Chapter 2

Gene Therapy for Cystic Fibrosis: Hurdles to Overcome for Successful Clinical Translation

Myriam Sainz-Ramos, Nuseibah AL Qtaish, Idoia Gallego, Ilia Villate-Beitia, Tania López, Gustavo Puras and José Luis Pedraz

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.79719

Abstract

Cystic fibrosis (CF) is a genetic disease that hampers the lung function. Despite that the main defective gene has been deeply characterized, some relevant concerns still need to be resolved before considering gene therapy as a realistic medical choice. One of the major issues that need to be strongly considered in order to succeed in the search for an effective gene therapy approach for CF is the design of the appropriate genetic material to be delivered. Other relevant factors to take into consideration include the design of safe and effective gene delivery systems, the biological barriers that need to be overcome in order to reach the nucleus of the target cells, and the problems related to the design of a drug formulation suitable for lung delivery purposes. Furthermore, some problems related to the commercialization of gene therapy products also need to be resolved. In this chapter, we discuss the up-to-date strategies to overcome such hurdles in order for gene therapy to become a routine treatment modality for CF.

Keywords: cystic fibrosis, gene therapy, drug delivery, biological barriers, drug formulation

1. Introduction

Cystic fibrosis (CF) is a rare disease with low prevalence caused by the dysfunction of the transmembrane conductance regulatory gene (CFTR). The most prevalent CFTR mutation consists of a deletion of a phenylalanine at position 508 [1]. The disease presents a heterogeneous distribution in the world population being more frequent in Northern Europe.

According to recent reports, in the European Union, 1 of every 2000–3000 newborns is affected by CF. In the USA, the incidence is 1 per 3500 births. In Asia, the predisposition to CF is low; however, there is evidence to show that this disease is severely underdiagnosed [2]. The basic characteristic of CF is the transport of defective ions in the apical membrane of most secretory cells, which leads to an altered secretion of mucus in the epithelium of the respiratory tract, the digestive tract, the pancreas, the liver, and the reproductive track [1]. The conventional treatments available on market, which include, among others, antibiotics, pancreatic enzyme supplements, high-fat diets, and even physiotherapy [3], afford the consequences derived from CFTR dysfunction and have significantly improved the mean life expectancy of patients affected by the disease up to 34 years [4]. However, their quality of life is severely compromised mainly due to side effects and interactions among such treatments [5]. Therefore, other therapeutic options such as gene therapy, in which the main goal is to restore the function of the mutated CFTR protein acting on the genetic cause of the problem, need to be considered. CFTR gene was cloned more than two decades ago, and the monogenic and autosomal recessive nature of CF disease means that the addition and expression of the corrected gene could reverse the underlying cause of the disease. Therefore, there is reasonable hope to consider gene therapy as a potential realistic medical option, and consequently, some clinical trials have been performed since 1993. However, despite the moderate optimism that emerged with the development of such clinical assays, there are still some hurdles to overcome before considering gene therapy a realistic medical option. Main concerns are related to the intrinsic properties of genetic materials, the development of safe and efficient gene delivery vectors able to deliver genetic materials to the nucleus of target cells, the design of a drug formulation suitable for pulmonary gene delivery applications, and the hurdles associated with the commercialization of such drugs (**Figure 1**).

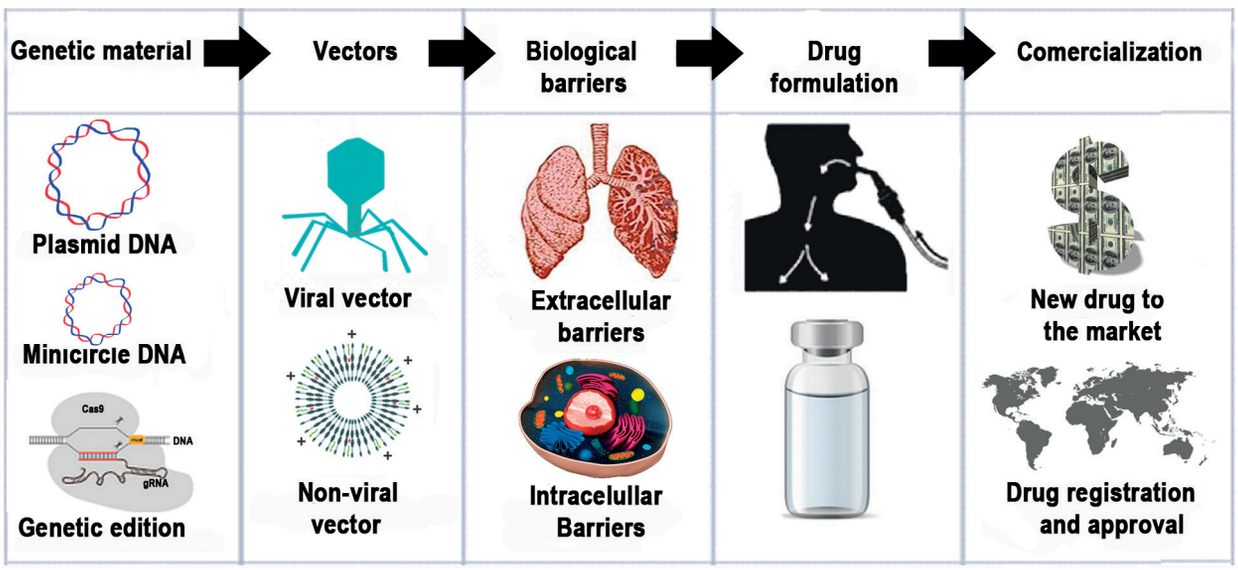

Figure 1. Hurdles that gene therapy should overcome in order to reach clinical practice in the treatment of CF disease.

In the next sections, we will analyze such barriers along with the most relevant approaches developed by the scientific community to circumvent them in order to cure CF with gene therapy.

2. Genetic material

2.1. Plasmid DNA

Bacterial plasmid DNA (pDNA) remains an interesting biomolecule for gene transfer, with several promising reports and clinical trials in progress worldwide [6]. In CF, pDNA has been successfully delivered by nonviral vectors to the sheep lung [7]. Additionally, when administered in multiple-dosage regimen, no loss of activity was observed [8]. In order to be produced in recombinant bacteria and to express their therapeutic gene of interest (GOI), pDNA needs a bacterial origin of replication sequence (bac-ORI). In addition, pDNA backbone includes a sequence with resistance to one/various antibiotics such as kanamycin, which allows to select the clone of bacteria transformed that expresses the plasmid. Finally, a eukaryotic promoter is needed to enhance GOI expression [9]. Usually, when pDNA reaches the nucleus of target cells, it remains in an episomal position, which means that it replicates independently from the host chromosomal DNA, avoiding the undesirable activation of oncogenic genes [10]. The main concerns of pDNA in gene therapy are related to safety issues. Classically, in eukaryotic cells, pDNA has been associated with the induction of undesired immune responses and secretion of proinflammatory cytokines [11]. For instance, a transient neutrophilic infiltration and an elevation in proinflammatory cytokines have been reported in mouse lung [12]. Although the episomal nature of pDNA could be an interesting advantage, the transfection efficiency remains compromised mainly by the transient and relatively low gene expression. Additionally, the size of the plasmid, determined by the number of base pairs, jeopardizes transfection efficiency [13, 14].

