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Strategies for delivery of non-viral plasmid DNA based gene therapy

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Abstract (for electronic indexing)

The delivery of a therapeutic gene inserted in a vector for somatic gene therapy holds great promise for the future. Encouraging results from the clinical trials of first-generation plasmid-based medicines/ vaccines have further validated the importance of molecular biology and biotechnology research. With newer developments in bioengineering for the mass production and purification of plasmid DNA, gene therapy using non-viral vectors offers opportunities for multiple therapeutic benefits in the healthcare industry. However, low therapeutic success rates due to inefficient delivery systems have blunted the success of gene therapy. This paper examines the status of plasmid DNA in the gene therapy clinical trials, challenges and advances in plasmid DNA based non-viral gene delivery through the different routes of administration, and focuses on the pulmonary route for aerosol gene delivery.

Keywords: plasmid DNA, gene therapy, formulation, gene delivery, pulmonary, aerosol, cystic fibrosis

INTRODUCTION

The advent of genetic engineering has resulted in an unprecedented elucidation of genetic data from genomic sequencing and gene chip analysis. Developments in bioprocess engineering have helped identify and mass produce therapeutic genes, leading to the development of new gene-engineered therapies. Application of human gene therapy has been the hope for the new therapeutic approach [1]. Somatic gene therapy refers to gene transfer targeted at the genetic material of tissues (muscles, lung, brain, bones, kidney, heart etc...) only, i.e., it does not contribute to hereditary transmission. In contrast, 'germ line' gene therapy refers to gene transfer to germ cells for the modification of the genome for transmission to subsequent generations. The objective of somatic gene therapy is to deliver functional groups of nucleic acids to target cells. The subsequent alteration in production of a specific protein or changes in protein expression results in a therapeutic benefit.

Over 4000 human diseases are essentially disorders of genes caused by inborn alterations in a single gene. While common therapeutic drugs often treat symptoms, gene therapy focuses on gene transfer for treating or eliminating the cause of a disease [2]. Gene-based medical interventions will be of critical importance in creating vaccines and antiviral therapies for HIV, hepatitis, herpes and other viral illnesses, as well as for developing new strategies for the prevention and treatment of emerging diseases [3]. The field of gene therapy has constantly evolved since its inception, moving from *ex vivo* to direct *in vivo* gene-based medicine [4]. Although newer delivery techniques are being developed, choosing the right approach for administration of gene medicine to the targeted cells still remains an issue and poses a significant constraint to the success of gene therapy.

The purpose of this paper is to review the different strategies for the delivery of plasmid DNA-based gene therapy. Plasmids are extra-chromosomal DNA capable of being transmitted from cell to cell. They are super-coiled, circular covalently closed (ccc) strands of

DNA ranging from 5 kb to 400 kb depending on the size of the therapeutic gene insert and replicate independently of the host DNA. The structure of the paper is as follows:

- (i) In the first part of the paper we outline, for the benefit of readers who may be less familiar with the subject, the basics of gene therapy and its status in the clinical trials, as well as the emergence of non-viral gene therapeutics based on plasmid DNA. We consider the steps involved in the production of plasmid DNA, and the physiological and biological barriers to non-viral gene delivery in the cells.
- (ii) The second part of the paper reviews strategies for the delivery of plasmid DNA by means of the pulmonary route. Aerosol gene delivery is the most obvious method for the treatment of chronic respiratory diseases, such as cystic fibrosis, lung cancer and influenza. Moreover, the lungs also provide a large tissue surface area with access to systemic circulation for the delivery of non-viral plasmid DNA formulations to tackle a wider range of conditions.

GENE THERAPY

Gene therapy essentially involves treatment of a disease by delivering therapeutic genes, in the form of viral or non-viral vectors to target cells in order to repair malfunctioning or missing genes. The viruses mainly used for viral gene transfer include retrovirus, adenovirus and adeno-associated virus as viral vectors. The main non-viral vectors used for gene therapy include plasmid/naked DNA and siRNA (short-interfering RNA). A comparison of the advantages and disadvantages of non-viral and viral vectors is given in **Table 1**. Viral vectors have higher gene transfection (introduction of therapeutic DNA into target cells) efficiency than non-viral vectors, so to-date most gene therapy experiments and clinical trials have used viral vectors for gene delivery. However, the disadvantages of viral vectors concerning insert-size limitation, safety for repeated administration (due to immunogenicity)

and problems with large-scale manufacture, have led to an increased focus on the use of non-viral vectors for gene therapy [5,6].

Status of gene therapy

The first application of gene therapy occurred with the transfer of naked DNA leading to the expression of the transgene in 1991 [7]. As of March 2008, a comparison of the vectors used in gene therapy clinical trials in 2005 and 2008 are shown in **Fig. 1** [8]. There is an increase in gene therapy clinical trials using naked/plasmid DNA compared to the other main vectors. Although the number of gene therapy clinical trials initiated/approved has declined from 2001 to 2007, plasmid DNA still represents more than one-third of the total number of main gene therapy vectors used in trials (**Fig. 2**). As of March 2008, 1379 gene therapy clinical trials were being carried out worldwide, with the USA and UK accounting for more than three-quarters of these trials [8]. With the results of the different phases of clinical trials expected soon (**Fig. 3**), research efforts are focused on achieving maximum productivity, high purity, best formulation and efficient delivery of plasmid DNA for gene therapy [9-16].

Non-viral gene therapeutics

Non-viral gene therapeutics have the potential to provide nucleic acid-based drugs that could be more effective than traditional pharmaceuticals. This overcomes the limitations associated with the direct administration of therapeutic proteins, which include low bioavailability, systemic toxicity, *in vivo* stability, high hepatic and renal clearance rates, and the high cost of manufacturing [17].

The simplest non-viral gene therapeutic system uses ‘naked’ plasmid DNA. Since plasmid DNA is susceptible to rapid degradation upon administration, protection from that degradation could be expected to lead to higher levels of gene expression. Plasmid DNA,

being anionic in nature, readily forms a complex with cationic substrates such as cationic lipids and cationic polymers to form lipoplexes and polyplexes respectively, or with both cationic lipids and polymers to form lipopolyplexes [18]. Another non-viral plasmid DNA approach employs a peptide nucleic acid (PNA) clamp to directly and irreversibly modify plasmid DNA, without affecting either its supercoiled conformation or its ability to be efficiently transcribed. This strategy helps to “functionalize” the gene by direct coupling of ligands (fluorophores, peptide, proteins, sugars or oligonucleotides) to plasmid DNA. The usefulness of this technique is that it provides versatile tools for specific targeting and efficient delivery, thereby overcoming the obstacles of synthetic non-viral gene delivery systems [19].

