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Review Article

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Non-Viral Vectors for Cystic Fibrosis Therapy: Recent Advances

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Abstract

Gene therapy, which is the transfer of genetic materials into the cells for therapeutic purposes, holds a huge promise in treating various hereditary diseases. Gene therapy tools are currently being used for wide range of monogenic and multigenic disorders including but not limited to cystic fibrosis. Cystic fibrosis is caused due to mutations in a cystic fibrosis trans-membrane conductance regulator (CFTR) gene and therefore, works as a model disease in gene therapy field. However, an ideal delivery vehicle, that ensures both efficient intracellular delivery and subsequent sufficient expression of transgene is still lacking. Fundamentally, two types of vectors are currently used to cross various cellular barriers broadly named as viral vectors and nonviral vectors. Viral vectors, as the name indicates, uses modified viruses for the transfer of genetic material into the cells. Viral vectors have greater efficiency in terms of transferring genetic materials; however, their toxic nature, immunogenicity and possible mutagenicity put their therapeutic applicability at question. On the contrary, nonviral vectors including cationic polymers and lipids, have recently gained more attention as alternatives to viral vectors. This can be attributed to their lesser immunogenicity, lower toxicity and ability to carry large nucleic acid fragments. In the current review we will first, briefly highlight various extracellular and intracellular barriers faced by nanocarriers during the process of gene delivery. Afterwards, we will pinpoint the most recent modifications made in these nanocarriers to cross these barriers efficiently which will subsequently allow us to use them in CF and other monogenic diseases therapy.

Keywords: Gene therapy; Cystic fibrosis; Nanocarriers; Cationic lipids; Cationic polymers

Introduction

Gene therapy is the transfer of genetic material in to the host cells for the treatment and prevention of inherited diseases and cancers. Since its time of discovery [1], gene therapy is been used for the treatment of genetic disorders through correction of mutated genes. Recent studies on other diseases such as cancers [2], autosomal dominant disorder, autosomal or X-linked recessive single gene disorder [3] and many other disorders had shown that they can potentially be treated through gene therapy approaches [4]. Monogenic, autosomal recessive disorders, for example cystic fibrosis, are good candidates for gene therapy trials. This can be attributed to the fact that only single gene needs to be corrected in a mutated cell to regain its normal function [5]. Since time, when CFTR gene was first discovered [6], many efforts have been made to develop gene delivery systems for somatic gene therapy of cystic fibrosis. Cystic fibrosis (CF) is a common life shortening genetic disorder, characterized by chronic lung inflammation resulting in reduced life expectancy to 45 years at best [7]. It is one of the most common autosomal recessive disorders among Caucasian population affecting 1 in 2000 individuals [8] and approximately 70,000 individuals worldwide each year [9]. The disorder is resulted from a mutation in CFTR gene located on chromosome 7q31.2. The gene encodes a protein of 1480 amino acid residues known as cystic fibrosis transmembrane conductance regulator (CFTR) [10,11] mainly located in the apical membrane of epithelial cells. Mutations in CFTR gene disrupt the cAMP (cyclin adenosine monophosphate) regulated chloride channel formed by CFTR protein and also interfere with the regulation of other ion channels [12]. This results in an imbalanced movement of ions and water across the airway epithelium thus leading to the accumulation of sticky mucus, chronic bacterial infection and inflammation of epithelial cells. Subsequently, these cause clinical aberrations and altered phenotype [13-16]. Presently, over 1500 mutations have been identified in CFTR gene [17] and are categorized into 6 classes shown in Table 1 [18-19].

Class	Common representative	CFTR defect	Frequency	Type of mutation
I	G542X	No functional CFTR protein	10%	Nonsense, splice
II	ΔF508	CFTR trafficking defect	70%	Missense; amino acid deletion
III	G511D	Defective channel regulation	2% - 3%	Missense; amino acid change
IV	R117H	Decreased channel conductance	< 2%	Missense; amino acid change
V	3349+10 Kb	Reduced Synthesis of CFTR	< 1%	Missense, splicing defect
VI	N287Y	Decreased CFTR stability	< 1%	Missense; amino acid change

Table 1: Six classes of mutations in CFTR protein that lead to CF phenotype [20-23].

The current review will first generally highlight various approaches that can be applied in the gene therapy of CF. The later part of the review will mainly focus on nonviral methods, particularly, cationic lipids and polymers that are currently used for CF therapy. Additionally, various extracellular and intracellular barriers that these nonviral vectors have to cross before they can show their therapeutic effects are highlighted. Finally summarizes the various recent biochemical approaches used to cross these barriers efficiently. Finding ways to increase the intracellular delivery and subsequently, gene

transfer efficiency of cationic nanocarriers will allow us to use them for therapeutic purposes of various inherited disease as well as cancers.

Gene Therapy for Cystic Fibrosis

Cystic fibrosis is widely regarded as a model genetic disorder for the gene therapy studies mainly because of four reasons; (1) it is a monogenic disorder (2) it is a recessive disorder in which heterozygotes are phenotypically normal (3) lungs are mainly affected that are comparatively easily accessible for treatment (4) it is a progressive disease with a nearly normal phenotype at birth, offering a therapeutic window. It is suggested that 5-10% of normal functioning CFTR gene expression is required for the treatment of disease [24]. However, it is not clear that whether majority of the airway epithelial cells have to express 5-10% CFTR function or just a small amount of cells expressing higher levels of CFTR would be sufficient for the treatment.

Nucleic acid fragment encoding CFTR protein should be transferred into the affected cell in order to regain its normal function. For the purpose, naked DNA have been delivered directly into the cells [25]. However, naked DNA molecules have several problems associated with them, when used for the therapy of CF. For example; they are larger in size and hydrophilic in nature which severely hampers their intracellular delivery. In addition, they are easily degraded by nuclease enzymes [26,27]. Therefore, people in the gene therapy field have always been interested in the development of ideal nanocarrier systems that ensure both, efficient delivery of CFTR gene into the target cells as well as maximum protection against both intra and extracellular enzymes.

