Chapter V

Linear polyethylenimine-Graft-Chitosan Copolymers as Efficient DNA/siRNA Delivery Vectors

In vitro and In vivo
1. INTRODUCTION

Cationic polymer-mediated gene delivery has emerged as a promising alternative to the use of viral vectors for gene therapy [1-3]. These polymers have the ability to condense DNA into a more compact form (i.e. nanoparticles) that allow easier transport through cellular membrane as well as provide protection to complexed DNA against nuclease digestion [4,5]. Chitosan has shown great potential as a gene delivery vehicle owing to its biodegradable nature and nontoxic property [6,7]. However, its extensive use in biomedical applications is restricted due to its poor transfection efficiency, which is mainly due to its i) low solubility at physiological pH [8], ii) poor endosomal escape due to lack of buffering amines [9], and iii) very strong binding with DNA resulting in inefficient unpackaging of genetic material in the cytoplasm [10].

In order to address these limitations, Chang et al. [11] modified chitosan by tethering histidine moieties onto the chitosan backbone and showed enhanced transfection efficiency due to increase in buffering capacity in the pH range of endosomes and lysosomes. However, the cellular uptake was found to be lower than Lipofectamine™, a commercially available transfection reagent. Jiang et al. [12] prepared chitosan-graft-polyethylenimine (CHI-g-PEI) copolymer via condensation of periodate-oxidized chitosan and bPEI(1.8 kDa) followed by reduction of the resulting Schiff’s bases. They demonstrated comparable transfection efficiency of grafted polymer with that observed with Lipofectamine™. A number of other modifications such as trimethylation [13], pegylation [14,15], depolymerization [16, 17] and galactosylation [18] have also improved the transfection efficiency of chitosan with limited success. Alternatively, in an elegant fashion, Chen et al. [19,20] have employed a dissimilar approach in exploring the transport mechanism of PEG-modified polylysine compacted DNA nanoparticles using lipid raft-mediated endocytosis to enter the cells.

To better the transfection efficiency of chitosan well above the reported success, it was decided to introduce a cationic polymer onto the chitosan backbone using a novel approach. The projected grafting would not only circumvent all the limitations (solubility, buffering and DNA binding properties) but also maintain the integrity of the chitosan backbone. For this purpose, here, low molecular weight linear polyethylenimine, IPEI(2.5 kDa), was deliberately selected, which possesses adequate amount of buffering capacity, but is a poor transfection reagent [21,22].

In this study, chitosan- IPEI(2.5 kDa) (CP) copolymer was prepared by partially converting chitosan hydrochloride into its chlorohydrin, which further partially reacts with 2º amines of IPEI(2.5 kDa) to convert them into 3º, by keeping the total number of amines intact
in the resulting copolymer. By doing so, the IPEI(2.5 kDa) grafted copolymer now has all types of amines (1°, 2°, 3°) that are required for good buffering, proper binding and release of DNA [23]. Adopting this strategy, a small series of graft copolymers (CP-1 to CP-5) was prepared by varying the amount of IPEI(2.5 kDa). The resulting CP copolymers, soluble at physiological pH, were subsequently characterized spectroscopically and evaluated in terms of their buffering capacity, DNA binding and release capability, ability to carry nucleic acids in vitro and in vivo, and cytotoxicity. One of the graft copolymers among the series, CP-4, demonstrated ~2-24 folds higher gene delivery ability in comparison to native chitosan, IPEI(2.5 kDa), bPEI(25 kDa) and selected commercial transfection reagents such as Superfect™ and Lipofectamine™.

2. EXPERIMENTAL

2.1. Preparation of chitosan chlorohydrin (CC)

Chitosan (100 mg) was dissolved in aqueous HCl (1%, 100 ml) by stirring it overnight at room temperature. The solution was then stirred at 65 °C and added epichlorohydrin (0.25 ml). Stirring was continued for ~20 h at the same temperature and then concentrated to dryness on a rotavapor to obtain chitosan chlorohydrin hydrochloride (CC) in ~95% yield as a white powder.

2.2. Preparation of chitosan-IPEI(2.5 kDa) copolymers (CP)

CC (25 mg) and IPEI(2.5 kDa) (25 mg) were suspended in water (25 ml, 1 mg/ml) and heated to 65 °C. An aqueous solution of sodium hydroxide (2 N, 2 ml) was added dropwise to the above rapidly stirred reaction mixture. The reaction was allowed to stir for ~20 h at 65 °C. Thereafter, resulting solution was concentrated on a rotary evaporator and dialyzed against water for 2 d with intermittent change of water (4x 12 h). The dialyzed solution was lyophilized in a speed vac to obtain CP-1 copolymer in ~85% yield. Similarly, other copolymers with increasing amount (weight ratio) of IPEI(2.5 kDa), viz., 1:2 (CP-2), 1:3 (CP-3), 1:4 (CP-4) and 1:5 (CP-5) were prepared in 78-85% yield and characterized by 1H-NMR (Bruker Avance, USA).