An alternative to the plasmid DNA-based non-viral system, RNA interference (RNAi) is gaining recognition as a powerful tool in gene therapy for post-transcriptional gene silencing [20,21]. With RNAi, small sequence specific, double-stranded, short interfering RNA (siRNA) molecules bind to a complementary portion of mRNA and either prevent it from being translated or trigger its destruction. The specific gene-targeting technology of RNAi to shut down the expression of a disease causing gene has added to the benefits of gene therapy [22]. Plasmid DNA-based non-viral gene therapeutic is the main focus of this review and is discussed in the next section.

Plasmid DNA based non-viral gene therapeutic

The use of plasmid DNA as a non-viral vector for gene therapy has shown promise in the development of new therapeutics [23]. The essential components of a non-viral plasmid DNA based gene therapeutic approach include (1) a therapeutic gene for a particular disease; (2) a gene expression plasmid controlling the function of the therapeutic gene within the

target cell; and (3) a gene delivery system controlling the delivery of gene expression plasmid to specific locations within the body.

1) Therapeutic gene

Over 240 clinical trials have been initiated using naked/plasmid DNA in the gene therapy clinical trials [24]. Clinical targets for non-viral plasmid DNA based gene therapy consists of a therapeutic gene for the treatment of cancer, cardiovascular, infectious, neurological, monogenic and ocular diseases. Around 57% of the diseases addressed using plasmid DNA in the clinical trials is for the treatment of cancer. Cardiovascular diseases account for 25% of clinical trials, while infectious and neurological diseases make up for 2.5% and 3.3% respectively.

2) Gene expression plasmids

Basically, gene expression occurs in two steps: (i) transcription – a process of converting information encoded in DNA into a molecule of RNA, the messenger RNA (mRNA) and (ii) translation – a process of converting information encoded in the nucleotides of mRNA into a defined sequence of amino acids in a protein. A gene expression plasmid is a plasmid DNA which is capable of expression of the therapeutic gene into the desired protein.

3) Production and processing of plasmid DNA

Preparation of plasmid DNA is a simpler manufacturing process than the viral packaging and purification method [25]. Although non-viral vectors are less effective than viral forms, the relative ease of plasmid DNA production means that it can be readily available in large quantities for gene therapy [26]. The production of a pharmaceutical grade ccc plasmid DNA [27] essentially involves the steps of: (i) cloning the therapeutic gene into a

plasmid vector, (ii) transforming the plasmid into *Escherichia coli*, (iii) cultivating *E. coli* cells in bioreactors for mass plasmid production and (iv) cell lysis and purification of plasmid DNA. Extensive purification procedures are required to ensure that the gene product contains a high percentage of plasmids in the super-coiled form. Interest in producing large quantities of super-coiled plasmid DNA has recently increased as a result of the rapid evolution of gene therapy and DNA vaccines [28].

Plasmid DNA is a very fragile molecule and is highly susceptible to shear during the manufacturing process. Medium to high shear processes such as mixing, turbulent flow during transport, filtration, lyophilization, and spray-drying are commonly encountered in the operations of a plasmid manufacturing process. Breakage in the DNA strand affects the quality and performance of the gene product, especially if the damage is in the promoter or gene-coding region. It is, therefore, necessary to address the potential of shear related damage that may occur during processing of the DNA [29]. A method for protection of plasmid DNA from high shear induced damage uses simple divalent cations and the lyophilizable alcohol, *tert*-butanol, to self-assemble DNA into condensed, shear-resistant forms [30]. In view of the economic considerations, the development of plasmid DNA production and purification strategies for gene-therapy vectors has largely been carried out in pharmaceutical companies within a confidential environment. Consequently, detailed information on large-scale plasmid purification is not available to the scientific community [31, 32].

4) *Quality of plasmid DNA for gene therapy*

The manufacturing of plasmids under current Good Manufacturing Practices (cGMP) compliance as required by the Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMA) is crucial to obtain a product that is consistent in

purity, potency, identity, efficacy and safety [13]. Possible contaminants in a plasmid DNA preparation include genomic DNA, RNA, protein, lipids and microflora. The level of contaminants in plasmid DNA preparations can be checked by quality assurance tests listed in **Table 2** to meet the specifications required for administration as a gene therapeutic agent [10].

5) *Gene delivery system*

The development of non-viral gene transfer methods requires proper formulations that are both effective *in vivo* and non-toxic. The development of non-viral gene vectors for therapeutic delivery must take into account the stability of the vector when exposed to physiological conditions. Aqueous formulations of non-viral vectors at the high concentrations necessary for clinical trials are very unstable compared to frozen formulations [33].

Cationic liposomes (lipoplexes) and cationic polymers (polyplexes) are the most frequently used non-viral gene transfer systems. Lipoplexes have been used as non-viral vectors in worldwide human clinical trials of gene therapy [34, 35]. Electrostatic interactions between the positive charges of the cationic lipid head-groups and the phosphate DNA backbones are the main driving force for the lipoplex formation [36]. Complexes between cationic lipids and plasmid DNA are typically prepared by mixing preformed cationic liposomes and DNA in an aqueous solution [37]. A scalable and extrusion-free method for efficient liposomal encapsulation of plasmid DNA for gene therapy has been reported [38]. Developing nonviral, pharmaceutical formulations of genes for human therapy is particularly important in functional tumor targeting of gene therapeutics. Ligand-directed lipoplex targeting enables dual expression of ligands such as folate, transferrin or anti-transferrin-receptor antibody, and lipoplexes. Such targeting methods have been used for gene delivery

and expression in human breast, prostate, head and neck cancers [39]. A schematic of cationic liposome mediated gene transfer is shown in **Fig. 4**.

