Chapter 6: Formulation Development and Characterization

6.1 Polyplex Preparation

As complexation ability of the modified polymers, depends on the charge density which is function of the pH of solvent or vehicle used for polyplex formulation. Hence, all the polyplexes formulations were prepared in 7.4 pH 20 mM sodium acetate buffer with batch volume of 20 µl. The appropriate quantity of polymer stock solution (1mg/mL) was added to sodium acetate buffer. Incubation time of polymer and siRNA as well as presence and absence of vortex mixing was studied to optimize the polyplex preparation at room temperature conditions. So for that, mixture was then gently mixed by vortex mixer for 2 min and then, siRNA (100 pmole) was added to it and kept for incubation for 20, 40, 60 mins at room temp. conditions.

6.2 Agarose Gel retardation assay (Gel Electrophoresis)

Interaction of the modified polymers and siRNA at different w/w ratio was studied by the agarose gel electrophoresis assay. Naked siRNA (uncomplexed) tend to move in the direction of anode under the influence of the electric field applied which can be detected through staining with nucleic acid strain. Briefly, all the incubated polyplexes (20 µl) were mixed with gel loading buffer (6X, 3 µl) Himedia, India) and loaded onto a 2 % agarose gel containing 0.5µg/mL EtBr (ethidium bromide) and electrophoresed at 100V in TBE buffer pH adjusted to 7.4. After 70% run siRNA was visualized by UV trans-illuminator using Bio-Rad Gel Doc System (Bio-Rad Lab., USA). siRNA retardation was determined at particular ratio of polymer to siRNA.

6.3 Complexation efficiency

To estimate the complexation efficiency at the weight to weight ratio resulting in retardation of the siRNA in gel electrophoresis, centrifugation-UV spectrophotometry was performed. polyplexes were ultracentrifuged at 30,000 rpm for 50 min by maintaining cooling at 4°-5°C. The aqueous supernatant was isolated siRNA content was estimated using Nano Drop.

6.4 siRNA Integrity determination

Heparin was used to assess the effect on integrity of siRNA by the polyanion competition assay. Polyplex formulation was mixed with Heparin sodium (1mg/mL) and incubated for 20-25 min. After incubation, displaced siRNA content was detected on agarose gel electrophoresis and analyzed.

6.5 Polyplex Size and zeta Potential Analysis

Dynamic light scattering (DLS) technique was used to determine the particle size of the polyplexes using Malvern Zetasizer. The formulated polyplexes were diluted suitably with nuclease free water and measured at 25^{o}C. Likewise, zeta potential of the polyplexes was measured by applying Smoluchowski's equation in the Malvern zeta sizer software. All the studies were performed in triplicates.

6.6 *In vitro* Cell line Studies

6.6.1 Introduction

In vitro cell lines studies are very essential as they provide method for preliminary evaluation of direct effects on the tissues and cells so as to set up clinical relevance in pathological states, screening and understanding of toxicity mechanisms. Cell suspension can be prepared by dispersing primary explant or any tissue either mechanically or enzymatically which then is may form monolayer or a substrate or as a suspension. The cells go through proliferation in cultures forming a monolayer or suspension, which constitutes a passage. After several generations cell can transform into a continuous cell line having high growth capacity and population uniformity. Monolayer culture is the most common mode of culturing, in which cell are usually anchorage dependent and need a substrate for cell attachment before cell proliferation. In contrast anchorage independent cells (suspension cultures) are proliferate without attachment, which is rare and is generally present in cells of hemopoietic system.

The cell culturing protocol also requires disaggregation of attached monolayer culture using some protease enzymes which can digest the extracellular matrix, thereby liberating the cell from matrix. Epithelial cells, if left long for confluency, are hold tightly by

desmosomes and tighter junctional complexes. Furthermore, Chelating agent are added to trypsin solution during disaggregation due to calcium ions dependency of adhesion molecules. After cell attachment, the space between cells is filled with ECM, the composition being determined by cell type. The ECM (extracellular matrix), stays in dynamic equilibrium with cells and its complexity is significant contributor to phenotypic expression of cells attached to it.

Cell motility is also obvious in cell culturing experiments. When the cell density is very low, cells have capability for movement on substrate. The motile cells can be recognized by the presence of polarity, as a result of polymerization of lammepodium due to polymerization of actin in the course of movement and sticks to the substrate. Consequently, the plasma membrane retracts opposite and cell moves. The cells migrate in erratic manner, however once attaining the confluence the migration stops under the influence of contact inhibition. Consequently, cells form patches and the entire patch may also demonstrate movement signs.

Primary cell cultures are often unsuitable due to their instability for studies i.e. they undergo continuous adaptive modifications and it's difficult to select a period of when entire cell population is homogenous or stable. After confluence, some cells may transform and become insensitive to contact inhibition and overgrow, therefore it is essential to keep the cell density low to maintain the original phenotype. Following first subculture or a passage, the culture is called cell line. In each subsequent subculture a population of cell having capacity to rapidly grow will predominate while slow growing cells dilute out. In most of the cases culture becomes stable after three passages. The ordinary cells split for limited times due to progressive telomerase shortening as these DNA terminal sequences are unable to replicate in each cell division. Nevertheless, and transformed cells, stem cells and germ cells express the telomerase which have a capacity to replicate terminal DNA sequences of telomeres.

Cell line propagation also requires culture media with defined chemical composition to make sure consistent quality and reproducibility. The physicochemical characteristics are taken into consideration to suit the requirements of purpose. Most of the cells grow well at pH 7.4. The CO_2 dissolves into medium to establish equilibrium with HCO_3-ions to

maintain the desire pH. Hence, atmospheric CO_2 tension regulates the pH and standard equivalent HCO_3-concentrations for different CO_2 have been provided in literature. Besides HCO_3-other ingredients like pyruvate, high concentration of amino acids is used as buffering agent in media. The cells also require oxygen, the depth of static culture should be kept within the range of 2-5 mm so as to maintain the rate of oxygen diffusion to the cells. The necessite of temperature depend upon animal body temperature from which cells were derived and therefore kept at 37^{o}C. They can tolerate a considerable drop i.e. can survive at 4^{o}C for several days and can be frozen at −196^{o}C, however; they cannot tolerate more than 2^{o}C above 37^{o}C for more than few hours and will die quickly at 40^{o}C and above. Hence, consistency of temperature is required to obtain reproducible results.

6.6.2 Cell Line Cultures

A549 alveolar epithelial cells were procured from NCCS, Pune. Cell protocol involved culturing in Dulbecco's Eagle's minimum essential medium (DMEM) (Himedia, Mumbai) with 2mM L-Glutamine, FBS(10%) and 1% antibiotic solution. Cells were maintained at 5% CO_2 at 37°C ± 2°C in incubator.

6.6.3 Cell Culturing Protocol

The A549 cells were maintained as a form of monolayer in T-25 cell culture flasks individually, and medium was replaced twice in a week. After attaining desired confluency, cells were sub-cultured in DMEM (Himedia, Mumbai, India) containing 1% penicillin/streptomycin, 10% FBS and 2mM L-Glutamine. The cell culture media was examined regularly during experimentation for freedom from any type of deterioration and contamination. The decision to subculture was taken Based on confluency, multi-layering, evidence of mitosis of the cells, sub culturing of the cell was done. Following sub-culturing protocol was used:

- The cell culture flask was taken to the sterile area and culture medium was separated

- The cells were washed with sterile PBS to eliminate the traces of serum which may inhibit trypsin action.
- 2 ml Trypsin-EDTA solution was added to the sides of the culture flask opposite to the cells and then turned down to ensure surface covering of the monolayer and left for 15-30 sec.
- All but few drops of trypsin were withdrawn before complete dislodgment and culture flask was incubated at 37 ^{o}C until the cells start to round up (5 min).
- 2 ml complete medium was added to disperse the culture cells, dispersion with pipetting as continuous cell line requires vigorous pipetting for disaggregation completely.
- Cells count was performed on haemocytometer.
- Then after suitable seeding concentration was added to the culture flask and then 10 mL of complete media was added.
- The culture flask was closed and incubated at 37 °C, 5% CO_2in incubator.

Each sub-culture represents one passage of the cells while the generation number was based on the split ratio. If the cells are growing perfectly they reach the same concentration after same time in each cycle, if the seeding cell concentration remains constant. Cell culture condition standardization was performed to maintain the phenotype stability which was achieved through regular maintenance and adherence to strict defined condition. The cell culture media was procured from Himedia, Mumbai, India during all experimentation. The serum is also a probable source of variability; hence a single batch was used throughout experimentation.

6.6.4 Cell Counting using Haemocytometer

1. Mix up the trypsinized cell suspension thoroughly so as to disperse the cell into individual.
2. The Haemocytometer was cleaned using 70% ethanol

3. A cleaned coverslip was kept over the semi-silvered surface. Newton's ring formation was observed to ensure proper attachment of coverslip and correct depth of the chamber.

4. A 50µl sample was taken using pipette and mixed with equal volume of trypan blue i.e. an exclusion dye for staining purpose to identify the dead cells. (The live cells exclude the water-soluble dye due to their impermeable lipoid membrane)

5. After staining a sample was taken after dispersing cells and immediately transferred to edge of the haemocytometer chamber.

6. Care was taken not to overfill the chamber as it may change the dimensions of the chamber.

7. The second chamber was also filled in the similar manner.

8. Using the 10X objective, the grid lines over the 16-corner square were focused.

9. The number of cells in the square was counted. Dead cells stained blue by trypan blue were excluded. Standard rule was followed not to count the same cell twice i.e. for each square the cells on top and left line were counted.

10. Viable cell count was performed on the on all 4 sets of 16 squares at each corner of Haemocytometer chamber.

11. No. of cells in 1 set of 16 corner squares is equal to the no. of cells in that set x 10^{-4} mL.

12. Average number of cells was calculated using following formula.

Total count from 4 sets of 16 corners = Average no. of cells per mL x 10^4 x 2. Where, 10^4 is conversion factor (0.1 mm^3 to ml) and dilution factor is 2.

6.6.5 In vitro cytotoxicity assay

Before the cellular internalization, destabilization of the cellular membrane or any DNA interaction after cell internalization could be the basis for the cytotoxicity of the polymer or formulation.

The synthesized TMC modified polymers and modified PEI as such were evaluated for cytotoxicity during initial screening described in previous chapters 4 and 5. Now the polyplexes finally optimized by siRNA binding and stability challenge studies were subjected to cytotoxicity characterization at increasing polymer to siRNA weight to weight ratio.

Study was performed in A549 alveolar epithelial cell line to assess the cytotoxicity of the polyplexes formulations. The A549 cells were seeded with 5×10^3 cells/well cell density in a 96 well microtiter plate using DMEM supplemented with 10% Fetal Bovine Serum. The cell culture was grown for 24 hrs in CO_2incubator maintained at 5%concentration and humidified with saturated Copper sulphate solution. After 24 hrs, the cells were exposed at different w/w ratio and evaluated for six hrs. After exposure time, cell the media was replaced with complete medium containing 1% solution of antibiotic and 10% Fetal Bovine Serum. After 24 h, cells were washed with phosphate buffer saline and MTT dye (5mg/mL) solution (20 µl) was added to each well plate. The MTT dye was permitted to react for 4 h under incubator condition, after that cell medium in each plate was replaced with 100 µl of DMSO (Himedia, Mumbai) and microtiter plate was shaken gently to dissolve the crystals of formazan. The color of the formazan was determined using Biorad microtiter plate reader (Biorad, California). PBS treated Cells were used as control cells. The absorbance values of cells treated with PBS were taken as 100% cell viability and all other treatments were expressed relative to it.

6.6.6 Cell Uptake studies

Cell uptake studies were performed using negative control siRNA labeled with FAM dye (FAM-NC-siRNA). The lyophilized pellet supplied was reconstituted with Nuclease free water in amber colored tubes and used for further in experiments. For quantitative cell uptake study was performed by flow-cytometry while qualitative evaluation was performed using confocal microscopy.

(i) Confocal microscopy

The laser scanning confocal microscope (LSCM) is an important element of modern day biomedical research. In a conventional microscopy, the entire specimen or samples

illuminated from mercury of xenon source. Nevertheless, in confocal microscopy the illumination is achieved through scanning one or more laser beams across the specimen to create an optical section of specimen in a non-invasive way. Further, It utilizes confocal pinholes that allow light coming only from the plane of focus to reach the photomultiplier tube detector and excludes the 'out of focus' light coming to the detector. This enables imaging of the living specimens and generation of 3-dimensional (3D) data in the form of Z-stacks.

The optical path is based on conventional reflected light wide-filed epi-fluorescence microscope with a point light source and a pinhole in front of detector which are confocal with each other. The specimens are labeled with one or more fluorescent probes. The confocal microscopy also offers the advantage greater resolution due to use of highly sensitive photomultiplier tube detectors. In cellular biology, confocal microscopy has been used for visualizing intracellular organelles, cellular uptake, intracellular localization of drugs and drug delivery systems using fluorescent probes. Cellular uptake studies were performed as per following section on A549 alveolar epithelium cell line.

Protocol: Cells were seeded at a density of 10^4cells/well in a 24 well plate containing 0.17 mm thick flame sterilized cover glass and were allowed to grow for 24 hr in DMEM at 37 °C temperature and 5% carbon dioxide. After 24 h cell medium was isolated and cells were washed with sterile PBS. After that the cells were exposed to optimized formulations containing FAM-NC-siRNA at 100 nM siRNA concentration. following 6 hr of exposure to the cells, cells were washed twice with PBS to confirm residual removal from formulation. The cells were fixed with 4% paraformaldehyde (1 mL/well) and incubated for 3-5 min at room conditions. The paraformaldehyde was instantly removed after exposure time and cells were washed with PBS thrice accompanied by intermittent shaking for each wash to eliminate the traces of paraformaldehyde. Then the nuclei of the cells were stained with DAPI at 1 µg/mL concentration with enough volume to cover the cells and kept for 15 min at room conditions for dye permeation under protection by aluminium foil. After that cells were washed once with PBS. The cells then mounted on glass slide using PBS:glycerin solution (50:50) with cover slips and confocal microscopy

was performed. The Naked FAM-NC-siRNA and Lipofectamine 2000 (L2K) complexed siRNA were used as negative and positive control, respectively for the experiment.

Sr No	Formulations	Cells	Treatment	Conditions
1.	Naked siRNA	A549 cells	100 nM FAM-NC siRNA	Incubation time=6 h Temperature = 37°C (5% CO_2)
2.	siRNA-TMC			
3.	siRNA-TMC-UAA			
4.	siRNA-TMC-UAB			
5.	siRNA-TMC-UAC			
6.	siRNA-TMC-PCA			
7.	siRNA-TMC-PCB			
8.	siRNA-TMC-PCC			
9.	siRNA-TMC-PAA			
10.	siRNA-TMC-PAB			
11.	siRNA-TMC-PAC			

Table 6.1: Treatment parameters for confocal microscopy and FACS for Modified TMCs based polyplex formulations in A549 Cell line

Sr No	Formulations	Cells	Treatment	Conditions
1.	siRNA-PEI-UAA	A549 cells	100 nM FAM-NC siRNA	Incubation time=6 h Temperature = 37°C (5% CO_2)
2.	siRNA-PEI-UAB			
3.	siRNA-PEI-UAC			
4.	siRNA-bPEI			
5.	L2K			

Table 6.2: Treatment parameters for confocal microscopy and FACS for modified PEI based polyplex formulations of A549 Cell line

(ii)Flow Cytometry (FACS Analysis)

Flow-cytometry is one of the useful techniques for characterizing cells in clinical diagnosis and biomedical research for quantifying aspects about their size, internal complexity and surface markers. In a flow cytometer the cell suspension is hydro-dynamically focused in a single cell wide stream of fluid containing a fast-moving sheath fluid around the slow moving cell suspension emerging through a 70 um nozzle.

