Chapter IV

1,4-Butanediol Diglycidyl Ether (BDE)-Crosslinked PEI-g-Imidazole Nanoparticles as Nucleic Acid-Carriers In vitro and In vivo
1. Introduction

Over the past few decades, bPEI (25kDa) based delivery systems have shown potential as transfection agents (Godbey et al., 1999; Remy et al., 1998). However, polymer associated cytotoxicity limits its applications in vivo (Gao and Liu, 2005; Seib et al., 2007). Reports indicate that higher charge density of bPEI contributes to its cytotoxicity, which has been circumvented by acylation (Nimesh et al., 2007), alkylation (Gao et al., 2009), PEGylation (Nimesh et al., 2006), coating with sugar moieties (Borchard et al., 2001; Kim et al., 2007) and incorporation of imidazolyl groups (Midoux, 2001; Germershaus et al., 2010, Swami et al., 2007a).

In one of the previous studies, bPEI was crosslinked with varying amounts of 1,4-butanediol diglycidylether (BDE) to address the concern of cytotoxicity with concomitant improvement in the transfection efficiency (Swami et al., 2007b). The resulting series of nanoparticles (PN-1 to PN-4 NPs) showed enhanced transfection efficiency and one of the formulations, PN-2/DNA complex, exhibited ~2.5-5 folds higher transfection efficiency than native PEI and showed minimal cytotoxicity. In order to further enhance the transfection efficiency and cell viability, the present study was undertaken to design and synthesize a novel linker, 2-(N-1-trylimidazol-4-yl)-N-(6-glycidyloxyhexyl)-acetamide (3), one end of which consists of an epoxy ring that would react partially with residual 1° and 2° amino groups in PN NPs and convert them into 2° and 3°, respectively, thereby maintaining the overall number of amines intact in the resulting PN-g-Imidazole nanoparticles (PNIm NPs). The other end of the linker bears an imidazole moiety, an endosomolytic agent, which would not only help in improving the transfection efficiency of the resulting particles but also incorporate a delocalized charge. As the beneficial role of imidazole in transfection is well documented (Midoux, 2001; Germershaus et al., 2010, Swami et al., 2007a), 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 (Midoux, 2001).

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)/DNA complex, scored ~2-3 and ~11 folds higher transfection efficiency than PN-2/DNA and bPEI/DNA complexes, 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.
2. Experimental section

2.1. Synthesis of IGA linker (3)

4-Imidazole acetic acid hydrochloride (5mmol) was dissolved in water and NaHCO₃ (10mmol) was added. The solution was stirred for ~1h and then dried on a rotary evaporator followed by in a vacuum desiccator. The sodium salt of 4-imidazole acetic acid was dissolved in dry THF (5ml) followed by addition of a solution of trityl chloride (5.5mmol), dissolved in dry pyridine (20ml). The reaction mixture was stirred for ~8h at 25±2°C. Then, the solvent was removed on a rotary evaporator to dryness and the residue triturated with diethylether (2x 10ml). The residual mass was dissolved in ethyl acetate (20ml) and washed with 5% aqueous solution of citric acid (2 x 10ml). The organic phase was separated, dried over sodium sulphate, filtered and concentrated to obtain (N-1-tritylimidazol-4-yl) acetic acid in almost quantitative yield, which was characterized by LC-MS: m/z = 368 (calculated), 367 (found, M-H⁺).

To a solution of (N-1-tritylimidazol-4-yl) acetic acid (4mmol) in dry THF (10ml), DCC (4.4mmol) and NHS (4.4mmol) were added and the reaction mixture was stirred for 4h at 25±2°C. 6-Aminohexan-1-ol (4.5mmol) was then added to the above reaction mixture and continued stirring for 4h. Subsequently, the reaction mixture was cooled to 4°C and solid dicyclohexylurea was filtered, which was washed with tetrahydrofuran (THF) (2 x 5ml) and the combined filtrate was concentrated on a rotary evaporator. The syrupy residue, so obtained, was dissolved in ethyl acetate (20ml) and washed successively with 5% aqueous solution of citric acid (2 x 10ml) and saturated brine (1 x 10ml). The organic phase was separated, dried over sodium sulphate, 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 = 467 (calculated), 466 (found, M-H⁺).

