Nucleic acid carriers for pulmonary gene delivery

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Abstract

The delivery of nucleic acids to the pulmonary route appears to be promising due to non-invasiveness, large area of epithelial surface lining the lung, easy accessibility and ability to provide a platform for local delivery. The therapeutic role of nucleic acids in various diseases pertaining to the pulmonary route e.g. cystic fibrosis, α1-antitrypsin deficiency, asthma, chronic obstructive pulmonary disease, lung cancer etc. have been realized due to the increase in the understanding of genetic pathways. Numerous potential therapeutic targets have been explored in pharmacotherapeutics of such pulmonary diseases for gene delivery. Viral and nonviral carrier mediated nucleic acids delivery has been investigated in the field. But gene delivery by viral vectors remains restricted due to their toxicity and immunogenicity. Nucleic acid delivery via nonviral vectors seems attractive to researchers due to safety, ease of synthesis and scale up. Successful delivery of nucleic acids by pulmonary route also remains challenging due to several anatomical and physical barriers, immune response, particle aggregation, etc. For overcoming the various challenges posed for achieving therapeutics benefits from gene delivery via pulmonary route, the problems that need to be addressed includes overcoming the mucus barriers by use of mucolytic agents, mucus penetrating particles; minimizing the immune response, improving *in vivo* stability, controlled release of the carriers, preventing aggregation of the carriers etc. The current review is focussed providing details on the various delivery strategies for overcoming the barriers for successful delivery and gives brief details on the therapeutic advancements made in the field.

Key words: Nucleic acids, gene delivery, pulmonary route, mucociliary clearance, nonviral carriers

1. INTRODUCTION AND CURRENT ASPECTS OF PULMONARY GENE DELIVERY

Nucleic acids are the prime constituent of cell in the form of DNA or RNA and control cellular functions and are inheritable (1). The realization of the concept of introducing foreign DNA into the host cell (coined as 'Gene delivery') led to the inception and exploration of idea of manipulating the genotypic expression of an individual which predisposes to and is primary cause of various diseases in human. With the tremendous research carried out in exploring genetic pathways and finding responsible gene and its defects in diseased condition, need arouse to targets and deliver potential gene therapeutic for achieving clinical benefits. The potential of gene therapy is evident through its application in treatment of genetic diseases like cystic fibrosis, haemophilia, etc. and also

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acquired diseases including carcinomas, cardiovascular diseases, neurological diseases, airway inflammatory disorders like asthma, chronic obstructive pulmonary disease (COPD) etc.

Lung remains attractive target for the delivery of drugs and nucleic acids due to the high morbidity of major pulmonary diseases including cystic fibrosis, asthma, chronic obstructive pulmonary disease (COPD), lung cancer, pulmonary arterial hypertension (PAH), acute lung injury etc. for which the current therapeutic interventions are largely insufficient for treatment purpose and associated mortality rate are very high for the patients. The Pulmonary route offers several advantages over delivery by systemic routes such as non-invasiveness, accessibility of high surface area of the lung epithelium, lower systemic exposure of administered therapeutics, rapid deposition in the target organ, avoidance of first pass effect etc.

Therapeutic gene delivery to lungs is always encouraging for the inherited genetic diseases like cystic fibrosis and α1-antitrypsin deficiency. Replacement or correction of defective cystic fibrosis transmembrane conductance regulator (CFTR) gene has been well studied and extensively researched for the gene therapy to cystic fibrosis treatment (2). Recently gene therapy approach by gene silencing mechanism of RNA interference (RNAi) is the thrust area of research for other pulmonary diseases and it has gained popularity (3).

Numerous targets have been recognized in pathophysiology of various pulmonary diseases and have a great potential as candidates for therapeutic interventions. Some of the potential candidates for gene delivery targets includes CFTR gene for cystic fibrosis, in addition epithelial sodium channel (4) which is upregulated in cystic fibrosis; oncogenes and tumour suppression gene like p53 in lung cancer (5); inflammatory mediators including cytokines, interleukins and other transcription factors, neurotropins like nerve growth factor (NGF) (6), brain derived neurotropic factor(BDNF) (7), matrix metalloproteinase (MMP) in asthma and COPD(8); growth factors like FGF (9), VEGF(10) in pulmonary arterial hypertension. Targeting of such therapeutic candidates would be promising approach for siRNA therapeutics and pDNA delivery.

Delivery of plasmid DNA or siRNA has always been challenging due to the anionic charge and high fragility to nucleases, and so, they require efficient carriers for intracellular delivery of therapeutic payloads. Several viral and nonviral carriers have been explored for pulmonary delivery of nucleic acids. Viral vectors like lentivirus, adeno virus, retro virus and adeno-associated virus are preferred over nonviral due to their high transfection efficiency. However high expression of therapeutic gene by the viral vectors, certain drawbacks limits their applicability such as immunogenicity, oncogenicity, inflammatory response and issues in scale up and production. Numerous clinical trials have been performed to access the efficiency and safety for pulmonary gene delivery vectors particularly for cystic fibrosis but till date success was not achieved for any formulation carriers due to several challenges to gene delivery *in vivo*. Most significant challenges for pulmonary gene delivery includes anatomical barriers, physical barriers, immune response, aggregation of the carriers etc.

Hence, research has been shifted towards the nonviral carriers which makes stable complexes with negatively charged nucleic acids. These carriers include cationic polymers, cationic lipids, liposomes, inorganic nanoparticles, micelles, dendrimers etc. Mostly cationic polymers like polyethylenimine (11), poly-l-lysine (12), Poly (amido amine) PAMAM dendrimers and cationic lipids like N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-

trimethylammonium chloride (DOTAP) (13), N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), etc. have been explored as carriers for pDNA and siRNA delivery to lungs and summarized in table 1.

Table: 1 List of various nonviral carriers explored in pulmonary gene delivery.

Non viral carriers	**pDNA/siRNA**	**Animal model/cell line**	**important findings**	**references**
Linear PEI and EDMPC:Chol with mucolytic agents	CAT and CFTR gene	Sheep tracheal epithelium model	Improved gene expression	(15)
PAMAM dendrimer (G4NH2)−siRNA complexes (dendriplexes)	EGFP siRNA	A549 lung alveolar epithelial cells	Dendriplexes encapsulated in mannitol and incorporated pMDI. Highly respirable fractions (~77%)	(16)
PEI complexes	Plasmid pCIKLux	Mouse	Higher gene expression	(17)
GL 67 and PEI complexes	CAT Reporter gene	Sheep animal model	Aerosolized gene delivery Reduced in dose related toxicity	(18)
PEI complexes	CAT Reporter gene	Mouse model	Nebulised delivery improved gene expression	(19)
PLL-conjugated PEG nanoparticles with mucolytics	CFTR gene	Mouse model	Enhanced penetration of carriers through CF sputum and higher gene expression	(20)
PEGylated poly-l-lysine –DNA nanoparticles	Firefly luciferase	Mouse	Improved stability of the formulation and efficient gene transfer to airway epithelium	(12)
PLGA-PEI nanoparticles	V1Jns encoding Antigen 85B of Mycobacterium tuberculosis	Human airway submucosal epithelial cells, calu-3 cells	Efficient vaccine carriers for TB	(21)
Chitosan-TPP-PEG nanoparticles	pDNA MB113	-	Stearically stabilized nanoparticles with potential for pulmonary delivery via pMDI	(22)

Spray dried powder containing pH responsive amphipathic peptides	NP-574,NP-1494,NP-1496 antiviral siRNA	A549 lung alveolar epithelial cells	Potential carriers for prophylaxis and treatment in H1N1 influenza virus infection	(23)
Cationic ethylphosphocholine based nanolipoplexes	myeloid cell leukemia sequence 1)-specific siRNA	Lung metastasis mouse model	Significantly silencing of target gene	(24)
Salbutamol modified Guanidinilyted chitosan	EGFP siRNA	A549 lung alveolar epithelial cells	Facilitated cellular internalization and improved gene silencing efficincy	(25)
Noncovalently PEGylated PDMAEMA-b-PMAPEG complex	CTGF siRNA	Sprague dawley rat model by orotracheal administration	Substantially gene silencing,reduction in inflammatory cytokines and collagendeposition Increased in survival rate	(26)
Dexamethasone conjugated PEI complex	MIF siRNA	BALB/c mice	Effectively reduction of particular matter(PM) induced airway inflammation	(27)
DOTAP modified PLGA nanoparticles	EGFP siRNA	human non-small cell lung carcinoma cell line H1299 stably expressing EGFP	Potential formulation as spray dried dry powder inhalation	(13)
PLGA nanoparticles	EGFP siRNA	H1299 cells stably expressing EGFP	Improved aerosolized properties siRNA biological activity preserved after spray drying	(28)

