Nanocomplexes for Gene Therapy of Respiratory Diseases: Targeting and Overcoming the Mucus Barrier

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ABSTRACT

Gene therapy, *i.e.* the delivery and expression of therapeutic genes, holds great promise for congenital and acquired respiratory diseases. Non-viral vectors are less toxic and immunogenic than viral vectors, although they are characterized by lower efficiency. The respiratory and airway epithelial cells, the main target of these vectors, are coated with a layer of mucus, which hampers the effective reaching of gene therapy vectors. This barrier is thicker in many lung diseases, such as cystic fibrosis. This review summarizes the most important advancement in the field of non-viral vectors has been achieved with the use of nanoparticulate (NP) systems, composed either by polymers or lipids, in the lung gene delivery. In particular, different strategies of targeting of respiratory and airway lung cells will be described. Then, we will focus on the two approaches that attempt to overcome the mucus barrier: PEGylation of the nanoparticulate system and treatment with mucolytics. Our conclusions are: 1) Ligand and physical targeting can direct therapeutic gene expression in specific cell types in the respiratory tract; 2) Mucopenetrating NPs are endowed with promising features to be useful in treating respiratory diseases and should be now advanced in pre-clinical trials.

Keywords: airway epithelium, mucolytics, mucus, non-viral vectors, poly(ethylene glycol), sputum, targeting, viscosity.

1. Introduction

Lung diseases represent a significant burden for the society. It has been estimated that diseases stemming from the respiratory tract will be the third cause of death in 2020. What is causing a plenty of studies in the treatment of these diseases is that there is not a cure for many of them. Gene therapy could be a resolutive approach to treat lung diseases, such as cystic fibrosis (CF), α1-antitrypsin deficiency (ATD), lung cancer, asthma, and chronic obstructive pulmonary disease (COPD) [1, 2].

Gene delivery has been revolutionized with the introduction of nanoparticles (NPs) which can carry drugs or genes into cells or tissues [3]. NPs have a size ranging from 1 to 1000 μm and can deliver their cargo by topical or systemic application. A variety of nanoparticulate gene delivery systems (nanocomplexes) has been evaluated in the lung. Although nanocomplexes have a gene delivery efficiency which is not so high as that observed with viral vectors, the former are safer than the latter. In the past few years, several improvements have been obtained in the study of the efficacy of nanocomplexes as potential tool for the treatment of diseases as CF and ATD. However, further preclinical studies are necessary to translate these delivery systems to human beings.

The application of these delivery systems by different administration routes (intratracheal injection, aerosolisation and systemic injection) has been evaluated with varying grades of efficiency in mice and humans. The level of transgene expression, in the lung tissue, after the application of nanocomplexe-based delivery systems, has gained significant levels. Nanocomplexes inhalation represents a noninvasive administration route, which can improve the targeting of a defined cell type. Pulmonary gene delivery is advantageous for many reasons such as reduced systemic side effects, no sequestration by serum proteins, and the use of a lower dose of topically administered formulations, as compared to the systemic ones. However, various physiological barriers, *i.e.* mucus in the airway lumen, makes very challenging the application of gene delivery to the lung.

In the first part of this review we will present NP systems for lung gene delivery as well as the targeting strategies aimed to enhance gene expression levels in the lung at the level of airway and respiratory lung cells. Further, we will discuss the role of mucus as extracellular barrier in respiratory gene delivery mediated by nanocomplexes. Also, some approaches that may improve the gene delivery across the airway mucus, will be described in the second part.

2. Structure and function of the respiratory tract

The development of new nano-delivery systems is strongly influenced by the anatomical structure of lungs. The respiratory apparatus can be divided into two regions: the conducting airways and the respiratory zone. The conducting airways work as an air transport system and consist of the mouth/nasal cavity, pharynx, larynx, trachea, bronchi and bronchioles. The gas exchange takes place in the respiratory zone, *i.e.* respiratory bronchioles and alveoli. The conducting airways are highly branched with 16 bifurcations which are followed by another 6 bifurcations of the respiratory bronchioles. A gradual reduction in the luminal diameter can be observed in the passage to the respiratory zone, where the alveolar ducts with alveolar sacs finally branch off.

Cells from the airway epithelium are very different from those of the alveolar epithelium. The airway pseudostratified epithelium is made of several cell types and principally of the ciliated columnar cell, the goblet or mucus-secreting cell, the basal cell and the Clara cell. Epithelial cells in the lung are intimately connected by several proteins forming tight junctions. The alveolar epithelial surface is covered for ~95% by type I pneumocytes, which are characterized by a very low thickness (≤ 200 nm) and a large extension (~200 μm) [4], whereas type II pneumocytes are cuboidal cells covering less than 5% of surface area.

The lung has evolved several barriers to avoid the uptake of any inhaled particulated compounds. It has been estimated that the human airways are exposed to >7 kg of pollutant a year. The thickness of the air-blood barrier gradually change in the passage from the tracheo-bronchial region (where

the wall is at least 10 μm tick), to the alveolar region (≤0.3–1 μm in thickness). The surface of the conducting airways is covered by a mucus layer (thickness: 5–55 μm) secreted by goblet and submucosal gland cells**.** Mucus contains 90-95% water by mass. The remaining mass consists of mucins (about 2%), DNA, lipids, electrolytes, proteins, cells and cell debris [5]. Mucins are high-molecular mass glycoproteins with alternating glycosylated and cysteine-rich regions, produced by the epithelial goblet cells and submucosal glands. Mucins are negatively charged owing to the abundant carboxyl groups at the termini of glycan entanglement and form networks via internal disulfide bonds, physical entanglement and non-covalent interactions [6]. Viscoelasticity of normal mucus is mainly attributable to mucins, predominantly MUC5AC and MUC5B [7]. Mucus is cleared from deep airways by the motion of cilia which is estimated to occur at a flow rate of about 5 mm/min. This process causes a "self-renewing" of mucus blanket which take place every 20 min in healthy subjects [4, 8]. Whereas gas, ions, nutrients and proteins easily diffuse through mucus, particulate substances can be entrapped and immobilized by the mucus and removed before they contact the underlying epithelial cells [9]. In this way, mucus protects the body from invasion of foreign substances such as toxins, pathogens and environmental ultrafine particles. The tip of a cilium sweeps the surface of the mucus layer by a shearing motion. The movement of cilia is small and fast enough to permit that mucus gel uses its elasticity rather than its viscosity. Cilia bath into the periciliary layer (PCL) whose viscosity is finely regulated and close to that of aqueous solution. Mucus lies atop PCL and is moved by the tips of the cilia and its secretion/composition is regulated to allow cilia to work correctly. Indeed, in some diseases, such as CF, mucus becomes so viscoelastic that cilia are not able to move it [10]. A successful delivery of nanocomplexes occurs when they cross the mucus layer before being cleared from the airways. Although mucus is transported by cilia motion, some lung diseases, such as CF, are characterised by a drastic change of the clearance rate [11].

