Chapter 8:

Summary and Conclusion

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Mounting environmental pollution, temperature changes, exposure to allergens, smoking habits, and continual respiratory infections have turned out to be leading causes for respiratory disorders such as chronic obstructive pulmonary disease, asthma, emphysema, and cystic fibrosis, chronic pulmonary infections and lung cancer. Among these, Cystic Fibrosis (CF) is one of the most common life-shortening, chronic disorder that even has a hereditary trait and gets worsened due to involvement of above mentioned etiological factors. This genetic disorder is characterized by its autosomal recessive nature, having defect in a single gene located on chromosome 7 which encodes for Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). The current average life expectancy of patient is about 35 to 40 years. In this context, the identification of novel therapeutic strategy to treat cystic fibrosis is highly pursued contemporary goal.

The disease affects organs comprising secretory epithelial cells, most predominantly the lungs. In normal lung the volume of the surface fluid covering the airways, required for effective muco-ciliary clearance, is maintained through a fine balance between ion and water secretion and absorption. In particular, Na^+ absorption through the epithelial Na^+ channel (ENaC), and Cl- secretion through the CFTR play major role (2). In CF, the mutations in the CFTR Cl- channel results in reduced Cl- secretion and Na^+ hyperabsorption due to which sodium ions and water accumulate within epithelial cells, leading to depletion of airway surface fluid (volume), consequently airways become obstructed by thick mucus, becoming inflamed, and gets frequently infected. Terminally, the lungs get colonized by opportunistic pathogens causing inflammation, leading to development of respiratory insufficiency and death. Traditionally the goal of therapy in CF is to reduce the signs and symptoms of airway infection, inflammation, and obstruction. Thus, antibiotic, anti-inflammatory, bronchodilator and mucolytic therapies are geared to treat the complications that compromise lung function. However; none of these therapies can cure the disease. Until recently, only few of the affected individuals' survived childhood due to lack of permanent therapy for CF lung disease.

Influenced by the revolutionary prospects offered by gene therapy in treatment of genetic diseases, a lot of initial work was directed towards restoration of Cl- transport by

administration of healthy CFTR gene to lung epithelial cells through viral and non-viral vectors. However; during 1993-2004, 29 clinical trial protocols were published, and all demonstrated a limited success. The main reason being that, ciliated lung epithelial cells are terminally differentiated and do not divide, which means that the nuclear membrane remains intact throughout the life of the cells, restricting the entry of endocytosed vectors from cytoplasm to nucleus (4). While the reported level of expression was very low (<10%), and were found sufficient to normalize Cl- flux, none was able to induce a correction to the Na^+ hyper-absorption present in CF. The Na^+ hyper-absorption is fundamental mediator of CF pathogenesis and must be normalized to get complete recovery. Further, it was reported that to normalize Na^+ hyper-absorption through CFTR activity, almost 100% of cells are required to express Cl-. As an alternative attempts were made by using Na^+ channel blockers (amiloride), which showed promising results to restore airway hydration and to restore the normal phenotype. However, the very short duration of effect due to rapid elimination and systemic side effects on kidneys were noted. Therefore, advanced therapeutic interventions directed to inhibit Na^+ hyper-absorption is presently the major focus of CF research.

In recent years, RNA interference has been identified as a powerful molecular process to downregulate gene expression by directing the endogenous RNAi machinery to carry out mRNA degradation. The siRNA is emerging as a promising tool because of various advantages. It provides a wide choice of targets based on Watson-Crick base paring interaction. The long duration of target suppression can decrease dosed frequency. Unlike gene therapy, no need to cross the nuclear barrier, since cytoplasm is the site of action, resulting into assured clinical response on successful transfection. Moreover, if combined with high potency and localized delivery of siRNA, the systemic exposure is minimal in contrast to small molecule inhibitors. It provides high selectivity for target and non-targets can give better therapeutic index and significantly improved quality of therapy than small molecule inhibitors.

