Chapter 7
Summary and Conclusion

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Cystic Fibrosis (CF) is the most common, life threatening disease in the Caucasian population. It is estimated that there are between 100000 and 700000 people with CF worldwide but it is difficult to state an accurate figure, as people with CF in countries without developed healthcare may die before diagnosis. There are around 30000 people with CF in the USA, over 7500 in the UK and aprox. 30,000 in EU. It is estimate that over 80,000 people may be living with CF in India alone. If both parents are carriers, there is a 1 in 4 chance that the baby will be born with CF. The average life expectancy is between 35 to 40 years. Therefore, a lot of research has been focused in the area of Cystic Fibrosis.

Cystic fibrosis is autosomal recessive genetic disorder. It is caused by a defect in a single gene located on chromosome 7, and is characterized by defective chloride ion transport in airway epithelial cells. This defective gene results in a mutation of the cystic fibrosis transmembrane conductance regulator (CFTR), the most common mutation being the ΔF508 mutation, which occurs on the surface of nucleotide-binding domain 1 (NBD-1). Following this mutation, lung pathology includes abnormal chloride ion transport, increased mucus secretion and viscosity, and decreased mucociliary clearance. These consequences lead to chronic inflammation by recurrent infection and obstructed airways. As a result of increased viscosity of the mucus in the respiratory tract, the clearance of microorganisms is reduced and chronic bacterial infections resulting in inflammation of lung tissue and fibrosis of the airways are a fact. *P. aeruginosa* (55.0%) infections play the greatest role in morbidity and mortality.

Until now, there is no permanent therapy for CF lung disease. Drug therapy is the only supportive therapy for the treatment of cystic fibrosis. Gene therapy represents the best hope for these individuals. The ultimate goal for gene therapy for CF is gene transfer of the intact CFTR gene to appropriate target cells with resultant genotypic and phenotypic correction of the disease. Although it is believed that gene-based therapies hold tremendous potential for the treatment but hindered by failure to deliver therapeutic gene safely and conveniently. Viral (Adenovirus, Adeno associated virus, Lentivirus) and non-viral vectors liposome (Lipoplexes), polycations (polyplexes) are commonly used as a carrier for the gene. Nonviral vectors are generally preferred due to flexibility in size of transgene to be delivered and relatively low

immunogenicity, have greater control of their molecular composition for simplified manufacturing and analysis.

Antibiotics are mainly used to treat the pseudomonas infection in cystic fibrosis. Different antipseudomonal agents used in treatment of CF include cephalosporin, broad-spectrum penicillins, polymyxins, fluoroquinolones, and the monobactams. Out of these agents, aminoglycoside are most preferred for treatment of P. aeruginosa infection in cystic fibrosis due to their concentration-dependent antibacterial activity with long term post-antibiotic effect. Various aminoglycoside antibiotics like amikacin, netilmicin, tobramycin, gentamicin shows good antibacterial activity against pseudomonas aerogenosa. However, low penetration of the aminoglycosides through CF mucus needs higher dose of the antibiotics to achieve concentration above the MIC. Also short duration of action of antibiotics need frequent dosing. This results in antibiotic resistance and increased adverse effects. Pulmonary drug delivery system seems to be very promising approach to overcome this drawbacks and deliver the antibiotics for treatment of bacterial infections.

The administration of therapeutic molecules and gene by pulmonary route gained attention due to the numerous advantages of pulmonary drug delivery over other delivery routes. These advantages include the large alveolar surface area suitable for drug absorption, low thickness epithelial barrier, extensive vascularization, and the relatively low enzymatic metabolic activity in addition to the absence of the first-pass effect. But still the pulmonary delivery of drug and gene is challenge for the formulation scientist due to its limitations. The respiratory tract, being in direct contact with the external environment, possesses a series of defenses against inhaled materials. In the conducting airways deposited particles are rapidly cleared by the mucociliary clearance into the pharynx. In addition to this barriers, specific and nonspecific immune response against gene transfer agents are an important problem, severely restricting the use of drugs and gene in CF. This can be overcome by the use of nonviral vectors.

To overcome these challenges encountered during pulmonary delivery, particulate drug delivery system has been proposed such as nanoparticles, microparticles, liposomes, polymerosomes, and micelles. However, these nanocarriers become ineffective due to the binding of plasma

complement, immunoglobulins, and other proteins (opsonization), leading to uptake by macrophages (absorptive process of pulmonary clearance). This clearance can be markedly delayed by the grafting of synthetic hydrophilic polymers such as poly (ethylene glycol) (PEG) onto the surface of the vehicles. PEG forms a hydrated shell hindering protein interaction with nanocarrier or drugs themselves, thereby greatly reducing opsonization and uptake by macrophages. PEGylation also helps in the improving the transfection efficiency of the gene in bronchial cells *in-vitro*. Such "stealth" DDS have improved pharmacokinetics and lesser side effects of activation of host defense (immune response, cytokine release, complement activation). Biodegradable polymeric carrier system has various advantages like non-toxic, biodegradable and biocompatible polymer with very interesting biological properties, such as permeation-enhancing and mucoadhesive properties. Thus, the Stealth polymeric nanocarriers may serve as suitable candidate for drugs and gene delivery formulations in the effective treatment of the Cystic fibrosis.

Several pre-clinical and clinical gene therapy studies using both viral and non-viral vectors have been performed since the CFTR gene was cloned, and pointed out the need to develop gene carriers with improved efficacy and reduced side-effects. Non-viral vectors like nanoparticles, liposomes are generally preferred due to flexibility in size of transgene to be delivered and relatively low immunogenicity, have greater control of their molecular composition for simplified manufacturing and analysis. Several recent studies have examined pDNA delivery by degradable nanoparticles (NPs) formed from poly(lactic-co-glycolic acid) (PLGA), which was selected due to its biodegradability, ability to encapsulate and protect nucleic acid payloads from enzymatic degradation..

