Chapter 1:

Introduction

1.1. Introduction

Growing rise in environmental pollution, temperature changes, exposure to allergens, smoking habits, and respiratory infections have turned into leading cause of respiratory disorders such as chronic obstructive pulmonary disease, asthma, emphysema, and cystic fibrosis, chronic pulmonary infections and lung cancer. Among these, Cystic Fibrosis (CF) has been identified as most common, life threatening disease in the Caucasian population. The current average life expectancy of patient is about 35 to 40 years. As per estimates, there are approximately 100000 to 700000 people with CF all over the world. Further as people with CF in developing countries may die may die before diagnosis it makes it challenging to state the correct number. Even in countries developed healthcare like in the USA and UK there are aproximately 30000 and 7500 people with CF, respectively. The numbers in EU is over 30,000 people. Even in India it is expected that more than 80,000 people might be existing with CF. With both parents as carriers, there is a 1 in 4 probability that the baby will inherit CF [1]. It is reported that 1 in every 25 of the Caucasian population harbors the defective gene that causes CF. The most responsible mutation, i.e. ΔF508, is said to occurs at a frequency of 19-34% in Indian children [2]. In this context, the identification of novel therapeutic strategy to treat CF is highly pursued contemporary goal.

CF is one of the most common life-shortening, chronic hereditary disease. The genetic disorders 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) [3]. CFTR is a chloride channel involved in the exchange of chloride ions between cytoplasm and the airway surface liquid (ASL). The disease affects organs containing secretory epithelial cells, most predominantly the lungs. In normal lungs the volume of ASL is dependent of the precise balance between secretion and absorption of ions and water. The Na^+ absorption, is mediated through the Na/K-ATPase in the basolateral membrane and epithelial Na^+ channel (ENaC) present in the apical membrane. On the other hand, Cl^- secretion is mediate through the CFTR and other Cl^- channels in the apical membrane, and the NKCC cotransporter in the basolateral membrane [4]. These secretion of Cl^- and absorption of Na^+ is under equilibrium. Apart

from this, the viscoelastic characteristics of mucus is properly maintained by appropriate volume of periciliary fluid and hydration. The effective mucociliary clearance is result of precise performance of these absorption and secretion processes. In CF, the mutations in the CFTR Cl^- channel leads to disturbance of equilibrium between absorption and secretion. As a consequence, Na^+ absorption is predominantly increased while Cl^- secretion is strongly supressed. This leads to accumulation of Cl^- and Na^+ ions within cells and inhibition of water transport across cell membranes. This finally culminates into causing the airways of CF patients to become dehydrated, clogged by thick mucus, inflamed, and often infected [5]. This condition may perhaps last several years; however, frequently the lungs become colonized by opportunistic pathogens such as *Pseudomonas aeruginosa* that are accountable for induction of inflammation and consequent destruction of the lungs leading to of respiratory inefficiency and death [6].

The desired therapeutic outcomes for CF are both long- and short-term. In the long-term, one apparently tries to halt or delay progression of the disease to allow for normal growth and development. In the short term, acute problems must be dealt with. The goal of therapy for the pulmonary component is to reduce the signs and symptoms of airway infection, inflammation, and obstruction. Thus, antibiotic, antiinflammatory, bronchodilator, and mucolytic therapies are geared towards treatment of complications that compromise lung function. For an acute pulmonary exacerbation, a return of pulmonary function to the pre-exacerbation status is the central goal of therapy [7]. However, none of these therapies are known to cure the disease. Very few of the affected individuals' manage to survive childhood due to lack of permanent therapy for CF lung disease [8].

