Chapter 1: Introduction

1.1 Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are the leading obstructive airway disorders globally, because of high prevalence and high burden on system of healthcare. Asthma is a chronic disorder of the airways generally featured by airway inflammation, airflow obstruction and airway hyper responsiveness. Allergen sensitization is significant risk for the asthma development. As per WHO report, it is estimated that currently 235 million people suffers from asthma and about 250,000 annual deaths worldwide and about 100 million people in India for asthma and COPD(1)and majorly common non-communicable disease in the children(2).Regardless of the development level, Asthma is a serious health issue among the all part of the world including lower middle and high-income countries. Further, Asthma generates significant burden to the individual persons and family, if under diagnosed and under treated and most of the time limits the individuals' activities for throughout life(2).

Asthma is characterized as recurrent reversible airway obstruction, with wheezing, breath shortness and frequently night-time cough and its main feature involves the airway inflammation, which causes the airway hyper-responsiveness and results into in reversible airway obstruction. Pathogenesis of the asthma involves, following the exposure of the allergens or stimuli activates the T cells with a Th2 profile of cytokine production, in mucosa, which attract inflammatory granulocytes following release of several other inflammatory mediators which cause damage to epithelium and one of the cause of airway hyper responsiveness.Key mediators such as leukotriene B_4 and cysteinyl leukotrienes (C_4 and D_4); interleukins IL-4, IL-5, IL-13; and tissue-damaging eosinophil proteins are released and mediates an strong, instantaneous inflammatory reactions (3).

Chronic obstructive pulmonary disease (COPD) is featured by airflow limitation progressively that is partially reversible and causes breathlessness, inflammation of airways and systemic effect. Study reports for the global burden of the COPD state that occurrence of 251 million cases of COPD in 2016 worldwide and it is estimated that in 2015, 3.17 million deaths were due to COPD. Major cause of the COPD is tobacco

smoke exposure and other factors include the occupational dusts and fumes, air pollution exposure. It is believed that disease is possibly augment in upcoming years owing to increase in smoking prevalence and aging populations in number of the countries globally (2) (4).

. COPD is mainly runs a progressive downward course (5) comprise the two major clinical situations, namely chronic bronchitis, defines as an inflammation of the bronchial tubes and occurrence of cough and mucous expectoration and second, emphysema, characterized by permanent damage to the alveoli. Following to the respiratory airway inflammation numbers of events occurs such as vasodilation, edema of the mucosa, congestion and hypertrophy of goblet cells. Subsequently, these events activate goblet cells to generate surplus amount of the mucus.

Several inflammatory mediators are involved in the inflammatory reactions of the asthma and COPD such as Chemokines are significant in the inflammatory cells recruitment into the respiratory airways and are principally expressed in airway epithelial cells (6-8), Cysteinyl leukotrienes, potent broncho-constrictors and pro-inflammatory mediators chiefly resultant from mast cells and eosinophils (9), released cytokines plan the inflammatory response in asthma. Chief Cytokines covers Interleulkine-1β and TNF-α and GM-CSF and Prostaglandin D2 is a bronchoconstrictor (10).

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

Neurotrophins, by modulating sensory nerve innervations, provoke the synthesis of neuropeptides namely or neurokinin A and B and substance P which are belongs to tachykinin family. Sensory neurons release these neuropeptides change range of responses of different cells, which leads to cell activation and differentiation. Such

neurotransmitters release in direct potentiation of cascade of inflammatory reactions called “neurogenic inflammation”

Neurotrophins, the key mediators in the neuronal plasticity and neurogenic inflammation, Asthmatic and patients with chronic obstructive disorders have been observed for enhanced levels of neurotrophins in BALF fluid. In preclinical model, Neurotrophins synthesis observed to be increased in macrophages and T cells infiltration airways. Initially it has been observed that neurotrophins have been enhanced in inflammation of airway came from clinical results. Bonini and coworkers have reported elevated level of NGF in asthmatic patients in 1996 (11). Indeed, using animal models one researchers group has evidently demonstrated that immune cells are one of the chief source of Brain derived neurotrophic factor and Nerve growth factor during airway inflammatory conditions (12, 13).Hence, neurotrophins can be potential targets in the airway inflammation and hyper-reactivity conditions to treat asthma and chronic airway obstructive conditions (8, 14, 15). High morbidity of the main pulmonary disorders such as airway inflammatory conditions of asthma and COPD, carcinoma, make pulmonary site as a promising target for drug and gene delivery for which current treatment therapy are mostly inadequate and high mortality of the patients as well.

Delivery of drugs and gene therapeutics via pulmonary route remains favorable and attractive route due to several advantages over other routes like non-invasiveness, large surface area of the lung epithelium, localized delivery, escaping first pass metabolism etc.

