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REVIEW PAPER

Barriers and recent advances in non-viral vectors targeting the lungs for cystic fibrosis gene therapy

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ABSTRACT

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in CFTR genes that affect chloride ion channel. The CF is a good nominee for gene therapy as the asymptomatic carriers are phenotypically normal, and the desired cells are accessible for vector delivery. Gene therapy shows promising effects involving the correction of gene or replacement of the mutant gene with the functional one. Accordingly, various viral and non-viral carriers have been investigated. Although viral vectors are efficient, they have some problems, including mutagenesis, host immune response, higher toxicity, and costliness. On the other hand, non-viral vectors have less toxicity and immunogenic response and are easier to prepare. For a successful gene therapy, the cargo must be delivered to the target site. However, various barriers are faced by non-viral vectors, which make the gene delivery to the target site difficult. Extracellular barrier, which is the first barrier, include nucleases, negatively charged serum proteins, blood cells, and activated immune system. Ciliated epithelium, mucus gel, apical surface glycocalyx, and plasma membrane come in the second category of the barriers. Furthermore, the third category, which is related to the intracellular barriers, includes endosome and lysosome, cytoplasmic nucleases, viscous environment of cytoplasm with different proteins, and finally nuclear membrane. Various approaches have been proposed to increase the systematic delivery of vectors and enhance their efficiency. Some of these approaches include surface coating with inert polymers, modification of surface charge with anionic polymers, and enhancement of endocytosis and reduction of toxicity by using polyethylene glycol. This review paper was conduct to highlight the barriers faced by non-viral vectors when carrying a genetic payload to the lungs. This study also involved the investigation of the strategies and different types of modifications targeted toward the improvement of the efficiency of non-viral vectors.

Keywords: *Cystic fibrosis, CFTR gene, Non-viral vectors, PEG*

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INTRODUCTION

Many human diseases, including cystic fibrosis, are caused by defective genes and improper protein functioning [1]. The CFTR gene within our DNA provides the blueprint for the production of chloride ion channels. Specific CFTR gene defects or mutations can result from a defect in the ion transport channel in the apical membrane of most of the secretory cells. This defect leads to the alteration of epithelial mucus secretion in the airway epithelia, digestive tract, pancreas, reproductive tract, and liver [2]. Cystic fibrosis is a recessive disease caused by mutations in CFTR gene located on the q arm of chromosome 7. This disorder is an attractive candidate for gene therapy as the carrier is phenotypically normal and target cells are accessible for vector delivery [1, 3].

Gene therapy involves the transfer of a gene through different vectors that target a precise cell to repair the mutated gene for coding a proper protein [4]. After entrance to the target cell, the carrier releases the copies of a gene that provides a blueprint for normal functioning protein or corrects the defective one. To achieve this end, the therapeutic gene is packaged within the vectors to restore the natural function of the disrupted protein.

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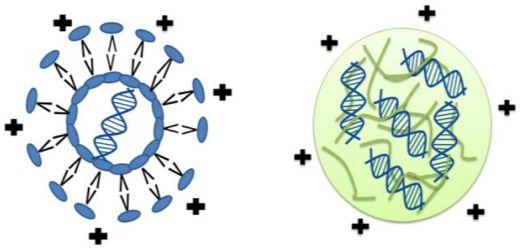

Fig 1. Schematic presentation of lipoplexes and polyplexes formed by the respective combination of DNA with liposome and polymer

However, the development of safer and more efficient vectors is the main challenge in gene therapy [5-7]. Different viral and non-viral carriers are used for carrying the genetic payload to the targeted site. However, various limitations are encountered in viral vectors, including immunogenicity and low efficiency [8]. On the contrary, non-viral carriers, such as lipoplexes and polyplexes, show promising results for gene therapy. These vectors have a safety profile, are more amenable for repeated administration, and are more easily synthesized on a large scale [9, 10].

