Chapter 2: Literature Review

2.1 Introduction

Bronchial asthma and Chronic Obstructive Pulmonary Disease (COPD) are the obstructive pulmonary disorders that affected millions of individuals globally. These two diseases have many likenesses and many differences which may further confuse therapists in diagnostics and treatment of these disorders which may affect more and more peoples every year globally.

2.2 Asthma

Asthma may be defined as the chronic inflammatory disorder of the respiratory tract. Chronic inflammation is related with the airway hyper-responsiveness and hyper-reactivity which may leads to frequent episodes of breathlessness, wheezing, coughing, chest tightness and particularly either at the night or in the early morning. These types of episodes are generally linked with prevalent, but the variable airflow obstruction of airways inside the lungs that is frequently reversible moreover spontaneously or with therapy (1,2).

Asthma is one of the severe global health problems of airways with a projected 300 million exaggerated individuals (2,3) Clinical expressions of asthma can be controlled with appropriate medicated therapy. Clinical spectrum of the disease is 1extremely variable, but then the inflammation of the airways leftovers a constant feature (1). Factors that may affect the risk of asthma could be divided into those that cause the growth of disease and those that triggering the asthmatic symptoms. The first one includes the host factors (mainly genetic) and other one is usually associated with the environmental factors (4, 5, 6).

2.2.1 Airway inflammation in Asthma

An inflammation of the respiratory airways in the asthma is persistent, however indications are episodic ones, and the association among the severity of the disease and the intensity of the inflammation is not clearly recognizable. The inflammation may affect all airways covering the upper respiratory airway and in nose in most of the cases but then again, its physiological properties are the most prominent in the medium sized

bronchi. The pattern of the inflammation in the respiratory airways seems to be comparable in all clinical form of the asthma, regardless of the cause of the disease whether it was allergic, non-allergic, or may aspirin-induced (1).

2.2.2 Inflammatory Cells in Asthmatic Airways

Bronchoconstrictor mediators i.e. histamine, prostaglandin D2 and cysteinyl leukotrienes release by the activated mucosal mast cells. Mast cells were activated by the allergens by means of osmotic stimuli or by IgE receptors (7). Numbers of eosinophils increases in the airways and may release the basic proteins that might damage the epithelial cells of the respiratory airway and may have a significant role in releasing of the certain growth factors (8). T-lymphocytes are also increases the release specific cytokines, with including IL-4, IL-5, IL-9, IL-13 that may orchestrate eosinophilic inflammation and the IgE production by the B lymphocytes (9). Furthermore, large amounts of T helper i.e. Th1 and Th2 cytokines are released by increased KT cells(10, 11), Macrophages and Dendritic cells increases, and release the inflammatory mediators and cytokines that may further intensify the inflammatory exacerbations(12, 13).In addition, numbers of neutrophils are increased in the respiratory airways and sputum of the patients with severe asthmatic conditions and also in smoking asthmatics, nevertheless the role of these cells are undefined and their increase may also due to the steroid therapy (12, 13, 14).

2.2.3 Inflammatory Mediators Involved in Asthma

Chemokines are significant in inflammatory cells recruitment into respiratory tract and are principally expressed in the respiratory airway epithelial cells (15, 16, 17). Eotaxin is selective for the eosinophils, although thymus and activation regulated chemokines (TARC) and macrophage-derived chemokines (MDC) recruits the Th2 cells (16, 17). Cysteinyl leukotrienes are the potent broncho constrictors and also the proinflammatory mediators chiefly derived from the mast cells and eosinophils (18). Chief Cytokines such as IL-1β, TNFα, and GM-CSF. In addition, Th2-derived cytokines such as IL-5, which is required for the eosinophil differentiation and survival; IL-4, which is imperative for the Th2 cell differentiation; and IL-13, required for the IgE formation (19). While histamine is released from the mast cells and contributes to the bronchoconstriction and

inflammation (15, 16). Nitric oxide (NO) is a potent vasodilator which is produced from the syntheses in the airway epithelium (20). Exhaled NO is wildly being used to monitor effectiveness of the asthma treatment (21). Prostaglandin D2 is the bronchoconstrictor and derived mostly from the mast cells and is involved in the Th2 cell recruitment to the respiratory airways (7). Moreover, structural cells of the airways involved in pathogenesis of the asthma are: airway smooth muscle cells, respiratory epithelial cells, fibroblasts, endothelial cells, airway nerves and myofibroblasts (13, 14, 22, 23).

2.3 Chronic Obstructive Pulmonary Disease (COPD)

COPD, major causes of chronic mortality and morbidity globally. Numerous individual suffers from this disease for many years and die prematurely due to its complications. COPD, pulmonary disease having some significant extra-pulmonary effects which may contribute to severity in the individual patient. Limitation of the airflow is generally progressive and may related with abnormal inflammatory responses of lung to the noxious particles or gases (24).

Impact of the COPD on patients may depends on severity of the symptoms particularly cough, mucous production, breathlessness, reduced exercise capacity, and any of the co-morbidity the patient might have not just on degree of the airflow limitation (24, 25, 26). COPD was characterized by the chronic airflow limitation with the range of pathological changes in lung, significant extra-pulmonary effects and also the important co-morbidities like loss in body weight, cachexia, osteoporosis, skeletal muscle wasting, cardiac arrhythmias, anemia, heart failure, cardiac ischemia, diabetes and cognitive deficits (27) that may contribute to severity of disease in the individuals patient (25, 27). Moreover, cigarette smoking is also the utmost frequently encountered risk factor for the COPD, while in many republics, air pollutions resulting from the wood burning and further biomasses fuels been recognized as a risk factor for COPD (24, 25,28).

2.3.1 Inflammatory Cells in COPD

In COPD, neutrophils are increased which are present in the sputum of smokers and associated to severity of the disease. These cells may be more important in mucus hyper-secretion and through the release of proteases enzymes. Macrophages, large in numbers present in lung parenchyma, airway lumen, and broncho-alveolar lavage fluid (BALF). Moreover, they produce inflammatory mediators and proteases and might display defective phagocytosis. T lymphocytes, both of the CD4+ and CD8+ cells are proliferated in the airway tract wall and lung parenchyma, in large ratio of CD8+/CD4+. Amplified CD8+ T-cells (Tc1) and Th1 cells that may secrete the interferon-γ and express the chemokine receptor, CXCR3. While CD8+ cells may be cytotoxic to the alveolar cells. B lymphocytes, increases in the peripheral airways and within the lymphoid follicles, probably as a response to the colonization and infection. In addition, eosinophils increase in the airway wall and eosinophil proteins in the sputum during exacerbations (29-33).

2.3.2 Inflammatory Mediators Involved in COPD

Lipid mediators and Chemotactic factors like leukotriene B4 attracts the neutrophils and T-lymphocytes, Chemokines: such as interleukin-8 attracts the neutrophils and monocytes. Proinflammatory cytokines: like tumor necrosis factor-(TNF-α), IL-1 β, and IL-6 intensify the inflammatory process and may contribute to the systemic effects of COPD. Growth factors, such as transforming growth factor-ß may tempt fibrosis in the small airways (29, 30, 32).

