Chapter 5: Development of Polyethylenimine (PEI) based non viral Vectors

5.1 Introduction

Among the Non-viral vectors, Cationic phospholipids such as DOTAP, DOTMA and cationic polymers like Chitosan, Poly-l-lysine(PLL), Polyethylenimine (PEI) have been explored which can complex to negatively charged siRNA as a delivery vector. Non-viral vectors for gene or siRNA delivery composed of cationic polymers are preferred over lipid based vectors when greater stability is desired in physiological environment(1). Positively charged polymers contain numerous amino groups in their backbone structure which interact with negatively charged siRNA leading to the spontaneous formation of nanosized complexes called as “Polyplexes”. Among these cationic polymers, polyethylenimine(PEI), especially branched PEI, is well-known as good transfection reagent due to its intrinsic proton sponge property (2). PEI has been studied extensively as a gene delivery vector as they have very good buffering capacity between pH 5.0 to 7.2 and due to protonable amino groups which absorb protons on intracellular uptake into endosomes and cause osmotically driven endosomes swelling followed by rupture of endosomes in the cytoplasm of the cell and release of the genes or siRNA of interest. Such escape of endosomes also assisted by the polymer expansion taking place due to intra molecular charge repulsion which helps to create pressure inside the endosomes.

However, due to high toxicity of the strong cationic nature of the amine groups and lack of biodegradability, and non-specific interactions with the serum proteins, it has limited or no clinical applications. Hence to reduce the cytotoxic potential while preserving the transfection efficiency has been the topic of interest.

PEI polymers is available in the two different forms i.e. linear PEI& Branched PEI, among the two types, branched PEI is very effective as gene delivery concern. Modification of amino groups of PEI has been approach to reduce the aforesaid drawbacks of PEI without affecting transfection efficiency. Several studies have been reported in order to enhance the transfection efficiency of the PEI via the modification of the polymer(3).

Modifications of the polymer PEI involves ligand conjugations targeted to specific receptors expressed on the cells of interest. Furthermore, efforts have been made to improve toxicity profile with maintained transfection efficiency. Amino group modification in the PEI structure is the most common approach to attach the pendent groups or ligand conjugation onto the amines of the polymer. In addition to this, PEI has been modified with amino acids, carbohydrates, grafted with cyclodextrins, PEGylated, Substituted with aliphatic acids, acetylated to improve the transfection and to reduce the toxicity profile(4).Several imidazole containing compounds have been use to conjugate with amines of PEI. Urocanic acid contains Imidazole in its structure. Imidazole ring has pKa of 6.15. Several studies demonstrated proton sponge effect of imidazole modified polymers(5).Polymers like Poly L-lysine and methacrylates also have substituted successfully with imidazolyl group. Such type of modifications has demonstrated the efficient transfection in mammalian cells and so eliminating the necessities of the endosomatic agents.

Here, in present work, imidazole ring containing Urocanic acid was conjugated on bPEI at different substitution levels. Primary objective of the work was to reduce cytotoxicity of the bPEI and improve the transfection efficiency intracellularly when polyplexes endocytosized into the cells to reach the target.PEI, well known for its proton sponge mechanism and thereby so escaping gene delivery vectors from endosomes and improving transfection efficiency.

5.2 Conjugation of Urocanic acid on PEI

25 kDa Branched Polyethylenimine (bPEI) was conjugated with Urocanic acid at different molar ratios by covalent conjugation using carbodiimide chemistry, i.e. EDC-NHS crosslinking chemistry. Briefly, appropriate quantity of Urocanic acid was dissolved in dimethyl formamide. Further, to this solution, EDC (2 mmole), NHS (2 mmole), and triethylamine (2 mmole), were added. This mixture was stirred for 30-40 min and then bPEI was added followed by borate buffer (100mM). The conjugation reaction was carried out for 24h at the room temperature and then reaction mixture purified by dialysis against MES buffer (50 mM). Resulting solution was lyophilized (Lyophilizer- Virtis Advantage Plus) to get dried product. Further, synthesized polymers were characterized

by^{1}H-NMR, FTIR, TNBS assay, buffering capacity etc. Nomenclature of the synthesized modified polymers was mentioned in the following table 5.1.

Table 5.1: Nomenclature of synthesized Polymers

Sr.No.	Quantity of Urocanic acid (mg)	Quantity of PEI (mmole)	Nomenclature
1	150		PEI-UAA
2	100	0.012	PEI-UAB
3	50		PEI-UAC

5.3 Nuclear Magnetic Resonance spectroscopy (NMR) Analysis

Proton NMR spectroscopy was carried out to determine the characteristics peaks of the functional group to confirm the modified polymers. ^{1}H-NMR spectra of PEI conjugated with Urocanic acid was recorded on Bruker 400 MHz spectrometers at frequency of 400MHz. Native PEI and modified PEI were dissolved in D2O in 5 mm NMR tubes and ^{1}H-NMR spectroscopy were performed individually.