Lipoplexes are the results of the formation of a complex entailing plasmid DNA and lipids. Lipoplexes are positively charged due to the high concentration of cationic lipids, facilitating the attachment to the cell surface [11, 12]. Similarly, polyplexes are formed by the combination of polymers with DNA. They are designed to protect DNA when being injected into the cells as a part of gene therapy [12] (Table 1).

with liposome and polymer For the successful treatment of cystic fibrosis, the genetic material has to be carried to the target cell, cross the cell membrane, and attain an adequate level of gene expression [13, 14].

This is not an easy task as the lungs have evolved multiple barriers for the entry of foreign particles to the airway cells. In order to access the airway cells, non-viral vectors have to cross the extracellular barriers, including nucleases, serum proteins, and immune system.

After successfully crossing the first line of barriers, they should cross the plasma membrane to enter into the cell. Then, the vectors must minimize the intracellular barriers and get access to the nucleus. For overcoming these barriers, several modifications have been made which are helpful for non-viral carriers in gene therapy [15].

The current review study was conducted to discuss the barriers faced by non-viral vectors in gene therapy for cystic fibrosis and investigate their modifications to overcome these barriers.

Extracellular barriers

A number of host systems on the exterior surface of the cell impair gene delivery and result in the destruction of nucleic acids by serum nucleases and lipases, serum proteins, and immune and inflammatory responses. The vectors enter the body through inhalation, intramuscular injection, intravascular injection, and other methods and face the first line of extracellular barriers [15].

Table 1. List of non viral vectors used for gene therapy in cystic fibrosis

	Physical-based delivery			Chemical-based delivery (non-viral vectors)		
Vector	Magnetofection	Electroporation	Ultrasound	Lipoplexes	Polyplexes	PEG-CK30 peptide
Transfecting component	Superparamagnetic nanoparticles	Electrodes	Waves	Lipids	Polymers	Peptides
DNA carrying capacity	High	Low	Low	High	High	High
Key mechanism	Magnetic force	High voltage current	Ultrasound waves	Electrostatic interaction	Electrostatic interaction	Cytoplasmic nucleolin interaction
Desired site	Brain, blood vessel, endothelium, lung, and liver	Skin, muscle, and lungs	Lungs, muscles, and skin	Airway epithelial and endothelial cells	Oral cavity and lungs	Liver, lung, and cardiac muscle
Advantages	Simple, efficient, and inexpensive	Reproducible	Safe and non-invasive	Low cytotoxicity	Low immunogenicity	High efficiency and low immunogenicity
Disadvantages	Lower efficiency and toxicity	Cell damaged	Low expression	Less immunogenic	Cytotoxic	Difficulty in preparation

Multiple factors are involved in the clearance of nanoparticles from the system before they reach the target site.

Nucleases are the first agents involved in the clearance of a naked DNA in 1.2-21 min depending on the topoform of DNA [16]. A similar observation has been made for plasmid DNA [17]. For increasing life expectancy, plasmid DNA is encapsulated within cationic lipids and polymers to be protected against nucleases. The other most important obstacle for gene delivery through liposome is the existence of lipases in serum, which degrade liposomes. The PEGlyation of cationic lipid and polymers shows effective results for enhancing the half-life of vectors and improving transfection efficiency [18-20].

Upon administration, cationic nanoparticles form aggregates, leading to the clearance of vectors from the blood [21]. This problem has been solved by combining cationic nanoparticles with palmitic acid (PA). The PA not only prevents nanocarriers from aggregation but also improves the transfection efficiency [22].

Another factor which restricts the utilization of non-viral carriers is the presence of proteins within the extracellular environment. Different types of proteins, including albumin, complement immunoglobulin, fibronectin, apolipoprotein, C-reactive protein, and beta-2 glycoprotein I, are present in serum. These proteins are involved in the clearance of non-viral vectors from blood [23]. These negatively charged proteins form aggregations with cationic lipids and polymers, which inhibit their biological activity. These complexes end up in the reticuloendothelial system and are removed by phagocytosis. Cationic lipids and polymers, modified with such co-lipids as polyethylene glycol (PEG) and cholesterol, help them to cover their positive charge and protect them from aggregation [18-20].

