Chapter 4:

Development of PEI Based siRNA Delivery System

4.1 Introduction

Non-viral vectors composed of cationic polymers are preferred over lipid based carrier when greater stability is desired in physiological environment [1]. Cationic polymers contain several amine groups in their backbone which interact with negatively charged siRNA leading to the spontaneous formation of nanosized complexes. Among these cationic polymers, PEI, especially branched PEI, is well-known as good transfection reagent due to its intrinsic proton sponge property [2]. However, but due to high toxicity and lack of biodegradability, it has no clinical applications. In contrast, linear PEI (LPEI) is comparatively less toxic, but it displays low transfection capacity [3]. The high transfection capacity of bPEI has been attributed to the unique ratio of 1^{o}:2^{o}:3^{o} amines of 1:2:1 imparting it a typical ionization behavior. However, LPEI, being consisted of only secondary amines, fails to behave similar to bPEI, as most of amines get protonated in pH range of 8.2 to 9.5 [4, 5]. The ionization behavior of polymers is major determinant of vector perfrormace as it governs the properties such as particle size and surface charge, proton sponge, transfection and toxicity. The reported values in literature LPEI ionization are inconsistent. Ogris et al. have reported that LPEI is more than 90% protonated at physiologic pH [6].

Chemical modification of LPEI may impart favorable properties to LPEI for efficient transfection and reduced toxicity. Recent studies have been focused on acetylation of PEI in order to reduce membrane disruptiveness and associated toxicity. However, this approach, though reduced toxicity, was found to reduce the buffering capacity required during endosomal capacity [7]. Further, PEGylation of PEI was also reported to reduce surface charge, increased dispersion stability at high concentrations, decrease protein binding and erythrocyte aggregation, and prolong blood circulation. However, such modification lead to decrease in binding affinity between DNA/siRNA and the polymer which may need higher quantities of polymer to deliver same required dose and subsequently higher body exposure to non-biodegradable polymer [8-10]. Kichler et al. prepared covalent conjugate of LPEI with polyethyleneglycol, they reported a decrease in binding affinity, however; observed improvements were not significant compared to naked siRNA [11]. Further some have reported backbone modification to

reduce the transfection efficiency [12]. Several modifications of LPEI have been tried for improved transfection efficiency, however most of them involved tedious and multistep synthesis [3, 11, 13-16]. Therefore, we devised a simple modification of LPEI to influence its ionization behavior and consequently the biological properties.

4.2 Experimental

4.2.1 Materials and chemicals

LPEI (22 kDa) was obtained from Polychemistry, USA. bPEI (25 kDa); 2-bromoethanol and high retention dialysis tubing (cut off = 12 kDa) were obtained from Sigma-Aldrich, Bangalore, India. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Agarose, Tris, ethidium bromide (EtBr) were purchased from Himedia, Mumbai, India; ethanol was purchased from Spectrochem, India, potassium carbonate (K_2CO_3) was from Qualigens, India.

4.2.2 Modification of LPEI

LPEI was modified to achieve different degrees of substituion on LPEI backbone to obtain hydroxyethyl susbtituted LPEI (HELPEI). Briefly, 500 mg of LPEI was dissolved in ethanol in a RBF. After complete dissolution 2-bromoethanol was addded to the solution at different molar ratios of amines in secondary amine content. To the reaction mixture 3.2 g potassium carbonate was added as proton abstractor. The RBF was kept for refluxing under stirring for 48 h and increamental quantity of 1.6 g K_2CO_3 was added at 12 and 24 h. After completion of reaction the mixture was centrifuged and supernatant collected and evaporated under vacuum (400 mmHg) on rotary evaporator at room temperature. The residue obtained was reconstituted in double distilled water and dialyzed against double distilled water to remove salts and any other low molecular impurities until the replaced dialysate no longer remains basic due to K_2CO_3, when measured using pH meter. The retentate of the dialysis were lyophilized to obtain the dry residue of HELPEI.

4.2.3 Characterization

4.2.3.1 Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra of HELPEI were recorded using the potassium bromide (KBr) disc technique. Minimal quantities of samples were mixed with potassium bromide using a clean glass pestle and a mortar. It was finally compressed to obtain a pellet and FTIR was performed on Bruker-αt FTIR spectrophotometer. Baseline was corrected and the samples were scanned against a blank KBr pellet background in the range of 4000-400 cm^{-1} wave number with a resolution of 1.0 cm^{-1}.

4.2.3.2 Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H-NMR spectroscopy was performed to study the structural changes in in polymer. ^{1}H NMR spectra of HELPEI were recorded on Bruker Avance II 400 NMR spectrometer at 400 MHz. Unmodified PEI and HELPEI were dissolved in D_2O and ^{1}H NMR spectroscopy was performed with a 5 mm probe.

4.2.3.3 Degree of substitution

The degree of substitution was determined from the NMR. All the reactions were set at mole to mole basis to get the desired degree of substitution (DS), while the DS of representative samples was confirmed by H1-NMR using the relation. The extent of substitution was determined by calculating percentage of peak integration value of C_2H_4 in hydroxyethyl substituent to C_2H_4 in ethylene backbone of LPEI.

For calculation the peak integration at chemical shifts of δ 2.3 to 2.9 were considered for C_2H_4 in ethylene backbone, and δ 3.25 to 3.61 for C_2H_4 in hydroxyethyl substituent. All integration were performed in triplicate.

4.2.3.4 Solubility

Although the monomeric unit of LPEI is identical to that of the much more familiar branched (bPEI), the physical properties of both polyelectrolytes differ considerably. The linear PEIs are solids at room temperature whereas bPEIs are liquids at all molecular weights. LPEI is soluble in hot water, cold water at low pH, methanol, ethanol and

chloroform. They are insoluble in cold water, benzene, ethyl ether, and acetone. They have a melting point of 73-75°C. They can be stored at room temperature.

Therefore, the effect of substitution on physico-chemical properties of HELPEI such as solubility was performed. For this, transmittance of LPEI and HELPEI solutions were measured at different pH values. Briefly, PEIs were dissolved in 0.1 N HCl (1 mg/ mL), the pH of solution was adjusted from 5.0 to 12.0 by using 0.1M NaOH solution. The transmittance of solution at wavelength of 600 nm as a function of pH value was recorded on a UV–visible spectrophotometer (UV-1800, Shimadzu).

4.2.3.5 Proton sponge

The buffering capacity of the LPEI and HELPEI from pH 10 to 3.5 was determined by acid–base titration as reported in literature [17, 18]. Briefly, 10 mg of the polymer was dissolved in 150 mM NaCl solution. The solutions were adjusted to an initial pH of 10.0 using a 1N NaOH solution and then titrated with 0.1 N HCl solutions. The pH values were recorded with a pH Meter (Lab India, Mumbai). The electrode was calibrated with buffer solutions of pH 4.0, 7.0 and 9.2. The buffering capacity was also determined as an indicator of endosomal escape capacity [19]. It was defined as the percentage of amine groups becoming protonated from pH 7.4 to 5.1 (mimic the pH change from the extracellular environment to the lower pH of the endosomes), and can be calculated from the following equation:

$$\text{Buffer capacity (\%)} = \frac{\Delta V X\ 0.1\ M\ X\ 100}{N\ mol}$$

wherein V is the volume of 0.1N HCl added to bring the pH of the polymer solution from 7.4 to 5.1, and N mol is the total moles of protonable amine groups in the given amount of cationic polymers [20].

Additionally, the ratio of protons consumed in intervals of 7.4-5.1 to 10-7.4 was also tracked as another indicator of effect of DS on basicity of amines and shift in pKa of LPEI.

4.2.3.6 Preparation of polyplex

Since the complexation efficiency of LPEI is function of charge density which is again a function of pH of vehicle used for polyplex preparation. Therefore, all the polyplex were prepared in 7.4 pH sodium acetate buffer (20 mM) with batch volume of 20 ul. The required amount of polymer stock solution (10 mg/mL) was added to vehicle. The effect of incubation time and presence and absence of vortexing was studied to optimize the polyplex preparation at room temperature. Therefore, mixture was then gently vortexed for 2 min and then, 100 pmole of siRNA was added to it and incubated for 20, 40, 60 mins at room temperature.

4.2.3.7 Gel retardation assay

To study the interaction of LPEI and HELPEI with the siRNA at different n/p ratio, agarose gel electrophoresis was applied. For LPEI and siRNA monomer weight of 43 and 330 were used for calculating n/p ratio. For HELPEIs n/p was calculated after compensation for increase in molecular weight of LPEI. Briefly, all the incubated polyplexes (20 µl) were mixed with 3µl of 6X DNA gel loading buffer (Himedia, India) and loaded onto a 4% agarose gel containing 0.5ug/mL ethidium bromide and electrophoresed at 50 V in TBE buffer pH adjusted to 7.4. After 70% run siRNA was visualized by UV trans-illuminator using Gel Doc System (Bio-Rad Lab., USA). The n/p ratio for complete retardation of siRNA were determined.