5.4 Fourier Transformed Infrared Spectroscopy (FTIR) Analysis

FTIR spectra were recorded for all the modified PEI polymers by potassium bromide (KBr) pellet technique. Appropriate quantity of the polymers was mixed with the KBr and compressed to obtain a pellet and analysis was performed by FTIR Spectrophotometer (Bruker and Shimadzu). Baseline correction was done and samples were then scanned against the blank KBr pellet background in range of 4000-600 cm^{-1} wave number with 1.0 cm^{-1} resolution.

5.5: 2, 4, 6- Trinitrobenzene 1-Sulphonic acid (TNBS) assay

Free primary amines of the modified PEI were estimated by TNBS assay. TNBS reacts with primary amines of the PEI making it possible to determine the percent of substituted primary amines in modified PEI with compared to unmodified PEI. Briefly, polymers were dissolved in reaction buffer,0.1 M Sodium bicarbonate buffer. 0.25 ml of 0.01% w/v TNBS was then added into 0.5 ml solution of each sample and mixed well and incubated at the 37 °C for 2 hr. Then, 0.25 ml of 10% SDS and 0.125 ml of 1 N HCL were added to

each sample and absorbance of the each sample was measured at 335 nm(6, 7). Experiment was performed in triplicate and percent of primary amines substitution was calculated accordingly. Calibration plot was prepared by using 5-30 ppm concentrations of histidine solutions.

5.6 Buffer Capacity/Acid base titration

Endosomal escape of the formulation is necessite for the successful transfection. This property directly relates to the buffering capacity of the vectors in the endosomal pH range. Several studies report that PEI has very good buffering capacity and proton sponge effect. Hence, proton sponge study was performed. Study was performed by titrating synthesized polymer solution from acidic pH to basic pH range. Briefly, 10 mg of polymer was dissolved in sodium chloride solution(150 mM) by hydrochloric acid by adjusting initial pH 10.0 and then this polymer solution was titrated with 0.1 N Hcl olution. pH values of the polymer solutions were recorded by pH meter (Lab India, Model:Pico^{+}). The amount of NaoH consumed was calculated and compared.

5.7 Hemolysis Study

Erythrocytes (RBCs) were collected from fresh heparinized rat blood by the centrifugation at 1000 g for 10 min at 4^{o}C for the hemolysis study. Obtained pellet was washed with PBS Ph 7.4 several times until supernatant became clear. RBCs were reconstituted in PBS pH 7.4 to obtained 2% v/v RBCs suspension. The 500 ul of cell suspension was added into different concentrations of the modified PEI prepared in eppendrof tubes. Then mixture was mixed by vortex mixer thoroughly and incubated at 37^{o}C for 1 hr under the constant shaking in Shaker incubator. After that, RBC suspension was centrifuged at 3000-3500 rpm for 10 min and after that the supernatant was estimated for hemoglobin content release by UV-Visible spectrophotometer and at 540 nm. (8-13).

5.8 Erythrocytes aggregation

Due to the cationic charge of the amino groups, PEI is known to induce aggregation of the erythrocytes *in vivo* and hence to evaluate biocompatibility of newly modified PEI conjugated with Urocanic acid, erythrocyte aggregation study was performed using rat blood cells. The erythrocyte suspension was prepared by the same procedure as that described above section for Hemolysis study. 500 µl of 2% erythrocyte suspension

containing modified polymers at different concentration were prepared in PBS pH 7.4 mixed thoroughly by vortexing and incubated for 2 hr at 37°C in the incubator. The erythrocyte aggregation was observed using inverted microscope (Nikon Eclipse TS100) in a phase contrast mode.

5.9 *In vitro* cytotoxicity assay (MTT Assay)

Modified PEI was accessed for *in vitro* cell cytotoxicity by MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-di-phenyl tetrazolium bromide) assay. As MTT assay is very rapid and versatile technique to access the cell viability in vitro(14). Other general method for the determination of cell viability like dye exclusion (trypan blue) can also be used.

MTT assay was performed in different concentrations of modified PEI polymers. The A549 cells were seeded at density of 5×10^3 cells/well in a 96 well microtiter plate (corning, New York) using DMEM supplemented with 10% Fetal Bovine Serum (FBS). The cell culture was grown for 24 hr in CO_2incubator maintained at 5%concentration and humidified with saturated Copper sulphate solution. After 24 hr, the cells were exposed to different polymer concentrations and evaluated for six hrs. After exposure time, cell the media was replaced with complete medium containing1% antibiotic solution and 10% Fetal Bovine Serum (FBS). After 24 hr, cells were then washed with phosphate buffer saline and 20 µl of MTT dye (5mg/mL) solution was added to each well plate. The MTT dye was allowed to react for 4 h under incubator condition, after that cell medium in each plate was then replaced with 100 µl of DMSO (Himedia, Mumbai) and microtiter plate was shaken gently to dissolve the formazan crystals. The color of the completely dissolved formazan was accessed by using microtiter plate reader (Biorad, California). Cells treated with DMEM media and 0.1% Triton X were used as negative and positive control. The absorbance values of cells treated with DMEM media were taken as 100% cell viability and all other treatments were expressed relative to it.