After evading from the nucleases and proteins, the vectors come in contact with negatively charged blood cells, such as erythrocytes, leukocytes, macrophages, and platelets [24]. Following the administration of cationic lipids and polymers in the body system, there is an electrostatic interaction between the positively charged particles and negatively charged cells, thereby decreasing their transfection efficiency and removing them from the blood system through the liver and spleen [25]. When these nanoparticles reach the lung, they have to face another major barrier which is alveolar macrophage [26]. These macrophages eat up all delivery agents, including viral and non-viral vectors before they transfer their cargo to the lung cells.

In vitro experiments have shown that glycol-coated modified nanoparticles are effectively taken up by the cells [27, 28]. Another adaptation is made by ligating the biodegradable agents, such as polyhydroxyethyl L-asparagine, attached by a hydrolyzable bond (e.g., ester), to the surface of nanoparticles resulting in the enhancement of their circulation in the serum [29, 30].

Activation of an immune response is another hinder in the way of transfection [31]. Viral vectors are mostly drawn in the activation of an immune response, whereas some of the non-viral vectors provoke it. As safer than viral vectors for gene therapy of cystic fibrosis, cationic lipids after intravascular administration, cause lung noxious due to stimulation of swelling, followed by the release of tumor necrosis factor and interferon gamma into the serum [32]. Cationic polymers also induce an immune response by activating both types 1 and 2 T helper cells [33]. The reasons for the immune system activation are the presence of unmethylated motif on plasmid DNA and identification by Toll-like receptors [34, 35].

PEGlytion of cationic lipids and polymers, as discussed earlier, is helpful in crossing the extracellular barriers, whereas anti-PEG IgM has the capability to impinge on the repetitive administration of vector [36-41]. There is room for various kinds of modifications in non-viral vectors for improving their efficiency through overcoming the extracellular barriers.

Cell surface barriers

After successfully crossing all extracellular barriers, nanoparticles approach the cell surface. The negative charge on cell surface is due to heparan sulfate proteoglycan, there is an electrostatic interaction between cationic lipids and polymer and plasma membrane which results in the internalization of nanocarriers. Given the high external charge of nanocarrier, several drawbacks are associated with nanoparticles, such as opsonization, attachment of various molecules to the particle face, and lack of specific targeting [42].

Several types of modifications have been implemented to make a vector target-specific. These modifications include the attachment of

folate, transferring, or monoclonal antibodies, which direct the nanoparticle to the cell, thereby expressing their moieties and facilitating receptor-mediated endocytosis [30, 43-45].

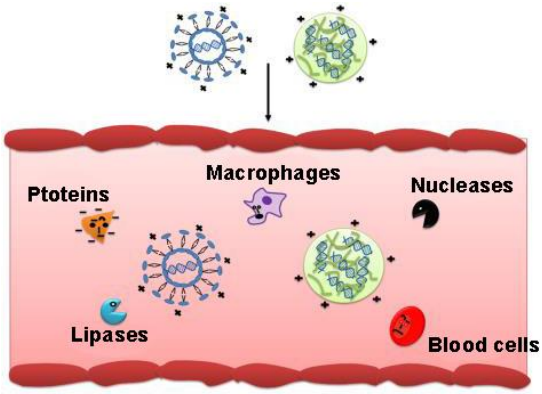

Fig 2. Reresent extra cellular barriers faced by nano carrier after administration into body

Before reaching the cell surface, various barriers are crossed by nanoparticles to get access to the targeted cell. Mucus is a dense gel covering the airway epithelium. It is recognized as a major barrier for the vectors before attaching to the cell surface [46]. The building block of mucus is mucin glycoprotein, which is composed of negatively charged glycans having hydrophobic regions [47]. Cationic nanoparticles are trapped in the mucus blanket through the electrostatic and hydrophobic interaction. Furthermore, mucin fiber is cross-linked to form a dense meshwork, which acts as a sticky net for inhaled therapeutics [48].

