum level of reporter gene expression was mediated by IPPL nanoparticles in both the series. The cytotoxicity, another pertinent problem with cationic polymers, was also negligible in case of IPP and IPPL nanoparticles.

Keywords: PEI; Imidazole; Transfection; Cytotoxicity; Lauric acid; PEG

1. Introduction

Recent years have witnessed a rapid development of non-viral vectors based on lipids and cationic polymers, which possess properties required to address the delivery problems in gene therapy. Many viruses have been genetically engineered to carry the fragments of foreign DNA inserted into their genome, owing to their natural ability to infect cells. The advantages and disadvantages of recombinant viral vectors are well documented in literature (Mulligan, 1993; Navarro et al., 1998). Cationic polymers mediated gene delivery hinges upon exploring new polymers or modify the existing ones to provide sufficient safety and transfection efficiency in clinical applications. The major advantages, gearing the use of cationic polymers in human gene therapies, include the facile manufacturing, scalability and biosafety (Kabanov, 1999; Zhang et al., 2004; De Smedt et al., 2000). The research groups have been constantly focusing on the development of polycation-based delivery systems with low toxicity and high transfection efficiency.

The pre-requisites for efficient gene delivery are condensation of DNA into particles, crossing the permeability barrier of cell and finally, the transfer of DNA into nucleus for protein expression. Polycations interact with DNA to form toroidal nanostructures, which can be effectively internalized (De Smedt et al., 2000). Among these polycationic carriers, polyethylenimine (PEI) has been a subject to numerous gene delivery studies and is one of the most efficient polymers reported to date (Park et al., 2005; Kim et al., 2005; Nimesh et al., 2006; Goula et al., 1998; Kunath et al., 2003). It has been shown to condense DNA for cellular internalization via endocytosis, prevents degradation by nucleases and subsequently aids the release from endosome to ensure efficient nucleic acid delivery. PEI has tremendous buffering capacity between pH 7.2 and 5.0, owing to the presence of a variety of protonable amines which could absorb the protons brought into the endosome by ATPase pump, cause osmotic swelling and thus induce rupture of the vesicle (Behr, 1997; Boussif et al., 1995; Kircheis et al., 2001). In addition, endosomal escape is aided by the expansion of polymer due to internal charge repulsion. Strong polycationic

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doi:10.1016/j.ijpharm.2006.11.033

nature of PEI is associated with certain drawbacks also, such as cytotoxicity (Tang et al., 2006; Chollet et al., 2002) and non-specific interactions with serum components (Walther and Stein, 1996). PEI per se exists in linear and branched forms, the later one being more effective gene delivery agent. Modifying amine structures in PEI is usually employed to reduce the cytotoxicity and enhance the transfection efficiency of DNA–polymer complexes (Thomas and Klibanov, 2002). A commonly used approach is to attach a pendent group onto the amines of PEI or to conjugate a ligand onto the amines of polymer for targeted delivery (Kircheis et al., 1997; Li et al., 2000; O'Neill et al., 2001). Beside this, PEI has been PEGylated (Peracchia et al., 1998; Kaul and Amiji, 2002; Bhadra et al., 2002), acetylated (Orrest et al., 2004), grafted with dextran (Tseng et al., 2004), cyclodextrin (Forrest et al., 2005) to improve the transfection efficiency and reduce the cytotoxicity of the carrier system.

Imidazole ring is present as a side chain in amino acid, histidine, with a pK_a of 6.15, and offers potent biocompatibility. The proton sponge capacity of imidazolyl moiety is also well documented (Pack et al., 2000; Kichler et al., 2003; Bello and Midoux, 2001; Midoux et al., 1998; Midoux and Monsigny, 1999). Poly histidine, in principle, can be used as a vehicle for gene delivery, but it is insoluble in aqueous solution at pH > 6, which is a major obstacle in its use. Imidazolyl groups have been chemically substituted on poly-L-lysine (PLL) and methacrylate-based gene delivery systems to introduce the endosomal buffering capacity in these polymers (Behr, 1997; Chen et al., 2005; Dubruel et al., 2004; Wattiaux et al., 2000). The presence of imidazole in these polymers enabled efficient transfer of nucleic acids into mammalian cells and eliminated the requirement of external endosomolytic agents. Protein expression level mediated by these imidazole containing polymers was manifold higher than the native polymer.

After understanding the structure–activity relationship of the PEI polymer, we attempted to maintain the subtle balance between charge density and endosomal escape potential, by substituting the amine functions with imidazolyl groups. Our earlier studies demonstrated that PEGylated PEI nanoparticles serve as better transfection agents compared to corresponding high molecular weight, branched PEI (Nimesh et al., 2006). Herein, we report the synthesis of a series of imidazolylated branched PEI (750 and 25 kDa) cross-linked with PEG to form nanoparticles (IPP). The encouraging results from imidazolyl substitution prompted us to further modify the nanoparticles by grafting with lauric acid (IPPL). The hydrophobicity thus introduced in the nanoparticles facilitated the interaction with cellular membranes. The surface charge on the nanoparticles was reduced but the buffering capacity remained unaltered by imidazolyl substitution. Interestingly, cytotoxicity was reduced to bare minimum and the transfection efficiency improved considerably. The strength of IPP or IPPL complex with plasmid DNA was lower than that of PEI, suggesting that the more efficient unpackaging of the DNA could be a contributing factor for the enhanced transfection efficiency.

2. Materials and methods

2.1. Materials

PEI (av. MW 750 and 25 kDa), PEG-8000 (av. MW 8 kDa), 4-imidazoleacetic acid hydrochloride, lauric acid, l-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), 3-(3-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), high retention dialysis tubing (cut off = 12 kDa), agarose, Tris–HCl, HEPES, ethidium bromide (EtBr), xylene cyanol and bromophenol blue were purchased from Sigma Chemical Co., St. Louis, MO, USA. Bradford reagent was procured from Bio-Rad Inc., Hercules, USA. Cell culture chemicals were purchased from Gibco-BRL Life Technologies, Web Scientific Ltd., UK. All other chemicals and reagents were purchased locally.

2.2. Cell cultures

The mammalian cell lines COS-1 (Simian virus 40 transformed kidney cells of an African green monkey) and HEK293 (Human embryonic kidney) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin.

2.3. Methods

2.3.1. Synthesis

2.3.1.1. Plasmid purification. The transfections were performed using the plasmid encoding enhanced green fluorescent protein gene (GFP) under the cytomegalovirus (CMV) immediate early promoter. The plasmid was transformed into *Escherichia coli* bacterial strain DH5α and extracted from the culture pellets using the Qiagen Maxi-prep Kit (Qiagen S.A. Courtaboeuf, France) as per manufacturer's instructions.

2.3.1.2. Preparation of polyethylene glycol (8000)-bis (phosphate). Polyethyleneglycol-bis(phosphate) (MW 8 kDa) was prepared following a reported procedure from our laboratory (Nimesh et al., 2006).

2.3.1.3. Preparation of imidazolyl-PEI. To an aqueous solution of 4-imidazoleacetic acid hydrochloride (14.1 mg, for 20% substitution, 1 mg/ml of dd water), sodium bicarbonate (7.33 mg) was added and swirled for 5 min. The resulting solution was added drop wise to an aqueous solution of PEI (25 kDa) (25 mg, 1 mg/ml) with vigorous stirring at room temperature. After 1 h, EDAC (32 mg) was added and the reaction was allowed to stir overnight at room temperature. The reaction mixture was concentrated on a rotary evaporator to one third of the original volume and poured into a dialysis bag. The dialysis was performed against water for 48 h with intermittent changes of water, followed by lyophilization to obtain imidazolyl (20%)-PEI (yield, 62%). Similarly, imidazolyl (20%)-PEI (750 kDa) was prepared. The 40%, 60%, and 80% imidazolyl substituted PEIs (25 and 750 kDa) were also prepared in a similar manner

varying the amount of 4-imidazoleacetic acid (yield, 49–67%). The extent of substitution of imidazole on PEIs was determined by ^{1}H NMR following the reported procedure (Orrest et al., 2004; von Harpe et al., 2000).