2.3. Formation of CP/DNA nanoplexes

All CP/DNA nanoplexes were freshly prepared prior to their use. Briefly, an aqueous solution of CP-1 (1 mg/ml) was added to 1 μl of pDNA (0.3 μg/μl) at various w:w ratios (1:1, 1.66:1, 2.33:1, 3.3:1, 4:1 and 5:1) in an aqueous 5% dextrose (5 µl) and final volume was made upto 20 μl with water. The resulting complexes were vortexed and incubated for 30 min at 25±1 °C prior to their use in biophysical studies or transfection experiments. Similarly,
other CPs, chitosan, IPEI(2.5 kDa) and bPEI(25 kDa)/pDNA polyplexes were also prepared at same w:w ratios.

2.4. Size and zeta potential of copolymer and their DNA complexes by dynamic light scattering (DLS)

The prepared CPs were characterized for size and zeta potential, as described in Chapter II.

2.5. DNA mobility shift assay

DNA condensation ability of the CP copolymers was confirmed by electrophoresis. Complex formation was induced at various w:w ratios of CPs:DNA (0.33:1, 1:1, 1.66:1, 2.33:1), and the assay was performed, as described in Chapter II.

2.6. Buffering capacity

Chitosan, bPEI(25 kDa), IPEI(2.5 kDa) and CP copolymers (3 mg each, separately) were dissolved in 0.1 N NaCl to obtain concentrations of 0.1 mg/ml. Aqueous HCl (0.1 M) and NaOH (0.1 M) were used as titrators. The buffering capacity of each of the polycations was determined using pH titration from low to high, as described in Chapter IV.

2.7. DNA release assay

CP-1 copolymer was complexed with pDNA (5 µl, 0.3 µg/µl) at w:w ratio (3.33:1) and incubated for 30 min, as described above. Likewise, other CPs, bPEI, IPEI(2.5kDa) and native chitosan were complexed with DNA. Heparin, a polyanion, was added in increasing amounts varying from 0.2-35 units for the release of plasmid DNA, which was bound to the polycations and the released DNA was examined on an agarose gel, as outlined in Chapter II.

2.8. Protection of pDNA against DNase I

Protection of plasmid DNA by CP-4 (at w:w ratio of 2.33:1) was evaluated using DNase I protection assay, as described in Chapter II.

2.9. Toxicity assessment

The cytotoxicity of DNA complexes of CP copolymers/bPEI(25 kDa)/IPEI(2.5 kDa)/chitosan as well as Superfect™, Fugene™, GenePORTER 2™, and Lipofectamine™ was evaluated on HEK293, CHO and HeLa cells by MTT colorimetric assay, as described in Chapter II.

2.10. In vitro transfection

The gene carrying efficiency of CP copolymers/bPEI(25 kDa)/IPEI(2.5 kDa)/chitosan was evaluated by complexing with pDNA (0.3 µg) at w:w ratios of 1:1, 1.66:1, 2.33:1, 3.33:1, 4:1 and 5:1. Similarly, pDNA complexes were prepared with the standard transfection reagents, viz., Superfect™, Fugene™, GenePORTER 2™, and Lipofectamine™ following
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manufacturers’ protocols in the presence and absence of serum. The transfection was performed and quantified, as described in Chapter II.

2.11. Fluorescence activated cell sorting (FACS) analysis

FACS analysis was performed to examine the GFP expression levels at the individual cell level at w:w ratios of 1:1, 1.66:1, 2.33:1, 3.33:1 and 4:1, as described in Chapter II.

2.12. Proton pump inhibition

Hela cells were seeded in a 24-well plate at an initial density of 4x10^4 cells per well, one day prior to the experiment and incubated at 37 °C in a humidified atmosphere (5% CO₂-air, 95% humidity). Bafilomycin A1 (200 nM) was added just before the addition of complexes. CP-4 (at a w/w ratio of 2.33:1), chitosan and bPEI (25 kDa) (at a w/w ratio of 1.66:1) were incubated with pDNA (1 µg) and added onto the cells. Treatments without Bafilomycin A1 served as controls. After 3 h incubation, the cells were washed with 1x PBS (1 x 0.5 ml) and the medium was replaced with 10% serum containing medium. After a further incubation of 36 h, the fluorescent intensity was quantified using FACS, as described in Chapter II.