In this study we have proposed delivery of siRNA through inhalation route to downregulate Na^+ channel. There are reports supporting the potential of siRNA against Na^+ channel to restore the phenotype in CF. They have very long duration, dependent on the life span of the transfected cells, i.e. 120 days of lung epithelial cells, which can

overcome the limitations of Amiloride therapy. Therapeutic siRNA has been tried in clinical trials in various other diseases and lung cancers which proves their clinical application. However; in case of lung diseases like CF no clinical trials with siRNA have started yet. The potential reason restricting the clinical application is the unavailability of the suitable local administration technique, preferably inhalational route. The inhalational route is highly convenient and patient compliant and it allows one to widely distribute the therapeutics agent along the airways. Further, it gives localized action and reduces the systematic side-effects.

The naked siRNA is prone to degradation by nucleases. Additionally; due to its large molecular weight and anionic nature, they cannot cross the cell membrane. Therefore, cationic polymers or lipids have been used as carriers which interact with negatively charged siRNA leading to the spontaneous formation of nanosized complexes which can interact with cell membrane and undergo internalization. The compact structure prevents the access of nucleases to the enclosed siRNA, thereby significantly improving the stability. Although; lipid and polymer based vectors can be used for complex formation but in context of the lung delivery it has been observed that lipid based vector experience strong interference from lung surfactants which makes cationic polymers as preferred agent for lung delivery. However, once endocytosed across the cell membrane, the polyplexes should escape from the endosomes to prevent 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 desired 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, PEI, especially high-molecular weight (25 kDa), is a well-known as good transfection reagent. PEI (25 kDa) has many advantages, but due to high toxicity and lack of biodegradability, the applications are limited as study tool for molecular biology. The toxicity is due to very high charge density. In contrast, high transfection of PEI (25 kDa) is due to its flexible branching and amino group 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 positive 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. Unfortunately, no such agent available for clinical applications with above property, which is non-toxic as well.

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 study, siRNA was delivered as a ready to use HPLC grade duplex with 1 mL 5x siMAX^{TM} buffer (30 mM HEPES, 100 mM KCl, 1mM $MgCl_2$, pH= 7.3). The obtained siRNA was checked for molecular weight and purity using MALDI-TOF and capillary gel electrophoresis.

UV absorbance ratio of A260/A280 and A260/A230 was also used to confirm the purity of nucleic acid. A260/A280 and A260/A230 values around ~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 plotted by recording absorbance of siRNA solutions of different concentrations at 260 nm on a NanoDrop UV spectrophotometer. The results showed acceptable linearity and reproducibility. The analytical method was validated to ensure good accuracy and precision as demanded for routine analysis.

Charge based migration of free siRNA on agarose gel using gel electrophoresis combined with sensitive densitometric detection system was used for quantification of siRNA useful in siRNA complexation efficiencies. The parameters of gel electrophoresis such as agarose concentration, electrophoresis voltage, tank buffer were optimized to obtain sharp elution bands of siRNA. Calibration curve was generated by determining relative band densities for different concentrations of siRNA. Curve was found to follow a linear equation $y = 0.0103x + 0.028$ with correlation coefficient of 0.99. The % recovery for the method was 98.20 ±0.014 and % bias was -1.8%, which was within the acceptable limits. The RSD values for all the densitometry analysis were < 3.0%.

The linear PEI (LPEI) suffers from drawback of high charge dependent toxicity, poor buffer capacity and transfection efficiency. The LPEI is solely composed of

secondary amines, which is reported to be > 50% ionized at physiologic pH. In order to develop LPEI based non-viral vector, LPEI was modified so as to influence its ionization behavior, hydrophilicity and toxicity. Therefore, secondary amines of LPEI were subjected to chemical modification to obtain hydroxyethyl substituted LPEI (HELPEIs) having different degrees of substitution (DS) on LPEI backbone. Briefly, LPEI was reacted with 2-bromoethanol at varying molar ratios to amine content in LPEI under reflux for 48 h using potassium carbonate as proton abstractor. The product obtained was reconstituted in double distilled water and dialyzed against double distilled water to remove salts and other low molecular impurities. The retentate of the dialysis were lyophilized to obtain dry residue of HELPEIs.