The nanoparticles entrapped in the mucus gel are then cleared through mucociliary clearance [49] or cough-driven clearance, which limits the efficient delivery of nanoparticles to the underlying cells. Recently, it has been shown that the nanoparticles, the surface of which are coated with dense PEG, are capable of effectively crossing the mucus layer [50-52]. Mucus-penetrating DNA-nanoparticles (DNA-mucus penetrating particles) are developed on the basis of PEG coating. In this regard, having a high density of PEG coating is associated with effective results regarding mucus penetration [53, 54]. It has been shown that the pretreatment of mucus with mucolytic agents increases the pore size, which facilitates the nanoparticle to overcome the mucosal barrier [55]. Furthermore, modifications with N-acetylcysteine and its derivative showed promising results by increasing gene delivery through mucus gel via lowering its viscosity and electricity [56]. The disulfide bonds between mucin subunits are reduced by N-acetylcysteine, thereby enhancing nanoparticle efficiency.

Periciliary liquid layer (PCL) is another barrier present beneath mucus gel. After crossing the mucosal layer, nanoparticles confront the PCL, which is a significant steric barrier to vector penetration [57]. The PCL has a well constricted network-like structure composed of cell-tethered mucins. It serves as an adhesive barrier for nanocarriers; therefore, nanoparticles need extra energy to cross the barrier of PCL [57].

Glycosylated proteins on the outer face of the plasma membrane form a carbohydrate coat known as glycocalyx. Glycocalyx is composed of carbohydrates, glycoproteins, and polysaccharides; accordingly, it is an obstacle in gene delivery. It attaches the invading nanocarriers and impedes them from binding to the cell surface [58]. A strategy for efficient gene delivery in cystic fibrosis patients involves the pretreatment of these patients with neuraminidase, which removes sialic acid residues from glycocalyx for the improvement of gene therapy [59].

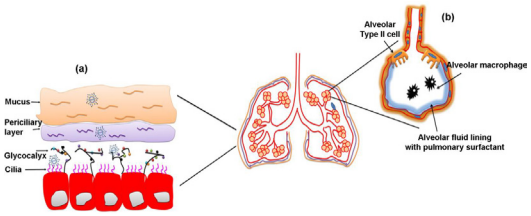

Fig 3. Cell surface barriers restricting the use of non-viral vectors, a) barriers to the delivery of nanoparticles to the lung after an intravascular release, b) airway barriers encountered by nanoparticles when delivered to the lung through inhalation

Inhaled gene delivery systems, directly transferring the vectors to the alveoli, overcome the barrier of mucus as it is not present in the alveolar sacs. The alveolar fluid contains a substance known as pulmonary surfactant, which reduces surface tension. Pulmonary surfactant is secreted from type II alveolar cells into the alveoli. Pulmonary surfactant consists of phospholipids, phosphatidylcholine, phosphatidylglycerol, and hydrophobic surfactant proteins. Vectors have to maintain their constancy and function in the existence of pulmonary surfactant [60].

Based on many investigations, gene delivery through cationic lipids are greatly reduced by pulmonary surfactant [61]. The presence of different surfactants, such as Alveofact (i.e., an extract from the bovine lung) and Exosurf (i.e., a synthetic surfactant), results in the inhibition of cationic-based nucleic acid transport; however, it induces lower effects on the cationic polymer-based delivery [62, 63]. The immune system also plays a role in impeding gene delivery. Alveoli macrophages are present in airspace which engulfs inhaled foreign substances directly or via an opsonin-dependent mechanism.

Alveolar macrophages release lysozyme and proteases and act as host defense against invading the nanoparticles by recruiting neutrophils, lymphocytes, and dendritic cells [64]. A recently designed cationic lipid, named GL67A, has shown promising results in gene delivery for cystic fibrosis for aerosol administration [65, 66]. to the lung after an intravascular release, b) airway barriers encountered by nanoparticles when delivered to the lung through inhalation.

