Pulmonary delivery of therapeutic siRNA

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ABSTRACT

Small interfering RNA (siRNA) has a huge potential for the treatment or prevention of various lung diseases. Once the RNA molecules have successfully entered the target cells, they could inhibit the expression of specific gene sequence through RNA interference (RNAi) mechanism and generate therapeutic effects. The biggest obstacle to translating siRNA therapy from the laboratories into the clinics is delivery. An ideal delivery agent should protect the siRNA from enzymatic degradation, facilitate cellular uptake and promote endosomal escape inside the cells, with negligible toxicity. Lung targeting could be achieved by systemic delivery or pulmonary delivery. The latter route of administration could potentially enhance siRNA retention in the lungs and reduce systemic toxic effects. However the presence of mucus, the mucociliary clearance actions and the high degree branching of the airways present major barriers to targeted pulmonary delivery. The delivery systems need to be designed carefully in order to maximize the siRNA deposition to the diseased area of the airways. In most of the pulmonary siRNA therapy studies *in vivo*, siRNA was delivered either intratracheally or intranasally. Very limited work was done on the formulation of siRNA for inhalation which is believed to be the direction for future development. This review focuses on the latest development of pulmonary delivery of siRNA for the treatment of various lung diseases.

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1. Introduction

RNA interference (RNAi) has huge therapeutic potential in treating many diseases by silencing the expression of the target gene in a post-transcriptional manner. The mechanism of RNAi has been extensively reviewed in the literature [1–6]. In brief, RNAi can be achieved artificially by three major ways: (i) introducing long double stranded RNA (dsRNA) which is cleaved into small interfering RNA (siRNA) by the enzyme Dicer in the cytoplasm, leading to the degradation of target mRNA; (ii) introducing plasmid DNA encodes for short hairpin RNA (shRNA) which is processed by Dicer into siRNA; and (iii) introducing small interfering RNA (siRNA) directly to initiate the mRNA degradation process.

For the last two decades, scientists have been investigating the use of nucleic acids including DNA and antisense oligonucleotide as therapeutic agents. The biggest hurdle we are encountering is delivery, and the same problem applies to RNAi therapy. Being negatively charged hydrophilic macromolecules that are highly susceptible to nuclease degradation, nucleic acids are incapable of crossing the biological membrane on their own to reach the site of action. A delivery vector is therefore required to protect the therapeutic nucleic acids from enzymatic degradation, facilitate cellular uptake and release nucleic acids at the site of action inside the cells. Amongst the types of nucleic acids that are involved in the RNAi, siRNA is the most popular candidate being studied in RNAi therapy. Introduction of long dsRNA (typically consist of 500 to 1000 base pairs) is known to induce interferon (IFN) response in mammalian cells [7,8], rendering it unsuitable for RNAi therapy, whereas siRNA (typically consist of 21–23 base pairs) can avoid the INF response. From the delivery perspective, siRNA has advantage over the plasmid DNA encoding shRNA. In order for DNA to be properly expressed, it must enter the nucleus where transcription takes place. Nuclear entry is an extremely inefficient process and this is indeed considered to be one of the biggest barriers to the success of gene therapy. On the other hand, siRNA targets the RNA-induced silencing complex (RISC), which is located in the cytoplasm. Although shRNA has a higher gene silencing potency than siRNA [9,10], the relatively ease of delivery makes siRNA a better candidate for RNAi therapy. In fact, delivery of siRNA has more success than other RNAi molecules. In 2004, the first human clinical trial of RNAi therapy was initiated for the treatment of age-related macular degeneration (AMD) with siRNA targeting VEGF-receptor 1 delivered intravitreally [11,12]. Since then a number of clinical trials of siRNA therapy are being conducted for different conditions [13], including solid tumor cancers [14] and respiratory syncytial virus (RSV) infection [15,16]. Table 1 shows the summary of clinical trials of siRNA therapy.

RNAi can be potentially used to treat or prevent diseases affecting the airways, such as lung cancer [26–30], various types of respiratory infectious diseases [16,31–36], airway inflammatory diseases [37–39] and cystic fibrosis [40]. siRNA delivery to the lungs could be achieved either by local delivery or systemic delivery. The former route offers several important benefits over the latter, such as a lower dose of siRNA is required, reduction of undesirable systemic side effects and improved siRNA stability due to lower nuclease activity in the airways than in the serum. Furthermore lungs could potentially serve as an interesting site for delivering siRNA for systemic effect due to its high vascularization, large surface area and ultra thin epithelium of the alveoli [41]. All these factors allow efficient and rapid siRNA absorption.

The process of pulmonary siRNA delivery is summarized in Fig. 1. There are several ways to administer siRNA locally into the lungs. Inhalation is the most common and the easiest method for pulmonary drug delivery and could be applied to siRNA delivery. siRNA for inhalation can be formulated into liquid aerosol or dry powder aerosol. The intranasal route is another common way to deliver siRNA into the airways due to the ease of administration and the intranasal siRNA preparation can be easily administered into the nasal cavity as nasal suspension. Intratracheal route of administration is also employed to deliver siRNA into the lungs. However this method of administration is relatively invasive and non-physiologic [42]. It is generally considered to be used in animal studies only rather than for clinical applications. Regardless of the administration route, it is very important to maintain siRNA stability and biological activity during manufacturing and delivery.

Similar to DNA delivery, both viral and non-viral vectors are being employed to deliver siRNA. Viruses are extremely efficient nucleic acids delivery vectors as they are evolved to transfer their genetic material into the host cells. To enable viruses to deliver nucleic acids for therapeutic use, the viruses must first be genetically engineered to remove their virulence. The main advantage of using viral vectors is their high transduction efficiency compared to transfection by non-viral methods. Viruses such as adenovirus [43–45], adeno-associated virus [46], and lentivirus [47,48] are being investigated to deliver siRNA to lung cells. Despite their high nucleic acids transfer efficiency, there are safety concerns regarding the use of viral vectors.

From the lessons we have learnt in DNA delivery, there are high risks associated with the utilization of viral vectors [49]. Immune response to viruses is the major challenge to viral delivery [50]. It

Table 1
Summary of clinical trials of siRNA therapy.

Latest stage development	Target disease	Route of administration/delivery agent	Company	Product name	Ref
III (terminated-unlikely to meet primary endpoint)	AMD	Intravitreal injection/naked siRNA	Opko Health	Bevasiranib (formerly Cand5)	[17]
II	AMD	Intravitreal injection/naked siRNA	Allergen & Sirna Therapeutics	AGN211745 (formerly Sirna-027)	[11]
II	RSV infection	Nasal spray/naked siRNA	Alnylam Pharmaceuticals	ALN-RSV01	[15,16]
II	Acute kidney injury	Intravenous injection/naked siRNA	Quark Pharmaceuticals	QPI-1002 (formerly I5NP)	[18]
II	AMD	Intravitreal injection/siRNA	Quark Pharmaceuticals, Pfizer	PF-4523665 (formerly REDD14NP & RTP801i)	
I/II	Ocular hypertension & glaucoma	Ophthalmic drops/naked siRNA	Sylentis	SYL040012	
I	Solid state tumors	Intravenous injection/cyclodextrin nanoparticles	Calando Pharmaceuticals	CALAA01	[14]
I	Solid cancers with liver involvement	Intravenous injection/lipid nanoparticles	Alnylam Pharmaceuticals	ALN-VSP02	[19]
I	Transthyretin mediated amyloidasis (ATTR)	Intravenous injection/lipid nanoparticles	Alnylam Pharmaceuticals	ALN-TTR01	[20,21]
I	Pachyonychia Congenita	Intradermal injection/naked siRNA	TransDerm	TD101	[22,23]
I	Chronic optic nerve atrophy & recent onset NAION	Intravitreal injection/naked siRNA	Quark Pharmaceuticals	QPI-1007	
I	Advanced solid cancer	Intravenous infusion/liposomal nanoparticles	Silence Therapeutics	Atu027	[24,25]

AMD: Age-related Macular Degeneration, DME: Diabetic Macular Edema, NAION: Non-Arteritic Anterior Ischemic Optic Neuropathy, RSV: Respiratory Syncytical Virus.
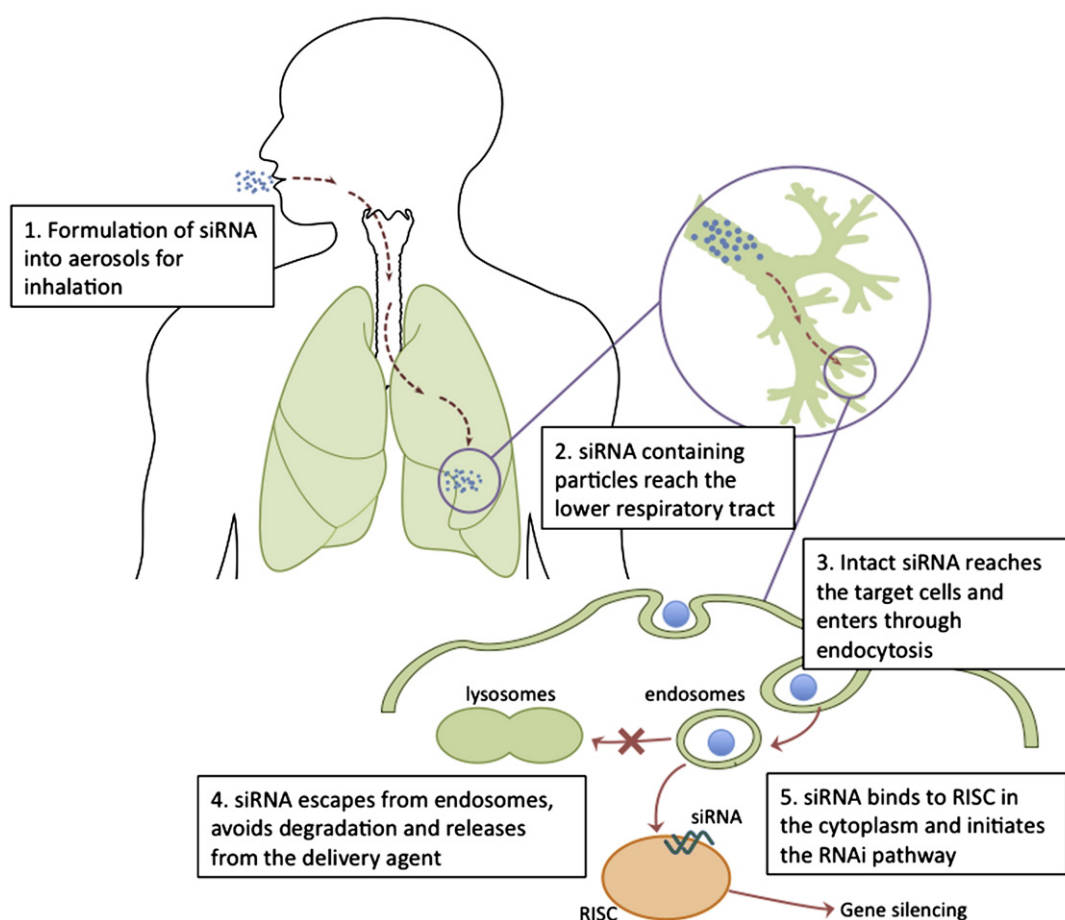
Fig. 1. Schematic illustrates steps involved in the delivery of siRNA into the lungs.

limits the effectiveness of therapy in repeated administration. Severe case of immune response can even lead to organ failure and the consequences could be fatal [51]. Furthermore, several types of virus may insert their genome randomly into the host chromosomes, disturbing gene function and resulting in insertional mutagenesis [52–54]. After the infamous Gelsinger case (18 years old volunteer Gelsinger died in a gene therapy trial using adenovirus) [51] and the French X-SCID case (one third of children developed leukemia in a gene therapy trial using retrovirus) [55], it is not hard to imagine that the chance of using viral vectors in any type of human nucleic acids therapy to get approval from the FDA will be extremely slim. The viral vectors are excellent agents to deliver siRNA for proof-of-concept studies, but appear to lack of real clinical application because of the safety issues. Alternative way to deliver siRNA is by non-viral methods which include any method that does not involve the use of viruses. The therapeutic siRNA are either administered directly into the site of action or delivered by non-viral vectors. Commonly used non-viral vectors for siRNA delivery include lipids, polymers and peptides.

Thomas et al. reviewed the non-viral siRNA delivery to the lung [56] and the review (published in 2007) focused on the delivery of siRNA for the treatment of three different lung virus infections: influenza, respiratory syncytial virus infection and severe acute respiratory syndrome (SARS). The field of siRNA delivery has rapidly expanded since then and a good number of *in vivo* studies were carried out in the past few years to investigate the local delivery of siRNA against various lung diseases including lung cancer [57], mycobacteria infection [36], pulmonary fibrosis [58,59], respiratory alphaherpesvirus infection [31], and other endogenous gene targets in the lungs [60,61]. In this review, we present the latest development of siRNA therapy for the treatment of various lung diseases. We discuss

the challenges to pulmonary drug administration and barriers to siRNA delivery to the lung, including both extracellular and intracellular barriers, with the focus on non-viral delivery methods. The findings of recent clinical studies of pulmonary siRNA therapy are also discussed.

2. Delivering siRNA to the lung

2.1. Challenges of pulmonary delivery

The pulmonary delivery of therapeutic macromolecules such as proteins and peptides has been investigated for over thirty years [41]. The challenges of siRNA delivery via the pulmonary route are similar to the delivery of other macromolecules. In order to develop an efficient siRNA pulmonary delivery system, it is important to understand the anatomical and physiological properties of the respiratory tract in the first place.