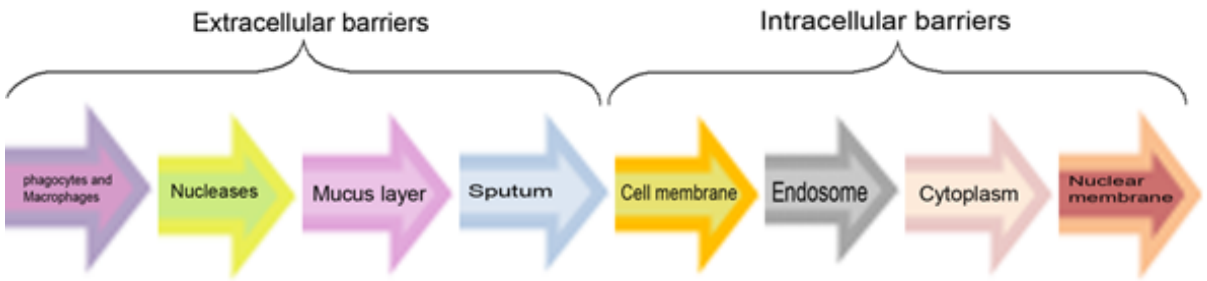

Figure 1: Summary of various extracellular and intracellular barriers faced by a nanocarrier while delivering genetic payload into the target cell.

Various types of vectors have been used in clinical trials to deliver CFTR cDNA into cells and *in vivo* (CFTR gene is 6.5 kb which can be reduced to 4.45 kb by using its cDNA [28]). These can broadly be grouped into viral vectors and nonviral vectors. In both cases plasmid DNA is used having three important components including; complementary CFTR DNA (cDNA), an upstream 5` promoter region which regulates transcription of gene of interest and downstream 3` terminator region which influences RNA splicing, polyadenylation and post-translational processing.

Therapeutic applicability of a particular vector is determined by numerous factors including but not limited to: vector carrying capacity, its efficiency, associated immunogenicity and possible mutagenesis. Viral vectors have higher efficiency of carrying nucleic acid fragments; however, their applicability is largely hampered by their higher immunogenicity, possible mutagenesis and potential risks in immune compromised individuals. Nonviral vectors, on the other hand, are lesser immunogenic and can carry large DNA fragments, however, their transfection efficiency is lower compared to their viral counterparts.

Barriers for Cystic Fibrosis Gene Therapy

In order to deliver their payload and consequently show their effects, the vectors have to cross various anatomical and cellular barriers in efficient manner. Extracellular matrix which surrounds the epithelial and endothelial cells lining are regarded as anatomical barriers while plasma membrane, endosomal membrane, cytoplasmic constituents and nuclear membrane are cellular barriers. Collectively, these two significantly affect the overall efficiency of gene transferring systems. For example, in blood circulation, DNA loaded nanocarriers are cleared by phagocytes such as Kupffer cells in liver and macrophages in the spleen. Moreover, nucleases present in blood and extracellular matrix can also degrade the unprotected nucleic acid fragments. Furthermore, the situation is worsened in case of CF, where a thin mucus layer covering the airway epithelium in lungs, provides an additional barrier. Although, the main role of mucus layer is to trap invading foreign particles, it significantly lowers the efficiency of gene delivery systems. Situations are further aggravated in the later stages of cystic fibrosis patients when lung airways are filled with sputum (mixture of saliva and mucus) thus further lowering the transfection efficiency of nanocarriers [29].

Among cellular barriers, cell membrane is a first barrier for various nonviral nanocarriers. Most of the nonviral nanocarriers exploit various cellular endocytic mechanisms to get entry into the cells, although, alternate mechanisms have also been suggested. For example, direct fusion of nanocarrier with plasma membrane, injecting its genetic payload into the cell, have been suggested [30]. Once inside the cells, through either of the endocytic mechanisms, the nanocarriers are enclosed by endosomal membrane and thus provides an additional barrier to the successful transfection process. Several escape mechanisms have been suggested for the release of macromolecules from endosomes. Nanocarriers that are unable to escape from endosomes are eventually digested in lysosomes thus further hampering their transfection efficiency. Although, endosomal escape

often leads to the release of free nucleic acid fragments, alternatively, escape of entire complex consisting of nucleic acids and nanocarriers into the cytoplasm has also been suggested [31,32]. Depends on the type of application and nucleic acid fragments, they should either stay free in cytoplasm in order to bind with target RNA (in case of siRNA etc.) or they should travel to the nucleus for efficient gene expression [33]. Cytoplasm by itself is greatly studded by huge number of proteins, nucleic acid digesting enzymes and network of cytoskeleton. Therefore, transport through cytoplasm is another huge task that a nanocarrier has to achieve. Nuclear envelope is another fundamental barrier that nanocarriers have to cross. The double membrane of nucleus greatly affects the transfection efficiency of nanoparticles. Overall this implies that gene therapy is a multistep process and nanocarriers have to efficiently cross various barriers in order to show their therapeutic effect (Figure 1). In following sections we will first briefly summarize various approaches adopted to efficiently cross these barriers. In later sections, we will discuss, exclusively, various chemical modifications made in nanocarriers to cross various cellular barriers in efficient manner.

Gene Therapy Systems

There are two essential components in current gene therapy protocol: 1) a therapeutic gene and 2) a vector system that delivers it to the target tissues or cells. Although an effective therapeutic gene construct with proper regulatory element is equally important, much efforts have been made to develop an efficient and safer vector system. Various vector systems used for gene delivery can be broadly grouped into viral and non-viral vectors [24,34].