The common cationic polymers used for complexing plasmid DNA include poly(ethylenimine) (PEI), poly(L-lysine) (PLL), Chitosan, Dendrimers and Poly(2-dimethylamino) ethyl methacrylate or pDMAEMA. The cationic lipids include *N*[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride) (DOTMA), 3 β -(*N,N*-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), 2,3-dioleyloxy-*N*-(2-(sperminecarboxamido) ethyl)-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and a neutral phospholipid, such as dioleoylphosphatidylethanolamine (DOPE) [40]. The DNA/cationic polyplexes are generally made in low salt solutions, because the complexes form micron-sized aggregates in either physiological saline or in blood. Polyethylenimine (PEI) is the most efficient nonviral gene vector for transfer of plasmid DNA. Gene transfer efficiency and cytotoxicity with PEI/ DNA complexes depend on the molecular weight of PEI. Smaller PEIs of size < 25-kDa, although less efficient are non-cytotoxic. Increase in gene transfer efficiency with minimal cytotoxicity can be achieved by cross-linking of small PEIs with potential biodegradable linkages [41].

Cyclodextrins are useful templates for further modification to produce molecular constructs capable of enhanced gene delivery. Polycationic cyclodextrin utility in promoting DNA cellular-uptake is dependent on proteoglycan-mediated binding to cells [42]. PEGylation is a common and effective means of conferring salt stability to polyplexes. PEGylated particles encounter and enter cells in a manner distinct from that of unmodified polyplexes [43]. Novel cationic pentablock copolymers possess better physical properties for effectively condensing DNA into polyplexes, and protecting plasmids from nuclease degradation, which is crucial for development of non-viral vectors for gene delivery [44]. The

most striking difference between cationic lipids and cationic polymers is the ability of the latter to more efficiently condense plasmid DNA.

Chitosan is a non-toxic biodegradable polysaccharide composed of two subunits, D-glucosamine and N-acetyl-D-glucosamine, linked together by $\beta(1,4)$ glycosidic bond. Cationic-charged chitosan interacts with negatively charged phosphate groups of DNA [45]. Because chitosan is a mucoadhesive polymer, chitosan/DNA complexes are attractive candidates for transfecting gastrointestinal epithelia and/or immune cells in gut-associated lymphoid tissue after being carried across the mucosal boundaries. Cells transfected with lactosylated chitosan [46] and galactosylated chitosan grafted with PEI [47] had a gene expression higher than PEI-mediated transfection.

Non-viral gene delivery

Recent developments in the formulation of plasmid DNA for production of non-viral gene therapeutics should pave the way for preparation of gene therapies to treat a wide range of inherited and acquired human diseases. However, the design of an optimal gene delivery system for effective non-viral gene therapy is limiting. Gene delivery systems should serve to protect the plasmid DNA from premature degradation in the extra-cellular milieu, mediate non-specific or cell-specific delivery to target cells and facilitate intracellular trafficking [48]. The main hurdle to the success of plasmid DNA based gene therapy is the lack of efficient, specific and safe DNA delivery systems that can permeate the physiological (extracellular) and biological (intracellular) barriers to gene transfer and expression [49,50]. The physiological barriers to gene delivery are mainly influenced by the methods of protection of DNA and the delivery route chosen for administration of the gene therapeutic (biodistribution). The biological barriers arise from intracellular events in the gene transfer route which include cell membrane entry, endosomal release, nuclear localization and gene

expression. The immunological barriers to non-viral DNA delivery result from the activation of the innate immune system by the plasmid DNA [51].

Barriers to non-viral gene delivery

Upon administration, non-viral vectors have to encounter extra-cellular barriers before they reach the targeted cells for gene transfer. However, every disease which can be treated by gene therapy has a unique set of physiological and biological barriers. Generally, most non-viral vectors are injected subcutaneously or intramuscularly. During intravenous injection, non-viral vectors must pass through capillaries to reach target cells, avoid recognition by mononuclear phagocytes, emerge from the blood vessels to the interstitial sites, and bind to the surface of the target cells [52]. Cationic vectors may attract serum proteins and blood cells when entering into blood circulation; this attraction results in dynamic changes in their physicochemical properties. Biological barriers compromise the delivery of plasmid DNA in the targeted cell to gene expression resulting in lower therapeutic efficacy. The biological barriers that pose obstacles to gene delivery [53] upon entry into the cell include: (i) endocytosis, (ii) dissociation of plasmid DNA from non-viral carrier, and (iii) pDNA uptake in nucleus for transgene expression.

(a) Endocytosis

After delivery to the targeted cell, viral/ non-viral vectors enter through endocytosis, an internalization process for the degradation of foreign/ extracellular material. For efficient gene transfer, the cytosolic release of plasmid DNA is a prerequisite for nuclear translocation. Since entrapment and degradation of plasmid DNA in endo-lysosomes constitute a major barrier to gene transfer, proper formulation is essential to encapsulate and protect the plasmid DNA. Formulation of lipoplex with a neutral lipid, DOPE has been shown to increase the

efficiency of gene transfer [54]. DOPE promotes the fusion of lipid/DNA particles with endosomal membranes, facilitating membrane disruption and increasing the amount of plasmid molecules released into the cytoplasm. Formulated non-viral vectors based on PEI and HVJ (Hemagglutinating Virus of Japan)-liposome has been characterized to escape endosomal degradation [55].

(b) Dissociation of plasmid DNA from non-viral carrier

Following internalization of the DNA-polycation complex by endocytosis, a large fraction is targeted to the lysosomal compartment by default. Once the plasmid DNA is released in the cytoplasm and before entering the nucleus, it can be quickly degraded by Ca-sensitive cytosolic nucleases. Only a small fraction of internalized plasmid DNA penetrates the cytoplasm. Hence the plasmid DNA should be imported into the nucleus quickly to be transcribed and avoid nuclease attack. It is estimated that at least 10^5 plasmids per cell are required in the extracellular compartment to ensure that a few DNA molecules are taken up into the nucleus of non-mitotic cells [54].

(c) Plasmid DNA uptake in nucleus

For cellular plasmid-based expression, nuclear import is a rate-limiting step, and intracellular trafficking of pDNA, either naked or complexed to synthetic vectors, is largely uncharacterized. After entry of plasmid DNA into the nucleus, a therapeutic gene has to be transcribed to generate an mRNA, with or without integration into the host's genome. This process would be expected to be common between viral and non-viral vectors. Gene regulation is very much dependent on the transcription and proper transcription control of a transgene is an important issue in gene therapy [56].