The final product was confirmed by FT-IR, ^{1}HNMR. The DS was determined by chemical shift integrations in NMR spectra. The HELPEI-15, HELPEI-25, HELPEI-35, HELPEI-45 were prepared having 15, 25, 35, 45 % DS, respectively. First, the modification of LPEI led to favorable effects on the hydrophobicity and solubility of the polymer. Unionized LPEI is insoluble in water at temperatures below 55^{o}C. The improvement in solubility was quantified using turbidimetry measured by transmittance of solution at different pH. In case of LPEI we observed drop in transmittance of LPEI at pH values greater than 8.0 due to its insoluble nature. Surprisingly, HELPEI with DS of 25% and above showed transmittance values > 94 % at all pH values and thus overcome pH dependent solubility of LPEI. This means that hydroxyethyl group introduces hydrophilicity to polymer backbone which avoids precipitation of LPEI even at higher pH where it is in unionized form, i.e. protonation of amine is no longer required, as in case of LPEI, to keep HELPEI dissolved.

In order to assess the quantitative effect of modification of LPEI on protonation behavior, the titration curves were obtained against volume of 0.1N HCl consumed from pH 10 to 3.5. The region 10 to 7.4 should be proportional to the number of protonated amines at physiologic pH. The greater protonation at physiologic pH results in charge dependent toxicity, therefore, consumption of more amount of HCl by LPEI makes it more cationic at physiologic pH and consequently more toxic in nature. The comparison of ratio of protons consumed in pH range of 7.4-5.1 (endosomal pH) to the protons consumed in pH range of 10-7.4, showed an increasing trend with increasing DS. The

polymer which consumes more protons in endosomal pH range after modification than at 10-7.4 would be less protonated at physiologic pH and would be efficient in endosomal escape. Finally, the buffer capacity calculations showed that, the buffer capacity of LPEI was lowest i.e. 10.32, which increased with increasing DS and it even crossed buffer capacity of bPEI.

The n/p ratios for complete retardation in gel electrophoresis were determined for polyplexes prepared. Both the LPEI and HELPEIs were able to condense more than 95% of siRNA at their optimized n/p ratios. However, n/p ratio were different for HELPEIs with different DS. HELPEIs showed a steady increase in n/p ratio required for complete retardation with increase in DS. Thus, with increase in DS the binding affinity of HELPEI to siRNA is reduced. The effect could be attributed to the reduction in charge density after substitution. However, as the DS is increased from 35% to 45% there was an anomalous increase in n/p required for complete retardation, with almost doubled amount of polymer required. This indicates that the charge reduction was non-linear with DS beyond certain point. This deviation could be attributed to the fact that with increase in DS, a significant suppression of protonation behavior of remaining nitrogen occurs.

The N/P was also optimized considering the size of polyplexes. At N/P showing complete gel retardation the polyplexes showed higher size, however, when N/P was increased the size was reduced. The size of the polyplexes formed from HELPEIs with lower DS were comparatively smaller than with higher DS at the given n/p ratio, showing that there is decrease in the affinity of polymer with higher DS. However, as the DS increased the zeta potential was found to decrease. The integrity of siRNA after complexation with polymers was studied using gel retardation and was found to retain as that prior to complexation.

The polymers were subjected to biocompatibility studies using hemolysis, erythrocyte aggregation and cell viability assay. In the present case, LPEI caused extensive hemolysis (> 10%) at concentration ranging from 10 to 2000 ug/mL. However, the HELPEI-15 to HELPEI-45 showed significant decrease in hemolysis. This can be attributed to combined effect of increase in hydrophilicity by incorporation of hydroxyethyl substituents, and decrease in charge density due to conversion of secondary amine into tertiary amine. Further, the HELPEI-15 and HELPEI-25 with degree of

substitution less than 25% caused considerable hemolysis meaning that level of substitution was not sufficient to bring influence biocompatibility of polymer. In continuation of same, HELPEI-35 and HELPEI-45 with higher DS were completely non-toxic, which caused < 3% hemolysis at all the concentrations. Erythrocyte aggregation was analyzed through microscopic observations. It showed that LPEI induced significant amount of aggregation at all the concentration selected. In case of HELPEIs the erythrocyte aggregation was found to be reduced with increase in DS.

