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PAPER

1,4-Butanediol diglycidyl ether (BDE)-crosslinked PEI-*g*-imidazole nanoparticles as nucleic acid-carriers *in vitro* and *in vivo*†

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Previously reported 1,4-butanediol diglycidyl ether (BDE) crosslinked PEI (branched polyethylenimine, 25 k) nanoparticles (A. Swami, R. Kurupati, A. Pathak, Y. Singh, P. Kumar and K. C. Gupta, A unique and highly efficient non-viral DNA/siRNA delivery system based on PEI-bisepoxide nanoparticles, *Biochem. Biophys. Res. Commun.*, 2007, **362**, 835–841) (PN NPs) were reacted with varying proportions of a novel linker, 2-(*N*-1-tritylimidazol-4-yl)-*N*-(6-glycidyloxyhexyl)-acetamide (IGA linker, **3**), to yield PN-*g*-imidazolyl nanoparticles (PNIm) with improved transfection efficiency. Here, the IGA linker (**3**) reacted through an epoxy ring to partially convert the residual 1° and 2° amines present in PN NPs to 2° and 3°, respectively, without altering the total number of amines and additionally incorporating the delocalized positive charge of the imidazolyl moiety. The resulting particles were characterized for their size, zeta potential and DNA complexing ability. PNIm/DNA nanoplexes, in the size range of 120–400 nm, were evaluated for transfection efficiency in HeLa, HEK293 and CHO cell lines, which was found to be ~11, ~2–3 and ~2–17 folds higher than PEI, PN-2 (the best working sample of the PN series) (A. Swami, R. Kurupati, A. Pathak, Y. Singh, P. Kumar and K. C. Gupta, A unique and highly efficient non-viral DNA/siRNA delivery system based on PEI-bisepoxide nanoparticles, *Biochem. Biophys. Res. Commun.*, 2007, **362**, 835–841) and commercial transfection reagents tested in this study, respectively. Also, flow cytometric analysis showed ~78% (*ca.* ~43% in PN-2) cells transfected with the PNIm 10(6)/DNA complex (the best working sample of the PNIm series) in HEK293 cells. Transfection of GFP specific siRNA in HEK293 cells suppressed the gene expression by ~90% (*ca.* ~70% in PN-2). All the cell lines treated with PNIm/DNA nanoplexes showed >90% viability. *In vivo* gene expression of luciferase enzyme in Balb/c mice showed highest expression in spleen after seven days.

Introduction

Non-viral vectors have emerged as promising candidates for the treatment of genetic diseases.[2–4] However, a challenge still remains where these vectors can be employed for *in vivo* therapeutic applications.[5] Over the past few decades, PEI based delivery systems have shown potential as transfection agents.[6–8] However, polymer associated cytotoxicity limits its applications *in vivo*.[9,10] Reports indicate that higher charge density of PEI contributes to its cytotoxicity, which has been circumvented by acylation,[11] alkylation,[12] PEGylation,[13] coating with sugar moieties[14–16] and incorporation of imidazolyl groups.[17–19]

Earlier, we had crosslinked PEI with BDE to address the concern of cytotoxicity with concomitant improvement in the transfection efficiency.[1] The resulting nanoparticles (PN NPs) exhibited 2.5–5 folds higher transfection efficiency than native PEI and showed minimal cytotoxicity. In the present study, we have tried to further improve the transfection properties of PN-2 using a novel IGA linker (**3**), one end of which consists of an epoxy ring that would react partially with residual 1^0 and 2^0 amino groups in PN NPs and convert them into 2^0 and 3^0, respectively, thereby maintaining the overall number of amines intact. The other end of the linker bears an imidazole moiety, which would incorporate a delocalized charge (*i.e.* an endosomolytic agent) that would also help in improving the transfection efficiency of the resulting particles. As the beneficial role of imidazole in transfection is well documented,[17–19] its accumulation inside the endosomes causes an increase in buffering capacity and thereby results in the increase of transfection efficiency by several orders of magnitude.[17]

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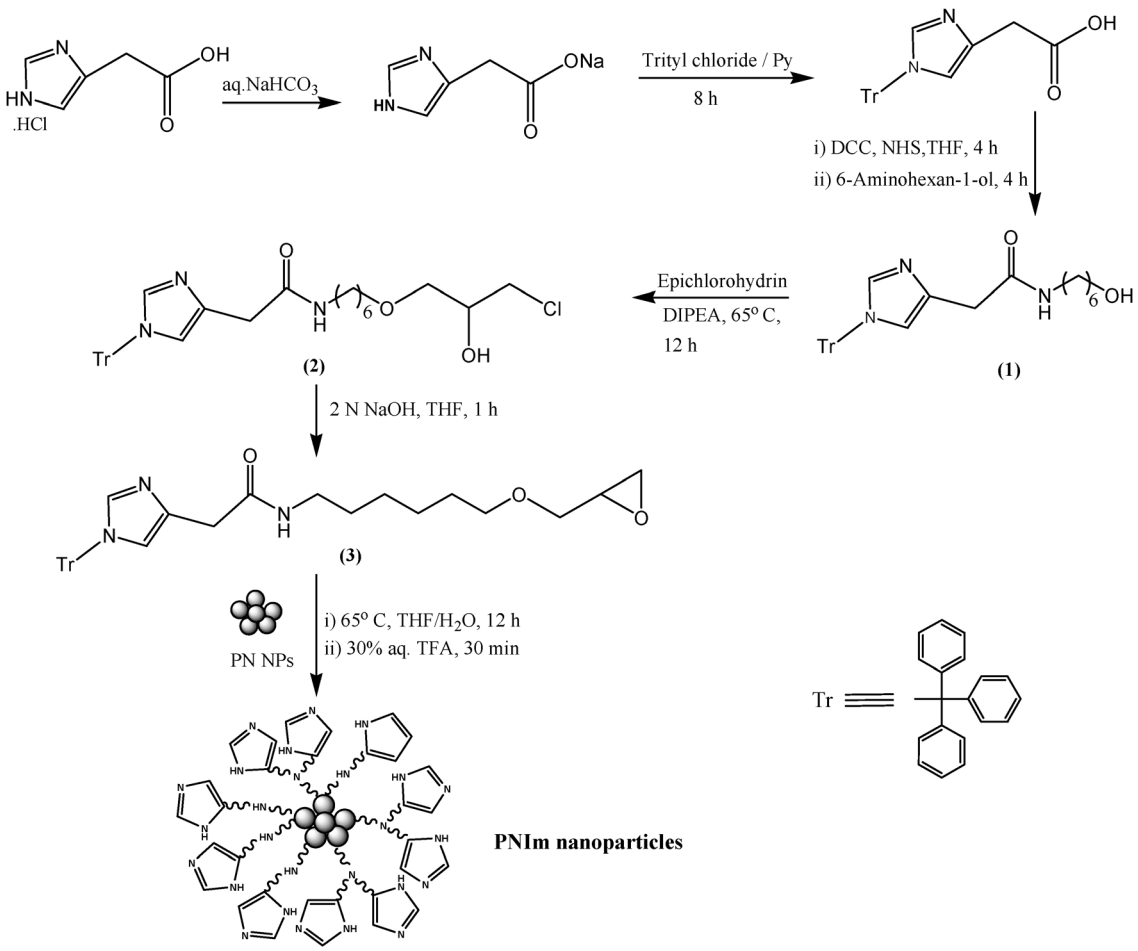
The synthesized PNIm NPs were evaluated in terms of their size, zeta potential, buffering capacity, cell viability, transfection efficiency, DNA release and *in vivo* gene expression. One of the formulations, PNIm 10(6), scored ~2–3 and ~11 folds higher transfection efficiency than PN-2 and PEI, respectively. *In vivo* gene expression studies revealed highest expression in spleen followed by heart and lungs. The PNIm NPs showed marked improvement over PN-2 NPs in terms of transfection and therefore, hold great potential as transfection agents.