During non-viral gene transfer, entry of exogenous DNA into the nucleus occurs only in cells that are actively dividing, i.e., when the nuclear envelope breaks down. This is consistent with the observation that well-differentiated, non-dividing airway epithelial cells show very low transfection efficiency. Hence delivery of therapeutic plasmid DNA to a non-proliferating cell nucleus is an inefficient process. Nuclear import of signal-mediated pDNA using nuclear localization signal (NLS) for non-viral gene delivery has been attempted [57-59].

DELIVERY SYSTEMS

Nucleic acid vaccines have the potential to replace conventional protein vaccines because of their ability to induce *de novo* production of antigens in a given tissue after DNA delivery. The routes for administration of plasmid DNA based gene therapy include: (i) parenteral, (ii) oral, (iii) nasal, (iv) transdermal, (v) ocular and (vi) pulmonary delivery. **Table 3** shows a comparison of the routes for administration of plasmid DNA for parenteral, oral, nasal, transdermal and ocular delivery. Each delivery route has its own barriers and advantages, and depends on the intended target application for safe and efficient delivery. Parenteral delivery through the intravenous route (hydrodynamic delivery) is simple and versatile over other parenteral forms. Oral delivery is preferred over parenteral due to ease in administration and reduced delivery device development costs. Nasal delivery is cheaper and induces both mucosal and systemic responses. Transdermal offers patient compliance and sustained or controlled delivery. Ocular delivery has an advantage of accessing therapeutic success by visualization. Pulmonary delivery offers a non-invasive route for delivery of therapeutics aimed at addressing respiratory and systemic diseases. With the availability of

proven respiratory drug delivery devices, pulmonary route offers tremendous potential over nasal delivery and will be discussed in detail in the following section.

Pulmonary delivery

The pulmonary epithelium has been an important delivery route for gene therapy in the last decade. The structure of the respiratory tract depicting the lower airway of the lungs consisting of trachea, bronchi and alveoli is shown in **Fig. 5**. The delivery of aerosol therapeutics into the pulmonary system has to be directed at the lower airway and is dependent on the aerosol particle size. Inhaled aerosols are effective therapeutic carriers capable of non-invasive systemic delivery of therapeutics [104, 105]. The aerosol characteristics for targeted therapeutic delivery to the different regions of the lung during inhalation are shown in **Table 4** [106]. Only delivery by breathing via the mouth is considered for aerosol dosage forms.

The main physiological barriers to gene delivery in the lungs [107] are mucus, pulmonary surfactant and alveolar macrophages. Mucus is the most frequently reported extracellular barrier to the delivery of genes to the cells of the upper respiratory tract. Respiratory mucus lines the luminal side of the tracheo-bronchial tree from the entrance of the trachea to the terminal bronchioles, humidifying inspired air and trapping small particles or microorganisms until they can be transported out of the lungs. Pulmonary surfactant synthesized by type II alveolar cells and non-ciliated epithelial cells reduces the surface tension in the lungs. The main components of surfactants are phospholipids, neutral lipids, serum proteins, and surfactant proteins. Surfactant proteins may reduce the efficiency of gene delivery when the DNA vectors contain carbohydrate moieties. Gene therapy targeted to the alveolar regions of the lung or to the systemic circulation via the alveoli may be limited by

the actions of the alveolar macrophages [106]. The physiological and intracellular barriers to gene delivery in the airway epithelial cells of the lungs are shown in **Fig. 6** [108].

Pulmonary delivery devices

The three types of pulmonary delivery devices commonly used in the treatment of respiratory ailments such as asthma/COPD, cystic fibrosis and anti-infectives include (i) metered dose inhalers (MDI), (ii) dry powder inhalers (DPI) and (iii) nebulizers. The most important parameters that define the site of deposition of aerosol drugs within the respiratory tract are the particle characteristics of the aerosol. The nature of the aerosol droplets is dependent on its MMAD (mass median aerodynamic diameter), which is a function of particle size, shape and density. The aerodynamic size of aerosol particles generated by various inhalers is fundamental to lung delivery since only the fine particle fraction (FPF) of approximately $<5\ \mu\text{m}$ diameter can reach target surfaces within the lung [109]. Pulmonary drug administration imposes stringent requirements on the delivery device, since particle size of a therapeutic powder or droplet aerosol greatly influences the accessibility to the delivery site, and ultimately the degree of drug absorption from the lungs [110].

(i) Pressurized metered dose inhalers

Pressurized metered dose inhalers (pMDIs) are the most popular vehicle for drug delivery into the lungs, and some 500 million units are manufactured each year. MDIs utilize propellants (chlorofluorocarbons and increasingly, hydrofluoroalkanes) to atomize the drug solution, which results in a uniform spray [111,112]. Macromolecules are not soluble in pMDI propellants and have to be formulated as dispersed systems i.e., solid particles dispersed in propellants. Dornase- α and salmon calcitonin have been formulated in pMDI systems, demonstrating good retention of primary and secondary structures [113]. However,

such dispersed formulations have an affinity for aggregation and particle growth, resulting in some macromolecules with an intrinsic instability in the environment of the propellant formulations [114]. Work in progress on the technology for producing drug nanoparticles for dispersion in HFA propellant for pulmonary gene therapy is reported in Birchall [115].

(ii) Dry powder inhalers

The development of macromolecular formulations for dry powder inhaler (DPI) devices has been prompted due to the potential for longer product shelf-life. In the 1980s and 1990s, passive systems - those where the powder aerosol is generated using only the inspiratory effort of the patient - were developed into Diskus (GSK, RTP, and NC) and Turbuhaler (Astra Zeneca) which are multi-dose, blister and reservoir systems, respectively. Recently, additional single dose devices such as the Aerolizer (Novartis) and the Handihaler (Boehringer Ingelheim) and multidose devices like Clickhaler (Innovata Biomed) have been approved [104]. The Nektar Pulmonary Inhaler and AspirairTM (Vectura) device are examples of active devices, which rely upon hand-assisted compressed air for aerosol generation. An inhaler and powder formulation developed by Nektar, Exubera® (human insulin of rDNA origin) insulin inhalation powder is approved in the US and EU for adults with Type 1 and Type 2 diabetes. Whilst dry powder formulations for DPIs have considerable potential for gene therapy in the lung, the issue of formulation remains a major obstacle to their practical use [116].