Moreover, sputum from patients with lung diseases such as cystic fibrosis or respiratory infections, is a complex mixture of biological substances (such as DNA and actin) derived from dead neutrophils,

epithelial cells and bacteria [12]. Both DNA and actin, by virtue of their anionic properties, can increase the viscosity of mucus by forming gels or interactions with mucus [13].Therefore, the alterations of mucus caused by some lung diseases may strike the transfection efficiency especially if the nanocomplexes cargo has to be delivered to the cells lining the conducting airways.

The epithelium covering the lumen of each alveolus is protected by a layer of surfactant fluid (20-80 nm in thickness) [14]. Pulmonary surfactant is secreted by type II pneumocytes and is composed of 80% phospholipids, 5-10% neutral lipids and 8-10% proteins [4]. The surfactant phospholipids are located at the air-liquid interface and reduce the work of breathing as well as avoid the collapse of the alveoli during expiration.

Alveolar macrophages (AM), which are located in the alveoli and alveolar ducts, act as phagocytic cells and appear to be another important barriers to lung gene transfer. They represent the first cells to encounter nanocomplexes in the lower respiratory tract. Human lungs are estimated to contain 2.3 x 10^{10} AM, with 50 to 100 AM per alveolus. Alveolar macrophages, localized in the deeper lung, can eliminate inhaled particles whose dimensions can influence the clearance process of itself. Indeed, particles having a size ranging from 1 to 3 μm are taken up by macrophages more efficiently [4, 15].

3. Gene delivery to the lung

The lung represents a proper target for gene therapy for the purpose to treat various lung diseases, such as inherited monogenic disorders, or bronchial tumours. There have been major advances in the understanding of the molecular pathogenesis of pulmonary diseases in past decades. However, effective translation of this knowledge into viable gene-based therapies and realization of their clinical potential is yet to be achieved.

The performance of a gene delivery system is influenced both by transgene expression and by reaching the correct target site in the lung. Indeed, many efforts have been made so as to improve gene delivery performances, such as considering the use of specific targeting ligands into the vector, in order to optimize gene delivery to the appropriate target cells and reduce the total dose of vector required. Similar hurdles hold for delivery of small interfering RNA (siRNA) to the lung cells [16, 17].

Gene delivery to the lung can be obtained by using two classes of non-viral vectors, so that polycations and cationic lipids have been applied in order to transfer gene to the lungs [18]. Both classes are quite efficient as gene transfer agents, but possess both a poor targeting capacity and a significant toxicity [19]. Therefore, targeting is supposed to increase efficiency in face of reduced toxicity.

3.1. Cationic polymers

Polycations studied in gene delivery experiments include synthetic aminoacid polymers such as polylysine, other cationic polymers such as polyethylenimine (PEI), dendrimers, or carbohydrate-derived polymers such as chitosan [20]. PEI is used in a broad range of molecular weight, each with its *in vitro* and *in vivo* gene transfer efficiencies. In view of its high transfection efficiency, PEI has been considered as a reference for other gene transfer vectors. Concerning the airways, many studies have shown that PEI can promote gene transfer *in vivo* with an efficiency corresponding up to 5% of pulmonary cells, when administered intravenously [21]. Administration of the PEI–DNA complexes through the airways resulted in transfection of various cell types such as epithelial cells, macrophages, and endothelial cells [22, 23]. Due to the lack of specific mechanisms involved in PEI polyplexes uptake, *i.e.*, occurring by adsorptive endocytosis, PEI has been modified (see below Section 3.1.1) in order to increase its targeting to the airways.

One of the first cationic polymers used in transfection experiments, was poly-L-lysine (PLL). This molecule has shown a poor transfecting capacity and its cytotoxicity as well as its tendency to form aggregate has discouraged its use [24].The cytotoxicity of PLL can be reduced by grafting of its backbone with various molecules such as biodegradable poly(lactic-co-glycolic acid) (PLGA) [25], biocompatible poly(ethylene glycol) (PEG) [26], and iron oxide (IONP) [27]. It is interesting to observe that IONP-PLL mediated the transfection with a much higher level in airways than in other organs. A nebulizing formulation of PLL and protamine has been shown to give a transfection efficiency 3- to 17-fold higher than PEI *in vitro,* whereas its toxicity *in vivo* was strongly reduced [28].

Another important category of cationic polymers is represented by natural polysaccharides, such as chitosans, cationic polymers derived by deacetylation of chitin. Chitosan is known as one of the most biocompatible polymers, having a low toxic effect but is affected by a poor transfection efficiency. Its low transfection efficiency is due to: 1) low solubility at physiological pH; 2) lack of buffering amines with reduced endosomal escape effect; 3) strong DNA and siRNA condensing ability with resulting inefficient unpacking of transgene in cytoplasm [29, 30].Various cell types have been transfected by chitosan and transfection efficiencies are extremely variable among cell types. Differences in the chitosan formulation as well as those in cell membrane structure (such as receptors and membrane charge) can cause this behaviour. The presence of degradative enzymes may also alter the intracellular trafficking and release of nanoparticle cargo. In differentiated epithelial cell lines, chitosan polyplexes showed a less efficient transfecting activity as compared to PEI [29]. Interestingly, in the airway epithelium of the lung, chitosan showed equal transfection efficiency to PEI [29]. When chitosan is grafted with PEG, both its biocompatibility and its solubility are improved. These PEGylated chitosan are improved in cellular uptake compared to unmodified chitosan [31]. A chitosan, characterized by trisaccharide branches, showed 4-fold higher marker gene expression, as compared to unmodified linear chitosan, following lung administration in mice [32]. Recently, it has been demonstrated that PEI could help chitosan for a favorable cellular

uptake. Nanoparticles containing PEI-conjugated chitosan (CS-PEI) showed much lower toxicity than PEI or CS separately [33]. This low toxicity may be due to the increase in the charge density due to a higher number of primary amine groups. Chitosans could have promising applications in the area of aerosolized gene delivery systems. The application of chitosan/DNA complexes, as dry powder formulation, has been tested *in vitro* and *in vivo*. Okamoto *et al.* showed that these kinds of therapeutic powders of DNA complexes are promising for pulmonary gene therapy [34, 35].

It must be said that one of the disadvantages of polymers, which can make difficult to assure the reproducibility of transfection experiments, is associated to their dishomogeneous molecular weight distribution. Indeed, the molecular weight of a defined polymer is, generally, conceived as a molecular weight average. Dendrimers, a relatively new class of compounds, characterized by a tree-like molecules with a specific molecular weight, have somehow permitted to overcome the limitations associated with such molecular dishomogeneity. In a primary normal human bronchial/tracheal epithelial cell line, a Starbust polyamidoamine (PAMAM) dendrimer, mixed with a surfactant preparation (Exosurf), enhanced the luciferase gene expression, as compared to dendrimer alone [36]. In this study, it was demonstrated that the Exosurf can act as penetration enhancer and induced uptake of DNA. Recently, these dendrimers have been shown to be able to modify the expression of various genes in treated cells [37]. A dendrimer generation 3, containing polypropylenimine diaminobutane (DAB), mediated high transfection efficiencies in cell lines, such as the A549 type-II penumocytes, but the tranfection was associated with up-regulation of EGFR (Epidermal Growth factor Receptor) [38]. Moreover, Starbust PAMAM induced acute lung injury, after *in vivo* application and this effect was correlated with the activation of autophagic cell death [39]. Therefore, novel biocompatible dendritic vectors need to be developed.