Results and discussion

Preparation of PNIm particles

In a previous publication from our laboratory, PEI was converted into nanoparticles by crosslinking with increasing amounts of BDE to synthesize a series of NPs (PN-1 to PN-4) and evaluated them in terms of transfection efficiency and cell viability properties.[1] It was observed that cell viability increased on increasing the percentage of crosslinking following the trend PN-1 < PN-2 < PN-3 < PN-4, and transfection efficiency increased upto PN-2 and then started decreasing on deviating from this percentage. Here, in order to further improve the cell viability of PN-2 (the best working system of the PN series) comparable to PN-3/PN-4 without compromising on the transfection efficiency, we have now designed a unique linker, which contains an epoxy group at one end, that could partially convert the residual 1° and 2° amines to 2° and 3° (*i.e.* bad charge of 1° amines to good charge of 2° amines), thereby improving the cell viability (comparable to PN-3 or PN-4). The selection of a group for the other end was done carefully, keeping the following points in mind, *viz.*, (i) it should help in improving the transfection efficiency of the modified NPs, (ii) it should not adversely affect the cytotoxicity of the system, (iii) it should not involve time consuming synthesis, and (iv) the required reagents should be commonly available. Having gone through the literature and keeping these points into consideration, we decided to have an imidazole moiety, which is known for its beneficiary properties in enhancing the transfection efficiency of the cationic polymers,[19] on the other end of the linker (Scheme 1). This linker (**3**), prepared from imidazole-4-acetic acid hydrochloride, was reacted with the preformed PN NPs (Scheme 1) in increasing percentage and a series (PNIm) was synthesized, which was evaluated in terms of transfection efficiency and cytotoxicity. The best working sample of the series, PNIm 10(6), dramatically enhanced the transfection efficiency over PEI (~10–11 folds), PN-2 (~2–3 folds) and commercial transfection reagents (~2–17 folds), which indicated that the



Scheme 1 Schematic representation of preparation of IGA linker (**3**) and PNIm nanoparticles.

projected linker not only improved the cell viability (comparable to PN-3/PN-4) of the grafted NPs but also enhanced the transfection efficiency by improving the endosomolytic properties of the resulting polymeric NPs.

Characterization of PNIm particles

The synthesized PNIm series was characterized by various techniques, *viz*., ^{1}H-NMR, IR, DLS and Zeta potential. The percentage grafting of linker (**3**) was analyzed on the basis of ^{1}H-NMR (Table 1). The peaks at δ 6.8 and 7.4 confirmed the presence of an imidazole moiety in the synthesized PNIm series. In IR, the peaks at 1620 cm^{-1} (amide stretching) and 1130 cm^{-1} (ether twisting) also confirmed the presence of linker (**3**) in the nanoparticles.

The size of PN NPs after grafting with (**3**) increased, although remained in the nano range (data not shown), as determined using DLS. This increasing trend in size also confirmed the grafting of the IGA linker on the pre-formed nanoparticles, which might be acting as a hanging pendant. The pDNA complexes of PNIm particles exhibited size in the range of 120–400 nm (Table 2). Further, in the presence of 10% FBS, the sizes decreased (49–212 nm), which might be due to adsorption of water molecules from the cationic surfaces by the serum proteins leading to dehydration of the particles.[20] The zeta potential of nanoparticles was found to increase with increase in percentage grafting of (**3**), confirming the conjugation of the same with PN NPs. On complexation with pDNA (at a w/w ratio of 1), the zeta potential decreased, which might be due to neutralization of some positive charge with the negatively charged phosphate backbone of DNA. However, in 10% FBS, the zeta potential became negative the reason for which is well documented in the literature.[20]

Table 1 Percent grafting of imidazole on PN NPs as estimated by ^{1}H-NMR spectroscopy

	PNIm	Attempted substitution of the IGA linker (%)	Found substitution of the IGA linker by ^{1}H-NMR (%)
PN-1	PNIm 5(2)	2	0.65
	PNIm 5(4)	4	1.25
	PNIm 5(6)	6	1.74
	PNIm 5(8)	8	2.11
PN-2	PNIm 10(2)	2	0.62
	PNIm 10(4)	4	1.19
	PNIm 10(6)	6	1.69
	PNIm 10(8)	8	2.07
PN-3	PNIm 15(2)	2	0.66
	PNIm 15(4)	4	1.29
	PNIm 15(6)	6	1.79
	PNIm 15(8)	8	2.12

DNA retardation assay

The amines present in nanoparticles interact with the negatively charged phosphate backbone of DNA electrostatically. This property is used to see the effect of nanoparticles in retarding the mobility of a fixed amount of pDNA on agarose gels. To determine the amount of nanoparticles needed to retard the mobility of a fixed amount (0.3 μg) of pDNA, the complexes were prepared at increasing w/w ratios of nanoparticle : DNA and loaded onto 1% agarose gel. PNIm particles retarded at w/w ratios of 0.33 or 0.66, while PEI retarded the mobility of the same amount of pDNA (0.3 μg) at a w/w ratio of 1. PN NPs retarded the mobility of DNA (0.3 μg) at a higher w/w ratio than PEI (Fig. 1).[1] These observations show that a lesser amount of PNIm particles and more amount of PN NPs are required in comparison to PEI in retarding the fixed amount of DNA. The reason may be attributed to the presence of extra amines available for binding with DNA in the imidazole ring in PNIm while conversely, in the case of PN NPs, some amount of charge is embedded inside the NPs, so a higher amount is required for retardation.

Cell viability assay

The synthesized PNIm series was evaluated in terms of cell viability using the MTT assay in three different cell lines, *viz*., HEK293, HeLa and CHO. The PNIm series was found to be almost non-toxic (cell viability ranged between 90–98%) in all the cell lines tested. However, PEI and Lipofectamine™ showed a significantly reduced cell viability (ranging between 45–60% in all the cell lines tested; $P < 0.01$) (Fig. S1, ESI†). For the commercial reagents, the cell viability was found to be in the range of 80–82% (Superfect™) and 87–92% (GenePORTER 2™). PN-2 showed the cell viability ranging between 84–91% in the cell lines tested in this study.

