4. POLYMER SYNTHESIS AND CHARACTERIZATION

Recently, gene therapy has been exploited as a promising strategy for the treatment of various incurable, inheritable and acquired diseases. Majority of gene delivery approaches developed so far have used viral vectors for intracellular delivery of therapeutic genes, but non-viral gene delivery vectors have gained attention in the last few decades. Their favourable characteristics, as compared to viral systems, like low toxicity, ease of manufacturing, high flexibility in order to accommodate varying sizes of therapeutic genes warrant their potential use in gene delivery applications (1). However, several anatomical and cellular barriers limit the overall efficiency of gene transfer by non-viral methods. Nucleic acids typically cannot pass through cell membrane unless their entry is facilitated by various active cell uptake mechanisms such as endocytosis, pinocytosis, or phagocytosis. In addition, the unpacking of nucleic acid–carrier complexes could constitute yet another rate-limiting step after transfection. Fast clearance of carrier from the circulation limits their utility in gene delivery to cells located beyond vascular endothelial cells. The major drawback they face is the low transfection efficiency as compared to viral vectors. Hence, we were trying to develop the gene delivery carrier which can provide good transfection efficiency along with lowest toxicity.

Among the non-viral vectors, various cationic phospholipids like DOTAP, DOTMA, and polymers like polyethylenimine (PEI), poly-arginine, poly-L-lysine (PLL), chitosan which can complex with anionic therapeutic genes have been explored. Cationic polymers interact ionically with genes and condense them into a colloidal carrier as well as, if not neutralized completely, help in endocytic uptake of so formed delivery vector. Among different cationic polymers, PEI has been recognized for its tremendous buffering capacity between pH 7.2 and 5.0, due to its protonable amines which absorb protons on intracellular uptake into endosome and cause osmotically driven swelling of the endosome followed by endosomal rupture and cytoplasmic release of genes of interest (2). Such endosomal escape is also aided by the expansion of polymer taking place due to intramolecular charge repulsion which helps to build pressure inside the endosome. However, strong cationic nature, also is known to cause other adverse effects like cytotoxicity and non-specific interaction with serum proteins (3). Therefore the reduction of this cytotoxic profile in a suitable manner so as to preserve the transfection efficiency has been the topic of much interest.

PEI is available in two forms namely linear and branched, among which branched PEI is more effective in gene delivery aspects. Modification of amine functional groups has been

the strategy since long to reduce aforesaid drawbacks without affecting its transfection efficiency. Modifications of PEI in order to improve its transfection efficiency have also been reported (4). Such modifications involve conjugation of ligands targeted to the receptors expressed on cells of interest. Additionally, attempts to improve toxicity profile, has many-a-times is associated with low transfection efficiency (5, 6).

Among the approaches used, commonly used ones are to attach pendent groups onto the amines of PEI or to conjugate a ligand onto the amines of polymer for targeted delivery (7). Besides this, PEI has also been PEGylated, acetylated, grafted with dextran, cyclodextrin etc. to improve transfection efficiency or to reduce cytotoxicity (8). Several amino acids have been used to conjugate with primary amine of polyethylenimine in different degree of substitution. Histidine, an essential amino acid, has an imidazole ring as its side chain. This imidazole side chain shows pKa of 6.15. The proton sponge effect of imidazole modified biopolymers is well established (9). Poly-histidine itself, in principle, can be used as a gene delivery vector, but its insolubility in aqueous media at pH >6 is a major obstacle for such use. Imidazolyl groups have been successfully substituted on poly-L-lysine and methracylate-based gene delivery systems to provide endosomal buffering capacity (2). Such modification has enabled in efficient transfection of gene in mammalian cells as shown by their manifold higher gene expression than native polymer and eliminated the requirement of external endo-osmolytic agents. Phenylalanine and leucine, other essential amino acids, have been demonstrated to provide PAMAM dendrimers modified using them with efficient gene transfection capability (10). 25 kDa PEI, after modification with alanine (a non-essential amino acid) through N-acylation, has also shown low toxicity and twice the transfection efficiency of native polymer, in presence of serum (4).

Alanine (Ala), Leucine (Leu) and Histidine (His) could therefore improve transfection efficacy of PEI *in vitro,* but little is known about what difference exist in the transfection efficacy of PEI modified with these amino acids among them. Similarly, little is known about their transfection efficacy *in vivo* after intravenous administration. To develop a model delivery nanosystem, we chose cationic polymer PEI, because it has been widely used for non-viral transfection *in vitro* and *in vivo,* and it combines strong nucleic acid compaction capacity with an intrinsic endosomolytic activity, also known as the proton sponge effect (11). The use of PEI as a transfection reagent is severely limited, however, by its cytotoxic effects (12). Shielding of PEI with polyethylene glycol moieties has overcome such

cytotoxicity, but at the expense of its gene transfection efficiency. Owing to the lipidic nature of plasma/cell membrane, incorporation of lipophilic properties to PEI has been sought as an alternative to improve the transfection efficiency as well as reduce toxicity. Lipophilic groups like BOC can be used to impart lipophilicity to pendant groups. In the present study, we have investigated whether the conjugation of Boc protected alanine, leucine and histidine residues onto the surface of a non-viral gene delivery system, PEI, could lead to improved transfection efficiency and reduced toxicity.

4.1 Methods

4.1.1 Polymer Synthesis

Coupling of Boc- amino acid to PEI was performed using condensation method. Different degree of substitution of amino acid was carried out by using different molar ratios of Boc-amino acids. The Boc-amino acids (Boc-alanine, Boc-histidine or Boc-leucine) were dissolved in 7 ml of dimethyl formamide (DMF). To this solution, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) (0.75 mmole), N-hydroxysuccinimide (NHS) (0.75 mmole), and triethylamine (0.75 mmole) were added. The reaction mixture was stirred for 30 min, and then polyethylenimine was added, followed by addition of borate buffer (7.0 mL, 100 mM). This reaction mixture was subjected to stirring for 24 hr and purified by dialysis with MES buffer (50 mM). The aqueous solution so obtained was lyophilized to obtain a solid form. The same procedure was used for coupling of polyethylenimine with all three Boc-amino acids at different mole ratios. Further, synthesized polymers were characterized by Infrared Spectroscopy (IR), Nuclear Magnetic Resonance Spectroscopy (NMR), trinitrobenzene sulfonic acid (TNBS) assay and Gel Permeation Chromatography (GPC).

Boc-alanine, Boc-leucine and Boc-histidine were used in different concentrations i.e. 1 mmole, 0.75 mmole and 0.5 mmole for 0.012 mmole of PEI to achieve different degree of substitution of Boc-amino acid on primary amine of PEI (**Figure 4.1, Figure 4.2, Figure 4.3**, and **Figure 4.4**). Different quantity of amino acids used in reaction were denoted by A, B and C for 1 mmol, 0.75 mmol and 0.5 mmol, respectively and different Boc-amino acids were denoted by A, L and H for Boc-alanine, Boc-leucine and Boc-histidine respectively. Hence, nomenclature mentioned in the **Table 4.1** below has been used here onward.

Table 4.1 Nomenclature of synthesized polymers

Sr No	Quantity of Amino Acid Used	Type of Amino Acid	Nomenclature
1.	1 mmol (A)	Boc-alanine (A)	AA
2.	0.75 mmol (B)	Boc-alanine (A)	BA
3.	0.5 mmol (C)	Boc-alanine (A)	CA
4.	1 mmol (A)	Boc-leucine (L)	AL
5.	0.75 mmol (B)	Boc-leucine (L)	BL
6.	0.5 mmol (C)	Boc-leucine (L)	CL
7.	1 mmol (A)	Boc-histidine (H)	AH
8.	0.75 mmol (B)	Boc-histidine (H)	BH
9.	0.5 mmol (C)	Boc-histidine (H)	CH

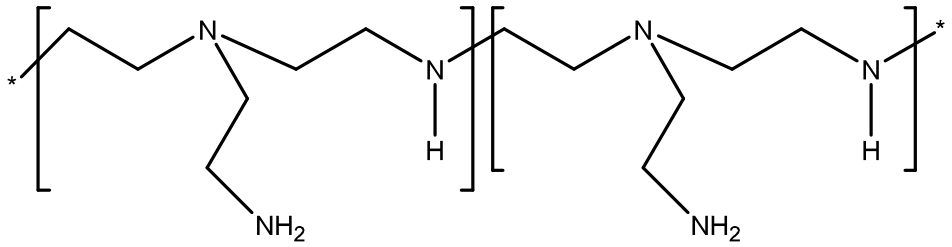

Figure 4.1 Structure of polyethylenimine (PEI).