2.2. Minicircle DNA

In order to overcome the previously mentioned disadvantages associated with the use of pDNA in gene therapy, small plasmidic cassettes known as minicircle DNAs (mcDNAs) have been recently developed [15]. Cameron and Scheleff first employed mcDNA terminology in 1995. Nowadays, this technology offers a potential alternative to enhance both transfection efficiency and safety of gene delivery [14]. Basically, mcDNAs are circular constructors similar to pDNA but significantly smaller, since mcDNAs contain a minimal expression cassette, of a promoter, a transgene, and a polyadenylation, signal but are devoid of bacterial pDNA elements. Thus, mcDNA technology allows sustained transgene expression mainly due to a lower activation of nuclear transgene silencing mechanisms and reduced immunogenic responses in vivo [16, 17].

In the lung, some promising results have been obtained with the use of small plasmidic cassettes [18]. In fact, results of a Phase IIb double-blind clinical trial for CF have been recently reported. These trials were performed with a plasmid encoding CFTR gene and lacking CpG

bacterial region, known as pGM169 [8]. In such study, treated patients exhibited modest but significant improvements in lung function compared to placebo-treated ones during 1-year follow-up [19]. In any case, despite the optimism generated, there are still some concerns that need to be considered, such as the reproducibility of the results; the intensity of the response, probably conditioned by the degradation of formulation after aerosolization process; or the number of patients that received such treatment.

2.3. Genome editing tools

Both previously mentioned approaches based on pDNA and mcDNA technologies allow to restore the function of the mutated CFTR gene, with the addition of normal copies, but they do not correct the mutation at their local chromosomal location. However, genome editing tools based on zinc-finger nucleases (ZFNs), or transcription activator-like effector nucleases (TALENs), can specifically correct CFTR gene mutations at their natural chromosomal location, and so, the corrected gene can remain under the control of its endogenous promoter [20].

ZFNs are synthetic restriction enzymes, which have three or more zinc-finger DNA-binding motifs linked to the FokI restriction enzyme that recognizes trinucleotides in a specific DNA sequence [21]. When FokI enzyme creates a double-strand break (DSB) near the mutation place, cellular DNA repair mechanisms are activated to maintain cell viability. In these conditions, a donor DNA sequence with high 5′ and 3′ homology with the DNA sequence where DSB has been generated can be exogenously supplemented to enhance the correction of the mutation by homologous recombination (HR) mechanism. This genome editing tool has been successfully used in vitro to correct CFTRΔF508 mutation in both human bronchial epithelial cells [22] and CF-induced pluripotent stem (IPS) cells [23].

TALEN technology is very similar to ZFNs. These nucleases were originally characterized in *Xanthomonas* bacteria, in which TALEN proteins are secreted when *Xanthomonas* infect a wide variety of plants, thus activating genes that help to develop the pathogenesis. This genome editing tool also produces a DSB around the mutation site of the target gene and consequently induces cellular DNA repair mechanisms [24]. TALENs are considered as a more efficient and cost-effective alternative to ZFNs [25]. In the case of ZFNs, each finger module recognizes three to four bases of the DNA sequence. However, in the case of TALENs, gene recognition is mediated by a more specific mechanism, where each module of 33–35 amino acid targets a single nucleotide. This technology has been recently applied to correct CFTRΔF508 mutations in CF patient-specific IPS cells [26]. Overall, such study reported correction of patient-specific IPS cells in less than 3 months, which could allow rapid scaling up for future applications.

Clustered regularly interspaced short palindromic repeats (CRISPR) methodology, originally described as an adaptive immune response in archaea, follows the same rationale described for ZFNs and TALENs, but instead of protein domains, short RNA molecules are used to drive the required homology [27]. In this case, an endonuclease called Cas9 is guided by a single guide RNA (gRNA) to hybridize specifically with the mutated sequence in the DNA; then, as described for ZFNs and TALENs, the resulting DSB triggers cellular DNA repair mechanism [28]. The main advantage of CRISPR technology is that it is an easy-to-synthesize cost-effective tool that is able to correct more than one mutation at the same time, if multiple-gene

targeted sgRNAs are delivered to target cell along with the Cas9 protein, which makes it an excellent option [24]. CRISPR technology has been applied to repair CFTRΔF508 mutations in intestinal stem cell organoids of CF patients [29].

This study represents an interesting proof of concept for CFTRΔF508 correction by HR using CRISPR/Cas9 technology in primary adult stem cells derived from patients with a single-gene hereditary defect and offers reasonable hope to be successfully applied to the lungs of patients affected by CF. However, some relevant concerns, mainly related to the frequency of undesirable off targets, still need to be resolved in order to reach clinical practice [19].

3. Vectors

One of the main concerns related to the clinical application of gene therapy is the design and development of safe and effective gene delivery vectors to introduce exogenous genetic material into the nucleus of target cells [30, 31]. In the absence of gene delivery vectors, naked genetic material is quickly degraded mainly by exogenous deoxyribonuclease enzymes, which clearly inhibit transfection efficiency [31]. Additionally, the negatively charged genetic material, mainly due to the phosphate groups, hampers the electrostatic interactions with cell membranes, which are negatively charged too. Therefore, the clinical application of gene therapy demands the design, characterization, and evaluation of efficient and safe carriers to mammalian cells.

3.1. Viral vectors

At present, viral-based carriers are the most appropriate from an effectiveness point of view. The natural evolution that viruses have undergone over many years has allowed them to face different intra- and extracellular barriers and, consequently, infect target cells with high efficiency.

In the CF field, a wide variety of viral-based vectors has been developed in clinical trials. The first one was performed in 1993 with adenovirus in three patients, where partial correction of the chloride transport in nasal epithelium was observed [32]. Some of the main advantages of adenoviruses include their non integrating nature and their natural tropism for the lung. However, despite such favorable properties, and the high transduction efficiency observed in most tissues, gene expression usually remains transient, and these viruses can induce strong immune and inflammatory responses in a dose-dependent manner, which clearly brings up safety issues and, therefore, limits their application in the clinical practice [8].

Initial clinical trials performed with adenovirus allowed the development of adeno-associated viruses (AAV), which have interesting characteristics for their application in gene therapy, such as broad tissue tropism, high transduction efficieny, and persistent episomal expression, which can last for years, even though it is a non integrating vector [33, 34]. In addition, recombinant AAV vectors have been shown to be safe in several clinical trials, as they are not related to any known human disease. However, these vectors also present relevant limitations, the main one being their low capacity to load genetic material (<5 kb) [35]. Between

1999 and 2007, six clinical trials were conducted with these kinds of vectors in CF [8]. Phase I clinical trials demonstrated that a single-dose administration of AAV in the respiratory tract of patients affected by CF was safe and well tolerated [36]. Nevertheless, subsequent studies, with repeated doses in more patients, did not report significant improvement in lung function [37]. This lack of efficacy was mainly attributed to the low DNA loading capacity of AAV, which prevented loading the 4.7 kb of the CFTR gene [8]. In addition, AAV capsid-specific immune responses limited repeated administrations in patients [8]. Nowadays, some interesting strategies are being developed in order to minimize adaptive immune responses after repeated administration, such as the design of hybrid AAV capsids or the removal of CpG bacterial regions from AAV vectors [38, 39].