Compound (1) (3mmol) was suspended in epichlorohydrin (10ml) and added N,N'-diisopropylethylamine (DIPEA) (100µl). The resulting reaction mixture was stirred overnight at 65°C followed by removal of unreacted epichlorohydrin on a rotary evaporator to obtain a syrupy residue, which was dissolved in ethyl acetate (20ml) and washed successively with 5% aqueous solution of citric acid (2 x 10ml) and saturated brine (2 x 5ml). The organic phase was separated, dried over sodium sulphate, 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 eluent. 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 = 559.5 (calculated), 558.5 (found, M-H⁺).
To a solution of compound (2) (2mmol), dissolved in THF (10ml), was added an aqueous solution of 2N NaOH (1.5ml) and stirred the reaction at 45°C. After 1h, the reaction mixture was diluted with water (10ml) and THF was removed on a rotary evaporator. The desired compound was extracted in dichloromethane (5 x 10ml), collected, dried over sodium sulphate, filtered and concentrated to obtain IGA linker (3), which was characterized by its $^1$H-NMR. $^1$H-NMR (CDCl$_3$), δ: 1.2-1.5 (8H, 4x -CH$_2$), 2.9-3.2 (5H, -CH-CH$_2$, -NH-CH$_2$), 3.75-3.85 (4H, 2x -OCH$_2$), 3.4 (2H, -CH$_2$CO), 6.75 (1H, -CH-N), 7.38 (1H, -N-CH-N), 7-7.2 (15 H, Ar-H).

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

bPEI was crosslinked with varying amount of BDE to generate PN-1 (5% crosslinking), PN-2 (10% crosslinking) and PN-3 (15% crosslinking) following a previously reported procedure (Swami et al., 2007b). The synthesized nanoparticles were grafted with 2, 4, 6 and 8% of IGA linker (3). Briefly, for 2% grafting, an aqueous solution of PN-1 (25mg in 10ml H$_2$O) was mixed with a solution of (3) (4.2mg in 1ml 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, 10ml). After stirring for 30min at 25±2°C, the reaction volume was reduced to half on a rotary evaporator and the aqueous phase was washed with diethyl ether (2 x 5ml). Subsequently, a saturated solution of sodium bicarbonate (2ml) was added to bring the pH of solution to ~7.5 and subjected to dialysis against water with intermittent changes of water for 2d. The dialyzed solution was lyophilized to obtain PNIm 5(2), which was characterized by biophysical techniques. Similarly, other imidazole-grafted nanoparticles in the series were prepared and characterized using DLS, zeta potential, $^1$H-NMR, IR and gel retardation assay.

2.3. DNA retardation assay

Aqueous solutions of bPEI and PNIm (1mg/ml) were mixed with 1μl of pDNA (0.3μg/μl) at w/w ratios 0.16, 0.33, 0.66 and 1.0 to form PEI/DNA and PNIm/pDNA complexes and the final volume was made up to 20μl with water. The resulting samples were gently vortexed and incubated at 25±2°C for 30min. All the complexes (20μl) were mixed with 2μl xylene cyanol and electrophoresed as described in Chapter II.

2.4. Physical characterization of nanoparticles

The hydrodynamic diameter of PNIm series (1mg/ml) and their DNA complexes, suspended separately in water and 10% serum, were measured by DLS in triplicates, as outlined in Chapter II.
2.5. Formation of nanoparticle/DNA complexes

To form nanoparticle/DNA complexes, an aqueous solution of PNIm nanoparticles (1.0mg/ml) was added to 1μl of DNA (0.3μg/μl) at various w/w ratios (0.33, 0.66, 1.0, 1.66 and 2.33) and the final volume was made up to 20μl with water. For in vitro transfection assay, 5.0μl of 20% dextrose solution was added before making up the final volume to 20μl with water. The resulting samples were gently vortexed and incubated for 30min at 25±1°C prior to their use in biophysical studies or transfection experiments.