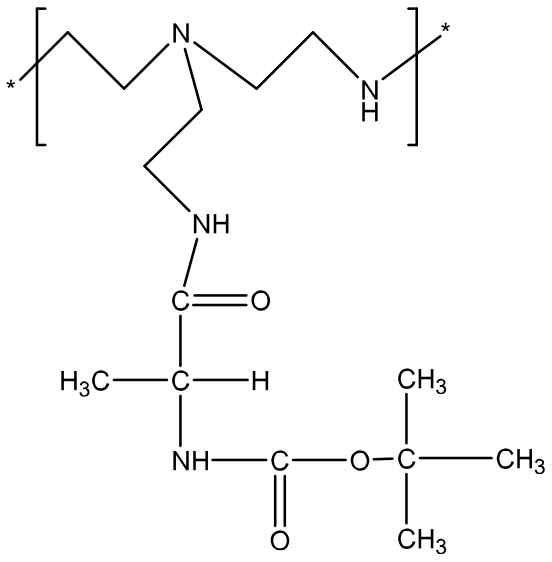

Figure 4.2 Structure of Boc-alanine modified PEI

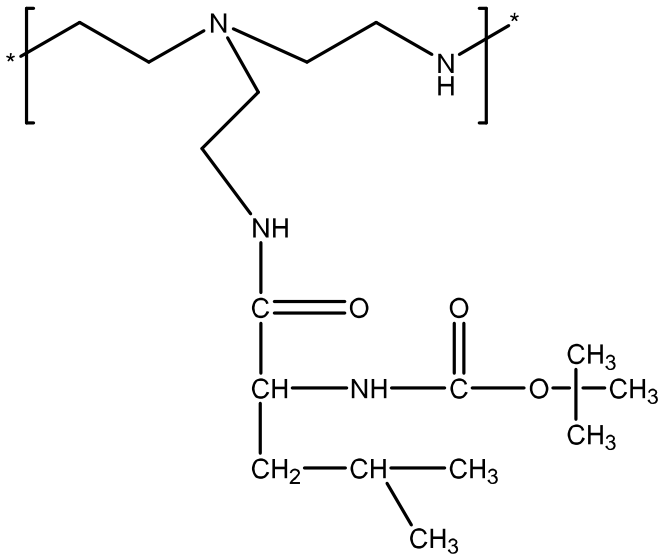

Figure 4.3 Structure of Boc-leucine modified PEI.

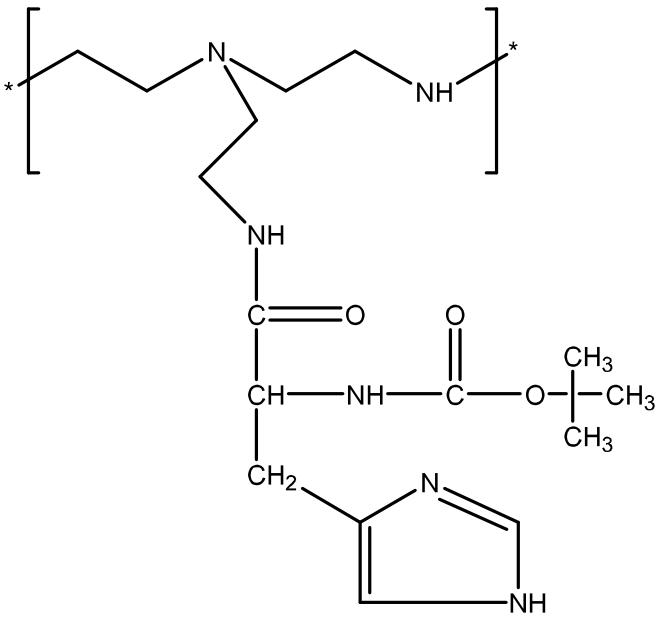

Figure 4.4 Structure of Boc-histidine modified PEI.

4.1.2 Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra of synthesized polymers were recorded using the potassium bromide (KBr) pellet technique. Minimal quantity of sample was mixed with KBr and then compressed to obtain a pellet and FTIR analysis was performed on Bruker-αt FTIR

spectrophotometer. Baseline was corrected and the sample was scanned against a blank KBr pellet background in the range of 4000-600 cm^{-1} wave number with a resolution of 1.0 cm^{-1}.

4.1.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{1}H-NMR spectroscopy was performed to determine the characteristic peaks of functional group to confirm the synthesis of suggested product. ^{1}H NMR spectra of Boc-amino acid conjugated PEI was recorded on Bruker DPX 400 MHz NMR spectrometer at 400 MHz. Unmodified PEI and modified PEIs were dissolved in D_2O in 5 mm NMR tubes and ^{1}H NMR spectroscopy was performed.

4.1.4 TNBS Assay

After the confirmation by IR and NMR, a technique based on UV–Vis spectroscopy was used to quantify the percentage substitution on primary amine of polyethylenimine. The quantification of amino groups is important in numerous biological fields. Many of the existing methods for this purpose are based on the nucleophilic character of the amino nitrogen. Among these, methods using nitrobenzenic derivatives such as 1-fluoro-2, 4-dinitrobenzene sulfonic acid and 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) are very popular, out of which, TNBS is specific for primary amino groups. Because the degree of modification plays a major role, in quantification, TNBS assay is often used to control the modification.

Free primary amine concentrations of the Boc-amino acid modified PEIs (mPEI) were determined by TNBS assay. TNBS reacts with primary amino groups of polymers making it possible to estimate the percentage of substituted primary amines in modified PEI as compared to unmodified PEI. Briefly, 20 µl of freshly prepared aqueous TNBS solution (15 mg/mL) was added to 600 µL of polyethylenimine (or modified PEI) dissolved in distilled water. After the addition of 200 µl sodium bicarbonate buffer (0.8 M, pH 8.5), the solutions were incubated at room temperature for 30 min. The absorbance was read at 410 nm using a UV spectrophotometer (13, 14). Experiment was performed in triplicate and % primary amine modification was calculated accordingly.

Calibration curve was developed by using 2.5 to 10 mg of polyethylenimine to find out the efficiency of the method. Later on, % free primary amine in modified

polyethylenimine was assessed by the same method and modification efficiency was calculated by following equation

$$\text{Modification percentage} = \left(1 - \frac{A_{mod}}{A_{unmodified}}\right) \times 100$$

where A_{mod} = absorbance of modified polymer and $A_{unmodified}$ = absorbance of unmodified polymer at 410 nm.

4.1.5 Gel Permeation Chromatography

Gel permeation chromatography (GPC/ Size exclusion chromatography-SEC) is a liquid chromatography technique accomplished as column chromatography. Stationary phase in GPC is made up of porous gel-like polymeric network of polyacrylamide or polystyrene, pore size distribution of which varies over several order or magnitude. In contrast to HPLC, the target molecules are always in a dissolved form in the mobile phase in GPC, so there doesn't take place any interaction between materials being separated and the stationary phase. Rather, separation of molecules in this stationary phase takes place depending on the molecular size of the molecules. As soon as the solution of polymer to be examined is injected in the column, smaller molecules distribute to the deepest in the polymer matrix as the mobile phase travels the column, while larger particles do not penetrate into the deepest pores of the stationary phase. This phenomenon makes smaller particles elute slowly while larger particles elute faster. Gel permeation chromatography is indirect and relative method for determination of molecular mass of a polymer since molecular mass is determined on the basis of the size of the polymer relative to a reference substance.

The molecular weight of the modified PEI was measured by a GPC equipped with a Waters 2414 separation module and refractive index detector. Waters Breeze 2 software was used to calculate the molecular weight based on a universal calibration curve generated by pullulan standards of narrow molecular weight distribution (15). Milli Q water containing 0.1 M $NaNO_3$ was used for elution at a flow rate of 0.5 mL/min from HSPgel AQ 4.0 column of size 6.0 mm X 150 mm. The column temperature was maintained as 35 °C.

4.1.6 Acid Base Titration

The buffering capacity of the polymers over pH range of 7.4 to 5.1 was determined by the acid– base titration as described by He et al. (16). The titration was performed at room

temperature and the pH was measured using a pH meter. Of each polymer, 15 mg was dissolved in 3 ml 0.9% (w/v) NaCl solution. The pH was adjusted to 10 with 1 M NaOH prior to titration. The polymer solution was titrated with 1 M HCl until pH 3. The volume of HCl consumed and the pH were recorded after each addition. Pure 0.9% NaCl solution was also titrated as reference (16).

4.1.7 Hemolysis Study

For hemolysis, fresh blood from six month old wistar rats was collected in heparinized tubes which was centrifuged at 4°C for 10 min at 1000 g and washed several times with phosphate-buffered saline (PBS pH 7.4) until the supernatant was colourless. A 500 µL amount of a 2.5% (v/v) suspension of erythrocytes was mixed with 500 µL of different concentrations of various polymer solutions in Eppendorf tubes. Tubes were incubated for 60 min at 37 °C in a shaker incubator, blood cells were removed by centrifugation and the supernatant was analysed spectroscopically for absorbance at 540 nm for the release of hemoglobin. PBS and 1% Triton X-100 solution were used as negative control and positive control, respectively. % hemolysis was calculated by following formula

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

where A_s is the absorbance of the cell supernatant treated with polymer sample, A_n is the absorbance of cell supernatant treated with the negative control PBS, A_p is the absorbance of cell supernatant treated with the positive control Triton X-100. All assays were performed in triplicate and data are reported as mean ± SD (14, 17).

4.1.8 Erythrocytes Aggregation Assay

To study polymer induced aggregation of erythrocytes, rat blood (see hemolysis test) was used. Ringer's solution (with the addition of sodium citrate, pH 7.4) was used to prevent coagulation. The blood was washed several times with Ringer's solution until the supernatant was colourless as described above. Finally, the erythrocytes were diluted with Ringer's solution 1:50. A 200 µL amount of this cell suspension was treated with 100 µL of polymer solution (100 µg/mL) in Eppendorf tube. Treatment with ringer solution was used as negative control. Afterwards, it was incubated for 2 h at 37°C, pictures of the cells were taken on an inverted microscope (Nikon TMS) in a phase contrast mode at 40 X magnification.