Protocol: cells were seeded with cell density of 5 X 10^5 cells/well in 24 well plate. The cells were permitted to grow for 24 h in DMEM culture media. After 24 h the cells were treated with polyplex formulations with FAM-NC-siRNA at a 100 nM concentration and kept for 6 h in incubator maintained with 5% CO_2 at 37°C in humidified conditions. During this time, the cells were supposed to internalize the formulations depending on the transfection efficiency. After incubation, the cells were washed with 7.4 pH cold PBS thrice to eliminate the residual polyplex formulations and collected using trypsin to obtain a cell suspension in 7.4 pH PBS. Before analysis the cell suspension was passed through 70 µm cell strainer to disperse any cell aggregates and analyzed for % cell uptake using fluorescence activated cell sorter (FACS BD, USA). The naked FAM-NC-siRNA and Lipofectamine 2000 (L2K) complexed siRNA were used as negative and positive control respectively for the experiment.

6.7 Electrolyte induced aggregation study

Polyplexes stability depends on the electrostatic interaction between cationic polymer and siRNA. In case of small molecules like siRNA, it depends on the number of the charge per molecule; stability is greatly affected by the electrolytes presence. Sodium chloride like salt can disrupt the electrolyte interactions causing aggregation or particle size change and then leading to polyplexes dissociation at particular concentration. Salt induced aggregation and polyplexes dissociation can be analyzed by the dynamic light scattering.

Mainly, electrolyte induced flocculation is used to evaluate to steric stability of the PEGylated liposomes. Attraction forces (Van der waals forces) and repulsion forces (electrostatic repulsions or steric stabilizing barrier) are majorly accountable for the physical stability of the dispersion systems. In addition to this, other interactions like steric interactions and depletion also demonstrate significant role in the formulation stability. Conventional Liposomes, when expose to electrolytes, face compression of electrostatic double layer around the liposomes and succeeding aggregation followed by flocculation. However, the system would remain stable if the formulations are stabilized by hydrated steric stabilizing barriers even if compression of the electrostatic double layers.

Method: The study was performed to estimate the polyplexes formulations stability of in electrolytes presence. Polyplexes formulations were diluted to obtain 100 nM siRNA concentration. The Particle sizes of all the formulations were estimated by Zeta Sizer (Malvern). Appropriate quantities of the sodium chloride were dissolved to obtain concentration range of 1% to 5% and particle sizes of the formulations were determined after each addition.

6.8 Heparin Polyanion Competition Assay

The polyplex formulations stability was checked by the heparin polyanion competition assay. After successful transfection through cell membrane, efficacy of the polyplexes *in vivo* can be achieved. However, siRNA transfection can be restricted due to unwanted exchange of siRNA with other polyanions found outside the cells. Low charged density of the complexes may be one of the causes behind the decreased siRNA complexes efficacy. So, it can give a noteworthy impact to determine the transfection ability of polyplexes *in vitro* by the polyanion competition assay.

Polyanions present outside the cell like sulphated glycosaminoglycans and siRNA complexes could deter gene delivery to the target cell and decrease cellular uptake. Positively charged polyplexes binds with negatively charged polyanions leading siRNA dissociation from the polyplexes or change in charge of surface and particle size to cause the dissociation of the complexes. Hyaluronic acid and Heparin are the main glycosaminoglycans which are present in extracellular matrix on different cell type surfaces at different concentrations(1).

Method:

The study was used to determine the siRNA polyplexes stability *in vivo* and to confirm that siRNA forms stable polyplexes with the polymers which resists the decomplexation by polyanions found in extracellular matrix. The developed polyplexes formulations at optimized w/w polymer/siRNA ratio were exposed to different concentration of heparin sodium. Then, dispersions are permitted to incubate for 20 min at room conditions. The heparin to siRNA ratio necessary to displace siRNA from polyplex was noted down for all formulations. siRNA release from the formulations was estimated by agarose gel

electrophoresis and observed using GelDoc^{TM} XR imaging system with staining by Ethidium bromide.

6.9 Serum stability study

The key objective of gene delivery system to deliver therapeutic gene to desired site of action in necessite concentration. For siRNA delivery, it is hard to preserve their functional activity, as formulations thereof must resist degradation by nucleases in vivo prior to cellular internalization. Several reports have demonstrated that half life of siRNA in vivo, when delivered as such or naked, is very less and vary from several minutes to about an hour. This problem impedes the achievement of the efficient therapeutic delivery of naked or unmodified siRNA on systemic application. Numerous novel approaches were invented to overcome this barrier which includes chemical modifications of the nucleotides or the backbone resulting in increased nuclease stability and prolonged half life of siRNA in serum maintaining efficacy of siRNA at target cell or site. Some studies demonstrated that siRNA degrades about 70% of the delivered within 1 min resulting in reduced therapeutic response. In addition to that, vectors like polyplexes and lipoplexes have demonstrated improved transfection efficiency of siRNA or plasmid as they are protected by these vectors. Nevertheless, with these vectors systems too, presence of serum cause affects the polyplexes stability. Hence, *in vitro* assessment of the protective effect of the vectors may give idea of their behavior *in vivo.*

Method: Study was done to determine the stability of polyplexes in presence of serum, as there is a chance for degradation of the siRNA during circulation *in vivo* and degradation owing to intracellular and extracellular RNAses enzymes. Naked siRNA and different polyplex formulations containing were incubated with 10 µl FBS at 37°C for different time points such that final incubation volume had 50% serum in concentration. Samples were then accessed for integrity of siRNA by gel electrophoresis. In order to remove any nuclease activity 0.5 M EDTA solution was added to the samples. The polyplexes formulations were treated with heparin (1 mg/mL) per sample after defined time period so as to dissociate the polyplex. The siRNA released at all the time points were compared with the free siRNA sample (untreated) to identify any type of degradation. Any type of siRNA degradation of would affect the band properties or intensity of the test.

Additionally, the densitometry quantification developed was used to quantify siRNA amount remaining at different time points.

6.10 Stability in bronchoalveolar lavage Fluid (BALF)

For formulations administered via pulmonary delivery to lungs, stability of the formulations to extracellular substances present in the airways fluid is important. Rats were euthanized using pentobarbital (75 mg/mL) intraperitoneal injection. Carefully, the trachea was exposed and cannulated with catheter (20-gauge). cold sterile phosphate buffer saline (0.5 ml) was instilled thrice through the trachea into the lung to recover BAL fluid at 50% to 60% percent of the original volume., BAL fluid was centrifuged at 1500 RPM for 10 min to separate any debris or cells.10 μL of formulation containing100 pmole siRNA was prepared and treated with 10 μL of BALF. Intactness of siRNA retained in each treated sample was estimated at intervals of 15 min over a period of 2 hr using agarose gel electrophoresis. In brief, polyplexes were analysed by heparin competition assay. After incubation, 0.5 M EDTA solution (1 μL of EDTA solution for 10 μL of BALF) was added to inactivate nuclease activity. Then, EDTA treated samples were exposed to heparin (1 mg/mL) to ensure 100% siRNA release from polyplexes, which were detected by gel electrophoresis as per the protocol.

6.11Transmission electron microscopy (TEM) analysis

To study the morphology of the polyplexes formed, transmission electron microscopy (TEM) was performed by Technai 20 Transmission Electron Microscope. Briefly, a drop of formulation was kept onto 300 # carbon coated copper grid. The surface water was removed from the grid by tapping with filter paper. After 5 min, using a probe the grid was placed in a sample holder and inserted into microscope and observed at 200 kV accelerating voltage with appropriate magnification.

6.12 Result and Discussion

Complexation efficiency

Conjugation of heterocyclic compounds on the primary amines of the chitosan and bPEI might influence the polymers capability to form complex with siRNA. Hence, we

observed the effect of conjugation of heterocyclic compounds on polyplexes formation by using agarose gel retardation assay. Further, optimized polyplex formulations were confirmed through Nanao Drop UV spectroscopic estimation of siRNA complexation ability. Charge dependent siRNA migration towards anode under electric current in agarose gel retardation assay facilitates to assess the complexation ability of the formulated polyplexes. In principle, when negatively charged siRNA is complexed with cationic polymers, siRNA migration is retarded and siRNA remains within wells. Nevertheless, un-complexed siRNA will migrate on the gel under electromotive force giving a band under UV light. Figures 6.1 to figure 6.14 have shown the optimized w/w ratios of siRNA to different polymers. Results for different weight ratio for all the polymers have been listed in table 6.3. (2)

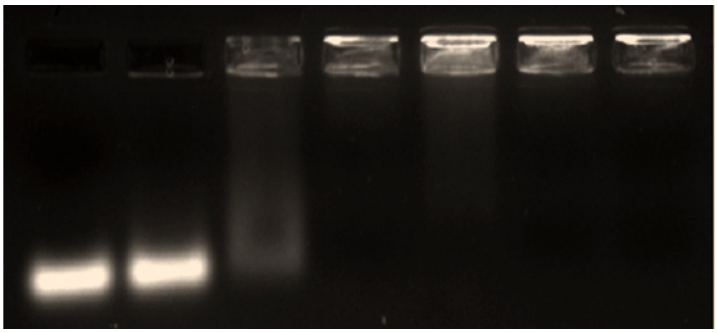


Figure 6.1: Gel electrophoresis of siRNA-TMC
Lane(L→R)(w/w ratio) Lane 1:Naked siRNA; Lane 2:2;Lane 3:4,**Lane 4:6**,Lane 5:8, Lane 6:10,Lane 7:12

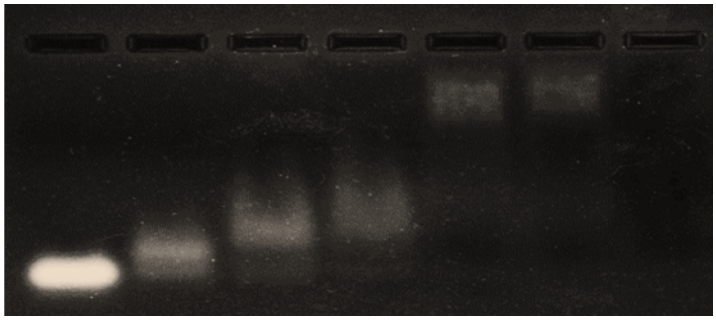


Figure 6.2: Gel electrophoresis of siRNA-TMC-UAA
Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:10;Lane 3:12,Lane 4:14,Lane 5:16,Lane 6:18,**Lane 7:20**

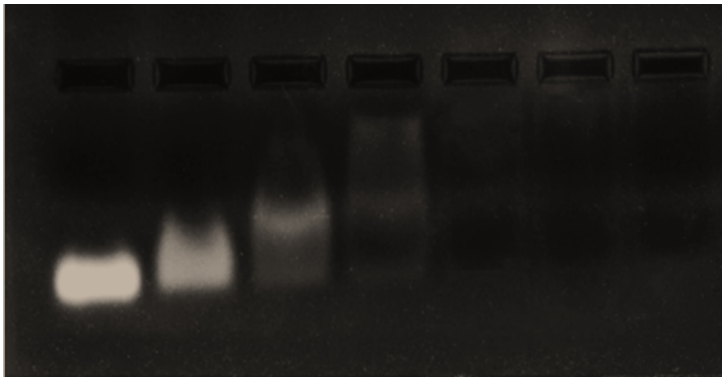


Figure 6.3: Gel electrophoresis of siRNA-TMC-UAB

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:10;Lane 3:12; Lane 4:14;**Lane 5:16**; Lane 6:18; Lane 7:20

Figure 6.4: Gel electrophoresis of siRNA-TMC-UAC

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:10;Lane 3:12;**Lane 4:14**;Lane 5:16; Lane 6:18; Lane 7:20

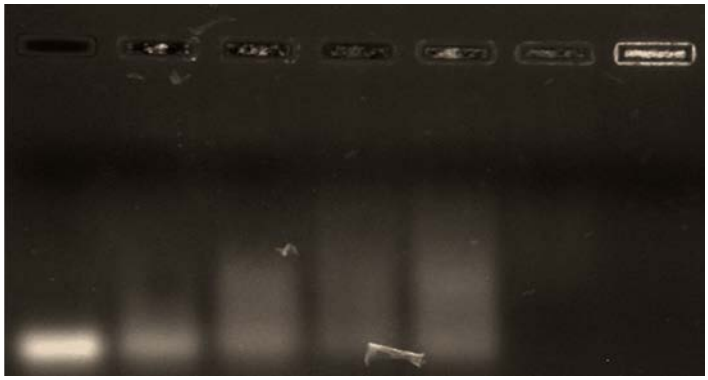


Figure 6.5: Gel electrophoresis of siRNA-TMC-PCA

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:2;Lane 3:4; Lane 4:8;Lane 5:12;**Lane 6:16**; Lane 7:20; Lane 8:24

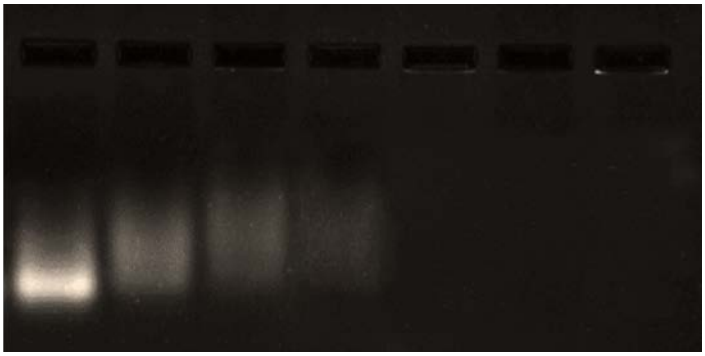


Figure 6.6: Gel electrophoresis of siRNA-TMC-PCB

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:2;Lane 3:4;Lane 4:8;**Lane 5:12**;Lane 6:16; Lane 7:20

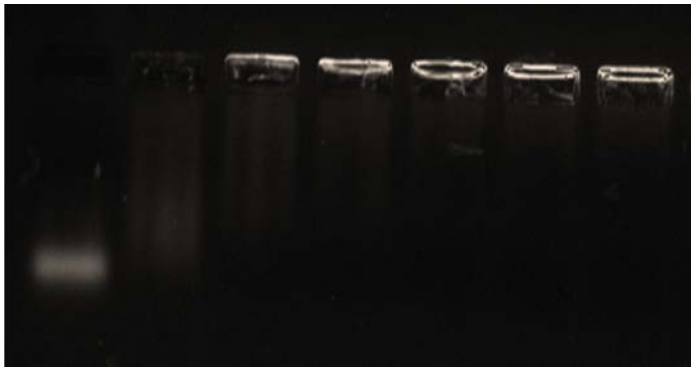


Figure 6.7: Gel electrophoresis of siRNA-TMC-PCC

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:2;Lane 3:4;**Lane 4:8**;Lane 5:12; Lane 6:16; Lane 7:20

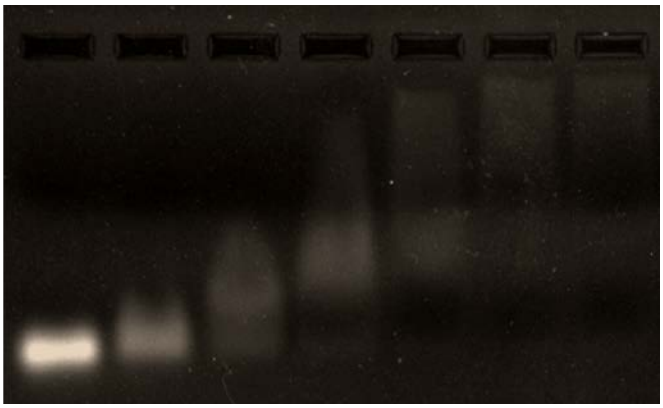


Figure 6.8: Gel electrophoresis of siRNA-TMC-PAA

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:5;Lane 3:10;Lane 4:15;Lane 5:20;**Lane 6:25**; Lane 7:30

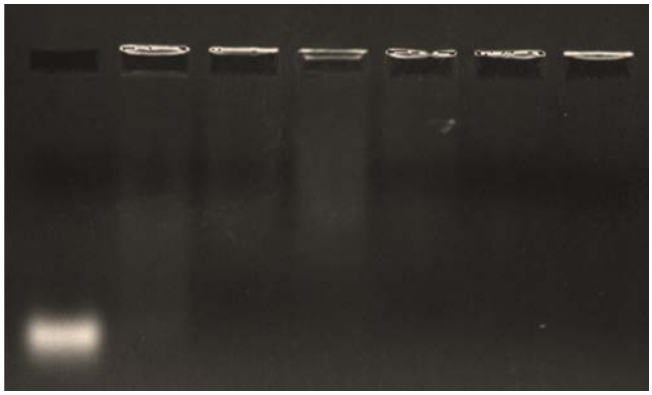


Figure 6.9: Gel electrophoresis of siRNA-TMC-PAB

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:5;Lane 3:10;Lane 4:15;**Lane 5:20**;Lane 6:25; Lane 7:30