2.6. Buffering capacity

The ability of PEI and PNIm (5, 10 and 15 series) to resist change in pH was experimentally demonstrated by acid titration assay following a method reported by Tseng et al. (2004). A suspension of PNIm 5(2) (6mg) in 0.1N NaCl (30ml) was adjusted to pH 10 with 0.1N NaOH and then the pH was brought to 3.0 by the addition of 50μl aliquots of 0.1N HCl. pH values were recorded after each addition. The slope in the plot between pH and the amount of HCl consumed indicated the intrinsic buffering capacity of the system. Similarly, the projected assay was repeated with the other formulations.

2.7. Cell viability assay

The cell viability of PEI/DNA and PNIm/pDNA complexes as well as Superfect™/DNA, GenePORTER 2™/DNA and Lipofectamine™/pDNA complexes was evaluated by MTT colorimetric assay on HEK293, CHO and HeLa cells, as described in Chapter II.

2.8. In vitro DNA/siRNA delivery

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

2.9. Fluorescence-activated cell sorting (FACS) analysis

FACS analysis was performed to examine the GFP expression at the individual cell level 36h post-transfection on HEK293 cells, following the procedure outlined in Chapter II.

2.10. DNA release assay

PEI was complexed with pDNA (1.5µg) at the w/w ratio, at which it exhibited the highest transfection efficiency, incubated for 30min and heparin was added in different amounts varying from 0.5-20U for the release of plasmid DNA, which was bound to the cationic carriers. The samples were then incubated for 20min, electrophoresed, as described in Chapter II. Likewise, the assay was repeated with PNIIm 10 series.

2.11. Cellular trafficking by confocal microscopy

Intracellular trafficking of dual labeled PNIIm 10(6)/DNA complex was monitored using confocal microscopy, as described in Chapter II.

2.12. DNase I protection assay

The ability of the PNIIm to protect the condensed pDNA from nuclease attack was assessed by DNase I assay. Native pDNA and PNIIm 10(6)/pDNA complex (at w/w ratio 1) were incubated at 37°C for 0.25, 0.5, 1 and 2h with DNase I (Sigma, USA) (1µl, 1unit/µl in a buffer containing 100mM Tris, 25mM MgCl₂ and 5mM CaCl₂). Subsequent to interaction by DNase I, the intact DNA was released by adding heparin and estimated following the procedure illustrated in Chapter II.

2.13. In vivo gene expression studies in Balb/c mice

For intravenous injection in Balb/c mice, 25µg pGL3 was complexed with PNIIm 10(6) and PEI at w/w ratio of 1:1 in normal saline (final volume of 100µl) and incubated for 30min at 25±1°C. These complexes along with naked DNA were injected through tail vein and extent of gene expression was monitored following the procedure detailed in Chapter II.

3. Results & discussion

3.1. Preparation of PNIIm particles

In a previous publication, 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 (Swami et al., 2007b). 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 up to 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 PN series) comparable to PN-3 or PN-4 or higher without compromising on the transfection efficiency, a novel linker was designed, which contains epoxy group at one end, that could partially convert the residual 1º and 2º amines to 2º and 3º amines (i.e. conversion of bad charge due to 1º amines to good charge of 2º or 3º amines), thereby improving the cell viability. 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 the required reagents should be commonly available.

Scheme-1: Schematic representation of preparation of IGA linker (3) and PNIm nanoparticles.
Having gone through the literature and keeping these points into consideration, it was decided to have an imidazole moiety, which is known for its beneficiary properties in enhancing the transfection efficiency of the cationic polymers (Swami et al., 2007a), on the other end of the linker (Scheme-1).

This linker (3), prepared from 4-imidazole acetic acid hydrochloride, was reacted with the preformed PN NPs (Scheme-1) in increasing amount and a series of PNIm nanoparticles was obtained, which was evaluated in terms of transfection efficiency and cytotoxicity. The best working sample of the series, PNIm 10(6) on complexation with DNA, dramatically enhanced the transfection efficiency ~10-11 folds with respect to PEI/DNA complex, ~2-3 folds with respect to PN-2/DNA complex and ~2-17 folds with respect to commercial transfection reagents, which indicated that the 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.