Viral vectors used in gene therapy

In most of currently available gene therapy tools, various viral vectors are predominant. Genetically altered viral vectors, in which amplification potential is removed, are used. Due to their intrinsic infection ability, modified viral vectors have enhanced efficiency and in case of insertion vectors, long term expression of desired gene. Recombinant viruses such as adenovirus, adeno-associated virus, lentivirus and helper-dependent adenovirus have been applied for gene delivery in cystic fibrosis (Table 2) [35,36].

Adenovirus vector: Adenovirus, used as a vector, has been isolated from a vast variety of species and has more than hundred serotypes. Among adenoviruses, serotype 2 and 5 are mostly used for gene delivery in cystic fibrosis patients. This can be attributed to the facts that humans are mostly exposed to these serotypes implying that they have higher gene-transfer efficiency [37]. Additionally, it also has the capacity to deliver large DNA fragments which is prerequisite in case of CFTR gene. Furthermore, since adenovirus serotype 2 and 5 have lower tissue specificity, until and unless targeted, they can be used to deliver genes to wide range of tissues [37]. However, due to severe host immunological responses and its ability to cause serious side effects in patients, the use of adenovirus vector in CF gene therapy is largely limited [38-40].

Adeno-associated viral vector: Adeno-associated viral vector are safer compared to adenovirus vectors since they are lesser toxic and are also replication deficient [41]. Moreover, AAV also has the ability to specifically integrate its genome into host DNA, therefore, it can be used to deliver gene to the targeted site. However, their main drawback in cystic fibrosis gene therapy is that they can carry only a small piece of nucleic acid (up to 4.8 kb) into the host genome which is not desired in case of CF [42,43].

Helper-dependent adenoviral vector: Helper-dependent adenoviral vector (HdAd) is consisted of two vectors, the helper vector and another vector. The helper vector contains viral genes required for replication and another vector consists of several fragments including: gene of interest ends of viral genome and packaging recognition signal. HdAd is superior version of adenovirus in which many limitations have been removed. Higher packaging capacity, lower immunogenicity and reduced toxicity are advantages of using HdAd vectors in gene delivery [44-46].

Lenti viral vector: Lentivirus is a subclass of retroviruses having higher efficiency of gene delivery in both dividing and non-dividing cells. Moreover, they have the greater capacity to deliver larger (8 kb) nucleic acid fragments. Furthermore, their ability to stably express gene of interest in cells and lower immunogenicity make them suitable candidates for gene therapy of cystic fibrosis [47-50].

Overall, recombinant viral vectors are effective tools in gene therapy for cystic fibrosis due to their higher transfection efficiencies. However, there are still many problems associated with viral vectors including (1) difficulty in production (2) increased immune response (3) limited packaging capacity (4) mutagenesis due to random insertion [51]. These all, lowers the therapeutic applicability of viral vectors for the cystic fibrosis.

Viral vector	Adenovirus (AdV)	Adeno-associated Virus (AAV)	Lentivirus	Helper-dependent adenovirus
Family	Adenoviridae	Parvoviridae	Retroviridae	
Genetic material	dsDNA	ssDNA	ssRNA	DNA
Geometry	Icosahedral	Icosahedral	Icosahedral	Icosahedral
DNA loading capacity	8 Kbp	5 Kbp	9 Kbp	36 Kbp
Pathogenicity	Low	Very low	High	Low
Nature	Non-integrating	Integrating	Integrating	Non-integrating
Expression	Short-term expression	Long-term expression	Long-term expression	Long-term expression

Target cells	Infect broad range of cell types	Transduce both dividing and non-dividing cells	Transduce both dividing and non-dividing cells	Transduce both dividing and non-dividing cells
Advantages	High gene-delivery efficiency, easy production	Stable transfection, Less immunogenic	Integration-defective vectors available, stable transfection, lower pathogenicity	Higher packaging capacity, reduced toxicity
Limitations	Highly immunogenic, Short-term transfection	Need co-infection by helper virus	Insertional mutagenesis	Innate immune response, Acute toxicity
Reference	36,39,52,53	38,42,54,55	35,49,56,57	45,58,59

Table 2: Various viral vectors that can be used for gene therapy of cystic fibrosis.

Nonviral Gene Delivery Methods

Nonviral gene delivery systems exploit all other methods except viruses for the delivery of genetic materials into the cells. These include both physical and chemical methods. Electroporation, gene gun, needle injection etc. are regarded as physical methods, while cationic lipids and polymers in addition to some others are grouped into the chemical methods.

Table 3,4 provides an overview of various physical and chemical methods that can potentially be applied in CF gene therapy.

Delivery methods	**Microin-jection**	**Electropora-tion**	**Jet injection**	**Gene gun**	**Needle injection**	**Sonoporation**	**Hydrodynamic gene transfer**	**Magnetofection**
DNA loading range	Low	Low	Low	Low	High	High	High	High
Functional components	Mechanical force, pressure	High-voltage electrical currents	High-speed\ pressure	High pressure helium gas	Mechanical force, directly injected	Ultrasound waves	Hydrodynamic force, high pressure	Magnetic force
Instrument	Micro needle Or microscopic injection	Electrodes	Jet injector	Biolistic gene gun and Gold tungsten (diameter 1–1.5um)	Needle, Syringes	Sonoporator	Hydrojector	Superparamagn-etic nanoparticles and magnetic
Procedure	Microsc-opic needle is used to deliver gene under microscope	Produce temporary pores in cell membrane allowing DNA delivery	DNA is delivered through pores formed by high pressuriz ed gas (e.g. CO2)	Delivery of DNA-coated heavy metal particles with high speed	Mechanic-al delivery of DNA to target site	Permeablize cell membrane thus temporary allowing cellular uptake of DNA	High hydrostatic pressure is used as driving force to deliver genes into internal organs.	DNA is coated on magnetic nanoparticles and then complexed with polymers or lipids. Cellular uptake is mediated through endocytosis
Target cells	Muscle, skin, liver, lung and cardiac muscle	Muscle, brain tumors, Skin, lung. and tumor treatments	Muscle, skin, fat and mammary tissues	Skin, muscle, mucosa, or tumor cells	Skeletal muscle, lung liver, skin, brain, tumors	Brain, cornea, kidney, peritoneal cavity, muscle, and heart	Liver, lung, kidney, spleen and heart	Brain, blood vessel endothelium, lung, liver
Factors affecting the efficiency	Form and size of the DNA, injection buffer, and the site of injection	Electric impulses, time interval and amount of DNA	Jet-injection volume, pressure, depth of jet penetratio n in the tissue, and DNA stability	Particle size, speed and dose	Type of organs, injection volume, injection speed	Amount of DNA, intensity of the pulses, frequency and duration	Type of organs, injection volume, injection speed and the total amount of the functional substance	Physico-chemical properties of superparamagn etic nanoparticles, as well as the magnetic field parameters – the magnetic field intensity