Table 2 Particle size and zeta potential measurements of PNIm particles and their corresponding DNA complexes in water and serum at a w/w ratio of 1 : 1 for PNIm particles and PEI

	Size ± SD (PDI)/nm		Zeta potential ± SD/mV		
	With DNA	With serum	Native particle	With DNA	With serum
PNIm 5(2)	396.0 ± 3.78 (0.165)	211.1 ± 4.61 (0.271)	30.6 ± 2.17	15.9 ± 1.83	−23.0 ± 1.07
PNIm 5(4)	386.7 ± 4.29 (0.276)	105.2 ± 2.91 (0.153)	33.9 ± 1.98	17.8 ± 1.12	−19.3 ± 1.67
PNIm 5(6)	330.0 ± 3.10 (0.292)	100.4 ± 3.79 (0.189)	36.4 ± 1.76	17.8 ± 1.95	−19.2 ± 1.92
PNIm 5(8)	321.1 ± 5.92 (0.189)	98.4 ± 4.76 (0.297)	40.6 ± 3.48	17.9 ± 2.63	−17.5 ± 2.72
PNIm 10(2)	280.0 ± 2.05 (0.156)	95.5 ± 5.39 (0.129)	29.2 ± 2.63	16.6 ± 1.98	−20.3 ± 2.02
PNIm 10(4)	248.6 ± 7.29 (0.208)	76.5 ± 2.07 (0.275)	30.2 ± 3.73	16.7 ± 2.76	−17.4 ± 2.83
PNIm 10(6)	226.8 ± 6.20 (0.143)	68.4 ± 3.87 (0.128)	32.4 ± 3.79	19.6 ± 3.65	−16.9 ± 2.67
PNIm 10(8)	202.1 ± 4.95 (0.187)	59.4 ± 3.92 (0.274)	36.4 ± 3.92	20.6 ± 2.93	−16.4 ± 1.29
PNIm 15(2)	169.4 ± 6.20 (0.387)	54.0 ± 4.73 (0.186)	26.4 ± 3.72	17.2 ± 1.98	−20.9 ± 2.90
PNIm 15(4)	168.0 ± 3.87 (0.170)	53.5 ± 4.93 (0.252)	28.3 ± 2.61	19.5 ± 2.75	−14.2 ± 1.83
PNIm 15(6)	148.2 ± 4.76 (0.186)	50.1 ± 4.27 (0.127)	29.6 ± 2.02	20.5 ± 3.81	−14.2 ± 1.28
PNIm 15(8)	123.5 ± 6.38 (0.241)	49.1 ± 3.68 (0.138)	32.8 ± 2.81	21.5 ± 2.72	−14.0 ± 1.29
PEI	287.8 ± 2.97 (0.675)	125.6 ± 2.95 (0.489)	31.8 ± 2.90	21.4 ± 2.81	−13.6 ± 1.98

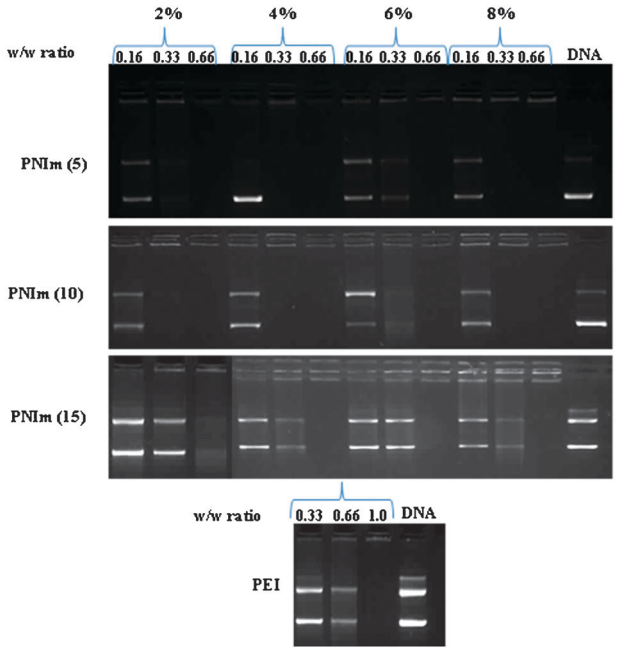


Fig. 1 DNA retardation assay of PNIm/DNA and PEI/DNA complexes. pDNA (0.3 μg) was incubated with increasing amounts of nanoparticles in 5% dextrose for 20 min. Samples were electrophoresed in a 1% agarose gel at 100 V for 45 min.

Therefore, it is evident from the figure that after grafting with the IGA linker (**3**), the cell viability improved in comparison to crosslinked nanoparticles (PN-2). The possible reasons for the increased cell viability of PNIm particles over PN-2 may be (i) further reduction in the number of toxic primary amino groups, and (ii) the presence of an imidazole moiety, as it is reported that a protonated imidazole ring is less cytotoxic than protonated amines.[17]

Buffering capacity

PEI exhibits a considerable buffering capacity in the pH range of endosomes and lysosomes,[21,22] however, displays considerable toxicity due to the presence of primary amines.[10] Therefore, PEI was crosslinked using BDE to result in well defined nanoparticles with reduced toxicity.[1] This process also enhanced the transfection efficiency of the PEI by several folds maintaining the buffering capacity of the resulting nanoparticles to the level of PEI. In this study, we have tried to enhance the buffering capacity of PN NPs by grafting the imidazole moiety on these nanoparticles through an epoxy linker (**3**).

The imidazole ring helps in the proper binding with DNA and also in the escape of pDNA from endosomes.[17] Due to the protonation tendency of the imidazole ring at pH 6.0, it is a weak base and, therefore, has been found to be suitable to modify the surface of cationic polymers.[17] To see the effect of imidazole grafting on the buffering capacity of PNIm NPs, an acid–base titration was performed in the pH range 3–10. Fig. S2 (ESI†) shows the buffering capacity of PNIm particles, PN-2 NPs and PEI under different pH conditions. It was found that the proton capturing tendency of the PNIm particles was slightly enhanced as compared to native PEI and PN-2 NPs. The enhanced buffering capacity of the PNIm series may be due to the presence of an imidazole moiety as this moiety is well documented in the literature to be a good buffering provider in the pH range of endosomes. We, therefore, analyzed the transfection efficiency of these particles. Interestingly, the transfection efficiency initially increased with increase in buffering capacity but showed a decreasing trend after attaining a maximum efficiency. This increase in buffering may be responsible for the enhanced transfection efficiency of the proposed nanoparticles.

In vitro transfection studies

Transfection efficiency of PNIm and PN-2 nanoplexes, PEI polyplexes and DNA complexes of Superfect™, GenePORTER 2™ and Lipofectamine™ was evaluated in HEK293, CHO and HeLa cells using EGFP (Enhanced Green Fluorescent Protein) as a reporter gene in the presence and absence of serum. The study was performed at various w : w ratios (0.33, 0.66, 1.0, 1.66, 2.33). It was found that PNIm 10(6) performed the best in terms of transfection efficiency and scored ~10.4, ~11.6 and ~12.7 folds higher in HEK293, CHO and HeLa cells, respectively, as compared to PEI. Also, the transfection efficiency of PNIm 10(6) was ~2.3, ~3.0, and ~2.0 folds higher in HEK293, CHO and HeLa cells, respectively, than PN-2 (Fig. 2a). Moreover, in the presence of 10% serum, the transfection efficiency did not reduce significantly ($P > 0.05$) (Fig. 2b), which further implicates the potential of modified nanoparticles for *in vivo* gene delivery applications.

Flow cytometry was employed to evaluate the reporter gene expression at the individual cell level. PNIm 10(6) showed maximum transfection efficiency, *i.e.* 78 ± 4.8 and 72 ± 3.9% in HEK293 and CHO cells, respectively. In contrast, PEI showed only 23 ± 2.3% and 20 ± 3.4% GFP positive cells in HEK293 and CHO cells, respectively (Fig. 3). Also, PN-2 showed 48 ± 5.3% in HEK293 and 45 ± 2.7% GFP positive cells in CHO cells. PEI and PNIm particles worked best at a w : w ratio of 1 : 1 (NP : DNA) while PN-2 worked best at 1.66 : 1. This might be due to the presence of an imidazole moiety, which provided an extra delocalized charge to bind pDNA. One of the plausible explanations for the enhanced transfection efficiency may be attributed to the enhanced buffering of PNIm nanoparticles in the pH range 3–10 due to the imidazole group. Secondly, PNIm particles are almost non-toxic (see Cell viability assay), which may further account for the enhanced transfection efficiency.