2.3.1.4. Preparation of covalently cross-linked imidazolyl-PEI-PEG (IPP) nanoparticles. For the preparation of nanoparticles, an aqueous solution of PEG8000-bis(phosphate) (1 mg/ml) was added drop wise with continuous stirring over a period of 45 min to an aqueous solution of imidazolyl (20%)-PEI (25 or 750 kDa) (1 mg/ml) at room temperature, to realize 5% cross-linking of PEI. Subsequently, EDAC (2 equiv) was added to the reaction mixture and incubated overnight for covalent cross-linking. The contents of reaction were dialyzed against water for 48 h with intermittent changes of water. The IPP nanoparticles, thus formed, were lyophilized in a speed vac (yield, 68%). Likewise, nanoparticles of 40%, 60% and 80% substituted PEIs (25 and 750 kDa) were prepared (yield, 68–73%). These nanoparticles were characterized by DLS, zeta potential and FTIR.

2.3.1.5. Preparation of lauryl substituted imidazolyl-PEI-PEG (IPP) nanoparticles. A solution of lauric acid (1.75 mg in 0.5 ml of DMF) was added drop wise to an aqueous solution of the above mentioned IPP nanoparticles (10 mg, 80%), followed by the addition of EDAC (2 equiv) to cover 5% amino groups of PEI and the reaction was allowed to stir overnight. Then the reaction mixture was dialyzed against water for 48 h with intermittent changes of water. The solution was subsequently lyophilized in a speed vac to obtain IPPL nanoparticles (lauryl substituted IPP in both the series) (yield, 69–76%), which were characterized by DLS, zeta potential, ^{1}H NMR and FTIR.

2.3.2. Instrumentation

^{1}H NMR spectra of the imidazolyl-PEIs were recorded on Bruker DRX-300 (300 MHz FT NMR) spectrometer using D_2O as a solvent. ^{1}H NMR (D_2O) of sample IPP-1 (25 kDa) δ: 2.45–3.50 (m, 2324H, –NCH_2CH_2-), 6.81 (s, 64H, –CH–N–), 7.35 (s, 64H, –N–CH–N–). FTIR (KBr) ν (cm^{-1}): 3436 (amino stretching), 2893, 1654 (carbonyl stretching), 1464, 1343.

The imidazolyl-PEI-PEG (IPP) nanoparticles were characterized by DLS, zeta potential and FTIR. The IR 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 scans, 16; resolution, 4.0 cm^{-1}; interval, 1.0 cm^{-1}; unit, %T. ^{1}H NMR (D_2O) δ: 2.51–2.78 (m, –NCH_2–), 3.46–3.69 (m, –CH_2– and –OCH_2–), 6.95 (s, –CH–N–), 7.35 (s, –N–CH–N–). FTIR (KBr) ν (cm^{-1}): 3432 (amino stretching), 2895, 1642 (carbonyl stretching), 1467, 1343, 1281 (ether twisting), 1113 (ether stretching).

^{1}H NMR of lauryl substituted imidazolyl-PEI-PEG (IPP) nanoparticles (D_2O) δ: 1.01 (t, –CH_3), 1.2–1.5 (m, –CH_2–), 2.49–2.82 (m, –NCH_2–), 3.52–3.72 (m, –CH_2– and –OCH_2–), 7.02 (s, –CH–N–), 7.57 (s, –N–CH–N–). FTIR (KBr) ν (cm^{-1}): 3435 (amino stretching), 2888, 1650 (carbonyl stretching), 1465, 1348, 1285 (ether twisting), 1111 (ether stretching).

2.3.3. DLS and zeta potential measurements

The size and zeta potential of nanoparticles and nanoparticle/DNA complexes were measured on Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) with the following settings: 15 measurements per samples, viscosity for water is 0.89 cP, temperature 25 °C. DNA complexes were prepared by incubating the various concentrations of nanoparticles and plasmid DNA (pDNA) for 30 min at room temperature.

2.3.4. Buffering ability of IPP and IPPL nanoparticles

Ability of PEI and PEI-modified nanoparticles to resist acidification was evaluated by acid titration assay following a reported method (Tseng et al., 2004). The samples were prepared by suspending nanoparticles at a concentration of 10 mg/ml in 150 mM NaCl. Initially, the pH of PEI, IPP and IPPL nanoparticles in both the series was adjusted to nearly 9.0 and then titrated by adding 0.1N HC1 until a pH of 3.5 reached. The slope of the curve, pH versus amount of HC1 consumed, provides an indication of the intrinsic buffering capability of the delivery vehicle.

2.3.5. DNA–polymer interactions measurement

The DNA/nanoparticle complex (at N/P ratio of 6.25) was prepared and then incubated with 2 ml of heparin buffer (20 mM HEPES, 5.2% glucose, 2 μg/ml EtBr and various concentrations of heparin, pH 7.0). The fluorescence intensity was measured at an excitation wavelength 520 nm and emission wavelength 605 nm with a slit-width of 5.0 nm (Tseng et al., 2004).

2.3.6. DNA retardation assay

A known amount of plasmid DNA (1 μg) was mixed with IPP and IPPL nanoparticles at different w/w ratio in a 20 mM HEPES buffer, pH 7.4 containing 150 mM NaCl and incubated for 30 min at room temperature. The IPP and IPPL/DNA complexes, thus formed, were mixed with the loading buffer containing tracking dye (Xylene cyanol) and loaded into individual wells of 0.8% agarose gel and electrophoresed at 100 V for 45 min. The gels were stained by ethidium bromide and the bands corresponding to plasmid DNA were visualized under UV light.

2.3.7. In vitro transfection studies

COS-1 cells were seeded into 96 well plates at a density 4.0×10^3 cells/well. After incubating for 16 h, the media was aspirated from the wells and the cells were washed with serum free DMEM. IPP and IPPL/DNA complexes were prepared at different w/w ratios, as described in the retardation assay. Subsequently, the complexes were diluted with serum free DMEM to a final volume of 80 μl (transfection medium) and gently added to each well, followed by incubation at 37 °C in humidified 5% CO_2 atmosphere. After 4 h, the transfection medium was replaced by 200 μl fresh growth medium (DMEM with 10% FBS) and the cells were incubated for 36 h. Thereafter, the cells, transfected with GFP reporter gene, were observed under Nikon Eclipse TE 2000-S Inverted microscope, Kanagwa, 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. Similar experiments were carried on HEK293 cell lines. All experiments were repeated atleast thrice.

2.3.8. Analysis of EGFP expression

The GFP, expressed in the cells, was quantified by measuring the fluorescence intensity. The wells of the plate containing cells were washed once with PBS, followed by lysis with 100 μl lysis buffer (10 mM Tris–HC1, pH 7.4, 0.5% SDS and 1 mM EDTA) and incubated in a shaker for 15–20 min at 25 °C. Lysate (10 μl) was used to estimate the expressed reporter gene product, green fluorescent protein (GFP), spectrofluorometrically at an excitation wavelength 488 nm and emission at 509 nm. Background fluorescence and auto-fluorescence were determined using mock treated cells (cells without naked DNA). The total protein content in cell lysate from each well was estimated using Bradford's reagent (BioRad), 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 extracts. The data is reported as arbitrary unit (A.U.)/mg of cellular protein and represent mean ± standard deviation for triplicate samples.

2.3.9. Cytotoxicity

To assess the cytotoxicity of IPP/DNA and IPPL/DNA complexes, the cells were treated, as described in the transfection experiments. After 36 h, MTT (0.5 mg/ml of DMEM) was added to COS-1 cells and incubated for 2 h. Then the supernatant was aspirated and the formazan crystals were suspended in 100 μl isopropanol containing 0.06 M HC1 and 0.5% SDS. Aliquots were drawn from each well and the intensity of color was measured spectrophotometrically in an ELISA plate reader at 570 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 were calculated by $(A_{sample}/A_{control}) \times 100$.