4.2.3.8 Size and zeta

The hydrodynamic diameter of the polyplexes was determined by using dynamic light scattering using Zetasizer, Nano ZS series (Malvern Instruments, Germany). The prepared polyplexes were diluted appropriately with NFW pH 7.4 and measured at 25°C. Similarly, zeta potential was measured by applying Smoluchowski's equation in the zeta sizer software. All the experiments were performed in triplicates.

4.2.3.9 Complexation efficiency

The complexation efficiency at the n/p ratio resulting in complete retardation in gel electrophoresis, centrifugation-UV spectrophotometric analysis was performed. The siRNA polyplex were centrifuged at 25000 rpm for 45 min at 4°C. The aqueous

supernatant of these centrifuged samples was separated and analyzed for siRNA content using NanoDrop UV spectrophotometer.

4.2.3.10 Determination of siRNA Integrity

The polyanion competition assay using heparin was used to assess the effect on siRNA integrity. The complex formulation after incubation was mixed with Heparin sodium (1mg/mL) and incubated for 20 min. After incubation, the content of siRNA displaced was detected on gel electrophoresis and analyzed as described in Chapter 3.

4.2.4 Biocompatibility studies

4.2.4.1 Hemolysis study

A fresh heparinized blood from rat was centrifuged at 1000g for 10 min at 4°C. The obtained erythrocyte pellet was washed with phosphate buffer saline pH 7.4 until the supernatant became clear. Finally, erythrocytes were reconstituted in PBS pH 7.4 to get 2% v/v suspension. Separate 500 ul cell suspension containing different concentrations of the polymer were prepared in eppendrofs. The mixture was thoroughly mixed by vortex and incubated at 37°C for 1 h under constant shaking. After incubation, cell suspension was centrifuged at 3000 rpm for 10 min and the supernatant was analyzed for hemoglobin release by its absorbance at 540 nm. PBS and 1% Triton was used as negative and positive control respectively, it was also ensured that polymer itself does not contribute to any absorbance at 540 nm. The % hemolysis was calculated using the formula:

$$\text{Haemolysis} = \frac{A_s - A_n}{A_p - A_n} \times 100$$

Where, A_s is the absorbance of supernatant, A_n is the absorbance of negative control treated cell supernatant, A_p is the absorbance of positive control treated cell supernatant. All assays were performed in triplicate and data are reported as mean ± SD [21, 22]

4.2.4.2 Erythrocytes aggregation

Cationic polymers are known to induce erythrocyte aggregation and can be used to evaluate biocompatibility of new polymers. Therefore erythrocyte aggregation study using rat blood cells was performed. The erythrocyte suspension was prepared by the

same protocol as that in Hemolysis study. 500 ul 2% erythrocyte suspension containing polymers at different concentration were prepared in PBS pH 7.4 mixed thoroughly by vortexing and incubated for 2 h at 37°C. The erythrocyte aggregation was visualized using inverted microscope (Nikon Eclipse TS100) in a phase contrast mode.

4.2.5 Cytotoxicity of polymer

Cell membrane destabilization before cellular internalization or chromosomal DNA interaction after cellular internalization could be the cause behind the cytotoxicity of free PEI [23]. Common method for determining cell viability such as dye exclusion (trypan blue) can be used. However 3-(4, 5-dimethylthiazole-2-yl)-2, 5-di-phenyl tetrazolium bromide (MTT; Himedia, India) assay is rapid and versatile method [24].

Therefore, MTT assay was carried out in varying concentrations of polymer. The CFBE41o- cell were seeded at density of 5000 cells/well in a 96 well microtiter plate (corning, New York) using EMEM supplemented with 10% Fetal Bovine Serum (FBS) [25]. The culture were grown for 24 hrs in incubator maintained at 5% CO_2 concentration and humidified with saturated CuSO4 solution. After 24 hr the cells were exposed to formulations at different polymer concentrations and evaluated for 6 hr. After exposure period the media was replaced with complete medium containing 10% FBS and 1% antibiotic. After 24 h, cells were washed with PBS and 20 ul of MTT reagent (5mg/mL) solution was added to each well plate. The reagent was allowed to react for 4 h under incubator condition, after which the medium in each plate was replaced with 100 ul of Dimethyl Sulfoxide (Himedia, Mumbai) and plate was shaken to dissolve the formazan crystals. The color of the dissolved formazan was measured using microtiter plate (Biorad, California). Cells treated with EMEM and 0.2% Triton X were used as negative and positive control. The absorbance values of cells treated with EMEM were taken as 100% cell viability and all other treatments were expressed relative to it.

4.2.6 Stability challenge studies

4.2.6.1 Salt induced aggregation

The electrolyte induced flocculation has been proposed to evaluate the steric stability of PEGylated liposomes [26]. The salts can neutralize the surface charge over the nanoparticles, required to maintain balance between competitive forces of van der Waals attraction and either electrostatic repulsive forces, leading to destabilization and aggregation [27]. PEGylation can compensate for aggregation due to the striping of electrostatic double layer by counterion adsorption [28, 29]. Further, the test also challenges the electrostatic affinity between the cationic polymer and anionic siRNA, through competition for binding as a counter-ion and through its effect on dielectric properties of the medium.

To assess the stabilizing effect of hydroxyethyl substitution against salt, polyplexes were prepared at 100 nM siRNA concentration. Accurately weighted quantities of NaCl were mixed to obtain concentration of 1%, 2%, 3%, 4%, 5% w/v NaCl and particle size of each formulation was measured using dynamic light scattering after each addition.

4.2.6.2 Heparin displacement assay

The extracellular matrix is replete with polyanionic glycosaminoglycan which may interact with the cationic carriers leading to displacement of siRNA. Therefore formulations were exposed to heparin competition assay to evaluate their susceptibility to displacement. The polyplexes were prepared at optimized n/p ratio. After that the polyplexes were exposed to increasing heparin concentrations and polyplex dissociation was monitored by gel electrophoresis. The heparin/siRNA ratio required to displace siRNA from polyplex was noted in each case. The amount of siRNA release at each ratio was quantified by densitometry.

4.2.6.3 Serum stability study

In order to simulate the *in vivo* conditions the developed polyplexes were analyzed for serum stability. Naked siRNA and polyplex prepared as per the previously stated protocol were incubated with 10 µL FBS at 37°C for such that final incubation volume had 50% serum concentration. The samples were analyzed at different time intervals through gel

electrophoresis to detect whether polyplexes were stable, partially stable or unstable. In order to eliminate any nuclease activity 0.5 M EDTA was added to the sample. The polyplexes were treated with 1 mg/mL heparin per sample after defined time interval so as to dissociate the polyplex. The released siRNA at all the time points were compared with the untreated free siRNA sample to detect any degradation effect. Any degradation of siRNA would affect the band properties or intensity of the test. Further, the densitometry quantification developed was used to quantify siRNA content remaining at different time points.

4.2.6.4 Stability in bronchoalveolar lavage Fluid

For formulations supposed to be administered through intravenous route the serum stability of polyplex is essential while for formulations administered to lungs stability to extracellular substances present in the airways fluid is essential. Rats were euthanized using pentobartital (75 mg/mL) intraperitoneal injection. Carefully, the trachea was exposed and cannulated with catheter (20-gauge). 1.5 mL of cold sterile PBS was instilled thrice through the trachea into the lung to recover BALF at 50% to 60% of the original volume. To separate any debris or cells, BALF was centrifuged for 10 min at 1500 rpm. The optimized 10 µL of formulation containing 100 pmole siRNA were prepared and it was treated with 10 µL of BALF. Intact siRNA retained in each treated sample was determined at intervals of 15 min over a period of 2 hr using gel electrophoresis. Briefly, polyplexes were analysed by heparin competition assay. After incubation the nuclease activity was inactivated by addition of 0.5 M EDTA solution (1 µL of EDTA solution for 10 µL of BALF used) [30]. EDTA treated samples were exposed heparin at a concentration of 1 mg/mL to ensure 100% release of siRNA from polyplexes, which were detected by gel electrophoresis as described in chapter 3.

4.2.7 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was performed to study the morphology of particles using Technai 20 Transmission Electron Microscope. Briefly, a sample drop was placed onto 300 # carbon coated copper grid. The surface water was removed from the grid by tapping with filter paper. After 5 mins, using a probe the grid was placed in a

sample holder and inserted into microscope and observed at 200 kV accelerating voltage with suitable 25x to 75000x.