Dexamethasone conjugated low MW PEI	EGFP pDNA	lipopolysaccharide (LPS) induced acute lung injury (ALI) model and L2 lung epithelial cells	Higher gene delivery efficiency and reduction in TNF-α and IL-6 in BALF and lung homogenate. Total protein and immunoglobulin reduction in BALF.	• (29)
Aerosolized chitosan-siRNA nanoparticles	EGFP siRNA	H1299 human lung cancer cell	Aerosolised form exhibited efficient gene silencing	(30)
Chitosan modified PLGA nanospheres	pGL3 firefly luciferase siRNA	A549 lung alveolar epithelial cells	Higher gene silencing activity than unmodified PLGA nanospheres Higher cellular uptake	(31)
Urocanic acid modified chitosan complex	PDCD4 tumor suppressor gene	K-ras null lung cancer mice model	Aerosolized complex effectively inhibit cell proliferation, supress tumor angiogenesis pathway and facilitate apoptosis	(32)
PEI-siRNA complex	Wilms tumor gene1(WT1)	B16F10 murine melanoma cell line and mice with B16F10 lung metastasis	Reduction in tumor angiogenesis and size of tumor foci Treated mice showed Prolonged survival time than control	(33)
Aerosolised Nanosized poly(ester amine) polymer	Akt1siRNA	$K\text{-}ras^{LA1}$ and urethane-induced lung cancer models	Suppression of lung tumorigenesis and alteration in akt signals and cell cycle	(34)
Aerosolized PEI-DNA complex	p53 gene	SAOS-LM6 cell line and mice model	Reduction in numbers and size of tumors of osteosarcoma lung	(35)

			metastases No signs of toxicity after repeat administration	

Abbreviations: PEI: Polyethylenimine, EDMPC:p-ethyldimyristoyl phosphatidyl choline cholesterol , Chol: Cholesterol, PAMAM: poly(amidoamine), eGFP: enhanced green fluorescent protein, CAT: Chloramphenicol acetyl transferase, pMDI:pressurised metered dose inhaler, PLL: Poly-l-lysine,WT1: Wilms tumor gene1, CTGF :connective tissue growth factor, MIF: Macrophage migration inhibitory factor, PDCD4: programmed cell death protein 4

Current research has been focused to enhance transfection efficiency into the target pulmonary cells and safety of the nonviral carriers. Currently, one clinical trial for the treatment of cystic fibrosis by the delivery of nebulised pGM169/GL67A gene-liposomal complex demonstrated promising findings with improved and stabilized pulmonary functions. Such type of clinical studies would be encouraging for the development of non-viral based pulmonary gene delivery systems (14).

Several novel strategies to overcome aforesaid challenges have been explored extensively by nonviral carriers such as gene delivery utilising mucolytic agents, mucus penetrating particles (MPPs) to overcome mucus barrier and mucociliary clearance. Further, Poly ethylene glycol (PEG) coated or grafted nanocarriers to improve stability, minimization of immune response and prevention of aggregation of carriers. Current review gives insights on the challenges of pulmonary gene delivery, various approaches to overcome the challenges associated with nonviral carrier-mediated gene delivery and future perspective of pulmonary gene therapy.

2. POTENTIAL TARGETS FOR DIFFERENT PULMONARY DISEASES

Pulmonary delivery offers noninvasive and direct route for the delivery of nucleic acids majorly to pulmonary epithelia or airways of the lungs (36). Local delivery of nucleic acids such as therapeutic DNA and siRNA to the lung represents a potential means of treating an array of pulmonary conditions including cystic fibrosis, COPD, asthma, lung cancer, pulmonary arterial hypertension, acute lung injury etc. (37). Theoretically, there are two approaches for gene therapy. First and easy one is to treat a monogenic disease for which only one gene is responsible such as cystic fibrosis. In this, the known therapeutic gene is introduced in the place of mutated gene using plasmid DNA or faulty expression of proteins could be downregulated by siRNA to perform its regular functions. While in case of polygenic diseases such as asthma and lung cancer, where multiple factors are responsible, application of gene therapy is not easy. In this approach, thorough knowledge and understanding of responsible causative factors and molecular level pathogenesis mechanisms to be explored in depth to select a candidate gene.Numerous such potential targets for the treatment of various pulmonary diseases by gene therapy are summarized in table 2.

Table 2: Potential targets for gene therapy for pulmonary diseases

Disease	Potential Targets	DNA/siRNA	Remarks	Ref.
Cystic fibrosis	CFTR gene	CFTR wild type gene	Restored a role of CFTR as a phosphorylation-regulated Cl^- channel and a regulator of other transporters	(38)
	BAP 31	siRNA	Augmented expression of wild-type as well as mutant CFTR and fairly restored the function of CFTR	(39)
	ENaC	siRNA	Counter regulation of water and ionic balance	(40)
Pulmonary arterial hypertension (PAH)	BMPR-II gene	BMPR-II wild type gene	Restored BMPR-II levels through gene delivery and reduced TGF-β response	(41)
	eNOS	eNOS gene	PAH can be corrected by in vivo gene transfer of eNOS to the lung	(42)
	CGRP	CGRP gene	Attenuate established PAH and exert reversal effects on pulmonary vascular remodeling	(43)
	PGIS (prostacycline synthase)	PGIS gene	Considerable evidence indicates that PGIS gene transfer is a promising approach for the stable production of endogenous PGI2 and has the potential to ameliorate progressive PAH	(44), (45)
	Adrenomedullin	Adrenomedullin gene	Intratracheal administration showed remarkable therapeutic efficacy with PAH animal models without compromising biocompatibility	(46)
	HGF	HGF gene	In response to acute lung injury, HGF plays a role in lung regeneration and protection	(47)

	Survivin	survivin gene with dominant-negative properties	Inhalation of an adenovirus vector encoding a mutant survivin gene with dominant-negative properties reverses established MCT-induced PAH	(48)
	VEGF	siRNA	It can be targeted to treat Pulmonary arterial hypertension therapy	(49)
	FGF_2	siRNA	Inhibition of increased expression of FGF_2 can treat PAH condition	(50)
COPD	TNF-α	siRNA	There is an important pathologic role of TNF- α in chronic bronchitis and suggest that greater inflammatory response may predispose an individual to this disease.	(51)
	IL-8, IL-8 receptor, chemokine receptor (CCR)1	siRNA	Inflammation gene sets become the most significantly affected in COPD	(52)
	MMP-12	siRNA	Plays a major role in COPD progression and can be a good target for antisense strategy.	(53)
	Transcription factor nuclear factor-kappa B (NFκB)	siRNA	The role of NF-kappaB in both diseases, will discuss its suitability as a target, and will highlight recent key studies that support the potential of NF-kappaB as a therapeutic target in these two important inflammatory lung diseases.	(54)
Asthma	IL-3,IL-4,IL-5 & chemokines	siRNA	Downregulation of these proinflammatory mediators is promising using antisense approaches	(55)