The respiratory tract can be divided into two regions: (i) the conducting region which consists of nasal cavity, pharynx, trachea, bronchi and bronchioles; and (ii) the respiratory region which consists of the respiratory bronchioles and alveoli. The conducting region is responsible for air conductance and the respiratory region is where the gaseous exchange takes place. The most prominent feature of the respiratory tract is the high degree of branching. According to the Wiebel's model of lung, there are 24 generations in total. This highly branched structure comprises airways with varying length and diameter presents an early barrier to targeted pulmonary delivery. Many lung diseases affect the lower region of the lungs. For the therapeutic agents to reach the diseased area, they must follow the airstream around the bend along the branched airway to the deep

lung area. The size of particles is an important factor in determining the site of deposition as illustrated in Fig. 2 [62]. In pulmonary delivery, size of particle is expressed in terms of aerodynamic diameter. Large particles (>6 μm aerodynamic diameter) carry high momentum and are more likely to be impacted on the airway wall at bifurcations instead of following the changing airstream. Therefore they are usually deposited higher up in the airway such as the back of the throat or pharynx. For small particles (<1 μm aerodynamic diameter), their movements are determined by Brownian motion. They are mostly exhaled during normal tidal breathing but pausing can enhance their deposition as the probability of the latter is proportional to the square root of time [63]. The optimal particle size for efficient deposition at the lower respiratory tract is found to be between 1 and 5 μm [41,64]. As the particle size further decreases towards the nanoscale, deposition in the lung increases again due to the increasing diffusional mobility [65]. For nanoparticles that are less than 100 nm, they appear to settle effectively to the alveolar region with a fractional deposition of around 50%. However, these ultrafine particles usually enter the lungs as larger agglomerates which can be broken down relatively easily into smaller particles on deposition. Various elimination pathways for nanoparticles exist in the lungs, including coughing, dissolution, mucociliarly escalator, translocation from the airways to other sites, phagocytosis by macrophages and neuronal uptake. What has not been established is the quantitative relationship of these pathways [66]. Very little is known about exactly how siRNA is cleared from the lungs.

Major barriers to pulmonary delivery include the mucociliary clearance action of the ciliated epithelial cells, and the presence of mucus, alveolar fluid and macrophages along different parts of the airways [67,68]. Particles that are deposited on the ciliated cells are rapidly removed by mucociliary clearance and are eventually being coughed up or swallowed. The mucus lines the respiratory epithelium from the nasal cavity to the terminal bronchioles [69]. The major component of mucus is mucins which are glycosylated proteins. Mucus constitutes a physical barrier as it increases the viscosity of the moist surface of the lung epithelial cells, thereby reducing drug penetration and diffusion rate. The alveolar fluid is found on the surface of alveoli epithelium as a thin layer of pulmonary surfactant which comprises phospholipids and other surfactant proteins. It has been reported that the pulmonary surfactant severely impeded the transfection efficiency of lipid-based nucleic acids delivery system, but not polymer-based system [70–72]. The alveolar macrophages located in the alveoli rapidly engulf the foreign particles by phagocytosis as a defense mechanism [73]. The siRNA that is taken up into the macrophages are subsequently degraded inside the cells.
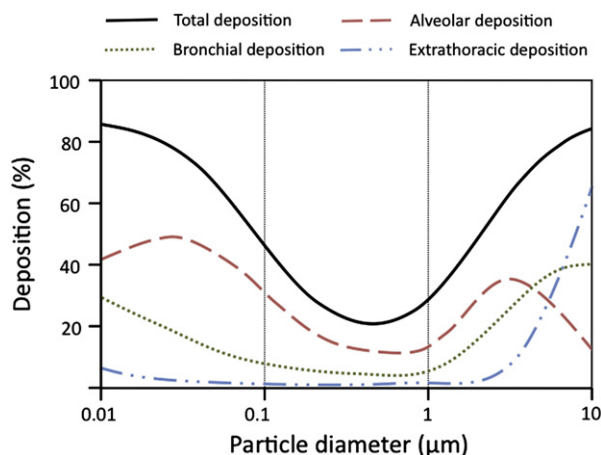
At disease state, the physiological conditions of the airways might be altered and pose a huge impact on the efficiency of the pulmonary delivery system. During infection and inflammation, there is an increase in mucus secretion and the mucociliary clearance is impaired [73,74]. The thickness, the viscosity and the composition of the mucus layer depend on the pathological condition and vary between individual [67]. It is important that these factors are being considered during the development of pulmonary delivery system for different therapeutic applications.

To overcome the anatomical and physiological barriers of the lungs, several delivery strategies can be incorporated. Besides using particles with small aerodynamic diameter suitable for deposition in the lower airways, it has been reported that the use of large porous particles can effectively avoid phagocytosis by the alveolar macrophages and prolong retention time in the lungs [75–77]. Porous particles over 10 μm in geometric diameter usually have a smaller aerodynamic diameter so that they are within the ideal aerodynamic size range for effective lung deposition, but their actual geometric size is too large to be removed by macrophages. To overcome the mucus barrier, the use of mucolytic agents, such as nacystelyn which breaks down the three dimensional gel network of mucus, or the use of mucus inhibitor, such as glycopyrrolate which inhibit mucus secretion, could be considered [78]. However their clinical benefits are limited [67,78]. Inhaled mannitol has been clinically proven to increase the mucus clearance in patients with cystic fibrosis or bronchietasis [79,80] and to improve the hydration and surface properties of sputum [81]. Reducing the mucus barrier by mannitol inhalation prior to the delivery of siRNA may thus be beneficial. The use of ultrasound and magnetic field has also been reported to direct and control the site of deposition of nucleic acids delivery systems in the airways [82–84].

2.2. Intracellular barriers to siRNA delivery

Once the siRNA has reached the surface of the target cells of the respiratory tract, assuming it successfully gets away with the extracellular barriers, it still has to cross the cellular membrane and gain access into the cytoplasm where RISC, the final target of siRNA, is located. siRNA is a negatively charged hydrophilic macromolecules with a molecular weight around 13 kDa. Base on its physicochemical properties, siRNA is not able to cross the biological membrane on its own. Therefore one of the main functions of a delivery vector is to facilitate the cellular uptake of siRNA.

Endocytosis is the major cellular uptake pathway known to be involved in non-viral nucleic acids delivery [85]. For efficient endocytosis to occur, particles should be under 150 nm in size. Particles within this size range could also avoid macrophage uptake and thereby delayed lung clearance [86]. For particles that enter the respiratory tract as large aggregates, they must be redispersed or deaggregated into the appropriate size before endocytosis could take place. Yet there are several different types of endocytic pathways which in turn affect the set of barriers the macromolecules may encounter, and the eventual fate of molecules could be very different [85]. Clathrin-mediated endocytosis is the major and the best-characterized endocytic pathway that occurs constitutively in all mammalian cells. Particles that are taken up by the cells through this pathway are enclosed in clathrin-coated vesicles. They are then transported into early endosomes, which fuse to form late endosomes and subsequently into the lysosomes. During the process, the pH inside the vesicles drops gradually to as low as pH 5.0 in the lysosomes where degradative enzymes including nuclease are present. Therefore the therapeutic siRNA must be able to exit from the endosomes/lysosomes into the cytoplasm before it is degraded by the nuclease (Fig. 1).

There are several strategies that can be employed to promote endosomal escape of siRNA. Polymers such as polyethylenimine (PEI) have the ability to trigger endosomal release. The 'proton sponge

Fig. 2. The effect of particle size on deposition in human respiratory tract following oral breathing of unit-density spheres at the reference pattern: 15-s breathing-cycle period and flow rate at 300 cm^3 s^{-1}.
Adapted from Ref. [62].

hypothesis' suggested that due to the high buffering capacity of PEI over a wide range of pH, the polymer becomes protonated as the pH drops inside the endosomes. This leads to the influx of chloride ions, protons and subsequently water into the endosomes. Eventually high osmotic pressure develops, causing the endosomes to burst open and release the contents into the cytoplasm [87]. However the cellular effect of 'endosomes bursting' has not been properly discussed or evaluated and the PEI is also known to be associated with toxicity problem. Another strategy is to incorporate pH sensitive fusogenic peptides to the delivery system. Fusogenic peptides such as GALA [88], KALA [89,90] INF7 [91] etc., have been employed in siRNA delivery both in vitro and in vivo. These peptides have the ability to undergo pH-dependent conformational change. At low pH, they adopt a 'membrane-disrupting' conformation and destabilize the endosomal membrane, thereby releasing the contents of endosomes into the cytoplasm.

Apart from clathrin-mediated endocytosis, caveolae-mediated endocytosis is another mechanism that is involved in the uptake of nucleic acids delivery systems [85]. After internalization, the delivery systems are enclosed in caveolin-coated vesicles called caveosomes which are non-acidic. Nuclease and other degradative enzymes are absence in caveosomes. The delivery systems can be directly transported to the Golgi and/or endoplasmic reticulum, thereby avoiding the lysosomal degradation. Since caveolin is abundantly expressed in many cell types including lung tissues [92,93], it is an important route of cell internalization in pulmonary delivery, and perhaps a more efficient route compared to clathrin-mediated endocytosis, especially with the delivery system that lacks the ability to escape from the acidic endosomal compartments.

The entrapment of siRNA inside the endosomes/lysosomes not only leads to the degradation of the nucleic acids, but may also trigger the activation of innate immunity. There has been evidence suggested that synthetic siRNA can be recognized by the toll-like receptors (TLRs), TLR7 and TLR8, inside the endosomal compartments, thereby stimulating the innate immune response [94–96]. 2′ modification of siRNA could circumvent the problem but this approach may adversely affect the gene silencing efficiency. Alternatively, early escape from or completely bypassing the endosomes during the intracellular delivery process could perhaps avoid this immunostimulatory activity of siRNA. Moschos et al. [61] reported that when TAT-conjugated siRNA was administered to the lung of the mouse, no immune response was observed. However when penetratin-conjugated siRNA was administered in the same way, innate immune response was observed, possibly through the activation of TLR. Since siRNA, TAT or penetratin alone did not induce innate immune responses, the different fate between TAT-conjugated and penetratin-conjugated siRNA systems could only be explained by their different cellular uptake mechanism. This study demonstrated the importance of the cellular uptake pathway and the intracellular trafficking in establishing the safety profile as well as the efficiency of siRNA delivery.

Lastly, phagocytosis is another cellular uptake mechanism that occurs in the respiratory tract. Since this route of uptake can only be performed by specialized cells such as alveolar macrophages, it is not expected to play an important role in siRNA delivery [85]. Unless the therapeutic siRNA is aimed to target the alveolar macrophages, e.g. treatment of mycobacterium infection, this pathway should be totally avoided, as molecules being taken up this pathway are eventually degraded in the phagolysosomes of the cells.

3. Pulmonary route of administration

Direct pulmonary delivery in humans is achieved by inhalation of aerosol generated by either an inhaler or nebulizer. Before a new therapeutic agent is used in a human clinical trial, it must demonstrate pre-clinical efficacy in suitable animal model with good translatability to humans [97]. There is no exception to therapeutic siRNA. In additional to inhalation, intratracheal and intranasal route are often used

to deliver therapeutic agents including siRNA to the lungs of animals because of the relatively simple setup. The summary of the in vivo studies of non-viral siRNA delivery to the lung is provided in Table 2, and the routes of administration commonly used in these studies are illustrated in Fig. 3. Intratracheal and intranasal methods may not be practical in the clinical setting. Intratracheal route is an invasive method of delivery which is not appropriate for human use. The success in delivering siRNA via intranasal route in rodents which is an obligate nose breathers cannot be extrapolated to human use because of the very different lung anatomy [98]. It is therefore important to take into consideration the route of administration in animal studies when assessing the delivery and therapeutic efficacy of a formulation for lung delivery.

3.1. Inhalation route

Inhalation is the most popular and a non-invasive way to deliver therapeutic agents into the lungs. Three main types of inhalation devices are currently available to deliver drug to the lung through inhalation. These include metered dose inhalers (MDIs), dry powder inhalers (DPIs) and nebulizers. With appropriate modification and optimization, these devices could be applied to pulmonary delivery of siRNA. To date, MDIs are the most commonly used inhalers. They are pressurized dosage form in which the therapeutic agents are either dissolved or suspended in propellants such as chlorofluorocarbons (CFCs) and hydrofluoroalkanes (HFAs). The propellants serve to provide a driving pressure to aerosolize the drug for inhalation into the respiratory tract. However both CFCs and HFAs are known to have environmental impact. The compatibility of formulation with propellants is another potential problem and the lung deposition is generally poor with MDIs. It may not be the best direction for developing inhalable siRNA.

DPIs are aerosol systems in which drugs are inhaled as clouds of dry particles. The use of DPIs appears to be a promising way to deliver siRNA to the lungs as they demonstrated successful in vivo delivery of other therapeutic macromolecules including insulin [108], parathyroid hormone [109] and low molecular weight heparin [110,111]. The formulation challenges and potential solutions for delivery of macromolecules such as proteins as powder aerosols have been reviewed [112]. Formulating biological macromolecules as powders for aerosol delivery is a challenge as it requires not only flowability and dispersibility of the powders but also biochemical stability of the macromolecules. To satisfy the latter requirement, proteins are usually formulated in amorphous glasses which are, however, physically unstable and tend to crystallize with inter-particulate bond formation and loss of powder dispersiblity. In addition, the biochemical stability requirement limits the manufacturing processes that can be used for protein powder production. Similar issues will be encountered for siRNA. Nevertheless, possible ways to tackle these challenges have been addressed [112]. The inhaled dry powder form of insulin (Exubera, marketed by Pfizer) was approved in Europe and the US for the treatment of diabetes in 2006 [113]. Although the product was withdrawn from the market in the subsequent year, Pfizer stressed that disappointing sales is the major reason for the withdrawal rather than safety or efficacy issues [114]. The safety profile of inhaled insulin was indeed reassuring and the efficacy was not inferior to the conventional injection formulations [115–119]. A second inhalable insulin product in dry powder form, Afresa, is currently awaiting for FDA approval. This encourages further development of dry powder form of macromolecules for inhalation.

There are different designs of DPI device and their delivery performance may vary. The key advantages of DPIs are the improved stability and sterility of biomolecules over liquid aerosols, and the propellant-free formulation [120]. Inhalable dry powder forms of proteins and peptides are commonly produced by spray-drying [121,122] and the same technique could be applied to siRNA [122,123]. Size of the spray-dried product must be carefully optimized

Table 2
Summary of *in vivo* study of non-viral siRNA delivery to the lung.