DNA release assay

The DNA carrier after carrying the desired therapeutic inside the cell must be able to release it to facilitate efficient transfection.[23] The extent of pDNA binding with the series of PNIm 10 nanoparticles was evaluated and compared with the release pattern of PEI. A fixed amount of PNIm 10 nanoparticle/DNA and PEI/pDNA complexes were incubated with the increasing units of heparin, a competitive anionic moiety, and the samples run on 1.0% agarose gel. On quantification by densitometry, it was observed that on descending the series (PNIm 10), the binding with DNA became stronger. With 5 U of heparin, the amount of pDNA released from

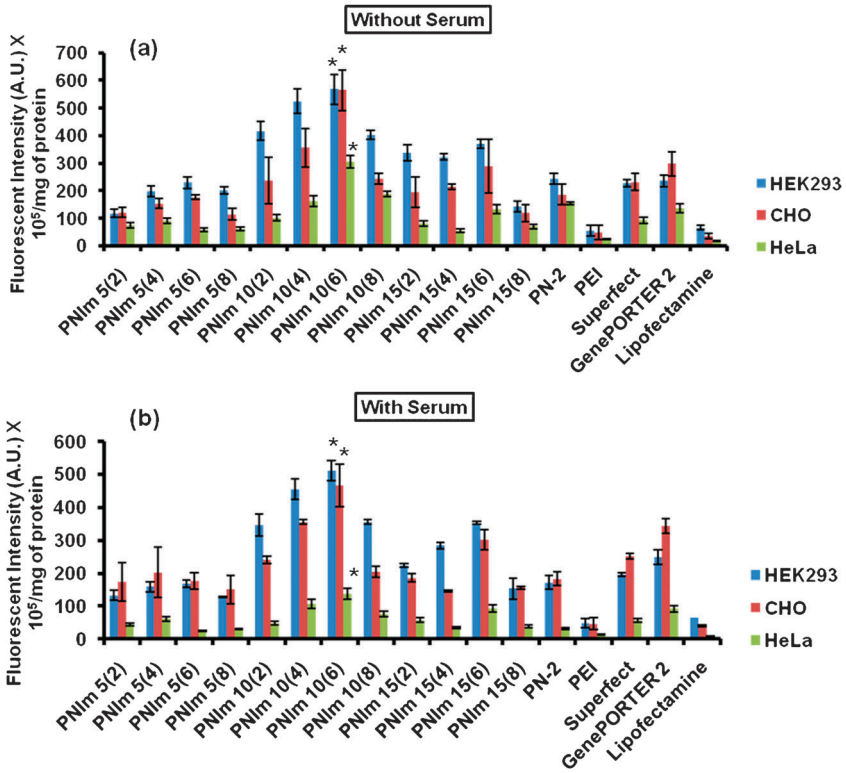


Fig. 2 GFP fluorescence intensity in HEK293, CHO and HeLa cells in the (a) absence of serum, and (b) presence of serum, transfected with PNIm/DNA, PEI/DNA, Superfect™/DNA, GenePORTER 2™/DNA and Lipofectamine™/DNA complexes. The transfection profiles show fluorescence intensity expressed in terms of arbitrary units per mg of total cellular protein obtained at a w/w ratio of 1 for PNIm/DNA and PEI/DNA polyplexes. Cells were incubated with the complexes 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 a spectrofluorometer. The results represent the mean of three independent experiments performed in triplicates. $*P < 0.05$ *vs.* PEI and Lipofectamine.

complexes decreased from 60 to 38% on increasing the percent of IGA linker (**3**), while under the same conditions, PEI released only 19% of bound DNA. Further, on increasing the heparin units (10 U), PNIm 10(2) and PNIm 10(6) released upto 98% and 75% of pDNA, respectively, while PEI released only 55% of it (Fig. 4). These results suggest that PEI binds very strongly to DNA, which may be due to the presence of a high amount of cationic charge (1° amines). In PNIm particles, some amount of charge is probably embedded inside the particles, which is not available to bind with DNA, and hence a comparatively loose complex is formed. Moreover, in a recent report, it has been demonstrated that transfection efficiency depends on the pDNA binding ability of NPs.[24] The synthesized nanoparticles not only carried the bound DNA efficiently to inside of the cell but also released it in sufficient amount in the cellular milieu to result in enhanced gene expression.

Intracellular localization of nanoplexes

The intracellular localization of DNA is an important factor for successful gene therapy. To determine the intracellular localization of PNIm 10(6)/pDNA nanoplexes after cellular uptake, HeLa cells were incubated with tetramethylrhodamine-labeled PNIm 10(6) nanoparticles (red fluorescence) complexed with YOYO-1 labeled DNA (green fluorescence) and observed by confocal laser scanning microscopy. DAPI (blue) was used to stain the nucleus. Within 30 min of the incubation, red and green particles were seen in the cells near the plasma membrane, and in the overlaid images yellow fluorescence was observed. Red and green fluorescence was also observed inside the cytoplasm and nucleus, which indicated the dissociation of the complex within the cytoplasm or nucleus. PNIm 10(6) was found to carry pDNA inside the cytoplasm within 1 h and to the nucleus within 2 h of the addition of complexes to the cells (Fig. 5). The nanoplexes were localized not only in the cytoplasm but also in the nucleus. This observation is in agreement with a previously reported study where PEI delivers nucleic acids to the nucleus.[25] The finding clearly demonstrates efficient intracellular delivery of DNA using PNIm 10(6).

Delivery of siRNA

RNA interference is a technology that allows fully or partially suppressing the expression of a specific gene, allowing targeted gene knockout and gene knockdown.[26,27] As PNIm 10(6) efficiently transfected pGFPDNA into the cell, its ability to knockdown this gene was evaluated using GFP specific siRNA in HEK293 cells and the results were compared with GenePORTER 2™. The cells were transfected first with the PNIm 10(6)/GFPDNA complex and after 3 h (the optimum time for cellular entry; see Confocal study)

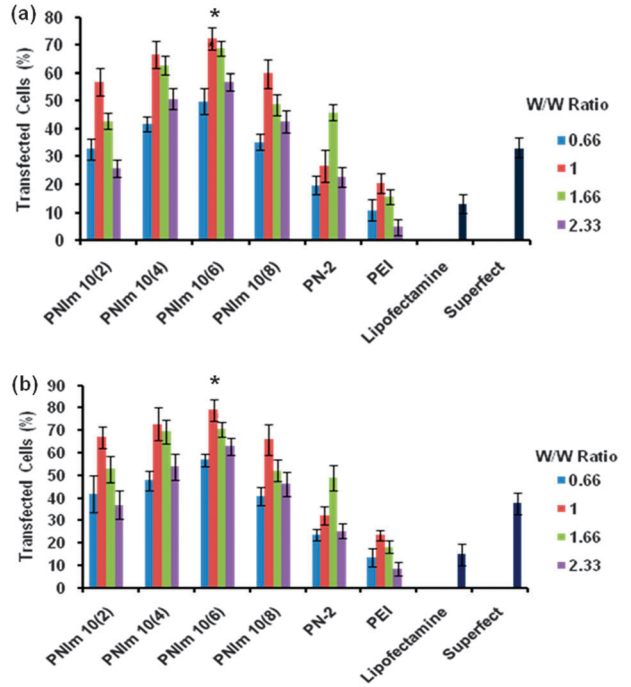


Fig. 3 Percent transfection efficiency of PNIm 10/DNA complexes determined using FACS in (a) CHO and (b) HEK293 at various w/w ratios compared with PN-2, PEI, Superfect™ and Lipofectamine™. $*P < 0.05$ *vs.* PEI and Lipofectamine.