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. Out of these, the non-viral vector systems proved to be advantageous over viral vectors due to negligible propensity for infection and immunostimulation. However, since 1993-2004, 29 clinical trial protocols were published, wherein all showed limited success [8]. 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 [9]. While the reported level of expression was very low <10% were found sufficient to normalize Cl^- flux but none was able to induce a correction to the Na^+ hyperabsorption present in CF. Further it was reported that to normalize Na^+ hyperabsorption, through CFTR activity, almost 100% of cells are required to express Cl^- [9]. Further, it has been suggested that downregulation of ENaC may help to restore airway hydration and mucus clearance, and to reverse, at least partially, the airway phenotype in patients with CF. The Na^+ hyperabsorption is fundamental mediator of CF pathogenesis and must be normalized to get complete recovery [10]. As an alternative, attempts were made to normalize Na^+ hyperabsorption directly by Na^+ channel blockers. Consequently, double-blind clinical trials with the ENaC blocker amiloride were conducted in CF patients few years ago. Amiloride was administered by local methods topically using aerosol. In study subjects with CF, amiloride was found to reversibly decrease the Na^+ reabsorption and increase the clearance of mucus; however, the benefit was transient and of short duration, attributed to the quick elimination of amiloride from the epithelium surface [11]. Thus, the very short duration of effect, because of rapid elimination and systemic side effects on kidneys have limited the further applications. *In vivo* pharmacodynamic studies, conducted using the more potent ENaC blocker benzamil, in sheep also demonstrated the short duration of action [12]. Therefore; advanced therapeutic interventions directed to inhibit Na^+ hyperabsorption are presently the major focus of CF research [13].

Recenlty, RNA interference (RNAi) has been recognized as a potent molecular method exploited by cells to alter the gene expression by guiding the RNA mechanism to degrade mRNA. RNAi is an endogenous machinery using which small dsRNA induce degradation of complementary mRNA and blocks the expression of a particular gene [14]. Since its finding in 1998, it has attracted attention of international scientific community [15]. Initially, it largely remained as a powerful tool to study gene functions in biological processes. The effect is mediated through small sequences of RNA, approximately 20 to 22 base pairs, that complement a target mRNA [16]. RNAi is also defined as the mechanism of degradation of mRNA in a sequence-specific manner by a double-stranded RNA (dsRNA). All eukaryotes, from yeast to mammals depict the

existence of RNAi. The ability and application of RNAi for explicitly silence a particular gene expression through a synthetically available sequence has attracted the interest of wide research community in application such as a device for reverse genetics in eukaryotic systems.

RNAi theory depends on the presence of a specialized enzyme, called Dicer, in the cell which can recognize dsRNA and cut it into small fragments of 21-25 base pairs in length. These smaller fragments, known as small interfering RNA, or siRNA are unique in that they can bind to a catalytic protein complex called RNA-induced silencing complex (RISC) [17, 18]. The differential thermodynamic stability at ends of the siRNAs decide the selectivity of strand loading into RISC. The activated form of RISC with siRNA binds to the corresponding mRNA using the antisense RNA. The enzyme present in RISC cleaves the bound mRNA into small pieces and is no longer available for translation [19].

The uniqueness of 21-25 bp siRNA, with symmetric 2-3 nucleotide 3' overhangs and 5' phosphate and 3' hydroxyl groups, lies in its specificity to the target mRNA and subsequent gene inhibition. [20]. The genetic interaction of nucleic acid based therapeutics has been concern from the possible adverse gene alteration. However, as the mechanism of RNAi is based on interference with translation, rather than transcription of DNA, which excludes the possibility of siRNA interaction with chromosomal DNA [21]. As the interaction is with mRNA and not protein, provides the advantage of reducing production of harmful proteins before synthesis. The first successful reports of siRNA application were for hepatitis C virus which further encouraged its applications in other diseases including influenza and HIV infection, cancer and genetic defects [22-24].

In brief, the siRNA are emerging as a promising tool because of advantages like,

- Wide choice of targets based on Watson-Crick base paring interaction.
- The long duration of target suppression resulting medicines which can be dosed infrequently and thereby increase patient compliance.
- Unlike gene therapy, no need to cross the nuclear barrier, since cytoplasm is the site of action, resulting into increase assurance 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.
- The high selectivity for target and non-targets can give better therapeutic index and significantly better quality of therapy than small molecule inhibitors.