3. Results

3.1. Preparation of IPP and IPPL nanoparticles

Though branched PEI is a more efficient transfection agent compared to linear one, the inherent toxicity of the former is also quite high (Weiss et al., 2006). Further, the toxicity is directly proportional to the molecular weight and becomes a major obstacle in the use of high molecular weight branched PEI. Several groups have tried to address this problem by adopting various approaches (Nimesh et al., 2006; Kunath et al., 2003; Tang et al., 2006). In this study, PEIs (25 and 750 kDa) were modified by covalently attaching imidazolyl moiety (20–80%) on primary as well as secondary amines and subsequently converted them into nanoparticles with the help of a polyethyleneglycol-based cross-linker (PEG8000-bis-phosphate). Lauric acid (5%) was substituted (e.g. on IPP 80%) to study the effect of hydrophobic alkyl chain on gene transfection. The extent of imidazolyl substitution was determined spectroscopically (^{1}H NMR) (Orrest et al., 2004; von Harpe et al., 2000) and found to be 11% (20), 21% (40), 31% (60) and 39% (80) (∼50% reaction yield) in case of 25 kDa and 12%, 20%, 29% and 41% in case of 750 kDa, as shown in Table 1. The values given in the parenthesis indicate the substitution attempted experimentally. Subsequently, IPP and IPPL nanoparticles were characterized by ^{1}H-NMR and FTIR. The peaks at ∼1281 and ∼1106 cm^{-1} are corresponding to ether twisting and stretching frequencies, respectively.

Table 1
Percentage substitution of imidazolyl moiety in IPs and solubility of IPPs

Samples	Attempted substitution (% Im)	Actual substitution (% Im)	Samples	Solubility in H_2O (mg/ml)
(A) 750 kDa PEI				
IP-1	20	12	IPP-1	100
IP-2	40	20	IPP-2	100
IP-3	60	29	IPP-3	60
IP-4	80	41	IPP-4	60
–	–	–	IPPL	40
(B) 25 kDa PEI				
IP-1	20	11	IPP-1	100
IP-2	40	21	IPP-2	100
IP-3	60	31	IPP-3	100
IP-4	80	39	IPP-4	60
–	–	–	IPPL	40

IP, imidazolyl-PEI; Im, imidazolyl.

In the text and figures, IPP-1, 2, 3 and 4 represent IPP 11%, 21%, 31%, 39%, in case of 25 kDa and 12%, 20%, 29%, 41%, in case of 750 kDa, respectively.

3.2. Size and zeta potential

The surface charge of a gene delivery agent is important for columbic interactions with DNA and later, in the cellular entry. IPP and IPPL nanoparticles retained sufficient positive surface charge even after substitution with imidazolyl moieties, as indicated by zeta potential studies. The zeta potential of all the samples decreased on complexation with DNA but still remained positive. As the degree of substitution is increased, the zeta potential decreased concomitantly in both the series, 25 and 750 kDa PEIs (Table 2). The decrease in zeta potential is diagnostic, as the amino functions are being replaced by imidazolyl moities, where a relatively weaker charge is delocalized within the ring structure. The zeta potential of nanoparticle/DNA complexes was found to be negative in the presence of serum.

The size distribution pattern of representative IPPL nanoparticles is shown in Fig. 1. The size distribution range is narrow, shows absence of aggregates and the majority population lies in nanometer range. The average size of IPP and IPPL nanoparticle/DNA complexes were found to be in the range of 180–230 nm for 25 kDa series and 200–300 nm for 750 kDa series. The smaller size of 25 kDa series suggests that the nanoparticles are more compact. When native PEI was allowed to interact with DNA, the self-assembled nanoparticles were larger in size. As the imidazolyl substitution was increased, an increase in particle size was observed in IPP and IPPL nanoparticles (Table 2). Also the size of IPPL nanoparticles is greater

Table 2
Particle size and zeta potential measurements of modified PEI nanoparticle/DNA complexes

S. No.	Samples	Average particle size (nm) (±S.D.)		Zeta potential (mV) (±S.D.)		Ratio of nanoparticles to DNA	
		DNA loaded nanoparticles (in H_2O)	DNA loaded nanoparticles (in serum)	DNA loaded nanoparticles (in H_2O)	DNA loaded nanoparticles (in serum)	(N/P)	(w/w)
(A) 750 kDa							
1	IPP-1	211.7 (1.7)	199 (6.3)	11.1 (1.1)	−14.3 (1.4)	74	10:1
2	IPP-2	249.6 (3.4)	140 (9.4)	5.28 (0.8)	−14.1 (1.1)	74	10:1
3	IPP-3	237.9 (5.6)	153 (8.4)	3.72 (0.9)	−13.8 (1.8)	74	10:1
4	IPP-4	246.5 (3.2)	155 (7.3)	3.18 (1.2)	−14.1 (1.1)	74	10:1
5	IPPL	292.4 (3.7)	167 (8.1)	3.15 (1.1)	−13.9 (0.9)	148	20:1
6	PEI (750 kDa)	437 (3.9)	181 (8.5)	16.8 (1.4)	−15.9 (1.2)	7	1:1
(B) 25 kDa							
1	IPP-1	183.9 (4.2)	108 (7.1)	9.68 (1.1)	−14.1 (1.6)	37	5:1
2	IPP-2	202.7 (3.2)	123 (8.1)	5.28 (0.8)	−14.3 (1.2)	37	5:1
3	IPP-3	192.9 (1.4)	134 (5.3)	3.72 (0.9)	−14.2 (0.9)	37	5:1
4	IPP-4	214.0 (2.1)	147 (6.9)	3.18 (1.2)	−13.9 (1.3)	37	5:1
5	IPPL	230.4 (4.8)	169 (8.7)	3.15 (1.1)	−14.0 (1.4)	74	10:1
6	PEI (25 kDa)	368.0 (5.4)	199 (7.6)	16.8 (1.4)	−15.2 (1.5)	3	0.5:1

than IPP nanoparticles in both the series (750 and 25 kDa PEI). When the size of nanoparticle/DNA complexes was measured in the presence of serum, the size of the particles was found to be reduced by nearly 30%, as shown in Table 2.

3.3. Buffering capacity

The buffering capacity of PEI is responsible for the release of DNA complexes from the endosome. The effect of imidazolyl and lauryl substitutions on buffering capacity of PEI was assessed by measuring the change in pH of the suspension (10 mg/ml) upon addition of increasing amounts of 0.1N HC1. As shown in Fig. 2, PEI has considerable buffering capacity over almost entire range of pH. Polymer with high buffering capacity would undergo a small change in pH when the same amount of

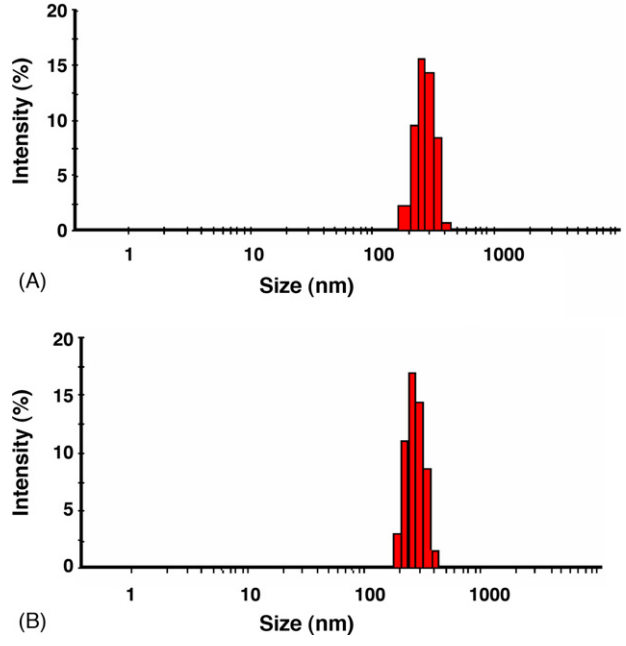


Fig. 1. Characterization of nanoparticles by Zetasizer Nano ZS. Size range determination of IPPL nanoparticles by Zetasizer shows a population of particles distributed between (A) 200–300 nm for 750 kDa series and (B) 180–230 nm for 25 kDa series of PEI.