								and magnetic field gradient.
Advantages	Simple, economical, effective, reproducible and lower toxicity	effective, reproducible and titratable modality	Higher gene transfer efficiency, reduce tissue damage by adjusting gas pressure	No receptor is required, size of DNA is not a problem, easy to produce, simple	Lower risks of toxicity, DNA vaccination is the major applications	Safer, non-invasiveness, transfer genes into the cells of internal organs without surgical procedure	Efficient gene transfer, safety, simplicity and effectiveness, reproducibility, minor toxic effects	Simple and efficient transfection, inexpensive
Drawbacks/ Limitations	Low expression level,	Difficulty in surgical procedures, high voltage damage the tissues	Localized pain, edema, and bleeding at the injection site	Causes greater immune response	Poor gene expression, degraded by the nucleases, lack of cellular specificity, transfection is limited to the site of injection	Low efficiency	Poor gene expression, large injection volume is the biggest hurdle	Lower efficiency and toxicity
References	32,60,61	62-64	65,66	25,67	67,68	69-71	72-74	75,76

Table 3: Various physical approaches used for gene therapy.

Physical methods

Several serious issues have been shown to be associated with viral vectors when used in gene delivery trials including their: potential immunogenicity and cytotoxicity, insertional mutagenesis, toxin production and limited nucleic acid packaging ability [32,82]. Alternatively, physical methods including naked DNA delivery through needle injection, gene gun and electroporation have been suggested as substitutes to the viral methods. Easy manufacturing, storing and potential safety benefits potentiate their application in cystic fibrosis therapy.

Needle injection: Genes of interest are delivered to the target site through needle injection, which are manipulated using atomic force microscope. The method has higher transfection efficiency and apparently there are no irreparable damages. In addition, since the technique does not utilize any vector for the nucleic acid transfer, it is widely regarded as one of the simplest and safest methods of gene transferring. However, there are still some issues associated with them, for example free nucleic acids are degraded intramuscularly, thus largely reducing its transfection efficiency [27]. Moreover, transfection achieved through this method is also limited to the needle surroundings.

Delivery methods	Cationic lipids	Cationic polymers
DNA loading range	High	High
Functional components	Electrostatic interaction	Electrostatic interaction
Instrument	Lipid	Polymer
Procedure	DNA is complexed with cationic lipid and particle uptake occurs through endocytosis	DNA is complexes with cationic polymer and particle uptake occurs through endocytosis, DNA condensation and Protein sponge effect
Target cells	Airway epithelial, endothelial, hepatocytes, muscles	Lung, oral cavity
Factors affecting the efficiency	Charge on particles, size, shape of complexes	Molecular weight of polymers, surface charge, charge density, hydrophilicity and the structure of cationic polymers
Advantages	Safe, lower cytotoxicity	Less immunogenicity, Fair transfection efficiency
Drawbacks/ Limitations	Low to medium efficiency, some types results in immunogenicity	Low efficiency, cytotoxicity
References	77-80	80,81

Table 4: Various chemical approaches used for gene therapy.

Gene gun: In this approach, the gene of interest is attached to heavy metal and then transferred to a specific site with speed achieved through helium pressure discharge [67,83]. Delivery of precise DNA doses to the targeted area is one of the main advantages of this system. However, greater cell damage during the process is the huge disadvantage of the method, hampering its applicability in CF therapy.

Electroporation: Electric pulses are applied in electroporation technique to generate transient pores in the plasma membrane so that the gene of interest can be transferred into the target cells. The method is efficient and reproductive if the associated parameters are optimized [84]. However, like other methods, electroporation also has limitations including but not limited to: huge cell damage due to high voltage,

lower transfection rate and limited accessibility of electrodes to internal organs [64,84].

The above mentioned physical methods used in gene delivery of cystic fibrosis minimize various side effects associated with viral vectors such as potential immunogenicity and carcinogenesis etc. In addition, these methods deliver gene of interest to single or multiple targeted cells at specific site effectively thus minimizing the risks of dispersion of transfected genes to other areas. However, in addition to these advantages, there are some problems linked with physical methods. For instance, in case of a gene that needs to be transferred to the nucleus, the transfection efficiency is significantly low as the nuclear membrane and potential nuclease degradation are the main limiting factors. In addition, higher cell damage, difficulty in large scale delivery and necessity of costly instruments critically limits the use of physical methods in gene therapy of cystic fibrosis.

Chemical-based nonviral vectors

Chemical-based nonviral gene delivery systems use synthetic or natural compounds for transferring of genes to the targeted area. Advantages of these chemical based vectors is that they are lesser toxic and lesser immunogenic compared to their viral counterparts. Additionally, they can be easily targeted to some specific cells or tissues by decorating their outer covering with cell or tissue specific ligands. Furthermore, they can be easily synthesized and importantly, due to their lower toxicity they can be repeatedly administered with minimum off-target effects.