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

siRNAs, which are small molecules of RNA with exogenous origin, have demonstrated gene silencing of the over expressed gene very effectively and specifically. siRNAs having greater gene-silencing ability demonstrated stability in biological fluids. Silencing

of the gene can be provoked by siRNAs by specific cleavage of complementary mRNA. RNAi based therapeutics provides several advantages such as it can inhibit every class of the targeted genes with high selectivity and potency, can offer personalized therapy. Hence, gene silencing by the RNA interference mechanism by siRNA is promising approach in the airway inflammatory conditions and hyper-reactivity by targeting the neurotrophin, Brain derived neurotrophic factor BDNF, which is greatly enhanced in airway inflammation (16).

siRNA can be delivered as such in naked form or through making electrostatic interaction with cationic lipids and cationic polymers to augment the intracellular release and stability *in vivo.* Therapeutic siRNA delivery has constantly been challenging task because of negative charge and very prone to degrade by nucleases and hence need efficient delivery vectors systems to deliver payload within target tissue or cell with maintain biological efficacy. Numerous viral and non-viral vectors have been explored for the delivery of siRNA or gene via pulmonary route. Viral vectors are favored for superior transfection efficiency. Nevertheless, disadvantages of viral vectors such as immune reactions, inflammation response, oncogenicity and production at large scale issue restricts their application as gene delivery vectors. i.e. Numbers of clinical trials have been performed for Cystic fibrosis as a efficient gene delivery carriers however till date none of the developed vectors achieved success because of *in vivo* challenges.(17,18).

Thus, in recent years, non-viral based delivery carriers have attracted increasing attention owing to advantages such as low immune response, ease of synthesis and can be tailor made in addition to potential advantages in terms of safety. Novel non viral vectors include cationic polymers and cationic lipid based complexes, liposomes, nanoparticles, dendrimers, inorganic nanoparticualtes. Liposomal vesicles have demonstrated relatively low entrapment efficacy, poor storage stability and fast clearance from the blood. Hence, non-viral systems based cationic polymers containing several amines in their structure backbone have been used extensively as gene delivery carriers like polycations polyethylenimine, poly-l-lysine, Chitosan, PAMAM dendrimers etc.

Novel approaches to conquer these problems have been studied extensively as pulmonary gene delivery such as mucus penetrating particles to cross mucosal layer and by modifying the hydrophilic -hydrophobic balance of the polycations, Conjugating the amino acids, synthesizing low molecular weight molecules to improve solubility at physiological pH (Chitosan), to improving buffering capacity of the vectors for endosomal escape etc.

Among the described above cationic polymers, Chitosan, a biodegradable polycation *in vivo*, has been studied extensively as drug delivery carriers. Chitosan, a natural polysaccharide obtain from deacetylation of the chitin, has advantageous characteristics such as biocompatibility, low toxicity and immunogenicity, high positive charge density. Owing to positive charge, chitosan can easily form polyelectrolyte complexes with anionic charged siRNA or pDNA by electrostatic interactions. Nevertheless, efficiency of the chitosan as a gene delivery vectors is considerably affected by formulation related parameters such as molecular weight, degree of deacetylation, N/P ratio, salt form of chitosan, plasmid concentration, stability against polyanions, etc. Nevertheless, its low aqueous solubility and poor stability at physiological pH in vivo, low cell specificity and subsequent low transfection efficiency limits its applications as a gene or siRNA delivery carriers *in vivo*(19). To overcome these limitations of chitosan as a gene delivery vector, several approaches and chemical modifications have been sought such as hydrophilic modification by PEG, carboxy methylation, succinylation, quaternization of the chitosan to improve the colloidal stability and solubility at physiological pH, conjugation with ligands for cell specific targets, grafting with PEI to improve transfection efficiency etc(19, 20).

Here, in present work, trimethylation of the chitosan has been sought to improve the aqueous solubility and stability at the physiological pH. As, stability of the vectors is most important for intracellular release of the gene therapeutics, endosomal escape and for dissociation of the gene or siRNA from the complexes. Furthermore, to improve the transfection efficiency conjugation of the heterocyclic moieties on the trimethylated chitosan containing imidazole, piperazine and pyridine rings in their structure has been sought as pKa of these moieties in between 5.0-6.5, so it may improve the buffering

capacity of the chitosan and may assist in endosomal escape of the formulations and release into cytoplasm.

Secondly, PEI is well-known as good transfection efficiency. PEI has many advantages, but due to high cytotoxicity and lack of biodegradability, the clinical applications are limited High charge density and non-biodegradability of the polymer reasons for high cytotoxicity. The high transfection of branched PEI (25 kDa) is due to its flexible branching and favorable amine ratio of 1^o:2^o:3^o of 1:2:1. It has been anticipated that cellular uptake depends on presence of cationic charge (proportional to 1^o amines) at physiological pH forcing the cell-vector interaction. While the endosomal escape depends on the presence of protonable amines of the polymer (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. Furthermore, the compactness of the polyplexes is governed by the polymer backbone flexibility and charge density. Unfortunately, there is no such agent available for clinical applications with blend of above property which is non-toxic as well. Hence, ideal vectors for siRNA delivery combining above characteristics have to be designed and should be validated in pulmonary inflammatory conditions.