Lentiviruses have an integrative nature and have shown long-term and stable transgene expression when administered in the respiratory tract of mice, which minimizes the need for repeated administration [40]. Additionally, the packaging of full-length CFTR gene and promoters is not limited by size. Therefore, nowadays they are considered promising vectors for the treatment of CF [41]. However, in order to consider its use in clinical practice, some concerns still need to be resolved, such as the scaling in the production of these vectors and the control of the place where the transgene is inserted into the genome of the pulmonary cells, which could increase the tumorigenicity potential of such viral vectors due to random integration [20]. Consequently, such viral vectors could be more suitable for ex vivo than for in vivo therapy. In any case, a promising study in three newborn CF pigs has recently shown that 2 weeks after lentiviral delivery by aerosolization, the anion channel defect can be corrected in a large animal CF model [42]. Other recent studies assessed with pseudotyped lentivirus vectors in both murine lungs and human air-liquid interface cultures showed that preexisting and acquired immune responses do not interfere with vector efficacy [43]. In such study, at least 14% of the airway cells were transduced. Interestingly, toxicological results, notably the integration site profile showing absence of integration near oncogenic loci, support further progression toward clinical trials.

3.2. Nonviral vectors

Although the use of viral-based vectors in clinical trials still predominates over that of nonviral vectors, in recent years, there has been a notable increase in preclinical studies using nonviral vectors [44]. The reason is that these systems represent a safer, cheaper, and easier to produce alternative to viral-based vectors [18]. The main advantages of nonviral vectors include, among others, the ability to produce them on a large scale with high reproducibility and low cost; their relative stability after storage; the possibility of multiple-dose regimen administration due to their low immunogenicity; their high capacity to carry genetic material, independently of the size [45]; as well as the possibility to modify them chemically in order to regulate important physicochemical parameters, such as size, charge, morphology, or polydispersion, which clearly influence their final biological properties. All these important advantages have raised the interest of the scientific community to develop new biocompatible materials of different structures, compositions, sizes, and characteristics to transport therapeutic genes into specific organs or cells, overcoming the different extra- and intracellular barriers [46].

Within the large variety of nonviral vectors developed, most of them are based on peptides as well as on cationic lipids and polymers, which form the corresponding complexes (polyplexes and lipoplexes) after electrostatic binding with DNA [47]. The resulting complexes protect nucleic acids from enzymatic degradation and facilitate cellular uptake by interactions with the cytoplasmic membrane [48]. The PEG-CK30 peptide, due to its low immunogenicity and its ability to be endocytosed by cells, is one of the most widely used, although the formulation must be optimized to allow its administration in aerosol form to reach the lungs [49].

Regarding cationic polymers, polyethylenimine (PEI) is one of the most used, since its chemical structure can be easily modified to increase the efficiency of transfection, for example, by incorporating lactose (Lac-PEI) to improve intracellular trafficking [50]. However, PEI has not yet been used in any clinical trial. The main limitation lies in the difficulty that exists to prepare PEI polyplexes at high DNA concentrations [49]. One of the most promising strategies that have been used to circumvent this problem is the use of ultrafiltration methods, through which PEI/DNA concentrates are prepared.

In the case of cationic lipids, some of the most widely used to develop nonviral formulations in the CF field are N-[1-(2,3-dioleyloxy) propyl]-N, N, N-trimethylammonium, dioleoylphosphatidyl ethanolamine, and dioleoyl trimethyl ammonium [51]. However, currently, the most promising nonviral vector in CF clinical trials is based on the cationic lipid GL6TA, which was synthesized to prevent DNA degradation in the lysosome and to be stable after pulmonary administration by aerosolization [8, 51]. In 2011, a preclinical comparative study of the use of PEI, PEG-CK30, or GL67A nonviral vectors in aerosols demonstrated that this last formulation was the best one to transport DNA to sheep's lungs [7]. Moreover, in an extensive preclinical study performed in 2014 [52], it was corroborated that the formulation based on the lipid GL67A, which had already been used for 15 years in CF clinical trials, was still suitable for administration in multiple-dose regimen, without any observed loss of activity [8].

4. Biological barriers

In order to reach the nucleus of target cells and initiate transgene expression, the genetic material must overcome some extracellular and intracellular barriers, which will be discussed in this section, along with the most relevant strategies that have been developed to make the transfection process more efficient.

4.1. Extracellular barriers

Even though intravenous injection is one of the most commonly used administration routes, especially for delivering genetic cargo into cancerous cells, some barriers still hamper its use in clinical practice, particularly in the CF disease [53]. First of all, the genetic material needs to be protected against extracellular enzymatic digestion, since DNA is quickly degraded when administered alone. To avoid such quick degradation, nonviral vectors based on both positively charged lipids and polymers offer the possibility to condense on their surface the genetic material by electrostatic interactions and minimize such undesirable effect [54].

However, the final positive charge of polyplexes or lipoplexes can interact in a nonspecific way, not only with target cell membranes but also with other negatively charged components such as serum proteins that jeopardize transfection efficiency [55]. These interactions could result in the formation of aggregates that classically are eliminated from the blood by the reticuloendothelial system. Interestingly, the incorporation of polyethylene glycol (PEG) motifs into the formulation of some nonviral carriers enhances the stability of complexes, since the highly hydrophobic nature of PEG chains creates a steric barrier to prevent both aggregation of complexes in blood circulation and extracellular enzymatic degradation by nucleases [55]. In any case, other relevant aspects related with both the length and the degree of PEGylation should be also considered, as they can also decrease DNA condensation efficiency with nanoparticles [56]. In addition to PEG, other polymers with hydrophobic nature such as poly(4-acryloylmorpholine) or poly(N,N-dimethylacrylamide) have recently emerged as interesting and promising alternatives to compensate or ameliorate the negative effects associated with PEGylation [57].

Since intravenous injections present relevant hurdles that hamper the delivery of genetic material into target lung cells, local administration into the lung seems to be a reasonable alternative. In this case, the presence of mucus and the clearance mechanism are the most relevant barriers to overcome [58]. To avoid such barriers associated with pulmonary administration, other interesting noninvasive routes of administration, such as intranasal instillation, can be used to target lung cells. Nevertheless, the main problem is the low amount of genetic material that can be administered by the intranasal route. From a technical point of view, aerosolized nonviral vector/DNA complexes, carefully designed for inhalation in combination with appropriate excipients to enhance both particle flow and aerodynamic diameter, could be an interesting option since they are needle-free systems able to deliver locally high cargo concentrations [53].

To circumvent the diffusion of complexes into lung cells due to unspecific interactions with the biopolymer network of the mucus, some mucolytic agents that hydrolyze mucins can be added [58]. Other strategies include the incorporation of N-acetylcysteine to reduce disulfide bridges between the subunits of mucin, and consequently the viscosity [59], or the functionalization of nonviral vector formulations with mucolytic agents.

4.2. Intracellular barriers

Once extracellular barriers are overcome, there is still a long way full of hurdles before reaching the nucleus of target cells. Firstly, complexes carrying the genetic material need to be endocytosed by target cells. The interaction between complexes and cell membranes can occur in an unspecific way or can be mediated by a specific ligand, which is the preferred one, especially for in vivo applications [60]. Of note, the choice of ligand to be incorporated into the nanoparticle formulation depends not only on the target cell but also on the type of cell entry pathway that will be used once the ligand binds to the desired receptor.