2.13. siRNA delivery

2.13.1. Sequential delivery of GFP siRNA

The potential of CP-4 to deliver siRNA was evaluated by transfecting pEGFP (1 µl, 1.4 nM) with CP-4 and Fugene™, as described above. Rests of the steps were followed, as described in Chapter II. All the experiments were performed in triplicates.

2.13.2. Delivery of JNKII siRNA

HEK293 cells were seeded in 6-well plates one day prior to transfection experiment. Subsequently, the cells were incubated with CP-4/JNK II siRNA (10 nM) (Cell Signaling Technology, USA) and CP-4/siRNA (scrambled sequence) in MEM (minus serum), and results evaluated, as reported in Chapter III.

2.14. Protein adsorption assay

The amount of protein adsorption onto the surface of chitosan and CP-4 (DNA complexes prepared at their best working w/w ratios) was examined, as described in Chapter IV.

2.15. Intracellular trafficking of CP-4 copolymer

Intracellular trafficking studies of CP-4 and chitosan were performed in HeLa cells. For this, pDNA was labeled with YOYO-1 iodide and CP-4 and chitosan were labeled with tetramethylrhodamine isothiocyanate (TRITC) and experiment was performed, as described in Chapter II.
2.16. Endosomal and lysosomal distribution of particles

Endosomal and lysosomal escape of complexes was also monitored by confocal microscopy on HeLa cells. Endosomes were labeled with Alexa Fluor 488 (100 µg/ml) and lysosomes with Lysosensor Green DND-189 (1 µM) and Lysotracker Green DND-26 (1 µM). These antibodies were added to the cells prior to addition of the chitosan and CP-4/DNA complexes containing tetramethylrhodamine-labeled pDNA and incubated for 20 min in Opti-MEM I medium. Subsequent to the addition of complexes, the cells were incubated for 1 h and then washed with 1x PBS (2 x 1 ml) followed by fixing with 4% paraformaldehyde. The images were recorded using a ZEISS LSM 510 inverted microscope.

2.17. In vivo gene expression

pGL3 (25 µg) was complexed with CP-4 at a w:w ratio of 2.33:1 and bPEI(25 kDa) at 1.66:1, and the final volume was made up to 100 µl using normal saline. Subsequent to intravenous injection, the animals were kept for 7 days on normal diet after which, they were sacrificed by cervical dislocation, all the vital organs were dissected out and experiment was performed, as described in Chapter II.

3. RESULTS AND DISCUSSION

In this chapter, the transfection efficiency of chitosan has been improved by chemical modification and while designing the strategy for the preparation of modified chitosan, the following parameters were kept into consideration, viz., (i) the modified chitosan should be soluble at physiological pH, (ii) it should have improved buffering capacity so that desired therapeutics is released in cytoplasm, (iii) it should efficiently release DNA into the cytoplasm for nuclear localization, and (iv) the modified analogue of chitosan should retain the property of chitosan with respect to biocompatibility and cell viability.

PEIs are known to possess very good buffering capacity, however, exhibit molecular weight dependent transfection efficiency. High molecular weight branched PEIs have better gene transfer capability but suffer from charge-associated toxicity. Low molecular weight PEIs are non-toxic but offer poor transfection efficiency. Taking advantage of the stated properties of low molecular weight PEIs and in order to satisfy all the above criteria, low molecular weight cationic polymers (bPEI1.8 kDa and IPEI 2.5 kDa) were selected for grafting on chitosan using a two-step methodology. In the first step, the chitosan hydrochloride was converted into its chlorohydrin derivative by the reaction with epichlorohydrin, which was subsequently reacted with 1° and 2° amines of bPEI(1.8 kDa) via in situ generation of epoxide under alkaline conditions followed by opening of epoxide ring...
to prepare a series of bPEI(1.8 kDa)-graft-chitosan copolymers by varying the amount of bPEI(1.8 kDa). The resulting copolymers were tested for their *in vitro* transfection efficiency and found to be incomparable to bPEI(25 kDa) (a gold standard). Subsequently, another series of graft-copolymers was prepared by replacing bPEI(1.8 kDa) with lPEI(2.5 kDa) in an analogous manner (Scheme-1). The percentage of grafting was determined by $^1$H-NMR, which confirmed that on increasing the concentration of IPEI(2.5 kDa), down the series, there is a gradual increase in the substitution of IPEI(2.5 kDa) on the chitosan backbone (Table-1). All the copolymers in the series were evaluated in terms of cell viability and transfection efficiency in a variety of cell lines. The transfection efficiency of this series was found to be dramatically improved i.e. ~2-24 folds higher than chitosan, lPEI(2.5 kDa), bPEI(25 kDa) and Lipofectamine™. Therefore, the study was proceeded with linear PEI to prepare chitosan-lPEI(2.5 kDa) copolymers and evaluated them for buffering capacity, DNA release and *in vitro* and *in vivo* gene expression.