5.10 Result and Discussion

NMR Analysis

Successful conjugation of Urocanic acid onto the PEI was confirmed by the ^{1}H-NMR spectra shown in the figure 5.1. PEI-UA showed characteristic peak of hydrogen of imidazole ring at around δ 8.5 ppm and peak of –CH at δ 4.7 ppm. Characteristics peaks of Polyethylenimine observed at δ 2.7 ppm to δ 3.5 ppm and those of ternary carbon appeared at δ 1.1 ppm. These^{1}H- NMR findings suggest the successful conjugation of Urocanic acid onto the 25 kDa branched PEI.

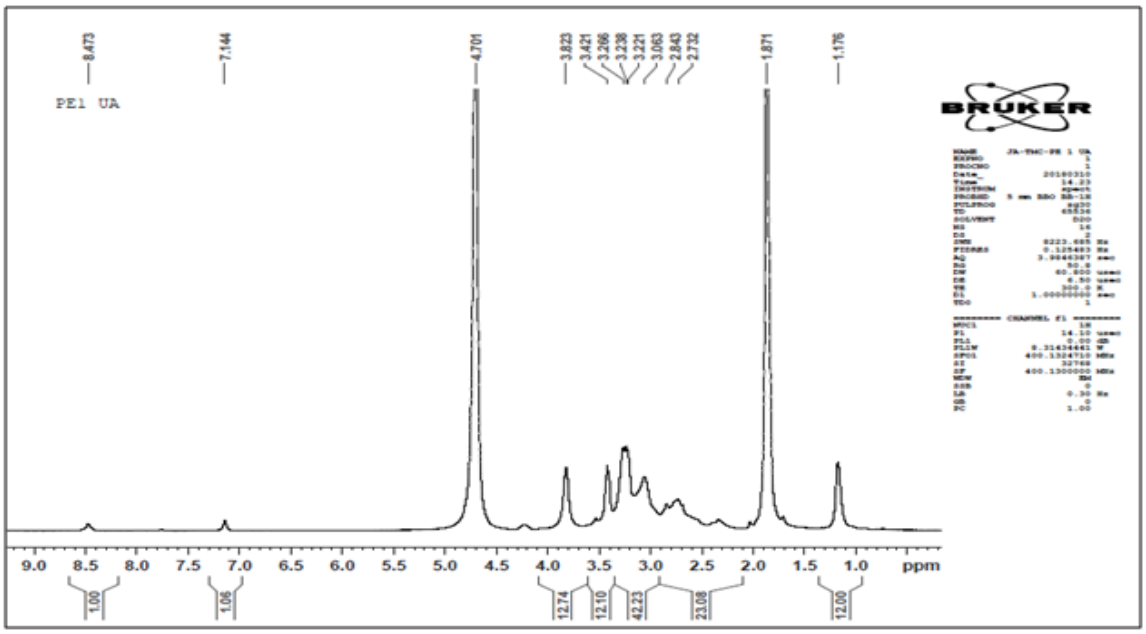

Figure 5.1:^{1}H-NMR spectrum of Urocanic acid Conjugated Polyethylenimine

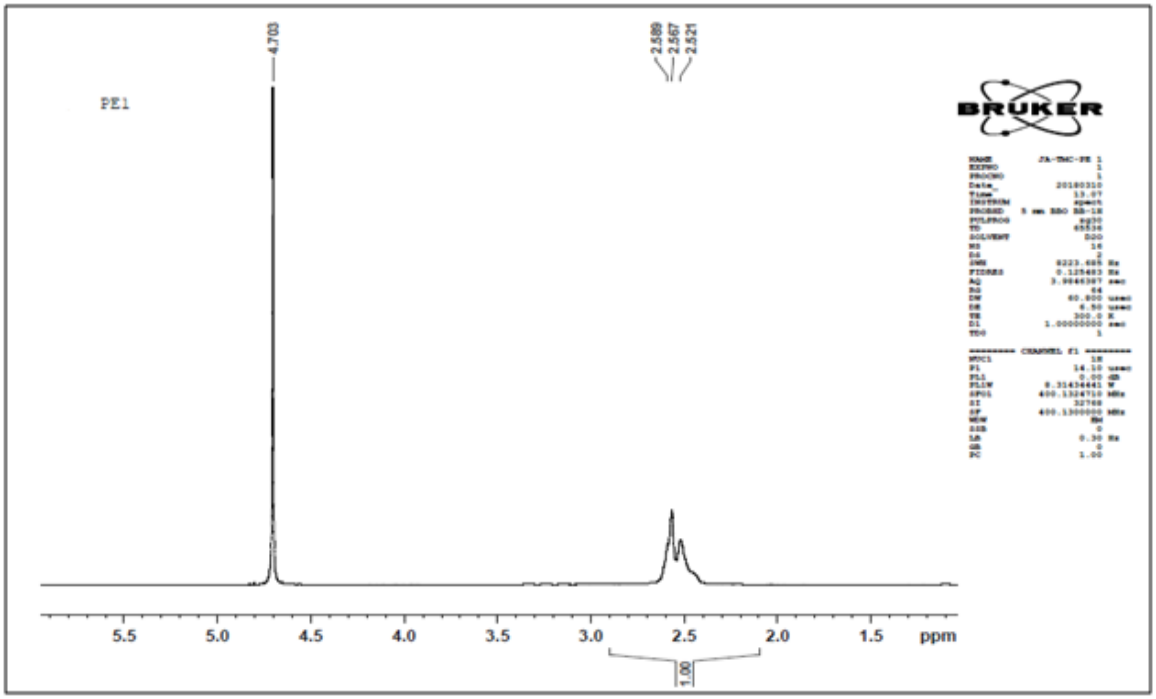

Figure 5.2: ^{1}H-NMR spectrum of Unmodified 25 kDa Branched Polyethylenimine

FTIR Analysis

Figure 5.3 and Figure 5.4shows the FTIR spectra of polyethylenimine and Urocanic acid conjugated Polyethylenimine respectively. Spectrum of later demonstrates Urocanic acid was conjugated on the PEI. Upon conjugation reaction, strong absorption around 1643 cm-1 ascribed to the amide linkage appeared in the spectra of PEI-UA in compared to spectrum of PEI. Additionally, synthesized PEI-UA illustrated strong and broad absorption of amines at 3414 cm-1 of N-H stretching and at around 1562 cm-1 of N-H bending characteristics of –NH group of PEI. Furthermore, other characteristic peaks were also seen in FTIR spectra of PEI-UA which include primary alkyl stretching around 2934 cm-1, alkyl bending at around 1396 cm-1 and CH2 bending at 1461 cm-1. These results are in concordance with the previous results of the modified PEI by using different conjugation moiety (15).