Route of administration	siRNA/siRNA target	Delivery vectors	Animal model	Notes	Year / Ref
Intranasal	PAI-1	Naked unmodified siRNA	C57BL/6 mice	Successfully reduced PAI-1 level in bronchoalveolar fluid	2010 [58]
Intranasal	siRNA-cy3	Naked unmodified siRNA	C57BL/6 mice	Low and inhomogeneous siRNA distribution in the lung	2010 [60]
Intranasal	GAPDH	Polymer (chitosan, chitosan-imidazole)	BALB/c mice C57BL/6 mice	~45% knockdown efficiency for both formulation	2010 [99]
Intratracheal	siGLO Green	Lipid (DharmaFECT)	C57BL/6 mice	siRNA distributed within epithelium cells of bronchi and bronchioles	2010
	SPARC			Successfully reduced inflammation in lungs	[59]
Intratracheal	siRNA-cy3	Naked modified siRNA (2′O-methyl modification)	C57BL/6 mice	High and homogenous siRNA distribution in lung	2010
	E-cadherin	Liposomes (AtuFECT01/ DPhyPE/DSPE-PEG)		Naked siRNA produced minor (~21%) knockdown of E-cadherin but not other targets	[60]
	VE-cadherin SFPD			Lipoplex evoked inflammation in lung	
Intratracheal	XCL1	Naked unmodified siRNA	C57BL/6 mice	Expression of XCL1 was suppressed by ~40–50% at mRNA and protein level	2009 [36]
Intranasal	EHV-1	Naked unmodified siRNA Lipid (Lipofectamine)	BALB/c mice	Successfully inhibit viral infection No significant difference between the naked siRNA and lipoplex	2009 [31]
Intratracheal	siGLO red	Liposomes (DOTAP)	Athymic nude mice	Longer retention in the lungs as compared to intravenous route of administration	2009 [100]
Intratracheal	EGFP	Polymer (PEI and PEI–PEG)	C57BL/6 mice	~42% knockdown efficiency of PEI–PEG formulation	2009 [70]
Inhalation (Nebulizer)	Akt1	Polymer (polyesteramine)	A/J mice K-ras^{LA1} mice	Successfully suppressed lung cancer progression	2008 [57]
Intratracheal	p38 MAP kinase	Naked siRNA	BALB/c mice	Peptide-siRNA formulations did not improve knockdown compared to naked siRNA and induced inflammatory response	2007
		Lipid (cholesterol) Cell penetrating peptide (TAT and penetratin)		Cholesterol-siRNA formulations extends duration but not magnitude of knockdown compared to naked siRNA	[61]
Intranasal	GFP	Polymer (chitosan)	C57BL/6 mice	Significant GFP knockdown in epithelial cells of bronchioles, ~37% expression of GFP compared to GFP mismatch	2006 [101]
Intranasal	RSV-P	Naked unmodified siRNA (C6-thiol modification) Lipid (TransIT-TKO)	BALB/c mice	Both formulations effectively inhibited RSV infection Transfection efficiency of naked siRNA was ~70–80% of lipoplex	2005 [102]
Intranasal	siSC2-5	Naked unmodified siRNA	Rhesus macaque	Successfully inhibit SCV replication in monkey respiratory tract	2005 [34]
Intratracheal	Fas	Naked unmodified siRNA	C57BL/6 mice	mRNA expression of Fas and caspase 8 were significantly reduced in lung tissue	2005
	Caspase 8			Animals were protected from hemorrhagic shock and sepsis-induced acute lung injury	[103]
Intratracheal	KC MIP-2	Naked unmodified siRNA	C57BL/6 mice	mRNA expression of KC and MIP-2 were reduced by ~40% in lung tissue, IL-6 and MPO activity were also reduced	2005 [104]
Intranasal	HO-1	Naked unmodified siRNA	C57BL/6 mice	Successfully silence endogenous gene expression in the lung	2004 [105]
Intranasal	GAPDH	Surfactant (InfaSurf)	C57BL/6 mice	GAPDH level in lung was inhibited to 50% in 24 h and 67% in 7 days	2004 [106]
Intranasal + intravenous	NP PA	Lipid (Oligofectamine)	BLAB/cAnNCR mice	Intranasal + intravenous delivery successfully inhibited viral replication at site of infection, less effective when only intravenous route was used	2004 [107]

for efficient delivery to the desirable site along the respiratory tract. A suitable delivery agent or formulation is required to protect the nucleic acids from degradation caused by the shear force and raised temperature during the drying process. The major drawback of DPIs is that drug deposition could be dependent on the patient inspiration flow rate. Therefore a suitable DPI device must be carefully designed to minimize such variation. In addition, the problem associated with de-aggregation of dry powders must be overcome [120].

Nebulizers are used to generate liquid aerosol and can be utilized to deliver large volumes of drug solutions or suspensions for inhalation. They are frequently used for drugs that are unsuitable to be formulated into MDIs or DPIs, and could be considered for siRNA delivery. During the process of nebulization, high shear stress is exerted on the siRNA which may lead to degradation of the nucleic

acids. This is a particular problem as 99% of generated aerosol droplets are recycled back into the reservoir [41] and the shear stress could be repeatedly exerted to the nucleic acids. In addition, biomolecules tend to be less stable in liquid form then in dry powder form. Stability is the prime concern in delivering siRNA with nebulizers. A suitable delivery vector is therefore required to protect siRNA from both physical and chemical degradation.

Although inhalation is a common way to deliver drug to the lungs, to our best knowledge, none of the *in vivo* study on siRNA therapy is intended for inhalation. Most of the *in vivo* studies use either intratracheal or intranasal route of delivery to the lungs. This could be due to the difficulty in formulating inhalable siRNA, especially in maintaining the stability and biological activity of siRNA during manufacturing and delivery process. Recently, Jensen et al. [123]

reported the production of siRNA loaded poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles by spray-drying. The integrity and biological activity of siRNA was successfully preserved during the drying process and the physicochemical properties of the particles are suitable for inhalation. It will be interesting to evaluate the *in vivo* performance of such formulation.

3.2. Intratracheal route

Intratracheal route is commonly used for lung delivery in animal studies for all kinds of drugs. However its clinical application is very limited. The method was initially described as an exposure technique to evaluate respiratory tract toxicity from airborne materials [42]. It was later adapted to assess pulmonary drug delivery, mainly in rodents [64]. Intratracheal route is a non-physiological and extremely uncomfortable delivery technique [42], it is therefore not employed as a routine route of drug administration for human use. Traditional method requires surgical procedure including tracheotomy, and the animal has to be anesthetized, usually with sodium pentobarbital or combination of xylazine and ketamine. The animal is then placed on a surgical board. The trachea is exposed and an endotracheal tube or needle is inserted through an incision made between the tracheal cartilaginous rings, projecting its tip at a defined position just before the tracheal bifurcation. The drug solution or suspension is instilled in the airways through the tube using a microsyringe (Fig. 3a).

A relatively non-invasive way to achieve pulmonary delivery via the intratracheal route was described by Bivas-Benita et al. [124]. No surgical procedure is required but the animal still needs to be anesthetized. While still under anesthesia, the tongue of the animal is gently pulled out and a microsprayer or similar device is carefully inserted endotracheally to deliver the aerosol into the lungs. Alternatively the animal is intubated through the mouth and trachea using a catheter or needle and the drug is being instilled in solution or suspension form. In these procedures, since the delivery is made through the mouth, it is also referred as oro-tracheal route [64], but correct insertion to the trachea may not be straight-forward (Fig. 3b).

Many *in vivo* studies employed the intratracheal route to deliver siRNA to the lungs [36,59,61,70,100]. The main advantage of intratracheal delivery is that it ensures high delivery efficiency with minimal drug lost, making it an excellent delivery method for proof-of-concept study when local delivery to the lung is desirable. The success of intratracheal siRNA delivery to the lungs of mouse was first reported by Perl et al. [103]. The group demonstrated that by using mice with over-expression of GFP, the intratracheal instillation of siRNA targeting GFP displayed a reduced fluorescent intensity in the lungs but not in the liver or the spleen. To investigate the gene knockdown efficiency, siRNA targeting fas and caspase-8 were delivered in the same way. The mRNA expression of fas and caspase-8 in lung tissues was found to be significantly reduced. This work also showed that the siRNAs did not induce lung inflammation as assessed by lung tissue interferon-α, tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 levels. Interestingly, delivery vector was not required to achieve *in vivo* siRNA transfection. Rosas-Taraco et al. [36] also reported successful delivery of naked siRNA through the intratracheal route. After the aerosolized siRNA targeting XCL1 was administered intratracheally to mouse infected with tuberculosis, the expression of XCL1 in the lungs was suppressed by 40–50% at both mRNA and protein level. Since the expression of XCL1 affects the formation of the lung granuloma which is known to be associated with mycobacterium tuberculosis infection, the local delivery of siRNA may be exploited as a novel therapy for the treatment of tuberculosis. The observation of successful *in vivo* gene knockdown mediated by naked siRNA is discussed later.

Some studies were performed to compare siRNA mediated silencing with or without delivery vector via the intratracheal route.

Moschos et al. [61] reported that naked siRNA failed to mediate any gene silencing *in vitro* in mouse fibroblast cell lines whereas conjugation of siRNA to cell penetrating peptides (TAT or penetratin), or lipid carrier (cholesterol) resulted in 20–40% gene silencing effect at mRNA level. Surprisingly when they proceeded to *in vivo* study, they found that naked siRNA managed to produce a 30–45% gene knockdown at mRNA level. Neither TAT nor penetratin improved the transfection efficiency as compared of naked siRNA. The use of cell penetrating peptides was even found to trigger inflammatory response in lung tissues. In a more recent study, Gutbier et al. [60] demonstrated that the epithelial E-cadherin was reduced moderately by 21% in mouse lung tissues following intratracheal delivery of naked target-specific siRNA, but no significant reduction of endothelial VE-cadherin or lamin B1 was observed. The use of lipid-based delivery vector (DPhyPE/DSPE-PEG) was found to evoke inflammatory response in the lungs.

Despite the success of intratracheal route of siRNA delivery in animal models, it must be noted that intratracheal route is an artificial way to deliver drug into the lungs. Drug deposition by this route tends to be less uniform as compared to inhalation [64]. Since intratracheal
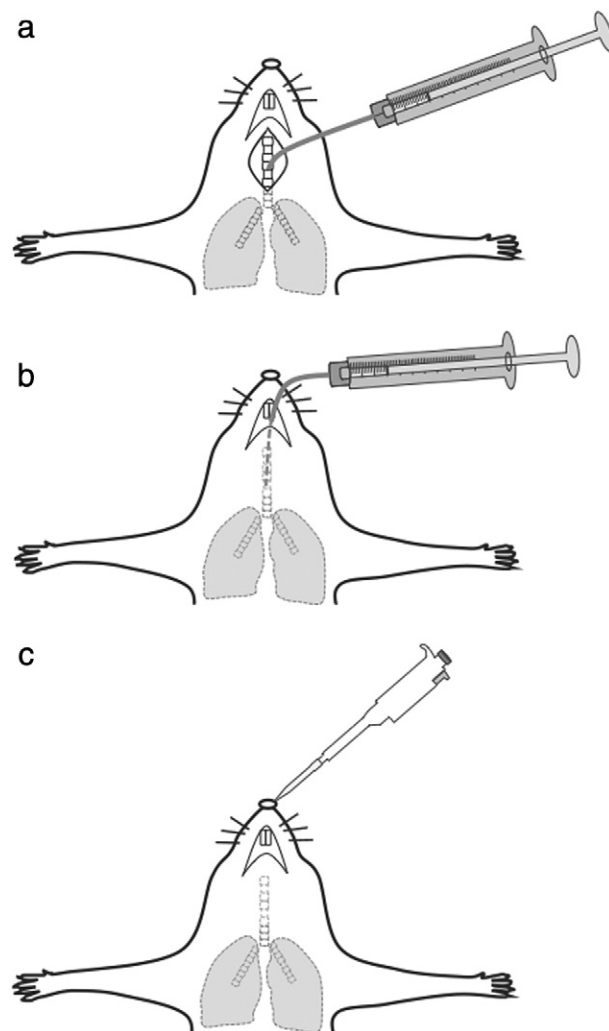

Fig. 3. Schematic illustrates the route of siRNA administration into the lungs used in *in vivo* studies. (a) Intratracheal route — trachea of the animal is exposed surgically and a tube is inserted through an incision made between the tracheal rings. The solution/suspension is instilled through the tube using a microsyringe. (b) Oro-tracheal route — the animal is intubated from the mouth to the trachea and the solution/suspension is instilled through the oral cavity to avoid the need of surgery. (c) Intranasal route — a micropipette or catheter containing the solution/suspension is inserted gently into the naris of the animal and the solution/suspension is slowly instilled into the nasal cavity.

route avoids the oropharynx deposition, it reduces drug loss. In addition, the effect of aerosol size which is the most critical factor affecting human lung deposition was not the focus of investigation in these studies. Therefore it is difficult to rely on this route of administration to accurately evaluate the *in vivo* delivery efficiency of a particular formulation.

3.3. Intranasal route

Intranasal route is another very popular way to deliver siRNA to the lungs in animal studies [31,34,58,60,99,101,102,105–107], partly because of its easy experimental setup. The animals are usually lightly to deeply anesthetized. The siRNA formulations are then instilled drop-wise to the naris to be breathed (Fig. 3c). There were a number of studies showed success in siRNA delivery to the lung *in vivo* through this route. Zhang et al. [105] were the first to demonstrate that lung-specific siRNA delivery could be achieved in mouse by intranasal administration without the need for any delivery vector. Naked siRNA targeting for heme oxygenase-1 (HO-1) effectively suppressed the expression of HO-1 gene in the injured lung models of mouse. Soon, the delivery of naked siRNA via intranasal route was shown to be successful in inhibiting lung viral infection in mouse [31,102] and rhesus macaque [34].

Since the anatomy and physiology of the lungs between mouse and human are very different, the success we see in intranasal siRNA delivery to the lungs could not be easily translated to human use. Mice are obligate nasal breathers, it is not surprising to see a high proportion of siRNA delivered through the nose was deposited in the lungs. In addition, the use of anesthetics in these animal studies may have impaired the mucociliary action [125], thereby overestimating the transfection efficiency of the formulations. In order to evaluate the feasibility of lung delivery via the nasal route in humans, Heyder et al. [126] performed an experiment on healthy adult volunteers who were asked to inhale mono-disperse particles through the nose at a fixed flow and volume. It was found that only about 3% of 1–5 μm particles were deposited in the bronchial airways through nose breathing. The majority of particles were deposited in the nose instead. For this reason, intranasal route is perfect for delivering siRNA to the nasopharynx area.

In fact one of the human clinical trials, which is currently at phase II stage, uses this route to deliver siRNA for the treatment of human RSV infection [15,16]. RSV is an upper respiratory disease. ALN-RSV01 is the siRNA, developed by Alnylam Pharmaceuticals, designed to inhibit the replication of RSV by interrupting the synthesis of the viral nucleocapsid protein. Since RSV replicates almost exclusively in the single outermost layer of cells of the respiratory epithelium including the lining of nasal passages and trachea, local delivery of ALN-RSV01 to nasopharynx could potentially treat the infections. ALN-RSV01 is delivered without a delivery vector as a nasal spray and targets the upper respiratory tract instead of the deep lung area. The author has also pointed out that the nasal spray of ALN-RSV01 would not be expected to reach the lower respiratory tract, and the development of an aerosolized drug that targets the lower respiratory tract will be desirable in future trials for the treatment of naturally infected patients [16].

