Chapter IV

Surface Modification of Crosslinked Dextran Nanoparticles Influences Transfection Efficiency of Dextran–Polyethylenimine Nanocomposites
1. INTRODUCTION

As discussed in the last chapters, PEI has attracted much attention as most of the essential features necessary for efficient transfection are intrinsic to branched PEI. However, to make it target specific and improve its biocompatibility, additional modifications have been incorporated, thereby addressing the issues related to charge associated toxicity arising mainly from primary amines and non-specific interactions with blood components for in vivo applications [1-3].

In addition to these, non-ionic and hydrophilic polymers like polyethylene glycol (PEG) and polysaccharides have also been used to reduce its overall surface charge, improve their efficacy as well as to confer physicochemical stability to the formed nanoparticles [4-6]. Dextran is one of such polysaccharides, which has repeat units of glucose with α-1,6 glycosidic linkages whereas branching occurs from α-1,3 linkages. It inhibits non-specific interactions with serum proteins and prolongs the biological half-lives of its conjugates by increasing their stability in vivo [7, 8]. Dextran grafting has been demonstrated to improve the cell viability and serum stability of PEI based systems, which have shown promising results in gene delivery applications [9,10]. Azzam et al. [11] showed that incorporating hydrophobicity in dextran-spermine conjugates resulted in improved transfection efficiency over the corresponding non-hydrophobic conjugates, yet not comparable to the efficacy of branched PEI. Tseng et al. [12] studied the effect of grafting branched and linear PEIs on the stability of dextran-PEI polymers and found branched PEI conjugates to be more stable under in vivo conditions. In yet another study, PEI-dextran conjugates were reported to adequately address the toxicity concern, however, transfection efficiency still remained a point of apprehension. Here, the decrease in transfection efficiency was mainly attributed to the loss in buffering capacity of branched PEI due to steric hindrance created by the formation of dextran gel network around PEI, thus inhibiting the access to amines of PEI for protonation and hence, diminished transfection efficiency. Moreover, formation of bigger sized nanoconjugates was another point of concern, which would have hindered their efficient entry in the cells resulting in the decreased transfection efficiency.

Here, in the present study, a different approach was adopted with an aim to generate dextran grafted bPEI 25 kDa (DP) nanocomposites with improved transfection efficiency and cell viability. For this purpose, first, dextran was converted into its nanoparticles by crosslinking with a low molecular weight crosslinker, 1,4-butanediol diglycidyl ether (BDE), followed by partial oxidation of these nanoparticles to generate aldehyde functionalities and finally, grafted onto PEI to generate DP nanocomposites. A series of the nanocomposites was synthesized by varying the amounts of dextran polyaldehyde (DPA) nanoparticles. The
resultant series was characterized and allowed to interact with plasmid to form complexes, which were physicochemically characterized by dynamic light scattering (DLS), zeta potential measurements and atomic force microscopy (AFM). Besides, the complexation was also evaluated by agarose gel electrophoresis and transfection efficiency examined in vitro and in vivo.

2. EXPERIMENTAL

2.1. Synthesis of crosslinked dextran nanoparticles (DN)

To an aqueous solution of dextran (1 g, 1 mg/ml), pre-heated at 60 ºC and acidified with 1N hydrochloric acid (1 ml), was added a solution of 1,4-butanediol diglycidyl ether (BDE) (0.248 g, 1 mg/ml in water, for 20% crosslinking) drop wise with continuous stirring. After completion of addition, the reaction mixture was stirred for 4 h at the same temperature after which its volume was reduced to one fourth on a rotary evaporator followed by dialysis against water (12 kDa, 4 x 12 h). The dialyzed solution was lyophilized to obtain crosslinked dextran nanoparticles (DN) as a white powder (0.84 g, ~85% yield). Similarly, 30%, 40%, 50% and 70% crosslinked dextran nanoparticles were prepared in ~80-86% yield and characterized by DLS. DN50, the smallest sized nanoparticle, was characterized by $^1$H-NMR (Bruker Avance 400 MHz) to determine the actual percent crosslinking.