4.3 Result and discussion

4.3.1 Characterization of LPEI

The FT-IR spectra showed the chemical changes in the LPEI. The intensity of the peak due stretching vibrations of secondary amine (-NH-) was reduced after substitution of the backbone, as they were converted to tertiary amines. The broad peak due to stretching vibrations of –OH was observed in HELPEI (Fig. 4.1).

The ^{1}HNMR spectrum showed that in case of LPEI, the protons of C_2H_4 in ethylene backbone shows peaks at chemical shift (δ) values of 2.3 to 2.9 while after modification the presence of protons of C_2H_4 in hydroxyethyl substituent in HELPEI show downfield shift in range of δ 3.25 to 3.61 due to presence of de-shielding effect of –OH group (Fig. 4.2).

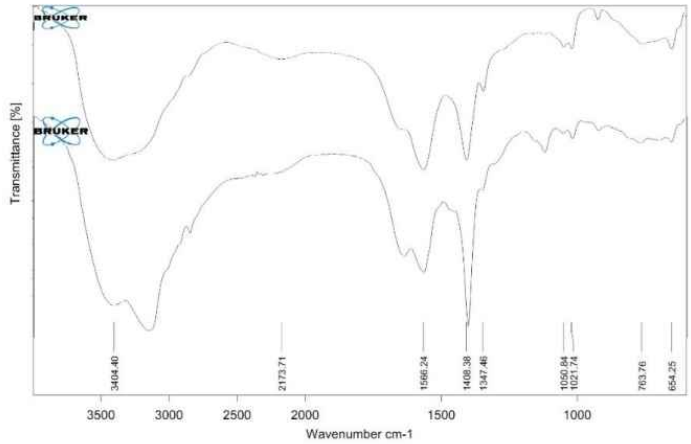

Fig. 4.1: IR overlay of LPEI and HELPEI

Based on the chemical shift integrations were performed in the scanned NMR spectra of HELPEIs and degree of substitution by hydroxyethyl group was determined. The results are shown in the Table 4.1.

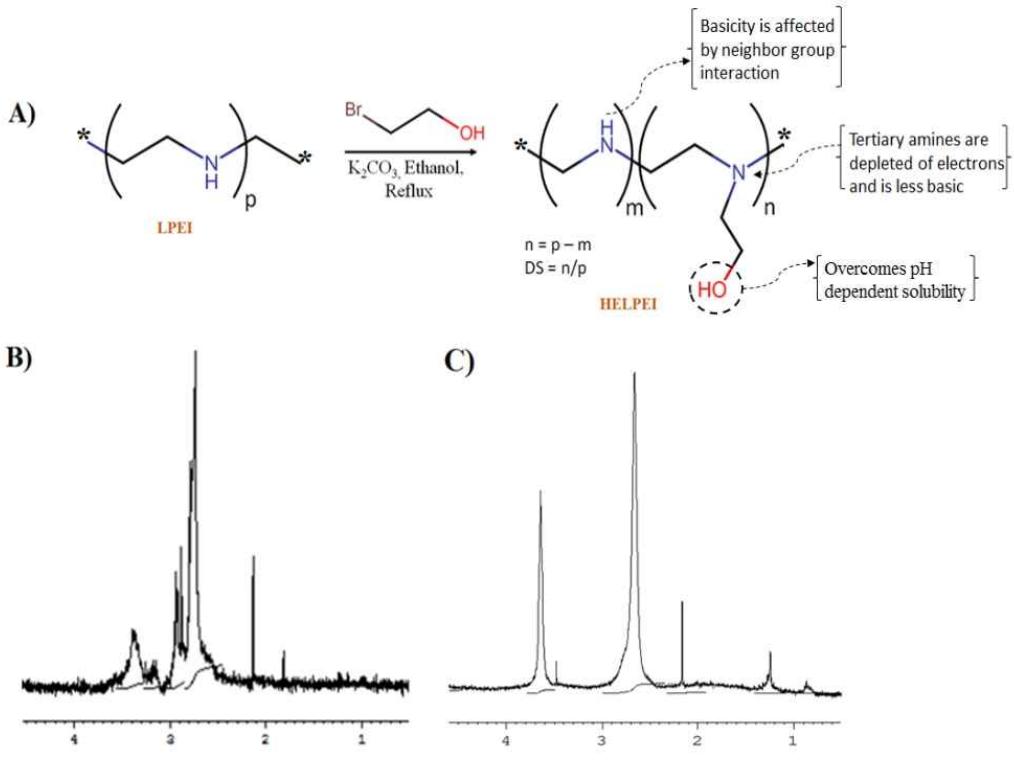

Fig. 4.2: A) Illustrative scheme for preparation B) ^{1}HNMR spectrum of LPEI; C) 1HNMR spectrum of HELPEI-45

Table 4.1: Degree of substitution determined by ^{1}HNMR

Polymer	Targeted DS	Obtained DS
LPEI	0	0
HELPEI-15	15	11.75
HELPEI-25	25	22.17
HELPEI-35	35	33.43
HELPEI-45	45	43.25

4.3.2 Solubility

The modification of LPEI led to favorable effects on the hydrophobicity and solubility of the polymer. The pH solubility profile of LPEI and HELPEIs was generated from pH 6.0 to 11.0 at room temperature, since LPEI is completely soluble in water below 6.0 pH, any improvements in solubility could be detected at pH greater than 6.0. Uncharged LPEI is insoluble in water at temperatures below 55°C, the pH of resulting solution is around 9.2.

The transmittance values at 600 nm showed that at pH values less than 8.0, LPEI has transmittance greater than 90%. However, as the pH was increased above 8.0 there is tremendous drop in the % transmittance to less than 20 (Fig. 4.3). As per literature reports, LPEI becomes soluble when it is more than 20% ionized, which occurs at pH values less than 9.0 which is the cause of observed drop in transmittance of unmodified PEI at pH values greater than 8.0. On the contrary all HELPEIs with DS of 25 % and above showed transmittance values greater than 94 % at all pH values. Fig. 4.3 shows the summary of transmittance of LPEI and HELPEI at different pH. This also showed that DS of 15% was not sufficient to impart hydrophilicity to overcome pH dependent solubility of LPEI. This implies that hydroxyethyl group introduces hydrophilicity to polymer backbone which overcomes precipitation of LPEI even at higher pH where it is un-ionized, meaning that protonation of amine is no longer required, as in case of LPEI, to keep HELPEI dissolved.

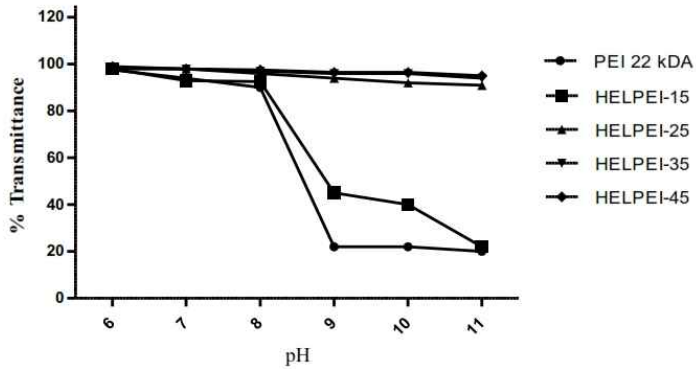

Fig. 4.3: Summary plot of transmittance of LPEI and HELPEI at different pH

4.3.3 Proton sponge

Buffer capacity is one of the most essential trait sought in a gene vector, which plays role in destabilization of endosome and releases the polyplexes from endosomes. Previous studies with PEGylated PEI reported that grafting of PEG to PEI have resulted in reduction in buffer capacity due to shielding effect of PEG [31, 32]. The pKa of the LPEI solution is influenced by the low molecular weight salt concentration added due to the screening of the electrostatic potential around LPEI chain by the salt therefore all the titrations were performed in 150 mM NaCl solution. The pKa shifts to higher values when increasing the NaCl concentration.

In the present investigation we studied the effect of increasing DS on the ionization behavior of LPEI. It is solely composed of secondary amines while primary amine only at the terminal end of linear chain. Therefore, all the properties of LPEI can be said to originate from secondary amine. The secondary amines have pKa in the range of 9.0. In present case, with increasing DS in HELPEIs the secondary amines are converted to tertiary amines, which are depleted of electron density and thereby have reduced basicity compared to primary and secondary amine. The order of basicity of amines in aqueous media is as follows:

Primary amine > Secondary amine > Tertiary amine

Further, all the secondary amines of LPEI are not protonated at same pH, since the titratable sites on a polyelectrolyte are close together, the protonation state of a site will electrostatically affect the protonation of nearby sites. The influence will be significant in LPEI, since only two methylene units separate the nitrogen. The analogy can be made from ethylenediamine, which requires a significantly lower pH (pK_2 of 7.08) for protonation of the second amine to occur after the first amine group (pK_1 of 9.89) has been protonated. Additionally, as a chain becomes increasingly charged, the cumulative build-up of multiple charge-charge interactions could lead to significant deviation in the protonation behavior from that of an independent single site.