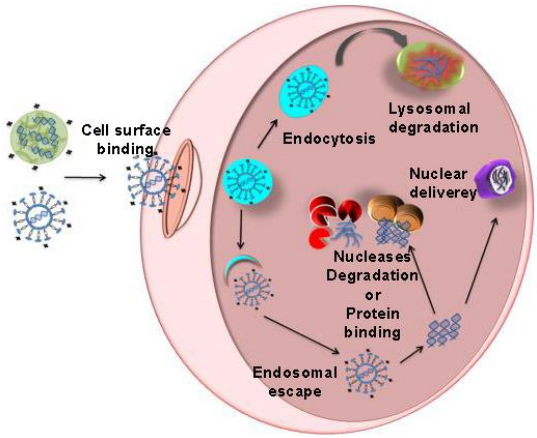

Fig 4. Intracellular barriers limiting the efficiency of nanocarriers during gene delivery

After successfully crossing all cell surface barriers, plasma membrane is another obstacle in the way of nanoparticles. Successful gene therapy depends upon the release of nucleic acid within the target cell.

In the absence of suitable carriers, naked DNA is unable to cross the plasma membrane due to the repulsion of the negatively charged vehicle. The enclosure of the genetic payload in the cationic nanocarriers creates an electrostatic attraction between the nanocarriers and plasma membrane.

Endocytosis is the main pathway for the internalization of nanoparticles. To make endocytosis independent entry, several types of modifications have been made with nanocarriers. Modifications of nanoparticles with cell penetrating peptides (i.e., Tat, antennapedia, and penetratin) and different proteins facilitate a direct access to the cell or energy-dependent macropinocytosis [67-69]. Alternative to these cell penetrating peptides, a domain from herpes simplex virus, peptide containing Wilms tumor protein is attached on the exterior to enhance the level of cellular uptake [70].

Intracellular barriers

The initial binding and entrance of the cationic vehicles occur by two main approaches. On the one hand, there is a direct attachment of cationic nanoparticles on the cell surface through electrostatic interactions, and entrance occurs by direct diffusion with the cell membrane. On the other hand, there is a specific attachment of nanocarriers to the target cell due to the presence of a specific ligand for the cell surface receptors. Therefore, the nanocarriers enter the cell through endocytosis [71].

After internalization, the most challenging step for nanocarriers is to cross the endocytic compartment, which is the first intracellular barrier. After endocytosis, the vector becomes reachable to early endocytosis and after late endosome, it forms phagolysosome by fusing with lysosome [72]. Due to the low pH and presence of hydrolytic enzymes in lysosomal environment, a broad range of nanocarriers is degraded along with the enclosure of the genetic material [73].

Several strategies have been adopted by cationic lipids and cationic polymers in order to release from endosome. Fusogenic characteristic is present in the cationic lipid utilized to escape through endosome.

Addition of dioleoylphosphatidylethanolamine helps in the conversion from a bilayer to an inverted hexagonal structure, which enhances the ability of cationic lipoplexes to fuse with endosomal membrane for escape [74]. However, cationic polymers, such as polyethylenimine (PEI), use a proton sponge mechanism for endosomal escape. The PEI is protonated within an acidic endosomal environment, which causes the influx of chloride ions within the endosomal compartment [75]. This results in the osmotic swelling; furthermore, the lysis of endosome increases the possibility of

DNA releasing in the cytoplasm [76]. Due to the cytotoxic effects of PEI, its clinical use is limited [75]. Currently, the use of PEI is facilitated by complex with lipid moieties, such as PEG or pluronic polycarbonate, which improve their biological properties [77, 78].

Recently, different types of peptides, such as GALA and KALA, which facilitate pore formation derived from influenza virus, have been attached to the vector surface which undergoes a pH-dependent conformational change and helps in endosomal escape by the disruption of the endosomal membrane [71, 79]. Many other membrane disrupting peptides have been derived from bacteria and animal to increase the transfection efficiency [80].

In another method, nanocarriers have been decorated with pH-sensitive fusogenic peptides, such as hemagglutinin HA-2 subunit, facilitating the endosomal escape [81]. In recent studies, TP10 with proton acceptors has been used for the delivery of the genetic payload to enhance the endosomal escape. A peptide-based vector has been developed in which TP10 is attached to the cell penetrating peptides, thereby facilitating both internalization and escape from the endosomal compartment [82].