2.2. Oxidation of crosslinked dextran nanoparticles

The vicinal hydroxyl groups of DN50 (0.5 g, 40 mg/ml in water) were oxidized via ring opening reaction to dialdehydes by adding drop wise an aqueous solution of sodium periodate (0.1 g, dissolved in 2.5 ml water) following a reported procedure [11]. The reaction mixture was vigorously stirred at 4 ºC for 8 h in the dark followed by the addition of ethylene glycol (1 ml) to consume the unused sodium periodate and stirring was continued for 4 h. Then the reaction mixture was poured into a dialysis bag and dialyzed extensively against ddH$_2$O (4 x 12 h) at 4 ºC. Purified dextran-polyaldehyde (DPA) nanoparticles were freeze-dried to obtain a white powder (0.44 g, ~89% yield). The so formed DPA nanoparticles were characterized by its FTIR, IR (Nujol) $\nu$ (cm$^{-1}$): 1780 (carbonyl stretching).

2.3. Synthesis of PEI grafted dextran nanocomposites (DP)

An aqueous solution of DPA nanoparticles (0.412 mg, 1 mg/ml), for 1% grafting with primary amines, was added drop wise to an aqueous solution of PEI (50 mg, 1 mg/ml) with vigorous stirring at room temperature. After 2 h, sodium cyanoborohydride (50 mg, NaCNBH$_3$) was added and the reaction was allowed to stir overnight at 25±2 ºC. The total
volume of the reaction mixture was reduced to one-fourth on a rotary evaporator and poured into a dialysis bag (cut off 12 kDa). The dialysis was performed against water (4 x 12 h), followed by lyophilization to obtain 1% Dextran-PEI (DP1) ~80% yield. Similarly, 3% (DP2), 5% (DP3), 7% (DP4), 10% (DP5), 15% (DP6) and 20% (DP7) dextran nanoparticles were grafted with PEI. The percentage of PEI grafted on to dextran nanoparticles was determined using $^1$H-NMR. Further, the so formed nanocomposite series was characterized by AFM, IR, DLS, TEM and zeta potential.

2.4. Determination of the degree of PEI grafting by TNBS assay

Branched PEI has a distribution of primary, secondary and tertiary amines in the ratio of 1:2:1 [13]. The percentage of primary amines on unmodified PEI and those on DP nanocomposites was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay. The extent of primary amines of PEI involved in the conjugation reaction with DPA nanoparticles was calculated by the differences in the amounts of primary amines on the unmodified PEI and those on the DP nanocomposite series following the standard protocol [14]. Three different concentrations of each DP nanocomposites were used to determine the percent grafting.

2.5. Formation of DP nanocomposite/DNA complexes

DP nanocomposite/DNA complexes (20 µl) were made by mixing aqueous solutions of DP nanocomposites (1 mg/ml) and DNA (1 µl, 0.3 μg/µl) at various N/P ratios (8, 12, 16, 20, 24 and 30) in 5% dextrose. The complexes were mixed thoroughly by vortexing and incubated for 30 min at 25±2 ºC prior to their use in biophysical studies or transfection experiments. Similarly, PEI/DNA polyplexes were prepared at different N/P ratios (5, 7, 10, 15, 20 and 24).

2.6. DNA mobility shift assay

DNA (0.3 µg/µl) was complexed with PEI (1 mg/ml) at N/P ratios of 1.5, 3 and 5, and with DP nanocomposites (1 mg/ml) at an N/P ratio of 2, 4 and 6, as described above. The assay was performed, following the procedure outlined in Chapter II.

2.7. Size and morphology of nanocomposites and their DNA complexes by dynamic light scattering (DLS) and atomic force microscopy (AFM)

The prepared series of DP nanocomposites and their DNA complexes, at their best working N/P ratio (20 for DP nanocomposites and 10 for PEI), were characterized for their size and zeta potential by DLS in triplicates using Zetasizer Nano-ZS. The morphology of DN nanoparticles, DP4 nanocomposite and DP4/DNA complex was analyzed with the help of AFM, as described in Chapter II.
2.8. Buffering capacity

Susceptibility of PEI and DP nanocomposites to resist acidification was evaluated by titration method reported in the literature (ref). Briefly, the samples were prepared by suspending 66.7 nmole amine containing DP nanocomposites and PEI at a concentration of 0.1 mg/ml in 0.1 N NaCl. Initially, the pH of PEI and DP nanocomposites was adjusted to nearly 10 and then titrated by adding 0.1 N HCl (25 µl at one time) until a pH of 3.0 was reached. A graph was plotted between pH and the amount of 0.1 N HCl consumed for bringing the pH of the solution from 10 to 3.