3.2. Characterization of PNIm particles

The synthesized PNIm series was characterized by various techniques, viz., $^1$H-NMR, IR, DLS and Zeta potential. The percent grafting of linker (3) on the nanoparticles was determined by $^1$H-NMR (Table-1).

**Table-1**: Percent grafting of imidazoyl-linker, IGA (3), on PN NPs as estimated by $^1$H-NMR spectroscopy.
The peaks at δ 6.8 and 7.4 confirmed the presence of 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 nano range (data not shown), as determined by DLS. This increasing trend in size also confirmed the grafting of 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).

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

<table>
<thead>
<tr>
<th>PNIm 5(2)</th>
<th>PNIm 5(4)</th>
<th>PNIm 5(6)</th>
<th>PNIm 6(6)</th>
<th>PNIm 10(2)</th>
<th>PNIm 10(4)</th>
<th>PNIm 10(6)</th>
<th>PNIm 10(8)</th>
<th>PNIm 15(2)</th>
<th>PNIm 15(4)</th>
<th>PNIm 15(6)</th>
<th>PNIm 15(8)</th>
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<td>Size ± S.D. (PDI)</td>
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<td>With DNA (nm)</td>
<td>With 10% Serum (nm)</td>
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<td>396.0 ± 3.78 (0.165)</td>
<td>211.1 ± 4.61 (0.271)</td>
<td>30.6 ± 2.17</td>
<td>15.9 ± 1.83</td>
<td>-23.0 ± 1.07</td>
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<td>306.7 ± 4.29 (0.276)</td>
<td>105.2 ± 2.91 (0.153)</td>
<td>33.9 ± 1.98</td>
<td>17.8 ± 1.12</td>
<td>-19.3 ± 1.67</td>
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<td>330.0 ± 3.10 (0.292)</td>
<td>100.4 ± 3.79 (0.189)</td>
<td>36.4 ± 1.76</td>
<td>17.8 ± 1.95</td>
<td>-19.2 ± 1.92</td>
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<td>321.1 ± 5.92 (0.189)</td>
<td>98.4 ± 4.76 (0.297)</td>
<td>40.6 ± 3.48</td>
<td>17.9 ± 2.63</td>
<td>-17.5 ± 2.72</td>
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<td>280.0 ± 2.05 (0.136)</td>
<td>95.5 ± 5.39 (0.129)</td>
<td>29.2 ± 2.63</td>
<td>16.6 ± 1.98</td>
<td>-20.3 ± 2.02</td>
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<td>248.5 ± 7.29 (0.208)</td>
<td>75.5 ± 2.07 (0.275)</td>
<td>30.2 ± 3.73</td>
<td>16.7 ± 2.76</td>
<td>-17.4 ± 2.83</td>
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<td>226.8 ± 6.20 (0.143)</td>
<td>68.4 ± 3.87 (0.128)</td>
<td>32.4 ± 3.79</td>
<td>19.6 ± 3.65</td>
<td>-16.9 ± 2.67</td>
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<td>202.1 ± 4.95 (0.187)</td>
<td>59.4 ± 3.92 (0.274)</td>
<td>36.4 ± 3.92</td>
<td>20.6 ± 2.93</td>
<td>-16.4 ± 1.29</td>
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<td>169.4 ± 6.20 (0.387)</td>
<td>54.0 ± 4.73 (0.185)</td>
<td>26.4 ± 3.72</td>
<td>17.2 ± 1.98</td>
<td>-20.9 ± 2.90</td>
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<td>168.0 ± 3.87 (0.170)</td>
<td>53.5 ± 4.93 (0.252)</td>
<td>28.3 ± 2.61</td>
<td>19.5 ± 2.75</td>
<td>-14.2 ± 1.83</td>
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<td>148.2 ± 4.76 (0.186)</td>
<td>50.1 ± 4.27 (0.127)</td>
<td>29.6 ± 2.02</td>
<td>20.5 ± 3.81</td>
<td>-14.2 ± 1.28</td>
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<td>123.5 ± 6.38 (0.241)</td>
<td>49.1 ± 3.68 (0.138)</td>
<td>32.8 ± 2.91</td>
<td>21.5 ± 2.72</td>
<td>-14.0 ± 1.29</td>
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<td>287.8 ± 2.97 (0.675)</td>
<td>125.6 ± 2.95 (0.489)</td>
<td>31.8 ± 2.90</td>
<td>21.4 ± 2.81</td>
<td>-13.6 ± 1.98</td>
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Further, in the presence of 10% FBS, the sizes decreased (49-212nm), which might be due to adsorption of water molecules from the cationic surfaces by the serum proteins leading to dehydration of the complexes (Swami et al., 2007a). Zeta potential of nanoparticles was found to increase with increase in percent grafting of (3), confirming the conjugation of the same with PN NPs. On complexation with pDNA (at w/w ratio of 1:1), the zeta potential decreased, which might be due to neutralization of some positive charge with negatively charged phosphate backbone of DNA. However, in 10% FBS, the zeta potential became negative due to the adsorption of negatively charged particles on the surface of these nanoparticles (Swami et al., 2007a).

