Abstract For gene delivery to the lung, the challenges are high, but successful treatment of cystic fibrosis or achieving immunity against the global infectious diseases provide an allure that cannot be ignored. This chapter summarizes and reviews nonviral DNA delivery for both gene therapy and DNA vaccination in the lung. Aerosolization of DNA is evaluated, and the stability during this process is discussed. Carriers for DNA are then discussed including lipoplexes and polyplexes, with a particular focus on systems that achieve good transfection and minimize potential toxicity. Then principles of DNA vaccination are introduced, and the advantages of pulmonary vaccination are discussed. Finally, the transport of plasmid DNA vaccines into the lungs is reviewed.

Keywords DNA delivery • DNA vaccination • Gene therapy • Non-viral gene delivery • Pulmonary route of application

16.1 Introduction

As such, the concept of gene delivery to the lung for therapeutic as well as preventive purposes appears to be straightforward: relatively easy application meeting with patient compliance, direct accessibility of the target tissue, low enzymatic activity (compared to the oral route of application) and absence of pH gradient. However, as with all “simple” concepts, the devil is in the details. In this case, as somebody put it correctly in the three challenges afflicting all efforts in drug development: Delivery, Delivery, and Delivery. The first delivery aspect is to deposit an aerosol DNA bearing particles at the correct site in the lung, maintaining DNA
integrity in lieu of shear forces necessary to produce aerosol droplet sizes suitable for inhalation. The second is to enable the uptake of DNA carrier systems by, if possible, the “correct” target cells. The last aspect is related to obtaining successful transfection, i.e., the successful expression and processing of the protein encoded in the DNA delivered. For all three of these stages, technologies have been developed over several decades. In this chapter, we would like to focus on supplying the reader with an overview over some aspects of – nonviral – DNA delivery for both gene therapy and DNA vaccination in the lung.

16.2 Aerosolization of pDNA

The first report of pulmonary delivery of aerosolized DNA, complexed with cationic liposomes, was described by Stribling et al. [63]. Using DOTMA (N-[1-(2,3)-dioleyl-oxy)propyl]-N,N,N-triethylammonium), in combination with the fusigenic lipid DOPE (dioleoyl-phosphatidylethanolamine) to form complexes with a pDNA encoding for chloramphenicol acetyl transferase (CAT), successful transfection of pulmonary tissues in mice was achieved in the absence of toxic side effects. In this case, the aerosolization was achieved by nebulization in what turned out to be a rather inefficient process. Application of aerosol had to be maintained for a total duration of 4–5 h, aerosolizing 6 mg of total DNA, of which only about 10 mg was finally deposited in the lung. However, this study sparked the interest in aerosol delivery of pDNA for a number of genetic diseases, including cystic fibrosis (CF) [2, 50], lung cancer [27], and inflammatory afflictions of the lung, such as asthma [2].

From these first trials, it became also clear that the nebulization of a large and relatively fragile molecule poses a challenge. Especially seen the forces necessary to produce droplet sizes suitable for inhalation (about 2 μm), shear stress occurring during nebulization is to be minimized in order to maintain structural integrity of the DNA delivered [15]. A recent paper by Lentz et al. [41] investigated the effect of the strain rate on the integrity of plasmid DNA (pDNA) aerosolized from different delivery systems (ultrasonic, vibrating mesh and jet nebulizer, electrostatic spray). The strain rate γ is defined as the derivative of velocity (du) in correlation to linear distance traveled (dy) [56]:

\[ \gamma = \frac{du}{dy}. \]

The study revealed the lowest destabilizing effect for pDNA aerosolized from electrostatic sprays, and the highest for jet nebulizers. In spite of a relatively low strain rate, also vibrating mesh nebulizers exerted a destabilizing effect on plasmid DNA, possibly due to the interaction of the molecule with the vibrating grid [41]. As was already shown, DNA degradation in ultrasonic nebulizers was due to cavitation, i.e., the collapse of air bubbles creating shock waves, which can damage the DNA [42]. In contrast to aerosolized “naked” DNA, complexation with positively
charged molecular entities, condensing and compacting DNA, has been shown to lead to a stabilization of the DNA delivered, besides resulting in a potential increase in transfection efficiency observed [57].