2.9. DNA release assay

DP4 nanocomposite and PEI was complexed with pDNA (5 µl, 0.3 µg/µl) at an N/P ratio of 20 and 10, respectively. Heparin, a polyanion, was added in different amounts varying from 0-10 units for the release of plasmid DNA, bound to the polycation, and rest of the steps carried out, as described in Chapter II.

2.10. Protection of pDNA against DNase I

DNase I protection assay was carried out with DP4/DNA complex (N/P 20) and pDNA (0.3 µg) alone, following the procedure, as described in Chapter II.

2.11. Toxicity assessment

The cytotoxicity of DP/DNA complexes at N/P ratios of 8, 12, 16, 20, 24, 30, PEI/DNA complexes at N/P ratios of 5, 7, 10, 15, 20, 24 and DNA complexes of commercial reagents, viz., Superfect™, Fugene™, GenePORTER 2™, and Lipofectamine™, prepared according to manufacturers’ protocols, was evaluated on HEK293, HepG2 and HeLa cells by MTT colorimetric assay following the standard method, as described in Chapter II.

IC<sub>50</sub> value or the concentration of nanocomposite/DNA complexes, at which the cell viability reaches 50%, was estimated for DP4/DNA (the best working formulation among the series in terms of transfection efficiency) and PEI/DNA complexes at N/P ratios of 20 and 10, respectively. The transfection efficiency of these formulations was found to be the best at these N/P ratios.

2.12. In vitro transfection

pDNA complexes of DP conjugates, PEI, Superfect™, Fugene™, GenePORTER 2™ and Lipofectamine™, prepared as above, were added gently on HEK293, HepG2 and HeLa cells in the presence and absence of serum. The assay was completed following the steps described in Chapter II.
2.13. Sequential delivery of GFP siRNA

For delivery of GFP specific siRNA, cells were sequentially treated with DP4/pDNA and DP4/siRNA at N/P ratio of 20 and compared with Fugene™/DNA/siRNA complex, as described in Chapter II.

2.14. Quantification of EGFP expression

EGFP expression in mammalian cells was quantitatively estimated by NanoDrop ND-3000 spectrofluorometer, as described in Chapter II.

2.15. Fluorescence activated cell sorting (FACS) analysis

DP4 nanocomposite was complexed with DNA (1.5 μg) at N/P ratios of 12, 16, 20, 24 and 30, PEI/DNA complexes were prepared at N/P ratio of 10 (best working concentration in terms of transfection) and DNA complexes of Superfect™, and Lipofectamine™ were prepared following the manufacturers’ protocols. Transfection using these complexes was performed on HEK293 in the absence of serum and analysis was carried out, as described in Chapter II.

2.16. Intracellular trafficking of DP4 nanocomposite

The monitoring of intracellular trafficking of dual labeled tetramethylrhodamine (TMR)-DP4/YOYO-1-DNA complexes was carried out after incubation of these complexes with HeLa cells at 30, 60 and 120 min time intervals and analysis was performed with the help of confocal microscopy, as described in Chapter II.

2.17. Protein adsorption assay

Protein adsorption on native PEI and DP4 nanocomposite was determined following a reported method. Briefly, PEI and DP4 nanocomposite were complexed with pDNA at their best working N/P ratios (i.e. 10 and 20, respectively). Samples were incubated with 6.6 μl standard bovine serum albumin (BSA, 5 mg/ml) for 3 h at 25±2 ºC. Final concentration of pDNA and BSA was kept at 80 μg/ml and 1 mg/ml, respectively. The mixtures were centrifugated at 14000 x g for 2 h at 4 ºC. The supernatant was decanted off and the pellet was washed with Milli Q water to remove unbound BSA. The pellet was resuspended in 6x loading buffer (0.2 M Tris-HCl pH 6.8, 10% w/v sodium dodecyl sulfate (SDS), 20% v/v glycerol, 0.05% w/v bromophenol blue and 10 mM dithiothreitol) and incubated at 100 ºC for 5 min to extract bound BSA from the samples. Extracted solutions were loaded onto a 12% denaturing SDS-polyacrylamide gel and electrophoresed for 2 h at 25 mA. Thereafter, gel was stained with easy blue stain (Fermantas International Inc., Canada) to visualize BSA that had adsorbed onto samples.
2.18. In vivo gene expression

DP4 nanocomposite was complexed with 25 μg pGL3 control vector at N/P ratio of 20 in normal saline for in vivo gene expression and experiment was performed, as described in Chapter II.