3.1. Preparation and characterization of CP copolymers

Chitosan-lPEI(2.5 kDa) copolymers (CP-1 to CP-5) were prepared by increasing the amount of IPEI(2.5 kDa). Chitosan chlorohydrin and CP copolymers were synthesized, in ~95 and ~78-85% yield, respectively. The degree of grafting of chlorohydrin followed by lPEI(2.5 kDa) on synthesized copolymers was determined by $^1$H-NMR spectroscopy. From integration of signals, the substitution of 3-chloro-2-hydroxypropyl groups onto chitosan backbone was found to be ~45%, i.e. ~ every two units of glucosamine contain one 3-chloro-2-hydroxypropyl group. Further, percent substitution of linear PEI onto chitosan chlorohydrin is shown in Table-1.

Size and surface charge of nanoparticles are the vital parameters to determine their cellular uptake and interactability with the membrane. The size and surface charge of the particles formed with DNA was measured by DLS and zeta sizer. The size of the CP-1 to CP-5/pDNA complexes was measured in water and 10% serum at a weight ratio of 2.33:1 (copolymer:DNA) for the CP series, 1.66:1 for chitosan and 2:1 for IPEI(2.5 kDa). In water, moving down the series from CP-1 to CP-5, size of the DNA complexes of the particles was found to be inversely proportional with the concentration of IPEI(2.5 kDa), decrease in size of the nanoplexes may be due to the formation of more compact complexes (Table-2). In presence of serum, an additional decrease in size of the particles was observed from CP-1 to CP-5. Such a decrease in the size of the complex in water might be due to the more compactness of complexes on increasing the amount of PEI in the series.
IPEI-g-chitosan copolymer as DNA/siRNA delivery vector

**Scheme-1.** Schematic representation of preparation of IPEI-graft-chitosan (CP) copolymers.

**Table-1.** Estimation of grafted IPEI(2.5 kDa) on chitosan chlorohydrin using $^1$H-NMR.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>IPEI-graft-chitosan copolymers</th>
<th>Attempted substitution of IPEI (%)</th>
<th>Actual substitution of IPEI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CP-1</td>
<td>100</td>
<td>15.5</td>
</tr>
<tr>
<td>2.</td>
<td>CP-2</td>
<td>200</td>
<td>22.4</td>
</tr>
<tr>
<td>3.</td>
<td>CP-3</td>
<td>300</td>
<td>25.9</td>
</tr>
<tr>
<td>4.</td>
<td>CP-4</td>
<td>400</td>
<td>27.7</td>
</tr>
<tr>
<td>5.</td>
<td>CP-5</td>
<td>500</td>
<td>28.8</td>
</tr>
</tbody>
</table>
**Table-2. Particle size and zeta potential measurements of CP/DNA nanoplexes in water and serum.**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Average particle size in nm ± S.D.</th>
<th>Zeta potential in mV ±S.D.</th>
<th>Weight Ratio of Nanoparticle/DNA complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA loaded Nanoparticles (in H₂O)</td>
<td>DNA loaded Nanoparticles (in DMEM)</td>
<td>Nanoparticles (in H₂O)</td>
</tr>
<tr>
<td>CP-1</td>
<td>100±1.52</td>
<td>85±7.2</td>
<td>44.7±0.87</td>
</tr>
<tr>
<td>CP-2</td>
<td>90.4±1.56</td>
<td>75±6.4</td>
<td>48.3±4.82</td>
</tr>
<tr>
<td>CP-3</td>
<td>85±3.68</td>
<td>68±1.5</td>
<td>50.3±0.96</td>
</tr>
<tr>
<td>CP-4</td>
<td>75.6±3.26</td>
<td>60±3.7</td>
<td>53.2±2.96</td>
</tr>
<tr>
<td>CP-5</td>
<td>65.5±3.52</td>
<td>55±4.4</td>
<td>56±1.73</td>
</tr>
<tr>
<td>Chitosan</td>
<td>136.3±5.38</td>
<td>60±4.3</td>
<td>30.5±2.0</td>
</tr>
<tr>
<td>IPEI (2.5 kDa)</td>
<td>128.7±4.21</td>
<td>61±7.0</td>
<td>25.7±1.17</td>
</tr>
</tbody>
</table>