16.3 Lipoplexes and Polyplexes

In nonviral delivery, DNA is usually condensed by electrostatic interaction with either cationic lipids to form so-called lipoplexes or cationic polymers (polyplexes). Examples for the former, in addition to the above mentioned DOTMA/DOPE liposomes, are 3-[N-[N′,N′-dimethylamino)ethane]carbamoyl]cholesterol (DC-cholesterol), used for the application of a plasmid expressing for the cystic fibrosis transmembrane conductance regulator (CFTR) protein in mice [1]. Since then, a number of lipoplex formulations have been developed and successfully tested in animals [26, 49].

In clinical trials, however, lipoplex-mediated transfection of pulmonary tissues has suffered from comparably low efficiency [6], as well as proinflammatory side effects [58]. The recent use of an improved lipid, the Genzyme lipid 67 (GL-67), called the “gold-standard” [25] in pulmonary gene delivery, has resulted in an increased transient transfection and expression of a reporter gene after instillation in mouse lungs [40]. The lipid, consisting of a spermine headgroup covalently attached to cholesterol “anchor,” used in a liposomal formulation including dioleoylphosphatidyl ethanolamine (DOPE), dimyristoylphosphatidyl ethanolamine (DMPE), and polyethylene glycol (PEG) has also been tested in clinical studies. In CF patients, treatment resulted in significant, though partial, restoration of the chloride channel activity disturbed in CF by the expression of the CFTR protein [2, 3].

Another cationic lipid, guanidinium-cholesterol-bis-guanidinium-tren-cholesterol (BGTC), showed relatively high transfection efficiency in vitro and in vivo when used in combination with DOPE [16]. Applied as an aerosol to mice, expression of the reporter gene CAT was however lower than for polyplexes prepared with polyethyleneimine (PEI), applied by the same route [22]. In addition, cytokine (TNF-alpha, IL-1 beta) levels measured in the bronchoalveolar fluid (BALF) after exposure were higher for BGTC-based systems than for the PEI polyplexes.

Inflammatory side effects in lipoplex-mediated gene delivery in the lung have not been attributed to the cationic lipids themselves, but more to the presence of (and exposure to) CpG sequences absent in mammalian, but present in bacterial plasmid DNA. These sequences have been identified as ligands of the Toll-like receptor (TLR) 9 [62]. TLR belongs to a group of pathogenic pattern recognition receptors that are a part of the innate immune system [30]. TLR ligands have therefore recently been introduced as novel adjuvants to boost the mucosal immune response following pulmonary application of vaccines (see below).

Polymers have long been used for condensing DNA, forming condensates (“polyplexes”) held together by electrostatic forces between the negatively charged DNA and positively charged polymers [36]. This condensation both reduces the size of the DNA delivered, and masks its negative charge, both factors making DNA amenable
for endocytosis by target cells. Cationic polymers, especially PEI, may also assist in the “endosomal escape,” i.e., the release of intact plasmid DNA from the endosomal compartment [12], as depicted in Fig. 16.1. In this case, the polymer acts as a “proton sponge,” by virtue of protonation of its multiple amino groups. This, in turn, buffers the pH in the endosomal compartment, causing the endosomal membrane to rupture and release the polyplex into the cytoplasm [53]. In addition, condensation with polymers was shown to stabilize DNA against enzymatic degradation [13].

Examples of widely used polymers in nonviral gene delivery are poly(l-lysine) (PLL) [39, 43], PEI [12], poly(2-(dimethylamino)ethylmethacrylate) (pDMAEMA) [67], as well as biodegradable polysaccharides such as chitosan and its derivatives [11, 48]. Among these, PEI, PLL, and chitosan (Fig. 16.2) and respective derivatives have been shown to successfully transfect lung epithelial cells in vitro and in vivo.
PEI shows a high density of primary, secondary, and tertiary amine function in its structure (Fig. 16.2), and is therefore suited to condense DNA plasmids and act as a proton sponge for lysosomal escape and facilitated intracellular trafficking of the vector. PEI has therefore been considered as a nonviral delivery system in gene therapy of CF [20], a genetic disease caused by the aberration of a chloride channel protein, the CFTR protein, expressed in mucosal epithelia in the intestinal tract and the lung [46]. As the cause of fatality is linked to the pulmonary effects of CF, gene therapy through application of the correcting gene by aerosolization was attempted early on. In this regard, PEI was shown to protect DNA against degradation during the aerosolization process [17], to lead to a higher transfection rates compared to most cationic lipids [16], with transfection using PEI polyplexes not inhibited by lung surfactant [19]. However, PEI is significantly cytotoxic, which appears to depend on the density of the positive charges as well as on the molecular weight of the polymer [33], and the degree of molecular branching [70]. Cytotoxicity of PEI and aggregation tendency of PEI/DNA complexes could greatly be reduced by grafting PEI with PEG, with reduction in cytotoxicity being dependent on the degree of PEG grafting, and not on the molecular weight of PEG [52, 55].