In order to assess the quantitative effect of modification of LPEI on protonation behavior, the titration curve was divided into different regions. The region 10 to 7.4

indicates the approximate amount of protonated amines at physiologic pH. Fig. 4.4 shows that LPEI curve was inclined in this region and therefore becomes most cationic at physiologic pH. While all HELPEIs curves were steep in this region due to reduced protonation in this region. Further, the comparison of ratio of protons consumed in pH range of 7.4-5.1 (endosomal pH) to the protons consumed in pH range of 10-7.4, showed an increasing trend with increasing DS. Finally, the buffer capacity calculations (Table 4.2) showed that, the buffer capacity of LPEI was lowest i.e. 10.32, which increased with increasing DS and it even crossed buffer capacity of bPEI (the standard used in proton sponge assays).

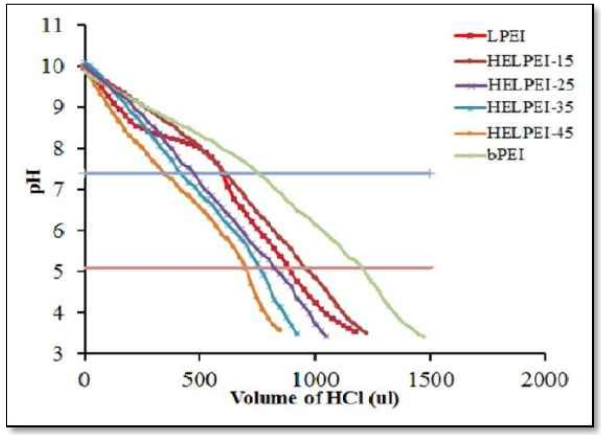

Fig. 4.4: Titration of LEPI and HELPEI with 0.1 N HCl

Table 4.2: Summary of polymer characteristics

Polymer	DS	Molecular weight (Da)	Ratio of HCl*	Buffering capacity
LPEI	0	22000	0.31 ± 0.022	10.32 ± 0.22
HELPEI-15	11.75	24645	0.34 ± 0.018	13.53± 0.35
HELPEI-25	22.18	26993	0.39 ± 0.029	15.33 ± 0.22
HELPEI-35	33.43	29514	0.44 ± 0.038	17.31 ± 0.34
HELPEI-45	43.25	31738	0.50 ± 0.028	18.28 ± 0.27
bPEI-25 kDa	---	----	0.38 ± 0.034	17.85 ± 0.25

*Ratio of HCl consumed in pH interval of 7.4-5.1 to 10-7.4,

4.3.4 Preparation of polyplex

Based on comparison of band density for sample incubated for times 20, 30, 60 min it was concluded that there was no significant effect of incubation time and vortexing. Therefore, in all experiments incubation time was kept as 30 min and vortexing for 2 min was performed. The n/p ratios for complete retardation was determined and complexation efficiency was determined. The results are reported in next section.

4.3.5 Gel retardation assay

The n/p ratios were determined for polyplex preparation (Table 4.3) by using amines of polymers and phosphate of siRNA. Obtained molecular weight of polymer DS of the polymer were used to find out number of amines involved in the formation of polyplexes.

The n/p ratio was different for different DS. For LPEI the n/p ratio for complete retardation was 4 (Fig. 4.6). This is because, LPEI typically have at least 55% of the amine groups protonated at physiological pH of 7.4 and the charges on LPEI chain are arranged in nearly alternating fashion which results in strong electrostatic interaction [33-35].

In contrast, HELPEIs showed a steady increase in n/p ratio required for complete retardation (Fig. 4.7, Fig. 4.8, Fig. 4.9, and Fig. 4.10). With increase in DS the binding affinity of HELPEI to siRNA is reduced. The effect could be attributed to the reduction in charge density after substitution. As discussed in proton sponge study, during substitution the secondary amines are converted to the tertiary amines which have lower ionization potential than that of secondary amines present in the LPEI. Literature also reports that after substitution with long chain PEG molecules there is a significant reduction in binding efficiency [36, 37]. This was attributed to shielding of portions of charges from PEI by PEG strands. This significant reduction in binding efficiency compels intake of higher amount of non-biodegradable polymer to deliver the desired dose of siRNA. The substitution with low molecular weight hydroxyl ethyl group could be advantageous as it has less screening effect than PEG, while the major reason for reduced affinity is because of reduced ionization of PEI backbone rather than screening of the ionized portion.

Table 4.3: The n/p ratio required for complete gel retardation

Sr No	Formulation	DS	N/P Ratio
1.	LPEI	--	4
2.	HELPEI-15	11.75	5
3.	HELPEI-25	22.17	6
4.	HELPEI-35	33.43	8
5.	HELPEI-45	43.25	15

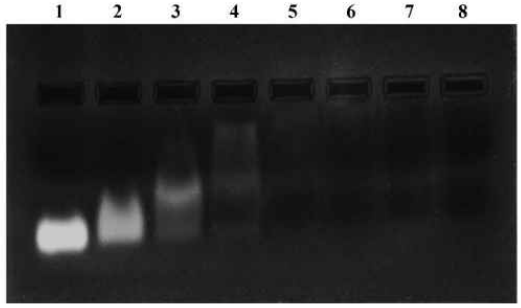

Fig. 4.5: Gel retardation of LPEI
N/P ratio→Lane 1: Naked siRNA; Lane 2: 1.0; Lane 3: 2.0 ; Lane 4: 3 ; **Lane 5: 4**; Lane 6: 5.0 ; Lane 7: 6.0 ; Lane 8: 7.0).

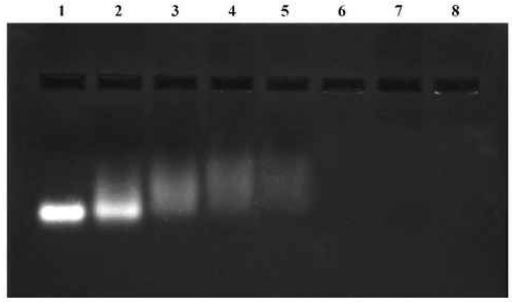

Fig. 4.6: Gel retardation assay of HELPEI-15
N/P ratio→Lane 1: Naked siRNA; Lane 2: 1.0; Lane 3: 2.0 ; Lane 4: 3 ; Lane 5: 4; **Lane 6: 5.0** ; Lane 7: 6.0; Lane 8: 7.0).

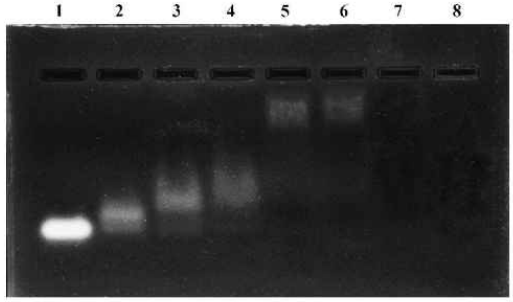

Fig. 4.7: Gel retardation assay of HELPEI-25
N/P ratio→Lane 1: Naked siRNA; Lane 2: 1.0; Lane 3: 2.0 ; Lane 4: 3 ; Lane 5: 4; Lane 6: 5.0 **Lane 7: 6.0** ; Lane 8: 7.0).

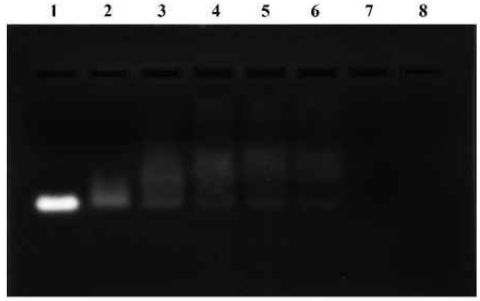

Fig. 4.8: Gel retardation assay of HELPEI-35
N/P ratio→Lane 1: Naked siRNA; Lane 2: 1.0; Lane 3: 2.0 ; Lane 4: 3 **;** Lane 5: 4; Lane 6: 6.0 ; Lane 7**: 8.0** ; Lane 8: 10).