3.1.1. Targeting strategies of cationic polymer vectors for the lung

Targeting strategies aimed at increasing gene transfer efficiency in airway and respiratory epithelial cells *in vitro* and *in vivo* are summarized in Table 1. Successful *in vitro* targeting in cell cultures with mannuronic acid- and PEG-modified PEI vectors has been demonstrated to give up to 1000-fold enhanced gene expression in target cells (immortalized bronchial epithelial 16HBE14o- cells) as compared to both transfection controls and cells treated with ligand-free complexes or free ligands [40]. In a more recent study, targeting of human bronchial epithelial cells, with purpose to obtain receptor-mediated gene delivery, has been studied by using lactoferrin as ligand [41]. Molecular conjugation of lactoferrin to branched-PEI (br-PEI) resulted in a significant increase in the transfection efficiency in human bronchial epithelial cells, whereas no effect could be observed on human alveolar epithelial cells. In addition to achieving cell-specific delivery, the cytotoxicity of the transfection complexes was also reduced significantly by conjugating lactoferrin to br-PEI.

Targeted polyplexes have also been evaluated in the lung *in vivo* [42, 43]. In order to improve the transfection efficiency, a cell receptor system has been used: the serpin enzyme complex receptor (sec-R), which is expressed on the apical surface of airway epithelial cells. Interestingly, when CF mice were administered with nanocomplexes consisting of plasmid DNA expressing CFTR (*i.e.*, the gene mutated in cystic fibrosis) and polylysine coniugated to the sec-R ligand, this resulted in correction of chloride channel activity, as demonstrated by measurements of *in vivo* nasal potential difference and immunohistochemical staining for CFTR. The presence of receptor–ligand was essential as unmodified complexes were ineffective [44].

β2–adrenergic receptors (β2-AR) have been demonstrated to be useful to improve the transfection efficiency in specific target lung cells. When bound to their agonists, these receptors are internalized by a clathrin-mediated process. Therefore, any β2-agonist coniugated to a non viral vector, may increase gene transfer by a receptor-mediated mechanism. Elfinger et al. studied the role of clenbuterol, which is a β2-AR agonist used as bronchodilator in clinic for asthmatic and COPD patients, in the "targeted" gene transfer by non-viral gene vectors. They demonstrated that alveolar epithelial cell lines (human A549 and murine MLE-12) do specifically express β2–

adrenergic receptors, and, therefore, could be targeted by clenbuterol-coniugated PEI/DNA complexes. The enhancement of transfection, using the ligand-PEI complexes was specific for alveolar but not bronchial epithelial cells in vitro [43]. Moreover, optimized transfection of human A549 and murine MLE-12 alveolar epithelial cells, in the presence of the ligand, was found higher as compared to unmodified PEI. After aerosolisation of clenbuterol-modified polyplexes, a 3-fold increase of transfection efficiency was observed as compared with unmodified PEI. Since the enhancement in gene transfer was observed in alveolar but not bronchial epithelial cells, it is worth to speculate that clenbuterol-containing PEI complexes may penetrate deeper into the lung due to the bronchodilatation effect of celnbuterol, although this hypothesis has not been proven yet. On this line of research, guanidinylated chitosan chemically conjugated with salbutamol showed improved siRNA uptake by HEK293 cells, and increased efficacy of gene silencing in vitro and in the lung of EGFP-transgenic mice in vivo using vibrating mesh nebulizer [45]. Another targeting strategy for alveolar epithelial cells was implemented by spontaneously self-assembled ternary PEI-pDNA-insulin nanoparticles resulting from adsorbing insulin on the surface of gene vectors [46]. PEI-pDNA-insulin nanoparticles PEI-pDNA nanoparticles increased transgene expression up to 16-fold on alveolar epithelial cells but not on bronchial epithelial cell compared to plain PEI-pDNA. Finally, Geiger *et al.* [47] explored the targeting capacity of PEI grafted with iloprost (ILO), a prostaglamdin I_2 analogue, which binds to the prostacyclin receptor. PEI-g-ILO increased 14- to 46-fold luciferase expression in human airway and respiratory epithelial cells and *in vivo* in the mice lung upon aerosol application compared to PEI.

Besides ligand-receptor targeting, physical targeting to the lung has been conceived. Targeted aerosol delivery may allow higher transgene expression in a specific lung region while avoiding toxic side effects in other regions of the lung and/or in other organs. Among different methods to achieve this task (*e.g.*, sonoporation, electroporation, etc.), magnetofection has attracted researchers working with nanoparticulate systems. Magnetosols are magnetized aerosols consisting of droplets comprising a gene transfer vehicle (or a soluble drug) together with superparamagnetic iron oxide

nanoparticles (SPIONs), and are guided by an external magnetic gradient field. Magnetosols comprised with PEI-pDNA complexes and SPIONs were aerosolized to the mice lungs obtaining 2-fold and several fold increase in DNA deposition and transgene expression, respectively, in the magnetized right lobe compared to the unmagnetized left lobe [48].

Transcriptional targeting has been the focus of one work. Dames *et al.* [49] chose to include glucocorticoid responsive elements (GRE) in pDNA encoding for luciferase and complexed this plasmid with PEI. An increase in the lung transgene expression with GRE-containing plasmid compared to plain plasmid was observed only when dexamethasone was given intraperitoneally.

3.2. Cationic lipids

The complexes formed by cationic liposomes consist of the positive charged side chains interacting with DNA and a hydrophobic lipid region which enhances fusion with cell membrane. Different mechanisms such as endocytosis, fusion with cell membrane and disruption of the cell membrane lipid bilayer have been proposed for internalization of lipoplexes (complexes of DNA and liposomes). Larger aggregated lipoplexes might also be internalized by phagocytosis [50].

In earlier studies, direct intratracheal administration of cationic liposome–DNA complexes led to efficient transfection of the mouse airways [51]. Successful transfection of the lungs *in vivo* was observed also when the lipoplexes were delivered intravenously [52]. In clinical setting, cationic cholesterol derivatives have, however, been hampered by their relatively low transfection efficiency *in vivo* and concerns regarding their pro-inflammatory activity [19, 53].

Lipoplexes directly administered into target tissue are able to transfect efficiently although their aspecificity do not allow a defined cell-targeting. Moreover, lipoplexes are prone to be inactivated since plasma or extracellular proteins can interact with their positively charged surface [54]. For this reason, a new class of lipoplexes, which is able to resist to the inactivation by proteins, have been developed [55]. Also, to facilitate specific uptake into the cells, targeting proteins have been

included into liposomes, for example, transferrin [56]. Numerous other approaches based on the use of site-directing ligands have been developed for liposome targeting [57].