Furthermore, intranasal route is being developed for a long time to administer macromolecules such as proteins and peptides for systemic delivery [125,127]. The relatively large absorptive surface area in the nasal cavity and the high vascularization of the nasal mucosa facilitate rapid absorption. The intranasal route is definitely a route to explore for systemic delivery of siRNA.

4. Non-viral delivery of siRNA to the lung

A delivery vector is often required to facilitate the cellular delivery of siRNA as the highly charged, hydrophilic natures of the macro-

molecules make it unable to cross the biological membrane to reach its target sites. Due to the safety concerns with viral-vector, many siRNA delivery studies focus on the development of non-viral vectors. An ideal siRNA delivery vector should consist of the following criteria: (i) condense siRNA into nanosized particles; (ii) protect siRNA from enzymatic degradation; (iii) facilitate cellular uptake; (iv) promote endosomal escape; (v) release siRNA into the cytoplasm where the RISC is located and (vi) negligible toxicity. In addition, optimal delivery should be achieved without compromising the gene silencing activity and specificity of siRNA. Commonly used non-viral siRNA delivery vector includes lipids, polymers and peptides. Interestingly, naked siRNA was also found to be successful in producing gene silencing effect *in vivo*. These vectors are illustrated in Fig. 4 and the pulmonary delivery using each of these systems is discussed in detail.

4.1. Naked siRNA

The term 'naked siRNA' or 'unformulated siRNA' refers to the delivery of siRNA without using any delivery agent. This includes the delivery of both unmodified siRNA and modified siRNA, formulated in saline or other simple excipients such as 5% dextrose. Since unmodified siRNA is susceptible to nuclease degradation, chemically modified siRNA was introduced initially to address this issue by increasing the nuclease resistance. The siRNA can also be chemically modified to improve potency, increase specificity, reduce immune response and reduce off-target effects. The subject of siRNA modification has been thoroughly reviewed by Watts et al. [128]. The major consideration of siRNA modification is to ensure that the gene silencing efficiency of siRNA is not adversely affected.

While the systemic delivery of naked siRNA generally failed to produce significant gene silencing effect [35,129,130], surprisingly there were some successes in delivering naked, unmodified siRNA locally including to the lung [31,34,36,58,60,102,103,105] (Table 1). Some of these studies have already been discussed in section 2.3.2 and 2.3.3. Usually the naked siRNA were administered either intranasally or intratracheally to the mouse and the siRNA were targeted against endogenous or viral genes. In some cases, the use of delivery vectors such as lipids or peptides showed no or only marginal improvement of gene silencing efficiency in the lung compared to naked siRNA [31,61,102]. These observations were truly intriguing, as the question of how the naked siRNA crossed the biological membrane in order to achieve the post-transcriptional gene silencing remains to be answered, although the reduced nuclease activity in the lung may provide part of the explanation [70]. Bitko et al. [102] reported that naked unmodified siRNA was able to prevent respiratory viral infection through siRNA-mediated gene knockdown when the naked siRNA was administered intranasally in the mouse. The authors suggested that the lung tissue is perhaps more receptive to exchange of molecules naturally or when infected, and this explanation is yet to be confirmed.

Nevertheless, the delivery of naked siRNA has been extended to clinical trials. As mentioned in the previous section, ALN-RSV01, which is a modified form of siRNA, were administered intranasally as nasal spray without the use of any delivery vector and the initial data confirmed its efficiency in reducing RSV infection [15,16]. Regardless of the mystery of how the naked siRNA can cross the cell membrane, gain access into the cytoplasm and remain intact to perform its biological action, the performance of naked siRNA so far in animal models and human studies are promising. The major advantage of using naked siRNA is simplicity without the need to concern about the toxicity and inflammatory responses associated with certain delivery vectors. However, it must be stressed that in order to make the formulation applicable for deep lung delivery in human, aerosol inhalation is still the administration route of choice. A particulate carrier will be required to deliver the siRNA in dry powder form, or a delivery agent will be needed to protect the siRNA from shear force
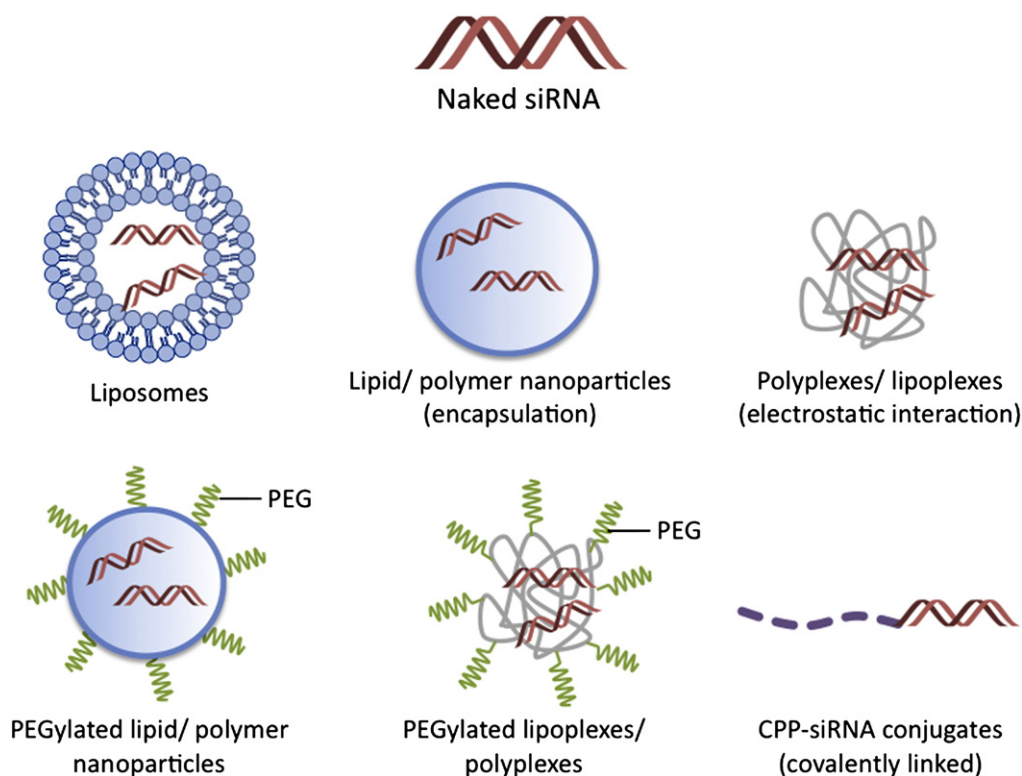
Fig. 4. Schematic illustration of different non-viral methods commonly used in siRNA delivery.

produced during the nebulizing process in suspension form. The use of delivery agents could also enhance specific cells targeting, improve pharmacokinetics and facilitate cellular uptake [131]. There were studies indicated that the use of optimized delivery vector could significantly improve lung delivery efficiency in animal models including nonhuman primates as compared to naked siRNA [132,133]. Therefore the development of an effective vector for pulmonary siRNA delivery is still the focus of research in the field.

4.2. Lipid-based delivery vectors

Lipid-based delivery systems are commonly used to deliver siRNA both *in vitro* and *in vivo* [134]. Typically cationic lipids or liposomes are used to form complexes with the negatively charged siRNA through spontaneous electrostatic interaction and the complexes are referred as lipoplexes. Many commercial siRNA transfection agents are lipids-based system, some of which are also employed for *in vivo* pulmonary delivery, *e.g.* Oligofectamine™ [107], TransIT-TKO [102] and DharmFECT [59]. The major challenges of using lipid-based delivery vectors in the clinic are their toxicity and the non-specific activation of inflammatory cytokines and interferon responses [135]. When the lipoplexes are to be aerosolized, special attention must be given to the stability [136] as they may undergo physical and chemical changes that may result in immature release and hence degradation of siRNA.

4.2.1. Cationic lipoplexes and liposomes

In order to maximize stability and delivery efficiency, and minimize toxic side effects, it is crucial to optimize the lipid composition, lipids to siRNA ratio and the lipoplexes preparation methods. Lipoplexes are easy to prepare and generally have good transfection efficiency due to their efficient interaction with the negatively charged cell membranes. However they also present some disadvantages such as poor stability and poor reproducibility [137]. Moreover, cationic lipids or liposomes are generally more toxic than their neutral counterparts. A study performed by Dokka et al. [138]

showed that pulmonary administration of cationic liposomes, Lipofectamine and DOTAP, elicited dose–response toxicity and pulmonary inflammation in mice. The effect was more pronounced with the multivalent Lipofectamine than the monovalent DOTAP. In addition, they found that neutral and anionic liposomes did not exhibit lung toxicity.

4.2.2. PEGylated lipids

To circumvent the problem associated with positive surface charge, hydrophilic polymer such as polyethylene glycol (PEG) has been employed to shield the surface charge of cationic lipids or liposomes, with the attempt to reduce the inflammatory response. PEG covalently linked to a phospholipid has been used for many years to prolong circulation in the bloodstream by reducing opsonization and subsequent cells capture by the mononuclear phagocyte system [139]. The cationic lipid, Genzyme Lipid (GL-67), was previously investigated for plasmid DNA delivery to the lung for the treatment cystic fibrosis in human clinical trials [140,141]. GL-67 consisted of DOPE:DMPE-PEG$_{5000}$ (at a molar ratio of 1:2:0.05) was complexed with DNA and the resulting lipoplexes were aerosolized and administrated to the patients with a jet nebulizer. For patients who received the treatment, their chloride abnormalities were found to be significantly improved and the bacterial adherence in the lungs was reduced. The promising results have led to the investigation of the same lipid-based system for siRNA delivery. Griesenbach et al. [142] assessed the *in vivo* efficiency of GL-67 in delivering siRNA into the lung of mice *via* the intranasal route. The siRNA targeting β-galactosidase (β-gal) was found to be localized in alveolar macrophages. β-gal mRNA was reduced by approximately 33%, but there was no significant change in β-gal protein expression. Perhaps the use of nebulizer could be considered for further investigation of this lipidic system.

4.2.3. Neutral lipids

Apart from PEGylation, another way to avoid toxicity and inflammatory response of lipid-based system is to use neutral lipid-

based systems. Although neutral systems have the more favorable safety profile, the lack of interaction with the negatively charged siRNA has limited their use as delivery vector. There are several methods to overcome this issue. One of the strategies is to link siRNA and lipids together through direct conjugation instead of electrostatic interaction. It has been demonstrated that chemical conjugation of siRNA to cholesterol can facilitate siRNA uptake *in vivo* after systemic administration [143]. Moschos et al. [61] applied this strategy to pulmonary delivery of siRNA. The cholesterol-siRNA conjugates were delivered to the mice by intratracheal administration. The duration of gene knockdown effect was extended by the cholesterol-conjugated system at mRNA level as compared to naked siRNA. However the magnitude of knockdown was not improved. Importantly, the cholesterol-siRNA conjugates did not elicit inflammation in mice tissues.

4.2.4. Lipids particles

An alternative strategy to improve the safety profile of lipid-based system is to encapsulate the siRNA inside the neutral lipid particles. Semple et al. [144] introduced the utilization of pH sensitive ionizable aminolipids to facilitate efficient encapsulation of antisense oligonucleotide in lipid vesicles. The important characteristic of these lipids is that they exhibit positive charge at acidic pH and neutral charge at physiological pH, thereby providing means for the lipids to efficiently interact with nucleic acids and the lipid vesicles can be rendered neutral at physiological pH. Semple et al. further developed this technique to produce stable nucleic acid lipid particles (SNALP) of uniform size with high siRNA encapsulation and delivery efficiency [145]. The two important parameters underlying lipid design for SNALP-mediated delivery are (i) the pKa of the ionizable cationic lipid, which determines the surface charge of the lipid particles under different pH conditions; and (ii) the ability, when protonated, to induce a nonbilayer phase structure when mixed with anionic lipids. This property of the lipids determines the membrane destabilizing capacity and the endosomolytic potential of the lipid particles. The SNALP demonstrated efficient *in vivo* gene silencing when administered intravenously in nonhuman primates [145,146]. These systems could be further exploited for pulmonary delivery.

4.2.5. Lipid-like molecules

Recently, a new class of lipid-like delivery molecules, termed lipidoids, was developed for siRNA delivery [132,147]. A combinatory approach was employed to allow the rapid synthesis of a large library of structurally diverse lipidoids. The library was screened for the ability to deliver siRNA *in vitro* and *in vivo*. The leading candidate for *in vivo* siRNA mediated gene knockdown was identified as $98N_{12}$-5. This system was tested for the ability to inhibit RSV replication in the lungs. After intranasal administration to the mouse model of RSV infection, the naked siRNA provided one log reduction in viral plaques in lung tissues, whereas the lipoidoid system at the same dose of siRNA provided greater than two log reductions in viral plaques [132]. This method provides the possibility of rapid design of new lipid-based materials for pulmonary siRNA delivery.

4.3. Polymer-based delivery vectors

One the attractive properties of polymer-based delivery vectors is their versatile nature that allows their physicochemical characteristics to be modified relatively easily to fit their purposes. In addition, it has been suggested that polymers generally do not evoke as strong an immune response as liposomes [13]. In general, polymer-based vectors can be divided into two categories: polycations and polymeric nanoparticles. Synthetic polycations such as polyethylenimine (PEI) [87], polyamidoamine (PAMAM) dendrimers [148] and natural polycations such as chitosan [149] are used for delivering DNA for a long time. These polymers have high cationic charge density and form polyplexes spontaneously with the negatively charged nucleic acids through electrostatic interaction. The size of polyplexes is affected by the molecular weight of polymers, the charge ratio, the pH and ionic strength of the medium. A net positive charge is required to maintain colloidal stability of the polyplexes. Because of the difference in physicochemical properties between plasmid DNA and siRNA, this approach is found to be less successful in delivering siRNA [150]. The stiffer structure of siRNA results in weaker interaction with polycations [151]. As a consequence, the polyplexes are less efficient in protecting siRNA against nuclease. Increase the amount of polycations may offer better protection but it might contribute to toxicity. The alternative way to deliver siRNA using polymer is to prepare polymeric nanoparticles which are usually solid nanoparticles made from hydrophobic polymers such as poly(D,L-lactide-co-glycolide) (PLGA) [122,152]. The siRNA is either dispersed throughout the polymer matrix, or surrounded by a polymeric shell. The polymeric nanoparticles could offer better protection to siRNA, but the loading efficiency is the main challenge to this delivery method.