	Spleen tyrosine kinase (Syk)	siRNA	Inhibition of inflammatory mediators was also achieved in a study using siRNA targeting Syk in airway epithelial cells. Excellair™ is being investigated in clinical trials have siRNA that targets Syk.	(56),(57)
	Signal transducers and activators of transcription (STAT6, GATA3, and NFκB)	siRNA	Small interfering RNAs to specifically inhibit the function of transcription factors and tyrosine kinases which are involved in orchestrating an allergic immune response.	(58)
Lung cancer	p53 tumor suppressor gene mutation	wild type p53 gene	tumour suppressor genes such as p53 which can normalize its function as a tumor suppressor	(59)
	k-*ras* oncogene	siRNA	downregulation of certain proteins such as K-*ras* oncogene to treat cancer	(60)
	WT1	siRNA	WT1 gene silencing in vivo by aerosol delivery of PEI-WT1 RNAi complexes is an effective therapeutic strategy for the treatment of lung metastases	(33)
	Akt1	siRNA	The use of poly (ester amine) serves as an effective carrier, and aerosol delivery of Akt1 siRNA may be a promising approach for lung cancer treatment and prevention	(34)
	IGF-1R	siRNA	Therapeutic potential of RNAi as a method for gene therapy in treatment of lung cancer	(61)
	ICAM-1	siRNA	ICAM-1(Intercellular Adhesion Molecule-1), which plays a crucial role in lung cell proliferation and tumor expansion, and offers	(62)

			an exciting target for treatment of lung cancers	
	NUPR1	siRNA	NUPR1 gene represents a promising target for gene silencing therapy in non-small cell lung cancer	(63)

Abbreviations:(BAP) 31: B-cell antigen receptor-associated protein, (ENaC): epithelial sodium channels, CFTR: Cystic fibrosis transmembrane conductance regulator, (BMPR2): Bone morphogenetic protein receptor type II, **(**TNF)-α : Tumor necrosis factor**,** (MMP-12): matrix metalloproteinase-12, Endothelial nitric oxide synthase (eNOS)-derived nitricoxide (NO), Calcitonin-gene-related peptide (CGRP), (VEGF): Vascular endothelial growth factor, (WT1): Wilms tumor 1, (IGF-1R): insulin-like growth factor receptor 1, FGF2: fibroblast growth factor 2, NUPR1: Nuclear protein transcriptional regulator

3. CONQUERING OBSTACLES OF PULMONARY GENE DELIVERY

Efficient and successful delivery carriers for nucleic acids must have to cross several barriers to reach at target cells for the desired therapeutic efficacy. Such barriers include anatomical barriers, physical barriers, immune response and aggregation of carriers. The barriers to delivery of gene therapeutics is outlined in the figure 1.

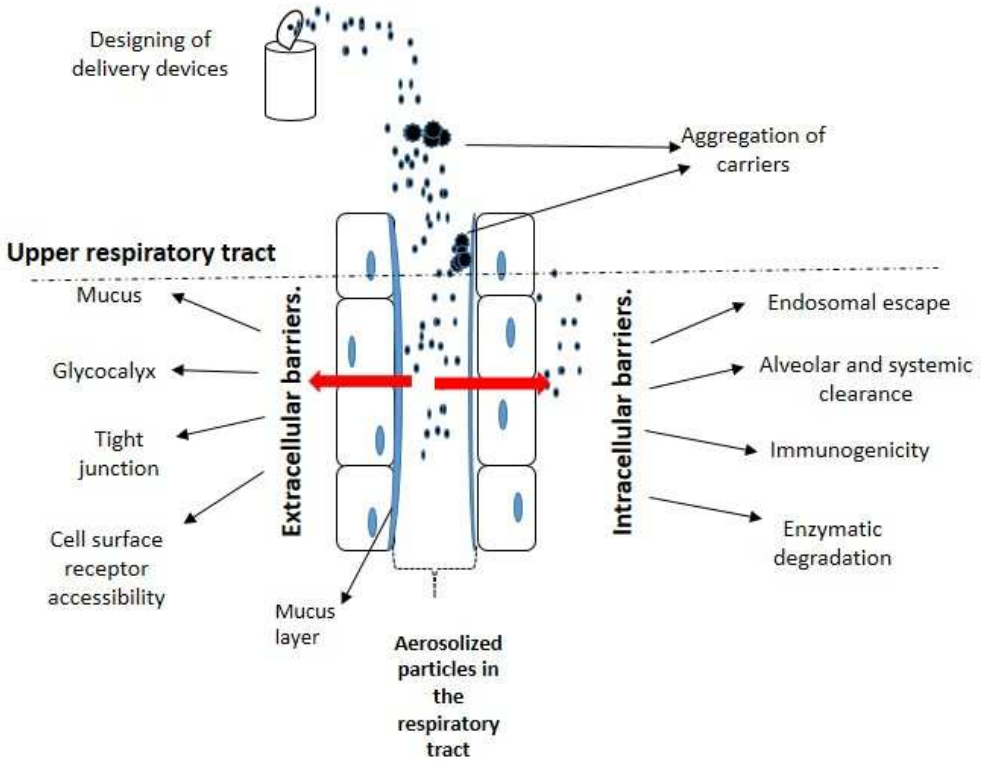

Figure 1: Hurdles to delivery via pulmonary route.

3.1. Anatomical barriers

Structure and anatomy of the lung airway is very complex architectural in nature.

Thus efficient targeting of therapeutic gene or siRNA to particular cells types appears challenging for gene delivery carriers. Delivery and deposition of the gene carriers as an aerosolised droplets or dry powder into the pulmonary airway regions depends on several factors including aerodynamic characteristics of the aerosolized droplets of gene delivery carriers such as droplet size, shape, density etc. and other factors such as breathing pattern and respiratory rate. Size distribution of particles is significant determinant for deposition of the particles into the lung. Particles having aerodynamic diameter between1-5 µm deposits in deeper lung which is most optimum size to efficient deposition to the deeper lung regions by the mechanism of gravitational sedimentation. While particles with > 5 µm size impacts on airway wall at upper airway region due to high momentum and particles with size < 1 µm are exhaled during breathing by brownian diffusion. These mechanisms of deposition of particles to the lung having different aerodynamic size are schematically represented in figure 2.

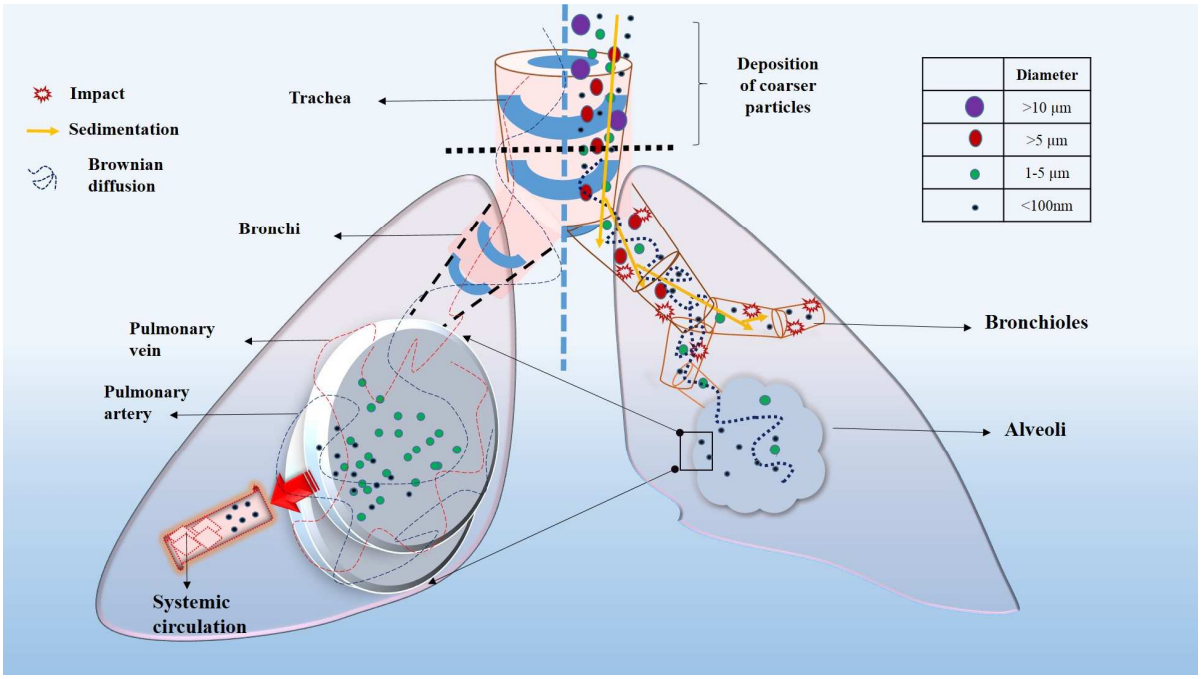

Figure 2: Schematic representation of different mechanisms of deposition of particle of different aerodynamic size to the lung.