On the other hand, Netilmicin sulfate (NS) an aminoglycoside antibiotic is mostly used for the treatment of *P. aeruginosa* infections in cystic fibrosis due to its concentration-dependent antibacterial activity and long term post-antibiotic effect. However their incorporation into a hydrophobic matrix is problematic because their amino groups and hydroxyl groups cause them to be polar, and their high water solubility inhibits their incorporation into the hydrophobic core of NPs. Moreover they penetrate into the sputum of CF patients poorly and their activity is reduced due to binding with sputum components such as glycoproteins and cations.

Consequently high systemic doses are needed to achieve effective concentrations of the drug at the infection site. High doses can result in nephrotic and ototoxic effects, with deafness, dizziness and unsteadiness, reducing the therapeutic window. NPs can provide controlled antibiotic release thereby maintaining a constant plasma concentration above the minimum inhibitory concentration (MIC) for a prolonged duration. This maximizes the therapeutic effect while minimizing antibiotic resistance, reduce dosing frequency, and also improves patient compliance.

Hence attempt has been made to deliver NS and pDNA in biodegradable NPs system and evaluated the designed drug delivery system for their physicochemical parameters, *in-vitro* release and *in-vitro* efficacy. It is hypothesized that stealth polymeric nanocarrier in dry powder formulation for nebulization after reconstitution containing therapeutic gene and drug will provide more efficient and direct delivery of the genes and drug in lung cells. PEGylation gives stealth effect to the nanocarrier, prevent it's uptake by macrophages and also helps in the improving the transfection efficiency of the gene in bronchial cells. Encapsulation of antibiotics in polymeric nanoparticles will provide sustained drug delivery will maintain the constant plasma drug concentration over MIC for a prolonged period, thus maximize the therapeutic effect while minimize antibiotic resistance, minimize the dosing frequency and thus dose related side effects. Co-administration of the drug and gene for treatment of the CF will increase possibility of better therapeutic response in patients.

Analytical methods were developed for characterization of drug and pDNA at different stage of formulation development. Different media like Acetonitrile: Phosphate buffer (ACN: PB) pH 7.4 (2:1), Phosphate buffer (PB) (pH 7.4), Simulated Lung Fluid (SLF) were evaluated for analysis of the netilmicin sulfate. The linearity range for netilmicin sulfate estimation was found to be 10-60 µg/mL (r^2 = 0.9994) in PB (pH 7.4), 10-60 µg/mL (r^2 = 0.9995) in SLF and 10-60 µg/mL (r^2 = 0.9998) in ACN: PB (pH 7.4) (2:1). Lower values of standard error (S.E.) of slope and intercept indicated high precision of the proposed methods. Also, the mean slope and intercept values are within the 95% confidence interval. Goodness of fit of the regression equations was supported by high regression coefficient values and lower calculated F-values. Absorption spectrum of pure drug sample was matching with the drug with excipients sample in all the selected media. The

calculated t-values were found to be less than that of the critical t-value, indicating that statistically there was no significant difference between mean absorbance of solutions prepared from pure drug sample and drug with common excipients. This indicates the selectivity and specificity of the proposed methods. All three QC level showed an accuracy (% bias) ranging from -0.39 to 0.24 for ACN: PB pH 7.4 (2:1), -0.58 to 0.69 for PB (pH 7.4), -0.36 to 0.37 for SLF. The mean percent recovery value and their low SD values represent the accuracy of methods. In ACN: PB pH 7.4 (2:1) mean percent analytical recoveries (± SD) for lower, intermediate and higher concentrations were found to be 100.57 (± 1.67), 102.45 (± 0.98), 99.00 (± 2.26) respectively. In PB (pH 7.4) mean percent analytical recoveries (± SD) for lower, intermediate and higher concentrations were found to be 101.43 (± 3.24), 101.02 (± 1.43), 100.99 (± 2.35) respectively. In SLF mean percent analytical recoveries (± SD) for lower, intermediate and higher concentrations were found to be 100.23 (± 2.50), 100.26 (± 1.73), 100.29 (± 1.22) respectively. Repeatability of the methods was determined through % RSD value. It was ranging from 0.73 to 1.86, 1.10 to 2.23, and 0.98 to 1.65 in the PB (pH 7.4), SLF, ACN: PB pH 7.4 (2:1) medium, indicates the precision of methods under the same operating conditions over a short period of time and inter-assay precision. % RSD values were found low for intermediate precision, with the intra-day variation % RSD value was less than 1.82% and % RSD value of inter-day variation was less than 1.96%. In ACN: PB pH 7.4 (2:1) Limit of detection (LOD) and Limit of quantification (LOQ) were found to be 0.9543 µg/ml & 2.8919 µg/ml, respectively. In PB (pH 7.4) LOD and LOQ were found to be 1.022 µg/ml & 3.098 µg/ml respectively. While, in SLF LOD and LOQ were found to be 1.022 µg/ml & 3.098 µg/ml respectively. The methods were found to be robust as variation of pH of phosphate by ± 0.2 units for PB (pH 7.4) and SLF; while in case of ACN: PB pH 7.4 ratio of solvents was changed by 0.25 unit. Variation in these media did not have any significant effect on absorbance. The mean % recovery (± S.D.) were found to be 99.68 ± 0.76, 100.09 ± 0.29 and 99.98 ± 0.51 in the PB (pH 7.4), SLF, ACN: PB pH 7.4 medium, respectively. The assay values of Netilmicin sulfate injection were 100.83 ± 1.18, 100.75 ± 1.13, 103.03 ± 2.54 in the PB (pH 7.4), SLF, ACN: PB pH 7.4 medium, respectively.

