Chapter 7: Nebulized siRNA delivery and Characterization

7.1 Introduction

Inhalation is a general route of drug delivery to patients with a variety of pulmonary diseases. Several drugs are available for pulmonary delivery(1) e.g. ß2-agonists, corticosteroids and anticholinergics. Next to these anti-asthma and COPD therapeutics, antibiotics inhalation is frequently applied for Cystic Fibrosis patients (CF) (2, 3). In recent time, drug delivery through pulmonary route is proposed to increasing extent for the administration of drugs with systemic action that can either not be absorbed by the GI tract or suffer from a first pass effect. In inhalation delivery, the drugs are administered directly to the site of action. And hence, onset of action of the therapeutics is little, less drug is required and systemic adverse effects are decreased.

There are different types of drug delivery devices are generally used for the delivery of drugs to the pulmonary tract: nebulizers, pressurized metered dose inhalers (pMDI) and dry powder inhalers (DPI). To make an appropriate selection between drug delivery devices, an sufficient understanding of the benefits and drawbacks of the different systems is necessary (4, 5). Since a long time, the MDI has been considered as a suitable device most commonly used in inhalation delivery(6, 7). MDIs contain the drug formulated in either suspension form, either emulsion form or solution to which a propellant has been added. When the MDI is activated, a metered dose is released at high velocity, which needs a concurrent inhalation by the user. Hence, a precise coordination of activation and inhalation by the patient is compulsory. Evidently MDI device as such is unsuitable for young children who lack the required coordination(8).The high aerosol velocity constitutes an inherent disadvantage of the system device because it leads to a significant oropharyngeal deposition of the formulation. To facilitate the coordination between activation and inhalation, breath actuated MDIs have been available in the market. In these devices the inspiratory flow triggers dose release. A disadvantage of MDIs is the so called “cold freon effect” caused by rapid evaporation of the propellant. The cooled aerosol can cause bronchoconstriction. The use of a spacer can partly overcome these drawbacks. Until now chlorofluorocarbons (CFC) were used as propellant in the aerosol formulation. But owing to environmental burden caused by the CFC propellants, most devices are being reformulated with HFA227 or 134A or exchanged by alternative devices.

A dry powder inhalation (DPI) system consists of a dry powder, a dosing principle and an inhalation device. In most of the systems the drug in micronized form is formulated with an inert excipient, like lactose, however excipient free DPIs have also been developed DPI use the inspiratory flow of the patient for dose entrainment and powder disintegration. The dose is delivered from a multiple dose reservoir or from a single dose unit (capsule or blister) during inhalation. Some DPIs require a moderately high inspiratory flow rate to deliver an acceptable mass fraction of the dose as fine particles. For other types of DPIs, the flow increase rate is rather the relevant flow parameter(9). The required inspiratory flow curve cannot always be attained, especially not by patients with severe broncho-obstruction or young children.

Nebulizers are used to aerosolize suspensions and solutions of drugs and for inhalation. They are normally used in conditions when severe airway obstruction or insufficient patient coordination which doesn't allow the use of other devices. Nebulizers are for example recommended for young patients who cannot manage other devices. Nebulization of ß2-agonists and anticholinergics is common practice in acute asthma.

Nebulization of the liquid effectively generates aerosols for pulmonary administration; atomization theories propose that aerosol size and characteristics of output are depends on operating principles, conditions and constructions of nebulizers and physiological characteristics of the nebulized liquids. Limitation associated with the nebulizer as a inhaler device is low deposition efficiency of the therapeutics in the target area of the lungs. However, nebulizers are used extensively for the inhalation, usually generating the aerosol droplets less than 5 µm in size can reach to the deep lung. Hence, Nebulization is a promising approach to deliver gene or siRNA directly to the affected airway epithelial cells in asthma and COPD patients. Key step towards this objective is choice of appropriate device and suitable formulation permits nebulization of much therapeutics including siRNA in broad range of doses. Developed non viral carrier systems should preserve their stability and properties, protecting the siRNA from the shear forces during nebulization so that its biological efficacy is preserved. Additionally, yield of nebulization must be maximized.

Nevertheless, proper understanding of the working mechanism and the factors that affects the nebulizer performance is important for an effective use. Various types of nebulizer devices are available in market including jet, ultrasonic and vibrating mesh nebulizers. In the present study we assessed the delivery of siRNA polyplexes with ultrasonic nebulizers.