Further, particle density also has effect on the aerodynamic deposition of the particles to the lung. Large particles with porous structure and lower density can be developed to make them respirable. Owing to large size and geometric diameter, phagocytosis by macrophages is avoided and at the same time smaller aerodynamic diameter leads to efficient deposition in the lung (64).Particle shape is also one of the significant determinant of aerodynamic depositions of particles into the lung. It has been reported that elongated and spherical shaped particles deposits in the deeper airways by mechanism of the interception (65). Elongated particles with high elongation ratio shows higher respiratory fractions to the airways and it has been also

demonstrated that elongated shaped particles significantly reduce the phagocytic uptake by the macrophages (66) Also patient centric factors like breathing pattern and flow rate also affects the aerodynamics of the particles. Further, efficacy of aerosolized dosage form of gene delivery carriers to airways depends on the choice of the delivery device and performance of the device.

Generally three types of delivery devices are being practiced including nebuliser, dry powder inhaler and pressurised metered dose inhaler (pMDI) for local pulmonary delivery due to high efficiency of delivery than systemic delivery. Currently, research have been focused on development of inhaled or aerosolised form for the local delivery of gene carriers due to certain advantages like maintenance of integrity and efficacy of gene delivery carriers, even distribution of aerosolised carriers to lung epithelia as most accessible local target, non-invasiveness etc. In addition, carriers administered through aerosolised or inhaled form elicit less immune response compared to other routes like direct instillation and intravenous where probabilities of aggregation of carriers. A better understanding and utilisation of the particle engineering and aerodynamic principles are required for expanding the research to overcome the anatomical barriers for the development of more efficient delivery methods and devices for gene via pulmonary route.

3.2. Physical barriers

The physical barriers hindering the entry and posing a challenge to the delivery of therapeutics include extracellular barriers like mucus barrier and mucociliary clearance and others are cell surface- and intracellular barriers. Such type of physical barriers for pulmonary gene delivery are represented in figure 3.

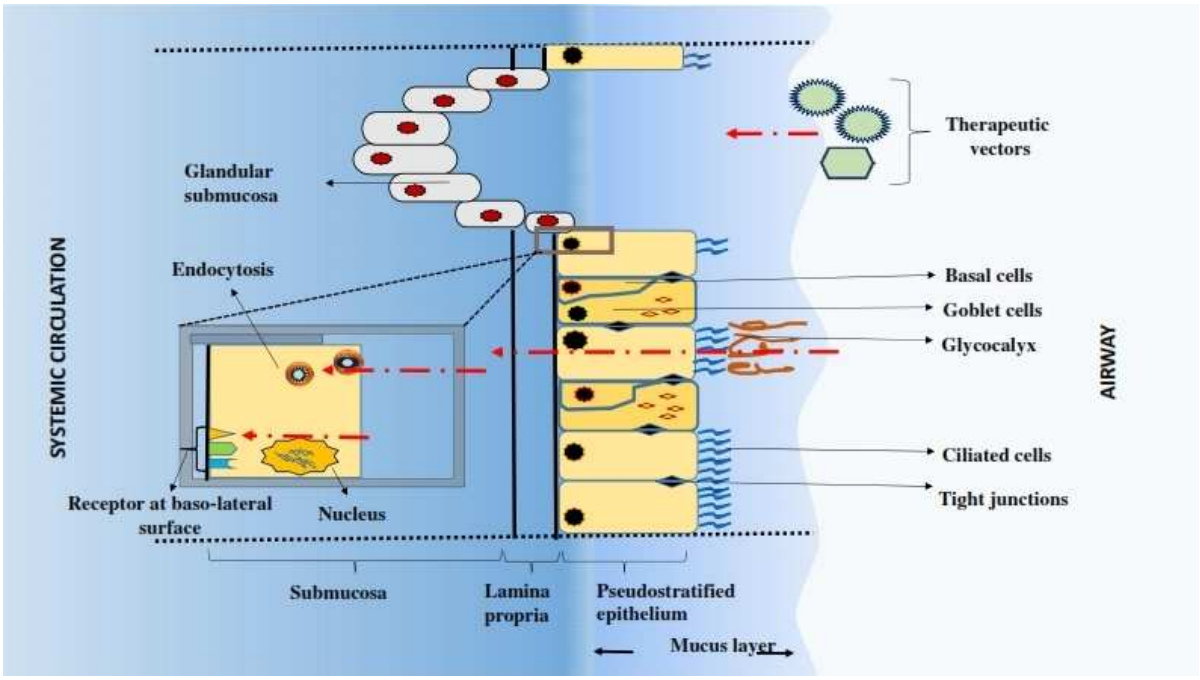

Figure 3: Schematic representation of extracellular and intracellular barriers for pulmonary gene delivery.

(i) *Extracellular barriers*

A well developed and complex set of defence mechanisms has been set up by airway epithelia to protect lumen and cellular compartment from the insults of foreign material including vectors for gene delivery. These barriers include mucus barrier, glycocalyx barrier and receptors accessibility at the apical cell membrane. Also, the presence of tight junctions across epithelial cell prevent the entry and transport of delivery vectors from lumen to interstitial spaces. The epithelial layer is also barred to the vector delivery during the diseased condition like cystic fibrosis wherein there is deposition of secretions containing inflammatory product and alteration in mucus composition which modifies its morphological characteristic and decrease cellular uptake of delivery system due to hindered transport (67).

Mucus layer overlaying the lung epithelia is the major obstacle for efficient delivery of carriers to target cells either administered as dry powder or in nebulized form via inhalation route or direct instillation of gene through nanocarriers.

Mucus secreted mainly by goblet cells and submucosal glands is amorphous and viscoelastic in nature, formed through cross linking of mucin fibers makes protective barrier to external environment.

In pathological conditions of cystic fibrosis and other inflammatory conditions, hypersecretion of mucus occurs in the upper respiratory tract which ultimately increases the chances of colonization of microorganisms and results in probability of infection thereby evading the health conditions and making airway obstruction (68). Mucociliary clearance is the first line defence mechanism of the airway system against the inhaled dust, microbes, antigens etc. Deposited particles may get cleared by functioning of cilia which beats synchronously and propel mucus and particles within mucus to pharynx. In inflammatory conditions of asthma and cystic fibrosis, mechanism of mucociliary clearance is highly impaired and such conditions lead to infection.

Some of the factors need to be considered to overcome the mucosal barrier or transport of gene delivery carriers across the barrier. Several strategies have been sought for the efficient gene delivery across the mucosal barrier including the modification of the surface properties of delivery carriers, delivery of nucleic acids carriers with certain mucolytic agents which disrupts the mucus layer and thereby improves the diffusion of carriers across the layer, coating of some inert and hydrophilic molecules e.g. PEG, over gene delivery carriers.

Surface properties of the nucleic acid carriers are greatly influencing factors to penetrate the pulmonary mucus barrier to reach the epithelial layer. Particle size and surface charge, affects the deposition of gene delivery carriers to lungs. With regarding to particle size, smaller the size of carriers suitably transport through mucus barrier due to lesser steric interactions than larger particles. For example, due to mucus having porous gel like network, exhibits different pore size of meshes, while cystic fibrosis sputum have 140±50 nm mesh size. (69) So, nanocarriers or gene transfer agents having size smaller than this can be penetrate through mucus effectively, while larger size molecules will have steric obstruction of the mucus. Transport across cystic fibrosis sputum was studied by polystyrene nanospheres with different sizes ranges from 124 nm to 560 nm and explained that water channels in mesh network of CF sputum was large enough to pass the smaller nanospheres while transport of larger nanospheres was strongly retarded due to strong steric obstruction (70).Surface charge is one of the important characteristics to be considered for gene delivery carriers to lung. Neutral surface charge of carriers minimizes the electrostatic interactions to mucus than

cationic and anionic charge surface. Coating of PEG renders the neutral surface charges to the carriers and facilitates the carriers in mucus penetration. Previously many researchers have reported PEG as a mucoadhesive while recently some studies reported that PEG with high dense coatings with neutral charge surfaces mimicking the conditions like muco-inert viruses which have high dense surface, showed better penetration in mucus layer by minimising the interpenetration and efficiently coating the hydrophobic pore.