Classically, there are four main pathways of endocytosis: clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CVME), phagocytosis, and macropinocytosis [61, 62]. CME is an energy-dependent mechanism widely studied and characterized [63]. Typically, this pathway is directly associated with lysosomes, where the genetic material needs to leave

such biological compartment quickly before being degraded by the acidic environment and the enzymes found in lysosomes [63]. To avoid this enzymatic degradation, some interesting strategies can be used, such as the formation of pores in the endosome membrane by incorporation of amphiphilic cationic peptides. This creates strong internal tensions in the membrane and enhances the exit of endosome content through such pores [64]. Another strategy is known as the "proton sponge effect," where the low pH within the endosome allows the protonation of trapped compounds that have a large capacity to absorb protons from the medium (buffer effect). Such protonation causes an important entry of ions (H^+ and Cl^-) and water in the endosome, which produce a swelling effect and its rupture. This effect has been observed in some cationic polymers with high buffering capacity over a wide pH range [64]. In CVME, internalized molecules go to the caveosome instead of lysosome [61] avoiding lysosomal degradation; however, there is still ongoing debate, with some authors claiming that CVME can fuse with lysosomes [65].

Phagocytosis is a special type of endocytosis used mainly by macrophages, monocytes, neutrophils, and dendritic cells, although other cell types can also use this cellular entry pathway [61]. Endocytosis mediated by phagocytosis comprises the formation of membrane extensions with certain forms to capture particles generally greater than 1 μm. In contrast, for endocytosis mediated by macropinocytosis, membrane extensions do not surround particles but form some kind of protuberances that finally fuse with the cytoplasmic membrane. In many cases, the physicochemical properties of nonviral vector-based nanoparticles, such as particle size, superficial charge, morphology, or polydispersity, directly influence the endocytosis mechanism and consequently the transfection efficiency.

Once the DNA is released into the cytosol of cells, it must enter the nucleus to produce its effect. This is considered a significant barrier that nonviral vectors must overcome in order to mediate a good transfection efficiency. One commonly used strategy to enhance nuclear import of genetic material is to incorporate a nuclear localization signal (NLS), such as polylysine or protamine [66]. NLS contains some amino acids that interact with some proteins of the cytoplasm known as importines. These importins enhance nuclear entry through the nuclear pore complex of the nuclear membrane through an energy-dependent mechanism [67].

5. Drug formulation

The airways seem to be the natural way to treat respiratory diseases and a good alternative to systemic and more invasive procedures. Currently, aerosolization is the prefered method of administration for airway targeting since it is a noninvasive route that induces little stress to patients. Moreover, high quantities of drug can be deposited directly and fast into the lungs, which circumvents the blood circulation and avoids the first-pass effect of the liver. However, the effectiveness of such approach strongly depends on the development of smart drug formulation strategies. One of the critical steps that need to be taken into account for a successful gene delivery approach by inhalation is the formulation of the drug molecules into an appropriate inhalable form with sufficient stability and adequate aerodynamic properties [68]. Highly susceptible molecules, such as nucleic acid, require special attention when delivered by this route of administration. The physicochemical constraints

such as the hydrodynamic shear forces generated during aerosolization can induce degradation of the nucleic acids, which will be more or less important depending on their size [69, 70]. Therefore, the need to develop a suitable formulation able to protect the material from degradation and at the same time ensure delivery of nucleic acid to the target cells in the lung needs to be deeply considered. In this sense, the commonly accepted aerodynamic size for pulmonary gene delivery is within the range of 1–5 μm. Larger particles (4–7 μm) tend to deposit in the airways, while smaller particles (1–3 μm) and those in submicron range (<1 μm) reach the lower airways and deeper lung [68]. The aerodynamic diameter of a particle can be modified not only by changing its size but also by varying its density or shape, which opens new possible strategies for gene delivery to the lung, such as the design of large porous hollow particles [71].

Suitable formulations for pulmonary delivery are mainly prepared either by dissolving or by suspending the therapeutic molecules in a liquid or formulating them into a dry powder for inhalation using liquid inhalers (including nebulizers), dry powder inhalers (DPIs), or pressurized metered dose inhalers (pMDIs); each of them is suitable for different applications. Once the aerosolized droplets or microparticles are deposited next to the target location into the lungs, they need to dissolve in the lung lining fluid for subsequent absorption and cellular uptake [72]. Nowadays, viral gene delivery to the lungs is limited to liquid formulations using a nebulizer [36], and there is no dry powder or metered dose inhaler formulation available for any vector-drug combination. In most cases, the gene transfer efficiency to lung cells using viral vectors is still too low with traditional nebulizer devices, probably due to the degradation of viral envelope by the shear forces caused during aerosolization [73] and the viscous mucus found in obstructive diseases, like CF [74]. Moreover, and as previously highlighted, the use of adenoviral or AAV vectors would likely induce an acute immune response upon the initial administration or result in low efficacy following repeat dosing. This is particularly relevant since aerosolized gene therapy might require repeat dosing because mucus clearance mechanisms and/or phagocytes may engulf and destroy the drug vector before it can be taken up by target cells [75]. In contrast, the simpler composition of nonviral vectors may have, in this case, an advantage over viral vectors, making readministration potentially more successful.

Although pulmonary gene-based therapies have not yet been granted marketing approval, numerous strategies are being tested both in vitro and in vivo, and various clinical trials are underway [19, 36]. **Table 1** summarizes some of the strategies used to date for the pulmonary delivery of nucleic acids by aerosolization.

Nowadays, the most studied approach for gene delivery to the lung involves the nebulization of the selected formulation [76], turning it from a liquid solution to microdroplets. Depending on the aerosolization system used, such as jet, ultrasonic, or mesh nebulizers, the implemented hydrodynamic stress that the therapeutic molecules would be subject to varies [77]. Interestingly, several strategies have been studied to reduce the damage to the genetic material during the aerosolization process, by condensing the nucleic acids with positively charged molecules, such as polyethylenimine (PEI), protamine, or poly-L-lysine (PLL), among others [78].

Description	Vector	Aerosolization	References
In vivo aerosol delivery of PEI-DNA comp	PEI	Nebulization	[68]
Randomized, double-blind, placebo-controlled, Phase II trial in CF patients with mild lung disease	AAV2 viral	Nebulization	[32]
Nebulization of receptor-targeted nanocomplexes for in vivo gene delivery to the airway epithelium	Receptor-targeting peptides and cationic liposomes	Nebulization	[69]
In vivo repeated aerosol delivery of pDNA/PEI complexes with CpG-free plasmids	PEI	Nebulization or instillation	[70]
In vivo aerosol delivery of DNA/liposomes to the lung	GL67A cationic liposomes	Multiple nebulizers	[71]
Randomized, double-blind, placebo-controlled, Phase IIb trial	GL67A cationic liposomes	Nebulization	[14]
In vivo intratracheal administration of pDNA-chitosan dry powders, obtained by SFD	Chitosan	Dry powder, obtained by SFD, administered by intratracheal syringe	[72]
Dry powder aerosols for in vivo gene delivery to the lung	PEI	Dry powder in insufflator lyophilization/ powderization with lactose, sucrose, or trehalose	[73]

Table 1. Pulmonary gene delivery strategies by aerosolization.