Table 7.1: Technical Parameters of Ultrasonic nebulizer B3-520

Parameters	Details
Ultrasonic frequency	2.4 MHz
particle size	0.5-5 micron
Capacity of Medicinal cup	10 ml
Capacity of Vessel	25 ml
Atomization volume 0.375/min	20 minutes timing automatically
Atomization volume 0.375/min	10 minutes timing automatically

7.2 In vitro aerosolization of formulations by Twin Stage Impinger

In order to deliver siRNA condensed polyplexes to lung, ultrasonic nebulizer was used to generate aerosol of polyplexes. siRNA polyplexes were solubilized in HEPES Buffer (30mM) containing KCl (100MM) and $MgCl_2$(1mM), pH 7.3. Ultrasonic nebulizer B3-520 is a handy aerosolization system , which can produce droplet by ultrasonication. The rate of spray droplet is above 0.1 ml/min. Further formulations were assessed for aerodynamic characteristics by Twin stage Impinger apparatus. Developed polyplexes formulations were pipetted directly into the nebulizer reservoir system and that was attached to twin stage apparatus with 7 ml of nuclease free water in the stage 1 and 30 ml of nuclease free water in the stage 2. The flow was adjusted to (60±5) L/min by vacuum pump and monitored throughout operation. For 10-15s, after vacuum pump was turned on, nebulizer was started to make aerosol and continued for some time. The vacuum pump was turned off at an interval of 5 seconds. After that the nebulized formulations collected in Stage1 and Stage 2 of Twin stage Impinger apparatus was disassembled and both stages, nebulizer reservoir and rubber mouthpiece were washed separately with nuclease free water and made up to the required volume using flask. Then these post nebulization samples were analyzed for their siRNA content by Nanodrop Spectrophotometer. Analytical method was developed for siRNA concentration

determination and validated for accuracy, precision as described in chapter 3 for analytical method development. Nebulized polyplex formulations were characterized for them aerosolize performance and Fine particle fraction (FPF), Emitted dose, MMAD, geometric standard deviation (GSD) were calculated.

Fine particle fraction (FPF) is denoted as the amount of siRNA available in Stage 2 (MMAD less than 6.4 μm) as percent of the total amount including nebulizer reservoir and two stages. Further, formulations were characterized after post Nebulization for the siRNA integrity, cellular uptake, Particle size and Zeta potential.

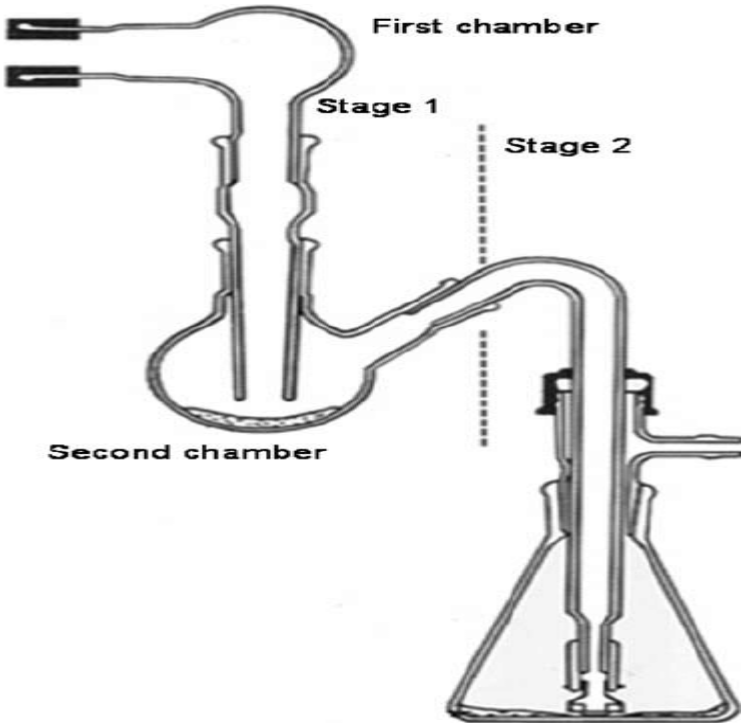


Figure 7.1: Schematic representation of Twin Stage apparatus

7.3 Stability of the formulations post Nebulization

7.3.1 siRNA Integrity

Nebulized polyplexes formulations were determined for the integrity of the siRNA by the agarose gel electrophoresis technique described in the previous chapter of formulation development.

