Chapter 9: Summary and Conclusion

9.1 Summary and Conclusion

Asthma and chronic obstructive pulmonary disease (COPD) are the leading obstructive airway disorders globally, because of high prevalence and high burden on system of healthcare. Asthma is a chronic disorder of the airways generally featured by airway inflammation, airflow obstruction and airway hyper responsiveness. Allergen sensitization is significant risk for the asthma development. As per WHO report, it is estimated that currently 235 million people suffers from asthma and about 250,000 annual deaths worldwide and about 100 million people in India for asthma and COPD and majorly common non-communicable disease in the children..Regardless of the development level, Asthma is a serious health issue among the all part of the world including lower middle and high-income countries. Further, Asthma generates significant burden to the individual persons and family, if under diagnosed and under treated and most of the time limits the individuals' activities for throughout life.

Asthma is characterized as recurrent reversible airway obstruction, with wheezing, breathshortness and frequently night-time cough and its main feature involves the airway inflammation, which causes the airway hyper-responsiveness and results into in reversible airway obstruction.

Chronic obstructive pulmonary disease (COPD) is featured by airflow limitation progressively that is partially reversible and causes breathlessness, inflammation of airways and systemic effect. Study reports for the global burden of the COPD state that occurrence of 251 million cases of COPD in 2016 worldwide and it is anticipated that in 2015, 3.17 million deaths were due to COPD. Major cause of the COPD is tobacco smoke exposure and other factors include the occupational dusts and fumes, air pollution exposure. It is believed that disease is possibly augment in upcoming years owing to increase in smoking prevalence and aging populations in number of the countries globally.

Several inflammatory mediators are involved in the inflammatory reactions of the asthma and COPD such as Chemokines are significant in the inflammatory cells recruitment into the respiratory airways and are principally expressed in airway epithelial cells(6-8), Cysteinyl leukotrienes, potent broncho-constrictors and pro-inflammatory

mediators chiefly resultant from mast cells and eosinophils (9), released cytokines plan the inflammatory response in asthma. Chief Cytokines covers Interleulkine-1β and Tumor necrosis factor and GM-CSF and Prostaglandin D2 is a bronchoconstrictor (10).

Neurotrophins, growth factors family includes Nerve growth factor, Brain Derived Neurotrophic Factors, NT-3 and NT-4/5 at first revealed in the nervous system, mediate inflammatory signals among neurons cells and immune cells and structural tissue cells. It has been also found that neurotrophins express outside the nervous system as well, particularly in immune cells. Nevertheless, majorly studies focused on inflammatory and allergic conditions and so their outcome reflects rather the underlying disease pathophysiology than the normal physiology. Neurotrophins trigger such neuroimmune interactions as well.

Neurotrophins, by modulating sensory nerve innervations, provoke the synthesis of neuropeptides namely or neurokinin A and B and substance P which are belongs to tachykinin family. Sensory neurons release these neuropeptides change range of responses of different cells, which leads to cell activation and differentiation. Such neurotransmitters release in direct potentiation of cascade of inflammatory reactions called " neurogenic inflammation"

Neurotrophins, the key mediators in the neuronal plasticity and neurogenic inflammation, Asthmatic and patients with chronic obstructive disorders have been observed for enhanced levels of neurotrophins in BALF fluid. In preclinical model, Neurotrophins synthesis observed to be increased in macrophages and T cells infiltration airways. Initially it has been observed that neurotrophins have been enhanced in inflammation of airway came from clinical results. Indeed, using animal models one researchers group has evidently demonstrated that immune cells are one of the chief source of Brain derived neurotrophic factor and Nerve growth factor during airway inflammatory conditions.Hence, neurotrophins can be potential targets in the airway inflammation and hyperreactivity conditions to treat asthma and chronic airway obstructive conditions.