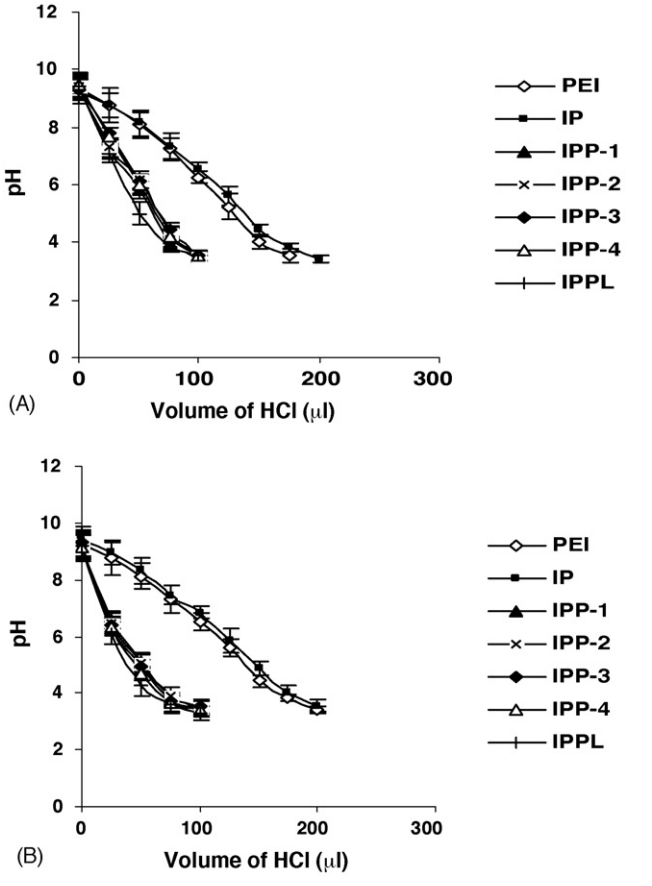


Fig. 2. Buffering capacity of IP, IPP and IPPL nanoparticles and native PEI: (A) 750 kDa series and (B) 25 kDa series. Acid titration curves for aqueous solution of nanoparticles, IP and native PEI (10 mg/ml) were obtained by adding equal aliquots of 0.1N HC1. (Error bars represent ±standard deviation from the mean.)

HC1 was added into the polymer solution during titration. The decrease in tendency of PEI to be protonated after conjugation depends on degree of substitution as well as type of substituent. A reasonably high extent of change in pH was recorded for IPP and IPPL nanoparticles (both 750 and 25 kDa series) on addition of HC1. We assumed that the observed reduction in buffering capacity of PEI was attributed to the presence of PEG not the imidazole. In order to confirm this, we prepared imidazolyl-PEI's (IP) without PEG and performed the same experiment. It was found that the buffering capacity of IP (80%) was greater than native PEI. Thus, the buffering capacity of IPP and IPPL nanoparticles is lower then the corresponding PEI because of PEG substitution, which blocks the charge of polycations. Also as expected, the buffering capacity of IPPL nanoparticles was slightly lower than the corresponding IPP nanoparticles (Fig. 2).

3.4. DNA retardation assay

The interaction between the nanoparticles and the pDNA results in the neutralization of the negative charge of DNA and consequently, the mobility of the DNA, under the influence of electric field, is retarded. Different weight ratios of IPP and IPPL nanoparticles were incubated with constant amount of pDNA to determine optimal concentration of nanoparticles required for the complete retardation of DNA. The retardation was analyzed on 0.8% agarose gel, as shown in Fig. 3. In case of native PEI polymer (25 and 750 kDa), pDNA was completely retarded at w/w ratio of 0.2, whereas a higher amount of nanoparticles, IPP and IPPL, was required for complete retardation of the same amount of DNA (Fig. 3). The results support previous findings for imidazolyl substitution on poly-L-lysine, which requires higher amounts of modified polymer for complete retardation compared to native polymer (Chen et al., 2005). However, the degree of imidazolyl substitution had very little effect on the amount of nanoparticles required for complete retardation but the presence of lauric acid in the nanoparticles increased the amount considerably. In other words, DNA loading capacity of IPPL nanoparticles was lower than IPP nanoparticles. In case of IPP and IPPL nanoparticles (25 and 750 kDa), the w/w ratios required to completely retard 1 μg pDNA were observed to be 3 (N/P = 22.22) and 4 (N/P = 29.6), respectively.

3.5. Measurement of DNA–nanoparticle interaction

After deciphering the stoichiometry of the nanoparticle/DNA complex formation, the physical attributes of the complex were determined. The extent of interaction of DNA with nanoparticles was studied through the changes in the fluorescence intensity of ethidium bromide (EtBr) in the presence of a competing polyanion (heparin). Heparin competes with DNA to bind the cationic polymer, where the binding affinity of nanoparticles for DNA governs the extent of replacement reaction by heparin. The amount of DNA dissociated from the complex will bind to the intercalator, EtBr and estimated by measuring the fluorescence intensity. As the degree of imidazolyl substitution was increased in nanoparticles, the amount of DNA released from the complex

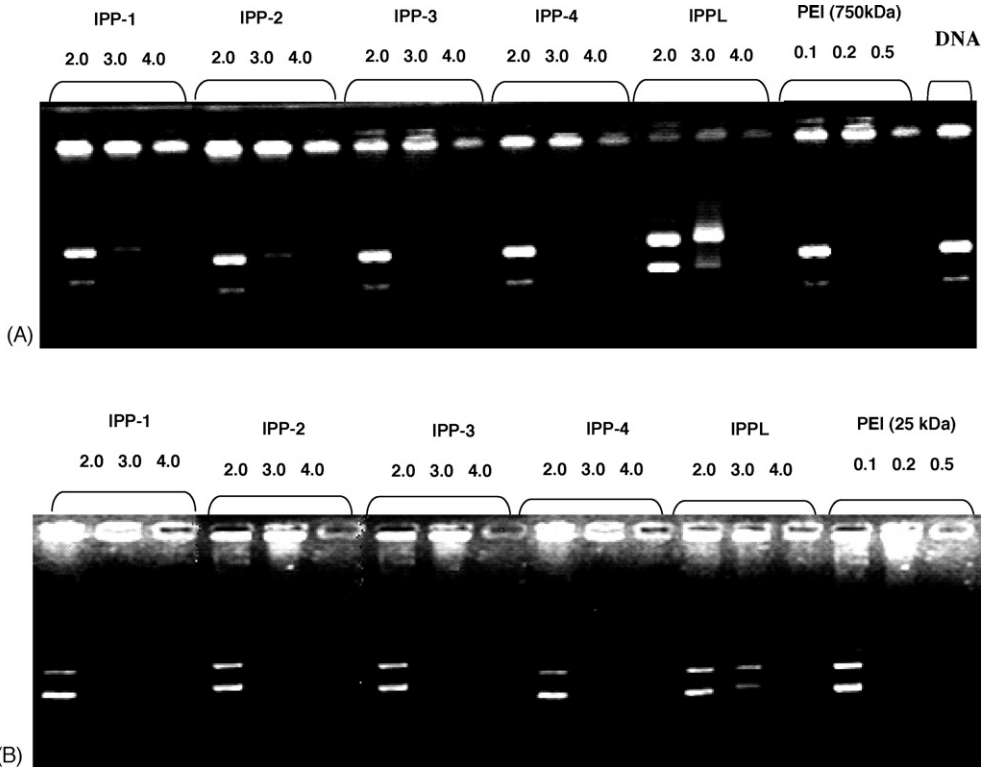


Fig. 3. Gel retardation assay of IPP/DNA and IPPL/DNA complexes. Plasmid DNA (1 μg) was incubated with increasing amounts of nanoparticles in a buffer containing HEPES/NaCl. The values mentioned correspond to the amount of nanoparticles (μg) used in a 20 μl reaction to condense pDNA: (A) 750 kDa PEI series and (B) 25 kDa PEI series.