7.3.2 Cell Uptake

Cellular uptake study of the nebulized formulations was performed as per same protocol described in the previous chapter 6 of formulation and development. Here, the cells were exposed to nebulized formulations containing FAM labelled negative control siRNA (FAM-NC-siRNA) at 100 nM siRNA concentration.

7.3.3 Particle size and zeta potential

Dynamic light scattering technique was used to determine the hydrodynamic diameter of the polyplexes after nebulisation using Malvern Zetasizer, Nano ZS series. The formulated polyplexes were diluted suitably with nuclease free water and measured at 25ºC. Likewise, zeta potential of the polyplexes after nebulization was measured by applying Smoluchowski's equation in the Malvern zeta sizer. All the studies were performed in triplicates.

7.4 Result and Discussion

The polyplex formulations when tested on twin stage Impinger apparatus, showed significant *in vitro* lung deposition. Nebulized formulations containing buffer demonstrated considerable fine particle fraction. The emitted dose, FPF, GSD and MMAD of the tested polyplex formulations have been tabulated in Table 7.2.

All the developed polyplexes formulations demonstrated emitted dose in the range of 84% to 90 %, mass median aerodynamic diameter less than 5µm, geometric standard deviation around 2.0-2.40 and Fine particle fraction between 34% to 41%. Results demonstrated that In vitro aerosolization performance of the polyplexes developed from modified polymers improved than polyplexes developed from the native polymers. All the polyplexes developed from modified TMC and modified PEI demonstrated almost similar in vitro aerodynamic characteristics of the nebulized formulations regardless of the conjugation moieties of heterocyclic compounds. Droplet sizes of 1–5 µm are considered optimal for delivery of therapeutics to alveolar regions, and nebulizers produce droplets within this range. Nebulized liquid reaching at stage 2 of twin stage Impinger have MMAD less than 6.4 µm and therefore expected to reach the peripheral area of lung. These findings propose that polyplexes formulations are appropriate for

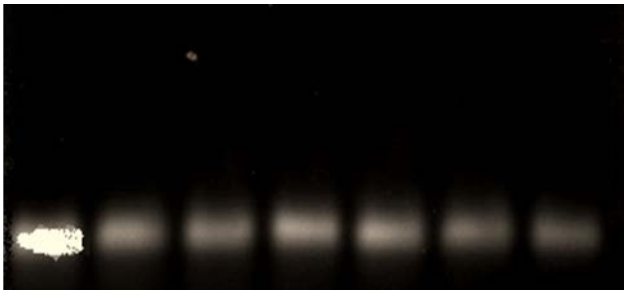
delivery to lower airways as well. Hence, *In vitro* analysis of the nebulized formulations performance from Ultrasonic nebulizer demonstrated high proportion of formulations was deposited on stage 2, then stage 1 of the twin stage impinge representing suitable aerosolization properties for pulmonary delivery of siRNA polyplexes.

Table 7.2: *In vitro* Aerosolization performance of nebulized formulations

Sr.No.	Formulations	Emitted Dose (%)	MMAD (µm)	GSD	FPF (%)
1	siRNA-TMC	84.14 ± 2.42	4.02 ± 0.38	2.40 ± 0.20	34.42± 1.89
2	siRNA-TMC-UAA	88.56 ± 2.85	3.54 ± 0.25	2.14 ±0.17	38.27 ±1.52
3	siRNA-TMC-PCA	87.27 ± 3.20	3.67 ± 0.16	2.05 ±0.12	37.50 ±1.48
4	siRNA-TMC-PAA	86.38 ± 3.34	3.86 ± 0.31	2.25 ±0.22	36.53 ±1.66
5	siRNA-bPEI	87.39 ± 2.76	3.70 ±0.18	2.17 ± 0.28	37.19 ± 1.58
6	siRNA-PEI-UAA	90.54 ± 2.76	3.24 ± 0.21	1.98 ±0.14	41.15 ±1.34