Cationic lipids: Cationic lipids-based gene transfer was first achieved by Felgner et al. [85] in 1987. Afterwards, many lipid-based gene delivery systems have been developed all sharing the same basic structure consisting of hydrophilic head, hydrophobic tail and a linker between them. The positively charged head groups are necessary for binding of lipids to the negatively charged phosphate groups in nucleic acids. Primary, secondary, tertiary amines and quaternary ammonium salts are predominantly used as head groups. Alternatively, several other head groups: for example guanidine and imidazole have also been suggested. Eighty nine pyridinium based compounds, with primary compound named 1-(2.3-dioleoyloxypropyl)-2, 4, 6-trimethyl pyridinium-lipid having a pyridinium group as its head group instead of amine or quaternary ammonium groups have been developed [86,87]. On the other hand, most of the hydrophobic tails are made up of aliphatic chains, cholesterol or other types of steroids rings. On the contrary, ether, carbamate and amide bonds are frequently used as linkers connecting hydrophilic heads with hydrophobic tails. Some commonly used cationic lipids in gene therapy with modifications are summarized in Table 5. Moreover, recent modifications made in these cationic lipids for enhanced gene delivery are diagrammatically shown in Figure 2.

Name of cationic lipid	Formula	Name after modification	Formula	Modification	Advantages
DOTMA	N-[1-(2,3-Dioleyloxy)propyl]N,N,N-trimethylammonium chloride	DORIE	1,2-dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide	Hydroethyl group incorporation in head group	Decreased in head group hydration results in stable lipid assembly
DOTAP	1,2-Dioleoyloxy-3-trimethylammonium-propane	DORI	1,2-dioleoyloxypropyl-3-dimethyl-hydroxyethyl ammonium chloride	Hydroethyl group incorporation in head group	Decreased in head group hydration results in stable lipid assembly
DC-Chol	3β-[N-(N′,N′-Dimethylaminoethane)-carbamoyl]cholesterol	Lipid 67	3-(N,N-dimethylamino) propanamine	Incorporation of polyamines in head group	Efficient for gene transfer to lungs and protect DNA from degradation
BGTC	Bis-guanidium-tren-cholesterol	BGDA	Pentacosa-10,12-diynoic acid (2[bis-(2-guanidino-ethyl)-amino]-ethyl)-amide	Modification in hydrophobic domain by incorporating diacetylene groups	Favorable degree of fluidity and destabilization. Significant transfection efficiency

Table 5: Some commonly used cationic lipids for gene delivery with some recent modifications made in them to enhance their transfer ability.

Liposomes and lipoplexes: Lipid platforms are suitable for carrying nucleic acid fragments by encapsulating them in liposomes core. Liposomes are easily prepared using reverse-phase evaporation. The method involves amphiphilic lipid hydration which leads to the multilamellar vesicles formation. Subsequently, the multilamellar vesicles are converted into unilamellar vesicles upon sonication. Liposomes when mixed with negatively charged nucleic acid fragments, form lipoplexes. Positively charged lipoplexes surrounds negatively charged DNA, due to electrostatic interactions, thus providing protection against extracellular and intracellular digestions. Transfection efficiency depends, among other factors, on the structure of liposomes (which includes geometric shape, number of charged groups per molecule, lipid anchor nature and linker), charged ratio used for DNA-lipid complexes formation and properties of colipids [88].

Colipids: Most commonly used colipids are dioleoylphosphatidylethanolamine (DOPE) and cholesterol. These colipids often act as "helper" lipids facilitating cationic lipids in transferring nucleic acid fragments on larger extent. Although some lipids require DOPE for transfection there are also others, which have double fatty acid chain capable of forming bilayer, do not require helper lipids for transfection. The presence of DOPE reduces cationic lipid to DNA charge ratio which is required for maximal transfection. Moreover, its addition also reduces cytotoxicity of cationic lipids. Furthermore, DOPE has also been shown to have fusogenic property, facilitating lipoplexes in endosomal escape. Cholesterol, another colipids, on the other hand, has been suggested to stabilize cationic

lipid membrane against destructive activity of serum components thus improving transfection (Figure 3) [88].

Disadvantages of cationic lipids: Cationic lipids carry large surface charge contributing to their very short blood circulation time [89]. Moreover, they can potentially form large aggregates which lead to their systemic elimination. This can be attributed to the facts that large positively charged aggregates interact strongly with negatively charged serum molecules and cellular components [90]. Lipoplexes, after injection, reaches to lungs cells in about 60 min. This also includes crossing of pulmonary vasculature, which offers a first passage effect. Consequently, pulmonary vascular endothelial cells and some airway epithelial cells are predominant cells that are transfected with lipoplexes [91-93].

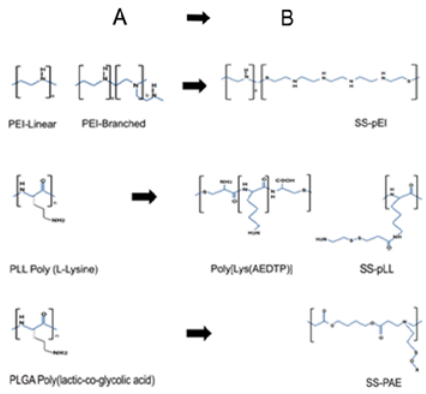

Figure 2: (A) Chemical structures of some commonly used polyplexes for gene therapy. **(B)** Introduction of disulfide bond in these polymers, making them primarily biodegradable.

Barriers based recent advances in lipoplexes: Lipoplexes have already been successfully used for the gene delivery to various cell lines both in *in vitro* and *in vivo* conditions. However, various problems have been shown to be associated with lipid based gene delivery systems. Among these, acute toxicity and triggered host-immune response are the major issues [93,94].