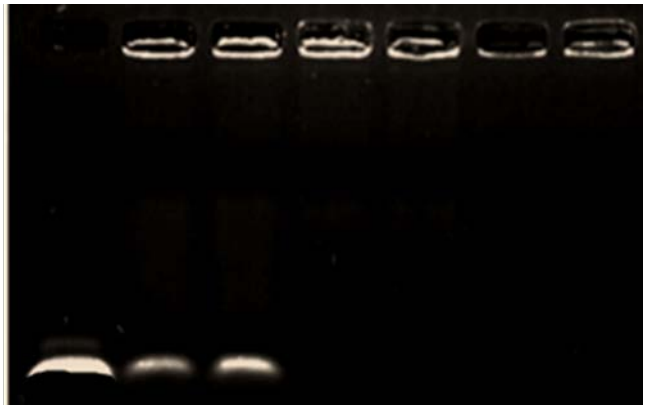


Figure 6.10: Gel electrophoresis of siRNA-TMC-PAC

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:5;Lane 3:10;**Lane 4:15**;Lane 5:20;Lane 6:25; Lane 7:30

Figure 6.11: Gel electrophoresis of siRNA-bPEI

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:0.2;Lane 3:0.4;**Lane 4:0.8**;Lane 5:1; Lane 6:1.2; Lane 7:1.4

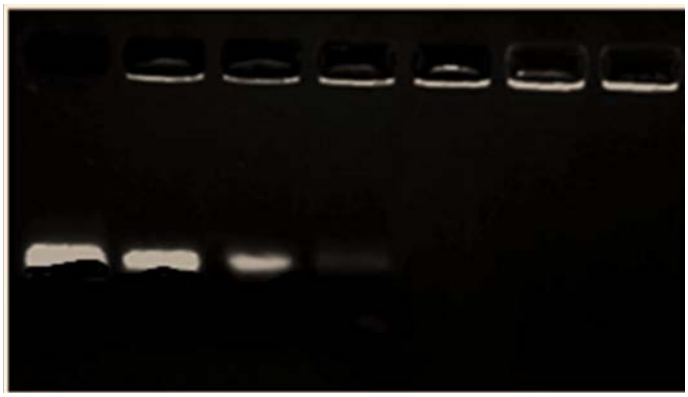


Figure 6.12: Gel electrophoresis of siRNA-PEI-UAA

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:0.5;Lane 3:1;Lane 4:2;**Lane 5:4**; Lane 6:6; Lane 7:8

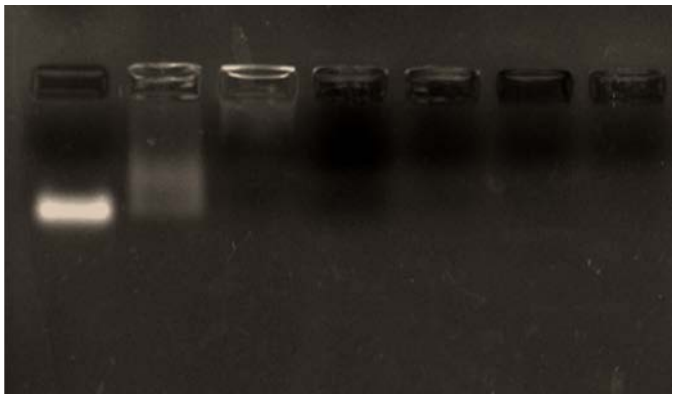


Figure 6.13: Gel electrophoresis of siRNA-PEI-UAB

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:0.5;Lane 3:1;**Lane 4:2**;Lane 5:4; Lane 6:6; Lane 7:8

Figure 6.14: Gel electrophoresis of siRNA-PEI-UAC

Lane(L→R)(w/w ratio) Lane 1:Naked siRNA;Lane 2:0.5;**Lane 3:1**;Lane 4:2;Lane 5:4; Lane 6:6; Lane 7:8

Table 6.3: Complexation efficiencies of polyplexes formulations

Sr.No.	Formulations	Optimized w/w ratio	Complexation* efficiency (%)
1	siRNA-TMC	6	96.80±1.24
2	siRNA-TMC-UAA	20	98.54±1.45
3	siRNA-TMC-UAB	16	98.20±0.88
4	siRNA-TMC-UAC	14	96.63±1.05
5	siRNA-TMC-PCA	16	97.42±0.79
6	siRNA-TMC-PCB	12	97.77±1.22
7	siRNA-TMC-PCC	8	96.38±1.39
8	siRNA-TMC-PAA	25	96.69±2.32
9	siRNA-TMC-PAB	20	97.53±2.14
10	siRNA-TMC-PAC	15	97.86±1.57
11	siRNA-bPEI	0.8	98.80±1.58
12	siRNA-PEI-UAA	4	98.91±1.34
13	siRNA-PEI-UAB	2	96.31±1.13
14	siRNA-PEI-UAC	1	97.45±1.36

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

All the synthesized TMC and PEI conjugated polymers were able to condense greater than 95% of siRNA at their optimized weight ratios of polymer to siRNA. For different degree of conjugation of heterocyclic compounds, obtained weight ratios were different.

As the degree of conjugation of modified TMCs and modified PEIs increased, the w/w ratio of for complexation increased. In case of PEI, it is reported that polymer has pKa around 10 for primary and 7.95 for secondary amino groups, so at only primary amino groups protonated at physiologic pH while secondary amines are protonated partially. As, the conjugation took place at different fractions of primary amines of PEI which are actively engaged in complexation of siRNA in PEI, conjugated amines would consequently not be present for binding and so required to be compensated by increasing w/w ratios. TMC-UAA able to retard siRNA completely at weight ratio of 20 while TMC-UAB and TMC-UAC requires weight ratio of 16 and 14 for complete retardation of

siRNA in gel electrophoresis, respectively. In case of, TMC-PCA, TMC-PCB and TMC-PCC, weight ration requires for complete complexation with siRNA was slight lower which might be ascribed to higher molecular weight of the piperazine-2-carboxylic acid compound conjugated on trimethylated chitosans. Urocanic conjugated PEI condensed siRNA at weight ratio of 4, 2 and 1 for descending degree of conjugation of Urocanic acid respectively as PEI is more cationic charged polymer, which complexes siRNA at lower weight ratio.

siRNA integrity

The siRNA integrity after complexation with polymers was maintained as it was before complexation with polymers as shown in figure 6.15 and figure 6.16. When polyplex formulations compared with naked siRNA, it did not show any instability or degradation of siRNA incorporated into the formulations.

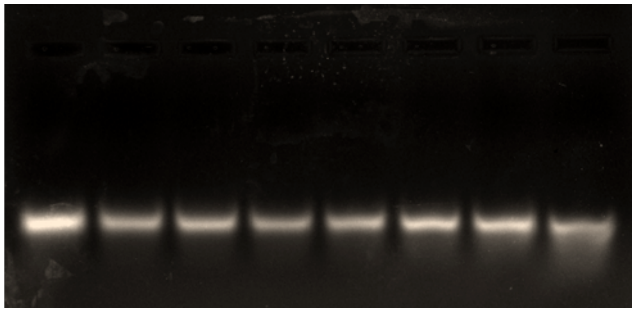


Figure 6.15: siRNA integrity after complexation

Lane (L→R)(**Lane 1**: Naked siRNA**, Lane 2**:siRNA-TMC **Lane 3**: siRNA-TMC-UAA,**Lane4**: siRNA-TMC-UAB,**Lane 5**:siRNA TMC-UAC, **Lane 6**: siRNA-TMC-PCA, **Lane 7**: siRNA-TMC-PCB,**Lane 8:**siRNA-TMC-PCC)

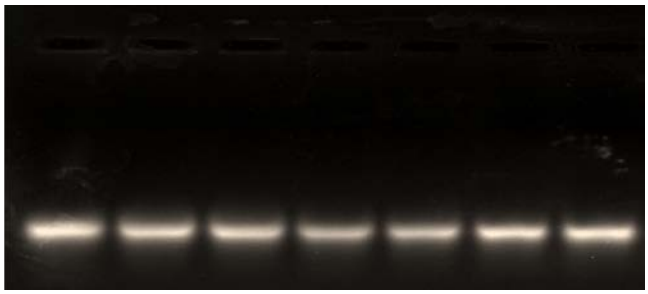


Figure 6.16: siRNA integrity after complexation

Lane (L→R) (**Lane 1**: siRNA-TMC-PAA, **Lane 2**: siRNA-TMC-PAB, **Lane 3** :siRNA TMC-PAC, **Lane 4:** siRNA-bPEI**,Lane 5**: siRNA-PEI-UAA, **Lane 6**: siRNA- PEI-UAB, **Lane 7**: siRNA- PEI-UAC)

Particle size and Zeta potential Analysis

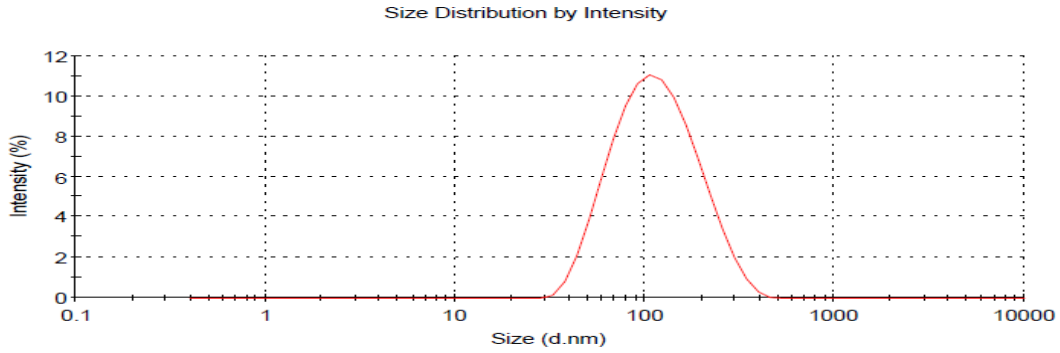
The charged surface of the therapeutic gene delivery carrier at physiological pH is significant its electrostatic interactions with the siRNA or gene and then, for intracellular entry of the formulation through interactions with anionic charged cellular membrane. Trimethylated chitosan and bPEI maintained adequate positive charge even after conjugation with heterocyclic compounds, as demonstrated by zeta potential values. At optimized w/w ratio of heterocyclic compounds conjugated TMC and bPEI polyplexes, a strong positive zeta potential was produced by surplus of cations which are not involved in complexation with siRNA. The net positive potential on the polyplexes formed with the modified polymers would cause electrostatic repulsion between complexes that would avoid the aggregation of the polyplexes *in vivo.* Positively surface charge of polyplexes furthermore assists electrostatic interaction to cell membrane. Table 6.4 shows particle size, particle size distribution and zeta potentials of the polyplexes formulations. Figure 6.17 (a-m) represents particle size distribution by intensity of the formulations measured by Malvern Zeta sizer (Nano ZS).

Table 6.4: Particle size and Zeta potential of formulations

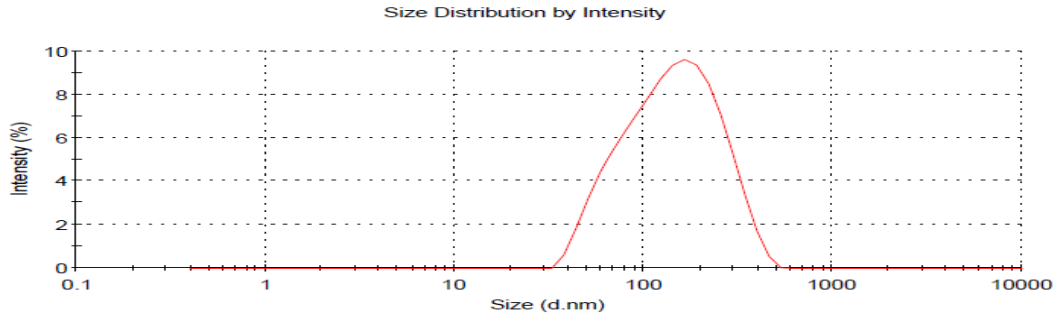
Sr.No.	Formulations	Particle size(nm)*	PDI	Zeta Potential(mV)*
1	siRNA-TMC	149.8±1.7	0.152	27.35±1.21
2	siRNA-TMC-UAA	188.5±1.9	0.114	13.36±2.57
3	siRNA-TMC-UAB	174.9±2.7	0.084	18.24±1.80
4	siRNA-TMC-UAC	165.3±4.4	0.136	24.51±2.32
5	siRNA-TMC-PCA	172.6±3.4	0.051	17.69±1.65
6	siRNA-TMC-PCB	163.4±2.8	0.106	20.86±1.34
7	siRNA-TMC-PCC	158.2±5.2	0.118	25.19±1.27
8	siRNA-TMC-PAA	227.3±5.9	0.169	16.33±2.07
9	siRNA-TMC-PAB	204.8±4.2	0.125	20.98±1.74
10	siRNA-TMC-PAC	195.5±7.6	0.094	22.85±1.15

11	siRNA-bPEI	100.2±2.4	0.109	34.63±1.20
12	siRNA-PEI-UAA	125.8±1.8	0.075	24.23±2.21
13	siRNA-PEI-UAB	112.3±1.8	0.120	27.34±1.46
14	siRNA-PEI-UAC	105.5±3.1	0.08	32.21±1.30

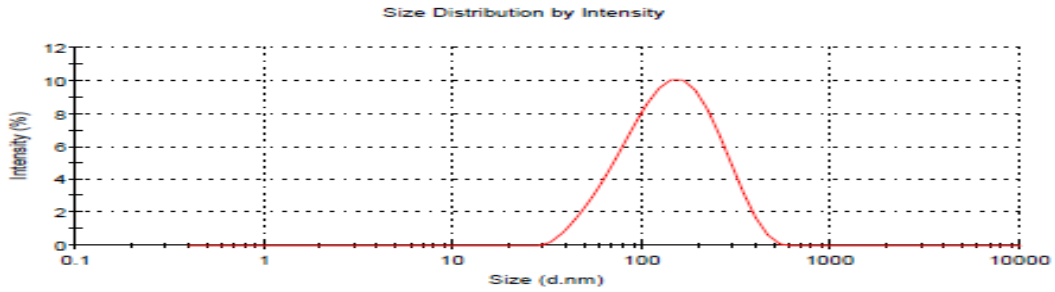
*Values are represented as Mean ± SD, n=3