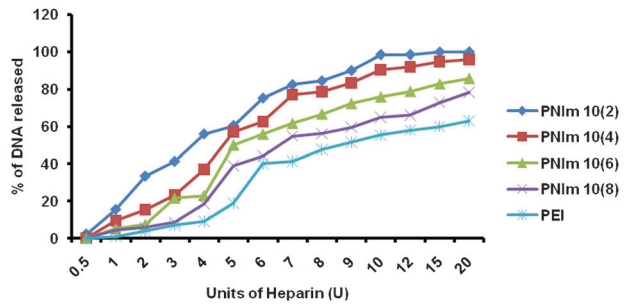


Fig. 4 DNA release assay of the PNIm 10 series and PEI. To a 20 μl of the PNIm/DNA nanoplex, heparin, in increasing concentration, was added and incubated for 20 min at 25 ± 1 °C. The samples were run on a 1% agarose gel at 100 V for 45 min. Error bars represent +/− standard deviation from the mean.

PNIm 10(6)/GFPsiRNA was added. After 36 h of treatment with the siRNA, the observed knockdown of GFP expression by PNIm 10(6)/siRNA formulation was ~90.2% (*ca.* 70% for PN-2)[1] (Fig. 6). In contrast, GenePORTER 2™/siRNA formulations knocked down GFP expression by ~53.2% only (Fig. 6). Hence, the delivery of GFP-specific siRNA oligonucleotides by selected nanoparticle formulation clearly down-regulated the GFP gene more efficiently than GenePORTER 2™. Therefore, the projected nanoparticles may serve as an effective carrier for the delivery of siRNA as well.

DNase I protection assay

An efficient gene delivery system is the one which provides sufficient protection to the gene of interest against nucleases present in the cellular environment from degradation.[28] The PNIm 10(6)/pDNA formulation and pDNA alone were incubated with DNase I for different time intervals (0.25, 0.50, 1 and 2 h) and run in 1% agarose gel. Quantitative analysis of the gel showed that in contrast to the free DNA (0.3 μg), which was degraded completely by DNase I within 15 min, PNIm 10(6) effectively protected bound DNA (Fig. 7) and only 15% of it was found to be degraded even after 2 h of treatment. Thus, the projected nanoparticles are suitable for *in vivo* administration of pDNA.

In vivo gene expression studies

In vivo transfection efficiency of the complexed PNIm 10(6) was examined in Balb/c male mice by luciferase activity in all the vital organs of the organism 7 d post intravenous injection. A significant ($P < 0.05$) increase in luciferase activity was observed in spleen and heart in PNIm 10(6) polyplexes compared to unmodified PEI (Fig. 8). Naked DNA performed least out of all the three tested. PEI also showed highest gene expression in the spleen for which the mechanism is still not clear.

Methods

Cell culture and materials

HEK293, HeLa and CHO cells were procured from NCCS, Pune, India. Cell cultures were maintained (37 °C, 5% CO_2) in Dulbecco's Modified Eagle's Culture Medium (DMEM) (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Life Technologies, UK) and 1% antibiotic cocktail of streptomycin and penicillin (Sigma, USA). Branched PEI (M_w 25k), tetramethylrhodamine isothiocyanate (TRITC), Bradford reagent, 4′,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), high retention dialysis tubing (cut off 12k), *N*-hydroxysuccinimide (NHS) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were procured from Sigma-Aldrich Chemical Co., USA. Standard transfection agents, Superfect™, Lipofectamine™ and GenePORTER 2™ were procured from Qiagen, France; Invitrogen, USA; and Genlantis, USA, respectively. YOYO-1 iodide was purchased from Invitrogen, USA, and plasmid purification kit and the plasmid pEGFPN3 were obtained from Qiagen, France, and Clontech, USA, respectively. Infrared spectra of nanoparticles 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 was recorded on a Bruker Avance 400 MHz instrument. Size and zeta potential measurements of the projected nanoparticles were carried out on Zetasizer Nano-ZS (Malvern Instruments, UK). Green fluorescent protein (GFP) in transfected cells was assayed (λ_{ex}: 488 nm; λ_{em}: 509 nm) on a NanoDrop™ ND-3300 spectrofluorometer (USA). The uptake and intracellular passage of nanoparticles/DNA complexes were monitored by confocal microscopy (LSM 510 Meta Zeiss, Germany). Fluorescence activated cell sorting (FACS) analysis was performed by Flow Cytometry (Guava® EasyCyte™

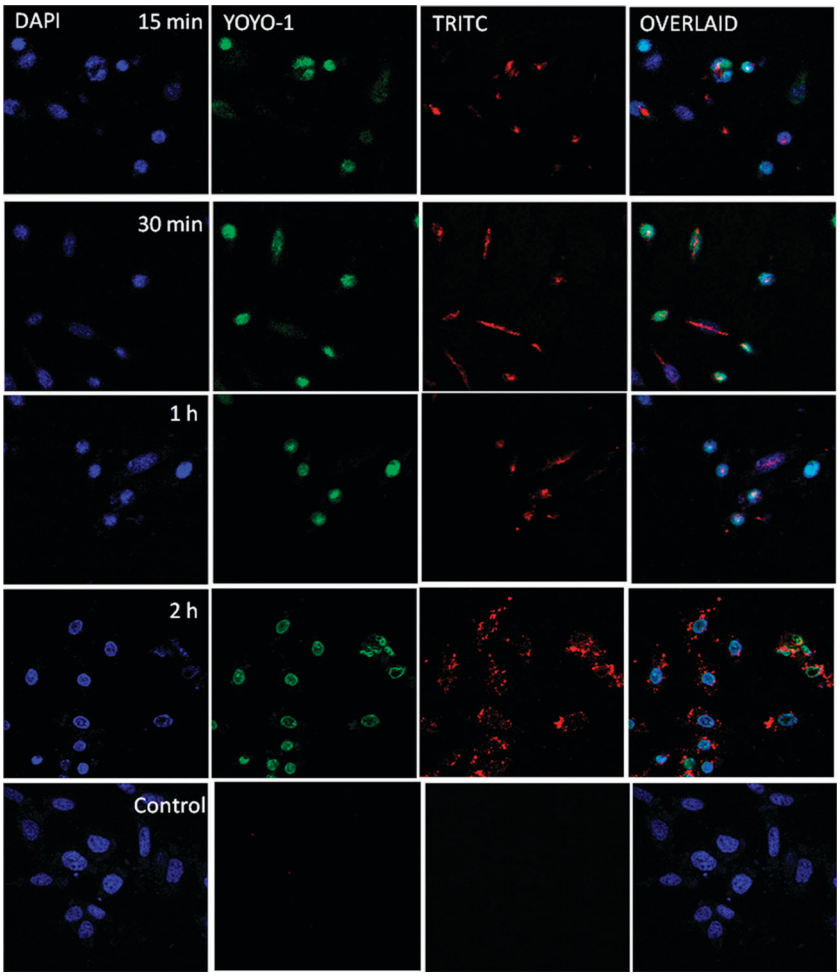


Fig. 5 Confocal microscopic images of HeLa cells treated with the tetramethylrhodamine–PNIm 10(6)/YOYO-1-pDNA nanoplex at different time points.