2.3.3 Airflow Limitation

The chronic airflow limitation is produced by the combination of small airway disease i.e. obstructive bronchiolitis and by the parenchymal destruction i.e. emphysema, the relative contributions and may vary from person to person. Further, Chronic inflammation grounds for the structural changes and small airways narrowing. Lung parenchyma destruction, also by the inflammatory processes, may leads to loss of alveolar attachments to small airways and reduces the lung elastic recoil; in turn these alterations lessen the ability of airways to remain open during expiration. Hence,

inflammation of the disease causes small airways inflammation, airway remodeling and lung parenchyma destruction that all prime to airflow limitation.

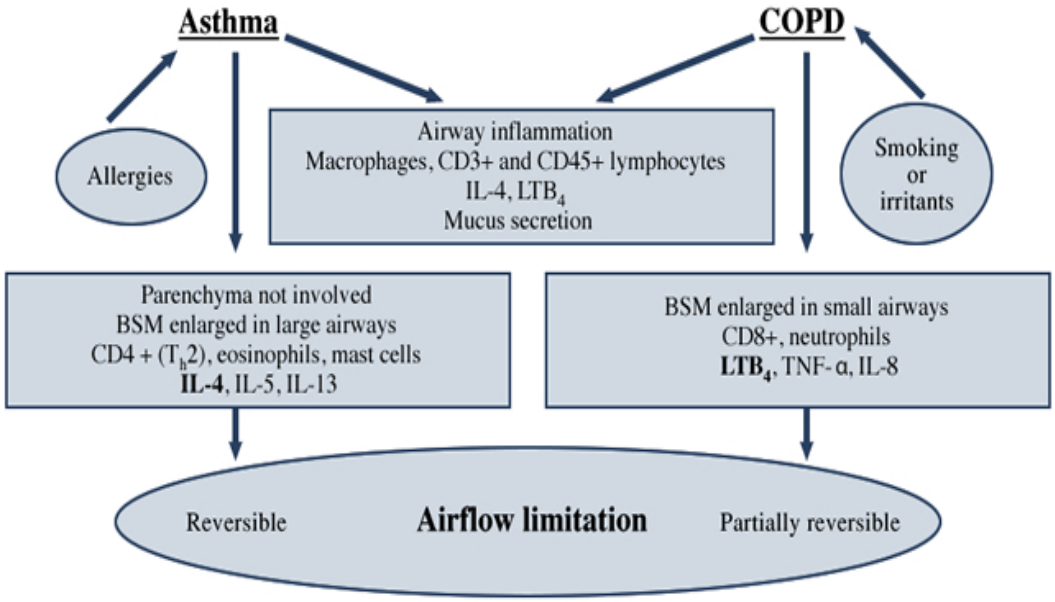

Figure 2.1: Airflow Limitation in Asthma and COPD

2.4 Current Medications

Asthma and COPD are a common respiratory problem globally, with recent evidence of suggesting an increasing prevalence in the developed countries. It is also projected that nearly one-half of the patients with asthma have underlying allergies. Medication therapy to treat asthma falls into 2 main categories, first, bronchodilators and second, anti-inflammatory drugs. Based on the symptoms and disease severity, therapy is escalated in a stepwise manner. National Asthma Education and Prevention Program current guidelines, deliver evidence-based recommendations in the diagnosis and management of the disease. Pharmacotherapy updates are provided covering new recommendations for utilization of the inhaled corticosteroids, β-agonists, cromolyns and leukotriene receptor antagonists etc. (Table 2.1)

Table 2.1 Current medications used in asthma and COPD

Bronchodilators (relieve bronchospasm)		
Adrenergic drugs	Non-selective	Adrenaline Isoprenaline Ephedrine
	Selective beta-2 agonists	Salbutamol Albuterol Terbutaline Levalbuterol Bambuterol Indacaterol Formoterol Salmeterol
Anti-cholinergic drugs		Ipratropium Tiotropium
Methyl-xanthine's		Theophylline Aminophylline
Anti-inflammatory drugs (prevent bronchospasm)		
Corticosteroids	Inhalational	Beclomethasone Budesonide Fluticasone Triamcinolone
Mast cell stabilizers		Sodium Cromoglycate Nedocromil Ketotifen
Anti-leukotriene drugs	Leukotriene synthesis inhibitor	Zileuton
	Leukotriene receptor antagonists	Montelukast Zafirlukast
Phosphodiesterase-4 (PDE-4) inhibitors		Roflumilast

2.5 Neurotrophins in lung diseases

Neurotropins are the growth factors that mainly found in the nervous system and works as survival and differentiation of the neurons. However, recent several studies evident those neurotrophin receptors are also expressed and released in the non-neuronal tissues as well. I.e. lung. Several research has been described that in the lung tissue, neurotropins involves in the inflammatory responses and immune reactions. In the airway inflammatory conditions, neurotrophins are mediated in the inflammatory actions and found enhanced in the BAL fluid in animal models of asthma. Additionally, salient features of the asthma like inflammation, bronchoconstriction and hyper-reactivity are mediated by the chief neurotrophins, Brain derived neurotrophic factor (BDNF) and nerve growth factor(NGF).Hence, BDNF and NGF are potential targets for pulmonary inflammatory conditions and airway obstruction.

2.5.1 Basis of BDNF expression and signaling

Growth factors of neurotrophin family especially brain derived neurotrophic factor control the survival and neurons differentiation, neuronal plasticity and nerve conductions as well (35-44). There are substantial facts that neurotrophins, especially BDNF are crucial factors in diseases such as cerebral tumors, spinal cord injury repair, depression, Alzheimer's disease (45-53). Here it is more and more apparent that BDNF is not only growth factor but its expression and signaling pathway are complicated entangled with other pathways. Documented evidence in airway inflammatory conditions, the number of pathways are significant (57-62). Following figures illustrates the BDNF production and its signaling pathway, which may give the ideas into airway inflammatory diseases. Following figure 2.2, figure 2.3 and figure 2.4 demonstrates production of BDNF, neurotrophins receptors and BDNF signaling, respectively.

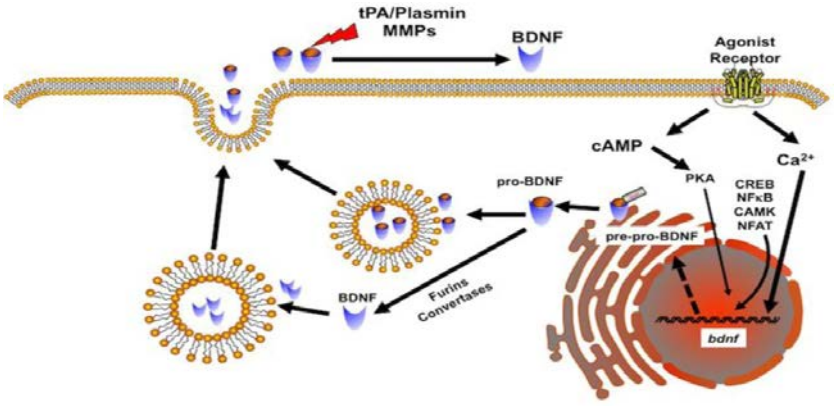

Figure 2.2: Production of brain-derived neurotrophic factor (BDNF)