Allon and colleagues [58] conjugated an high-affinity 7-amino-acid peptide, targeting endothelin receptor to liposomes with high phosphatidyl serine content. These liposomes were shown to give increased gene transfer to A549 respiratory epithelial cells *in vitro* and in the mouse lung after intratracheal instillation.

In addition to the targeting ligands, the choice of the lipid also plays a pivotal role in cell transfection. Tagalakis *et al*. [59] compared the behaviour of two different receptor-targeted lipoplexes and other non-viral vectors, such as GL67, PEI 22 kDa and PEI 25 kDa. The two receptor-targeted nanocomplexes were obtained by self-assembling of the peptide K(16)GACSERSMNFCG with liposomes DHDTMA/DOPE or DOSEP3/DOPE, on mixing with plasmid. Interestingly, the lipoplexes containing DHDTMA/DOPE liposomes transfected the airway epithelial cells, whereas GL-67 and the DOSEP3/DOPE containing liposomes transfected alveolar cells.

4. Respiratory mucus: a barrier towards pulmonary gene therapy

4.1. Biochemical aspects of respiratory mucus in health and disease

The collection of respiratory mucus, from normal subjects, is very difficult. For this reason, many studies on the biochemical properties of respiratory mucus were carried out by sampling mucus from patients whose lung diseases are characterized by mucus hypersecretion (*e.g.* subjects with COPD and CF).

The visco-elasticity of respiratory mucus is due to a three dimensional mesh of cross-linked mucin chains. The structure of mucins is characterized by 4 to 5 subunits which are bound together by disulfide bridges [60]. Each subunit has a length of about 500 nm and consists of a highly glycosylated protein backbone with non-glycosylated ends [61].

Mucins are characterized by sugar chains containing molecules (*e.g.* sialic acid and sulphated monosaccharides) which impart a negative charge to these proteoglycans [60, 61]. The sugar chains can contain from 1 to 20 sugar monomers and they are covalently linked through O-glycosidic bonds between N-acetylgalactosamine and serine or threonine residues. It must be said that each epithelial layer secretes its own characteristic mucins so as to have different location of our body having different biophysical properties of mucus. The concentration of mucin in the sputum obtained from a normal subject is about 20 g/l [62]. CF expectorate is rich of mucus having a narrowly lower mucin concentrations [63]. However, CF patients whose mucus is much more tenacious can have a higher mucin concentration up to 47 g/l [64]. Recently, it has been demonstrated that mucin levels in patients with asthma are comprised between 100 and 1000 g/l [65].

Infection and inflammation can drastically change the characteristics of mucus. Inflammation is associated to mucus hypersecretion, ciliary dysfunction, and alterations in the physicochemical properties of airway secretions [66]. In the inflamed site, necrotic neutrophils release deoxyribonucleic acid (DNA) and cytoskeleton derived actin (F-actin). These two molecules are able to copolymerize to shape up a second stiff network within airway secretions [67]. Such interaction may make the mucin chains more hydrophobic. Such hydrophobicity can strongly influence the delivery of NPs: some studies, conducted by polystyrene nano-beads, have demonstrated an entrapment within mucosal network, probably because of the hydrophobic polystirene bead-forming chemical bonds with hydrophobic domains distributed along mucin fibers [68].

4.2. Interactions of nanocomplexes with respiratory mucus and their role on the gene transfer efficiency

Respiratory mucus is characterized by a biopolymeric network which may delay the diffusion of nanocomplexes by sterical hindrance or by interacting with the nanocomplexes. Moreover, non cross-linked macromolecules may also bind to the surface of the nanocomplexes causing an aggregation which can make difficult their movement through the mucus. It has been reported that, even when no aggregation occurs, the interaction of extracellular components to the surface of nanocomplexes may induce a drastic reduction of their gene transfection efficiency [69]. Therefore, the nanocomplexes will move to the epithelial cell surface in dependence on various factors: the mobility of the nanocomplexes in the mucus, the thickness of the mucus layer and the mucus clearance speed.

When nanocomplexes interact with mucus components, many detrimental effects on nanocomplexes can be produced, including the entrapment of nanocomplexes in the mucus, the neutralization of their surface charge with the formation of aggregates, release of their DNA cargo, and a reduced cellular uptake, which is caused by masking of their positive charges or their receptor ligands (Figure 1).