Molecular weight of PEG coating also affect the mucus penetration of particles. It was revealed that molecular weight 5-10 kDa PEG with higher dense coating of nanoparticles become mucoadhesive (71) while 2 kDa molecular weight PEG penetrates better in mucus. Suk et.al investigated penetration of nanoparticles through cystic fibrosis sputum and demonstrated that nanoparticles having 200 nm in diameter coated with low MW PEG at high density penetrate 90 times faster than uncoated particles, while transport of particles with 500 nm size is hindered (69).In context of the gene delivery carriers composed of cationic polymers or cationic lipids, gene transfer efficiency significantly reduces in the mucus due to the electrostatic interactions with anionic charged mucin components and aggregation of carriers by mucus (72).This problem can be circumvent by PEGylation of cationic charged carriers and thus charge shielding of carriers and prevention of aggregation (72). Additionally, nanocarriers can be formulated with copolymers of PEG with aim to better mucus penetrating property. Tang et al prepared biodegradable nanoparticles using diblock copolymer of poly (sebacic) acid and PEG, which exhibited that better diffusion and penetration through cystic fibrosis thick sputum than unmodified particles. High resolution multiple particle tracking analysis showed transport rates of PSA-PEG nanoparticles 50 times higher than uncoated latex nanoparticles measured as mean squared displacements(MSD) (73).

Further, viscoelasticity of the mucus is mainly regulated by the mucin fibre content and other factors including actin, DNA, cell debris and serum proteins also contribute in regulation of viscoelasticity. Viscoelasticity significantly increases in the inflammatory conditions rendering gene transfer more difficult through mucus barrier. Gene delivery with mucolytic agents that disrupt the mucus barrier significantly increases the gene expression. Mucolytic agents usually include N-acetyl-L-cysteine (NAC) (20, 74), recombinant human DNAse (rhDNase) (75), Nacystelyn (15), glycopyrrolate (15) etc. can be administered with gene delivery carriers to cross the mucus barrier. Ferrari et al showed the delivery of gene to airway epithelium for cystic fibrosis with mucolytic agent Nacystelyn and results revealed an increase in reporter gene expression *in vivo* in mouse lung. Nacystelyn acts by breaking of the hydrogen and disulphide bonds in the mucus network which are responsible for maintaining the three dimensional mucosal structure (15).Stern et.al investigated effect of different mucolytics on the gene transfer efficiency through the cystic fibrosis sputum and it was revealed that presence of sputum significantly inhibits the gene delivery efficiency, while rhDNase pretreatment improves transfection of DC-Chol/DOPE cationic liposomes and adenovirus mediated gene transfer(75, 76). In another study, aerosolized rhDNase was found to be well tolerated and safe in reducing exacerbations of respiratory infections and improving pulmonary functions in cystic fibrosis patients. (77). Mucociliary clearance also affects the transfection efficiency and thus gene expression due to the lesser contact time of the carriers to the cell surface. Thus to improve cell surface contact and so cell uptake, a novel approach called magnetofection is explored for gene transfer using magnetic nanoparticles, by applying the magnetic field to magnetic particles associated

with delivery vectors and thereby to improve gene delivery efficiency and target cellular uptake. Gersting et al studied gene transfer by the magnetofection and reported higher gene expression in airway epithelial cells within shorter period of time than lipofection and polyfection. This might be due to accumulation of particles at cellular surface under magnetic force (78).

The glycocalyx composed of highly glycosylated tethered mucin along with carbohydrate rich molecules like glycolipids, glycoproteins and proteoglycans is a complex barrier which binds to the inhaled particles and prevents them from reaching the cell surface receptors (79). This barrier can be infringed by use of various enzymes like nonspecific proteases (80) or more conveniently by O-glycosylation inhibitors which provide benefits of enhanced gene transfer efficiency (67). Recent progress in the development of the various delivery carriers like mucus penetrating nanoparticles (MNPs) and carriers with mucolytic agents to overcome the mucosal barriers would be encouraging as they improve the delivery efficiency, sustained release and kinetics in comparison to the conventional particles.

(*ii) Cell surface barriers*

The initial studies by employing poorly differentiated cell culture model that inaccurately mimicked the *in vivo* epithelial cell morphology which provided relative ease in transduction resulted in limited success in eliciting the desired therapeutic response as evidenced from the *in vitro* studies. The result was due to poor extrapolation of the *in vitro* study to the *in vivo* behaviour of the formulated gene delivery system due to the lack of cell surface specific barriers which the *in vitro* cell cultures failed to express. However, with the advent of well differentiated cell cultures, it became clear that the cell surface specific barriers that need to be tackled for effective gene delivery consist of columnar cells joined by tight junctions and epithelial cells that have a basal and stimulated rate of endocytosis. Also, the receptor accessibility specially in case of certain viral vector delivery systems is very difficult as the receptor are located at the basolateral membrane (81), although targeting of receptor with nonviral vectors may prove to be equally challenging. To circumvent this issue of poor permeability across tight junctions, various agents can be utilized such as detergents (non-ionic), ion chelators, fatty acids etc. which disrupt the integrity of tight junction temporarily (82-85). A similar strategy was utilized for basolateral delivery of recombinant adeno-associated viral vector serotype 2 (rAAV2) by utilizing cytochalasin D, a fungal toxin that inhibits actin polymerization (86). Targeting to receptors that are highly expressed on the epithelial cell apical surface and has the capacity to internalise vector have been utilized; for example, stimulation of endocytosis on the apical surface of the endothelial cells by receptor binding of urokinase plasminogen activator or its peptide enhanced gene transfer using adeno or adeno associated viral vectors (87). Other available receptors include purino receptor P2Y2-R (88) serpin enzyme complex receptor (62) etc. Studies with nonviral vectors also have shown success in delivery to transfect air epithelial cells with the use of cationic lipid complexes with detection level of the gene expression even after months along with expression in extravascular parenchymal cell of other organs (spleen, lymph node etc.) without any apparent toxicity related to the treatment (89-91).

(*iii) Intracellular barriers*

Overcoming extracellular barriers doesn't solve the problem, however, second to this, one has to think of strategies that would help to overcome intracellular barriers which may affect the ultimate transfection abilities of nucleic acid carriers. It's requisite for any therapeutic gene delivery carrier to reach into the cell for their efficacy. Any drug or gene carrier gets uptaken into the cell via process

called internalization via different endocytosis mechanisms named as receptor mediated endocytosis, clathrin mediated endocytosis, phagocytosis, micropinocytosis etc. and delivered to endosomes which further fuse with lysosomes which degrades the carriers or particles. Hence endosomal escape is the most important step for any nucleic acids carriers to deliver their therapeutics payloads into the cytoplasm before it gets degraded by endosomes. Thus, not only the cellular uptake through cell membrane but also intracellular availability of gene cargo at target site is most vital for efficacy of gene delivery carriers.