In recent years, natural endogenous mechanism called RNA interference (RNAi) for gene silencing has been the focus of interest for the researchers for its promising potential use in novel drug delivery and gene delivery. Particular gene expression can be regulated by different mediators namely small interfering RNA (siRNA), short hairpin RNA (shRNA), microRNA (miRNA).

siRNAs, which are small molecules of RNA with exogenous origin, have demonstrated gene silencing of the over expressed gene very effectively and specifically. siRNAs having greater gene-silencing ability demonstrated stability in physiologic fluids and mammalian cells. Silencing of the gene can be provoked by siRNAs by specific cleavage of sequence complementary messenger RNA (mRNA). RNAi based therapeutics provides several advantages such as it can inhibit every class of the targeted genes with high selectivity and potency, can offer personalized therapy. Hence, gene silencing by the RNA interference mechanism by siRNA is promising approach in the airway inflammatory conditions and hyper-responsiveness by targeting the neurotrophin, Brain derived neurotrophic factor BDNF, which is highly upregulated in airway inflammation.

In this study we have proposed delivery of siRNA through inhalation route to downregulate neurotrophin, brain derived neurotrophic factor (BDNF). There are reports supporting the potential of siRNA against neurotropins to alleviate airway inflammation. Therapeutic siRNA has been tried in clinical trials in various other pulmonary diseases which prove their clinical application. The inhalation route is highly suitable and patient compliant and it permits one to widely distribute the therapeutics agent along the airways. Further, it gives localized action and reduces the systematic adverse-effects.

The naked siRNA is very prone to degradation by nuclease enzymes *in vivo*. In addition; due to its large molecular weight and anionic nature, they can't cross the cell membrane. Hence, cationic charged polymers or lipids have been used as vectors which interact electrostatically with negatively charged siRNA leading to the spontaneous formation of nanosized complexes which can interact with cell membrane and undergo internalization by endocytosis mechanism. The compact structure restricts the access of nucleases to the enclosed siRNA, in that way significantly improving the stability.

Although; cationic lipid and polymer based vectors can be used for complex formation but in context of the pulmonary delivery it has been found that lipid based vectors experience strong interference from pulmonary surfactants which makes cationic polymers as preferred agent for pulmonary delivery. Nevertheless, once endocytosed across the cell membrane, the polyplexes should escape from the endosomes to avoid the subsequent lysosomal degradation, and unload the siRNA into the cytoplasm, where the endogenous RNAi machinery will be utilized to down regulate the target mRNA by enzymatic cleavage before expressing the preferred protein.

Cationic polymers such as chitosan, polyethylenimine (PEI), poly(L-lysine), Poly(arginine), polyphosphoester, and dendrimers have been used for non-viral vectors. Among these polymers, we have studied chitosan and Polyethylenimine as a non viral vectors for pulmonary siRNA delivery.

siRNA is available in two purification grades i.e. desalted grade or HPLC grade, either as predesigned siRNA molecules or as custom synthesized 21 mer or 27 mer duplexes. For this work, siRNA was delivered as a ready to use HPLC grade duplex. The obtained siRNA was checked for purity by Nanodrop spectrophotometer.

UV absorbance ratio of A260/A280 and A260/A230 was also used to verify the purity of nucleic acids. A260/A280 and A260/A230 values about ~2 suggested good purity of siRNA. This siRNA was used to establish relationship between the results of Nanodrop and siRNA concentration. Once purity was confirmed, correlation curve was drawn by recording absorbance of siRNA solutions of different concentrations at 260 nm on a NanoDrop UV spectrophotometer. The results demonstrated acceptable linearity and reproducibility. The analytical method was validated to make sure good accuracy and precision as demanded for routine analysis.

Charge based migration of free siRNA on agarose gel by gel electrophoresis combined with sensitive densitometric detection system was used for siRNA quantification useful to determine siRNA complexation efficiencies. The parameters of gel electrophoresis such as agarose concentration, electrophoresis voltage, tank buffer were optimized to get sharp elution bands of siRNA. Calibration curve was plotted by

determining relative band densities for different concentrations of siRNA. Calibration curve was found to follow a linear equation y = 0.106x + 0.466 with correlation coefficient of 0.998. The % recovery for the method was 98.80 ±0.023 which was within the acceptable limits. The RSD values for all the densitometry analysis were less than 3.0%.