4.2 Results and Discussion

4.2.1 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra showed that amino acids were grafted on the PEI chains. Upon grafting reaction, strong absorption around 1644 cm^{-1}, 1649 cm^{-1} and 1646 cm^{-1} attributed to the amide linkage appeared in the spectra of Boc-alanine, Boc-histidine, Boc-leucine modified PEIs respectively when compared with the spectrum of polyethylenimine (**Figure 4.5**, **Figure 4.6**, **Figure 4.7** and **Figure 4.8**). In addition, all the synthesized polymers showed strong and broad absorption of amines between 3410 cm^{-1} to 3420 cm^{-1} (N–H stretching) and around 1560 cm^{-1} (N-H bending) characteristic to the –NH group of PEI.

In addition some more characteristic peaks were also observed in FTIR spectra which include primary alkyl stretching around 2975 cm^{-1}, alkyl bending around 1400 cm^{-1} and CH_2 bending around 1457 cm^{-1} in FTIR spectra of all modified polyethylenimine. These results are in concordance with the previous report of modified polyethylenimine by using different conjugation moiety (18).

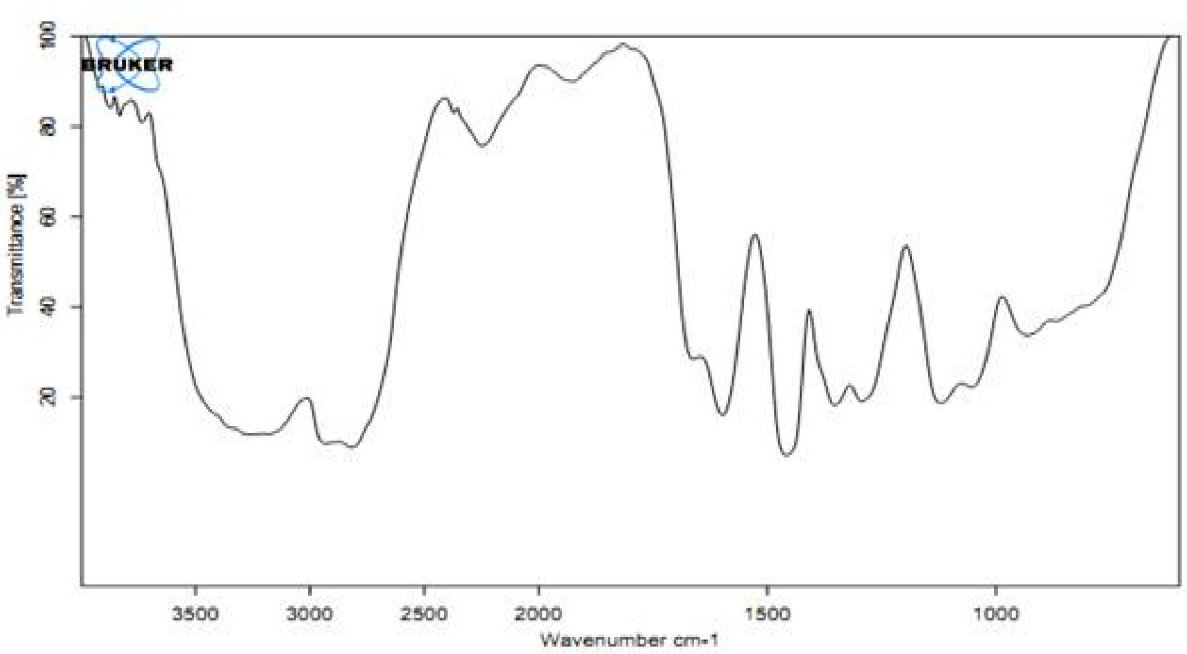

Figure 4.5 IR spectra of PEI.

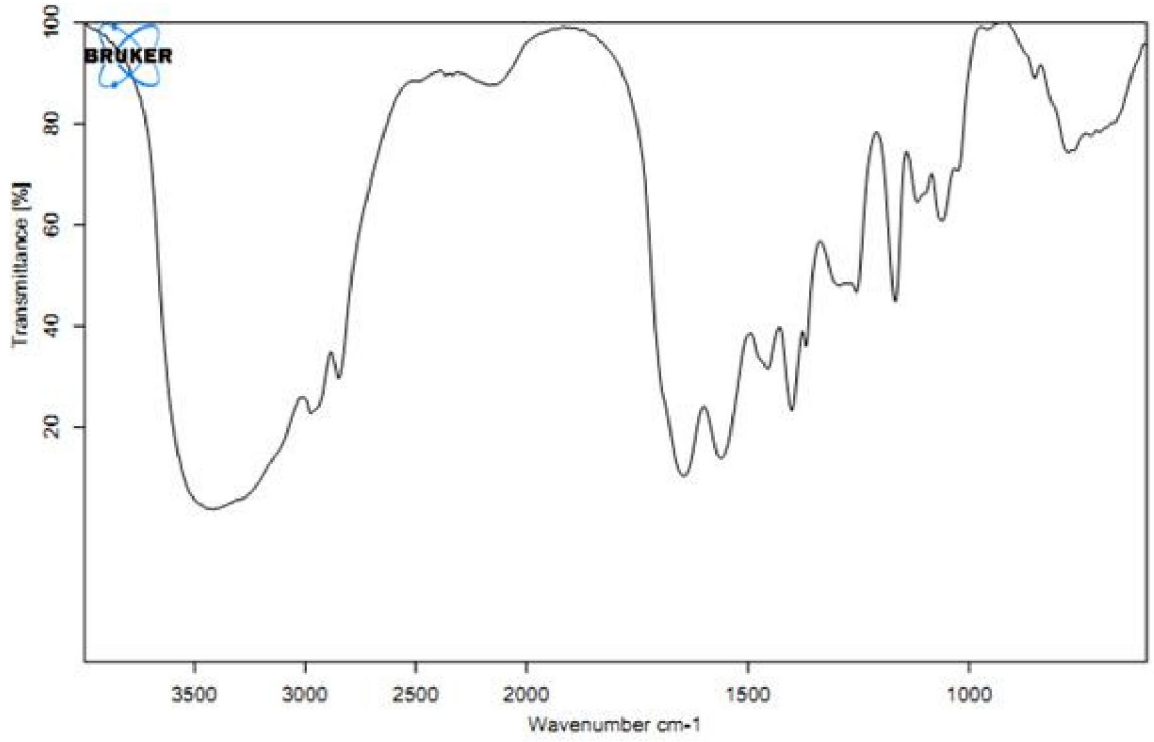

Figure 4.6 IR spectra of Boc-alanine modified PEI.

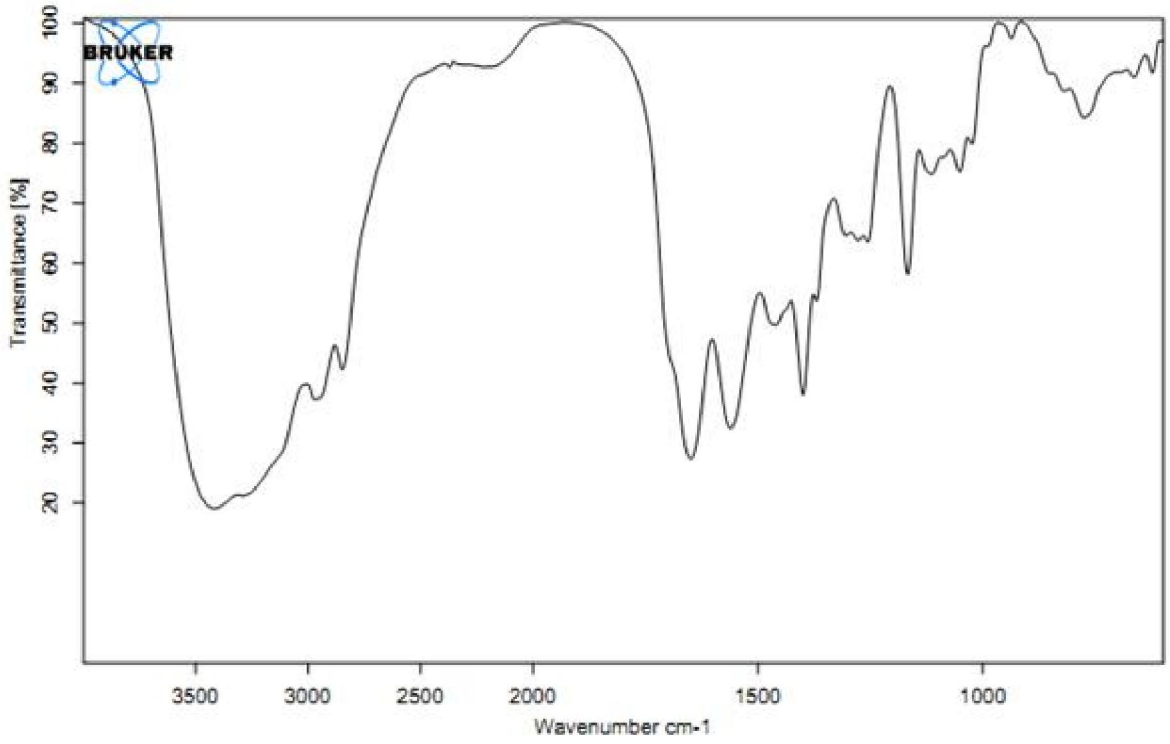

Figure 4.7 IR spectra of Boc-histidine modified PEI.