Currently several approaches have been sought for the nonviral carriers to overcome effectively the challenges of intracellular trafficking. Owing to larger size and anionic charged nucleic acids mainly pDNA and siRNA their singular transport through cell membrane remains difficult, therefore by making stable complex with cationic polymers or lipids, pDNA and siRNA can be delivered efficiently. Nanocarriers containing fusogenic lipids, cationic charged lipids, polycations, pH sensitive peptides would be promising strategies for intracellular gene delivery. Lipid like DOPE having fusogenic or membrane destabilization activity and can be used in the cationic liposomes as a helper lipids. At endosomal acidic pH, DOPE transits its bilayer structure to inverted hexagonal phase yielding endosomal membrane destabilization and releases the contents into the cytoplasm (92) As mentioned earlier, cationic polymers named polyethylenimine (PEI), poly-l-lysine (PLL) and cationic lipids have been investigated as a pulmonary gene delivery carriers. Polyethylenimine is a polycation having a proton sponge effect and buffering capacity at endosomal acidic pH and escape from endosomes which is crucial step to prevent endolysosomal degradation. Unprotonated amino groups of PEI attracts the protons resulting in the influx of water and chloride ions within the vesicles which leads to increase in osmotic pressure and subsequently causes bursting of endosomes. A number of studies have been performed utilising PEI- based nucleic acid complexes for pulmonary delivery. (93) Also a number of approaches for PEI modifications have been devised with aim to reduce the toxicity of carriers and to improve the transfection efficiency. Grafting of PEG on PEI molecules is one of the approach to fulfil the objective. PEI-g-PEG-DNA polyplexes exhibited high transfection efficiency and low cytotoxicity in bronchial and alveolar cells studied by Kleemann et al. In addition to PEI, others cationic polymers or polycations such as chitosan, poly-l-lysine have been scrutinised to achieve the better transfection efficiency as a pulmonary gene delivery carriers. Conjugation of these polymers with PEG and PLGA also studied with view to reduce the toxicity and to improve biocompatibility (12, 22, 31, 94).In addition, pH responsive peptides contains pH responsive residues, which makes complexes with nucleic acids. At endosomal acidic pH, they do conformational change releasing from complex and destabilizes the membrane and discharge nucleic acids to the cytoplasm. Liang et al developed spray dried powder containing pH responsive peptides demonstrating the efficient transfection of DNA overcoming the pulmonary surfactant liquid barrier, which is one of the hurdle for pulmonary gene delivery (95).

However, to develop efficient and successful gene delivery carriers for pulmonary route numerous difficulties are necessitated to be resolved with respect to the delivery aspects, to achieve specific intracellular targets focusing on the toxicity and transfection efficiency of the carriers, challenges to cellular internalisation, endosomal escape, performance of the delivery devices in terms of the patient compliance, dose accuracy etc. At present, polymer-lipid based gene delivery nano-carriers are being comprehensively explored to develop efficient aerosolized delivery to cellular target for the treatment of various pulmonary diseases.

3.3 Immunological barriers

In addition to mucus and mucociliary clearance barriers to the gene transfer to the lung, Innate and acquired immune responses is another challenging obstacle for the viral and nonviral gene transfer carriers. Nonviral vectors and mostly viral vector mediated gene delivery associated with higher probabilities of immunological responses including cellular response, humoral response, non-specific inflammation and also innate mechanism of lung itself provoke defence mechanism to remove foreign particles. Alveolar macrophages eliminate the particles by phagocytosis significantly reducing the gene expression (96). Further, repeated administration of gene delivery carriers is limited by the generation of neutral antibodies by helper T cells dependent responses. CFTR gene delivery for cystic fibrosis by the viral vectors have been greatly associated with immune responses, generation of neutralised antibodies which are found in clinical studies (97-99).

To overcome such immune responses, several approaches have been devised by researchers. Immunosuppressant drugs like cyclosporine, cyclophosphamide prevent the neutral antibodies formation and also improve gene expression (100). While corticosteroid drug like budesonide has been addressed for reducing the neutralised antiviral antibodies in BALF and serum and thus improves the gene expression and permits re-administration of carriers (101). Interferon-γ and interlukin-12 co-administration to reduce Th_2 cells activity and neutralising antibody formation, by blockage of CD4+ cells is important in cellular and humoral responses and prolongs gene expression.

Nonviral carriers mediated nucleic acids delivery to lungs is generally safer and less immunogenic than viral vectors. Several researchers have addressed the immunogenicity and inflammatory response by the unmethylated CpG motif through plasmid DNA. Generally, plasmid DNA contains unmethylated CpG motifs which is known to be immunostimulatory and cause adverse effects by activating the innate and acquired responses through involvement of mainly Toll like receptors (TLR) family. A number of reports have shown that unmethylated CpG motifs of pDNA complexed with cationic lipid carriers induced inflammatory responses with of release of IL-6, TNFα and IFN-γ like proinflammatory cytokines and influx of cellular infiltrate in BAL fluid which further exaggerated by lipid vectors (102-104).Hence newer strategies are needed to develop safe and less immunogenic gene delivery carriers. Modification of the CpG motif sequence from DNA vectors and elimination or methylation of plasmid DNA are different ways to avoid or remove the immunostimulatory effects of unmethylated CpG motifs. Yew et al studied that modification of CpG nucleotide sequence in modified plasmid vector (CpG-reduced pGZA-CAT) administered in BALB/c mice intranasally or intravenously demonstrated substantially less immunostimulatory actions with significant reduction in proinflammatory cytokines in BAL fluid. (105) They have also studied the inhibition of CpG signalling pathways by the chloroquine and quinacrine and observed almost 50% inhibition of cytokine production (105).Methylation of CpG motifs of plasmid DNA is another strategy to prevent the immune response by the vectors. CpG methylated motifs inhibits the stimulation of immune response and thereby improving the gene expression (106). Alveolar macrophages have key role in the inflammatory response in the respiratory diseases like asthma and COPD. Tumor necrosis factor (TNFα), secreted by macrophages is proinflammatory cytokine having important role in the innate immune response. So targeting TNFα by siRNA is promising strategy to treat respiratory inflammatory conditions. Kelly et al developed siRNA encapsulated in PLGA microparticles

with DOTAP and optimized for aerodynamic parameters for inhalation and targeting to alveolar macrophages. Formulation exhibited around 45% decreased in expression of TNFα in human monocytic cell line THP-I over 48 h. (107).

Several cationic polymers like Polyethylenimine (PEI) and poly l-lysine (PLL) based gene delivery carriers also have been reported for eliciting immunological response. Conjugation of cationic polymer/DNA complexes to PEG, minimizes the toxicity potential by shielding the cationic charge of the polymers and making particles enabled to mucus penetrating by rendering the high dense surface coating with small sized particles. Such type of highly compacted mucus penetrating particles demonstrated penetration through CF mucus *ex vivo* without causing the inflammatory response and holds great potential as a gene delivery vectors. (108),(109). Delivery of nucleic acid carriers either by aerosolized form or direct instillation also have influence on the immune response in the lung. It has been reported that delivery of nucleic acids carriers in aerosolized form is most efficient technique for gene therapeutics. Aerosolized droplets of the carriers have been reported less immunogenic, reduction in dose related toxicity due to the evenly distribution of therapeutics (18) with localized higher gene expression (17, 19) than direct instillation and intravenous administration.

3.4 Aggregation of Carriers

From the formulation aspects the charge ratio of the lipid and DNA employed determines the resulting zeta potential of the complex formed and the presence of electrostatic repulsive forces that plays an important role in preventing aggregation. In case of neutral complexes formed by a 1:1 charge ratio mixing, the complexes exhibit much less colloidal stability as well as heterogenous size distribution (110, 111) whereas highly positively and negatively charged complexes exhibit homogenous size distribution and reduced tendency to aggregation (112, 113). The formation of lipoplexes by interaction of DNA and cationic liposomes are enhanced by preparation in solution with low ionic strength which prevents aggregation and sedimentation of the complexes. The incorporation of PEGPE (poly(ethylene glycol) phospholipid conjugates) in cationic liposomes has been investigated to prevent aggregates and increasing stability of the complexes (114).

The aggregation of certain types of lipids in circulation and their role in the formation of aggregates has been investigated which suggest a higher amount of interaction of large size vector aggregates with endothelial cells in the lung capillaries. Interactions which arises by using higher amount of helper lipids like cholesterol or DOPE with cationic lipid vector is beneficial for attaining maximum level of gene expression due to a balance between rate of aggregation of lipid vector with the serum and subsequent disintegration of the aggregates from the target cells in the lungs leading to high transfection efficiency (115).The passive interaction of negatively charged endothelium with the cationic lipid vector play a significant role in pulmonary gene transfer. The aggregation of cationic lipids induced by its passive interaction with the serum proteins (116) lead to increase in the size of lipidic vectors which gets entrapped in the pulmonary vasculature due to their larger capillary bed post systemic administration (117). The aggregation behaviour of siRNA loaded nanogels was evaluated in the presence of a pulmonary surfactant which imparted colloidal stability to the surfactant coated particles as compared to the uncoated ones. Though the coated nanogels showed decreased intracellular internalization, a comparable amount of gene silencing was achieved in both the cases (118).