In the presence of serum, a further decrease might be due to absorption of water from the complexes by the negatively charged serum proteins. The zeta potential of the particles increased on increasing the amount of lPEI(2.5 kDa) (ranging from ~ +44.7 - +56 mV) in the copolymers. Also, the increase in zeta potential on descending the series further substantiated the attachment of IPEI(2.5 kDa) with chitosan. The decrease in size (due to increasing crosslinking leading into compactness) and increase in zeta potential (due to incorporation of more positive charge of PEI) on increasing the percent crosslinking of PEI in going down the series from CP-1 to CP-5. However, zeta potential of particles decreased after complexation with pDNA and it became negative in presence of serum.

### 3.2. DNA mobility shift assay

To establish the pDNA binding ability of the copolymers, gel retardation assay was carried out at different weight ratios. Binding of CP copolymers to DNA neutralizes the negative charge in the phosphate backbone, which resulted in the decreased mobility of DNA under the influence of current. Here, it was found that on increasing the amount of IPEI(2.5 kDa) in CP copolymers (CP-1 to CP-5), the electrophoretic mobility of DNA decreased with lesser amount of copolymers and at copolymer:DNA ratio of 1:1 (w:w) in case of CP-4 and 5, the mobility of pDNA was completely retarded (Figure-1). IPEI(2.5 kDa) also retarded the DNA mobility at the same weight ratio.
**Figure-1. DNA retardation assay of CP/DNA, chitosan/DNA and lPEI/DNA complexes.**

### 3.3 Buffering capacity

The high transfection efficacy of PEI polyplexes is attributed to their excellent buffering capacity in the endosomes [24]. The endosomal release of DNA nanoplexes in the cytoplasm is one the important parameters that depends upon the intrinsic buffering capacity of the vector system, which is measured by acid-base titration. The standard acid-base titration of lPEI(2.5 kDa) showed a considerable buffering capacity over a pH range of 3 to 10, however, due to limited solubility of CP copolymers at higher pH, the titration was carried out over a pH range of 3 to 7.5. The volume of NaOH required for bringing pH from 3 to 7.5 in case of lPEI(2.5 kDa) and bPEI(25 kDa) was significantly higher (P<0.05) than chitosan (Figure-2). Interestingly, for CP copolymers, volume of NaOH required was comparable to that of lPEI(2.5 kDa) and bPEI(25 kDa). Due to the poor buffering capacity of chitosan, their polyplexes are unable to rupture the endosomes and are degraded into lysosomes resulting in poor transfection efficiency [21]. Whereas, CP copolymers were found to possess improved buffering capacities (comparable to PEIs) and these observations were indicative of improved transfection efficiency of CP copolymers over lPEI(2.5 kDa), bPEI (25 kDa) and chitosan.
**lPEI-g-chitosan copolymer as DNA/siRNA delivery vector**

3.4. Effect of $H^+$-ATPase inhibitor

Effect of Bafilomycin A1 (a proton pump inhibitor) was observed on the transfection efficiency of CP-4, bPEI(25 kDa) and chitosan on HEK293 cells using FACS. A considerable decrease in transfection efficiency was noticed in case of CP-4 (47% ca. 8% with Bafilomycin A1) and bPEI(25 kDa) (25% ca. 6% with Bafilomycin A1), while the decrease in case of chitosan (2% ca. 1.6% with Bafilomycin A1) was insignificant (Figure-3).

**Figure-2. Buffering capacity of lPEI, chitosan and CP copolymers.**

**Figure-3. Effect of Bafilomycin A1.**

Further, to confirm the effect of buffering capacity, transfection assay was carried out in the presence of Bafilomycin A1 (a proton pump inhibitor), which resulted in the substantial decrease in the endosomal mediated transfection efficiency of PEI (~76%).
Similar results were obtained with CP-4 formulation (~83% decrease in the transfection efficiency), however, insignificant change in transfection efficiency was observed with chitosan formulation. The results indicated that CP-4/DNA complex, having comparable buffering capacity to branched PEI(25 kDa), followed pathways identical to PEI, while chitosan, which does not possess any buffering capacity, could not rupture the endosomes.