However, the effective delivery of siRNA into target cells has been the bottleneck in application of RNAi-based therapeutics. Due to its highl molecular weight and charge of phosphate residues makes it difficult to cross the cytoplasmic membrane by free diffusion. siRNA is highly susceptible to degradation by RNase in the circulation and interstitial space. Though a direct or local delivery of siRNA to target cells can be accomplished in specific cases, a carrier system is obligatory in majority of applications to shield siRNA from degradation and to assist its entry into target cells. Therefore, several viral and non-viral vectors have been utilized for siRNA transfection. Viral vector are naturally most effective form of gene transfection, however, the potential to cause immunogenicity and infection to other tissues remains an impending limitation for their application. Therefore, non-viral vectors, though have a lower transfection efficiency, are preferred over viral vectors due to less toxicity. The non-viral gene delivery methods include injection of naked DNA only or using physical techniques like gene gun, electroporation, hydrodynamic delivery, sonoporation and magnetofection. However, these methods are less efficient is systemic gene delivery in humans. Therefore, many synthetic non-viral vectors were developed as carrier systems such as cationic lipids and liposomes, cationic polymers, dendrimers, polysaccharides, polymerrosomes, cell penetrating peptides and inorganic nanoparticles etc [25].

Cationic polymers such as chitosan, polyethylenimine (PEI), poly(L-lysine), poly(arginine), polyphosphoester, and dendrimers have been used for non-viral vectors [26]. The cationic polymers contain several amine groups in their backbone and interact with negatively charged siRNA leading to the spontaneous formation of nanosized complexes. 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 the lung delivery, it has been observed that lipid based vector experience strong interference from lung surfactants

which makes cationic polymers as the preferred agent for lung delivery. 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 [27].

Among these polymers, PEI, especially high-molecular weight (25 kDa), is 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 and has no clinical applications. The high toxicity is due to non-biodegradable nature and very high charge density. The high transfection of branched PEI (25 kDa) is due to its flexible branching and favorable 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 (proportional to 1^{o} amine content) 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 indirectly means 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. Further the compactness of the polyplex is governed by the polymer backbone flexibility and charge density [28]. Unfortunately there is no such agent available for clinical applications with blend of above property which is non-toxic as well. Finally, the ultimate efficacy is also dependent on cell type in transfection indicating the importance of studies of vector application in disease relevant cell lines [29]. Therefore, ideal vector combining above properties has to be designed and should be validated in CF relevant applications.

As the ability of polymers to condense DNA and transfect cells is strongly dependent on the favorable amino group ratio, charge density and backbone flexibility of polymer. In this context an accurate balance of these properties when introduced into the non-viral vectors by different polymer modification and vector design approaches, can achieve high transfection efficiency and better therapeutic effect. By restricting the choice of polymers to the biodegradable nature, small molecular weight (if non-

biodegradable), optimizing charge density (25 - 35 % ionization at physiological pH), decreasing the amount required for transfection, can decrease the toxicity of polymer.

There has been a lot of research carried out to modify the gene delivery vector to induce favourable properties such as reduced toxicity, improved biocompatibility, improved transfection etc [30-33]. The key to these modifications lies in understanding of basics of transfection and cation charge mediated toxicity. The factors such as buffer capacity of polymer play an important role in endosomal escape of vector, therefore parameters affecting this property is also an active area of research [34]. The amine group ratio of polymers like chitosan, gelatin, poly (amino acids), can be altered to mimic properties of PEI. Further, the properties of two different polymers can be combined through crosslinking. To further improve the transfection of polymers which do not have amino groups or low buffer capacity, the grafting of amino residues with pKa in the range of 6-7 (e.g. histidines, imidazoles) can be carried out. By varying the quantities of crosslinking agent and substituents a library of modified polymer can be prepared. The charge density required for cell uptake is function of all changes affecting polymer structure and ionization. The backbone flexibility can be modulated by grafting of long chain alkyl amines (e.g. spermine, adipic acid, stearyl amine etc.) into the polymer backbone.

The pulmonary route of delivery has been the active area of research due to its advantage for localized delivery at sufficiently high concentrations, with minimal systemic side effects [35]. Therefore, proteins and gene therapeutics are preferred for delivery through pulmonary route. However, the unique anatomical organization of respiratory tract puts specialized requirements for the delivery systems claiming to be efficient at in delivering to lungs. Among them the three major inhalation platforms are: nebulizers, meter dose inhaler (MDI), and dry powder inhaler (DPI). The differences in underlying physical principles for each of the device provides distinct advantages leading to different applicability. However, the particulate behavior of the bulk systems decides the deposition characteristics and therefore stringent requirements have been laid down by the regulatory agencies for evaluation of the same. Further, all of these are amenable for combining their merits with advantages of carrier systems such as liposomes, micelles, nano- and polymers based microparticles to seek superior advantages in drug

delivery systems. These particulate based systems can provided improvements in the therapeutic index of new or conventional drugs by altering their absorption, decreasing metabolism, extending biological half-life or decreasing toxicity, increase bioavailability, better drug targeting and delivery. Specifically, the drug distribution and disposition can be controlled by characteristics of the carrier and no longer by physicochemical characteristics of the drug substance only.