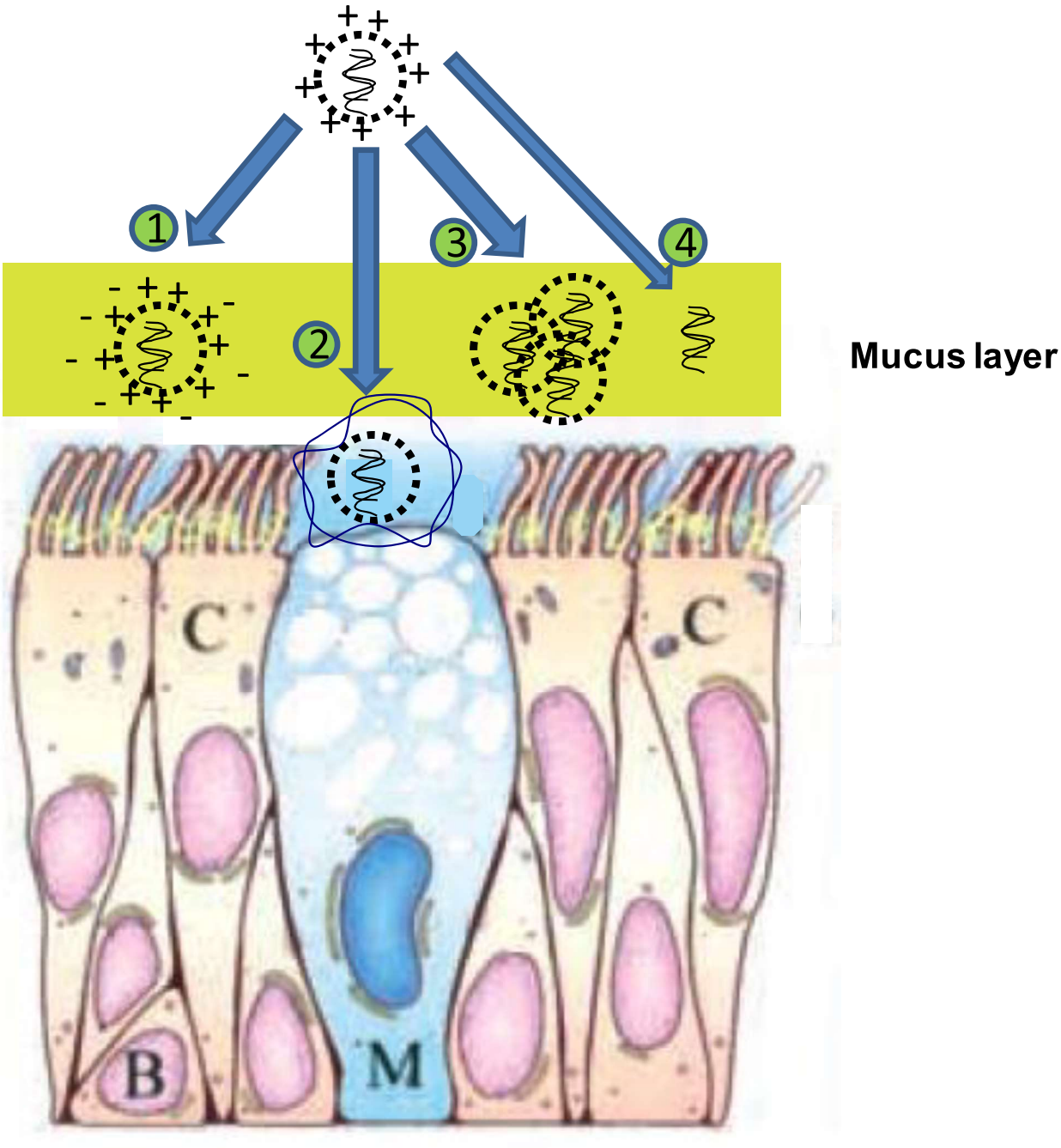

Figure 1.

Several studies were performed to detect the role of sputum (mainly from CF patients) on the structure and transfecting efficiency of nanocomplexes. When DOTAP/DOPE lipoplexes were mixed with growing quantities of mucin, linear DNA, or albumin, the surface charge of these nanocomplexes decreased or reversed [64]. This process is due to the interaction between the

charged mucus components and the cationic nanocomplexes. Surprisingly, higher amounts of albumin and linear DNA caused an anionic shield around the lipoplexes with an increase of their protection against aggregation. Moreover, the *in vitro* transfection efficiency of these linear DNA or albumin coated lipoplexes did not decrease [64]. On the contrary, mucins coated lipoplexes, although do not form large aggregates or dissociate, have a reduced transfecting ability. This indicates that mucins can influence the intracellular trafficking of complexes during the transfection process. Accordingly, we showed that albumin-coated PEI polyplexes reached gene transfer efficiencies higher than unmodified PEI, also in the presence of CF sputum [70].

Endogenous DNA is another important mucus component which can strongly reduce the transfection efficiency of nanocomplexes. Using cell cultures covered with diluted CF sputum, Alton and colleagues [71] have shown that the gene delivery, mediated both by lipoplexes and by adenoviral vectors, can be reduced by binding of CF sputum DNA to epithelial cell surface. Indeed, this group observed that a massive decrease in gene transfer occurred in cells pre-covered with highly diluted sputum as well as those washed to remove this diluted sputum from surface, before the transfection. Interestingly, the transfecting capacity of both vectors was partly recovered after pre-treating cells with rhDNase.

4.3. PEGylation as method for shielding nanocomplexes

An ideal non-viral gene delivery system should mediate efficient cell delivery through exhibiting weak interaction with extracellular components, such as the mucus, and avoiding macrophage recognition.

All studies concerning the application of nanocomplexes for gene delivery have in common that a positive overall surface charge of the polyplexes a prerequisite to yield stable complexes and high transfection rates. However, a positive overall surface charge of the polyplexes poses serious limitations with respect to their interactions with extracellular anionic components. Gene transfer to

the lung is adversely affected by interaction with mucus components such as proteoglycans, glycosaminoglycans and to certain extent by constituents of the surfactant layer, for example, phospholipids.

Self-aggregation of non-viral gene carriers and interaction with extracellular components was decreased by various shielding strategies (Figure 2). Among these, attachment of hydrophilic, uncharged polymers like PEG [72] seems to be a crucial factor for effective pulmonary gene delivery, leading to more stable polyplexes and reducing interactions with sialic acid, a major component of mucus. The idea of using PEG to modify drugs, proteins and particles is not new: PEG has been conjugated to various drugs and particulate drug carriers to increase their circulation time after intravenous injection [73].

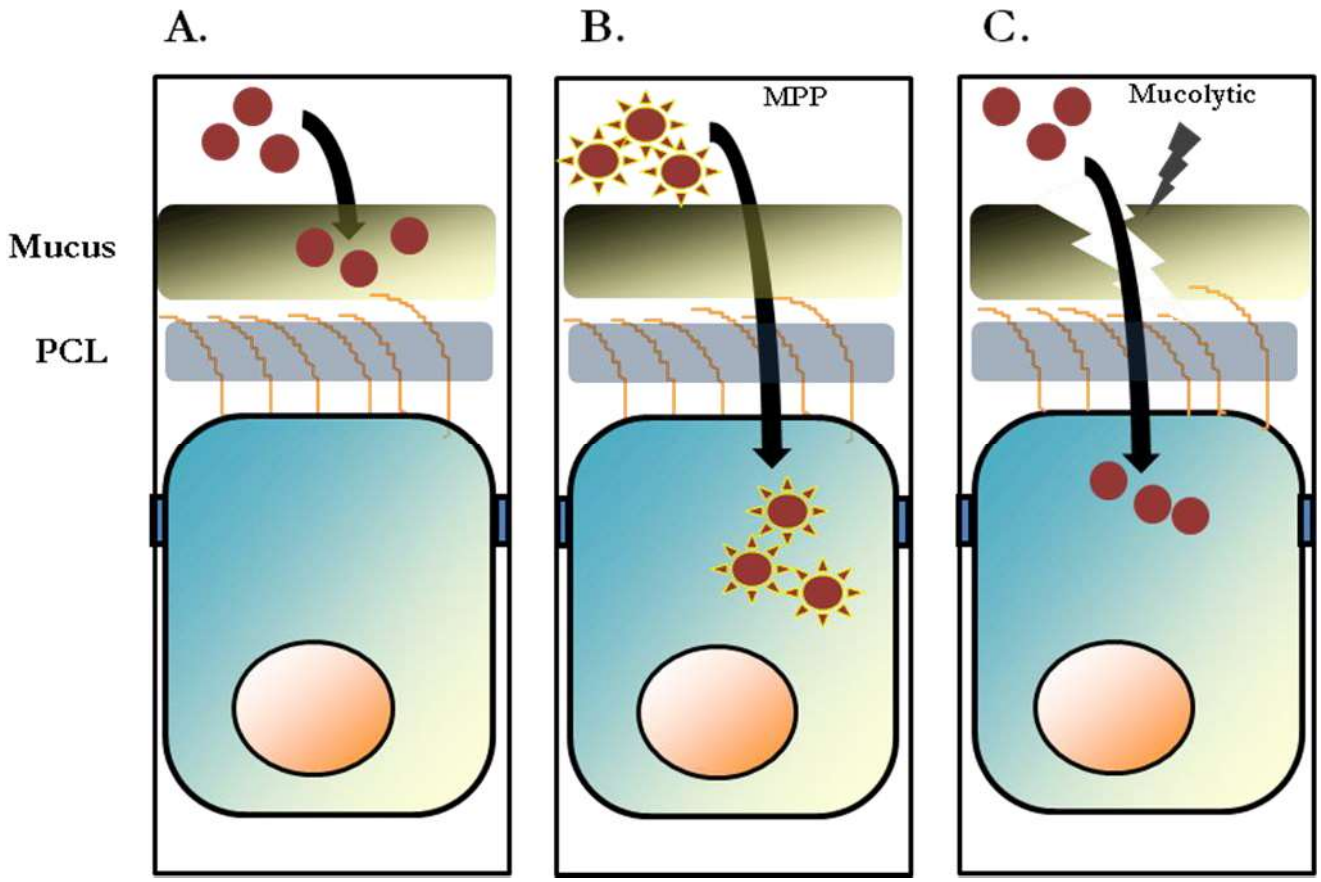

Figure 2.