3. RESULTS AND DISCUSSION

Dextran-PEI conjugates have been shown to possess low cellular uptake and diminished buffering capacity mainly due to their larger sizes and poor accessibility to PEI amines for protonation, which account for their low transfection efficiency [9-12]. In order to address these concerns, the present methodology was developed in such a way that i) the particle size should remain smaller, ii) buffering capacity should increase, and iii) the available charge should be accessible for DNA binding. To achieve these criteria, first, dextran was converted into its smaller sized nanoparticles using a commercially available cross linker, BDE, which were, subsequently, subjected to partial oxidation with periodate to generate aldehyde functionalities. The step resulted in the oxidation of the vicinal diols in dextran, which were then reacted with the primary amines of bPEI (25 kDa) followed by in situ reduction of the resulting Schiff’s bases with sodium cyanoborohydride (NaCNBH₃) leading to generation of DP nanocomposites (Scheme 1). Using this strategy, a series of nanocomposites was prepared and characterized physicochemically. They were then evaluated for their cytotoxicity and in vitro and in vivo transfection efficiency, respectively, and finally, compared with the standard transfection reagents.

3.1. Preparation and characterization of DP nanocomposites

Dextran was first crosslinked with varying amount of BDE (20, 30, 50 and 70%) to obtain dextran nanoparticle (DN). The step was carried out to fix the size of the particles as well as to increase the surface area for the subsequent reaction with PEI. By employing this strategy, the smallest particle size of DN was obtained, when it was 50% crosslinked with BDE (DN ~300 nm in DLS and ~171.5 nm in AFM). Therefore, subsequent studies were carried out with DN50 particles.

The percent crosslinking in dextran nanoparticles was determined spectroscopically using ¹H-NMR. Peaks centred at δ 4.84-4.85 ppm correspond to anomic protons while at δ 1.45-1.6 to methylene protons of the crosslinker BDE. The percent crosslinking was obtained by the ratio of the integrated area of anomic protons of dextran to the integrated area of methylene protons of the crosslinker. From this ratio, the percent crosslinking was found to be ~20% (attempted crosslinking was 50%). Partial oxidation with sodium periodate (NaIO₄) generated aldehydic functionalities on the DN50 nanoparticles with insignificant change in the size of the particles.
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(~320 nm) in ~85% yield. IR analysis revealed the peak at 1780 cm\(^{-1}\), which confirmed the presence of a carbonyl group in dextran-polyaldehyde (DPA) nanoparticles.

Scheme-1: Schematic presentation of preparation of DP nanoparticles.
A positive 2,4-dinitrophenylhydrazine test further established the presence of aldehydic groups on DPA nanoparticles. Subsequently, PEI (25 kDa) was reacted with varying amount of DPA nanoparticles to convert 1º amines into 2º amines to obtain dextran-PEI nanocomposites (DP1 to DP7) in ~75-80% yield after in situ reduction of Schiff’s bases with sodium cyanoborohydride. The percent grafting of dextran onto PEI was estimated using trinitrobenzenesulphonic acid (TNBS) assay and is shown in Table 1. The results of TNBS assay showed that the substitution on PEI was in the range of ~50-70 % of the attempted substitution.

**Table 1.** Extent of substitution of PEI in DP nanocomposite via TNBS assay.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Attempted substitution (%)</th>
<th>Realized substitution (%) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP1</td>
<td>1</td>
<td>0.67 ±0.03</td>
</tr>
<tr>
<td>DP2</td>
<td>3</td>
<td>2.03 ±0.16</td>
</tr>
<tr>
<td>DP3</td>
<td>5</td>
<td>3.02 ±0.15</td>
</tr>
<tr>
<td>DP4</td>
<td>7</td>
<td>4.71 ±0.27</td>
</tr>
<tr>
<td>DP5</td>
<td>10</td>
<td>5.34 ±0.38</td>
</tr>
<tr>
<td>DP6</td>
<td>15</td>
<td>8.03 ±0.86</td>
</tr>
<tr>
<td>DP7</td>
<td>20</td>
<td>10.17 ±0.67</td>
</tr>
</tbody>
</table>