3.3. DNA retardation assay

The amines present in nanoparticles interact with 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 fixed amount (0.3µg) of pDNA, the complexes were prepared at increasing w/w ratios of nanoparticle:DNA and loaded onto 1% agarose gel.

![DNA retardation assay of PNIm/DNA and PEI/DNA complexes on 0.8% agarose gel.](image)

Figure-1. DNA retardation assay of PNIm/DNA and PEI/DNA complexes on 0.8% agarose gel.
PNIm nanoparticles retarded at w/w ratios of 0.33 or 0.66, while PEI retarded the mobility of same amount of pDNA (0.3µg) at a w/w ratio of 1:1. PN NPs retarded the mobility of DNA (0.3µg) at a higher w/w ratio than PEI (Fig. 1) (Swami et al., 2007b). These observations showed that a lesser amount of PNIm particles and more amounts of PN NPs were required in comparison to PEI in retarding the fixed amount of DNA. The reason might be attributed to the presence of extra amines available for binding with DNA in the imidazole ring in PNIm, while conversely, in case of PN NPs, some amount of charge got embedded inside the NPs, so a higher amount was required for retardation.

3.4. Cell viability assay

The synthesized PNIm series was evaluated in terms of cell viability using MTT assay on three different cell lines, viz., HEK293, HeLa and CHO. PNIm/DNA complexes were found to be almost non-toxic (cell viability ranged between 90-98%) in all the cell lines. However, PEI/DNA and Lipofectamine\textsuperscript{TM}/DNA complexes showed a significantly reduced cell viability (ranging between 45-60% in all the cell lines; $P<0.01$) (Fig. 2). For the other commercial reagents, the cell viability was found to be in the range of 80-82\% (Superfect\textsuperscript{TM}) and 87-92\% (GenePORTER 2\textsuperscript{TM}). PN-2/DNA complex showed the cell viability ranging between 84-91\% in all the cell lines. Therefore, it is evident from figure 2 that after grafting with IGA linker (3), the cell viability has improved in comparison to crosslinked nanoparticles (PN-2). The possible reasons for the increased cell viability of PNIm/DNA complexes particles over PN-2/DNA complexes may be (i) further reduction in number of toxic primary amino groups, and (ii) presence of imidazole moiety, as it is reported that protonated imidazole ring is less cytotoxic than protonated amines (Midoux et al., 2009).