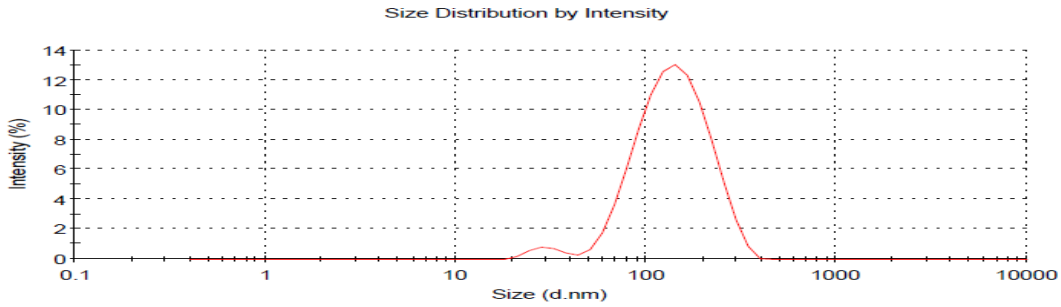
(a) siRNA-TMC



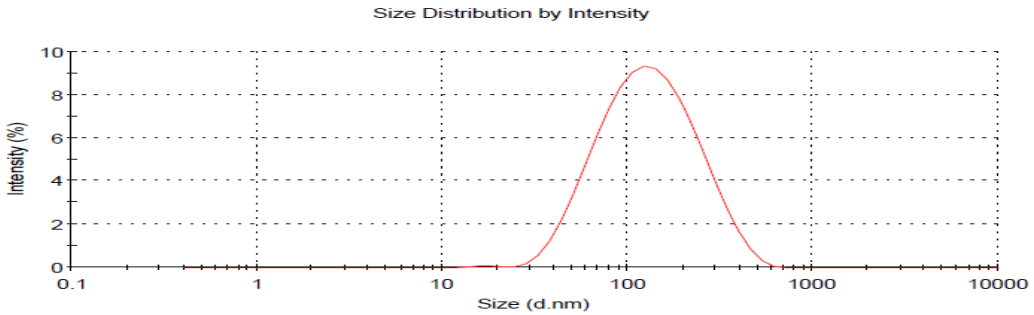
(b) siRNA-TMC-UAA



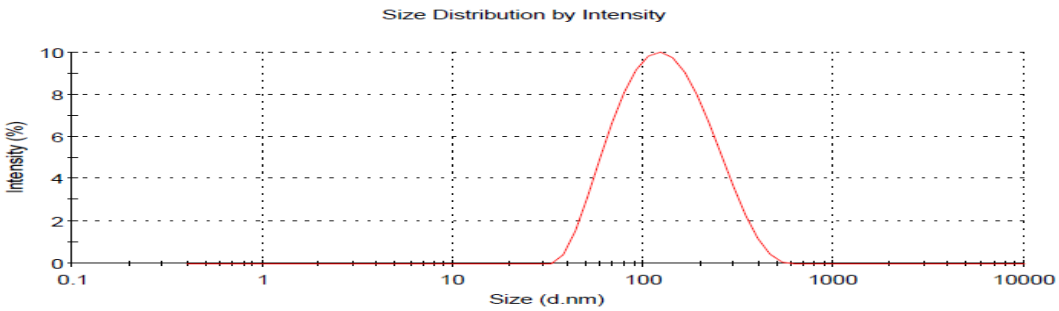
(c)siRNA-TMC-UAB



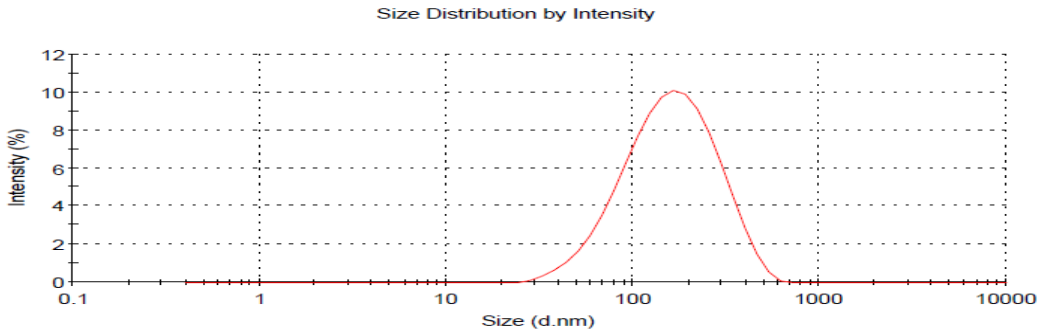
(d)siRNA-TMC-UAC



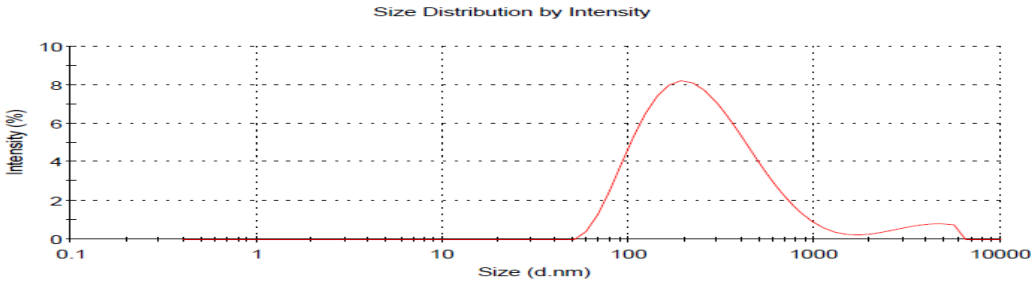
(e)siRNA-TMC-PCA



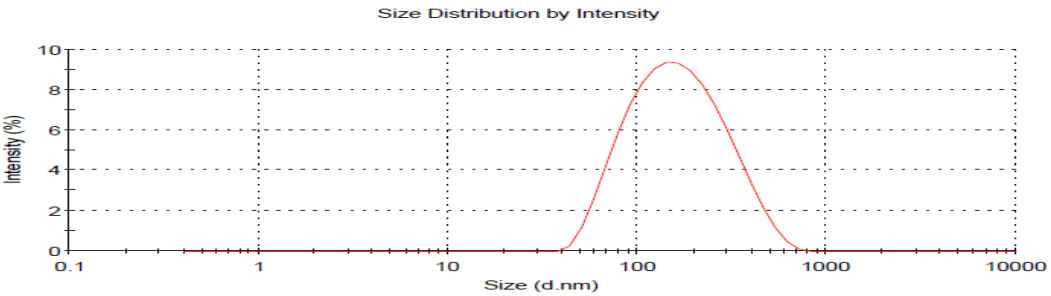
(f)siRNA-TMC-PCB



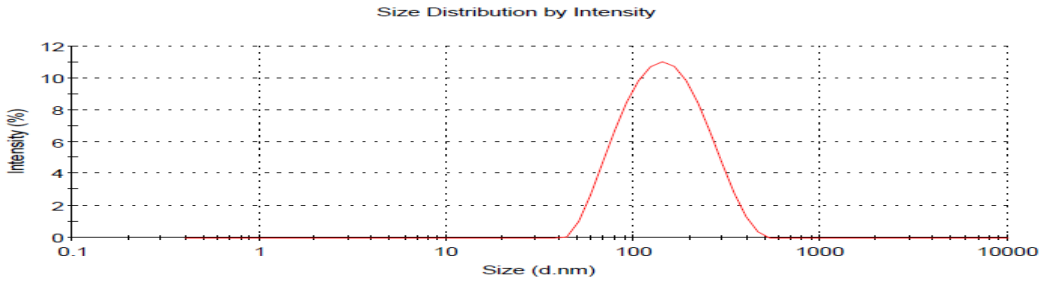
(g)siRNA-TMC-PCC



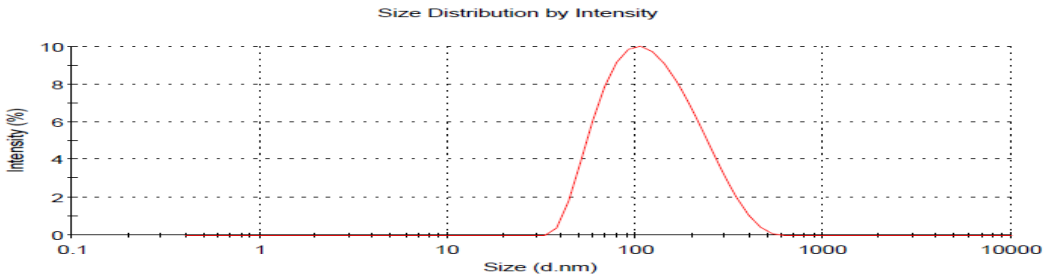
(h)siRNA-TMC-PAA



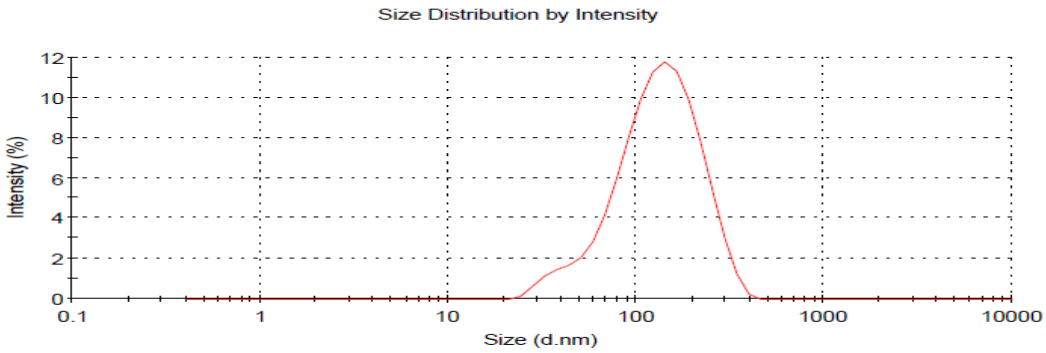
(i)siRNA-TMC-PAB



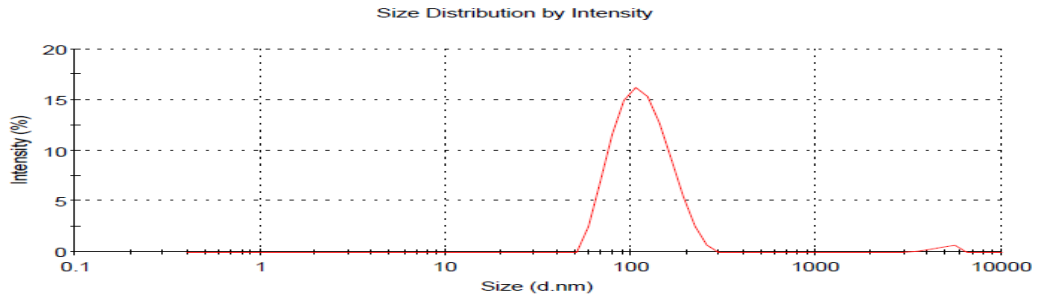
(j)siRNA-TMC-PAC



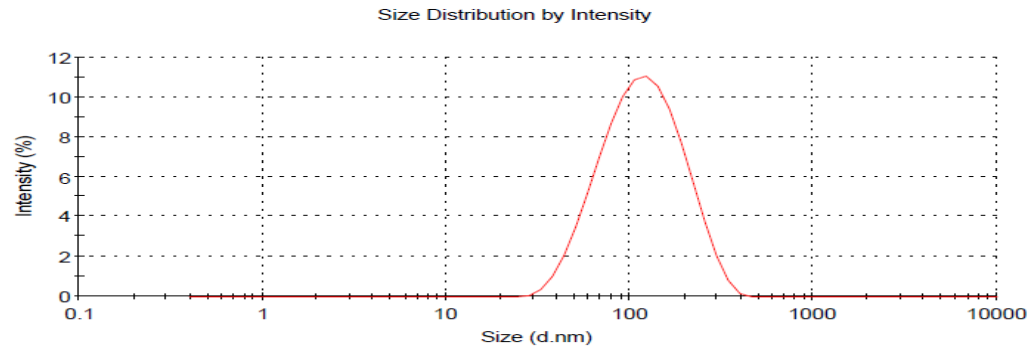
(k)siRNA-bPEI



(l)siRNA-PEI-UAA



(m)siRNA-PEI-UAB



(n)siRNA-PEI-UAC

Figure 6.17 (a-n): Particle size of different polyplex formulations

As the degree of conjugation increased, the zeta potential values decreased alongside in all of heterocyclic compound modified TMCs and PEI. The decline in zeta potential can be elucidated by shielding of primary amines which act as cationic sites on the polymers. After conjugation with heterocyclic compounds, surface charge of polymers remained enough positive which was needed for efficient interactions with siRNA and afterward with cell membrane.

The particle size results demonstrated that degree of conjugation also influence the particle size of the polyplexes. As the conjugation increased, the size of the particles also increased which can be attributed to shifting in charge and hydrophobicity balance of the polymers. Conjugation is supposed to adjust to the surface of the polymers leading to positive charge shielding & hydrophilic elements. Reduced charged of the polymers would reduce the ability for condensation to siRNA efficiently making loose complexes. Previous findings show the influence of various substitution, nature of substitution and conjugation degree on the polyplex particle size also favors the results(3).

Mean particle size of siRNA-TMC polyplex was 149.8± 1.7 nm while mean particle size of modified TMC polyplexes were obtained in the range of 158 nm-188 nm while mean size of modified PEI polyplexes was found between 105 nm-125 nm. Polyplexes within this range of particle size would enter the cells via endocytosis mechanism.

***In vitro* cytotoxicity study**

In vitro cytotoxicity study for the developed polyplexes formulations were performed in A549 alveolar epithelial cells. PBS treated control was used as a blank as background. Graphical representation of percent cell viability against the increasing w/w ratio of polymer to siRNA, after formulation treatment was studied by MTT assay as demonstrated in Figures 6.18-6.21. Cell viability of L2KL, commercial transfection agent was also studied for comparison of different polyplexes formulations. TMC-UAA, TMC-PCA and TMC-PAA polyplexes demonstrated cell viability of 85.24±1.38%, 84.20± 1.24 % and 81.20± 1.62 % at w/w ratio of polymer to siRNA 24,20 and 30, respectively. While, TMC polyplexes showed 96.51±1.24 % viability at w/w ratio of 6. On the other side, PEI polyplexes, PEI-UAA, PEI-UAB and PEI-UAC polyplexes demonstrated cell viability of 87.21 ±1.25 %, 94.22±1.57%, 93.11±1.36%,91.94±2.34 % and 94.37±1.32% at 0.8 w/w ratio of polymer to siRNA. While, at the w/w ratio of 4, cell viability was decreased significantly ($p<0.05$) for PEI polyplexes to 67.95± 2.11%. L2KL has demonstrated 90.14±1.68 % viability at w/w ratio of 4.

From the results, it can be said that as the degree of conjugation of modified TMC and modified PEI polyplexes increased, cell viability was increased. Furthermore, there was

no significant difference in percent cell viability among the different heterocyclic moieties used for conjugation to the polymers to prepare polyplexes which means that decrease in overall cationic charge by conjugation and hydrophobicity imparted by the heterocyclic moieties are the major contributor for decrease in cell cytotoxicity irrespective of types of heterocyclic moieties used. These findings explained polymers toxicity was strongly depends on the degree of the conjugation of the amines of the polymers. Cell cytotoxicity of the positive charged polymers is probably due to aggregation of polymers on the cellular surfaces, damaging important cellular functions (4).

Modification of cationic polymers by hydrophobic moiety has been shown to be advantageous for gene delivery for a variety of reasons. Recent examples on hydrophobic modification of polymer demonstrated improved the product but too much hydrophobicity can reduce the efficacy(5). Therefore, hydrophilic/hydrophobic balance in the gene delivery vectors should be optimum, as it can influence the transfection efficiency.