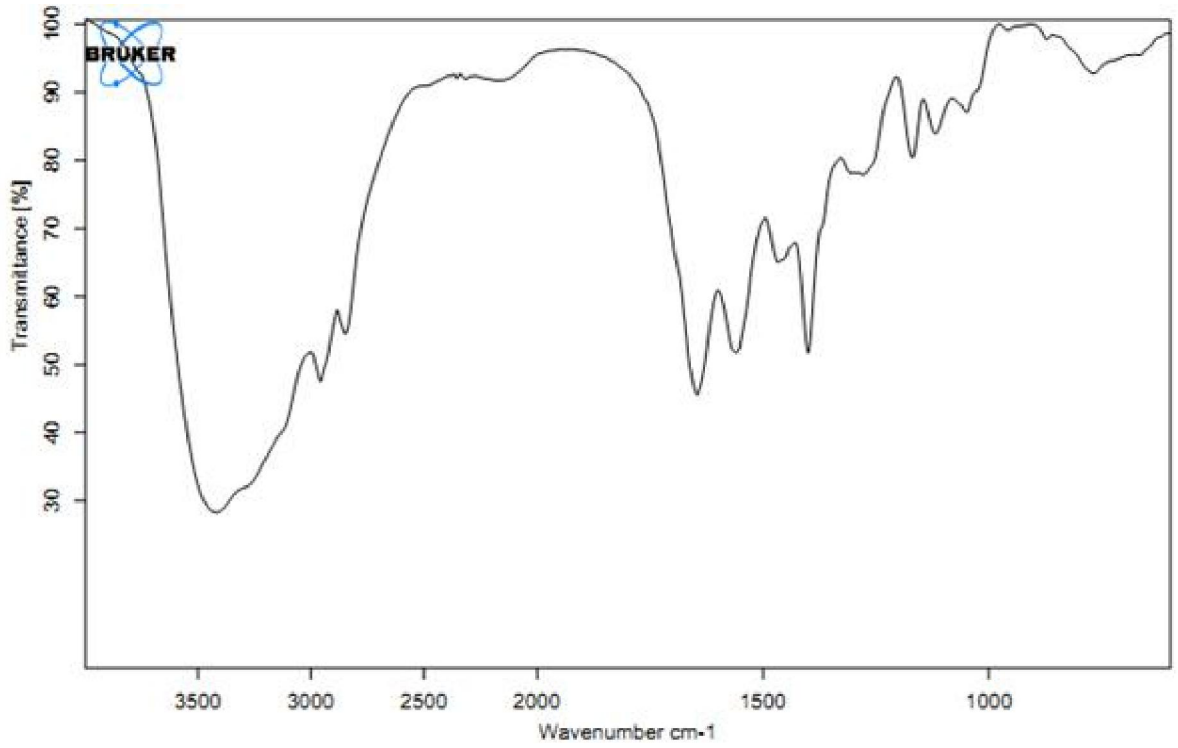

Figure 4.8 IR spectra of Boc-leucine modified PEI.

FT-IR spectra proved the synthesis of Boc-amino acid conjugated PEIs. Identity of the synthesized polymers was further confirmed by nuclear magnetic resonance spectroscopy.

4.2.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

Successful synthesis of Boc-alanine-PEI, Boc-histidine-PEI and Boc-leucine-PEI was confirmed using ^{1}H-NMR spectra, as shown in **Figure 4.9, Figure 4.10** and **Figure 4.11**. The characteristic peaks of PEI appeared at δ 2.2 ppm to δ 3.2 ppm and those of tertiary carbon (≡C<u>H</u>) appeared at δ1.2 ppm to δ 1.4 ppm for all synthesized polymers (19). In case of Boc-alanine-PEI, characteristic peak of -C<u>H</u>$_3$ was observed at δ 1.17 ppm. While Boc-histidine-PEI showed a peak of amine group of imidazole ring and peak of –C<u>H</u> near to carboxamide group at around δ 4.0 ppm. Moreover, in case of Boc-leucine-PEI, peak of -C<u>H</u>$_3$ and -C<u>H</u> of isopropyl group were observed at δ 0.8 ppm and δ 1.35 ppm, respectively. These findings suggest the successful conjugation of Boc-alanine, Boc-histidine and Boc-leucine on polyethylenimine (**Figure 4.9, Figure 4.10** and **Figure 4.11**).

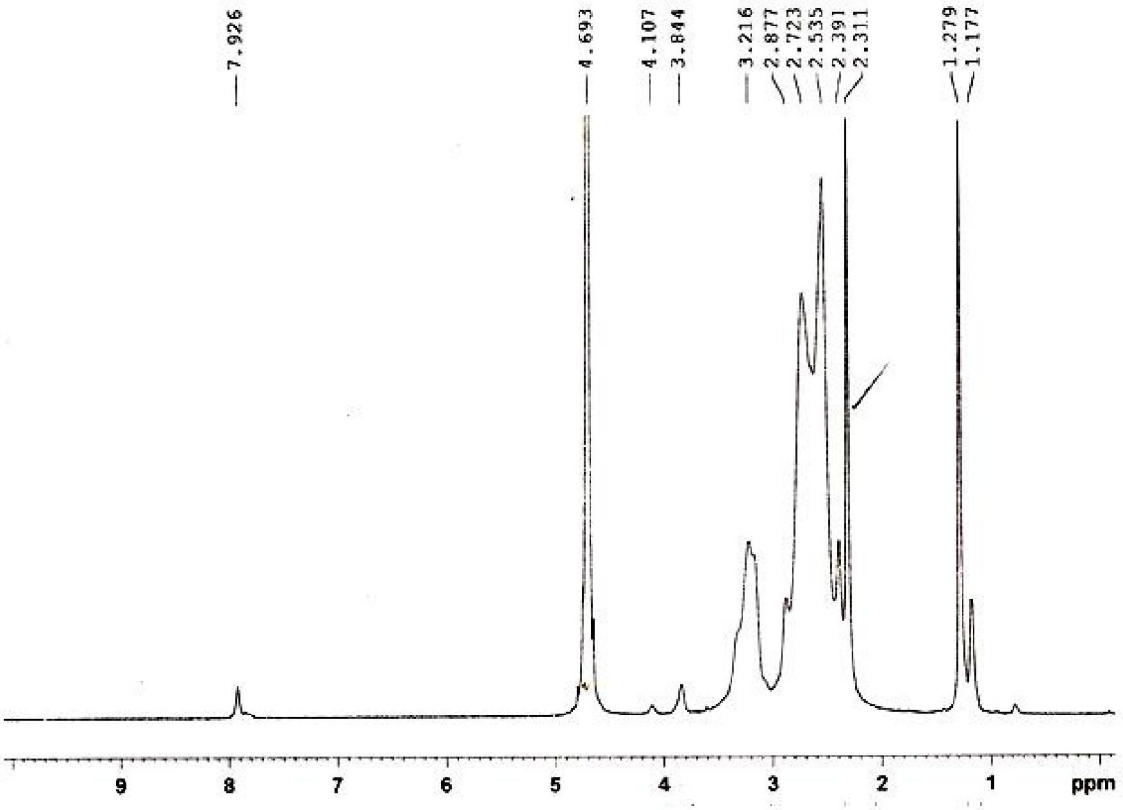

Figure 4.9 [1]HNMR spectrum of Boc-alanine modified PEI.

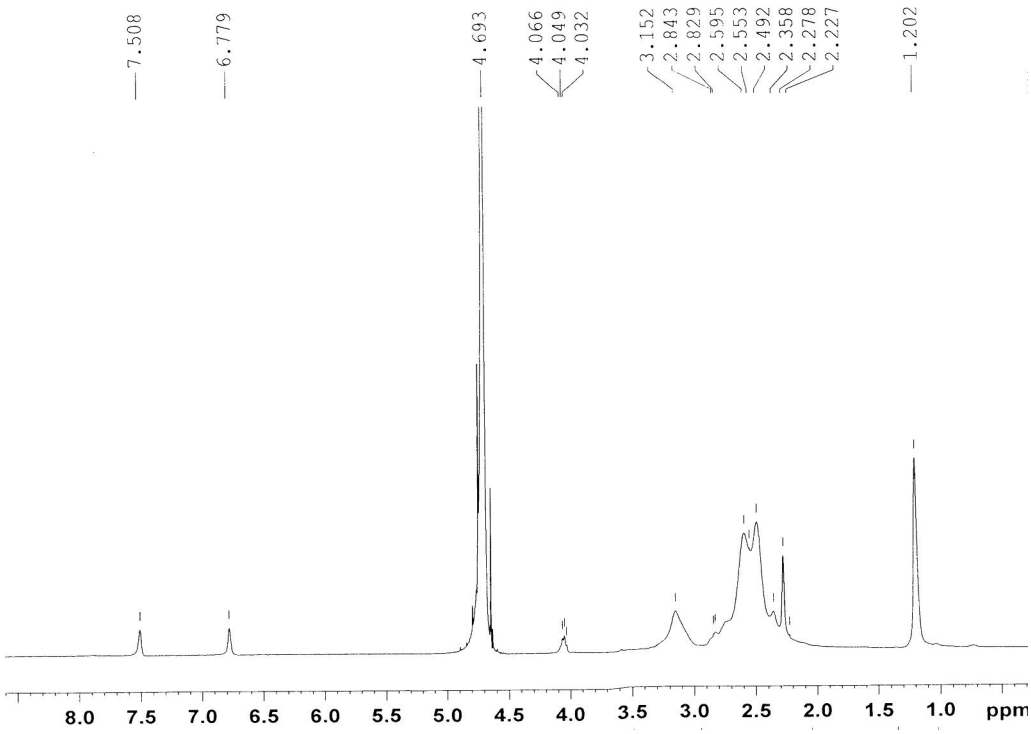

Figure 4.10 [1]HNMR spectrum of Boc-histidine modified PEI.