(iii) Nebulisers

An alternative to pMDIs and DPIs for delivery of biopharmaceuticals is the nebuliser, which can generate respirable aerosols from the liquid with less formulation requirements and

a wider dose range. The types of nebulisers commonly used for respiratory drug delivery are Pneumatic or Jet nebulisers and Ultrasonic nebulisers [117,118].

(a) Jet nebuliser

The operation of a pneumatic nebuliser requires a pressurized gas supply as the driving force for liquid atomization [121]. Compressed gas is delivered through a jet, causing a region of negative pressure. The solution to be aerosolized is entrained into the gas stream and sheared into an unstable liquid film, which breaks into droplets because of surface tension forces. The aerosol is delivered into the inspiratory gas stream of the patient.

(b) Ultrasonic nebuliser

The ultrasonic nebuliser uses a piezoelectric transducer to produce pressure waves at ultrasonic frequencies that pass through the solution and aerosolize it at the surface of the solution. The frequency of the ultrasonic waves determines the size of the particles, with an inverse relationship between frequency and particle size. Small-volume ultrasonic nebulisers are commercially available for delivery of bronchodilators. Large-volume ultrasonic nebulisers are used to deliver inhaled antibiotics in patients with cystic fibrosis [122]. Ultrasonic nebulisers produce a large number of droplets per unit volume, which in the absence of air circulation through the nebuliser will tend to aggregate and settle in the case of a low velocity aerosol [123]. The conventional ultrasonic nebuliser with two tanks utilizes the cavitation effect of ultrasonic waves for nebulisation. The mesh-type of ultrasonic nebuliser is seen as the next-generation of nebulisers. The liquid passes through a mesh with an array of hundreds or thousands of micron-sized holes.

- *Mesh type nebulisers*

Mesh type nebulisers may be classified into two types based on their mode of vibration as active or passive vibrating mesh. An active vibrating mesh indicates the mesh itself is vibrated directly by a piezoelectric crystal. For a passive vibrating mesh, the vibrations of an ultrasonic horn force liquid through the mesh, which vibrates in sympathy with the horn [125]. The key mesh nebulisers either in development or on the market following regulatory approval [126] include:

Active vibrating mesh:

- (i) e-Flow[®] (PARI, Germany), Touchspray[®] (Odem, UK)
- (ii) AeroNeb[®]/ Aerodose[®] (Aerogen, USA),

Passive vibrating mesh:

- (i) MicroAir[®] (Omron, Japan)
- (ii) Microflow[™] (Pfeiffer, Germany)

The advantages of ultrasonic mesh nebulisers include little requirement for patient coordination, small dead volume and quiet operation, high dosage delivery, no chlorofluorocarbon release and fast drug delivery. It has the potential for smaller volume fills, lower or no residual volumes of drug, higher lung deposition and shorter nebulisation times. The disadvantages which have limited their acceptance are that they are expensive, prone to electrical and mechanical breakdown, not recommended for all drug formulations available and requirement of drug preparation. Some ultrasonic nebulisers may be used with the solution to be nebulised placed directly over the transducer. The use of nebulisers to administer biopharmaceutical agents has many important limitations. Such drugs are often very unstable in aqueous solutions, and are easily hydrolyzed. In addition, the process of nebulisation exerts high shear stress on the labile compounds, which can lead to product denaturation or degradation. Other important characteristics of nebuliser performance include

nebulisation time, cost, ease of use, and requirements for cleaning and sterilization. Technical profiles of the next-generation aerosolisation devices for pulmonary delivery and their regulatory/market status are compared and shown in **Table 5** [127-132].

(c) Other aerosolisation devices

The AER_x[®] delivery device (Aradigm) is an electro-mechanical extrusion device for aerosolisation of liquid. This device is not yet approved and essentially consists of a single-use nozzle contained in each disposable AER_x Strip[™] created as a laser-machined array. The nozzle exit is approximately 1 µm in diameter with a uniform shape which is expected to deliver consistent, fine-particle dosing from the blister/strip containing 50 µl of medication. The aerosol is generated by extruding the formulation under pressure through an array of holes. Aerosolisation of unformulated plasmid DNA (pCMV-SEAP with an approx. size of 7.5 kb) has shown it is degraded upon passage through the AER_x nozzle system [131]. However, formulation of the plasmid DNA with cationic lipoplex has resulted in no damage to the sc structure on delivery. Aradigm's AER_x insulin Diabetes Management System (iDMS) has been licensed to Novo Nordisk for Phase 3 testing of Type 1 and Type 2 diabetes.

The electro-hydrodynamic (EHD) process applies an electric field over a flowing conductive liquid. The electric field is generated by transferring high voltage DC (direct current) through an array of electrodes, creating a field of discharge ions in front of a multi-spray site nozzle. These cations induce an accumulation of charge at the liquid's surface causing a Taylor cone to form at each spray site. As the surface charge overcomes the surface tension of the liquid, a fine mist of nearly mono-dispersed droplets is formed. As the droplets pass through the field of ions, their charge is subsequently neutralized, causing the cloud to disperse into aerosol droplets. A device presently in its development using the electro-

hydrodynamic technology (Ventaria, USA) has been reported to efficiently reproduce aerosol droplets in the 1-5 μm range [133].

Aerosol delivery of plasmid DNA

The field of aerosol gene delivery began to decline until it was discovered that complexation of pDNA with polymers (PEI) resulted in far better transfection within the lungs than did lipids or naked DNA [134]. A number of potential obstacles to the intrapulmonary delivery of genes via conventional jet nebulisation include shearing effects associated with nebulisation and requirements to produce aerosol droplet sizes appropriate for optimal delivery to the peripheral lung and to maximize the dose of DNA delivered to lung surfaces. Non-viral polyplexes are emerging as suitable candidates for use in pulmonary inhalation gene therapy. PEI-based formulations appear to be good candidates for aerosol delivery of genes for the treatment of a variety of genetic pulmonary disorders, including lung tumors. Aerosolisation of plasmid DNA formulations with PEG/PEI in a jet and ultrasonic nebuliser resulted in change in polyplex structure due to jet nebulisation [135]. Aerosol-delivered PEI-based formulations are very effective in transfecting the lungs, but produce relatively low levels of transfection in the nasal passages of mice [136].