The elaboration of DPIs, composed of drug-based dry powders and an aerosol-generating device, also presents important advantages such as high physicochemical stability, easy handling, and propellant-free aerosols. In order to transform the therapeutic nucleic acids into stable dry powders, several techniques, such as freeze-drying (FD) [79], spray-drying (SD) [80], and spray freeze-drying (SFD) [81], are being investigated. In addition, the incorporation of suitable stabilizing agents/thermal protectors such as polysaccharides (sucrose [79], trehalose [79], agarose [82], lactose [83], mannitol [81], or chitosan [84]), amino acids (leucine [84] or glycine [82]), or proteins (BSA [85]) is critical.

6. Commercialization

In addition to the above concerns, other relevant issues specifically related to the commercialization of gene therapy medicinal products (GTMP) must also be considered. Commercially

available medical products based on gene therapy along with cell therapy and tissue engineering are classified as advanced therapy medicinal products. Although highly promising, their translation into clinical practice is nowadays hampered by major critical issues such as complex regulatory and ethical aspects, along with the intrinsic difficulties to scale up these products to an industrial level [20].

Regarding the regulatory concerns of GTMP that affect clinical applications, the economical investments, along with their manufacture and control, demand more attention than chemically synthesized small molecules [86]. Therefore, a deep analysis of both costs and benefits needs to be done before considering the commercialization of such therapies [87].

Another relevant concern that jeopardizes the clinical use of GTMP in CF is the ethical aspect of clinical trials. Since the early 1990s, more than 25 Phase I gene therapy clinical trials have been conducted. These trials have been carried out largely to assess the safety and feasibility of gene transfer methods and their expression in the host, reporting variable successes for both viral and nonviral approaches. Gene therapy products designed for the treatment of CF must meet certain requirements in order to become a viable therapeutic option. For instance, their clinical efficacy must be demonstrated by analyzing appropriate variables of the lung function such as the patient´s vital capacity that they are able to expire in the first second of forced expiration (FEV1), their age, sex or body composition, and the therapeutic efficacy which must be maintained with repeated administrations. In addition, the GTMP must demonstrate an acceptable profile when it comes to side effects, and other considerations such as treatment of early versus established lung disease must also be analysed.

Since a high percentage of patients affected by CF are children, clinical trials involving these patients must carefully balance the potential benefits of these therapies and the associated risks [88]. Regarding this controversial issue, the Gene Therapy Advisory Committee recommends that clinical trials on children should only be performed under specific circumstances, whereby: (i) it has been demonstrated that the research is necessary to promote the health of the trial population, (ii) the research cannot be done in adults, and (iii) there is a high potential of therapeutic benefit [88]. In fact, owing to a demonstrated benefit of early gene therapy intervention, the age of enrolment of children in clinical trials has progressively reduced over the years from 18 to 12 years old. However, parents should have legal rights to make the final decision on behalf of their children.

Another critical hurdle that strongly compromises the clinical application of gene therapy products for the treatment of CF is the difficulty to scale up formulations that were originally developed for basic clinical research [89]. Most of these products are usually developed by small- and medium-sized enterprises, in collaboration with academic groups, which are usually highly engaged in preclinical activities, but have limited manufacturing experience at industrial level. For instance, the normal procedure for preparing nonviral-based gene therapy products is by simply mixing and pipetting the negatively charged genetic material and the positively charged polymer - or lipid-based nonviral vector formulations, which are often produced in the laboratory at small volumes that usually oscillate between 1 and 5 mL. However, the standardization of this procedure at industrial level to produce high and stable levels of complexes under GMP conditions

represents a great challenge that needs to be overcome for successful clinical application. In this sense, pilot plants, which employ small volumes of the product, represent an interesting option to gain knowledge on the technical process before full scale up production.

7. Conclusion

Despite the fact that the CFTR gene was cloned two decades ago, the current, conventional treatments for CF focus on masking the main symptoms, rather than addressing the underlying genetic cause of the disease. In this sense, gene therapy represents a promising alternative to tackle CF, considering the autosomal recessive nature of the most relevant ΔF508 mutation. Although the main objective of gene therapy seems simple, there are some hurdles that need to be overcome before gene therapy for CF becomes a realistic treatment option. In any case, the increase in knowledge and recent advances in biopharmaceutical technology offer reasonable hope for the treatment of this devastating disease. The minicircle technology, along with the new gene editing tools, offer important advantages compared with classical plasmids used to add functional copies of the gene. Additionally, intense research in novel nonviral vectors functionalized to overcome both extra- and intracellular barriers and the possibility to aerosolize such formulations without losing activity merit special attention.

Acknowledgements

This project was supported by the Basque Country Government (Department of Education, University and Research, predoctoral grant PRE_2016_2_0302 and Consolidated Groups, IT907-16) and by the University of Basque Country UPV/EHU (predoctoral grant PIF17/79). Authors wish to thank the intellectual and technical assistance from the ICTS "NANBIOSIS," more specifically by the Drug Formulation Unit (U10) of the CIBER in Bioengineering, Biomaterials, and Nanomedicine (CIBER-BBN) at the University of the Basque Country (UPV/EHU).

Author details

Myriam Sainz-Ramos[1,2†], Nuseibah AL Qtaish[1,2†], Idoia Gallego[1,2], Ilia Villate-Beitia[1,2], Tania López[1,2], Gustavo Puras[1,2*] and José Luis Pedraz[1,2*]

*Address all correspondence to: joseluis.pedraz@ehu.eus and gustavo.puras@ehu.eus

1 NanoBioCel Group, University of the Basque Country (UPV/EHU), Spain

2 Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria-Gasteiz, Spain

† These authors contributed equally to the work.

References

[1] Wang Y, Wrennall JA, Cai Z, Li H, Mint SDN. Understanding how cystic fibrosis mutations disrupt CFTR function: From single molecules to animal models. The International Journal of Biochemistry & Cell Biology. 2014;**52**:47-57. DOI: 10.1016/j.biocel.2014.04.001

[2] Ronan NJ, Elborn JS, Plant BJ. Current and emerging comorbidities in cystic fibrosis. La Presse Médicale. 2017;**46**(6, Part 2):e138. DOI: 10.1016/j.lpm.2017.05.011

[3] Lane MA, Doe SJ. A new era in the treatment of cystic fibrosis. Clinical Medicine. 2014; **14**(1):76-78. DOI: 10.7861/clinmedicine.14-1-76

[4] Edwards J, Clarke A, Mint GD. Adults with cystic fibrosis—Responding to a new ageing population. Chronic Illness. 2013;**9**(4):312-319. DOI: 10.1177/1742395313479982

[5] Davies JC, Ebdon A, Mint OC. Recent advances in the management of cystic fibrosis. Archives of Disease in Childhood. 2014;**99**(11):1033-1036. DOI: 10.1136/archdischild-2013-304400

[6] Mayrhofer P, Schleef M, Mint JW. Use of minicircle plasmids for gene therapy. Gene Therapy of Cancer. 2009;**542**:87-104. DOI: 10.1007/978-1-59745-561-9_4