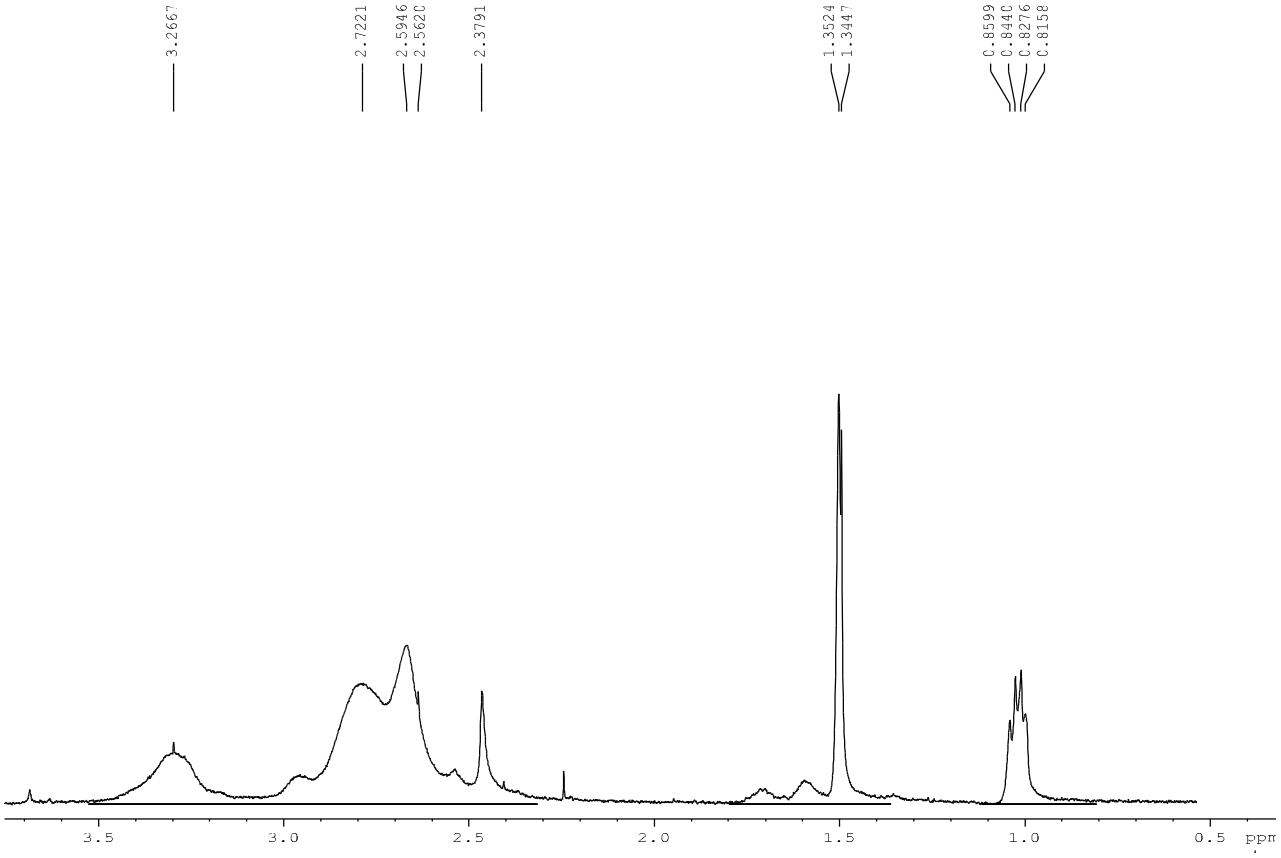

Figure 4.11 [1]HNMR spectrum of Boc-leucine modified PEI.

4.2.3 TNBS Assay

PEI is an attractive carrier for intracellular gene delivery because of its well-established ability to condense nucleic acids via electrostatic interaction between anionic DNA or RNA and the cationic primary, secondary, and tertiary amines of the polymer. Due to the presence of primary (25%), secondary (50%), and tertiary (25%) amino groups PEI are amenable to diverse and selective chemical modifications (20). Primary site for chemical modification of PEI is primary amine group as it can be easily modified and also plays an important role in complexation with siRNA, transfection efficiency and toxicity as well. Hence, presence of % primary amines in modified polymers can be assessed by TNBS assay and % of substitution on primary amine of modified polymers can be calculated against % primary amines of native PEI.

Calibration curve (**Figure 4.12** and **Figure 4.13**) showed a straight line expressed by equation y=0.0596x + 0.2668 with correlation coefficient of 0.9992. The developed

calibration curve was used further to find out the degree of substitution made in synthesized polymers as compared to polyethylenimine.

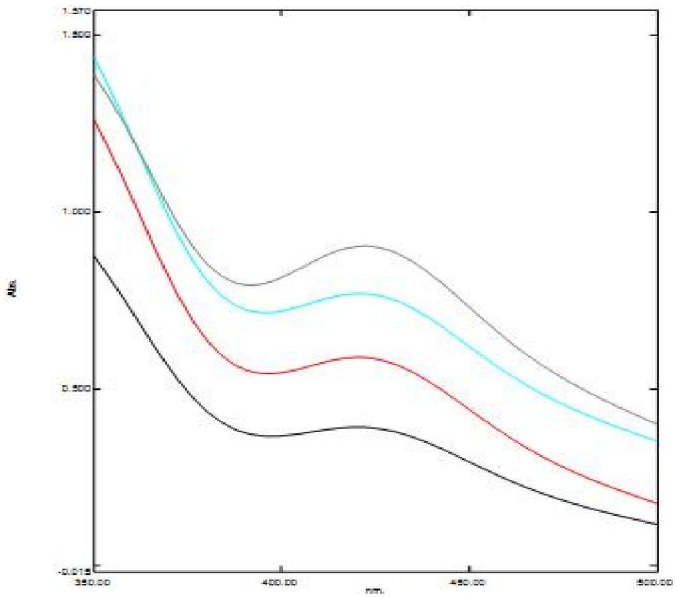

Figure 4.12 UV spectra of TNBS assay for determination of primary amine in PEI.

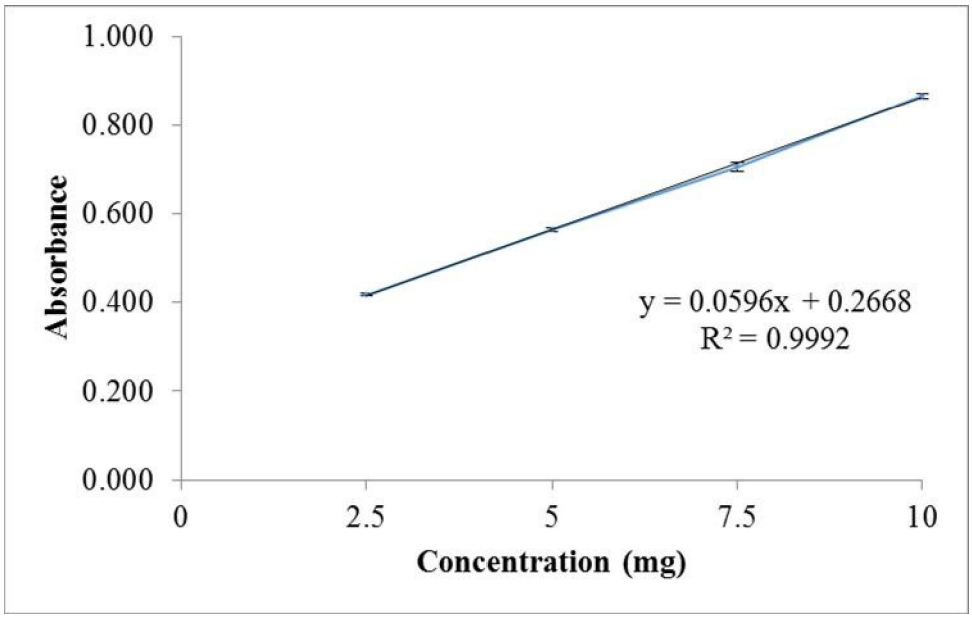

Figure 4.13 Calibration curve for determination of primary amine in PEI.