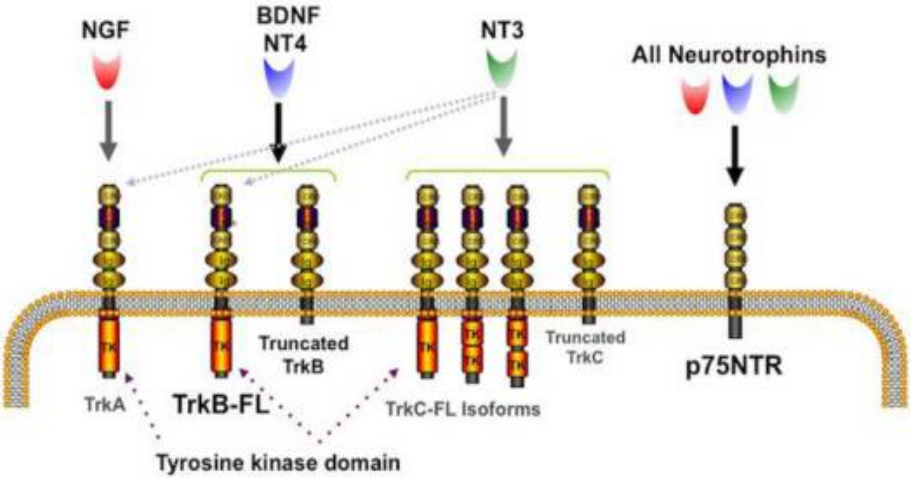

Figure 2.3 : Neurotrophins and their receptors

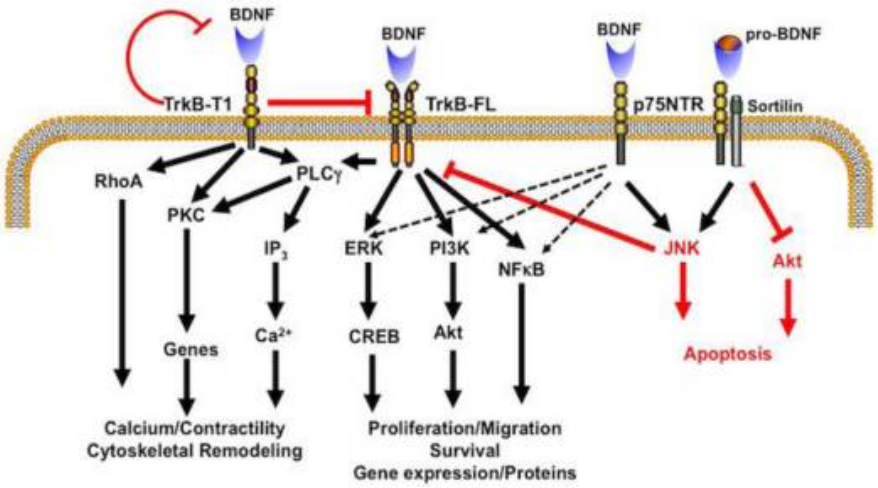

Figure 2.4: BDNF signalling

2.5.2 BDNF in the lung

Now it is obvious that Trk receptors are distributed in non-neuronal tissues as well. It is evidenced that neurotrophins and their receptors are expressed in the lung tissue at different levels in different cells. i.e. airway epithelium, fibroblasts, smooth muscle and immune cells as well. In recent study demonstrated that BDNF levels is augmented in the pulmonary inflammatory conditions and also evidenced that its receptors expression is also increased which can be suggested by clinical findings of BALF and sputum, as these samples are confirmed for elevated levels of BDNF in the patients of COPD which may be due to cigarette smoke, allergy or any other means (63-65). These results undoubtedly suggest that enhanced BDNF level is correlated with the airway inflammatory diseases.

2.5.3 BDNF and Pulmonary inflammatory conditions

Inflammatory cascade triggered by the allergy or other causes, presents the airway hyper-reactivity, broncho-constriction, anatomical and structural changes in airways leading to obstruction of the airways in asthma and COPD.

Tetsuya Watanabe et al have demonstrated that BDNF is associated with type-2 inflammatory responses and severity of the disease. Findings suggested noticeably increased amount of BDNF in asthmatic sputum in comparison to healthy groups (65). In another study in asthmatic children demonstrated the elevated level of BDNF and eosinophils in the blood and sputum specimens. it is believed that BDNF controls the crosstalk in between immune system and neuronal systems which performs major role in pathophysiology of inflammation of airways (66). Armin Braunet al confirmed in the allergic mice model of airway inflammation that BDNF play a role in the neuronal dysfunction and consequently enhanced the hyper-reactivity of the airways and increase the airway tone continually (67). BDNF also regulate the signaling of calcium and contributes in hyper-responsivess by interactions with cytokines. Furthermore, BDNF may proliferates the airway smooth muscles explaining its significance in the airway remodeling (68,69). From above all clinical and preclinical reports suggested that BDNF plays significant role in asthmatic airway inflammation and airway hyper-reactivity and hence can be promising target for the future therapy of the asthma and COPD.

2.5.4 BDNF as a potential target for treatment of asthma and COPD

BDNF and its receptors present potential targets from the point view that understanding the airway functions and mechanisms and investigating alterative therapeutics for the pulmonary inflammatory diseases. Nevertheless, BDNF displayed the pleiotropic actions in the airways which would be more attractive with respect to treatment therapeutics but also would be confront to achieve cell specificity; in particular, those are difficult to access.

2.6 Neurotrophins in inflammatory conditions

2.6.1 Neurogenic inflammation by neurotrophins

Allergy induced inflammation cause release of the several inflammatory mediators such as which modulate the structurally and functionally sensory innervations in the lung tissue. It is much evidenced that released inflammatory mediators during the course of inflammation activate the immune cells following to the nerve innervations and neuropeptide production. Recent results explained that neurotrophins plays a role in crosstalk between nervous system and immune system (70-73).

Neurotrophins, by modulating functions of sensory nerve, stimulates neuropeptide synthesis i.e. Neurokinin A & B and Substance-P. Sensory nerves releases neuropeptides that amend the immune system response and consequently activates and differentiate the immune cells (74-79). Release of growth factors or neurotransmitters which potentiates the inflammatory response in the lungs directly called **“neurogenic inflammation”.**

2.6.2 Allergic immune response amplification

It is apparent that structural cells release the neurotrophins in inflammation of airways by crosstalk interaction with immune cells. Results of the recent study, demonstrated elevated neurotrophins in the BALF after challenging the mice with the known allergen, Ovalbumin; BDNF and NGF founds majorly in the epithelial cells in inflamed lung chronically (80, 81). Furthermore, lung eosinophils co-culture with airway epithelial cells yielded in elevated release of BDNF and NGF and in extended eosinophils survivals in culture.

2.6.3 Angiogenesis and microvascular remodeling

In chronically inflamed lung, produced cytokines and growth factors locally favors angiogenesis and microvascular remodeling. These events mostly result from proliferation of endothelial cell and often occur simultaneously (82). In addition, it has been shown that nerve growth factor causes MMP 9 expression in vascular smooth muscle cells. One of the study finding suggested that BDNF survives the endothelial cells and provoke the neoangiogenesis in ischemic tissues (83). Amplified vascularization, fibrosis, hypertrophy of the smooth muscles, hyper-innervation of sensory nerves are characteristic features of the airway remodeling. It is evidenced that neurotrophins contributes to the airway remodeling. Furthermore, NGF provokes the collagen production in fibroblast cells and deposition in the airways during inflammation (84).