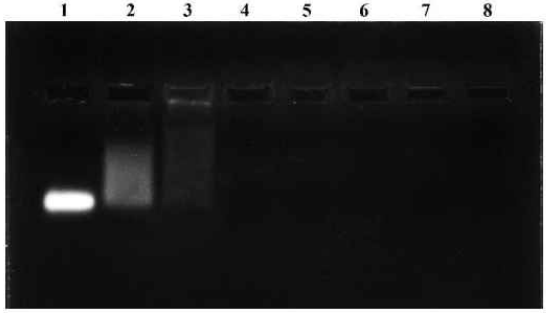

Fig. 4.9: Gel retardation assay of HELPEI-45
N/P ratio→Lane 1: Naked siRNA; Lane 2: 5.0; Lane 3: 10; **Lane 4: 15**; Lane 5: 20; Lane 6: 25; Lane 7: 30; Lane 8: 35).

However, as the DS is increased from 35% to 45% there was an anomalous increase in n/p required for complete retardation, with almost doubled amount of polymer requried. This indicates that the charge reduction was non-linear with DS. This deveation could be attributed to the fact that with increase in DS the chain becomes increasingly charged, causing a cumulative build-up of multiple charge-charge interactions leading to significant suppression of protonation behavior of remaining nitrogens [5].This also means that to deliver same does of siRNA, higher quantity HELPEI-45, a non-biodegradable substance would be introduced in the body. Therefore, optimum delivery system should be balance between siRNA stability and adverse effects brought by vector.

4.3.6 Size and zeta potential

The purpose of the study was to confirm whether the polymers can form polyplexes in the desired size rage with sufficient positive zeta potential for efficient cell attachment and internalization. At n/p ratio showing complete gel retardation all the polyplexes showed particle size 150-350 nm. The size of polyplexes formed from HELPEIs with lower DS were comparatively smaller than with higher DS at the given n/p ratio, showing that there is decrease in the affinity of polymer with higher DS. However, as the n/p was increased the sizes were reduced to <200 nm due to increased condensation. The PDI values should reflect the stoichiometry in interaction of siRNA and PEI polymer and were within acceptable range. Therefore, optimized n/p ratios were greater than n/p ratio required for complete gel retardation to account for smaller size and higher zeta potential.

Since zeta potential is strongly influenced by pH, all the measurements were made at physiologically relevant pH 7.4. The results showed that polyplexes were able to maintain the positive charge after complexation with anionic siRNA molecules. However, as DS increased the zeta potential was found to decrease. The decrease in zeta potential could be the result of reduced ionization of the amines on the polymeric backbone at measurement pH of 7.4. As expected the increase in n/p values led to increase in zeta potential.

Table 4.4: Particle size and zeta potential of LPEI and HELPEI polyplexes

Formulation	N/P ratio	Particle Size (nm)	Mean PDI	Zeta Potential (mV)
LPEI	4	168.7±4.5	0.219±0.013	26.2±1.54
	6	129.8±2.3	0.147±0.009	29.4±1.61
HELPEI-15	5	185.3±3.8	0.215±0.018	25.8±2.50
	7	135.2±3.2	0.152±0.014	28.1±1.68
HELPEI-25	6	213.5±4.6	0.237±0.017	24.1±1.84
	8	138.8±2.5	0.171±0.012	26.6±1.55
HELPEI-35	8	248.9±4.7	0.259±0.025	18.3±2.79
	10	156.5±5.6	0.175±0.015	21.2±1.70
HELPEI-45	15	311.2±6.2	0.287±0.028	17.3±2.81
	17	195.7±4.4	0.213±0.019	17.5±2.87

*Values are represented as mean±SD, n=3

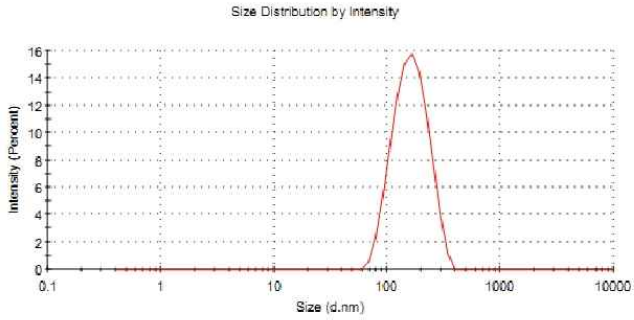

Fig. 4.10: Malvern Zeta sizer report of HELPEI-35 polyplex

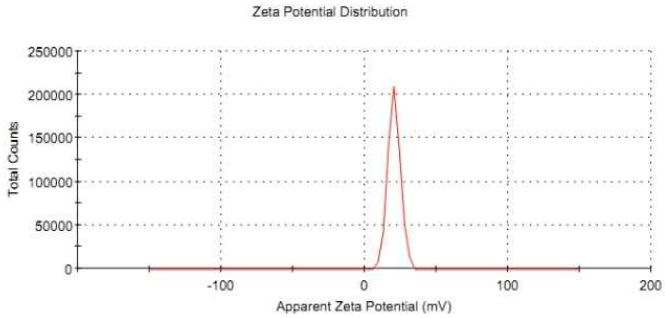

Fig. 4.11: Malvern Zeta Potential Report of HELPEI-35 polyplex

4.3.7 Complexation efficiency

Both the LPEI and HELPEIs were able to condense more than 95% of siRNA at their optimized n/p ratios (Table 4.5).

Table 4.5: Complexation efficiency at the optimized n/p ratio

Sr No	Formulation	N/P Ratio	Complexation Efficiency (%)
1.	LPEI	6	97.29±1.36
2.	HELPEI-15	7	98.54±2.04
3.	HELPEI-25	8	97.37±1.29
4.	HELPEI-35	10	97.09±2.44
5.	HELPEI-45	17	95.48±2.62

*Values are represented as mean±SD, n=3

4.3.8 Determination of siRNA integrity

The integrity of siRNA after complexation was retained as it was before complexation as shown in Fig. 4.12. When we compared with fresh naked siRNA it did not show any degradation or instability of siRNA incorporated.

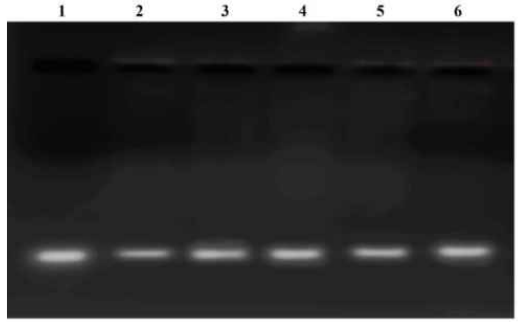

Fig. 4.12: Figure showing the integrity of siRNA

Lane 1: Naked siRNA; Lane 2: LPEI; Lane 3: HELPEI-15; Lane 4: HELPEI-25; Lane 5: HELPEI-35; Lane 6: HELPEI-45.

4.3.9 Biocompatibility study

4.3.9.1 Hemolysis

Though cationic charge is a facilitator of cell membrane attachment and subsequent endosomal uptake, however too much of cationic charge leads to membrane destabilization and hemolysis [38-40]. During hemolysis polymers produce nano-sized pores in cell membrane inducing an influx of solutes into the cells which causes rupture by destabilization of membrane and release of hemoglobin molecules. Along with charge, the polymer structure and conformation also plays important role in mediating toxicity, wherein rigid and helical conformations are said to be more membrane permeabilizing [41-43].

In the present case LPEI caused extensive hemolysis (>10%) at concentration ranging from 10 to 2000 ug/mL (Fig. 4.13). As stated earlier LPEI is more than 50% ionized at pH 7.4 leading to charge dependent toxicity [5, 34, 37]. We also included bPEI to understand the behavior of two native PEIs available. It was evident that the bPEI exhibited less hemolysis compared to LPEI at all concentrations, which could be because of bPEI being comparatively less ionized at pH 7.4, as only 25% of the total amine content is 1^{o} and 50% is of secondary amine which are partially ionized [44, 45]. Besides charge the linear verses branched nature might also be responsible for the observed difference.

However, the polymer modification from HELPEI-15 to HELPEI-45 led to significant decrease in hemolysis as shown in Fig. 4.15 (P-value < 0.05). This can be attributed to combined effect of increase in hydrophilicity by incorporation of hydroxyethyl substituents, the decrease in charge density due to conversion of secondary amine into tertiary amine. Besides charge reduction the backbone substitution may also make polymer more flexible affecting the conformation which is responsible for membrane adsorption and permeabilization [41]. Further, the HELPEI-15 and HELPEI-25 with DS less than 25% caused considerable hemolysis meaning that level of substitution was not sufficient to bring about significant changes in biocompatibility of polymer. In continuation of same, HELPEI-35 and HELPEI-45 with higher DS were completely non-toxic which caused < 3% hemolysis at all the concentrations (Fig. 4.14).