Finally, the cytotoxicity of polymers was studied in CFBE 41-o cells by MTT assay and it was found that, LPEI was very toxic and it reduced the cell viability to 15 % at 80 µg/mL and was less than 55% at all the concentrations tested. In case of HELPEIs, it was observed that the cytotoxicity decreased with increase in DS. At DS of 15% and 25% there was significant toxicity meaning that the charge density was still above the minimum toxic levels. The HELPEI-35 and HELPEI-45 showed significant improvement in cell viability compared to all other polymers with almost ~90% and still higher at all concentrations tested.

Further, the polyplexes stability was evaluated by resistance in different stability challenge studies. The salt induced aggregation was performed to study the balance of factors influencing colloidal stability of polyplexes. It revealed that stability of LPEI was could be governed by the positive surface charge and balance between attractive and repulsive force, which resulted in a behavior similar to lyophobic colloid under salt addition. The addition of counter-ions from salt neutralize those charges leading to particle aggregation and colloidal system becomes unstable. LPEI showed a dramatic ~1.76 fold increase in size after salt additions. However, the HELPEI polyplexes retained size to considerable extent. HELPEI derived significant portion of its stability from the hydrophilic hydroxyethyl chains, rather than ionized functional groups, which are not affected by salt addition. The behavior becomes similar to that of lyophilic colloids, which derive stability from hydrophilic substituents and are less sensitive to salt additions.

Stability challenge with heparin was performed to study the resistance to displacement of siRNA by poly-anions *in vivo*. The resistance to displacement up to heparin/siRNA weight ratio of >1 is considered appropriate for achieving adequate *in*

vivo stability. LPEI formulations showed displacement from w/w ratio of 2. HELPEI-35 started releasing siRNA from heparin/siRNA weight ratio of 1.5 and completely released at 3. HELPEI-45 started releasing siRNA from heparin to siRNA weight ratio of 1.0 and completely released at 2. Thus HELPEI-35 was having higher resistance to heparin displacement than HELPEI-45. This can be attributed to low charge density of HELPEI-45 than HELPEI-35. The serum stability studies were performed to study the protection from nucleases. Both the PEI based formulations showed good stability in serum challenge study. The HELPEI-35 showed excellent stability at the optimized n/p ratio which was evidenced as absence of degraded siRNA in gel electrophoresis. HELPEI-45 showed less retention of intact siRNA as compared to HELPEI-35 though it retained more than 80% of siRNA. This indicates that the association between siRNA and HELPEI-45 is weaker than that in the HELPEI-35.

The morphology of the HELPEI-35 and HELPEI-45 polyplexes was observed through TEM. The TEM images showed that polyplexes were spherical and compact which is the result of strong electrostatic interaction. They were discrete with no visible evidence of aggregation, proving the homogenous state of system.

Therefore, based on the results of size, zeta, complexaton efficiency, biocompatibility and stability challenge studies. It was observed that HELPEI-35 and HELPEI-45 were equally non-toxic and had higher buffer capacity and colloidal stability. However, the results of size showed that HELPEI-45 forms higher size polyplex due to lower charge density. At the same time the zeta potential values were less than of HELPEI-35. Finally, the complex formed with HELPEI-45 showed lower resistance to salt and heparin challenge studies. Therefore, HELPEI-35 was considered a better vector for siRNA delivery.

The optimized polyplexes were also evaluated *in vitro* cell line studies. The cytotoxicity results of polyplex showed that, at the optimized composition they were non-toxic in delivering increasing siRNA dose. It was observed that there was no significant difference in % cell viability for naked siRNA, HELPEI-35, HELPEI-45 formulations at increasing dose of siRNA when compared with untreated control ($P > 0.05$).