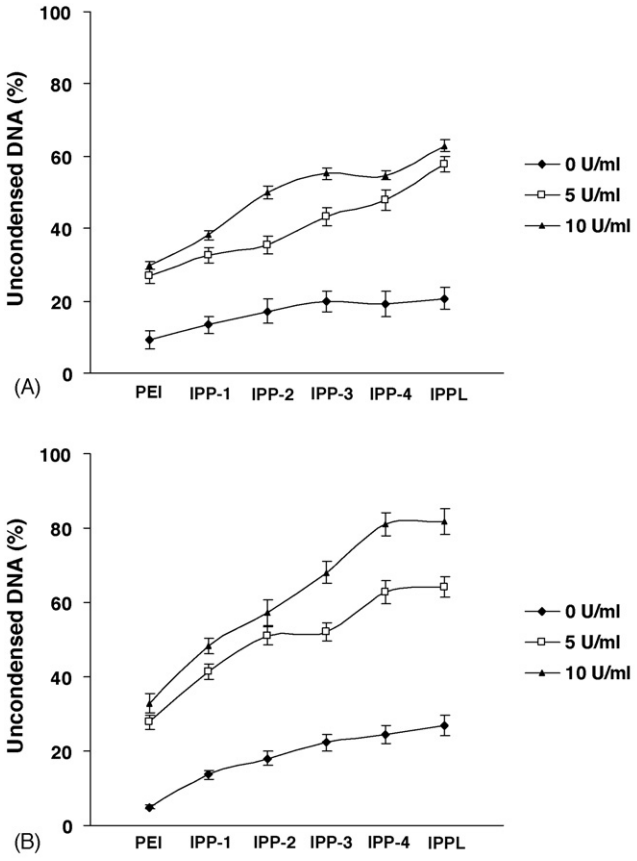


Fig. 4. Measurement of nanoparticle/DNA interactions. Interactions between DNA and polymer, at N/P ratio of 6.25, is indicated by the percentage of uncondensed DNA after the DNA–polymer complexes were exposed to various heparin concentrations (0, 5 and 10 U/ml) The fluorescence intensity of naked DNA was taken as 100%. Ordinate axis represents relative fluorescence intensity of DNA released from the nanoparticles/DNA complexes: (A) 750 kDa PEI series and (B) 25 kDa PEI series. (Error bars represent ±standard deviation from the mean.)

also increased, as indicated by an increase in the fluorescence intensity (Fig. 4). At higher concentrations of heparin, a major fraction of free DNA was detected in the solution. The imidazole ring forms a loose complex with DNA compared to native PEI, where the charge is localized and easily available.

3.6. Cytotoxicity

The synthesis of PEI-based nanoparticles was accomplished to reduce the cytotoxicity for *in vivo* applications. The cytotoxicity of IPP and IPPL nanoparticles was assessed in the presence of complexed pDNA over the concentration range relevant to gene delivery by MTT assay. The level of toxicity was found to be a function of the degree of substitution and it decreased with increase in the imidazolyl substitution on PEI. Cytotoxicity was further reduced on lauryl substitution in IPP nanoparticles (Fig. 5). Native PEI, 750 kDa, was found to be most toxic to the cells with 37% cell viability and imidazolyl modifications increased the cell viability to 65%, which was further improved to 68% on substituting the nanoparticles with lauric acid. Similarly, in native PEI 25 kDa, cell viability was restored to 78% on imidazolyl substitution and 81% on lauric acid grafting to the nanoparticles. The concentration of nanoparticles in DNA complexes, as high as 500 μg/ml was well tolerated by the cells (Fig. 5A). The IC_{50} values were calculated at the DNA:nanoparticle ratio resulting in optimal transfection for various nanoparticle preparations, as shown in Fig. 5B and Table 3. The effects were almost similar on both the cell lines studied.

Table 3
IC50 values at optimum transfection efficiency

S. No.	Samples (25 kDa PEI)	IC_{50} values (N/P = 74)	Samples (750 kDa PEI)	IC_{50} values (N/P = 74)
1	IPP-1	20	IPP-1	12
2	IPP-2	24	IPP-2	14
3	IPP-3	26	IPP-3	15
4	IPP-4	29	IPP-4	18
5	IPPL	31	IPPL	24

3.7. In vitro transfection studies

The various physio-chemical parameters studied so far emphasize the suitability of IPP and IPPL nanoparticles as transfection agents. The transfection efficiency was evaluated on COS-1 (Fig. 6) and HEK293 (Fig. 7) cell lines using a plasmid containing a reporter gene encoding green fluorescence protein (GFP). The cells were incubated with various nanoparticle/DNA complexes for 4 h and afterwards, the quantification of GFP expression was used as an index of transfection efficiency. As reported earlier, PEI (25 kDa) was found to be an efficient transfection agent in the study at all the concentrations tested in comparison to PEI (750 kDa). Fig. 6A shows the influence of nanoparticle/DNA ratio on the GFP fluorescent intensity. The degree of substitution of imidazole in nanoparticles was found to be directly proportional to the transfection efficiency. The incorporation of lauric acid in the IPP nanoparticles resulted in further improvement in transfection efficiency. IPPL nanoparticles of PEI (750 kDa) delivered DNA with a remarkably improved efficiency of nine- to ten-folds. The transfection efficiency of PEI (25 kDa), which is a gold standard for cationic polymer-based delivery systems, was also improved by three- to four-fold upon substitution with imidazole and lauric acid. The amount of IPPL nanoparticles required in DNA complex to achieve maximum transfection was higher compared to IPP. From Fig. 6B and C, it became clear that the IPPL nanoparticles, in both the series (25 and 750 kDa), gave maximum transfection efficiency. The fluorescent images of transfected cells corroborated the expression analysis and also demonstrated that the cell density was high in cells transfected with IPPL nanoparticles (Fig. 6F). The cells transfected with naked DNA barely expressed the reporter gene. The transfection with nanoparticles was also carried out in the presence of 10% serum and the efficiency was found to be almost similar to that obtained without using serum, in both the series (25 and 750 kDa), as shown in Fig. 6D and E. However, in case of unmodified PEI, the transfection efficiency was reduced in the presence of serum (Fig. 6D and E). The enhancement in transfection efficiency was evident in both the cell lines studied, showing thereby, that the effect is specific to the delivery agent.