The Spectrofluorimetric method was employed to estimate rhodamine B and 6-coumarin in nanoparticles and for estimating distribution of rhodamine B and 6-coumarin loaded

nanoparticles in the pulmonary tissue & CFBE 41o- cells). Calibration curve was developed in Chloroform:Methanol (1:1) with Spectrofluorometer (RF-5301PC, Shimadzu) and Synergy™ Mx Microplate Reader. With Spectrofluorometer (RF-5301PC, Shimadzu) linearity range for rhodamine B was found to be 1000-8000 pg/mL (r^2 = 0.9981), while that for 6-coumarin was found to be 1000-8000 pg/mL (r^2= 0.9987). With Synergy™ Mx Microplate Reader, linearity range for rhodamine B was found to be 20-160 ng/mL (r^2= 0.9969), while that for 6-coumarin was found to be 20-160 ng/mL (r^2=0.9985).

To estimate accurate DNA the QuantiFluor® dsDNA System, Promega was used. The QuantiFluor® dsDNA System contains a fluorescent double-stranded DNA-binding dye that enables sensitive quantitation of small amounts of double-stranded DNA (dsDNA) by using fluorescence plate reader. Based on the application difference media was used for development of calibration curve of the plasmid DNA. To estimate the entrapped DNA in NPs 1X TE buffer was used while to estimation of released DNA from the NPs PBS pH 7.4, and Acetate buffer pH 5 were used. At all concentration levels the SD was low and the % RSD did not exceed 7.59 for 1X TE buffer, 8.82 for PBS pH 7.4 and 6.15 for Acetate buffer pH 5. Linearity range in all media was found to be 10-70 ng/mL, while regression coefficient (R^2) was 0.9997, 0.9994, and 0.9992 in 1X TE buffer, PBS pH 7.4, and Acetate buffer pH 5 respectively.

To estimate the proteins in cell lysate samples Pierce BCA Protein Assay Kit, Thermo Scientific was used. Calibration curve was developed with a bovine serum albumin (BSA). At all concentration levels the SD was low and the % RSD did not exceed 2.67. Linearity range was found to be 10-60 µg/ml with a regression coefficient (r^2) of 0.9997. In conclusion, the proposed methods were simple, rapid, specific, accurate, precise. Thus, these methods can be used for the estimation of netilmicin sulfate, pDNA, rhodamine B, 6-coumarin either in bulk or in the dosage formulations without interference with commonly used excipients and related substances.

The piRIS2-EGFP CFTR plasmid & pCDNA-3 LUC-WT plasmids were transformed in bacterial cells using CaCl2 method and transformation was confirmed by the growth of the bacterial cells on agar plates containing antibiotics. From the transformed colonies pDNA was isolated using

alkaline lysis method and was confirmed for correct transformation by restriction endonuclease digestion. Confirmed plasmids were amplified using maxi-precipitation technique using the principle of alkaline lysis. The isolated pDNA was purified using phenol chloroform extraction followed by PEG 8000-Lithium Chloride precipitation. The purity of the pDNA was assessed by calculating the ratio of UV absorbance at 260 and 280 nm. The ratio in between 1.8-2.0 indicates pure pDNA devoid of protein and RNA.

While developing NPs formulations, the high solubility of Netilimicin leads to its diffusion into aqueous phase during preparation of the NPs, resulting in low entrapment. Thus systematic study has been employed to improve entrapment efficiency (EE) of Netilmicin sulfate (NS) in PLGA NPs. A 2^{5-1} fractional factorial design (FFD) was used to determine the effect of these process parameters on NP characteristics. Based on the preliminary experiments the chosen independent process parameters included: the volume ratio between the inner water phase and the oil phase (V_{w1}/V_O) (X1), the PLGA concentration (mg/mL) (C_{PLGA}) (X2), the surfactant concentration (%) ($C_{Pluoronic\ F68}$) (X3), the NS-to-DS charge ratio (DS:NS Charge ratio) (X4), and the sonication time (sec) of the primary emulsion ($t_{sonication}$) (X5). The X2, X4 and X5 independent variables had positive correlations with the EE. The electrostatic interaction between DS and NS form hydrophobic complex and their hydrophobicity enhances the incorporation of NS in NPs. It was also observed that EE increased with increasing polymer concentration due to increased viscosity of the organic phase, resulting in reduced diffusion of the drug towards the aqueous solution through polymer drops. It was observed that a lower volume ratio enhances encapsulation. Increasing the inner water phase volume reduces the NS concentration gradient between the inner and outer water phase, limiting the diffusion of NS from the inner to the outer water phase to promote its encapsulation in the PLGA core. Sonication time plays important role in determining the particle size of the primary emulsion droplets. A smaller difference in droplet size between primary and secondary emulsion results in the formation of a thin oil layer around the water droplet allowing easy pass for drug from inner aqueous phase can through oil phase resulting in lower EE. Increasing sonication time results in larger size difference between primary and secondary emulsions, which enhances encapsulation of the drug.

Particle size is responsible for the biopharmaceutical properties of the NPs formulation. The variables X1, X2 and X4 showed positive correlations with particle size while the variables X3, X5 showed negative correlation with the particle size. A large interaction was observed between the ratio of the inner water phase to the organic phase and sonication time (X1 & X5), concentration of the surfactant and sonication time (X3 & X5), drug charge ratio and sonication time (X2 & X5), and PLGA concentration and DS: Drug charge ratio (X2 & X4). These provided a positive synergistic effect between the respective two factors. The interaction between the ratio of the PLGA concentration and sonication time (X2 & X5) may indicate that a longer sonication time is required to obtain a stable emulsion with small particle size when PLGA is added at high concentration.