Two strategies have been employed to create nanocomplexes bearing inert polymers at their surface. The first strategy is based on the covalently coupling between the non-viral cationic carrier with the shielding polymer and subsequent mixing with DNA [74]. However, in this procedure the shielding polymers may hinder the self-assembling process between the cationic carrier and DNA. Therefore, the second strategy involves the covalent attachment of the shielding polymer to pre-formed non-viral nanocomplexes [75]. Since PEGylation may prevent endosomal escape, different groups are currently developing several strategies in which the nanocomplexes are deprived of their PEG-chains outside the cell or in acidifying endosomal compartments [76]. Synthetic cationic lipids, such as GL67, are among the most successful nanocomplexes for lung gene delivery. PEGylated GL67/DOPE lipoplexes were not affected in their gene transfer activity by the presence of molecules present in CF mucus (linear DNA, albumin, phospholipids and mucin), while this was not true for the cationic DOTAP/DOPE lipoplexes [77]. Additionally, aggregation of GL67/DOPE lipoplexes by the CF mucus compounds was prevented by the PEG-chains.

Many studies on the effect of PEGylation were carried out with PEI, which, although protects DNA from nuclease-mediated degradation, is endowed with high cellular toxicity, which can be reduced *e.g.* by decreasing the molecular weight or by grafting with PEG [78]. A comparison among different types of PEI in terms of gene transfer to the lung demonstrated that low molecular weight (LMW) PEI (5 kDa) mediated transgene expression mostly in both bronchial and alveolar cell, while high molecular weight PEI (25 kDa) transfected only bronchial cells [79]. On the other hand, 25 kDa PEI grafted with PEG (5 kDa) failed to deliver DNA *in vivo*, which may have been caused by a reduction of nanocomplexes interaction with the cell surface [80]. To avoid this reduced interaction, TAT, a cell-penetrating peptide derived from the human immunodeficiency virus (HIV), was coupled to 25 kDa PEI grafted with PEG (3.4 kDa). TAT–PEG–PEI polyplexes were more efficient in *in vivo* gene delivery to the lung than PEI alone [72].

Sanders *et al.* [81] showed that particles should be smaller than 560 nm to quantitatively penetrate through viscous mucus of CF patients. Accordingly, Nguyen *et al.* [82] demonstrated that

PEGylation of PEI conjugated to various cell-penetrating peptides allows the formation of nanocomplexes whose size remain below 300 nm, allowing them to penetrate through the mucus barrier and reach the cells. In this study, the exposure of PEI 25 kDa, PEG-PEI and all bioconjugates to increasing amounts of mucin (sialic acid) was studied in terms of nanoparticles aggregation by measuring the hydrodynamic diameter of polyplexes. While 25 kDa PEI nanocomplexes strongly interacted with the negatively charged sialic acid and aggregated up to 1200 nm, by contrast, PEGylated bioconjugates were shielded against anionically charged mucus compounds or sialic acid. PEI-PEG-siRNA nanoparticles increased siRNA uptake and luciferase knockdown compared with PEI-siRNA in polarized, fully differentiated airway epithelial Calu-3 cell monolayers [83].

Cytotoxicity and inflammatory responses are responsible for side effects in nanoparticle delivery. Recently, Uchida *et al.* [84] have shown that finding balanced PEG shielding of nanocomplexes reduces inflammatory responses in the lungs. In this study, the researchers used ternary nanocomplexes formed by DNA, a PEG-block-copolymer and a non PEGylated copolymer. Interestingly, they found that an optimal combination of the two forms of copolymers was effective in achieving high transfection efficiency in lungs with minimal toxicity. Further, this study elucidated that the aggregation of polyplexes may significantly affect the pro-inflammatory activity of macrophages. Indeed, nonPEGylated polyplexes tended to aggregate during incubation in bronchoalveolar lavage fluid, whereas PEGylated polyplexes did not aggregate, indicating that aggregated polyplexes might have induced uptake by macrophages and their pro-inflammatory response. In general, these observations highlight that transgene expression is not the only parameter that should be considered in gene therapy studies when nanoparticles are applied to the lung. In conclusion, even when no appreciable changes are detected in *in vitro* physicochemical characterization of nanoparticles, the therapeutic outcome of these studies can be influenced by slight structural modifications.

The group of Hanes and collaborators has recently focused its work on the concept of mucus-penetrating particle (MPP) formulations. Since airborne particles interact and are immobilized by mucus barrier and then are rapidly cleared from the mucosal tissue through the mucociliary escalator, the design of biocompatible and biodegradable MPP systems capable of nucleic acid delivery is mandatory. Initially, Wang and colleagues [85] used either 2 kDa or 10 kDa PEG to coat the surface of negatively charged 220-nm diameter polystyrene particles that usually diffuse poorly in mucus. By multiple particle tracking (MPT) microscopy, which can be used to study the movement of a relatively small number of particle in solution, they demonstrated that polystyerene beads coated with short length PEG molecules diffuse readily in mucus gel. In contrast, a high density coating of 10 kDa PEG molecules reduced the diffusion ratio by three orders of magnitude, likely due to their strong adhesion to mucin fibers. In agreement with these findings, the same group demonstrated that 200-nm polymer-based particles can be engineered to rapidly penetrate sputum from CF patients by coating them with a dense low MW PEG moieties [86]. These findings suggest that the muco-inert particles (MIPs) can be rendered suitable to penetrate the CF sputum presenting openings between structural elements that are filled with a low viscosity fluid. Indeed, Tang and colleagues [87] prepared 173-nm nanoparticles composed of a biodegradable diblock copolymer of poly(sebacic acid) (PSA) and 5 kDa PEG, which diffused at an average speed in human cervicovaginal mucus only 12-fold lower than in pure water. PSA-PEG particles also rapidly penetrated CF sputum, with speeds similar to those that were reported for 200 nm non-degradable PEG-coated latex beads, *i.e.* they were slowed approximately 66-fold in CF sputum compared to water [86]. Altogether, these studies indicate that a net neutral surface to avoid binding and a small radius to avoid entanglement with the mucin network are key factors responsible for improving particle transport in mucus, indeed mimicking the properties of viruses able to efficiently infect mucosal surfaces.