Table 4.2 % Primary amine modification in modified PEIs

Sr. No.	Polymer	% Primary amine modification*
1.	AA	45.03 ± 3.33
2.	BA	37.62 ± 3.93
3.	CA	28.65 ± 4.51
4.	AH	40.88 ± 3.38
5.	BH	33.53 ± 4.02
6.	CH	23.82 ± 4.82
7.	AL	41.04 ± 1.4
8.	BL	32.35 ± 4.16
9.	CL	23.88 ± 3.08

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

The percentage of primary, secondary and tertiary amines in the polyethylenimine has been reported previously (21). The results showed increasing substitution percentage on the polymer with increasing molar ratios of the Boc-amino acid used (**Table 4.2**). Substitution of Boc-amino acids at higher (40-45%), moderate (32-38%) and lower (23-29%) levels was of similar extent for each Boc-amino acid type.

4.2.4 Gel Permeation Chromatography

Gel permeation chromatographs were recorded for standard with seven different molecular weight and retention time for each was noted (**Table 4.3**). Calibration curve was constructed by plotting log molecular weight against retention time. It gave a straight line with equation y= -0.8666x+9.0856 and correlation coefficient of 0.9951 (**Figure 4.15**). Molecular weights of the developed polymers were determined by the same method.

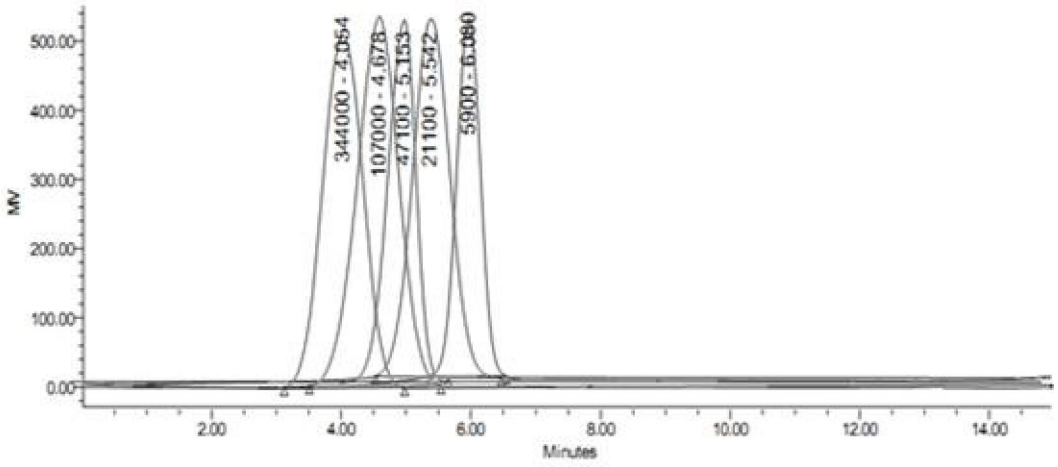

Figure 4.14 Chromatograph for calibration curve of gel permeation chromatography.

Table 4.3 Retention time of standards of different molecular weight

Sr. No.	Molecular Weight (Da)	Mean Retention Time (min)
1.	5900	6.065±0.015
2.	21100	5.542±0.002
3.	47100	5.147±0.006
4.	107000	4.689±0.011
5.	344000	4.052±0.002

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

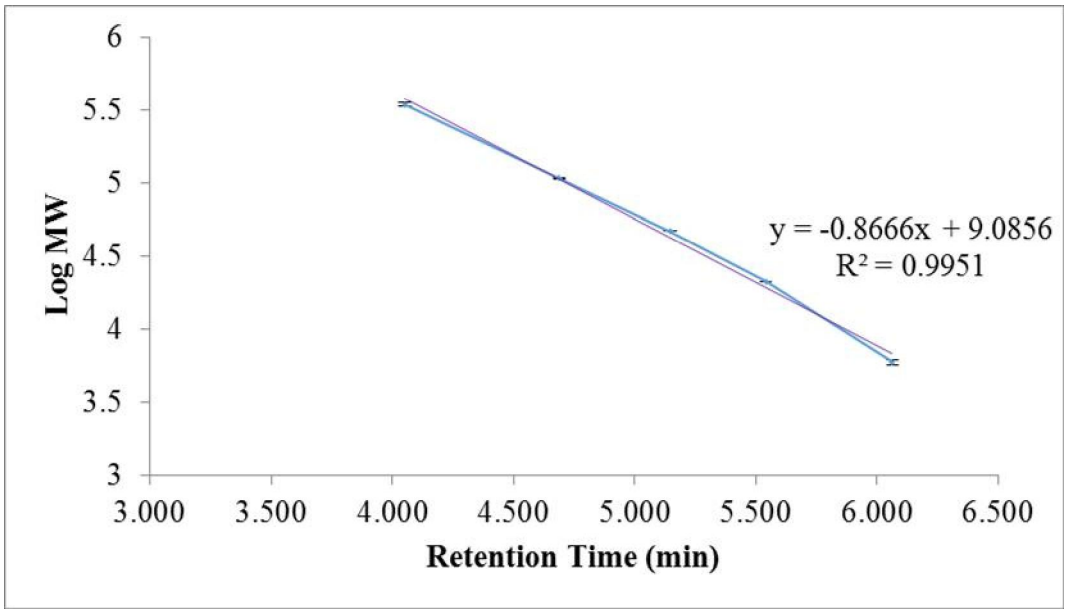

Figure 4.15 Calibration curve of gel permeation chromatography.

Table 4.4 Molecular weights of modified PEIs

Sr. No.	Polymer	Molecular Weight (Da)	% PEI	% Boc Amino Acids
1.	AA	37078.4	65.77	34.23
2.	BA	35346.8	69.50	30.50
3.	CA	32812.5	74.81	25.19
4.	AL	39730.4	61.25	38.75
5.	BL	36492.0	66.58	33.42
6.	CL	33251.4	73.13	26.87
7.	AH	38586.0	63.47	36.57
8.	BH	35914.8	68.22	32.04
9.	CH	32725.5	74.81	25.42

The nomenclatures and the characteristics of the synthesized polymers are presented in **Table 4.4**. For the copolymer samples used in this work, Boc-amino acid-grafted PEI, the molecular masses were calculated using the gel permeation chromatography (**Figure 4.14, Figure 4.15** and **Table 4.3**). According to the data of TNBS assay, Boc-amino acid-grafted PEIs contained an average of 23% to 45% substitution on primary amine of polycationic chain. By combining the results of both studies, % of Boc-amino acid and % PEI present in the modified polymer was calculated which is given in **Table 4.4**.

4.2.5 Acid Base Titration

Cationic carriers used for gene delivery ensures high intracellular uptake of gene through endocytosis. However, prime requirement for a gene delivery system is that they should escape endosomes to avoid exposure of therapeutic genes to high pH of late endosomes. High transfection efficiencies of polymers such as PEI, imidazole containing polymers, lipopolyamines like DOSPA, Starburst Dendrimers™ etc. have been extrapolated to their high buffering capacity that help in endosomolysis at acidic pH of endosomes through ionization of secondary and tertiary amines (22). Endosome maintains relatively low pH (7.4 to 5.1) inside through ATPase proton pump. The so called “proton sponge effect” theory can be explained using example of PEI which when uptaken inside the endosome, takes up the proton through ionization of its secondary and tertiary amines. This in turn causes influx of more protons to maintain the pH and this proton influx drives the accumulation of chloride

inside the endosomes. This inflow of protons and chlorides raises the osmotic pressure inside the endosomes causing rupture of endosomes and cytosolic release of PEI-gene complex. Additionally, high charge density created on the polymer causes its expansion inside the endosome, which also augments the endosomolytic effect of polymer. Capture of protons by polymer also protects therapeutic genes from detrimental effects of pH. Given that our polymer structure was rich in PEI motifs, we hypothesized that Boc-amino acid-PEI would retain the same proton buffering capacity as native PEI. We used acid–base titration to obtain the titration curve of polymers (**Figure 4.16**).

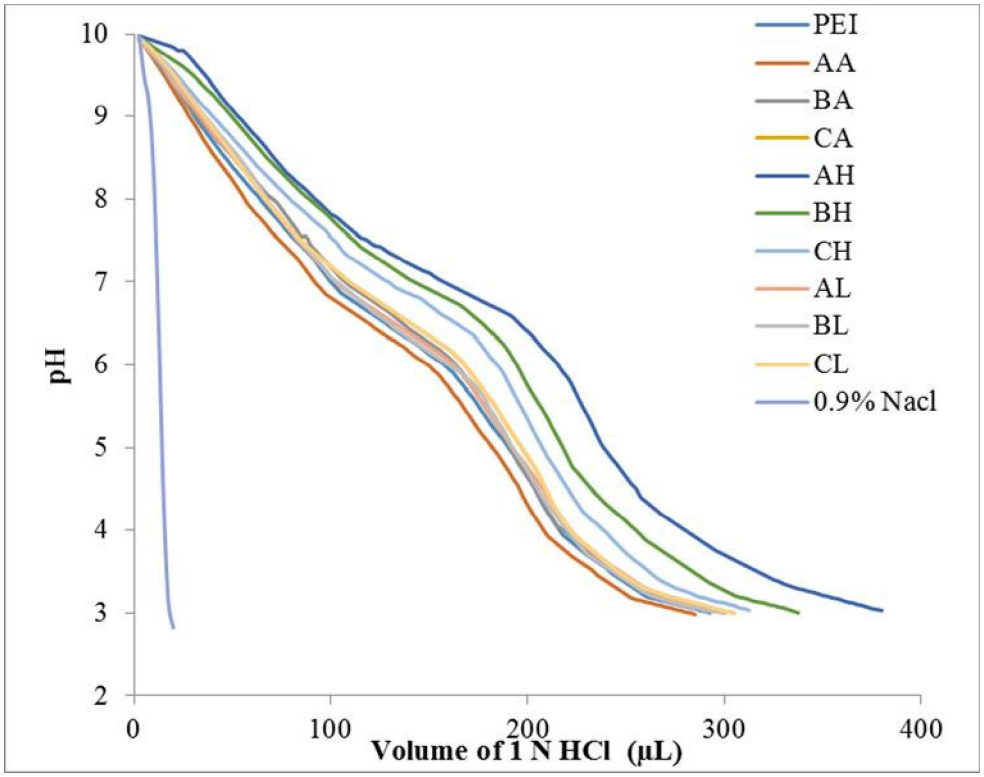

Figure 4.16 Acid base titration curves for different polymers.