Chitosan is such natural polysaccharide for siRNA delivery applications. Chitosan based gene delivery has emerged as a safe and biocompatible carrier for gene therapy, though, the potential applications of chitosan are restricted due to low transfection efficiency. This has been ascribed to lack of colloidal stability at physiologic pH and premature dissociation of polyplexes. Thus, in order improve the colloidal stability, chitosan was quartenized (trimethylated) methyl iodide. Trimethylated chitosans with different degree of quaternization were synthesized and characterized. Furthermore, Trimethylated chitosan selected based on cytotoxicity was conjugated with different heterocyclic moieties containing imidazole, piperazine and pyridine ring for improving cell uptake and subsequent buffer capacity.

Trimethylation of the native chitosan improves the solubility of the polymer at the physiological pH and assist intracellular release of the DNA or siRNA at particular target and could be potential non viral vectors for the gene delivery. Supplementary, modification or conjugation of amines of the chitosan remains promising strategy to improve the transfection efficiency and intracellular release of the vectors. To enhance the chitosan buffering capacity in the range of endosomal pH and thereby so assisting the endosomal escape of the polymer complexes by the osmotic burst, heterocyclic compuonds with different pKa values (Urocanic acid, Piperazine 2-Carboxylic acid, 3-Pyridyl acetic acid) were conjugated through their –COOH (Carboxyl) groups onto remaining amines of trimethylated chitosan by amide linkage by known EDC/NHS Chemistry.

Trimethylated chitosan were synthesized with different degree of the quaternization (DOQ) by varying reaction time and varying concentration of the reactants. Chitosan with different DOQ were obtained were TMC12, TMC-23, TMC31 and TMC 39.All the synthesized trimethylated chitosan with different DOQ were characterized for NMR,

FTIR, cloud point and PH50 for solubility improvement at physiological pH by transmittance value, *in vitro* cytotoxicity assay.

NMR spectra confirmed characteristic peaks of the quartenized amino groups appeared at δ 2.8 ppm and 3.4 ppm for all the quartenized chitosans. In case of low degree quartenized chitosan peak intensity was low while higher degree quartenized chitosan demonstrated intense characteristic peaks. Further, The FTIR spectrum of the trimethylated chitosan gives the evidence for the occurrence of the methylation particularly in the region between 1,700–1,200 cm-1. Band centered at 1455-1475 cm^{-1} in the FTIR spectra of the trimethylated chitosan attributed to the C–H bonds of methyl group asymmetric angular deformation of the TMCs, which is absent in the Chitosan spectrum.

The Trimethylated chitosans (TMCs) were characterized for solubility profile using turbidimetry analysis to study the effect of quaternization on colloidal stability of trimethylated chitosans. With change in degree of quaternization the pH dependent solubility was changed which was detected by change in transmittance value by UV-Vis spectrophotometer. A correlation was observed for transmittance and pH for all TMCs. As expected the pH50 and cloud point increased with degree of quaternization due to increase in solubility. Solubility of chitosan polymer is a function of degree of polymerization. The Cloud point of TMC was 6.5 and with increase in quaternization, cloud point was increased upto 7.2. While, pH50 values were from 6.8 to 7.82. For chitosan, values of cloud point and pH 50 were 5.1 and 5.5.

TMCs were characterized for in vitro cytotoxicity study by MTT assay in A549 alveolar epithelial cells. TMC 12, TMC 23 and TMC 31 showed negligible toxicity i.e. > 90% after 24h at concentration of 1, 10, 100, 1000 µg/mL in A549 cells, while TMC 39 demonstrated the cell viability at concentrations of 100 and 1000 µg/mL was ~85% and 78 % after 24h. Which means that increased DOQ, increases the cytotoxicity. Hence, TMC 31 was found appropriate for desired vector development due to superior solubility, colloidal stability and greater cell viability and thus chosen for further experiments.