The cell uptake potential was studied through confocal microscopy and FACS. Naked siRNA showed negligible cellular uptake, while LPEI polyplexes showed marked

cellular uptake. LPEIs association with the cells had a typical pattern, in which small particles were observed bound to the cell membrane high-lighting the periphery of the cell. The cell association was also in the form of few large aggregates. The HELPEI-35 and HELPEI-45 showed higher uptake compared to both naked siRNA and LPEI. Both the formulations were able to form stable formulations. In contrast to LPEI, the fluorescence in confocal images of both HELPEIs was throughout the cytoplasm, which is a good indicator of cytoplasmic intake of polyplexes. The results of FACS corroborate the qualitative observations of confocal microscopy. Thus, based on FACS data the order of cell uptake could be stated as:

HELPEI-35 > HEPEI-45 > L2KL > LPEI > Naked siRNA

When comparing the cell uptake of HEPEI-35 and HELPEI-45, the cell uptake in case of HELPEI-35 was higher than that of HELPEI-45 ($p < 0.05$). This means that increase in substitution beyond certain point does not lead to increase in the cell interaction and cell uptake.

The polyplexes were also evaluated for gene knockdown efficacy. With the optimized culture conditions, the CFBE 41o- cells were transfected with siRNA concentration at 25 nM, 50 nM and 100 nM. As the ENaC gene knockdown was dependent on the concentration of siRNA and highest knockdown was observed at highest concentration of siRNA. The HELPEI formulations showed significant mRNA knockdown and it was also higher than the LPEI. RT-PCR study revealed that the buffer capacity present in HELPEI-35 was sufficient to show comparable gene knockdown. Further, it was expected that the unloading characteristic of the HELPEI may differ due to differences in charge density. However, as HELPEI-45 used at higher n/p ratio might have resulted in similar binding affinity between siRNA and polymer. Although HELPEI are non-toxic, they contain the PEI backbone which is non-biodegradable, therefore, it is always preferred to reduce the polymer load in to the tissues. Thus, HELPEI-35 turns out to be a better vector compared to HELPEI-45 also due to its higher transfection efficiency on mass basis.

Chitosan is another such polymer for siRNA delivery applications. Chitosan based non-viral vectors have emerged as a safe and biocompatible carrier for gene therapy, however, the potential applications are limited due to low transfection efficiency. This

has been attributed to lack of colloidal stability at physiologic pH and premature dissociation of polyplexes. Therefore, in order improve the colloidal stability, chitosan was depolymerized using nitrous acid. The concentration of nitrous acid with respect to glucosamine was optimized to achieve desired viscosity-average molecular weight (Mv) of 30, 60, 90 kDa. The product was isolated by freeze-drying and then subjected to characterization. The depolymerized chitosan was characterized for Mv by using Mark–Houwink's equation for determination of intrinsic viscosity.

The effect of depolymerization on pH solubility profile was studied by turbidimetry. pH_{50} and cloud point increased with decrease in Mv due to increase in solubility resulting from reduced inter-chain interactions between short chains of LMWCs. The objective was to obtain LMWCs with good colloidal stability around physiologic pH. From the transmittance values it was observed that only LMWC-29 had good solubility near physiologic pH. The cloud point was 7.2 while the pH_{50} was well above 8.0. Therefore, only LMWC-29 was found appropriate for desired vector development.

Though, depolymerization of chitosan resulted in improved colloidal stability with good buffer capacity, it reduces its affinity for nucleic acid and fails to protect them from degradation by nucleases and serum components leading to poor transfection efficiency. Incorporation of a binding partner for negatively charged siRNA may overcome this problem. Therefore, here we combined the nucleic acid binding property of protamine (PS) with buffer capacity of colloidally stable LMWC-29. LMWC-PS conjugates were prepared through EDC-NHS carbodimide chemistry.

For determination of conjugation efficiency, Sakaguche reaction was used. The physical interference of chitosan during the reaction can be removed by depolymerizing it using sodium nitrite at the beginning of the sample preparation. The gel retardation of MMWC showed retardation at weight ratio of 4 and more. On the other hand, the LMWC-29 was able to condense the siRNA at weight ratios of 28. However, PS also showed very good binding affinity due to high charge density. It was able to retard siRNA at polymer/siRNA weight ratio of 1.5. The conjugates prepared with different degree of conjugation from 5, 12 and 17% PS on weight basis. The conjugate containing 5% PS was unable to retard the siRNA and required 24 weight ratio for complete

retardation. While conjugates containing 12 and 17% protamine were able to retard siRNA at 16 and 10 weight ratio respectively. Thus, the conjugation of protamine with LMWC was able to overcome the low complexation capacity of LMWC. The complexation efficiency was more than 93% in all the cases and was acceptable.