The optimal parameters for NS-loaded PLGA NPs were found to be using inner aqueous phase (W_1) (0.35 mL), PLGA concentrations (15 mg), organic phase volume (0.5 mL), secondary aqueous phase (7 mL), DS: NS charge ratio (2.5). Optimized NPs showed EE of 93.23±2.7%, size 140.83±2.4 nm, with a polydispersity index (PDI) of 0.130±0.018 and zeta potential -23.45±3.06 mV. The TEM image of NPs reveals that NPs are spherical in shape and uniform in size. Finally optimized formulation was surface modified using DSPE-PEG at a 3 % w/w lipid to-polymer ratio. The PEGylated NPs were successfully prepared with essentially spherical morphology and mean particle diameter of ~ 150 nm. PEGylation doesn't affect EE of NS in PLGA NPs. Blank and non PEGylated PLGA NPs had similar particle sizes indicating that the encapsulation of NS did not change the particle size. Zeta potential was significantly increased as compared with blank PLGA NPs. The high negative surface charges on the NS-PLGA NPs were due to presence of the anionic charge on the DS chains, which further supported the role of electrostatic interaction with NS. However slight reduction in zeta potential was observed in case of PEGylated NPs, it might be due the presence of the PEG moiety on the surface of the NPs. Further optimized formula was used to prepare fluorescently-labelled NPs (Rhodamine B-loaded NPs) for *in-vitro*/*in-vivo* deposition studies. They were prepared in a same way by replacing NS with Rhodamine B. The obtained particles were uniform in size with similar characteristics as that of drug loaded NPs.

DSC analysis is useful for demonstrating possible interactions between different compounds in a mixture. Absence of endothermic peak of NS in DSC curve of NS-loaded NPs, indicating that NS was present in an amorphous phase in the NPs. The FTIR study showed that all typical bands of PLGA and NS were also present in the FTIR spectrum of their physical mixture. However, the FTIR spectrum of NS loaded NPs had only the characteristic peaks of the polymer. The absence of characteristic drug peaks in the NPs sample indicates that non encapsulated drug was not present. In case of PEGylated NPs presence of DSPE PEG 2000 peaks in NPs which confirms the PEGylation of NPs.

In vitro release study was performed in phosphate buffer (pH 7.4) and simulated alveolar fluids (SLF) to simulate the properties of the delivery site. In both media, bi-phasic release behavior was observed, characterized by initial burst release followed by sustained release of NS from NPs. Lower burst release was observed due to the uniform distribution of the NS in NPs rather than just on the surface of the NPs. About 25% of drug content was released within the first 24h, followed by sustained drug release. However significant difference was observed in release behavior of NS DS PLGA and PEG NS DS PLGA NPs ($p= 0.00001$; $p < 0.05$ & $p=0.00001$; $p< 0.05$ for PBS pH 7.4 & SLF respectively). Being a hydrophilic molecule DSPE-PEG enable a greater influx of water into the NP matrix, promoting greater degradation of PLGA and improved NS release from NPs. Interestingly, no significant difference in NS release rate was observed in simulated lung fluids as compared to results achieved in buffer at pH 7.4, suggesting that ion concentration in SLF does not affect NS release properties. Projecting these results in humans, the fact that the developed NP release NS according to a biphasic kinetics and a prolonged release helps to maintain NS level above MIC to treat bacterial infection effectively.

The antibacterial activity of NS-loaded NPs was determined as MIC on *P. aeruginosa*. A MIC value of NPs was found approximately equal to 18 μg/mL, for control free NS was 2.4 μg/mL, while the complete eradication of the bacteria was observed at 44 μg/mL and 10 μg/mL for NS loaded NPs and native NS, respectively. The higher MIC value for NS-loaded NPs was attributed to the progressive release of the drug from the NPs; i.e. the amount of drug released by the particles after 12 h was ~ 25 %, which should result in a concentration of available drug similar to the MIC observed for the NS alone. Therefore, it could be estimated that the MIC and MBC

value for the native NS and NS released from NPs were similar. In case of PEG NSDS NPs MIC & MBC values were found 14 µg/mL & 36 µg/mL respectively. Similar eradication rates of encapsulated NS and native NS indicate encapsulation of NS in NPs doesn't affect the antibacterial efficiency of NS. To mimic the *in-vivo* condition, CFBE41o- cells were incubated with free NS or NS-loaded NPs, and antimicrobial activity was tested every 24 h for 5 days. It was observed that NPs were more effective in eradicating the bacterial growth as compare to free NS. The cations present in the airway surface fluid antagonized the activity of the NS when incubated with bronchial cells and decreased its antibacterial efficiency. However improved efficacy was observed with NPs due to the protection of the NS by NPs during incubation with the bronchial cells. NS was released slowly from NPs over days and retained its antibacterial activity against *P. aeruginosa*. Further, it was observed that PEGylated NPs were found more effective than non-PEGylated NPs. Reduced negative charge of PEGylated NPs could promote the NPs penetration in bacterial cells. Also faster release of drug from PEGylated NPs as compare to non-PEGylated NPs could enhance it's antibacterial activity.

Mucus penetration study was performed to assess how the composition of the formulation affected NP diffusion through lung lining fluids. It was observed that PEGylated NPs diffused faster through mucus as compare to non-PEGylated NPs. Non PEGylated NPs can be electrostatically repelled by the anionic barrier of mucin due to strong negative surface charge which retard their diffusion through the mucus. While PEGylation of NPs enhance their interaction with mucin through formation of hydrogen bonding thus promote their diffusion through mucus. Thus PEGylated NPs shows the great potential to act in vivo as a drug reservoir which slowly releases the active agent to the target to have a prolonged therapeutic effect.