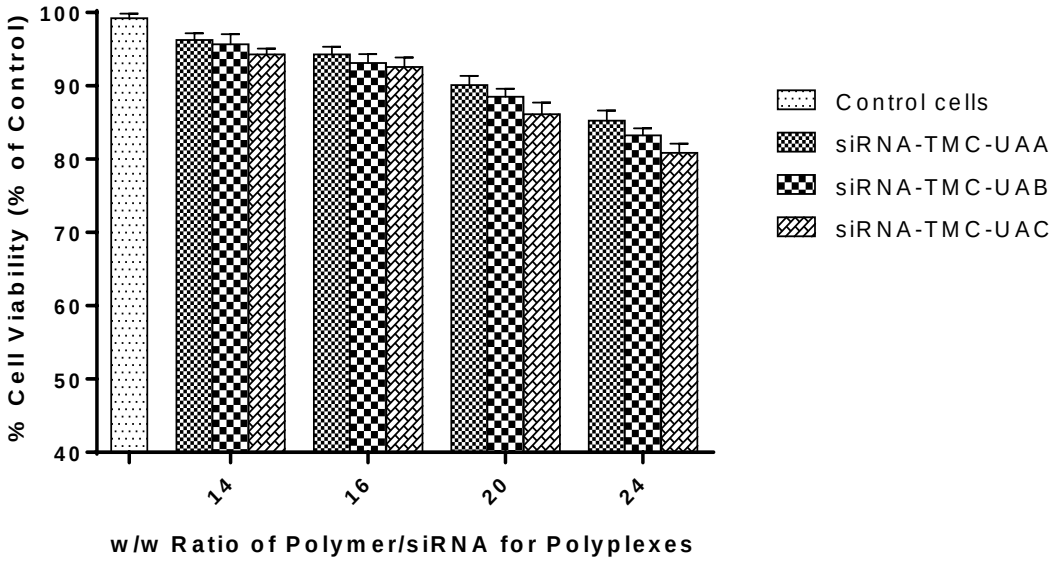


Figure 6.18: Cytotoxicity of siRNA-TMC-UAA, siRNA-TMC-UAB, siRNA-TMC-UAC

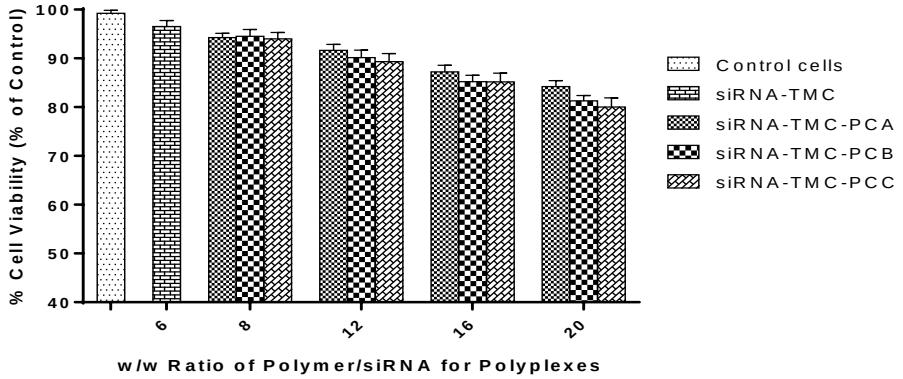


Figure 6.19: Cytotoxicity of siRNA-TMC, siRNA-TMC-PCA, siRNA-TMC-PCB, siRNA-TMC-PCC

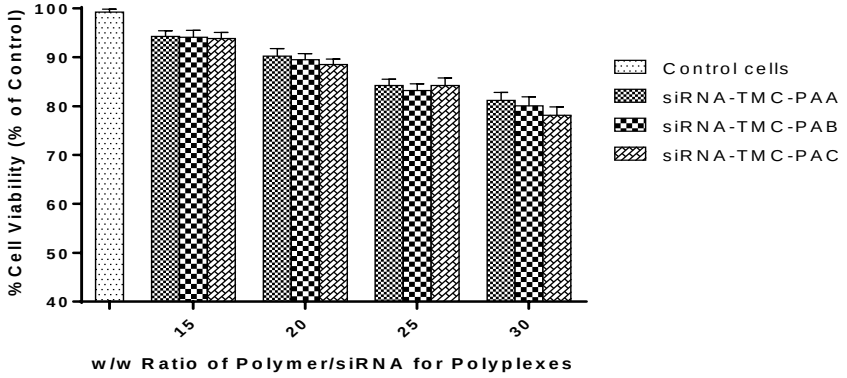


Figure 6.20: Cytotoxicity of siRNA-TMC-PAA, siRNA-TMC-PAB, siRNA-TMC-PAC

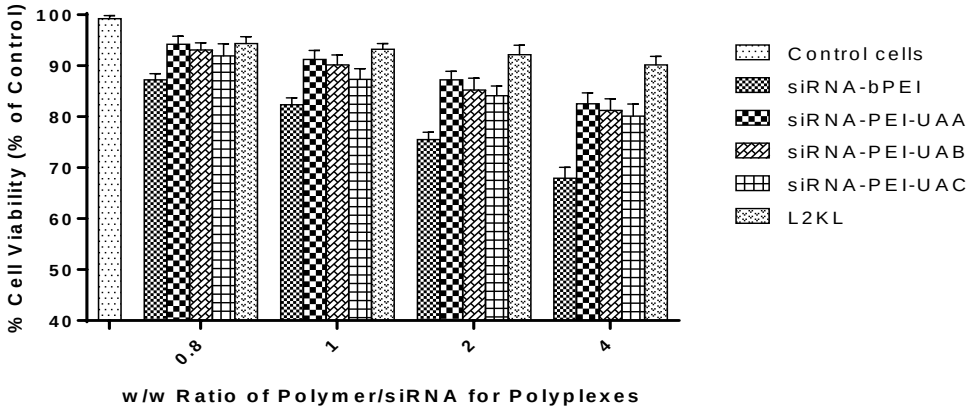


Figure 6.21: Cytotoxicity of siRNA-bPEI, siRNA-PEI-UAA, siRNA-PEI-UAB, siRNA-PEI-UAC and L2KL

Polyplex formulations from different polymers at all concentrations exhibited their potential as a novel siRNA vector with improved safety margin. Nevertheless, in order to assess whether reduced cytotoxicity of the developed polyplexes has led to any changes in cell uptake, uptake studies to be performed to verify the transfection of polyplexes. Furthermore, transfection ability of the developed polyplexes was evaluated by qualitative and quantitative uptake studies. (6)

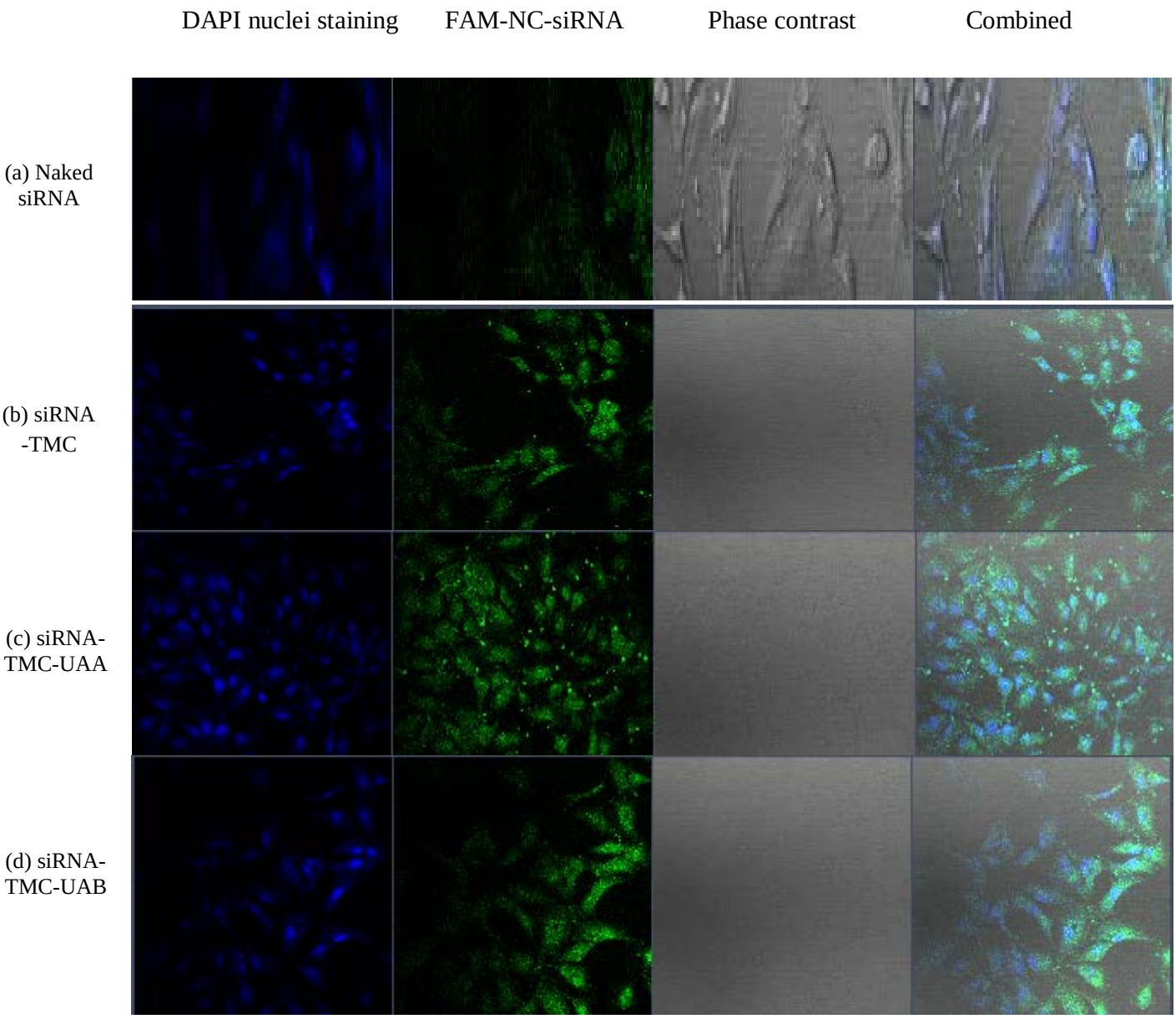
***In vitro* uptake by Confocal Microscopy**

Figures illustrates confocal microscopy images demonstrating cellular uptake of naked siRNA, different polyplexes formulations prepared from synthesized TMCs and PEI and commercial transfection agent, L2KL. Thoughtful selection of heterocyclic moieties was done in order to observe the influence of conjugation on cellular uptake and transfection by balance between hydrophobicity as well as lipophilicity.

As it can be observed from the figure 6.22, naked siRNA exhibited negligible cellular uptake, whereas modified TMCs and modified PEI polyplexes exhibited significant cellular uptake. Very low cellular uptake of naked siRNA can be attributed to its higher molecular mass as well as higher hydrophilicity that restricts its passive diffusion through cellular membrane. There are also probabilities of degradation of naked siRNA through hydrolysis or by nucleases. L2KL was incorporated for the comparison purpose and furthermore to ensure the suitability of the experimental procedures and protocols. L2KL,commercial transfection agent also demonstrated considerable cellular uptake as shown in figure 6.25. TMCs and PEI being positively charged assists to condense the anionically charged siRNA while residual cationic charge further augments its cell uptake through interaction with anionic charged cellular membrane. Such type of interaction would lead to polyplexes endocytosis.

Influence of different modified polymers on cell uptake is demonstrated in figure 6.22 to 6.25. It can be noted that all the polyplex formulations exhibited higher cell uptake and this can ascribed to the conjugation of heterocyclic moieties that provided hydrophobic modifications. It is also evident from the figures that with increasing the degree of conjugation for each heterocyclic moiety, the cell uptake increased. The figures also note

the difference between types of conjugation i.e. at each conjugation level, the cell uptake was higher for Urocanic acid conjugated TMCs and PEI polyplexes followed by piperazine-2 carboxylic acid conjugated TMCs and PEI polyplexes followed by 3-pyridyl acetic acid conjugated TMCs and PEI polyplexes. This difference in cellular uptake can be attributed to influence of imidazole ring of Urocanic acid, partial ionization of which under physiologic conditions, would have supplement the cell uptake of Urocanic acid conjugated TMCs and PEI than piperazine and pyridine moieties.



(e) siRNA-TMC-UAC

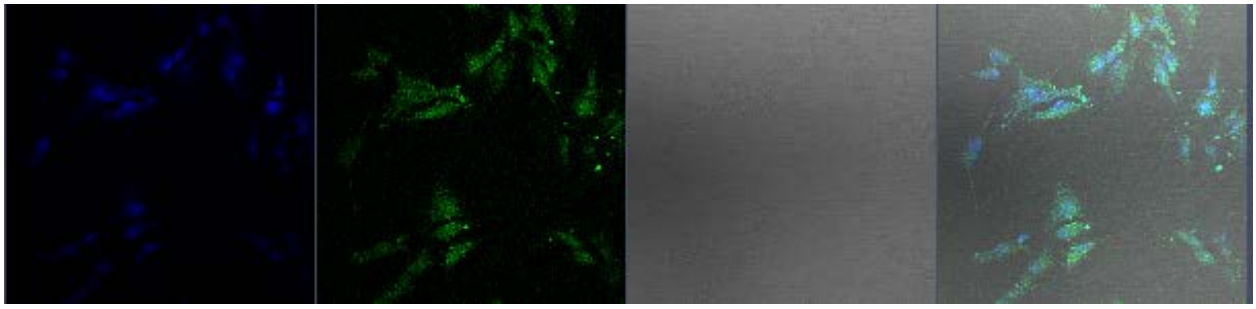


Figure 6.22: Cell uptake of (a) Naked siRNA (b) siRNA-TMC (c) siRNA-TMC-UAA (d) siRNA-TMC-UAB(e) siRNA-TMC-UAC by Confocal Microscopy

(a)siRNA-TMC-PCA

(b) siRNA-TMC-PCB

(c) siRNA-TMC-PCC

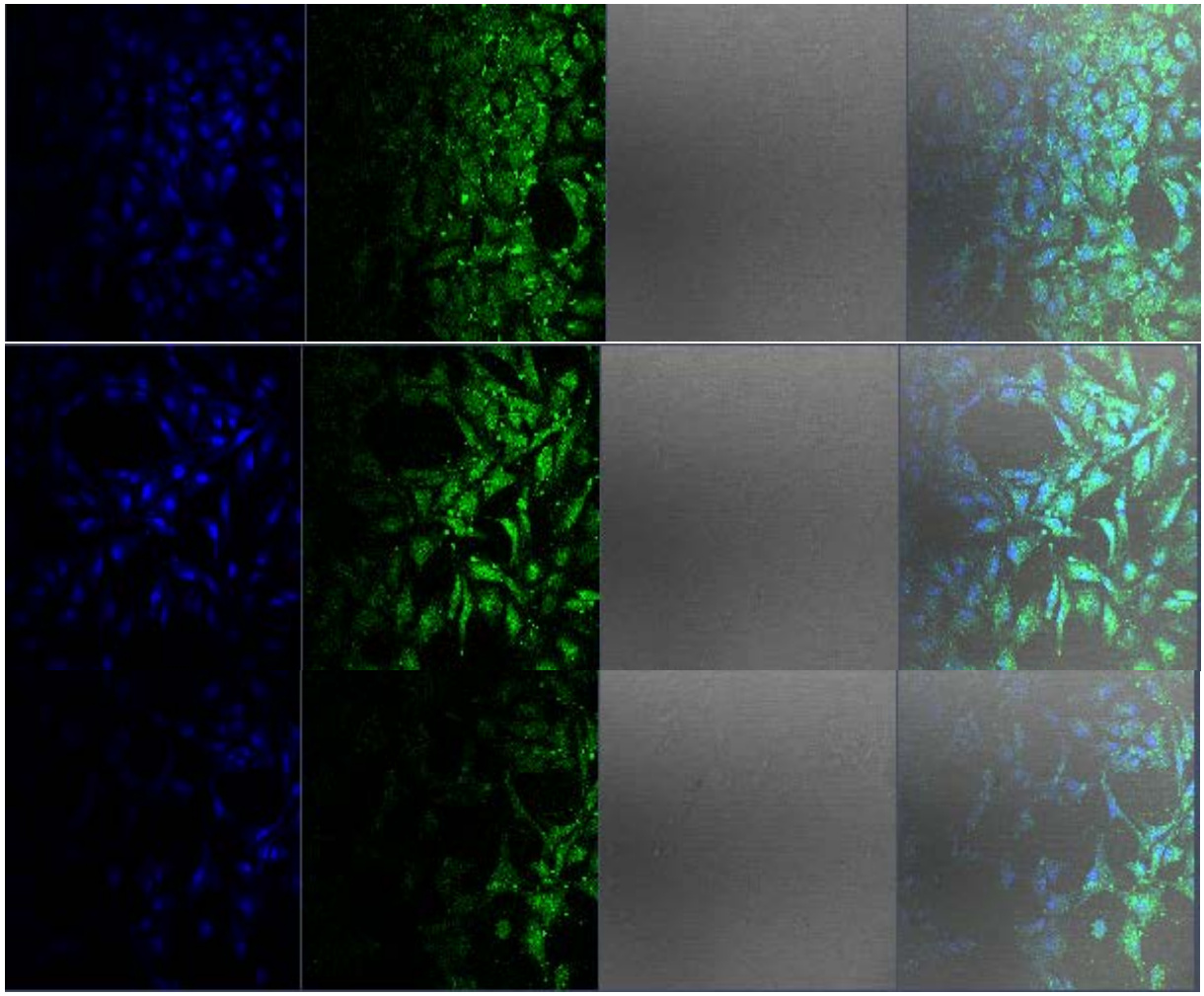


Figure 6.23: Cell uptake of (a) siRNA-TMC-PCA (b) siRNA-TMC-PCB(c) siRNA-TMC-PCC by Confocal Microscopy