siRNA Integrity post nebulization

In order to verify the siRNA integrity after Nebulization, polyplexes were assessed by heparin competition assay by agarose gel electrophoresis. Figure 7.2 showed gel electrophoresis image of the polyplex formulations after Nebulization. It was observed that band density of siRNA from polyplex formulations after nebulization was less than naked siRNA before nebulization, it may be because of dilution effect of the formulations within the impinger. These results propose stability and successful aerosolization of polyplexes. Report findings says that stability of formulations may be compromised during course of nebulization due to shearing forces within the nebulizer device and surface effects at interface of liquid droplet/air (10). Association with the polymer complexes has ensured siRNA integrity is preserved during process of nebulization. Previously, siRNA condensation with PEI was found to be essential to preserve stability while nebulization (11).These results, hence, confirm the successful nebulization using a nebulizer and delivery to stage 2 of the twin stage impinger using novel modified TMC and PEI based carriers for siRNA, indicates their potential for in vivo studies.

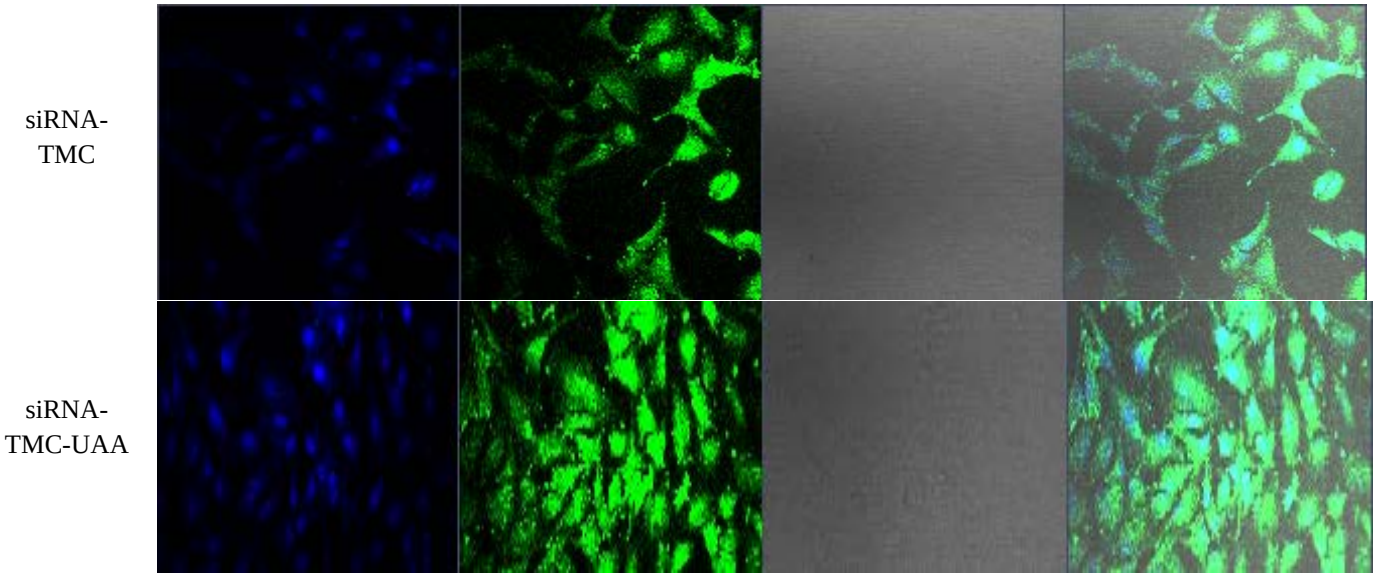


Lane(L→R)(Lane 1: Naked siRNA(control before Nebulization),Lane-2: siRNA-TMC,Lane-3: siRNA-TMC-UAA, Lane 4 : siRNA-TMC-PCA, Lane 5: siRNA-TMC-PAA, Lane 6: siRNA-PEI, Lane 7: siRNA-PEI-UAA

Figure 7.2: siRNA integrity post nebulization

Cell uptake post nebulization

Figure 7.3 demonstrated cellular uptake of the formulations post nebulization. It can be said that polyplexes shows remarkable cellular uptake even after nebulization demonstrating formulation stability and maintenance of the physicochemical properties against the sheer force of the Nebulization process.