NS-loaded NPs and blank NPs formulations both demonstrated cell viability of ~80% even after 24h at concentrations as high as 5mg/mL compared to control. Results showed that NPs exhibit cell toxicity in a dose-dependent fashion. Further incorporation of DSPE-PEG in formulation helps in improving cell viability due to reduction in surface charge, suggesting their safe use in these formulations. This results were further supported by Transepithelial electrical resistance (TEER) measurements. The PEGylated NPs doesn't show any significant difference in the TEER value when compared to the control ($p=0.22$; $p > 0.05$). While in case of the non-PEGylated NPs

the decrease in TEER value observed. Taken together, these results suggest the safety of NPs *in vitro* and need *in vivo* evaluation for further confirmation.

PEGylated NPs shows significantly higher cell uptake than non-PEGylated NPs. The uptake of PEGylated NPs in the cells was significantly higher than non-PEGylated NPs. The results shows that PEGylated NPs has lower zeta potential as compare to non PEGylated NPs, which seems to provide better cell uptake. Also PEGylated particles followed clathrin-independent endocytosis pathway, while non-PEGylated particles follow clathrin-dependent route for endocytosis. Thus, PEGylation promote the uptake of the NPs. These findings will opens new door to treat intracellular bacterial infections in CF.

The stability of NPs was assessed as suspension in 0.9% saline simulated lung fluid & 10% fetal bovine serum by evaluating particle size measurements at fixed time intervals. Serum proteins (particularly albumin) bind with NPs which also responsible for enhanced particles size when incubated with serum. Addition of PEG in NPs preparation gives a significant advantage in shielding the NPs from adsorption of proteins. DLS sizing measurements demonstrate that the PEGylated NPs remain stable over 48 h with no significant change in size. Based on this study, non- PEGylated NPs are less favorable, compared to PEGylated NPs, for pulmonary administration as their instability in media may lead to reduced therapeutic efficacy due to faster clearance from the lung.

pDNA loaded NPs were prepared by using water/oil/water (w/o/w) emulsion method. The effect of sonication amplitude and sonication time were evaluated on structural integrity of the pDNA and extracted plasmid was then analyzed by using agarose gel electrophoresis. The results of gel electrophoresis showed intact nature of the pDNA extracted from both 1^{o} and 2^{o} emulsion when compared to untreated pDNA, indicating that the used amplitude for emulsification process and sonication time doesn't disrupt pDNA.

The PEI-modified PLGA NPs were prepared by inclusion of PEI in the organic phase at different PEI to PLGA weight ratios. The size of the NPs was affected by inclusion of PEI into the PLGA matrix, and a significant concentration-dependent decrease in size was observed at increased PEI

concentrations ranging from 246.8 ± 7.59 nm for the unmodified NPs to 166.2 ± 2.55 nm at 12.5% (w/w) PEI. The PDIs were below 0.216 for all compositions, suggesting relatively mono-disperse suspensions. The incorporation of increasing amounts of the cationic polymer PEI into the PLGA matrix resulted in a significant reduction of the particle size and PDI. The zeta-potential was also significantly affected by the inclusion of PEI and a PEI-ratio dependent increase in the zeta-potential was observed ranging from - 06.53±1.00 mV for unmodified PLGA NPs to + 34.00±2.44 for particles containing 12.5% (w/w) PEI. This negative charge for unmodified PLGA NPs is due to uncapped end carboxyl groups of PLGA present on the surface of the particles. However, such negatively charged particles are unable to encapsulate negatively charged pDNA. To render the PLGA NPs with a positive zeta potential, in the present study, we dissolved PEI in the organic phase along with PLGA. The zeta-potential of the PLGA NPs was increased from negative to positive at increasing content of PEI, suggesting that PEI molecules are present on the surface of the NPs.

The pDNA encapsulation efficiency was also positively affected by the addition of PEI. The results showed a significantly increased pDNA encapsulation efficiency as PEI increase. ~100 % pDNA loading was obtained at 10 % PEI. pDNA is negatively charged and highly hydrophilic in nature make it difficult to entrap into negatively charged highly hydrophobic polymer. Addition of the positively charged PEI molecule in the system results in the formation of hydrophobic complex between positively charged PEI with negatively charged pDNA. This hydrophobic cationic complex get easily entrapped in PLGA core thus enhances pDNA loading. The TEM image of NPs further reveals that NPs are spherical in shape and uniform in size.
FTIR study was used to confirm the presence of PEI on NPs surface. When PEI added to organic solution containing PLGA, process involves the electrostatic interactions of PEI with the negatively charged surface of PLGA NPs, but possibly PEI also had reacted with PLGA by aminolysis. The FTIR spectrum of PEI PLGA NPs showed characteristic peaks of PEI confirms the presence of PEI on NPs surface.

Release of pDNA from NPs were performed in PBS pH 7.4 for 24 h and then the half of NPs were transferred to a solution having pH 5.0 to mimic endolysosomal condition. The incorporated pDNA were released rapidly from NPs within 48 h, followed by very slow release

until day 10. Initial higher burst of pDNA release was observed for the unmodified PLGA NPs systems than that observed for PLGA-PEI NPs. Increasing concentration of PEI in NPs system significantly reduces burst release due to the strong electrostatic interactions between PEI and pDNA. Also being a hydrophilic molecule located at the NPs surface, PEI may facilitate degradation of the NPs by increasing hydration and thus accelerate PLGA hydrolysis. However, the cationic nature of PEI may retard pDNA release due to the electrostatic attractions between them thus sustain the PEI release. In both the cases, at the initial phases no significant change in release rate was detected even though the samples were incubated at pH 5.0. But, the release rates were abruptly accelerated after 24 h only at pH 5.0 for all formulations. This result could be attributed to the pH dependent degradation nature of PLGA. These results indicates that the release of the pDNA from PLGA-PEI or PLGA-NPs is depends on the on the degradation kinetics of PLGA matrix after initial burst of the pDNA that located at the NPs surface.