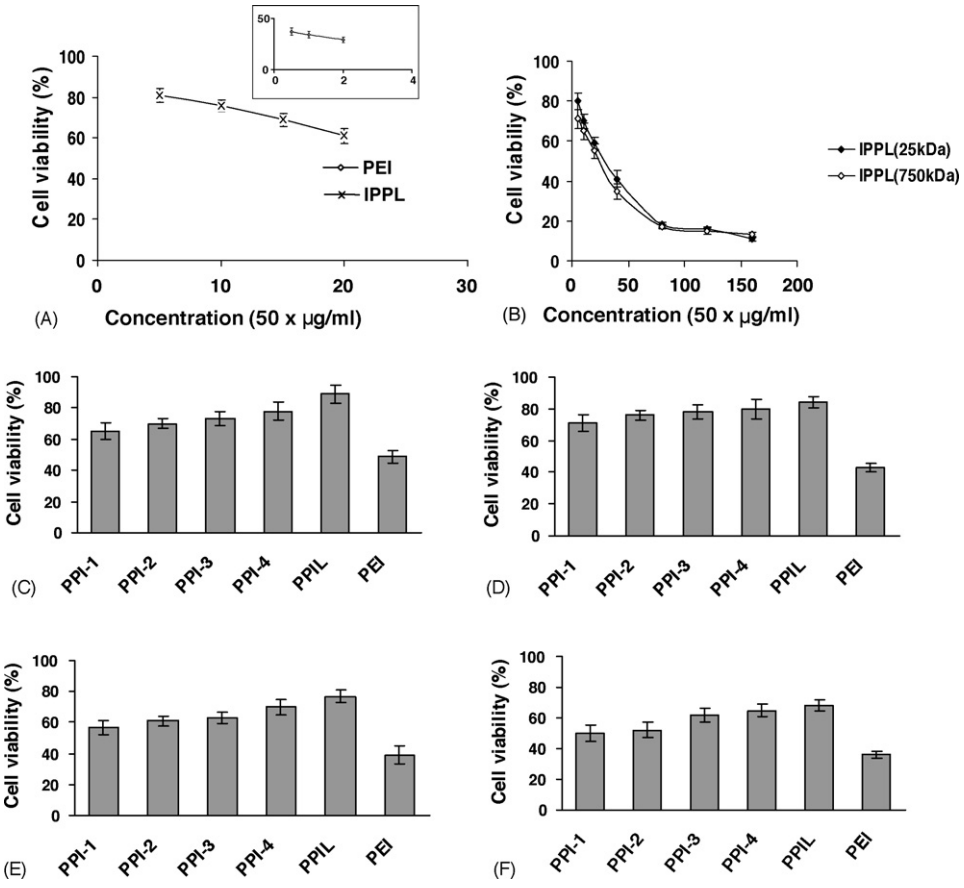


Fig. 5. Cytotoxicity of IPP/DNA and IPPL/DNA complexes on COS-1 cells. Cells were treated with IPP/DNA and IPPL/DNA complexes under conditions described in Section 2, and cytotoxicity was determined by MTT assay. Percent viability of cells is expressed relative to control cells. Each point represents the mean of two independent experiments performed in triplicates for IPP/DNA and IPPL/DNA complexes mediated cytotoxicity at various concentrations. (A) Dose-dependence curve for cytotoxicity mediated by PEI/DNA and IPPL/DNA complexes for 25 kDa series prepared at various weight ratios. The abscissa represents the concentrations (μg/ml) of nanoparticles used to condense 1 μg pDNA. The concentrations of unmodified PEI used were 0.5, 1, 2 μg, respectively, as shown in the inset. Cytotoxicity associated with IPP/DNA and IPPL/DNA complexes at maximum transfection efficiency, (B) representative dose-dependence curve for cytotoxicity of IPPL (750 and 25 kDa) at N/P = 74 for calculation of IC_{50} values, (C) 750 kDa series on COS-1, (D) 25 kDa series on COS-1, (E) 750 kDa series on HEK293, and (F) 25 kDa series on HEK293. N/P ratios are indicated in Table 2. (Error bars represent ±standard deviation from the mean.)

4. Discussion

Cationic polymers have emerged as a leading class of transfection reagents owing to their molecular diversity that enables to manipulate their physico-chemical properties by chemical modifications. The various derivatives of cationic polymers like PEI, poly-L-lysine (PLL) have been shown to have superior transfection ability over the native polymers (Benns et al., 2000; Merdan et al., 2005). In the current study, we selected branched PEI (25 and 750 kDa) for chemical modifications. Branched PEI (25 kDa), though known to be the most efficient transfection agent, has associated cytotoxicity and PEI (750 kDa) has been almost abandoned due to its highly toxic nature. The PEI-based nanoparticles described here, imidazolyl-PEI-PEG (IPP) and imidazolyl-PEI-PEG-lauric acid (IPPL) (Scheme 1), are designed to preserve the buffering capacity of PEI and reduce the charge associated cytotoxicity. In the present study, a series of PEI-based nanoparticles were synthesized with a varying degree of imidazolyl substitution, keeping the amount of PEG cross-linker constant. PEGylation is known to reduce the aggregation of particles by preventing the non-specific interactions with serum proteins (Olbrich et al., 2001). Lauric acid was incorporated in the best sample among the IPP nanoparticles to impart some hydrophobic character to them. The lauric acid blocked only 5% amino groups so that the cationic charge density is not reduced substantially, which is essential for the electrostatic interactions with DNA and the cell membrane (already 5% of amino groups have been utilized in cross-linking with PEG to form nanoparticles). The samples are soluble in water (Table 1), partially soluble in DMSO and insoluble in all organic solvents. The nanoparticles, thus formed, were soluble in water at a concentration 10 mg/ml, which is higher than the routinely used concentrations (0.1–2 mg/ml) in *in vivo* studies. The association of IPP and IPPL nanoparticles with DNA at physiological ionic strength resulted in homogenous particles with a positive surface charge. While the zeta potential of the nanoparticles showed minimal reduction on varying the imidazolyl substitution, a decline was observed on introducing the lauryl moiety. Modified PEI nanoparticles contain protonable imidazolyl moieties and a variety of amino functions, which have been proposed to initiate membrane destabilization after protonation in the acidic medium (Pichon et al., 2000). Pichon and co-workers clearly

demonstrated by confocal microscopy that the protonated imidazole helps in delivery of DNA into cytosol. In this study, it was observed that PEI-based nanoparticles had lower extents of protonation than unmodified PEI, when the same amount of acid was added (Fig. 2). The substitution by imidazolyl groups marginally increased the buffering capacity of PEI at the same time slightly reducing the surface charge density in IPP nanoparticles. Nevertheless, the presence of PEG and lauric acid in the nanoparticles reduced the buffering capacity to some extent. The amount of IPP nanoparticles required for the complete neutralization of DNA was also similar, reflecting that the binding capacity is not altered by varying the degree of imidazolyl substitution. DNA loading capacity of IPPL nanoparticles was considerably lower than IPP ones.

The binding affinity of IPP and IPPL nanoparticles and DNA condensation are other major contributing factors for efficient gene delivery. It is widely accepted that the release of DNA from polymeric carrier occurs by a competitive reaction between the cationic components of gene delivery and charged intracellular species (Chen et al., 2005). In competition with heparin, DNA was released from relatively loose nanoparticle–DNA complexes. The binding affinity of PEI-based nanoparticles was found to decrease on increasing the imidazolyl content. At neutral pH, the apparent pK_a of imidazole groups is higher than the amines of PEI, so the electrostatic contribution to the relative binding affinity presumably decreases with increase in imidazolyl content in nanoparticles. As the number of imidazolyl moieties increase at neutral pH, the polymer becomes