3.2. Characterization of nanoparticles and their DNA complex

Further characterization of nanocomposites was carried out on the basis of their size and zeta potential. The size of DP nanocomposites, as determined using DLS, was found to be in the range 150-223 nm (Table-2), with polydispersity index < 0.3. This observation clearly revealed the formation of well-defined uniform nanocomposites. Moving down the series from DP1 to DP7, a concomitant decrease in the size of nanocomposites was observed, which might be attributed to the formation of more complex networks on increasing the amount of DPA in the nanocomposites. The size of DP nanocomposites decreased further on complexing with plasmid DNA (~194-128 nm) (Table-2). This might be due to interactions between two oppositely charged polymers i.e. DP nanocomposites and DNA, which resulted in the formation of more compacted structures. Further, in the presence of 10% FBS, the pDNA complexed DP nanocomposites showed an additional decrease in the size (~130-90 nm). The morphology and size of DN, DP4 nanocomposite and DP4/DNA complex were further analyzed using AFM, which revealed nearly spherical shaped crosslinked dextran.
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nanoparticles (~171.5 nm; Figure-1a), DP4 nanocomposite (~102.5 nm; Figure-1b) and DP4/DNA nanoplexes (~73 nm; Figure-1c). However, the size of these nanocomposites was smaller than that determined by DLS. This difference in size might be due to hydrodynamic diameter of the swollen nanocomposites in DLS measurements.

Figure 1. AFM images of (a) dextran nanoparticles (~171.5 nm), (b) DP4 nanocomposites (~102.5 nm) and (c) DP4/DNA complex (~73 nm).

Zeta potential measurements of nanocomposites showed a decline with increasing amount of DPA in the nanoparticles. This might be due to the fact that some of the 1° amines on PEI got converted into 2° / 3° amines during conjugation reaction with DPA nanoparticles, which was also observed in the previous studies [15]. A further decrease in zeta potential was also noticed when DP nanocomposites were allowed to interact with the negatively charged DNA (Table-2), which might be due to the charge neutralization. Conversely, in 10% serum, zeta potential of the DP nanoplexes and PEI polyplexes was found to be negative.

The decreased size and zeta potential with increasing concentration of dextran in the nanocomposites is probably due to the compactness and charge masking after grafting of dextran polyaldehyde nanoparticles onto PEI. The decreased particle size in the presence of serum was probably due to the loss of water to 10% serum leading to their compaction. Though the zeta potential of the DP nanocomposites was found to be lower than that of PEI (+36.5 mV), it was still sufficient to interact directly with the inner surface of the endosomal membrane and promote its disruption. Having estimated the zeta potential of DP nanocomposites (+32 mV to +18.4 mV), their interactions with negatively charged DNA were visualized by agarose gel electrophorsis.
**Table-2. Particle size and zeta potential measurements of DP nanoparticles and their corresponding DNA complexes.**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Average particle size in nm ± S.D (PDI)</th>
<th>Zeta potential in mV ±S.D.</th>
<th>Ratio of Nanoparticle:DNA (N/P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nanoparticle (in H$_2$O)</td>
<td>DNA loaded Nanoparticle (in H$_2$O)</td>
<td>DNA loaded Nanoparticle (in DMEM)</td>
</tr>
<tr>
<td>DP1</td>
<td>223±3(0.3)</td>
<td>194±2(0.18)</td>
<td>130±5(0.13)</td>
</tr>
<tr>
<td>DP2</td>
<td>208±2(0.25)</td>
<td>180±3(0.21)</td>
<td>125±3(0.17)</td>
</tr>
<tr>
<td>DP3</td>
<td>180±4(0.22)</td>
<td>165±5(0.22)</td>
<td>114±5(0.11)</td>
</tr>
<tr>
<td>DP4</td>
<td>170±3(0.18)</td>
<td>148±4(0.19)</td>
<td>110±6(0.13)</td>
</tr>
<tr>
<td>DP5</td>
<td>167±2(0.22)</td>
<td>140±4(0.21)</td>
<td>100±4(0.18)</td>
</tr>
<tr>
<td>DP6</td>
<td>158±2(0.19)</td>
<td>137±3(0.26)</td>
<td>95±5(0.16)</td>
</tr>
<tr>
<td>DP7</td>
<td>150±2(0.2)</td>
<td>128±4(0.2)</td>
<td>90±4(0.19)</td>
</tr>
<tr>
<td>PEI</td>
<td>-</td>
<td>267±6(0.36)</td>
<td>89±7(0.11)</td>
</tr>
<tr>
<td>Dextran</td>
<td>1600±24(0.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DN</td>
<td>300±6(0.3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DPA</td>
<td>320±3(0.24)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.3. DNA retardation assay

DNA retardation assay was carried out to determine the amount of DP nanocomposites required to retard the mobility of a fixed amount of DNA. It was observed that the amount of nanocomposites required to retard the mobility of a fixed amount of pDNA increased in comparison to PEI. Complete retardation of 0.3 μg of pDNA occurred at N/P ratio of 3 in case of PEI, whereas, DP nanocomposites retarded at slightly higher N/P ratios i.e. 6-9 (Figure-2), because of the charge conversion from primary amines to secondary or tertiary amines. Therefore, on increasing the percent grafting in the DP series (DP1 to DP7), the higher amounts of nanocomposites were required to retard the fixed amount of DNA.