![Figure-2](image-url). Cell viability profile of PNIm/DNA complexes, PEI/DNA complex (w/w ratio of 1:1), Superfect\textsuperscript{TM}/DNA, GenePORTER 2\textsuperscript{TM}/DNA and Lipofectamine\textsuperscript{TM}/DNA complexes on HEK293, CHO and HeLa cells determined by MTT assay. Percent viability of cells is expressed relative to control cells. Each point represents the mean of three independent experiments performed in triplicates. *$P<0.05$ vs PEI and commercial transfection reagents.
3.5. Buffering capacity

bPEI exhibits a considerable buffering capacity in the pH range of endosomes and lysosomes (Demeneix and Behr, 2005; Akinc et al., 2005), however, displays considerable toxicity due to the presence of primary amines (Seib et al., 2007). Therefore, bPEI was crosslinked using BDE to result into well defined nanoparticles with reduced toxicity (Swami et al., 2007b). 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, the buffering capacity of PN NPs was optimized for enhancement by grafting imidazole moiety on these nanoparticles through IGA linker (3).

Imidazole ring helps in the escape of pDNA from endosomes (Midoux et al., 2009). As imidazole is weak base (pKa ~6.5), it has tendency to get protonated at neutral pH, therefore, it has been found to be suitable to modify the surface of cationic polymers. To investigate the effect of imidazole grafting on the buffering capacity of PNIIm NPs, an acid-base titration was performed in the pH range 3-10. Figure 3 shows the buffering capacity of PNIIm particles, PN-2 NPs and PEI under different pH conditions.

![Figure 3](image.png)

**Figure-3.** Acid-base titration curve for PN-2, PEI and PNIIm nanoparticles.

It was observed from the figure-3 that proton capturing tendency of the PNIIm particles got slightly enhanced as compared to native PEI and PN-2 NPs. The enhanced buffering capacity of the PNIIm series might be due to the presence of imidazole moiety as
this moiety has already been well documented in literature to be a good buffering provider in the pH range of endosomes. Therefore, transfection efficiency of these particles was evaluated. 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.

3.6. In vitro transfection

Transfection efficiency of PNIm/DNA and PN-2/DNA complexes, PEI/DNA complex and DNA complexes of Superfect™, GenePORTER 2™ and Lipofectamine™ was evaluated on 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), and it was found that PNIm 10(6)/DNA complex performed the best in terms of transfection efficiency scoring ~10.4, ~11.6 and ~12.7 folds higher transfection efficiency on HEK293, CHO and HeLa cells, respectively, as compared to PEI/DNA complex. Also, the transfection efficiency of PNIm 10(6)/DNA complex was 2.3, 3.0, and 2.0 folds higher in HEK293, CHO and HeLa cells, respectively, than PN-2/DNA complex (Fig. 4a). Moreover, in the presence of 10% serum, the transfection efficiency did not decrease significantly (P>0.05) (Fig. 4b), which further implicates the potential of modified nanoparticles for in vivo gene delivery applications.

Flow cytometry was employed to quantify the reporter gene expression at the individual cell level. PNIm 10(6)/DNA complex showed maximum transfection efficiency, i.e. 78±4.8 and 72±3.9% cells transfected in HEK293 and CHO cells, respectively. In contrast, PEI/DNA complex showed only 23±2.3% and 20±3.4% GFP positive cells on HEK293 and CHO cells, respectively (Fig. 5). Also, PN-2/DNA complex showed 48±5.3% cells transfected in HEK293 and 45±2.7% GFP positive cells in CHO cells. PEI/DNA and PNIm/DNA complex worked best at w:w ratio of 1:1 (NP:DNA), while PN-2/DNA complex worked best at 1.66:1. This might be due to the presence of imidazole moiety, which provided an extra delocalized charge to bind pDNA. One of the plausible explanations for the enhanced transfection efficiency might be attributed to the enhanced buffering of PNIm nanoparticles in the pH range 3-10 due to the presence of imidazole group. Secondly, PNIm/DNA complexes were almost non-toxic (see cell viability assay), which might further account for the enhanced transfection efficiency.
Figure 4. GFP fluorescence intensity in HEK293, CHO and HeLa cells in (a) absence of serum, and (b) presence of serum, transfected with PNIm/DNA, PEI/DNA, Superfect\textsuperscript{TM}/DNA, GenePORTER 2\textsuperscript{TM}/DNA and Lipofectamine\textsuperscript{TM}/DNA complexes. Fluorescence intensity is measured on spectrofluorometer, expressed in terms of arbitrary units/mg of total cellular protein obtained at a w/w ratio of 1:1 for PNIm/DNA and PEI/DNA complexes, respectively. The results represent the mean of three independent experiments performed in triplicates. *P<0.05 vs PEI and Lipofectamine\textsuperscript{TM}.
Figure 5. Percent transfection efficiency of PNIm 10/DNA complexes determined using FACS in (a) CHO and (b) HEK293 cells at various w/w ratios and compared with the percent transfection efficiency of PN-2/DNA, PEI/DNA, Superfect™/DNA and Lipofectamine™/DNA complexes. *P<0.05 vs PEI and Lipofectamine™.