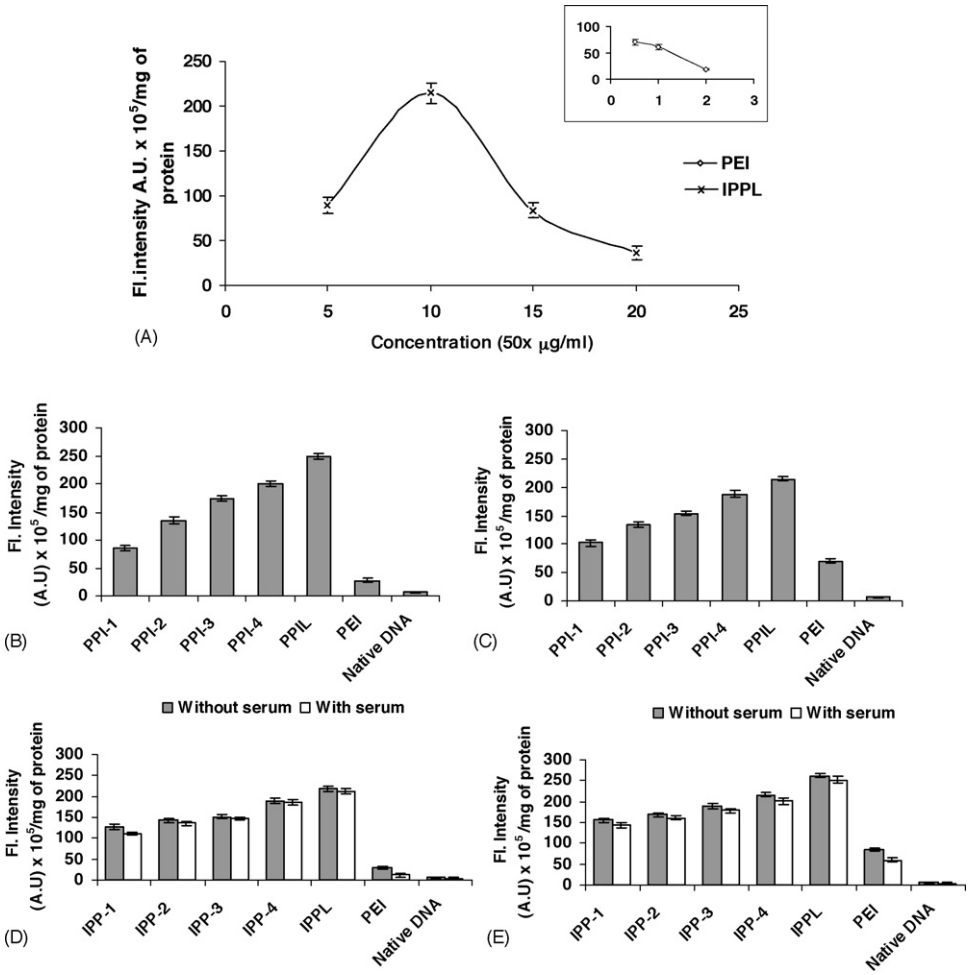


Fig. 6. GFP fluorescence intensity of COS-1 cells transfected with IPP/DNA and IPPL/DNA complexes. The fluorescent intensity of GFP fluorophore in the cell lysate was measured on spectrofluorimeter and the results are expressed in terms of A.U./mg total cellular protein. The results represent the mean of two independent experiments performed in triplicates. (A) Transfection efficiency with PEI/DNA and IPPL/DNA complexes for 25 kDa series prepared at various weight ratios. The abscissa represents the concentrations (μg/ml) of nanoparticles used to condense 1 μg pDNA. The concentrations of unmodified PEI used were 0.5, 1, 2 μg, respectively, to condense 1 μg pDNA, as shown in the inset, (B) fluorescent intensity of COS-1 cells transfected with IPP/DNA and IPPL/DNA complexes, 750 kDa series at maximum transfection efficiency, (C) fluorescent intensity of COS-1 cells transfected with IPP/DNA and IPPL/DNA complexes, 25 kDa series at maximum transfection efficiency, (D) fluorescent intensity of COS-1 cells transfected in presence and absence of serum, with IPP/DNA and IPPL/DNA complexes, 750 kDa series at maximum transfection efficiency, (E) fluorescent intensity of COS-1 cells transfected in presence and absence of serum, with IPP/DNA and IPPL/DNA complexes, 25 kDa series at maximum transfection efficiency, and (F) fluorescent microscopy of COS-1 cells transfected with IPP/DNA and IPPL/DNA complexes, 25 kDa and 750 kDa series at maximum transfection efficiency. Images were recorded at 10× magnification. COS-1 cells transfected with respective nanoparticle/DNA complexes, as observed under UV, C-Fl epifluorescence filter of fluorescent microscope. N/P ratios are indicated in Table 2. (Error bars represent ±standard deviation from the mean.)

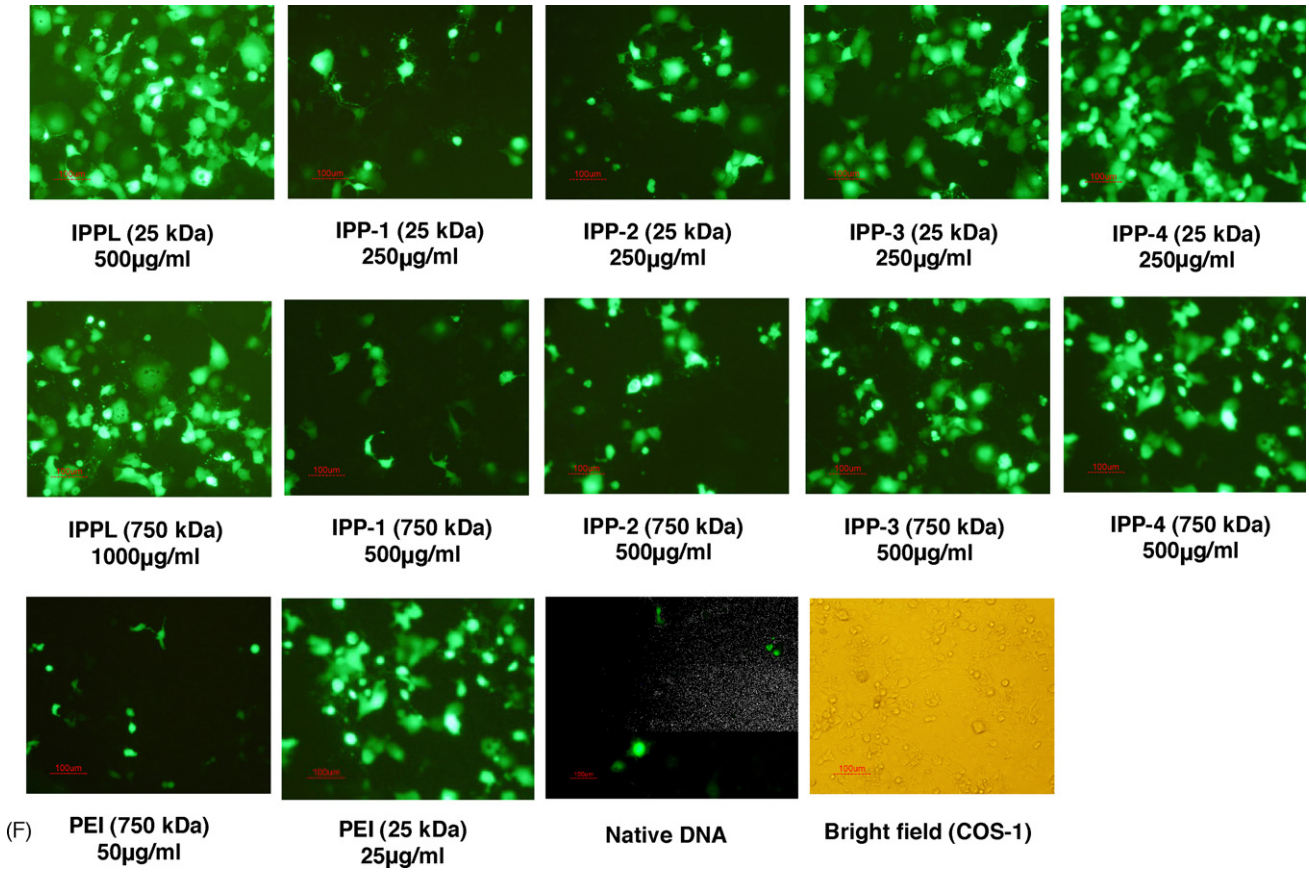


Fig. 6. (*Continued*).

less accessible for electrostatic interaction with DNA and consequently loose complexes are formed with lesser degrees of DNA condensation, which provides a better opportunity for the release of DNA in the intracellular milieu. A recent report also suggests that a polycation's high affinity for DNA may be a limiting step for successful transfection due to the difficulty in release of DNA from the gene carrier itself (Chen et al., 2005).

The cytotoxicity of IPP nanoparticles decreased with increasing imidazolyl content. The trend is clearly reflected in the IC_{50} values, where the cells can tolerate greater amounts of substituted PEIs as the degree of imidazolyl substitution was increased (Fig. 5B and Table 3). The incorporation of lauryl moiety further decreased the toxicity with a remarkable cell viability of >80%. The study on transfection efficiency was accomplished by measuring the fluorescence intensity of reporter gene encoding green fluorescent protein. The nanoparticles were able to mediate successful transfection in COS-1 and HEK293 cell lines. Transfection with reporter gene plasmids using these nanoparticles resulted in transfection profile, as shown in Figs. 6 and 7. Transfection with PEI alone resulted in minimal levels of GFP fluorescence intensity regardless of nanoparticle/DNA ratio. GFP expression increased as nanoparticle/DNA ratio and the imidazolyl substitution were increased in a non-linear relationship. IPPL nanoparticles mediated the maximum level of gene expression amongst all the members of the series synthesized. Current study indicates the role of