For gene expression, the genetic material has to move to the nucleus through cytoplasm where various barriers are present. The nuclease is the first barrier, which degrades the free DNA. In the experiments in HeLa and Cos cells, it was shown that free DNA is present with a half-life of 50-90 min in the cytoplasm [83]. Along with the problem of dissociation, a diffusional barrier is also present in the cytoplasm. Viscous milieu with crowded proteins decreases the mobility of DNA to the nucleus [84-86]. After releasing from vectors, there is a lot of distance to the nucleus covered by DNA. It has been observed that in case the DNA unbinds in the cytoplasm, it cannot proceed towards its desired location [87, 88]. Microtubule network and molecular motor are present in the cytoplasm and help the DNA move towards the nucleus [89, 90].

There are multiple proteins including transcription factors which help in the binding of DNA with a molecular motor, such as dynein [91]. In an experiment, by adding transcription binding sites in plasmids, such as cAMP responsive element binding protein, the velocity of DNA was improved [92]. Recently, it has been observed that the acetylation of microtubules also increases the mobility of DNA to the nucleus. Modulation of deacetylase 6 (HDAC6) increases the velocity of DNA as acetylation status is controlled by this enzyme [93]. In another strategy, the attachment of dynein association sequence improved the mobility of DNA to the nuclear region [94]. Some of the researchers have observed that the use of urea facilitates the movement of lipoplexes in the cytoplasm and helps the genetic material get closer to the nuclear section [95].

After successfully crossing the cytoplasmic barriers, the genetic material has to cross the nuclear membrane barrier in order to get access to the transcription machinery. The nuclear membrane cannot allow the DNA to have a size greater than 300 bp and a molecule of size greater than 50 kDa [96, 97]. One strategy is to transfer nucleic acid to the dividing cells as the nuclear membrane is noncontinuous during cell division [96].

Recently, the decoration of nanocarriers with a Nuclear localization Signal (NLS) has shown promising results for gene delivery to the nucleus. These NLSs are utilized by different proteins for getting access to the nucleus. In this regard, minimal NLS that is PKKKRKV132 of simian virus 40 has been frequently used [98]. In another study, it has been shown that by conjugating T-ag NLS peptide (tumor antigen residue of 126-135) at the end of the plasmid, a nuclear uptake can be persuaded [99]. In addition, the conjugation of other NLSs, such as GAL4 and opT-NLS, has promising results in gene expression [100, 101].

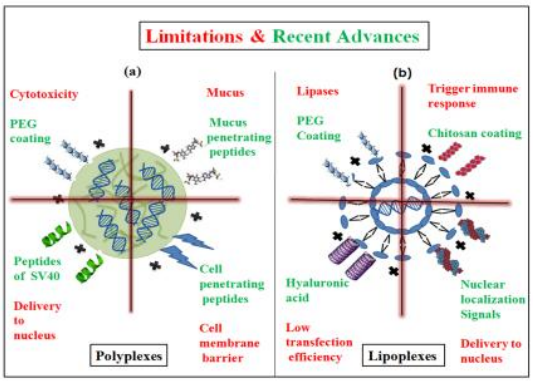

Fig 5. Various modifications in polyplexes (a) and lipoplexes (b) (Limitations are highlighted in red and modifications are highlighted in green.)

CONCLUSION

Over the last 20 years, nanoparticles have been used in the trails for the treatment of cystic fibrosis. In order to obtain the expression of a therapeutic

gene, it should be delivered to the desired location. Lipoplexes and polyplexes show promising results in the gene therapy of genetic diseases. Therapeutic genes must be expressed at a high level for the treatment of a disease. However, many barriers lower their efficiency of the nanoparticle by limiting their access to the required location. These barriers, including extracellular, cell surface, and intracellular barriers, prevent an efficient gene transfer. The key purpose of this review paper was to highlight the barriers faced by lipoplexes and polyplexes. For successful gene therapy, sufficient DNAs should be delivered to the target cell. By understanding and characterizing these barriers, we can overcome these barriers through making various modifications in nanocarriers to make them efficient for gene delivery.

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