Building up on these interesting results, subsequent work was carried out to prepare polymeric gene therapy vectors that could penetrate human mucus. It is known that a high degree of PEG

conjugation leads to less efficient DNA compaction by cationic polymers, resulting in loosely compacted larger particles [88] and possibly interfering with mucus penetrating properties. In order to tackle this drawback, Kim and colleagues [89] used single-site-functionalized dendrons to achieve a dense PEG (5 kDa) coating on the surface of either PAMAM or PEI. These carriers were shown to condense plasmidic DNA into compacted nanoparticles rapidly penetrating human CF mucus: dPEG-PAMAM/DNA and dPEG-PEI/DNA were slowed at lower rate than uncoated cationic polymer gene vectors. Interestingly, the functionalized dendrons were able to transfect efficiently human respiratory epithelial cell lines and to increase the expression of CFTR mature band in CF cells of bronchial origin. Suk *et al.* [90] developed a formulation process that allows highly dense PEG coating on small, stable gene nanocarriers based on PEI or PLL. This was achieved by using a mixture of free and PEG- conjugated cationic polymers, which led to more neutral PEG 5k-PEI primarily localized to the surface, while cationic PEI is localized in the core of nanoparticles. Highly compacted MPP protected cargo DNA against DNase degradation, did not induce acute lung inflammation or toxicity, and mediated a higher gene expression levels in the lungs of mice than block copolymers of poly-L-lysine and 10 kDa PEG (CK30PEG10k). Furthermore, MPP carrying the wild-type CFTR plasmid DNA enhanced expression of CFTR in the mouse lungs, human primary airway epithelial cells grown at air-liquid interface and in CF cells stably expressing F508del, the most frequent mutation in CF patients.

In summary, these studies have shown that shielding of nanoparticles by highly dense PEG favours their physicochemical stability and their gene transfer ability in the presence of respiratory mucus.

4.4. Mucolytic agents as enhancers of gene delivery to airway epithelium

Two broad approaches to altering the mucus barrier can be envisaged, namely a change in its composition by a mucolytic or a reduction in amount by a mucoregulator (Figure 2). Mucolytics

"lyse", or reduce the viscosity of, mucus. The term mucolytic refers to compounds with sulfydryl groups that are able to dissociate disulphide bonds and potentially reduce mucus viscosity. Typically, mucolytic compounds have either "exposed" or "free" sulfydryl groups that directly break disulphide bonds (for example, N- acetylcysteyne, NAC), or have "blocked" sulfydrylgroups that are exposed upon metabolism in the body (for example, carbocysteine). Other compounds, such as proteolytic enzymes and recombinant human deoxyribonuclease (also known as dornase alfa or rhDNase) are not strictly mucolytic since their mechanism of action is not based on breaking up mucus, but do so via alternative mechanisms. Indeed, rhDNase, known to reduce sputum viscoelasticity, is known to improve lung function in CF patients. Dornase acts by cleaving neutrophil-derived DNA in infected sputum [91].

Mucoregulators are drugs that do not directly have any great influence on airway mucus, but they may reduce the process of chronic mucus hypersecretion, for example, by inhibiting a particular aspect of mucus physiology (*e.g.*, anti-cholinergic drugs that inhibit cholinergic nerve-induced mucus secretion, in addition to their anticholinergic bronchodilator activity).

Mucolytic agents like NAC or rhDNase, have been used to increase the mobility of gene carrier systems in the lung [92]. The reduction of disulfide bonds between the mucin subunits represent the main mechanism by which, NAC and its derivatives, decrease the viscosity and elasticity of mucus. Furthermore, NAC is characterized by other important properties than that mucolytic activity. Two studies have shown that the lysine salt of NAC can inhibit the maturation of dendritic cells and possesses anti- inflammatory properties [93, 94]. These studies elucidated a possible role of NAC as inhibitor of the immune response against especially viral vectors. In a sheep trachea organ culture model, explicit removal of mucin increased the transfection efficiency of liposomes by 25–fold [95]. Subsequently, Ferrari *et al.* [96] have shown in this model that the gene transfer by lipoplexes (ethyldimyristoyl-phosphatidylcholine/cholesterol) and linear PEI was respectively about 20 and 10 fold higher when mucus covered epithelium was treated with NAC before the application of nanocomplexes. In the same study, this group has shown that the intramuscular injection of

glycopyrrolate (an inhibitor of mucus secretion) 30 minutes before perfusion with NAC followed by transfection resulted in an increase of gene delivery.

There is a controversial debate on the use of mucolytic agents to enhance transport of nanocomplexes through mucus. It was observed unexpectedly by Sanders *et al.* [97] that nanoparticles experienced a lower diffusivity in low viscoelastic sputum than in a more viscoelastic sputum, likely due to a more heterogeneity of macroporous network in this last condition. Thus, microviscosity might be more important than macroviscosity in determining the mobility of nanoparticles in sputum.

rhDNAse treatment of CF sputum-covered cells increased the transgene expression mediated by cationic liposomes or adenoviruses, whereas this positive effect was not gained by pre-treatment with NAC, alginase or lysine [71]. On the other hand, Dawson and colleagues [98] have showed that, by using 100- and 200-nm particles, CF sputum microviscosity was 7-15-fold lower than its macroviscosity, suggesting that nanoparticles may diffuse in such high viscoelastic matrix within pores with low viscoelastic gel. However, they did not find a positive effect by rhDNase treatment in the transport of 200-nm nanoparticles in CF sputa, as studied by MPT. This result may reflect the hydrolytic cleavage of DNA into smaller fragments that diffuse freely into micropores which may increase the microviscosity within the pores.

Conversely, Sanders and colleagues showed that rhDNase treatment of sputum increased the transport of 270-nm polystyrene nanospheres and DOTAP/DOPE based lipoplexes of 2.5- and 1.4-fold as compared with untreated sputum [92, 97]. Moreover, Shute and collaborators [99] found that the lack of rhDNase effect on the transport of the same nanoparticles through 'synthetic CF sputum', *i.e.* a mixture of DNA, mucin and actin, was due to the inhibition of rhDNase by actin monomers expected to be present in such matrix. When gelsolin, that can dissociate DNase–actin complexes, was simultaneously added along with rhDNase to the synthetic CF sputum, it did not restore the rhDNase activity nor increased the transport of nanospheres [99].

In regard to gene therapy, highly compacted DNA nanoparticles, composed of block copolymers of poly-L-lysine and 10 kDa PEG ($CK_{30}PEG_{10k}$) mediated effective gene transfer to lungs in mice and nasal epithelium of CF patients. However, these DNA NPs do not efficiently penetrate human CF sputum, being at least ~3000-fold slower in CF sputum than their theoretical speed in water [100, 101]. To improve transport of gene carrier vectors across sputum, Suk and colleagues tested adjuvant regimens consisting of NAC, rhDNase and NAC together with rhDNase [101]. Although rhDNase induced a marked decrease in bulk viscosity of CF sputum, it failed to enhance gene carrier diffusion. On the other hand, NAC and NAC + rhDNase allowed greater diffusivity rates, which were 6-fold and 13-fold higher, respectively, than in untreated sputum. In order to mimic *in vivo* the CF lung, the effect of NAC was tested in a mucus hypersecretory mouse model with mice challenged with *Pseudomonas aeruginosa* lipopolysaccharide (LPS). Pretreating mice with NAC prior to administration of DNA-nanocomplexes enhanced gene expression by up to about 12-fold compared to saline control, restoring the marker gene expression to levels comparable to that of mice not challenged with LPS. These findings suggest that mucolytic therapy with NAC or NAC-rhDNase combined treatment may be envisioned as a promising pre-treatment that would allow more efficient nanocomplex-based gene transfer to lungs of CF patients.