Pure 0.9% NaCl solution was titrated as negative control. When HCl was added, pH of the NaCl solution rapidly dropped to less than 5. In contrast, the pH of PEI solution went down gradually by continuous addition of HCl, suggesting that PEI has a strong proton capturing ability. The titration curves of Boc-alanine-PEI and Boc-leucine-PEI were very close to PEI and thus have almost equal buffering capacity to that of PEI. It has been reported that secondary and tertiary amines play a key role in proton sponge effect and that's why the substitution of Boc-amino acids on primary amine of PEI could not affect the proton sponge effect of PEI adversely. Degree of substitution and substituting moiety (Boc-alanine and Boc-leucine) didn't improve the proton sponge effect of modified PEIs as the amine group of both

Boc-amino acid do not take part in protonation due to resonance effect of carbonyl group. However, titration curves of Boc-His-PEI for each degree of substitution showed more consumption of 1 M HCl as compared to native PEI. As the degree of substitution of Boc-histidine was increased on PEI, consumption of 1 M HCl was also increased. The results indicate that imidazole ring of Boc-histidine took part in improvement of proton sponge effect of modified PEI and it will help to promote cytoplasmic release of siRNA polyplexes by endosomal escape. The results of our study are congruent with the previous reports which also showed that imidazole derivatives like histidine plays a crucial role in improvement of proton sponge effect as imidazole ring nitrogen which has pKa of around 7.0 (9).

Therefore, the buffer capacity of PEI is dependent on the secondary and tertiary nitrogen functionalities of the polymer.

4.2.6 Hemolytic Potential and Erythrocyte Aggregation Study

The compatibility of any vector with blood is clearly important and indicates its suitability for introduction into the systemic circulation. The results of the hemolysis study are presented in **Figure 4.17**. Results showed that there was concentration dependent rise in hemolytic potential of all the polymers suggesting the increased amounts of primary cationic amines that would confer higher blood cell destruction. However, synthesized polymers showed reduced hemolytic potential as compared to that obtained with PEI. All modified polymers exhibited less than 6% hemolysis at all concentrations, while non-modified PEI showed ~7% hemolysis at lowest concentration of 10 μg/mL which rose to about 20% at 1000 μg/mL concentration. The results obtained with PEI are in agreement with the results of Brownlie et al. (23)

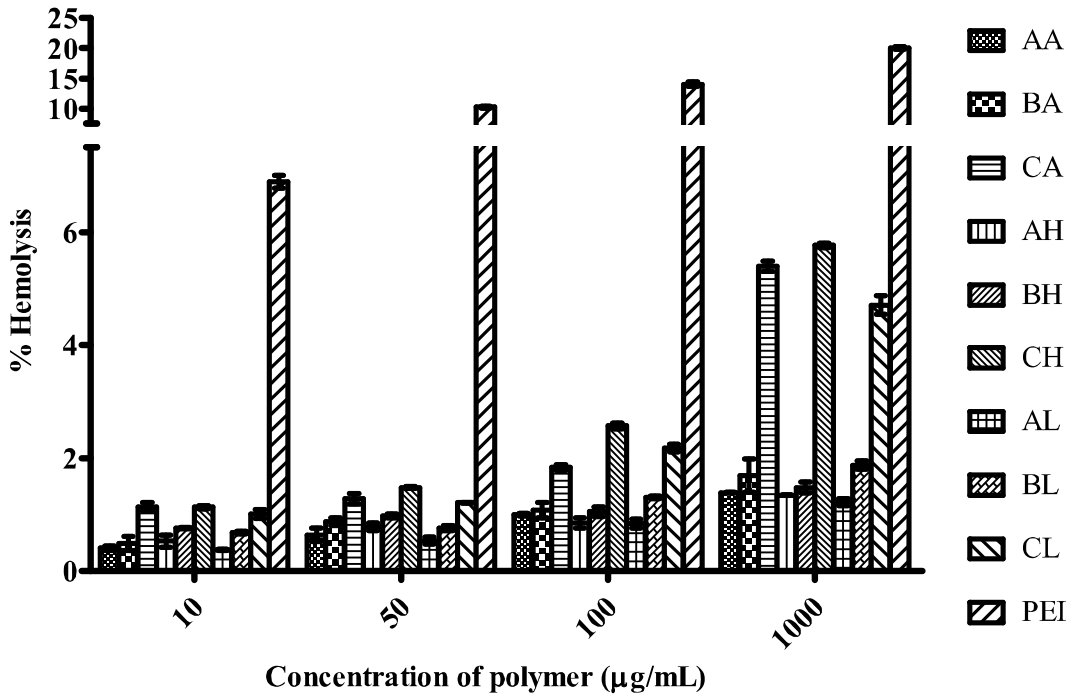

Figure 4.17 Hemolytic potential of different modified PEIs at different concentrations.

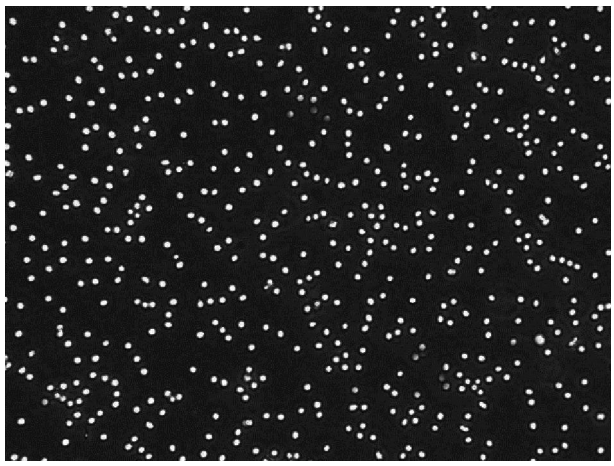

Figure 4.18 Erythrocyte aggregation by negative control.

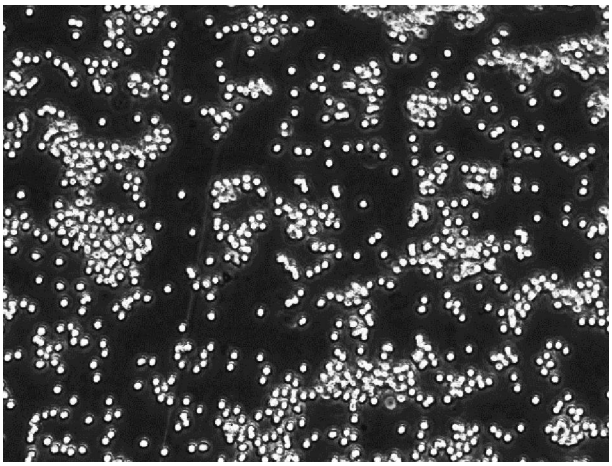

Figure 4.19 Erythrocyte aggregation by PEI.

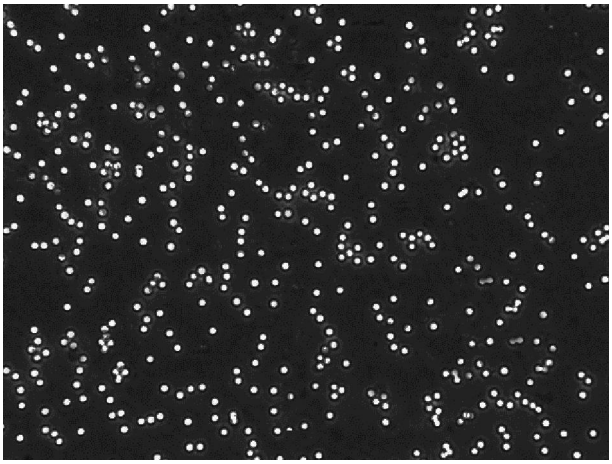

Figure 4.20 Erythrocyte aggregation by AA.

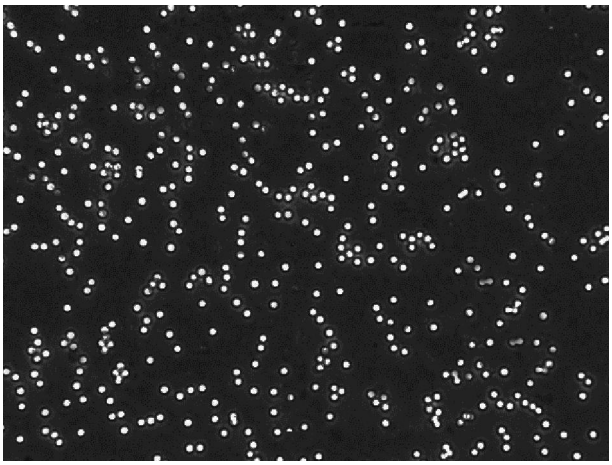

Figure 4.21 Erythrocyte aggregation by BA.