2.7 Gene/siRNA delivery in pulmonary diseases

Human lungs are generally bared to several pollutants, smoke and volatile organic compounds. This increase the human susceptibility to many inflammatory pulmonary disorders such as asthma, COPD, cystic fibrosis; infections like tuberculosis, Respiratory Syncytial virus infection and lung carcinomas. Such pulmonary diseases constitute a principal reason of morbidity and mortality in many patients around the globe. Mild conditions of pulmonary diseases weaken the quality of human life, whereas severe cases result in death. A high death rate related with pulmonary diseases is chiefly due to the fact that many of these conditions are tough or impossible to cure (85, 86).

Seeing the severity of the lung diseases, development of effective approaches to overcome these, has become very important. Asthma and COPD are the diseases related to persistent inflammation of airways. Corticosteroids still remain as the pioneering therapeutics for treating the airways inflammation and airway obstruction. Nevertheless, they are used with carefulness considering their possible adverse effects. Patients with refractory lung disorders also depend on corticosteroids but have to bear their systemic side effects such as weight gain and hyperglycemia. In this scenario, gene delivery approaches represent novel strategies to alleviate various lung diseases and could possibly be regarded as the future therapy (87-89).

Gene delivery is the novel promising approach defined as delivery of therapeutic nucleic acids using appropriate carriers as a therapeutic by gene expression of mutated gene or interfering the protein expression for treatment of various diseases including airway inflammatory conditions in asthma and COPD. Drug and gene delivery through pulmonary route remains favorable and attractive owing to several advantages over other routes. Owing to advantages of avoidance of clearance, high absorption area of the airway epithelium, local delivery of therapeutics, pulmonary route is preferred.

2.8 Basic of RNA interference

RNA interference (RNAi) unique mechanism of post-transcriptional gene silencing and is a pathway involved in cellular defenses against viral infections. RNAi is a molecular biology tool for gene functions and signaling pathways determinations, with huge potential in diagnostics and therapeutic area. RNAi-based therapies mostly depends on three main mediators: (a) dsRNA (double stranded RNA), which is cut into short small interfering RNA (siRNA) by the Dicer within the cellular cytoplasm, (b) a plasmid which encodes short hairpin RNA (shRNA) which is cleaved by Dicer into siRNA and (c) siRNA itself, which directs gene silencing of its complementary mRNA (messenger RNA). As compared to traditional drugs, RNAi based therapies exhibit superior therapeutic effects due to their high selectivity and potency. Furthermore, they may be exclusively designed to provide personalized therapy (90).

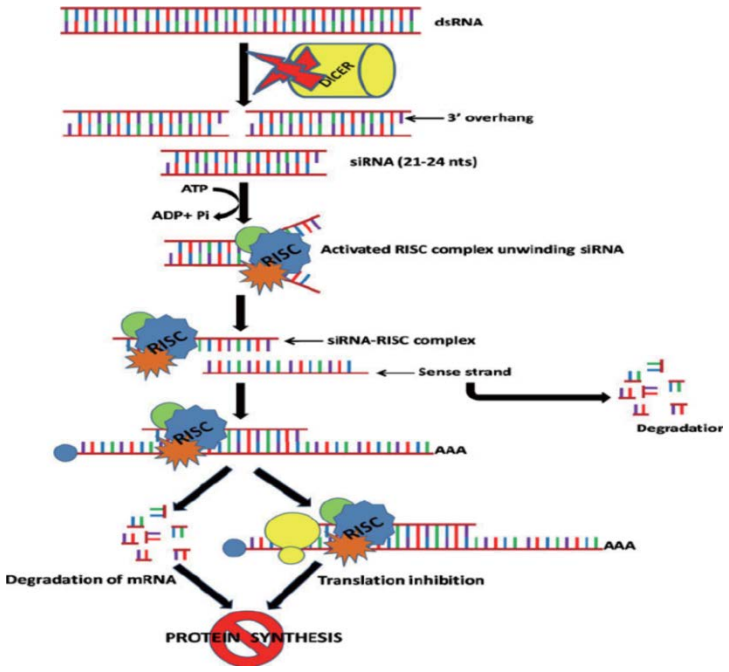

Figure 2.5: RNA interference mechanism

Since the advent of RNAi, investigators have investigated the therapeutic potential of siRNA molecules in a range of disorders and diseasic conditions. siRNA establish most popular choice among aforementioned types of nucleic acids used in RNAi therapy. A Key reason for this is that the siRNAs target the RISC (RNA-Induced Silencing Complex) present within cytoplasm. The RISC-loaded siRNAs act as a guide to recognize the complementary mRNA targets for nucleolytic cleavage and therefore down-regulate the associated therapeutic protein.

2.9 Other Potential targets for pulmonary gene delivery

Pulmonary route offers several advantages such as non-invasive route, lung epithelium for absorption, local delivery of the therapeutics etc. There are numerous potential therapeutic targets for gene delivery for pulmonary diseases. Based on the thorough knowledge of the molecular pathophysiology and cause factors of the disease, potential target can be identified. Basically two gene delivery approaches are there first, delivery of therapeutic gene in place of mutant gene by pDNA .i.e. In cystic fibrosis

transmembrane conductance regulator gene is responsible for cause of the disease. In recent years, several studies have been explored for gene therapy in cystic fibrosis by CFTR gene. Second approach is to downregulate the particular mRNA by RNA interference mechanism is very established approach for the treatment of the diseases.RNA interference, specially by silencing RNA is very promising. Here we have discussed the potential therapeutic targets in the asthma and chronic obstructive airway conditions, which can be targeted by the gene delivery by pDNA or siRNA.

Asthma is featured by airway inflammation, obstruction and hyper-reactivity of the respiratory airways. Pro-inflammatory mediators such as cytokines, interleukins etc releases upon any allergy or by other causative factors. IL-3, IL-5, IL-4 and other chemokines are major mediators in asthma pathophysiology. Targeting such interleukins by antisense approach seems promising for airway inflammation. Khaitov MR et al demonstrated that IL-4 siRNA considerably down regulates the mRNA expression of IL-4 and decreased the total cellular counts and specifically, eosinophils in the BALF and hyper reactivity of the airways as well (91). In another example, IL-5 siRNA has been studied and findings are same as previous example. IL-5 siRNA delivered through lentivirus effectively reduced the expression of mRNA and appreciably reduced the airway inflammation, eosinophils in the blood and BALF in animal model (92).

Number of reports has established that proteins, suppressor of cytokines signaling is found to be enhanced during asthmatic conditions. These proteins are mainly control release of cytokines. SOCS siRNA in the mouse model displayed the reduced hyper-responsiveness, furthermore decreased secretion of mucus and decreased in collagen.JAK/STAT and RhoA/Rho-kinase pathway involved in this mechanism (93).