**3.5. Toxicity assessment**

The cytotoxicity of CP copolymers /DNA complexes at different weight ratios was investigated using MTT assay on HEK293, HeLa and CHO cells and compared with IPEI(2.5 kDa), bPEI(25 kDa), chitosan and the commercial transfection reagents. bPEI(25 kDa) showed significant cytotoxicity (P<0.05) in comparison to IPEI(2.5 kDa), chitosan and CP copolymers. However, IPEI(2.5 kDa) showed minimal cytotoxicity at its best working concentration on all the three tested cell lines (Figure-4). Chitosan and CP copolymers did not show any cytotoxicity as evident by healthy growth of the cells with more than 100% cell viability. The CP copolymers were found to possess almost negligible toxicity in comparison to bPEI(25 kDa) in all the cell lines tested. Since both the polymers used in this study are by themselves non-toxic to the cells, the resulting copolymers also exhibited a similar non-cytotoxic effect. bPEI(25 kDa) was found to be toxic to the cells as it is also reported that primary amino groups present in bPEI(25 kDa) cause cytotoxicity through perturbation of protein kinase activity.

![Figure-4](image-url)
3.6. In vitro transfection efficiency

Having confirmed the non-cytotoxic nature of the CP copolymers, the gene transfer ability of these copolymers was evaluated on a variety of cells, viz., HEK293, CHO and HeLa cells, using enhanced green fluorescent protein (EGFP) as the reporter gene. Here, transfection efficiency of CP copolymers was compared with IPEI(2.5 kDa), bPEI(25 kDa), chitosan and selected commercial transfection reagents, viz., Superfect™, Fugene™, GenePORTER 2™ and Lipofectamine™. The same w:w ratios of copolymer:DNA (see gel retardation assay) were used in all the transfection experiments. Transfection efficiency of CP copolymers at w:w ratio 2.33 was found to be significantly higher (P<0.05) than that of IPEI(2.5 kDa), bPEI(25 kDa) and chitosan at w:w ratio of 1.66, in the presence and absence of serum (Figure 5). Interestingly, CP-4 among the series of synthesized copolymers displayed ~20 and 24 folds (P<0.05) higher GFP expression in HEK293 cells in comparison to chitosan and IPEI(2.5 kDa), respectively, both in the presence or absence of serum. Further, with CP-4, EGFP expression was ~1.3-3.0 folds higher (P<0.05) in this particular cell line as compared to bPEI(25 kDa), GenePORTER 2™, Superfect™, Fugene™ and Lipofectamine™ both in the presence or absence of serum (Figure 5). A similar trend was observed in the other two tested cells, viz., HeLa and CHO.

The enhanced transfection efficiency of CP copolymer might be due to increased solubility (~10 mg/ml) at physiological pH and improved buffering capacity due to IPEI(2.5 kDa) grafts. Higher transfection efficiency of CP-4/DNA complexes in the presence or absence of serum was well supported by an observation wherein little or negligible quantity of BSA was adsorbed onto the surface of CP-4/DNA complex in comparison to IPEI(2.5 kDa)/DNA and chitosan/DNA complexes (see protein adsorption assay). Therefore, diminishing interaction between serum and CP-4 could be due to the presence of hydrophilic chitosan, a sugar moiety, known for its properties to reduce interactions with serum proteins. It was further observed that transfection efficiency increased with increase in the weight ratio of IPEI(2.5 kDa), reached up to a maximum for CP-4 and thereafter started decreasing indicating CP-4/DNA as the best carrier among the series of synthesized copolymers. Thus, in this study, w:w ratio was found to have an important impact on the transfection ability of the synthesized copolymers and CP-4/DNA, among the tested transfection agents, emerged as the best formulation.
IPEI-g-chitosan copolymer as DNA/siRNA delivery vector

**Figure-5.** GFP fluorescence intensity in HEK293, HeLa and CHO cells in (a) absence of serum, and (b) presence of serum. **P<0.01 vs. PEI, chitosan and commercial transfection reagents in all the cell lines.

### 3.7. FACS analysis

To find out the cell population expressing GFP, FACS analysis was performed with the HEK293 cells transfected with copolymer:DNA/complex over a range of w:w ratios. The maximum percentage (~45%) of GFP positive cells transfected with CP-4 was at a w:w ratio of 2.33:1; however, GFP expression regressed with a deviation from the above ratio (Figure-6). In contrast, chitosan and IPEI(2.5 kDa) showed only 3 and 2% GFP positive cells, respectively, at a w:w ratio of 1.66:1. While only ~26% GFP positive cells were scored after transfection with bPEI(25 kDa), a gold standard for the transfection studies, at
a w:w ratio of 1.66:1; ~16% and ~35% GFP positive cells were observed following transfection with selected commercial reagents, viz., Lipofectamine™ and Superfect™, respectively.