(a) siRNA-TMC-PAA

(b) siRNA-TMC-PAB

(c) siRNA-TMC-PAC

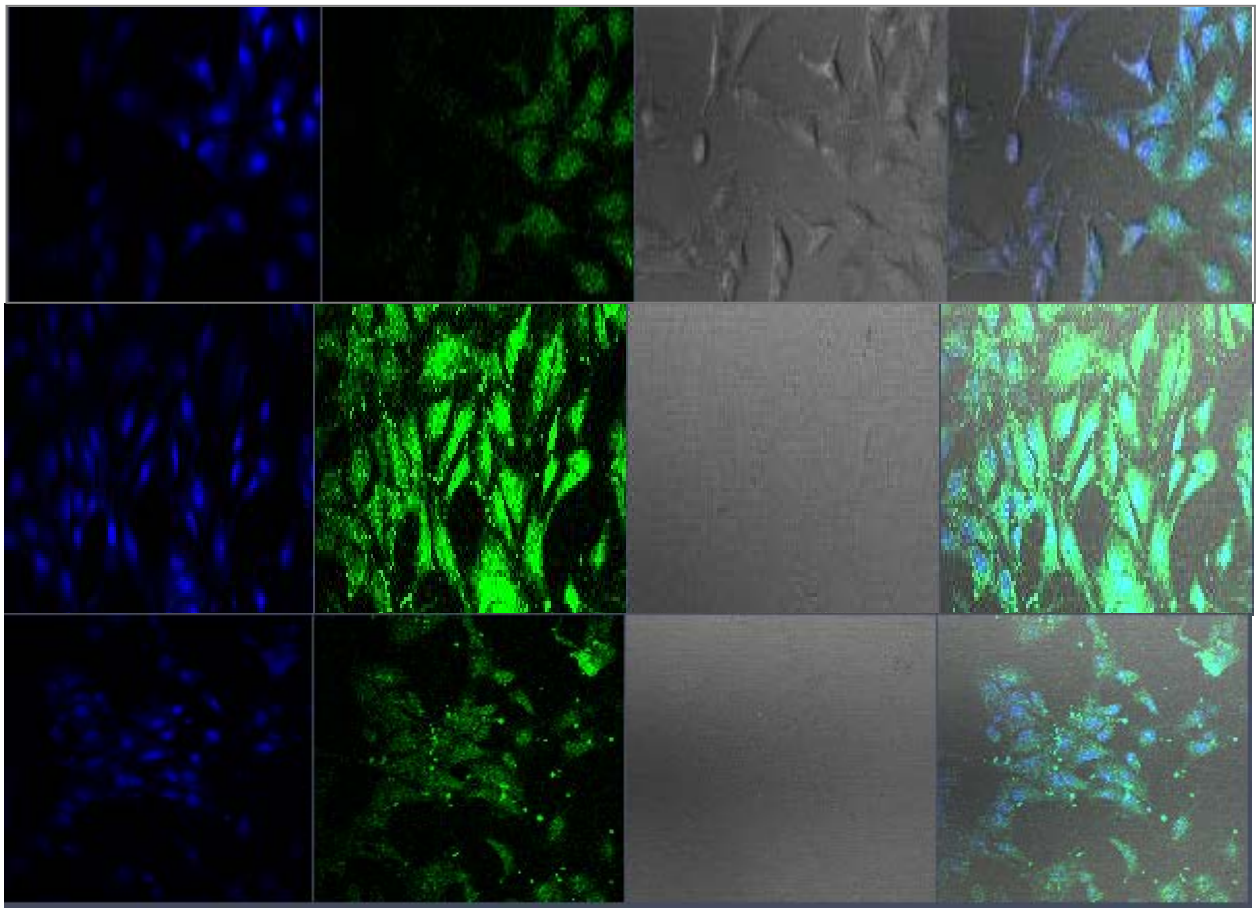


Figure 6.24: Cell uptake of (a) siRNA-TMC-PAA (b) siRNA-TMC-PAB(c) siRNA-TMC-PAC by Confocal Microscopy

(a) siRNA-PEI

(b) siRNA-PEI-UAA

(c) siRNA-PEI-UAB

(d) siRNA-PEI-UAC

(e) L2KL

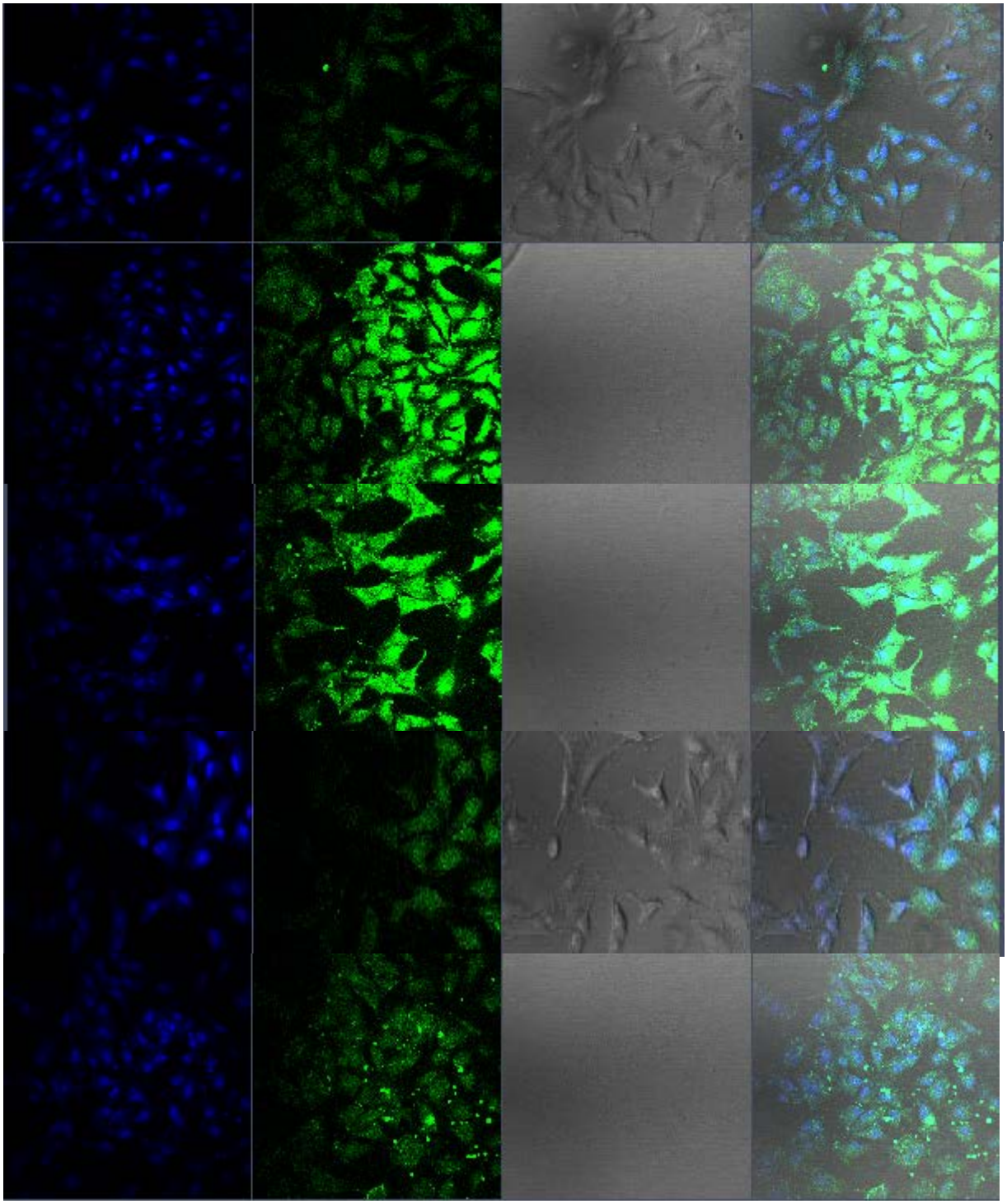


Figure 6.25: Cell uptake of (a) siRNA-PEI (b) siRNA-PEI-UAA (c) siRNA-PEI-UAB (d) siRNA-PEI-UAC(e)L2KLby Confocal Microscopy

***In vitro* cellular uptake by FACS**

Cell uptake of FAM-NC-siRNA at 100 nM concentration and different developed polyplexes at equivalent concentrations were subjected for analysis by flow Cytometry for cell uptake quantification. Figures 6.26 to figure 6.31 show 2D histograms of FACS of cell uptake of formulations, naked FAM-NC-siRNA and commercial transfection agent. L2KL results of which are reported in table 6.5 and represented in figure 6.31(a).

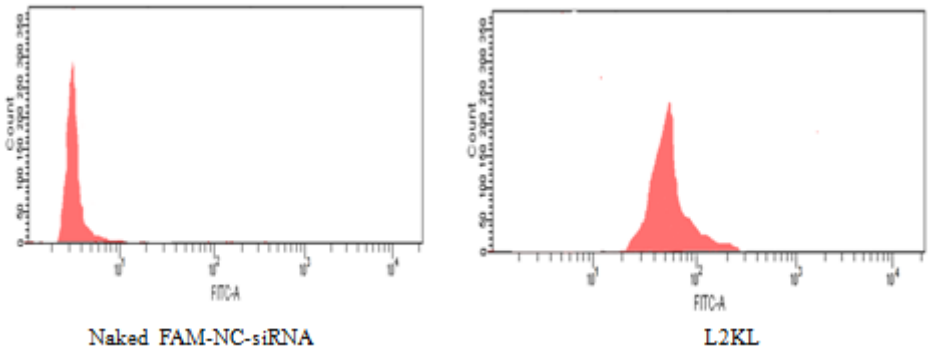


Figure 6.26: FACS analysis of Naked FAM-NC-siRNA and L2KL

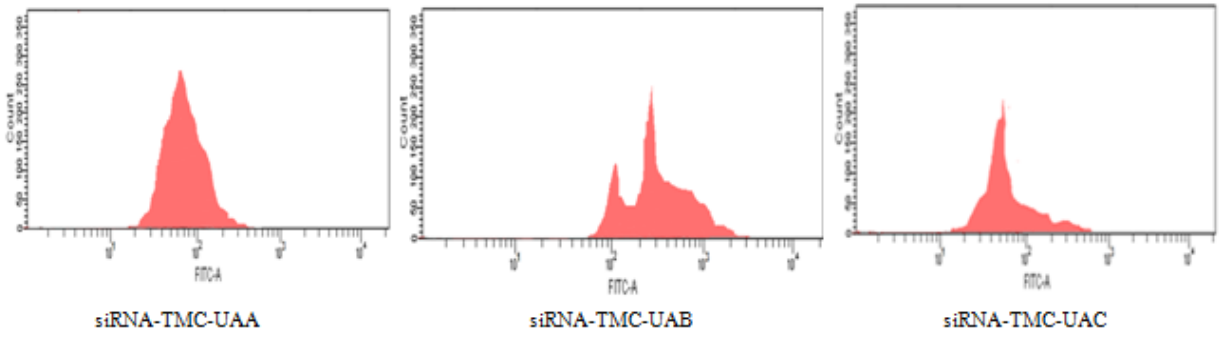


Figure 6.27: FACS analysis of siRNA-TMC-UAA, siRNA-TMC-UAB and siRNA-TMC-UAC

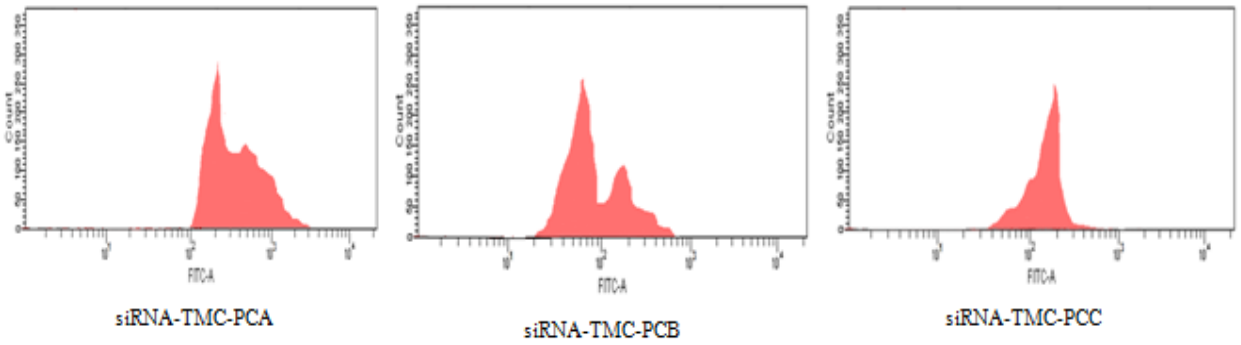


Figure 6.28: FACS analysis of siRNA-TMC-PCA, siRNA-TMC-PCB and siRNA-TMC-PCC

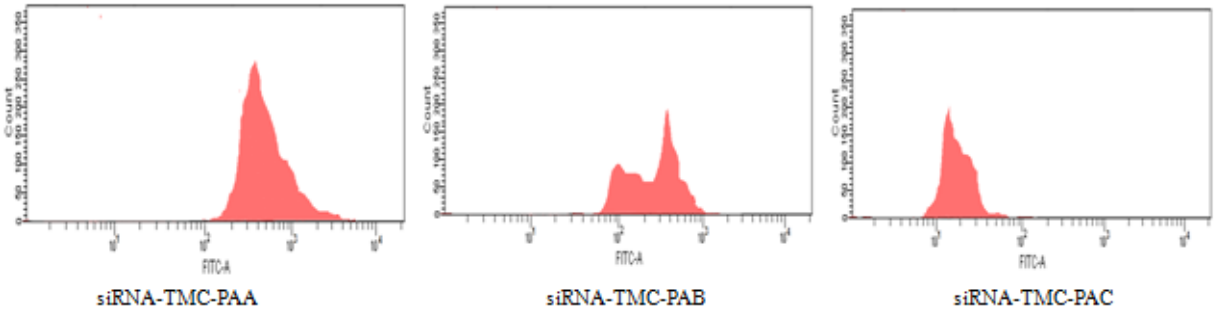


Figure 6.29: FACS analysis of siRNA-TMC-PAA, siRNA-TMC-PAB and siRNA-TMC-PAC

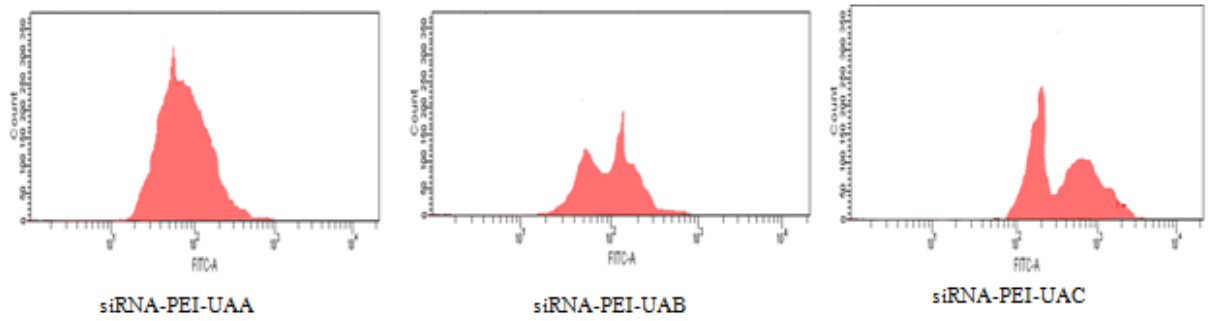


Figure 6.30: FACS analysis of siRNA-PEI-UAA, siRNA-PEI-UAB and siRNA-PEI-UAC

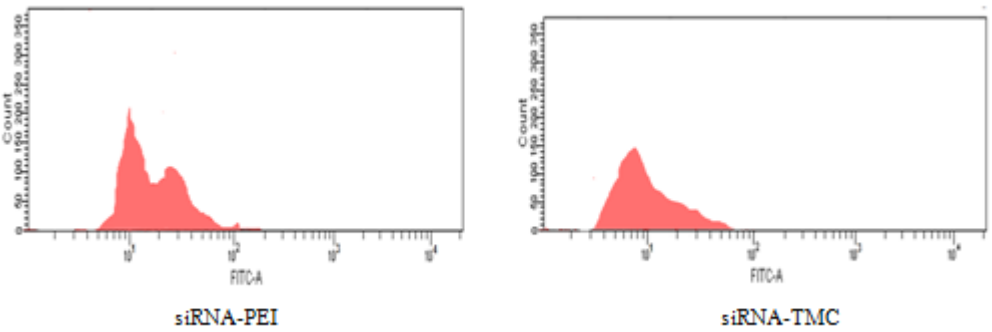


Figure 6.31: FACS analysis of siRNA-PEI and siRNA-TMC

Table 6.5: Cell Uptake of different Polyplexes Formulations

Sr. No.	Formulations	Mean Fluorescent Intensity*
1	Naked FAM-NC-siRNA	12.15±0.55
2	L2KL	66.35 ±0.71
3	siRNA-TMC	61.28 ±0.70
4	siRNA-TMC-UAA	78.64 ±1.05
5	siRNA-TMC-UAB	73.15 ±1.12
6	siRNA-TMC-UAC	70.39 ±1.20
7	siRNA-TMC-PCA	74.22 ±1.09
8	siRNA-TMC-PCB	71.94 ±0.89
9	siRNA-TMC-PCC	68.87 ±0.57
10	siRNA-TMC-PAA	71.25 ±0.49
11	siRNA-TMC-PAB	66.26 ±0.68
12	siRNA-TMC-PAC	64.21 ±0.74
13	siRNA-PEI	70.19 ±0.91
14	siRNA-PEI-UAA	83.17 ±1.32
15	siRNA-PEI-UAB	77.54 ±1.25
16	siRNA-PEI-UAC	73.25 ±0.95