Recent approaches, for example, surface coating of lipoplexes with hydrophilic and charge neutral polymers such as chitosan have significantly reduced their nonspecific interactions thus leading to their reduced toxicity [95]. Moreover, multiple issues associated with lipoplexes have recently been resolved using modified form of lipid-based carrier system called Vaxfectin. Vaxfectin is synthesized by combining cationic lipid (GAP-DMORIE) with neutral phospholipid (DPyPE). The liposomes thus formed, have shown improved gene delivery as well as DNA-based vaccination to the targeted sites [96]. Additionally, surfaces of liposomes and PEI have also been decorated with cell penetrating peptides also referred to as Protein Transduction Domain (PTDs) to improve their gene delivery efficiency [97,98]. Currently, over 25 clinical trials, utilizing PTDs-mediated lipid based gene delivery systems are approved as safer nanocarriers for gene delivery [99].

Lipoplexes, have been shown to form aggregate and non-specifically adsorb to non-targeted tissues thus severely hampering their applicability in cystic fibrosis therapy. These can be attributed to overall massive positive charge present on their surface [100]. For the purpose, PEGylation of cationic lipoplexes have been shown to reduce their interaction with serum protein in blood stream and also helps them in avoiding their recognition by immune system[101,102]. Subsequently, these resulted in their increased circulation time and facilitated their targeted delivery of genes [103]. Alternatively, surface of cationic liposomes is also covered with polyanions which ultimately reduced their nonspecific interactions thus leading to their increased *in vivo* circulation [104].

Among various prerequisites for gene therapy, targeted gene delivery is fundamentally important. For the purpose, various modifications are applied. Surfaces of liposomes, for instance, are decorated with iron-saturated transferrin [77], folic acid [105,106] and RGD [78] for specific gene delivery. In addition, protamine sulphate, has recently been applied to condense genetic material encoding for desired protein. Condensed DNA thus obtained is subsequently encapsulated in PEGylated liposomes. In this case, Calf thymus DNA is used which provide extra negative charge thus facilitates the formation of compact nanoparticle in the core, while PEGylated liposomes provided surface protection against digestion and aggregation, improving overall transfection efficiency [107,108]. In agreement, decorating the surface of liposomes with MPG resulted in enhanced expression of HPV16 E7 gene *in vitro* [109]. In addition, a cardiac glycoside such as Strophanthidins, are also attached to the surface of liposomes. Attachment of Strophanthidins is helpful in binding nanoparticles to the cell surface by targeting Na+/ K+ ATPase receptor. Subsequently, this improves delivery of nucleic acids to a number of cells originating from different organs such as lungs, prostate, ovary, breast, and pancreas [79].

Once inside the cells, the lipoplexes has to escape from endosomes. This implies that endosomal degradation and entrapment is among the major obstacles for the successful application of lipoplexes. Various modifications have been made in lipoplexes in order to promote their endosomal escape and the transfection efficiency. For example, Arukuush et al. [110] has recently modified lipoplexes with endosomal membrane penetrating peptide TP10, named as NickFects. The resulting complex can efficiently transfect a large variety of cell lines thus offering a potential applicability in CF therapy [110]. Notably, this modified particle appears not only efficient in crossing endosomal membrane but is also suggested to be useful for mammalian protein production system to express and produce recombinant proteins [110]. More recently tocopherol-based cationic lipids are prepared having pH sensitive dipeptide head groups. Introduction of cationic dipeptide head groups appreciably affects the physicochemical and biological properties of cationic lipids. Moreover, cationic head group of lipids are modified using imidazole group with pH buffering ability while using histidine as a building block in the preparation. The resulted nanocarrier has the ability to deliver nucleic acid with high efficiency and also reduces cytotoxicity [111].

Finally, the delivery of nucleic acids should reach to the nucleus, in order to replace mutated gene with normal functioning one. Lipoplexes are mostly decorated with NLS (nuclear localization signals), derived from short peptide sequences of viral proteins, in order to transfer gene to the nucleus [67]. Additionally, cell-specific modifications have also been made in lipoplexes in order to enhance their efficiency in particular types of cells. For instance, liposomes are modified with hyaluronic acid (HA), instead of PEG, for their applications in ocular gene therapy [112]. HA is a glycosaminoglycan,

ubiquitously expressed in mammalian cells and have several sites of chemical modifications making it more attractive chemical in gene delivery applications. Electrostatic HA-coating increases intravitreal mobility of cationic nanocarriers without affecting its cellular uptake and transfection efficiency [113]. In addition, arginine rich lipids are synthesized which were later on modified with guanidine or imidazole-rich margin. The resulting lipoplexes have been shown to have increased DNA binding abilities, decreased cytotoxicity and nineteen folds higher transfection efficiency compared to unmodified lipoplexes [114].

Most of these lipoplexes have shown their increased applicability in specific types of cells, implying that CF affected cells can potentially be targeted using specific nanocarriers. Therefore, it is urgently required to synthesize lipoplexes keeping in mind the physicochemical properties of CF cells.

Cationic polymers: Cationic polymers have been extensively used for gene delivery mainly because of their versatility and easy manipulation. Although, mostly cationic polymers are used for gene therapy, other types such as hydrophobic biodegradable polymers and negatively charged alginate have also been applied [115,116]. Polymers differ from cationic lipids both in structure and chemical composition, lacking hydrophobic tails and are completely soluble in water. Moreover, they interact totally differently with DNA and their intracellular behaviors also vary from their cationic lipids counterparts [117,118]. Synthetic polymers are attractive tools for gene therapy because of their easy and low cost production as well as their availability in wide range of structures [118]. Importantly, when required, they can easily be modified. Furthermore, modified polyplexes reduces various side effects including cytotoxicity and nonspecific binding, indicting their utility in gene delivery systems with higher transfection efficiency [119].