The synthesized TMC 31 was conjugated with different heterocyclic compounds such as Urocanic acid, Piperazine 2-Carboxylic acid, 3-Pyridyl acetic acid with an objective to improve the buffer capacity of the TMC based vectors of siRNA in the endosomal range and so to assist in intracellular release of siRNA and transfection of the cells. Modified TMCs were characterized for NMR, FTIR, TNBS assay, buffer capacity, % hemolysis, erythrocyte aggregation, In vitro cytotoxicity etc.

Heterocyclic compounds Conjugated TMCs demonstrated improved buffer capacity as imidazole, piperazine and pyridine rings having pka in the range of endosamal pH. Among the these modified TMCs, Urocanic acid conjugated TMC (TMC-UAA) showed higher buffering capacity as it is synergist the buffer capacity of the chitosan as pKa of imidazole ring of Urocanic acid is in range of 6.0-6.5 took part in the proton sponge effect and it will assist to release therapeutic genes into the cytoplasm by endosomal escape. Furthermore, developed heterocyclic compound conjugated trimethylated chitosan polymers shows negligible or no hemolytic potential at different conjugated levels. However, increase in concentration of the polymers, increase in hemolytic potential was observed from 0.5 % to 1.8 % hemolytic activity and exhibit no any types of aggregation or flocculation of the erythrocytes. These results prove the biocompatibility of the synthesized polymers to the cells. TMC- UA, TMC-PCA and TMC-PAA demonstrated cell viability of greater than 90% after 24h at each concentration in A549 cells at each conjugation levels.

PEI, especially high-molecular weight (25 kDa), is a well-known as good transfection reagent. PEI (25 kDa) has many advantages, but owing to high cytotoxicity and lack of biodegradability, the applications are limited as study tool for molecular biology. The cytotoxicity is due to very high charge density of the amines. In contrast, high transfection of PEI (25 kDa) is due to its flexible branching and amines ratio of 1^{o}:2^{o}:3^{o} of 1:2:1. In case of PEI, it has been proposed that cell uptake depends on presence of cationic charge at physiological pH forcing the cell-vector interaction. While, the endosomal escape depends on the presence of protonable amines (2^{o}& 3^{o} Amines), as required for buffer capacity against endosomal acidification from pH 7-5 (Proton Sponge Effect). This implies that polymer with pKa in range of 7-5 (for buffer capacity) and

sufficient charge density at physiological pH (for cell uptake) could be very good candidate. Unluckily, no such agent available for clinical applications with above property, which is non-toxic as well. In present work, we have conjugated Urocanic acid, which having imidazole ring, to PEI for reducing cytotoxicity and improving transfection efficiency. As, Imidazole ring having pKa in the range of physiological pH, would assist in endosomal uptake by improving buffer capacity.

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 the 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. Developed PEI-UA was characterized for NMR, FTIR, TNBS assay, buffer capacity, hemolytic activity, erythrocyte aggregation and *in vitro* cytotoxicity. From the NMR spectrum, 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 demonstrating successful conjugation of Urocanic acid on PEI.

Further, TNBS assay results 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 was 11.89 ±1.54%. Acid/base titration curve explained 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.

Modified PEI was characterized for hemolytic activity and erythrocyte aggregation. 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 1000 µg/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.

MTT assay showed that modified PEI 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 10 and 100 µg/mL concentration, respectively. Modified PEIs shows around 80-83% cell viability at 100 µg/mL concentration. As the concentration of conjugated PEI increases from the 1 µg/mL to 1000 µg/mL, there was decrease in the cell viability.