In a recent study [65], branched PEI was compared to a newly developed copolymer from oligo(ethylene glycol) methyl ether methacrylate (OEGMA) and poly N,N-dimethylaminoethylmethacrylate (pDMAEMA) in terms of toxicity and transfection efficiency after intratracheal application in Balb/c mice. The copolymer could prevent polyplex aggregation at high concentrations in isotonic solutions, rendering formulations useful for in vivo application. Gene expression in vivo apparently improved by sevenfold when compared to branched PEI.

Condensation of plasmid DNA by poly-L-lysine (PLL) appears to be dependent on the interaction of the primary amine groups of the polymer with negatively charged DNA. For the formation of stable polyplexes, the number of amine groups available appears to be of crucial importance. Polyplexes formed with PLL below a molecular weight of 3 kDa were shown to be unstable [38]. On the other hand, polyplexes prepared with high molecular weight PLL tended to form aggregates in isotonic solutions [43] and exerted considerable cytotoxicity [14]. To overcome these challenges, PLL was grafted with PEG, resulting in an A–B-type block copolymer [71]. Polyplexes formed with these copolymers maintained their stability in serum, and can be applied in suspensions at a concentration exceeding 12 mg/ml DNA in isotonic saline [44]. Successively, PEG-PLL polymers were employed in preclinical studies in mice, transfecting 60–75% of epithelial cells lining the bronchial tract after a single intratracheal application [75]. Based on these favorable data, a first clinical trial was initiated [35], testing the application of a plasmid expressing for the CFTR protein and condensed with PEGylated PLL (CK30) in a total of 12 CF patients in a placebo-controlled, double-blind, dose-escalation intranasal study. Primary endpoints – safety and tolerability – were met, and the secondary endpoint – gene transfer efficiency – was assessed as well. A partial to complete response was seen in eight subjects, and correction persisted for 6 days, for 1 patient even up to 4 weeks after single nasal application of the polyplex formulation.
Chitosan is a linear, cationic polysaccharide consisting of randomly distributed β-(1-4)-linked d-glucosamine (deacetylated unit) and N-acetyl-d-glucosamine (acetylated unit). It is industrially produced by alkaline deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans [61]. The degree of deacetylation of chitosan ranges from 40 to 98%. Different molecular weights (range of >100,000 to <2,000 Da) and viscosity grades are on the market. The primary amino function affords the possibility of easily altering the chemical modification of chitosan. The pK_a value of this amino group was determined to be around 6.5 and does not vary significantly, even for different degrees of N-acetylation. Hence, chitosan is positively charged and soluble in acidic solutions, whereas unmodified chitosan is insoluble at physiological pH values (7.2–7.4). Chitosan has been considered to serve as a vector for gene delivery [60] due to biocompatibility and biodegradability [5]. A study dedicated to unravel the relationship between the structure of chitosan, i.e., its molecular weight and degree of deacetylation, its toxicity in vitro, and transfection efficiency after intratracheal administration in mice was performed recently [34]. It was shown that the percentage of deacetylation must be greater than 65% to achieve stable complexes with plasmid DNA, and achieve transgene expression. In terms of acute toxicity, measured by incubation with 293 cells, an IC50 value of 630 mg/ml was measured for ultrapure chitosan (UPC) of a deacetylation degree of 83%, in comparison to an IC50 value of just 75 µg/ml for PEI of a molecular weight of 800 kDa [24]. After intratracheal instillation, polyplexes were mostly found in the airways of the central lung. PEI transfection was seen to be more effective and longer lasting than with UPC, which itself was comparable to cationic lipids. It was considered to be safe for pulmonary application, provided chitosan is used in its ultrapure form [31].