[7] McLachlan G, Davidson H, Holder E, Davies LA, Pringle IA, Sumner-Jones SG, et al. Preclinical evaluation of three non-viral gene transfer agents for cystic fibrosis after aerosol delivery to the ovine lung. Gene Therapy. 2011;**18**(10):996. DOI: 10.1038/gt.2011.55

[8] Griesenbach U, Alton E. Moving forward: Cystic fibrosis gene therapy. Human Molecular Genetics. 2013;**22**(R1):R58. DOI: 10.1093/hmg/ddt372

[9] Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. DNA cloning with plasmid vectors. Molecular Cell Biology. 4th edition. New York: W. H. Freeman; 2000. Section 7.1

[10] Thibault T, Degrouard J, Baril P, Pichon C, Midoux P, Mint MJ. Production of DNA minicircles less than 250 base pairs through a novel concentrated DNA circularization assay enabling minicircle design with NF-κB inhibition activity. Nucleic Acids Research. 2017;**45**(5):e26. DOI: 10.1093/nar/gkw1034

[11] Yew NS, Zhao H, Wu I, Song A, Tousignant JD, Przybylska M, et al. Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs. Molecular Therapy. 2000;**1**(3):255-262. DOI: 10.1006/mthe.2000.0036

[12] McLachlan G, Stevenson BJ, Davidson DJ, Porteous DJ. Bacterial DNA is implicated in the inflammatory response to delivery of DNA/DOTAP to mouse lungs. Gene Therapy. 2000;**7**(5):384. DOI: 10.1038/sj.gt.3301097

[13] Gill DR, Pringle IA, Mint HSC. Progress and prospects: The design and production of plasmid vectors. Gene Therapy. 2009;**16**(2):165. DOI: 10.1038/gt.2008.183

[14] Gaspar V, Melo-Diogo D, Costa E, Moreira A, Queiroz J, Pichon C, et al. Minicircle DNA vectors for gene therapy: Advances and applications. Expert Opinion on Biological Therapy. 2015;**15**(3):353-379. DOI: 10.1517/14712598.2015.996544

[15] Kay MA, He C, Mint CZ. A robust system for production of minicircle DNA vectors. Nature Biotechnology. 2010;**28**(12):1287. DOI: 10.1038/nbt.1708

[16] Ahmad-Nejad P, Häcker H, Rutz M, Bauer S, Vabulas R, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. European Journal of Immunology. 2002;**32**(7):1958-1968. DOI: AID-IMMU1958>3.0.CO;2-U

[17] Gracey Maniar LE, Maniar JM, Chen Z, Lu J, Fire AZ, Kay MA. Minicircle DNA vectors achieve sustained expression reflected by active chromatin and transcriptional level. Molecular Therapy. 2013;**21**(1):131-138. DOI: 10.1038/mt.2012.244

[18] Montier T, Delépine P, Pichon C, Férec C, Porteous DJ, Midoux P. Non-viral vectors in cystic fibrosis gene therapy: Progress and challenges. Trends in Biotechnology. 2004;**22**(11):586-592. DOI: 10.1016/j.tibtech.2004.09.009

[19] Alton EWFW, Armstrong DK, Ashby D, Bayfield KJ, Bilton D, Bloomfield EV, et al. Repeated nebulisation of non-viral CFTR gene therapy in patients with cystic fibrosis: A randomised, double-blind, placebo-controlled, phase 2b trial. The Lancet Respiratory Medicine. 2015;**3**(9):684-691. DOI: 10.1016/S2213-2600(15)00245-3

[20] Villate-Beitia I, Zarate J, Puras G, Pedraz JL. Gene delivery to the lungs: Pulmonary gene therapy for cystic fibrosis. Drug Development and Industrial Pharmacy. 2017;**43**(7):1071-1081. DOI: 10.1080/03639045.2017.1298122

[21] Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. Proceedings of the National Academy of Sciences of the United States of America. 1996;**93**(3):1156-1160

[22] Lee CM, Flynn R, Hollywood JA, Scallan MF, Harrison PT. Correction of the ΔF508 mutation in the cystic fibrosis transmembrane conductance regulator gene by zinc-finger nuclease homology-directed repair. Biores Open Access. 2012;**1**(3):99-108. DOI: 10.1089/biores.2012.0218

[23] Crane A, Kramer P, Bui J, Chung W, Li X, Gonzalez-Garay M, et al. Targeted correction and restored function of the CFTR gene in cystic fibrosis induced pluripotent stem cells. Stem Cell Reports. 2015;**4**(4):569-577. DOI: 10.1016/j.stemcr.2015.02.005

[24] Kormann MSD. In vivo gene correction of cystic fibrosis. In: Cystic Fibrosis in the Light of New Research. InTech; 2015. DOI: 10.5772/60697

[25] Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK. FLASH assembly of TALENs for high-throughput genome editing. Nature Biotechnology. 2012;**30**(5):460. DOI: 10.1038/nbt.2170

[26] Camarasa MV, Gálvez VM. Robust method for TALEN-edited correction of pF508del in patient-specific induced pluripotent stem cells. Stem Cell Research & Therapy. 2016;**7**:1-27. DOI: 10.1186/s13287-016-0275-6

[27] Mojica FJM, Montoliu L. On the origin of CRISPR-Cas technology: From prokaryotes to mammals. Trends in Microbiology. 2016;**24**(10):811-820. DOI: 10.1016/j.tim.2016.06.005

[28] Dominguez AA, Lim WA, Qi LS. Beyond editing: Repurposing CRISPR–Cas9 for precision genome regulation and interrogation. Nature Reviews. Molecular Cell Biology. 2016;**17**(1):5-15. DOI: 10.1038/nrm.2015.2

[29] Schwank G, Koo B, Sasselli V, Dekkers J, Heo I, Demircan T, et al. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell Stem Cell. 2013;**13**(6):653-658. DOI: 10.1016/j.stem.2013.11.002

[30] Rolland A. Gene medicines: The end of the beginning? Advanced Drug Delivery Reviews. 2005;**57**(5):669-673. DOI: 10.1016/j.addr.2005.01.002

[31] Aliño SF, Bobadilla M, Crespo J, Lejarreta M. Human α1-antitrypsin gene transfer to in vivo mouse hepatocytes. Human Gene Therapy. 1996;**7**(4):531-536. DOI: 10.1089/hum.1996.7.4-531

[32] Zabner J, Couture LA, Gregory RJ, Graham SM, Smith AE, Welsh MJ. Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. Cell. 1993;**75**(2):207-216. DOI: 10.1016/0092-8674(93)80063-K

[33] Duan D, Sharma P, Yang J, Yue Y, Dudus L, Zhang Y, et al. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. Journal of Virology. 1998; **72**(11):8568-8577

[34] Schnepp BC, Jensen RL, Chen C, Johnson PR, Clark KR. Characterization of adeno-associated virus genomes isolated from human tissues. Journal of Virology. 2005;**79**(23):14793-14803. DOI: 10.1128/JVI.79.23.14793-14803.2005

[35] Chamberlain K, Riyad JM, Weber T. Expressing transgenes that exceed the packaging capacity of adeno-associated virus capsids. Human Gene Therapy Methods. 2016;**27**(1): 1-12. DOI: 10.1089/hgtb.2015.140