The use of spray drying technique using mannitol as antiaggregant, for preparation of cationic micro-particles of PLGA nanospheres (namely PEI, DOTMA, DC-Chol or CTAB) containing plasmid DNA

was investigated to confer positive charge to the formulation and prevent aggregation (119). Spray drying of drug loaded nanosuspension lead to the formation of large hollow carriers with their shells composed of nanoparticle aggregate having large geometric diameter and small aerodynamic diameter making them suitable for DPI application. Such nanoparticle aggregate with small size or thin shells were found to easily dissociate in contact with aqueous microenvironment to yield primary nanoparticles as investigated by Hadinoto et al (120). Laouini et al investigated the aggregation behaviour of nebulization of different nanocarriers for vitamin E namely liposomes, micelles, nano-emulsion and solid lipid nanoparticles and the extent of their retention in lung. It was concluded that for nanosystems with solid as a dispersed phase, the extent of aggregation was less as compared to those with liquid as dispersed phase. Such systems in which nebulization lead to the formation of large size aggregates led to selective retention and deposition in the upper respiratory tract or the broncho-alveolar region and can similarly be investigated for pulmonary gene delivery as well (121). z-DNA molecule exist in negative supercoiled state in the biological systems thus has torsional strength as compared to relaxed DNA and a higher free energy lending the form highest immunogenicity as compared to other forms (122). For achieving highest immunogenicity of the vaccine formulation for eliciting antibody production, it is mandatory that the pDNA retains its supercoiled structure during the process of nebulization and do not form aggregates. For this the morphological change of the supercoiled structure of the DNA at the time of nebulization needs to be addressed properly. Use of sound acoustic wave (SAW) for nebulization provided for retention of the structure of pDNA >90 % with minimal distortion of the structural integrity after repeated nebulization cycles and presence of a low amount of aggregated structures (123).

The delivery of siRNA through intravenous route has been utilized to knockdown pulmonary endothelial specific genes to treat respiratory tract diseases as opposed to direct delivery via inhalation or intranasal route. PEGylation of the delivery carriers is a promising strategy for intravenous delivery carriers. As being a hydrophilic in nature, PEG shields the charged surface of the carriers and prevents RES uptake and also it prevents the aggregation of carriers in the serum. McCaskill et al developed the i.v. liposomal delivery system using PEGylated-PEI lipoplexes based on same fundamentals (124). Also, condensation of the lipoplexes with polycations such as protamine or poly (l-lysine) with DNA at an optimized charge ratio produced complexes without the issue of aggregation and enhanced transfection of cell line along with protection from nucleases (125, 126).

3.5 Controlled or Sustained Release of carriers:

Sustained gene expression in lung is required for any gene delivery carriers due to the limitation of development of host immune response when administered frequently. Even after designing of delivery system that shows sustained expression of gene, the expression is limited by highly variable epithelial cell turn-over rate. This can be counteracted by transient immunosuppression for avoiding the immune response and re-administration of viral or nonviral vectors with long action as seen in mice administered with helper dependent adveno viral vector (127). Also designing a suitable vector that incorporates therapeutic gene that can be used for integration to airway progenitor cells and engineering site specific endonucleases for selecting a safe integration site for the designed vectors is a fascinating area to explore the potential for sustaining the gene expression of the therapeutics (128, 129).

It is important to meticulously design the delivery vector for efficient gene transfer.

Alteration of the nucleotide bearing region in the plasmid DNA vector was found to be useful in sustaining the expression of the therapeutic gene and preventing immune response. Hyde et al. investigated the sustained trans-gene expression of CpG free nonviral vector containing pDNA and cationic liposomes complexes (GL67A) for treatment of cystic fibrosis in the absence of lung inflammatory response, in which the transgene was a cDNA for cystic fibrosis transmembrane regulator protein with no CpG motifs and delivered sustained expression of mRNA for 56 days in murine lung model (130). The presence of even a single CpG in plasmid was sufficient to elicit an immune response and altering the gene expression along with inflammatory response.

The most prominent factors that are required for the sustained gene expression of therapeutic gene is to deliver the genetic load inside the nanosystems by solving the issue of decreased permeability, prevention of clearance and enzymatic degradation, since once inside the nucleus of cell, the products of the cell transfection process gets expressed for days to months (131-133). The use of polymeric carriers for sustaining the release from the nanosystems appears to be the most convenient method. The delivery aspects of plasmid discussed here can be extrapolated to delivery of siRNA or nucleotides to pulmonary route. The weak interaction between the negatively charged plasmid and negatively charged polymers leads to repulsive interactions resulting into modulation of rate of release by the properties of polymer employed. Yun et al investigated hyaluronic acid microspheres incorporating DNA using adipic dihydrazide chemistry for controlled release application. The prepared microspheres had the capability of *in vitro* and *in vivo* transfection with selectin receptor specificity after conjugation to targeting ligands and exhibited sustained release for months (134). Other natural polymers investigated include albumin, gelatin, alginate, collagen, cyclodextrin, chitosan etc and synthetic polymers investigated include PLGA polymers, ethylene vinyl co-acetate polymers, polyanhydrides, polyacylates etc that provided sustained release profile from days to months (135-140).

For successful pulmonary gene delivery, performance of devices to deliver gene or siRNA is important consideration for efficacious delivery of the therapeutics. Pulmonary gene or drug delivery can be done via intranasal, intratracheal or inhalation route. Even though, intratracheal and intranasal administration demonstrated efficacy in preclinical models, clinical applications are limited. Due to the several advantages of inhalation route like non-invasiveness, local delivery to pulmonary region for targeting local diseases, avoidance of first pass effect, aerosolised delivery systems seems attractive for the treating pulmonary disorders than other routes. However, development of the efficient gene delivery technique is challenging due to the presence of the several barriers as described earlier, immunogenic response, presence of the pulmonary surfactants which results in reduction in the transfection efficacy. Also several factors need to be considered to develop the delivery techniques which includes stability of the delivery vectors, stability against the force of the nebulisation, aggregation or agglomeration of carriers etc. Gene delivery vectors can be administered through various forms like aerosol, dry powder inhaler and nebulisation. The widely used delivery devices were metered dose inhalers (MDI) but due to the drawbacks such as patient incompliance due to difficulty in breathing coordination during dose instillation in children, oropharyngeal deposition of dose, use of toxic propellants etc. limited their applications. Currently several studies and investigations have been done for the development of more efficient delivery through nebulisation and dry powder inhaler. Nebulisation is one of the most practical

technique for the frequent applications of the gene therapeutics. During nebulisation, maintenance of the physical stability and biological activity is one most challenging task for the development of the delivery systems. However, several studies have demonstrated the potential applications of the nebulisation in pulmonary delivery. Manunta MD et al studied that gene delivery through nebulisation have great potential applications for treating diseases like cystic fibrosis. Authors have demonstrated that plasmid DNA complexed with the cationic liposomes revealed integrity of pDNA against the shear force of the nebulisation, maintenance of aerodynamic properties of the formulation and improved *in vivo* and *in vitro* transfection efficiency (141) (93).

Another approach for safe and stable delivery of therapeutics is by dry powder inhaler (DPI). Drug or gene delivery vectors are formulated with the inert materials like lactose, trehelose, mannitol, sucrose etc. as a dry powder form to be inhaled. DPI exhibited improved in vivo delivery of the anti-asthmatics (142) and biomolecule like insulin (143) (144) and seems promising to deliver siRNA therapeutics or pDNA. Improved stability as a dry form and propellant free dosage form makes DPI more promising as an inhaler device. Dry powder can be processed by the techniques like spray drying, spray freeze drying, supercritical fluid technology etc. Jensen DK et. al prepared DOTAP modified PLGA nanoparticles for the siRNA delivery to the lung and spray dried using mannitol demonstrating improved stability, maintenance of siRNA intactness, with sustained release characteristics. Such other examples are listed in Table 1 (145). Hence, choice of method or delivery device for the nanocarriers is one of the significant contributing factor for achieving efficient gene delivery via pulmonary route.