Tumor necrosis factor alpha (TNF-α), one of the chief inflammatory mediators in the airway inflammation. TNF-α is another potential therapeutic target to treat inflammatory conditions. Adam Bohr et al developed cationic phosphorus-based dendrimer complexes for delivery of TNF-α siRNA and showed that upon intranasal administration of the formulations in model of acute lung injury, better gene silencing activity and further dendriplexes demonstrated the efficient complexation, elevated cell uptake (94). Other potential targets for asthma and COPD includes GATA3, nuclear factor-kappa B, Matrix

metalloproteinase, IL-8, spleen tyrosine kinase etc. and targets for other pulmonary diseases such as lung carcinoma includes RRMP, p53 gene, intracellular adhesion molecule-1, nuclear protein transcription factor; targets for pulmonary arterial hypertension such as fibroblast growth factor, vascular endothelial growth factor, surviving, BMPR-II gene; targets for cystic fibrosis includes CFTR, epithelial sodium channel etc.

2.10 Non viral vectors based gene delivery

However, delivery of the gene therapeutics via pulmonary route suffers the drawback of several barriers to reach that target tissue or cells. Such barriers include such as mucosa layer, intra and extracellular barriers, mucociliary clearance, cell surface barriers and chances of aggregation of delivery vectors in *in vivo* environment. Therefore, current research is going on to overcome such barriers and to make gene therapeutics efficacious. As previously discussed potential therapeutic targets for pulmonary diseases can be explored by the non-viral based delivery vectors as well. Several cationic polymers and lipids-based systems, nanocomplexes, liposomes-based delivery, polymer-based nanoparticles etc. have been utilized for gene delivery to lung with an objective to improve transfection efficiency, to overcome extracellular and intracellular barriers like mucus layer, intracellular endosomes, mucocilliary clearance etc**.** Delivery of nucleic acid can be made by well-established delivery devices such as metered dose inhalers, nebulization devices, and dry powder inhalers. Lung anatomy and physiology understanding is foremost necessities for development of efficient non-viral based gene delivery vectors.

Gene delivery approach has been extensively explored for cystic fibrosis by viral and non-viral vector delivery. In a study which demonstrated the nebulized delivery of receptor targeted peptide-based complexes using jet nebulizer (95). Results explained that aerodynamic size of the droplets in range of 5.5 µm to 1.4 µm demonstrating deposition of the formulation in central and deep lungs. Airway inflammation by allergy or by other means, also leads to the mucus secretion to airway epithelium. To alleviate the problem, siRNA delivery with targeting peptide by silencing the gene IL-4R-alpha over expressed in the inflammatory reactions has been done by (Hyo Sung Cho et al). Targeting peptide

binds to the nicotinic Ach receptors present on airway epithelium. Reduced inflammation has been confirmed by histopathological analysis demonstrating promising non-viral based vectors for inflammation conditions in lungs. Other strategies to overcome the mucosal barrier include the delivery with mucus penetrating nanoparticles are other potential carriers for penetration of mucosa layer. Surface charge of the particles and hydrophobicity has significant effect on mucus penetration of the nanoparticles. Some of the approaches to achieve the better mucus penetration includes by modification of the surface properties of the particles i.e. PEG coating on the particles better penetrates the mucus as PEG molecule is neutral charged and molecule is enough hydrophilic. Other reason for mucus penetration of PEG as its molecular weight is small too hold up the mucin contents by polymer chain penetration and also density of the PEG shields the hydrophobicity. In other hand, Pluronic F-127 is also utilized for the mucus penetrating particles by coating the hydrophobic surface by its hydrophobic segments while hydrophilic segments of PEG, stick out to the surface of the particles (96). Viscoelasticity of the mucus can be hindrance for the gene delivery vectors and hence delivery with mucolytic agents i.e. N-acetyl-L-cysteine that rupture the mucosa and reaches to the epithelium is another approach to cross the mucosal layer. Aerodynamic properties of the delivered formulation as a dry powder or liquid droplets are most significant parameters to understand the particle deposition mechanism as larger particles will deposited in the upper respiratory tract and particles having size less than 5 µ deposits in deeper lung by gravitational sedimentation. Particle engineering is the concept to formulate the particles to make it respirable my tuning the particle shape, size and density. In the development of the dry powders for the inhalation, such approach is promising.

Intracellular delivery of the gene therapeutics is also challenging as vectors has to cross the negatively charged cell membrane, cellular uptake, must be protected from the endosomes and must be disassociates from the complexes completely and all the payload must release at the site. To overcome such challenges, lots of the work has been done by the researchers. Cationic polymer PEI, known for proton sponge effect and its very good buffering capacity it assists in the endosomal escape of the vectors. Non viral vectors containing pH sensitive moieties or peptide or molecules having fusogenic properties are the promising to escape the carriers from endosomes to release the payload into the cell

cytoplasm. i.e. DOPE has a membrane destabilization activity, at lower pH of endosomes, DOPE transits its structure from bilayer to invert hexagonal phase and thereby release the contents into the cytoplasm.

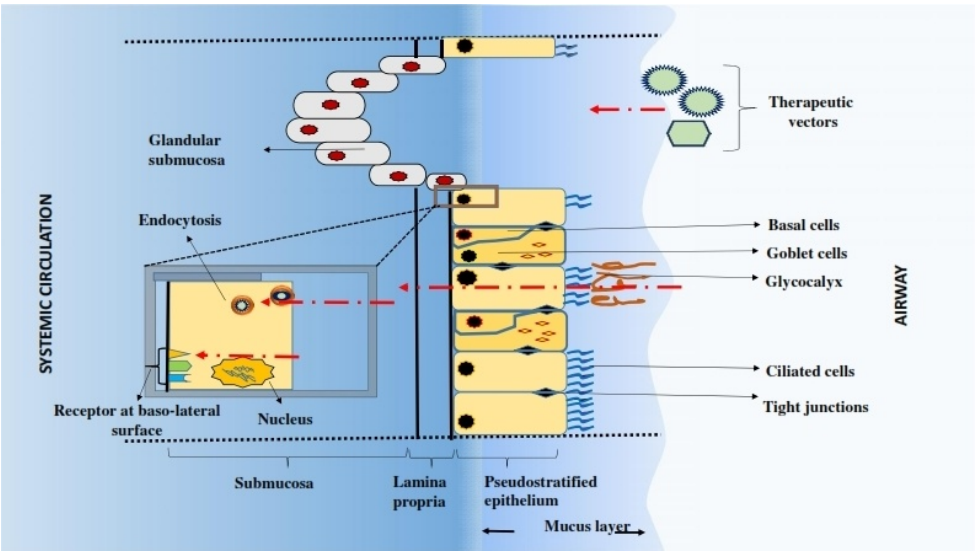

Figure 2.6: Intracellular and extracellular barriers in pulmonary delivery

Gautam A et al have studied the aerosol-based gene delivery based on the PEI complexes with DNA. Findings revealed improved gene expression in mice model and histopathology of the lung tissue showed no inflammation (97). Nevertheless, PEI is being studied extensively as a transfection agent but cytotoxicity of the polymer restricts its applications. High molecular weight and higher branching in the polymer cause more toxicity than lower branching. Several approaches have been studied to reduce the cytotoxicity of the polymer thorough modification of the structures specially amines groups. Dagmar Fischer et al studied the effect of low molecular weight PEI on the cytotoxicity and transfection and found positive toxicity profile and efficiency of the transfection.

Biodegradable vectors and PEG coated PEI are another way to alleviate challenge of toxicity of the polymers. Holger Petersen et al developed biodegradable PEI with linking the polymer to L-lactic acid-co-succinic acid. Developed vectors showed lower toxicity profile in L929 mouse fibroblasts cells and improved transfection compared to its low molecular weight native PEI (98).