Aerosol delivery of plasmid DNA holds considerable promise for the treatment of many demanding respiratory diseases, such as cystic fibrosis, influenza or lung cancer. The delivery of plasmid constructs in the size range of 5 to 20 kb is particularly challenging. The retention of the supercoiled structure (sc) of the plasmid during aerosol delivery is essential for its use as a genetic drug and to comply with regulatory requirements on product quality. In order to assess the level of damage experienced by pDNA due to the aerosolisation process, the integrity of the supercoiled structure in the aerosols after aerosolisation has been studied and a summary of findings of a number of studies is presented in **Table 6**. From the

table, jet nebulisation resulted in damage to a 5 kb and 9.8 kb plasmid, the loss of the sc content being attributed to hydrodynamic shear and shock waves [137]. Damage to a small plasmid of size 4.8 kb in the aerosols of a jet nebuliser was reported by Kleemann et al. [138] due to the shearing effects of aerosolisation. Aerosolisation based on electrohydrodynamic (EHD) delivery did not damage supercoiled pDNA. Although there is no damage to the sc structure of the plasmid in the aerosols, the EHD device is yet to be commercialized for pulmonary delivery in the clinic [139]. A press release from Ventaria Pharmaceuticals [dated March 21, 2006] stated the device based on EHD technology (MysticTM) is in the manufacturing stage with Nypro Inc., a medical device manufacturing company [Ventaria website]. A commercial mesh-based nebuliser (eFlow[®]) has also been reported to damage extensively plasmid DNA [140]. However, the aerosol delivery of a naked gWizTM Luc plasmid has resulted in an intact supercoiled structure in the aerosols using a miniaturized nebulisation catheter device [141]. Our own recent work [142] has shown that the sc structure of the 5.7 kb plasmid and a formulated 20 kb plasmid can be safely aerosolized using a commercially available mesh nebuliser (MicroAIR[®], Omron Healthcare, Japan). Although the results from gene therapy clinical trials are expected soon, developing an aerosol gene delivery which would be as safe and effective as intravenous delivery will be a considerable challenge to the healthcare industry.

Magnetofection

Magnetofection is the delivery of genes using magnetic forces and has been shown to enhance transfection efficiency of non-viral systems up to several-hundred-fold. In this technique non-viral gene carriers, such as polyethylenimine (PEI), are associated with super para-magnetic nanoparticles and complexed with plasmid DNA. Gene delivery is targeted by the application of a magnetic field leading to an accelerated sedimentation of magnetofectins

on the cell surface and increase in contact time. They do not adversely affect the intracellular uptake mechanism and could lead to efficient targeting of gene expression into the desired organ and tissue *in vivo* [143]. The greatest potential for using magnetic nanoparticles to treat paediatric respiratory illness lies in the nanoparticle-facilitated delivery of therapeutic genes for cystic fibrosis [144]. TransMAG^{PEI} (Chemicell, Germany), a PEI-coated iron oxide particle, complexed to Genzyme Lipid 67 (GL67) mixed with luciferase plasmid DNA was tested in mouse mammary epithelial cells. Although an increase in gene transfer *in vitro* was observed, there was no increase in the transfection efficiency *in vivo* [145]. Biocompatible magnetic nanoparticles enable targeted gene delivery and show much promise for *in vitro* and *in vivo* transfection studies. Magnetofection promises to overcome the fundamental limitations of non-viral gene transfer to the airways [146].

Nebulisation for cystic fibrosis gene therapy

Cystic fibrosis is a common lethal hereditary disease causing chronic lung inflammation. CF, a common genetic disease occurring due to a recessive genetic mutation, causes deficiencies in the transport of salt across the membranes of secretory cells. This abnormal metabolic transport causes the accumulation of thick, sticky mucus in the respiratory and digestive tracts, leading to recurrent lung infections, pulmonary damage and difficulties in food intake. Clinical trials in CF patients have been conducted using cationic liposomes carrying the genes for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). In order to improve the efficacy of CFTR gene delivery using plasmid DNA for cystic fibrosis patients, it is essential that (i) the mucus layer is degraded using a mucolytic agent to enable the gene therapeutic to reach the epithelial cells and (ii) the integrity of the fragile gene therapeutic is not damaged during the aerosolisation process.

Cationic liposomes have been used successfully for DNA delivery to airway cells *in vitro* and are being tested in human clinical trials for their efficacy in CFTR gene delivery in cystic fibrosis patients [147]. However, liposomes are not as effective for gene delivery to human airway cells *in vivo* when compared with transfection of airway cells *in vitro*. The physiological barriers to gene delivery in cystic fibrosis lungs are the main reasons for the reduced expression levels [148]. A potential gene therapy-based application for the cure of cystic fibrosis (CF) is being pursued [149-151]. With the approval in the delivery of inhaled insulin [152], the pulmonary delivery route also offers promise for the treatment of systemic diseases.

To summarize, testing the efficacy of a plasmid DNA-based gene therapeutic at the pre-clinical/clinical stage is crucial towards achieving the promise of gene therapy. The application of mesh nebulisation technology for the aerosol delivery of shear-sensitive therapeutics such as plasmid DNA and siRNA into and via the airways offers immense potential for the treatment of unmet medical needs such as influenza, SARs, lung cancer.

Conclusions

Gene therapy is gaining credibility and is an emerging area in medicine today. Plasmid-based gene therapy has been proven to be well-tolerated and safe for administration by a variety of routes (e.g., intramuscular, intratumoral, pulmonary, transdermal) but efficiency has often been limited with current technologies. A rational approach towards the design of synthetic gene delivery systems is necessary taking into account the route of administration and the targeted site at which gene expression is expected to take place. A major milestone in plasmid DNA delivery for gene therapy is the requirement of highly pure plasmid DNA formulations capable of passage through the delivery device and route. This

could contribute to a great extent to significantly reduce the dosages required for administration. With greater attention to the production of efficient expression systems, as well as stable and viable formulations for gene therapy, the utilization and development of state-of-the-art delivery devices hold considerable importance for the success of plasmid-based medicines and vaccines as innovative therapies for the future.

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Figure Captions

Fig. 1: Comparison of vectors used in gene therapy clinical trials initiated/approved in 2008 and 2005 (Source: www.wiley.co.uk/genetherapy/clinical).

Fig. 2: Year-wise distribution of major gene therapy vectors in gene therapy clinical trials (Source: www.wiley.co.uk/genetherapy/clinical).

Fig. 3: Percentage of open gene therapy clinical trials for the major gene therapy vectors (Source: www.wiley.co.uk/genetherapy/clinical).