It was noted that the increased PEI concentration increases luciferase transfection in CFBE 41o- cells. This could be attributed to the increase positive charge on particles which promote cell uptake and thus luciferase expression. This results are in agreements till PLGA PEI NPs 10%. At 12.5% PEI luciferase expression was reduced as compared to 10 % PEI due to cell toxicity. Surface adsorbed PEI chain promote cellular uptake, results in large amount of cell death and results in decreased transfection efficiency of PLGA-PEI NPs 12.5 %. After 48 h lipofectamine plus shows highest luciferase expression than NPs. But following 48 h luciferase expression was reduced as compared to NPs. While in case of NPs luciferase expression was observed consistent even till 144 h.

Particle toxicity was evaluated by using the MTT assay after exposure to controlled doses of particles. There was a substantial increase in particle toxicity which increases in dose and PEI content. NP-10 % and NP-12.5 % formulations produced considerable cell toxicity especially at high doses. These cytotoxic effects are likely due to the high positive surface charge imparted by the PEI, which is apparent from the increased zeta potential with increasing PEI content. Thus, we aimed to reduce the toxicity of the formulations and thus to improve the transfection efficiency.

PEGylation of particles performed to reduce the toxicity effect. We chose modification to PLGA-PEI NPs 10 % particles with DSPE-PEG (3 % w/w) because they showed the most promise as a transfection vehicle in CFBE41o– cells. The PEGylated NPs were successfully prepared with essentially spherical morphology and mean particle diameter of ~ 190 nm. It was observed that PEGylation doesn't affect EE of pDNA in NPs. Surface modification with PEG significantly reduces zeta potential as compared with PLGA-PEI NPs due to the shielding effect imparted by PEG. Further presence of PEG on NPs surface further confirmed by FTIR spectroscopy. All these characteristic peaks of DSPE PEG 2000 were observed in PEGylated NPs which confirms the PEGylation of NPs. Further these formulations were used to prepare fluorescently-labelled NPs (6-coumarin loaded NPs) for *in-vitro*/*in-vivo* studies. The obtained particles were uniform in size with similar characteristics as that of pDNA loaded NPs.

It was observed that PEGylation significantly reduces toxicity of the particles. Further it was confirmed from TEER analysis on cells under liquid/liquid culture conditions. The progressive decrease in TEER value was observed over the period of time with significant difference between PEG-PEI PLGA NPs and control cells ($p=0.011$; $p < 0.05$ & $p=0.027$; $p< 0.05$ for cells &PEG-PEI PLGA NPs respectively). While there was no significant difference in TEER value of cells and PEG-PEI PLGA. Taken together, these results suggest that the PEGylated NPs do not interfere with the integrity of the airway epithelial cells *in vitro* and may not have toxicity effect when used *in vivo* to treat lung disorders.

6-coumarin loaded NPs used to assess cellular association and uptake of NPs. Both PEGylated and non-PEGylated NPs showed cell uptake, as revealed by fluorescence microscopy. PEI-PLGA NPs shows significantly higher cell uptake than PLGA NPs. The uptake of PEI-PLGA NPs in the cells was significantly higher than PLGA NPs ($P= 0.001$; $P <0.05$). The presence of PEI on NPs surface imparts a positive charge to the NPs, which helps in the electrostatic interaction with the negatively charged cell membrane thus promote their uptake. Further PEGylation of these particles again improved their uptake ($P<0.05$; $P=0.003$ against PEI-PLGA NPs & PEG PEI-PLGA NPs; $P=0.001$ against PLGA NPs & PEG PEI-PLGA NPs). Though presence of PEI shows enhanced uptake but their cell uptake is limited as serum proteins (particularly albumin) bind with PEI prior to cell uptake which abrogate the uptake of NPs. Addition of PEG in NPs

preparation gives a significant advantage in shielding the NPs from adsorption of proteins and promote the uptake of the PEGylated NPs.

In release study it was observed that there was a significant difference in release from non-PEGylated and PEGylated NPs in both media ($p= 0.00001$; $p < 0.05$ & $p=0.012$; $p< 0.05$ for PBS pH 7.4 & Sodium acetate buffer pH 5.0 respectively). However it was observed that the incorporation of PEG enable a greater influx of water into the NPs matrix, accelerate PLGA hydrolysis and thus promoting greater degradation of PLGA and improved DNA release from NPs.

Further PEGylated particle formulations showed improved luciferase activity. Results of luciferase expression were further confirmed with western blot analysis. The western blot analysis showed that the monoclonal antibody directed against the C-terminal of the CFTR protein detected two bands: one band around 170 kDa corresponded to the fully processed and fully glycosylated form of CFTR; another band at about 150 kDa referred to the immature, core-glycosylated form of CFTR that is retained in the endoplasmic reticulum (ER) or in an intermediate ER-associated compartment. While sodium potassium ATPase was used as internal control. The most intense signals were reported with a PEG PEI-PLGA NPs (10%) followed by PEI-PLGA NPs (10%), PLGA NPs, Lipofectamine plus. Absence of any CFTR band in cells treated with DNA only indicated the need of the delivery vector. The Western blot observations shows similar results when correlated with the GFP expression. Highest GFP expression was obtained with PEG PEI-PLGA NPs (10%).