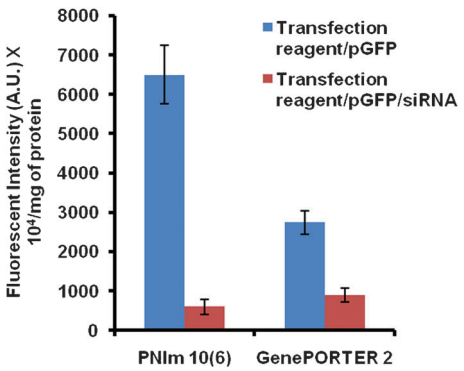


Fig. 6 siRNA delivery in HEK293. PNIm 10(6) mediated delivery of siRNA suppressed the expression of GFP in cells by more than 90% in comparison to GenePORTER 2™/pGFP DNA/siRNA, which suppressed GFP expression by only 53.2%, as monitored by measuring fluorescence on a spectrofluorometer. All the experiments were performed at least thrice and error bars represent the standard deviation.

Plus System, USA). Unless otherwise stated, milliQ water filtered through 0.22 μm sterile filters was used in the present study.

Animals

Six to seven week old male Balb/c mice (25 ± 3 g), from the animal breeding colony of Indian Institute of Toxicology Research (IITR), Lucknow, were acclimatized under standard laboratory conditions and given a commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water *ad libitum*. Animals were housed in plastic cages with rice husk bedding and maintained at 22 ± 2 °C with 12 h dark/light and 50–60% humidity as per rules laid down by the Animal Welfare Committee of IITR.

Synthesis of siRNA

The following oligonucleotide sequences were synthesized using phosphoramidite chemistry and purified on RP-HPLC:

T7 primer: d (TAA TAC GAC TCA CTA TAG)

GFP sense: d (ATG AAC TTC AGG GTC AGC TTG CTA TAG TGA GTC GTA TTA)

GFP antisense: d (CGG CAA GCT GAC CCT GAA GTT CTA TAG TGA GTC GTA TTA)

After annealing the T7 primer sequence to GFP sense or antisense oligonucleotides, complementary strands were synthesized by T7 RNA polymerase.[29] The sense and antisense

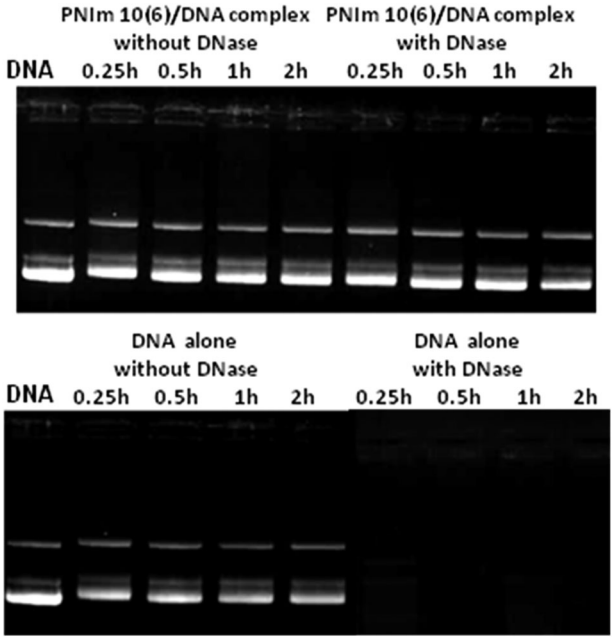


Fig. 7 DNase I protection assay. The PNIm 10(6)/DNA nanoplex (w/w ratio 1) was treated with DNase I for different time intervals. The complexed DNA was released by treating the samples with heparin. The amount of DNA protected (%) after DNase treatment was calculated as the relative integrated densitometry values (IDV) quantified and normalized by that of pDNA values (untreated with DNase I) using a Gel Documentation system (Syngene, UK).

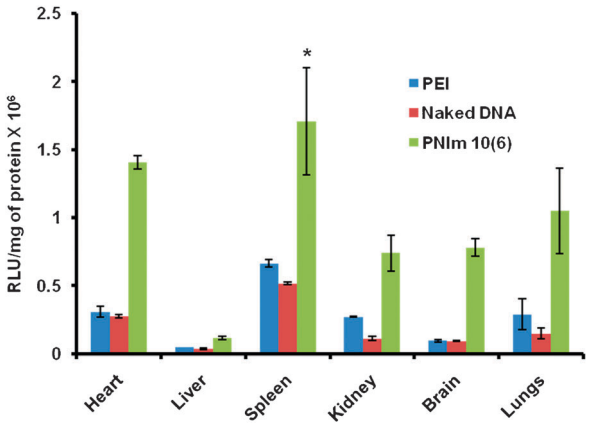


Fig. 8 *In vivo* gene expression analysis in Balb/c mice 7 d post intravenous injection using the pGL3 control vector as a reporter gene. The mice were sacrificed post 7 d injection and all the vital organs were dissected out. The organs were homogenized in a 1 × lysis buffer and the lysate was read on a luminometer to quantify the luciferase gene expression. $*P < 0.05$ *vs.* PEI and naked DNA in the respective organs.

RNA strands were annealed and double stranded siRNA was used for transfection.

Synthesis of IGA linker (3)

4-Imidazole acetic acid hydrochloride (5 mmol) was dissolved in water, $NaHCO_3$ (10 mmol) was added and the solution was stirred for ~1 h. The resulting sodium salt was dried in a vacuum desiccator and dissolved in dry THF (minimum amount). Trityl chloride (5.5 mmol), dissolved in dry pyridine (20 ml), was added to the reaction mixture and stirred for ~8 h at room temperature. Then, the reaction mass was concentrated on a rotary evaporator, dried completely to remove all traces of solvents and triturated with diethylether. The residual mass was dissolved in ethyl acetate (20 ml) and washed with a 5% aqueous solution of citric acid (2 × 10 ml). The organic phase was separated, dried over sodium sulfate, filtered and concentrated to obtain (*N*-1-tritylimidazol-4-yl) acetic acid in almost quantitative yield and characterized using LC-MS (*m*/*z*: 367, $M - H^+$).

To a solution of (*N*-1-tritylimidazol-4-yl) acetic acid (4 mmol) in dry THF (10 ml), DCC (4.4 mmol) and NHS (4.4 mmol) were added and the reaction mixture was stirred for 4 h at room temperature. 6-Aminohexan-1-ol (4.5 mmol) was then added to the above reaction mixture and stirring was continued for 4 h at room temperature. Subsequently, the reaction mixture was cooled to 4 °C and solid dicyclohexylurea was filtered, which was washed with THF (2 × 5 ml) and the combined filtrate was concentrated on a rotary evaporator. The syrupy residue, so obtained, was dissolved in ethyl acetate (20 ml) and washed successively with a 5% aqueous solution of citric acid (2 × 10 ml) and saturated brine (1 × 10 ml). The organic phase was separated, dried over sodium sulfate, filtered and concentrated to obtain 2-(*N*-1-tritylimidazol-4-yl)-*N*-(6-hydroxyhexyl) acetamide (**1**) in ~85% yield, which was characterized using LC-MS (*m*/*z*: 466, $M - H^+$).