Active targeting approach is also used for delivery of gene therapeutics by non-viral vectors via pulmonary route. Some of the examples are conjugation of the sugar moieties on PEI to accomplish the tissue specific delivery e.g. epithelial cells of airways. Uronic acid conjugated on PEI and showed receptor mediated endocytosis confirmed by fluorescence microscopy and FACS analysis (99). To enhance the transfection efficiency of the non-viral vectors, targeting with ligand or other elements is promising approach in that vectors binds efficiently on the receptor to specific cells. R G Jenkinset al developed LID vectors for delivery of gene by integrin targeting which is glycoprotein expressed on the cell surface specially epithelial cells, fibroblast etc. above work demonstrated better transfection efficiency of the developed vectors than cationic liposomes demonstrating as promising vectors for gene delivery to respiratory diseases (100). Another example for lung carcinoma is siRNA delivery by cationic liposomes conjugating with cRGD peptide and inorganic nanoparticles demonstrated good transfection efficiency *in vitro* and safety profile. Furthermore, researchers have been studied the chemo sensitization of with gemcitabine in drug resistance. Other ligands used for active targeting to lung tissue are folate, lactoferrin, trisaccharide, different carbohydrate moieties etc.

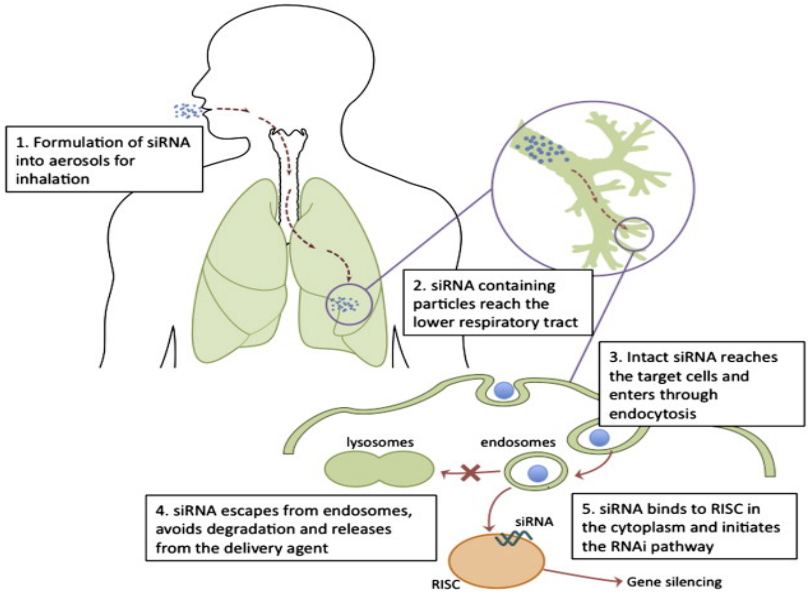

Figure 2.7: siRNA therapeutics delivery via pulmonary route

2.11 Pulmonary delivery platforms

Inhalation delivery is of the most accepted one because of non-invasive nature of administration. Aerosolization of gene delivery is very promising approach to treat pulmonary disorders**.** There are three main aerosolization systems; Meter Dose Inhaler (MDI), and Dry Powder Inhaler (DPI) and Nebulizers. With appropriate modifications these delivery devices can be made compatible for siRNA therapeutic delivery.

2.11.1 Metered dose inhalers (MDI)

MDIs are designed to deliver discrete doses to airways in the form of aerosol. It utilizes an actuator to dispense a metered dose of 25 - 100 µL of liquid solution or suspension containing suitable amount of active ingredient (101) The propellant used undergoes flash evaporation from discharged liquid droplets to produce drug having preferred aerodynamic size (102). MDIs are considered to be “the most complicated dosage form used in medicine today” as device performance is result of combination of formulation, container, metering valve and actuator performance (103). Though, propellant must be compatible with the formulations. The formulation is usually presented in the suspension or solutions forms. The suspensions are the favored one, as propellants are non-polar liquids in which most drugs have limited solubility. MDIs also gives high shear to the formulation, and for that reason, may not be encouraging for the development of inhalable siRNA.

2.11.2 Dry Powder Inhaler

DPIs represent drugs for inhalation in the form dry particles in air stream which is drawn through the device by patient’s inspiratory action. In contrast to MDIs they are devoid of dependence of coordination between drug aerosolization and inspiration. DPIs has been fruitfully used to deliver therapeutic macromolecules such as insulin(105) parathyroid hormone(104)Nevertheless, formulating as DPI for siRNA delivery presents significant challenges as it demands not only flowability , dispersibility of the powders but preserving biochemical efficacy of the conformationally sensitive biomacromolecules as well (106).The difficulty can be addressed by formulating macromolecules using

lyophilization or spray drying and then processing them into flowable and dispersible powder.

2.11.3 Nebulizers

Nebulization devices are the oldest aerosol devices and still have the significance for generating constant liquid droplets stream for easy penetrability in size range of 1-5 um. Devices offer ease of use because of no requirement of synchronization of actuation and inhalation which also eliminates any training for users (107). It is most favored method for administering high dose antibiotics. Nebulization of the liquid effectively generates aerosols for pulmonary administration; atomization theories propose that aerosol size and characteristics of output are depends on operating principles, conditions and constructions of nebulizers and physiological characteristics of the nebulized liquids. Limitation associated with the nebulizer as inhaler device is low deposition efficiency of the therapeutics in the target area of the lungs. However, nebulizers are used extensively for the inhalation, usually generating the aerosol droplets less than 5 μm in diameter that can reach to the deep respiratory tract. Hence, Nebulization is a promising approach to deliver gene or siRNA directly to the affected airway epithelium in the patients with asthma and COPD. Key step towards this objective is choice of appropriate device and suitable formulation permits nebulization of much therapeutics including siRNA in broad range of doses. Developed non-viral carrier systems should maintain their stability and biophysical properties, protecting the siRNA from the shear forces during nebulization so that its biological efficacy is preserved. Additionally, yield of nebulization must be maximized.

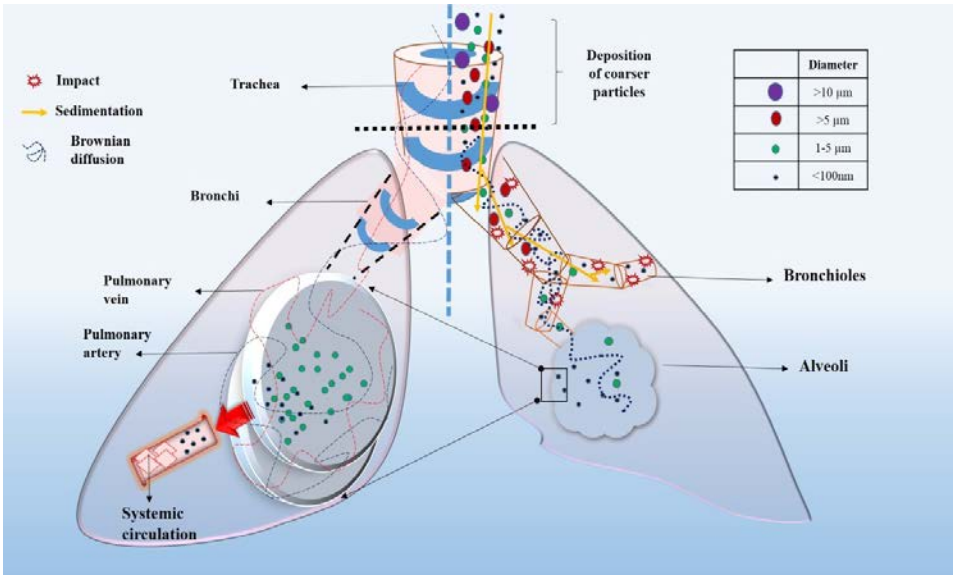

Figure 2.8: Particles depositions mechanism in pulmonary delivery

Nevertheless, proper understanding of the working mechanism and the factors that affects the nebulizer performance is important for an effective use. Various types of nebulizer devices are available commercially including jet, ultrasonic and vibrating mesh nebulizers. In following table 2.2 nebulizers with novel advanced technologies are represented.