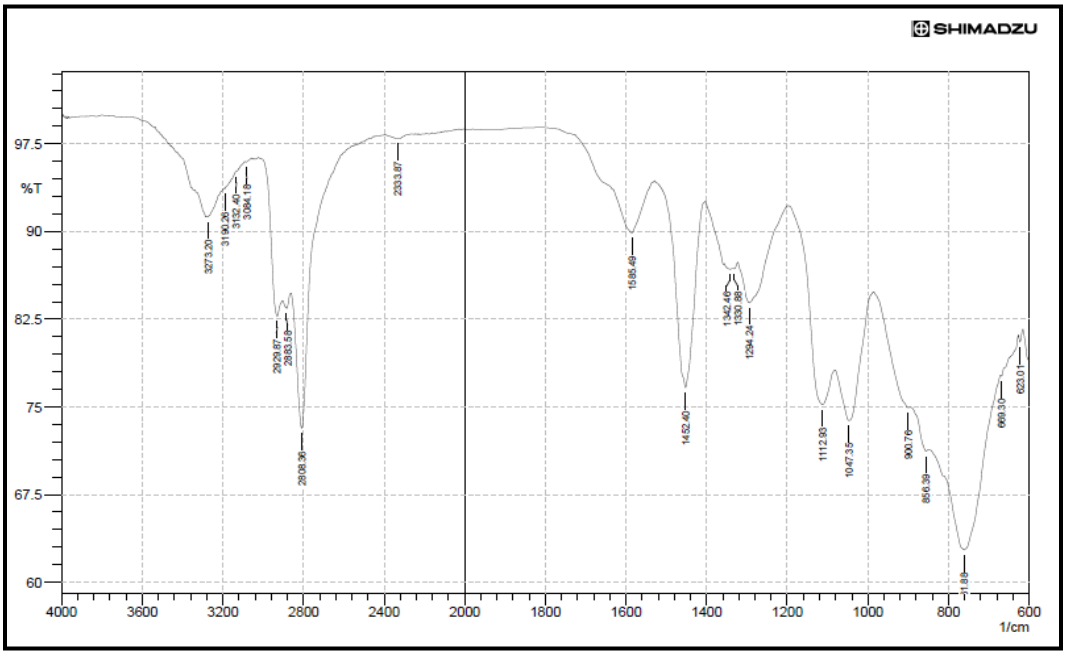

Figure 5.3: FTIR spectrum of Polyethylenimine

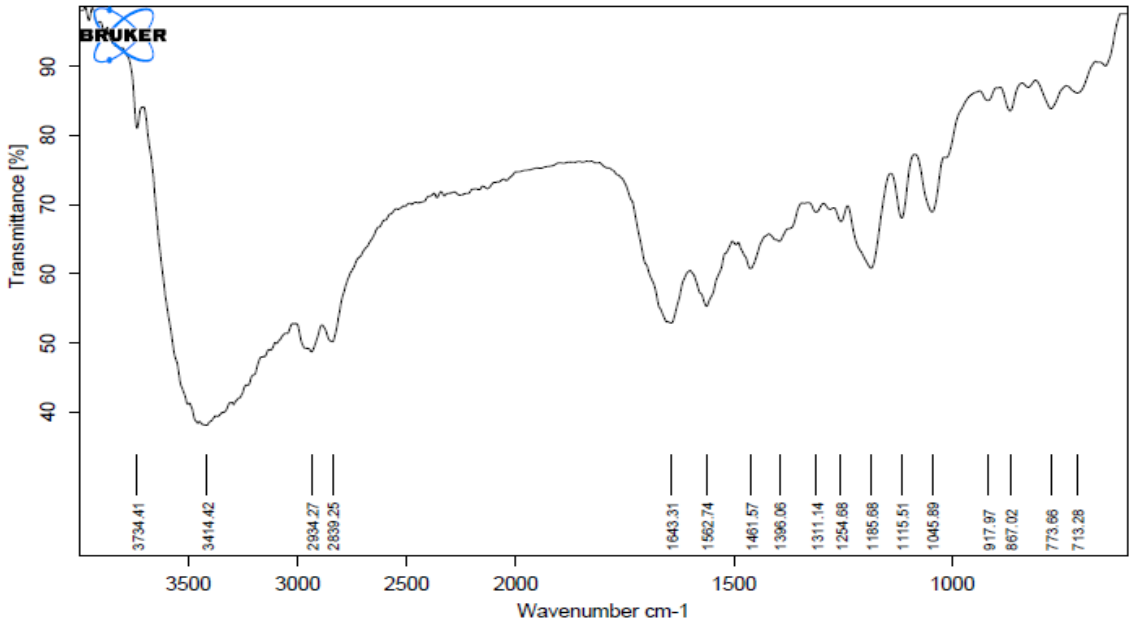

Figure 5.4: FTIR spectrum Urocanic acid conjugated Polyethylenimine (PEI-UA)

Determination of conjugation by TNBS assay

Same as chitosan discussed in the previous chapter, polyethylenimine is also an attractive non-viral vector for the gene delivery due to its well-known capability to condense the siRNA or DNA through the electrostatic interaction between the negatively charged

nucleic acids and the primary amines of the chitosan polymer. Presence of the primary, secondary and tertiary amino groups, PEI is amenable to diverse and selective modifications or any type of conjugation on the structure. Primary site for PEI modification is primary amine group as it can be modified easily and plays a significant role in siRNA complexation and