3.7. DNA release assay

The DNA carrier after carrying the desired therapeutic inside the cell must be able to release it to facilitate efficient transfection (Ganesh and Sastry, 2002). 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% agarose gel. On quantification by densitometry, it was observed that on descending the series (PNIm 10), the binding with DNA became stronger. With 5U of heparin, the amount of pDNA released from 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 (10U), PNIm 10(2) and PNIm 10(6) released up to ~98% and ~75% of pDNA, respectively, while PEI released only 55% of it (Fig. 6). 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 (Vijayanathan and Thomas, 2002). The synthesized nanoparticles not only carried the bound DNA efficiently to insides of the cell but also released it in sufficient amount in the cellular milieu to result into enhanced gene expression.

![Figure-6. DNA release assay of PNIm 10 series and PEI.](image)

### 3.8. Intracellular localization of complexes

The intracellular localization of DNA is an important factor for successful gene therapy. To determine the intracellular localization of PNIm 10(6)/pDNA complexes after cellular uptake, HeLa cells were incubated with tetramethylrhodamine (TMR)-labeled PNIm 10(6) nanoparticles (red fluorescence) complexed with YOYO-1 labeled DNA (green fluorescence) and observed under confocal laser scanning microscope. 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 (Fig. 7).
Red and green fluorescence was also observed inside the cytoplasm and nucleus, which indicated the dissociation of complex within the cytoplasm or nucleus. PNIm 10(6) was found to carry pDNA inside the cytoplasm within 1h and to the nucleus within 2h of the addition of complexes to the cells (Fig. 7). The complex was localized not only in the cytoplasm but also in the nucleus. The finding clearly demonstrates efficient intracellular delivery of DNA using PNIm 10(6).
3.9. Delivery of siRNA

As PNIm 10(6) efficiently transfected pGFPDNA into the cell, its ability to knockdown this gene was evaluated using GFP specific siRNA on HEK293 cells and compared these results with GenePORTER 2™ mediated siRNA delivery. The cells were transfected first with PNIm 10(6)/GFPDNA complex and after 3h (the optimum time for cellular entry; see intracellular trafficking), PNIm 10(6)/GFPsiRNA was added. After 36h 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) (Fig. 8). In contrast, GenePORTER 2™/siRNA formulation knocked down GFP expression by ~53.2% only (Fig. 8). Hence, the delivery of GFP-specific siRNA by selected nanoparticle formulation clearly down-regulated GFP gene more efficiently than the commercially available transfection reagent, GenePORTER 2™. Therefore, the projected nanoparticles may serve as an effective carrier for the delivery of siRNA as well.

![Figure-8](siRNA delivery on HEK293 cells. All the experiments were performed at least thrice and error bars represent the standard deviation.)

3.10. DNase I protection assay

The PNIm 10(6)/pDNA formulation and pDNA alone were incubated with DNase I for different time intervals (0.25, 0.50, 1 and 2h) and after release by heparin, the samples were run on a 0.8% agarose gel. Quantitative densitometric analysis of the gel image showed that in contrast to the free DNA (0.3μg), which was degraded completely by DNase I within ~15min, PNIm 10(6) effectively protected bound DNA (Fig. 9) and only 15% of it was found to be degraded even after 2h of treatment. Thus, the projected nanoparticles are suitable for in vivo administration of pDNA.
3.11. In vivo gene expression

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