Compound (**1**) (3 mmol) was suspended in epichlorohydrin (10 ml) and *N*,*N*′-diisopropylethylamine (DIPEA) (100 µl) was added. The resulting reaction mixture was stirred at 65 °C for overnight followed by removal of unreacted epichlorohydrin on a rotary evaporator to obtain a syrupy residue, which was dissolved in ethyl acetate (20 ml) and washed successively with a 5% aqueous solution of citric acid (2 × 10 ml) and saturated brine (2 × 5 ml). The organic phase was separated, dried over sodium sulfate, filtered and concentrated to obtain crude 2-(*N*-1-tritylimidazol-4-yl)-*N*-[6-(3-chloro-2-hydroxypropoxy) hexyl] acetamide (**2**), which was purified by silica gel column chromatography using the solvent system, ethylene dichloride : methanol (8 : 2, v/v), as an eluant. The fractions containing the desired compound were pooled together and concentrated to obtain (**2**) in ~80% yield, which was characterized using LC-MS (*m*/*z*: 558.5, $M - H^+$).

To a solution of compound (**2**) (2 mmol), dissolved in THF (10 ml), was added an aqueous solution of 2 N NaOH (1.5 ml) and the reaction mixture was stirred at 45 °C. After 1 h, the reaction mixture was diluted with water (10 ml) and THF was removed using a rotary evaporator. The desired compound was extracted in dichloromethane (5 × 10 ml), dried over sodium sulfate, filtered and concentrated to obtain IGA linker (**3**), which was characterized by its ^{1}H-NMR ($CDCl_3$): δ 1.2–1.5 (8H, 4 × –C**H**$_2$), 2.9–3.2 (5H, –C**H**–C**H**$_2$, –NH–C**H**$_2$), 3.75–3.85 (4H, 2 × –OC**H**$_2$), 3.4 (2H, –C**H**$_2$CO), 6.75 (1H, –C**H**–**N**–), 7–7.2 (15 H, Ar–**H**), 7.38 (1H, –N–C**H**–**N**–).

Synthesis of BDE crosslinked PEI-*g*-imidazole nanoparticles (PNIm)

PEI was crosslinked with varying amounts of BDE to generate PN-1 (5% crosslinking), PN-2 (10% crosslinking) and PN-3

(15% crosslinking) using a previously reported procedure from our laboratory.[1] The synthesized nanoparticles were grafted with 2, 4, 6 and 8% of IGA linker (**3**). Briefly, for 2% grafting, PN-1 (25 mg, 10 ml H_2O) was mixed with (**3**) (4.2 mg, 1 ml THF). The reaction mixture was stirred overnight at 65 °C and concentrated to obtain a syrupy residue, which was suspended in 30% aqueous trifluoroacetic acid (TFA, 10 ml). After stirring for 30 min at room temperature, the reaction volume was reduced to half on a rotary evaporator and the aqueous phase was washed with diethyl ether (2 × 5 ml). Subsequently, a saturated solution of sodium bicarbonate was added (~2 ml) to bring the pH of the solution to ~7.5 and subjected to dialysis against water with intermittent change of water for 2 d. The dialyzed solution was lyophilized to obtain PNIm 5(2), which was characterized by biophysical techniques. Similarly, the whole series was prepared and characterized using DLS, zeta potential, ^{1}H-NMR, IR and gel retardation assays.

DNA retardation assay

An aqueous solution of PEI/PNIm (1 mg ml^{-1}) was added to 1 µl of pDNA (0.3 µg µl^{-1}) at w/w ratios 0.16, 0.33, 0.66 and 1.0 to form PEI/DNA and PNIm/pDNA polyplexes and the final volume was made up to 20 µl with water. The resulting samples were gently vortexed and incubated at room temperature for 30 min. All the polyplexes and nanoplexes (20 µl) were mixed with 2 µl xylene cyanol (in 20% glycerol), electrophoresed (100 V, 1 h) in 1% agarose, stained with ethidium bromide and visualized on a UV transilluminator using a Gel Documentation system (Syngene, UK). From the gel, the amount of sample required for complete retardation of 0.3 µg of pDNA was obtained.

Physical characterization of nanoparticles

The hydrodynamic diameters of the PNIm series (1 mg ml^{-1}) and nanoplexes, suspended separately in water and 10% serum, were measured by DLS in triplicate. The data analysis was performed in automatic mode and measured sizes were presented as the average value of 20 runs.

Zeta potential measurements of nanoparticles and nanoplexes in water and 10% serum were carried out on a Zetasizer Nano ZS, carried out 30 runs in triplicate and the average values were estimated by the Smoluchowski approximation from the electrophoretic mobility.

Buffering capacity

The ability of PEI and PNIm (5, 10 and 15) series to resist change in pH was experimentally demonstrated by an acid titration assay following a method reported by Tseng *et al.*[30] A suspension of PNIm 5(2) (6 mg per 30 ml) in 0.1 N NaCl was adjusted to pH 10 with 0.1 N NaOH and then the pH was brought to 3.0 with 50 µl aliquots of 0.1 N HCl. pH values were recorded after each addition. The slope in the plot between pH and the amount of HC1 consumed indicates the intrinsic buffering capacity of the system. Similarly, the projected assay was repeated with the whole series.

Cell viability

The cell viability of PEI and PNIm/pDNA nanoplexes as well as Superfect™, GenePORTER 2™ and Lipofectamine™/pDNA complexes was evaluated by the MTT colorimetric assay. HEK293, CHO and HeLa cells were seeded in 96-well plates one day prior to performing the assay. Nanoparticle/pDNA nanoplexes were prepared by adding an aqueous solution of PNIm nanoparticles (1 mg ml^{-1}) to 1 µl of pDNA (0.3 µg µl^{-1}) at various w/w ratios (0.33, 0.66, 1.0, 1.66 and 2.33) and 5 µl dextrose (20%) was added before making up the final volume to 20 µl with water. The complexes were gently vortexed, incubated for 30 min at 25 ± 1 °C, diluted with serum-free DMEM (60 µl) and gently added to HEK293, CHO and HeLa cells. After 4 h, the transfection medium was replaced by 200 µl fresh DMEM containing 10% FBS and cells were incubated for 36 h. After 36 h, MTT (100 µl, 0.5 mg ml^{-1} in DMEM) was added to the cells and incubated for 2 h at 37 °C. Then the supernatant was aspirated, the cells were rinsed with PBS and the formazan crystals, so formed, were suspended in 100 µl of isopropanol containing 0.06 M HCl and 0.5% SDS. Absorbance of the resulting solution was measured spectrophotometrically in an ELISA plate reader (MRX, Dynatech Laboratories) at 540 nm. Untreated cells were taken as the 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 the formula $[Abs]_{sample}/[Abs]_{control} \times 100$.

In vitro transfection

PEI/DNA, PNIm/DNA complexes were prepared and transfected to HEK293, CHO and HeLa cells, as described above. Similarly, transfection was performed in a 10% serum containing medium. DNA complexes of Superfect™, GenePORTER 2™ and Lipofectamine™ were also prepared following manufacturers' protocols and transfected onto the cells for comparison studies.