transfection efficiency. Successful conjugation of any group on the primary amine group of PEI can be assessed by the TNBS assay and degree of the conjugation or substitution can be estimated. Figure 5.5 showed overlay spectra of calibration explained by the equation y=0.031x+0.007 with correlation coefficient of 0.998.

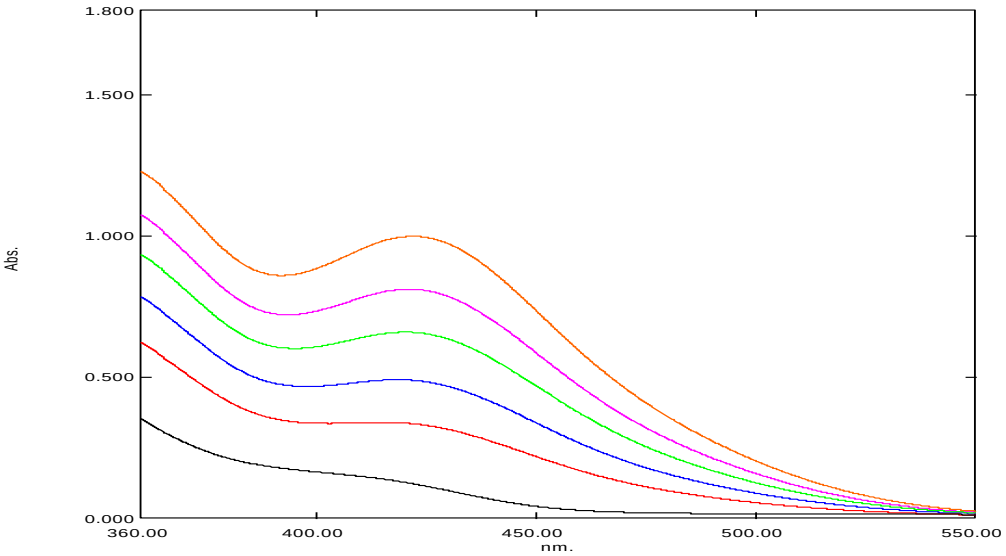

Figure 5.5: UV spectra of Calibration for TNBS assay

Table 5.2: Degree of conjugation of Urocanic acid on PEI

Sr. No.	Modified Polymers	Degree of conjugation* (%)
1	PEI-UAA	33.12±3.57
2	PEI-UAB	24.87±2.23
3	PEI-UAC	11.89 ±1.54

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

Above results of TNBS assay showed increasing conjugation percentage on the PEI with increasing molar ratio of the Urocanic acid used. Urocanic acid conjugation at higher molar ratio was 33.12±3.57% and at lower molar ratio was11.89 ±1.54%.