As complexation ability of the modified polymers, depends on the charge density which is function of the pH of vehicle used for formulation of polyplex. Hence, all the polyplexes formulations were prepared in sodium acetate buffer. Developed Polyplexes were characterized for complexation efficiency, heparin competition assay, particle size, zeta potential analysis, in vitro cell like studies like in vitro cell cytotoxicity, cellular uptake by Confocal microscopy and Flow Cytometry, stability challenge studies like electrolyte induced flocculation, serum stability, formulations in Bronchoalveolar lavage fluid and TEM microscopy etc. 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.

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 NanaoDrop UV spectroscopic estimation of siRNA complexation ability. All the synthesized TMC and PEI conjugated polymers were able to condense greater than 95% of siRNA at their optimized polymer to siRNA weight ratios. For different degree of conjugation of heterocyclic compounds, obtained polymer to siRNA weight ratios were different. As the degree of conjugation of modified TMCs and modified PEIs increased, the w/w ratio of for complexation increased. 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. In addition, when polyplex formulations compared with naked siRNA, it did not show any instability or degradation of siRNA incorporated into the formulations.

Developed polyplexes were characterized for particle size and zeta potential analysis. 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. 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 the shielding of primary amino groups which act as cationic sites on the polymers.

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 orientate to the surface of the polymers leading to positive charge shielding and hydrophilic elements. Average particle size of siRNA-TMC polyplex was 149.8± 1.7 nm while mean particle size of the modified TMC polyplexes was 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 for the developed polyplexes formulations were performed in A549 alveolar epithelial cells. 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. Polyplex formulations from different polymers at all concentrations exhibited their potential as a novel siRNA vector with improved safety margin.

Cellular uptake by confocal microscopy demonstrates that 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. 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 confocal microscopy that with increasing the degree of conjugation for each heterocyclic moiety, the cell uptake increased. 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.

Flow Cytometry analysis to quantify the cell uptake showed that naked siRNA demonstrated very less 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. 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 induced flocculation study showed that 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. siRC-TMC-UAA, siRNA-TMC-PCA and siRNA-TMC-PAA showed 1.36, 1.35 and 1.27 fold increased in particle size after sodium chloride incubation, respectively. While siRNA-PEI-UAA showed 1.34 fold increased in particle size of polyplexes.

Heparin competition assay was performed to assess the stability of polyplexes formulations on exposure to in vivo conditions where polyplexes are face polyanions. Results demonstrated that 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. 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/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.

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 results for stability of polyplexes formulations demonstrated that polyplexes were able to protect BDNF siRNA from serum nuclease mediated degradation. Experiment findings 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.

Polyplexes formulations stability in present of bronchoalveolar lavage fluid was assessed by agarose gel electrophoresis. 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.TEM studies demonstrate that modified TMC and modified PEI based polyplexes showed that polyplexes were spherical in shape and condensed due to strong electrostatic interaction. TEM images show particle size of polyplexes below 250 nm without sign of aggregation confirming the homogenous systems.

Nebulization is a promising approach to deliver gene or siRNA directly to the affected airway epithelial cells in the patients with asthma and COPD. 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 maintain their stability and biophysical properties, protecting the siRNA from the shear forces during nebulization so that its biological efficacy is preserved. Hence, polyplexes formulations were formulated as nebulized formulations in buffer solution. Nebulization of the formulations effectively generates aerosols for pulmonary administration. 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. Further, formulations were assessed for in vitro aerodynamic performance by Twin stage Impinger apparatus.

Nebulized polyplex formulations were characterized for their aerosolize performance and Fine particle fraction (FPF), Emitted dose, MMAD, and geometric standard deviation (GSD). 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. All the developed polyplexes formulations demonstrated emitted dose in the range of 84% to 90 %, mass median aerodynamic diameter less than 5 µm, geometric standard deviation around 2.0-2.4 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. 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 has MMAD less than 6.4 μm and therefore expected to reach the peripheral lung. These findings propose that polyplexes formulations are suitable for delivery to lower airway tract as well. Additionally, polyplex formulations were characterized for siRNA integrity, cellular uptake, particle size, zeta potential after nebulization. Association with the polymer complexes has ensured siRNA integrity is maintained during nebulization. Polyplexes show remarkable cellular uptake even after nebulization demonstrating formulation stability and maintenance of the physicochemical properties against the shear force of the nebulization process. 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.