Table 2.2: List of nebulizers with novel advanced technologies

Nebulizer Device	Type	Company
AeroEclipse® II BAN	Breath-actuated jet nebulizer	Monaghan Medical Corporation
AKITA2 APIXNEB	Vibrating mesh nebulizer	Activaero GmbH
I-neb AAD System	Vibrating mesh nebulizer with metering chambers and adaptive aerosol	Koninklijke Philips NV
Micro Air® NE-U22	Vibrating mesh nebulizer	Omron
CompAIR™ NE-C801	virtual valve technology in jet nebulizer	Omron
SideStream Plus	Breath-enhanced jet nebulizer	Koninklijke Philips NV
PARI LC® Plus		PARI international
PARI eFlow® rapid	Perforated oscillating membrane	PARI international

Stability of the formulations and maintaining integrity of the therapeutic gene are significant consideration while developing the delivery systems based on nebulization**.** E.Kleemann et al studied the PEG-g-PEI complexes as non-viral based vectors for nebulization and explored for structure of the complex and stability during nebulization by ultrasonic nebulizer (108). PEG-g-PEI based polymer complexes didn't change the particle size and maintained the structure after ultrasonic nebulization. Charles L.Densmore et al have been developed PEI based DNA complexes for immunization through aerosolized delivery. Findings exhibited that complexes resisted the nebulization process and suitable for pulmonary delivery.PEI based complexes demonstrated high transfection and stability during nebulization process (109). Chitosan based nanoparticles for siRNA delivery through aerosolization showed better gene silencing activity in H1299 cells in transgenic mice model of EGFP gene (110).

2.12 Cationic polymers based non-viral vectors

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 polycation polyethyleneimine, poly-l-lysine, Chitosan, PAMAM dendrimers etc.

Previously, low transfection and gene expression held non-viral methods are at a disadvantage; though, recent advances in vector technology are being developing molecules and techniques with good transfection efficiencies and low cytotoxicity.

2.12.1 Polyethylenimine

PEI available in two forms, either as a branched polymer, and linear form with molecular weight from <1 kDa to 1600 kDa range. PEI mol. weight between 5 and 25 kDa are most

appropriate for gene transfer ranges. PEI Structure is different from other polymers, such as poly-lysine, in that at physiological pH only an amines fractions are in protonated conditions. Whenever endosomal compartment becomes acidic, PEI capture protons followed by swelling of endosomes by osmotic pressure subsequent lysis. This show the way to the release of endocytosed DNA/siRNA into the cytosol. At physiological pH, PEI is partially protonated but when it is exposed to acidic conditions within endosomes or endolysosomes, it triggers passive chloride ion influx by proton sponge mechanism which ultimately leads to endosomallysis and escape. Moreover, structural tunability and the molecular mass of PEI are imperative for proficient delivery of gene therapeutics. From several studies it was found that although low molecular weight PEIs (800 Da, 2 kDa) has ability to condense DNA they were not capable to show efficient transfection; while PEIs with above 10 kDa molecular weight demonstrated high transfection efficiency. In addition, 800 kDa PEI exhibits high toxicity and aggregation of erythrocytes *in vivo* as compared to lower molecular weight PEIs and so 20–25 kDa PEIs are favored for *in vivo* applications (111-113).

The cationic charge of complexes generally also serves to bind cells by electrostatic interactions with the negatively charged cell membrane. The binding of Cationic charged complexes to sulfated proteoglycans on the cell membrane has been verified. The call fora surplus positive charge for proficient complexation of DNA and cell binding can, though, pose major troubles, principally for many *in vivo* applications. High molecular weight PEI can cause aggregation of the cationic polymer inducing necrosis and in so doing cytotoxicity. Contrastingly, low molecular weight PEI has showed low cytotoxicity in cell culture experiments. Linear PEI based transfection agents are already available in market (e.g. ExGen500, jet PEI) (114-116). Thus, the current research is focused on furnishing safe and biocompatible derivatives of polyethylenimine while maintaining the transfection efficacy.

Inefficiency of polymers compared to viral vectors, cytotoxicity, non-biodegradability, are cornerstones for the development of other derivatives of these polymers.

Various chemical modifications to the basic PEI backbone can be made in order to get various physicochemical characteristics and following alteration of safety profile. Forrest

et al. have demonstrated that conjugates of 14–30 kDa by coupling low molecular weight 800 Da PEIs through short diacrylate linkages resulted in the favorable properties like low toxicity, higher transfection efficiency. According to Kramer et al. PEI demonstrated lowest cytotoxicity at 60% degree of branching (117) while additional increase in degree of branching increases the cytotoxicity *in vitro*, as well as the hemolysis of the erythrocytes (118). Therefore, thorough knowledge of the polymer structure is needed for establishing structure-function relationships, reducing cytotoxicity and improving biocompatibility.

Different findings proved that higher pKa corresponds to higher protonation of the primary and secondary amines present, hence producing a higher number and density of charges (118). PEIs can also explain buffer capacity in the pKa range between 4 to 6 by which it can buffer the interior of endosomes resulting in their osmotic swelling and endosomal membrane rupture (119). In recent many years 'proton sponge' hypothesis has gained widespread approval. Funhoff et al. suggested that for polymers having buffer capacity at low pH values of around 5, hypothesis of the proton sponge may not be relevant (114). Still differences evidences like living cell confocal microscopy are available to substantiate the proton sponge theory (120). In addition, decelerated acidification, high chloride accumulation, increase in the relative volume of PEI-containing endosome vesicles and the concept of reduction in transfection efficiency by removal of protonable amines by quaternization, supports the proton sponge hypothesis (121, 122).

Physicochemical characteristics of the polymer like as the molecular weight and branching ratio can extensively influence the transfection ability and cytotoxicity which concludes that, structure notably affects the efficacy of PEI-based vectors. Thus, appropriate selection of polymer based on the carrier systems like plasmids, oligonucleotides or siRNA is a very sophisticated task.