Buffer capacity/Acid base titration

As discussed in the previous chapter, positively charged vectors ensures high cellular uptake of gene or siRNA by endocytosis. Though, key requirement for nucleic acid delivery systems is that they should escape the endosomes to circumvent exposures of therapeutic siRNA or DNA to higher pH of the late endosomes. High transfection efficiency of the cationic polymers like polyethylenimine has been extrapolated to their higher buffering capacity that aid in lysis of endosomes at lower pH of the endosomes by secondary and tertiary amines ionization. In the cell, endosomes have comparatively low pH (5.1 to 7.4) by ATPase proton pump. And so, called “proton sponge” mechanism can be explained by the example of PEI which once uptaken within the endosomes, takes up the protons through secondary and tertiary amines ionization which results in more proton influx to maintain pH and these influx of the protons forces the chloride ion accumulation within endosomes. Proton and chloride influx increase the osmotic pressure inside the endosome vesicles causing endosomes rupture and release of the therapeutic genes from the vectors or complex to the cell cytoplasm. In addition, high charged density developed on the polymer causes its expansion within the endosome vesicles, which further supplements the endosomatic effect of the developed polymer, polyethylenimine. Proton sponge by modified polyethylenimine too protects the therapeutic genes from unfavorable pH effects.

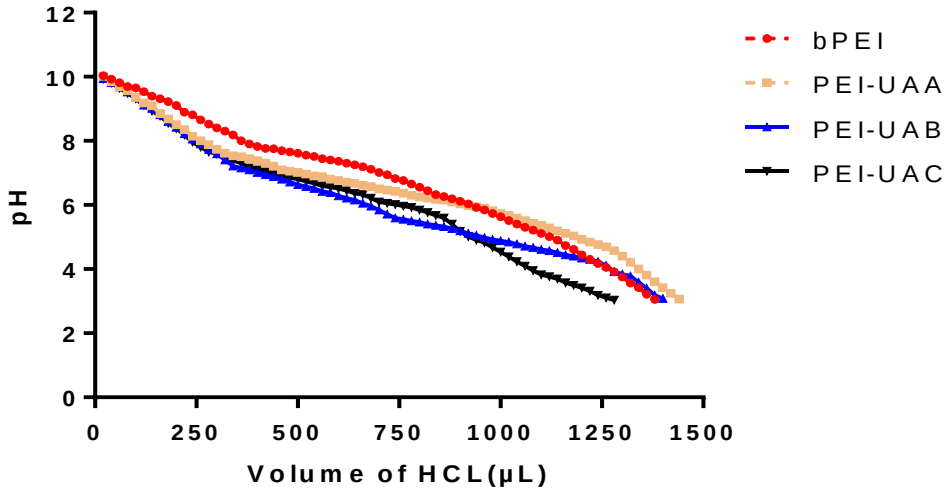

Figure 5.6: Acid/Base titration of modified Polyethylenimines

Figure 5.6 demonstrates that pH of polyethylenimine solution went down slowly by continuous HCl addition, which explains that polymer has a strong proton sponge ability. PEI-UAA has an improved buffering capacity than bPEI while PEI-UAB and PEI-UAC demonstrates comparable buffering capacity to PEI. Titration curve explains that increased degree of conjugation of Urocanic acid consumed higher amount of HCl compared to bPEI. The results demonstrate that imidazole ring of Urocanic acid took part in enhancement of proton sponge activity of the conjugated PEI and it will assist to promote release of siRNA polyplexes into the cytoplasm by endosomal escape. The findings of our experiments are congruent with the previous reports which explained that imidazole derivatives like histidine performs significant role in improvement of proton sponge effect as imidazole ring nitrogen has pKa of around 6.0-6.5. (5)

Hemolysis and Erythrocyte aggregation

Compatibility of any vectors of gene delivery with blood is undoubtedly imperative and indicates its appropriateness administration into the systemic circulation. The findings of hemolysis study are represented in figure 5.7. Results demonstrated that concentration dependent increase in hemolytic potential of the modified PEIs suggesting the higher primary amine amount would confer higher cell destruction or damage. Nevertheless, modified polymers exhibited reduced hemolytic activity as compared to

that found with PEI. From the above figure 5.7 it can be said that bPEI exerted 2%- 20% hemolysis activity at 1 µg/ml to 1000 µg/ml concentration. While modified polymers demonstrated less hemolytic potential. PEI-UA showed about 5% hemolysis potential at the highest concentration of 1000µg/ml studied. Hemolytic potential of polyethylenimine can be ascribed to cell membrane damaging and pore forming ability owing to ionized primary amines of the PEI. Polyethylenimine can interact with the negatively charged cell membrane and like phospholipids and proteins and yield in membrane disruption.