Polyplexes: Polyplexes are formed due to electrostatic interactions between cationic polymers and anionic DNA. They are more stable and relatively smaller in size as compared to their lipoplexes counterparts [120]. Among various synthetic polymers, Polyethylenimine (PEI) is fundamentally important for various gene therapy applications including CF. This can partially be attributed to the fact that PEI is rich in amine groups which are unprotonated at neutral pH. Upon acidification in endosomal compartments, excessive protonation of amine groups takes place leading to their swelling and subsequent endosomal escape [121,122]. Cytotoxicity and gene transfer ability of PEI depends, among many factors on, molecular weight of PEI, polymer structure (liner or branched), and ratio of amine group of PEI to phosphate of DNA (N/P ratio). For example, PEI with low molecular weight and optimum N/P ratio has been shown to be more efficient and lesser toxic compared to high molecular weight PEI [123]. Moreover, branched PEI have been shown to have high toxicity and low transfection efficiency compared to linear PEI [123,124]. In addition to PEI, poly(L-lysine) (PLL) is another cationic polymer can potentially be used for CF gene therapy. Along with physical properties similar to PEI, PLL also show has high transfection efficiency (Table 6) [125,126].

Polymer	Abbreviation	Feature
Poly(ethylene)glycol	PEG	Inert
Polyethylenimine	PEI	Cationic
Poly(L-lysine)	PLL	Cationic
Poly(propylenimine)	PPI	Dendrimer
Poly(amidoamine)	PAMAM	Dendrimer

Table 6: Some commonly used polymers for gene delivery.

Disadvantages of Polyplexes: Polyplexes used in CF gene therapy systems have various issues associated with them thus limiting their therapeutic applicability. For example, branched polyplexes possess higher toxicity and lower transfection efficiency as compared to linear polyplexes[80,127]. Moreover, polyplexes have been shown to form large aggregates upon *in vivo* administration, further questioning their applicability in CF gene therapy [80]. Furthermore, non-targeted polyplexes bind to various non-specific areas thus showing potential off-target effects [128]. This can be attributed to their net positive charge, which can efficiently bind to the negatively charged cell surfaces of various cells [81]. Keeping in mind these multiple issues, various modifications have been made in these polyplexes to ensure their future applicability in CF therapy. Some of these recent modifications are summarized in the following section.

Recent advances in polymers: The fundamental properties of polymers which make them potential alternatives to viral vectors are their easier functionalization, protectability of cargo and biodegradability upon administration. In earlier trials, polymer like PLL (poly-L-lysine) was used as vector. However, its poor transfection efficiency seriously limits its *in vivo* applicability. This can be attributed to the fact that the characteristic amine-groups of PLL are positively charged at physiological pH. Consequently, this lowers the buffering capacity of PLL thus severely reducing its endosome escape ability [33]. Among various modifications of PLL, PEGylation is fundamentally important. It ensures both enhanced endocytosis and reduced cytotoxicity of PLL. PEGylated PLL used in trials of cystic fibrosis has already shown more promising results compared to its unmodified form [129]. Earlier, it has been shown that PLL tends to form aggregate upon administration to the cells resulting in huge cell toxicity and subsequent cell death [80]. More recently this problem was solved by combining PLL with palmic acid (PA). Mixing of PA with PLL not only prevent PLL from forming aggregates but also improve its transfection efficiency. In fact an increase in transfection efficiency due to addition of PA can be attributed to the fact that PA increases the interaction of nanocarriers with plasma membrane [130].

PEI (poly-ethyleneimine) is another widely used cationic polymer used for gene delivery. PEI is routinely used as transfection agent in *in vitro* experiments. However, their huge positive charge makes them cytotoxic and non-biodegradable [118] making their *in vivo* applications in question. Particularly, low molecular weight PEI (LMW PEI), which is routinely used, is less efficient as compared to high molecular weight forms of PEI(HMW PEI) [131]. Recently, a strategy has been developed in converting LMW PEI into HMW PEI. The idea is to allow the cells to convert the modified HMW PEI, after transfection, into segments that would be easily eliminated from the cells [132,133]. For the purpose, phenyl boronic acid (PBA) conjugated with sugar molecule is linked to LMW PEI, through cross linking, thus converting it into HMW PEI [134]. The resulting particles are then transferred into target cells where the bond between PBA and sugar is disrupted by acidic pH in endosomes or intracellular ATP thus leading to efficient release of genetic payload [135]. Additionally, in order to protect nucleic acid from nuclease degradation, PEI has been conjugated with polyamidoamines leading to enhanced efficiency [136].

Cell membrane is among the major barriers to the gene therapy of CF. For the purpose, surface of the PEI is decorated with cell penetrating peptides (CPPs). The basic amino acid present in the structure of CPPs hugely increases the intracellular delivery of Polyplexes [137]. Alternatively, a domain from herpes simplex virus, WT peptide has been attached to the surface of polyplexes leading to their increased cellular uptake [138].

PEI is proved to be an efficient gene delivery vehicle that triggers endosomal escape via pH buffering-effect. The so called proton-sponge effect results both in; endosomal escape and dissociation of cargo from the polymers [139]. However, cationic property of PEI can potentially induce cytotoxicity which hampers its therapeutic applicability. For the purpose, a promising approach is to adjoin anionic polymer with cationic PEI [140]. In agreement, incorporation of γ-PGA group to PEI resulted in enhanced gene delivery with minimal cytotoxicity [141].