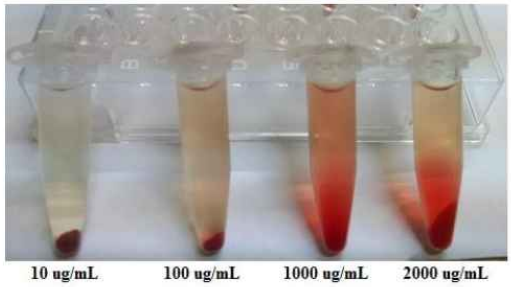

Fig. 4.13: Result of hemolysis study of LPEI

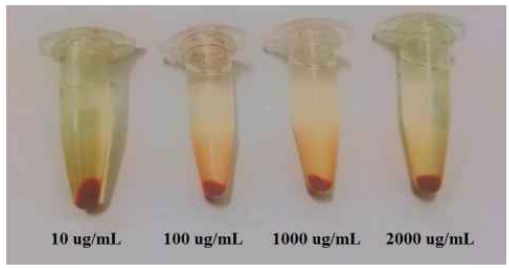

Fig. 4.14: Result of hemolysis study of HELPEI 35

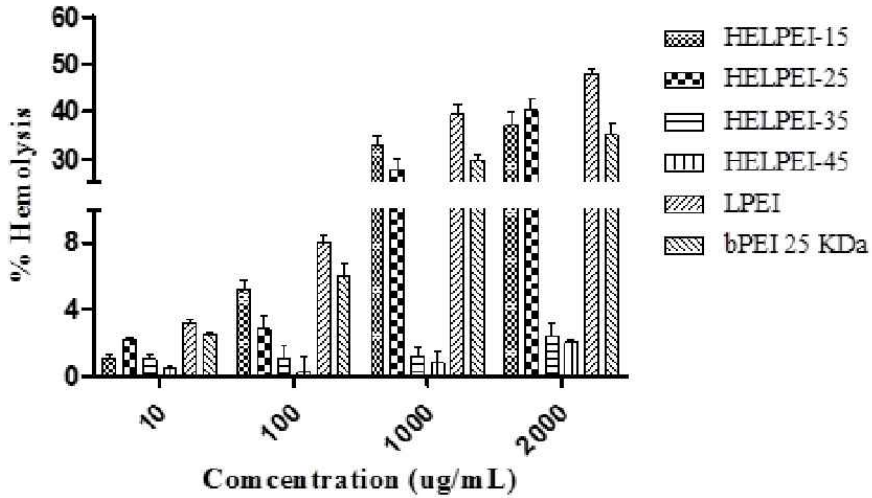

Fig. 4.15: Hemolytic potential of different HELPEIs at different concentrations.

4.3.9.2 Erythrocyte aggregation study

During aggregation process the polymer molecules first interact with the surface of colloids and then bridge/chains inter-tangle with each other leading to flocculation and aggregation [46-48]. As shown in **Fig. 4.16**. It was observed that LPEI induced significant amount of erythrocyte aggregation at all concentrations selected. LPEI, owing to its significant positive charge and long linear structure can interact with anionic cell membranes surface glycoprotein and bridges cells into aggregates.

However, in case of HELPEIs the erythrocyte aggregation was found to be reduced with increase in DS. The HELPEI-15 and HELPEI-25 showed aggregation as that of LPEI indicating that the cationic charge present even after 25% DS were sufficient to interact with cell membrane and induce aggregation. When DS reached 35% and 45%, resultant decrease in charge density and favorable change in conformation could be responsible for HELPEI-35 and HELPEI-45 not interacting with the cell membrane and therefore no aggregation was observed. Similar reduction in erythrocyte aggregation as well as plasma protein binding upon PEGylation of PEI based DNA complexes [21, 49].

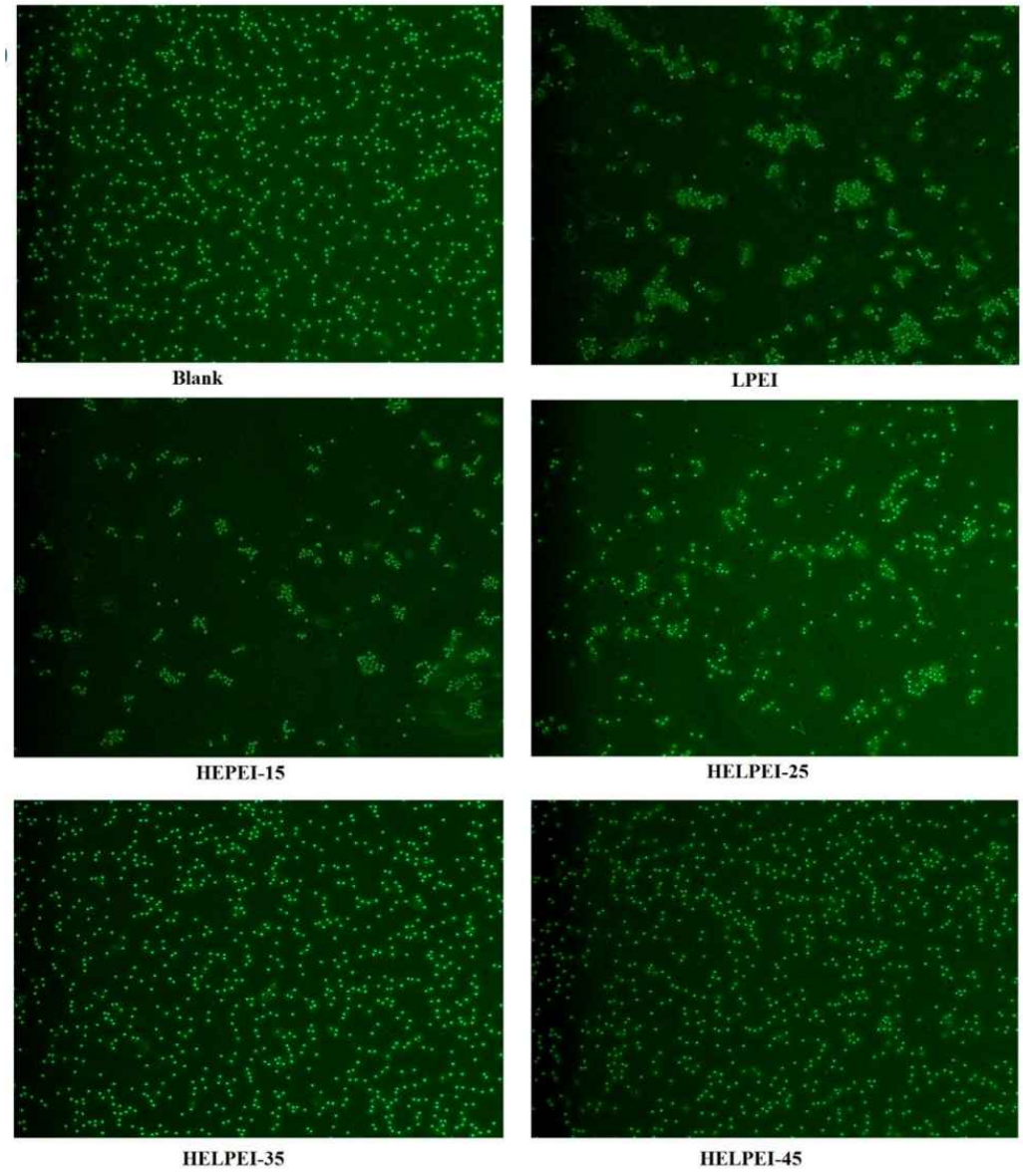

Fig. 4.16: Results of erythrocyte aggregation study for LPEI and HELPEIs

4.3.9.3 Cytotoxicity of polymer

The cytotoxicity of LPEI and HELPEIs was determined *in vitro* in CFBE41o- cell line. The biocompatibility of any polymer is reported to be governed by polymer properties such as molecular weight, charge density and type of charge bearing functionality, structure (block, random, linear, and branched) and conformational flexibility [42, 50, 51]. Different methods to reduce cytotoxicity are based on charge reduction and surface coating with hydrophilic polymer such as PEG [52]. As shown in Fig 4.17, LPEI was found to be very toxic and it reduced the cell viability to ~15 % at 80 µg/mL and was less than 55% at all the concentrations tested. PEI exhibits toxicity in two stages: first rapid phase (30 min), affecting the membrane integrity due to phosphatidylserine translocation from inner plasma membrane to outer cell surface; second delayed phase (24h), due to formation of channels in outer mitochondrial membrane and alteration of mitochondrial membrane potential [43, 53, 54].