The polyplexes were evaluate for particle size and zeta potential. The MMWC formed very large size polyplex due to long linear chains. PS forms much smaller polyplexes. LMWC-29 also forms comparatively larger polyplexes. The conjugates showed size comparable to LMWCs. As the chitosan is unionized at 7.4 pH the MMWC and LMWC-29 showed very low values of zeta potential. The conjugates also showed higher zeta potential as they contain PS fraction which was around +14.33 mV for LMWC-29-PS-12 and +15.28 mV for LMWC-29-PS-17.

All the chitosan based polymer and conjugate were subjected to cytotoxicity evaluation using MTT assay. All showed negligible toxicity i.e. > 90%, in CFBE1o- cells after 24 h at all tested concentration. In case of PS the cell viability was ~70% after 24 h. Which means that PS as such is toxic to the cells, however; when used in the form of conjugate the toxicity is significantly reduced as only small amounts are associated with LMWC-29. Thus, it was observed that conjugate displayed better physicochemical properties compared to MMWC, LMWC-29 and PS alone. There wasn't significant benefit of increasing PS from 10 to 15% in complexation, size and zeta potential. Therefore, 12% conjugation was concluded to be optimum for carrier properties.

The higher charge density of PS than chitosan and its ionized state relative to chitosan which is unionized at 7.4 pH can be used to induce binding of siRNA preferentially to PS fraction, and leaving negligible portion on the chitosan. This can also overcome the pH dependent binding affinity of LMWC, and may overcome premature release of siRNA at physiologic pH conditions. Therefore, during polyplex preparation, siRNA addition and incubation with polymer solution was performed at 7.4 pH where only PS was ionized and then pH was reduced to 5.5. The siRNA bound to LMWC can be released by exposure to $NaNO_2$ but not the siRNA bound to PS. The $NaNO_2$ treatment could not release the siRNA from the LMWC-29-PS-12 conjugate, indicating that siRNA was bound to PS fraction of conjugate.

The stabilized polyplexes of LMWC-29-PS-12 and siRNA was cross-linked with TPP to reduce size and make polyplex rigid. TPP did not negatively affect the binding affinity of siRNA to cationic polymer. In presence of TPP also, the complete retardation was observed at optimized weight ratio. Further, the polyplexes prepared were evaluated for entrapment efficiency (97.67±1.55), size (143.7±4.56) and zeta potential (+12.8±1.38).

The improvement in stability was studied through stability challenge studies. The heparin challenge study revealed that, LMWC-29 formulation showed displacement from heparin/siRNA weight ratio of 0.5. Finally, 100% dissociated at weight ratio of 1.5-2. Thus, LMWC-29 had low resistance to heparin competition due to lower affinity. The LMWC-29-PS-12 also showed improved resistance to heparin competition as it started to displace siRNA from weight ratio of 1, while complete release occurred at weight ratio of 2. However, TPP cross-linked LMWC-29-PS-12 polyplex showed further increase in resistance, as it started to release siRNA from weight ratio of 1.5, while complete displacement occurred at weight ratio of 2-3.

Similarly, serum stability studies showed that the formulations were able to retain more than 80% of siRNA up to 24h, specifically the TPP cross-linked LMWC-29-PS-12 polyplex had > 90% retained siRNA**,** while LMWC-29-PS-12 polyplex retained about 77% of the siRNA after 24 h. The naked siRNA was degraded in 6h. This shows that TPP cross linked LMWC-29-PS-12 polyplex showed greater nuclease protection of siRNA in serum than LMWC-29-PS-12 and the protection was very significant compared to naked siRNA. Thus, as observed in the heparin resistance study the stabilization through TPP cross-linking led to improved serum stability.