4.3.1. PEI

Synthetic polymer PEI is commonly used in siRNA delivery due to its high intracellular delivery efficiency. According to the proton sponge hypothesis as mentioned earlier in Section 2.2, the high buffering capacity of PEI inside the acidic endosomes leads to the swelling and rupture of endosomes, thereby promoting endosomal escape [87]. PEI is considered as the 'gold standard' for *in vitro* gene delivery and its transfection efficiency depends on the molecular weight and degree of branching. Merkel et al. [70] investigated the use of PEI and PEI–PEG polyplexes for siRNA delivery to the lung. The polyplexes containing siRNA targeting EGFP were administered to the actin-EGFP expressing mice through intratracheal instillation. The result showed that the PEI–PEG system displayed a better delivery efficiency with a 42% knockdown of EFGP expression in the lung tissues. However the PEI–PEG polyplexes also produced a moderate proinflammatory effect as the levels of TNF-α and IL-6 were elevated. PEI also associates with problems such as relatively high toxicity and the lack of biodegradability [86]. To tackle these problems, Xu et al. developed a new degradable PEI derivatives, poly(ester amine)-*alt*-PEG copolymer for *in vivo* siRNA delivery [57]. A previous study confirmed the biodegradability and low toxicity of this polymer [152]. For *in vivo* pulmonary delivery, the polyplexes containing siRNA targeting Akt1 were aerosolized and administered to mouse models of lung cancer through a nose-only inhalation system. The lung cancer progression of treated mice was significantly suppressed without showing any significant sign of toxicity. To facilitate rapid development of PEI derivatives for siRNA delivery, Thomas et al. [153] employed a high throughput synthesis and screening method to create a combinatorial library of biodegradable PEI derivatives to identify a novel vectors for gene delivery. The study focused on DNA delivery through intravenous injection to the mice and the technique could be easily adapted for the investigation of pulmonary siRNA delivery.

4.3.2. Chitosan

Chitosan has attracted much attention as potential vector for siRNA delivery due to its natural, biocompatible, biodegradable and low toxicity nature. In addition, the mucoadhesive and mucosa permeation properties of chitosan make it particularly favorable for pulmonary delivery. Unfortunately, it only shows moderate *in vitro* and *in vivo* transfection efficiency, possibility due to its relatively weak ability to promote endosomal escape [86,149]. Chitosan-based formulation for siRNA delivery has been thoroughly reviewed by Mao et al. recently [154]. The feasibility of using chitosan to deliver siRNA into the lungs was demonstrated by Howard et al. [101]. Chitosan/siRNA nanoparticles were prepared by the addition of small volume of siRNA into chitosan stock solution in sodium acetate buffer.

After the nanoparticles were administered intranasally to EGFP-expressing transgenic mice, the number of EGFP expressing epithelial cells in the bronchioles was reduced by 43% (compare to untreated control) and 37% (compared to mismatch control) [101], and the stability of the system increased with the molecular weight and degree of deacetylation [155]. A recent study conducted by the same group described the aerosolization of chitosan/siRNA nanoparticles for intratracheal administration with the attempt to improve lung deposition in mice [133]. The aerosol was generated by an AeroProbe™ nebulizing catheter which was inserted non-invasively into the animal below the vocal cords. This delivery route was compared with the intranasal route and, as expected, the results confirmed that the aerosolized formulation improved the lung deposition of the same siRNA dosage, and the EGFP expression in the transgenic mice model was reduced by 68%. However the author also pointed out that catheter insertion ensured sufficient delivery and the effect of size in lung deposition was not investigated. The size of aerosol droplet generated in this study was around 20 μm which is not suitable for inhalable formulation. It is critical to reduce droplet diameters in the range of 1–5 μm to allow optimal lung deposition in human following inhalation. To enhance the siRNA delivery efficiency of chitosan, Katas et al. [151] described the ionic gelation method using sodium tripolyphosphate to produce siRNA nanoparticles. Compare to electrostatic complexation, the chitosan/siRNA nanoparticles produced by ionic gelation method showed a better *in vitro* gene silencing effect due to better stability and higher loading efficiency. It would be interesting to assess the *in vivo* performance of such system through pulmonary delivery.

4.3.3. PLGA

PLGA is a biodegradable and biocompatible polymer that is being investigated to provide controlled release of therapeutic macromolecules such as peptides, proteins and plasmid DNA [156,157]. The versatility of PLGA allows chemical modification and many derivatives of PLGA with tailored properties are designed to fit their delivery purposes. Unlike polycations, PLGA does not form polyplexes with nucleic acids. Instead the nucleic acids are encapsulated in the PLGA nanoparticles. By manipulating the degradation rate of PLGA, sustained release of the encapsulated nucleic acids could be achieved. To design an inhalable formulation, particle size is of extreme importance. Double emulsion-solvent evaporation is conventionally used to prepare PLGA nanoparticles and the dry particles are then collected by lyophilization [157]. However large specific surface area of the particles often leads to aggregation and the ice formation during lyophilization process also facilitates aggregation. As a result particles prepared by this method are not desirable for inhalable formulation as large particle size is likely to be impacted out in the oropharynx area. Takashima et al. [158] demonstrated that aggregation of PLGA particles containing plasmid DNA could be effectively minimized without loss of *in vitro* transfection efficiency using spray-drying technique. This method was soon adapted by Jensen et al. [123] who demonstrated the production siRNA containing PLGA nanoparticles by spray-drying with controlled size distribution that are intended for inhalation. PLGA nanoparticles were co-spray-dried with a carbohydrate excipient such as lactose, trehalose and mannitol to protect the siRNA containing nanoparticles against the shear forces and raised temperature during the process of spray-drying, and to ensure the particle size is suitable for inhalation. The physicochemical properties and the powder yield of the formulation could be optimized by varying the amount of excipients and the nanoparticles to excipients ratios. The study showed that the integrity and biological activity of the siRNA were preserved during the spray-drying process. The potential of this technique to generate inhalable siRNA formulation will be confirmed by further investigation on lung deposition and *in vivo* gene silencing efficiency.

4.4. Peptide-based delivery vectors

Since the discovery of TAT protein from HIV-1 which is responsible for the cellular uptake of the virus [159], a variety of cell-penetrating peptides (CPPs) have been derived or synthesized. CPPs are frequently employed to facilitate the transport of therapeutic macromolecules into the cells and this strategy has been extended to the delivery of siRNA [160,161]. CPPs and derivatives that are investigated for siRNA delivery included TAT [61,162,163], penetratin [163–165], transportan [163], MPG [166,167], CADY [168,169] and LAH4 [170]. The peptides are either covalently attached to siRNA through disulphide bond formation, or bind to siRNA through electrostatic interaction to form complexes in a non-covalent manner. Due to the sequence diversity of different CPPs, their mechanism of action is also expected to vary. Some of these peptides improve cellular delivery by efficient transport of their cargo across the biological membranes, whereas others promote endosomal escape and prevent lysosomal degradation. However the exact mechanisms of these peptides are still controversial. The activities of different CPPs have been extensively reviewed [161,167,171–174].

Although a variety of CPPs have been investigated for siRNA delivery, very few studies reported their use for *in vivo* pulmonary delivery. To date only one group has described the use of CPPs for *in vivo* delivery of siRNA. Using siRNA targeting p38 MAP kinase, Moschos et al. [61,175] compared the gene silencing effect between naked siRNA, TAT-siRNA conjugates and penetratin-siRNA conjugates both *in vitro* and *in vivo*. The peptides were conjugated to the siRNA through disulfide linkage. All siRNA conjugates, but not naked siRNA, showed small but significant reduction (20–36%) of p38 MAP kinase expression at mRNA level in mouse fibroblast L929 cell lines. Interestingly, when the systems were administrated intratracheally to mouse model, naked siRNA was able to produce a 30–45% knockdown of p38 MAP kinase in the lung of the animal despite the absence of knockdown effect *in vitro*. Although moderate gene knockdown was observed in TAT- and penetratin-siRNA conjugated systems, the TAT or penetratin peptide alone also produced similar knockdown level, suggesting that the CPPs could be the modulators of p38 MAP kinase expression and the gene knockdown effect was not produced by the siRNA. Furthermore, penetratin-siRNA conjugates were found to induce an *in vivo* innate immune response, but not with the naked siRNA, TAT-siRNA conjugates, penetratin or TAT alone. The understanding of the mechanism of CPPs activity was crucial to determine their application in siRNA delivery. In addition, it has been suggested that covalent attachment of CPP to siRNA may have a negative effect on cellular delivery as the biological activity of the peptides may have been altered during chemical modification [161]. The non-covalent complexing method provides an alternative strategy but the effect in lung delivery to animal model remains to be seen. More work needs to be done in this area to develop an effective peptide-based siRNA vector system for *in vivo* pulmonary delivery.

5. Conclusions

Since the discovery of siRNA, its therapeutic potential has been rapidly recognized. Many studies have been carried out in the past few years in delivering siRNA to the lungs for the treatment of various lung diseases. However the majority of these investigations focus on the design of siRNA molecules to target a specific disease instead of looking into the delivery perspective. In order to make siRNA therapy practical in treating human lung diseases, we believe that the inhalation route, especially in dry powder form, is the best of choice. Unfortunately there is very few literature described siRNA formulation specifically designed for inhalation for clinical use. Very recently, Jensen et al. [123] reported the spray-drying method to produce PLGA based siRNA nanoparticles with physiochemical

properties that are intended for inhalation, but they have not reached the stage of *in vivo* study yet.

The major challenges to pulmonary delivery of siRNA include: (i) there is no obvious correlation between *in vitro* and *in vivo* study (*e.g.* the naked siRNA failed to show gene silencing effect *in vitro* in many studies, but the *in vivo* studies often proved otherwise); (ii) it is very hard to translate the information from animal (especially rodents which are the most frequently used model) to human as the anatomy and physiology of the respiratory tract between animal and human are very distinct; and (iii) the administration routes used for animal studies are not suitable for human use, and it is extremely difficult to evaluate the delivery efficiency of the formulation before it enters the clinical study. Since many studies have successfully proved the therapeutic efficiency of siRNA on various lung diseases, the final hurdle we need to overcome is the development of inhalable and stable siRNA formulations for human use in a practical way before siRNA therapeutics for lung diseases become available in the clinic.

Acknowledgements

References

[1] P.D. Zamore, RNA interference: listening to the sound of silence, Nature Structural Biology 8 (2001) 746–750.

[2] G.J. Hannon, RNA interference, Nature 418 (2002) 244–251.

[3] S.M. Elbashir, W. Lendeckel, T. Tuschl, RNA interference is mediated by 21- and 22-nucleotide RNAs, Genes & Development 15 (2001) 188–200.

[4] J.Y. Yu, S.L. DeRuiter, D.L. Turner, RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells, Proceedings of the National Academy of Sciences of the United States of America 99 (2002) 6047–6052.

[5] N. Agrawal, P.V.N. Dasaradhi, A. Mohmmed, P. Malhotra, R.K. Bhatnagar, S.K. Mukherjee, RNA interference: biology, mechanism, and applications, Microbiology and Molecular Biology Reviews 67 (2003) 657–685.

[6] M.T. McManus, P.A. Sharp, Gene silencing in mammals by small interfering RNAs, Nature Reviews. Genetics 3 (2002) 737–747.

[7] J. Gil, M. Esteban, Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action, Apoptosis 5 (2000) 107–114.

[8] M. Kumar, G.G. Carmichael, Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes, Microbiology and Molecular Biology Reviews 62 (1998) 1415–1434.

[9] M.A. McAnuff, G.R. Rettig, K.G. Rice, Potency of siRNA versus shRNA mediated knockdown in vivo, Journal of Pharmaceutical Sciences 96 (2007) 2922–2930.

[10] A.V. Vlassov, B. Korba, K. Farrar, S. Mukerjee, A.A. Seyhan, H. Ilves, R.L. Kaspar, D. Leake, S.A. Kazakov, B.H. Johnston, shRNAs targeting hepatitis C: effects of sequence and structural features, and comparison with siRNA, Oligonucleotides 17 (2007) 223–236.

[11] P.K. Kaiser, R.C.A. Symons, S.M. Shah, E.J. Quinlan, H. Tabandeh, D.V. Do, G. Reisen, J.A. Lockridge, B. Short, R. Guerciolini, Q.D. Nguyen, I. siRNA-027 study, RNAi-based treatment for neovascular age-related macular degeneration by siRNA-027, American Journal of Ophthalmology 150 (2010) 33–39.

[12] J. Shen, R. Samul, R.L. Silva, H. Akiyama, H. Liu, Y. Saishin, S.F. Hackett, S. Zinnen, K. Kossen, K. Fosnaugh, C. Vargeese, A. Gomez, K. Bouhana, R. Aitchison, P. Pavco, P.A. Campochiaro, Suppression of ocular neovascularization with siRNA targeting VEGF receptor 1, Gene Therapy 13 (2006) 225–234.

[13] V. Brower, RNA interference advances to early-stage clinical trials, Journal of the National Cancer Institute 102 (2010) 1459–1461.

[14] M.E. Davis, J.E. Zuckerman, C.H.J. Choi, D. Seligson, A. Tolcher, C.A. Alabi, Y. Yen, J.D. Heidel, A. Ribas, Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles, Nature 464 (2010) 1067–U1140.

[15] J. DeVincenzo, J.E. Cehelsky, R. Alvarez, A. Elbashir, S. Elbashir, J. Harborth, I. Toudjarska, L. Nechev, V. Murugaiah, A. Van Vliet, A.K. Vaishnaw, R. Meyers, Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV), Antiviral Research 77 (2008) 225–231.

[16] J. DeVincenzo, R. Lambkin-Williams, T. Wilkinson, J. Cehelsky, S. Nochur, E. Walsh, R. Meyers, J. Gollob, A. Vaishnaw, A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus, Proceedings of the National Academy of Sciences of the United States of America 107 (2010) 8800–8805.

[17] N.S. Dejneka, S.H. Wan, O.S. Bond, D.J. Kornbrust, S.J. Reich, Ocular biodistribution of bevasiranib following a single intravitreal injection to rabbit eyes, Molecular Vision 14 (2008) 997–1005.