![DNA mobility shift assay of DP nanocomposites/DNA and PEI/DNA complexes.](image)

This indicated that the degree of grafting affected the complexing ability of the nanocomposites with DNA. Having calculated the N/P ratio of nanocomposite/DNA at which DNA completely neutralized the charge on cationic polymer (i.e. N/P ratios 6-8 in case of DP nanocomposites), toxicity studies were carried out at N/P ratios, where overall charge on the nanoplexes remained positive (i.e. N/P ratios ranging from 8-30).

3.4. Toxicity assessment

Cytotoxicity is a major concern for cationic vectors to be used essentially as transfection agents for successfully delivering the DNA inside a cell. To confirm whether the formed nanocomposites fulfill these criteria, in vitro cytotoxicity of DNA complexes of DP nanocomposites was evaluated by MTT assay at various N/P ratios (8-30). Figure-3 shows that the DP/DNA complexes have low cytotoxicity ranging between 91-97% in all the three cell lines (HEK293, HeLa and HepG2).
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**Figure-3.** Cell viability profile of DP/DNA complexes at an N/P ratio of 20, PEI/DNA polyplex at an N/P ratio of 10, Superfect™/DNA, Fugene™/DNA, GenePORTER 2™/DNA and Lipofectamine™/DNA complexes in HEK293, HeLa and HepG2 cells. *P<0.05 vs. PEI and Lipofectamine™ in all the cell lines.

In comparison, cells exposed to PEI or Lipofectamine™ showed a significantly reduced viability (P<0.01). For the other commercial reagents, the cell viability was found to be in the range of 74-78% (Superfect™), 83-84% (Fugene™) and 84-86% (GenePORTER 2™) in all the three cell lines. IC₅₀ values when compared with PEI showed nearly non-cytotoxic properties of DP nanocomposites (Table-3).

**Table-3.** IC₅₀ values for PEI and DP nanoparticles in HEK293 cells.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sample Id</th>
<th>IC₅₀ mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DP4</td>
<td>253</td>
</tr>
<tr>
<td>2</td>
<td>PEI</td>
<td>19.6</td>
</tr>
</tbody>
</table>

The cationic polymers are well known to interfere with critical intracellular processes of cells, particularly, primary amines, which disrupt protein kinase C (PKC) function due to altered protein kinase activity [16]. Therefore, the reduced cytotoxicity of DP/DNA complexes might be due to the reduced positive charge on PEI (bad charge of 1° amines was converted into beneficial charge of 2°/3° amines) in DP nanocomposites. This trend was further supported by the increased cell viability of DP nanocomposites down the series on increasing the percent grafting of dextran (i.e. conversion of more and more 1° amines to 2°/3° amines). Following toxicity studies, transfection properties were evaluated in these cell lines.
3.5. **In vitro transfection**

After observing that the nanocomposites fulfilled the criterion of a potential transfection agent, i.e. being almost non-cytotoxic, transfection at N/P ratios ranging from 8 to 30 (see gel retardation assay) was carried out, as described in chapter II. After 36 h of incubation, cells were observed under fluorescence microscope for EGFP expression. After quantifying fluorescence, it was observed that the transfection efficiency of DP nanocomposites (DP1-DP7) first increased with increasing N/P ratio and then decreased beyond an optimal value (Figures 4a and 4b).

![Figure 4a](image_url)

**Figure 4a.** GFP fluorescence intensity in HEK293, HeLa and HepG2 cells in (a) absence of serum, and (b) presence of serum. *P<0.05 vs. PEI and commercial transfection reagents in all the cell lines.
Among all the tested DPs, DP4/DNA formulation exhibited the highest level of gene expression. The fluorescent intensity of GFP expression in case of DP4/pDNA formulation at N/P ratio of 20 varied significantly (P<0.01) with the fluorescent intensity in case of PEI/DNA (at N/P ratio 10). Further, the transfection efficiency was found to be cell line dependent showing maximum efficiency in HEK293 followed by HeLa and HepG2 cells. DP4/DNA complex exhibited the gene transfer efficacy ~3-7.2 folds in the absence of serum as compared to PEI (branched, 25 kDa, gold standard) and commercially available transfection reagents in HEK293 cells (Figures 4a and 4b). Likewise, the transfection efficiency of DP4/DNA complex was found to be ~4.9 and ~3.7 folds higher in comparison to PEI/DNA complex in HeLa and HepG2 cells, respectively. While transfection efficiency of the nanocomposite/DNA complexes was non-significantly affected in the presence of serum (10%), PEI-mediated transfection was indeed affected significantly (P<0.05).