Efficacy of the polyplex formulations were studied *in vivo* by Ovalbumin induced inflammatory mice model. The OVA-challenged asthma mice model is the industry gold standard model used to assess pulmonary inflammation. Inhalation of ovalbumin has been having been confirmed to induce an immune response associated with increased airway hyper-responsiveness (AHR). In addition, this model also demonstrates a number of other features characteristic of human asthma covering, cellular infiltration into the lungs, increase in cell counts, and elevated levels of inflammatory cytokines in bronchoalveolar lavage fluid.

BDNF expression was estimated by RT-PCR in isolated lungs from mice of normal control, positive control, and from treatment groups. Levels of BDNF mRNA were also measured in mice lungs in all the groups on day 42nd after ovalbumin administration. Results of the *in vivo* mRNA knock down efficiency of the siRNA demonstrated that BDNF siRNA efficiently down regulates the BDNF mRNA. Polyplexes formulations administered animal groups exhibited gene silencing up to 64 %

in vivo. Modified TMC and Modified PEI based polyplexes showed higher gene silencing compared to the polyplexes prepared from the native polymers.

Results of the BDNF mRNA expression showed that modified TMC based polyplexes showed 51 % to 57 % gene knockdown efficiency. While, Only TMC based polyplexes demonstrated about 46 % gene silencing. On the other hand, PEI based and PEI-UAA polyplexes showed 60% and 64% gene silencing efficiency. From the results, it can be said that developed novel cationic polymers based non viral vectors based siRNA systems efficient gene knock down activity which show very promising potential as a vectors for siRNA therapeutics for the treatment of pulmonary inflammatory conditions.

Bronchoalveolar fluid examination shows picture of the airway inflammation as in the inflammatory conditions increased in the cells, cellular infiltrates in the lung, release of the pro-inflammatory mediators like cytokines and interleukins. Analysis of the inflammatory cells in the BAL fluid samples showed that total cell counts were significantly enhanced by ovalbumin sensitization in the mice model. However, total cell counts were decreased by siRNA polyplexes formulations treatment. Particularly, the eosinophils counts were elevated in the Ovalbumin challenged mice than in the control animal group and lower in the polyplex formulations treated animals than in the Ovalbumin challenged group of animals.

Supplementary, the findings of the histopathological examination showed that less or no inflammation was present in the lungs of the mice in the saline control group. In opposition, the lungs of the mice in the Ovalbumin challenged animals (Positive control) showed inflammation of epithelium remarkably as well as extensive cellular infiltration. Nevertheless, the lungs of the mice in the formulations treated groups of animals displayed considerably better lung pathology in comparison with those of the mice in the Ovalbumin challenged animals demonstrating potential of gene silencing RNA therapeutics for the treatment of obstructive airway conditions.

The results demonstrate that polymeric vectors with useful characteristics for nucleic acid delivery such as adequate binding affinity, lower cytotoxicity, endosomal escape capacity can be derived by modifications or conjugation so as to balance individual attributes of ionization, hydrophilicity, buffer capacity, colloidal stability. The

approaches for reduction in cytotoxicity and improvement in efficiency of cationic carrier such as bPEI and to increase efficiency of biocompatible vectors such as chitosan is much investigated research endeavors. The present research work provides a feasible solution to achieve the same. The positive outcomes of *in vitro* and *in vivo* studies (cell uptake and gene expression) prove the potential for effectiveness of the vectors in airway inflammation and obstructive airway disorders. It also provides proof of concept for feasibility of convenient way of delivery of siRNA to lung in the form of nebulization formulation. This bodes for further potential improvements in preclinical and clinical efficacy.