16.4 Principles of Plasmid DNA Vaccination

With an increasing knowledge of the immune system and its molecular answers to infections, it becomes more and more obvious that antibody inducing vaccines might not be the appropriate solution to intracellular infections (such as tuberculosis, influenza, hepatitis, or HIV/AIDS) requiring for their prevention a potent cytotoxic T lymphocyte response (CTL). Plasmid deoxyribonucleic acid (DNA) vaccination might be an answer to that due to its hallmark of inducing a strong CTL response in orchestration with CD4+ T helper cells (cellular immunity) as well as its generation of antibodies (humoral immunity).

In general, a plasmid DNA vaccine consists of a bacterial plasmid vector into which a gene is inserted encoding for one or more antigenic protein(s). When compared to gene therapy, plasmid DNA vaccination is supposed to be already efficient at a relatively low level of gene expression. Plasmid DNA vaccines are produced in bacteria (such as Escherichia coli) and after purification injected into the host [29].

Wolff et al. [72] reported for the first time that an injection of plasmid DNA (encoding for the bacterial enzyme beta-galactosidase) into muscle cells can result
in lasting protein expressions, even after 2 months of injection. This technique of in situ expression of protein(s) was then applied for eliciting an antibody response by Tang et al. [64]. They were the first to demonstrate that an injection of plasmid DNA by gene gun is able to elicit an immune response in vivo against the delivered human growth hormone. Further studies in mice and chicken injected with influenza plasmid DNA demonstrated protection against following viral challenge [21, 66]. Starting from these days, several plasmid DNA vaccines were found to provide protective immunity in various animal models [37]. In comparison to current protein vaccines, plasmid DNA vaccination exhibits many advantages [9, 37], such as:

1. Favoring a cellular immune response, which is most desired for the prophylaxis against intracellular pathogens. This cell-mediated immunity is developed thanks to an induction of major histocompatibility complex (MHC) 1-restricted CTLs, also killer T cells termed. CTLs are a result of in situ DNA transfection, subsequent intracellular processing of polypeptides, followed by a presentation of the corresponding epitope(s) to antigen-presenting cells (APC).

2. Allowing the genetic construction of multiple antigens of choice included into the same vector, which in turn leads to transcription of different antigenic proteins (epitopes) in situ by one single vaccination.

3. Possessing an intrinsic adjuvant, unmethylated 5’-deoxycytidine-phosphate-guanosine (CpG)-motifs, which are stimulating the innate immune system via the TLR-9. Activation of TLR-9 leads to a favorable T helper cell type 1 (Th1) biased immune response with induction of proinflammatory cytokines.

4. Prolonging the expression of the antigenic protein(s) enables a continuous stimulation of the immune response.

5. Being easily produced, up-scaled and stored at higher temperatures without causing loss of activity. These properties render DNA vaccines economically very attractive and are certainly an advantage for their transport, especially to countries in the southern hemisphere.

6. DNA vaccination is considered as safe immunization for immunocompromised hosts as it does not induce vector immunity (in contrast to viral or bacterial delivery systems) and therefore can be used for repeated boosting.

However, in order to successfully transfect host cells, plasmid DNAs have in general to overcome a couple of extra and intracellular barriers before desired antigen(s) can be expressed (see Fig. 16.3). In addition to these barriers, potential safety issues raised [9, 37] and have to be addressed at preclinical and clinical level:

- Integration of plasmid DNA into the host genome
- Immunological tolerance to the encoded antigen
- Development of anti-DNA antibodies or autoimmunity disorders against DNA
- Development of antibiotic resistance

Despite promising results of plasmid DNA immunizations in preclinical trials [28, 37, 66], studies in nonhuman primates and humans have failed so far in achieving protective immunity [32, 68, 69]. Consequently, amelioration strategies
of DNA vaccination were exploited, ranging from plasmid optimization over coformulation with adjuvants to changing the route of administration to, e.g., pulmonary vaccination.