Fig. 4: A schematic for the preparation of lipoplex for non-viral gene transfer

Fig. 5: A human respiratory system showing anatomy of the lower airways comprising trachea, bronchi and alveoli (Image courtesy: Wikimedia commons; http://commons.wikimedia.org/wiki/Image:Respiratory_system_complete_en.svg)

Fig. 6: Barriers limiting gene transfer efficiency of non-viral vectors to airway epithelial cells; extracellular barriers – gel layer containing mucus and aqueous layer containing a thin layer of low-viscosity fluid critical for effective ciliary movement; intracellular barriers - Endocytosis, endosomal release and nuclear import of plasmid DNA..

Table Captions

Table 1: Viral and non-viral vectors for gene therapy

Table 2: Quality assurance tests of plasmid DNA preparation for gene therapy

Table 3: Comparison of the common routes for administration of plasmid DNA other than pulmonary delivery

Table 4: Targeting aerosols to the lung via inhalation

Table 5: Technical profile of next-generation aerosolisation devices

Table 6: Aerosol delivery of plasmid DNA in some reported aerosolisation devices

Table 1: Viral and non-viral vectors for gene therapy

<i>Factors</i>	<i>Positive attributes</i>		<i>Reasons for positive impact</i>
	<i>Viral</i>	<i>Non-viral</i>	
Transfection efficiency	+++	+	Viral vectors achieve higher gene transfer rates <i>in vivo</i> than non-viral.
Repeated administration	-	+++	Non-viral vectors are non-immunogenic
Transgene capacity	+	+++	Non-viral ability to handle and transfect with larger DNA sequences (> 100 kb).
General safety	+	+++	Non-viral systems generally non-infectious and non-toxic.
Production cost	+	+++	Less expensive GMP production for non-viral vectors.
Increase in gene therapy clinical trials	++	+++	Increase in plasmid DNA over retroviral and adenoviral vectors
Time to develop a DNA vaccine to treat pandemic diseases and unmet medical needs	+	+++	More time required to develop a viral vector compared to non-viral. In view of the above, von-viral vectors such as pDNA and siRNA have a better chance to reach the clinic over viral vectors.

Table 2: Quality assurance tests of plasmid DNA preparation for gene therapy

S.No.	Test	Analytical Method
1	DNA concentration	UV-absorption (260 nm)
2	General purity	UV-scan (220–320 nm)
3	Homogeneity (ccc content)	CGE (capillary gel electrophoresis)
4	Purity (visible)	Visual inspection
5	Purity (genomic DNA)	Agarose gel (visual); Southern blot; quantitative PCR (polymerase chain reaction)
6	Purity (RNA)	Agarose gel (visual); fluorescence assay; quantitative PCR
7	Purity (protein)	BCA (Bicinchoninic acid) test
8	Purity (LPS)	LAL (Lymulus amebocyte lysate) test
9	Purity (microorganisms)	Bioburden test; sterility test
10	Identity (vector structure)	Restriction fragment length conforms to reference in AGE (1–3 enzymes)
11	Identity (sequence)	Sequencing (double strand)

Table 3: Comparison of the common routes for administration of plasmid DNA other than pulmonary delivery

Delivery route	Delivery principle	Advantages/ barriers to plasmid DNA delivery	Formulation and plasmid DNA delivery strategy	Ref.
Parenteral <ul style="list-style-type: none"> Sub-cutaneous 	Delivery other than digestive tract aimed at systemic action by cutaneous administration	DNA injection into skin tissues results in low transfection efficiency due to rapid degradation by endogenous nuclease action within tissues	<ul style="list-style-type: none"> Administration of pDNA and a competitive nuclease inhibitor, aurintricarboxylic acid (ATA) followed by electroporation was observed to be more effective than the administration of naked DNA Entrapment of plasmid DNA in dehydration/rehydration vesicles (DRV) Radio frequency ablation to create transient micro-conduits in human skin, allowing DNA delivery, inducing systemic immune response and resulting cutaneous gene expression. 	[62] [63] [64,65]
<ul style="list-style-type: none"> Intravenous 	Hydrodynamic delivery involving large-volume, high-speed intravenous injection	Attractive in terms of efficiency, simplicity and versatility	<ul style="list-style-type: none"> Demonstrated proof-of-principle of repeated delivery of erythropoietin (EPO) expressing pDNA doses for the correction of anaemia, hitherto treated using recombinant human EPO protein formulations Intravenous interferon gene delivery with naked plasmid DNA in murine metastasis better than subcutaneous delivery Dendritic poly(L-lysine) of the 6th generation as non-viral plasmid DNA carrier for <i>in vivo</i> intravenous administration in tumor-bearing mice better than DOTAP/Chol liposomes & PEI. 	[66-68] [69] [70]
<ul style="list-style-type: none"> Intramuscular 	Delivery of plasmid DNA through intramuscular injection	Lipoplex plasmid DNA is resistant to serum enzymatic digestion and induced an inhibition of gene expression	<ul style="list-style-type: none"> Delivery of plasmid DNA coding for glutamic acid decarboxylase (GAD) resulted in less effective disease suppression compared to intradermal or oral administration, with uptake of pDNA <i>in vivo</i> in cells of bone marrow and lymph node Administration of plasmid vectors engineered for gene delivery into mammalian muscle did not induce the production of anti-ds DNA and anti-nuclear auto-antibodies in mice. Peripheral intramuscular immunization in mice with PEI/pDNA documented gene transfer in neurons of central nervous system. 	[71-73] [74] [75]
Oral	Oral delivery is significant due to availability of a large mucosal surface housing the tissues in the GI tract	Advantages over parenteral approach due to ease in administration, patient convenience and compliance, and reduced delivery development costs	<ul style="list-style-type: none"> PLGA microparticle carrier containing PEI polyplexes for the deposition of intact polyplexes in intestinal lymphoid tissue yielded transgene expression Plasmid DNA vaccine for treating gastrointestinal diseases orally delivered to the intestines using N-acetylated chitosan as carrier Oral administration of pDNA-chitosan microparticles as an indicator of exogenous gene expression in <i>in vivo</i> animal studies Disadvantage of oral delivery is the disintegration of the complex in the GI tract due to mechanical, chemical and enzymatic barriers. 	[76,77] [78]