The delivery through inhalation route, can provide localized delivery of siRNA to lungs with long duration of Na^+ channel inhibition and may lead to restoration of normal phenotype in CF patients. The respiratory tract, due to its direct exposure to the external environment, harbors several defense mechanisms against inhaled objects. The mucociliary clearance, coughing, and alveolar clearance are among the 3 major physical mechanisms for removing deposited foreign particles. Mucociliary clearance in conducting airways quickly clears the deposited particles into the pharynx. The absorptive or non-absorptive routes in the terminal airways (alveoli) can also remove deposited particles. The absorptive process include direct penetration into the epithelial cells or uptake and clearance by the alveolar macrophages. The particle transport to the ciliated region (conducting airways) forms the non-absorptive process which is followed by mucociliary clearance [36-38].

1.2. Objective of the proposed work:

The objective of the proposed investigation is development and characterization of therapeutic siRNA delivery system for cystic fibrosis.

1.3. Rationale

The goal of therapy in CF is to reduce the signs and symptoms of airway infection, inflammation and obstruction. However, none of these therapies can cure the disease. As the pathogenesis of CF lung disease is dominated by ENaC mediated sodium uptake by the respiratory epithelium, it has been proposed that a suppression of ENaC activity could lessen CF lung disease. Pharmacological approaches directed to inhibition of ENaC have been attempted. Therapeutic siRNA has been tried in clinical trials in various other diseases. However; in case of lung diseases like CF no clinical applications with siRNA have been started yet. The potential reason restricting the clinical application is the

unavailability of an efficient and non-toxic vector as a carrier for siRNA delivery and a suitable local administration technique, preferably inhalational route [39]. Chemical modification of polymeric carriers through substitution or conjugation can alter the transfection and toxicity behavior of the polymeric carriers. 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 [40]. These combined attributes indicate that inhaled siRNA therapeutic targeting Na^+ channel, is the most relevant form for future clinical application in CF treatment.

1.4. Hypothesis:

It is hypothesized that the carrier mediated delivery of therapeutic siRNA trough pulmonary route will improve the therapeutic benefits in treatment and will significantly reduce the patient treatment burden.

1.5. Research Plan: Aims & Objective

Development of delivery system for siRNA delivery in CF, having ideal properties of non-viral vector required for efficient transfection with reduced toxicity and in dosage form amenable for delivery through inhalation route. Thus objectives of this work are,

i. To develop a polymeric vector by polymer modifications focused at improving transfection capacity and low toxicity.
ii. To evaluate the developed vector by quantitative assays and structural elucidation.
iii. To characterize the vectors for physico-chemical properties, proton sponge effect/buffer, siRNA condensation capacity using Agarose Gel Electrophoresis.
iv. To evaluate the developed polyplex for biocompatibility, in vitro toxicity and in vitro efficacy in suitable cell line models.
v. Development of dry powder inhalation dosage form of siRNA loaded polyplex.
vi. To evaluate the optimized formulation in vivo.

1.6. Expected Results

Development of non-viral vector as a carrier for siRNA delivery for delivery through inhalational route having ideal properties of non-viral vector required for efficient

transfection with reduced toxicity and preserving stability. Favorable results from present study would provide for improved clinical applicability of siRNA therapeutics. The study would provide literature base for insights into efficacy and safety by furnishing effect of newer variables.

1.7. Work Plan

- Literature survey
- Selection & procurement of siRNA, polymer and other excipients
- Analytical method development
- Development and optimization of polyplexes
- Physico-chemical characterization of vector
- In vitro cell line studies
    - ✓ Cytotoxicity study
    - ✓ Intracellular uptake by confocal microscopy and flow cytometry
    - ✓ Gene expression study
- Development and optimization of dry powder for inhalation
- In-vivo evaluation

1.8. References

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