[18] A.O. Gaber, A. Patel, M.S. Polinsky, E.C. Squiers, Safety and tolerability of intravenous QPI-1002, a siRNA, for prophylaxis of delayed graft function (DGF) in deceased donor renal allograft recipients at increased risk of DGF: initial

[19] I. Toudjarska, A. Judge, J. Brodsky, K. McClintock, S.D. de Jong, E. Ambegia, T. Buck, A. Akinc, T. Racie, L.B. Jeffs, E. Yaworski, J. Gollob, I. MacLachlan, D.W. Sah, D. Bumcrot, Development of ALN-VSP: an RNAi therapeutic for liver malignancies, Hepatology 50 (2009) 1829.

[20] R. Alvarez, T. Borland, Q. Chen, S. Milstein, T. Nguyen, G. Hinkle, S. Kuchimanchi, J. Costigan, V. Ristoiu, G. Wang, G. Cole, R. Dorkin, A. Akinc, L. Nechev, V. Kosovrasti, L. Tchangov, M. Tracy, L. Jeffs, I. MacLachlan, P. Lutwyche, D. Martins, S. Costelha, M.J. Saraiva, D.W.Y. Sah, ALN-TTR, an RNAi therapeutic for the treatment of transthyretin-mediated amyloidosis, Amyloid: Journal of Protein Folding Disorders 17 (2010) 51–52.

[21] S. Costelha, R. Alvarez, T. Borland, L. Nechev, L. Jeffs, D.W.Y. Sah, M.J. Saraiva, Evaluation of ALN-TTR on transthyretin tissue deposition in a FAP V30M animal model, Amyloid: Journal of Protein Folding Disorders 17 (2010) 52-52.

[22] S.A. Leachman, R.P. Hickerson, M.E. Schwartz, E.E. Bullough, S.L. Hutcherson, K.M. Boucher, C.D. Hansen, M.J. Eliason, G.S. Srivatsa, D.J. Kornbrust, F.J.D. Smith, W.H.I. McLean, L.M. Milstone, R.L. Kaspar, First-in-human mutation-targeted siRNA phase Ib trial of an inherited skin disorder, Molecular Therapy 18 (2010) 442–446.

[23] R.P. Hickerson, F.J.D. Smith, R.E. Reeves, C.H. Contag, D. Leake, S.A. Leachman, L.M. Milstone, W.H.I. McLean, R.L. Kaspar, Single-nucleotide-specific siRNA targeting in a dominant-negative skin model, The Journal of Investigative Dermatology 128 (2008) 594–605.

[24] M. Aleku, P. Schulz, O. Keil, A. Santel, U. Schaeper, B. Dieckhoff, O. Janke, J. Endruschat, B. Durieux, N. Roder, K. Loffler, C. Lange, M. Fechtner, K. Mopert, G. Fisch, S. Dames, W. Arnold, K. Jochims, K. Giese, B. Wiedenmann, A. Scholz, J. Kaufmann, Atu027, a liposomal small interfering RNA formulation targeting protein kinase N3, inhibits cancer progression, Cancer Research 68 (2008) 9788–9798.

[25] A. Santel, M. Aleku, N. Roder, K. Mopert, B. Durieux, O. Janke, O. Keil, J. Endruschat, S. Dames, C. Lange, M. Eisermann, K. Loffler, M. Fechtner, G. Fisch, C. Vank, U. Schaeper, K. Giese, J. Kaufmann, Atu027 prevents pulmonary metastasis in experimental and spontaneous mouse metastasis models, Clinical Cancer Research 16 (2010) 5469–5480.

[26] S.D. Li, L. Huang, Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells, Molecular Pharmaceutics 3 (2006) 579–588.

[27] D. Jere, C.X. Xu, R. Arote, C.H. Yun, M.H. Cho, C.S. Cho, Poly(beta-amino ester) as a carrier for si/shRNA delivery in lung cancer cells, Biomaterials 29 (2008) 2535–2547.

[28] A.W. Tong, Small RNAs and non-small cell lung cancer, Current Molecular Medicine 6 (2006) 339–349.

[29] X.L. Ren, Y.M. Xu, W. Bao, H.J. Fu, C.G. Wu, Y. Zhao, Z.K. Li, J. Zhang, S.Q. Li, W.Q. Chen, T. Wang, R. Zhang, L.H. Zhang, G.S. Qian, S.Y. Chen, L.T. Jia, A.G. Yang, Inhibition of non-small cell lung cancer cell proliferation and tumor growth by vector-based small interfering RNAs targeting HER2/neu, Cancer Letters 281 (2009) 134–143.

[30] D.E. Zamora-Avila, P. Zapata-Benavides, M.A. Franco-Molina, S. Saavedra-Alonso, L.M. Trejo-Avila, D. Resendez-Perez, J.L. Mendez-Vazquez, J. Isaias-Badillo, C. Rodriguez-Padilla, WT1 gene silencing by aerosol delivery of PEI-RNAi complexes inhibits B16-F10 lung metastases growth, Cancer Gene Therapy 16 (2009) 892–899.

[31] A. Fulton, S.T. Peters, G.A. Perkins, K.W. Jarosinski, A. Damiani, M. Brosnahan, E.L. Buckles, N. Osterrieder, G.R. Van de Walle, Effective treatment of respiratory alphaherpesvirus infection using RNA interference, PLoS ONE 4 (2009).

[32] R. Alvarez, S. Elbashir, T. Borland, I. Toudjarska, P. Hadwiger, M. John, I. Roehl, S.S. Morskaya, R. Martinello, J. Kahn, M. Van Ranst, R.A. Tripp, J.P. DeVincenzo, R. Pandey, M. Maier, L. Nechev, M. Manoharan, V. Kotelianski, R. Meyers, RNA interference-mediated silencing of the respiratory syncytial virus nucleocapsid defines a potent antiviral strategy, Antimicrobial Agents and Chemotherapy 53 (2009) 3952–3962.

[33] K. Zhou, H.X. He, Y.Y. Wu, M.X. Duan, RNA interference of avian influenza virus H5N1 by inhibiting viral mRNA with siRNA expression plasmids, Journal of Biotechnology 135 (2008) 140–144.

[34] B.J. Li, Q.Q. Tang, D. Cheng, C. Qin, F.Y. Xie, Q. Wei, J. Xu, Y.J. Liu, B.J. Zheng, M.C. Woodle, N.S. Zhong, P.Y. Lu, Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in rhesus macaque, Nature Medicine 11 (2005) 944–951.

[35] Q. Ge, L. Filip, A.L. Bai, T. Nguyen, H.N. Eisen, J. Chen, Inhibition of influenza virus production in virus-infected mice by RNA interference, Proceedings of the National Academy of Sciences of the United States of America 101 (2004) 8676–8681.

[36] A.G. Rosas-Taraco, D.M. Higgins, J. Sanchez-Campillo, E.J. Lee, I.M. Orme, M. Gonzalez-Juarrero, Intrapulmonary delivery of XCL1-targeting small interfering RNA in mice chronically infected with *Mycobacterium tuberculosis*, American Journal of Respiratory Cell and Molecular Biology 41 (2009) 136–145.

[37] C.C. Lee, B.L. Chiang, RNA interference: new therapeutics in allergic diseases, Current Gene Therapy 8 (2008) 236–246.

[38] R.M. Seguin, N. Ferrari, Emerging oligonucleotide therapies for asthma and chronic obstructive pulmonary disease, Expert Opinion on Investigational Drugs 18 (2009) 1505–1517.

[39] M. Ulanova, M. Marcet-Palacios, S. Munoz, S. Asfaha, M.K. Kim, A.D. Schreiber, A.D. Befus, Involvement of Syk kinase in TNF-induced nitric oxide production by airway epithelial cells, Biochemical and Biophysical Research Communications 351 (2006) 431–437.

[40] N. Vij, S.Y. Fang, P.L. Zeitlin, Selective inhibition of endoplasmic reticulum-associated degradation rescues Delta F508-cystic fibrosis transmembrane

regulator and suppresses interleukin-8 levels, The Journal of Biological Chemistry 281 (2006) 17369–17378.

[41] R.U. Agu, M.I. Ugwoke, M. Armand, R. Kinget, N. Verbeke, The lung as a route for systemic delivery of therapeutic proteins and peptides, Respiratory Research 2 (2001) 198–209.

[42] K.E. Driscoll, D.L. Costa, G. Hatch, R. Henderson, G. Oberdorster, H. Salem, R.B. Schlesinger, Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations, Toxicological Sciences 55 (2000) 24–35.

[43] C.X. Shen, A.K. Buck, X.W. Liu, M. Winkler, S.N. Reske, Gene silencing by adenovirus-delivered siRNA, FEBS Letters 539 (2003) 111–114.

[44] T. Hosono, H. Mizuguchi, K. Katayama, Z.L. Xu, F. Sakurai, A. Ishii-Watabe, K. Kawabata, T. Yamaguchi, S. Nakagawa, T. Mayumi, T. Hayakawa, Adenovirus vector-mediated doxycycline-inducible RNA interference, Human Gene Therapy 15 (2004) 813–819.

[45] C.T. Yang, J.M. Li, H.H. Weng, Y.C. Li, H.C. Chen, M.F. Chen, Adenovirus-mediated transfer of siRNA against survivin enhances the radiosensitivity of human non-small cell lung cancer cells, Cancer Gene Therapy 17 (2010) 120–130.

[46] Z.C. Han, M. Gorbatyuk, J. Thomas, A.S. Lewin, A. Srivastava, P.W. Stacpoole, Down-regulation of expression of rat pyruvate dehydrogenase E1 alpha gene by self-complementary adeno-associated virus-mediated small interfering RNA delivery, Mitochondrion 7 (2007) 253–259.

[47] H. Sumimoto, S. Yamagata, A. Shimizu, H. Miyoshi, H. Mizuguchi, T. Hayakawa, M. Miyagishi, K. Taira, Y. Kawakami, Gene therapy for human small-cell lung carcinoma by inactivation of Skp-2 with virally mediated RNA interference, Gene Therapy 12 (2005) 95–100.

[48] H.Y. Huang, C.C. Lee, B.L. Chiang, Small interfering RNA against interleukin-5 decreases airway eosinophilia and hyper-responsiveness, Gene Therapy 15 (2008) 660–667.

[49] U. Griesenbach, E. Alton, U.K.C.F.G.T. Co, Gene transfer to the lung: lessons learned from more than 2 decades of CF gene therapy, Advanced Drug Delivery Reviews 61 (2009) 128–139.

[50] N. Bessis, F.J. GarciaCozar, M.C. Boissier, Immune responses to gene therapy vectors: influence on vector function and effector mechanisms, Gene Therapy 11 (2004) S10–S17.

[51] S.E. Raper, N. Chirmule, F.S. Lee, N.A. Wivel, A. Bagg, G.P. Gao, J.M. Wilson, M.L. Batshaw, Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer, Molecular Genetics and Metabolism 80 (2003) 148–158.

[52] U.P. Dave, N.A. Jenkins, N.G. Copeland, Gene therapy insertional mutagenesis insights, Science 303 (2004) 333-333.

[53] S. Hacein-Bey-Abina, C. von Kalle, M. Schmidt, F. Le Deist, N. Wulffraat, E. McIntyre, I. Radford, J.L. Villeval, C.C. Fraser, M. Cavazzana-Calvo, A. Fischer, A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency, The New England Journal of Medicine 348 (2003) 255–256.

[54] D.B. Kohn, M. Sadelain, J.C. Glorioso, Occurrence of leukaemia following gene therapy of X-linked SCID, Nature Reviews. Cancer 3 (2003) 477–488.

[55] E. Marshall, Gene therapy: second child in French trial is found to have leukemia, Science 299 (2003) 320-320.

[56] M. Thomas, J.J. Lu, J.Z. Chen, A.M. Klibanov, Non-viral siRNA delivery to the lung, Advanced Drug Delivery Reviews 59 (2007) 124–133.

[57] C.X. Xu, D. Jere, H. Jin, S.H. Chang, Y.S. Chung, J.Y. Shin, J.E. Kim, S.J. Park, Y.H. Lee, C.H. Chae, K.H. Lee, G.R. Beck, C.S. Cho, M.H. Cho, Poly(ester amine)-mediated, aerosol-delivered Akt1 small interfering RNA suppresses lung tumorigenesis, American Journal of Respiratory and Critical Care Medicine 178 (2008) 60–73.

[58] T. Senoo, N. Hattori, T. Tanimoto, M. Furonaka, N. Ishikawa, K. Fujitaka, Y. Haruta, H. Murai, A. Yokoyama, N. Kohno, Suppression of plasminogen activator inhibitor-1 by RNA interference attenuates pulmonary fibrosis, Thorax 65 (2010) 334–340.

[59] J.C. Wang, S.L. Lai, X.J. Guo, X.F. Zhang, B. de Crombrugghe, S. Sonnylal, F.C. Arnett, X.D. Zhou, Attenuation of fibrosis in vitro and in vivo with SPARC siRNA, Arthritis Research & Therapy 12 (2010).

[60] B. Gutbier, S.M. Kube, K. Reppe, A. Santel, C. Lange, J. Kaufmann, N. Suttorp, M. Witzenrath, RNAi-mediated suppression of constitutive pulmonary gene expression by small interfering RNA in mice, Pulmonary Pharmacology & Therapeutics 23 (2010) 334–344.

[61] S.A. Moschos, S.W. Jones, M.M. Perry, A.E. Williams, J.S. Erjefalt, J.J. Turner, P.J. Barnes, B.S. Sproat, M.J. Gait, M.A. Lindsay, Lung delivery studies using siRNA conjugated to TAT(48–60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity, Bioconjugate Chemistry 18 (2007) 1450–1459.

[62] H. Bisgaard, C. O'Callaghan, G.C. Smaldone, Drug Delivery to the Lung, Marcel Dekker, New York, 2002, p. 38.

[63] F. Morén, M.T. Newhouse, M.B. Dolovich, Aerosols in Medicine: Principles, Diagnosis, and Therapy, second ed.Elsevier, Amsterdam, 1993.

[64] M. Sakagami, In vivo, in vitro and ex vivo models to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery, Advanced Drug Delivery Reviews 58 (2006) 1030–1060.

[65] W. Yang, J.I. Peters, R.O. Williams, Inhaled nanoparticles — a current review, International Journal of Pharmaceutics 356 (2008) 239–247.