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

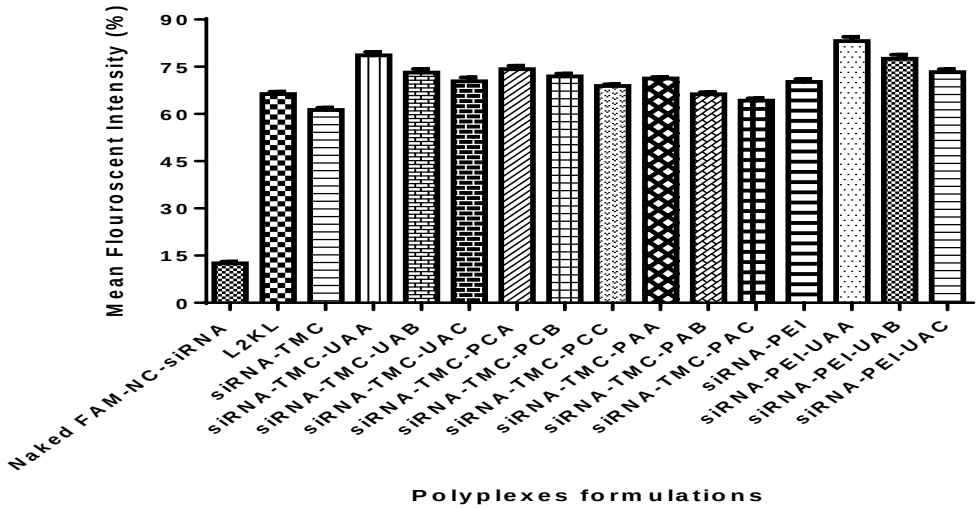


Figure 6.31(a): Mean Fluorescent Intensity of different Polyplexes formulations

Figure 6.31(a) exhibits mean fluorescent intensity treated with naked FAM-NC-siRNA and developed polyplex formulations thereof. Naked siRNA demonstrated very little cell uptake while modified TMC and PEI based polyplex formulations led to elevated uptake within the cells owing to positive charged mediated endocytosis mechanism. All modified polymers demonstrated higher cell uptake in comparison to native polymers. From the results, it can be said that as the conjugation of the heterocyclic moieties increased, the cell uptake within the cells was found to enhanced, regardless of the type of heterocyclic moiety i.e. cell uptake of Urocanic acid modified TMC and PEI revealed pattern of TMC-UAA>TMC-UAB>TMC-UAC>TMC and PEI-UAA> PEI-UAB>PEI-UAC>PEI, respectively.

Among the different heterocyclic moieties, at each degree of conjugation, the cell uptake revealed pattern like UAA>PCA>PAA. Differences in cell uptake in heterocyclic moieties can be ascribed to the hydrophobicity and positive charge; both are important factors that affect the cell uptake of the formulations. Modification of the polymers through hydrophobic compounds increases the cell uptake. Furthermore, the polyplex formulations with highest cell uptake among the different conjugation level of different heterocyclic moiety were selected for stability challenge studies and further characterization.

Electrolyte induces flocculation study

Sodium chloride solution was used to study the stability of the polyplexes in order to have the estimation of formulation stability in existence of high salt concentration conditions that to be happening *in vivo*. Figure 6.32 shows that changes in the particle size of the polyplexes at various concentration of sodium chloride. Increasing sodium chloride concentrations led to increase in the particle size of the polymer complexes.

Polyplexes demonstrated linear rise in the particle size after incubation with sodium chloride solution. Nevertheless, all the polyplexes maintained the particle size below the 300 nm. After that higher salt concentrations led to sudden increase in particle size. Literature shows that polyplexes start to dissociate after certain salt concentration leading to decrease in intensity of scattering up to certain level of NaCl solution. However, same case was not viewed with any polyplex formulations. Improved stability of the polyplex formulations can be attributed to conjugation of heterocyclic compounds modified TMC and bPEI which would have decreased the closer approach of electrolytes. Nevertheless, sudden rise in particle size at higher salt concentration might be owing to electric double layer suppression by sodium chloride which would induce hydrophobic interaction between particles causing aggregation of the polyplexes. Results of particles size after incubation with electrolyte sodium chloride solution are shown in the table 6.6. siRC-TMC-UAA, siRNA-TMC-PCA and siRNA-TMC-PAA showed 1.36,1.35 and 1.27-foldincreased in particle size after sodium chloride incubation, respectively. While siRNA-PEI-UAA showed 1.34-fold increased in particle size of polyplexes.

Table 6.6: Electrolyte induced flocculation of polyplexes formulations

NaCl Concentration (%)	Particle size of polyplexes(nm)*			
	siRNA-TMC-UAA	siRNA-TMC-PCA	siRNA-TMC-PAA	siRNA-PEI-UAA
0	191.2±1.5	170.5±2.7	230.2±4.1	128.9±1.4
1	203.8±2.2	182.5±2.4	239.5±3.8	135.6±1.2
2	217.1±1.9	193.6±2.8	251.2±3.5	142.9±1.8
3	229.3±2.4	205.2±2.0	267.1±4.2	150.3±2.1
4	241.4±2.7	213.7±3.2	280.5±4.6	163.8±2.4
5	260.2±3.1	230.8±3.5	293.4±3.9	172.6±1.9
Increase in folds	**1.36**	**1.35**	**1.27**	**1.34**

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

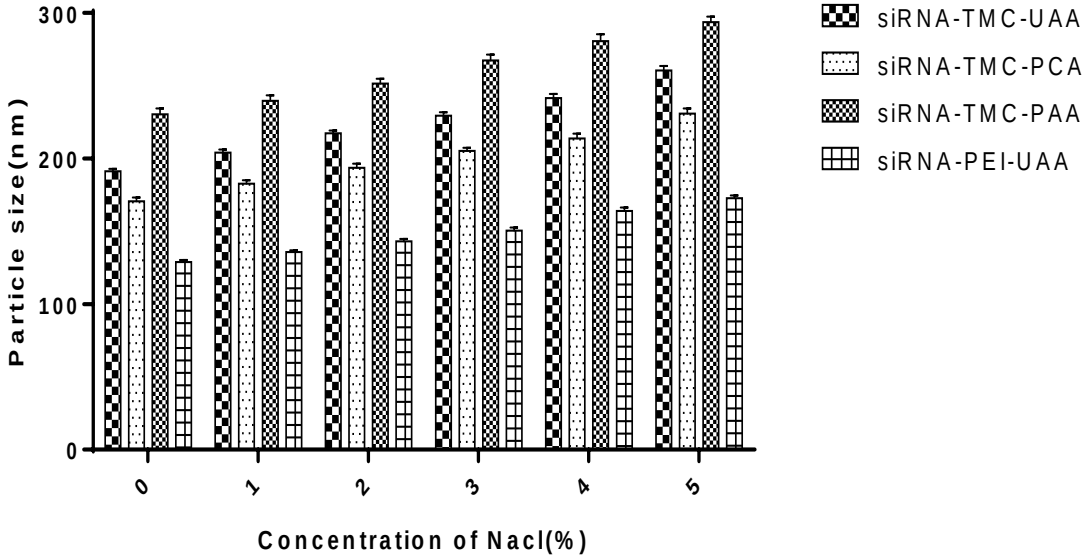
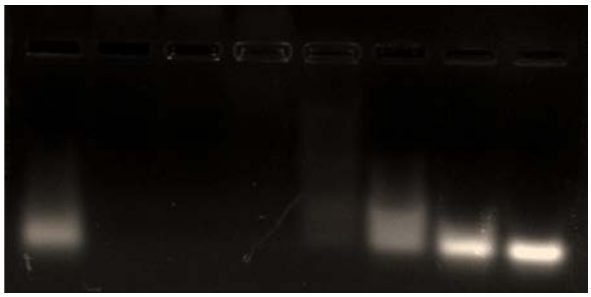


Figure 6.32: Electrolyte induced flocculation of polyplexes

Heparin Polyanion Competition Assay

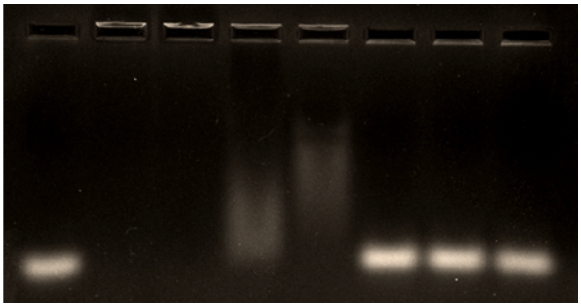
Negatively charged siRNA and cationic polymers electrostatic interaction provides stability to polyplexes. It has been reported that heparin causes concentration dependent dissociation of DNA from polyplexes(7).Similar could be the case polyplexes with siRNA. Nevertheless, the polyplexes stability depends on the charge per complexed molecule(8).In addition, it has been demonstrated that polymeric siRNA sustained stable polyplexes with polyethylenimine in present of heparin(9).

This makes obvious that siRNA-based formulation would be much affected by the polyanions presence such as heparin. Therefore, heparin competition assay was performed to assess the stability of polyplexes formulations on exposure to in vivo conditions where polyplexes are face polyanions. Moreover, this would give idea about whether the polyplexes were made at appropriate polymer to siRNA w/w ratio as it directly extrapolates to transfection efficiency (7).



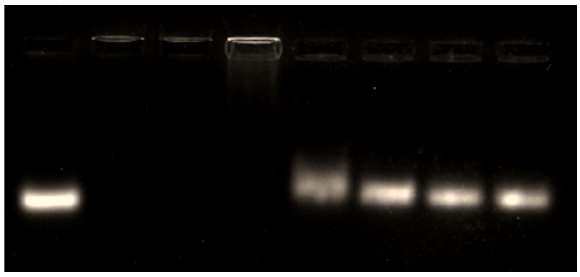
Heparin/siRNA w/w ratio (L→R) Lane 1: naked siRNA, Lane 2: 0.5, Lane 3: 1, lane 4: 1.5, Lane 5: 2.0, Lane 6: 2.5, Lane 7: 3.0, Lane 8: 3.5

Figure 6.33: Heparin competition of siRNA-TMC-UAA



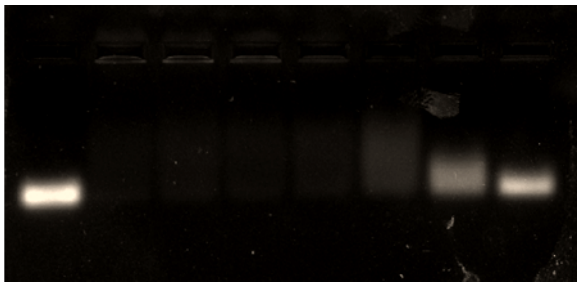
Heparin/siRNA w/w ratio (L→R) Lane 1: naked siRNA, Lane 2: 0.5, Lane 3: 1, lane 4: 1.5, Lane 5: 2.0, Lane 6: 2.5, Lane 7: 3.0, Lane 8: 3.5

Figure 6.34: Heparin competition of siRNA-TMC-PCA



Heparin/siRNA w/w ratio (L→R) Lane 1: naked siRNA, Lane 2: 0.5, Lane 3: 1, lane 4: 1.5, Lane 5: 2.0, Lane 6: 2.5, Lane 7: 3.0, Lane 8: 3.5

Figure 6.35: Heparin competition of siRNA-TMC-PAA



Heparin/siRNA w/w ratio (L→R) Lane 1: naked siRNA, Lane 2: 0.5, Lane 3: 1, lane 4: 1.5, Lane 5: 2.0, Lane 6: 2.5, Lane 7: 3.0, Lane 8: 3.5

Figure 6.36: Heparin competition of siRNA-PEI-UAA

After incubation with various heparin concentration, the quantity of heparin necessitate for releasing the total siRNA quantity from polyplexes was assessed by agarose gel electrophoresis. As it can be noticed, siRNA was observed in the sample wells when it was not released from the formulations at that particular heparin/siRNA ratio. Increase in heparin concentration led to increase in siRNA band density demonstrating concentration dependent siRNA release from polyplex formulations. This might be ascribed to the displacement of siRNA by heparin molecules.

Figure 6.33 to 6.36 demonstrates that siRNA starts releasing from TMC-UAA polyplexes at w/w heparin/siRNA ratio of 2.0 and complete siRNA was released from polyplexes at heparin to siRNA ratio of 3.0-3.5. While, 2.5 and 2.0 w/w heparin/siRNA ratio was required for complete release of siRNA from TMC-PCA and TMC-PAA polyplexes. In case of PEI-UAA polyplexes, siRNA started releasing at w/w heparin to siRNA ratio of 2.5 and complete siRNA released from the polyplexes at w/w heparin to siRNA ratio of

3.5. GAG concentration in human serum at its higher end is 20 µg/ml. Amount of heparin needed to release siRNA from polyplexes formulations was way far higher than present in human blood demonstrating their apparent stability *in vivo*. In addition, Various GAGS presents in lungs include heparin sulphate, hyaluronan, dermatan sulphate and chondroitin sulphate(10). Nevertheless, polyplexes formulations stability at high heparin levels justifies their stability in BALF as well. As evident for TMC-UAA and PEI-UAA polyplexes, modified polymers could hinder release of siRNA by heparin, probably through entrapping siRNA inside strong complexes with imidazole ring than piperazine and pyridine that provided some additional protection. Furthermore, hydrophobic modification with heterocyclic compounds could also be accounted for polyplexes stability. Hence, developed polyplexes exhibited good resistance to disruption by heparin representing their stability *in vivo*.

Serum stability study

Polyplexes like non-viral vectors should have the property to guard siRNA from degradation by RNAses enzymes, particularly, those enzymes present in the serum if administration is meant for I.V. administration or in organs where anionic charged proteins influence the polyplex formulation stability. in order to assess the potential of developed polyplexes formulations to protect siRNA from degradation, serum stability studies were performed by incubation of polyplexes formulations with high serum concentrations. Agarose gel electrophoresis images illustrating siRNA analysis of various polyplexes formulations at different time periods is shown in following table 6.7 and figure 6.37.