Along with intact delivery of the nucleic acid and reduction of adverse effects, ideal gene delivery system should also have capability for cell specific targeting. Passive targeting of PEI or its modifications is one of the simplest approaches to achieve this goal. PEI-grafted –PEG polyplexes of DNA showed high transfection efficiency and low

cytotoxicity in bronchial and alveolar cells studied by Kleemannet al. Pluronic 123 or Pluronic 85 grafted targeted hepatocytes cells while eight PEG chains grafted onto 2kDa PEI targeted the kidneys (123). While, active targeting relies on receptor-mediated uptake of polymer complexes to particular target tissues, such as hepatocytes and dendritic cells by carbohydrates; tumor tissue by folate receptor, integrin or transferring targeting; and to tissues expressing specific receptors with antibodies or their fragments. i.e. PEI conjugated with galactose is used for liver- targeting which acts on asiloglycoprotein receptor expressed on hepatocytes cells. The transfection efficiencies of such galactose-modified PEI increase with increase in grafting ratio upto 5% but further increase up to 31% lead to reduction in the transfection efficiency which may be due to steric shielding effect impairing complete DNA condensation (124, 125).

Recently, it has been showed that some peptide known as transduction domains contain cationic charged amino acid such as arginine and lysine, which have been known to have cell-penetrating ability (126-128). polyamidoamine (PAMAM), poly-propylenimine, and poly(lysine)polymers conjugated with arginine improves the *in vitro* transfection efficiency compared to native polymers.

Another promising approach is the PEI conjugation with the moiety which having pKa in the range of pH so it can assist the endosomal escape of the vectors and release payload in cytoplasm. Imidazole ring containing amino acid histidine and other Imidazole derivatives have been reported to improve the transfection efficiency of the PEI. Thorough knowledge of physicochemical characteristics assists to formulate gene delivery that can overcome various barriers for *in vivo* application of carriers which are challenged in various ways. In order to overcome these barriers several factors including stability *in vivo*, target cell surfaces interaction, cell uptake, release from endosome vesicles, nuclear uptake as well as vector dissociation should be considered. The information regarding these processes is still limited but PEI based polyplexes present upper edge that can subside such problems. Nucleic acid condensation with PEI offers several benefits that include enhanced stability against degradation in extracellular environment, improved cell uptake and augmented endosomal release as per proton sponge mechanism.

2.12.2 Chitosan

Chitosan, a natural polysaccharide obtain from deacetylation of the chitin, has advantageous characteristics such as biocompatibility, low toxicity and immunogenicity, high charge density. Owing to cationic charge, chitosan can easily form polyelectrolyte complexes with anionic charged nucleic acids by electrostatic interactions. Nevertheless, efficiency of the chitosan as a gene delivery vectors is considerably affected by formulation related parameters such as, deacetylation degree, N/P ratio, salt form of chitosan, plasmid concentration, stability against polyanions, molecular mass etc.

Numerous studies have demonstrated the prospectus of chitosan as a carrier for nucleic acids and influencing expression of reporter genes in vitro and in vivo (129). The higher susceptibility of RNA to enzymatic degradation as compared to DNA may cause problems to chitosan based RNA transfer. Furthermore, the structure and size of siRNA are less than the plasmid DNA that also can affect the performance of vector and hence different formulation parameters necessities to be optimized with respect to the physicochemical and biological characteristics of the developed siRNA complex.

In spite of number of benefits of chitosan as a non-viral gene delivery vector, its application limited by poor solubility and poor stability at physiological pH, low cell specificity and subsequent low transfection efficiency. For that reason, numerous approaches are taken to address these limitations such as hydrophilic modification by PEG, quertenization of the chitosan to improve the colloidal stability and solubility at physiological pH, glycol chitosan, thiolation of chitosan, guanidinylated chitosan etc. chitosan structure modifications normally either by grafting of molecules or polymer into the chitosan backbone structure or by the amines quertenization were examined extensively. Modification in the structure of chitosan can be explored at these site : , two –OH groups (1°or 2°) and one 1° amine by glucosamine units. The modification site is dictated through the preferred application of the chitosan derivative. Different chitosan derivatives have come out for gene delivery. These approaches resulted in improved aqueous solubility, transfection ability and stability.

Trimethylation of the chitosan is one of the approaches to make it soluble at physiological pH. Trimethylated chitosan, quaternized chitosan derivative, is of huge interest due to its well-defined structure, improved solubility and ease of preparation

(130). As Trimethylated chitosan can more efficiently condense DNA at physiological pH, transfection ability of complex has been increased 30 times than that of chitosan only (131). Some studies have reported about the ability of trimethylated chitosan with different degrees of quaternization to transfect carcinoma cells (131, 135). Table 2.3 and Table 2.4 describes some of the examples of modification of chitosan for gene delivery and clinical trials of RNAi therapeutics in the respiratory diseases.

Table 2.3: Different types of modifications of Chitosan for gene delivery

Type of modification	Type of derivatives	Inference	References
Hydrophilic Modification	Quartenized Modification	Soluble at physiological pH. Enhanced transfection efficiency	136
	PEG-Chitosan	Improved stability	137
	Arginine-chitosan	Improves aqueous solubility and transfection	138
	PEI grafted Chitosan	improved transfection diminished Cytotoxicity	139
Modifications by hydrophobic moieties	chitosan modified with Deoxycholic acid	greater gene condensation ability Protection from endonuclease enzymes	140
	Alkylated Chitosan	Easier decomplexation of gene Increase perturbation of membrane and cell entry	141
	Thiolated Chitosan	Improved mucoadhesiveness and cell penetration	142
	Stearic acid chitosan	enhance endosomal escape	143
	N-Dodecylated Chitosan	Improved thermal stability of DNA	144
Ligand Conjugation	Chitosan modified with galactose group	increased cell permeability transfection into Hep G2 cells	145

	Chitosan modification by mannose	Receptor mediated delivery showed improved gene transfection in macrophages	146
	Folate- chitosan	Folate receptor mediate endocytosis High transfection efficiency	147
	Transferrin-Chitosan	efficient receptor mediated endocytosis	148
	Galactosylated Chitosan grafted PEG	Improved solubility and hepatocyte targeting	149
Thermosensitive Modification	TMC grafted poly(N-isopropyl acrylamide)	Better transfection efficiency No apparent toxicity	150

Table 2.4: Clinical trials of RNAi therapeutics in respiratory diseases

Disease	RNAi target	Delivery or formulation	Status	Company/Sponsor	Year
Asthma	syk siRNA (Excellair)	Inhalation, Naked siRNA	Phase I completed	ZaBeCor Pharma.	2010
RSV infection in lung transplant patient	N protein siRNA (ALN-RSV01)	Inhalation, Naked siRNA	Phase II completed	Alnylam Pharma.	2011
RSV infection	N protein siRNA (ALN-RSV01)	Inhalation, Naked siRNA	Phase II completed	Alnylam Pharma.	2012
Solid tumor including NSCLC	miR-34 (MRX34)	Intravenous, Liposomes	Phase I continue	Mirna Therapeutics	2013
Recurrent MPM and NSCLC	miR-16 (TargomiRs)	Intravenous, Nanoparticles	Phase I continue	University of Sydney	2015
Brain tumor	ATN-RNA	Naked	Phase I	Department of Neurosurgery and Neurotraumatology of University of Medical Sciences in Poznan	2004-2008

Abbreviations: MPM: malignant pleural mesothelioma, NSCLC: non small cell lung cancer;RSV: respiratory syncytial virus;syk: spleen tyrosine kinase;

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