### 16.4.1 Advantages of Pulmonary Vaccination

Most known pathogens invade the human body through mucosal epithelia (such as nasal, oral, and pulmonary). Hereby, airborne bacterial and viral infections in the lung tract (e.g., influenza, measles, and tuberculosis) are major reason for high rate of deaths per annum [74]. In fighting against such lung infections, pulmonary delivery of vaccines mimics the natural way of infection and might therefore be an appropriate way for their prevention. In animal models, aerosol delivery involves intratracheal instillation and insufflation or the use of exposure chambers, whereas for clinical trials a delivery device is required. Microsized particles (1–5-μm in size) are generated by dry-powder inhalers and aerosols from liquid-suspended particles by nebulizers, which are then delivered into the respiratory tract [47]. In history, aerosol vaccination was applied in human subjects for more than a century and includes aerosol vaccines against anthrax, plague, tularemia, smallpox, tetanus, and
botulism [23]. When compared to common parenteral immunizations, the following benefits of aerosolized vaccines are matter of discussions:

1. Delivery of vaccines into the respiratory tract can trigger the secretion of local IgA antibodies, which are in turn capable of crossing epithelia and preventing further entrance of pathogens.
2. The particular noninvasive nature of antigen delivery into the lungs circumvents the common use of needles and syringes, which are the main cause for unsafe injections (e.g., needle-stick injuries). According to WHO sources [51], those unsafe injections cause 8.2 million cases hepatitis B, 2.3 million cases of hepatitis C, and 0.1 million cases of HIV/AIDS around the world.
3. The application of pulmonary dry-powder vaccines could stop the common imperative of an intact cold chain for storage, what is mandatory for conventional vaccines.
4. For the administration of vaccines by using inhalers, no specially trained medical personnel will be required.

In addition, two independent studies in infants underline the potential of aerosol vaccination and will be highlighted in more details. First, 4,327 schoolchildren (age 5–14) in South Africa received a measles vaccines (either Schwarz or Edmonston–Zagreb (EZ) vaccine) by aerosol or by subcutaneous (s.c.) administration. After 1 month of treatment, aerosol vaccination with the EZ strain caused seroconversion of 326 (84.7%) schoolchildren, which was superior to 257 (78.8%) who received the subcutaneous EZ vaccine or 176 (62.2%) who received subcutaneous Schwarz vaccine. The authors stated that this method of aerosol vaccination might also be suited for mass campaigns in pediatric populations [18].

In a second study, a measles vaccine (EZ) or measles-rubella (Edmonston–Zagreb with RA27/3) was administered to Mexican schoolchildren via inhalation or injection. Interestingly, titers of neutralizing antibodies for the aerosol group were around fourfold greater (52–64%) in comparison to the group, which received the injected vaccine (4–23%). Moreover, fewer side effects were observed after aerosol than injection administration of vaccines [7]. Besides, Wong-Chew et al. [73] reported that a measles aerosol vaccine in 9-month-old Mexican infants stimulated strong cellular immunity as measured by the Th1 cytokine interferon-gamma (IFN-γ).

Considering reported findings, pulmonary delivery of antigens holds certainly promise as an immunogenic and safe way of vaccination for the prevention of airborne pathogens. Moreover, it is an exceedingly attractive approach for developing countries in the south, where not always cold chain, correct syringe disposal, and trained personal can be guaranteed.

16.4.2 Transport of Plasmid DNA Vaccines into the Lungs

Pulmonary plasmid DNA vaccination is a rather new and promising concept of vaccination and might enable in the future immunizations against intracellular pulmonary pathogens, such as Mycobacterium tuberculosis, respiratory syncytial
virus (RSV), and severe acute respiratory syndrome coronavirus (SARS) [9]. Until now, most in vivo studies focused mainly on gene therapy to the lung [4] and expectations raised that the most efficient and safe pulmonary gene delivery systems will find their application also for transport of plasmid DNA vaccines.

Regarding the delivery of plasmid DNA into the lung, in addition to common intracellular barriers for gene therapy (see Fig. 16.1), further extracellular obstacles have to be anticipated and overcome, e.g., withstanding shear forces during aerosolization and crossing the respiratory mucus layer, which is covering conducting airways or the liquid layer in the alveoli [59].

So far, pulmonary delivery of plasmid DNA vaccines was only reported for a very few antigens (see Table 16.1) by different research groups and will be discussed in the following.