In case of HELPEIs, it was observed that the cytotoxicity decreased with increase in DS. As there was no significant increase in molecular weight of polymer compared LPEI (Table 4.2), the reduction in toxicity can be attributed to the decrease in the charge density at physiologic pH of 7.4, alteration in structural and conformational property, as well as increased hydrophilicity due to presence of hydroxyethyl substituents. At DS of 15% and 25% there was significant toxicity meaning that the charge density is still above the minimum toxic levels. However, after further increase in DS, the HELPEI-35 and HELPEI-45 showed significant improvement in cell viability compared to others and showed cell viability of ~90% and more at all the concentrations tested.

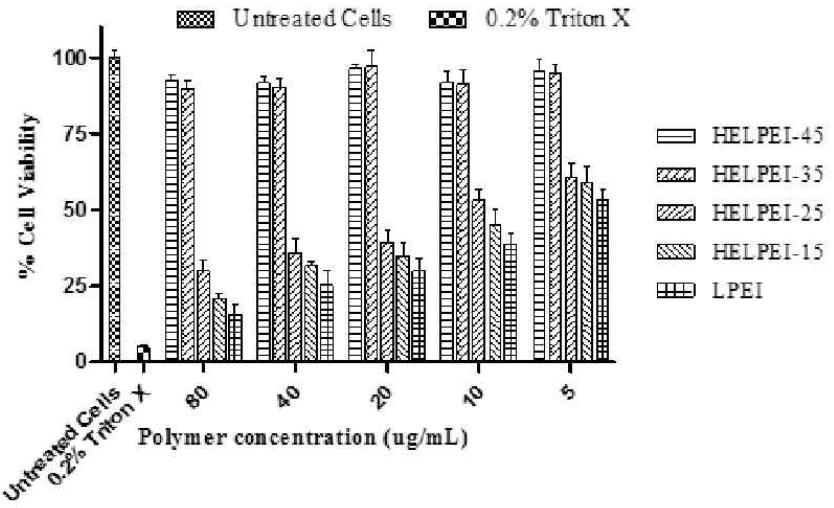

Fig. 4.17: Comparison of cytotoxicity of polymers

4.3.10 Stability challenge studies

4.3.10.1 Salt induced aggregation

Table 4.6 and Fig. 4.18 shows the results of electrolyte induced aggregation. The polyplexes were diluted with using NaCl. The LPEI polyplexes showed a dramatic ~1.76 fold increase in size after salt additions. This means that the stability of LPEI was largely governed by the positive surface charge and balance between attractive and repulsive force, and showing a behavior similar to lyophobic colloid under salt addition [28]. The addition of counterions from salt neutralize those charges leading to particle aggregation. However, the HELPEI polyplexes retained size to considerable extent and fold increase in size decreased with increase in DS. HELPEI-35 and HELPEI-45 showed only 1.19 and 1.17 fold increase in initial size. HELPEI derived significant portion of its stability from the hydrophilic hydroxyethyl chains, rather than ionized functional groups, which are not affected by salt addition [55]. The behavior becomes similar to that of lyophilic colloids, which derive stability from hydrophilic substituents and are less sensitive to salt additions.

Table 4.6: Electrolyte induced flocculation of polyplex

Concentration of NaCl	Particle size (nm)				
	LPEI	**HELPEI-15**	**HEPEI-25**	**HEPEI-35**	**HEPEI-45**
0%	135.0±2.62	138.2±2.50	145.3±2.70	149.2±2.48	178.4±2.66
1%	150.4±2.22	154.2±3.47	156.0±3.37	152.8±2.26	181.5±3.09
2%	161.5±2.98	164.1±3.19	165.4±4.81	156.3±3.43	189.6±3.52
3%	189.7±2.30	178.0±4.68	178.8±3.00	164.4±4.62	198.5±4.18
4%	205.3±4.46	194.8±4.13	185.5±4.47	172.0±4.15	204.0±3.88
5%	238.1±6.86	214.3±5.15	197.6±4.66	178.2±5.61	208.6±5.17
Increase in folds at 5%	1.76	1.55	1.35	1.19	1.17

Values are represented as mean±SD, n=3

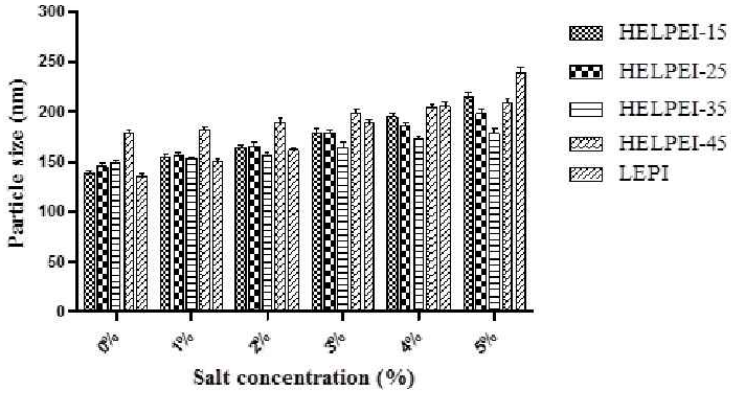

Fig. 4.18: Electrolyte induced flocculation of polyplex.

4.3.10.2 Heparin displacement assay

The resistance to heparin competition assay depends on ratio of siRNA to heparin. Additionally, it also give idea about whether the formulations were made at appropriate n/p ratio as it directly extrapolates to their transfection efficiency [30, 56, 57]. Stability up to heparin/siRNA weight ratio of >1 is considered appropriate for achieving adequate in vivo stability [58, 59]. LPEI formulations showed displacement from heparin to siRNA weight ratio of 2 (Fig. 4.19), and 100% dissociated for heparin/siRNA ratio of 4.

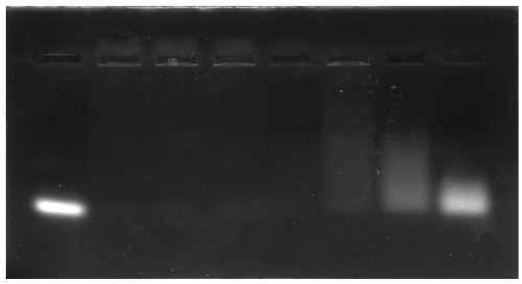

Fig. 4.19: Heparin displacement of LPEI

Heparin/siRNA weight ratio Lane1: siRNA, lane 2: 0.25, lane 3: 0.5, Lane 4: 1.0**,** Lane 5: 1.5, **Lane 6: 2**, Lane 7: 3, Lane 8: 4.

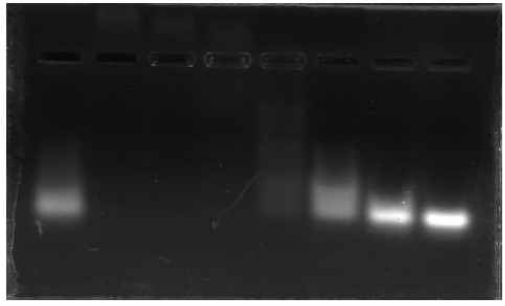

Fig. 4.20: Heparin displacement of HELPEI-35

Heparin/siRNA weight ratio Lane1: siRNA, lane 2: 0.25, lane 3: 0.5, Lane 4: 1.0, **Lane 5: 1.5**, Lane 6: 2, Lane 7: 3, Lane 8: 4.

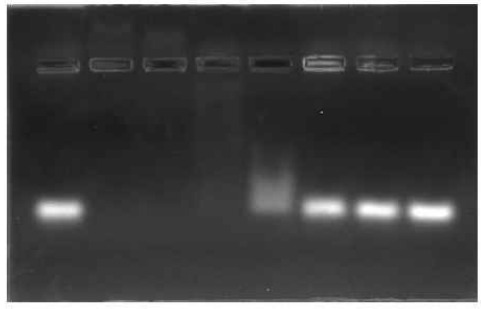

Fig. 4.21: Heparin displacement of HELPEI-45

Heparin/siRNA weight ratio Lane1: siRNA, lane 2: 0, lane 3: 0.5, **Lane 4: 1.0**, Lane 5: 1.5, Lane 6: 2, Lane 7: 3, Lane 8: 4

The HELPEI-35 and HELPEI-45 were evaluated at their optimized n/p ratio. It was found that HELPEI-35 started releasing siRNA from heparin/siRNA weight ratio of 1.5 and completely release at 3 (Fig. 4.20). HELPEI-45 started releasing siRNA from heparin to siRNA weight ratio of 1.0 and completely release at 2 (Fig. 4.21). Thus HELPEI-35 was having higher resistance to heparin displacement than HELPEI-45. This can be attributed to low charge density of HELPEI-45 than HELPEI-35

4.3.10.3 Serum stability study

The ability of polymer to protect siRNA can be evaluated by incubation with serum to simulate the cell culture media. The serum stability is essential since the polyions present in the extracellular matrix *in vivo* and *in vitro* cell culture media can compete with siRNA and displace them, thereby exposing to the degradation conditions [60].