Table 6.7: Serum stability of polyplexes

Time(hr)	siRNA Retained *(%)				
	Naked siRNA	**siRNA-TMC-UAA**	**siRNA-TMC-PCA**	**siRNA-TMC-PAA**	**siRNA-PEI-UAA**
0	100.0	100.0	100.0	100.0	100.0
1	76.23±1.47	98.25±2.87	97.51±2.58	98.11±2.13	98.75±2.24
2	44.13±2.54	97.17±2.65	95.11±2.32	97.24±2.44	97.27±2.49
4	20.14±3.32	96.21±3.12	92.13±1.98	94.31±3.27	96.84±1.90
8	-	91.65±4.11	89.22±3.30	91.66±3.78	92.15±3.24
16	-	88.32±3.85	86.91±2.67	87.82±2.63	89.16±3.47
20	-	85.45±2.94	82.78±3.09	83.29±1.95	85.65±2.90
24	-	81.72±3.45	78.24±2.57	77.52±2.10	82.11±2.54

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

Agarose gel electrophoresis results for stability of polyplexes formulations demonstrated that polyplexes were able to protect BDNF siRNA from nuclease mediated degradation present in serum. Figure 6.37 showed that naked siRNA started to degrade significantly in serum from the start with about 24% degradation within an hour and about 80% degradation within 4 hrs which reached almost complete siRNA degradation within 5-6 hrs incubation. In case of polyplexes, degradation was slow as compared to naked siRNA demonstrating the inaccessibility of serum nucleases to degrade siRNA. Even after extensive incubation with serum, polyplexes were stable with siRNA. Polyplexes retained greater than 75% of siRNA after 24 hr. This type of shielding presented by polyplexes can be ascribed to the condensed polyplexes structure that holds siRNA that is located within the interior of the polyplexes. (11)

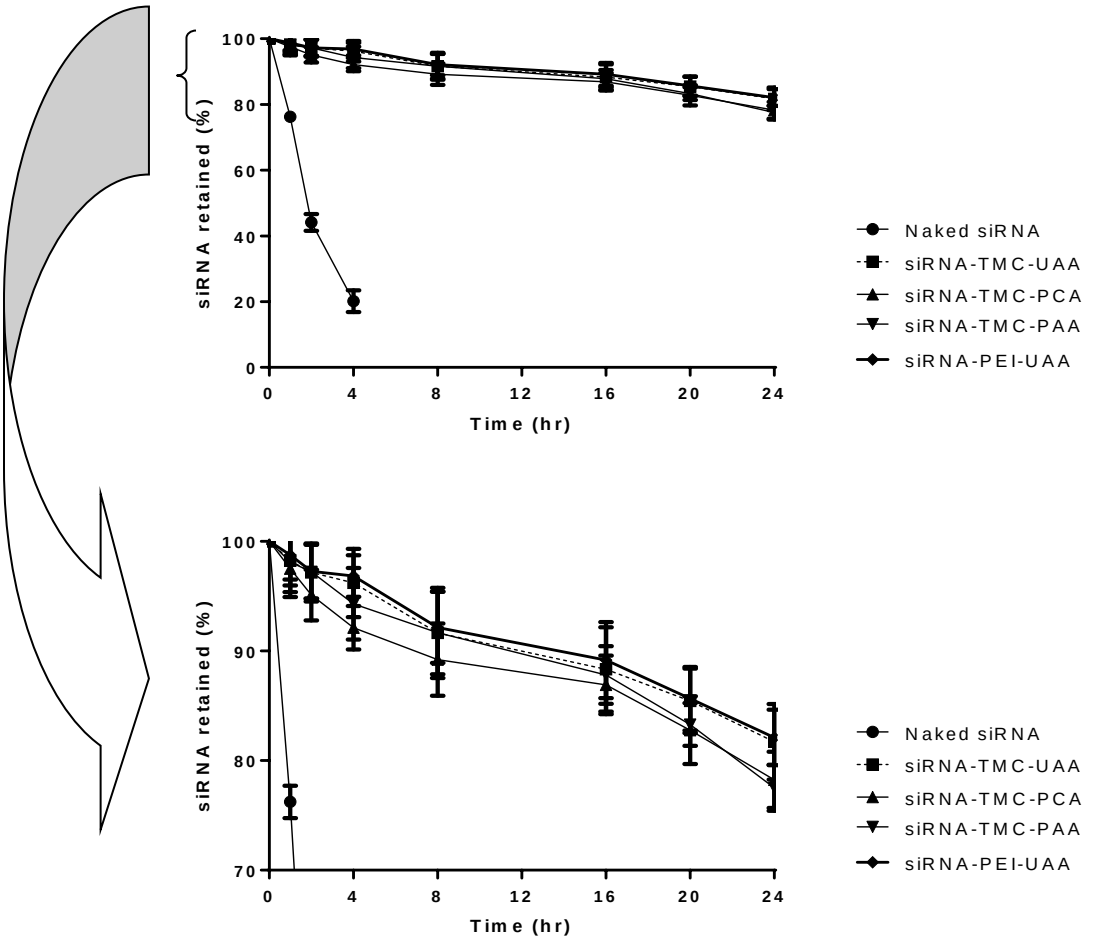


Figure 6.37: Serum stability of polyplexes

Stability study in Bronchoalveolar lavage fluid

Polyplexes formulation stability in present of bronchoalveolar lavage fluid was assessed by agarose gel electrophoresis. Band density of incubated formulations for different time points with BALF relative to that at 0 min time point was estimated and percent of siRNA retained was estimated. In present of BALF, mean siRNA retained in the polyplexes formulations was seen to be reducing demonstrating that siRNA was release in presence of BALF with the loss of 5-6% after 2 hr. This can be ascribed to the presence of pulmonary surfactants and anionic charged proteins in the BALF that might have affected the displacement of complexed siRNA. Though, the reduction in siRNA retention at each time point was non-significant illustrating the polyplexes stability in

BALF. Nevertheless, the release was of very low amplitude for each formulation, as seen by similar band densities of siRNA obtained for formulations exposed to BALF**.**

Table 6.8: Polyplexes stability in Bronchoalveolar lavage fluid

Time (Min)	siRNA Retained* (%)			
	siRNA-TMC-UAA	siRNA-TMC-PCA	siRNA-TMC-PAA	siRNA-PEI-UAA
0	100.0	100.0	100.0	100.0
15	99.25±1.37	99.52±2.10	98.88±2.56	99.38±1.89
30	98.57±1.44	98.25±2.32	98.34±3.21	98.80±2.17
45	98.10±2.02	97.80±1.74	97.78±3.05	98.12±2.65
60	97.83±2.15	97.42±1.45	96.35±1.90	97.37±2.44
75	96.24±1.87	96.59±1.92	95.86±1.57	96.44±1.74
90	95.69±1.65	95.77±2.30	95.24±3.20	95.91±1.82
120	95.10±2.25	95.23±2.45	94.78±2.80	95.26±2.63

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

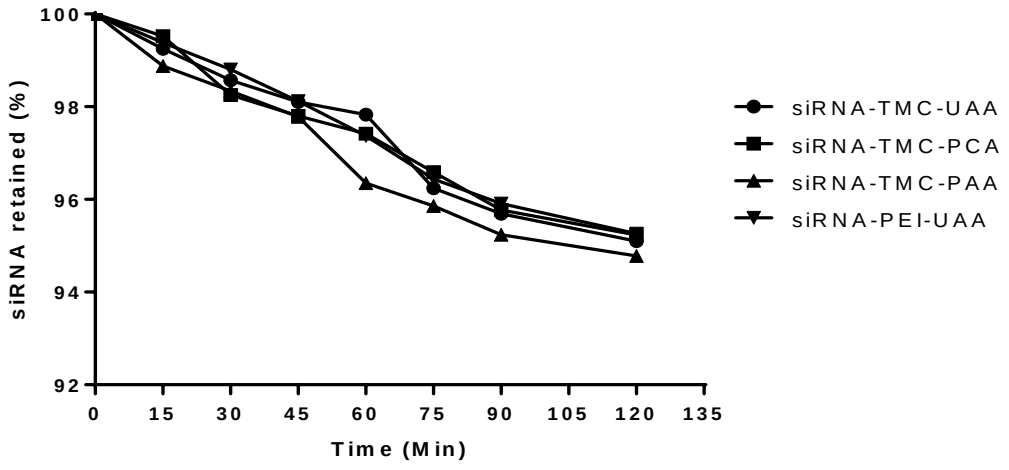


Figure 6.38: Stability of polyplexes in Bronchoalveolar lavage fluid

Transmission Electron Microscopy (TEM)

TEM images of modified TMC and modified PEI based polyplexes showed that polyplexes were spherical in shape and condensed due to strong electrostatic interaction as demonstrated in figures. TEM images show particle size of polyplexes below 250 nm without sign of aggregation confirming the homogenous systems. Acquired images are in concordance with the results obtained from the dynamic light scattering technique (DLS) and it confirmed the spherical shape of developed polyplexes.

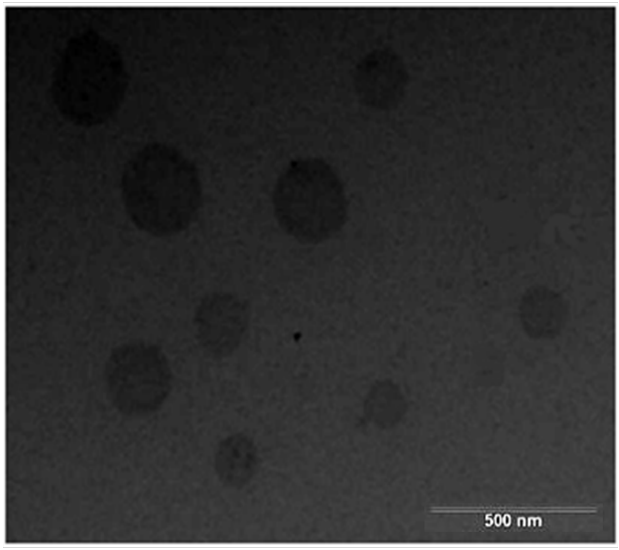


Figure 6.39: TEM image of siRNA-TMC-UAA polyplexes

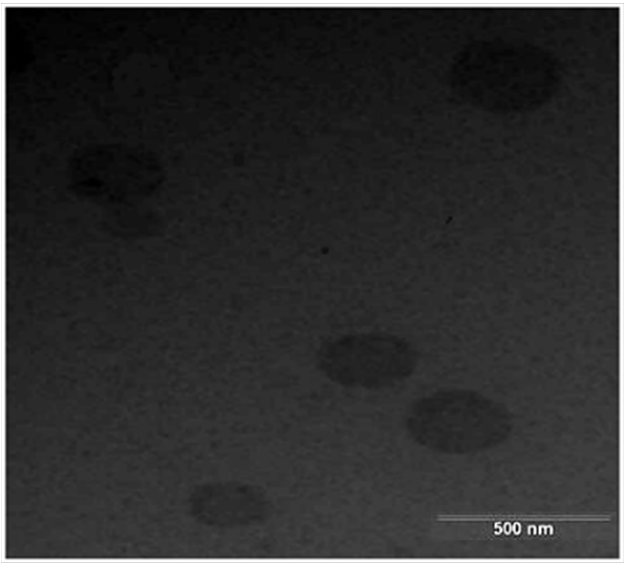


Figure 6.40: TEM image of siRNA-TMC-PCA polyplexes

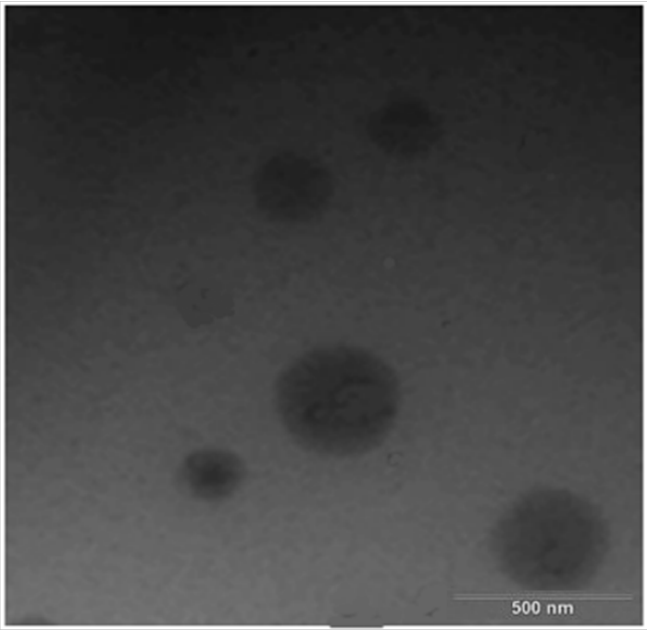


Figure 6.41: TEM image of siRNA-TMC-PAA polyplexes

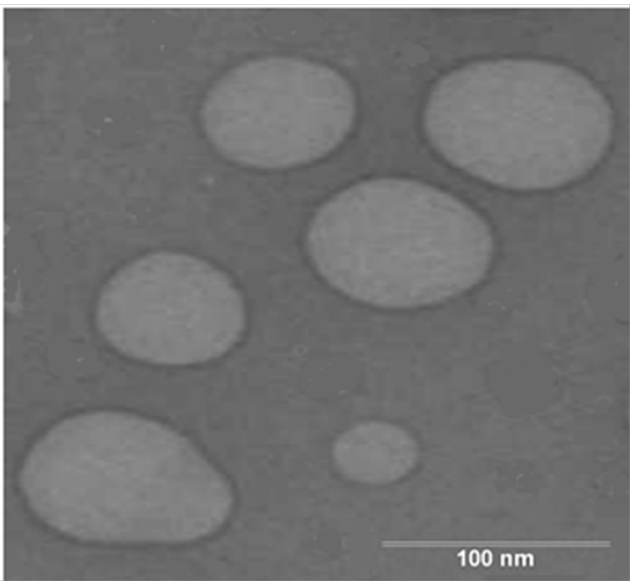


Figure 6.42: TEM image of siRNA-PEI-UAA polyplexes

6.12 References

1. Danielsen S, Maurstad G, Stokke BT. DNA–polycation complexation and polyplex stability in the presence of competing polyanions. Biopolymers. 2005;77(2):86-97.

2. Vader P vdAL, Engbersen JF, Storm G, Schiffelers RM. . 2012; 29(2): 352-61. Physicochemical and biological evaluation of siRNA polyplexes based on PEGylated poly (amido amine) s. Pharmaceutical research. 2012;29(2):9.

3. Kunath K, von Harpe A, Fischer D, Kissel T. Galactose-PEI–DNA complexes for targeted gene delivery: degree of substitution affects complex size and transfection efficiency. Journal of Controlled Release. 2003;88(1):159-72.

4. AC. H. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. Advanced Drug Delivery Reviews. 2006; ;58(14):8.

5. Sunshine JC, Akanda MI, Li D, Kozielski KL, Green JJ. Effects of base polymer hydrophobicity and end-group modification on polymeric gene delivery. Biomacromolecules. 2011;12(10):3592-600.

6. Parhamifar L LA, Hunter AC, Andresen TL, Moghimi SM. . 2010; 6(17): 4001-09. Polycation cytotoxicity: a delicate matter for nucleic acid therapy—focus on polyethylenimine. Soft Matter 2010;6(17):8.

7. Moret I, Peris JE, Guillem VM, Benet M, Revert F, Dasí F, et al. Stability of PEI–DNA and DOTAP–DNA complexes: effect of alkaline pH, heparin and serum. Journal of Controlled Release. 2001;76(1-2):169-81.

8. Zintchenko A, Philipp A, Dehshahri A, Wagner E. Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity. Bioconjugate chemistry. 2008;19(7):1448-55.

9. Lee S-Y, Huh MS, Lee S, Lee SJ, Chung H, Park JH, et al. Stability and cellular uptake of polymerized siRNA (poly-siRNA)/polyethylenimine (PEI) complexes for efficient gene silencing. Journal of Controlled Release. 2010;141(3):339-46.

10. Schmid K, Grundboeck-Jusco J, Kimura A, Tschopp FA, Zollinger R, Binette JP, et al. The distribution of the glycosaminoglycans in the anatomic components of the lung and the changes in concentration of these macromolecules during development and aging. Biochimica et Biophysica Acta (BBA)-General Subjects. 1982;716(2):178-87.

11. Ogris M BS, Schuller S, Kircheis R, Wagner E. . 1999; 6(4): 595-605. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther.6(4):10.