![Graph showing transfection efficiencies](image)

**Figure-6.** Percent transfection efficiencies of CP-3, CP-4, and CP-5/DNA nanoplexes determined using FACS at various w/w ratios and compared with IPEI, chitosan, Superfect™ and Lipofectamine™. **P<0.01 vs. PEI (at all w:w ratios), Superfect™ and Lipofectamine™.**

### 3.8. siRNA Delivery

The versatility of the test copolymer, CP-4, as an efficient carrier for nucleic acids was established using two types of siRNAs, viz., GFP specific and JNKII on HEK293 cells. CP-4 was able to deliver both as could be seen by the suppression in the expression of targeted genes. The delivery of two types of siRNAs, viz., GFP specific and JNKII, was tested on HEK293 cells. The transfection was performed in a sequential manner using CP-4/GFP system, as described in chapter II. The observed knockdown of GFP expression by CP-4/siRNA system was found to be ~77%, whereas only ~49% knockdown was recorded with Fugene™/siRNA system (Figure-7a). As a proof of principle, another siRNA, JNKII, which is activated by a variety of environmental stresses, was delivered using CP-4 copolymer [30]. Using western hybridization, nearly 60% knockdown of JNKII was observed after 36 h of treatment, which was comparable to that observed with the commercial transfection reagent, Lipofectamine™ (~55%) (Figure-7b). This further boosts the potential of CP-4 as a versatile gene delivery agent.
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3.9. DNA release assay

To achieve successful transfection, it is a prerequisite that the bound pDNA must be released from the polyplex. The extent of binding of DNA to the cationic polymer is one of the major factors influencing transfection efficiency. The binding ability of CP-4, chitosan and IPEI(2.5 kDa) nanocomposites with pDNA was studied by incubating the polyplex solution with increasing amounts of heparin, a competitive anionic moiety, and subsequent release pattern of pDNA was recorded on gel electrophoresis. As expected, only 30% of bound DNA was found to be released from chitosan even after addition of 35 U of heparin, whereas ~96% DNA got released from IPEI(2.5 kDa) in presence of 2.5 U heparin and 72% DNA was released from bPEI(25 kDa) with 5 U heparin. Interestingly, 92% of DNA released was observed from CP-4 copolymer in presence of 5 U of heparin (Figure 8). Chitosan has been reported to interact with pDNA via strong electrostatic/hydrogen bonding or via hydrophobic interactions [11]. The interactions between chitosan and pDNA are quite strong and do not get affected even at very high salt/detergent concentration as well as in presence of high concentration of heparin [25]. The binding ability was found to be in the order Chitosan>>>bPEI(25 kDa)>CP-4>IPEI(2.5 kDa). These observations suggested that binding capability of CP copolymer was neither too strong nor loose, which further accounted for its high transfection efficiency.
IPEI-g-chitosan copolymer as DNA/siRNA delivery vector

Figure-8. DNA release assay of CPs, lPEI(2.5 kDa), bPEI(25 kDa) and chitosan.

3.10. Protection of pDNA against nucleases

Having evaluated the binding and release of DNA from the copolymer, CP-4, it becomes mandatory to examine the integrity of pDNA bound to copolymer against nucleases. DNase I protection assay was performed and the results analyzed by 0.8% agarose gel electrophoresis. Figure-9 clearly depicts that while naked DNA (0.3 μg) was found to be degraded within 15 min of incubation with DNase I, pDNA complexed with CP-4 copolymer did not show any significant degradation (~15% after 2 h). Thus, the present finding suggests that the synthesized copolymer, CP-4, has potential to be used for in vivo administration of nucleic acids.

Figure-9. DNase I protection ability of CP-4 copolymer.
3.11. Nuclear distribution of nanoparticles through CLSM

After successful transfection was achieved by CP copolymers, it was rationalized to track the path of cellular entry of DNA through confocal microscopy. To investigate the path of cellular entry, tetramethylrhodamine-labeled CP-4 was complexed with YOYO-1-labeled pDNA and the resulting complex was added on to HeLa cells, which were fixed with paraformaldehyde at specified time intervals. It was observed that CP-4 was able to carry pDNA to the insides of the cells (cytoplasm) within 30 min of the addition onto the cells. Further, both pDNA and CP-4 were observed inside the nucleus after 1h of the addition of the complexes, and after 2h, maximum amount of fluorescence was observed inside the nucleus only (Figure 10).