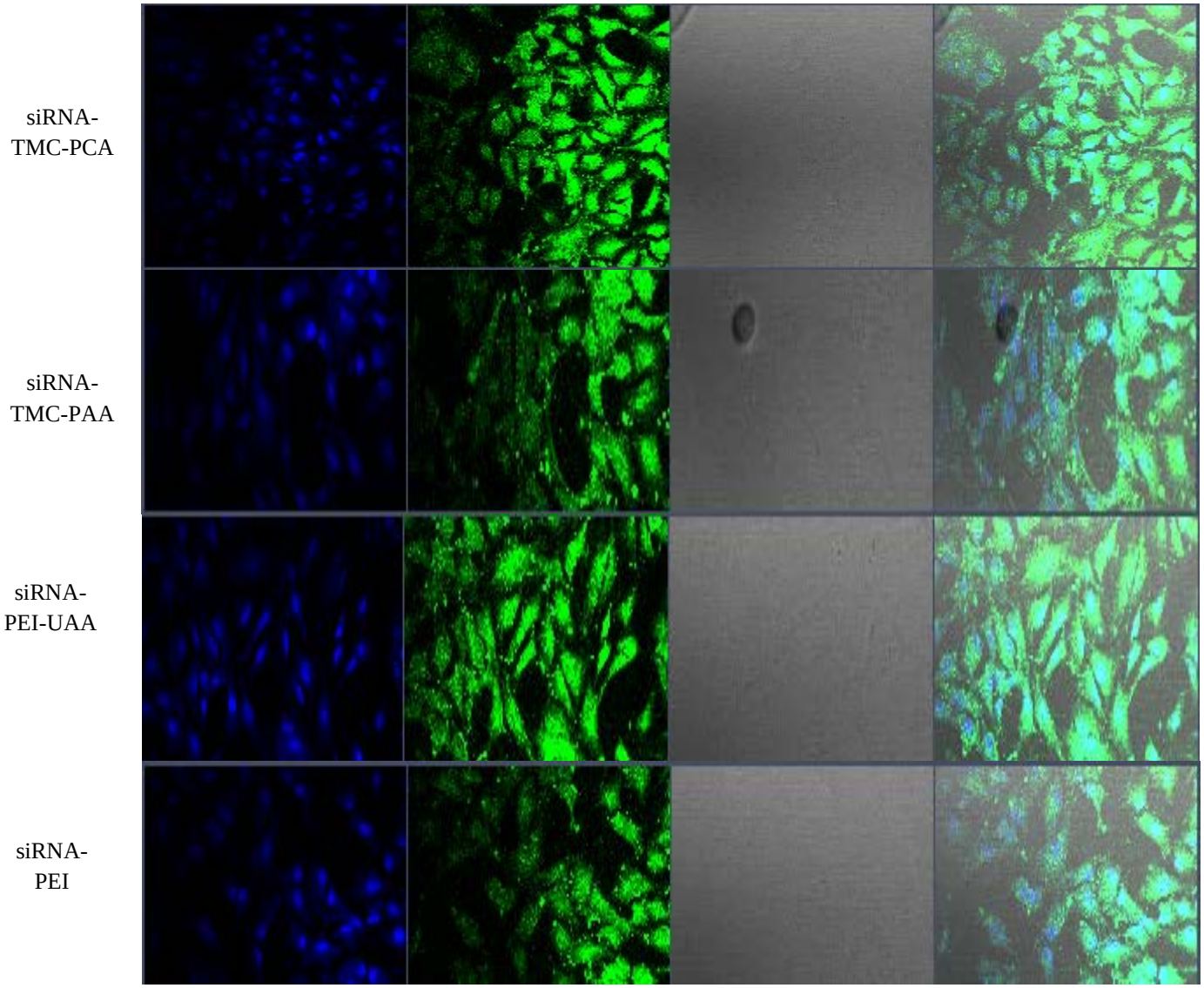


Figure 7.3: cell uptake of TMC,TMC-UAA, TMC-PCA, TMC-PAA, PEI and PEI-UAA based polyplexes post nebulization

The nebulized polyplexes not only maintained the transfection ability, but the integrity of siRNA was also retained in accordance with what was previously reported by others researchers (12, 13). Those polyplexes demonstrated increased colloidal stability, hence were less chances to aggregation or flocculation.

Particle size and Zeta potential post nebulization

Table 7.3 describes the results of the particle size and zeta potential post nebulization of the polyplexes. There was no significant change in particle size and zeta potentials of the polyplex formulations after nebulization demonstrating the colloidal stability of the polyplexes. Slightly increased of 3-4 nm size was observed after post nebulization it may be ascribed that during aerosolization, passage of the formulation through stages. Positive surface charge of the formulations remained unaffected during aerosolization process demonstrating there was no sign of any aggregation of the polyplexes.