For delivery of GFP specific siRNA, pGFP DNA (1 µl, 1.4 nM) was transfected with PNIm 10(6) as above, and after 3 h, the transfection medium was replaced by GFP specific siRNA (2 µl, 2.5 µM) complexed with PNIm 10(6) in a 80 µl reaction mixture containing 60 µl of DMEM. Post 3 h, the medium was again replaced with fresh DMEM containing 10% serum and the cells were incubated for 36 h. The PNIm 10(6)/DNA nanoplex alone was used as the control. Lipofectamine™/DNA/siRNA and Lipofectamine™/DNA complexes were also prepared and all of these formulations were added on to seeded HEK293 cells. The experiment was carried out as described above. The expression levels were monitored by quantifying GFP. All experiments were performed at least in triplicate.

Quantification of EGFP expression

The quantification of EGFP expression was made using a NanoDrop ND-3300 spectrofluorometer. After 36 h of transfection, cells in each well were washed with PBS (2 × 100 µl) and incubated with cell lysis buffer (10 mM Tris, 1 mM EDTA and 0.5% SDS, pH 7.4, 100 µl) for 45 min at 37 °C. The cell

lysates were centrifuged and EGFP was estimated spectrofluorometrically by taking 2 μl of lysate. The fluorescence intensity values for background and auto-fluorescence were determined using mock-treated cells. The total protein content in cell lysate from each well was estimated using the Bradford reagent (Bangalore Genei, India), taking BSA as the standard. The level of fluorescence intensity of GFP was calculated by subtracting the background values and normalized against protein concentration in the cell extract. The data are reported as arbitrary units (AU) mg^{-1} of cellular protein and represent mean ± standard deviation for triplicate samples.

Fluorescence activated cell sorting (FACS) analysis

FACS analysis was performed to examine the GFP expression at the individual cell level 36 h post-transfection. Briefly, HEK293 cells were seeded in 24-well plates, and after 16 h, the medium was aspirated and cells washed with phosphate buffer saline (1 × PBS, twice). The transfection was performed as described above. Similarly, pDNA complexes were prepared with Lipofectamine™ following manufacturers' protocols, added onto the cells and incubated for 36 h. Subsequently, transfected cells were washed with PBS (1 × 1 ml) and harvested by trypsinization. Cells were recovered by centrifugation at 5000 rpm for 5 min at 4 °C, the supernatant was removed, the pellet was washed with 1 × PBS (2 × 500 μl) and resuspended in 1 × PBS (1.0 ml). The percentage of EGFP-expressing cells was determined to quantify transfection efficiency by flow cytometry equipped with Cytosoft Software (Guava® EasyCyte™ Plus Flow Cytometry System, USA). The percentage of transfected cells was obtained by determining the statistics of cells fluorescing above the control level, where non-transfected cells were used as the control. Approximately, 5000 cells were analyzed to generate data for statistical purpose.

DNA release assay

PEI was complexed with pDNA (1.5 μg) at a w/w ratio, at which it exhibited the highest transfection efficiency, and was incubated for 30 min, as described above. Subsequently, an aqueous solution of heparin, an anionic polymer, was added in different amounts varying from 0.5–20 U for the release of plasmid DNA, which was bound to the cationic polymer. The samples were then incubated for 20 min, electrophoresed (100 V, 1 h) on 1% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator using a Gel Documentation system. The amount of DNA released from complexes after heparin treatment was estimated by densitometry. Likewise, the assay was repeated with the PNIm 10 series.

Confocal laser scanning microscopy (CLSM)

The path of cellular entry was monitored using confocal microscopy. The best working sample PNIm 10(6) was labeled with TRITC to block nearly ~1% of total amines in the nanoparticles using a reported procedure from the lab.[20] pDNA (0.3 mg) was labeled using YOYO-1 iodide (2 μl, 1 mM solution in DMSO) and stored at −20 °C. HeLa cells were seeded (1.5×10^5 cells per well) on circular glass coverslips in a 6-well plate, grown overnight to ~70% confluence. A solution of nanoplex (500 μl) containing 2 μg of pDNA in DMEM was added to each well. After incubation for 0.25, 0.5, 1, and 2 h, the cells were washed with 1 × PBS (3 × 500 μl) and fixed with 4% paraformaldehyde solution for 10 min at 4 °C. Then, the fixed cells were counter-stained with a blue nuclear dye, DAPI, and the coverslips were mounted on glass slides with a fluorescence-free Mounting Medium (UltraCruz™, Santa-Cruz Biotechnology, USA). All confocal images were captured using a Zeiss LSM 510 inverted laser-scanning confocal microscope.

DNase I protection assay

The ability of the PNIm to protect the condensed pDNA from nuclease attack was assessed by the DNase I assay. Native pDNA and PNIm 10(6)/pDNA nanoplexes (at w/w ratio 1) were incubated at 37 °C for 0.25, 0.5, 1 and 2 h with DNase I (Sigma, USA) (1 μl, 1 unit μl^{-1} in a buffer containing 100 mM Tris, 25 mM $MgCl_2$ and 5 mM $CaCl_2$). Treatment with PBS alone served as a control. After incubation, a solution of EDTA (5 μl, 100 mM) was added and the mixture incubated at 75 °C for 10 min to inactivate DNase I. The mixture was further incubated for 2 h (room temperature) with heparin (10 μl, 5 mg ml^{-1}) to release bound DNA from the cationic polymer. Subsequently, samples were electrophoresed and visualized, as described above (DNA release assay). The amount of pDNA released from complexes after heparin treatment was estimated by densitometry.

In vivo gene expression

For intravenous injection in Balb/c mice, 25 μg of the Luciferase vector (pGL-3 control vector) and DNA complexes of PNIm 10(6) and PEI at a w/w ratio of 1 using normal saline as the medium (final volume of 100 μl) were incubated for 30 min at 25 ± 1 °C. These complexes were injected through tail vein using an insulin syringe (40 U, needle size of 0.3 × 8 mm). After 7 d on normal diet, the mice were sacrificed and liver, spleen, kidney, lung, heart and brain were dissected out, washed with chilled normal saline, weighed, chopped and homogenate was made (25% w/v, 1 × lysis buffer). After three cycles of freezing and thawing, the homogenates were centrifuged at 5000 rpm for 10 min at 4° C and 100 μl supernatant was used to measure luciferase activity. Luciferase activity was represented as RLU per mg of total protein. All the experiments were carried out in duplicate.

Statistical analysis

Statistical analysis wherever needed was carried out by one-way analysis of variance followed by Student's *t*-test after ascertaining homogeneity of variance and normality of data. A value of $P < 0.05$ was considered statistically significant. JMP version 6.0.0, Statistical Discovery™ from SAS was used for analysis.

Conclusions

In this study, we showed the effect of imidazole grafting through an epoxy end-based linker on BDE crosslinked PEI nanoparticles. The grafting was induced in such a way so as not to reduce the overall charge on PEI but to convert one

form to another. Additional delocalized charge was introduced in the form of an imidazole ring in order to decrease the cytotoxic effect and impart more buffering into the system. The effect of increased buffering on the transfection efficiency of the resulting nanoparticles was studied. It was found that introduction of imidazole groups resulted in enhancement of transfection efficiency of crosslinked nanoparticles. This enhancement was attributed to the increased buffering in the pH range 3–10. Among the series of nanoparticles prepared in this study, PNIm 10(6) was found to be ~2–17 folds superior compared to PN-2, PEI and commercial transfection reagents tested in this study, in terms of transfection efficiency *in vitro*. siRNA studies involving PNIm 10(6) led to ~90% suppression of gene expression, indicating the efficient delivery of siRNA. The projected particles were also able to efficiently carry the desired gene *in vivo*. These studies thus suggest that PNIm 10(6) tested in the present study has a potential for future applications in gene delivery.

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