Lombry et al. [45] studied the immunogenicity of two protein antigens, ovalbumin (OVA) and hepatitis B surface antigen (HBsAg) and their encoding plasmid DNAs, pOVA and pHBsAg, after intratracheal instillation or injection into mice. Pulmonary immunizations induced equipotent cellular and humoral immune responses when related to injected vaccines. Interestingly, antigen and plasmid DNA immunizations favored a Th2 and a Th1 response, respectively.

Moreover, Bivas-Benita et al. [8] encapsulated a plasmid DNA vaccine encoding eight T-cell epitopes of *M. tuberculosis* into chitosan nanoparticles and applied them intratracheally into HLA-A2 transgenic mice. DNA nanoparticles elicited maturation of dendritic cells (DC) and stimulated an increased secretion of IFN-γ cytokines in comparison to pulmonary delivery of the plasmid DNA in solution or via intramuscular immunization.

The same group evaluated the in vitro and in vivo immunogenicity of a plasmid DNA vaccine encoding for the latency antigen Rv1733c of *M. tuberculosis* [10]. pRv1733c was formulated in poly(d,l-lactide-co-glycolide) (PLGA)-PEI nanoparticles. DNA nanoparticles matured human DCs and induced secretion of two Th1 cytokines, TNF-α and IL-12, to a similar extent as the positive control lipopolysaccharide (LPS). Priming of mice with pRv1733c nanoparticles and boosting after 3 weeks with Rv1733c protein enabled superior levels of T cell proliferation as intramuscular immunization controls. In addition, the same trend was observed for IFN-γ secretions, where aerosol delivery of nanoparticulates in conjunction with the protein boost triggered the highest release of IFN-γ.

**Table 16.1** Overview of pulmonary DNA vaccination studies

| Encoded protein               | Delivery route            | Delivery system | References |
|-------------------------------|---------------------------|-----------------|------------|
| Hepatitis B surface antigen   | Intratracheal (instillation) | None            | [45]       |
| Eight epitopes of *M. tuberculosis* | Intratracheal (microsprayer) | Chitosan NP | [8]        |
| Hemagglutinin                 | Aerosol (nebulizer)       | PEI NP         | [54]       |
| Rv1733c                      | Intratracheal (microsprayer) | PGLA-PEI NP   | [10]       |

*PLGA* poly(d,l-lactide-co-glycolide); *PEI* polyethylenimine; *NP* nanoparticulates; *RSV* respiratory syncytial virus
So far, the only study of pulmonary DNA vaccination demonstrating next to immunogenicity also protective efficacy was reported by Orson et al. [54]. An influenza antigen, hemagglutinin (HA; from viral strain A/PR8/34), expressing plasmid DNA (pHA) was incorporated in PEI particles and aerosolized into mice. When compared to intravenous delivery of the same HA plasmid in macroaggregated albumin (MAA)-PEI particles, less virus neutralizing antibodies were found after 2 weeks postimmunization. However, when plasmids encoding the cytokines IL-12 and granulocyte-macrophage colony stimulating factor (GM-CSF) were co-aerosolized in pHA PEI particles, a significant increase in neutralizing titer was remarked together with protection against subsequent influenza challenge.

16.5 Conclusions

Fallbacks – sometimes fatal – have sent gene therapy from the bedside back to the bench. In this scenario, the application of nonviral gene delivery systems appears as an advantageous alternative to viral carriers. However, the dilemma remains that non-viral delivery systems are either toxic and therefore not safe for human use, or much less efficient in transfecting target cells than their viral counterparts. The same holds true for genetic vaccines: while quite successful in animal studies, DNA vaccine trials in human subjects fail to reach their endpoints of establishing protective immunity.

On top of this, the administration site of the lung is a difficult one. Though relatively accessible by inhalation, toxicity and thus safety concerns play a major role when designing clinical trials for pulmonary vaccine delivery, although safety for measles vaccines applied by inhalation was shown. The introduction of novel excipients, such as ultrapure chitosan or modified PEI, will prove difficult in this context. However, research must continue to develop novel gene therapy and DNA vaccine carrier systems, especially for the lung. The challenges are high, but successful gene therapy of CF and achieving immunity against the global killer tuberculosis are too big benefits to be missed.

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