Efficiency of NPs to protect encapsulated pDNA against nuclease digestion was evaluated by exposing them to DNAse I followed by agarose gel electrophoresis. It was observed that naked plasmid DNA was completely digested within 5 min of incubation, while DNA encapsulated in NPs remained intact up to 4 h when incubated with DNAse I. When compared with controlled pDNA, pDNA extracted from NPs doesn't showed any degradation while naked DNA was completely degraded, which shows the efficiency of the encapsulation process against the degradation effect of the enzyme. Thus, hydrophobic PLGA matrix can be served as an effective protection layer to encapsulated pDNA when administered in-vivo from protein and enzyme.

To assess how the composition of the formulation affected NP diffusion through lung lining fluids, the amount of 6-Coumarin permeated through an artificial mucus layer was measured. It was observed that the PEGylated NPs diffused faster through mucus. PEGylation of NPs reduces their electrostatic interaction with mucin but promote formation of hydrogen bonding with mucin thus promote their diffusion through mucus. Taken together, these results suggest the great potential of the PEGylated NPs to act in vivo as a drug reservoir which slowly releases the active agent to the target to have a prolonged therapeutic effect.

The stability of NPs was assessed by suspension in 0.9 % saline simulated lung fluid & 10% fetal bovine serum by evaluating particle size measurements at fixed time intervals. The PEI PLGA NPs showed increased particle size over the period of time. The enhanced particle size is due to the flocculation effect induced by ions present in the solutions. However serum proteins (particularly albumin) bind with surface PEI of NPs which is also responsible for enhanced particles size when incubated with serum. The resulted aggregates limits their cell uptake. Addition of PEG in NPs preparation gives a significant advantage in shielding the NPs from adsorption of proteins. DLS sizing measurements demonstrate that the PEG PEI PLGA NPs remain stable over 48 h with no significant change in size. From the all results obtained, it is observed that PEGylated NPs system in most promising delivery system hence, they were used for development of dry powder inhaler formulations.

Freeze drying technique was used for preparation of the dry powder inhalation formulation by using suitable cryoprotectant. The nanoparticulate formulations were lyophilized with sugars such as Lactose, Sucrose and Trehalose at a weight ratio of 1:0.5, 1:1, 1:1.5 and 1:2 of polymer: sugar. After lyophillization at different Polymer: cryoprotectant ratio, particle size of the NPs in the cake was determined after resuspension in 2 ml of water followed by vortexing to break the lumps. Both drug loaded and pDNA loaded NPs showed comparatively lower particle size with Trehalose than Lactose as well as sucrose and at ratio 1:1. Further increase in ratio of Trehalose results in non-significant improvement in particle size, hence NPs: Trehalose ratio of 1:1 was optimized and used for all formulations and these formulations were further used to develop DPI formulation.

The Polymer: Trehalose ratio of 1:1 was used for further evaluations. Formulations further mixed with lactose carrier (Lactohale 201) in varying mass ratios from 1:0.5 to 1:3 and evaluated for aerodynamic properties of the formulations. In case of NS loaded NPs, Dry powder formulation without lactose carrier particles, only 75.96 ± 3.03 % of the particles are successfully emitted off. The low ED is caused due to the agglomeration of the particles and may be due to their small diameter, making them difficult to be aerosolized off the percentage of initial dose. Even for the drug particles that are successfully aerosolized, only a small fraction of them (Dispersibility: 17.36 ± 0.69 %) possess below effective cut-off diameter < 4.7μm indicating that a majority of the aerosolized drug particles remain agglomerated entering the ACI resulting in MMAD 6.53±0.29 μm. The combination of the low ED and low dispersibility also leads to a low FPF (13.19±0.86 %), which is much lower than the typical FPF of ≈ 20 –30% for commercial DPI products. It was observed that, addition of the inhalable lactose significantly improve ED, dispersibility and thus % FPF, while lower the MMAD bellow 5 μm. Carrier to mass ratio of 1:1.5 show high %FPF (34.64±0.42 %) and MMAD (3.49±0.1 μm); however further increased in carrier to mass ratio doesn't significantly improved %FPF, thus carrier to mass ratio 1:1.5 was used for further preparation of the dry powder formulations. Dry powder formulations of PEGylated NPs and Rhodamine loaded NPs also showed similar aerosolization properties.

Similar results were also observed with pDNA loaded NPs. Dry powder formulation without lactose carrier showed low ED (71.72±2.54 %), as well as dispersibility (17.41±3.23 %). The combination of the low ED and low Dispersibility also leads to a low FPF (12.43±1.94 %) and high MMAD (6.64±0.46 μm). While further addition of the inhalable lactose at carrier to mass ratio of 1:1.5 show high %FPF (34.25±0.50 %) and MMAD (3.82±0.04 μm). Dry powder formulations of PEGylated NPs and 6-Coumarin loaded NPs also showed similar aerosolization properties. The lyophilized powder of the NPs were adsorbed onto the surface of the inhalable lactose carriers during the mixing process and easily separated from the surface of the inhalable lactose carriers under the airstream generated by patients and deposited in the deep lung. Subsequently the lyophilized PLGA NPs could be reconstituted in the lung fluid, and release the therapeutic moiety.

SEM image of nanocomposite particles showed NPs powders were adsorbed on the surface of inhalable lactose carriers and form nanocomposite particles. The residual water content plays

crucial role in long-term stability of formulation, both physically and chemically. Water content in all formulations was less than 1.5 % which was apt for long term stability.