[36] Moss RB, Rodman D, Spencer LT, Aitken ML, Zeitlin PL, Waltz D, et al. Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: A multicenter, double-blind, placebo-controlled trial. Chest. 2004;**125**(2):509-521. DOI: 10.1378/chest.125.2.509

[37] Moss RB, Milla C, Colombo J, Accurso F, Zeitlin PL, Clancy JP, et al. Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: A randomized placebo-controlled phase 2B trial. Human Gene Therapy. 2007;**18**(8):726-732. DOI: 10.1089/hum.2007.022

[38] Faust SM, Bell P, Cutler BJ, Ashley SN, Zhu Y, Rabinowitz JE, et al. CpG-depleted adeno-associated virus vectors evade immune detection. Journal of Clinical Investigation. 2013;**123**(7):2994. DOI: 10.1172/JCI68205

[39] Mays LE, Wang L, Tenney R, Bell P, Nam H, Lin J, et al. Mapping the structural determinants responsible for enhanced T cell activation to the immunogenic adeno-associated virus capsid from isolate rhesus 32.33. Journal of Virology. 2013;**87**(17):9473-9485. DOI: 10.1128/JVI.00596-13

[40] Stocker AG, Kremer KL, Koldej R, Miller DS, Anson DS, Parsons DW. Single-dose lentiviral gene transfer for lifetime airway gene expression. The Journal of Gene Medicine. 2009;**11**(10):861-867. DOI: 10.1002/jgm.1368

[41] Griesenbach U, Inoue M, Meng C, Farley R, Chan M, Newman NK, et al. Assessment of F/HN-pseudotyped lentivirus as a clinically relevant vector for lung gene therapy. American Journal of Respiratory and Critical Care Medicine. 2012;**186**(9):846-856. DOI: 10.1164/rccm.201206-1056OC

[42] Cooney AL, Abou Alaiwa MH, Shah VS, Bouzek DC, Stroik MR, Powers LS, et al. Lentiviral-mediated phenotypic correction of cystic fibrosis pigs. JCI Insight. 2016;**1**(14):1-9. DOI: 10.1172/jci.insight.88730

[43] Alton EWFW, Beekman JM, Boyd AC, Brand J, Carlon MS, Connolly MM, et al. Preparation for a first-in-man lentivirus trial in patients with cystic fibrosis. Thorax. 2017; **72**(2):137-147. DOI: 10.1136/thoraxjnl-2016-208406

[44] Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG. Non-viral vectors for gene-based therapy. Nature Reviews Genetics. 2014;**15**(8):541. DOI: 10.1038/nrg3763

[45] Davis ME. Non-viral gene delivery systems. Current Opinion in Biotechnology. 2002; **13**(2):128-131. DOI: 10.1016/S0958-1669(02)00294-X

[46] Keles E, Song Y, Du D, Dong W, Lin Y. Recent progress in nanomaterials for gene delivery applications. Biomaterials Science. 2016;**4**(9):1291-1309. DOI: 10.1039/C6BM00441E

[47] Kinsey BM, Orson CLD, Orson FM. Non-viral gene delivery to the lungs. Current Gene Therapy. 2005;**5**(2):181-194. DOI: 10.2174/1566523053544254

[48] Al-Dosari MS, Gao X. Nonviral gene delivery: Principle, limitations, and recent progress. The AAPS Journal. 2009;**11**(4):1-35. DOI: 10.1208/s12248-009-9143-y

[49] Pringle IA, Hyde SC, Gill DR. Non-viral vectors in cystic fibrosis gene therapy: Recent developments and future prospects. Expert Opinion on Biological Therapy. 2009;**9**(8):991-1003. DOI: 10.1517/14712590903055029

[50] Grosse S, Aron Y, Honoré I, Thévenot G, Danel C, Roche A, et al. Lactosylated polyethylenimine for gene transfer into airway epithelial cells: Role of the sugar moiety in cell delivery and intracellular trafficking of the complexes. The Journal of Gene Medicine. 2004;**6**(3):345-356. DOI: 10.1002/jgm.515

[51] Eastman SJ, Lukason MJ, Tousignant JD, Murray H, Lane MD, George JAS, et al. Mint: A concentrated and stable aerosol formulation of cationic lipid: DNA complexes giving high-level gene expression in mouse lung. Human Gene Therapy. 1997;**8**(6):765-773. DOI: 10.1089/hum.1997.8.6-765

[52] Alton E, Stern M, Farley R, Jaffe A, Chadwick SL, Phillips J, et al. Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: A double-blind placebo-controlled trial. The Lancet. 1999;**353**(9157):947-954. DOI: 10.1016/S0140-6736(98)06532-5

[53] Villate-Beitia I, Puras G, Zarate J, Agirre M, Ojeda E, Pedraz JL. First insights into non-invasive administration routes for non-viral gene therapy. In: Gene Therapy—Principles and Challenges. InTech; 2015. DOI: 10.5772/61060

[54] Agirre M, Zarate J, Puras G, Rojas LA, Alemany R, Pedraz JL. Strategies for improving the systemic delivery of oncolytic adenoviruses and plasmids: Potential application of non-viral carriers. In: Atta-ur-Rahman M, Chouldhary I, editors. Frontiers in Anti-Cancer Drug Discovery. Bentham Science; 2013. pp. 190-225

[55] Parhiz H, Hashemi M, Ramezani M. Non-biological gene carriers designed for overcoming the major extra- and intracellular hurdles in gene delivery, an updated review. Nanomedicine Journal. 2015;**2**(1):1-20. DOI: 10.7508/nmj.2015.01.001

[56] Amoozgar Z, Yeo Y. Recent advances in stealth coating of nanoparticle drug delivery systems. Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology. 2012; **4**(2):219-233. DOI: 10.1002/wnan.1157

[57] Ishihara T, Maeda T, Sakamoto H, Takasaki N, Shigyo M, Ishida T, et al. Evasion of the accelerated blood clearance phenomenon by coating of nanoparticles with various hydrophilic polymers. Biomacromolecules. 2010;**11**(10):2700-2706. DOI: 10.1021/bm100754e

[58] Sanders N, Rudolph C, Braeckmans K, De Smedt SC, Demeester J. Extracellular barriers in respiratory gene therapy. Advanced Drug Delivery Reviews. 2009;**61**(2):115-127. DOI: 10.1016/j.addr.2008.09.011

[59] King M, Rubin BK. Pharmacological approaches to discovery and development of new mucolytic agents. Advanced Drug Delivery Reviews. 2002;**54**(11):1475-1490. DOI: 10.1016/S0169-409X(02)00156-4

[60] Medina-Kauwe LK, Xie J, Hamm-Alvarez S. Intracellular trafficking of nonviral vectors. Gene Therapy. 2005;**12**(24):1734. DOI: 10.1038/sj.gt.3302592

[61] Xiang S, Tong H, Shi Q, Fernandes JC, Jin T, Dai K, et al. Uptake mechanisms of non-viral gene delivery. Journal of Controlled Release. 2012;**158**(3):371-378. DOI: 10.1016/j.jconrel.2011.09.093

[62] Hillaireau H, Couvreur P. Nanocarriers' entry into the cell: Relevance to drug delivery. Cellular and Molecular Life Sciences. 2009;**66**(17):2873-2896. DOI: 10.1007/s00018-009-0053-z