In synthesis, adjuvant treatment with mucolytics or DNA-liquefying drugs may improve nanocomplexes-mediated gene transfer to the lung, although more is to be understood about the microrheology of nanoparticle transport across pathological mucus as well as the rigidity of mucus.

5. Conclusions and Perspectives

Nanocomplexes show great potential for the gene delivery to the lung but successful gene therapy requires an appropriate transfection efficiency. Targeting of specific cell types in the respiratory tract is thought a valid approach to increase gene expression with no adverse events linked to inappropriate expression in other cell types and/or organs. While ligand-receptor and physical

targeting are being explored, transcriptional targeting, which would give regulated gene expression in space and time, is not the focus of ongoing research concerning airway and respiratory epithelial cells. Anyhow, targeting should be accompanied by strategies aimed to overcome the mucus barrier in pathological conditions. Recent research points to the feasibility of penetrating the mucus layer by appropriately constructed nanoparticle complexes bearing plasmid DNA or siRNA. However, these studies should be now completed with the pre-clinical testing of the nanoparticulate systems in animal models. It is envisioned that detailed information on macro- and micro-rheology of the mucus layer will benefit research on mucopenetrating nanoparticles likely allowing a further step to clinical application. The better comprehension of the structure and function of mucus in pathological respiratory conditions will likely bring to the development of novel mucolytics and mucoregulators which ultimately will further increase the efficacy of gene therapy vectors. Finally, some of the chronic respiratory diseases, such as CF, are characterized by bacterial growth in the so-called biofilm which represents a further barrier to the gene therapy vectors and should be another field of active investigation if we want to bring a viable gene therapy-based treatment to the patient.

Although gene therapy for respiratory diseases is at the forefront of gene delivery since the beginning of the 21st century, it has still to overcome many hurdles to be a reality in the clinical scenario. This is illustrated by the efforts in the cystic fibrosis lung disease with non-viral vectors, which after a growing phase in vector development are now focused on the safety and discovery of clinical endpoints rather than on efficacy. siRNA may offer a novel platform for modulate therapeutically various respiratory diseases, including those which are not of genetic origin such as lung infection and tumors. To-date no siRNA-based therapeutics have been approved as commercial products for the treatment of lung and other diseases although many are in the clinical trial stage of development. Nevertheless, there is presently a steep development in advanced formulations overcoming respiratory disease-associated lung barriers (including the mucus) as concerning lipid-based carriers, polymer-based carriers and solid lipid particles for drug delivery.

As a matter of fact, advanced clinical studies are ongoing on liposomal-based antibiotic formulation for the treatment of CF patients with lung infection by *Pseudomonas aeruginosa* and for non-tubercolous mycobacteria lung infection [102]. These advancements should be also applied to the field of gene therapy.

Another critical point is represented by the nebulization device which will be used in the clinics. Due to their superior handling, metering and reliability, it is envisioned that dry powder inhalers will substitute conventional nebulizers. As a consequence, much knowledge is still to be acquired on cellular uptake of nanocomplexes bearing pDNA or siRNA molecules, the pharmacokinetics and lung biodistribution profiles of nanocomplexes and extension of lung retention times, this last issue of enormous importance for chronic lung diseases which need repeated administrations.

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Table 1. Strategies to enhance nanocomplex-mediated gene delivery to the lung.

Strategy	Nanocomplexes	In vitro/in vivo model	References
Ligand-receptor Targeting			
	PEI-mannuronic acid	Human bronchial epithelial cells (16HBE14o-)	[40]
	PEI-lactoferrin	Human bronchial epithelial cells (BEAS-2B)	[41]
	PEI-clenbuterol	Human A549 and murine MLE-12 alveolar epithelial cells/ Aerosolisation to the mice lung	[43]
	Poly-lysine-serpin enzyme complex receptor	Intratracheal instillation to the mice lung	[44]
	PEI-iloprost	Human A549 alveolar epithelial cells, human bronchial epithelial cells (16HBE14o- and BEAS-2B)/ Aerosolisation to the lung mice	[47]
	Insulin adsorbed to PEI-pDNA nanoparticles	Human A549 alveolar epithelial cells, human bronchial epithelial cells (BEAS-2B)	[46]
	Guanidinylated chitosan-siRNA	Human A549 alveolar epithelial cells, human bronchial epithelial cells (16HBE14o-)/ Aerosolisation to the lung mice	[45]

	Cationic liposomes targeting endothelin receptor	Human A549 alveolar epithelial cells/ Intratracheal instillation in the rat lung	[58]
	Cationic liposome targeting ICAM-1	Human airway epithelial cell lines, of non-CF (16HBEo− and 1HAEo−) and CF origin (CFBE41o− and CFTE29o−)/ Nebulisation to the mice lung	[59]
Physical Targeting (magnetofection)			
	PEI-pDNA and magnetosols	Aerosolisation to the mice lung	[48]
Transcriptional Targeting			
	PEI-pDNA with glucocorticoid responsive elements	Aerosolisation to the mice lung	[49]

Figure Legends

Figure 1. Detrimental effects of mucus on cationic vector-mediated gene delivery tot he airway epithelial cells. Cationic vector/DNA complesexes are typically represented as negatively charged double strand DNA embedded into the cationic vector presenting a surplus of positive charges, which are involved in cell binding and uptake. The effects of interaction of mucus components with such complexes can eventually lead to: 1) the entrapment of nanocomplexes in the mucus; 2) reduced cellular uptake, caused by masking of their positive charges or their receptor ligands; 3) the neutralization of their surface charge with the formation of aggregates; or 4) the release of their DNA cargo. A pseduostratified epithelium, typical of the upper aiways, is represented, with ciliated (C), mucus-producing (M), and basal (B) cells.

Figure 2. Strategies for overcoming the mucus barrier in the respiratory tract by non-viral gene transfer vectors. (**A**) The top of airway epithelial cells is covered by the watery periciliary liquid layer (PCL), layered by the viscous mucus layer, together representing the main barrier to the penetration of non-viral gene therapy vectors. (**B, C**) Two main strategies are being developed to overcome the mucus barrier. (**B**) Mucus-penetrating particles(MPP), which are prepared by a dense coating of low molecular weight PEG, allow to escape the mucociliary clearance. (**C**) Mucolytics reduce the viscosity of mucus allowing better transport of gene delivery vectors.