[66] J. Zhang, L. Wu, H.-K. Chan, W. Watanabe, Formation, characterization, and fate of inhaled drug nanoparticles, Advanced Drug Delivery Reviews 63 (2011) 441–455.

[67] N. Sanders, C. Rudolph, K. Braeckmans, S.C. De Smedt, J. Demeester, Extracellular barriers in respiratory gene therapy, Advanced Drug Delivery Reviews 61 (2009) 115–127.

[68] I. Roy, N. Vij, Nanodelivery in airway diseases: challenges and therapeutic applications, Nanomedicine: Nanotechnology, Biology and Medicine 6 (2010) 237–244.

[69] M.R. Knowles, R.C. Boucher, Mucus clearance as a primary innate defense mechanism for mammalian airways, The Journal of Clinical Investigation 109 (2002) 571–577.

[70] O.M. Merkel, A. Beyerle, D. Librizzi, A. Pfestroff, T.M. Behr, B. Sproat, P.J. Barth, T. Kissel, Nonviral siRNA delivery to the lung: investigation of PEG–PEI polyplexes and their in vivo performance, Molecular Pharmaceutics 6 (2009) 1246–1260.

[71] J. Roesnecker, S. Naundorf, S.W. Gersting, R.W. Hauck, A. Gessner, P. Nicklaus, R.H. Muller, C. Rudolph, Interaction of bronchoalveolar lavage fluid with polyplexes and lipoplexes: analysing the role of proteins and glycoproteins, The Journal of Gene Medicine 5 (2003) 49–60.

[72] J.E. Duncan, J.A. Whitsett, A.D. Horowitz, Pulmonary surfactant inhibits cationic liposome-mediated gene delivery to respiratory epithelial cells in vitro, Human Gene Therapy 8 (1997) 431–438.

[73] J.S. Patton, Mechanisms of macromolecule absorption by the lungs, Advanced Drug Delivery Reviews 19 (1996) 3–36.

[74] D.A. Groneberg, P.R. Eynott, S. Lim, T. Oates, R. Wu, I. Carlstedt, P. Roberts, B. McCann, A.G. Nicholson, B.D. Harrison, K.F. Chung, Expression of respiratory mucins in fatal status asthmaticus and mild asthma, Histopathology 40 (2002) 367–373.

[75] D.A. Edwards, J. Hanes, G. Caponetti, J. Hrkach, A. BenJebria, M.L. Eskew, J. Mintzes, D. Deaver, N. Lotan, R. Langer, Large porous particles for pulmonary drug delivery, Science 276 (1997) 1868–1871.

[76] A. Ben-Jebria, M.L. Eskew, D.A. Edwards, Inhalation system for pulmonary aerosol drug delivery in rodents using large porous particles, Aerosol Science and Technology 32 (2000) 421–433.

[77] M.M. Arnold, E.M. Gonnan, L.J. Schieber, E.J. Munson, C. Berkland, NanoCipro encapsulation in monodisperse large porous PLGA microparticles, Journal of Controlled Release 121 (2007) 100–109.

[78] S. Ferrari, C. Kitson, R. Farley, R. Steel, C. Marriott, D.A. Parkins, M. Scarpa, B. Wainwright, M.J. Evans, W.H. Colledge, D.M. Geddes, E. Alton, Mucus altering agents as adjuncts for nonviral gene transfer to airway epithelium, Gene Therapy 8 (2001) 1380–1386.

[79] E. Daviskas, S.D. Anderson, J.D. Brannan, H.K. Chan, S. Eberl, G. Bautovich, Inhalation of dry-powder mannitol increases mucociliary clearance, The European Respiratory Journal 10 (1997) 2449–2454.

[80] E. Daviskas, S.D. Anderson, S. Eberl, I.H. Young, Effect of increasing doses of mannitol on mucus clearance in patients with bronchiectasis, The European Respiratory Journal 31 (2008) 765–772.

[81] E. Daviskas, S.D. Anderson, A. Jaques, B. Charlton, Inhaled mannitol improves the hydration and surface properties of sputum in patients with cystic fibrosis, Chest 137 (2010) 861–868.

[82] S. Xenariou, U. Griesenbach, S. Ferrari, P. Dean, R.K. Scheule, S.H. Cheng, D.M. Geddes, C. Plank, E. Alton, Using magnetic forces to enhance non-viral gene transfer to airway epithelium in vivo, Gene Therapy 13 (2006) 1545–1552.

[83] P. Dames, B. Gleich, A. Flemmer, K. Hajek, N. Seidl, F. Wiekhorst, D. Eberbeck, I. Bittmann, C. Bergemann, T. Weyh, L. Trahms, J. Rosenecker, C. Rudolph, Targeted delivery of magnetic aerosol droplets to the lung, Nature Nanotechnology 2 (2007) 495–499.

[84] S. Xenariou, U. Griesenbach, H.D. Liang, J. Zhu, R. Farley, L. Somerton, C. Singh, P.K. Jeffery, S. Ferrari, R.K. Scheule, S.H. Cheng, D.M. Geddes, M. Blomley, E. Alton, Use of ultrasound to enhance nonviral lung gene transfer in vivo, Gene Therapy 14 (2007) 768–774.

[85] I.A. Khalil, K. Kogure, H. Akita, H. Harashima, Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery, Pharmacological Reviews 58 (2006) 32–45.

[86] T. Lebhardt, S. Roesler, M. Beck-Broichsitter, T. Kissel, Polymeric nanocarriers for drug delivery to the lung, Journal of Drug Delivery Science and Technology 20 (2010) 171–180.

[87] O. Boussif, F. Lezoualch, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in-vivo polyethylenimine, Proceedings of the National Academy of Sciences of the United States of America 92 (1995) 7297–7301.

[88] H. Hatakeyama, E. Ito, H. Akita, M. Oishi, Y. Nagasaki, S. Futaki, H. Harashima, A pH-sensitive fusogenic peptide facilitates endosomal escape and greatly enhances the gene silencing of siRNA-containing nanoparticles in vitro and in vivo, Journal of Controlled Release 139 (2009) 127–132.

[89] S.W. Choi, S.H. Lee, H. Mok, T.G. Park, Multifunctional siRNA delivery system: polyelectrolyte complex micelles of six-arm PEG conjugate of siRNA and cell penetrating peptide with crosslinked fusogenic peptide, Biotechnology Progress 26 (2010) 57–63.

[90] S.H. Lee, S.H. Kim, T.G. Park, Intracellular siRNA delivery system using polyelectrolyte complex micelles prepared from VEGF siRNA-PEG conjugate and cationic fusogenic peptide, Biochemical and Biophysical Research Communications 357 (2007) 511–516.

[91] S. Oliveira, I. van Rooy, O. Kranenburg, G. Storm, R.M. Schiffelers, Fusogenic peptides enhance endosomal escape improving siRNA-induced silencing of oncogenes, International Journal of Pharmaceutics 331 (2007) 211–214.

[92] N.R. Yacobi, N. Malmstadt, F. Fazlollahi, L. DeMaio, R. Marchelletta, S.F. Hamm-Alvarez, Z. Borok, K.J. Kim, E.D. Crandall, Mechanisms of alveolar epithelial translocation of a defined population of nanoparticles, American Journal of Respiratory Cell and Molecular Biology 42 (2010) 604–614.

[93] R.G. Parton, A.A. Richards, Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms, Traffic 4 (2003) 724–738.

[94] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. McClintock, I. MacLachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, Nature Biotechnology 23 (2005) 457–462.

[95] M. Sioud, Innate sensing of self and non-self RNAs by Toll-like receptors, Trends in Molecular Medicine 12 (2006) 167–176.

[96] M. Sioud, RNA interference and innate immunity, Advanced Drug Delivery Reviews 59 (2007) 153–163.

[97] P.C. Chiang, J.W. Alsup, Y.R. Lai, Y.D. Hu, B.R. Heyde, D. Tung, Evaluation of aerosol delivery of nanosuspension for pre-clinical pulmonary drug delivery, Nanoscale Research Letters 4 (2009) 254–261.

[98] A.J. Hickey, L. Garcia-Contreras, Immunological and toxicological implications of short-term studies in animals of pharmaceutical aerosol delivery to the lungs: relevance to humans, Critical Reviews in Therapeutic Drug Carrier Systems 18 (2001) 387–431.

[99] B. Ghosn, A. Singh, M. Li, A.V. Vlassov, C. Burnett, N. Puri, K. Roy, Efficient gene silencing in lungs and liver using imidazole-modified chitosan as a nanocarrier for small interfering RNA, Oligonucleotides 20 (2010) 163–172.

[100] O.B. Garbuzenko, M. Saad, S. Betigeri, M. Zhang, A.A. Vetcher, V.A. Soldatenkov, D.C. Reimer, V.P. Pozharov, T. Minko, Intratracheal versus intravenous liposomal delivery of siRNA, antisense oligonucleotides and anticancer drug, Pharmaceutical Research 26 (2009) 382–394.

[101] K.A. Howard, U.L. Rahbek, X.D. Liu, C.K. Damgaard, S.Z. Glud, M.O. Andersen, M.B. Hovgaard, A. Schmitz, J.R. Nyengaard, F. Besenbacher, J. Kjems, RNA interference in vitro and in vivo using a chitosan/siRNA nanoparticle system, Molecular Therapy 14 (2006) 476–484.

[102] V. Bitko, A. Musiyenko, O. Shulyayeva, S. Barik, Inhibition of respiratory viruses by nasally administered siRNA, Nature Medicine 11 (2005) 50–55.

[103] M. Perl, C.S. Chung, J. Lomas-Neira, T.M. Rachel, W.L. Biffl, W.G. Cioffi, A. Ayala, Silencing of Fas, but not caspase-8, in lung epithelial cells ameliorates pulmonary apoptosis, inflammation, and neutrophil influx after hemorrhagic shock and sepsis, The American Journal of Pathology 167 (2005) 1545–1559.

[104] J.L. Lomas-Neira, C.S. Chung, D.E. Wesche, M. Perl, A. Ayala, In vivo gene silencing (with siRNA) of pulmonary expression of MIP-2 versus KC results in divergent effects on hemorrhage-induced, neutrophil-mediated septic acute lung injury, Journal of Leukocyte Biology 77 (2005) 846–853.

[105] X.C. Zhang, P.Y. Shan, D.H. Jiang, P.W. Noble, N.G. Abraham, A. Kappas, P.J. Lee, Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis, The Journal of Biological Chemistry 279 (2004) 10677–10684.

[106] D. Massaro, G.D. Massaro, L.B. Clerch, Noninvasive delivery of small inhibitory RNA and other reagents to pulmonary alveoli in mice, American Journal of Physiology. Lung Cellular and Molecular Physiology 287 (2004) L1066–L1070.

[107] S.M. Tompkins, C.Y. Lo, T.M. Tumpey, S.L. Epstein, Protection against lethal influenza virus challenge by RNA interference in vivo, Proceedings of the National Academy of Sciences of the United States of America 101 (2004) 8682–8686.

[108] L.D. Mastrandrea, T. Quattrin, Clinical evaluation of inhaled insulin, Advanced Drug Delivery Reviews 58 (2006) 1061–1075.

[109] V. Codrons, F. Vanderbist, R.K. Verbeeck, M. Arras, D. Lison, V. Preat, R. Vanbever, Systemic delivery of parathyroid hormone (1–34) using inhalation dry powders in rats, Journal of Pharmaceutical Sciences 92 (2003) 938–950.

[110] S.H. Bai, V. Gupta, F. Ahsan, Inhalable lactose-based dry powder formulations of low molecular weight heparin, Journal of Aerosol Medicine and Pulmonary Drug Delivery 23 (2010) 97–104.

[111] A. Rawat, Q.H. Majumder, F. Ahsan, Inhalable large porous microspheres of low molecular weight heparin: in vitro and in vivo evaluation, Journal of Controlled Release 128 (2008) 224–232.

[112] H.K. Chan, Formulation challenges: protein powders for inhalation, in: M.J. Rathbone (Ed.), Modified-Release Drug Delivery Technology, Informa Healthcare, New York, 2008, pp. 867–878.

[113] J. Lenzer, Inhaled insulin is approved in Europe and United States, British Medical Journal 332 (2006) 321–321.

[114] J. Kling, Inhaled insulin's last gasp? Nature Biotechnology 26 (2008) 479–480.

[115] T. Quattrin, A. Belanger, N.J.V. Bohannon, S.L. Schwartz, I.I.I.S.G. Exubera Phase, Efficacy and safety of inhaled insulin (Exubera) compared with subcutaneous insulin therapy in patients with type 1 diabetes — results of a 6-month, randomized, comparative trial, Diabetes Care 27 (2004) 2622–2627.

[116] P.A. Hollander, L. Blonde, R. Rowe, A.E. Mehta, J.L. Milburn, K.S. Hershon, J.L. Chiasson, S.R. Levin, I.I.I.S.G. Exubera Phase, Efficacy and safety of inhaled insulin (Exubera) compared with subcutaneous insulin therapy in patients with type 2 diabetes — results of a 6-month, randomized, comparative trial, Diabetes Care 27 (2004) 2356–2362.

[117] J. Rosenstock, W.T. Cefalu, P.A. Hollander, S.S. Klioze, J. Reis, W.T. Duggan, Safety and efficacy of inhaled human insulin (Exubera) during discontinuation and readministration of therapy in adults with type 2 diabetes: a 3-year randomized controlled trial, Diabetes Technology and Therapeutics 11 (2009) 697–705.

[118] V. Alabraba, A. Farnsworth, R. Leigh, P. Dodson, S.C.L. Gough, T. Smyth, Exubera inhaled insulin in patients with type 1 and type 2 diabetes: the first 12 months, Diabetes Technology and Therapeutics 11 (2009) 427–430.

[119] R.A. DeFronzo, R.M. Bergenstal, W.T. Cefalu, J. Pullman, S. Lerman, B.W. Bode, L.S. Phillips, I.I.I.S.G. Exubera Phase, Efficacy of inhaled insulin in patients with type 2 diabetes not controlled with diet and exercise — a 12-week, randomized, comparative trial, Diabetes Care 28 (2005) 1922–1928.

[120] S.A. Shoyele, A. Slowey, Prospects of formulating proteins/peptides as aerosols for pulmonary drug delivery, International Journal of Pharmaceutics 314 (2006) 1–8.

[121] M. Ameri, Y.F. Maa, Spray drying of biopharmaceuticals: stability and process considerations, Drying Technology 24 (2006) 763–768.