Table 7.3: Particle size and zeta potential post nebulization

Sr. No.	Formulations	Particle size*(nm)	PDI	Zeta potential*(mV)
1	siRNA-TMC	155.2 ± 3.6	0.130	26.20 ±1.05
2	siRNA-TMC-UAA	191.2 ± 1.8	0.122	13.10 ± 1.32
3	siRNA-TMC-PCA	175.8 ± 2.8	0.069	18.23 ±1.65
4	siRNA-TMC-PAA	231.5 ± 4.2	0.158	15.98 ±2.05
5	siRNA-bPEI	106.2 ± 2.5	0.116	35.73 ±1.27
6	siRNA-PEI-UAA	127.3 ± 3.7	0.090	25.30 ±2.54

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

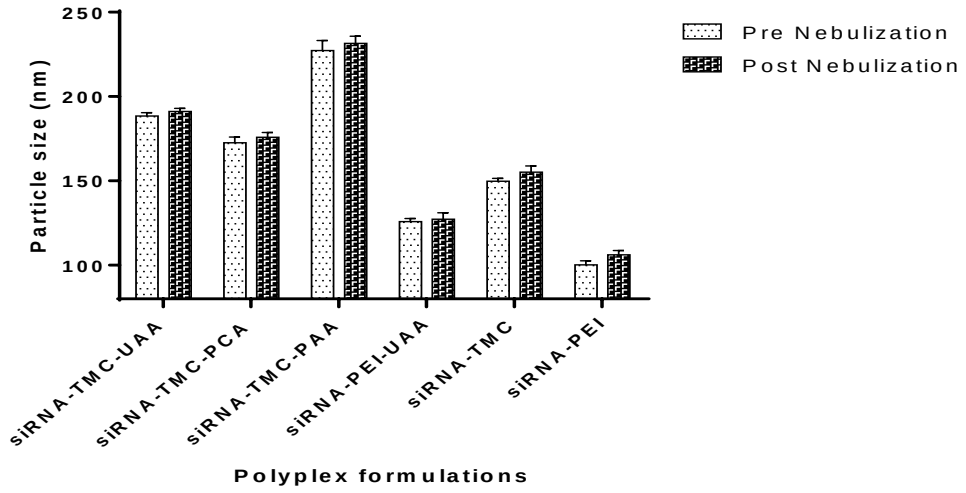


Figure 7.4 : Particle size of polyplexes at pre nebulization and post nebulization