Chitosan, owing to its pKa in the range of 6.5-6.8, harbours high buffer capacity in the endosomal pH range. The proton sponge study showed that, LMWC-29-PS-12 has very good buffer capacity. This was due the presence of LMWC-29. Further, TPP crosslinking had no effect on buffer capacity. However, PS alone shows negligible proton sponge being peptide rich in arginine amino groups that are all protonated at endosomal pH leading to poor transfection. Thus, conjugation also overcomes the limitations of PS as a delivery vector. TPP cross-linked LMWC-29-PS-12 polyplex were subjected to TEM evaluation. The TEM images revealed the relatively homogenous and spherical shape of

the particles. Thus stabilized or TPP cross-linked LMWC-29-PS-12 polyplexes were considered optimized LMWC-29-PS-12 polyplexes for further evaluation.

The polyplexes were evaluated for efficacy in *in vitro* cell line studies. The confocal microscopy was performed to study cell uptake. The cells exposed to unformulated FAM-NC-siRNA showed negligible cell uptake, neither the non-specific binding to cells. However, in case of MMWC, the fluorescence was observed around the cell membranes. It could be attributed to self-aggregation and precipitation in the culture conditions, as observed in solubility studies. Similarly, LMWC-29 showed less fluorescence in confocal images. This may be due to unionized nature of chitosan even after improved colloidal stability, which makes it difficult to interact with cell membrane. The stabilized LMWC-29-PS-12 polyplexes showed significant cell uptake compared to MMWC and LMWC-29. PS was also used as control for proposed modification. It showed significant cell uptake, comparable to conjugate. It could be due to the formation of stable polyplex with siRNA and positive surface charge. L2K, as positive control, showed comparable uptake to that of LMWC-29-PS-12 conjugate.

To more precisely understand the cell internalization event, we quantified the internalized FAM-NC-siRNA using FACS analysis. The naked siRNA used as control showed negligible levels of uptake (<14%) while formulations led to higher uptake inside cells. Both the MMWC and LMWC-29 polyplexes showed cell uptake of ~45% and 36% respectively at the optimized weight ratios. LMWC-29 polyplexes showed the least level of cell uptake, indicating that apart from physical stability the unionized nature of chitosan would be the limiting factor for low transfection with chitosan based systems. In contrast, polyplexes of PS showed cell uptake (~63%) greater than of MMWC and LMWC-29. This could be due to stability of complex, small size and presence of positive surface charge were sufficient to interact with the cell and undergo internalization. Finally, among all the formulations TPP cross-linked LMWC-29-PS-12 polyplex**,** which had combined physicochemical attributes of LMWC and PS, showed cell uptake (~70%) comparable to that of transfection standard, L2K (~68%). The results of FACS were in full agreement with the confocal laser scanning study.

Based on the results of cytotoxicity, confocal, FACS the formulations were subjected to RT-PCR analysis. The mRNA expression levels were estimated as % of

ENaC-mRNA expression of untreated control. The NC-siRNA loaded polyplex did not cause knockdown of ENaC mRNA, which confirmed the sequence specificity of siRNA to inhibit ENaCα. While comparing remaining formulations, at each siRNA concentration tested, the maximum activity was attained at highest concentration of siRNA treated. At the highest dose, naked siRNA reduced mRNA expression at very low extent (~14%) and commercial transfection standard L2K showed significant mRNA knockdown of 44.46 % at the highest dose. LMWC-29 polyplexes showed very less mRNA knockdown, which could be due to lack of cell interaction as observed in FACS studies. However, in case of PS polyplexes, in spite of showing greater uptake in FACS studies, the subsequent gene expression was very less i.e. ~67% at the highest dose. This could be attributed to the lack of endosomal escape capacity in PS-siRNA complex, as observed in poly-L-lysine, poly-l-arginine based vectors. The stabilized LMWC-29-PS-12 polyplexes showed significant mRNA knockdown of 42.63%**.** They were able to interact with the cell surface, due to presence of PS fraction, and undergo cell internalization through endocytosis. Further, subsequent to endocytosis, the associated LMWC fraction contributes to endosomal escape and cytosolic release of siRNA from the formulation.