Condensation ability and transfection efficiency of the polymer is govern by charged density, available amines and backbone flexibility (21). By keeping in mind these context, different polymer modification and vectors design approaches have been sought in the recent many years for improved transfection efficiency and low cytotoxicity. There has been extensive research work carried out to modify the gene delivery carriers to induce favorable characteristics such as low toxicity, improved biocompatibility, improved transfection etc. (22-25). Buffer capacity of polymer play a significant role in endosomal escape of vector systems. So, factors affecting this property is also an active area of research in recent time (26). Furthermore, to improve the transfection of polymers which do not have amino groups or low buffer capacity, amino residues grafting with pKa in the range of 6-7 (e.g. histidines, imidazoles) can be carried out. In present work, polyethylenimine modification by Urocanic acid, containing

imidazole moiety have been sought to improve the transfection efficiency and to reduce the cytotoxicity of the polymer.

Drug delivery via pulmonary route has been the active research area due to its advantage for local delivery at adequately high concentrations with minimal systemic side effects(27). For that reason, proteins, peptides and gene therapeutics are favored for delivery through pulmonary administration. Nevertheless, the unique anatomical organization of respiratory tract puts specialized necessities for the delivery systems or devices claiming to be efficient at in delivering to lungs. Among them the three major inhalation platforms are: dry powder inhaler (DPI), nebulizers, meter dose inhaler (MDI). The differences in underlying physical principle mechanisms for each of the device present distinct advantages leading to different applicability. Though, 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. Additional, all of these delivery devices are amenable for combining their merits with advantages of carrier systems such as nanoparticles, liposomes, micelles; polymers based microparticles to seek better advantages in drug delivery systems. These particulate based systems can provide improvements in the therapeutic index of new or conventional drugs by altering their absorption, decreasing metabolism, extending half-life or reducing toxicity, increase bioavailability, better drug targeting and delivery.

Delivery via inhalation route can provide local siRNA delivery to lungs with inhibition of inflammatory cascade in the asthma and chronic airway disorders. The respiratory tract, towing to its direct exposure to the external environment, harbors several defense mechanisms against inhaled stuff. The mucociliary clearance, alveolar clearance and coughing are major physical mechanisms for removing 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 can also remove particles deposited. The absorptive process includes direct penetration into the airway epithelium or uptake and alveolar macrophage clearance. The particle transport to the ciliated region (conducting airways) forms the non-absorptive process which is followed by mucus clearance(28, 29).

1.2 Objective of the research work

The objective of the research work to develop nontoxic cationic polymers based non-viral vectors for therapeutic siRNA delivery to treat obstructive airway disorders via pulmonary route.

1.3 Rationale

The goal of therapy in asthma and COPD is to reduce the signs and symptoms of airway inflammation obstruction of airways and airway hyper-reactivity or hyper-responsiveness. Nevertheless, none of these therapies can cure the disease. As the pathogenesis of these pulmonary inflammatory diseases is dominated by inflammatory cascade including release of the inflammatory mediators and neurotrophin mediated neurogenic inflammation. It has been proposed that a suppression of neurotrophins, specifically brain derived neurotrophic factor could lessen the airway inflammation and thereby so reducing airway hyper-responsiveness. Therapeutic siRNA has been tried in clinical trials in various other diseases. However; in case of lung diseases like asthma and COPD no clinical applications with siRNA have been started yet. The probable reason restricting the clinical application is the unavailability of an efficient and non-toxic vector as a carrier for siRNA delivery and an appropriate local administration technique, preferably inhalational route. Modification of cationic polymers through substitution or conjugation can change the transfection and toxicity behavior of the polymeric carriers. The inhalation route is highly convenient and patient compliant and it permits one to broadly distribute the therapeutics along the airways. Furthermore, it gives localized action and reduces the systematic adverse effects (30). These combined attributes indicate that inhaled siRNA therapeutic targeting brain derived neurotrophic factor, is the most relevant form for future clinical application in obstructive airway disorders treatment.

1.4 Hypothesis

It is hypothesized that development of safe non-viral vectors or carriers to deliver siRNA therapeutics by pulmonary route will improve the therapeutic efficacy of the treatment of obstructive airway disorders with minimum toxicity.

1.5 Research Plan

Development of non-viral vectors or carriers-based siRNA therapeutic delivery for treatment of inflammatory conditions of asthma and COPD requires the higher transfection efficiency with minimum toxicity and preserving stability of formulation during aerosolization.

So, the objectives of the research work are:

1. Development of the suitable non-viral vectors or carriers focusing the safety and complexation ability of the carriers.
2. Characterization of the developed carriers in terms of buffering capacity, proton sponge effect, condensation ability to siRNA by gel retardation assay and physicochemical characteristics.
3. Development and evaluation of the formulations containing siRNA in terms of *In vitro* cytotoxicity by MTT assay using suitable cell line and cellular uptake.
4. Development of nebulised form of siRNA formulation and evaluation in terms of siRNA integrity and formulation stability and performance during nebulisation.
5. Evaluation of *in vivo* efficacy of the formulation in suitable animal model.

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