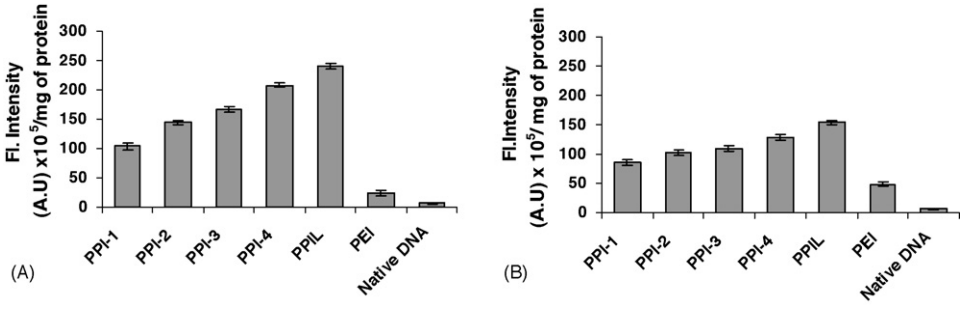
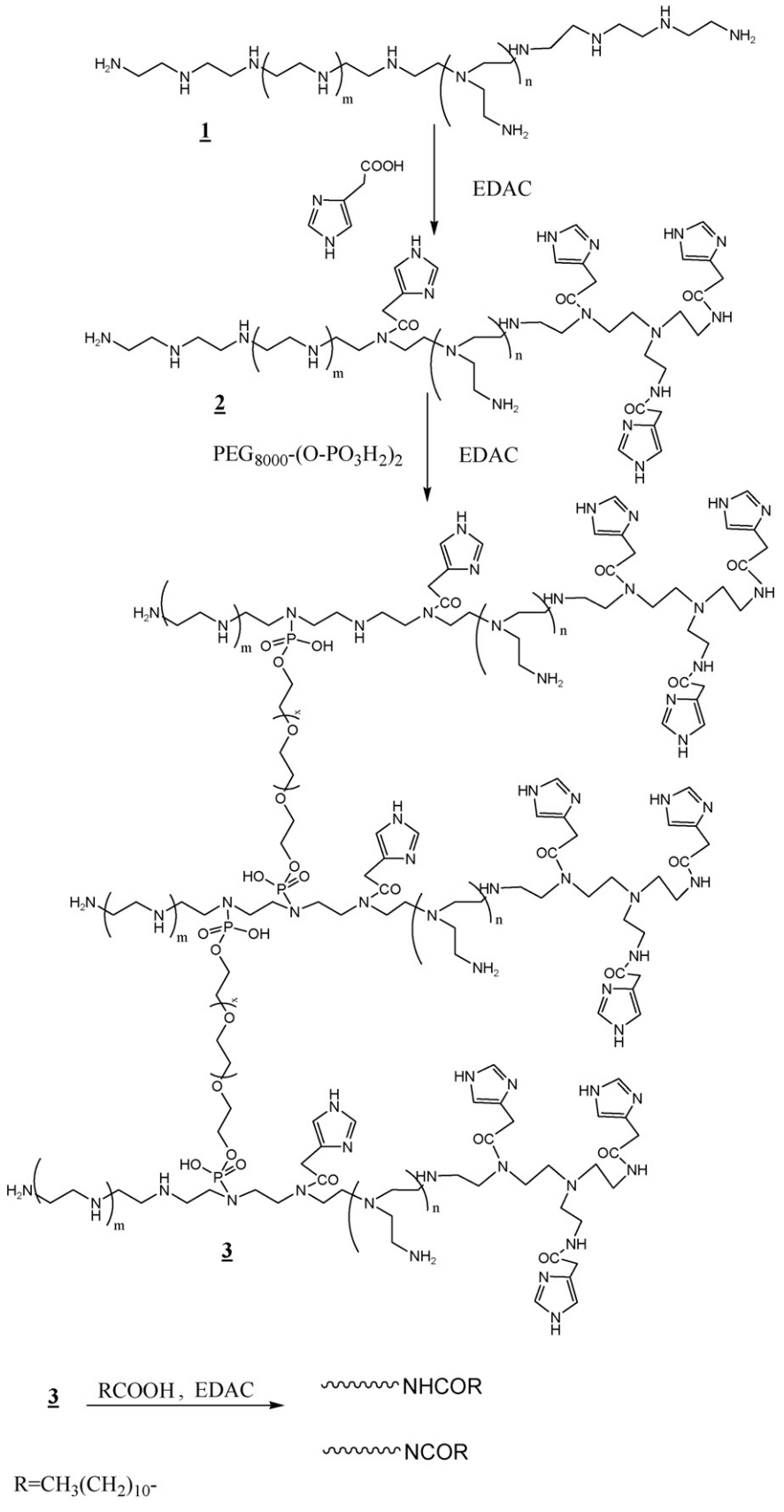


Fig. 7. Maximum transfection efficiency of IPP/DNA and IPPL/DNA complexes on HEK293 cell line: (A) 750 kDa series and (B) 25 kDa series. N/P ratios are indicated in Table 2. (Error bars represent ±standard deviation from the mean.)



Scheme 1. Schematic representation of preparation of IPP and IPPL nanoparticles.

fatty acid (hydrophobicity) in transfection and future efforts are directed to optimize the concentration of fatty acid in the nanoparticles for maximum efficiency and minimum toxicity. At optimal transfection ratio, the net surface charge on the nanoparticle/DNA complexes was positive and hydrodynamic diameter was in the range of 185–295 nm for both the series of IPP and IPPL nanoparticles (Table 2). The lauric acid has been grafted on the pre-formed nanoparticles of imidazolyl-PEI polymer, cross-linked with polyethyleneglycol-bis(phosphate). This would increase the size of the nanoparticles, because the lauryl substitutions hang on the surface of already well-defined nanoparticles. Such observation was also recorded earlier (Lee et al., 2002). The size of nanoparticle/DNA complexes decreased by nearly 30% in the presence of serum, which is in accordance with earlier results. The observed feature may be a consequence of absorption of anionic serum proteins on the nanoparticle/DNA complex surface, thereby, segregating the particles to greater extent. The global charge of serum-stabilized nanoparticle/DNA complexes was also reversed in polarity, substantiating the above hypothesis (Pichon et al., 2001; Ogris et al., 1998). The high N/P ratio resulting in optimal transfection appears to be a consequence of DNA loading on the highly compact nanoparticles, where some of the amino functionalities may be buried and not available for interactions with DNA. Another interesting feature is that though the N/P ratios are very high in nanoparticle/DNA complexes, yet the zeta potentials are lower than those of PEI/DNA ones. This also suggests that DNA binding to the surface of nanoparticles follows a different mode of complexation than is observed for PEI. High N/P ratios have been earlier reported in literature in context of modified PEI–DNA complexes (Jung et al., 2004; Bieber et al., 2002). Collectively, imidazole and lauric acid substitutions enabled the efficient delivery of DNA with efficiencies superior to PEI and reduced cytotoxicity to a minimal level. Similarly, imidazole conjugation in PLL has also been shown to mitigate the toxicity of the polymer and enhance the transfection efficiency (Chen et al., 2005).

5. Conclusions

In spite of several efforts towards development of an ideal gene delivery system, research is still moving around cellular entry, endosomal escape, cytoplasmic transport and uptake by nucleus. The study shows that by modification of PEI with imidazole, the number of protonable amines at neutral pH as well as the capacity to interact with DNA decreases; however, the transfection efficiency increases many fold. Answer to this may lie in the remarkable decrease in cytotoxicity of the system and more efficient unpacking of the nanoparticle/DNA complexes within the cells. Improved transfection efficiency may be a consequence of reduction in charge while maintaining the buffering capacity. These results suggest that imidazole and lauric acid substituted PEIs serve as better transfection reagents compared to PEI. The study reaffirms the importance of DNA packaging and hydrophobicity in designing biocompatible cationic polymer-based gene carriers. Outcome of the experiments are promising and may be suitable to use the proposed systems *in vivo*.

Acknowledgements

We wish to thank Indian Institute of Technology, New Delhi and Central Drug Research Institute, Lucknow for their assistance in NMR. Authors are also grateful to Ambedkar Center for Biomedical Research, University of Delhi, Delhi for their help in IR analysis. Financial supports received from Council of Scientific and Industrial Research (CSIR), New Delhi and Lady Tata Memorial Trust (LTMT), Mumbai are duly acknowledged.

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