To determine the percentage of cells that actually expressed the transgene, quantification of the EGFP-positive cells was carried out by flow cytometry 36 h post-transfection in HEK293 cells. It was observed that PEI/DNA showed 22±3.1% cells transfected at N/P ratio of 10, while DP4/DNA nanocomposites showed a significant increase (P<0.05) in the percentage of cells that expressed EGFP i.e. ~65±5.2% cells at N/P ratio of 20. Also, the transfection efficiency of DP4/DNA complex was found to be significantly higher (P<0.05) than that of Lipofectamine™ and Superfect™, which transfected 14±2.8% and 32±3.2% cells, respectively. Moreover, it was observed that on increasing the N/P ratio, the transfection efficiency first increased and then decreased beyond an optimal value (Figure 5). These data indicated that the transfection efficiency of DP/DNA complexes was significantly enhanced after the incorporation of dextran polyaldehyde nanoparticles. Among all the formulations, the cells transfected with DP4/DNA complex at N/P ratio of 20 had the highest gene expression level.

Figure 5. Flow cytometry analysis of DP4/DNA (at N/P ratios of 12, 16, 20, 24, 30), PEI/DNA (at N/P ratio of 10), Lipofectamine™/DNA and Superfect™/DNA in HEK293 cells.
3.6. Sequential delivery of siRNA

Having achieved the successful DNA delivery using DP4 nanocomposite, the efficacy of DP4 as a carrier for siRNA using sequential delivery method was evaluated. Here, ~82% knock-down of GFP expression was observed using DP4/pDNA/siRNA as a carrier in comparison to 53% knock-down scored by Fugene™/pDNA/siRNA complex (Figure 6). The data clearly demonstrates the potential of DP4 as a safe and efficient carrier for a variety of nucleic acids (i.e., DNA and siRNA). A number of factors may affect the transfection efficiency of the polymeric systems, i.e. buffering capacity, ability to protect DNA, the extent of DNA binding and interaction with serum proteins. Collectively, effects of these factors decide the transfection ability of the polymeric system under observation and hence are important to determine.

![Figure 6. Sequential siRNA delivery in HEK293 cells.](image)

3.7. Buffering capacity

The proton-sponge effect has been widely speculated to account for cytosolic appearance of polyplexes like PEI [13,17]. The hypothesis is based on the proton buffering capacity of PEI in the low pH range of endosomes and lysosomes, which, in turn, triggers the influx of chloride ions thus increasing the net ion concentration in the endosomes. As a result, the osmotic pressure and water influx would lead to rupture of the endosomes, releasing the polyplex in the cytosol preventing subsequent lysosomal degradation of the same [13,17]. An acid base titration was used to examine whether the grafting of dextran affected the buffering of PEI. Figure 7 shows the buffering capacity of different DPs (3, 4 and 5) and PEI in the pH range 3-10. We found that the amount of 0.1 N HCl required in bringing the pH from 10 to 3...
increased with percent grafting with DP7 showing the maximum buffering capacity. In previous studies, buffering of the nanoparticles it was found to be reduced due to steric hindrance after conjugation of dextran with PEI. Contrary to this, here, all the DPs showed enhanced buffering capacity than the native PEI. As rationalized, this might be due to an easy accessibility to PEI in DP nanocomposites for protonation. Dextran nanoparticles may have provided more surface area for the PEI to react on the surface and hence, most of the amines may be available on the surface only. This could be the basis for the enhanced buffering of DP nanocomposites over PEI.