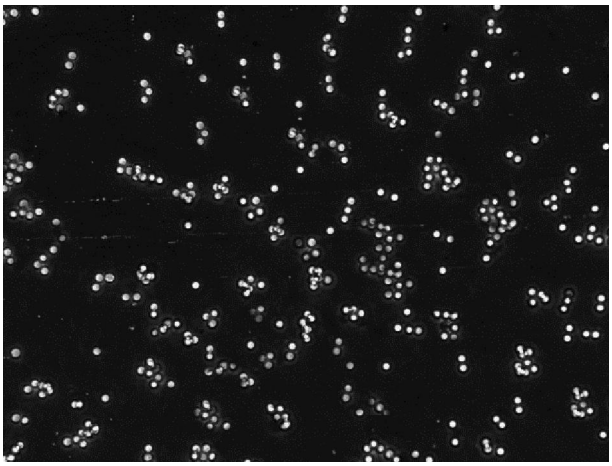

Figure 4.22 Erythrocyte aggregation by CA.

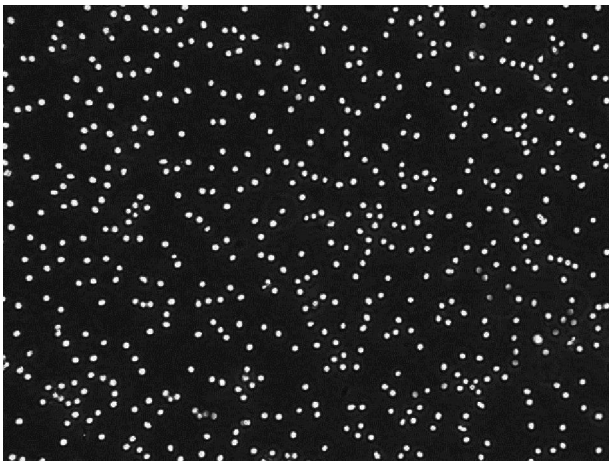

Figure 4.23 Erythrocyte aggregation by AH.

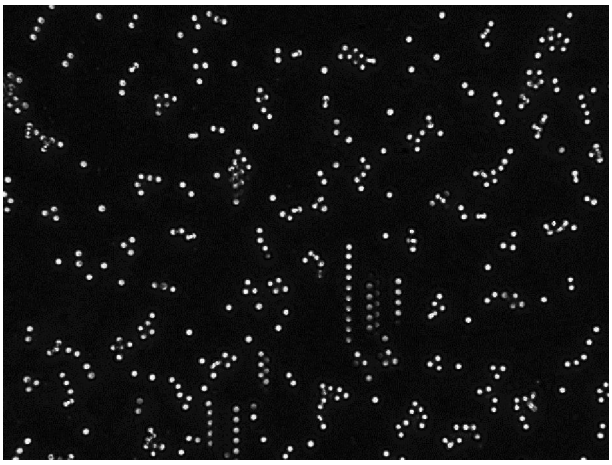

Figure 4.24 Erythrocyte aggregation by BH.

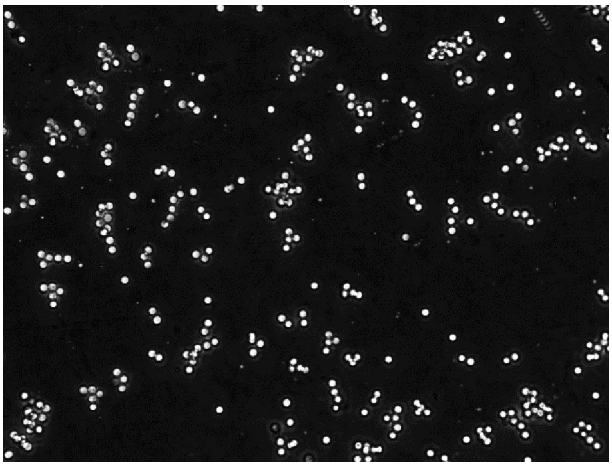

Figure 4.25 Erythrocyte aggregation by CH.

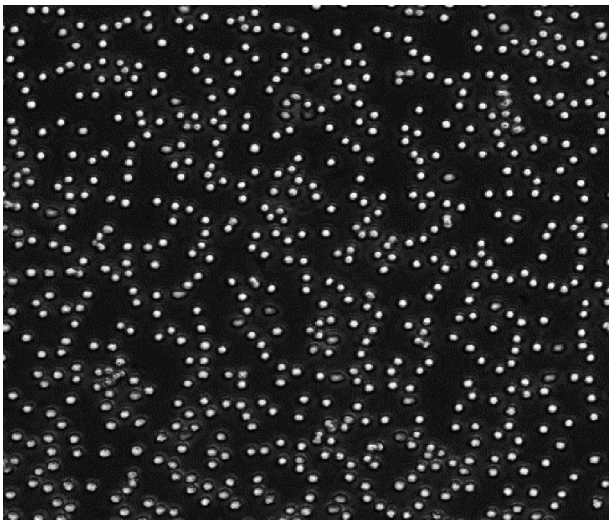

Figure 4.26 Erythrocyte aggregation by AL.

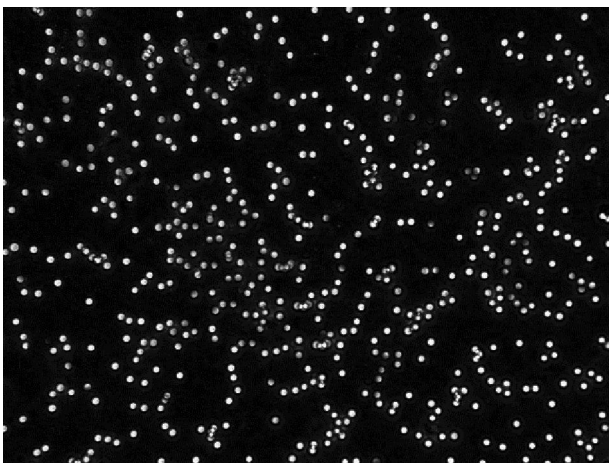

Figure 4.27 Erythrocyte aggregation by BL.

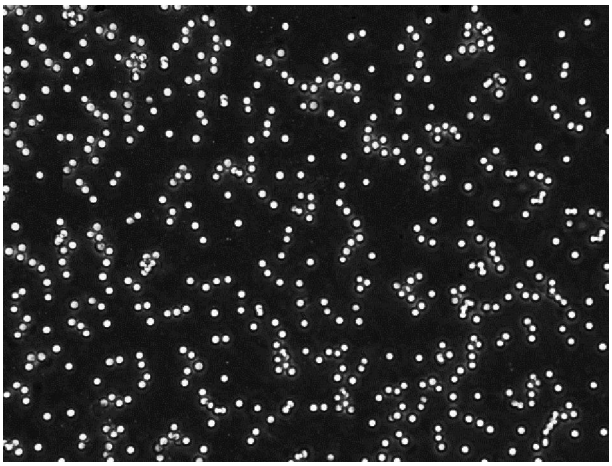

Figure 4.28 Erythrocyte aggregation by CL.

Hemolytic activity of PEI can be attributed to the membrane damaging and pore forming capability of cationic PEI due to constitutively ionized primary amines of the PEI. Such PEI can interact with negatively charged cell membrane components and like proteins and phospholipids and resulting in disruption of membrane (24, 25). From the results it is apparently clear that modified PEIs caused minimal damage to the erythrocytes leading to very low hemoglobin release. This could be attributed to the blockade of aforesaid primary amine groups with Boc-amino acids leading to reduced surface charge density which in turn would have reduced the interaction of modified PEIs with erythrocyte cell membranes.

Apart from hemolysis, the interaction of modified PEI with other particulate/cellular elements in the blood stream was evaluated using the aggregation of erythrocytes as a model (26). Such erythrocyte aggregation assay allowed semiquantitative evaluation of hemocompatibility of synthesized PEIs. Results of this study are given in **Figure 4.18, Figure 4.19, Figure 4.20, Figure 4.21, Figure 4.22, Figure 4.23, Figure 4.24** and **Figure 4.25, Figure 4.26, Figure 4.27, Figure 4.28,**. Only representative microscopic images of the erythrocytes are shown in these figures. Ringer's solution did not show any aggregation of erythrocyte (control experiment).

Positive control, PEI, induced a considerable amount of aggregation bunching a large number of cells in long chain and large cluster like aggregates as it can be seen in image. This can be ascribed to the high number of cationic primary amines of branched PEI which would cause high electrostatic interaction between PEI and erythrocytes. The cell walls between adjacent cells were also only partially visible suggesting the possibility of membrane fusion events. However, in the case of the modified PEIs, aggregation tendency was found to be less

as compared to non-modified PEI where small chains and clusters of erythrocytes were seen. Within different polymers, it was observed that as the concentration of free primary amines decreased with increasing substitution of Boc-amino acids (from polymer A to C), the erythrocyte aggregation was also decreased. This effect also gave a gross idea of the substitution pattern i.e. higher the substitution, lower the primary amines and hence lower will be erythrocyte aggregation.

4.3 References

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