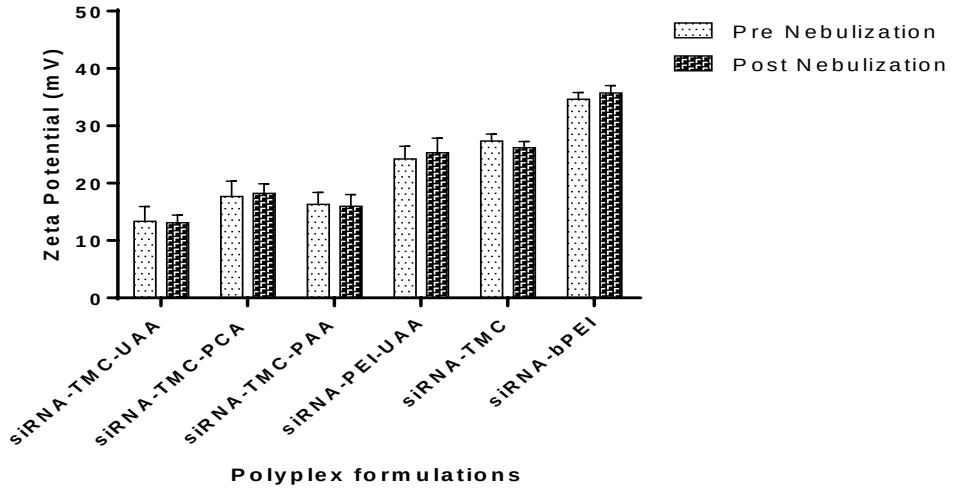
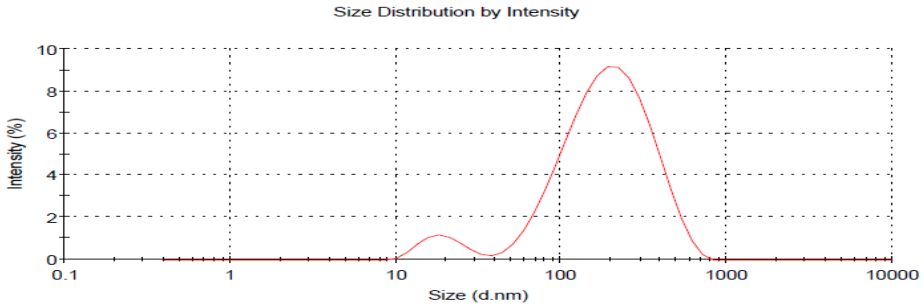
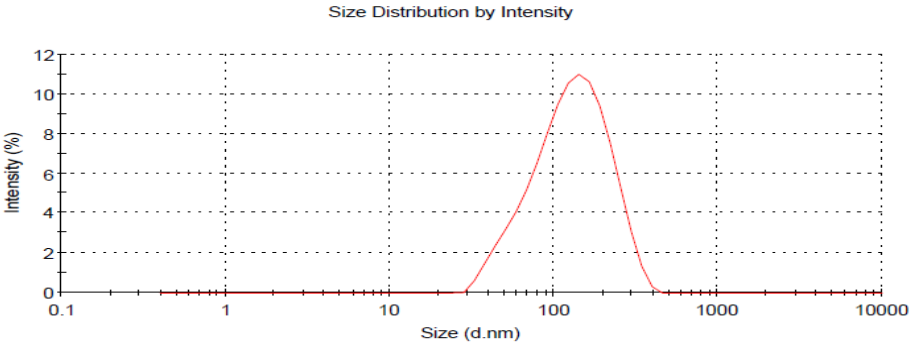


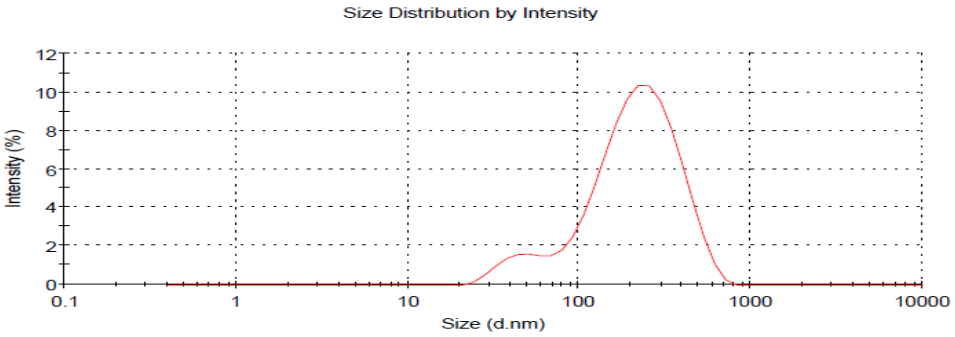
Figure 7.5: Zeta Potential of polyplexes at pre-nebulization and post nebulization



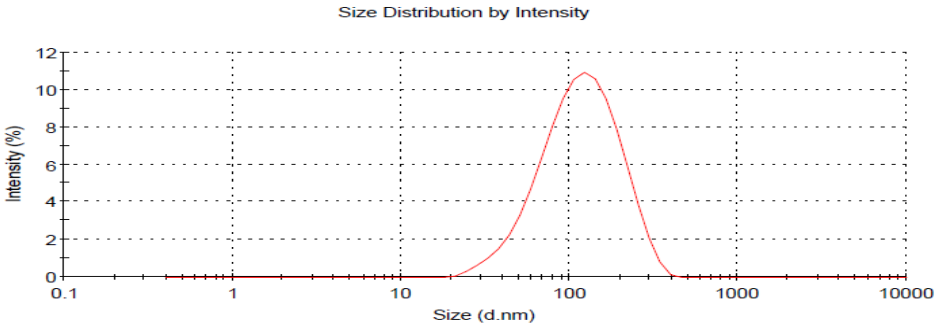
(a) siRNA-TMC-UAA



(b) siRNA-TMC-PCA



(c) siRNA-TMC-PAA



(d) siRNA-PEI-UAA

Figure 7.6: Particle size of different polyplexes post nebulization

7.5 References:

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