![Confocal microscopic images of HeLa cells treated with TMR CP-4/YOYO-1-pDNA nanoplex at different time points: In each image, the first quadrant (i) shows images captured under fluorescein filter, the second quadrant (ii) shows images captured under rhodamine filter, the third quadrant (iii) shows the cells observed under DAPI filter and the fourth quadrant (iv) represents the overlaid images.](image)

The observations clearly state that the CP copolymers are capable of efficiently carrying the desired gene inside the cells. The buffering capacity was also evaluated through confocal microscopy for the CP-4/DNA and chitosan/DNA complexes [11]. HeLa cells, pre-labeled with endosomal and lysosomal dyes (green), were incubated with tetramethylrhodamine (TMR) labeled (pDNA)/chitosan and CP-4 complexes (red). After
imaging through confocal microscopy, it was observed that in case of chitosan, red and green regions were overlapping, showing the entrapment of these complexes within these compartments (Figure-11), while, in case of CP-4, separate red and green regions were observed indicating the release of these complexes from these compartments. The merged green and red regions (Figure-11), in case of chitosan, showed that these complexes could not escape the endosomes and lysosomes, whereas, in case of CP-4/DNA complex, independent scattered red and green areas showed that these particles came out of endosomes efficiently.

Figure-11. Endosomal and lysosomal escape of complexes of chitosan/TMR-pDNA and CP-4/TMR-pDNA. HeLa cells were used as models to observe the endosomal escape. After 24 h incubation, medium of 100 mg/mL Alexa Fluor 488 transferrin conjugate (a), 1 μM LysoTracker Green DND-189 (b), or 1 μM LysoSenser Green DND-26 (c) was added to each well before complex administration. The distribution of fluorescence was observed by confocal microscopy.
3.12. Protein adsorption assay

Sugars are known to reduce the non-specific interactions with blood serum proteins and increase the blood circulation time. To validate the observation, the amount of protein adsorbed on to the surface of CP-4/DNA was evaluated on SDS-PAGE taking BSA as a standard. The amount of protein adsorbed on to the surface of CP-4 was evaluated on SDS-PAGE taking BSA as a standard. It was found that binding of BSA significantly reduced with test copolymer CP-4, as evident on the gel in comparison to lPEI(2.5 kDa) (Figure-12). Interestingly, CP-4 displayed much reduced interactions with serum proteins in comparison to PEI. Reduced binding with serum proteins (BSA) suggests that the synthesized copolymer, CP-4, has potential to be used for in vivo administration of nucleic acids.

![Figure-12. Protein adsorption onto surface of CP-4 compared to adsorption onto native lPEI(2.5kDa). Lane 1: Protein Marker, Lane 2: CP-4 with PBS, Lane 3: CP-4 with BSA, Lane 4: lPEI with PBS, Lane 5: lPEI with BSA, and Lane 6: BSA.](image1)

3.13. In vivo gene expression

Having achieved the significant level of transfection in vitro, the efficacy of CP-4/DNA was further examined for its in vivo applications and used firefly luciferase expression-reporter system in Balb/c mice. The maximum luciferase activity in spleen (P<0.05) followed by heart and brain in the mice after seven days as compared to bPEI(25 kDa) (Figure-13). Chitosan and lPEI(2.5 kDa) were not used in this study, since none of them showed any significant transfection in vitro. The higher uptake and transfection efficiency of CP-4 might be due to presence of sugar moieties which helped in reducing the interactions with serum proteins present in the blood and hence, also prolonged the circulation time in the blood. [26] Luciferase activity was majorly observed in spleen, brain and heart. Spleen and liver have irregular fenestrations and also endothelia with large meshes, which allow
extravasation of molecules up to 0.1-1 μm. [27] Surprisingly, negligible luciferase activity was observed in liver and at present, no plausible explanation can be provided for the same. The encouraging results of improved gene expression in the brain may be useful for brain targeting but increased gene expression in spleen indicated higher clearance of the vector by this organ and as a result shortened gene expression in the other organs. Although several reports are available on the expression and tissue distribution of the test gene(s), [17, 28] little is known about the mechanism of distribution.

**Figure-13.** In vivo gene expression analysis in Balb/c mice 7 days post intravenous injection using pGL3 control vector as a reporter gene. *P<0.05 vs PEI in the respective organs.
IPEI-g-chitosan copolymer as DNA/siRNA delivery vector

4. REFERENCES

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