![Buffering capacity of PEI and DP3, DP4, DP5 nanocomposites.](image)

**Figure-7. Buffering capacity of PEI and DP3, DP4, DP5 nanocomposites.**

### 3.8. Protection of pDNA against nucleases

A successful gene delivery system must have the ability to protect the complexed DNA from degradation by the nucleases present in the serum, extracellular matrix and at mucosal surfaces [18]. In this study, the ability of DP4 to provide protection to bound DNA, against DNase I, at different time intervals was investigated on an agarose gel. The gel analysis showed that naked DNA was completely degraded within 15 min while the released DNA remained appreciably intact (82.9%) in case of DP4 even after 2 h of treatment with DNase I (Figure-8). This implicated that the projected DP4 nanocomposite efficiently condensed DNA and provided sufficient stability against nucleases, which is considered as an important requisite for efficient gene delivery in vivo.
3.9. DNA release assay

To facilitate efficient gene expression, cationic polymers should not only condense plasmid DNA extracellularly, but also efficiently release DNA from polyplexes intracellularly [19]. To examine the DNA binding ability of the synthesized nanocomposites, DNA release assay was performed. It was observed that DP4 nanocomposite released up to 95±2.1% pDNA with 10 units of heparin (Figure 9), while with the same amount of heparin, only 65±3.1% of DNA was released in the case of PEI.

The easy release of DNA from nanocomposite in comparison to that observed with PEI might be due to conversion of primary amines (high charge) into secondary/tertiary amines. The compounded nanocomposites prepared by incorporating DPA onto PEI not only offered protection to loaded DNA during the course of delivery but also released it efficiently within cells.
3.10. Protein adsorption assay

Serum proteins present in the body fluid may affect the release of DNA due to their binding with the carrier systems [3]. To observe this effect, the extent of binding of serum proteins with DP4 was examined by carrying out protein adsorption assay using BSA as a standard. The results of this assay revealed that binding of BSA with DP4 reduced up to ~56% compared to branched PEI, indicating a reduction in non-specific binding of DP4 to serum proteins, thereby a possibility of improved gene carrier property of the nanocomposites over PEI (Figure-10). These observations further suggest that DP4 has a great potential as a gene carrier for *in vivo* applications.

![Figure-10](image-url). *Protein adsorption onto surface of DP4 nanocomposite compared to adsorption onto native PEI. Lanes 1) ladder, 2) only BSA, 3) DP4 incubated with BSA and 4) PEI with BSA.*

3.11. Confocal laser scanning microscopy (CLSM)

Along with the successful delivery of nucleic acids, it is pertinent to track the path of cellular entry followed by the delivery agent. In this context, confocal microscopy was performed after incubating HeLa cell with dual labeled DP4/pDNA complex at specified time intervals. Nuclear staining was carried out using DAPI (blue). Nanocomposite/DNA complex formation was evidenced by the yellow fluorescence in the overlaid images at some places (Figure-11). Red fluorescence (TMR-DP4 nanocomposite) and green fluorescence (YOYO-1-DNA) near the plasma membrane of HeLa cells within 30 min of the administration of complexes to cells, indicating the dissociation of complexes after entering the cell (Figure-11). DP4 nanocomposite was also found to carry pDNA inside the cytoplasm in less than an hour and in the nucleus within 2 h of the addition of complexes to the cells, as evidenced by the red and green fluorescence inside the cytoplasm and nucleus (Figure-11). These observations clearly show the efficient intracellular delivery of DNA using DP4, which is in agreement with a previous study reporting that PEI delivers nucleic acids to the nucleus [20].
Dextran-PEI nanocomposites as gene delivery vector

3.12. In vivo gene expression

In vivo gene expression studies were performed to extend the in vitro studies using DP4 as a gene delivery vehicle. For comparison, bPEI/DNA and naked DNA (pGL3) were used. Of all these formulations tested for gene expression, the naked DNA group exhibited the least efficacy. DP4/DNA showed maximum expression in spleen followed by lungs and brain of mice (luciferase activity in spleen and heart was ~4 and ~3.7 folds higher respectively, as compared to PEI). In lungs, brain, liver and kidney, observed luciferase activity remained non-significantly different from the other tested groups (P>0.05, Figure-12). The higher efficacy of DP4/DNA over PEI/DNA is consistent with our in vitro results.
While spleen based gene expression is poorly understood at present, it is speculated that such expression may be attributed as a consequence of immunogenic response of the said tissue. Further studies are warranted to understand the basis of such higher gene expression and their eventual consequences in target delivery.

**Figure 12.** *In vivo* gene expression analysis of DP4, PEI and naked DNA in Balb/c mice 7d post intravenous injection using pGL3 control vector as a reporter gene. *P<0.05 vs. PEI and control in the respective organs.*
4. REFERENCES