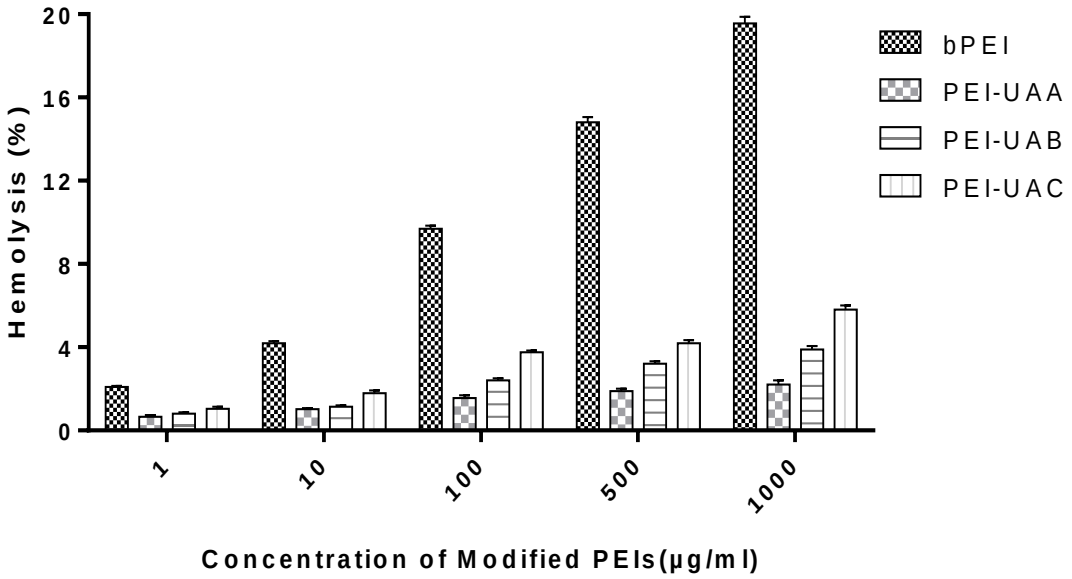

Figure 5.7: % Hemolysis of different modified PEIs

From the findings of the study it seems that modified polymers caused minimal damage to erythrocytes leading to low amount of hemoglobin release. This could be ascribed to the blockage of primary amine groups with Urocanic acid leading to decreased charge density which in turn would have decreased the interaction with erythrocyte cell membrane of modified polymers(16, 17).

Separately from hemolysis study, interaction of modified polymers with other cellular elements in the blood was assessed by erythrocyte aggregation as a model(18).Such type of study allowed semi-quantitative evaluation of hemocompatibility of the modified PEI based polymers. Results of the study are shown in the figure 5.8 (a-d). Only representative images of microscopy of erythrocytes are shown in figures.

PEI induced a substantial amount of aggregation bunching large number of cells in long chain and aggregates as it can be observed in figures. This can be attributed to the high numbers of primary amines of PEI which would cause high electrostatic interaction between polymer and erythrocytes. The cells wall between cells were too partially visible demonstrating probability of membrane fusion events. Nevertheless, in case of modified polymers, erythrocyte aggregation tendency was found to be less as compared to native PEI where small chains and aggregates of the erythrocytes were observed. Within different polymers, increasing conjugation of Urocanic acid, aggregation of erythrocytes was decreased as primary amines concentration was reduced.