Fig. 4.22 and Table 4.7 show the results of serum challenge study. Both the PEI based formulations showed good stability in serum challenge study. The HELPEI-35 showed excellent stability at the optimized n/p ratio which was evidenced as absence of degraded siRNA in gel electrophoresis. The band of the siRNA release after heparin treatment of samples had comparable intensity to free siRNA at all-time points. On the other hand naked siRNA showed degradation in 6h. HELPEI-45 showed less retention compared to HELPEI-35 though it retained more than 80% of siRNA. This indicates that the association between siRNA and HELPEI-45 is weaker than that in the HELPEI-35.

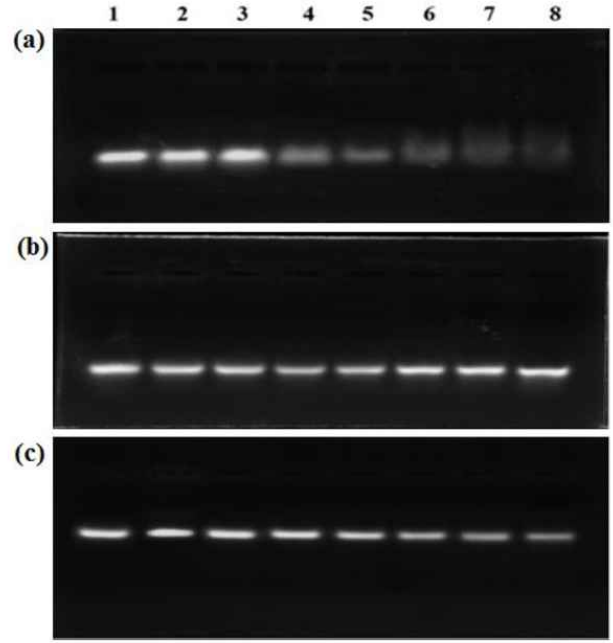

Fig. 4.22: Gel electrophoresis of serum stability

(a) Naked siRNA; 1=0h, 2=0.5h, 3=1.0h, 4=2.0h, 5=3.0h, 6=4.0h, 7=5.0h, 8=6.0h

(b) HELPEI-35 polyplex; 1=0h, 2=1.0h, 3=2.0h, 4=4.0h, 5=8.0h, 6=16h, 7=20h, 8=24h

(c) HELPEI-45 polyplex; 1=0h, 2=1.0h, 3=2.0h, 4=4.0h, 5=8.0h, 6=16h, 7=20h, 8=24h

Table 4.7: Serum stability of polyplex as compared to naked siRNA

Time (hr)	% siRNA Retained	Time (hr)	% siRNA Retained	
	Naked siRNA		HELPEI-35	HELPEI-45
0	100.00	0	100.00	100.00
0.5	86.38±1.89	1	98.19±1.18	98.35±1.78
1.0	72.44±2.31	2	96.55±2.61	96.32±2.11
2.0	42.39±2.46	4	93.43±3.19	92.89±2.79
3.0	29.58±3.78	8	94.48±3.84	88.54±3.30
4.0	18.58±3.54	16	94.14±2.28	86.22±3.09
5.0	11.72±4.85	20	95.54±3.22	83.61±2.85
6.0	6.09±4.21	24	95.12±4.16	80.29±2.36

4.3.10.4 Stability in bronchoalveolar lavage fluid

siRNA released from formulation after incubation for different time periods with BALF was determined using densitometry. The results are shown in Table 4.8, it was observed that with increase in incubation time siRNA retained was decreasing Fig. 4.23. This was attributed to the competitive displacement from negatively charged surfactants present in BALF. However, the release was very low and in all the cases more than 92% of siRNA was retained. HELPEI-35 showed higher retention and it was attributed to the higher charge density than HELPEI-45. Thus, form the results of the BAL fluid stability study, developed polyplexes were found to be stable in presence of pulmonary fluids.

Table 4.8: Stability of polyplexes in BALF

Time (min)	% siRNA Retained	
	HELPEI-35	HELPEI-45
0	100.00	100.00
15	99.29±1.74	99.19±1.24
30	98.58±2.17	98.21±2.16
45	98.03±2.68	97.09±3.21
60	97.78±2.13	96.33±2.53
75	97.40±3.44	95.49±2.37
90	96.82±2.53	94.18±2.08
120	96.56±3.22	92.72±2.45

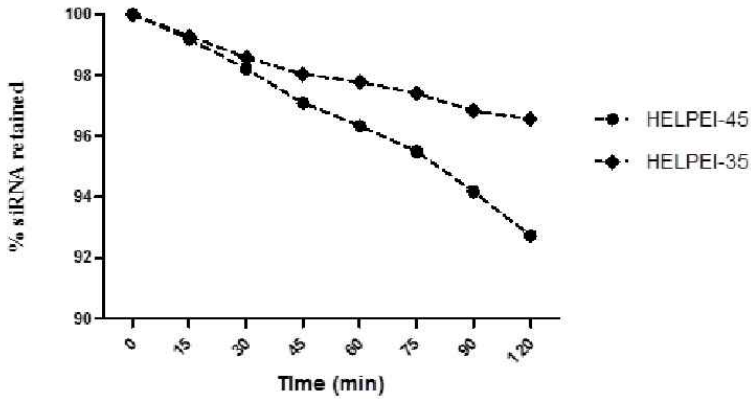

Fig. 4.23: siRNA retained in polyplexes at different time points in presence of BALF

4.3.11 TEM

Fig. 4.24 shows the morphology of the HELPEI-35 and HELPEI-45 polyplexes as observed through TEM. The TEM images showed that polyplexes were spherical and compact which is the result of strong electrostatic interaction [61]. They were discrete with no visible evidence of aggregation, proving the homogenous state of system. The images support the light scattering data however, the size were relatively smaller than that of in DLS measurement. The effect might be result of recording of hydrodynamic radius of the particles in the DLS which is more than the actual size [62].

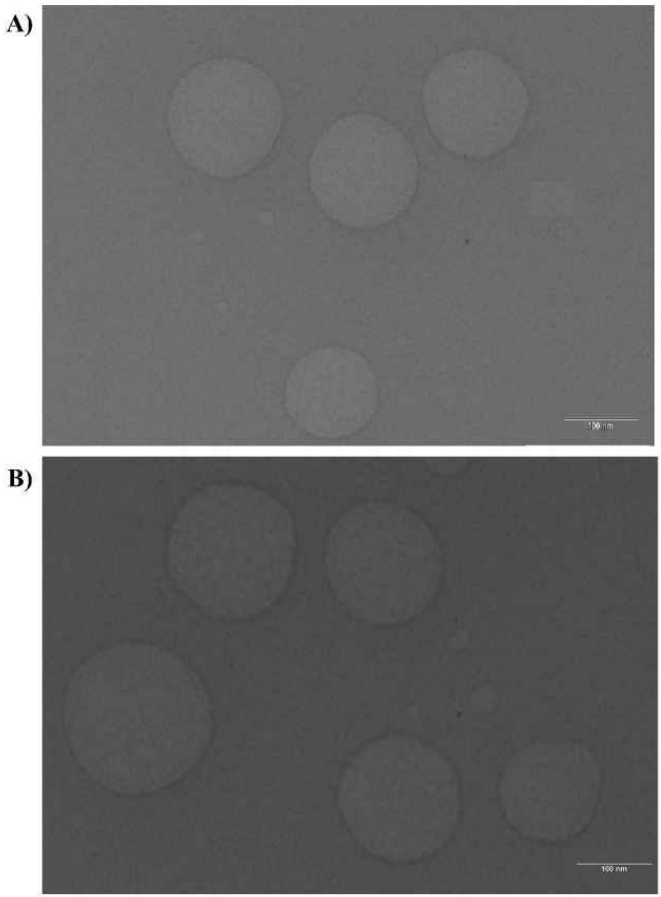

Fig. 4.24: TEM image of A) HELPEI-35 polyplex, B) HELPEI-45 polyplex

Therefore, based on the results of size, zeta, complexaton efficiency, biocompatibility and stability challenge studies, it was observed that HELPEI-35 and HELPEI-45 were equally non-toxic and had higher buffer capacity and colloidal stability. However, the results of size showed that HELPEI-45 forms higher size polyplex due to lower charge density. At the same time the zeta potential values were less than of HELPEI-35. Finally the complex formed with HELPEI-45 showed lower resistance to salt and heparin challenge studies. Therefore, HELPEI-35 was considered a better vector for siRNA delivery.

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