[122] M. Irngartinger, V. Camuglia, M. Damm, J. Goede, H.W. Frijlink, Pulmonary delivery of therapeutic peptides via dry powder inhalation: effects of micronisation and manufacturing, European Journal of Pharmaceutics and Biopharmaceutics 58 (2004) 7–14.

[123] D.M.K. Jensen, D. Cun, M.J. Maltesen, S. Frokjaer, H.M.r. Nielsen, C. Foged, Spray drying of siRNA-containing PLGA nanoparticles intended for inhalation, Journal of Controlled Release 142 (2010) 138–145.

[124] M. Bivas-Benita, R. Zwier, H.E. Junginger, G. Borchard, Non-invasive pulmonary aerosol delivery in mice by the endotracheal route, European Journal of Pharmaceutics and Biopharmaceutics 61 (2005) 214–218.

[125] M. Hinchcliffe, L. Illum, Intranasal insulin delivery and therapy, Advanced Drug Delivery Reviews 35 (1999) 199–234.

[126] J. Heyder, J. Gebhart, G. Rudolf, C.F. Schiller, W. Stahlhofen, Deposition of particles in the human respiratory tract in the size range 0.005–15 μm, Journal of Aerosol Science 17 (1986) 811–825.

[127] A.A. Hussain, Intranasal drug delivery, Advanced Drug Delivery Reviews 29 (1998) 39–49.

[128] J.K. Watts, G.F. Deleavey, M.J. Damha, Chemically modified siRNA: tools and applications, Drug Discovery Today 13 (2008) 842–855.

[129] B. Urban-Klein, S. Werth, S. Abuharbeid, F. Czubayko, A. Aigner, RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo, Gene Therapy 12 (2005) 461–466.

[130] A. Pal, A. Ahmad, S. Khan, I. Sakabe, C.B. Zhang, U.N. Kasid, I. Ahmad, Systemic delivery of RafsiRNA using cationic cardiolipin liposomes silences Raf-1 expression and inhibits tumor growth in xenograft model of human prostate cancer, International Journal of Oncology 26 (2005) 1087–1091.

[131] M.A. Behlke, Progress towards in vivo use of siRNAs, Molecular Therapy 13 (2006) 644–670.

[132] A. Akinc, A. Zumbuehl, M. Goldberg, E.S. Leshchiner, V. Busini, N. Hossain, S.A. Bacallado, D.N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. de Fougerolles, J.R. Dorkin, K.N. Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K.G. Rajeev, D.W.Y. Sah, J. Soutschek, I. Toudjarska, H.P. Vornlocher, T.S. Zimmermann, R. Langer, D.G. Anderson, A combinatorial library of lipid-like materials for delivery of RNAi therapeutics, Nature Biotechnology 26 (2008) 561–569.

[133] E.J.B. Nielsen, J.M. Nielsen, D. Becker, A. Karlas, H. Prakash, S.Z. Glud, J. Merrison, F. Besenbacher, T.F. Meyer, J. Kjems, K.A. Howard, Pulmonary gene silencing in transgenic EGFP mice using aerosolised chitosan/siRNA nanoparticles, Pharmaceutical Research 27 (2010) 2520–2527.

[134] Y.C. Tseng, S. Mozumdar, L. Huang, Lipid-based systemic delivery of siRNA, Advanced Drug Delivery Reviews 61 (2009) 721–731.

[135] S.Y. Wu, N.A.J. McMillan, Lipidic systems for in vivo siRNA delivery, The AAPS Journal 11 (2009) 639–652.

[136] M.M. Gaspar, U. Bakowsky, C. Ehrhardt, Inhaled liposomes — current strategies and future challenges, Journal of Biomedical Nanotechnology 4 (2008) 245–257.

[137] A.R. De Fougerolles, Delivery vehicles for small interfering RNA in vivo, Human Gene Therapy 19 (2008) 125–132.

[138] S. Dokka, D. Toledo, X.G. Shi, V. Castranova, Y. Rojanasakul, Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes, Pharmaceutical Research 17 (2000) 521–525.

[139] G. Storm, S.O. Belliot, T. Daemen, D.D. Lasic, Surface modifications of nanoparticles to oppose uptake by the mononuclear phagocyte system, Advanced Drug Delivery Reviews 17 (1995) 31–48.

[140] E. Alton, M. Stern, R. Farley, A. Jaffe, S.L. Chadwick, J. Phillips, J. Davies, S.N. Smith, J. Browning, M.G. Davies, M.E. Hodson, S.R. Durham, D. Li, P.K. Jeffery, M. Scallan, R. Balfour, S.J. Eastman, S.H. Cheng, A.E. Smith, D. Meeker, D.M. Geddes, Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial, Lancet 353 (1999) 947–954.

[141] F.E. Ruiz, J.P. Clancy, M.A. Perricone, Z. Bebok, J.S. Hong, S.H. Cheng, D.P. Meeker, K.R. Young, R.A. Schoumacher, M.R. Weatherly, L. Wing, J.E. Morris, L. Sindel, M. Rosenberg, F.W. van Ginkel, J.R. McGhee, D. Kelly, R.K. Lyrene, E.J. Sorscher, A clinical inflammatory syndrome attributable to aerosolized lipid-DNA administration in cystic fibrosis, Human Gene Therapy 12 (2001) 751–761.

[142] U. Griesenbach, C. Kitson, S.E. Garcia, R. Farley, C. Singh, L. Somerton, H. Painter, R.L. Smith, D.R. Gill, S.C. Hyde, Y.H. Chow, J. Hu, M. Gray, M. Edbrooke, V. Ogilvie, G. MacGregor, R.K. Scheule, S.H. Cheng, N.J. Caplen, E. Alton, Inefficient cationic lipid-mediated siRNA and antisense oligonucleotide transfer to airway epithelial cells in vivo, Respiratory Research 7 (2006).

[143] J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R.K. Pandey, T. Racie, K.G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, H.P. Vornlocher, Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs, Nature 432 (2004) 173–178.

[144] S.C. Semple, S.K. Klimuk, T.O. Harasym, N. Dos Santos, S.M. Ansell, K.F. Wong, N. Maurer, H. Stark, P.R. Cullis, M.J. Hope, P. Scherrer, Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures, Biochimica Et Biophysica Acta-Biomembranes 1510 (2001) 152–166.

[145] S.C. Semple, A. Akinc, J.X. Chen, A.P. Sandhu, B.L. Mui, C.K. Cho, D.W.Y. Sah, D. Stebbing, E.J. Crosley, E. Yaworski, I.M. Hafez, J.R. Dorkin, J. Qin, K. Lam, K.G. Rajeev, K.F. Wong, L.B. Jeffs, L. Nechev, M.L. Eisenhardt, M. Jayaraman, M. Kazem, M.A. Maier, M. Srinivasulu, M.J. Weinstein, Q.M. Chen, R. Alvarez, S.A. Barros, S. De, S.K. Klimuk, T. Borland, V. Kosovrasti, W.L. Cantley, Y.K. Tam, M. Manoharan,

M.A. Ciufolini, M.A. Tracy, A. de Fougerolles, I. MacLachlan, P.R. Cullis, T.D. Madden, M.J. Hope, Rational design of cationic lipids for siRNA delivery, Nature Biotechnology 28 (2010) 172-U118.

[146] T.S. Zimmermann, A.C.H. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M.N. Fedoruk, J. Harborth, J.A. Heyes, L.B. Jeffs, M. John, A.D. Judge, K. Lam, K. McClintock, L.V. Nechev, L.R. Palmer, T. Racie, I. Rohl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A.J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H.P. Vornlocher, I. MacLachlan, RNAi-mediated gene silencing in non-human primates, Nature 441 (2006) 111–114.

[147] K.T. Love, K.P. Mahon, C.G. Levins, K.A. Whitehead, W. Querbes, J.R. Dorkin, J. Qin, W. Cantley, L.L. Qin, T. Racie, M. Frank-Kamenetsky, K.N. Yip, R. Alvarez, D.W.Y. Sah, A. de Fougerolles, K. Fitzgerald, V. Koteliansky, A. Akinc, R. Langer, D.G. Anderson, Lipid-like materials for low-dose, in vivo gene silencing, Proceedings of the National Academy of Sciences of the United States of America 107 (2010) 1864–1869.

[148] A. Bielinska, J.F. KukowskaLatallo, J. Johnson, D.A. Tomalia, J.R. Baker, Regulation of in vitro gene expression using antisense oligonucleotides or antisense expression plasmids transfected using starburst PAMAM dendrimers, Nucleic Acids Research 24 (1996) 2176–2182.

[149] W.F. Lai, M.C.M. Lin, Nucleic acid delivery with chitosan and its derivatives, Journal of Controlled Release 134 (2009) 158–168.

[150] D. Cun, L.B. Jensen, H.M. Nielsen, M. Moghimi, C. Foged, Polymeric nanocarriers for siRNA delivery: challenges and future prospects, Journal of Biomedical Nanotechnology 4 (2008) 258–275.

[151] H. Katas, H.O. Alpar, Development and characterisation of chitosan nanoparticles for siRNA delivery, Journal of Controlled Release 115 (2006) 216–225.

[152] M.R. Park, K.O. Han, I.K. Han, M.H. Cho, J.W. Nah, Y.J. Choi, C.S. Cho, Degradable polyethylenimine-alt-poly(ethylene glycol) copolymers as novel gene carriers, Journal of Controlled Release 105 (2005) 367–380.

[153] M. Thomas, J.J. Lu, C.C. Zhang, J.Z. Chen, A.M. Klibanov, Identification of novel superior polycationic vectors for gene delivery by high-throughput synthesis and screening of a combinatorial library, Pharmaceutical Research 24 (2007) 1564–1571.

[154] S.R. Mao, S. Sun, T. Kissel, Chitosan-based formulations for delivery of DNA and siRNA, Advanced Drug Delivery Reviews 62 (2010) 12–27.

[155] X.D. Liu, K.A. Howard, M.D. Dong, M.O. Andersen, U.L. Rahbek, M.G. Johnsen, O.C. Hansen, F. Besenbacher, J. Kjems, The influence of polymeric properties on chitosan/siRNA nanoparticle formulation and gene silencing, Biomaterials 28 (2007) 1280–1288.

[156] J. Panyam, V. Labhasetwar, Biodegradable nanoparticles for drug and gene delivery to cells and tissue, Advanced Drug Delivery Reviews 55 (2003) 329–347.

[157] R.C. Mundargi, V.R. Babu, V. Rangaswamy, P. Patel, T.M. Aminabhavi, Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives, Journal of Controlled Release 125 (2008) 193–209.

[158] Y. Takashima, R. Saito, A. Nakajima, M. Oda, A. Kimura, T. Kanazawa, H. Okada, Spray-drying preparation of microparticles containing cationic PLGA nano-spheres as gene carriers for avoiding aggregation of nanospheres, International Journal of Pharmaceutics 343 (2007) 262–269.

[159] A.D. Frankel, C.O. Pabo, Cellular uptake of the TAT protein from human immunodeficiency virus, Cell 55 (1988) 1189–1193.

[160] A. Eguchi, S.F. Dowdy, siRNA delivery using peptide transduction domains, Trends in Pharmacological Sciences 30 (2009) 341–345.

[161] B.R. Meade, S.F. Dowdy, Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides, Advanced Drug Delivery Reviews 59 (2007) 134–140.

[162] Y.L. Chiu, A. Ali, C.Y. Chu, H. Cao, T.M. Rana, Visualizing a correlation between siRNA localization, cellular uptake, and RNAi in living cells, Chemistry & Biology 11 (2004) 1165–1175.

[163] J.J. Turner, S. Jones, M.M. Fabani, G. Ivanova, A.A. Arzumanov, M.J. Gait, RNA targeting with peptide conjugates of oligonucleotides, siRNA and PNA, Blood Cells, Molecules & Diseases 38 (2007) 1–7.

[164] A. Muratovska, M.R. Eccles, Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells, FEBS Letters 558 (2004) 63–68.

[165] T.J. Davidson, S. Harel, V.A. Arboleda, G.F. Prunell, M.L. Shelanski, L.A. Greene, C.M. Troy, Highly efficient small interfering RNA delivery to primary mammalian neurons induces MicroRNA-like effects before mRNA degradation, The Journal of Neuroscience 24 (2004) 10040–10046.

[166] F. Simeoni, M.C. Morris, F. Heitz, G. Divita, Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells, Nucleic Acids Research 31 (2003) 2717–2724.

[167] S. Veldhoen, S.D. Laufer, A. Trampe, T. Restle, Cellular delivery of small interfering RNA by a non-covalently attached cell-penetrating peptide: quantitative analysis of uptake and biological effect, Nucleic Acids Research 34 (2006) 6561–6573.

[168] L. Crombez, G. Aldrian-Herrada, K. Konate, Q.N. Nguyen, G.K. McMaster, R. Brasseur, F. Heitz, G. Divita, A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells, Molecular Therapy 17 (2009) 95–103.

[169] K. Konate, L. Crombez, S. Deshayes, M. Decaffmeyer, A. Thomas, R. Brasseur, G. Aldrian, F. Heitz, G. Divita, Insight into the cellular uptake mechanism of a secondary amphipathic cell-penetrating peptide for siRNA delivery, Biochemistry 49 (2010) 3393–3402.

[170] B. Langlet-Bertin, C. Leborgne, D. Scherman, B. Bechinger, A.J. Mason, A. Kichler, Design and evaluation of histidine-rich amphipathic peptides for siRNA delivery, Pharmaceutical Research 27 (2010) 1426–1436.

[171] M. Lindgren, M. Hallbrink, A. Prochiantz, U. Langel, Cell-penetrating peptides, Trends in Pharmacological Sciences 21 (2000) 99–103.

[172] H. Brooks, B. Lebleu, E. Vives, Tat peptide-mediated cellular delivery: back to basics, Advanced Drug Delivery Reviews 57 (2005) 559–577.

[173] S. Futaki, Membrane-permeable arginine-rich peptides and the translocation mechanisms, Advanced Drug Delivery Reviews 57 (2005) 547–558.

[174] S. Deshayes, M.C. Morris, G. Divita, F. Heitz, Cell-penetrating peptides: tools for intracellular delivery of therapeutics, Cellular and Molecular Life Sciences 62 (2005) 1839–1849.

[175] S.A. Moschos, A.E. Williams, M.A. Lindsay, Cell-penetrating-peptide-mediated siRNA lung delivery, Biochemical Society Transactions 35 (2007) 807–810.