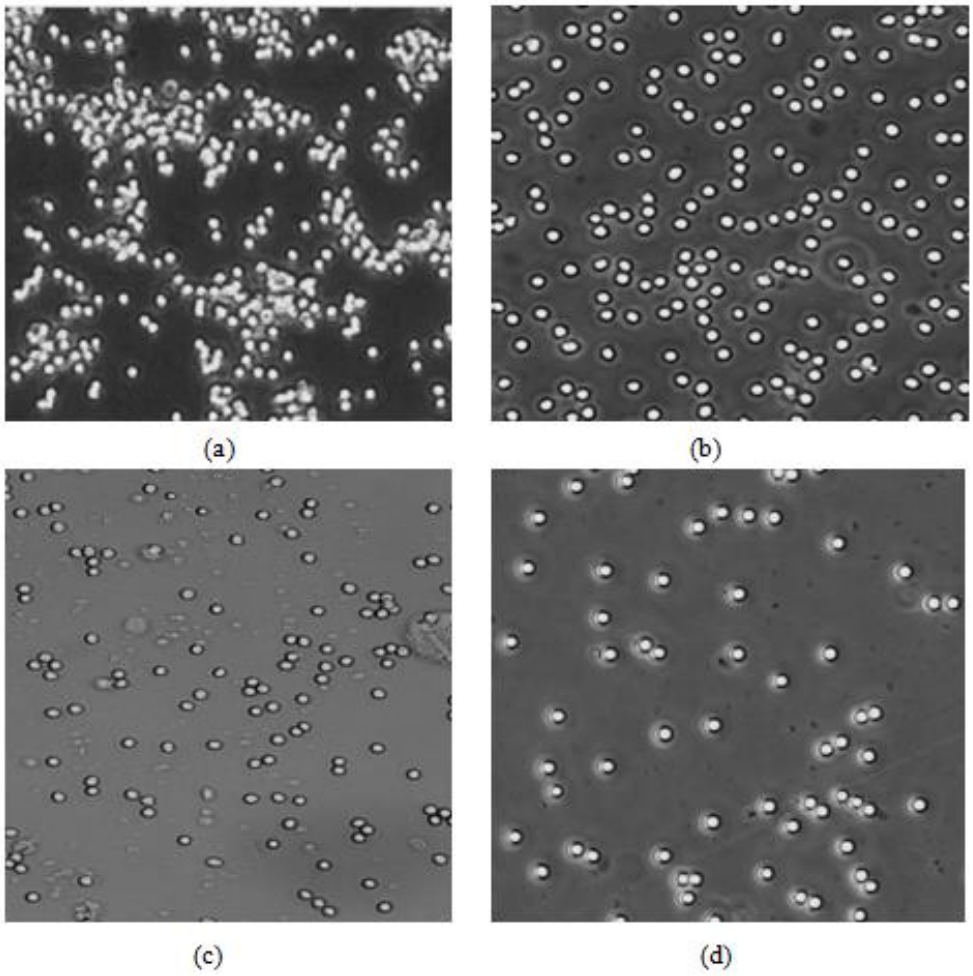

Figure 5.8: Erythrocyte aggregation by (a) bPEI (b) PEI-UAA (c) PEI-UAB (d) PEI-UAC

***In vitro* cytotoxicity study**

Figure 5.9 elucidates the viability of A549 alveolar epithelial cells after 24h incubation with different concentrations of the synthesized polyethylenimine conjugated with Urocanic acid. The concentration ranged from 1, 5, 10, 20, 50, 100 µg/mL. Modified polymers exhibited cell viability of greater than 90% after 24h at concentration of 10 µg/mL in A549 cells at each conjugation levels. While bPEI showed about 82.12% and 62.15 % cell viability at 10and 100 µg/mL concentrations, respectively. Modified PEIs show around 80-83% cell viability at 100 µg/mL concentration. As the concentration of conjugated PEI increases from the 1µg/mL to 1000 µg/mL, there was decrease in the cell viability. Cell viability was assessed by considering the cell viability of the control cells.

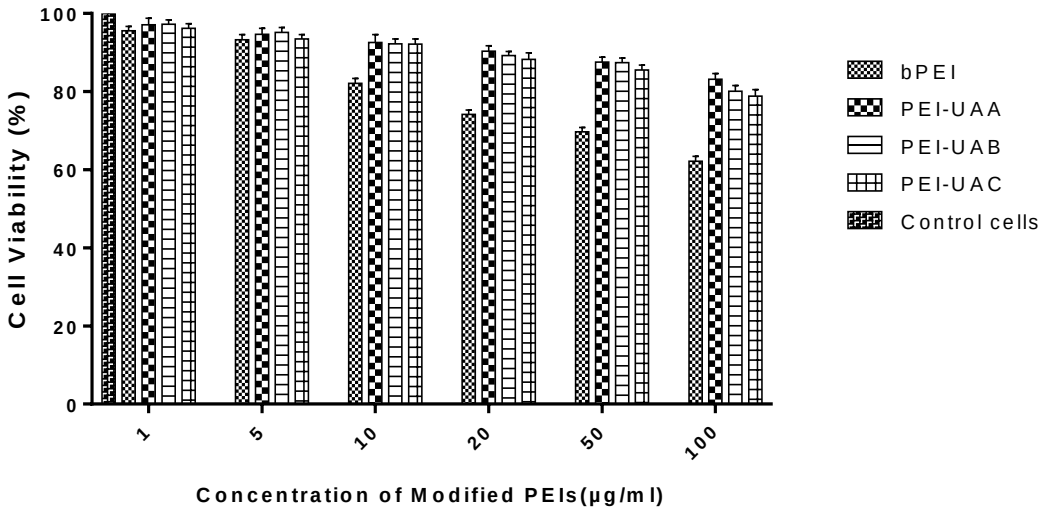

Figure 5.9: Cell viability of modified PEIs

From the results, it can be said that conjugation of Urocanic acid reduced the cationic charge of bPEI as density of the primary amino group decreases. Higher the conjugation of the amines, higher the cell viability and thereby so reduced cytotoxicity of the polymers.

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