However, lyophilization and powderization doesn't affect antibacterial activity of the NS loaded NPs. A MIC value for NSDS PLGA NPs (Initial) and Formulation D (Reconstituted) was found to ~18 μg/mL and ~17 μg/mL respectively while complete eradication of the bacteria was observed at 44 μg/mL and 42 μg/mL for NSDS PLGA NPs (Initial) and PEG NSDS PLGA NPs + Carrier (1:1.5) (Reconstituted), respectively. MIC value for PEG NSDS PLGA NPs (Initial) and Formulation F (Reconstituted) was found to be ~14 μg/mL and ~15μg/mL respectively, while the complete eradication of the bacteria was observed at 36 μg/mL and 38 μg/mL for NSDS PLGA NPs (Initial) and PEG NSDS NPs + Carrier (1:1.5) (Reconstituted), respectively. Also lyophilization and powderization doesn't affect the luciferase expression activity of the pDNA loaded NPs. Both dry powder formulations and initial formulations showed similar transfection efficiencies in the cell lines used. In general, dry powder formulations seemed to increase gene expression slightly, but this increase was not significant.

PEGylation of particles resist their uptake by macrophage. Fluorescent markers (Rhodamine b and 6-Coumarin) loaded particles were used for this purpose. Rho-DS PLGA NPs (Formulation G), PEG Rho-DSPLGA NPs (Formulation H), 6-C-PEI PLGA NPs (Formulation O), PEG 6-C-PEI PLGA NPs (Formulation P) were reconstituted in HBSS and used for evaluation of the macrophage uptake. PEGylation of the particles reduces the macrophage uptake and thus their removal. Also being a hydrophilic molecule, PEG reduces NPs electrostatic interaction with mucin, promote formation of hydrogen bonding with mucin thus promote NPs diffusion through mucus and deliver active molecule to the epithelial cells. Thus PEGylation promote the mucus penetration of the inhaled particles and also prevent the removal by the phagocytic uptake.
The deposition pattern and clearance of the nanocomposite particles was assessed in vivo after administration of the selected formulations in rats by a syringe. It was observed that PEGylated formulations were distributed more extensively in the lung tissues as compared with non-PEGylated particles. In case of Rhodamine B loaded particles, ~18% of PEGylated particles (Formulation H) were eliminated from the lungs, while ~35 % of non-PEGylated particles (Formulation G) were cleared off one day post administration of particles. While in case of 6-

Coumarin loaded particles, ~14% of PEGylated particles (Formulation P) were eliminated from the lungs, while ~37 % of non-PEGylated particles (Formulation O) were cleared off one day post administration of particles. However, on 6^{th} day, about ~ 86 % of nonPEGylated particles were out of the lungs while about ~70 % of PEGylated particles were cleared out. It is well known that inhaled particles are cleared from lung mainly by macrophage uptake and mucociliary clearance results in low therapeutic efficiency. These defense mechanism are main hurdles in effective lung delivery. Based on the data presented it is clear that PEGylated particles present desirable lung retention pattern, can produce sustained therapeutic effects with reduced accumulation of particles exhausted of drug.

Cytotoxicity of the DPI formulations was assessed by MTT assay in CFBE 41o- cells. All formulations showed cell toxicity in dose-dependent fashion. These doses used were too high than the required dose. In case of drug formulations, PEGylated formulation (Formulation F) showed improved cell viability but it is not statistically significant. While in case of pDNA loaded formulations, it was observed that PEGylation significantly improved cell viability of formulation N. This effect could be due to reduced surface charge of the cationic particles by DSPE-PEG addition. PEG shield the particle surface, reduces surface charge thus improve the cell tolerance. TEER analysis on cells under air-interfaced culture (AIC) conditions were in agreement with MTT assay.

Further in vivo safety was assessed by Bronchoalveolar lavage (BAL) studies. Saline treated animals, lung weight was 0.47 while that for LPS treated animals was 0.89. The increased weight for LPS treated animals suggested formation of edema due to accumulation of extracellular fluid into the epithelial cells of the respiratory wall. For formulations D and L, L/ B ratio were 0.61 and 0.63 respectively, indicating considerable lung injury or edema formation, while for formulation F and N L/B ratio were 0.50 and 0.49 respectively indicating no substantial lung injury. The enzymatic activities of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) (injury markers) in BAL collected from the treated animals supports the obtained data of L/ B ratio. For Formulation D and L, activity of LDH and ALP in BAL was observed significantly different from that of control treatment, while that for Formulation F and N, no significant difference was observed between control and test formulation. However, level of ALP and LDHs

in LPS treated animals were ~ 2.75 fold higher than those observed after administration of saline. Overall, data observed indicate the safety profile of PEGylated formulation. Histopathological analyses were also performed to evaluate the toxicity of the particles. Histopathological results showed good correlations with BAL analysis. An increase in the infiltration of inflammatory cells was observed with Formulation D and L as compared to the Formulations F and N and saline. While high level of inflammation was observed with LPS.

The stability studies of the developed formulations showed that the critical parameters i.e. mean particles size, water contents, % EE doesn't affected during storage. All formulations were easily dispersed and showed no significant change in particle size, % EE & moisture content of formulations on storage. The lower moisture content in formulation on storage indicate the long term stability of formulations.

To conclude NS and pDNA loaded nanoparticulate formulations were prepared successfully. The use of helper polymer seems worthwhile option for improvement of EE of highly water soluble drug. Also addition of the cationic polymer in pDNA loaded NPs showed viable outcomes in terms of the transfection efficacy in cell lines. Further PEGylation of the NPs helps to reduce cell toxicity, improved mucus penetration, and reduces macrophage uptake of the particles. Therefore, direct lung delivery as dry powder inhaler will help in opening a new strategy to treat CF. Use of the inhalable lactose carrier significantly improved the aerosolization properties of the developed formulations depicting their usefulness in delivery of the dry powder formulation. Co-administration of the drug and gene formulations for treatment of the CF will increase possibility of better therapeutic response in patients. Thus, this study provides a thinking which may result in development of new platform technology for better therapeutic treatment to cure CF.

Future Scope

Although the work shows excellent *in-vitro* efficacy, the preclinical and clinical testing must be executed for actual performance of the formulation in the *in-vivo* condition.