Nasal	Nasal delivery is targeted at the nasal-associated lymphoid tissue (NALT)	An attractive vaccination route since it is easier and cheaper for patients, and it induces both mucosal and systemic responses	<ul style="list-style-type: none"> • Different formulation strategies used for nasal vaccine delivery include particulate antigen delivery systems such as emulsions, liposomes, microspheres, where the antigenic material is entrapped or presented on the surface of a particle, and solution systems, where the antigen is dissolved or suspended within a simple solution vehicle • With nasal inoculation of liposome/DNA complexes, liposomes are trapped within the lung epithelium and the cationic lipid to DNA ratio is changed during transit through the lung epithelial cells • Administration by nasal instillation of PEI-PEG/DNA complexes in mice resulted in significant levels of transgene expression. 	<p>[80,81]</p> <p>[82]</p> <p>[83]</p>
Transdermal	Skin is the largest area of the human body. The principle barrier to penetration through the skin is the "stratum corneum", due to its "brick and mortar" structure of proteins and intercellular lipids.	Transdermal gene transfer provides a painless and patient-friendly interface for systemic administration. An inherent advantage over injections and oral routes due to increased patient compliance as well as sustained and controlled delivery	<ul style="list-style-type: none"> • Transdermal delivery can be achieved by using two different methods to render the skin permeable to drugs, namely physical and electrical forces • The three physical modes which have been adopted to enhance skin permeability for transdermal drug delivery include photoacoustic waves, low-frequency sonophoresis, and microneedles. The electrical means of delivering drugs transdermally include iontophoresis and electroporation. • Topical gene therapy requires penetration of the vector-gene complex to the target cells within the skin, as well as for systemic circulation • Electroporation is a versatile and efficient method of enhancing gene transfer which involves the application of electric field pulses after the injection of nucleic acids (DNA, RNA and/or oligonucleotides) into tissues • Microneedle delivery of plasmid DNA encoding hepatitis B surface antigen induced stronger immune responses compared to hypodermic injection, requiring fewer immunizations for full seroconversion. 	<p>[84]</p> <p>[85-89]</p> <p>[90-92]</p> <p>[93,94]</p> <p>[95]</p>
Ocular	Delivery through the eye is easily accessible and occupies an "immune-privileged" site	Advantage of ocular gene therapy is the possibility of assessing the success of the treatment in a non-invasive manner by directly measuring visual function	<ul style="list-style-type: none"> • Naked DNA/non-viral delivery to the cornea has the potential to alter the treatment of a wide variety of corneal and anterior segment diseases • Efficient and stable transfer of the functional gene was achieved with PEO-PPO-PEO polymeric micelles through topical delivery in mice and rabbits • Encouraging results in the non-viral gene transfer of compacted DNA nanoparticles to ocular tissues were shown in a human clinical trial. 	<p>[96-101]</p> <p>[102]</p> <p>[103]</p>

Table 4: Targeting aerosols to the lung via inhalation [106]

Target region	Particle diameter	Primary deposit mechanism	Inhalation method	Potential target diseases for gene therapy
Extrathoracic	$> 8 \mu\text{m}$	Impaction	High inspiratory flow velocity	Cancer
Tracheobronchial	$4\text{-}8 \mu\text{m}$	Impaction and sedimentation	Slow inspiratory flowrate	CF, COPD, cancer
Alveolar	$2\text{-}5 \mu\text{m}$	Sedimentation and diffusion	Slow inspiratory flowrate	Vaccines, cancer cytokine therapy
	$0.02\text{-}0.05 \mu\text{m}$	Diffusion		

Table 5: Technical profile of next-generation aerosolisation devices

Nebuliser type/ driving force/ Company	Trade Name (Status)	MMAD * (μm)	Aerosol mass fraction < 5μm (%)	Aerosol Condition	Technical information	Ref.
Vibrating mesh: OnQ technology / Aerogen	Aeroneb Pro (<i>Market</i>)	3.1	83	Nebulized medium – 0.083% Albuterol (3mL); Freq. 128 kHz	Adult % Dose deposited :13; Minimum vol. 0.3 mL; (Autoclaveable)	Aerogen website [127]
Vibrating mesh: Ultrasonic / Odem	Touch-spray (<i>NA</i>)	4.39 (1) 5.32 (2)	62.3 (1); 43.4 (2)	Nebulized medium – (1) - CC plasmid DNA & (2) - linear plasmid; Temp - 23°C; RH - 50%;	Intact DNA following aerosolisation: (1) CC – 10% (2) CC – 50%; Minimum vol. 0.3 mL	[128]
Vibrating mesh: Ultrasonic / Pari	Pari eFlow (<i>Market</i>)	GSD 1.5	63	NA	Single pass; no recirculation; Minimum vol 0.73 mL	CF website [129]
Vibrating mesh: Ultrasonic / Omron	MicroAir (<i>Market</i>)	5.0	80	Temp – 10-40°C; RH – 30-85%; Freq. 180 kHz	Lung deposition (% vol fill): 18.1 (8.0); Minimum vol. 0.5 mL	Omron website [130]
Electro-Mechanical Extrusion / Aradigm	AERx (<i>NA</i>)	2.95	NA	Nebulized medium – Aq. Formul.	Dia -1 μm ; Unit dose; Minimum vol. 0.045 mL	[131]
Electro-Hydrodynamic device / Battelle	Mystic Inhalation device (<i>NA</i>)	2.85	90-95	Nebulized medium – NaCl in ethanol	Minimum vol. 0.02 mL	[132]

* MMAD – Mass Median Aerodynamic diameter; NA – Not available

Table 6: Aerosol delivery of plasmid DNA in some reported aerosolisation devices

Aerosolisation device (clinical status)	Plasmid size (kb)	Percentage damage to sc DNA in the aerosols	Reference
Jet nebuliser (<i>approved</i>)	5.0	40%	[137]
	9.8	90%	
	4.8	> 75%	[138]
EHD device (<i>in trials</i>)	4.6, 9.2, 15.3	No damage	[139]
Mesh nebuliser (<i>approved</i>)	4.5	50%	[140]
	11.0	90%	
	5.7	No damage	[142]
	Formulated 20 kb	No damage	
Nebulisation catheter device (<i>preclinical trials</i>)	6.7	No damage	[141]

Figure 1.

Figure 2

Figure 3

Figure 4

Figure 5.

Figure 6