[63] Khalil IA, Kogure K, Akita H, Harashima H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacological Reviews. 2006;**58**(1):32-45. DOI: 10.1124/pr.58.1.8

[64] Varkouhi AK, Scholte M, Storm G, Haisma HJ. Endosomal escape pathways for delivery of biologicals. Journal of Controlled Release. 2011;**151**(3):220-228. DOI: 10.1016/j.jconrel.2010.11.004

[65] Kiss AL, Botos E. Endocytosis via caveolae: Alternative pathway with distinct cellular compartments to avoid lysosomal degradation? Journal of Cellular and Molecular Medicine. 2009;**13**(7):1228-1237. DOI: 10.1111/j.1582-4934.2009.00754.x

[66] Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: The importance of the entry pathway. European Journal of Pharmaceutics and Biopharmaceutics. 2011;**79**(3):495-502. DOI: 10.1016/j.ejpb.2011.06.005

[67] Jans DA, Hubner S. Regulation of protein transport to the nucleus: Central role of phosphorylation. Physiological Reviews. 1996;**76**(3):651-685. DOI: 10.1152/physrev.1996.76.3.651

[68] de Kruijf W, Ehrhardt C. Inhalation delivery of complex drugs—The next steps. Current Opinion in Pharmacology. 2017;**36**:52-57. DOI: 10.1016/j.coph.2017.07.015

[69] Lentz YK, Worden LR, Anchordoquy TJ, Lengsfeld CS. Effect of jet nebulization on DNA: Identifying the dominant degradation mechanism and mitigation methods. Journal of Aerosol Science. 2005;**36**(8):973-990. DOI: 10.1016/j.jaerosci.2004.11.017

[70] Jr DJC, Fogg JM, Ii DES, Gilbert BE, Zechiedrich L. Supercoiled minivector DNA resists shear forces associated with gene therapy delivery. Gene Therapy. 2012;**19**(1):94. DOI: 10.1038/gt.2011.77

[71] Gharse S, Fiegel J. Large porous hollow particles: Lightweight champions of pulmonary drug delivery. Current Pharmaceutical Design. 2016;**22**(17):2463-2469

[72] Patton JS, Brain JD, Davies LA, Fiegel J, Gumbleton M, Kim K, et al. The particle has landed—Characterizing the fate of inhaled pharmaceuticals. Journal of Aerosol Medicine and Pulmonary Drug Delivery. 2010;**23**(S2):71. DOI: 10.1089/jamp.2010.0836

[73] Griesenbach U, McLachlan G, Owaki T, Somerton L, Shu T, Baker A, et al. Validation of recombinant Sendai virus in a non-natural host model. Gene Therapy. 2011;**18**(2):182. DOI: 10.1038/gt.2010.131

[74] Hida K, Lai SK, Suk JS, Won SY, Boyle MP, Hanes J. Common gene therapy viral vectors do not efficiently penetrate sputum from cystic fibrosis patients. PLoS One. 2011;**6**(5):e19919. DOI: 10.1371/journal.pone.0019919

[75] Laube BL. Aerosolized medications for gene and peptide therapy. Respiratory Care. 2015;**60**(6):806-824. DOI: 10.4187/respcare.03554

[76] LGd R, Svolos M, Hartwig B, Windhab N, Young PM, Traini D. Inhaled gene delivery: A formulation and delivery approach. Expert Opinion on Drug Delivery. 2017;**14**(3):319-330. DOI: 10.1080/17425247.2016.1214569

[77] Ari A. Jet, ultrasonic, and mesh nebulizers: An evaluation of nebulizers for better clinical outcomes. Eurasian Journal of Pulmonology. 2014;**16**(1):1-7. DOI: 10.5152/ejp.2014.00087

[78] Dailey LA, Kleemann E, Merdan T, Petersen H, Schmehl T, Gessler T, et al. Modified polyethylenimines as non viral gene delivery systems for aerosol therapy: Effects of nebulization on cellular uptake and transfection efficiency. Journal of Controlled Release. 2004; **100**(3):425-436. DOI: 10.1016/j.jconrel.2004.08.031

[79] Mohammed-Saeid W, Michel D, El-Aneed A, Verrall RE, Low NH, Badea I. Development of lyophilized Gemini surfactant-based gene delivery systems: Influence of lyophilization on the structure, activity and stability of the lipoplexes. Journal of Pharmacy & Pharmaceutical Sciences. 2012;**15**(4):548-567

[80] Seville PC, Kellaway IW, Birchall JC. Preparation of dry powder dispersions for non-viral gene delivery by freeze-drying and spray-drying. The Journal of Gene Medicine. 2002;**4**(4):428-437. DOI: 10.1002/jgm.282

[81] Liang W, Kwok PCL, Chow MYT, Tang P, Mason AJ, Chan H, et al. Formulation of pH responsive peptides as inhalable dry powders for pulmonary delivery of nucleic acids. European Journal of Pharmaceutics and Biopharmaceutics. 2014;**86**(1):64-73. DOI: 10.1016/j.ejpb.2013.05.006

[82] Kuo JS. The effect of protective agents on the stability of plasmid DNA by the process of spray-drying. Journal of Pharmacy and Pharmacology. 2003;**55**(3):301-306. DOI: 10.1211/002235702702

[83] Pfeifer C, Hasenpusch G, Uezguen S, Aneja MK, Reinhardt D, Kirch J, et al. Dry powder aerosols of polyethylenimine (PEI)-based gene vectors mediate efficient gene delivery to the lung. Journal of Controlled Release. 2011;**154**(1):69-76. DOI: 10.1016/j.jconrel.2011.05.006

[84] Mohajel N, Najafabadi AR, Azadmanesh K, Amini M, Vatanara A, Moazeni E, et al. Mint: Drying of a plasmid containing formulation: Chitosan as a protecting agent. Daru. 2012; **20**(1):22. DOI: 10.1186/2008-2231-20-22

[85] Tsukamoto M, Okuda T, Okamoto H, Higuchi Y, Kawakami S, Yamashita F, et al. Bovine serum albumin as a lyoprotectant for preparation of DNA dry powder formulations using the spray-freeze drying method. Biological & Pharmaceutical Bulletin. 2012; **35**(7):1178-1181

[86] Flory E, Reinhardt J. European regulatory tools for advanced therapy medicinal products. Transfusion Medicine and Hemotherapy. 2013;**40**(6):409-412. DOI: 10.1159/000356364

[87] (cat) The Committee for Advanced TherapiesSchneider CK, Salmikangas P, Jilma B, Flamion B, Todorova LR, et al. Mint: Challenges with advanced therapy medicinal products and how to meet them. Nature Reviews Drug Discovery. 2010;**9**(3):195. DOI: 10.1038/nrd3052

[88] Jaffé A, Prasad SA, Larcher V, Hart S. Gene therapy for children with cystic fibrosis—Who has the right to choose? Journal of Medical Ethics. 2006;**32**(6):361-364. DOI: 10.1136/jme.2005.012740

[89] Davies LA, Nunez-Alonso GA, Hebel HL, Scheule RK, Cheng SH, Hyde SC, et al. A novel mixing device for the reproducible generation of nonviral gene therapy formulations. BioTechniques. 2010;**49**(3):666-668. DOI: 10.2144/000113498