After delivery to the target site, the efficiency of nanocarriers is also dependent upon the dissociation of polymer from the nucleic acid fragments. For example, early release may result in the degradation of nucleic acid by cytoplasmic nucleases while the late release will results in non-efficient gene expression [142]. Therefore, optimized conditions should be maintained in order to ensure enhanced nucleic acids release from polymers and subsequent higher gene expression [142]. Nuclear membrane is one of the fundamental barriers in gene therapy strategies which should be efficiently crossed by nanocarriers for the optimum gene expression [143]. In recent studies, a modification has been made in PEI by coupling it to large T-antigen peptide of SV40 which acts as NLS (nuclear localizing signals). Resulted PEI has shown improved nuclear transport and hence higher gene expression [144].

PPA (polyallylamine) is another polymer used for gene delivery to target cells. Although, currently used for various delivery applications, PPA still carries various issues that need to be addressed. For example, it is less efficient in both crossing the plasma membrane and escaping from endosomes. Moreover, it also shows increased toxicity once supplied to the cells [145]. These issues are recently solved by modifying PAA with bromoalkane derivatives. Inclusion of bromoalkane derivatives into PAA increases its hydrophobicity thus leading to its improved interaction with both cell and endosomal membrane. Subsequently, these greatly enhances the transfection efficiency of PAA [146].

Dendrimers are type of polymers having highly branched structures. PAMAM (polyamidoamine) is a type of dendrimer which is widely used for gene delivery because of its high transfection efficiency. However, toxicity is a major problem associated with PAMAM hampering its therapeutic applicability in CF therapy [147,148]. Recently, researchers have formulated various nitrogen-core poly(propyl etherimine) (PETIM) dendrimer-DNA formulations. Complexes thus obtained were investigated for their applications in clinical trials. Overwhelmingly, they showed lower toxicity and efficient gene delivery compared to their unmodified counterpart [149]. In addition to PETIM dendrimers, normal dendrimers have also been modified using tri-ethanolamine, which greatly increased its transfection efficiency [150].

PLGA (poly-lactic-co-glycolic acid) is biodegradable polymers used for the gene delivery mainly because of its stability and ability to protect DNA from degradation during *in vivo* application [132]. Nucleic acid fragments are encapsulated in PLGA using double-emulsion solvent evaporation method. Lower cellular interaction and less efficient endosomal escape are the main problems faced by PLGA polymer during gene delivery process [151]. To solve these problems, recently a modification has been made in PLGA. The polymer is modified with biocompatible chitosan and other cationic components which increases its overall positive charge. This lead to the enhanced PLGA cellular interaction subsequently increasing its transfection efficiency [151-154].

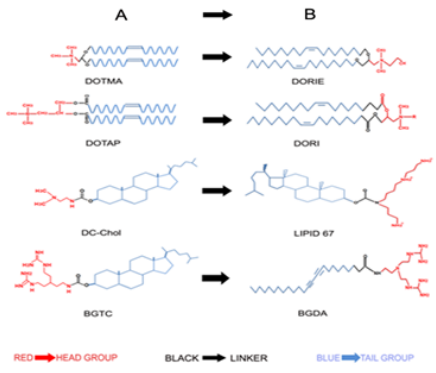

Figure 3: Structural representations of modifications made in some commonly used cationic lipids. (**A**) Unmodified structures of cationic lipids. (**B**) Modified forms of cationic lipids.

Increasing the hydrophobicity of nonviral vectors enhances their interactions with cells thus enhancing their cellular uptake and subsequent transfection efficiency [155]. For example, synthesis of chitosan with a number of hydrophobic modifications improved its cellular uptake [156]. More recently, researchers have utilized trimethylated-chitosan, a chitosan based nanoparticle, for transferring nucleic acid into the cells. The modified complexes have shown high transfection efficiency even compared to PEI/DNA Polyplexes [157,158].

Besides to different modification made in the various already available polymers such as PLL, PEI, PLGA, chitosan, dendrimers, and PPA, other formulations have been made to develop novel polyplexes with improved transfection ability. For crossing cell membrane barrier effectively, membrane disruptive peptides, such as melittin, are attached on the surface of polymers. Moreover decoration of polymers with virally derived peptides such as TAT, Antennapedia and HGP have also showed promising results in crossing cell membrane. Overall these all resulted in polymers with improved gene delivery [159,160].

Release from endosome is a key step for the successive gene delivery because the nanoparticles that is unable to escape from endosomes and subsequently degraded there. In order to help nonviral vectors to escape efficiently from endosome, recently researches have ligated the KALA sequences with nonviral vectors. The KALA has been shown to facilitate endosomal escape by disrupting its membrane [161]. However, how these disruptions affects the cell physiology is a fundamental question that needs to be answered.

Nuclear membrane along with nuclear pore complex (NPC) is considered as selective barrier that severely limit the overall transfection efficiency of polyplexes for those nucleic acid fragments that need to be delivered into the nucleus. Recently Liashkovich et al. [162] have functionalized the surface of polyplexes with trans-1,2-cyclohexanediol (CHD) and importin β. Importin β has been shown to

help nanocarriers in guiding towards NPC while CHD helps in leaking of NPC. Combined effects of both have greatly improved the overall efficiency of the modified Polyplexes [162].

Conclusion and Future Perspectives

Various strategies have been developed to deliver CFTR gene efficiently into target cells through carrier vectors. In recent years nonviral vectors have attracted much attention because of having the ability of gene delivery without the complication of immunogenicity and insertion mutation seen in viral vectors. In past few years the work continued in developing new nonviral vectors for gene delivery in cystic fibrosis. Further improvements are made to increase the efficiency and reduce toxicity of nonviral vectors are needed before clinical implication can be met. Many improvements are made in changing the composition of existent nonviral vectors to overcome the problems such as large surface charge, formations of aggregates, early clearances while other strategies are apply to improve gene delivery in cystic fibrosis by combining of nonviral vectors with different chemicals. It is imaginative that strategies that combine nonviral and viral vectors might be helpful to achieve more, efficient, long-lasting, and nontoxic gene delivery systems used for the treatment of cystic fibrosis.

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