The siRNA polyplex were prepared and dispersed in nuclease free water to which different cryo-protectant (mannitol, trehalose, sucrose) were added and filled in glass vial. The vials were partially closed with double slotted grey bromo butyl rubber stopper and lyophilized. After lyophilization, the formulations were tested for particle size, zeta potential and physical appearance on hydration of cake. It was observed that, lyo-cycle selected resulted in aesthetic appearance of cake with good redispersibility. At the end of the cycle the water content was < 2% w/w in all the cases. Although all the cryoprotectants were successfully lyophilized to yield an elegant dry cake, there were differences in physical nature of cake. The cake from sucrose showed shrinkage. On the other hand, trehalose and mannitol behaved equally in terms of cake formation, but the mannitol was superior to trehalose. The cake formed by mannitol was more homogenous and porous in nature. Therefore, it was expected that mannitol would show better product characteristics. Further, mannitol is known as osmogen and could help in reducing viscosity of mucous secretions through transfer of water to airways.

Therefore, lyophilized formulation containing mannitol was subjected to powder processing using our previously developed techniques. The lyophilized bulk was passed through 120# and 240# to convert it to fine size. The particle size was evaluated over Malvern Mastersizer 2000, Malvern UK. The obtained fine powder was blended with different inhalational carriers such as inhalac 230 and respitose SV001 at different weight ratios of lyophilized bulk/carrier, ranging from 1/1 to 1/6 to improve the dispersibility of the powder during inhalation process. The dry powder formulation was filled in capsule size 3 and subjected to evaluation of aerodynamic characteristics using Anderson Cascade Impactor. The FPF and consequently the MMAD observation revealed differences in inter-particulate forces when using two different carriers. Inhalac 230 always led to higher FPF than the respitose e.g. 37.48 ±3.82% and 28.10 ± 1.12 respectively at carrier mass ratio of 1:6. This clearly, indicated the obvious choice of inhalac 230 as carrier for present formulation. Further, in case of inhalac 230 there was no significant difference in FPF at carrier mass ratio of 1:5 and 1:6, therefore, 1:5 carrier mass ratio was considered optimal for the developed formulation. The developed DPI formulations of polyplex retained the integrity of the siRNA after the lyophilization and powder processing.

The in vivo safety was tested in female Sprague–Dawley rats. The animals were dosed intra-tracheally with saline, LPS, LPEI formulation, HELPEI-35 formulation, LMWC-29-PS-12 formulation. All animals were found healthy and no mortality was observed, neither any sign of clinical toxicity was observed. The weight of wet lungs in formulation treated animals was similar to saline treated group. The L/B ratio for LPEI was 0.65, indicating lung injury or edema formation, while for HELPEI-35, it was 0.53 with no significant difference from saline control.

The enzymatic activities of LDH and ALP in BAL collected from the treated animals were in accordance with L/B data. The levels of ALP and LDH in LPS-treated animals were ~2.6 fold higher than that of saline control. For LPEI also, the activity of LDH and ALP in BALF was significantly different from that of the control ($p<0.05$), while for HELPEI-35 and LMWC-29-PS-12 there was no significant difference ($p>0.05$). Histopathological analyses performed to evaluate the toxicity of particles yielded results

that were in good agreement with those obtained by BAL analysis. A high level of inflammation was observed with LPS as expected, it shows bronchial epithelial with infiltrated leucocyte migration, epithelium shows degeneration with necrosis and exudation. On the other hand saline treated samples showed no inflammatory infiltration. The LPEI formulation showed comparatively more inflammatory cell migration while HELPEI-35 and LMWC-29-PS-12 were similar to control. Finally, the stability studies showed that the critical physicochemical parameters such as particle size, complexation efficiency, water content were maintained during stability. The formulations can be proposed for long term at refrigerated conditions in dry state.

The results show that polymeric vector with useful properties for nucleic acid delivery such as adequate binding affinity, low toxicity, 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 toxicity and improvement in efficiency of cationic carrier such as LPEI and to increase efficiency of biocompatible carriers such as chitosan is much investigated research endeavours. The present research provides a feasible solution to achieve the same. The positive outcomes of *in vitro* studies (cell uptake and gene expression) prove the potential for effectiveness of the vectors in cystic fibrosis disease state. It also provides proof of concept for feasibility of convenient way of delivery of siRNA to lung in the form of dry powder inhalation. This bodes for potential improvements in preclinical and clinical efficacy.