4. FUTURE PERSPECTIVE AND CHALLENGES

Despite great excitement arising from basic research and large number of animal model studies, acceptance of gene/siRNA as major therapeutic modalities to revolutionize treatment of pulmonary diseases faces so many challenges as represented in figure 4.

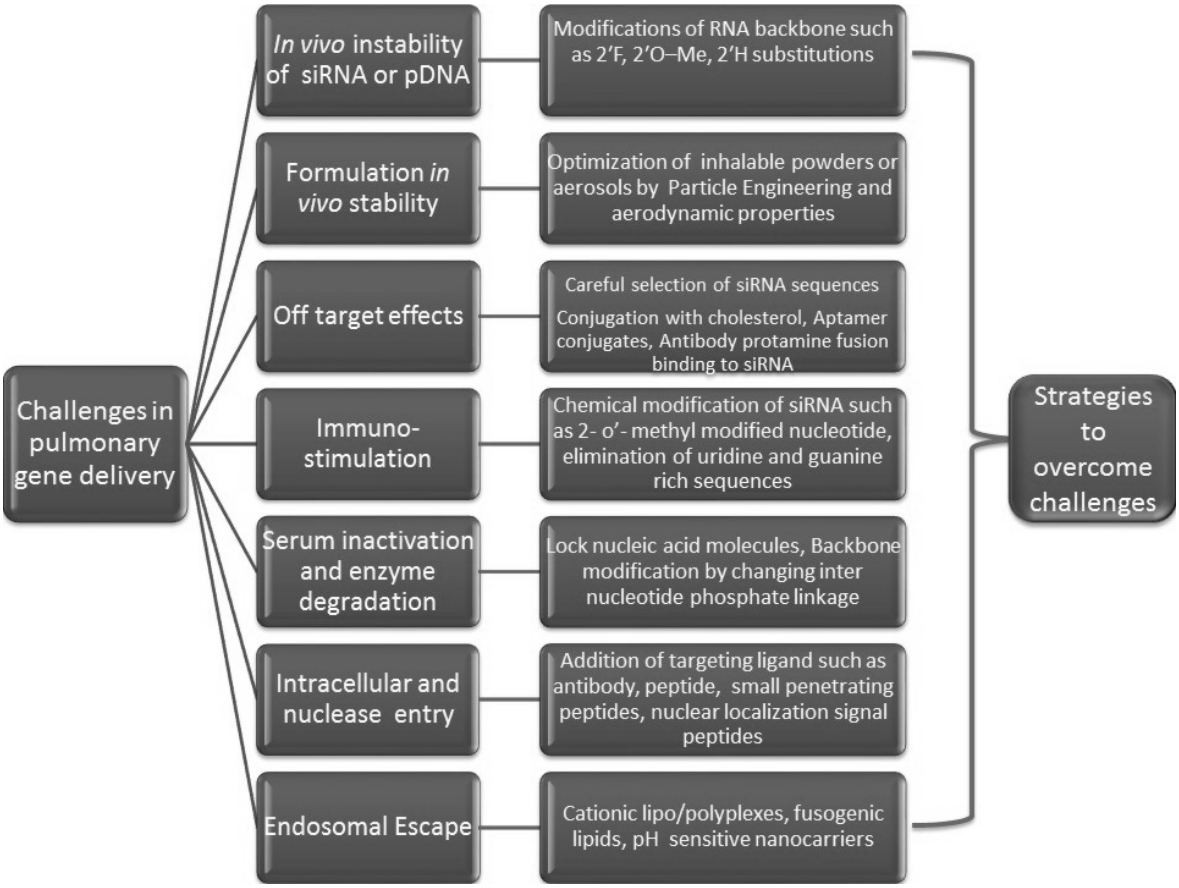

Figure 4: Challenges to pulmonary gene delivery.

In literature, naked siRNA delivery has also exhibited therapeutic effectiveness when administered *in vivo* but it is severely criticized for its *in vivo* stability. Therefore, research needs to focus on modification in RNA backbone such as 2′F, 2′O–Me, 2′H substitutions to increase its half-life and serum stability without compromising target expression profile (146). Further, to improve *in vivo* site specific delivery and reduce off target effects various conjugation strategies such as conjugation of siRNA sense strand to cholesterol, antibody protamine fusion binding to siRNA, aptamer siRNA conjugates and co expression of argonaute protein needs to be explored (147-149). Novel computational technology that can identify and screen hyper functional siRNA and predict its effect on target protein expression should be designed to increase specificity and it will boost research in this therapeutic arena (150). To encounter problem of nuclear entry of pDNA, newer nuclear localization signal peptide (NLS) such as large tumor antigen of simian virus 40 should be identified. DNA sequences having nuclear import activity based on their ability to bind to cell-specific transcription factors such as the SMGA promoter and flk-1 promoter should also be identified.

Although viral vectors seem to be promising approach, the major problem associated with viral vector gene delivery is unwanted immunotoxicity. It is believed that uridine and guanosine rich sequences with either UG dinucleotide or 5′-UGU-3′motifs potently activate TlR7 and TlR8, and elimination of such sequences decreases innate immune response (151). Identification of such sequences in viral vectors eliciting innate

immune response and their elimination will remove immune response triggered problems.

Pulmonary administration of nonviral vectors also faces many problems such as change in conformation and integrity resulting into decrease in bioactivity of gene due to solvent exposure and processing condition. A mild development method preserving biological activity of gene having suitable physicochemical parameters for inhalation of nanoparticles would boon the research in this arena. Detailed optimization of nonviral vectors concerning size, chemistry, surface charge, shape, biocompatibility, and their efficacy is also necessary for their pulmonary administration. If aerosols or dry powders for inhalation are formulated for pulmonary delivery, additional requirements such as the compatibility with excipients, for example propellants or lyoprotectants, freeze drying process optimization, achievement of optimal aerodynamic diameter for disposition of carrier need to be factored in. Minko et al. delivered combinatorial local inhalation delivery of doxorubicin along with BCL2 and MRP1 gene for treatment of lung cancer and found high antitumor activity and low adverse side effects in comparison to individual components administered separately (152). Such strategies for co-administration of therapeutic agent along with gene should be explored for treatment of other pulmonary diseases such as asthma and cystic fibrosis.

Recently Novel delivery agents such as lipidoids, synthetic lipid like materials, developed using multidisciplinary approach of computational technology, biotechnology, chemistry and pharmaceutical technology for gene delivery seems to be promising approach. Akin et al have developed novel lipidoids 98N12-5 (153) for liver targeting after systemic administration which induced fully reversible, long-duration gene silencing without loss of activity following repeat administration (153). Development of such lipidoids should be encouraged for treatment of pulmonary disease.

5. CONCLUSION

With the increase in demand of an alternative therapeutic intervention in pulmonary diseases and the emergence of novel delivery methods for genetic material through the use of novel vectors, new delivery methods along with the advancements in genetic engineering, an era of genetic manipulation and its utilization in various difficult to treat ailments has provided a new horizon. Though the current prospects of pulmonary delivery are limited in terms of achieving the desired result of receptor specificity, high transfection, stability and low immunogenicity, further research efforts are needed to ensure safety, in *in-vivo* applications and the development of process that can be easily scaled up. Through the realization of target specificity and means to achieve it along with the inherent advantage of non-invasiveness, the pulmonary route is emerging as a potential for treatment of not only localized but systemic diseases as well. However, the need to effectively and efficiently deliver genetic material for the said purpose and obtaining clinical benefits still requires a more comprehensive outlook.

List of abbreviations:

COPD Chronic obstructive airway disease

PAH Pulmonary arterial hypertension

RNAi RNA interference

CFTR Cystic fibrosis transmembrane conductance regulator gene

MMP Matrix metalloproteinase

DOTAP N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride

DOPE 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine

DOTMA N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride

PLGA	Poly(lactide)-co glycolide
pDNA	Plasmid deoxyribonucleic acid
PEG	Poly ethylene glycol
CpG	Cytosine-phosphate-guanine
DPI	Dry powder inhaler
PEI	Polyethyleneimine
CTAB	Cetyl trimethylammonium bromide
PLL	Poly-l-lysine
TNFα	Tumor necrosis factor α

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